

EARLY-LIFE EXPOSURE TO 5G ELECTROMAGNETIC RADIATION ALTERS LOCOMOTION AND STRESS MARKERS IN ZEBRAFISH EMBRYOS

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ABSTRACT

The fifth generation (5G) wireless communication technology which is being globally deployed, operates in the frequency range of 3.5 GHz to 28 GHz. Given the extensive and increasing use of mobile phones day by day, their effect on the health of all living organisms needs to be carefully monitored. Here, we examined the impact of 5G mobile phone EMR exposure on zebrafish embryos, specifically the possibilities of stress and cortisol changes during development. Zebrafish embryos, in three separate groups were exposed to mobile phone EMR starting from 1, 6 and 24-hour post fertilization (hpf) for 7days, with daily exposure period of 6 hours. The results revealed significant alterations in locomotion patterns in two batches of embryos in which EMF exposure started before 24hpf. Also, a raise in levels of glutathione S-transferase, lipid peroxidation and cortisol were observed. A significant reduction in the level of acetylcholine esterase was also noted. However, while assessing the impact of EMF on primary motor neuron development, the axonal lengths showed no significant alteration. Also, no significant morphological variations were observed. To conclude, EMF exposure at the current intensity of our study induces stress and neurobehavioral changes only when initiated prior to 24hpf. The same exposure when started from 24hpf onwards has shown no significant alteration in the parameters observed. Although no overt developmental damage was detected under the present studied conditions, the potential for subtle, long-term biological impacts on development need to be closely monitored especially in the early stages of development.

Keywords: 5G; Electromagnetic radiation; Zebrafish embryo; Locomotion behavior; Motor neuron

INTRODUCTION

The rapid proliferation and ubiquity of mobile phones and wireless devices has heightened concerns regarding the potential adverse effects of radio frequency electromagnetic radiation (RF-EMR) on biological systems. As Telecom Regulatory Authority of India (TRAI) reported Government of India (GOI) in 2024, India alone had 1.06 billion mobile phone users. A study conducted by International Telecommunication Union (2023) reported that 5.4 billion people were using internet globally, showcasing the increasing dependence on digital connectivity. Since the advent of 5G wireless technology, operating in frequencies ranging from 3.5 GHz to 28 GHz, has intensified, a rigorous scientific evaluation is imperative.

Previous studies have demonstrated various physiological and neurological effects of RF-EMR exposure. A review mentions that the interaction of EMFs with biological tissue, especially at higher frequencies or intensities, can influence the movement of ions across cell membranes. The ion gradient is critical for maintaining cell function, signaling, and overall tissue health. Any disruption to this balance could therefore lead to potential consequences (Kivrak *et al.*, 2017). As EMFs can alter intracellular calcium dynamics, independent of membrane-bound ion channels, leading to calcium ion oscillations (Luo *et al.*, 2014). A study reported that EMF can alter neural function in the human brain (Croft *et al.*, 2002). Experimental evidence from rodent models suggests that prolonged exposure to 900 MHz EMR leads to neurobehavioral deficits, including impaired spatial memory and increased permeability of the blood-brain barrier (Tang *et*

al., 2015). A study on murine models exposed to 2.4 GHz EMR modulated by 100 Hz square pulses reported heightened wakefulness, indicating potential neurophysiological disruptions (Liu *et al.*, 2021). Another study revealed that the electromagnetic radiation can cause variations in immune system by altering the adhesion ability of peripheral blood mononuclear cells (Atasoy *et al.*, 2009). According to a report, intermittent exposure to 5G (3.5 GHz) signals resulted in altered basal activity of HSF1 in human keratinocytes and fibroblasts (Joushomme *et al.*, 2023). Similarly, exposure to 2100 MHz EMR in human amniocytes altered gene expression related to neurogenesis and Wnt signaling pathways, suggesting potential developmental consequences (Uzun *et al.*, 2023).

Despite increasing evidence of RF-EMR-induced biological alterations, studies on the developmental impact of 5G EMF remain limited. The zebrafish (*Danio rerio*) serves as an established vertebrate model in developmental biology due to its rapid external embryogenesis, transparency, and well-characterized neurobehavioral responses. While our previous study has explored the effects of RF-EMR from 4G mobile phone (Khira and Uggini, 2024), our current study focuses on the specific physiological consequences of RF-EMR exposure from 5G mobile phone during early embryonic development. Here, we have selected 7 days post fertilization for the analysis because it represents established behavioral responses and developed sensory motor functions (Basnet *et al.*, 2019; Joseph *et al.*, 2020). Through a comprehensive analysis of morphological, behavioral, and biochemical endpoints, this study aims to explore the effects of RF-EMR on embryonic development. It addresses gaps in current knowledge with respect to the consequences of early-life EMF exposure, thereby offering essential data for public health considerations and environmental safety regulations.

MATERIALS AND METHODS

Study model

Wild type zebrafish were procured from a certified supplier and maintained and bred as per standard laboratory protocol by the guidelines mentioned in CCSEA, Registration number - 827/GO/Re/S/04/CCSEA. Protocols followed in the current study were approved by the Institutional Animal Ethics Committee (IAEC No. Z/IAEC-3/10-2019) of the Department of Zoology, Faculty of Science, The Maharaja Sayajirao University of Baroda.

RF-EMR exposure setup

The RF-EMR exposure setup was adapted from our previous study (Khira & Uggini, 2024), with modifications to accommodate 5G EMR exposure through a mobile phone with 1.31W/kg specific absorption rate (SAR) value. Zebrafish embryos (n=200) were kept in 6-well plate containing embryo medium. To ensure the absence of background electromagnetic radiation interference, an Electromagnetic Field Tester (Smiledrive) was used to monitor the experimental room. The mobile phone used for exposure was placed in silent mode (with vibration off) and positioned 16 mm from the 6-well plate. The control group (B1) was kept in an isolated room without any kind of EMR radiations. The sham group (B2), was kept in a similar setup with a switched off mobile phone (6h/7 days). In order to investigate whether the effects of RF-EMR exposure vary depending on the developmental timeline, the exposure was timed and initiated at three distinct stages of development: 1hpf (B3), 6hpf (B4), and 24hpf (B5). These time points were selected to represent different phases of embryonic development, allowing for an assessment of potential vulnerabilities at various stages of growth and differentiation. After completion of 7days of exposure, the assessments were made through the methods mentioned below.

Cardiac rate measurement

To assess the 5G mobile phone electromagnetic radiation effects on cardiac system, the cardiac rate of zebrafish embryos was calculated at 7dpf, with a sample size of 10 in each group. Atrial and ventricular contractions of the heart were observed and recorded under a microscope for a duration of 1 minute. The resulting data was used to create a graph illustrating the average heart rate, measured in beats per minutes (bpm).

Locomotion behavior analysis

10 zebrafish embryos from each group were pooled into a petri plate and their movements were tracked for 5 minutes through video recording. An individual embryo's total distance travelled (in millimeters) was measured (Norton, 2013). The locomotion tracking was done with the help of Panlab Smart v 3.0 software (free version) (Harvard Apparatus, US).

Analysis of oxidative stress and cortisol levels

To assess oxidative stress, Glutathione-S-transferase activity, Catalase activity and Lipid peroxidation levels were analyzed using microplate method (Domingues and Gravato, 2018). 10 embryos from each batch were taken for analysis. The cortisol levels were determined using the Human salivary cortisol kit (Salimetrics New market, UK) with a pool of 30 embryos for each group.

Determination of Acetylcholinesterase Activity

The levels of AChE activity were assessed as per the protocol (Gravato, 2021), the embryos (n=10) from each batch were euthanized and homogenized. The methodology involves combining the sample suspension with a reaction mixture of DTNB (5,5'-Dithiobis (2-nitrobenzoic acid) in potassium phosphate buffer (pH 7.2). This mixture was incubated at 25°C for 20 minutes. Following the incubation, acetylcholine iodide was added to the mixture, and the absorbance shift at 412 nm was measured using a microplate reader at 1-minute intervals. Enzyme activity was determined by utilizing the molar extinction coefficient of acetylcholine.

Motor neuron morphometry

Motor neurons were immunostained as per the protocol (Rebecca, 2021). Embryos (n=10) were fixed in 4% paraformaldehyde at 4°C, overnight. After washing them with PBS, the samples were incubated overnight with the primary antibody mouse IgG2a anti-synaptotagmin (1:100 znp-1, DSHB, University of Iowa) at 4°C. The next day, samples were washed and incubated in fluorescent secondary anti-mouse antibody conjugated to Alexa flour 488 (1: 1000 dilution PBST). Followed by washing step, samples were analyzed using a fluorescent microscope. Imaging of znp-1-labeled motor neurons is focused on a few (six) representative somatic hemisegments from the approximate 30-34 segments present along the zebrafish spinal cord (Feldner *et al.*, 2005). The images captured revealed the bilateral symmetry typical of motor neuron arrangement and the organization of primary motor neuron networks. The measurement of axon length was done in Image J software. The final region of interest was obtained by subtracting seven pixels around the initial region to restrict the analysis to the region of interest and to reduce detection of artifacts (Lea *et al.*, 2022).

Statistical analysis

The statistical analysis was done by using GraphPad prism (9.3.1 version). All estimations were analyzed using one-way ANOVA followed by Dunnette's post-hoc test. 95% confidence interval was set and significance level at $p < 0.05$.

RESULTS AND DISCUSSION

Results

Cardiac rate measurement

The results indicated no statistically significant variation amongst the exposed groups when compared to the control groups (Fig. 1).

Locomotion behavior analysis

The results of locomotion behavior showed that embryos in B3 and B4 travelled more distance than the rest of the batches (B1, B2 & B5) ($p < 0.05$). Also, B3 and B4 preferred peripheral area more than the central area. The statistical analysis showed significant increase in the total distance and peripheral distance travelled by B3 and B4 embryos ($p < 0.05$) (Fig. 2 and Fig. 3).

Oxidative stress and cortisol level analysis

The levels of Glutathione-S-transferases (GST), Catalase, and lipid peroxidation when analyzed, showed a significant increase in GST and lipid peroxidation levels in B3 compared to the control batch. However, catalase levels remained unchanged in the exposed embryos (Fig. 4).

Also, the cortisol levels were significantly increased in B3 batch of embryos compared to the control batch (Fig. 5).

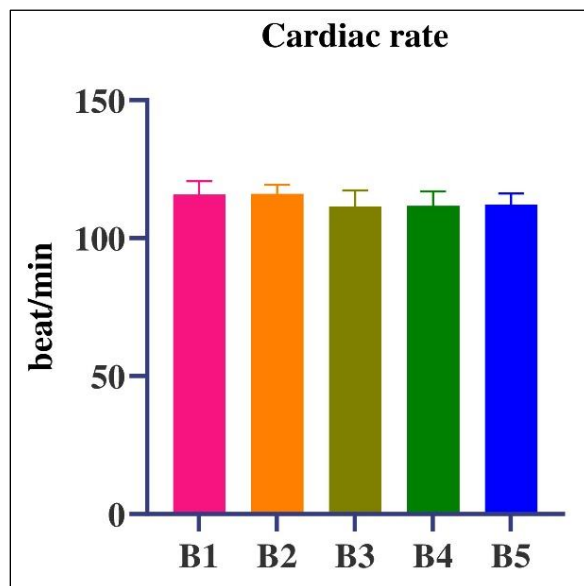


Figure 1: Cardiac rate of zebrafish embryos on 7dpf. Data expressed as mean±SD, n=10.

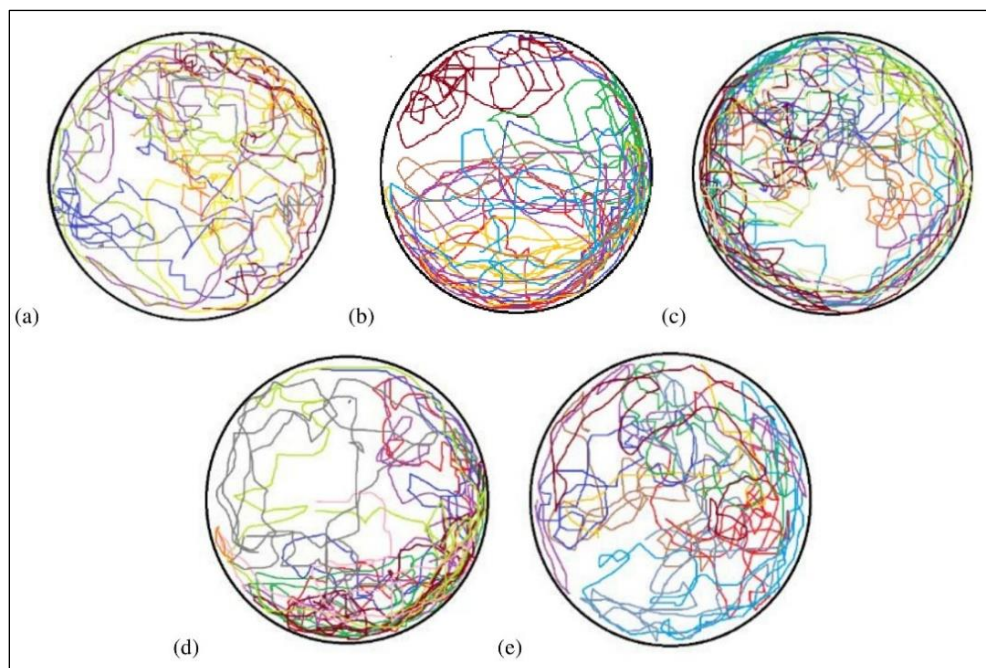


Figure 2: Trajectory images of zebrafish embryos on 7dpf. (a) B1, (b) B2, (c) B3, (d) B4, and (e) B5. Each color indicates the locomotion trajectory of an individual embryo, (n=10/batch).

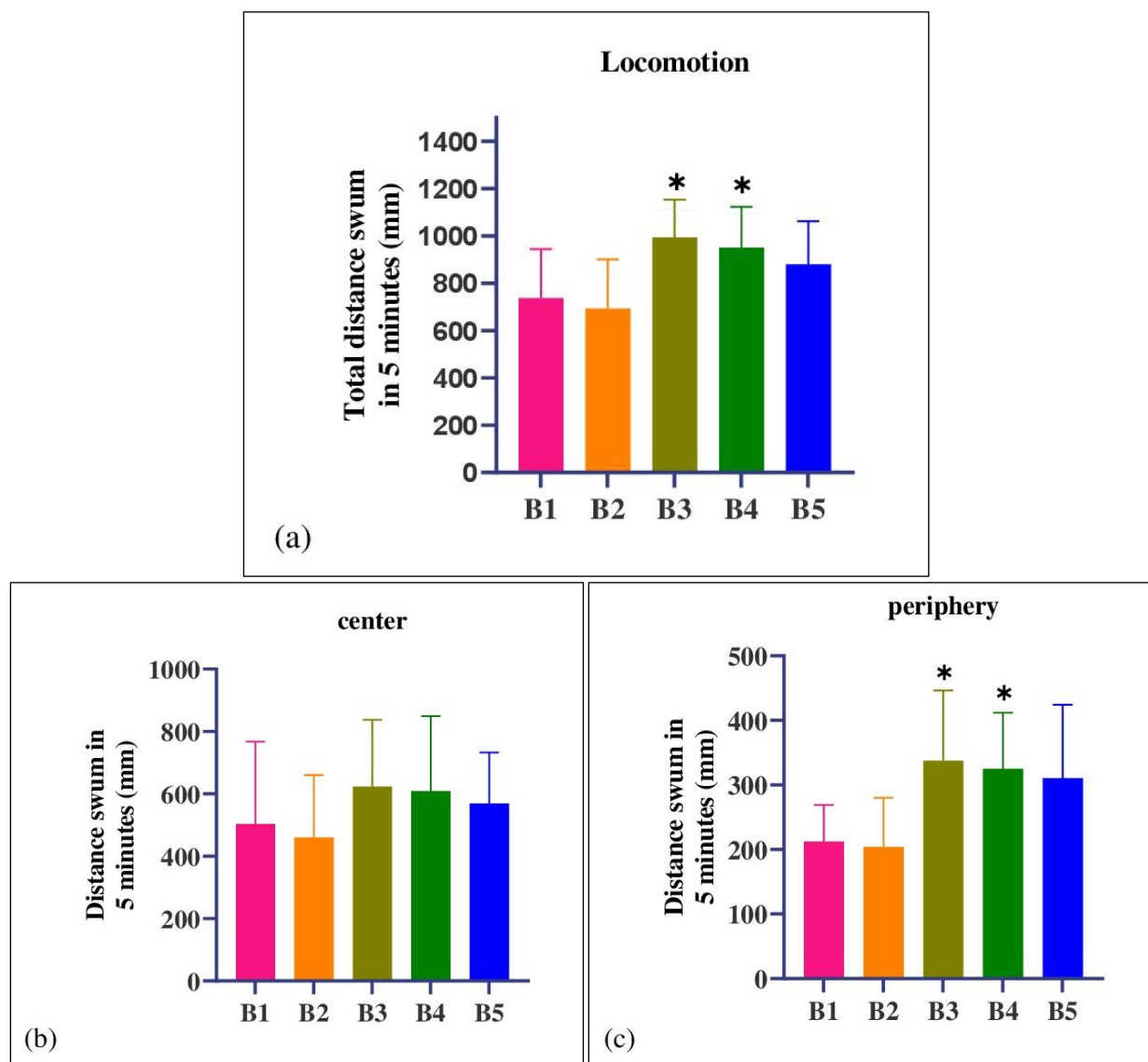


Figure 3: Locomotion behavior. (a) total distance travelled, (b) distance covered in central zone, and (c) distance covered in peripheral zone. Data expressed as mean±SD, n=10, * $p \leq 0.05$ (one way ANOVA+ Dunnette's post-hoc test).

AChE activity

The results of quantitative analysis of AChE activity showed a statistically significant decline in B3 and B4 batches in comparison to control batch (Fig. 6).

Motor neuron morphometry

Motor neuron development was examined in embryos at 7dpf and visualized by IHC/immunostaining with the motor neuron marker anti-synaptotagmin (znp-1). The antibody binding results showed that the axonal length in the exposed batches (B3 and B4) was shorter than that of the control batch (B1). However, the observed difference was not statistically significant (Fig. 7 and Fig. 8).

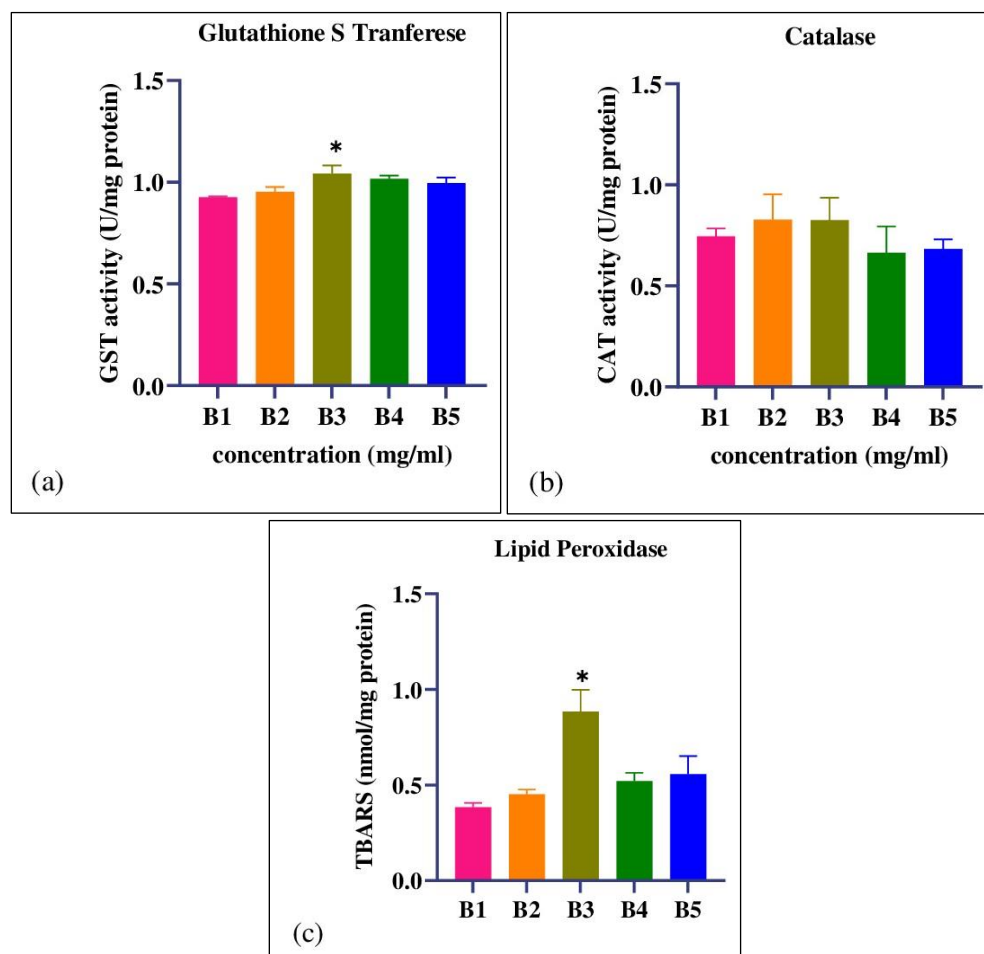


Figure 4: Oxidative stress parameters on 7dpf, (a) GST activity, (b) catalase, and (c) lipid peroxidation levels. Data expressed as mean±SD, n=10. $*p \leq 0.05$ (one way ANOVA+ Dunnette's post-hoc test).

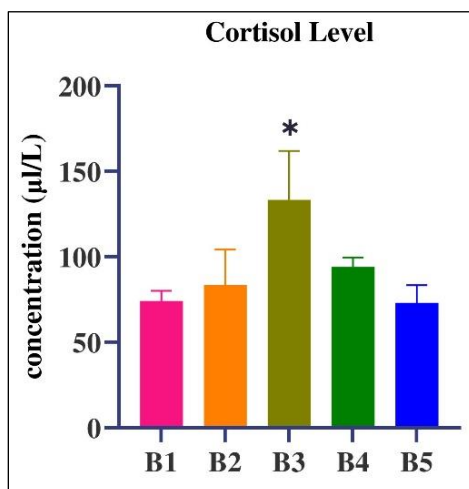


Figure 5: Whole body cortisol levels on 7dpf. Data expressed as mean±SD, n=30. $*p \leq 0.05$ (one way ANOVA+ Dunnette's post-hoc test).

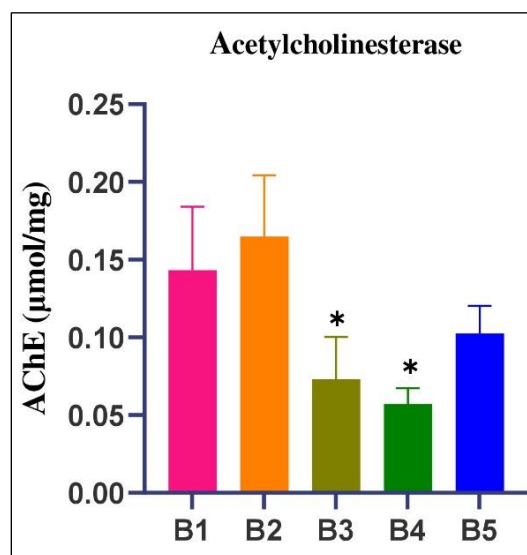


Figure 6. Acetylcholinesterase levels of zebrafish embryos on 7dpf. Data expressed as mean±SD, n=30. * $p \leq 0.05$ (one way ANOVA+ Dunnette's post-hoc test)

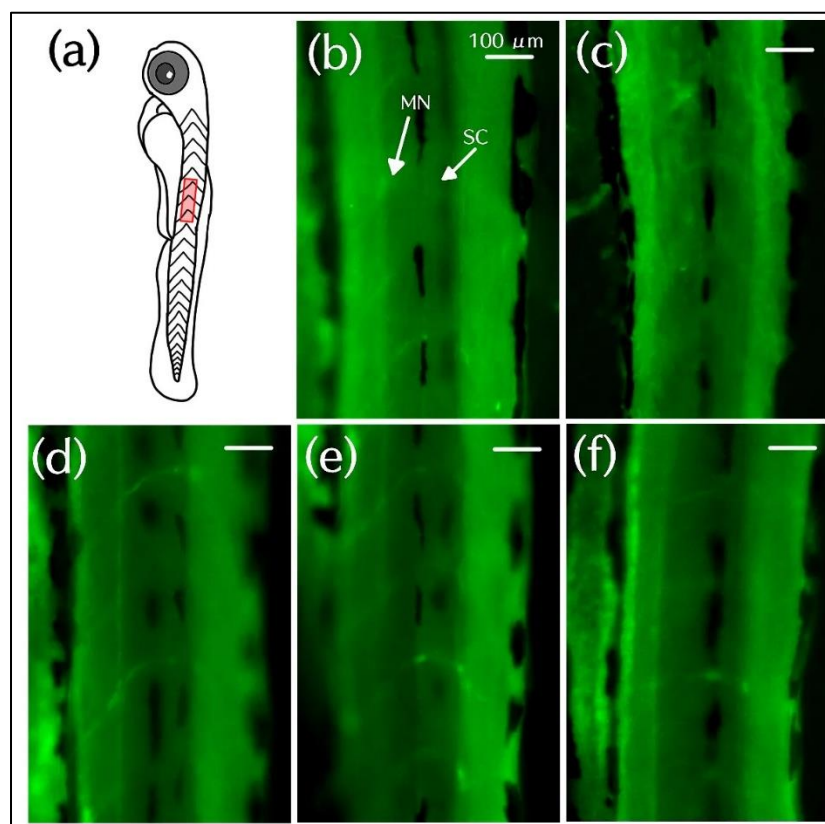


Figure 7. Zebrafish embryos labelled with znp-1 antibody at 7dpf. (a) diagrammatical representation of the embryo - lateral view (anterior to the top, and dorsal to the left), (b) B1, (c) B2, (d) B3, (e) B4, and (f) B5. MN- motor neuron; SC- spinal cord. Red box shows the selected area of localizing the znp-1 antibody along motor neuron.

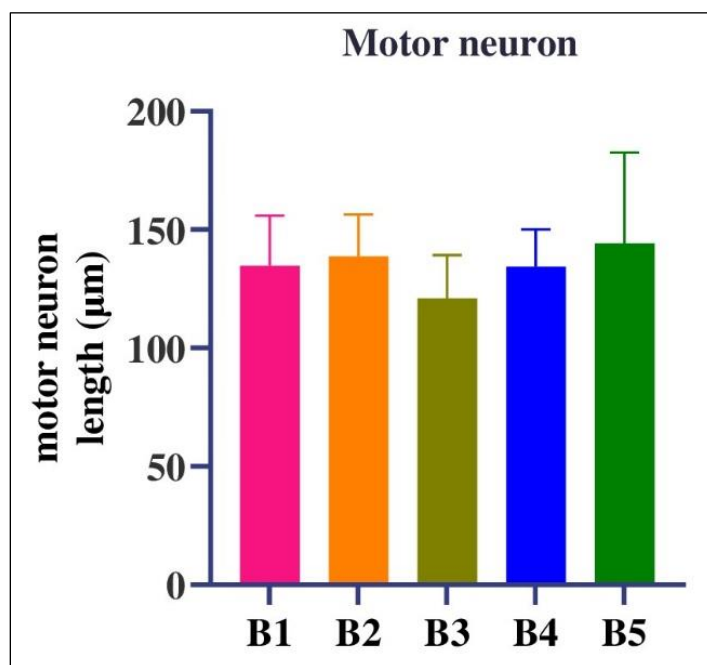


Figure 8. Motor neuron length of zebrafish embryo on 7 dpf. Data expressed as mean±SD, n=30. * $p \leq 0.05$ (one way ANOVA+ Dunnette's post-hoc test).

Discussion

The present study investigated the locomotion behavior, stress markers, and motor neuron development in embryos exposed to 5G mobile phone EMR given for a duration of 6 hours/day till 7dpf. Changes with respect to the mortality rate, hatching rate or cardiac rate across the exposed batches of embryos were insignificant. However, the locomotion assays indicated a statistically significant increase in the distance traveled by embryos in B3 and B4, which highlights the potential of RF EMR to modify the locomotor behavior, which is a key indicator of neurological and developmental status. These embryos also exhibited a preference for peripheral areas (wall clinging), which may reflect heightened stress levels and potential impacts on spatial navigation. This behavior could be linked to alterations in the nervous system or sensory input, warranting further investigation into the underlying mechanisms. Similar studies earlier have reported altered neurobehavior in zebrafish embryos and adults following EMF exposure (Dasgupta *et al.*, 2020; Nirwane *et al.*, 2016; Piccinetti *et al.*, 2018). Zebrafish embryos show mature swimming at 4–5dpf after the development of a swim bladder. In zebrafish it is regulated by the activities of reticulospinal neurons in the brainstem and descending vestibulospinal or neuromodulatory projections, a mechanism that is evolutionarily conserved across all vertebrates (Grillner and Jessell, 2009; Grillner and El Manira, 2019) and crucial for their survival. Therefore, alterations in their locomotor patterns could be used as an indicator of stress.

To determine if these behavioural changes were associated with morphological alterations, we examined the overall body morphology. No abnormalities were detected in external morphology across the exposed batches. These findings are consistent with previous studies, which also reported no external morphological damage in zebrafish embryos exposed to 50 Hz and 100 MHz EMF, respectively (Fey *et al.*, 2009; Piccinetti *et al.*, 2018).

Despite the absence of morphological defects, we observed significant changes in oxidative stress markers. Specifically, there was an increase in glutathione S-transferase (GST) activity and lipid peroxidation (LPO) levels in the EMF-exposed embryos, while catalase (CAT) activity remained unchanged. This suggests an imbalance between reactive oxygen species (ROS) production and antioxidant defences, leading to

oxidative stress. Our findings align with a previous study, who reported elevated LPO and GST levels in zebrafish embryos exposed to EMF in the frequency range of 15 to 3000 MHz (Üstündağ *et al.*, 2020). Additionally, in a report they observed increased plasma lipid peroxidation levels in healthy adult males exposed to RF fields from cell phones in standby mode (Moustafa *et al.*, 2001). A study reported that 900 MHz radiofrequency radiation significantly increased malondialdehyde (MDA) levels while decreasing reduced glutathione (GSH) levels (Yürekli *et al.*, 2006). An investigation demonstrated increased lipid peroxidation and oxidative stress in the liver of rats following long-term exposure to 900 MHz frequency (Dasdag *et al.*, 2008). Further, elevated cortisol levels were also detected in the B3 group, indicating an activated stress response. This is consistent with findings, who reported higher salivary cortisol levels in individuals residing near mobile phone towers (Mahila *et al.*, 2020). Stress responses, such as increased cortisol levels, are critical indicators of physiological impact and are often correlated with behavioural changes.

Given the observed locomotor changes and elevated stress parameters, we assessed acetylcholinesterase (AChE) activity, which plays a crucial role in regulating motor activity and modulating neurotransmitter release involved in stress regulation. AChE is essential for hydrolyzing acetylcholine (ACh), thereby terminating signal transmission at cholinergic synapses and ensuring proper synaptic function. In our study, AChE activity was significantly reduced in B3 embryos, potentially contributing to the observed alterations in locomotor behavior. Similar results were observed in a study, where 700 and 3500 MHz EMF exposure caused behavioral alterations and decreased AChE activity in zebrafish embryos (Torrez-Ruiz *et al.*, 2024). To further explore the impact on motor function, we analysed the primary motor neurons. Spontaneous motor activity in developing zebrafish begins between 18- and 27 hours post-fertilization (hpf). At around 19.5 hpf, motor output is likely driven by primary motor neurons releasing acetylcholine onto muscle pioneers and adjacent muscle fibers (Melancon *et al.*, 1997). Their axons extend past the horizontal myoseptum, forming functional connections within the myotome, providing a model for studying motor neuron axonal development (Feldner *et al.*, 2007). For analyzing primary motor neurons, immunostaining with the znp-1 antibody was employed. This antibody specifically labels motor neuronal axons, including their main trunks extending to the myotomes (Fashena, 1999; Trevarrow *et al.*, 1990). We observed axonal branches along the myosepta. When measuring individual axonal lengths, no significant differences were found between the exposed and control embryo batches. This suggests that the exposure did not cause major developmental abnormalities in motor neurons, but may have affected the integration of sensory or motor inputs, as seen in locomotion assays.

Conclusion

This study examined the effects of 5G mobile phone electromagnetic radiation (EMR) on zebrafish embryos exposed for 6 hours daily up to 7 dpf. No significant changes were observed in mortality, hatching, cardiac rates, or general morphology. However, locomotor behavior showed marked alterations, with increased travel distances and wall-clinging behavior in exposed groups (B3 and B4), indicating heightened stress and altered spatial navigation. Elevated levels of oxidative stress markers (GST, LPO), cortisol and reduced acetylcholinesterase (AChE) activity further highlighted neurodevelopmental and stress-related impacts. Despite these changes, morphological assessments and motor neuron analysis showed no significant differences between the control and exposed. These findings suggest that EMR exposure does not impair overall development but induces measurable stress and neurobehavioral effects, warranting further investigation into its biological implications. Further research is needed to fully explore the molecular mechanisms underlying the observed changes and assess the long-term implications of prolonged RF-EMR exposure on neural function and developmental trajectories.

CRedit authorship contribution statement

Rifat Khira: Methodology, Formal analysis, Investigation, Writing– original draft. Swati Fumakiya: Methodology, Formal analysis, Investigation. Jahnavi Mehta: Methodology, Formal analysis, Investigation. Gowri K. Uggin: Conceptualization, Resources, Supervision, Validation, Writing – review & editing.

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Data availability

Data will be made available on request.

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