Pertanika J. Sci. & Technol. 27 (4): 1615 - 1624 (2019)

PERTANIKA JOURNALS

SCIENCE & TECHNOLOGY

Journal homepage: http://www.pertanika.upm.edu.my/

MRN Complex and ATM Kinase Inhibitors Impacts towards UVC-Treated Zebrafish Embryonic Development

Shazrul Fazry[1,2]*, Malina Kumaran[1], Nahid Khalili[1], Ibrahim Mahmood[1], Herryawan Ryadi Eziwar Dyari[1,2], Nur Hidayah Jamar[1,2], Douglas Law[3], Nik Marzuki Sidik[3], Nor Azian Abdul Murad[4] and Yi Chieh Lim[5]

[1]Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 UKM, Bangi, Selangor, Malaysia
[2]Chini Lake Research Center, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 UKM, Bangi, Selangor, Malaysia
[3]Faculty of Agro Based Industry, Universiti Malaysia Kelantan, Jeli Campus, Locked Bag 100, 17600 UMK, Jeli, Kelantan, Malaysia
[4]UKM Medical Molecular Biology Institute, UKM Medical Center, Jalan Ya'acob Latiff, Bandar Tun Razak, 56000 Cheras, Kuala Lumpur, Malaysia
[5]dkDanish Cancer Society, Research Centre Strand Boulevard, 49DK-2100 Copenhagen, Denmark

ABSTRACT

The zebrafish (*Danio rerio*) has become a prevailing vertebrate model for developmental biology studies due to its ease of care, rapid embryogenesis stages development and translucent embryos. In this studies, ATM Kinase and MRN complex role as DNA damage response proteins during embryogenesis was examined by using specific MRN complex (Mirin) and ATM Kinase inhibitors (Ku60019 and Ku55933). To create DNA lesions in zebrafish, embryos at mid-blastula transition (MBT) stage were exposed to inhibitors (Mirin, Ku60019 or Ku55933) and later exposed to UVC irradiation wavelength of between 100 to 280 nm. Hatching but with visible physical deformation was observed for embryos treated with Mirin, Ku60019 or Ku55933 and UVC exposure at concentration of 3μM, 1.5 nM and 3nM or lower, respectively up to 72 hours-post fertilisation (hpf). On the other hand, no deformities were observed for all control as well as mock treated embryos. This study confirmed that DNA damage response proteins are crucial during embryo development to prevent undesired abnormal

ARTICLE INFO

Article history:
Received: 24 April 2019
Accepted: 02 July 2019
Published: 21 October 2019

E-mail addresses:
shazrul@ukm.edu.my (Shazrul Fazry)
malina.kumaran@gmail.com (Malina Kumaran)
nahidkhalili599@gmail.com (Nahid Khalili)
ibrahim_m_mahdy@yahoo.com (Ibrahim Mahmood)
herry@ukm.edu.my (Herryawan Ryadi Eziwar Dyari)
njamar@ukm.edu.my (Nur Hidayah Jamar)
douglas.law@gmail.com (Douglas Law)
nikmarzuki@UMK.edu.my (Nik Marzuki Sidik)
nor_azian@ppukm.ukm.edu.my (Nor Azian Abdul Murad)
yilim@cancer.dk (Yi Chieh Lim)
* Corresponding author

ISSN: 0128-7680
e-ISSN: 2231-8526

biological development. Thus, it is proven that protein inhibitors are a cheaper alternative in valuating specific protein roles during embryogenesis compared to both genomic and transcription modification tools.

Keywords: ATM Kinase, *Danio rerio*, DNA damage, MRN complex, zebrafish

INTRODUCTION

DNA lesion can occur consequently from cell exposure to endogenous and exogenous genotoxic pressure. Aside from that, DNA also prone lesion during the polymerisation of single strand DNA due to collapse of the replication forks (Lavin, 2007). The MRE11-RAD50-NBN (MRN) complex which consists of meiotic recombination 11 (MRE11), DNA repair protein RAD50 (RAD50) and nibrin (NBN) has been identified to play important role in distinguishing the DNA double strand breaks and activates DNA repair proteins mechanism through ataxia telangiectasia mutated (ATM) kinase (Gatei et al., 2011; Lavin, 2007). Null mutation of either one of the MRN complex proteins disrupt proper embryo development which is lethal (Luo, 1999; Williams et al., 2002; Zhu et al., 2001), signified the crucial function of every single MRN components during this process. The RAD50 gene significance in sustaining genomic stability was recorded through a female patient having a RAD50 deficiency (Waltes et al., 2009). The RAD50 deficiency disorder was later renamed as Nijmegen Breakage Syndrome-like Disorder (DBSLD). DBSLD patients is having similar characterisation usually observed in NBS patients such as short stature, bird-like face, microcephaly and mental retardation (Waltes et al., 2009).

The importance of RAD50 and the implications of any of its mutations studies have largely been *in vitro*. It is important to note that *in vitro* study does not give a clear picture of how disruption of RAD50 function could affect the phenotype of an organism. Some *in vitro* studies even fail to replicate the precise cellular conditions of a mutated organism (Bender et al., 2002). Although previous researches using mice could shed some light on the implications of RAD50 deficiency on the function of the MRN complex, there are many more aspects that remain elusive. Bender et al. (2002) had shown that the RAD50s/s mutant mice exhibited growth defects and cancer predisposition and the outcomes of the RAD50s/s phenotype were not associated with overt defects in the MRN complex. Indicating that subtle alteration of the MRN complex might have deleterious effects. However, the actual importance of RAD50 have yet to be fully understood. Knowing how important the correct expression of RAD50 is in ensuring MRN complex proper functioning leads to another question of how important is RAD50 in ensuring genomic stability.

While studies on RAD50 have been established in mice previously, mainly due to their evolutionary proximity and homology to human, zebrafish (*Danio rerio*) are yet to be utilised in RAD50-related researches. Zebrafish can be used to explore the genetic and physiological mechanisms involved in the functioning of various systems (Meyers, 2018). Zebrafish embryos are particularly an attractive model for *in vivo* studies to their

optical transparency and rapid embryonic development; making it possible to observe embryogenesis and organogenesis in real time (Bladen et al., 2005). This can be exploited to observe the effects of the inhibitors on their development and bodily functions (Dale et al., 2009; Driever et al., 1996; Golling et al., 2002; Segner 2009). The aim of this study was to investigate the effects of MRN complex, ATM and ATR Kinase inhibitors on UVC-treated zebrafish embryogenic development.

METHODS

Zebrafish Husbandry

Adult wild type zebrafish were kept at standard condition of 14:10 hour light-dark photoperiod. The water pH and temperature were maintained at 7.4 and 28.5°C, respectively. Eggs produced were screened for fertilization under light microscope 1 h post fertilization (hpf). Selected fertilized eggs was incubated in E3 solution (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4). Animal care and all experimentation were conducted as previously reported by Fazry et al., (2018) and was approved by the UKM ethics committee (Animal ethic approval number: (FST/2015/SHAZRUL/25-MAR./670-MAR.-2015-DEC.-2016).

Treatment with Inhibitors

Selected single cell zebrafish embryos at mid-blastula transition mid-blastula transition (MBT) stage were treated individually with 200 µL using Mirin (MRN complex inhibitor) (Sigma-Aldrich, USA), Ku60019 (ATM Kinase inhibitor)(Sigma-Aldrich, USA) and Ku55933 (ATM and ATR Kinase inhibitor)(Sigma-Aldrich, USA) prior to UVC irradiation at room temperature. Inhibitor concentrations used in the treatment were 3, 6 and 12 µM for Mirin; 1.5, 3 and 6 nM for Ku60019; and 3, 6 and 12 nM for Ku55933. The concentration of inhibitors used were based on the half maximal inhibitory concentration (IC50) of the inhibitors (Dupré et al., 2008; Negi & Brown, 2015; Williams et al., 2013).

Irradiating the Embryos with UVC

The embryos that were exposed to the inhibitors were then incubated at 28.5°C for 1 hour prior to UVC radiation to ensure that DNA damage indeed occurs. UVC has a wavelength of 100-280 nM which represents the most lethal form of radiation in comparison to UVB and UVA. UVC is utilized to ensure maximum exposure of UV ray that will induce DNA damage in the developing embryos (Clemente et al., 2014). Embryos were exposed to radiation using a UVC light (4.56 watt/m^2) in a customized box. A dosage of 800 Joules/m^2 was given to the inhibitor-treated embryos by manipulating the duration of the UVC exposure (1 watt/m^2 = 1 Joules/second/m^2) (Ledo, 1993; Kaur et al., 2018). The non-UVC

treated embryos were maintained under standard laboratory lighting. The embryos were left to be incubated at 28.5°C and their survival and development were evaluated at 24, 48 and 72 hpf, under light microscope at 4× magnification.

Statistical Analysis

Values of experimental results shown in figures were the mean of at least three determinations (±standard deviation).

RESULTS AND DISCUSSIONS

In this study, we observe significant changes in the phenotypes of inhibitor and UVC-treated embryos at 3 days' post fertilisation (dpf) in comparison with control embryos. The data collected were distributed into analysing the survivability of the embryos and the characterisation of deformities observed in embryos that had survived the treatment at the end of 3 dpf. A total of 15 embryos were used in triplicates of 5 embryos (Figure 1).

Zebrafish embryos that were exposed to inhibitors within the MBT phase without prior exposure to UVC radiation had a good survivability (Figure 1A). All the embryos treated with different concentration of Mirin had 100% survivability. The ATM kinase inhibitor Ku60019-treated embryos had slightly lower survivability than Mirin, with the 1.5 nM displaying the lowest survivability at 86%. The Ku55933 treated embryos had the least survivability with 80% of embryo alive at the lowest concentration 3 nM, and 86% for both 6 and 12 nM treated embryos. This result indicates that the Ku55933 is a more potent inhibitor in comparison to Mirin and Ku60019 as the lowest concentration of 3 nM can result in the lowest survivability of embryos. This may be contributed by the mechanism at which Ku55933 works since it inhibits both the ATM and ATR kinase activity (Chwastek et al., 2017). Hence, it can halt the downstream signalling of the DNA damage response more effectively than Mirin and Ku60019.

In the UVC treated embryos, the survivability dropped significantly as observed in the control embryos (Figure 1B). The survivability of Mirin-treated embryos was the highest at 53% when the lowest concentration of 3 μM is used. The lowest survivability was observed at 20% when the embryos were treated with 6 μM Mirin. In the Ku60019 treated embryos, the survivability drops from 46% to 40% as the concentration increases from 1.5 to 6 nM, respectively. Embryos treated with 12 nM Ku55933 had the lowest survivability of only 26%. Based on this data, we can conclude that the survival of inhibitor treated embryos decreases when exposed to UVC. However, percentage of survived inhibitors treated embryos did not portray the actual effect of UVC on MRN complex inhibited zebrafish embryos. This was due to the nature of random pyrimidine photodimers created by UV irradiation (Mullenders, 2018; Nair & Loppnow, 2019; Sugiyama & Chen, 2019), which affected the survivability rate of the embryos.

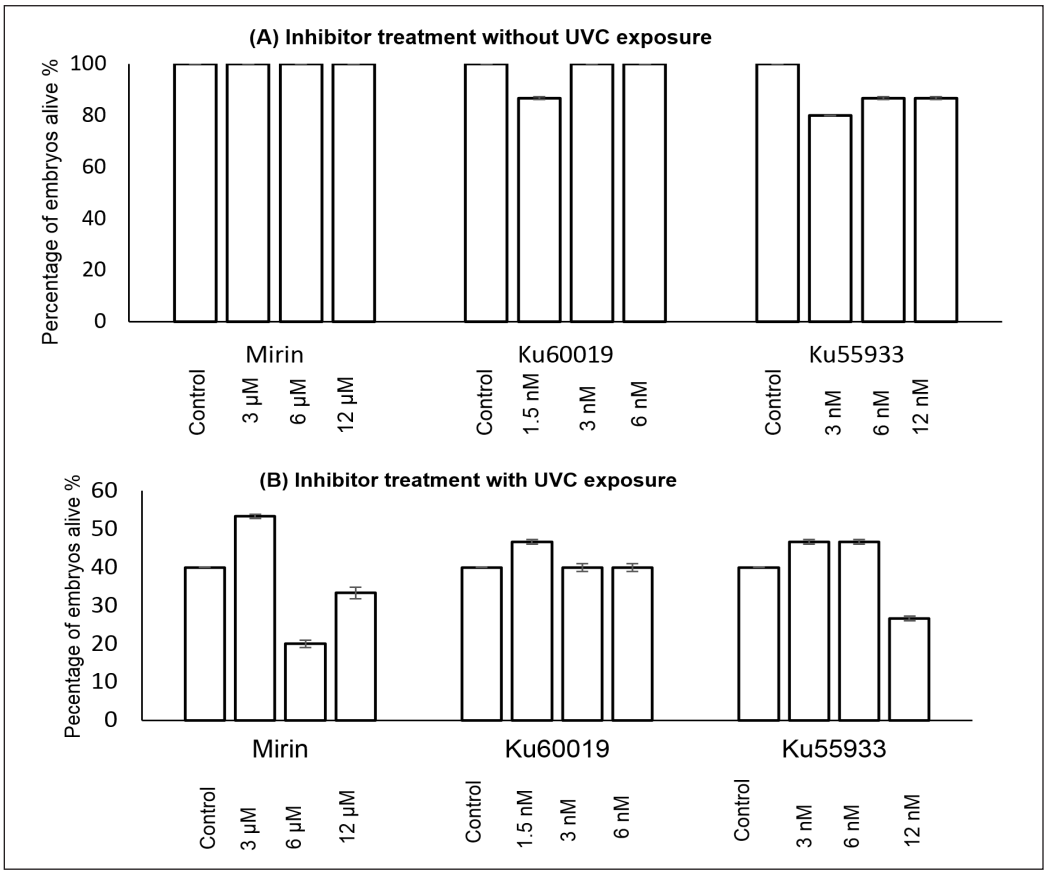

Figure 1. Survivability of embryos with and without UVC exposure at the end of 72 hpf. (A) Graph shows the survivability of embryos without UVC exposure. Embryos were treated with inhibitors at different range of concentration within the MBT stage and were incubated at 28.5°C under standard laboratory lighting. (B) Graph shows the survivability of embryos with UVC exposure. Briefly, the inhibitor treated embryos were incubated for 1 hour at room temperature before being irradiated with UVC. The embryonic development was evaluated at 24, 48 and 72 hpf. Experiments were performed in triplicate and the data are expressed as mean ± SD

Embryos which were alive by the end of 72 hpf were evaluated for the manifestation of any morphological abnormalities (Figure 2). On the other hand, embryos which displayed any deformed phenotype were tabulated as having abnormal development. In this study, only the embryos treated with UVC developed deformities while the non-UVC treated embryos developed normally with or without protein inhibitors (data not shown). The control embryos which were treated with 0.1% DMSO have the lowest rate of deformed embryos, excluding the embryos treated with 6 µM Mirin. The rest of the embryos treated with varying concentration of the inhibitors developed into phenotypically deformed embryos by 72 hpf, with embryos treated with Mirin (3 µM) having the highest rate of deformed embryos at 53%.

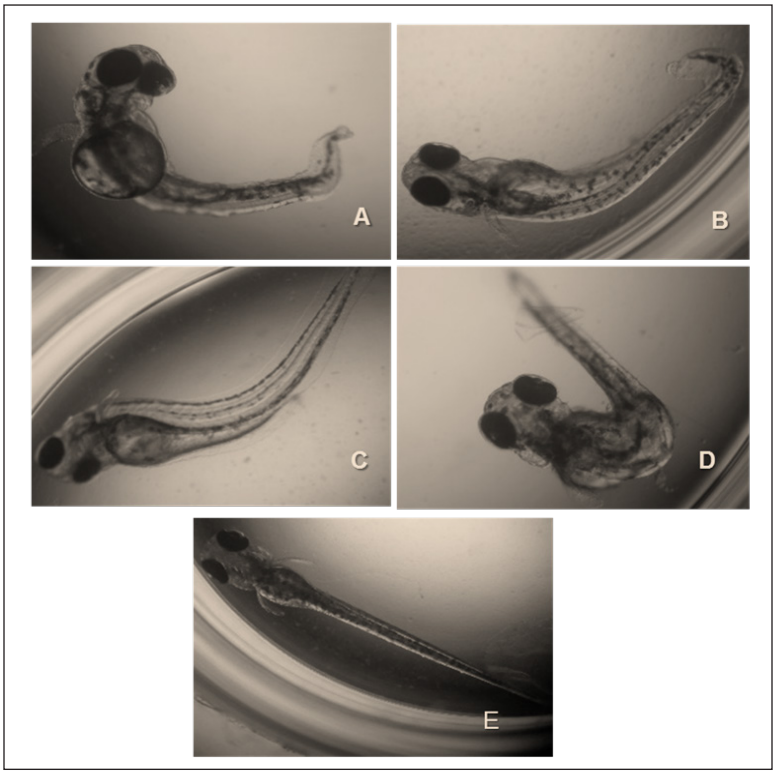

Figure 2. The figure illustrates the deformed phenotypes and the normal phenotypes observed at 72 hpf. (A) Enlarged yolk sac (B) Bent caudal fin (C) Curved-body (D) Bent floor plate and (E) Normal. *The data represents the % of deformed embryos from total embryos which are still alive at 72 hpf.

The majority of embryos treated with UVC developed enlarged yolk sac by the end of 72 hpf. A hundred (100) percent of alive control embryos had enlarged yolk sac, similarly with the rest of the Mirin, Ku60019 and Ku55933 inhibitors with varying concentration (Figure 3A). Interestingly, treatment with 3 μM Mirin and 3 nM Ku60019 showed a decrease of 12.5% and 28.6% in the formation of enlarged yolk sac, respectively. Based on this result, we can assume that the DNA damage inflicted by the UVC affects the development of the yolk sac. The damage is prominent enough to cause the phenotype to have almost 100% manifestation across the range of inhibitors at varying concentrations used.

In the UVC treated embryos, 75% of embryos alive developed bent caudal fin phenotype, indicating that the development of zebrafish embryos caudal tail during embryogenesis were sensitive to DNA damage (Figure 3B). Unexpectedly, the formation of the bent caudal tail phenotype decreased with the additional of Mirin, Ku60019 and Ku55933. This may be due to the inhibition of apoptosis of the damage cells when treated with these inhibitors. Previous studies have reported that both MRN and ATM induced apoptosis on DNA damaged cells (Chwastek et al., 2017, Dubois & Gerber, 2016). We speculate that cells with compromised DNA structure in UVC treated embryos (without

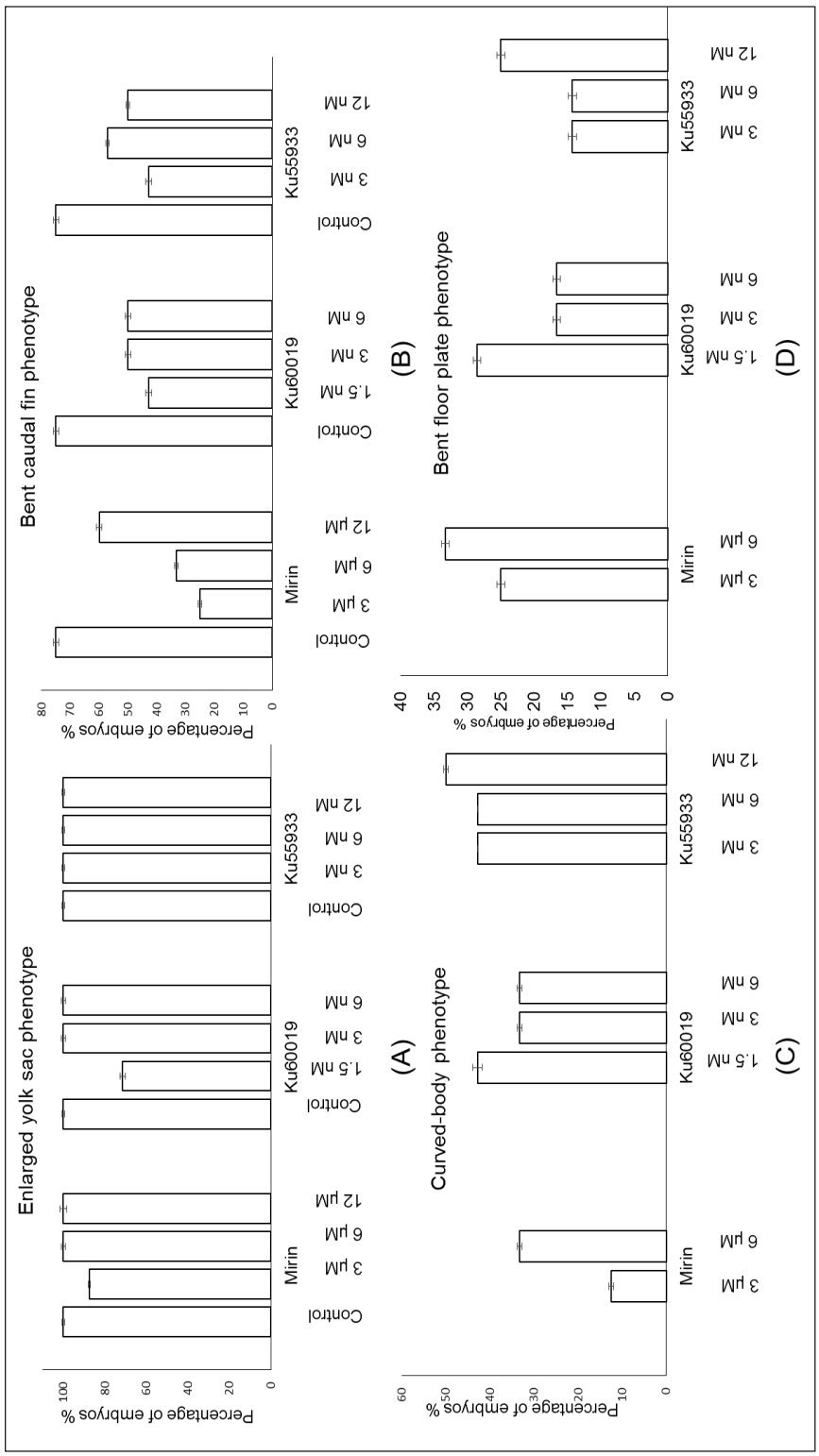

Figure 3. Percentage of embryos that develop deformed phenotypes. These phenotypes are observed only in the UVC treated embryos. (A) Graph illustrates the percentage of embryos that develop enlarged yolk sac by 72 hpf. (B) Graph shows the rate of embryos which have develop bent caudal fin. (C) The manifestation of curved-body phenotype among the alive embryos is shown across the inhibitor type and concentrations. (D) Graph shows the % of embryos that develop bent floor plate phenotype. Alive and morphologically deformed embryos at 72 hpf were evaluated for characteristic differences in the type of deformities observed. The deformities were then categorised into these 4 deformed phenotypes. Experiments were performed in triplicate. Error bar showed the standard error of the mean. Asterisk (*) showed the significant ($p < 0.05$) difference between treated group and control. Experiments were analysed using student t-test.

the presence of inhibitors) undergo apoptosis which stunted the growth of the tail at the region of damage, while the healthy cells adjacent to them grow normally, producing the bent phenotype.

For both the curved-body and bent floor-plate phenotypes, none of the control group embryos had developed these phenotypes (Figure 3C and 3D).

The manifestation of these phenotype was of the same pattern across the range of inhibitors' concentrations. In both Mirin and Ku55933 treated embryos, as the concentration increases, the rate of embryos developing these phenotypes increased with 50% being the highest for the curved body phenotype and 33% for the bent floor plate phenotype. However, embryos treated with Mirin at 12 μM did not develop any of these phenotypes and the embryos treated with Ku60019 had decreasing rate of the phenotype manifestation as the concentration increases. The data indicates the severity of these two phenotypes is independent of the concentration of inhibitors used.

Our findings indicate that embryos can survive and hatch phenotypically normal when treated with inhibitors alone. However, with the occurrence of DNA damage inflicted by the UVC radiation, majority of the inhibitor treated embryos that are alive pass the hatching stage, develop into phenotypically deformed embryos. This data is statistically significant when compared to the control group.

CONCLUSION

We found that specific protein inhibitors such as Mirin, Ku60019 and Ku55933 increased the possibilities of deformities formation particularly in enlarged yolk sac, curved-body and bent floor-plate phenotypes among the UVC treated embryos. In the presence of protein inhibitors however, we observed that embryos without UVC exposure did not show any abnormal phenotypes. This suggests that RAD50 and ATM do not involve directly in zebrafish embryogenesis but may be crucial in safeguarding the whole embryogenesis process as a whole.

ACKNOWLEDGEMENTS

This work was supported by the Ministry of Science, Technology and Innovation (MOSTI) of Malaysia (Grant no.: ScienceFund 02-01-02-SF1279), the Ministry of Higher Education (MOHE) of Malaysia (Grant no.: FRGS/2/2014/ST04/UKM/03/1) and Pusat Penyelidikan Tasik Chini (PPTC), UKM (LIV-2015-05).

REFERENCES

Bender, C. F., Sikes, M. L., Sullivan, R., Huye, L. E., Le Beau, M. M., Roth, D. B., … & Petrini, J. H. J. (2002). Cancer predisposition and hematopoietic failure in Rad50S/S mice. *Genes and Development, 16*(17), 2237-2251.

Bladen, C. L., Lam, W. K., Dynan, W. S., & Kozlowski, D. J. (2005). DNA damage response and Ku80 function in the vertebrate embryo. *Nucleic Acids Research, 33*(9), 3002-3010.

Chwastek, J., Jantas, D., & Lasoń, W. (2017). The ATM kinase inhibitor KU-55933 provides neuroprotection against hydrogen peroxide-induced cell damage via a γH2AX/p-p53/caspase-3-independent mechanism: Inhibition of calpain and cathepsin D. *International Journal of Biochemistry and Cell Biology, 87*, 38-53.

Clemente, Z., Castro, V. L. S. S., Moura, M. A. M., Jonsson, C. M., & Fraceto, L. F. (2014). Toxicity assessment of TiO_2 nanoparticles in zebrafish embryos under different exposure conditions. *Aquatic Toxicology, 147*, 129-139.

Dale, R. M., Sisson, B. E., & Topczewski, J. (2009). The emerging role of Wnt/PCP signalling in organ formation. *Zebrafish, 6*(1), 9-14.

Driever, W., Schier, A. F., Neuhauss, S. C. F., Malicki, J., Stemple, D. L., Stainier, D. Y. R., … & Boggs, C. (1996). A genetic screen for mutations affecting embryogenesis in zebrafish. *Development, 123*, 37-46.

Dubois, E., & Gerber, S. (2016). UV-dependent phosphorylation of COP9/signalosome in UV-induced apoptosis. *Oncology Reports, 35*(5), 3101-3105.

Dupré, A., Boyer-Chatenet, L., Sattler, R. M., Modi, A. P., Lee, J. H., Nicolette, M. L., & Gautier, J. (2008). A forward chemical genetic screen reveals an inhibitor of the Mre11–Rad50–Nbs1 complex. *Nature Chemical Biology, 4*(2), 119-125.

Fazry, S., Noordin, M., Sanusi, S., Noor, M., Aizat, W., Lazim, A., … & Law, D. (2018). Cytotoxicity and toxicity evaluation of xanthone crude extract on hypoxic human hepatocellular carcinoma and zebrafish (*Danio rerio*) embryos. *Toxics*, *6*(4), 60-70.

Gatei, M., Jakob, B., & Chen, P. (2011). ATM-dependent phosphorylation of Rad50 regulates DNA repair and cell cycle control. *Journal of Biological Chemistry, 286*(36), 31542-31556.

Golling, G., Amsterdam, A., Sun, Z., Antonelli, M., Maldonado, E., Chen, W., … & Hopkins, N. (2002). Insertional mutagenesis in zebrafish rapidly identifies genes essential for early vertebrate development. *Nature Genetics, 31*(2), 135-140.

Kaur, S., Kaushik, S., & Kedarisetti, K. C. (2018). Lasers in glaucoma. In H. V. Nema & N. Nema (Eds.), *Gems of Ophthalmology: Glaucoma* (pp. 334-354). New Delhi, India: Jaypee Brothers Medical Publisher.

Lavin, M. F. (2007). ATM and the Mre11 complex combine to recognize and signal DNA double-strand breaks. *Oncogene, 26*(56), 7749-7758.

Ledo, E. (1993). Photodermatosis part I: photobiology, photoimmunology, and idiopathic photodermatoses. *International Journal of Dermatology*, *32*(6), 387-396.

Luo, G. (1999). Disruption of mRad50 causes embryonic stem cell lethality, abnormal embryonic development, and sensitivity to ionizing radiation. *Proceeding of the National Academy of Sciences, 96*(13), 7376-7381.

Meyers, J. R. (2018). Zebrafish: development of a vertebrate model organism. *Current Protocols Essential Laboratory Techniques, 16*(1), 1-26.

Mullenders, L. H. (2018). Solar UV damage to cellular DNA: from mechanisms to biological effects. *Photochemical and Photobiological Sciences, 17*(12), 1842-1852.

Nair, S. G., & Loppnow, G. R. (2019). Comparison of K-Ras and N-Ras mutagenic hot spots for UVC damage. *ACS Omega*, *4*(2), 3469-3475.

Negi, S. S., & Brown, P. (2015). rRNA synthesis inhibitor, CX-5461, activates ATM/ATR pathway in acute lymphoblastic leukemia, arrests cells in G2 phase and induces apoptosis. *Oncotarget, 6*(20), 18094-18104.

Segner, H. (2009). Zebrafish (*Danio rerio*) as a model organism for investigating endocrine disruption. *Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology, 149*(2), 187-195.

Sugiyama, T., & Chen, Y. (2019). Biochemical reconstitution of UV-induced mutational processes. *Nucleic Acids Research, 2019*, 1-14.

Waltes, R., Kalb, R., Gatei, M., Kijas, A. W., Stumm, M., Sobeck, A., … & Dörk, T. (2009). Human RAD50 deficiency in a Nijmegen breakage syndrome-like disorder. *The American Journal of Human Genetic, 84*(5), 605-616.

Williams, B. R., Mirzoeva, O. K., Morgan, W. F., Lin, J., Dunnick, W., & Petrini, J. H. J. (2002). A murine model of Nijmegen breakage syndrome. *Current Biology, 12*(8), 648-653.

Williams, T. M., Nyati, S., Ross, B. D., & Rehemtulla, A. (2013). Molecular imaging of the ATM kinase activity. *International Journal of Radiation Oncology Biology Physics, 86*(5), 969-977.

Zhu, J., Petersen, S., Tessarollo, L., & Nussenzweig, A. (2001). Targeted disruption of the Nijmegen breakage syndrome gene NBS1 leads to early embryonic lethality in mice. *Current Biology, 11*(2), 105-109.