Effects of physical stresses on zebrafish samples

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Abstract
Zebrafish (*Danio rerio*) embryos were subjected to different physical stresses, consisting of a static and two low frequency magnetic fields, and two radiofrequencies. Embryos were exposed to static (Bo, $0\,Hz$), very low frequency (VLF, $0.2\,Hz$), low frequency (LF, $270\,kHz$), very high frequency (VHF, $100\,MHz$), and ultra-high frequency (UHF, $900\,MHz$) magnetic field irradiation for up to 5 days. The field intensities were $40\,mT$, $40\,mT$, $470\,\mu T$, $240\,nT$ and $240\,nT$, respectively. Untreated embryos were used as control. (n = 10 for each condition).

I. INTRODUCTION

Human population lives surrounded by electronic instruments. Electric currents origin magnetic fields and the Ampère law governs their intensity. Variable magnetic fields generate electric fields, called inducted fields and different from the electrostatic ones; they are governed by the Faraday law. Understanding the magnetic effects on live organisms may have a dual value, i.e. to understand how magnetism can influence, positively and negatively, the animal biological functions and to discover the role of magnetism on living beings behavior [1-3].

The electric field interacts with the electric charges, stopped or moving, applying a force according to the Coulomb law [4]:

$$\vec{F} = q\vec{E} \qquad (1)$$

changing the system energy. Differently, the behavior of the magnetic field consists in interacting only with moving charges, applying a force according to the Lorentz law[4]:

$$\vec{F} = q\vec{v} \times \vec{B} \qquad (2)$$

For what concerns biological matter, the charges are closely bound to atoms but are responsible of the magnetic moment of the biological matter itself. The exhibited magnetic moment of this matter is regulated by quantum mechanics and it is responsible of the ferromagnetism and paramagnetism of molecules, that are very evident phenomena. Less evident is the diamagnetism, a characteristic of all molecules, which is sensitive to the derivative of the magnetic flux due to the Lentz law. Therefore, only fields of high frequency can get to significant results.

Generally, the biological matter can be considered paramagnetic, but generally the interaction of the magnetic field with the matter applies a mechanical moment corresponding to [5]:

$$\vec{N} = \vec{M} \times \vec{B} \qquad (3)$$

where $\vec{M}$ represents the magnetic moment of matter and $\vec{B}$ the magnetic field. Inside the matter the mechanical moment causes a variation of the orientation and of the energy of the same molecule expressed according to [5]:

$$E_B = -\vec{M} \cdot \vec{B} \qquad (4)$$

The study of the living matter exposed to fields is very difficult due to the complexity of the cell membrane structure. In these cases, it is reasonable to suppose that the mechanical moment induced by the magnetic field can influence the charge transport through the membrane and the energetic state. The magnetic field could interact directly with the DNA, but this is to be discovered. Considering the only magnetic moment due to the electron whose spin is ± ½, the energy variation according to Eq. 4 can be positive or negative, that is:

$$E_B = \pm g \mu_B B m \qquad (5)$$

where g is the factor of Landé which is close to 2.00 for free electrons and for most organic radicals [6], μ_B is the magnet of Bohr, B is the magnetic field and m is the quantum constant.

It is known that on the earth a magnetic field is present and that it is very relevant for many living organisms even if its intensity is very low, about $50\ \mu T$ as well as its variation. In general, more intense fields are used in laboratory experiments and are expressed by the term of moderate field and range from a few 10^{-3} to 1 Tesla.

II. EXPERIMENTAL APPARATUSES

Different suitable experimental set ups were made, based on the use of the teleost fish zebrafish (*Danio rerio*), a widely used model of the biology of vertebrates, also in research contexts such as eco-toxicology and effects of magnetic fields [7,8]. The samples were zebrafish fertilized eggs immersed in standard egg water [9] contained in Petri cells. During the exposure the Petri dishes were fixed, and did not move, but being the fertilized eggs subjected to Brownian moto, the eventual mechanical moment changed continually, in direction and intensity. Please note that the fertilized eggs did develop to embryos and larvae during the experiment. Therefore, we measured the effects of magnetic fields in an organism which cell number has been increased and organogenesis has occurred for the time of the experiment.

II:1 Static magnetic field (Bo) irradiation

The static magnetic field is easily obtained by means of a simple magnet. In this case, we used a magnetic disc $4\ cm$ in diameter. To irradiate the samples contained in Petri dishes ($5.5\ cm$ in diameter), we placed it on the magnet on the North late. The magnetic field intensity was about $40\ mT$ at about $0.5\ cm$ from the magnet center. *Fig. 1* shows the experimental set up.

Figure 1: Photo of the static magnetic set up.

II:2 Very low frequency (VLF) magnetic field irradiation

The low frequency magnetic field is an alternation field by $0.2\ Hz$ frequency ($40\ mT$ peak). It is constructed with four static magnets made of discs of $4\ cm$ in diameter. The magnets are placed along a circumference supported by two ferromagnetic strips, (see *Fig. 2*). The support moved by a low frequency motor which frequency was of about $0.1\ Hz$.

Figure 2: Photo of VLF magnetic field set up.

II:3 Low frequency (LF) magnetic field irradiation

Variable and intense magnetic fields are difficult to to get due to the fact that we need rather high, about $10\ A$, and variable currents. Since the common RF generators are low current generators, it was necessary to create a generator *ad hoc*. Therefore, we made a solenoid with an internal diameter of about $5\ cm$ and a height of $11\ cm$ with a number of turns of 18 in order to get low inductance and low resistance to the electric current. The value of the inductance is about $4\ \mu\ H$. The inductor is coupled in parallel with a capacitor. The coupling of a capacitor with an inductor forms an oscillating LC circuit. To have a frequency $\omega = 2\pi f$ it is necessary to use a capacitor of about $C = 1/(L\omega^2)$. To reach a frequency of about $270\ kHz$ we used a capacitor of about $100\ \mu F$. The picture of the coil is in *Fig. 3*.

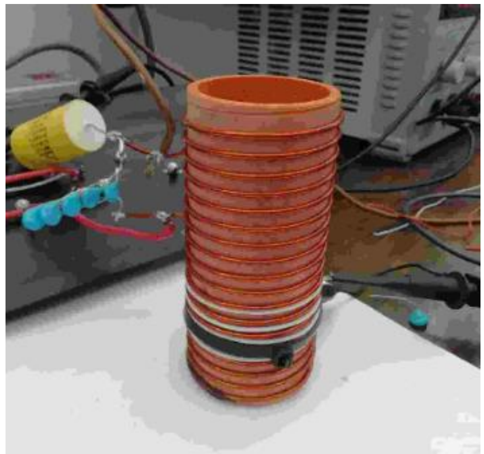

Figure 3: LF magnetic field set up.

All real LC circuits are dispersive, that is they are unable to maintain continuum oscillations. Therefore, it is necessary to restore the energy lost per cycle. To achieve this goal, the LC circuit was fed by a continuum voltage and was connected to ground by a switch circuit formed by four transistors in the emitter mode (*Fig. 4*).

All transistor gates were connected to a RF generator, which signal was in resonance with the current of the LC circuit. This signal was also used to trigger the oscilloscope.

Moreover, in order to control the magnetic field, as there are no gaussometers for magnetic fields variable to hundreds of kHz, we opted to measuring the current value in the inductor of a secondary inductor concatenated with the principal and closed on an infinite impedance. We call it probe. The latter consists of 4 turns and the attenuation coefficient depends on frequency. At $270\,kHz$ the attenuation coefficient was $A_{tt} = I_B\,/\,V_{out} \approx 0.38\ A/V$. According to the Ampère law, the magnetic field inside the inductor is controlled by the relation $B = I \cdot 206\ x\ 10^{-6}$ T. Therefore, with 6 V of output voltage V_L the maximum magnetic field was $470\ \mu T$.

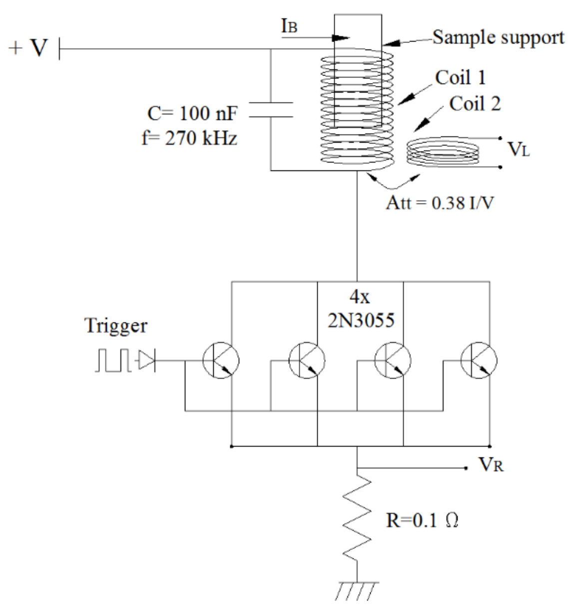

Figure 4: Electronic circuit for the oscillator.

The output signal from probe V_L is shown in *Fig. 5*. In the same figure, it is possible to see the trigger signal.

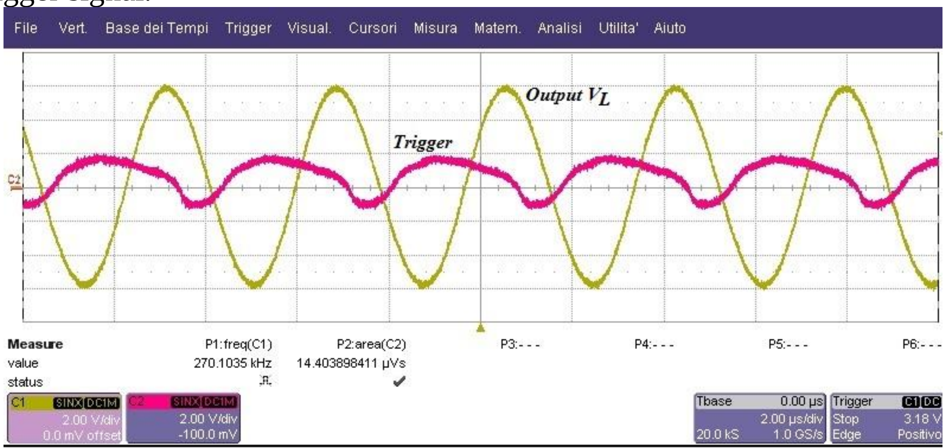

Figure 5: Waveform of the output signal V_L and of trigger signal.

II:4 Very high frequency (VHF) magnetic field irradiation

One of our goals was to carry out measurements in the range of hundred MHz and for this reason we created a transmission line. It was designed with a height $h = 1.4\ cm$, width $a = 9\ cm$ and with a length of $20\ cm$. The length of the line does not affect the characteristic impedance but allows to treat more samples. The RF generator was a RHODE & SCHWARZ SM 300. Its output power is $20\ mW$. The expression of the characteristic impedance of the flat line, excluding the external radiation and pointing out with L and C, inductance and capacitance for length unit, respectively, is looked at the following formula[10]:

$$R_o = \sqrt{\frac{L}{C}} = \sqrt{\frac{\mu_o}{\varepsilon_o}}\frac{h}{a} \tag{5}$$

where ε_o and μ_o are the electrical permittivity and magnetic permeability, respectively. From Eq. 5 it is deduced that our line has an impedance of about $50\ \Omega$ like that of the generator output impedance. As a consequence, the line input was connected to the generator via a high frequency $50\ \Omega$, while the other line output was connected to a $50\ \Omega$ utilizing 4, $200\ \Omega$ resistors (*Fig. 6*).

The wavelength at $100\ MHz$ in vacuum was about $3\ m$, while makes secure the transversal uniformity of the fields.

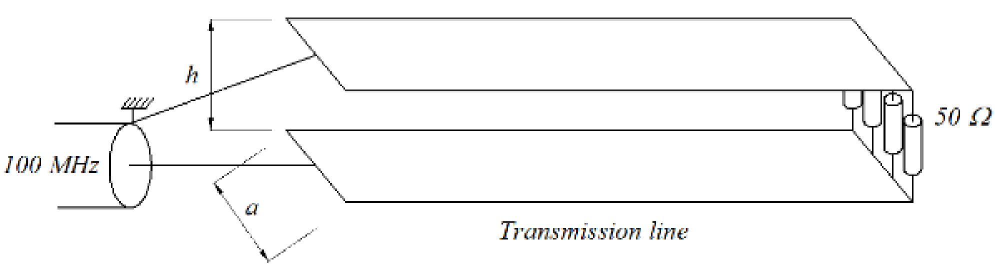

Figure 6: Plane transmission line for Very High Frequency (VHF)

The applied input signal was $1\,V$ at $100\,MHz$ and the correspondent electric field was about $71\ V/m$, while the magnetic one was about $240\,nT$.

All measurements were made with a Le Croy Wavepro 7100 fast oscilloscope, 200GS/s with 1 GHz band limitation.

II:5 Ultra high frequency (UHF) magnetic field irradiation

To perform treatments at $900\,MHz$ we used a short transmission line in order to limit the effects of stray irradiations. To preserve the characteristic impedance of the system, the dimensions of the line were again $h = 1.4\,cm$, $a = 9\,cm$, while the length was $12\,cm$. The RF generator was a RHODE & SCHWARZ SMF 100A having an output power of 20 W. The expression of the characteristic impedance of the flat line is express by $Eq.\,5$ excluding the external radiation.

The wavelength at $900\,MHz$ in vacuum is about $0.33\,m$. So, to limit the irradiation, it is necessary to modify the conductors bending the lateral outline and the final line width is about $0.10\,m$ (*Fig. 7*).

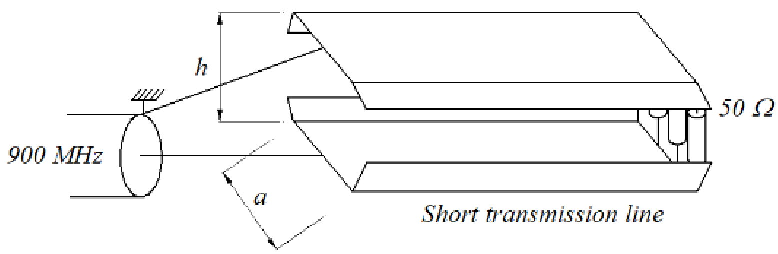

Figure 7: Short transmission line for Ultra High Frequency (UHF).

The applied input signal was again $1\,V$ and the correspondent electric field was about $71\ V/m$, while the magnetic field was again about $240\,nT$.

All measurements were made with a Le Croy Wavepro 7100 fast oscilloscope, 200GS/s with 1 GHz band limitation.

II:6 Zebrafish maintenance and treatments

Adult zebrafish were maintained in plastic tanks with fresh water at 27-28 °C. Fishes were subjected to a constant 14 h light:10 h darkness photoperiod and fed daily.

Fertilized eggs were collected after natural spawning; then, they were transferred in Petri dishes with egg water [9].

Within 12 hours post fertilization (hpf), developing embryos were exposed to static (Bo, $0\,Hz$), very low frequency (VLF, $0.2\,Hz$), low frequency (LF, $270\,kHz$), very high frequency (VHF, $100\,MHz$), and ultra high frequency (UHF, $900\,MHz$) magnetic field irradiation. At the same time, untreated embryos were utilized as control (n=10 for each condition).

Zebrafish embryos/larvae were maintained under the magnetic fields and radio frequencies for 5 days. At the end of the experiment, treated larvae were anesthetized in MS-222 and observed at low magnification (4X, 8X) using a Nikon AZ100 stereomicroscope equipped with the Nikon NIS-Elements D suite for image capture.

For each group, morphological abnormalities and movement behavior (activity and type of movement, i.e. straight lines or in circles) were analyzed.

III. RESULTS

At the end of the experiment, we observed growth retardation in each group of larvae: larvae came out of the chorion at 5 days post fertilization (dpf) and their anatomy appeared similar to 3 dpf zebrafish larvae but with evident cardiac edema, *Fig. 8*.

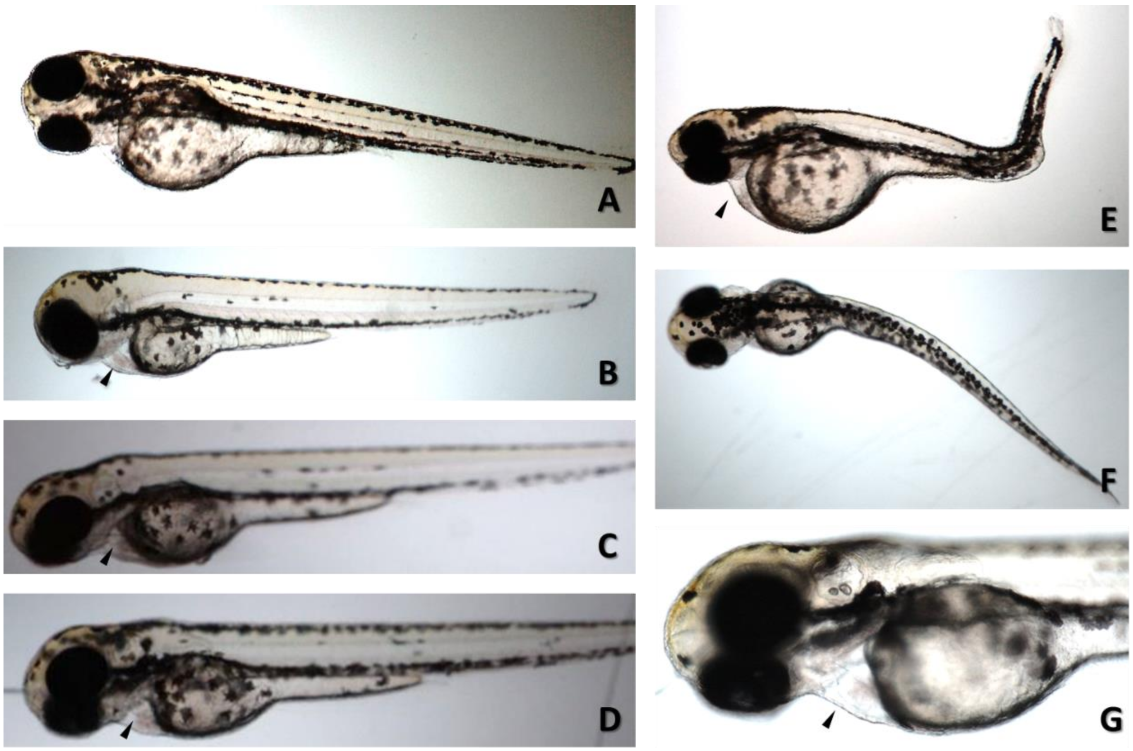

Figure 8: Effects of magnetic field irradiations on zebrafish larvae. A: untreated control larvae; B: very low frequency ($VLF, 0.2\ Hz$) exposure; C: very high frequency ($VHF, 100\ MHz$) exposure; D: ultra high frequency ($UHF, 900\ MHz$) exposure; E: low frequency ($LF, 270\ kHz$) exposure; F: static ($Bo, 0\ Hz$) exposure; G: static ($Bo, 0\ Hz$) exposure, detail of the rostral region. Black arrowheads depict the cardiac edema occurring in all treatments. Stereoscope magnification 4X except for G (8X).

The number (percent) of survivor and non-survivor larvae is shown in *Table 1*.

To characterize the effect of the magnetic fields and radio frequencies on the development of the somatomotor system, we performed a movement analysis on zebrafish larvae at 5 dpf by means of the touch-and-response test (*Table 2*). Compared to controls, zebrafish larvae exposed to static magnetic fields showed reduction in swimming activity and circling motion associated – in the 25% of larvae – at hyperactivity and twirching. In addition, a static magnetic field caused the appearance of malformations in the spine development and the larvae appeared curved (*Fig. 8F*).

Table 1: Percentage of survival zebrafish larvae at 5 dpf after exposure to magnetic fields and radio frequencies.

	Total observed	*Total failed*	*Total censored*
Control	100%	60%	40%
Bo, 0 Hz	100%	20%	80%
VLF, 0.2 Hz	100%	20%	80%
LF, 270 kHz	100%	60%	40%
VHF, 100 MHz	100%	20%	80%
UHF, 900 MHz	100%	40%	60%

The exposure to the ($VLF, 0.2\ Hz$) fields had less effect on the locomotive behavior: 75% of larvae had normal movements, the remaining 25% showed hypolocomotor activity.

The effects of radio frequencies were proportional to their intensity. All larvae exposed to $LF, 270\ kHz$ had normal movement, nevertheless some evident malformations occurred in terms of spinal curvatures (*Fig. 7E*). Instead, ($VHF, 100\ MHz$) and ($UHF, 900\ MHz$) caused an abnormal swimming behaviour consisting of hypolocomotion activity, slow swim speed and failure to maintain posture, even in the absence of spinal curvatures (*Fig. 7 C,D*).

The results herein indicated the damaging impacts of magnetic fields and radio frequencies on the biology of zebrafish in the early embryonic/larval stages, with potential involvement of the dynamics of neurodevelopment.

Table 2: movement behaviour of zebrafish larvae at 5 dpf exposure to magnetic fields and radio frequencies.

	Spontaneous movement	*Hypolocomotor activity*	*Twirching*
Control	100%	-	-
Bo, 0 Hz	-	75%	25%
VLF, 0.2 Hz	75%	25%	-
LF, 270 kHz	100%	-	-
VHF, 100 MHz	25%	75%	-
UHF, 900 MHz	-	100%	-

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