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RESEARCH ARTICLE

Radiofrequency Electromagnetic Field Exposure Amplifies the Detrimental Effects of Fetal Hyperglycemia in Zebrafish Embryos

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ABSTRACT

Objective: Radiofrequency electromagnetic field (RF-EMF) exposure during the embryonic period can cause defects in the development of the fetus. The study's aim is to evaluate the effects of RF-EMF on the lipid accumulation, oxidant-antioxidant system parameters, locomotor activities, and gene expressions of insulin and leptin as genes related to insulin resistance in fetal hyperglycemia-induced zebrafish embryos.

Materials and Methods: The study exposed zebrafish embryos to RF-EMF (60 min) and glucose (5%) every day until 96 hours post fertilization (hpf). The study measured lipid peroxidation (LPO), superoxide dismutase, nitric oxide (NO), glutathione *S*-transferase (GST), and glutathione (GSH) levels to observe the oxidative stress status. The study monitored the development of the zebrafish embryos under a microscope, performed a locomotor activity analysis, measured acetylcholinesterase activity, and conducted oil red O staining to determine lipid accumulation. The study used reverse transcription polymerase chain reactions (RT-PCRs) to determine the expressions of *ins* and *lepa* by using RT-PCR.

Results: Both the glucose and RF-EMF applications decreased locomotor activity and increased the LPO and NO levels as oxidative damage indicators. Applying RF-EMF alone increased GST and GSH levels, while applying RF-EMF and glucose showed a decrease in the antioxidant defense systems. *ins* expression increased in the glucose and RF-EMF groups, while *lepa* expression increased in the glucose group and decreased in the RF-EMF group.

Conclusion: The harmful effects of hyperglycemia and RF-EMF exposure during the fetal period on embryo development need to be supported by studies to confirm the changes the current study has identified at the gene and protein levels.

Keywords: Radiofrequency electromagnetic field, Zebrafish embryos, Fetal hyperglycemia, Insulin resistance

INTRODUCTION

Despite the benefits of using radiofrequency electromagnetic fields (RF-EMF) through different sources (e.g., wi-fi, mobile phones, television), the negative effects of RF-EMF exposure are a matter of concern, as RF-EMF exposure has also been associated with undesirable effects on cell components, causing differentiation and abnormalities in cell proliferation, DNA damage, cancer, and birth defects.[1] EMFs are classified based on their respective frequencies, with low frequency EMFs being below 300 Hz, intermediate frequency EMFs occurring between 300 Hz-10 MHz , and high frequency EMFs ranging between 10 MHz-3 GHz. Mobile phones emit high-frequency RF-EMFs.[1]

EMFs have a high penetrating capacity, and the electrons they emit have the ability to move macromolecular ions and charged particles. Consequently, they can have devastating effects on the body.[2,3] Many devices used daily (e.g., computers, televisions, radios, mobile phones) cause the formation of magnetic fields. Daily exposure to the radio waves that mobile phones emit has been associated with infertility, congenital anomalies, and stillbirths.[4,5] In addition to all these, exposure to RF-EMF during pregnancy adversely affects fetal development, through studies on this subject have not been conclusive.[4-6]

Hyperglycemia in pregnancy is called gestational diabetes and causes excess glucose transport from the mother to the fetus through the placenta. As a result, it frequently increases

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the risk of developing metabolic disorders such as fetal macrosomia, excess weight, insulin resistance, and type 2 diabetes.[7] The mechanism underlying the relationship between fetal exposure to maternal hyperglycemia and increased disease risk in adulthood has not yet been elucidated. Functional changes occurring in the target tissues of diabetes (e.g., adipose tissue) have been suggested to play an important role in epigenetic mechanisms. Zebrafish embryos subjected to glucose represent an effective model for fetal hyperglycemia associated with gestational diabetes.[7]

High RF-EMF causes membrane depolarization, changes in calcium ion diffusion, and nerve and muscle stimulation. A fetus has many stem cells and the fetus' immunity has not yet developed. Environmental exposure to such things as RF-EMFs cause changes in gene expression in stem cells (e.g., HSP70). This situation causes the development of oxidative stress, disrupting the oxidant-antioxidant balance in the organism. Moreover, once the antioxidant system is disrupted, the organism cannot provide adequate defense against RF-EMF exposure, resulting in increased oxidative stress.[1]

Zebrafish have a 71% genetic similarity to humans and are an important experimental model for developmental biology, human genetics, and diseases. Due to external fertilization and the optical transparency of their embryos, their embryonic development can be easily observed live under a microscope. These factors facilitate the use of zebrafish embryos in genetic, toxicological, and many other studies. In addition to being widely used for developmental biology and toxicology studies, zebrafish have also been frequently used in researching metabolic diseases in recent years.[8] The pancreas of zebrafish embryos completes its development between 48-72 hours post fertilization (hpf). The energy required for the cell divisions occurring during embryogenesis is provided through the use of maternal glycogen stores.[9] As the size of the yolk decreases toward 96 hpf, the energy needs of the zebrafish embryo are met by gluconeogenesis.[10] However, increased glucose flow to the fetus as a result of maternal hyperglycemia or various environmental exposures during the embryonic period may cause various defects in embryonic development.[11] No study is found in the literature to have examined the effects of glucose and RF-EMF exposure during the embryonic period on embryonic development or on the genes that play a key role in insulin resistance. Based on this, the current study aim of the study is to determine the effects RF-EMF has on gene expressions related to the development of insulin resistance in fetal hyperglycemia-induced zebrafish embryos. The research also aims to determine the effects of glucose and RF-EMF exposures on lipid accumulation and oxidant-antioxidant system parameters and examines acetylcholinesterase (AChE) activity and locomotor activities in relation to behavioral and developmental impairments.

MATERIALS AND METHODS

Zebrafish Care

Wildtype AB/AB strain zebrafish were maintained under apparently disease-free conditions. Husbandry and egg laying were carried out in accordance with the protocols approved by the Marmara University Institutional Animal Care and Use Committee. Fish were kept in an aquarium rack system (Zebtec, Tecniplast, Italy) at a temperature of 27 ± 1 ºC on a 14/10 h light/dark cycle and fed twice a day with live Artemia as well as flake fish food. Following natural spawning, fertilized embryos were collected and staged according to their development and morphology.[12] These were then included in the experiments. 100 embryos from each group were used for the biochemical parameters, 40 embryos for the reverse transcription polymerase chain reaction (RT-PCR) analyses, and 20 embryos for the oil red O method.

RF-EMF and Glucose Exposure

Zebrafish embryos at 0–2 hpf were divided into four groups: control, RF-EMF-exposed group (EMF), 5% glucose-exposed group (G), and both EMF- and 5% glucose-exposed group (EMF+G). EMF exposure was applied for 60 min once a day at 0-2, 24, 48, 72, and lastly 96 hpf. For this exposure, the study used a special exposure system installed by the Istanbul Technical University Faculty of Electronics. The 5% glucose exposure was applied at 0-2, 24, 48, and 72 hpf. Embryos were maintained in E3 medium (15 mM NaCl, 0.5 mM KCl, 1.0 mM $MgSO_4$, 0.15 mM KH_2PO_4, 0.05 mM Na_2HPO_4, 1.0 mM $CaCl_2$, 0.7 mM $NaHCO_3$, pH 7.2) throughout their exposure period.

Locomotor Activity Analysis

At the end of 96 hpf, behavioral analyses were performed with 10 randomly selected embryos from each group. The embryos were placed one by one in a petri dish with a diameter of 20 cm. The tail was then stimulated laterally with a sharp object, and this process was recorded with a camera. Next, the camera recordings were analyzed using the semi-automated system, and numerical data was obtained from the images.[13] The results of these analyses provided measurements for the average speed, total distance, and exploration rate parameters.

Biochemical Analyses

For each group, 10% homogenates were prepared in three replicates using physiological saline for biochemical analyses from the pools of zebrafish embryos. The supernatant was separated for analysis. Lowry's method was used for determining total protein,[14] Yagi's method for lipid peroxidation (LPO),[15] Mi-

randa's method for nitric oxide (NO),[16] Habig's method for glutathione s-transferase (GST),[17] Mylorie's method for superoxide dismutase (SOD),[18] Ellman's method for AChE,[19] and Beutler's method for glutathione (GSH).[20] Protein levels were measured to present biochemical data as values per mg of protein.

RT-PCR Analysis

Three biological replicates were made from each pool of zebrafish embryos, with each replication consisting of 40 embryos. These were homogenized with a 350 µL lysis buffer. The RNeasy Mini Kit and the QIAcube RNeasy kit (Qiagen, Hilden, Germany) were used to isolate the RNA from the embryos. The BlasTaq™ 2X qPCR MasterMix kit (Applied Biological Materials Inc. (abm) , Richmond, Canada) was used to perform the RT-PCRs. Beta-actin (*actb1*) is a housekeeping gene and is used as a reference gene. Expressions for the *lepa* and *ins* genes were determined through the RT-PCR analyses (Table 1). Relative transcription levels were calculated based on the normalization of values using the housekeeping gene.[21]

Table 1. Forward and reverse primers used in the study.

Genes	Primers (forward/reverse)
actb1	5′-AAGCAGGAGTACGATGAGTCTG-3′
	5′-GGTAAACGCTTCTGGAATGAC-3′
ins	5′-GCTCTGTTGGTCCTGTTGGT-3′
	5′-GGGCAGATTTAGGAGGAAGG-3′
lepa	5′-CTCCAGTGACGAAGGCAACTT-3′
	5′-GGGAAGGAGCCGGAAATGT-3′

Oil Red O Methods

At 96 hpf, the embryos were subjected to the following procedures for oil red O (ORO) staining. All embryos were fixed in 4% paraformaldehyde overnight. After fixation, embryos (10-15 embryos for each microcentrifuge tub) were transferred to a 1.5 mL microcentrifuge tube and washed three times (5 min each) with 1X phosphate buffered saline/0.5% Tween-20 (PBS-Tween). After PBS-Tween removal, the embryos were then stained with a mixture of 300 µL of 0.5% ORO and 200 µL of distilled water in 100% isopropyl alcohol for 15 min. The embryos were then washed three times in 1X PBS-Tween and twice in 60% isopropyl alcohol for 5 min each. Lastly, the embryos were then washed in PBS-Tween and fixed in 4% paraformaldehyde for 10 min. Embryos were mounted in glycerol before imaging, and ORO staining of the embryos was recorded under a Zeiss Sterio Discovery V8 microscope.[22]

Statistical Analysis

GraphPad 9 was used to evaluate the differences among the groups. First, one-way analysis of variance (ANOVA) was applied, followed by Tukey's multiple comparison test. A value of $p < 0.05$ is considered statistically significant.

RESULTS

Results of Morphological Analysis and ORO Staining

Embryonic development was observed under a microscope to monitor the malformations that had occurred as a result of the experimental exposures at 24, 48, 72, and 96 hpf. Scoliosis was observed in zebrafish embryos given a 5% glucose solution at 48 hpf. At 72 hpf, scoliosis was detected in the G and EMF+G groups, while cardiac edema was detected in the EMF and G groups. Eye development retardation was also detected in the EMF, G, and EMF+G groups. Head hemorrhage was seen in the G group, and yolk edema was seen in the G and EMF+G groups. Scoliosis, yolk edema, delay in eye development, and cardiac edema were observed in all exposure groups at 96 hpf (Figure 1). ORO stain was used to detect lipid accumulation in the zebrafish embryos, an increase in lipid accumulation in the liver being found at 96 hpf in the EMF and EMF+G groups (Figure 2).

Results of Locomotor Activity

The study has found average speed, total distance, and exploration rate to have decreased in all groups compared to the control. Furthermore, all locomotor parameters were lower in the EMF+G group compared to the G group (Figures 3a-c).

Results of Biochemical Analysis

As a result of the 5% glucose and RF-EMF exposures, lipid peroxidation levels were seen to have increased significantly in the EMF, G, and EMF+G groups compared to the control group. LPO increased significantly in the G group compared to the EMF group (Figure 4a). In addition, NO levels had increased significantly in the EMF, G, and EMF+G groups compared to the control group. Furthermore, NO levels were significantly lower in the EMF+G group than in the EMF group (Figure 4b). Compared to the control group, GST activities had increased significantly in the EMF and EMF+G groups. Compared to the G group, GST activities had again increased significantly in the EMF and EMF+G groups (Figure 4c). SOD activities declined significantly in the EMF and EMF+G groups compared to the control group. SOD activity also decreased significantly in the EMF and EMF+G groups compared to the G group (Figure 4d). When compared to the control, AChE activities were found to have significantly decreased in the EMF, G, and EMF+G groups. AChE activity was also seen to have decreased in the EMF and EMF+G groups compared more than in the G group (Figure 4e). GSH levels were higher in the EMF group compared to the control group. The EMF group showed a

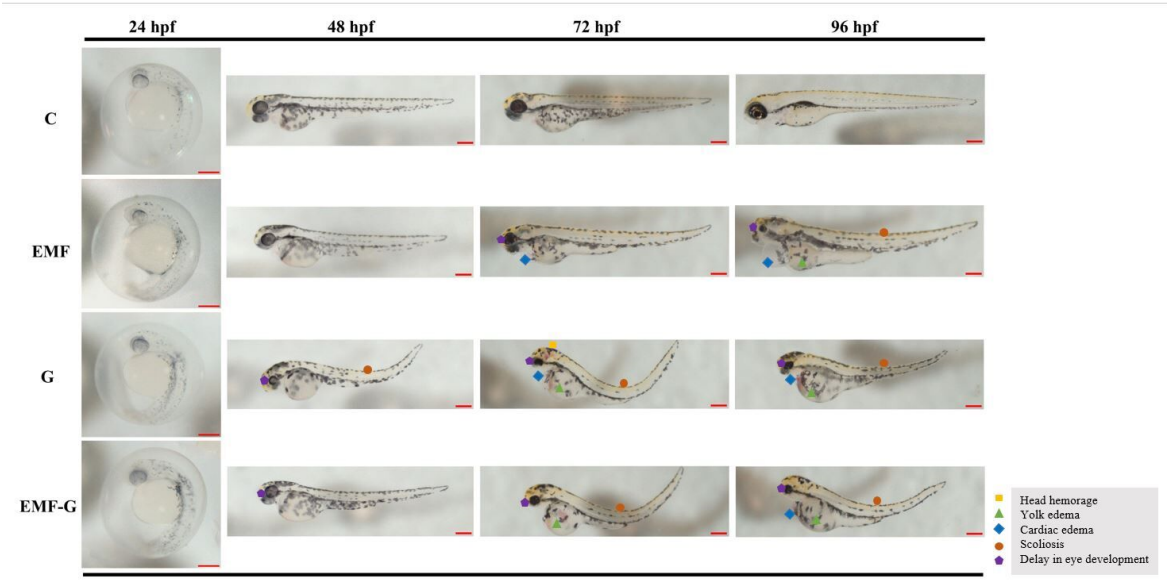

Figure 1. Effects of RF-EMF and 5% glucose exposure on embryonic development. Representative figures of zebrafish embryos at 24, 48, 72 and 96 hpf: A Zeiss Sterio Discovery V8 microscope was used for measurements. 6.3X magnification was used for embryos in the chorion and 3.2X magnification was used for hatching embryos. Scale bar: 500µm.

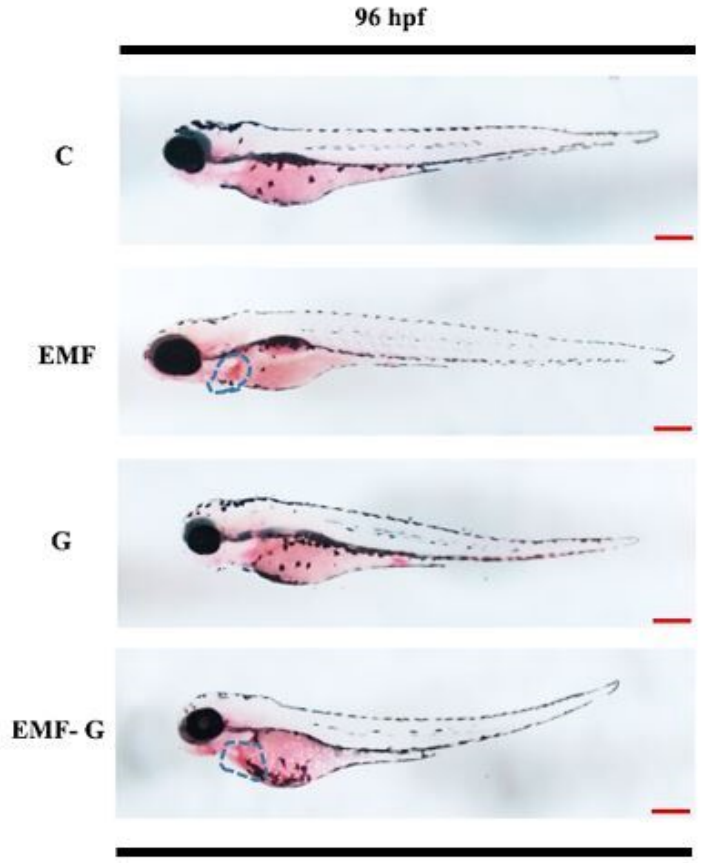

Figure 2. Comparison of lipid accumulations of the groups with oil red O staining. Representative figures of zebrafish embryos at 24, 48, 72 and 96 hpf; A Zeiss Sterio Discovery V8 microscope was used for measurements. 3.2X magnification was used for embryos. Scale bar: 500µm.

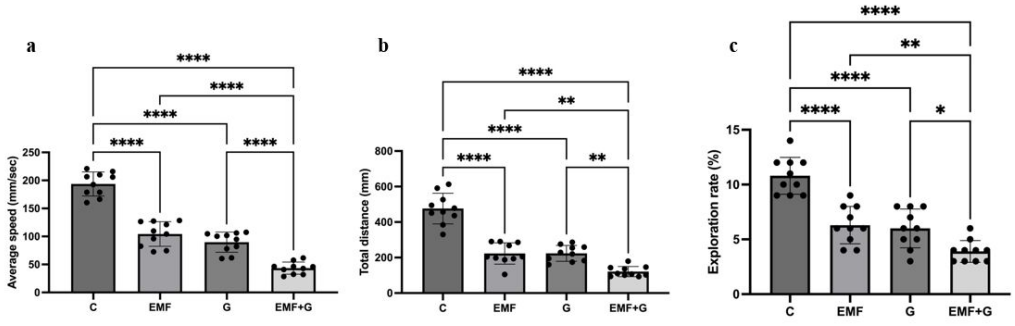

Figure 3. Bar graph presentation of the locomotor activity: a) Average speed analysis results of the groups; b) Total distance measurement results of the groups; and c) Exploration rates of the groups. Data are expressed as *Mean* ± *SD* (*n* = 10). * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$.

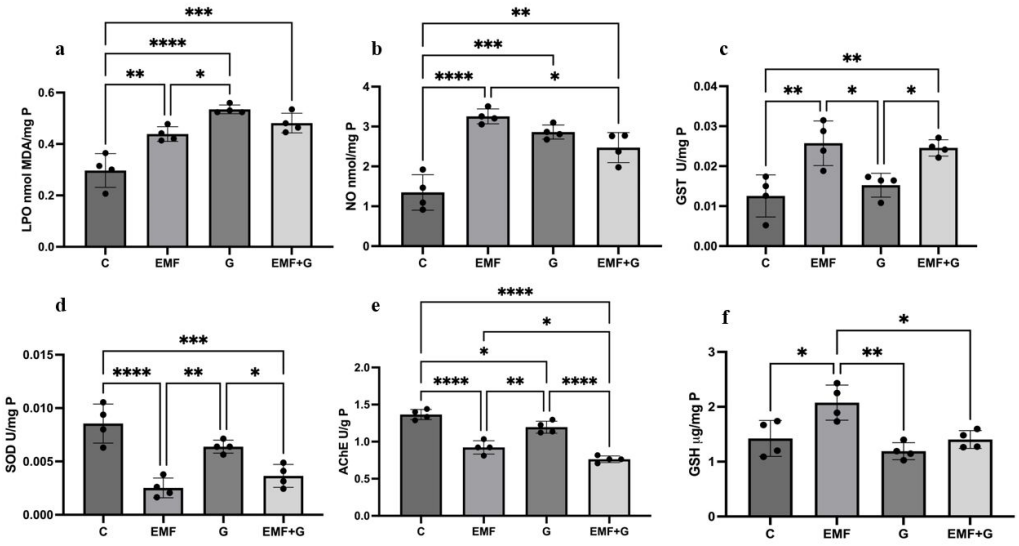

Figure 4. Bar graph presentation of the comparison of the biochemical parameters of the groups': a) lipid peroxidation (LPO) levels; b) nitric oxide (NO) levels; c) glutathione S-transferase (GST) levels; d) superoxide dismutase (SOD) levels; e) acetylcholinesterase (AChE) activities; and f) glutathione (GSH) levels. The data from the four independent experiments are expressed as *Mean* ± *SD* (*n* = 4, 4 biological replicates for each group, 50 embryos per pool); *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

greater increase in GSH levels compared to the G group, while the EMF+G group showed no statistical difference. GSH levels had declined more in the EMF+G group compared to the EMF group (Figure 4f).

Results of RT-PCR Analysis

The RT-PCR analysis was performed to determine the *lepa* and *ins* mRNA expression levels as a result of RF-EMF and 5% glucose exposure. As a result of these analyses, *lepa* expression levels were lower in the EMF and EMF+G groups compared to the control, while these levels increased significantly in the G group (Figure 5a). *ins* expression levels were higher in the EMF, G, and EMF+G groups compared to the control. Furthermore, INS expression levels were lower in the EMF and EMF+G groups compared to the G group (Figure 5b).

DISCUSSION

During the fetal period, exposure to various stress factors can lead to various defects in fetuses, whose metabolic development is inadequate. Exposure to various environmental factors (e.g., radiation, heavy metals, air pollution) increase the risk of developing various diseases during prenatal and early infancy when rapid development occurs.[23]

The present study has examined the effect of RF-EMF exposure on glucose-exposed zebrafish embryos as a model of fetal hyperglycemia. In order to evaluate whether RF-EMF exposure may have an effect on gene expressions related to the development of insulin resistance, the study determined the mRNA expressions of insulin and leptin and performed an oil red O staining to determine lipid accumulation. Developmental parameters, oxidant-antioxidant status, and locomotor activity

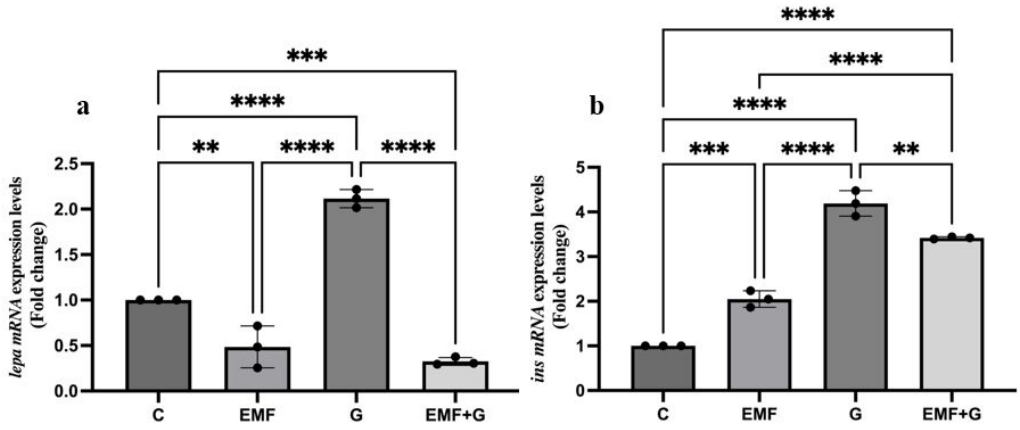

Figure 5. Bar graph presentation of the fold change of the RT-PCR-quantified transcripts of a) *lepa* and b) *ins*. All RT-PCR results are expressed as changes from their respective controls after being normalized to the housekeeping gene *actb1*. Three studies ($n = 3$; 3 biological replicates for each group, 50 embryos per pool) were used to calculate the average values. Data presented are expressed as *Mean* ± *SD.* $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

were also assessed to find out how these exposures affected normal embryonic development.

According to the Pedersen hypothesis, an increase in glucose flow to the fetus causes fetal hyperinsulinemia, which may cause changes in the development and growth of the fetus.[11] The 5% glucose exposure-induced hyperglycemia model in zebrafish embryos revealed defects in eye development.[24] Another study found that zebrafish embryos that had been exposed to glucose developed scoliosis and edema.[25] Consistent with these studies, edema, scoliosis, and retardation in eye development were determined in the groups exposed to RF-EMF and glucose.

The present study assessed LPO levels to detect oxidative stress status. According to the results, 5% glucose and RF-EMF exposure have been revealed to lead to an increase in oxidative stress. Furthermore, lipid accumulation was observed in the liver of the RF-EMF-applied groups as a consequence of staining with oil red O. Guru and Arockiaraj performed oil red o staining to observe lipid accumulation in the fetal hyperglycemia model induced by bisphenol A in zebrafish embryos and identified lipid accumulation in the abdominal area.[26] The accumulation of specific lipid metabolites and increased lipid levels within the cell are related to hyperglycemia.[27] Hyperglycemia can damage vascular structures by stimulating oxidative stress, interfering with NO production, and triggering the formation of lipid-containing foam cells by macrophages.[28] As a result of the disruption in the integrity of the endothelial structure, lipid accumulation accelerates.[28] Kaplan et al. revealed an increase in NO and LPO levels in zebrafish embryos that had been administered 5% glucose.[7] In light of this information, the increase in LPO and NO levels in this study's exposure groups may be associated with lipid accumulation and hyperglycemia.

Kaplan et al. demonstrated an increase in GST activities in the fetal hyperglycemia model they created regarding zebrafish embryos.[7] While GST activity in the present study did not change significantly in the group given only 5% glucose, increased GST activity was observed in the RF-EMF groups, which may be related to the increased LPO in these groups. The GST enzyme is effective in xenobiotic detoxification. This enzyme aims to prevent cellular damage by conjugating the reactive species created by Phase I enzymes with glutathione.[29] In both RF-EMF groups, glucose exposure caused a decrease in GSH and GST activities. This demonstrates that glucose exposure suppresses the elements of the antioxidant defense system that activate under increased oxidative stress in the case of RF-EMF exposure. A significant decrease in SOD activity was observed to have occurred in the group that was administered 5% glucose.[11] Consistent with the findings of Hansen et al., the decrease observed in SOD activity in the glucose and RF-EMF groups in the present study is an indicator of the decreased antioxidant defense system in the face of increasing oxidative stress in these groups and supports the findings regarding the disruption of embryonal development in these groups.

Aerobic organisms need oxygen to survive. While a large portion of oxygen molecules turn into water in the electron transport chain, a very small portion may cause radical formation. The increases in *ins* mRNA levels and in LPO and GST activity in this study may be indicators of oxidative stress formation. Increased oxidative stress and mitochondrial dysfunction have been reported in the glucose-induced hyperglycemia model in zebrafish.[25] The glucose-stimulated increase in insulin mRNA expression in the present study was accompanied by increased LPO (an indicator of oxidative stress), as well as the stimulation of antioxidant defense systems in response to this situation. Mitochondrial dysfunction that develops as a result of hyperglycemia can cause a disruption in the electron transport chain and ROS formation. Although this situation was observed in both the RF-EMF and glucose groups, the increase in insulin gene expression was much more pronounced in the glucose group. On the other hand, when glucose was administered to

the RF-EMF group, an increase in insulin gene expression was detected, which was expected.

Torres-Ruiz et al. discovered RF-EMF exposure to cause a decrease in AChE activity as well as locomotor activity in zebrafish embryos.[30] In the present study, locomotor activity decreased in all exposure groups, with the G group showing an increase in AChE enzyme activity, which hydrolyzes acetylcholine, while the EMF and EMF+G groups showed a decrease.

In the present study, *ins* mRNA expression increased in all exposure groups compared to the control. Furthermore, *lepa* mRNA expression increased in the glucose group compared to the control group while decreasing in the EMF and EMF+G groups. Insulin is one of the most important hormones for ensuring glucose control. Therefore, the present study analyzed the expressions of the *ins* and *lepa* genes to understand how these two hormones act in case of EMF exposure in a fetal hyperglycemia model, as well as to evaluate whether EMF exposure affects the gene expressions related to the development of insulin resistance that may develop in fetal hyperglycemia. The increase in *ins* mRNA levels in the present study indicates that RF-EMF exposure during the fetal period may affect glucose hemostasis. Glucose is known to stimulate the accumulation of insulin mRNA. In accordance with this information, the study found increased insulin mRNA levels in the glucose-exposed groups.[31] Consistent with the current study, Kaplan et al. reported an increase in *ins* mRNA levels in their hyperglycemia model induced with 5% glucose.[7] Meo and Rubeaan's study revealed fasting blood glucose and insulin resistance to increase in Wister Albina rats that were exposed to mobile phone radiation for more than 15 minutes a day over three months.[32]

Leptin hormone is important in energy homeostasis and regulates the hunger and satiety mechanism.[33] The current study detected a decrease in *lepa* mRNA levels. Leptin levels have been positively correlated with body fat, with obese subjects being more hyperleptinemic than lean subjects.[34] Reduced leptin mRNA levels through RF-EMF exposure may be related to the lipid accumulation observed in these groups. However, in order to support these findings, the changes this study has identified in insulin and leptin expressions at the gene level need to be confirmed at the protein level.

CONCLUSION

Hyperglycemia in a mother during pregnancy increases glucose flow to the fetus, which can cause ROS accumulation and lipid accumulation in addition to developmental disorders. This study's results have shown that, in cases of fetal hyperglycemia, RF-EMF exposure increases the harmful effects caused by glucose exposure on zebrafish embryo development. In addition, this study supports the suitability of the zebrafish embryo for studies on fetal hyperglycemia and exposure to environmental factors, including RF-EMF.

Ethics Committee Approval: Zebrafish embryos can be worked with for up to 120 hours after fertilization without requiring ethical permission.

Peer Review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- D.C., M.B., G.E., S.I., Z.M., I.U., S.P., A.A.A.; Data Acquisition- D.C., M.B., G.E., S.I., Z.M., I.U., S.P., A.A.A.; Data Analysis/Interpretation- D.C., M.B., G.E., S.I., Z.M., I.U., S.P. A.A.A. ; Drafting Manuscript- D.C., E.E.A.; Critical Revision of Manuscript- E.E.A.; Final Approval and Accountability- D.C., M.B., G.E., S.I., Z.M., I.U., S.P., A.A.A., E.E.A.

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