Apoptotic cell death during *Drosophila* oogenesis is differentially increased by electromagnetic radiation depending on modulation, intensity and duration of exposure

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Abstract

Present generations are being repeatedly exposed to different types and doses of non-ionizing radiation (NIR) from wireless technologies (FM radio, TETRA and TV stations, GSM and UMTS phones/base stations, Wi-Fi networks, DECT phones). Although there is controversy on the published data regarding the non-thermal effects of NIR, studies have convincingly demonstrated bioeffects. Their results indicate that modulation, intensity, exposure duration and modulation system are important factors determining the biological response to irradiation. Attempting to address the dependence of NIR bioeffectiveness on these factors, apoptosis in the model biological system *Drosophila melanogaster* was studied under different exposure protocols. A signal generator was used operating alternatively under Continuous Wave (CW) or Frequency Modulation (FM) emission modes, at three power output values (10 dB, 0, –10 dB), under four carrier frequencies (100, 395, 682, 900 MHz). Newly emerged flies were exposed either acutely (6 min or 60 min on the 6th day), or repeatedly (6 min or 60 min daily for the first 6 days of their life). All exposure protocols resulted in an increase of apoptotic cell death (ACD) observed in egg chambers, even at very low electric field strengths. FM waves seem to have a stronger effect in ACD than continuous waves. Regarding intensity and temporal exposure pattern, EMF-biological tissue interaction is not linear in response. Intensity threshold for the induction of biological effects depends on frequency, modulation and temporal exposure pattern with unknown so far mechanisms. Given this complexity, translating such experimental data into possible human exposure guidelines is yet arbitrary.

Keywords

Apoptosis, continuous wave, *Drosophila melanogaster*, frequency generator, frequency modulation, frequency window, oogenesis, radiofrequencies

Introduction

More and more devices in our modern society generate and make use of electromagnetic fields (EMFs): cellular phones (GSM-Global System for Mobile Communications) or smartphones (3G/UMTS-Universal Mobile Telecommunications System), digital enhanced cordless telecommunications (DECT) wireless telephones, tablets, i-pads, i-pods, laptops, notebooks and netbooks. In some cases such a device can be equipped with three different communication systems requiring three different transmitters and antennas, i.e. smartphones with cellular, Bluetooth and Wi-Fi (Wireless-Fidelity) connections simultaneously. Human exposure to these artificial sources can be higher than the corresponding from natural fields of the earth, the sun and space.

Therefore, the possible risks of EMFs for human health have become a growing concern for our society. For example, a maximal whole-body absorbed energy (in units of Specific Absorption Rate (SAR) expressed in terms of W/kg) of 0.08 W/kg and a maximal peak average power of 1.6 mW in 1g of tissue over a 30-min period were imposed as an exposure limit in the USA by the Federal Communications Commission. SAR is defined as the time rate of energy absorbed in an incremental mass, divided by that mass and is an important parameter for characterizing exposure to microwaves (MW).

In spite of the enormous efforts of research groups, controversy remains over the possibility of adverse health effects from exposure to low-level EM fields, such as those associated with the use of the above-mentioned devices, daily and constantly used with exponentially increasing numbers, over the entire population on the globe. This controversy is fuelled in part by the lack of an established non-thermal interaction mechanism.

Electromagnetic exposures differ largely based on the characteristics of the emitted microwaves, i.e. frequency, modulation, intensity, etc. However, whether those characteristics influence in bio-effectiveness has not yet been clarified.

Frequency modulation (FM) is a form of analog angle modulation which conveys information over a carrier wave by
varying its instantaneous frequency. It is commonly used in radio, telemetry, radar, earthquake prediction, for broadcasting music and speech, two-way radio systems, magnetic tape-recording systems and some video-transmission systems. In contrast, a continuous wave (CW) is an electromagnetic wave of constant amplitude and frequency of infinite duration in which a carrier wave is switched on and off. Information is carried in the varying duration of the on and off periods of the signal.

There are several studies that compare bio-effectiveness of CW signals with modulated ones and especially of the pulsed types used in wireless technology devices.

Cell death, particularly apoptosis, may occur after MW exposure. Some in vitro studies concerning radiofrequency (RF)-induced apoptosis were made using different cell types (Capri et al., 2004a,b; Caraglia et al., 2005; Hook et al., 2004; Maeda et al., 2004). These studies did not give the same results, indicating that sensitivity to MW may be different according to the cell type. For example, no apoptosis was induced by RF-exposure in human peripheral blood mononuclear cells (Capri et al., 2004a), in human lymphocytes (Capri et al., 2004b), or in lymphoblastoid cells (Hook et al., 2004), whereas human colon cancer cells (Maeda et al., 2004) and human epidermoid cancer cells (Caraglia et al., 2005) were forced into apoptosis by MW treatment.

Cell-dependent response to RF-EMFs was also shown by two studies of the same research group using originally a cell line as a model system and then primary cells. Specifically, human neuroblastoma cell line exposed to 900 MHz for 24 h did not reveal any apoptosis in either CW (SAR: 2 W/kg) or GSM exposure (SAR: 0.25 W/kg) (Joubert et al., 2006). However, these authors showed subsequently that CW exposure of rat primary neuronal cultures, under the same as above conditions, revealed a significant increase in apoptosis (Joubert et al., 2008).

Furthermore, exposure to 900 MHz CW or GSM-modulated signal (SAR: 0.4, 2.0 and 3.6 W/kg), did not significantly increase the expression of stress proteins HSP70 and HSP27 in human leukocytes (Lim et al., 2005). Comparing CW and pulsed waves at 2.45 GHz with a wide range of high SAR values 5, 10, 20, 50 and 100 W/kg, Komatsubara et al. (2005) did not find statistically significant differences in chromosomal aberrations in mouse m5S cells after 2 h of exposure.

Again, no detectable changes in cell proliferation kinetics after CW exposure, but a statistically significant increase in micronuclear induction after Gaussian minimum shift keying (GMSK) phase modulated irradiation of human peripheral blood cultures were found at 1.748 GHz, for 15 min with a maximum SAR of 5 W/kg (d’Ambrosio et al., 2002).

One more in vitro report studied comparatively the bioeffects of CW and amplitude modulated signals. Franzellitti et al. (2010) exposed human trophoblasts (HTR-8/SVneo cell line) to either continuous or amplitude-modulated (GSM-217, GSM-Talk, i.e. intermittently: 5 min field on, 10 min field off), 1800 MHz signal at SAR 2 W/kg. Exposure duration was 4, 16 and 24 h. There was a significant increase in DNA damage and/or strand breaks after the exposure to the amplitude modulation (AM) signal, while the CW exposure was found to be ineffective.

Another aspect studied by Salford’s research group was the correlation of EMF strength (or SAR values) with actual bio-effect and CW/modulated signals. In this in vivo study, it was found that low SAR exposure of rats (~1 mW/kg) had the strongest effect on the disruption of blood brain barrier (BBB) at modulation of 8–50 Hz (carrier frequency 915 MHz) and the weakest effects after CW exposure (Persson et al., 1997). However, at higher SAR values (>10 mW/kg) the damage was less, regarding pulse modulation exposure and interestingly the difference between the bioactivity of CW and modulated signals varied among the diverse SAR levels examined.

Different tissues of the same animals responded differently in an assay for enzyme activity after exposure to CW or to 50 Hz amplitude modulated (50/50% on, off) waves (2.45 GHz, 100 min per day); in the liver tissues, an increase was found after both CW and modulated signals, whereas in the brain a decrease was detected after CW exposure and no-effect after the modulated signal exposure (Kubinyi et al., 1996). In this study, in utero exposure of mice was applied with relatively high power density values (3 mW/cm², SAR: 4.23 W/kg). AminoAcyl-Transferase activity was measured at post-natal day 24 of the offspring.

Concerning studies in humans, reduced reaction speed and increased accuracy in a working-memory task of 24 healthy, young volunteers was found after pulse-modulated exposure, while CW had no impact on the performance of these cognitive tasks. The exposure lasted for 30 min, at 900 MHz with calculated SAR value of 1 W/kg (Regel et al., 2007).

Analyzing these publications superficially we may come to wrong conclusion that they are controversial or conflicting. However, this may not be the case if all parameters of each experiment are to be taken into account. To pursue this issue further and to find out whether the final bioeffect is dependent on (a) SAR, (b) duration scheme, (c) model system and (d) frequency, we examined apoptosis in a biological system, very well established as an EMF biological marker (Margaritis et al., 2014), namely, oogenesis in Drosophila melanogaster. During this process, apoptotic cell death occurs physiologically in order to compensate for environmental stress factors reducing the number of vital eggs (Drummond-Barbosa and Spradling, 2001; McCall, 2004; Nezis et al., 2000, 2001, 2002). Normally, a small percentage (~2–4%) of the egg chambers within the ovaries, at developmental stages up to 10 exhibit apoptotic features with condensed, fragmented nurse cell and follicle cell nuclei and disorganized actin network (Nezis et al., 2006). Such phenotypes are detectable after dissection of the flies, removing the ovaries and stain with acridine orange which binds to fragmented DNA and then fluoresces (Hayashi et al., 1983). Using a fluorescent microscope the number of apoptotic egg chambers compared to the normal ones within the ovaries removed by a certain number of flies, can be measured. Using this model system of which the developmental biology we have studied extensively (Margaritis, 1985, 1986; Margaritis et al., 1980), we have been exposing the flies mainly with mobile phone radiation and have detected a raise in apoptotic cell death as well as a decrease in fecundity (Chavdoula et al., 2010; Margaritis et al., 2014; Panagopoulos and Margaritis, 2010).
been found to be associated with pulsed DECT exposures (Manta et al., 2014). Recently, by applying a variety of EMF sources we came across the conclusion that *Drosophila* oogenesis can be used as a biomarker in EMF studies (Margaritis et al., 2014).

In the present work, in order to answer the above addressed questions we have applied four different frequencies that are of special interest, (i) 100 MHz used by broadcast FM stations, (ii) 395 MHz, used in TETRA communication, (iii) 682 MHz, used in TV broadcasting and finally (iv) 900 MHz widely used in mobile telephony. We applied CW (constant, non-modulated carrier waves) and 50 kHz FM modulated signals through a signal generator, in three largely different intensities; power output of 10 dB, 0 dB and −10 dB. Corresponding E-field intensities as measured by spectrum analyzers were far below existing guidelines. For 100 MHz: 4.8 V/m, 1.5 V/m and 0.47 V/m, for 395 MHz: 9.2 V/m, 3.1 V/m and 1.1 V/m, for 682 MHz: 5.7 V/m, 1.5 V/m and 0.48 V/m and for 900 MHz: 3 V/m, 0.8 V/m and 0.23 V/m, respectively.

**Materials and methods**

**Model biological system-culturing of *D. melanogaster***

The experiments were performed with the dipteran flies *D. melanogaster* (Diptera, Drosophilidae) Oregon R, wild type. Control insects were kept in a 25°C culture room, totally protected from electromagnetic radiation, with 50–70% relative humidity and 12:12 h light/dark cycle. Exposed flies were kept in a separate room (for long-term exposure) but cultured under similar conditions as the control group, 12:12 h light/dark cycle, 50% relative humidity and temperature of 23–25°C. Newly emerged flies (approximately 4 h after eclosion) were collected and separated in groups with light anesthesia. Each group consisted of five males and five females. Control and exposed flies were fed on a standard diet (agar, yeast, sugar, rice flour, tomato paste, ethanol and propionic acid) and kept in plastic culture tubes (8 cm height and 3 cm diameter).

**Exposure system**

Flies were exposed from the side of the culture tube to the near field of a multi-directional antenna (14.5 cm long) that was placed in between two of them. A signal generator (Agilent-HP 8924E Mobile Communications Test Set 30–1000 MHz, Palo Alto, CA) was used, emitting either CW or FM/50 kHz deviation signals (Figure 1) in various frequencies, intensities and durations. The distance of the antenna to the fly samples was approximately 0.5–3 cm, depending on the position of the free moving flies. The environment temperature was fixed at 23–25°C.

We used the following exposure scenarios in newly emerged flies:

- **(A)** Acute exposure for 6 min at the 6th day of their adult life
- **(B)** Acute exposure for 60 min at the 6th day of their adult life
- **(C)** Repeated exposure for 6 min per day starting from the first day of their lives
- **(D)** Repeated exposure for 60 min per day starting from the first day of their lives

Frequencies used:

- **(i)** 100 MHz (corresponding to an FM radio station)
- **(ii)** 395 MHz (corresponding to TETRA station communication)
- **(iii)** 682 MHz (corresponding to TV broadcasts)
- **(iv)** 900 MHz (corresponding to GSM900 communication)

The output power of the signal generator was set at 10, 0 and −10 dB. Spectra were recorded by the FSL/6 ROHDE AND SCHWARZ (Munich, Germany) spectrum analyzer. The corresponding electrical field intensity in each experimental setup was measured for 6 min time period according to ICNIRP (1998) guidelines with the NARDA SRM3000 (NardaSafety Test Solutions, Inc., Pfullingen, Germany).

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**Figure 1. Emission spectra and spectrographs under 1 MHz span and 87 ms sweep rate of the CW (A) and FM/50 kHz (B) modes of the signal generator as recorded by the FSL/6 ROHDE AND SCHWARZ spectrum analyzer.** CW: Continuous Wave, FM: Frequency Modulation. The time-dependent characteristics of the FM signal is demonstrated in lower half of the right image, compared with the uniform lane of the CW signal (left image).
Table 1. Presenting E-field intensities (V/m) at the various frequencies used, under CW and FM/50 KHz, as measured with the NARDA SRM3000 spectrum analyzer at the position of the fly vials during exposure and at the three power settings of the generator (10, 0 and −10 dB).

| Frequency | 100 MHz | 395 MHz | 682 MHz | 900 MHz |
|-----------|---------|---------|---------|---------|
|            | CW      | FM      | CW      | FM      | CW      | FM      | CW      | FM      |
| Output power | 10 dB    |         |         |         |         |         |         |         |
| E-Field (V/m) | 4.790    | 4.584   | 9.253   | 9.270   | 5.777   | 5.590   | 3.000   | 3.067   |
| SAR (W/kg) | 0.027    | 0.025   | 0.102   | 0.102   | 0.040   | 0.037   | 0.011   | 0.011   |
| Output power | 0 dB     |         |         |         |         |         |         |         |
| E-Field (V/m) | 1.510    | 1.530   | 2.954   | 3.152   | 1.479   | 1.525   | 0.922   | 0.800   |
| SAR (W/kg) | 0.003    | 0.003   | 0.010   | 0.012   | 0.003   | 0.003   | 0.001   | 0.001   |
| Output power | −10 dB   |         |         |         |         |         |         |         |
| E-Field (V/m) | 0.477    | 0.480   | 0.975   | 1.100   | 0.481   | 0.454   | 0.262   | 0.232   |
| SAR (W/kg) | 0.0003   | 0.0003  | 0.0011  | 0.0014  | 0.0003  | 0.0002  | 0.0001  | 0.0001  |

Values were confirmed with near field probes inserted within the vials and measured with the FSL/6 Rohde & Schwarz spectrum analyzer. Specific absorption rate (SAR) was calculated in W/kg for the flies with electrical conductivity $\sigma = 1.19 \text{ S/m}$ and mass density $\rho = 1000 \text{ kg/m}^3$ values according to Lee et al. (2008). CW: Continuous Wave, FM: Frequency Modulation.

Acridine orange stain

Apoptosis was estimated through acridine orange (AO) staining, which has been used as a fluorescent stain indicator of apoptosis in Ceratitis and Drosophila (Abrams et al., 1993; Velentzas et al., 2007; White et al., 1994). In previous studies of ours, the results derived from AO staining were also confirmed with TUNEL assay and the percentage of ovarian apoptosis, compared to control samples, was found to be the same between the two assays used (Chavdoula et al., 2010).

Four to 5 h after the last irradiation, female flies were cryo-anesthetized in ice and rapidly dissected in Ringer’s solution and their ovaries were removed under fiber optics cold illumination. The 4–5 h post-irradiation timing has been found as optimum for the ovarian tissue to exhibit the maximum number of apoptotic follicles (Chavdoula et al., 2010). Ovaries were then separated into individual egg chambers and incubated in 200 μl 1.6 μM Acridine Orange dye (Invitrogen, Carlsbad, CA) in Ringer’s solution for 5 min in the dark with constant shake. They were washed in 200 μl Ringer’s solution for 5 min in the dark and immediately mounted onto Vidal glass slides in fresh Ringer’s solution. Elapsed time from dissection to the end of the viewing was restricted to 20 min in order to maintain good fluorescence activity. Samples were then examined at a Nikon Eclipse TE-2000U fluorescent microscope (Nikon Instruments, Tokyo, Japan).

Egg chambers from gerarium to stage 10 b showing positive acridine orange staining signal (DNA fragmentation) were counted compared to the total number of the unstained (negative) normal, non-apoptotic follicles.

Statistical analysis

All data from AO fluorescent images were gathered on Excel spreadsheet and were analyzed by SPSS v.22.0 software (SPSS Inc., Chicago, IL). Statistical evaluation was performed using the one-Way analysis of variance (ANOVA) followed by the least significant difference (LSD) post-hoc statistics. Differences were considered significant at $^*p \leq 0.05$ and $^{**}p \leq 0.01$.

Results

A signal generator was used operating alternatively under CW or FM emission modes and three power output values 10 dB, 0 dB and −10 dB under four carrier frequencies (100, 395, 682, 900 MHz). The corresponding E-field values and SAR is shown in Table 1. Newly emerged flies were exposed either acutely (6 min or 60 min at the 6th day of their adult life) or repeatedly (6 min or 60 min daily for the first 6 days of life). In every exposure protocol more than three independent experiments were performed. Individual experiments were done in duplicates and every sample, for assaying apoptosis, consisted of five pairs of ovaries. Apoptotic follicles were detected due to their spotted fluorescence pattern following AO staining (representative fluorescent images are shown in Figure 2). The number of apoptotic follicles at stages below 10b compared to the total follicles denotes the percentage of apoptosis shown in Figures 3–6.

Acute exposure, of adult flies for 6 min or 60 min at the 6th day of their lives, or repeated exposure, for 6 min or 60 min daily for the first 6 days of life, resulted in an increase of apoptotic cell death, observed in egg chambers. Generally, frequency modulated waves seem to have a stronger effect in apoptotic cell death than continuous waves as described below. Statistically significant difference between CW and FM signals indicates that FM is more bioeffective. In cases where the opposite is true this is stated. For each of the four different frequencies used, under the three power settings and the four exposure scenarios, the detailed findings of the 96 experimental cases are as follows.

Exposure at 100 MHz

The overall data at this frequency are presented in Figure 3 and Table 2.

An acute exposure for 6 min, revealed a statistically significant increase ($p \leq 0.001$) in ovarian cell death

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compared to control samples at all used intensities (10, 0 and
−10 dB) in both wave forms. Comparing the two wave forms, CW and FM, there was a statistically significant difference
between them at the low output power of −10 dB (p < 0.05) (Figure 3A).

A 60-min acute exposure, resulted in a statistically
significant increase in apoptotic cell death compared to
control samples, in both wave forms, CW or FM, at output
power densities of 10 and −10 dB with p < 0.001 and at 0 dB
exposure with p < 0.05 (Table 2). Comparing the two
wave forms, FM produced a statistically significant higher
increase (p < 0.001) at the output power of 0 and −10 dB
(Figure 3B).

After repeated exposure for 6 min daily (for the first 6 days
of life) to CW or FM signals, statistically significant increase
(p < 0.05 for CW and p < 0.01 for FM), compared to control
samples, was observed at power values of 10 and 0 dB. The
lowest −10 dB output power had also a statistical significant
increase in both waves (p < 0.001), but comparing the two
wave forms CW and FM, no statistically significant difference
was found in apoptotic cell death caused by any of the three
exposure intensities (Figure 3C).

Repeated daily exposure to CW or FM waves for 60 min for
6 days led to a statistically significant increase (p ≤ 0.001) in
ovarian cell death, at all power intensities in both wave
types compared to control (Table 2). Comparing the two
wave forms, a statistically significant increase (p < 0.05) was
detected only at the highest power of exposure (10 dB)
(Figure 3D).

Exposure at 395 MHz

The overall data at this frequency are presented in Figure 4
and Table 3.

A statistically significant increase in ovarian cell
death compared to control was noticed after acute exposure
for 6 min, at the intensities of 10 dB (p < 0.001) and
0 dB (p < 0.001) in both wave forms. Exposure to the lowest
intensity (−10 dB) had a statistically significant increase
in ACD induced by CW (p < 0.05) but not by the FM signal.
No statistically significant difference in apoptotic cell
death increase was observed between the continuous and
the modulated wave in any exposure intensity (Figure 4A).

A 60-min acute exposure, resulted in a statistically
significant increase (p < 0.001) in apoptotic cell death
compared to control samples, at all power outputs used,
in both wave forms, CW or FM. The ACD percentage
between CW and FM exposure was statistically significant
at 10, 0 (p ≤ 0.05) and −10 dB (p < 0.001), respectively
(Figure 4B).
Figure 3. Bar graph presentation of the ACD percentage mean values. SEM measured at the exposed and the control samples. The frequency of exposure was set at 100 MHz with two wave forms (CW and FM), three output power levels of 10, 0 and −10 dB and four exposure scenarios applied to the Drosophila flies: (A) Acute exposure for 6 min at the 6th day of their adult life; (B) Acute exposure for 60 min at the 6th day of their adult life; (C) Repeated exposure for 6 min per day starting from the first day of their lives; (D) Repeated exposure for 60 min per day starting from the first day of their lives. All of the exposure schemes, either CW or FM mode created a statistically significant increase in the apoptotic cell death percentage compared to controls. Between the two wave forms, FM versus CW, a statistically significant difference was found after exposure at 10 dB in the cases of repeated 60 min exposure, at 0 dB after acute 60 min exposure and finally, at −10 dB after acute exposure of 6 and 60 min (*p ≤ 0.05, **p ≤ 0.01). CW: Continuous Wave, FM: Frequency Modulation.

Figure 4. Bar graph presentation of the ACD percentage mean values. SEM measured at the exposed and the control samples. The frequency of exposure was set at 395 MHz with two wave forms (CW and FM), three output power levels of 10, 0 and −10 dB and four exposure scenarios applied to the Drosophila flies: (A) Acute exposure for 6 min at the 6th day of their adult life; (B) Acute exposure for 60 min at the 6th day of their adult life; (C) Repeated exposure for 6 min per day starting from the first day of their lives; (D) Repeated exposure for 60 min per day starting from the first day of their lives. Nearly all of the exposure schemes either to CW or to FM mode gave a statistically significant increase in the apoptotic cell death percentage compared to controls, except for the acute 6 min exposure at −10 dB. Between the two wave forms, a statistically significant difference was found after exposure: at 10 dB in all protocols except the acute 60 min exposure, at 0 dB after acute 60 min and repeated 60 min exposure and at −10 dB in all protocols except the acute 6 min exposure (*p ≤ 0.05, **p ≤ 0.01). CW: Continuous Wave, FM: Frequency Modulation.
Figure 5. Bar graph presentation of the ACD percentage mean values. SEM measured at the exposed and the control samples. The frequency of exposure was set at 682 MHz with two wave forms (CW and FM), three output power levels of 10, 0 and −10 dB and four exposure scenarios applied to the Drosophila flies: (A) Acute exposure for 6 min at the 6th day of their adult life; (B) Acute exposure for 60 min at the 6th day of their adult life; (C) Repeated exposure for 6 min per day starting from the first day of their lives; (D) Repeated exposure for 60 min per day starting from the first day of their lives. All of the exposure schemes either to CW or to FM mode gave a statistical significant increase in the apoptotic cell death percentage compared to controls. Between the two wave forms, a statistical significant difference was found: (a) at 10 dB in the cases of repeated 6 and 60 min exposure and (b) at 0 dB after acute 6 and 60 min and also after the repeated 60 min exposure. The power of −10 dB did not gave a significant difference between CW and FM exposure (*p ≤ 0.05, **p ≤ 0.01). CW: Continuous Wave, FM: Frequency Modulation.

Figure 6. Bar graph presentation of the ACD percentage mean values. SEM measured at the exposed and the control samples. The frequency of exposure was set at 900 MHz with two wave forms (CW and FM), three output power levels of 10, 0 and −10 dB and four exposure scenarios applied to the Drosophila flies: (A) Acute exposure for 6 min at the 6th day of their adult life; (B) Acute exposure for 60 min at the 6th day of their adult life; (C) Repeated exposure for 6 min per day starting from the first day of their lives; (D) Repeated exposure for 60 min per day starting from the first day of their lives. In nearly all exposure schemes either to CW or to FM mode gave a statistically significant increase in the apoptotic cell death percentage compared to controls, except for the CW −10 dB acute 6 and 60 min and the repeated 6 min exposure. Between the two wave forms, a statistically significant difference was found after exposure at 10 dB and 0 dB in all exposure protocols and at −10 dB only in the acute 60 min exposure (*p ≤ 0.05, **p ≤ 0.01). CW: Continuous Wave, FM: Frequency Modulation.
After repeated exposure for 6 min daily for 6 days to CW or FM signals, the exposed samples revealed a statistically significant increase in ovarian apoptosis ($p<0.001$), compared to control samples (*$p \leq 0.05$, **$p \leq 0.01$). The difference of the FM effect versus CW is significant in four out of the 12 cases. The samples that did not show any statistical significance are designated as >0.05. CW: Continuous Wave, FM: Frequency Modulation.

Acute 60-min exposure at the 6th day, showed a statistically significant increase ($p<0.01$) in apoptotic cell death compared to control samples, at all output powers, in both wave forms (CW or FM). No significant difference was found comparing the induction of apoptotic cell death between FM and CW except under the intensity of 0 dB ($p<0.01$) (Figure 5B).

After repeated exposure for 6 min daily for 6 days to CW or FM signals, a statistically significant increase in ACD ($p<0.001$), compared to control samples, was observed at all tested power values (Table 4). Comparing the two wave forms, in regard to ovarian apoptosis, the modulated wave provoked a larger increase ($p<0.001$) at the highest power of exposure (10 dB) only (Figure 5C).

At the repeated exposure protocol to the frequency of 682 MHz for 60 min daily for 6 days, using either CW or FM waveform, led to a statistically significant increase ($p<0.001$) in ovarian cell death at all three power intensities for both wave types compared to the control samples. Statistically significant difference in ACD was detected between the CW and FM exposure only at 10 and 0 dB power densities ($p<0.01$) with the modulated signal to be more bioactive (Figure 5D).

### Exposure at 900 MHz

The overall data at this frequency are presented in Figure 6 and Table 5.

Acute exposure for 6 min at the 6th day, revealed a statistically significant increase ($p<0.001$) in ovarian cell...
Table 4. Statistical analysis (one-way ANOVA) of apoptotic follicles at a frequency of 682 MHz under three power values (10, 0 and −10 dB) and four exposure durations.

| Exposure power | CONTROL/CW | CONTROL/FM | CW/FM |
|----------------|------------|------------|-------|
| Exposure duration | 6 min only the 6th day | 60 min only the 6th day | 6 min daily for 6 days | 60 min daily for 6 days |
| 10 dB | ** | ** | >0.05 |
| 0 dB | ** | ** | >0.05 |
| −10 dB | ** | ** | >0.05 |
| Exposure duration | 6 min only the 6th day | 60 min only the 6th day | 6 min daily for 6 days | 60 min daily for 6 days |
| 10 dB | ** | ** | >0.05 |
| 0 dB | ** | ** | >0.05 |
| −10 dB | ** | ** | >0.05 |
| Exposure duration | 6 min only the 6th day | 60 min only the 6th day | 6 min daily for 6 days | 60 min daily for 6 days |
| 10 dB | ** | ** | >0.05 |
| 0 dB | ** | ** | >0.05 |
| −10 dB | ** | ** | >0.05 |

All of the exposed samples either to CW or to FM, showed statistically significant increase in apoptotic cell death percentage, compared to control samples (*p ≤ 0.05, **p ≤ 0.01). The difference of the FM effect versus CW is significant in five out of the 12 cases. The samples that did not show any statistical significance are designated as >0.05. CW: Continuous Wave, FM: Frequency Modulation.

Table 5. Statistical analysis (one-way ANOVA) of apoptotic follicles at a frequency of 900 MHz under three power values (10, 0 and −10 dB) and four exposure durations.

| Exposure power | CONTROL/CW | CONTROL/FM | CW/FM |
|----------------|------------|------------|-------|
| Exposure duration | 6 min only the 6th day | 60 min only the 6th day | 6 min daily for 6 days | 60 min daily for 6 days |
| 10 dB | ** | ** | ** |
| 0 dB | ** | ** | ** |
| −10 dB | >0.05 | * | >0.05 |
| Exposure duration | 6 min only the 6th day | 60 min only the 6th day | 6 min daily for 6 days | 60 min daily for 6 days |
| 10 dB | ** | ** | ** |
| 0 dB | ** | ** | ** |
| −10 dB | >0.05 | * | ** |
| Exposure duration | 6 min only the 6th day | 60 min only the 6th day | 6 min daily for 6 days | 60 min daily for 6 days |
| 10 dB | ** | ** | ** |
| 0 dB | ** | ** | ** |
| −10 dB | >0.05 | * | >0.05 |

All of the exposed samples either to CW or to FM, showed statistically significant increase in apoptotic cell death percentage, compared to control samples (*p ≤ 0.05, **p ≤ 0.01) except of some samples exposed to very low power (−10 dB). The difference of the FM effect versus CW is significant in nine out of the 12 cases. The samples that did not show any statistical significance are designated as >0.05. CW: Continuous Wave, FM: Frequency Modulation.

Discussion

In the last few decades, a serious concern is expressed about the biological effects of the electromagnetic fields of non-ionizing radiation, which are constantly growing with the development of telecommunication systems. The question of whether or not modulated waves are more bioactive than their CW counterparts remains open until now, since the available data offer controversial conclusions. Several factors may be responsible for the inconclusive research outcomes, as cell responses may vary according to the duration of the exposure and/or the signal characteristics, such as modulation scheme and waveform. In particular, the modulation of the emitted radiofrequency has been an object for investigation for many years (for a review see Juutilainen et al., 2011).

The present study having this issue as one of its goals has demonstrated that the vast majority of the experimental cases (discussed below) using frequency-modulated signals coming from a generator are indeed more bioactive under the specific conditions used; the model biological system and the exposure parameters. Thus, as shown in Tables 6 and 7, in 25 out of 48 experimental cases of our study there is a greater statistically significant bioeffect after FM exposure compared to CW exposure (the other conditions being the same). It is interesting to point out that nine of these cases come from exposure to 900 MHz denoting the greater bioactivity of this frequency band although the E-field and SAR values are very low (0.23–3.0 V/m and 0.1–11 mW/kg; see Table 1).
Regarding the other frequencies used, eight of the cases for which FM is more bioactive than CW belong to the 395 MHz exposure, whereas 682 MHz results in five and 100 MHz in as low as four such cases. Thus, 900 MHz regardless of power and duration produces the largest difference in apoptotic effects between FM and CW (Table 7) in the biological system of *D. melanogaster* oogenesis. Overall, only in one case does the CW signal appear more bioeffective, statistically significant, than the FM one (395 MHz, repeated 6 min, 6 days, −10 dB; see Figure 4) the importance of which cannot be evaluated so far.

These results confirm other investigations which imply that modulated waves are more bioactive than continuous ones (d’Ambrosio et al., 2002; Guler et al., 2011; Juutilainen et al., 2011; Lai and Singh, 1997; Markkanen et al., 2004), although, there are studies supporting the opposite; that CW signals are more hazardous under certain exposure conditions (Persson et al., 1997; Salford et al., 1994).

Not having other data associated with these exposure conditions it is hard to explain why the modulated radio-frequency wave of a signal at 50 kHz (routinely used in FM broadcasting of speech and music) would be more bioactive than a CW one. We can only postulate that the target biological molecules (ion channel proteins, heat shock proteins, ROS creating molecules, etc.) react more readily to an oscillating (or pulsed) wave compared to a steady one as found in several publications using different model systems, frequencies and modulations (Capri et al., 2004b; d’Ambrosio et al., 2002; Franzellitti et al., 2010; Regel et al., 2007).

In fact, our previous studies in *Drosophila* oogenesis have indicated ROS induction in ovaries following exposure to EMFs (Manta et al., 2014).

Another aspect that has been of concern to EMF scientists and also an objective of this work has to do with the actual intensity threshold at which modern techniques can detect biological changes. A number of controversial publications has studied this question and in some of them new exposure guidelines have been proposed (Fragopoulou et al., 2010). Supporting evidence to our data comes from Persson et al. (1997), who showed that exposed rats had their BBB affected by low-to-middle SAR values (1–10 mW/kg). Interestingly, our data show maximum apoptotic effect in EMFs used (100, 395, 682 and 900 MHz) varies non-linearly with modulation (CW or FM), frequency, power intensity (or SAR) and exposure scenarios.

A more thorough survey of our results indicates that there is a differential impact between the various frequencies tested. It seems that in general the effect is more pronounced at 900 MHz followed by 395 MHz, 682 MHz and 100 MHz (Table 7). These observations of the different effect noted between the frequencies can indicate the possible presence of “frequency windows” concerning bioeffects of modulated signals in comparison to the CW signals. Thus, 900 MHz signals regardless of power and duration produces the larger difference in apoptotic effects between FM and CW.
Dependence of bio-effectivity on intensity appears to be non-linear. An “expected” behavior (lower effect at lower intensity) is followed only in the longest exposure scenario (repeated exposure – 60 min/daily for 6 days) with the intensity lowered by 20 dB. In most cases (as shown in Figure 7) exposing the flies with 10 dB less power (i.e. from 10 dB to 0 dB or from 0 dB to \(-10\) dB), apoptosis either remains the same or as in the case mentioned above, is rising (100 MHz, 395 MHz – acute exposures 6 min and 60 min and also 900 MHz acute 6 min – see Figures 7A, B and D, respectively). It is worth pointing out that all exposures at 682 MHz regardless of power and exposure scenario have the same moderate apoptotic fold-up (Figure 7C). The only case in which there is a clear reduction of effect by lowering the power is the 60-min acute exposure at 900 MHz (Figure 7D); in which case, however, FM exposure produces a statistically significant result of apoptosis above CW value (Figure 6B, Table 5).

Induction of ovarian apoptosis does not depend linearly on the duration of exposure (Figures 8 and 9). At the lowest generator power intensity (\(-10\) dB) the effect is more pronounced at the repeated exposures, whilst higher intensities do not seem to present any motif between duration and the biological impact induced. Repeated 60-min exposure is more bio-effective than repeated 6 min, for the highest intensity used (10 dB), something that is not observed at lower intensities (0 dB, \(-10\) dB). On the other hand, there is a correlation between the duration of irradiation and the bioactivity of FM signal in comparison to CW signals. Thus, the acute 6-min exposure produces 4+ score (FM versus CW), the acute 60-min exposure produces 9+ (Table 6). Also the repeated 6 min daily for 6 days exposure has 5+ scores, whereas the repeated 60 min for 6 days exposure produces 8+ scores (Table 6).

Finally, this work demonstrates that at the frequency of 900 MHz, an E-field as low as 250 mV/m can induce...
Figure 8. Line graphs showing the correlation between the percentages (%) fold-up of ACD induced by CW exposure compared to controls, as a function of power, frequency and exposure scenarios applied to the Drosophila flies: (A) Acute exposure for 6 min at the 6th day of their adult life; (B) Acute exposure for 60 min at the 6th day of their adult life; (C) Repeated exposure for 6 min per day starting from the first day of their lives; (D) Repeated exposure for 60 min per day starting from the first day of their lives. An intensity window is revealed at 0 dB only after the acute 6 min-exposure at the lower frequencies of 100 and 395 MHz (A), not visible neither after acute 60 min-exposure (B) nor after the repeated protocols (C, D). This result could mean that low dose-short exposures do not allow the biological system to fire its defense mechanisms. In repeated exposure a stable more or less effect is found (although a frequency dependency is visible) regardless of the 10-fold reduction of EMF intensity (C, D). CW: Continuous Wave.

Figure 9. Line graphs showing the correlation between the percentages (%) fold-up of ACD induced by FM exposure compared to controls, as a function of power, frequency and exposure scenarios applied to the Drosophila flies: (A) Acute exposure for 6 min at the 6th day of their adult life; (B) Acute exposure for 60 min at the 6th day of their adult life; (C) Repeated exposure for 6 min per day starting from the first day of their lives; (D) Repeated exposure for 60 min per day starting from the first day of their lives. The intensity window seen under CW exposure (see Figure 8A) is also present under FM exposure conditions at nearly all frequencies (395, 682, 900 MHz), unlike the response of the system to the repeated 60 min which is nearly the same in all EMF intensities regardless of the 10-fold reduction (C and D). FM: Frequency Modulation.
apoptotic cell death in the ovaries of *D. melanogaster* depending on the exposure duration (Tables 8 and 9, Figures 7–9). It is true then that, depending on the model, biological system used the threshold is different and it also depends on the exposure conditions. Therefore, the statement that ‘‘testing low SAR values would be indicated in cases of positive findings at high SAR’’ (Luukkonen et al., 2009) may not be correct and in fact it may have discouraged research at low SAR exposures.

In conclusion, our experiments reveal that there are intensity and frequency windows of bioeffects at certain values below and above which the effect is lower (Figure 7). This behavior suggests that EMF-biological tissue interaction is not linear in response and it seems plausible to propose at this point that translating such experimental data into possible human exposure guidelines is highly arbitrary and impossible using available scientific tools. This work also emphasizes the need for reconsideration of published data that are either inconclusive or controversial under the scope that different organisms, respond entirely differently under the same exposure conditions. It would be ideal for unraveling the mechanisms associated with EMF-living matter interaction to have the same model system studied under the same exposure conditions by different laboratories using complementary approaches through international, concerted research activities.

**Declaration of interest**

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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**Table 8.** Percentage (%) fold up of apoptotic cell death of the CW exposed samples compared to the controls, as a function of power and frequency.

| E-Field compared to the output value | 10 dB | 0 dB | −10 dB | Frequency of exposure | 6 min | 60 min | 6 min | 60 min |
|-------------------------------------|------|-----|-------|----------------------|------|-------|------|-------|
| 4.79                                | 1.51 | 0.48| 4.80  | 100 MHz              | 100  | 100  | 100  | 100   |
| 9.25                                | 2.95 | 0.98| 9.34  | 395 MHz              | 200  | 100  | 200  | 100   |
| 5.78                                | 1.48 | 0.48| 5.86  | 682 MHz              | 200  | 100  | 200  | 100   |
| 3.00                                | 0.92 | 0.26| 3.07  | 900 MHz              | 200  | 100  | 200  | 100   |

**Table 9.** Percentage (%) fold up of ACD of the FM-exposed samples compared to the controls, as a function of power and frequency.

| E-Field compared to the output value | 10 dB | 0 dB | −10 dB | Frequency of exposure | 6 min | 60 min | 6 min | 60 min |
|-------------------------------------|------|-----|-------|----------------------|------|-------|------|-------|
| 4.58                                | 1.53 | 0.48| 4.62  | 100 MHz              | 100  | 100  | 100  | 100   |
| 9.27                                | 3.15 | 1.10| 9.38  | 395 MHz              | 200  | 100  | 200  | 100   |
| 5.59                                | 1.53 | 0.45| 5.64  | 682 MHz              | 200  | 100  | 200  | 100   |
| 3.07                                | 0.80 | 0.23| 3.10  | 900 MHz              | 200  | 100  | 200  | 100   |

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Supplementary material available online

Supplementary Table S1.