Malaysian Applied Biology (2024) 53(4): 27-42
https://doi.org/10.55230/mabjournal.v53i4.3077

Research

RAD50 Deficiency and Its Effects on Zebrafish Embryonic Development and DNA Repair Mechanisms

Nahid Khalili[1], Shazrul Fazry[1,2,3]*, Ibrahim Mahmood[4], Ahmed Najm[1], Ahmad Azfaralariff[1], Douglas Law[5]

1. Department of Food Science, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600, Bangi, Selangor, Malaysia
2. Centre of Excellence, Innovation Centre for Confectionery Technology (MANIS), Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor, Malaysia
3. Tasik Chini Research Center, The Centre for Natural and Physical Laboratory Management UKM, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor, Malaysia
4. Dentistry department, Al-Rafidain University College, Baghdad, Iraq
5. Faculty of Health and Life Sciences, Inti International University, 71800 Nilai, Negeri Sembilan, Malaysia
*Corresponding author: shazrul@ukm.edu.my

ABSTRACT

The MRE11-RAD50-NBS1 (MRN) complex is essential in detecting, signaling, and repairing DNA double-strand breaks (DSBs), thus maintaining genomic integrity. Mutations in RAD50 are linked to severe conditions such as microcephaly, mental retardation, and growth retardation in humans. This study investigates the developmental impact of RAD50 protein disruption in zebrafish embryos. Zebrafish embryos were treated with MIRIN (35 μM) to inhibit RAD50 and subsequently exposed to gamma-ray irradiation (15 Gy) to analyze the role of RAD50 in managing DNA damage during embryogenesis. Time-point analysis indicated that inhibiting RAD50 and ATM proteins during early embryonic stages (at 1 hpf) leads to increased embryonic mortality and abnormalities. These adverse effects were exacerbated by irradiation, underscoring the critical role of RAD50 in DNA DSB repair. The study concludes that RAD50 deficiencies can lead to embryonic lethality and human deformities due to the inability of tissues to repair DNA DSBs effectively.

Key words: ATM protein, DNA damage, human health, MRN Complex, RAD50 gene

Article History
Accepted: 26 August 2024
First version online: 27 October 2024

Cite This Article:
Khalili, N., Fazry, S., Mahmood, I., Najm, A., Azfaralariff, A. & Law, D. 2024. RAD50 Deficiency and its effects on zebrafish embryonic development and DNA repair mechanisms. Malaysian Applied Biology, 53(4): 27-42. https://doi.org/10.55230/mabjournal.v53i4.3077

INTRODUCTION

DNA double-strand breaks (DSBs) are the most serious hazard to the integrity and survival of cells when they occur. Cell exposure to DNA damage causes a variety of lesions. Chromosomal rearrangements such as translocations and deletions can be caused by improperly repaired DSBs, and these might result in neoplastic transformation or cell death (Shibata *et al*., 2014). DSBs in DNA can arise from a range of factors, which are broadly categorized into endogenous and exogenous factors. Endogenous factors include Reactive Oxygen Species (ROS), replication stress, and topoisomerase activity. Exogenous factors encompass ionizing radiation, chemicals, ultraviolet (UV) light, physical stress, and environmental toxins. To repair DNA, a broken DNA strand must be inserted into a homologous DNA duplex and then duplicates must be synthesized (Paull & Lee, 2005). MRE11-RAD50-NBS1 (MRN) is a critical protein complex that reacts mainly to hairpins, (DSBs), and other aberrant terminal DNA structures. Through the Ataxia Telangiectasia Mutated (ATM) kinase, this complex controls both signal transduction and DNA repair (Hopfner *et al*., 2000). ATM phosphorylates RAD50 at a specific location (Ser-635), essential for RAD50's adaptor function in signaling DNA repair and cell cycle regulation. In RAD50-deficient cells,

mutations in this signaling region led to an S-phase checkpoint deficiency, an inability to repair (DSBs), and radiosensitivity (Gatei *et al*., 2011).

ATM plays a crucial role in DNA damage response and maintaining genomic stability, with significant implications for cancer and neurodegenerative disorders. Zebrafish serve as an invaluable model for studying vertebrate brain development, offering insights into the genetic and cellular processes underlying neurodevelopmental disorders and potential therapeutic approaches (Shiloh *et al*., 2013).

(DSBs), which arise during meiosis, are one of the DNA repair pathways in which RAD50 is involved in meiotic recombination in the mouse testis (Enguita-Marruedo *et al*., 2019). As a component of the MRN complex, RAD50 is vital for identifying and repairing DNA damage, including (DSBs), which are necessary for preserving genomic integrity during meiotic recombination (Lamarche *et al*., 2010). Some of this role may involve aiding in the process of homologous recombination, which fixes broken ends of DNA by using homologous sequences as templates. The roles of RAD50 in meiotic recombination are essential for correct chromosome segregation and the production of genetically diverse gametes, which in turn guarantee successful reproduction (Hunter *et al*., 2015).

In mouse embryonic fibroblasts, the MRN complex plays a crucial role in responding to telomere dysfunction, indicating a shared mechanism with the response to interstitial DNA damage (Syed A. *et al*., 2018). The impact of melatonin as a radioprotective agent on the expression of RAD50, a component of the MRN complex protein, in the peripheral blood of rats subjected to radiation has been investigated. Treating rat embryos with melatonin before irradiation led to increased levels of RAD50 expression (Enguita-Marruedo *et al*., 2019). During Drosophila embryogenesis, the MRN complex is necessary for telomere capping. Disruption of RAD50 in Drosophila results in lethality, with mutant flies failing to reach adulthood. Late pupae with RAD50 deficiency exhibit morphological abnormalities such as abdominal segmentation defects, black spots, and epithelial tumors (Gao et *al*., 2009).

In this study, we utilized *Danio rerio* as a model to explore the role of the RAD50 gene in embryonic development. Microcephaly, a characteristic sign of RAD50 deficiency, was investigated by observing defects in head growth under conditions of gene inactivation. *Danio rerio*, with its short lifespan and rapid progression to the reproductive stage, provides a visible platform to monitor embryonic development, facilitating the detection of morphological changes and growth rate alterations. Our findings reveal how gene dysfunction impairs DNA damage repair and the cell cycle, consequently leading to abnormal embryonic development and inhibiting early-stage head and brain growth.

MATERIALS AND METHODS

Ethics statement

All in vivo experimental procedures were performed under the EU-directive 2010/63/EU on the protection of animals used for scientific purposes, and all procedures were carried out under project approval number FST/2018/MOHD SHAZRUL/28-MAR.-2018-DEC. -2021, approved by the UKM Animal Ethical Committee.

Zebrafish maintenance

Every experiment was conducted using a wildtype zebrafish, AB strain that was bred in the Molecular Genomic Lab 1 (UKM). The zebrafish were kept in an aquarium (PH 6.8-7.2) with a 14-hr light cycle and a 10-hr dark cycle, kept at 28°C. Once or twice a day, a mixture of ground flake food, and egg yolk was fed to the adult fish. Adult zebrafish were purchased from Brain Research Institute Monash Sunway (BRIMS), and adult fish were stocked at a maximum density of one fish per 200 milliliters.

Embryo production

Male zebrafish can be differentiated from females by their distinctive swollen abdomens, more compact body morphology, and the orange-to-reddish hue observed in the silvery bands encircling their bodies. On the day preceding mating, males and females were introduced into breeding tanks at a ratio of 2:1, timed just before the onset of darkness. A translucent barrier was placed within the tank to separate the sexes, and a small quantity of marbles was added to the section containing females to simulate natural spawning substrates. Mating, spawning, and fertilization occurred within 30-60 min after the onset of light in the morning. Mesh egg traps were installed in the breeding tanks to prevent egg displacement. Approximately 30-60 min after spawning, the eggs were transferred to a 92 mm plastic Petri dish containing 30 mL of fresh 1X E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$). Eggs were identified based on their transparency, ideally by placing the spawning dish on a black pad and using transverse light. Any eggs showing morphological abnormalities during cleavage or those that failed to undergo the cleavage process were discarded.

Inhibition of MRN complex and ATM proteins in zebrafish embryo

To further investigate the MRN and ATM protein functions in zebrafish embryo development, the protein complexes were inhibited with specific inhibitors, MIRIN and KU55933, respectively. (Fazry *et al*., 2019).

Embryo exposure and irradiation

Healthy embryos were subjected to treatment with various concentrations of the MIRIN inhibitor (SIGMS M9948), including 0, 5, 10, 15, 25, 35, 45, and 55 µM, dissolved in 0.1% DMSO. Treatment commenced at 8 hr post-fertilization (hpf) and continued until the completion of 72 hpf in 24-well plates format, with each well containing 1 embryo submerged in 240 mL of the inhibitor solution. Media replenishment with inhibitors was done every 24 hr. All concentrations were concurrently tested in parallel wells with up to 3 independent replications. DMSO was utilized as a control solution at a final concentration of 0.1% v/v. Comparative phenotypic analysis between embryos treated with 0.1% DMSO solvent and those treated with embryo water revealed no observable phenotypic alterations at the designated developmental stages. The irradiation protocol for the embryos was carried out by the Nuclear Science Department at Universiti Kebangsaan Malaysia. Subsequently, the embryos were irradiated at room temperature using Gammacell 220® (MDS Nordion, Ottawa, Canada) or exposed to 137Cs gamma irradiation. In each well of a 6-well plate filled with 5 mL of E3 medium, 8 embryos aged 8 hpf were placed for irradiation, with two other parallel wells undergoing equal treatment. Various doses of irradiation (1, 5, 10, 15, & 20 Gy/min) were administered to the embryos. Subsequently, all treated and irradiated embryos were incubated at 28°C to monitor their development until 72 hpf.

Effect of inhibitors on the development of zebrafish embryo

To assess the inhibition effect of MRN and ATM proteins, chemical inhibitors were employed (Fazry *et al*., 2019). The MRN inhibitor MIRIN and the ATM inhibitor KU-55933 (SML1109) were utilized to inhibit MRN and ATM proteins, respectively. Embryos were exposed to 35 µM of the MRN inhibitor MIRIN, 10 µM of the ATM inhibitor KU-55933, and 0.1% DMSO was used as the control group.

Each treated group was divided into two subsets: one subset was irradiated at 15 Gy/min, while the other remained non-irradiated. Each group contained eight embryos, with three replicates, totaling 24 embryos per group. To examine the roles of MRN and ATM proteins, treatments and irradiations were performed at three embryonic stages. In the first analysis, embryos were treated at 1 hpf and irradiated at 4 hpf. In the second, treatment occurred at 4 hpf and irradiation at 8 hpf. In the third, treatment was at 12 hpf, with irradiation at 24 hpf. Embryonic development was assessed morphologically at 24, 48, 72, and 120 hpf. Embryos were anesthetized with ice-cold Tricaine Methanesulfonate (MS-222) at 200 mg/L. Images were taken at 4x and 10x magnifications using a Leica macroFluo™ microscope with a monochrome camera and analyzed using Adobe ImageJ software. Measuring the heart rate of zebrafish can be a critical indicator of their physiological health and the effects of various treatments. First, anesthetize the embryos or larvae using an appropriate anesthetic, such as Tricaine Methanesulfonate (MS-222), at a concentration of 200 mg/L. Then, transfer the anesthetized zebrafish to a petri dish containing the anesthetic solution. Position the petri dish under a stereomicroscope or a compound microscope equipped with a video recording system. Record the heartbeats and count them within a known time frame (e.g., 20 seconds).

Histology of zebrafish embryo

Wild-type zebrafish embryos underwent treatment with MIRIN (35 µM) and KU55933 (10 µM) at one hpf, followed by irradiation at four hpf with 15 Gy of gamma rays. Subsequently, embryos at 120 hpf were fixed with Neutral Buffered Formalin 10% (NBF) overnight at 4°C. The dehydration and clearing of all zebrafish embryos were accomplished using a series of ethanol and xylene solutions. This process involved immersing the specimens sequentially in ethanol solutions [70% (2X), 80%, 95%, & 100% (2X)] for 45 min each. The tissue processing continued with Xylene 2 (45 min) and Paraffin 2 (1 hr). Paraffin infiltration steps were conducted in an oven incubator at 60°C to prevent solidification. Subsequently, a stainless-steel mold (Leica Biosystems, 39LC-700-1) was filled with liquid paraffin, and the specimen was carefully placed in the mold according to the intended plane of the section. A labeled cassette (Leica Biosystems, 39LC-500-1), previously used for tissue processing, was placed on top of the mold and topped up with more paraffin before allowing it to solidify on a cold plate. To facilitate reduced access to paraffin, the paraffin blocks containing specimens were trimmed. Specimens were then cut at a thickness of 4–5 µm to minimize overlap between cells in a section and ensure optimal histological detail. Paraffin ribbons containing the tissue sections were expanded in a water bath and

then scooped onto labeled microscope slides using the fishing method. The slides were dried in an incubator at 37°C overnight. For histological staining, the dried sections of the slides were subjected to H&E staining. Initially, paraffin was removed with xylene for 10 min, followed by rehydration with a decreasing concentration of ethanol series [100% 2(X), 90%, 80%, & 70%] for 5 min each. After rinsing the slides in distilled water (30 dips), the sections were immersed in hematoxylin solution for 8 min, followed by rinsing with tap water for 5 min. The slides were then stained with Eosin for 2 min and rinsed in running tap water for one min. Subsequently, they were dehydrated in an increasing ethanol series [70%, 80%, & 100% (2X)] for 5 min each and finally cleared in xylene solution for 10 min.

Acridine orange assay

Acridine orange staining was employed for visualizing cell death in vivo, utilizing this vital stain renowned for its nucleic acid-selective metachromatic staining technique, particularly valuable for studying apoptosis (Guo *et al*., 2015). Embryos were subjected to the inhibitors (MIRIN & KU55933) at one hpf and irradiated at six hpf. At 48 hpf, embryos were dechorionated with pronase (1 mg/mL), and 20 larvae from each group were rinsed with E3 medium. Subsequently, they were incubated with 5 µg/mL acridine orange (AO) (Sigma-Aldrich, St Louis, MO, USA) dissolved in E3 medium for 30 min at room temperature. Following incubation, embryos were washed with E3 medium three times for 10 min each. Throughout the staining process, embryos were shielded from light, and the visualization and photography of apoptotic cells were conducted in a dark room. Apoptotic cells were discerned under a fluorescence stereomicroscope (Leica macroFluoTM, Wetzlar, Germany) and captured with a monochrome camera (Imaging, Surrey, BC, Canada) for fluorescence imaging. To quantify the number of dying cells from the photographs, the Image J program was utilized. The count of dying cells was determined from each embryo selected for assessment. Results were presented with error bars (mean ± s.d), and P values were included for statistical analysis.

Data analysis

Statistical analysis was conducted using SPSS version 25.0 (IBM Corp., Armonk, NY, USA). The data were analyzed for normality and homogeneity of variances. Parametric tests such as one-way ANOVA followed by Tukey's post-hoc test were used to compare multiple groups when assumptions were met. Non-parametric data were analyzed using the Kruskal-Wallis test followed by Dunn's post-hoc test. A significance level of P=0.05 was used for all analyses. Results are presented as mean ± standard deviation.

RESULTS AND DISCUSSION

Inhibition of MRN complex and ATM proteins in zebrafish

To assess the dose-response relationship of the MIRIN inhibitor, zebrafish embryos at 8 hpf were exposed to eight different concentrations (0, 5, 10, 15, 25, 35, 45, & 55 µM) of the inhibitor. Following exposure, the lethality of the embryos was evaluated at 72 hpf. Notably, at higher concentrations (45 & 55 µM), over 30% of embryos were deceased, and the remaining live embryos exhibited abnormalities. Conversely, at lower concentrations (5 & 15 µM), approximately 80% of embryos appeared normal, with 10% showing abnormalities. Similarly, the condition persisted at 25 µM; however, at 35 µM, fewer than 60% of embryos were normal, with 30% exhibiting abnormalities and over 10% being deceased. The objective of this study was to assess changes in zebrafish embryos resulting from inhibition of the RAD50 protein. As depicted in Figure 1, 35 µM concentration demonstrated the presence of three distinct embryo types, thus serving as an acceptable optimal concentration for evaluating the effectiveness of inhibiting the MRN complex protein.

Survival analysis in time point treatment and irradiation

To assess the significance of RAD50 in zebrafish embryo survival, we conducted experiments involving three distinct sets of treatments and irradiations at different developmental stages. Zebrafish embryos were subjected to inhibitors (MIRIN & KU55933) at varying time points (1 hpf, 4 hpf, & 12 hpf) and exposed to gamma-ray radiation at different time points (4 hpf, 8 hpf, & 24 hpf) throughout their embryonic development. Morphological and functional changes attributable to the inhibitors and gamma-ray radiation were observed at specific time points up to 72 hpf. Treatment and irradiation protocols were standardized to induce a predetermined level of lethality and abnormality. In the first set, embryos were treated at 1 hpf and irradiated at 4 hpf; in the second set, treatment occurred at 4 hpf followed by irradiation at 8 hpf; and in the third set, treatment was administered at 12 hpf with irradiation at 24 hpf.

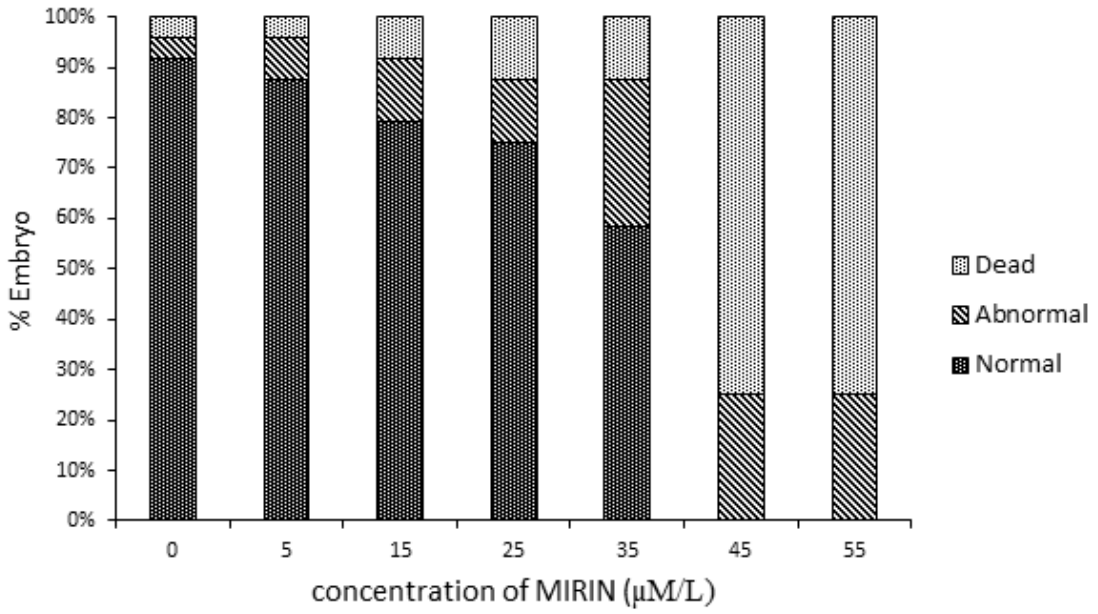

Fig. 1. Dose administration *in vivo* quantification. MIRIN inhibitor was applied to zebrafish at 6–72 hpf. The survivability of the embryos was determined at eight different (0-55 µM/L) concentrations. A significant difference between groups (n=24, p<0.001).

Figure 2b illustrates the impact of MRN and ATM protein inhibitors on zebrafish embryo survival. Embryos treated with MIRIN (35 µM/L) and KU55933 (10 µM/L) at 1 hpf and subsequently irradiated with gamma radiation (15 Gy) at 4 hpf exhibited significant differences in survival rates compared to non-irradiated groups (n=24, p<0.05). In the control group, 92% of embryos were normal, with 8% deceased, while in the irradiated group, 25% were normal, 45% showed abnormalities, and 30% were deceased. Notably, no normal embryos were observed in irradiated and inhibitor-treated groups, indicating the severe impact of irradiation Observations at 72 hpf revealed that non-irradiated groups had successfully hatched, whereas treated and irradiated groups did not hatch, displaying major phenotypic abnormalities. This outcome underscores the detrimental effects of irradiation on embryonic development.

Gamma-ray irradiation induces (DSBs) of DNA, leading to cell survival challenges until repair. DSBs trigger the phosphorylation of histone variant H2AX and the formation of γ-H2AX nuclear foci at DSB sites, processes in which the MRN complex plays a crucial role. However, irradiation of embryos treated with MIRIN and KU55933 did not activate ATM, suggesting impairment in DNA damage response pathways. Previous studies have demonstrated that exposure to gamma rays accelerates hatching rates, induces oxidative stress, and causes DNA damage in zebrafish embryos. Continuous gamma irradiation during early gastrula has been associated with changes in gene expression linked to developmental defects in zebrafish embryos. Overall, these findings highlight the essential role of RAD50 in zebrafish embryo survival and underscore the impact of DNA damage and repair mechanisms on developmental outcomes.

The second set of embryos was treated with MIRIN (35 µM/L), KU55933 (10 µM/L) and 0.1% DMSO at 4 hpf and irradiated with gamma radiation (15 Gy) at 8 hpf. Embryo survival was determined with 72 hpf as the endpoint, more than 95% were normal in the non-irradiated control group, 30% were normal, and 66% were abnormal in the irradiated control group. The non-irradiated MIRIN group shows 50% normal, less than 10% abnormal, and 40% dead embryos and 75% of the embryos in the irradiated MIRIN group are visible as abnormal and 25% as dead. In the unirradiated and irradiated KU55933, 12% of dead embryos were found, with 70% of embryos in the unirradiated group normal and abnormal in the irradiated group. A significant difference between irradiated and non-irradiated groups was *P 0.001* at n=24. Figure 3a shows the morphological details of embryos after treatment and irradiation in the second set. All groups did not hatch after 24 hpf, but after 48 hpf only the non-irradiated control group hatched. After 72 hpf, all groups had hatched except the irradiated and treated KU55933 group. In the irradiated groups, the morphological abnormalities were more obvious. The repair of DNA damage in zebrafish embryos can vary, but studies indicate that significant repair processes can occur within a few hr post-irradiation. For example, the zebrafish embryo has been observed to activate DNA damage response mechanisms and repair DNA within 2 to 6 hr after exposure to ionizing radiation.

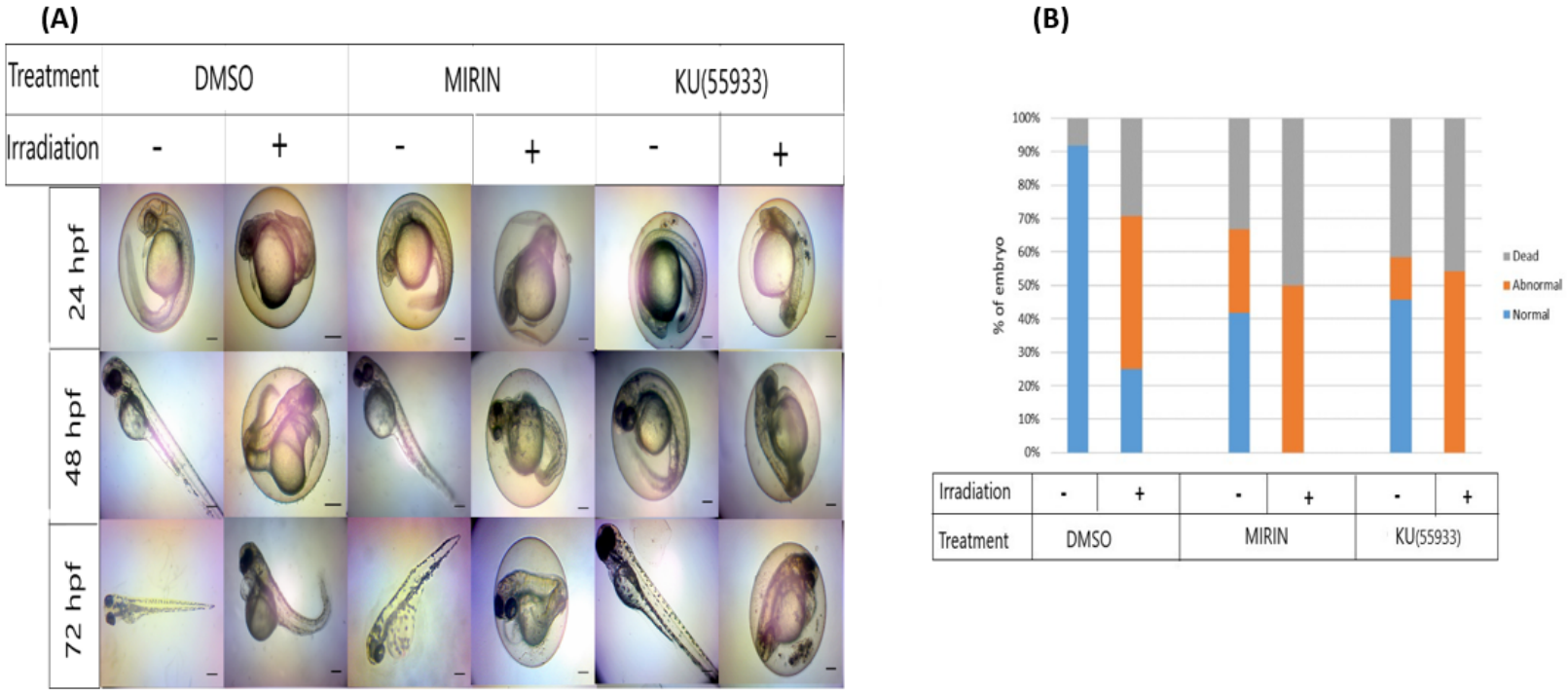

Fig. 2. (**A**) Photomicrograph representing the developmental deformities induced and irradiated zebrafish embryos. Embryos were immersed in MIRIN (35 µM/L), KU55933 (10 µM/L), and 0.1% DMSO at 1 hpf and were irradiated with Gamma ray (15 Gy) at 4 hpf. The survivability of the embryos was determined by taking 72 hpf as the endpoint. Magnification 4x. Scale bars: 100 µm. (**B**) Effect of MRN and ATM protein inhibitor on survival of zebrafish embryo. Embryos were immersed in MIRIN (35 µM/L), KU55933 (10 µM/L), and 0.1% DMSO at 1 hpf and were irradiated with Gamma ray (15 Gy) at 4 hpf. The survivability of the embryos was determined by taking 72 hpf as the endpoint. The significant difference between irradiated and no-irradiated groups (n=24, p<0.05). The scale 20x.

To ensure that untreated embryos are given sufficient time to heal and to assess the effects of treatments accurately, it would be prudent to wait at least 6 hr post-irradiation before conducting morphological examinations. This timeframe allows for the activation of DNA repair pathways and the resolution of some DNA damage, providing a clearer distinction between the effects of treatment and the natural repair processes in untreated embryos.

For the third group, embryos were treated with MIRIN (35 μM/L), KU55933 (10 μM/L) and 0.1% DMSO at 12 hpf and irradiated with gamma radiation (15 Gy) at 24 hpf. Embryos in the non-irradiated control group were normal (more than 95%), in the irradiated control group more than 50% were normal, 30% were abnormal and more than 10% were dead (Figure 4b). Embryos were around 50% normal, more than 20% abnormal, and more than 10% dead (non-irradiated and inhibitor-treated groups). The irradiated MIRIN group showed more than 30% normal, more than 50% abnormal, and about 10% dead embryos. Embryos in the irradiated KU55933 group were more than 30% normal, 45% abnormal, and 20% dead. Figure 3b shows the photomicrographs of the induced and irradiated zebrafish embryos of the third set with developmental deformities. At 48 hpf, embryos had hatched in all groups except the irradiated KU55933 group. At 72 hpf, the irradiated groups have a normal appearance, but abnormalities are observed in embryos in the irradiated groups. There was a significant difference between the irradiated and non-irradiated groups (n=24, p<0.001). The deformation rates of zebrafish embryos improved, while the survival and hatching rates of zebrafish embryos decreased after γ-irradiation treatment (Zhao *et al*., 2019).

The most important result that can be seen in early-stage embryonic development due to inhibition of MRN and ATM proteins and gamma irradiation. A study by Hudson *et al*. showed that the tissues of younger animals are much more susceptible to DNA damage from irradiation. Younger animals exhibited higher formation of γ-H2AX, which was partially associated with cellular proliferation and expression of DNA repair proteins (Hudson *et al*. 2011). These results are consistent with other studies of early growth stages and radiation toxicity (Costa & Reagan, 2019), which showed that the lethal consequences of irradiation are inversely correlated with developmental age.

Zebrafish embryos also provide an opportunity to better identify effects on cardiac function. The zebrafish heart is usually observed in toxicity experiments and usually appears as a change in heart rhythm. Assessment of cardiac function involves quantification of the heart rates of treated and irradiated embryos. The heart rate of the first set of zebrafish embryos was evaluated at 120 hpf. Embryos were treated with MIRIN (35 μM/L), KU55933 (10 μM/L), and 0.1% DMSO at 1 hpf and irradiated at 4hpf. Heart rates in beats per min (bpm) were counted in irradiated groups (n=15) and non-irradiated groups (n=20). Embryo heart rates were affected by irradiation and inhibitor treatment. As shown in Figure 5, the highest heart rate appeared in the non-irradiated control group (180 beats/min), and the lowest heart rate was observed in the irradiated MIRIN group (50 beats/min). A significant difference was observed between irradiated and non-irradiated groups (p<0.001). The error bars indicate the standard deviations, the inhibitors do not affect RAD50 phosphorylation. Effect of MIRINe inhibitor on ATM phosphorylation. This inhibitor turns off the MRN complex protein so that the inactive MRN is not active ATM and cannot be phosphorylated. MIRIN inhibits MRE11-associated exonucleus activity, prevents MRN-dependent ATM activation, and abrogates the G2/M checkpoint and homology-dependent repair in mammalian cells (Dupré *et al*., 2008).

Brain developmental defects irradiated and induced by MIRIN and KU55933 inhibitors

The effects of inhibition of RAD50 and ATM proteins on embryos at each time point were examined. The results showed that embryos were most damaged in the early hr (treatment at 1 hpf & irradiation at 4 hpf). We used this time frame to examine the effects of inhibition of these two proteins on the fetal brain. Figure 6a shows a representative morphological evaluation of embryos at 120 hpf. Embryos were treated with MIRIN (35 μM/L), KU55933 (10 μM/L), and 0.1% DMSO at 1 hpf and irradiated at 4 hpf. The yellow line shows the area of the skull. The treatment and irradiation caused abnormalities in the embryos, the embryos became shorter and the head became smaller. The embryos Figure 6a shows the greatest effect while the development of the midbrain was delayed and Figure 6b proves this the head size was reduced in the irradiated and inhibitor-treated groups. The head size in the untreated and non-irradiated group was 187.6 μm, but in the treated and non-irradiated group it was nearly 150 μm, and in the treated and irradiated group, it was nearly 120 μm. It became clear that the brain in the irradiated groups showed a greater reduction than in the non-irradiated groups.

To obtain a detailed structure of the brain of embryos with damaged DNA, embryos at 1 hpf were treated with MIRIN (35 μM/L), KU55933 (10 μM/L), and 0.1% DMSO and irradiated at 4 hpf. These samples were used for sectioning and H&E staining. Histological lateral and transverse sections

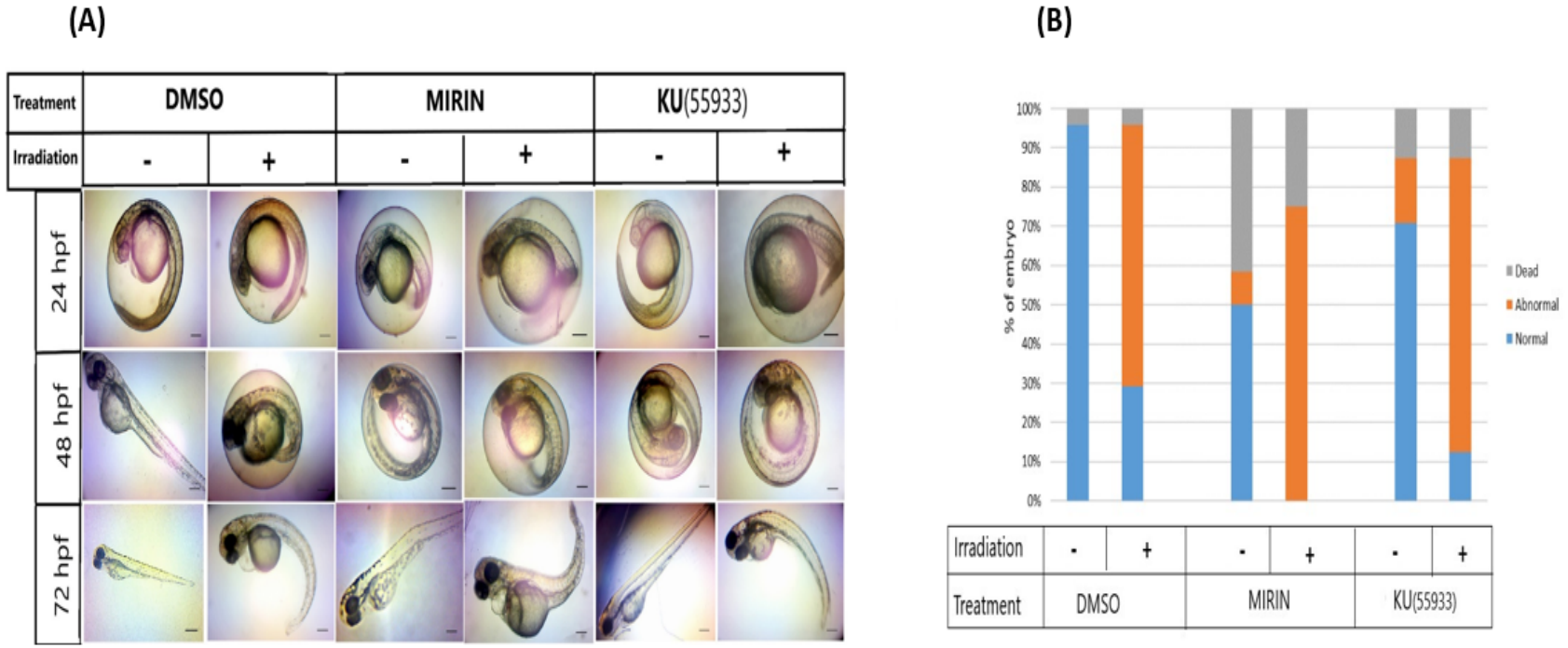

Fig. 3. (A) Photomicrograph representing the developmental deformities induced and irradiated zebrafish embryos. Embryos were immersed in MIRIN (35 µM/L), KU55933 (10 µM/L), and 0.1% DMSO at 4 hpf and were irradiated with Gamma ray (15 Gy) at 8 hpf. The survivability of the embryos was determined by taking 72 hpf as the endpoint. Magnification 4x. (Bar= 100µM). (**B**) Effect of MRN and ATM protein inhibitor on survival of zebrafish embryo. Embryos were immersed in MIRIN (35 µM/L), KU55933 (10 µM/L) at and 0.1% DMSO 4 hpf and were irradiated with Gamma ray (15 Gy) at 8 hpf. The survivability of the embryos was determined by taking 72 hpf as the endpoint. Significant differences between irradiated and no-irradiated groups (n=24, p<0.001) were noted. The scale 20x.

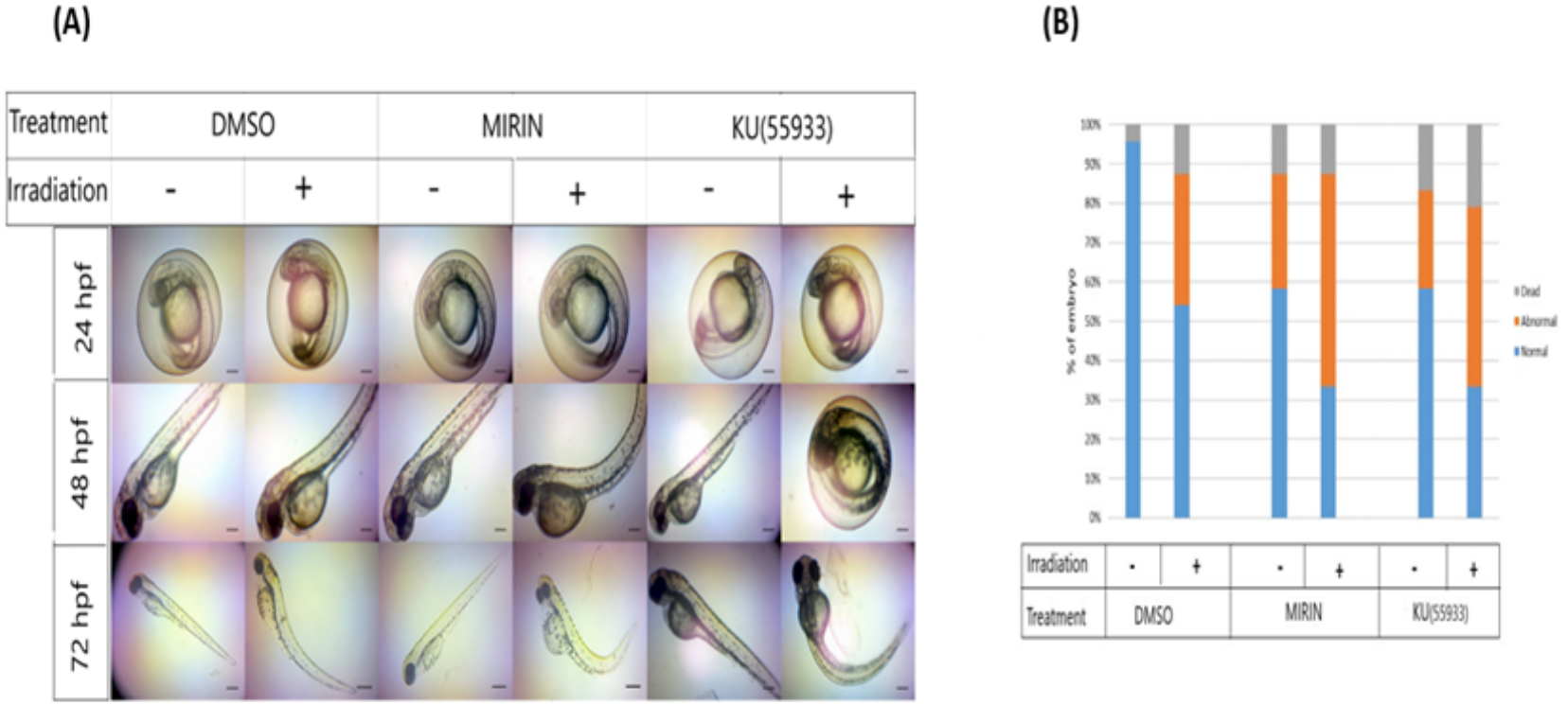

Fig. 4. (A)Photomicrograph representing the developmental deformities induced and irradiated zebrafish embryos. Embryos were immersed in MIRIN (35 µM/L), KU55933 (10 µM/L), and 0.1% DMSO at 12 hpf and were irradiated with Gamma ray (15 Gy) at 24 hpf. The survivability of the embryos was determined by taking 72 hpf as the endpoint. Magnification 4x. Scale bars: 100 µm. (B) Effect of MRN and ATM protein inhibitor on survival of zebrafish embryo. Embryos were immersed in MIRIN (35 µM/L), KU55933 (10 µM/L), and 0.1% DMSO at 12 hpf and were irradiated with Gamma ray (15 Gy) at 24 hpf. The survivability of the embryos was determined by taking 72 hpf as the endpoint. A significant difference between irradiated and non-irradiated groups (n=24, $p<0.001$) was reported. The scale 20x.

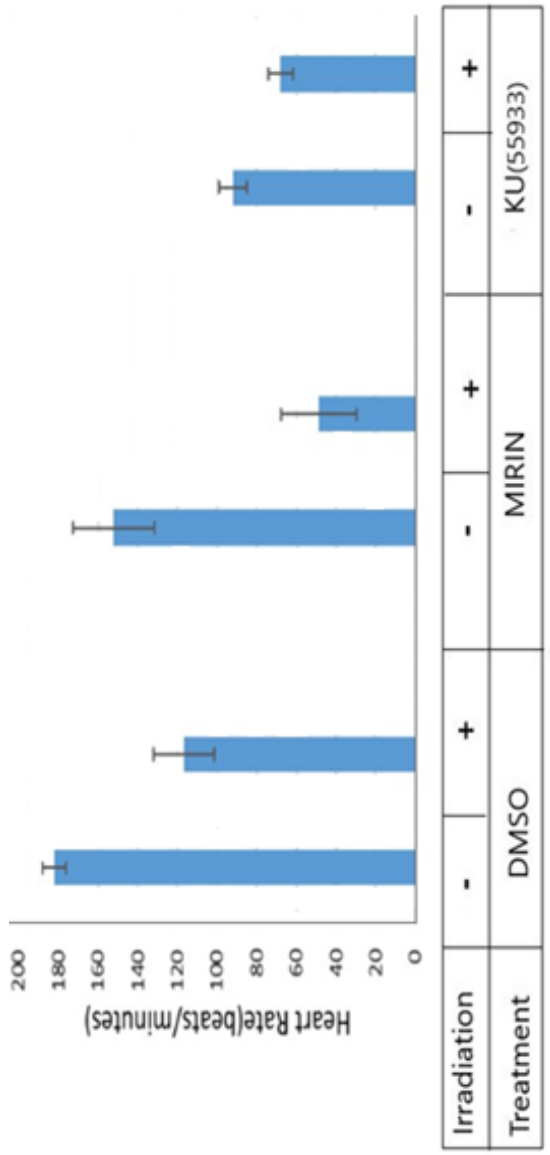

Fig. 5. Heart rate of zebrafish embryo at 120 hpf. Embryos treated with MIRIN (35 µM/L), KU55933 (10 µM/L), and 0.1% DMSO at 1 hpf and irradiated 4hpf. Heart rates in beats per min (bpm) were counted in irradiated groups (n=15) and non-irradiated groups (n=20). Significant differences between irradiated and non-irradiated groups (p<0.001) were noted). The error bars indicate standard deviations.

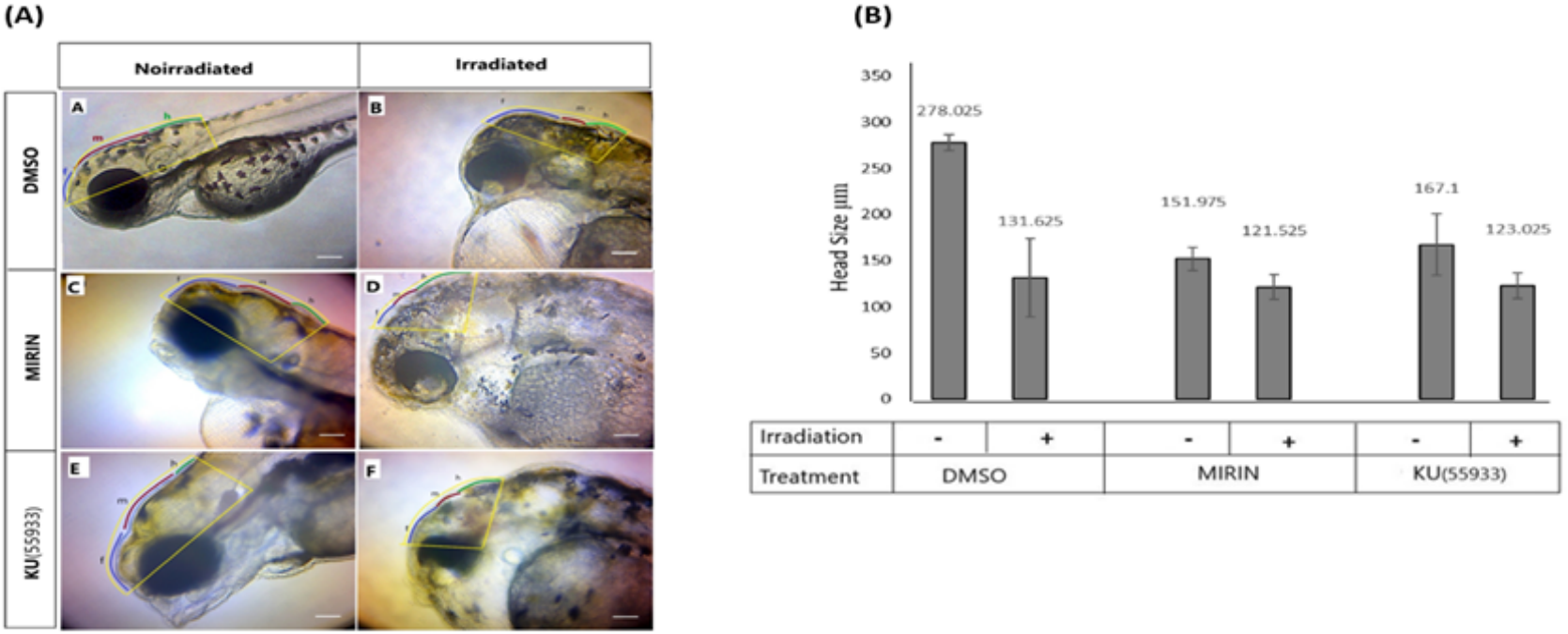

Fig. 6. (A) Treated with inhibitors and irradiated embryos display cranial defects. Representative morphological evaluation of embryos at 120 hpf embryos was treated with MIRIN (35 µM/L), KU55933 (10 µM/L), and 0.1% DMSO at 1 hpf and irradiated 4 hpf. The yellow line indicates the area of the skull. Different colors, forebrain (f) blue, midbrain (m) red, and hindbrain (h) green represent different parts of the brain. Magnification 10x. Scale bars: 100 µm. (**B**) MRN complex protein-inhibited embryos display cranial defects. Embryos treated with MIRIN (35 µM/L), KU55933 (10 µM/L) and 0.1% DMSO at 1 hpf and irradiated at 4 hpf. Head size (µM) was measured at 120 hpf embryos in irradiated groups (n=15) and no-irradiated groups (n=20). A significant difference between irradiated and non-irradiated groups (p<0.001) was observed. The error bars indicate standard deviations. The scale 20x.

prepared from treated and irradiated embryos show dilated ventricles, reduced tissue mass, and weak midbrain and hindbrain structure (Figures 7a & 8a). The results of Hudson *et al*. (2011) showed that the brain of young animals may contribute to the deleterious effects of radiation on the brain, cell death, unrepaired damage to living brain cells, and neuroinflammation are known to be three radiation-induced CNS pathology features. (Belka *et al*., 2001; Acharya *et al*., 2010) irradiated embryos at 4 hpf of age, and embryo morphology showed reduced head size in the irradiated group. Figure 7b To verify the role of RAD50 as one of the important proteins in the DNA damage repair pathway, the samples were treated with a MIRIN inhibitor for 3 hr before irradiation. The reduction in brain size was greater in these groups. To check the histological results of the embryos, RAD50, and ATM proteins were disrupted to inhibit the repair of the damaged DANA. Parts of the brain were lost, and extensive holes were formed. DDR signaling and radiosensitivity in neural stem cells (NSCs) have been studied by several researchers. Schneider *et al*. (2013) discovered that NSCs stimulate canonical DDR after exposure to ionizing radiation. Remarkably, these cells were found to be radioresistant as they lacked functional DDR signaling and repressed key DDR genes such as ATM at the transcriptional level. (Schneider *et al*., 2013). In the brain, neural stem cells (NSCs) differentiate and produce neural progenitor cells that eventually replicate mature neurons and glial cells to maintain the stem cell population. The initial steps of neurogenesis are the induction of neural progenitor cells and cell division, which increases the reservoir of progenitor cells. This is accompanied by a list of criteria for active progenitors and postmitotic neuron differentiation. Each of these steps is spatially and temporally organized to produce the various forms of neuronal and glial cells that will eventually form the mature central nervous system (CNS) (Schmidt *et al*. 2013). Inhibition of MRN and ATM involves inhibition of the activity of these proteins in signaling pathways involved in DNA repair, cell cycle regulation, and apoptosis induction, which in turn results in cell division and survival. In the group samples where RAD50 and ATM were disrupted, perhaps normal neurogenesis had been lost. All these results suggest that the RAD50 and ATM proteins are required for normal brain development in the zebrafish embryo.

Several possible mechanisms, such as reduced cell proliferation and increased cell death, could cause defects in brain development. To evaluate cell death as a possible mechanism, apoptotic cells in embryos were analyzed by acridine orange staining. Acridine orange staining of live embryos showed increased staining of brain cells in irradiated embryos (Figure 8a). As shown in Figure 8b, there were increased apoptotic cells in irradiated embryos, which were inhibited by RAD50 and ATM proteins, by MIRIN and KU55933. Gamma irradiation can cause DNA damage and mitochondrial lesions and induce apoptosis (Zhao et al., 2019). Inhibition of RAD50 protein-induced apoptosis in zebrafish embryos. The group of gamma-irradiated embryos with inhibited RAD50 and ATM proteins shows more apoptosis cells in contrast to the non-irradiated embryos. An increase in DNA damage can also lead to apoptosis (Gagnaire et al., 2015) In the study of the apoptotic response of caspase-2 to DNA damage, non-irradiated p53+/+ embryos treated with KU55933 or the Chk2 inhibitor II showed strong AO labeling preferentially localized to the brain and eyes (Sidi et al., 2008). Other studies showed cell death mainly in the head and eye region after irradiation of zebrafish larvae (Mena et al., 2010). Rhee et al. (2013) reported that irradiation of K. marmoratus larvae at 4-6 Gy also induced p53 gene expression and DNA damage.

Microcephaly is a common feature of DDR-defective diseases, suggesting that normal DDR is critical during brain development. A study by Murat et al. (2019) reported the effect of irradiation on neuronal migration. The possibility that progenitor cell populations change in the developing central nervous system (neural tube) and muscles (called somites in the embryo) of zebrafish embryos was investigated (Murat et al., 2019). Microcephaly is most likely caused by the loss (increased cell death) or failure of developing neural stem cells or their progenitors to divide, suggesting a fundamental role for the DDR in maintaining proliferative potential in the developing nervous system. (O'Driscoll & Jeggo, 2008). On the other hand, specific roles of NBS1 and RAD50 in ATR-mediated signaling pathways may contribute to the microcephaly that has been correlated with defects in ATR function (Walters et al., 2009). Radiation-induced DNA damage causes observable morphological changes in zebrafish embryos, such as spinal curvature, shortening of body length, yolk sac or pericardial edema, abnormalities of the eyes (microphthalmia), or abnormal development of the head (microcephaly). The severity of these abnormalities is dose-dependent (Geiger et al., 2006).

CONCLUSION

MIRIN was used as a RAD50 protein inhibitor in zebrafish embryos, while KU55933 was employed to independently evaluate the ATM protein. Irradiation significantly impacted embryonic survivability. Three different treatment and irradiation time points were applied to zebrafish embryos. Notably,

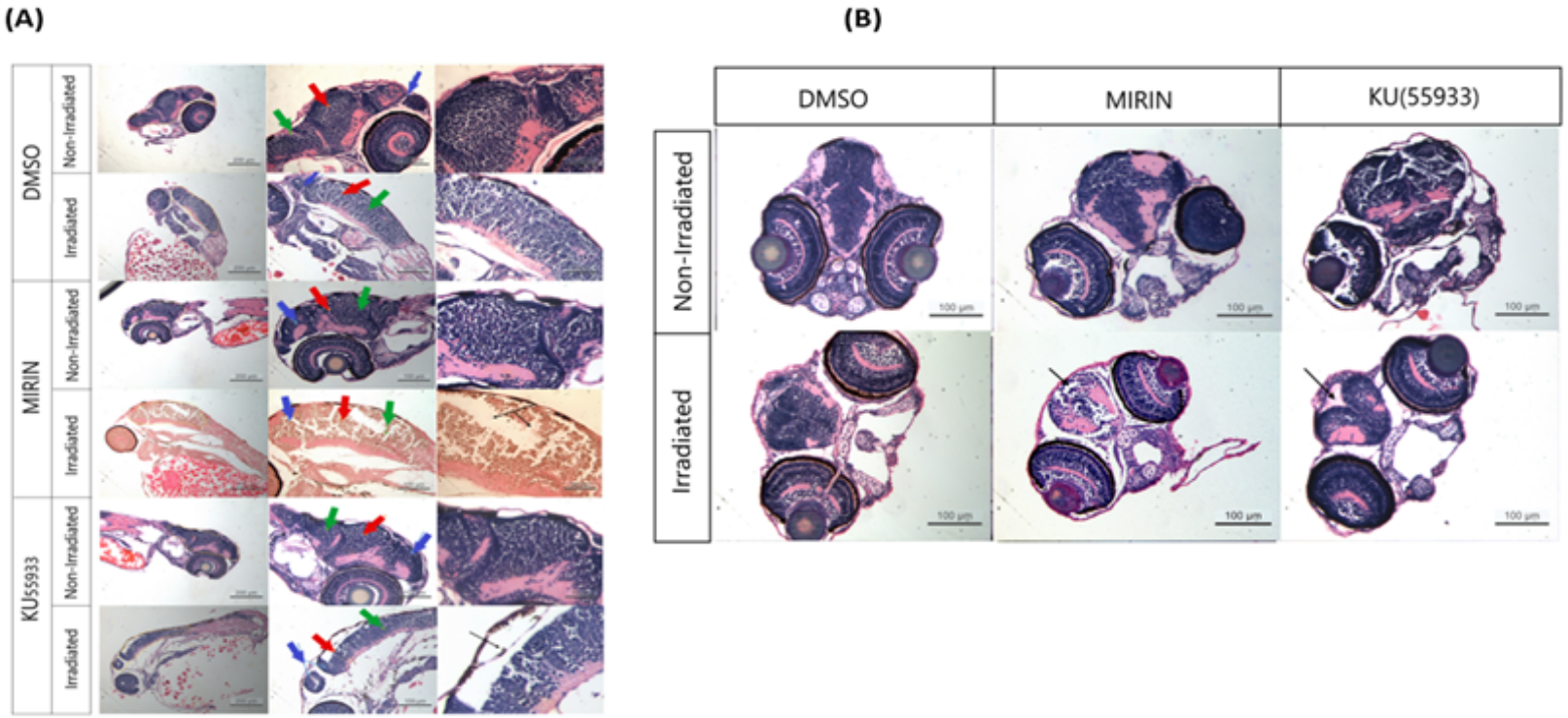

Fig. 7. (A) Histological lateral sections of zebrafish embryo brain anatomy at early stage. Embryos treated with MIRIN (35 µM/L), KU55933 (10 µM/L), and 0.1% DMSO at 1 hpf and irradiated at 4 hpf. Three parts of the brain have been shown with different color arrows, forebrain (f) blue, midbrain (m) red, and hindbrain (h) green. Black arrows indicate the brain cavities created. (**B**) Histological transverse sections of zebrafish embryo brain anatomy at an early stage. Embryos treated with MIRIN (35 µM/L), KU55933 (10 µM/L), and 0.1% DMSO at 1 hpf and irradiated 4 hpf. Black arrows indicate the brain cavities created. The scale 20x.

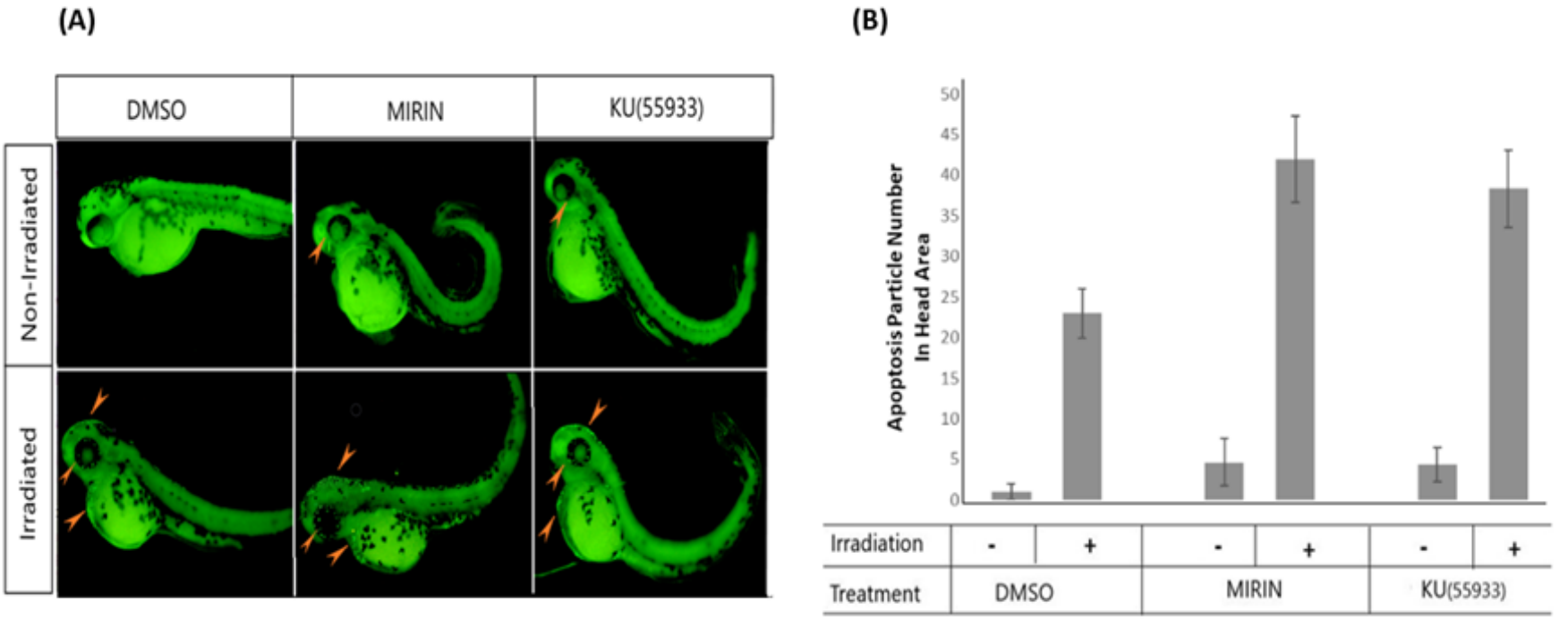

Fig. 8. (A) Analysis of apoptosis in live embryos with acridine orange staining. Embryos treated with MIRIN (35 µM/L), KU55933 (10 µM/L), and 0.1% DMSO at 1 hpf and irradiated 6 hpf. Apoptotic cells were identified as green punctate dots and indicated by orange arrows, and Apoptosis in the brain region was identified. (B) Cell death in zebrafish embryos increases in irradiated groups as shown by the number of AO positive. Embryos treated with MIRIN (35 µM/L), KU55933 (10 µM/L), and 0.1% DMSO at 1 hpf and irradiated 6 hpf. Apoptosis particles were counted at 48 hpf (n=10) and compared between the irradiated and non-irradiated groups. A significant difference between irradiated and non-irradiated groups ($p<0.05$) was observed. Error bars indicate standard deviations. The scale 20x.

the combined irradiation and inhibition of both the MRN complex and ATM protein had pronounced effects on early-stage embryos. Embryos treated with MIRIN and KU55933 at 1 hpf and irradiated at 4 hpf failed to hatch, with 50% exhibiting cranial defects. Histological sections showed clear evidence that irradiation and inactivation of RAD50 and ATM proteins adversely affected brain development in zebrafish embryos, with apoptotic cells observed in embryos displaying microcephaly.

Understanding these mechanisms and their effects on embryonic development can benefit mankind by providing insights into the roles of RAD50 and ATM proteins in DNA repair and cellular response to damage. This knowledge can lead to the development of targeted therapies for diseases involving DNA damage, such as cancer, and improve strategies for radioprotection and treatment of radiation-induced damage. Additionally, it can inform genetic research and contribute to preventing congenital disabilities related to DNA repair deficiencies.

ACKNOWLEDGEMENTS

This project is funded by Ministry of Science, Technology and Innovation of Malaysia (MOSTI) (02-01-02-SF1279); Malaysian Ministry of Higher Education (MoHE) (FRGS/1/2023/STG01/UKM/02/5); Universiti Kebangsaan Malaysia (ST-2019-013; UKM-TR-018; GGPM-2014-006; and ST-2021-010); and Inti International University (INTI-FHLS-01-26-2023; and INTI-FHLS-01-03-2022).

ETHICAL STATEMENT

All in vivo experimental procedures were performed under the EU-directive 2010/63/EU on the protection of animals used for scientific purposes, and all procedures were carried out under project approval number FST/2018/MOHD SHAZRUL/28-MAR.-2018-DEC. -2021, approved by the UKM Animal Ethical Committee.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Costa, S. & Reagan, M.R. 2019. Therapeutic irradiation: Consequences for bone and bone marrow adipose tissue. Frontiers in Endocrinology, 10: 587. https://doi.org/10.3389/fendo.2019.00587

Dupré, A., Boyer-Chatenet, L., Sattler, R.M., Modi, A.P., Lee, J.-H., Nicolette, M.L. & Kopelovich, L. 2008. A forward chemical genetic screen reveals an inhibitor of the Mre11-Rad50-Nbs1 complex. Nature chemical biology, 4(2): 119-25. https://doi.org/10.1038/nchembio.63

Enguita-Marruedo, A., Martín-Ruiz, M., García, E., Gil-Fernández, A., Parra, M.T., Viera, A., Rufas, J.S. & Page, J. 2019. Transition from a meiotic to a somatic-like DNA damage response during the pachytene stage in mouse meiosis. PLoS Genetic, 15: e1007439. https://doi.org/10.1371/journal.pgen.1007439

Fazry, S., Kumaran, M., Khalili, N., Mahmood, I., Dyari, H.R.E. & Jamar, N.H. 2019. MRN complex and ATM kinase inhibitors impacts towards UVC-treated zebrafish embryonic development. Pertanika Journal of Science and Technology, 27: 1615-24.

Gagnaire, B., Cavalié, I., Pereira, S., Floriani, M., Dubourg, N., Camilleri, V. & Adam-Guillermin, C. 2015. External gamma irradiation-induced effects in early-life stages of zebrafish, *Danio rerio*. Aquatic Toxicology, 169: 69-78. https://doi.org/10.1016/j.aquatox.2015.10.005

Gao, G., Bi, X., Chen, J., Srikanta, D. & Rong, Y. S. 2009. Mre11-Rad50-Nbs complex is required to cap telomeres during *Drosophila* embryogenesis. Proceedings of the National Academy of Sciences of the United States of America, 106(26): 10728-10733. https://doi.org/10.1073/pnas.0902707106

Gatei, M., Jakob, B., Chen, P., Kijas, A.W., Becherel, O.J., Gueven, N., Birrell, G., Lee, J. H., Paull, T.T., Lerenthal, Y., Fazry, S., Taucher-Scholz, G., Kalb, R., Schindler, D., Waltes, R. & Dörk, T. 2011. ATM protein-dependent phosphorylation of Rad50 protein regulates DNA repair and cell cycle control. Journal of Biological Chemistry, 286(36): 31542-31556. https://doi.org/10.1074/jbc.M111.258152

Geiger, G.A., Parker, S.E., Beothy, A.P., Tucker, J.A., Mullins, M.C. & Kao, G.D. 2006. Zebrafish as a "biosensor"? Effects of ionizing radiation and amifostine on embryonic viability and development. Cancer Research, 66(16): 8172-8181. https://doi.org/10.1158/0008-5472.CAN-06-0466

Guo, P., Huang, Z., Tao, T., Chen, X., Zhang, W., Zhang, Y. & Lin, C. 2015. Zebrafish as a model for studying the developmental neurotoxicity of propofol. Journal of Applied Toxicology, 35(12): 1511-1519. https://doi.org/10.1002/jat.3183

Hopfner, K.P., Karcher, A., Shin, D.S., Craig, L., Arthur, L.M., Carney, J.P. & Tainer, J.A. 2000. Structural

biology of Rad50 ATPase: ATP-driven conformational control in DNA double-strand break repair and the ABC-ATPase superfamily. Cell, 101(7): 789-800. https://doi.org/10.1016/S0092-8674(00)80890-9

Hudson, D., Kovalchuk, I., Koturbash, I., Kolb, B., Martin, O.A. & Kovalchuk, O. 2011. Induction and persistence of radiation-induced DNA damage is more pronounced in young animals than in old animals. Aging, 3(6): 609-20. https://doi.org/10.18632/aging.100340

Hunter, N. 2015. Meiotic recombination: The essence of heredity. Cold Spring Harb Perspect Biology, 7(12): a016618. https://doi.org/10.1101/cshperspect.a016618

Lamarche, B.J., Orazio, N.I. & Weitzman, M.D. 2010. The MRN complex in double-strand break repair and telomere maintenance. FEBS Letters, 584(17): 3682-3695. https://doi.org/10.1016/j.febslet.2010.07.029

Mena, P., Allende, M. & Morales, J.R. 2010. Survival study of zebrafish embryos under gamma irradiation. AIP Conference Proceedings, 1265: 455-456. https://doi.org/10.1063/1.3480235

Murat El Houdigui, S., Adam-Guillermin, C. & Loro, G. 2019. A systems biology approach reveals neuronal and muscle developmental defects after chronic exposure to ionising radiation in zebrafish. Scintefic Reports, 9: 20241. https://doi.org/10.1038/s41598-019-56590-w

O'Driscoll, M. & Jeggo, P.A. 2008. The role of the DNA damage response pathways in brain development and microcephaly: Insight from human disorders. DNA Repair (Amst), 7(7): 1039-50. https://doi.org/10.1016/j.dnarep.2008.03.018

Paull, T.T. & Lee, J.-H. 2005. The Mre11/Rad50/Nbs1 complex and its role as a DNA double-strand break sensor for ATM. Cell Cycle, 4(6): 737-740. https://doi.org/10.4161/cc.4.6.1715

Rhee, H.W, Zou, P., Udeshi, N.D, Martell, J.D, Mootha, V.K., Carr, S.A. & Ting, A.Y. 2013. Proteomic mapping of mitochondria in living cells via spatially restricted enzymatic tagging. Science, 339(6125): 1328-1331. https://doi.org/10.1126/science.1230593

Schmidt, R., Strähle, U. & Scholpp, S. 2013, February 21. Neurogenesis in zebrafish - from embryo to adult. Neural Development, 8(1): 1-13. https://doi.org/10.1186/1749-8104-8-3

Schneider, L., Pellegatta, S., Favaro, R., Pisati, F., Roncaglia, P., Testa, G., Nicolis, S.K., Finocchiaro, G. & d'Adda di Fagagna, F. 2013. DNA damage in mammalian neural stem cells leads to astrocytic differentiation mediated by BMP2 signaling through JAK-STAT. Stem Cell Reports, 25;1(2):123-138. https://doi.org/10.1016/j.stemcr.2013.06.004

Shibata, A., Moiani, D., Arvai, A. S., Perry, J., Harding, S. M., Genois, M.M. & Maity, R. 2014. DNA double-strand break repair pathway choice is directed by distinct MRE11 nuclease activities. Molecular Cell, 53(1): 7-18. https://doi.org/10.1016/j.molcel.2013.11.003

Shiloh, Y. & Ziv, Y. 2013. The ATM protein kinase: Regulating the cellular response to genotoxic stress, and more. Nature Reviews Molecular Cell Biology, 14(4), 197-210. https://doi.org/10.1038/nrm3546

Sidi, S., Sanda, T., Kennedy, R.D., Hagen, A.T., Jette, C.A., Hoffmans, R., Pascual, J., Imamura, S., Kishi, S., Amatruda, J.F., Kanki, J.P., Green, D.R., D'Andrea, A.A. & Look, A.T. 2008. Chk1 Suppresses a Caspase-2 apoptotic response to DNA damage that bypasses p53, Bcl-2, and Caspase-3. Cell, 133(5): 864-877. https://doi.org/10.1016/j.cell.2008.03.037

Syed, A. & Tainer, J.A. 2018. The MRE11-RAD50-NBS1 Complex Conducts the orchestration of damage signaling and outcomes to stress in DNA replication and repair. Annual Review of Biochemistry, 87: 263-294. https://doi.org/10.1146/annurev-biochem-062917-012415

Waltes, R., Kalb, R., Gatei, M., Kijas, A. W., Stumm, M., Sobeck, A. & Wieland, B. 2009. Human RAD50 deficiency in a nijmegen breakage syndrome-like disorder. The American Journal of Human Genetics, 84(5): 605-616. https://doi.org/10.1016/j.ajhg.2009.04.010

Zhao, M., Smith, L., Volpatti, J., Fabian, L. & Dowling, J.J. 2019. Insights into wild-type dynamin 2 and the consequences of DNM2 mutations from transgenic zebrafish. Human Molecular Genetics, 28: 4186-4196. https://doi.org/10.1093/hmg/ddz260