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RESEARCH ARTICLE

Effects of low-dose ionizing radiation on the molecular pathways linking neurogenesis and autism spectrum disorders in zebrafish embryos

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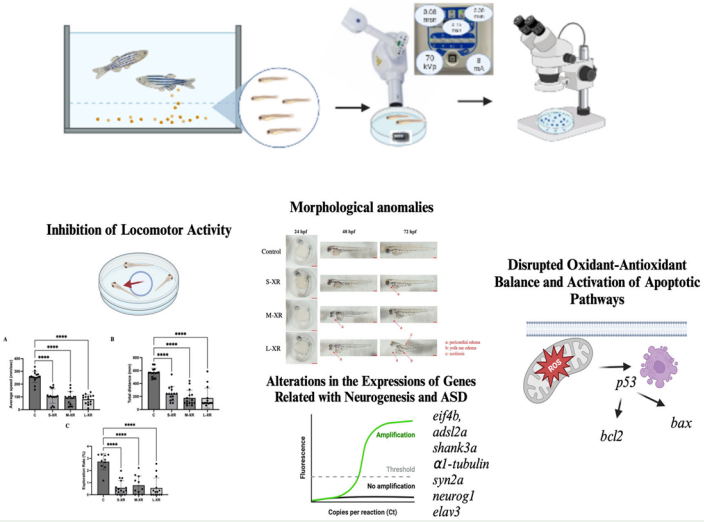
ABSTRACT

Prenatal exposure to environmental factors may play an important role in the aetiopathogenesis of autism spectrum disorder (ASD). We aim to investigate the potential effects of low-dose x-rays from dental diagnostic x-rays on neurodevelopment and molecular mechanisms associated with ASD in developing zebrafish embryos. Zebrafish embryos were divided into four groups and exposed using a dental x-ray unit: control, 0.08, 0.15 and 0.30 seconds, which are exemplary exposure settings for periapical imaging. These exposure times were measured as 7.17, 23.17 and 63.83 mSv using optical stimulated luminescence dosimeters. At the end of 72 hours post-fertilization, locomotor activity, oxidant-antioxidant status, and acetylcholine esterase (AChE) activity were analyzed. Expression of genes related to apoptosis (*bax, bcl2a, p53*), neurogenesis (*α1-tubulin, syn2a, neurog1, elavl3*) and ASD (*eif4eb, adsl2a, shank3*) was determined by RT-PCR. Even at reduced doses, developmental toxicity was observed in three groups as evidenced by pericardial edema, yolk sac edema and scoliosis. Deleterious effects of dental x-rays on neurogenesis through impaired locomotor activity, oxidative stress, apoptosis and alterations in genes associated with neurogenesis and ASD progression were more pronounced in the 0.30s exposure group. Based on these results we suggest that the associations between ASD and low-dose ionizing radiation need a closer look.

GRAPHICAL ABSTRACT



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KEYWORDS

Low-dose ionizing radiation; zebrafish embryo; neurogenesis; autism spectrum disorder; diagnostic radiology

Introduction

Autism spectrum disorder (ASD) is a complex set of neurodevelopmental disorders characterized by cognitive deficits and impaired social and communicative development (American Psychiatric Association, 2023). The causes and mechanisms of ASD have not yet been fully elucidated, but it has been reported that genetic background, epigenetic modification, and environmental risk factors all contribute to the development of ASD (Cohen et al., 2005; Werling & Geschwind, 2013).

According to a report published by the US Centers for Disease Control and Prevention in 2020, the prevalence of

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ASD has increased significantly, reaching 18.5 (1/54) per 1000 children (American Psychiatric Association, 2023). Due to the rapidly increasing prevalence of ASD, research has focused on potential environmental factors influencing ASD development and prenatal exposure to endogenous and exogenous factors has been suggested to affect neurodevelopment and behavior (Hu, 2013; Hollander et al., 2018). At the genetic level, there are genes directly linked to ASD, known by cell growth and migration regulations (Polšek et al., 2011).

Dental diagnostic x-rays are crucial in dental practice. Certain types of dental diagnostic imaging, such as periapical and panoramic radiographs are frequently used. As individuals are exposed to dental diagnostic x-rays throughout their lifetime, even a slight increase in health risks could be significant for public health (Cervantes, 2016). The radiation doses of radiographs used in dentistry have been reduced over time with improved digital imaging and modern digital sensors has remarkably enabled the reduced exposure doses. The average dental radiation dose required for a periapical radiograph is approximately 3 μSv (Aps, 2013). The maximum annual allowable dose is considered to be 1 millisievert (ICRP, 2007).

When cells and tissues absorb ionizing radiation, it causes biological effects. These biological effects can lead to cell damage at the molecular level, affecting bio-molecules like DNA. Low doses of ionizing radiation can lead to significant morphological variations, affect cardiovascular and neurological development, and alter the expression of genes associated with a variety of diseases (Freeman et al., 2014). It is therefore necessary to determine the effects of low-dose radiation exposure on gene expression in order to investigate the specific effects, especially on neurogenesis, of exposure to radiation in the developmental period and the associated life-long consequences.

Previous investigations have focused on elements such as DNA damage mechanisms in the adult organism, free radical generation, and elevated cancer markers at low doses of ionizing radiation (Si et al., 2013). Cells with high levels of mitotic activity are known to be hypersensitive to radiation. Many short and long term effects could result from an excessive increase in a cell's apoptosis and DNA damage mechanisms during the embryonic phase. The process by which neural precursor cells differentiate into mature neurons during the development of the central nervous system is known as neurogenesis. Neurogenesis can be affected by both internal and external factors. Although organogenesis and in particular neurogenesis in the early fetal period are the most susceptible periods to radiation for the fetus, research on low-dose ionizing radiation exposure during the embryonic stage is limited (Williams & Fletcher, 2010). Animal models have been used to study the effects of prenatal exposure because human participants are difficult to manage (Driscoll et al.,1990).

In recent years, the zebrafish (*Danio rerio*) has become the model of choice in biomedical research, mainly because of its homology with the human genome and its ease and affordability (Çalışkan & Emekli-Alturfan, 2021). The majority of the organs, including the nervous system, are identical in terms of anatomy and physiology (Vaz et al., 2018). Measuring the levels of expression of different biostimulants in zebrafish and embryos is an effective and fast way to detect changes in gene expression sequences when exposed to neurotoxins (Çalışkan & Emekli-Alturfan, 2021). Therefore, to study the molecular effects of ionizing radiation during organogenesis, the zebrafish embryo is an excellent model.

We have previously shown the deterioration of the oxidant-antioxidant balance, suppression of immune response, induction of inflammation and apoptosis in low-dose diagnostic dental x-rays exposed zebrafish embryos (Karagöz et al., 2023). However, there is no study in the literature regarding the effects of dental diagnostic radiation on neurogenesis and the development of ASD. Therefore, based on the hypothesis that exposure to ionizing radiation during embryonic development affects oxidative stress-induced apoptotic pathways and changes the expression of genes that play a role in neurogenesis and the development of ASD, we aimed to investigate the possible effects of low-dose diagnostic dental X-rays in developing zebrafish embryos. For this purpose, molecules involved in the oxidant-antioxidant system (malondialdehyde, nitric oxide, superoxide dismutase, glutathione S-transferase and catalase) and apoptotic pathways (bax, bcl2a and p53) were examined to determine the molecular mechanisms of the effects of ionizing radiation. In addition, to assess whether ionizing radiation-induced changes in oxidant-antioxidant and apoptotic systems affect embryonic development, locomotor activities, acetylcholinesterase activities, expression of neurogenesis (syn2a, neurog1, α1-tubulin) and ASD-related genes (eif4eb, adsl, shank3) were determined.

Methods

Maintenance of zebrafish

The Zebrafish (AB/AB Strain) were kept in a Zebtec aquarium rack system from Tecniplast, Italy, in a disease-free environment. The temperature was maintained at 27 ± 1 °C, and a light/dark cycle of 14/10 h was followed. The fish were fed commercial fake fish food twice a day, supplemented with live Artemia. The pH level of the water in the system ranged between 6.9 and 7.2. We used reverse osmosis water containing 0.018 mg/L Instant Ocean™ salt for all experiments. After natural spawning, we collected fertilized embryos and staged them based on their development and morphology, as previously described (Westerfield, 1995). Zebrafish embryos of the AB/AB strain were selected under the stereomicroscope after fertilization and divided into four groups.

Embryo exposure

A dental x-ray unit (New Life Evolution X3000 2 C, Grugliasco, Italy) was used at three different representative exposure settings for periapical imaging (0.08, 0.15 and 0.30 seconds); (70 kVp, 8 mA, equipped with 2 mm Al eq total filtration and 8 inch (20.32 mm) cone) with the same projection geometry. The equipment of the dental x-ray unit consists of an x-ray tube (0.7 mm Toshiba DG-073B-AC and 0.8 mm Kailong

KL2-0.8–70 G), an articulated arm with wall support and a timer integrated in the wall plate. The timer on the control panel has digital buttons with a maximum time setting of 3s and a minimum time setting of 40 ms.

During the experiment, three OSL dosimeters (Epsilon Landauer, Istanbul, Turkey) were used for each exposure group. These dosimeters had a sensitivity range of 0.05 mSv to 10.0 Sv with an uncertainty of 8% for deep and shallow dose Hp, which was done for comparison of dose measurement reliability. Each OSL dosimeter was placed in the center of a Petri dish to measure absorbed doses. The same conditions but without embryos were used for the exposure of two other OSL dosimeters. This procedure was repeated for each x-ray group. One dosimeter in each group was not exposed and served as a control. The exposed OSLs were then processed using an InLight Auto200 reader (Epsilon Landauer, Istanbul, Turkey) and the numbers read by the reader were recorded.

After only one x-ray exposure for each setting (0.08, 0.15 and 0.30 seconds) at 2 hpf (hours post-fertilization), developmental parameters were tracked using a stereomicroscope (ZEISS Discovery V8, Germany) and recorded every day until 72 hpf.

Locomotor activity

The locomotor activity of the zebrafish embryos at 72 hpf was evaluated as described previously (Goody et al., 2012). This was performed by placing a 60 mm Petri dish containing embryo medium on top of the motility wheel which is on the microscope stage. Then, by using an embryo poker tool the zebrafish embryo was positioned in the middle of the motility wheel and the time it took for an embryo to swim a predetermined distance was recorded and the average escape response was calculated.

Biochemical assays

At 72 hpf , the zebrafish embryos were grouped into replicate pools consisting of 100 individuals. There were four pools in total. Each pool was homogenized in 1 ml of PBS and then briefly centrifuged. The resulting supernatant was utilized to determine biochemical parameters (Üstündağ et al., 2019).

Total protein assay

To determine the total protein levels in the samples, the Lowry method was utilized (Lowry et al., 1951). The process involves reacting proteins with copper ions in an alkaline solution, and then reducing them using a Folin reagent. The absorbances are measured at 500 nm and the results are presented per protein.

Acetylcholinesterase (AChE) activity assay

The Ellman et al. method was used to measure the activity of acetylcholinesterase in the supernatants (Ellman et al., 1961). Acetylcholinesterase generates thiocholine in this mechanism, which combines with 5.5′-dithiobis (2-nitrobenzoic acid) to produce a yellow hue. At 412 nm, the yellow color's intensity is evaluated and directly correlated with the sample's enzyme activity.

Lipid peroxidation assay

We used the Yagi method to assess the amounts of malondialdehyde (MDA), which is the by product of lipid peroxidation (LPO). Using an extinction value of $1.56 \times 105\,M\text{-}1\,cm\text{-}1$, the MDA levels were identified as thiobarbituric acid reactive compounds and quantified as nmol MDA/mg protein (Yagi, 1984).

Nitric oxide assay

After converting nitrate to nitrite with vanadium (III) chloride, nitric oxide (NO) was detected at 540 nm using a spectrophotometer. The results were given in units of nmol NO/mg protein (Miranda et al., 2001).

Glutathione-s-transferase assay

By calculating the absorbance of the by-product of the conjugation of GSH and CDNB at 340 nm, glutathione-s-transferase (GST) activity was determined (Habig et al., 1974).

Superoxide dismutase assay

The ability of superoxide dismutase (SOD) to improve the outcome of riboflavin-sensitized photo-oxidation of o-dianisidine was employed as a method to assess SOD activity. A fluorescent lamp was used to illuminate a reaction mixture including riboflavin and O-dianisidine dihydrochloride, which resulted in superoxide activity. Riboflavin sensitized O-dianisidine oxidation and SOD increased it in a concentration-dependent way. Using a spectrophotometer, the absorbances at 0 and 8 minutes of illumination were measured at 460 nm, and the net absorbances were computed. U/mg protein was used to represent the findings (Mylroie et al., 1986).

Catalase assay

Using Aebi's approach, the catalase activity was assessed in this study. The technique assesses the enzyme's ability to convert hydrogen peroxide into water (Aebi, 1974).

Reverse transcription (cDNA synthesis) and quantitative Real-Time PCR

After the experiment, the embryos from each group had their RNA isolated. The Rneasy Mini Kit and Qiacube, both from Qiagen in Hilden, Germany, were used in accordance with the manufacturer's instructions. A single-stranded cDNA was created using RT2 Profiler PCR Arrays from 1 microgram of total RNA. RT-PCRs were performed using the DNA Master SYBR Green kit, also from Qiagen. Beta-actin was used as the housekeeping gene, and the

ΔΔCT method was used to calculate the relative levels of transcription. This was done by normalizing the values using the housekeeping gene (Livak & Schmittgen, 2001). The primers used are listed in Table 1.

Statistical analysis

One-way analysis of variance (ANOVA) was used to assess the effects of ionizing radiation on zebrafish embryos, taking into account variables including hatching and mortality rates, locomotor activity, AChE, LPO, SOD, GST, CAT, and NO. GraphPad Prism 9 was used for the statistical analysis, and a significant p values of less than 0.05 used.

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

Bax	Forward primer	5′-AGCAGGAGTACGATGAGTCTG-3′
	Reverse primer	5′-GGTAAACGCTTCTGGAATGAC-3′
bcl2a	Forward primer	5′-TGGAGGTTGGGATGCCTTCG-3′
	Reverse primer	5′-ATTGGCATGGAGACCGCAGA-3′
p53	Forward primer	5′GGGCAATCAGCGAGCAAA-3′
	Reverse primer	5′-ACTGACCTTCCTGAGTCTCCA-3′
syn2a	Forward primer	5′-TCCTGACAGGCTGCGTTCAA-3′
	Reverse primer	5′-CCCTGAAGAGACGACCGCTT-3′
neurog1	Forward primer	5′-ACGTCGTGAAGAAGAACCGCAGG-3′
	Reverse primer	5′-CCCTGCTTCTGGTCTGCGATCCG-3′
α1tübülin	Forward primer	5′-AGTCGAGTGTGAGAGCGTTT-3′
	Reverse primer	5′-ATCTGGCTGGATGCCATGCT-3′
β actin	Forward primer	5′-AAGCAGGAGTACGATGAGTCTG-3′
	Reverse primer	5′-GGTAAACGCTTCTGGAATGAC-3′
elavl3	Forward primer	5′-CACCTCACGCATCCTGGTAA-3′
	Reverse primer	5′-GGCTGTCTGGTAGAGCTGGG-3′
Adsl	Forward primer	5′-CGTTTAACCGCTGCAGGCAT-3′
	Reverse primer	5′-AGCATCGCTTATGGGGAGGC-3′
eif4eb	Forward primer	5′-ACTCTGCTGTGCCTCATCGG-3′
	Reverse primer	5′-TTCATAGGGACGCCCAACCG-3′
shank3	Forward primer	5′-CTGTTTTACGGAGCGGACAT-3′
	Reverse primer	5′-CCCTGAAAGGCACAACATCT-3′

Table 2. Absorbed doses (mSv) for the exposed zebrafish embryos.

Exposure time	Deep dose (mSv)	Shallow dose (mSv)
0.08 s (S-XR)	7.17	8.33
0.15 s (M-XR)	23.17	27.67
0.30 s (L-XR)	63.83	78.00

Results

Results of absorbed doses

The dose measurements for individuals were calculated by taking the average of three exposures in each x-ray group. In Table 2, the absorbed deep and shallow doses are shown. The standard periapical group had body doses of 7.17 mSv, 23.17 mSv, and 63.83 mSv for 0.08 s (S-XR), 0.15 s (M-XR), and 0.30 s (L-XR) exposure times respectively (Katsoni et al., 2011).

Results of developmental analyses

The representative images of the zebrafish embryos are presented in Figure 1. Yolc sac edema, pericardial edema, decreased body pigmentation and scoliosis were observed in the low-dose ionizing radiation-exposed zebrafish embryos at 24, 48 and 72 hpf.

At 72 hpf pericardial edema was observed in the 0.08 s (S-XR), 0.15 s (M-XR) and 0.30 s (L-XR) group of ionizing radiation-exposed embryo. In addition, pericardial edema was observed at 48 hpf in the M-XR and L-XR groups. In the L-XR group, yolk sac edema and scoliosis were observed at 72 hpf.

Results of locomotor activities

Average speed, total distance swam and exploration rates were determined to evaluate the effects of x-ray radiation on locomotor activity. Average speed, total distance swam, and exploration rate were found to be decreased by x-ray radiation at all applied periods. No statistically significant change was detected between the x-ray applied groups depending on the application time (Figure 2).

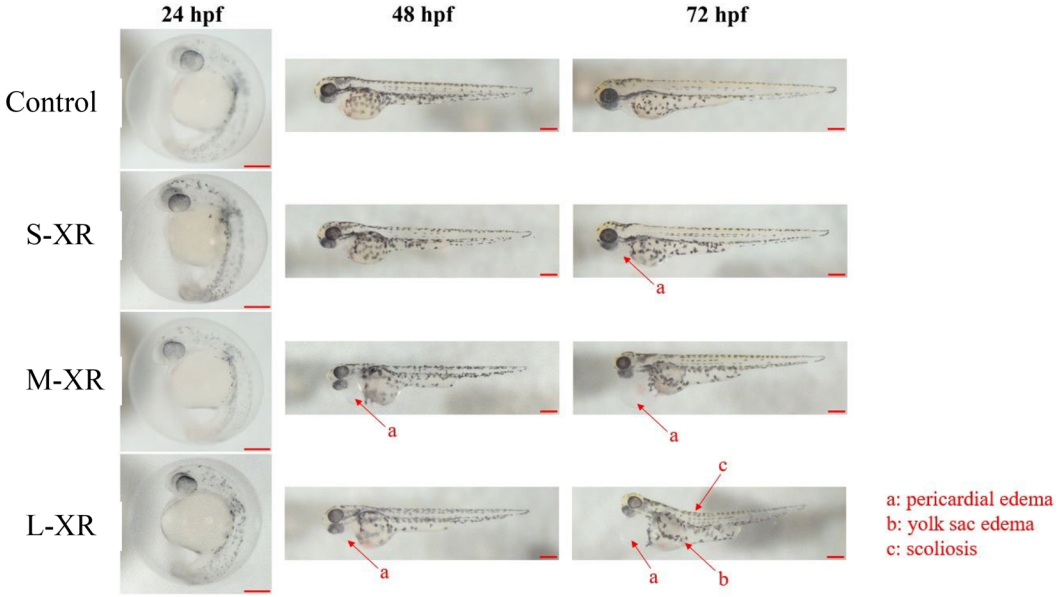

Figure 1. Representative images of the zebrafish embryos in the groups. a: Pericardial edema, b: Yolc sac edema, c: Scoliosis. (exposure time; 0.08 s = S-XR, 0.15 s = M-XR, 0.30 s = L-XR).

Results of oxidant-Antioksidant analyses

Figure 3(A) shows the outcomes of lipid peroxidation analyses for the control, 0.08 (S-XR) seconds, 0.15 (M-XR) seconds, and 0.30 (L-XR) seconds groups. Compared to the control group, malondialdehyde (MDA) concentrations significantly increased in the 0.08, 0.15, and 0.30-second groups ($p<0.05$; $p<0.05$; $p<0.01$, respectively).

The results of Glutathione S-Transferase (GST) analyses of the control, S-XR, M-XR and L-XR groups are given in Figure 4(B). Accordingly, GST values significantly increased in S-XR, M-XR, L-XR groups compared to the control group ($p<0.01$; $p<0.0001$; $p<0.0001$, respectively)

We determined CAT activity as it is an important antioxidant enzyme that breaks down hydrogen peroxide into oxygen and water. The results of CAT analyses of the control, S-XR, M-XR and L-XR groups are given in Figure 3(C). CAT activities significantly decreased in the S-XR group compared to the control group ($p<0.0001$). In the M-XR group, CAT activities decreased significantly compared to the control group ($p<0.01$). Compared to the S-XR group, the CAT activities of the M-XR group significantly increased ($p<0.001$). Compared to the M-XR and S-XR groups, the activities of the L-XR group increased significantly ($p<0.05$ and $p<0.0001$, respectively).

In our study SOD activities decreased significantly in the S-XR, M-XR, L-XR groups compared to the control group ($p<0.05$; $p<0.01$; and $p<0.01$ respectively) (Figure 3D).

Result of AChE analysis

The results of Acetylcholine esterase (AChE) analyses of the control, S-XR, M-XR and L-XR groups are given in Figure 4(A). AChE activity significantly decreased in the S-XR group compared to the control group ($p<0.01$). In the M-XR group, AChE activity decreased significantly compared to the control group ($p<0.001$). AChE activity of the L-XR group decreased significantly compared to the Control, S-XR and M-XR groups ($p<0.0001$, $p<0.01$, and $p<0.05$).

Result of NO analysis

The results of nitric oxide (NO) analyses of the control, S-XR, M-XR and L-XR groups are given in Figure 4(B). Accordingly, NO values significantly increased in the S-XR, M-XR, L-XR groups compared to the control group ($p<0.05$; $p<0.01$; and $p<0.001$ respectively). When compared with the S-XR group, NO values of the L-XR group were significantly higher ($p<0.05$).

Result of gene expression analysis

The results of *bax* expression analyses of the control, S-XR, M-XR and L-XR groups are given in Figure 5(A). *bax* expression increased significantly in the S-XR, M-XR, L-XR groups

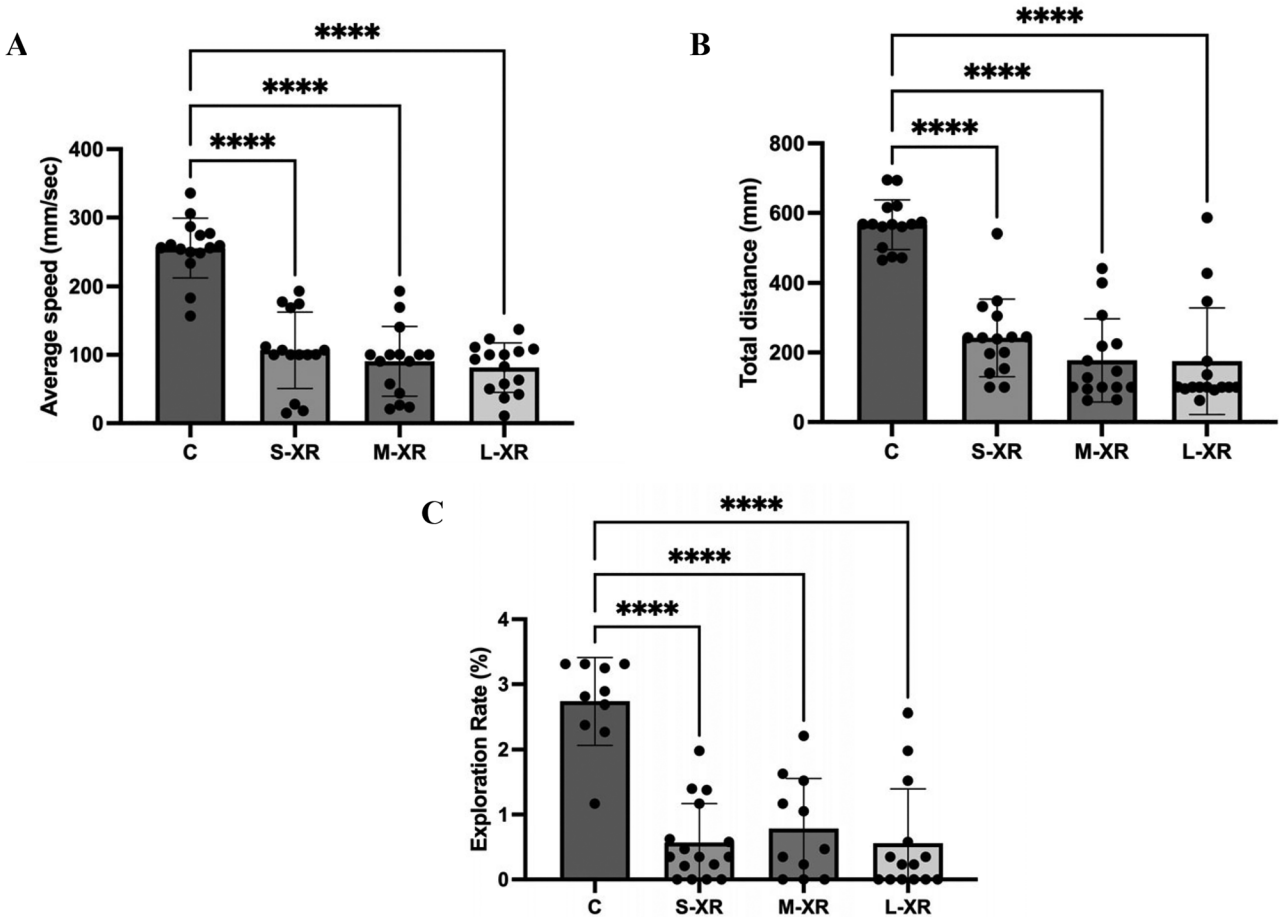

Figure 2. (A) Average speed (mm/sec); (B) Total distance swam (mm) (C) Exploration rates (%) of the groups ($n=15$) ****$p<0.0001$ C: Control; SD: standard deviation. (exposure time; 0.08 s = S-XR, 0.15 s = M-XR, 0.30 s = L-XR).

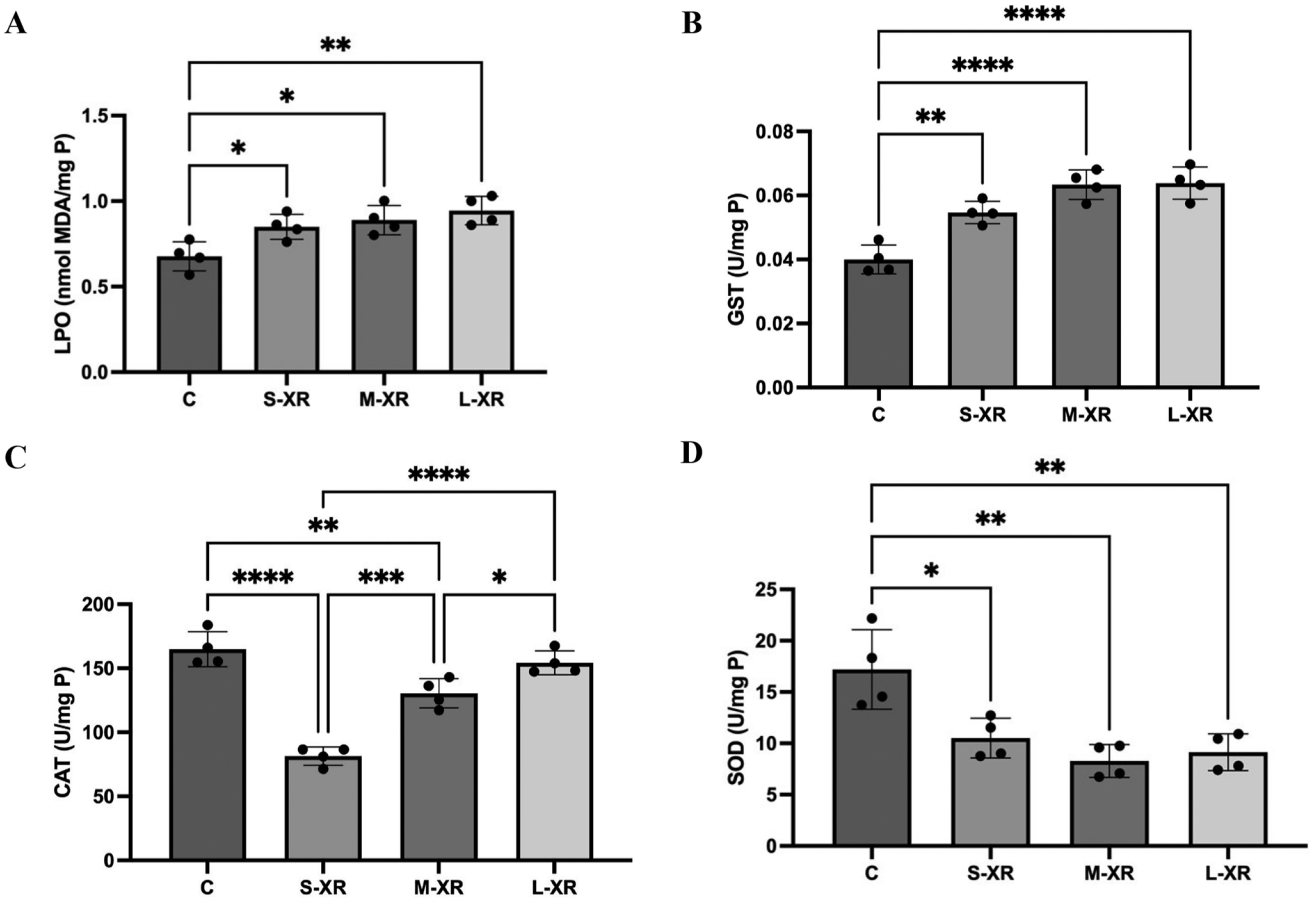

Figure 3. (A) Malondialdehyde (MDA) levels as an index of lipid peroxidation (LPO); (B) Glutathione S-transferase activities of the groups; (C) Catalase activities of the groups (D) Superoxide dismutase activities of the groups. Data are expressed as mean ± SD from the four independent experiments ($n = 4$, 4 biological replicates for each group, 50 embryos/Pool). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ C: Control; SD: standard deviation. (exposure time; 0.08 s = S-XR, 0.15 s = M-XR, 0.30 s = L-XR).

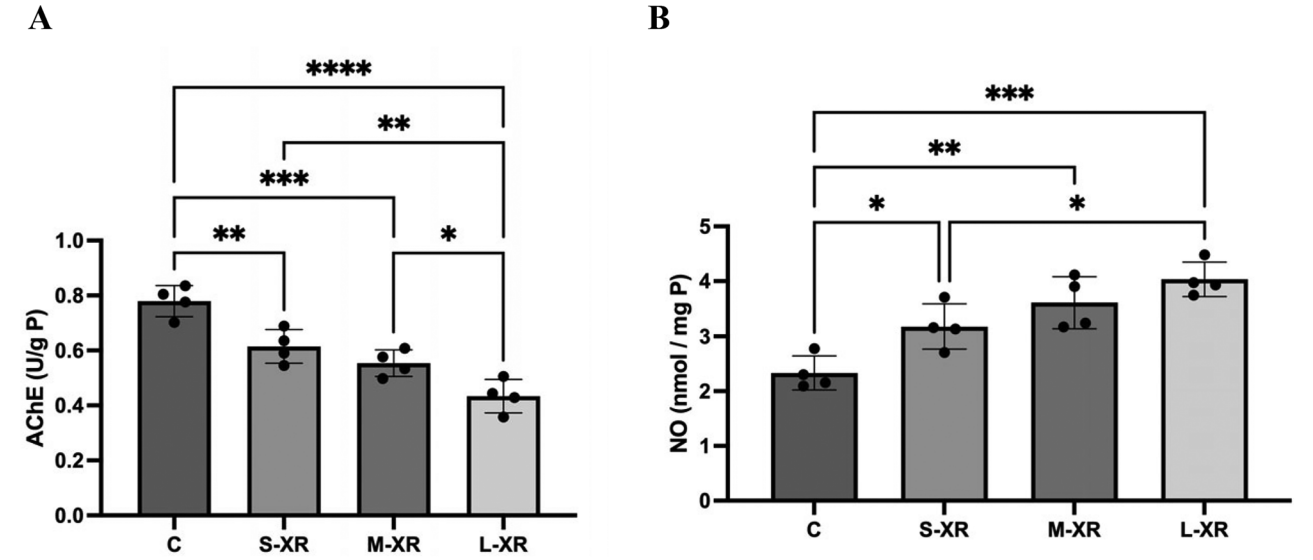

Figure 4. (A) Acetylcholine esterase activities of the groups (B) Nitric oxide levels of the groups Data are expressed as mean ± SD from the four independent experiments ($n = 4$, 4 biological replicates for each group, 50 embryos/Pool). *$p < 0.05$; **$p < 0.01$; *** $p < 0.001$; ****$p < 0.0001$ C: Control; SD: standard deviation. (exposure time; 0.08 s = S-XR, 0.15 s = M-XR, 0.30 s = L-XR).

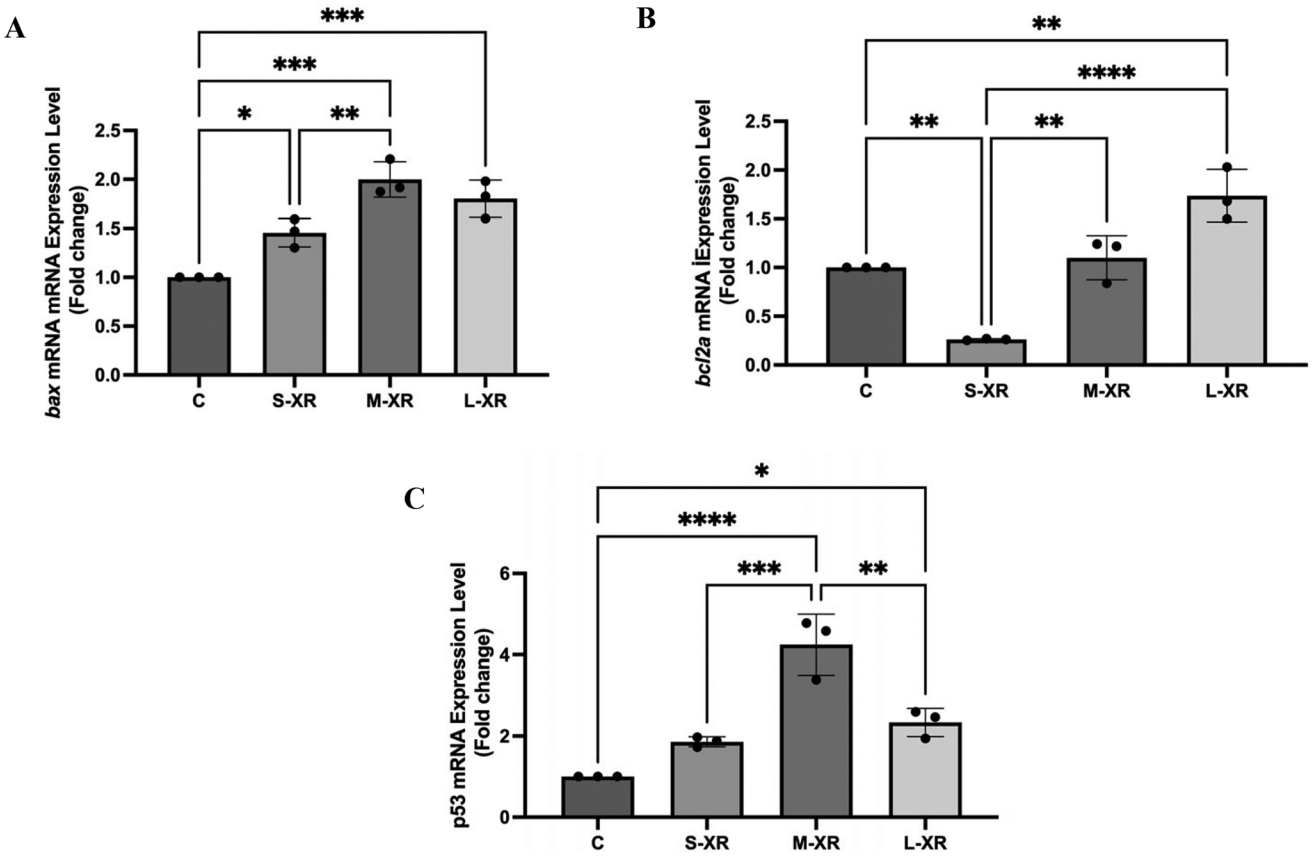

Figure 5. Bar graph presentation of the fold change of (A) *bax* (B) *bcl2*, and (C) *p53* transcripts quantified by RT-PCR. All RT-PCR results are normalized to β-actin, the housekeeping gene, and expressed as change from their respective controls. The average values were obtained from three experiments ($n = 3$, 3 biological replicates for each group, 50 embryos/Pool). Data presented are mean ± SD. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ C: Control; SD: standard deviation. (exposure time; 0.08 s = S-XR, 0.15 s = M-XR, 0.30 s = L-XR).

compared to the control group (respectively, $p < 0.05$; $p < 0.001$; $p < 0.001$). Compared to the S-XR group, the *bax* expression of the M-XR group was significantly higher ($p < 0.01$). The results of *bcl2a* expression analyses of the control, S-XR, M-XR and L-XR groups are given in Figure 5(B). *bcl2a* expression decreased significantly in the S-XR group compared to the control, M-XR, and L-XR groups, ($p < 0.01$, $p < 0.01$ and $p < 0.0001$ respectively). Moreover when compared to the control group, the *bcl2a* expression of the L-XR group was significantly higher ($p < 0.01$). *p53* expressions increased significantly in the M-XR and L-XR groups compared to the control group ($p < 0.0001$ and $p < 0.05$ respectively). In the M-XR group, *p53* expression was significantly higher than the S-XR and L-XR groups ($p < 0.001$ and $p < 0.01$ respectively), (Figure 5C).

The results of *α1-tubulin* expression analyses of the control, S-XR, M-XR and L-XR groups are given in Figure 6(A). *α1-tubulin* expressions increased significantly in the S-XR and M-XR groups compared to the control group ($p < 0.001$ and $p < 0.01$ respectively). In the L-XR group, *α1-tubulin* expression values decreased significantly compared to the control group ($p < 0.05$). Compared to the S-XR group, the *α1-tubulin* expression values of the L-XR group were significantly decreased ($p < 0.0001$). Compared to the M-XR group, the *α1-tubulin* expression values of the L-XR group were significantly decreased ($p < 0.0001$).

syn2a expressions significantly decreased in the S-XR, M-XR, L-XR groups compared to the control group ($p < 0.0001$). In the M-XR group, *syn2a* expression decreased significantly compared to the S-XR group ($p < 0.05$). Compared to the S-XR group, the *syn2a* expression of the L-XR group was significantly lower ($p < 0.01$) (Figure 6B).

The results of *neurog1* expressions of the control, S-XR, M-XR and L-XR groups are given in Figure 6(C). *neurog1* expressions decreased significantly in the M-XR and L-XR groups compared to the control group ($p < 0.05$ and $p < 0.05$ respectively). *elavl3* expression decreased significantly in the S-XR, M-XR, L-XR groups compared to the control group ($p < 0.01$; $p < 0.001$; and $p < 0.001$ respectively) (Figure 6D).

eif4eb expressions increased significantly in the S-XR, M-XR, L-XR groups compared to the control group ($p < 0.001$; $p < 0.0001$; and $p < 0.0001$ respectively). In the M-XR group, *eif4eb* expression increased significantly compared to the S-XR group ($p < 0.05$). Compared to the S-XR group, *eif4eb* expression of the L-XR group was significantly higher ($p < 0.05$), (Figure 7A).

Accordingly, *adsl* expression decreased significantly in the S-XR and M-XR groups compared to the control group ($p < 0.0001$ and $p < 0.0001$ respectively). In the L-XR group, *adsl* expression decreased significantly compared to the control group ($p < 0.001$). In the M-XR group, *adsl* expression increased significantly compared to the S-XR group ($p < 0.001$). Compared to the S-XR group, the *adsl* expression of the L-XR group increased significantly ($p < 0.001$) (Figure 7B). *shank3a* expression values significantly decreased in the M-XR and

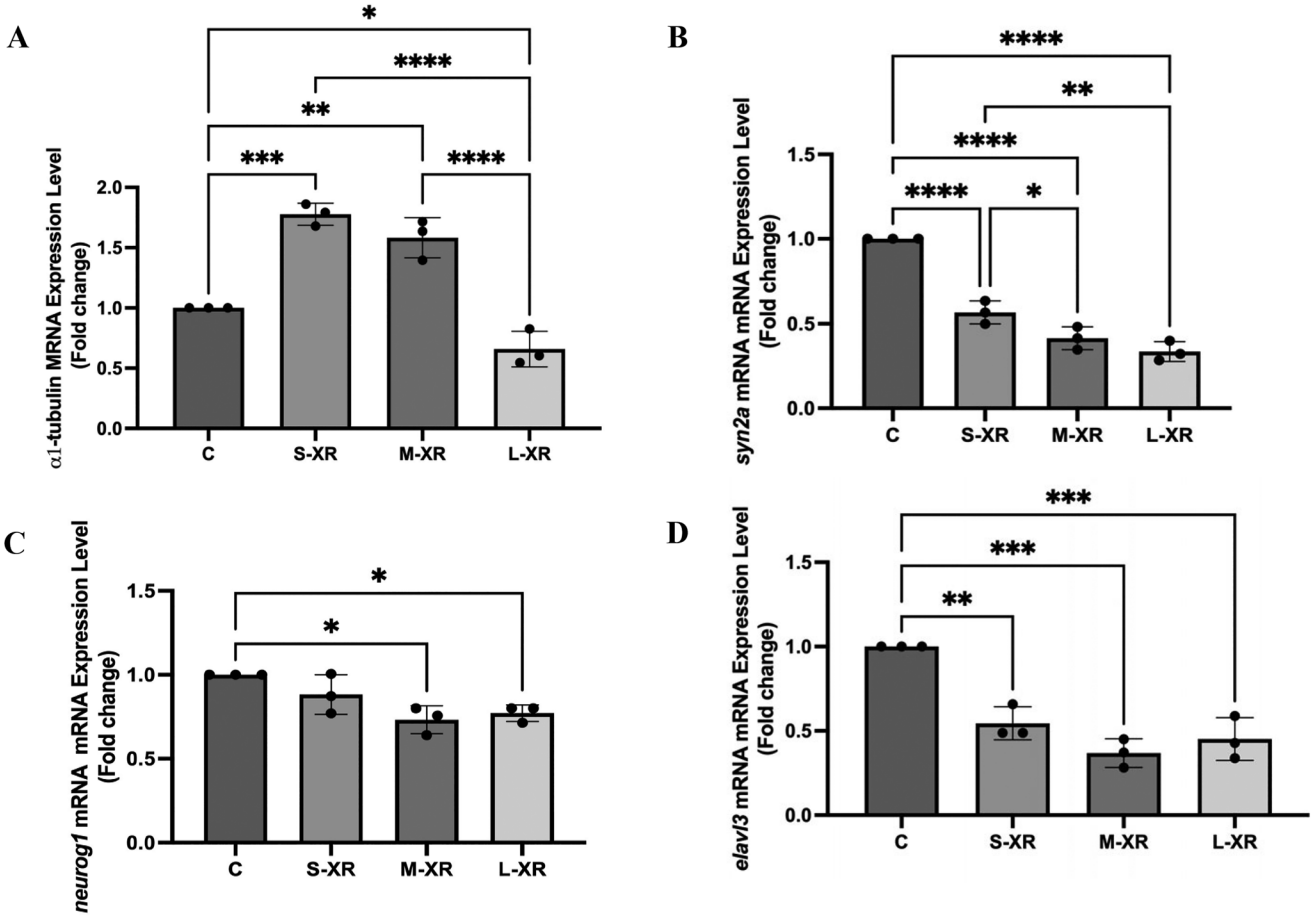

Figure 6. Bar graph presentation of the fold change of (A) *α1-tubulin* (B) *syn2a* (C) *neurog1*, and (D) *elav3* transcripts quantified by RT-PCR. All RT-PCR results are normalized to β-actin, the housekeeping gene, and expressed as change from their respective controls. The average values were obtained from three experiments ($n = 3$, 3 biological replicates for each group, 50 embryos/Pool). Data presented are mean ± SD. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ C: Control; SD: standard deviation. (exposure time; 0.08 s = S-XR, 0.15 s = M-XR, 0.30 s = L-XR).

L-XR groups compared to the control group (respectively, $p < 0.0001$; $p < 0.0001$). In the M-XR group, *shank3a* expression values decreased significantly compared to the S-XR group ($p < 0.0001$). Compared to the S-XR group, the *shank3a* expression of the L-XR group decreased significantly ($p < 0.0001$) (Figure 7C).

Discussion

Our study is the first to determine the effects of low-dose ionizing radiation from a dental x-ray unit and different exposure times on developing zebrafish embryos focusing on neurogenesis and autism-related genes. The results of our study showed that dental x-ray exposure altered the expression of neurogenesis and autism-related genes in zebrafish embryos at all different exposure times examined.

The prevalence of ASD is rapidly increasing, and there has been a growing number of studies focusing on the potential influential environmental factors in the development of ASD (Hu, 2013). The maternal and infant microbiome, antibiotic therapy and epigenetic changes have all been linked to the development and progression of ASD pathogenesis (Sgritta et al., 2019). X-rays are one of the most common environmental factors. Although the nervous system is resistant to radiation, neuroblasts are quite sensitive (McNerlin et al., 2022). Low-dose ionized radiation has been shown to affect neurological cells in the developing hippocampus, with a decrease in neurogenesis and an increase in cell apoptosis (Peissner et al., 1999). In order to investigate the effects of radiation exposure during the developmental period, in particular on neurogenesis, and the associated lifelong consequences, it is necessary to determine the effects of low-dose radiation exposure on gene expression.

In our study, low dose x-ray exposure led to changes in the expressions of *syn2a*, *neurog1*, *α1-tubulin* and *elavl3*, which are genes related to neurogenesis. Expressions of *syn2a*, *neurog1,* and *elavl3* decreased on the exposure periods. On the other hand, *α1-tubulin* expression was found to increase in the S-XR and M-XR exposure groups, while a decrease was observed in the L-XR group. Although the increases in *α1-tubulin* in the S-XR and M-XR exposure groups can be explained as the activation of protective systems in response to inhibitory stressors during embryogenesis, it can be suggested that the protective systems are disabled in the L-XR group.

The zebrafish's proneural genes, which are apparent in the late gastrulation stage, preserve the start of neurogenesis in the neural plate. One of the earliest proneural genes to produce transcription factors is *neurog1* (Çalışkan &

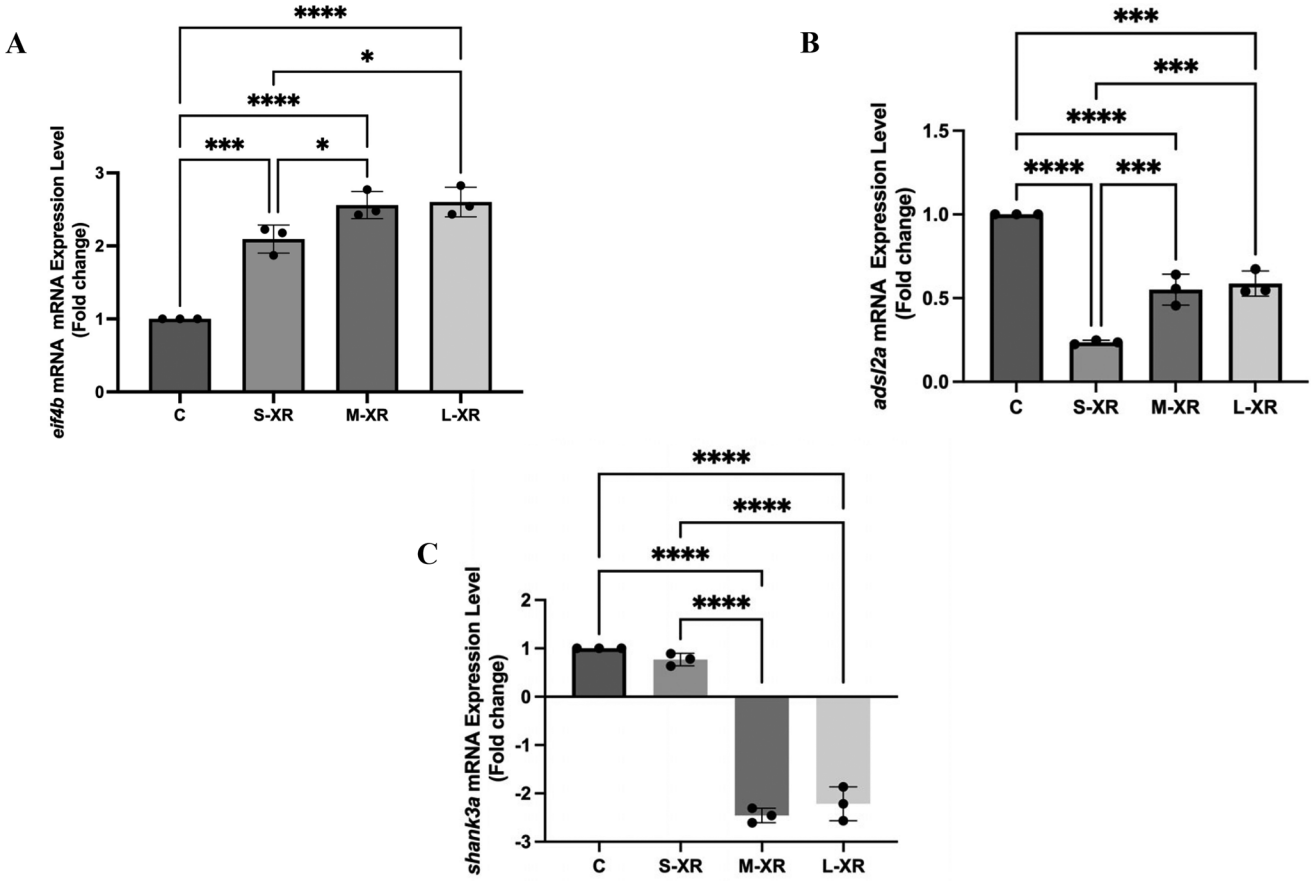

Figure 7. Bar graph presentation of the fold change of (A) *eif4b* (B) *adsl2a,* and (C) *shank3a* transcripts quantified by RT-PCR. All RT-PCR results are normalized to β-actin, the housekeeping gene, and expressed as change from their respective controls. The average values were obtained from three experiments ($n = 3$, 3 biological replicates for each group, 50 embryos/Pool). Data presented are mean ± SD. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ C: Control; SD: standard deviation. (exposure time; 0.08 s = S-XR, 0.15 s = M-XR, 0.30 s = L-XR).

Emekli-Alturfan, 2021). Similar to the results of our study, different environmental toxins have been shown to affect the expression of neurogenesis genes in zebrafish embryos. As an important neuro-developmental gene *neurog1* has been reported to be affected by ibuprofen and diclofenac exposure in zebrafish embryos (Xia et al., 2017). *syn2a* and *α1-tubulin* are also among the neurodevelopmental genes that have been found to be downregulated in chlorpyrifos and 1,3-dichloro-2-propyl-phosphate exposed zebrafish embryos (Jeong et al., 2006). The expression of *elavl3* has also been suggested to be inhibited in zebrafish embryos exposed to triphenyl phosphate (Shi et al., 2018). In a previous study, *Microcystis aeruginosa*, a freshwater cyanobacterium, was found to decrease the expressions of *elavl3* and *syn2a* and affect neurogenesis in zebrafish (Qian et al., 2018). Furthermore, bisphenol A, the endocrine disrupting chemical, has also been shown to reduce the expression of *elavl3* and *syn2a* (Costa & Cairrao, 2024).

In our study, we detected changes in the expressions of *eif4eb*, *adsl*, and *shank3*, which are associated with autism, as well as changes in genes effective in neurogenesis in the x-ray radiation groups. eIF4 is involved in the control of synaptic plasticity as a mediator between neuronal activity and protein translation, and in our study, *eif4b* expression was found to increase with increasing x-ray exposure time. This finding is noteworthy as the overexpression of the translation initiation factor eIF4E to increase protein synthesis in brain cells has been shown to lead to autism-like behavior in mice (Bettegazzi et al., 2017). *adsl2* gene encodes the enzyme adenylosuccinate lyase (ADSL), which is necessary for *de novo* purine production (Lee et al., 2018). We detected decreases in *adsl2a* expressions in all x-ray groups, most notably in S-XR group. On the other hand, decreases in *shank3a* expression were especially evident in the M-XR and L-XR groups. In an experimental autism model induced by valproic acid (VPA) in zebrafish, the expressions of these genes changed compared to the control group, while *adsl* expression increased, *shank3* expression decreased (Lee et al., 2018). In contrast to this study, in which zebrafish embryos were exposed to VPA for 120 hours, our study involved a single exposure for each group at relatively low doses. Accordingly, the decreased *adsl* expression in our study can be considered as the first response of the organism to the short-term exposure to ionizing radiation.

In both laboratory animals and clinical research for investigating psychiatric and developmental disorders, locomotor dynamics may provide a valuable objective and quantifiable measure for translational research, since behavioral index investigation is less invasive and can be carried out for an extended period of time (Nakamura et al., 2008). Ogino et al. (2018) evaluated the relationship between locomotor dynamics and clinical features such as autistic traits,

emotional and behavioral problems in children with ASD and reported negatively altered all-day locomotor activity, which could be a potentially helpful measurable behavioral measure for ASD, particularly for social awareness in autism. Accordingly, one of the striking findings of our study, consistent with the change in expression of genes associated with neurogenesis and autism, was the decrease in locomotor activity in the x-ray exposed groups, as evidenced by decreased average speed, distance traveled and areas explored. It was also notable that the decrease in locomotor activity was accompanied by a decrease in AChE activity. AChE hydrolyzes acetylcholine, resulting in the formation of choline and esterases. Acetylcholine spreads from the neurons into the synaptic cavity where the signal is formed. Excessive accumulation of acetylcholine leads to increased muscle contraction with clinical signs such as tremor (Klaassen, 1985). Studies have shown that exposure to environmental factors such as pesticides has led to increased cholinesterase levels and organophosphate poisoning (Yücesan et al., 2012). Inhibition of locomotor activity may be associated with the deleterious effects of X-rays on cholinergic function, as improvement of cholinergic function through inhibition of AChE has been shown to enhance locomotion (Holschneider et al., 2011). Although the molecular pathogenesis of ASD is still unclear, it has been suggested that oxidative stress and apoptosis play important roles in the pathogenesis of ASD (Kaushik & Zarbalis, 2016). Few studies in the literature show that oxidative stress contributes to the development of ASD (Chen et al., 2021). Low-dose ionized radiation is absorbed by the cell, resulting in increased oxidative stress as apoptosis mechanisms become active. We have previously shown the deterioration of the oxidant-antioxidant balance, suppression of immune response, induction of inflammation and apoptosis in dental panoramic x-rays exposed zebrafish embryos (Karagöz et al., 2023). Consistent with our previous study, in the present study exposure doses for periapical imaging disrupted the oxidant-antioxidant balance as evidenced by increased LPO, and decreased SOD activities. On the other hand, significant increases in GST and CAT activities were detected in response to oxidant stress, proportional to the exposure times. Several studies have examined the effects of ionizing radiation on oxidative systems and free radicals (Salaberria et al., 2009; Srivastava et al., 2012; Tharmalingam et al., 2017). Both low and high dose ionizing radiation have been shown to disrupt oxidant-antioxidant balance by inducing oxidative stress and exceeding the antioxidant capacity of cellular antioxidants (Shao et al., 2012; Si et al., 2013).

Radiation-induced damage is associated with an increase in ROS and free radicals (Nuszkiewicz et al., 2020; Hogg & Kalyanaraman, 1999). In our study increased LPO was more pronounced at the maximum exposure time setting (L-IR) than the other groups. Similarly, NO levels increased significantly with increasing exposure time. Consistent with the results of our study, rats showed an increase in LPO levels as a result of exposure to 3 Gray (Gy) of x-rays (Nishi et al., 1986). Another study found a significant increase in NO levels in rabbits following exposure to 550 rads of x-rays (Dede et al., 2009). In our study, increased NO levels in the ionizing radiation groups may be associated with the observed pericardial edema findings. Besides being an endogenous free radical, NO is a potent vasodilator. The edema associated with radiation has been reported to indicate an increase in the permeability of the capillary wall. Vessel dilation is one of the most common early changes observed in the capillaries and pre-arterioles after irradiation. Naturally, compared to the endothelial cells in older capillaries, the rapidly growing endothelial cells in newly formed capillaries are more sensitive to radiation (Hatjikondi et al., 1996).

GST, part of the antioxidant defence system which prevents xenobiotics from combining with antibodies to damage the cell, was found to increase with increasing exposure. Previous studies have shown an increase in GST levels due to oxidative stress and environmental stimuli (Kumar & Prabodh, 2018). The results of our study showed an increase in GST activity as the x-rays exposure time increased. SOD is suggested to be responsible for combating oxidative stress caused by ionized radiation. A significant decrease in SOD activity with increased exposure time was observed in the x-ray groups. Decreased SOD activity was also reported in rats exposed to different x-ray doses between 2 and 25 Gy (Xu et al., 2011). In our study a significant decrease in CAT activity was observed in all x-ray groups although it was most evident in the S-XR group, and increases in CAT activity was observed with increasing duration of exposure reflecting the response to increased oxidant damage. Accordingly, it is possible that CAT activity increases due to oxidative stress as reported significantly higher CAT activities in hospital workers exposed to low-dose ionized radiation (Gao et al., 2020).

Cellular redox change caused by ROS is part of the apoptosis signal transduction pathway (Dong et al., 2018). Inhibition of apoptosis during early neurogenesis has been reported to result in the expansion of neuronal cell types, leading to disruption of normal neurogenesis (Yeo & Gautier, 2003). It has been suggested that the increase in brain size in autism may result from excessive neuronal production due to disruption of apoptosis pathways (Kaushik & Zarbalis, 2016).

The *p53* pathway induces apoptosis by up-regulating the transcription of pro-apoptotic genes, especially bax, and down-regulating anti-apoptotic genes, including bcl-2 (Li, 2021). Consistent with this, in our study, pro-apoptotic *bax* expression increased in all x-ray groups along with increases in *p53* expressions, but the most significant increase was in the M-XR group. On the other hand, the decrease in antiapoptotic *bcl2* expression was evident only in the S-XR group. The function of *p53* is to activate the apoptosis mechanism by increasing the production of *apaf-1* phase genes and reducing the production of bcl-2 secreted from the mitochondrial membrane as a result of DNA or cell damage. When *p53* function is compromised, *bcl-2* is overproduced, the apoptosis mechanism is not activated, and aged or cancerous cells proliferate. In a previous study, Zhou et al. showed that cell apoptosis increased with increasing exposure dose (Zhou et al., 2014).

In our study, low dose ionizing radiation also caused morphological changes such as yolc sac edema, pericardial edema, decreased body pigmentation and scoliosis in zebrafish embryos. Like human embryo, zebrafish embryo also has

an extending yolk sac acting as a nutrient cache. Low dose ionizing radiation led to a larger yolk sac area compared with the control embryos indicing impaired yolk uptake. A pathology named "Blue sac syndrome" characterized by yolk sac and pericardial edema was observed in the L-XR group at 72 hpf (Hill et al., 2004). Abnormal shapes of yolk sac and pericardial edema have been reported in fish embryotoxicity studies mostly reported after exposure to toxic substances (Sant & Timme-Laragy, 2018) The most direct way that toxicants can affect the deposition of yolk is by interfering with the formation of vitellogenin, main substance found in zebrafish yolks (Hanisch et al., 2010). Numerous critical mechanisms govern the storage and utilization of nutrients, including the Peroxisome Proliferator-Activated Receptor (PPAR) pathway and PPAR-disrupting factors can directly affect vitellogenesis. Zaghloul et al. reported that irradiation affected vitellogenin expression in *Galleria mellonella* which may be related with the malformations observed in our study due to ionizing radiation (Zaghloul et al., 2017). Moreover, abnormal circadian rhythm and consequent defects in melatonin expression caused by radiation has been reported which is associated with defecrs in pigmentation and also development of scoliosis (Girardo et al., 2011; Zhao et al., 2023).

In our previous study we have shown the deterioration of the oxidant-antioxidant balance, suppression of immune response, induction of inflammation and apoptosis in panoramic x-rays exposed zebrafish embryos (Karagöz et al., 2023). To our knowledge, the present study is the first to investigate how developing zebrafish embryos are affected by different exposure settings of periapical dental x-rays. The results of our study show that x-rays used in periapical dental imaging caused oxidative stress, activated apoptotic pathways, and altered the expressions of genes related with neurogenesis and ASD even at reduced exposure modes of the device. Although these findings demonstrate the deleterious effects of ionizing radiation even at low doses on neurodevelopment, the fact that the expressions of markers related to neurogenesis and ASD development were determined only at the gene level can be considered a limitation of our study. Moreover, in our study MDA levels were measured as an index of lipid peroxidation and oxidative stress however, the fact that ROS levels were not directly measured can be considered as another limitation of our study.

We found that the effects of 0.30 s periapical x-ray exposure on zebrafish embryos were more severe than those of 0.08 and 0.15 s periapical x-ray exposure. Furthermore, the results of the OSL dosimeter measurements were consistent with this observation. These were 7.17, 23.17 and 63.83 mSv for the 0.08 s (S-XR), 0.15 s (M-XR) and 0.30 s (L-XR) x-ray exposed groups.

The development of digital technology has led to a reduction in radiation dose. While analogue film sensors require higher doses, digital sensors require lower doses (International Atomic Energy Agency, 2023). The doses were chosen and modified as exemplary representative doses required for periapical film taking (Charged Coupled Device (CCD), Phosphor Plate System (PPS) and analogue film (E or F speed film) sensors, i.e., 0.08 s, 0.15 s, 0.30 s respectively (Ludlow et al., 2008; Tamam et al., 2021; Yalcinkaya et al., 2006, 2012).

The Life Span Study of Japanese atomic bomb survivors has shown how the risk of different cancers varies with radiation dose after immediate external exposure, and how these risks can vary with factors such as age at exposure and time since exposure. The risk of thyroid cancer and leukemia has also been associated with nuclear weapons testing and nuclear power plant accidents (Preston et al., 2007; Muirhead, 2001; Hwang et al., 2018). Dental x-rays have been associated with an increased risk of cancer, and an increased risk of cancer is also associated with repeated exposure (Hall & Brenner, 2008; Hwang et al., 2018; Zielinski et al., 2009). In addition, stochastic effects such as cancer and genetic defects occur as the intensity of radiation increases. Therefore, the aforementioned association requires a closer look from the perspective of the lifetime frequency of dental diagnostic x-rays and the potential risks of low-dose ionizing radiation. Accordingly, the results of our study emphasize the importance of further consideration of biological radiation hazards and selection of optimal periapical modes prior to dental image acquisition.

Conclusion

Although the level of exposure in dentistry is lower than that of medical radiation, there is an inherent risk of radiation exposure that cannot be ignored. Even at the lowest possible doses, the use of x-rays from dental x-ray units should be used with caution. The use of advanced digital sensors, which require shorter exposure times, may reduce these effects and potentially reduce the dose-dependent effects on neurogenesis for the possibility of an unknown pregnancy. The results of this study showed dose-related deleterious effects of dental x-rays on neurogenesis through impaired locomotor activity, oxidative stress, apoptosis and alterations in genes associated with neurogenesis and ASD progression. We believe that further research on the subject is needed, such as examining the changes in neurogenesis and ASD-related genes shown in our study at the protein level and confirming the relationship using proteomic and metabolomic approaches.

Ethical approval

As the zebrafish embryos used were no older than 5 days old, no ethical approval was required for the protocols applied as stated by the Council of Europe (1986), Directive 86/609/EEC.

Disclosure statement

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

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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