Article

Multimarker approach to evaluate the exposure to electromagnetic fields at 27 GHz (5G) on *Danio rerio* larvae

Roberta Pecoraro[1§*], Santi Concetto Pavone[2,3§], Elena Maria Scalisi[1], Sara Ignoto[1], Carmen Sica[1], Fabiano Capparucci[4], Carmelo Iaria[4], Antonio Salvaggio[5], Gino Sorbello[2,3], Loreto Di Donato[2,3§§] and Maria Violetta Brundo[1§§]

[1] Department of Biological, Geological and Environmental Science, University of Catania, Via Androne 81, 95124, Catania, Italy, roberta.pecoraro@unict.it, elena.scalisi@phd.unict.it, sara.ignoto@phd.unict.it, carmensica93@gmail.com, mariavioletta.brundo@unict.it

[2] Department of Electrical, Electronics, and Computer Engineering (DIEEI), University of Catania, Viale Andrea Doria 6, 95125 Catania, Italy, santi.pavone@unict.it, gino.sorbello@unict.it, loreto.didonato@unict.it

[3] Consorzio Nazionale Interuniversitario per le Telecomunicazioni (CNIT), Viale G.P. Usberti, 181/A Pal. 3, 43124 Parma, Italy, santi.pavone@unict.it, gino.sorbello@unict.it, loreto.didonato@unict.it

[4] Department of Chemical, Biological, Pharmacological and Environmental Science, University of Messina, via F. Stagno D'Alcontres 31, Messina 98166, Italy, fcapparucci@unime.it; carmelo.iaria@unime.it

[5] Experimental Zooprophylactic Institute of Sicily "A. Mirri", Via Gino Marinuzzi, 3, Palermo 90129, Italy, antonio.salvaggio@izssicilia.it

§ These authors have contributed equally to this work and share first authorship

§§ These authors have contributed equally to this work and share last authorship

* Correspondence: roberta.pecoraro@unict.it; Tel.: +390957306040

Abstract: 5G technology is evolving to satisfy several service requirements favoring high data-rate connections and lower latency times than current ones (<1ms). 5G systems use different frequency bands of the radio wave spectrum, taking advantage of higher frequencies than previous mobile radio generations. In order to guarantee a capillary coverage of the territory for high reliability applications, it will be necessary to install a large number of repeaters because higher frequencies waves have a lower capacity to propagate in free space. Following the introduction of this new technology, there has been a growing concern about possible harmful effects on human health. The aim of this study is investigating possible short term effects induced by 5G-millimeter waves on embryonic development of *Danio rerio*. We have exposed fertilized eggs to 27 GHz frequency, 9.7 mW/cm^2 incident power density, 23 dbm and have measured several endpoints every 24 hours. The exposure to electromagnetic fields at 27 GHz (5G) caused no significant impacts on mortality nor on morphology because the exposed larvae showed a normal detachment of the tail, presence of heartbeat and well-organised somites. A weak positivity on exposed larvae has been highlighted by immunohistochemical analysis.

Keywords: millimeter waves; zebrafish; DanioScope; biomarkers of exposure; SAR.

1. Introduction

Nowadays, telecommunications represent an increasingly important component of our society infrastructure and they are the subject of a radical revolution. The arrival of a new fifth generation (5G) technological standard, in fact, has had a deep impact on economy and society, revolutionizing wireless communications by transforming existing market sectors and industries [1]. Moreover, the 5G system will drive future economic resiliency by untethering more workers from physical workstations, triggering growth in new industries that are digital at their core and increasing efficiency and productivity across a variety of other industries. No less significant will be the impact that the advent of the 5G

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will have in the consumer's daily life, who will be able to enjoy new ones totally digitalized and interconnected experiences. The fifth generation will guarantee a considerable increase in connectivity, in the volume of traffic and network reliability, in terms of latency and density [2].

Electromagnetic waves are an integral part of the environment in which we live and work, and their origin is partly artificial (radio waves, radar and telecommunications), and partly natural (visible light, X-rays or gamma rays). Therefore, everybody are constantly surrounded by electromagnetic fields, an inevitable phenomenon and from the physical point of view absolutely unitary, because all fields and their effects are based on the same principles. The determining parameter is the frequency, i.e. the number of oscillations of the electromagnetic wave per second. The biological effect of electromagnetic waves essentially depends on their intensity and their frequency. Consequently, the electromagnetic spectrum can be divided in two main types: ionizing radiations (for example X and gamma rays) and those non-ionizing ones, such as radio waves and microwaves [3]. Radiations differ from each other due to the different ability to interact with atoms and the molecules that make up matter. The main effect that non-ionizing radiations can produce on molecules is to make them oscillate producing friction and consequently heat; heating is precisely the main effect of non-ionizing radiation, but their biological effect depends so much on their frequency [4,5].

Regarding the effects of electromagnetic fields, it's possible to distinguish between thermal and athermic effects [6]. The thermal effects of high-frequency fields are related to the absorption of energy and the consequent increase in temperature in the irradiated tissue. Thermal effects are usually caused by short and intense exposures. To measure the radiant energy absorbed from the human body in the unit of time is used the Specific Absorption Rate (SAR) expressed in watts per kilogram body mass (W/Kg). The value of the SAR has a direct correspondence with the biological effects of electromagnetic exposure. In the presence of high absorption rates, little organs particularly vascularized are at risk, i.e. those with poor blood circulation and therefore decongestion slower thermal, such as eyes or testicles. They faster heat up so therefore they are more exposed to risk than other areas of the body. In addition to thermal effects, electromagnetic radiations cause biological effects on humans associated with lower SAR values (<0.01 W/kg), which cannot be explained by just heating the tissues. This is why they are usually called athermic effects. Usually, these are long-term exposures but of low intensity [6]. There are few studies in literature which have not yet shed full light on the real consequences induced by athermic effects on human health; moreover, the results appear often contradictory. In particular, bioeffects studies performed considering high-band at 25–39 GHz are particularly scarce [4, 5, 7- 9].

The aim of the study is to clarify biological effects on embryonic development of *Danio rerio* induced by the exposure to electromagnetic fields at 27 GHz (5G). The reason why is that 5G high band frequencies will be increasingly used by communications technologies so it is reasonable thinking a bigger exposure in next future [1]. In order to decrease the impact of the experimental assays on live animals, the European Guidelines suggest to use zebrafish embryo toxicity test (ZFET) as an alternative tool to acute test with adult fish [10, 11]. By means this test, it has been possible to evaluate the effects induced by 5G-millimeter waves on the embryonic stages of zebrafish. We have used a multimarker approach analyzing: hatching failure and post-hatching death, four endpoints for ZFET (embryo coagulation, lack of somite formation, lack of detachment of the tail-bud from the yolk sac and lack of heartbeat), the heart frequency with DanioScope software, intracellular reactive oxygen species (ROS) by 2,7-dichlorodihydrofluorescein diacetate (DCFH 2-DA) immunohistochemical assay, apoptosis analysis by Acridina Orange assay, Heat Shock Proteins 70 (HSP70) and P540 Aromatase (Cyp19b).

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2. Materials and Methods

2.1 Exposure Setup Description and Numerical Dosimetry

The experiments at 27 GHz were conducted by using an in house produced high gain pyramidal horn antenna feeder for parabolic antennas and satellite power pattern measurements applications. The dimension of the antenna aperture are 8.02 x 6.02 cm and the maximum gain is 24.9 dBi. Moreover, the antenna is fed by a RF signal generation (R&S SMB100A) with +23dBm output power through a coaxial cable that introduce attenuation of 3dB. As a result, the distance between the antenna aperture and Petri dishes was set to 15 cm assuming normal incidence of the antenna main beam (Figure 1a), in order to ensure density incident power level comparable with the exposure limit imposed by the international guidelines [12].

To evaluate the specific absorption rate (SAR), a numerical simulation has been performed by means of the electromagnetic simulator CST Microwave Studio. The simulation was performed accurately by considering six Petri dish holder, the antenna and a reflecting plane behind the sample holder resembling the plane of the agitator at 1 cm fat from the bottom of the polystyrene holder. The foam, between the metallic plane and the polystyrene basis (used in the experimental setup to prevent metallic plane too much close to aqueous samples) has not been considered in the simulation since the foam electromagnetic parameters are very close to that of the air. The electric parameters of the materials adopted for the simulation at the working frequency of 27 GHz are reported in Table 1.

Table 1. Electromagnetic parameter of the full wave CAD model used in the numerical simulation.

Component	Materials	Dielectric Constant	Loss Tangent
Horn Antenna	PEC	-	∞
Sample	Water	28.5	1.25
Petri	Polystirene	2.5	0
Ground plane	PEC	-	∞

The local SAR (W/Kg) was evaluated from the electric field according to formula:

$$SAR = \frac{1}{2}\frac{\sigma|E|^2}{\rho}$$

where E is the peak value of the electric field in each cell of the discretization grid, σ is the medium effective conductivity and ϱ is the mass density (1000 Kg/m^3). Indeed, taking into account the very low concentration of zebrafish and its negligible dimension with respect to the wavelength in the aqueous sample (5 fishes per Petri), we have considered the medium as homogeneous with the above reported parameters.

Only numerical dosimetry has been performed because possible experimental thermal monitoring during the long exposure time was not viable due to invasiveness of probes at these working frequencies, that can significantly affect the field distribution inside biological samples. On the other hand, the resulting incident power density (9.7 mW/cm^2 in the maximum gain direction of the antenna) was comparable with the power density limit of 10 mW/cm^2 set by the international guidelines as limit for nonthermal effects above 6 GHz [12].

2.2 Experimental Procedure

The Zebrafish Embryo Toxicity (ZFET) test was performed according to the OECD (2013) guidelines for testing chemicals [13]. Fertilized eggs, coming from Centre for

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Experimental Fish Pathology of Sicily (CISS) located at University of Messina, were used for ecotoxicological assay. Zebrafish adults were maintained only in the zebrafish breeding room and reared in a ZebTEC Active Blue Stand Alone system (Tecniplast). In this housing system, the water derives from reverse osmosis treated city water (disinfected by ultraviolet treatment). Environmental conditions at the primary enclosure are maintained at 26 ± 1°C, pH 7.2 ± 0.3, and a dissolved oxygen content of 6.00 ppm for freshwater species. Moreover, animals are exposed to a light/dark cycle (14 light/10 dark) and fed twice daily with *Artemia* nauplii (JBL Artemio Pur, BL GmbH & Co. KG, Germany). Following mating, the fertilized eggs were collected by Pasteur pipettes under stereomicroscope, while the unfertilized eggs were discarded.
Healthy embryos were placed in 6-well microplates (five embryo/well) in 5ml solution/well. Control samples (negative control) were incubated only with stock embryo medium and a positive control with 3,4-dichloroaniline (DCA) was also done. A controlled room temperature has allowed to maintain 26 ± 1°C in wells. Five replications were performed.

2.3 Observation of endpoints

During the exposure period, started within 180 min from fertilization of the eggs and finished at 96 h, four endpoints were analyzed every 24 h by a stereomicroscope: embryo coagulation, lack of somite formation, lack of detachment of the tail-bud from the yolk sac and lack of heartbeat. Hatching failure and post-hatching death were also recorded, this is why failure to hatch represents an important sub-lethal effect.

2.4 Cardiology measurements

Cardiological measurements were recorded using the DanioScope™ software. This software analyzes the videos to provide quantitative data of the investigated endpoint, such as heart rate and intervals between beats. Daily, after the observation of acute endpoints the embryos were immobilized on dish of agarose and acclimated for at least 3 min before to record videos. The videos were made with the E200 MV-R LED microscope (Nikon) equipped with CMOS camera (Nikon). After selecting the heart area in the video imported, the cardiological activity has been detected automatically by DanioScope™ software. The software applies an algorithm to detect changes in pixel density during ventricular contractions and this changes are directly correlated with cardiac muscle contraction. DanioScope ™ software provides the number of beats per second (BPS) and beats per minute (BPM).

2.5 Evaluation of intracellular reactive oxygen species (ROS)

Intracellular ROS content in exposed larvae, has been detected by 2,7-dichlorodihydrofluorescein diacetate (DCFH 2-DA), a fluorescent probe useful to measure the reactive oxygen species (ROS). At 96hpf, a number of 2 exposed larvae to electromagnetic fields and two unexposed larvae were stained with ROS-detection solution as described by Mugoni et al. (2014) [14]. Collected embryos were washed with Hank's Balanced Salt Solution (HBSS) (Thermo Fisher Scientific) twice for 2 minutes each in a small tubes. ROS-detection solution (5µM DCFH 2-DA in HBSS) has been added to each tube, which was incubated in the dark for 15 min at 28 °C to avoid light exposure. At the end of the incubation time, ROS-detection solution has been removed and the larvae have been washed twice for 2 minute each with HBSS. Embryos were put on a glass slide, then the fluorescence was observed using a fluorescence microscope (NIKON ECLIPSE Ci), equipped with camera NIKON DS-Qi2.

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2.6 Acridina orange staining

Acridine orange is a dye useful to apoptosis analysis in whole-mount of embryos or tissue [15]. After exposure, zebrafish larvae were transferred to Eppendorf tubes, washed twice with phosphate buffered saline (PBS, pH 7.4, 0.1 M) and stained with 5µg/mL of acridine orange dissolved in PBS solution for 20 minutes at room temperature [16]. Embryos were quickly washed in PBS and mounted on slide with a drop of glycerol. Acridine orange staining was also carried out on control larvae. Measurement of the fluorescence intensity was performed by florescence microscope (NIKON ECLIPSE Ci), equipped with camera NIKON DS-Qi2.

2.7 Immunohistochemical analysis

The immunofluorescence protocol was performed according to Pecoraro et al. (2017) [11] on exposed larvae to 5G-millimeter waves, including controls, to detect positivity to HSP70 and P540 biomarkers. Larvae were incubated with anti-rabbit-HSP70 (GeneTex®, 1:1000) and anti-rabbit-P540 Aromatase polyclonal (Creative Diagnostics®, 1:1000) primary antibodies; secondary antibodies were goat anti-rabbit IgG antibody, pre-adsorbed (rhodamine) (GeneTex®, 1:1000). Samples were mounted with DAPI solutions (Bioptica) and sealed with rubber cement. The observations were made with the NIKON ECLIPSE Ci fluorescence microscope and the images captured with the NIKON DS-Qi2 camera.

2.8 Statistical analyses

In order to process the images obtained by fluorescence microscope, Image J software [17] has been used. It calculates the mean value (the sum of the values at all pixels divided by the number of pixels) of a specified area. In each photo, for control and exposed larvae, the same area (macro) was set to obtain density histograms. The mean values were compared with GraphPad Software by T-Student test to detect significant differences between the photos of exposed larvae and the photos of control groups ($p < 0.05$).

3. Results

3.1 Numerical dosimetry analyses

The computed SAR level (averaged over 1g of mass, i.e. SAR (1g)) on the surface of the aqueous samples is shown in Figure 1b. At a first glance, the SAR distribution seems to be very non homogeneous among the sample (intra-samples) and among the different Petri dishes (inter-samples). However, as Zebrafish larvae move randomly inside the sample, they will experience a medium SAR values of 0.115 W/Kg for the peripheral samples (Petri number 1, 3, 4, 6) and about 0.18 W/Kg for the innermost sample (Petri number 2 and 5) (Figure 1c). On the other hand, it is interesting to note that besides the different SAR mean values between the peripheral Petri and the innermost one, the average level of SAR in the different numerical layer along z direction (sample depth) keeps very uniform (Figure 1d). This is mainly due to the presence of the metallic screen under the Petri dishes. As expected, at the top layers of the samples, different levels of SAR are expected (with respect to the innermost layers) due to strong electromagnetic discontinuities. However, these density power levels are below the recently released ICNIRP guidelines for frequency above 6 GHz and below 30 GHz (200 W/m^2) [12]. The SAR level, not applicable for frequency larger than 6GHz, is anyway far below the threshold value of 4 W/kg indicated by ICNIRP in the frequency range 100 KHz-300GHz [12].

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However, temperature measurements of the aqueous sample during the water refilling, have sensed a temperature increase of 0.2°C, with respect to the sham samples. This very low temperature increase cannot be accountable of any thermal effect for the study at hand. Therefore, the dosimetric analysis suggests that no relevant thermal energy is deposited on the system by the MMW radiation.

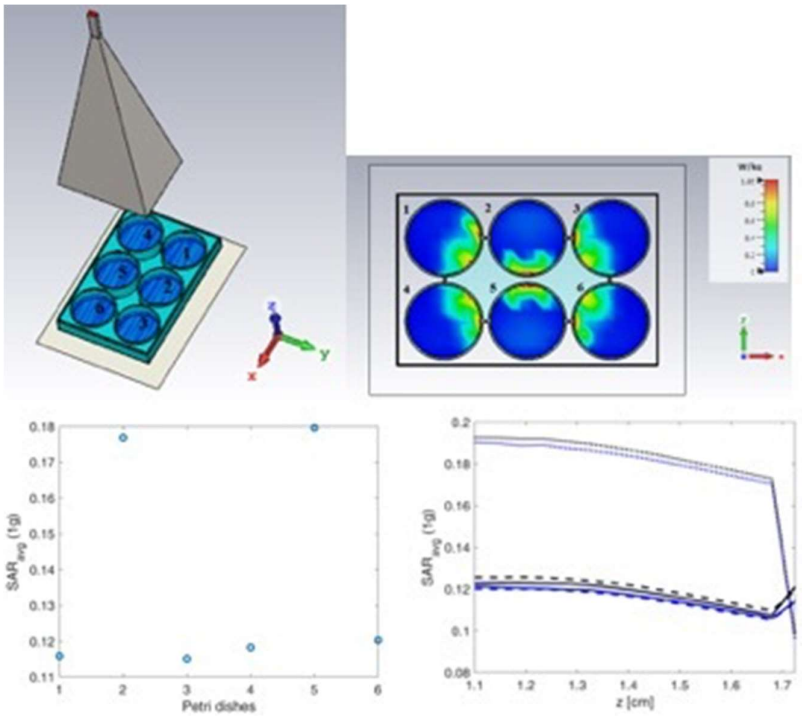

Figure 1. Experimental setup and numerical dosimetry. (a) CAD model accounting for very accurate modelling of the sample holder (six Petri dishes) and pyramidal horn antenna. (b) SAR distribution at the air-water interface at the top of the aqueous samples. (c) average SAR for each aqueous sample. (d) average SAR along the depth of the aqueous samples (z direction): blue continuous line Petri n.1, blue dotted line Petri n.2, blue dashed line Petri n.3, black continuous line Petri n.4, black dotted line Petri n.5, black dashed line Petri n.6.

3.2 Endpoints and biomarkers analysis

The evaluation of the endpoints defined by the ZFET test was useful for investigating the effect of 27 GHz on embryonic development. Exposure to 27 GHz did not cause the coagulation of eggs. Both exposed and unexposed embryos have completed embryonic development, in fact a normal development of the head, notochord, fin, pigmentation, heart and eyes has been observed (Figure 2) [18]. The hatching of the larvae was observed at 48hpf for the exposed groups, while at 72hpf for the unexposed (Figure 3), however there was no statistically significant difference ($p > 0.05$).

Instead, a statistically significant difference was observed for heart rate. Thanks to the DanioScope software, it was highlighted that the exposure to 27 GHz caused an increase of heartbeat rate on exposed embryos at 48h than control group, but this increase has not been more shown at 72-96h as shown in Figures 4 and 5. Heart rate variability is of the utmost significance parameter for the study of cardiac function; in zebrafish, the heart rate is physiologically around 120-180 bpm, but its alteration is associated to cardiotoxicity [19, 20]. Nevertheless, post-hatching death was not observed. All embryos were vital until the end of test. By immunohistochemical investigation, we have observed a higher expression

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of P540 biomarker in the exposed larvae compared to controls (Figure 6c, 6d). Concerning HSP70, the expression was not increased in exposed larvae compared to the controls (Figure 6a, 6b). The analyze has confirmed a statistically significant difference ($p < 0.05$) for P540 biomarker between control group and exposed larvae response. However, as regard HSP70 biomarkers, it has not been founded a statistically significant difference ($p > 0.05$). Conversely, no production of ROS has been detected by DCFH2-DA assay, maybe because the change of this indicator is an important tool to highlight the toxicity of many pollutants, as demonstrated by some studies on neurons or neural cells where the ROS formation and impairment of antioxidative protection measures occurred after exposure to electromagnetic fields (EMF) [21, 22]. Equally, the acridine orange staining did not show apoptosis in the body of larvae. No fluorescent intensity has been observed under fluorescent microscope.

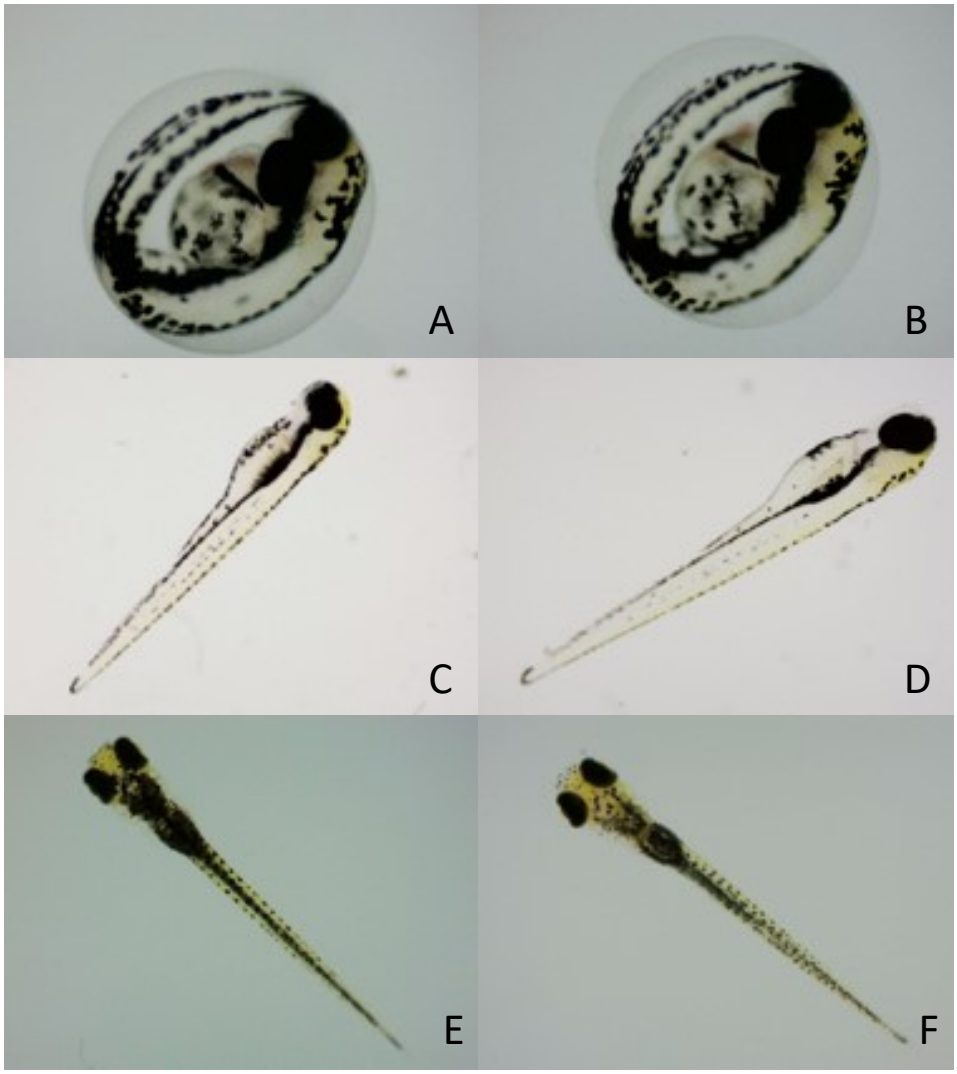

Figure 2. Phenotypes of unexposed (A, C and E) and exposed (B, D and F) larvae to 27 GHz at 48hpf (A and B), 72hpf (C and D) and 96hpf (E and F).

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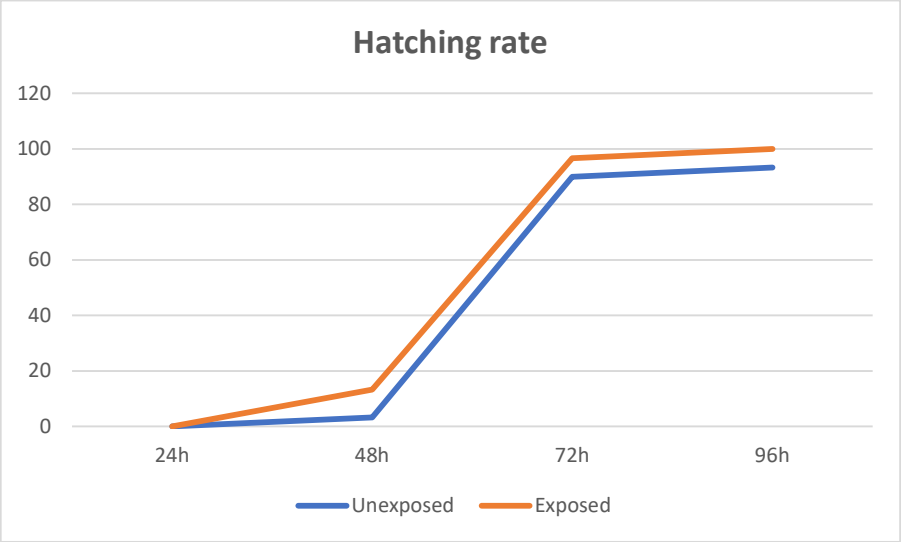
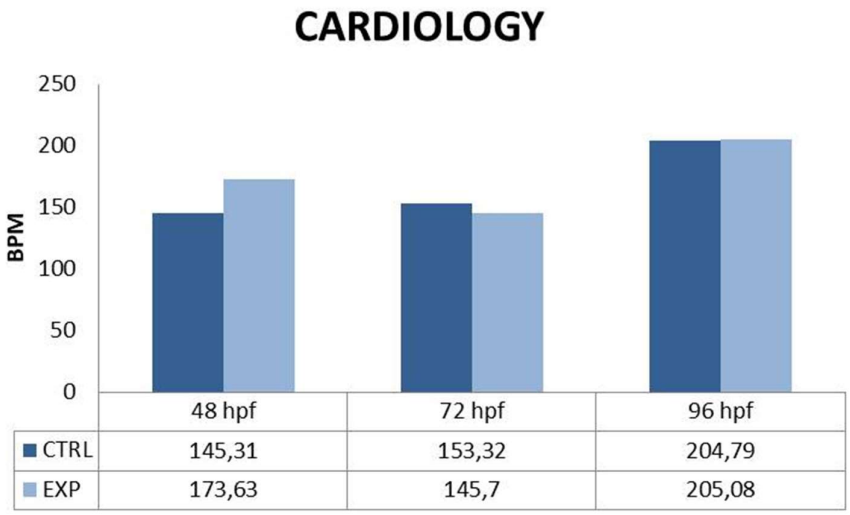


Figure 3. Hatching rate of unexposed and exposed embryos to 27 GHz.



	48 hpf	72 hpf	96 hpf
CTRL	145,31	153,32	204,79
EXP	173,63	145,7	205,08

Figure 4. Beats per minute (BPM) values of control group and exposed embryos to 27 GHz obtained by DanioScope software.

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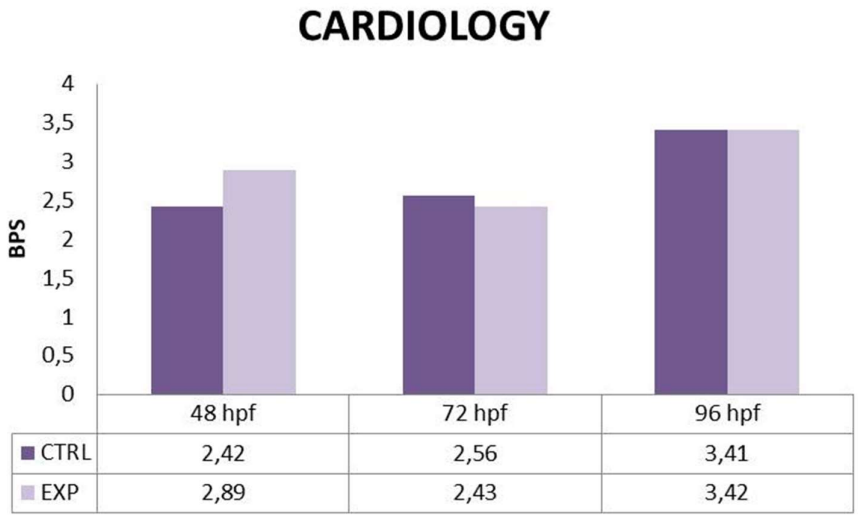

Figure 5. Beats per seconds (BPS) values of control group and exposed embryos to 27 GHz obtained by DanioScope software.

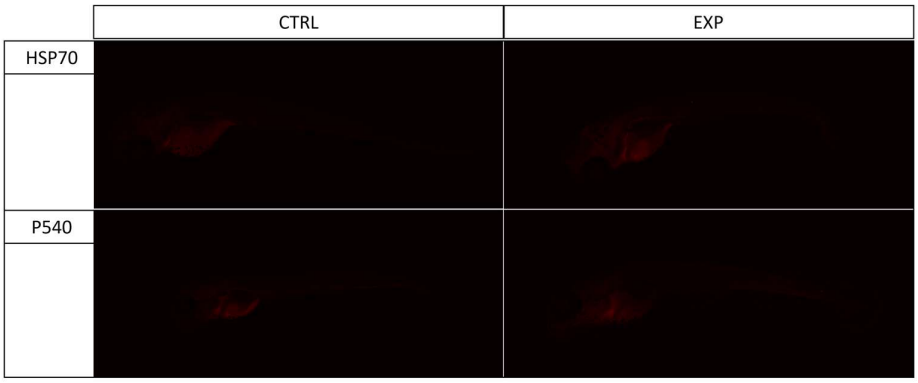

Figure 6. Immunohistochemical investigation of HSP70 and P540 markers in unexposed (CTRL) and exposed (EXP) larvae to 27 GHz.

4. Discussion

The new fifth generation technology (5G), which should favor high data speed connections (1Gbps) and latency times lower than the current ones (<1ms), has the characteristic of working on different frequency bands of the radio wave spectrum (700 MHz, 3.6-3.8 GHz and 26.5-27.5 GHz), thus also exploiting higher frequencies than previous generations of mobile radios (1G-4G). The higher frequency waves, on the other hand, have a lower ability to propagate in free space and therefore, to ensure a capillary coverage of the territory for high reliability applications, it will be necessary to install a large number of repeaters. Following the introduction of this new technology, there has been a growing concern about possible harmful effects on human health. Generally, the exposure to electromagnetic fields (EMF) has been linked to the production of reactive oxygen species (ROS) [23]. Several *in vitro* studies have shown that the production of ROS leads to cellular or systemic oxidative stress [24]; and also oxidative stress in the brain in

studies concerning laboratory animals after EMF exposure [25]. However, in addition to oxidative stress, others radiofrequency exposure endpoints can help to assess biological effect. In this context, the best animal model is the zebrafish. Commonly, zebrafish is the gold standard for evaluate the harmful effects of many xenobiotics such as chemicals compounds or nanomaterials [26], but recently it has been considered a predictive model able to evaluate radio frequency effects. For example, Kim and colleagues (2018) have investigated the pro-pigmentation effect of pulsed electromagnetic fields (PEMFs) in zebrafish model [27]. Their results suggest that PEMFs, at an optimal intensity and frequency, promote pigmentation and then PEMFs are useful tool for treating gray hair with reduced melanin synthesis in the hair shaft, or hypopigmentation-related skin disorders. Instead, other studies have investigated the effect of higher radiofrequency radiation (RFR) on neurobehavior in adult zebrafish and few studies have investigated the effects of electromagnetic fields on zebrafish embryonic development [28]. The results obtained in our investigation demonstrated that exposure to 27 GHz caused an increase of heartbeat rate on exposed embryos at 48h than control group, but this increase has not been more shown at 72-96 h. According to Piccinetti et al., (2018) [29], biological effects were no more visible at hatching time, because of the activation of specific detoxification biological pathways. Dasgupta and colleagues (2018) [28] have tried to assess whether the exposure to 3.5 GHz radio frequency radiations (RFR) is associated with any developmental perturbations during embryogenesis of zebrafish. Their results did not reveal any large-scale effects of RFR exposure on embryonic survival or development but revealed a modest depression of sensorimotor function. Therefore, our results are in accord with Dasgupta and colleagues (2018) [28], because also the higher frequency used in our study has not caused an alteration of embryonic development, neither mortality after hatching. At the light of these results and literature data, it will need to investigate the EMF dose-response effects to have an clearer overview on electromagnetic pollution.

5. Conclusions

By using embryos and larvae of zebrafish as a model system, we have shown that exposure to 27 GHz frequency does not interfere with the embryonic development of zebrafish, although an increase of heart rate was observed at 48hpf which fades at 72-96h, confirming that the first stages of development are always particularly sensitive. These results demonstrate that zebrafish is an efficient *in vivo* model system for studying the EMF effects on embryonic development. This study can be useful to investigate the potential ecological impact of the EMFs on aquatic animals, that currently are poor investigated. Future experimental studies should enrich the knowledge about EMF effects and also to understand if they can be hazardous to human health.

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