

Measurement of the 100 MHz EMF radiation *in vivo* effects on zebrafish *D. rerio* embryonic development: A multidisciplinary study

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ABSTRACT

The augmented exposure of both environment and human being to electromagnetic waves and the concomitant lack of an unequivocal knowledge about biological consequences of these radiations, raised public interest on electromagnetic pollution. In this context, the present study aims to evaluate the biological effects on zebrafish (ZF) embryos of 100 MHz radiofrequency electromagnetic field (RF-EMF) exposure through a multidisciplinary protocol.

Because of the shared synteny between human and ZF genomes that validated its use in biomedical research, toxicology and developmental biology studies, ZF was here selected as experimental model and a measurement protocol and biological analyses have been set up to clearly discriminate between RF-EMF biological and thermal effects.

The results showed that a 100 MHz EMF was able to affect ZF embryonic development, from 24 to 72 h post fertilization (hpf) in all the analyzed pathways. Particularly, at the 48 hpf stage, a reduced growth, an increased transcription of oxidative stress genes, the onset of apoptotic/autophagic processes and a modification in cholesterol metabolism were detected. ZF embryos faced stress induced by EMF radiation by triggering detoxification mechanisms and at 72 hpf they partially recovered from stress reaching the hatching time in a comparable way respect to the control group.

Data here obtained showed unequivocally the *in vivo* effects of RF-EMF on an animal model, excluding thermal outcomes and thus represents the starting point for more comprehensive studies on dose response effects of electromagnetic fields radiations consequences.

1. Introduction

Over the last decades, the population exposure to electromagnetic fields (EMFs) has progressively increased with the concomitant intensification of technological tools from both domestic and industrial uses (Tomitsch and Dechant, 2012; Gajšek et al., 2015). More recently, the rapid development of wireless technologies together with the EMFs use in diagnostic and therapeutic medicine (magnetic resonance imaging, radiofrequency ablations, etc.) amplified human exposure to radiofrequency fields (RF) (Consales et al., 2012; Vijayalaxmi, 2016).

Although these new technologies appear to improve the quality of life, doubts about their effect on human health, as well as on the environment, are on the rise, producing a growing number of studies on possible biological effects of EMF exposure (Funk and Monsees, 2006; Tenorio et al., 2012; Gajšek et al., 2015).

Various effects have been described on cell biology, mainly related to DNA damages and its consequences including primary DNA damage in the form of single and double strand breaks, chromosomal aberrations and mutations (Phillips et al., 2009; Choy and Brannigan, 2012; Zalata et al., 2015; Dasdag and Akdag, 2016), but, to date, evidences of a clear association between EMF exposure and cancer in human and animals are limited. On the other hand, public anxiety about the potential health consequences of electromagnetic pollution is getting higher and authorities have set safety boundaries on the subject to protect the community against EMFs exposure. Particularly, the international reference guidelines for time-varying electromagnetic fields [International Commission on Non-Ionizing Radiation Protection (ICNIRP, 1998, 2010)] ruled out that limits are not constant with the frequency since they are related to the dimensions of a human being.

ICNIRP differentiates between low frequencies (LF, 1 Hz to 100 kHz)

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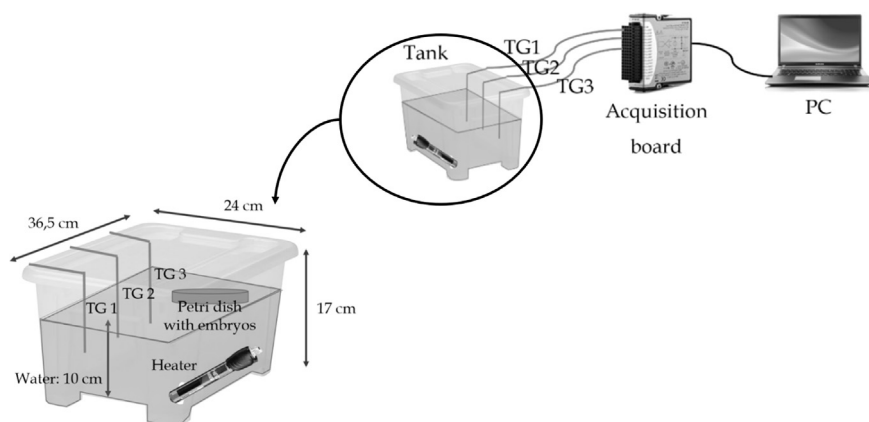


Fig. 1. Acquisition system. Water tank with heater, temperature sensors, Petri dishes and data acquisition system - tridimensional view (example for tank no. 1).

where the quantity used to specify the restrictions is the induced current density (J), and high frequencies (HF, 100 kHz to 300 GHz), where the monitored parameter is the Specific Absorption Rate (SAR).

The Italian exposure limits [Decreto del Presidente dei Ministri (DPCM, 2003)] are instead frequency independent: the quality objective for new installations states that the electric field strength must be lower than 6 V/m from 100 kHz to 300 GHz.

Several studies were performed to investigate the effects at both LF and HF. Unfortunately, data so far available for *in vivo* and *in vitro* studies are conflicting, especially regarding their biological effects and still need to be unequivocally interpreted also considering that they are primarily related to the arising thermal effects and not on the radiation per se. The heterogeneity of exposure systems, the different magnetic stimulation parameters, the inadequate dosimetry and the variability of responses depending on the used experimental models are often responsible for this kind of contrasting results (Paffi et al., 2010; Vijayalaxmi, 2016).

Nevertheless, most of the studies conducted so far, concurred that the crucial event in biological responses elicited by EMFs exposure is the onset of oxidative stress with overproduction of ROS (reactive oxygen species), which may result in cell death, reproductive decline or even cancer induction in both mammalian and non-mammalian species (Harakawa et al., 2005; Juutilainen, 2005; Phillips et al., 2009; Kong and Lin, 2010; Li et al., 2014; Dasdag and Akdag, 2016).

Particularly, the establishment of both stress response and oxidative stress, is strictly conserved in vertebrates and relies on specific pathways defined as hypothalamus-pituitary-adrenal axis (HPA) in mammals and hypothalamus-pituitary-interrenal (HPI) axis in teleosts (Alsop and Vijayan 2008, 2009a, 2009b; Alsop and Aluru, 2011; Farrell, 2011; Spiers et al., 2015). As a consequence, several cellular and physiological modulations ranging from apoptosis/autophagy trigger (Kannan and Jain, 2000; Kroemer et al., 2010) to changes in growth, lipid metabolism and reproduction are carried out by vertebrates to cope with stress and survive it.

Thus, considering the crucial importance of these issues for human health and environmental safety, the present study aims to better understand the biological effects induced by EMFs during the embryonic development of zebrafish (ZF) *Danio rerio* (Cornet et al., 2017) by performing a specific protocol that unequivocally defines instrumental methods and biological analysis. In particular, the EMF here adopted is a 100 MHz radio-broadcasting frequency, with a field strength of 6 V/m, (ICNIRP limits are higher (61 V/m for occupational and 28 V/m for general public) and were produced by means of the use of a Transverse ElectroMagnetic (TEM) cell, that allowed to generate a well-defined electromagnetic field. Zebrafish embryos were exposed to the above described EMF and oxidative stress pathways, cholesterol metabolism and apoptotic/autophagic processes were analyzed. Finally, in order to unequivocally discriminate between RF-EMF biological effects and

thermal or indirect effects, measurements of temperature were made during the whole experiment by means of K-type thermocouples.

2. Materials and methods

2.1. Ethics

All procedures involving animals were conducted in line with European and Italian legislation on experimental animals (Directive, 2010/63/EU, 2010; Decreto Legislativo 26/14, 2014), optimal rearing conditions were applied throughout the study and all efforts were made to minimize animal suffering in accordance with recommendations by “Ministero della Salute” applying the guidelines for the Care and Use of Laboratory Animals. As stated by Article 1.3 of the Directive 2010/63/EU, teleosts embryos are not included in the list of organisms that require a specific authorization, so no approval was requested.

2.2. Fish maintenance and embryo collection

Adult AB wild-type ZF > 5 months old (0.85 ± 0.07 g) were kept and bred in a ZF system (Tecniplast, Italy) at 28 °C, 14 L/10D photoperiod, and fed a commercial diet (BlueLine, Italy) twice daily (2% body weight). After spawning, 0 h post fertilization (hpf) embryos were collected from breeding tanks, carefully rinsed and vitality and stage of development were confirmed under a dissecting microscope (Leica wild M3B).

2.3. Measurement procedure and experimental design

Newly fertilized ZF embryos (3000 ± 30) 0 hpf were assigned to a control group and a 100 MHz EMF-exposed group.

The experiment was carried out in triplicate so embryos were randomly placed in different Petri dishes assigned to each experimental group (9 Petri dishes for control and 9 for exposed group) and, as illustrated in Fig. 1, Petri dishes were located on plastic parallelepiped tanks (dimensions: 36.5 cm length, 24 cm width, 17 cm depth), filled with water up to a height of 10 cm.

Exposed groups were kept in a TEM cell and exposed to a 100 MHz EMF from 0 hpf to hatching time (72 hpf) and control groups were reared under the same conditions but without EMF exposure. Rearing parameters of each experimental group were maintained constant in terms of photoperiod (24/0 h of light and dark, respectively) and temperature (28 ± 1 °C). Inside each Petri dish, ZF embryos were kept in 30 ml of sterilized (autoclaved) water containing Methylene Blue accordingly to Jarvis and Knowles (2003).

Twice a day, 2/3 of the Petri dishes water was changed, the bottom was syphoned and cleaned, and dead embryos were collected and counted for survival rate analysis. Embryos were sampled at 24, 48 and

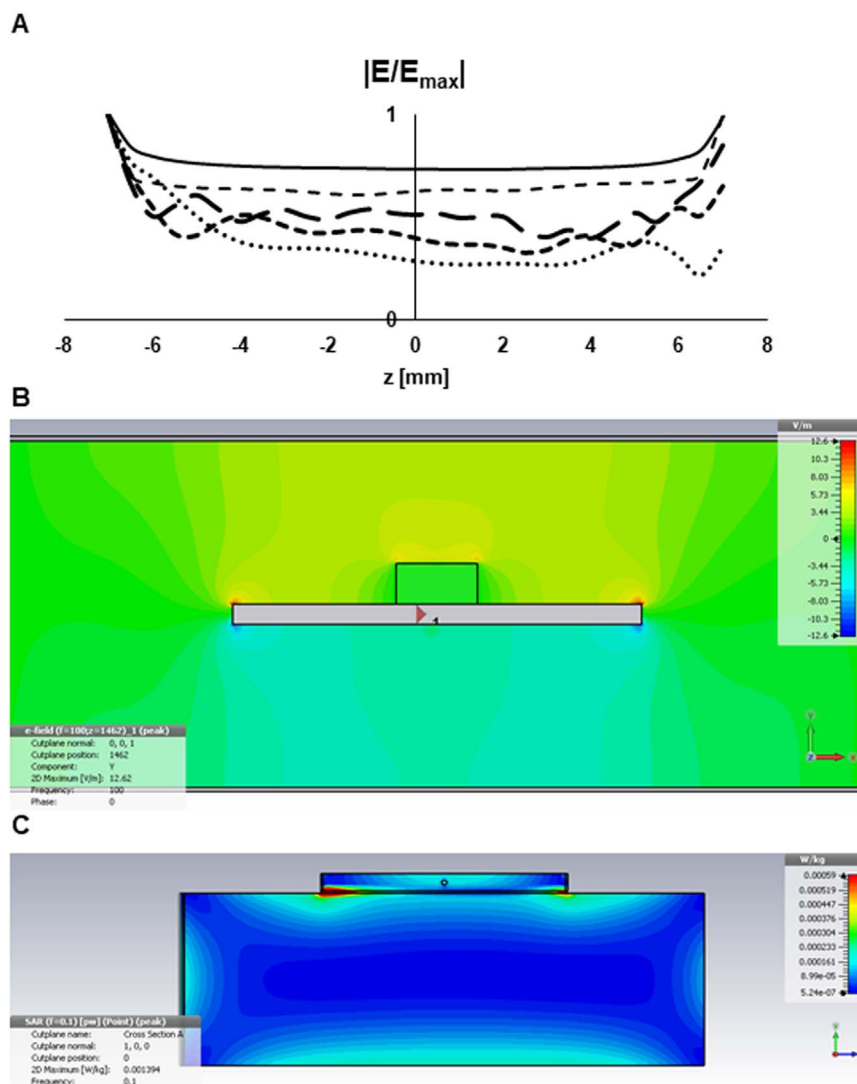


Fig. 2. A–C. Electromagnetic exposure set up. A) Electric field distribution along the electromagnetic wave propagation direction in the Petri dish filled with water at frequencies typical of telecommunication services. B) Electric field distribution in a transversal section of the working volume of the TEM cell. A water tank was placed on the central conductor of the cell. C) SAR distribution in W/kg in the water tank and in the Petri dish.

72 hpf. At each sampling time, a significant number of embryos was randomly collected from the control and EMF-exposed groups Petri dishes, for morphometric analysis, histology, Real-Time PCRs, BODIPY staining and TUNEL assay. A detailed description of sample size for the different analysis carried out in this study is explained in the specific section.

The experiment was repeated three times.

2.4. Temperature measurement system

Tank water was maintained in the optimal temperature range (*i.e.* $28 \pm 1^\circ\text{C}$) through heaters (25 W Hydor Theo Heater). These were calibrated and adjusted in order to maintain the desired temperature since factory calibration data were not available.

To such purpose, the water temperature was continuously measured by means of 3 temperature gauges, realized with K-type thermocouples.

At first, temperature gauges were calibrated in a calibration micro bath (Hart Scientific 7102 Microbath) and a platinum resistance thermometer (Hart Scientific 5680) was used as reference instrument. Then, they were adjusted and the computed mean uncertainty (2σ , where σ is the standard deviation of the residuals) was of 0.02°C , so the measurement instrument can be considered very accurate.

Then, heaters were calibrated and the temperature was monitored during the whole test duration.

Three temperature measurement points were realized in each tank:

- Temperature Gauges no. 1, 2, 3 (TG1, TG2 and TG3) in the thermostated tank with fish embryos exposed in the TEM cell;
- Temperature Gauges no. 4, 5, 6 (TG4, TG5 and TG6) in the thermostated tank with fish embryos of the control group, outside the TEM cell;
- Temperature Gauges no. 7, 8, 9 (TG7, TG8 and TG9) in the tank without embryos and not thermostated.

A further Temperature Gauge (*i.e.* TG10) was used to monitor the environment temperature.

All the gauges were fixed by means of nylon threads and immersed in water for a depth of 5 cm so that precise points were monitored in each tank.

Data acquisition system (Fig. 1) was based on a National Instrument board NI 9123 (24-bit ADC, temperature sensitivity up to 0.02°C , suitable for K-type thermocouples, accurately operating in the range -40 to 70°C), able to acquire up to 16 channels simultaneously at a sampling frequency of 75 Hz. The acquisition board was controlled via a

software developed in LabVIEW environment.

Through a proprietary algorithm, data were down-sampled at 1 Hz and band-pass filtered to eliminate artefacts.

2.5. Electromagnetic exposure set up

Two main aspects were considered during ZF exposure to EMF.

The first aspect concerns the exposure limits that in this experiment were set up considering the Italian legislation.

The Italian exposure limits (DPCM, 2003) are frequency independent: the quality objective for new installations states that the electric field strength must be lower than 6 V/m from 100 kHz to 300 GHz.

The second aspect concerns the fact that Petri dishes used for ZF embryos exposure contained water, and this may affect field distribution.

Information provided by simple expressions, as the penetration depth of a plane electromagnetic wave impinging into a lossy medium, are not accurate enough, therefore a more realistic scenario was considered and implemented into a numerical code (CST Microwave Studio, 2011). A “lossy medium” is characterized by a finite, non-zero value of conductivity, so the electric currents induced by the external electric field generates a power dissipation transforming the electromagnetic energy into heat.

A cylindrical Petri dish (radius = 45 mm, height = 10 mm) with 30 ml volume of incubation media, illuminated by a linearly polarized plane wave with electric field perpendicular to the water surface was simulated. Fig. 2A shows the electric field normalized to the maximum value inside the water, at a depth of 5 mm from the surface, due to a wave travelling along the positive direction of the z-axis, in the Petri dish cross-section at five different frequencies.

The frequencies chosen are typical of telecommunication services: 100 MHz for the radio FM spectrum, 940 MHz for Global System for Mobile Communication (GSM), 1830 MHz for Long GSM/Long Term Evolution (GSM/LTE), 2150 MHz for Universal Mobile Telecommunications Service (UMTS) and 2650 MHz for the GSM/LTE upper band. We can observe that the higher the frequency the more irregular field distribution, affected by the medium and by the Petri dish walls.

After this preliminary analysis, the field intensity for exposition was set at 6 V/m, the most restrictive condition, at the frequency of 100 MHz where the electric field has the highest amplitude into the water and the most uniform distribution.

2.6. TEM cell

In order to recreate in our laboratory a well defined electromagnetic exposure system, a TEM cell was used. Such a structure can be seen as a parallel plate structure connected to a coaxial cable, suitable to contain the embryos inside a Petri dish, the water tank and the heating system. The side walls of the cell are removed to allow air circulation and light penetration. The cell conductors are properly tapered to maintain the characteristic impedance of the guiding structure constant in all its longitudinal sections and equal to 50 Ω .

The working volume is composed of two parallelepipeds (dimensions: 800 mm $\times$ 1000 mm $\times$ 1000 mm), placed in the region where the central conductor and the two shields are parallel. In this space, the electric field is mainly vertical (directed along y axis) and constant in amplitude ($E = V/h$) where V is the voltage between the central conductor and the shield, and h is the distance between them.

Considering the physical dimensions of the TEM cell, the only TEM mode is in propagation up to 200 MHz, so at 100 MHz the higher order modes can be neglected. Some simulations were carried out to check the field uniformity inside the cell, with and without the tank.

Fig. 2B reports the electric field in a transversal section of the TEM cell, where a water pan (dimensions 300 mm $\times$ 400 mm $\times$ 200 mm)

was inserted on the central conductor to simulate the temperature control system.

It can be observed that the presence of water does not significantly modify the electric field distribution: the average of the module of the electric field inside the working volume is 6.32 V/m with a standard deviation of 1.35 V/m.

Also the transmission coefficient S21 between the two ports of the cell was measured, to check the cell matching conditions because standing waves can affect longitudinal field uniformity: the measurements show that the electromagnetic wave travels unperturbed along the structure (no difference between curves with and without water tank), up to 200 MHz, when higher order modes are excited.

The time stability of the generator was also checked: the field variation in 72 h was lower than 0.3 dB.

2.7. Field generation set up

The following instrumental setup was used to generate and monitor the electric field inside the TEM cell:

- A signal generator HP8620C;
- A 10 dB attenuator;
- A power amplifier, Amplifier Research Model 10W1000;
- A 50 dB directional coupler, Amplifier Research Model DC2600, connected to a spectrum analyzer (SA) HP 8591A;
- A 50 Ω matched load;
- An electric field probe, Aeritalia TE 307;

The signal generator produces a 0 dBm (1 mW), 100 MHz continuous harmonic wave. This signal is amplified by a power amplifier, whose input port is protected by an attenuator, to achieve the 6 V/m electric field intensity inside the TEM cell. The feeding network also includes a directional coupler connected to a SA for an accurate power and frequency measurement and control, without affecting the exposition conditions. In this way, the TEM cell is surely fed by the proper electromagnetic radio frequency signal.

A further check is carried out with the electric probe that measures the field intensity inside the cell to show possible field variation during the exposure. Finally, a 50 Ω load terminates the structure with a matching impedance to prevent reflections inside the TEM cell, and assure field uniformity along the longitudinal direction.

2.8. SAR simulation

According to ICNIRP statement, the physical quantity used to specify exposure restrictions at 100 MHz is the Specific Absorption Rate (SAR) that is the power absorbed per mass unit of tissue and measured in watts per kilogram; in particular for general public exposure the whole body averaged SAR limit is 0.08 W/kg.

To evaluate SAR involved in the set-up, a full wave numerical tool was used to simulate the scenario, consisting of a fish tank containing Petri dishes with zebrafish embryos. Fig. 2C shows the SAR distribution due to the wave travelling inside the cell along the positive direction of the z-axis, polarized in the y-direction, and intensity of 6 V/m. We can observe that the distribution is not uniform and in particular the edge effect increases the field around the corners of the dish.

However, this effect is unavoidable because it depends on the presence of different materials and their geometry.

The water is modelled as a lossy dielectric (Stogryn, 1971) with relative permittivity $\epsilon_r = 78$ and conductivity $\sigma = 1.59$ S/m, and the plastic as a lossless dielectric with $\epsilon_r = 3.6$. (Gabriel et al., 1996; Gabriel and Corthout, 1996; Lau and Gabriel, 1996). Permittivity is a macroscopic parameter accounting for the interaction between the field and the microscopic structure a medium.

Even if the edge effect causes localized hot spots, considering a density of 1000 kg/m³, we can observe that the SAR value are three

orders of magnitude lower than ICNIRP basic restrictions, and therefore thermal effects due to the electromagnetic fields can be excluded.

A zebrafish embryo is simulated as a small volume immersed in the water. Since in literature, to the authors' knowledge, the electromagnetic properties of the tissues of these biological beings is not available, a reasonable maximum value of 2.5 S/m for the conductivity and 100 for relative permittivity is assumed, considering the corresponding values for human tissues. This situation can be assumed as the worst case for SAR computation.

Small circle in Fig. 2C highlight the presence of an embryo inside the water. Since the electrical conductivity of the embryos is not very different from that of the water, SAR distribution is almost the same. In conclusion, simulations exclude also for embryos the presence of hot spots that could affect the biological analysis with thermal effects.

2.9. Morphometric analysis

At 24, 48 and 72 hpf, ten embryos (in triplicate) were randomly collected from the control and exposed groups Petri dishes for a total number of thirty embryos/group/sampling.

Total length (TL) was measured with a STEMI 2000 micrometric microscope. Embryos were first observed under a dissecting microscope (Leica wild M3B) and then dechorionated to carry out the measurements. Yolk sac size, represented by area, was measured for all experimental groups, using ImageJ software. ZF embryos were imaged using a Leica TM IRB inverted microscope connected to a digital camera.

2.10. Histology

For general morphological examinations, ten embryos (in triplicate) were randomly collected from Petri dishes of control and exposed groups for a final number of thirty embryos/group/sampling at 24, 48 and 72 hpf. Embryos were fixed by immersion in neutral 4% paraformaldehyde, prepared in phosphate buffered saline (PBS, 0.1 M, pH 7.4) at 4 °C overnight, washed in PBS, dehydrated through a graded ethanol series and embedded in paraffin. Then, 4 µm consecutive sections were cut with a microtome, stained with Mayer's hematoxylin and eosin, dehydrated, mounted in Eukitt, and examined under an Olympus Vanox photomicroscope.

2.11. RNA extraction and real-time PCR

Total RNA extraction and cDNA synthesis from triplicate pools of thirty ZF embryos randomly collected from control and 100 MHz-exposed Petri dishes, for a final number of ninety embryos/group per sampling point, were optimized as already described (Olivotto et al., 2004; Piccinetti et al., 2014).

Real-time PCRs were performed with the SYBR green method in an iQ5 iCycler thermal cycler (Bio-Rad Laboratories). Triplicate PCRs were carried out for each analyzed sample. The reactions were set following Maradonna et al. (2013). Modification of gene expression is reported with respect to the control sample. Relative quantification of the genes expression was performed using *actb1* and *rplp* (Piccinetti et al., 2014; Bustin et al., 2009) as the housekeeping genes to standardize results by removing variations in mRNA and cDNA quantity and quality. Amplification products were sequenced and homology was verified. No amplification product was detected in negative controls and no primer-dimer formation was found in control templates. Data were analyzed using the iQ5 optical system software version 2.0, including Genex Macro iQ5 Conversion and Genex Macro iQ5 files (all from Bio-Rad). Primer sequences were designed using Primer3 (210v. 0.4.0) starting from ZF sequences available in ZFIN. The primer sequences are described in Table 1 of Supplementary material.

2.12. TUNEL assay

Apoptotic cells in the embryos were detected by the TUNEL assay following Celeghin et al. (2011). The TUNEL assay (TdT-mediated fluorescein-dUTP nick end labelling) was performed using alkali stable digoxigenin-dUTP and TdT (terminal deoxynucleotidyl transferase) (Roche). Fifteen embryos (in triplicate) were randomly collected from Petri dishes of control and exposed groups for a final number of forty-five embryos/group/sampling, were fixed in 4% PFA (overnight, 4 °C), treated with methanol and stored at 20 °C. Methanol-stored embryos were rehydrated in methanol/PBS series and permeabilized by proteinase K (10 µg/ml). Afterwards, the embryos were washed in PBT (5 × 5 min, RT) and in ethanol/acetic acid (2:1) (20 min RT). After incubation with TUNEL buffer (30 min, RT), embryos were incubated in 100 µl TUNEL reaction mixture (overnight, RT). The reaction was stopped by washing samples with PBT/EDTA 1 mM (2 × 1 h, RT). TUNEL-labelled embryos were imaged using a Leica TM IRB inverted microscope equipped with a digital camera. The number of apoptotic cell bodies in TUNEL stained embryos were quantified within the whole eye, the forebrain, mid-hindbrain using ImageJ software, as described by Mahmood et al., 2013.

2.13. BODIPY FL C₁₂ staining

Fifteen embryos were randomly collected from the Petri dishes of control and exposed groups for a final number of forty-five embryos/group per sampling point. Embryos at 24, 48 and 72 hpf were placed into 12-well plastic dishes (3 larvae per well) and soaked in 100 mM BODIPY® FL C₁₂ (4,4-Difluoro-5,7-Dimethyl-4-Bora-3a,4a-Diaza-s-Indacene-3-Dodecanoic Acid) (Invitrogen) diluted in 2% DMSO, then incubated for 1 h at 28 °C in the dark. Fluorescent images were obtained with an epi-fluorescence Nikon microscope ECLIPSE Ni (Japan). Bodipy FL C₁₂ was excited at 505 nm (blue light) and emitted a spectrum of wavelength light which peaked at 515 nm. Regions of Interest (ROI) of 15 yolk sacs per group were selected. Fluorescence intensity (arbitrary units a.u.) was determined in 15 yolk sacs using Image J software (Falcinelli et al., 2015).

2.14. Statistical analysis

ZF survival rate, morphometric data and real-time PCR data were examined by two-way analysis of variance followed by Tukey's post-test (100 MHz exposure/hpf). The statistical software package Prism5 (GraphPad Software) was used for analyses. Significance was set at $p < 0.05$.

With regard to the temperature measurements, the considerations were made following the indications of the Guide to the Expression of Uncertainty Measurement (JCGM, 2008).

3. Results

3.1. Temperature monitoring

In Fig. 3A an example of the temperature monitoring during the whole test duration (i.e. 72 h, mean values). The black curve represented the room temperature, while the others were the measurements done by means of the three temperature gauges in tank no. 1. It can be noted that the water temperature was maintained fairly constant thanks to the heaters, while the ambient temperature varied according to the normal oscillation in the work environment.

In Fig. 3B results of temperature monitoring averaged on the three expositions, for each tank (i.e. tank 1, thermostated, exposed embryos; tank 2, thermostated, control embryos; tank 3, not thermostated and without embryos) and for the ambient temperature. The bars represented the mean values of the temperatures measured by means of the three gauges, with a vertical line representing the variation (2σ,

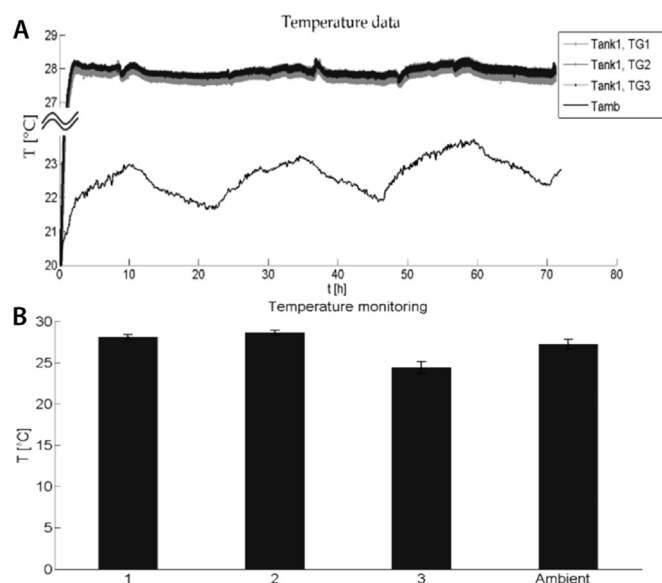


Fig. 3. A, B. Temperature data. A) Temperature monitoring in tank 1, compared with ambient temperature. B) Temperature monitoring in tanks 1, 2 and 3 and ambient temperature; values averaged on the three tests (reported as $m \pm 2\sigma$, where m is the mean value, σ the standard deviation) are the following ones: (28.1 ± 0.3) °C in Tank no. 1, (28.6 ± 0.3) °C in Tank no. 2, (24.4 ± 0.7) °C in Tank no. 3, (27.2 ± 0.6) °C in Air.

where is the standard deviation and k the coverage factor, indicating that the 95% of the values are in the specified range). It is observable that the temperature values in tanks 1 and 2 were comparable since the confidence intervals (computed according to the GUM) intersect.

Numerical results were averaged on the three tests and computed from 2 h after the heaters ignition (*i.e.* after the transient time) to the end of the test (approximately 72 h after the start). Values from the three temperature gauges in each tank were mediated to obtain a singular range for the tank.

Temperatures in tank no. 1 (the one with the embryos exposed to the electric field in the TEM cell) and in tank no. 2 (outside the TEM cell) were comparable: both the test and the controls embryos were maintained at the same temperature conditions, included in the optimal range. Thus, it was possible to state that final biological results were not affected by temperature.

3.2. Morphometric and histological analysis

Embryos from control and 100 MHz EMF-exposed groups did not exhibit significant differences in survival rate at the end of the experiment with a survival rate at 72 hpf of $49 \pm 1.5\%$ ($P < 0.05$) for the control and $51 \pm 1.45\%$ ($P < 0.05$) for the EMF exposed group, respectively.

While at 24 and 72 hpf, significant differences between experimental groups were not noticeable, in animals exposed to the EMF at 48 hpf morphometric and histological analysis showed evidences of a general delay in embryonic development in particular in terms of total length, yolk sac area and pigmentation.

As concerns TL, 100 MHz exposed embryos showed a significant lower length with respect to the control group (3.48 ± 0.1 mm vs 3.87 ± 0.09 mm, respectively) at 48 hpf as illustrated in Fig. 4A. At the same time, yolk sac area measurements (Fig. 4B) displayed a significantly larger yolk sac surface in EMF exposed animals (0.326 ± 0.03 mm²) respect to the control group (0.165 ± 0.04 mm²). Moreover, microscope investigations and histological examination of EMF exposed specimens at 48 hpf showed a general lower pigmentation, (melanophores of dorsal and ventral stripes) and a lower degree of development (cephalic area and eye) respect to unexposed ones. However, no relevant histological damages were observed in control

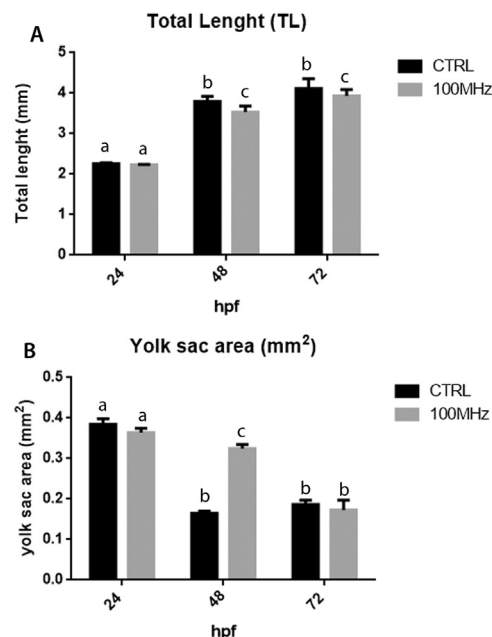


Fig. 4. A, B. Morphometric results. A) Graph of TL of zebrafish embryos from control and 100 MHz EMF exposed groups collected at 24, 48 and 72 hpf. Length measurements were referred to the dechorionated animals. Values with different superscript letters are significantly different ($p < 0.05$). B) Yolk sac area (mm²) of zebrafish embryos from control and 100 MHz EMF exposed groups collected at 24, 48 and 72 hpf. The area was calculated by ImageJ software. Values with different superscript letters are significantly different ($p < 0.05$).

and treated animals (Fig. 5D–I).

3.3. 100 MHz EMF affects detoxification and apoptosis-autophagic processes

3.3.1. Gene expression

Embryos gene expression was evaluated at 24, 48 and 72 hpf by Real-time PCR.

As regards oxidative stress biomarkers, *glutathione S-transferase (gstl)* and *superoxide dismutase 2 (sod2)* showed a very similar increasing trend during embryonic development: in both treated and control groups, gene expression level increased at each sampling point reaching the highest level at 72 hpf (Fig. 6A, B). Significant differences between the control group and the exposed one were found only at 48 hpf, where transcripts of *gstl* and *sod* were significantly ($p < 0.05$) more abundant in 100 MHz EMF exposed embryos with respect to control (Fig. 6A, B).

Stress response was described in Fig. 6C. Glucocorticoid receptor transcript (*nr3c1*) did not show significant differences from 24 to 72 hpf in the control group while 100 MHz exposed group showed a significant increase ($p < 0.05$) of *nr3c1* transcript from 24 to 72 hpf, reaching its highest level at the last sampling point. The comparison between the two groups evidenced a significant ($p < 0.05$) increase in *nr3c1* gene expression at 48 and 72 hpf in exposed group with respect to control, similarly to *gstl* transcript trend.

Autophagy was investigated by monitoring *beclin1 (becn1)* and *microtubule-associated protein 1-light chain 3 (map1lc3b)* gene expression (Fig. 6D, E). Relative levels of *becn1* did not exhibit significant differences during embryonic development, from 24 to 72 hpf in the control group whereas, in 100 MHz exposed group a significant ($p < 0.05$) up-regulation of the gene was detected at 48 and 72 hpf respect to the control. Differently from *becn1*, transcript levels of *map1lc3b* showed an increasing trend from 24 to 72 hpf both in the control and exposed group but, at 48 and 72 hpf *map1lc3b* gene expression was significantly higher in exposed group ($p < 0.05$) respect to control.

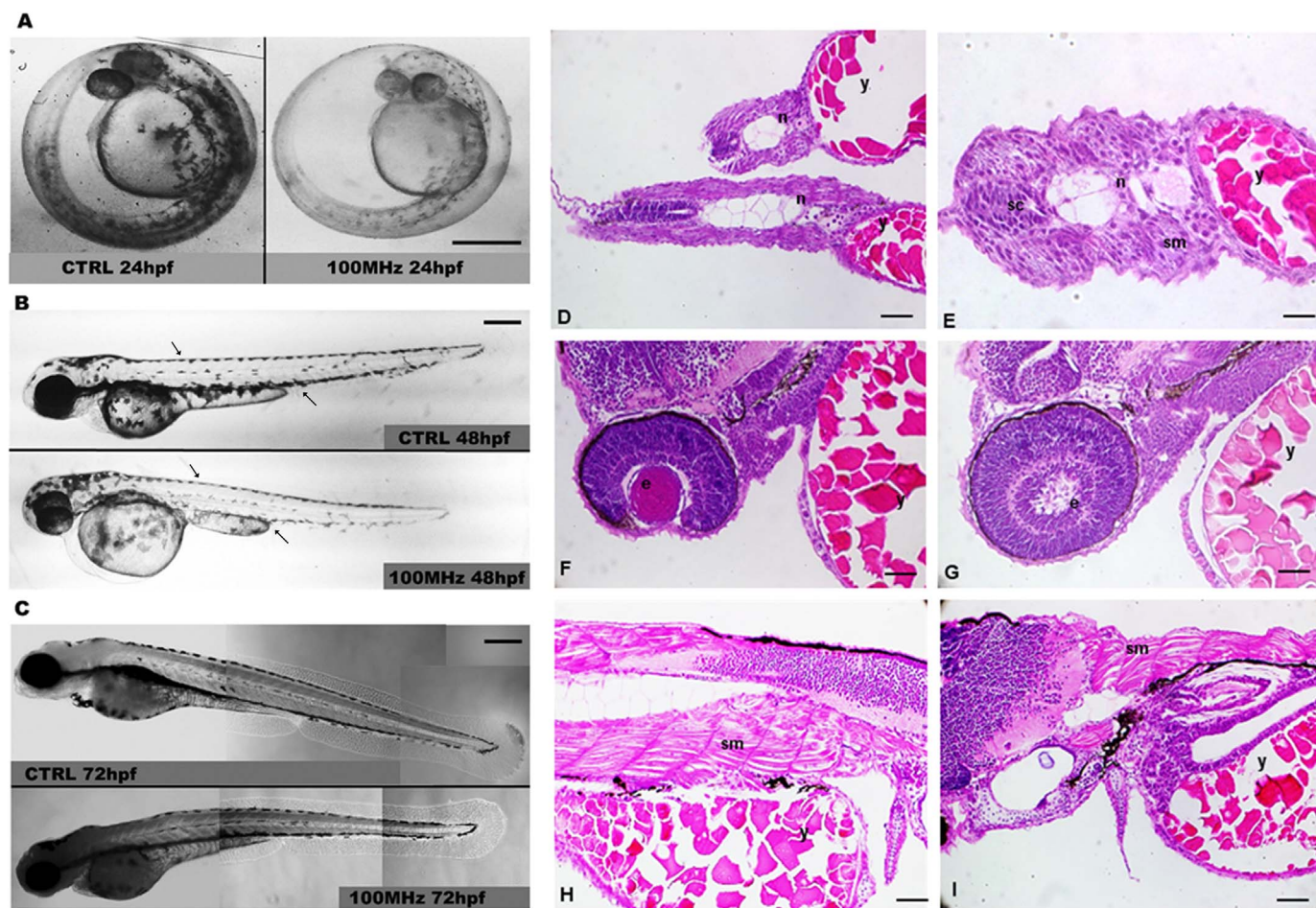


Fig. 5. A–I. Phenotypic observation of ZF embryos. A) 24 hpf embryos, scale bar = 200 μ m; B) 48 hpf embryos, scale bar = 300 μ m; C) hatching time (72 hpf), scale bar = 200 μ m. On the right, from D to I, Histological evaluation of ZF embryos aged 24, 48 and 72 hpf. All panels are stained with haematoxylin and eosin. Panels D, F and H represent control animals at 24, 48 and 72 hpf respectively; panels E, G and I represent treated animals at 24, 48 and 72 hpf respectively. n: notochord; y: yolk; sc: spinal cord; sm: skeletal muscle; e: eye. Scale bar = 30 μ m.

In Fig. 6F, G the analysis of *caspase 3* (*casp3*) and *bcl2-associated X protein* (*bax*) transcripts, encoding for apoptosis signals, were reported. Interestingly, both genes showed a very similar trend during embryonic development that was highly comparable to that observed for autophagic genes.

Casp3 and *bax* gene expression did not show significant differences from 24 to 72 hpf in the control group. In the 100 MHz exposed group, firstly a significant reduction in gene expression level was recorded (24 hpf) and secondly a significant increase was observed at 48 and 72 hpf, respect to control ($p < 0.05$).

3.3.2. Apoptosis detection

TUNEL assay carried out on the ZF embryos cephalic area evidenced a significantly ($p < 0.05$) higher number of positive apoptotic bodies at 48 and 72 hpf in 100 MHz EMF exposed ZF with respect to the control, in perfect agreement with *casp3* and *bax* gene expression data. In detail, at 24 hpf the number of apoptotic bodies did not show statistical differences between control and 100 MHz EMF exposed group (Fig. 6 I, L).

Apoptotic cells were mostly detected in the eye, lens and retina (arrows), trigeminal ganglion, anterior lateral line and otocyst. From 24 hpf apoptosis was overall reduced. However, 48 hpf TUNEL assay revealed a significantly higher number of apoptotic bodies, mainly located in olfactory organ and statoacoustic ganglion, in the exposed group with respect to the control (Fig. 6M, N). A very similar result in numbers of apoptotic bodies and their distribution was observed at 72 hpf where, also in this case, the number of apoptotic bodies was

significantly higher in the treated group as regard to control (Fig. 6O, P).

3.4. 100 MHz EMF modulation of cholesterol metabolism

3.4.1. Gene expression

Different genes implicated in lipid metabolism were selected to investigate cholesterol pathways, particularly *cytochrome P450, family 7, subfamily A, polypeptide 1* (*cyp7a1*), *apolipoprotein A-1a* (*apoa1a*) and *Niemann-Pick C1-like 1* (*npc1l1*).

From 24 to 72 hpf *cyp7a1* showed an increasing trend, reaching the highest expression level at 72 hpf for both control and exposed group. At 24 hpf the gene expression levels were almost undetectable (Fig. 7A) and no significant differences were observed between the control and the exposed groups ($p < 0.05$). At 48 hpf gene transcription levels were significantly higher in the treated group respect to control, at 72 hpf no significant differences were detected between groups.

The analysis of *apoa1a* (Fig. 7B) expression levels revealed significant differences only at 48 hpf in which the abundance of the gene was higher in the treated group with respect to the control, reaching its highest value of expression. No statistical differences between the groups considered were found at 24 and 72 hpf.

The *npc1l1* transcript analysis (Fig. 7C) showed a similar trend to that of *cyp7a1*; at 24 hpf it was nearly undetectable, slightly increasing at 48 hpf, and at 72 hpf, the highest level of expression. Was reached. Significant differences ($p < 0.05$) were found at 48 and 72 hpf, where an increase of the gene abundance was recorded at 48 hpf, followed by

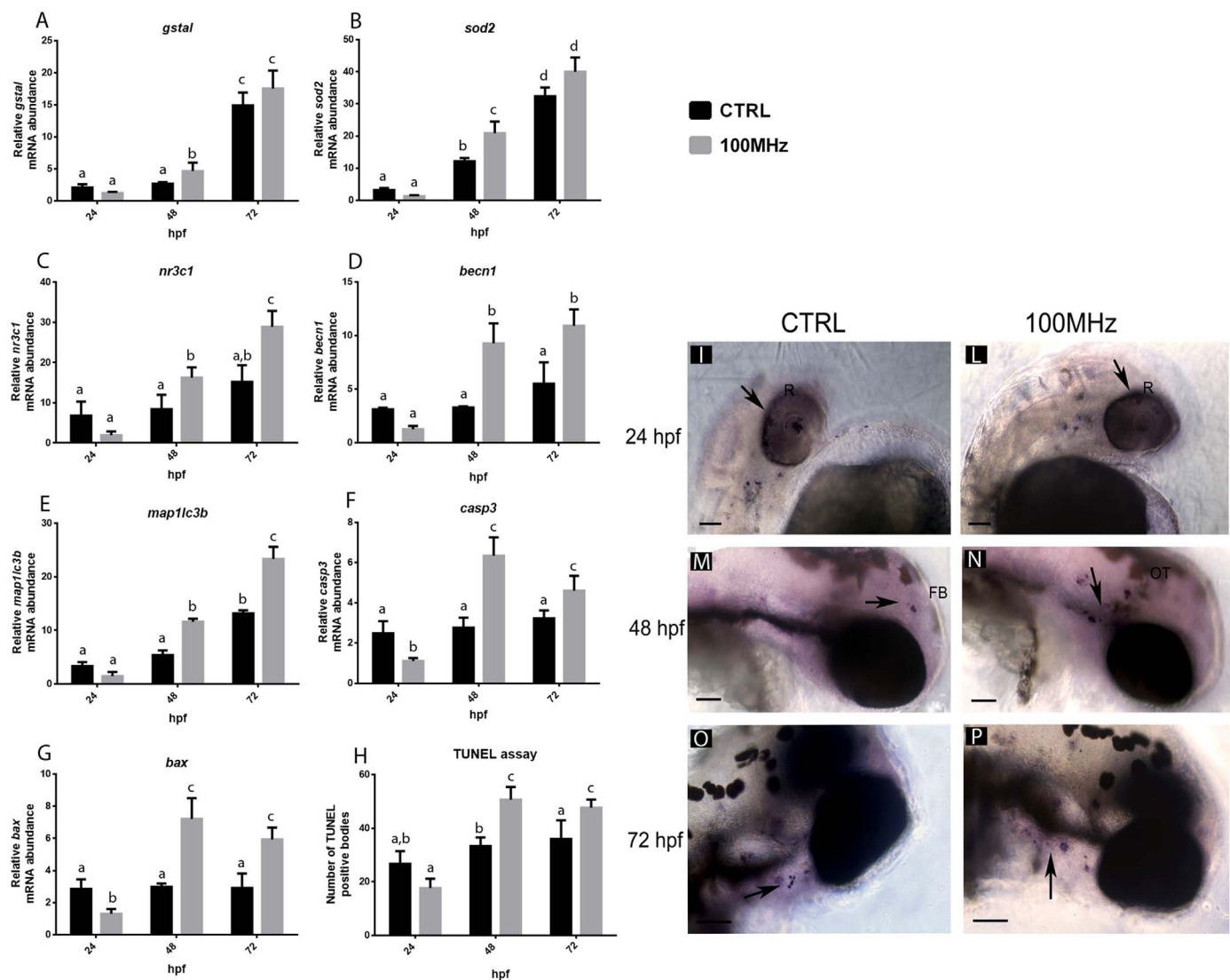


Fig. 6. A–P. From A to G, qRT-PCR analysis. Relative *gsta1*(A), *sod2* (B), *nr3c1* (C) *becn1* (D), *map1lc3b* (E), *casp3* (F), *bax* (G), gene expression normalized against *actb1* and *rplp10*, in pools of 30 zebrafish embryos from control and 100 MHz EMF exposed groups collected at 24 hpf, 48 hpf and 72 hpf. Assays were performed in triplicate. Values with different superscript letters are significantly different ($p < 0.05$). In H–P TUNEL assay images and relative graph. TUNEL was performed using alkali stable digoxigenin-dUTP and TdT (terminal deoxynucleotidyl transferase) (Roche) in control and 100 MHz EMF treated ZF embryos to detect apoptosis at various time periods and apoptotic bodies were quantified by ImageJ software. In I, M and O images of control embryos at 24, 48 and 72 hpf respectively. In L, N and P treated ZF embryos at 24, 48 and 72 hpf. Significant ($p < 0.05$) differences were found at 48 and 72 hpf in treat group that exhibit a higher number of apoptotic bodies respect to control. Scale bar 200 μ m. R = retina, FB = forebrain, OT = optic tectum.

a down-regulation at 72 hpf in treated with regard to the control group.

3.4.2. In vivo yolk sac cholesterol localization with BODIPY FL C_{12} staining

BODIPY FL C_{12} staining was used in the present study as a non-invasive *in vivo* method in order to visualize the dynamics of cholesterol in embryonic living tissues. Images revealed similar green fluorescence intensity in the yolk sac of both control and 100 MHz EMF exposed group (Fig. 7E, F) at 24 hpf. Then, the treated group exhibited, at 48 hpf, significantly ($p > 0.05$) higher fluorescent signal in the yolk sac with respect to the control (Fig. 7G, H) highlighting an accumulation of cholesterol. On the contrary, at 72 hpf a significant reduction in fluorescence and therefore in cholesterol content, was observed in the treated group with respect to the control (Fig. 7I, L).

4. Discussion

The present study intended to assess, with the best possible certainty, the outcomes of high frequency (100 MHz) EMF exposure on zebrafish embryonic development.

The use of a specific acquisition system allowed to monitor and maintain temperature throughout the duration of the whole experiment and the analysis of SAR values confirmed EMF incidence within ICNIRP limits. As a consequence, the values obtained allowed to exclude any thermal effect.

Furthermore SAR simulation, exempted the onset of hotspots: the results here obtained on ZF embryo development were thus uniquely due to the EMF exposure.

Generally, the present study evidenced no significant differences in ZF embryo mortality or abnormality; however, differently from observations in medaka (Lee and Yang, 2014), ZF embryos exposed to 100 MHz EMF showed a general delay in development between 24 hpf and 48 hpf. However, at 72 hpf the same embryos reached a similar development with respect to control ones and hatch rate did not show any statistically difference with respect to the control (data not shown).

Accordingly to Kimmel et al. (1995), the most impacted developmental stage was the 48 hpf time. For zebrafish embryos this is a very delicate and sensitive period to external cues: embryos undergo an intense and rapid morphogenesis process of primary organs, and, as

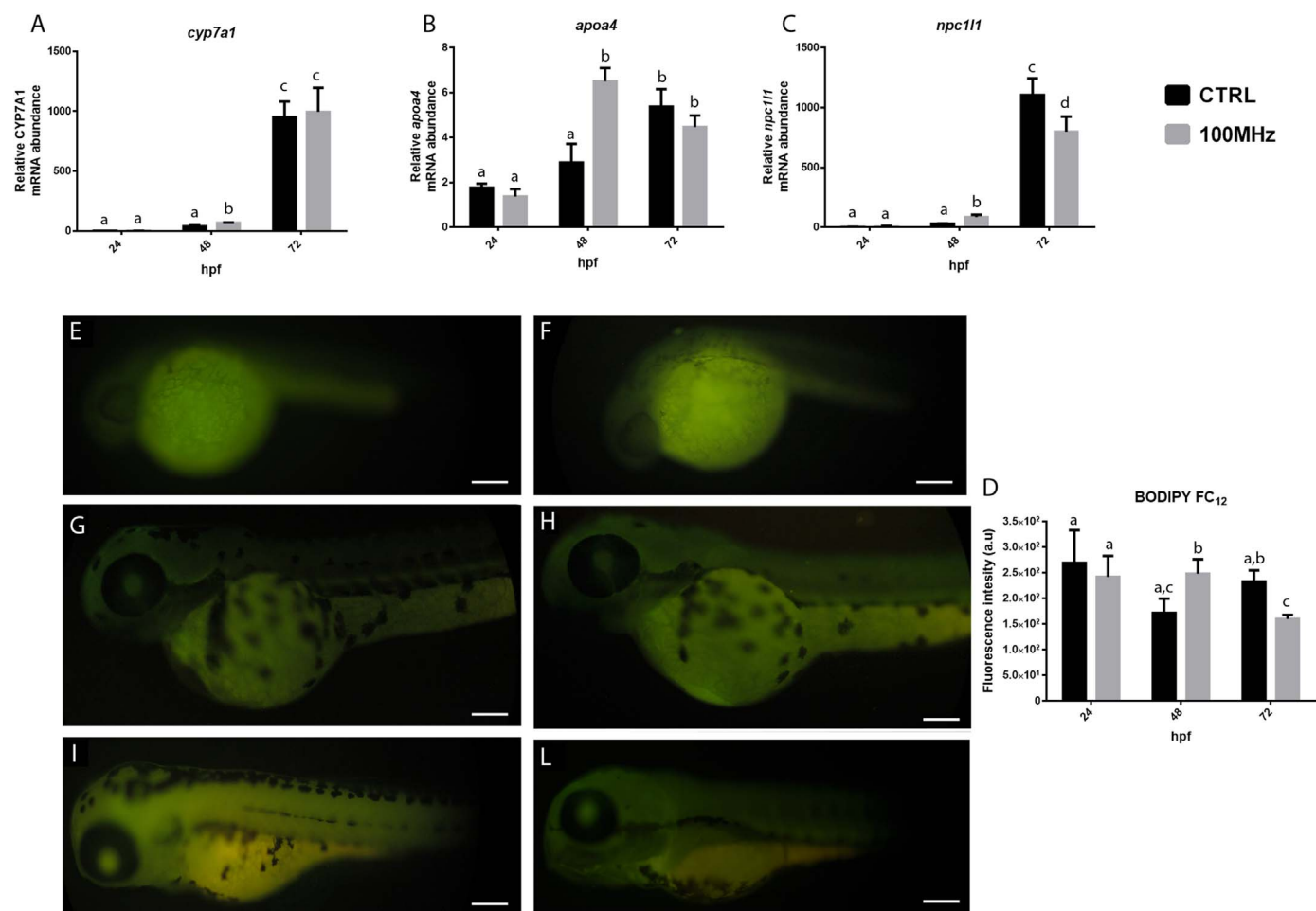


Fig. 7. A–L. From A to C, qRT-PCR analysis. Relative *cyp7a1* (A), *apo4* (B), *npc1l1*, gene expression normalized against *actb1* and *rplp10*, in pools of 30 zebrafish embryos from control and 100 MHz EMF exposed groups collected at 24 hpf, 48 hpf and 72 hpf. Assays were performed in triplicate. Values with different superscript letters are significantly different ($p < 0.05$). From D to L, representative fluorescent images and relative graph of live 24, 48 and 72 hpf zebrafish embryos soaked in BODIPY FL C₁₂ for 1 h. Images revealed green fluorescence in the yolk sac of control (E, G, I) and 100 MHz EMF exposed groups (F, H, L). Scale bar: 500 μ m.

described by Field et al., 2003, liver completes its development at this stage.

Several studies demonstrated that EMF exposure often results in ROS production by biological systems (Harakawa et al., 2005; Juutilainen, 2005; Phillips et al., 2009; Kong and Lin, 2010; Li et al., 2014). The connections among ROS, growth, muscle development and tissue integrity remains poorly understood, but a direct role of ROS in development was assessed in mammals and fish (Holt, 2011; Randazzo et al., 2017). Moreover, Dennerly (2007) demonstrated how redox status affects multiple cellular processes including cell proliferation, differentiation, and signalling. Consequently, the delay in embryo development evidenced in this study (24 and 48 hpf) is possibly related to a ROS production induced by EMF exposure.

This hypothesis is fully supported by the molecular analysis performed in the present study, which showed how RF-EMF exposure was able to induce transcriptional changes in genes involved in detoxification and stress response.

Particularly, *sod2* and *gstal* genes were significantly up-regulated by EMF exposure in zebrafish embryos at 48 hpf, with respect to the unexposed ones. These results demonstrated the onset of detoxification processes by the exposed embryos and were in agreement with the delay recorded in embryonic developmental described by morphometric data. Interestingly, glucocorticoid receptor (*nr3c1*) gene expression trend was very similar to detoxification genes transcript. During embryogenesis, components of HPI axis are functional, but embryos have access to cortisol because of a maternal deposit in the

oocyte; this amount of cortisol is continuously depleted reaching its minimum level around hatching, then the larva begins to synthesize cortisol by its own (Alsop and Vijayan, 2008; Piccinetti et al., 2017; Olivotto et al., 2002).

Glucocorticoid receptor may thus be not critical in stress response during embryonic development. However, our results revealed a clear increase in *nr3c1* mRNA levels in exposed embryos: were the embryos already able to respond to an EMF stress? The answer was not obvious since glucocorticoid receptor in embryos is also involved in basal metabolism and osmoregulation recovery (Alsop and Aluru, 2011). Likely, animals exposed to EMF have faced the stress resulting from a redox status by the activation of detoxification processes for the recovery of homeostasis. The development of reactive radical species, therefore, remains at the heart of the matter.

Phillips et al. (2009) described the connections between electromagnetic field and DNA damage, asserting that the energy delivered by EMF exposure is not enough to cause direct breakage of chemical bonds within molecules, but the pivotal responsible for DNA, protein and lipid membranes impairment is the formation of free radicals inside cells aroused by EMF exposure (Lai and Singh, 1997a, 1997b; Oral et al., 2006; Simko, 2007). Oxidative stress generated by free radicals culminates in the induction of apoptosis. Apoptosis is an important event during embryogenesis, but increasing apoptosis, triggered by external factors, can cause an adverse impact on the embryonic processes. Morphologically, apoptosis is identified by nuclear condensation and fragmentation without major ultrastructural changes of cytoplasmic

organelles (González-Polo et al., 2005).

Our study detected an up-regulation of genes involved in apoptosis activation mechanisms in those animals exposed to EMF, demonstrating the onset of an oxidative stress status by the embryos.

While control group *casp3* and *bax* showed a constant pattern in their gene expression during embryonic development, a strong increase of their transcript was detected in embryos exposed to EMF at 48 and 72 hpf.

These data corroborated the results obtained by TUNEL assay in which the number of positive apoptotic bodies was higher in the cephalic region of exposed animals at 48 and 72 hpf with respect to control. Cole and Ross (2001) described the normal pathway of apoptosis in developing zebrafish embryo. Accordingly to this study, the present control group embryos showed a very similar trend in olfactory organ, retina, lens, statoacoustic organ, trigeminal ganglion and anterior lateral line ganglion formation processes. On the contrary, 100 MHz exposed embryos showed an increased number of apoptotic bodies in the cephalic region.

Apoptosis together with autophagy constitute the two processes through which superfluous, damaged or aged cells or organelles are eliminated (Maiuri et al., 2007). In our study, levels of autophagy-related transcript, Beclin1 (*becn1*) and microtubule-associated protein 1 light chain 3 beta (*map1lc3b*) were higher in EMF exposed embryos than in the control ones, showing a very comparable trend to apoptotic signals expression. Also in this case, the control group did not show significant differences during embryonic development, only at 72 hpf an increase of the transcripts was observed.

The functional relationship between apoptosis and autophagy is complex because in many conditions autophagy is a process of adaptation to stress that protects against cell death; other times, however, autophagy becomes an alternative way of cell death (Amelio et al., 2011). The same cellular stress can activate both the apoptotic pathway and the autophagic mechanism, resulting in a synergistic effect (González-Polo et al., 2005). Results here obtained showed a combined action of the two mechanisms and interestingly, as described by González-Polo et al. (2005), autophagy vacuolization may have acted as a compensatory process allowing embryos to recover and complete embryonic development normally at 72 hpf.

Histological examinations endorsed the results previously described: a delay in development in embryos exposed to 100 MHz EMF was observed but no evidence of tissue damages was recorded. Thus, it could be speculated that EMF exposed embryos were directing most of their energy to the recovery from oxidative stress rather than to the growth.

A further crucial point during the embryonic development of zebrafish, is the yolk lipid processing (Miyares et al., 2014). Particularly, genes involved in lipid and lipoprotein metabolism are frequently expressed early in embryonic development where they can be critical for early metabolic processes, proper embryonic development and/or viability (Willnow et al., 2007). These mechanisms are well conserved among vertebrates and zebrafish express most of the classes of apolipoprotein and enzymes for cholesterol metabolism (Babin et al., 1997; Imai et al., 2012).

Remarkably, the EMF adopted in this study was able to induce transcriptional modulation to a suite of genes involved in cholesterol transport, absorption and conversion in bile acids, during zebrafish embryonic development. Apolipoprotein a1a (*apo1a*), in mammals and lower vertebrates, is involved in cholesterol transport, from the yolk to the developing embryonic tissues (Anderson et al., 2011), through the yolk syncytial layer (YSL). This area expresses several apolipoproteins during embryogenesis to sustain embryonic and larval development.

Otis et al. (2015) demonstrated that in ZF *apo1a* mRNA starts to be expressed at gastrulation stage playing a key role in the regulation of cholesterol efflux from cells to HDL particles, the first step of the process which carries cholesterol to the liver for excretion in bile. In this

study, *apo1a* transcription was strongly increased in 48 hpf embryos by 100 MHz EMF exposure.

Furthermore, in embryos exposed to the EMF, transcript of *cholesterol 7 alpha-hydroxylase* (*cyp7a1*) and Niemann-Pick C1-Like 1 (*npc1l1*) were modulated too. *Cyp7a1* catalyzes the first step in the conversion of cholesterol in bile acid synthesis, while *npc1l1* encodes for a protein that plays a key role in cholesterol absorption (Walters et al., 2012). Both genes were upregulated by EMF at 48 hpf whereas at 72 hpf significant changes were only detected for *npc1l1* that decreases compared to the control.

Interestingly, these data are consistent with those detected by BODIPY® FL C12 staining; at 48 hpf embryos exposed to EMF evidenced an intense and significant fluorescent signal indicating a cholesterol buildup in the yolk sac that, by contrast, declined in the last sampling point. Thus, in the light of the data here described, it is possible to assume that oxidative stress aroused by EMF exposure interacted with cholesterol metabolism, oxidizing LDL and HDL, as already described in mammals (Gesquière et al., 1999; Rosenblat et al., 2015).

After an accumulation of cholesterol (48 hpf) in the yolk sac, through the stimulation of *apo1a* and *npc1l1* mRNAs, a reduction of its content was observed at 72 hpf. Furthermore, since cholesterol is known as cortisol precursor (Alsop and Aluru, 2011), data here obtained matched with glucocorticoid receptor transcript at 48 hpf, demonstrating the probable onset of a early stress response by the embryos exposed to EMF.

5. Conclusion

The results obtained in this study demonstrated unequivocally that 100 MHz EMF had biological effects on ZF embryonic development, particularly evident at 48 hpf. However, these effects were no more evident at hatching time, because of the activation of specific detoxification biological pathways.

In the light of the results obtained, future research on EMF dose-response effects is needed to achieve a complete overview of electromagnetic pollution outcomes.

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Disclosure statement

No competing financial interests exist.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.ecoenv.2018.02.053>.

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