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RESEARCH ARTICLE

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Effects of non-ionizing radio frequency electromagnetic radiation on the development and behavior of early embryos of *Danio rerio*

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ABSTRACT

Biological effects of radio frequency electromagnetic radiation (RF-EMR) in the range of 900–1800 MHz emerging from the mobile phone were investigated and were found to influence the locomotor pattern when exposure was initiated from 1 hour post fertilization (hpf) in zebrafish embryos (ZE), *Danio rerio*. Mobile phones and other wireless devices offer tremendous advantages. However, on the flipside they are leading to an increased electromagnetic energy in the environment, an excess of which could be termed as electromagnetic pollution. Herein, we tried to understand the effects of RF-EMR emerging from the mobile phone, on the development and behavior of ZE, exposed to RF-EMR (specific absorption rate of 1.13 W/kg and 1800 MHz frequency) 1 hr daily, for 5 days. To understand if there could be any developmental stage-specific vulnerability to RF-EMR, the exposure was initiated at three different time points: 1hpf, 6hpf and 24hpf of ZE development. Observations revealed no significant changes in the survival rate, morphology, oxidative stress or cortisol levels. However, statistically significant variations were observed in the batch where exposure started at 1hpf, with respect to locomotion patterns (distance travelled: 659.1 ± 173.1 mm Vs 963.5 ± 200.4 mm), which could be correlated to anxiety-like behavior; along with a corresponding increase in yolk consumption (yolk sac area: 0.251 ± 0.019 mm^2 Vs 0.225 ± 0.018 mm^2). Therefore, we conclude that RF-EMR exposure influences the organism maximally during the earliest stage of development, and we also believe that an increase in the time of exposure (corresponding to the patterns of current usage of mobile phones) might reveal added afflictions.

PLAIN LANGUAGE SUMMARY

Mobile phones and other wireless devices are on a rampant usage worldwide. They work by radiating low energy radiofrequency electromagnetic radiations. An excessive usage of wireless devices is leading to increased presence of these radiations in our surroundings. Since these radiations are not physically sensed by the organisms, its impact stays elusive. Nevertheless, the interaction of these radiations with biological systems may produce some unwarranted effects. When we exposed the ZE to the mobile phone radiation daily 1hr for 5days, our observations revealed that the youngest of the experimental group showed susceptibility. The effect was evident through haphazard movements and stressed behavior. So, it is important to be aware of the potential effects and take necessary precautions by following safety guidelines, especially when the organism is in its early life stage.

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KEYWORDS
Non-ionizing RF-EMR; zebrafish embryo; mobile phone radiation; cortisol; neurobehavior

Introduction

Today, while we highly applaud the accomplishments brought through the gadgets of technology and communication, we even need to ponder upon the consequences of their prolific usage, especially wireless technologies. Wireless communication has gone through a vast advancement in terms of its generation technology from 1 G to 5 G. From 1 G using a frequency of 824–894 MHz to 4 G operating at a range of 2100 MHz, the frequency band has noticeably increased. The current 5 G operates at a frequency band of 2–8 GHz. The biological effects of non-ionizing RF-EMR have drawn considerable scientific scrutiny during the past two decades (Havas 2017). The epidemiological studies were complemented by *in-vivo* and *in-vitro* studies that explored the biological effects of electromagnetic fields (Phillips et al. 2009). Among the biological effects elicited by RF-EMR exposure, the onset of oxidative stress, cell death, reproductive decline and even cancer induction were shown in most of the studies conducted on both mammalian and non-mammalian species (Panagopoulos 2019; Piccinetti et al. 2018). Nevertheless, some studies reported no effects or minimal effects in organisms exposed to RF-EMR (Dasgupta et al. 2020). However, the radiofrequency exposure given at a specific

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stage in an organism's life, the duration and intensity of the exposure might as well bring in variable effects (Bartosova et al. 2021). The consequences of such exposures especially need a very meticulous monitoring when the organism is in its very early stage of life, i.e., embryonic development; for the reason that, all the systems are yet naive and still developing at that point of time and could be easily prone to environmental stress. Some studies have reported high mortality rate and teratogenic effects in chick embryos (Augustianath et al. 2023; Batellier et al. 2008) and asynchronous growth and mortality in frog tadpoles (Balmori 2010). Also (Koohestanidehaghi et al. 2023), have reported oxidative stress, delayed development and decrease in blastocyst viability in mouse preimplantation embryos exposed to RF-EMR. The absorption of RF-EMR by an organism relies on its cellular water content, ions, and biochemical composition (Christ et al. 2010), which keeps changing as the organism matures. Studies indicating the embryonic stage-specific vulnerabilities to RF-EMR are limited. We therefore selected ZE of three different stages, i.e. 1hpf (prior to midblastula transition, maternal gene expression stage), 6hpf (during midblastula transition stage) and 24hpf (active zygotic gene transcription stage) to understand the developmental stage-specific vulnerability to RF-EMR.

In a review by (Bartosova et al. 2021), the importance of accurate setting of RF-EMR source and description of dosimetry were mentioned. A devised RF-EMR set up in the laboratory could be programmed to emit a specific radiofrequency in regular pulses. This could be used to understand the effects with respect to a specific EMR frequency. However, such an exposure will be quite different from the real-world exposure, as that from a mobile phone. The RF-EMR emerging from a mobile phone is considered to be relatively more bioactive due to its extreme and unpredictable variability (Panagopoulos et al. 2015). Therefore, we chose to use a mobile phone directly as a source of radiation in our experiment.

Methodology

Study model

Wild-type zebrafish were obtained from a certified local supplier, identified and separated into male and female sex, fed and maintained as per the standard laboratory protocols. Breeding was accommodated shortly after dawn. Eggs when laid, were siphoned and rinsed thrice in the standard embryo medium and were transferred to a 6 well plate.

The protocols followed herein were approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (registration number 827/GO/Re/S/04/CPCSEA).

RF-EMR exposure setup

ZE ($n = 200$) were placed in 6-well plate with embryo medium. Care was taken that there were no sources of electromagnetic radiations inside or in close proximity, which was regularly monitored through an Electromagnetic Field Tester (Smiledrive). Temperature of the embryo medium and humidity of the room were kept under constant monitoring.

The embryos were divided into five batches – B1, B2, B3, B4 and B5. B1 was control batch, isolated in Faraday cage to prevent exposure to any kind of RF-EMR. B2 served as sham batch, housed within Faraday cage setup mirroring the experimental conditions, but with a switched off mobile device. This was to check whether the presence of the device in close vicinity could cause any effect in the embryos. The three batches B3, B4 and B5 were separately exposed to RF-EMR initiated at three different timepoints: 1hpf, 6hpf and 24hpf respectively (Figure 1). Exposure was given for a period of one hour per day for five consecutive days.

For the exposure, a 4 G mobile phone with SAR (specific absorption rate) value of 1.13 W/Kg and 1800 MHz

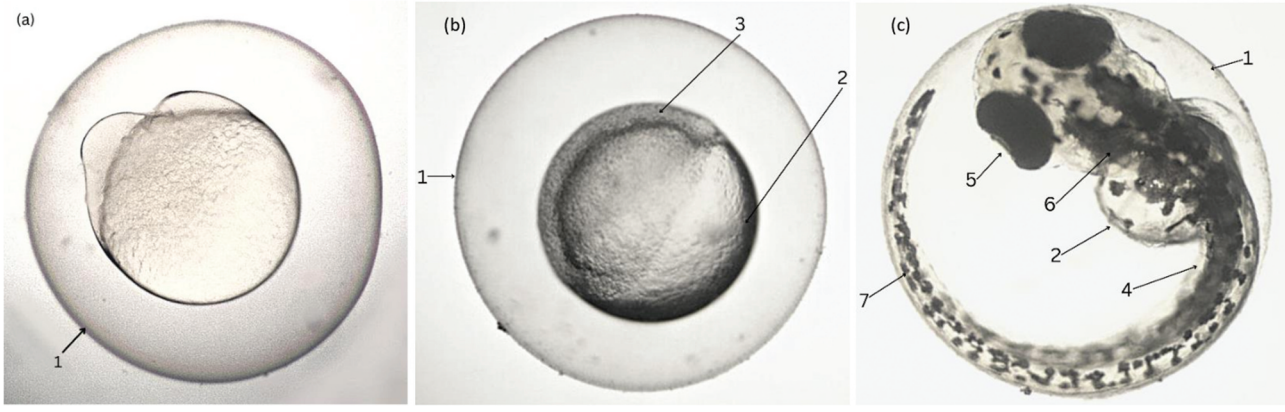


Figure 1. ZE stages (a) B3: 1 hpf (b) B4: 6 hpf (c) B5: 24 hpf. 1-chorion, 2-yolk, 3-germ ring in shield stage (50% epiboly), 4-yolk extension, 5-lens, 6-otic vesicle, 7-melanin pigment.

frequency was used. The mobile phone was set on silent mode with no vibration and was placed on two plastic tripods at the distance of 16 mm from a 6-well plate.

Survival and hatching rate

Every day, from 0hpf to 6dpf the number of dead or live embryos were noted and separated. Hatching rate was monitored from 48-72hpf.

Morphological measurements

Images of 5dpf larvae from all the batches were captured under a compound microscope with the help of Catymage software. Standard length (SL), yolk sac size (YSS), eye length (EL) and eye width (EW) were measured, where the SL was to the nearest 1 mm, yolk sac size was measured to the nearest 0.1 mm (Figure 2).

Locomotion behavior analysis

On 6dpf, 10 ZEs from each batch were carefully transferred to a petri plate, acclimatized for 30 min. The central area of 4 cm diameter of the petri plate is marked as the central zone and the rest as the peripheral zone. Their movements were recorded for 5 min (Sony 9.2MP handycam). The total distance travelled by individual embryo as well as the distance covered in the central and peripheral zones were analysed with the help of Panlab SMART v3.0 software, free version (Harvard Apparatus, US). The readings were then used to interpret the behavior type, i.e., normal or stress/anxiety (Norton 2013).

Stress analysis

The 6dpf control and exposed embryos were analysed for Catalase, Glutathione-S-transferase activity and lipid peroxidation by microplate method (Domingues and Gravato 2018). Control, sham and different groups of exposed embryos as separate batches (pool of 10) were rinsed in embryo medium, homogenized, and centrifuged at 9000×*g* for 20 min at 4°C to isolate the post-mitochondrial supernatant (PMS). The supernatants were used for immediate analysis of CAT and GST activities. CAT activity was assessed by monitoring H_2O_2 decomposition at 240 nm, while GST activity was determined by observing glutathione conjugation with CDNB at 340 nm. For LPO, in homogenized sample, TCA and TBA were added, followed by centrifugation at 6000×*g* for 10 min at 25°C. The level of LPO were assessed by measuring the thiobarbituric acid-reactive substances (TBARS) at 535 nm. The protein concentration was measured by the Bradford's method. For measurement of cortisol levels, the samples were prepared as per (Alderman and Bernier 2009) and analysed using the cortisol kit (Salimetrics Newmarket, UK).

Statistical analysis

Statistical analysis was done using GraphPad prism (9.3.1 version). For the analysis of various morphological, behavior endpoints and other estimations, one-way ANOVA followed by Dunnette's post-hoc test with 95% confidence interval was performed. For survival rate and hatching rate, binary responses were computed.

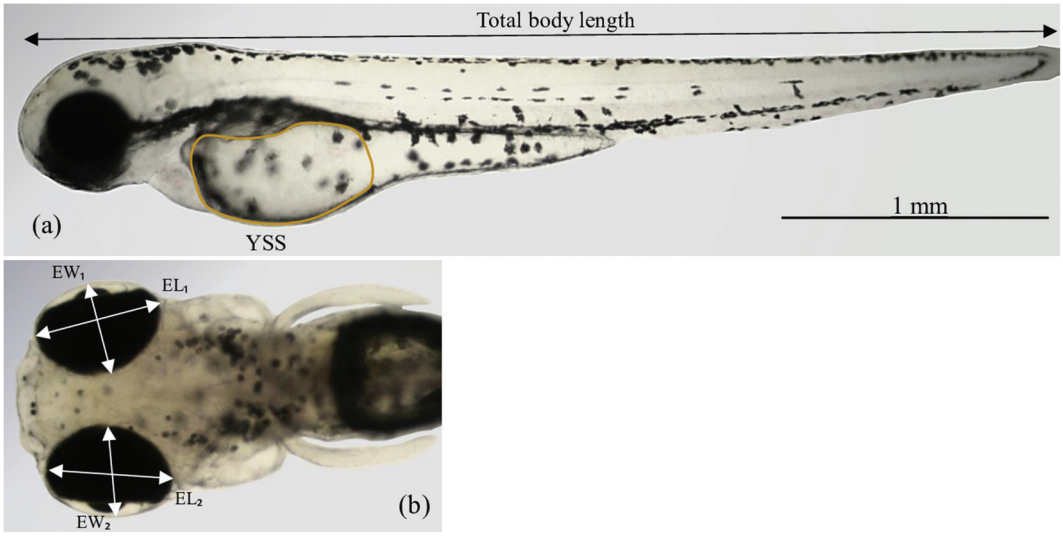


Figure 2. Morphological measurements (a) total body length and yolk sac size (YSS) (b) eye length (EL) & eye width (EW) on 5dpf.

Results

The results were analysed by comparing the B1 with each of the other batches.

Survival and hatching rate

The results showed that there was a slight decline in survival rate in B3 relative to the control on 2dpf. However, no statistically significant results were obtained with respect to survival or hatching rates in the control and RF-EMR exposed batches (Figure 3).

Morphological assessments

No significant changes in standard body length were observed in the exposed batches relative to the control. However, a significant reduction was observed in the YSS in B3 ($p < 0.05$), while in B2, B4 and B5, YSS had no significant variations. The eye sac width and eye sac length of all sham and exposed batches showed no significant variations relative to control (Figure 4).

Locomotion behavior analysis

The total distance covered by B3 was significantly more (963.5 mm) than B1, B2, B4, or B5 ($p < 0.05$). Also, in the centre of the petri plate, B3 significantly swum more than the other batches ($p < 0.05$) (Figures 5 and 6). Nevertheless, we also observed that few of the B3 larvae were found with limited motility and spent more time along the walls of the petri plate.

Stress analysis

No significant changes were recorded in catalase, GST activity, lipid peroxidation or cortisol levels in RF-EMR exposed batch relative to control batch (Figures 7 and 8).

Discussion

RF-EMR exposure in ZE had no significant effects on survival or hatchability as per few earlier reports (Dasgupta et al. 2020; Fey et al. 2019). However, on continuous exposure to 100 MHz from 0hpf to 72hpf, ZE showed delay in embryonic development (Piccinetti et al. 2018). In our study, no major effects on embryonic development or behavior were noticed, except in B3. We noted a highly significant decrease in the YSS in B3 along with a significant increase in locomotion. This could be due to relatively faster utilisation of the yolk to generate energy for their increased locomotory movements. Locomotion patterns or neurobehavior is often used to assess anxiety and stress. The significantly altered locomotory pattern of B3 larvae in our study could be considered as an anxiety-like behavior driven by the effects of RF-EMR on sensorimotor development. Similar results were stated in an earlier study (Dasgupta et al. 2020) where subtle sensorimotor effects were reported as a consequence to RF-EMR exposure to ZE. Kim et al. (2019) have reviewed several studies wherein RF-EMR induced changes in neuronal cells, neurotransmitter levels, electrical activity of the brain and lead to cognitive impairment. Such effects on the central nervous system could be a possible explanation to the neurobehavioral changes we observed. Apart from anxiety-like behaviour with increased locomotion, we also observed a few B3 larvae with limited locomotion and restriction to the peripheral area. We suspect these larvae as those which were maximally affected, energy exhausted and showed a depressed behavior. Similar results were reported earlier (Nirwane et al. 2016), where RF-EMR exposed zebrafish showed decreased locomotion and preferred the peripheral area of the petri plate.

Further, maintaining redox balance is crucial for internal homeostasis. Any disruption in this balance

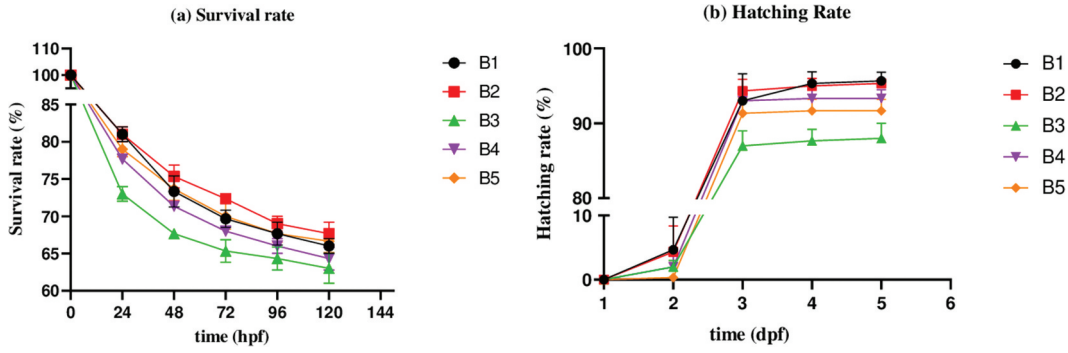


Figure 3. (a) Survival rate (b) hatching rate, B1-control; B2-sham; B3-exposure initiated at 1 hpf; B4- exposure initiated at 6 hpf; B5-exposure initiated at 24 hpf.

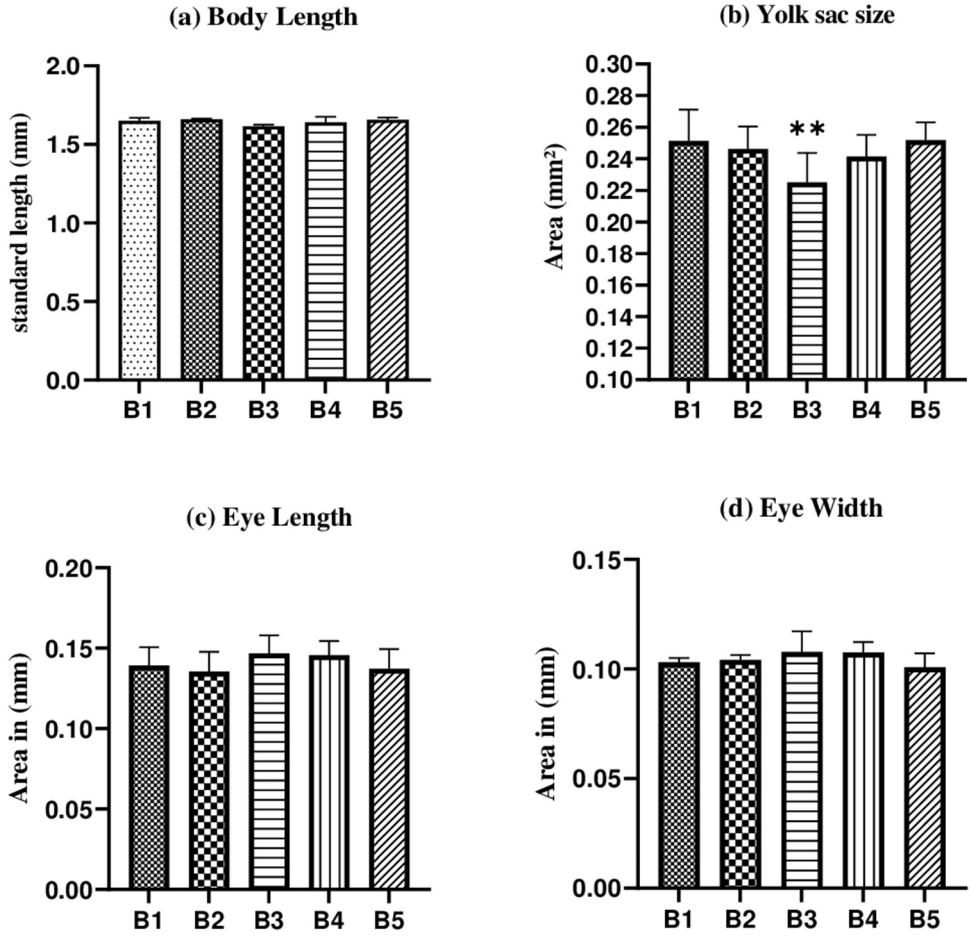


Figure 4. (A)body length, (b)yolk sac-size (YSS), (d)eye length (EL) and (e)eye width (EW) measured on 5dpf. Data expressed as mean ±SD ($n = 10$/batch). $^{**}p \leq 0.01$, (one-way ANOVA+ Dunnette's post-hoc test).

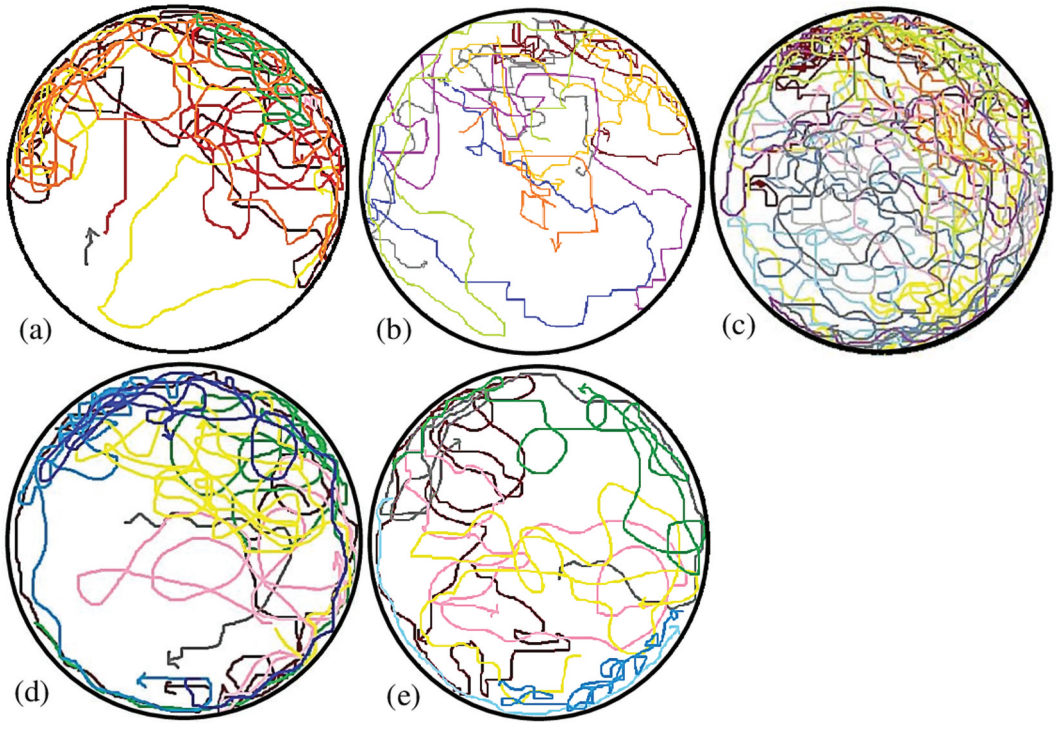


Figure 5. Trajectory images 6dpf ZE. B1 (a), B2 (b), B3 (c), B4 (d) and B5 (e). Locomotion trajectory of individual larva is indicated by a distinct color. $n = 10$.

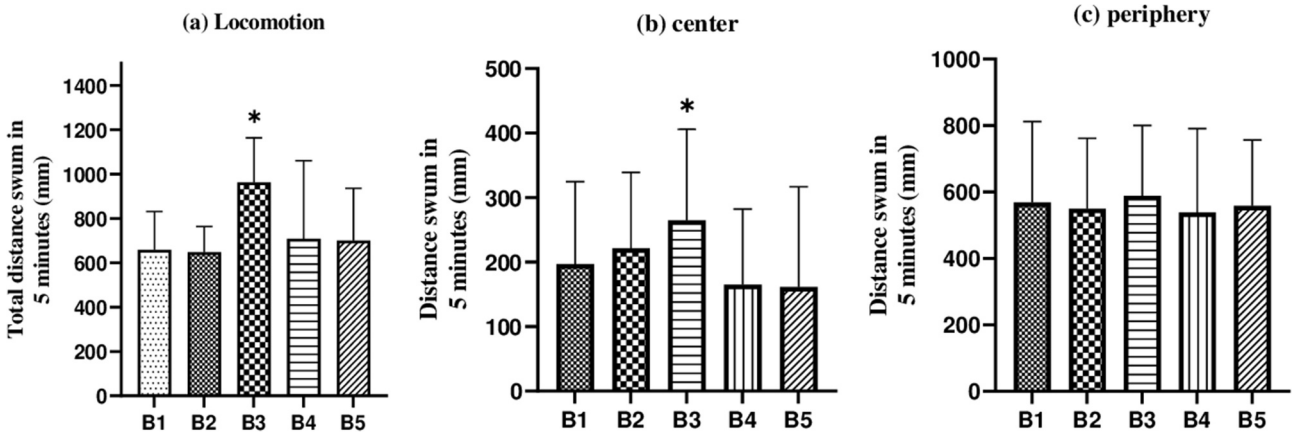


Figure 6. Locomotion behavior (a) total distance travelled, (b) distance covered in central zone, (c) distance covered in peripheral zone. Data expressed as mean±SD, $n = 10$, *$p \leq 0.05$ (one-way ANOVA+ Dunnette's post-hoc test).

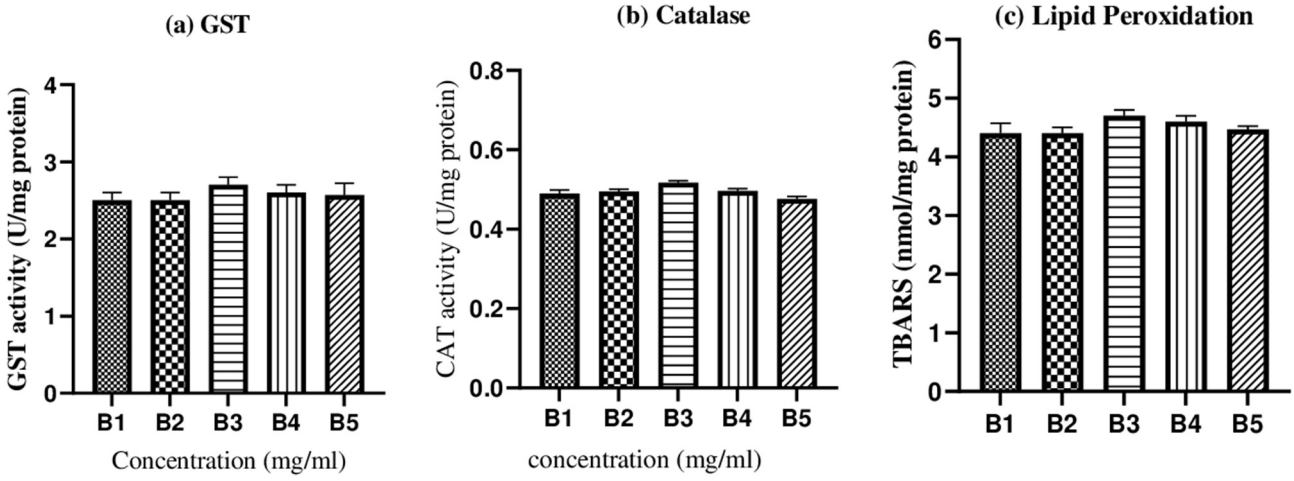


Figure 7. Oxidative stress (a) GST activity (b) catalase activity (c) lipid peroxidation levels. Data expressed as mean±SD, $n = 10$.

due to oxidative stress can have detrimental consequences on the developing embryo. The RF-EMR were reported to induce oxidative damage in zebrafish embryos and adults (Nirwane et al. 2016; Piccinetti et al. 2018), and in rats (Dasdag et al. 2012). However, we found no significant changes to indicate oxidative stress in our study. Also, it is possible that, at the RF-EMR exposure level and duration of our study, the oxidant–antioxidant balance could be maintained by the ZE.

Exposure to 900 MHz RF for 1 hr/day for 50 days, in Syrian hamsters (Shahryar et al. 2009) resulted increased cortisol levels. In another study, EMF exposure from a smart phone led to increased cortisol levels in human females (Schneider 2022). Also, in a review article (Singh and Neeru 2014), several mentions were made with respect to increased cortisol levels subsequent to EMR exposure. While in some other studies no effects (Eskander et al. 2012) or decrease in cortisol

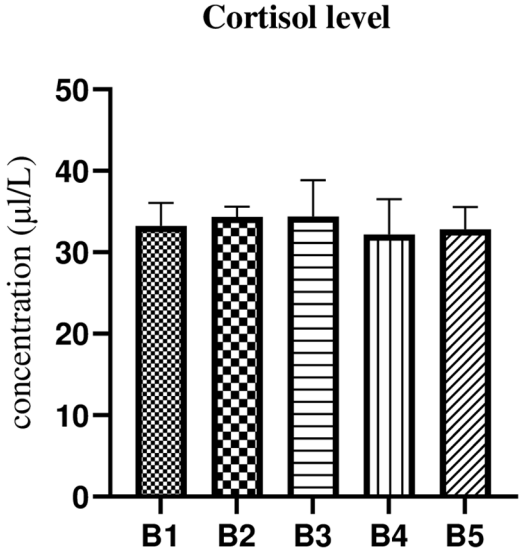


Figure 8. Whole body cortisol levels. Data expressed as mean±SD, $n = 30$.

levels (Djeridane et al. 2008) were reported. In our study, no significant alterations in cortisol levels were observed in any of the exposed batches. The probable reasons to these ambiguities in the effects could be due to the differences in radiation device settings and variations in the dosimetry.

To conclude, our study did not reveal any large-scale effects of EMF on embryonic survival or development but showed significant changes in the pattern of locomotion in the batch where exposure was initiated at the earliest, i.e., 1hpf, which can be correlated to stress or anxiety-like behavior. The level of absorption of RF-EMR by the biological systems depends not only on the frequency and strength of the radio waves but also on its own water and ion content as it leads to change in the dielectric property or conductivity and therefore the absorption levels of the RF energy (Christ et al. 2010). During the early developmental stages of an organism, the content of water and ions and therefore the whole-body electrical conductivity would be high, leading to absorption of more RF radiation (Belyaev 2005). Hence, the early embryonic stages could be the most vulnerable periods of life to the of RF-EMR exposure. In agreement with this, we observed that B3 was the effected batch in our study where exposure was initiated at 1hpf. If an exposure time of 1 hr for 5 days could show effects, the possibility that prolonged periods of exposure may produce detrimental effects cannot be denied and needs scrutiny with prominence. Therefore, further studies with increased dose and exposure time (corresponding to the average mobile phone usage by an individual) to understand the most susceptible groups is highly suggested.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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