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Non-induction of radioadaptive response in zebrafish embryos by neutrons

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ABSTRACT

In vivo neutron-induced radioadaptive response (RAR) was studied using zebrafish (*Danio rerio*) embryos. The Neutron exposure Accelerator System for Biological Effect Experiments (NASBEE) facility at the National Institute of Radiological Sciences (NIRS), Japan, was employed to provide 2-MeV neutrons. Neutron doses of 0.6, 1, 25, 50 and 100 mGy were chosen as priming doses. An X-ray dose of 2 Gy was chosen as the challenging dose. Zebrafish embryos were dechorionated at 4 h post fertilization (hpf), irradiated with a chosen neutron dose at 5 hpf and the X-ray dose at 10 hpf. The responses of embryos were assessed at 25 hpf through the number of apoptotic signals. None of the neutron doses studied could induce RAR. Non-induction of RAR in embryos having received 0.6- and 1-mGy neutron doses was attributed to neutron-induced hormesis, which maintained the number of damaged cells at below the threshold for RAR induction. On the other hand, non-induction of RAR in embryos having received 25-, 50- and 100-mGy neutron doses was explained by gamma-ray hormesis, which mitigated neutron-induced damages through triggering high-fidelity DNA repair and removal of aberrant cells through apoptosis. Separate experimental results were obtained to verify that high-energy photons could disable RAR. Specifically, 5- or 10-mGy X-rays disabled the RAR induced by a priming dose of 0.88 mGy of alpha particles delivered to 5-hpf zebrafish embryos against a challenging dose of 2 Gy of X-rays delivered to the embryos at 10 hpf.

KEYWORDS: radioadaptive response, neutrons, zebrafish embryos, hormesis, NASBEE

INTRODUCTION

In normal environments, the majority of neutron exposure of the general public is contributed by cosmic radiation. Higher neutron exposures can be received by airline crew members, well loggers, nuclear power plant workers, and medical doctors and patients involved in clinical radiotherapy. Interest in the biological effects of neutrons has been increased by advances in space research. The biological effects resulting from neutrons are less well understood and seem to differ from other those of other ionizing radiation sources such as X-ray photons, gamma-ray photons and alpha particles. For example, while radiation-induced bystander effects (RIBEs) can generally be induced by gamma radiation and alpha-particle radiation [1–4], all previous *in vitro* and *in vivo* attempts have failed to demonstrate neutron-induced bystander effects [5–7]. Moreover, neutron fluxes from a variety of neutron sources are invariably contaminated by gamma-ray photons, which some suggest may trigger gamma-ray hormesis, thus mitigating the neutron-induced damages by generating high-fidelity DNA repair and removal of aberrant cells through apoptosis [8, 9].

Another interesting biological effect generated by ionizing radiation is the radioadaptive response (RAR), which refers to the phenomenon that exposures of cells, tissues or organisms to low doses (referred to as the 'priming dose' or 'adapting dose') of ionizing radiation can lessen the genotoxic effect arising from a subsequent larger

dose (referred to as the 'challenging dose') of ionizing radiation. The phenomenon was first reported by Olivieri *et al.* [10], who found that pretreating human lymphocytes with low doses of radioactive thymidine significantly lowered the frequencies of chromatid aberrations resulting from subsequent exposure to a large challenging dose, when compared with non-primed cells. Numerous previous studies have demonstrated RARs in mammalian systems using a variety of endpoints, including micronuclei formation [11, 12], cell proliferation [13], chromosomal aberration [14, 15], apoptosis [16, 17] and cell killing [18]. On the other hand, non-induction of RAR has also been reported in human lymphocytes [19], mouse preimplantation embryos [20] and rat fetal brains [21].

While characterization of RARs is important for understanding and providing realistic risk assessments and radiation protection [22, 23], studies on neutron-induced RARs have been relatively rare. Early research showed that neutrons failed to induce RARs in human lymphocytes [24]. On the other hand, Marples and Shov [25] showed that neutrons could induce RARs in Chinese hamster V79 cells, protecting against subsequent X-ray irradiation. Apparently, more carefully designed studies, in particular *in vivo* studies, would be needed to get a better understanding of neutron-induced RARs. To the best of our knowledge, up till now, there has been no previous *in vivo* study of neutron-induced RARs. The closest to it was an *ex vivo* study by Gajendiran *et al.* [26] on the RARs induced in whole blood samples collected from 10 volunteers (including 2 atomic-bomb survivors, who had received 1.5–2 Gy *in vivo* exposure). In the initial screening test (with a priming dose of 10 mGy and a challenging dose of 1 Gy of γ rays from ^{137}Cs, separated by 4 h), a RAR was clearly detected only in the blood samples from Donor 3 (a female aged 27, who was the only female in the 'young' group). Her blood samples were then employed for more in-depth studies: they were first exposed to a priming dose of either 10 mGy of ^{137}Cs γ-rays or 2.5 mGy of ^{252}Cf neutrons and 4 h later to a corresponding challenging dose of 1 Gy of ^{60}Co γ-rays or 250 mGy of ^{252}Cf neutrons, or first exposed to a priming dose of 10 mGy of ^{137}Cs γ-rays and 4 h later to a challenging dose of 250 mGy of ^{252}Cf neutrons. All these treatments led to significant reduction in the initial DNA damages.

The pioneer study of Gajendiran *et al.* [26] provided valuable insights and information on neutron-induced RARs. The present work aimed to extend the study to an *in vivo* situation using embryos of the zebrafish (*Danio rerio*) as the vertebrate model, which has been widely employed for assessing the biological effects of ionizing radiation [27–36]. Zebrafish and human genomes share considerable homology, including conservation of most DNA repair–related genes [37]. Other advantages of this model include rapid development and high fecundity, which allow short turn-around time for experiments. Moreover, zebrafish embryos have previously been shown to develop RARs upon exposure to high linear-energy-transfer (LET) radiations other than neutrons [36, 38, 39]. All the studied zebrafish embryos were at the same stage of development in our experiments, and the time of final assessment was 25 h post fertilization (hpf), which avoided the potential influence from the heterogeneity and long life history encountered in the subjects (22–80 years old) employed by Gajendiran *et al.* [26]. It had been established that people with varying exposures to radiations, either due to different environments or durations, develop different RARs [40–42]. The complications would be exacerbated if we took into account non-specific cross-adaptation for RARs [43], and if we included stressors other than ionizing radiations in the consideration of multiple stressor effects (e.g. refs. [44–47]).

The present work used neutrons with a mean energy of 2 MeV from the Neutron exposure Accelerator System for Biological Effect Experiments (NASBEE) facility at the National Institute of Radiological Sciences (NIRS) for our irradiations [48]. The gamma-ray contamination in the neutron beam was as low as 14%. Such low gamma-ray contamination could help avoid the complications involved in using neutrons from the ^{252}Cf source, which emitted a much higher γ-ray contamination (~33%) [26] and alpha particles. In the present work, neutron doses ranging from 0.6 to 100 mGy were employed as the priming dose, which spanned all the different neutron dose–response zones (comprising the neutron hormetic and toxic zones, and the gamma-ray hormesis zone) [49]. Instead of a neutron dose, an X-ray dose of 2 Gy was chosen as the challenging dose to avoid potential complications caused by gamma-ray hormesis. The number of apoptotic signals within the whole embryos was adopted as the biological endpoint in the present study ('apoptosis signals' referring to the observed numbers of cells that were undergoing apoptosis). The number of apoptotic signals has been commonly employed as the biological endpoint to assess the effects of radiation in zebrafish embryos [32, 50–52].

We hypothesize that a RAR is not induced in zebrafish embryos due to neutron-induced hormesis and gamma-ray hormesis. The present work further examined the suppression of a RAR by high-energy photons through separate (alpha-particle and X-ray) experiments. We showed that X-ray photons (with a small dose of 5 or 10 mGy) were able to disable the alpha-particle–induced RAR successfully induced by a priming dose of 0.88 mGy of alpha particles against a challenging dose of 2 Gy of X-rays.

MATERIALS AND METHODS

Ethics statement

Proposed animal experiments for this study (Proposal No. 09–1021–6) in the NIRS were approved by the Animal Research and Ethics Committee at the NIRS and were performed in accordance with the guidelines for animal care in Japan. The animal studies in Hong Kong were approved by the Department of Health, Government of the Hong Kong Special Administrative Region, under Ref: 13–7 in DH/HA&P/8/2/5 Pt.1 and were performed in accordance with the guidelines.

Neutron irradiation facility

As described in the Introduction, for the studies on neutron-induced RAR, the NASBEE facility at NIRS was employed to provide neutrons with a mean energy of 2-MeV neutrons for our irradiations [48]. A high-flux neutron beam was generated by bombarding a 4-MeV deuteron beam onto the surface of a Be target, with the latter installed inside a target shield made of iron plates and polyethylene walls to collimate the neutron beam. The gamma-ray contamination in the neutron beam was reduced to 14% by a shutter installed at the beam port [48]. To maintain uniform experimental conditions, all neutron irradiations in the current study made use of neutrons with an average energy of 2 MeV delivered at a single dose rate of 220 mGy/h. The same neutron energy and dose rate were also employed in our previous study [49].

Alpha-particle irradiation setup

To demonstrate suppression of alpha-particle–induced RAR by X-ray photons, we adopted a setting for alpha-particle irradiation of zebrafish embryos similar to that designed by Yum *et al.* [33]. A planar ^{241}Am source with alpha-particle energy of 5.49 MeV under vacuum and an activity of 4.26 kBq was employed. In order to minimize the uncertainty in the energy of alpha particles hitting the cells of the embryos, all embryos were irradiated with the alpha particles coming from bottom after passing through a 3.5-μm thick Mylar film (Dupont, Hong Kong). All embryos were orientated carefully so that the cells of the embryos were facing directly towards the Mylar film and the alpha-particle source. With such a setting, the absorbed dose rate was ~1.1 mGy/min [33].

X-ray irradiation facilities

For the studies on neutron-induced RAR, an X-ray generator (TITAN, Shimazu Corporation, Kyoto, Japan) with the voltage and current set at 200 kVp and 20 mA, respectively, was employed to irradiate the zebrafish embryos. The generated X-ray photons passed through 0.5-mm thick filters made of aluminum and copper. With such settings, the effective X-ray energy was ~83 keV. The same conditions were adopted in our previous studies [35, 53]. For the studies on alpha-particle–induced RAR, the X-ray doses were delivered using an X-ray irradiation system (X-RAD 320, Precision X-Ray (PXi), Connecticut, USA). The voltage was always set at 200 kVp, and the current was set at 2 mA to provide the supplementary priming dose and 12.5 mA to provide the challenging dose. The X-ray photons generated passed through 2.5-mm thick filters made of aluminum, copper and tin. With such settings, the effective X-ray energy was ~132 keV.

Zebrafish embryos

Adult zebrafish with mixed gender were kept in 45-l glass water tanks maintained at 28°C. The zebrafish were maintained under a 14–10 h light–dark cycle to facilitate a stable and good production of embryos. The fish were fed four times a day with commercial tropical fish food (TetraMin, Melle, German) or brine shrimp (Brine Shrimp Direct, Ogden, Utah, USA). Spawning was stimulated at the beginning of the photoperiod. To ensure synchronization of developmental stages of the collected embryos, the embryos were collected 15–30 min after the start of the light period. All embryos were then kept in Petri dishes with E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, 0.1% methylene blue) and were transferred to a 28°C incubator for development. At 4 hpf, the zebrafish embryos were examined under stereomicroscope (Model SZH, Olympus Co., Shinjyuku-ku, Tokyo, Japan, or Nikon, Chiyoda-ku, Tokyo, Japan). Healthily developing embryos were selected and transferred into new Petri dishes with 5 ml E3 medium and a thin layer of biocompatible agarose gel lining the bottom. The chorion of each embryo was then carefully removed with a pair of sharp forceps (Dumont, Hatfield, PA, USA).

Irradiation protocols for studies on neutron-induced RAR

A total of 5 neutron doses (0.6, 1, 25, 50 and 100 mGy) were employed as the priming doses for examining the effects of neutron exposure against a subsequent X-ray challenging dose on zebrafish embryos. For each priming dose, two independent experiments were performed, with at least 31 dechorionated embryos employed in each experiment. At 4 hpf, the embryos were dechorionated and then separated into three groups, namely, the adaptive (***AR***) group, which received both the neutron priming dose and the X-ray challenging dose at a later stage, the adaptive control (***C***) group, which received only the X-ray challenging dose at a later stage, and the dechorionated control (***D***) group, which did not receive any further radiation dose for monitoring purposes. These three groups of embryos were accommodated in separated wells in a 6-well cell-culture dish (3516, Corning Life Science Inc.) with a layer of biocompatible agarose lining the inner well bottoms. At 5 hpf, the desired neutron priming dose was delivered to the embryos using the NASBEE facility. The irradiation procedures were described by Ng *et al.* [49]. Briefly, the zebrafish embryos in the ***AR*** group were placed within the uniform dose irradiation field (with a diameter of 26 cm ± 2%) on the movable bed of NASBEE, with the source-to-target distance set to 1835 mm. During and after irradiation, the embryos were accommodated in wells containing 3 ml of E3 medium. Before the embryos were returned to the 28°C incubator for further development, both the medium and samples had to be checked by a Geiger–Müller (GM) survey meter (TGS-133, Hitachi Aloka Medical, Ltd, 6–22–1, Mure, Mitaka-shi, Tokyo 181–8622 Japan) to see that they were not activated by neutrons.

After a further 5 h of incubation, i.e. at 10 hpf, the embryos in the ***AR*** group were further exposed to the X-ray challenging dose of 2 Gy. Our group had previously employed such a challenging dose on zebrafish embryos to study the RAR induced by microbeam protons [35]. Choi *et al.* [35] also demonstrated that such an X-ray dose alone increased the number of apoptotic signals in zebrafish embryos. The dishes holding the embryos were irradiated with X-rays at a dose rate of ~0.65 Gy/min, with the source-to-target distance set at 700 mm. After being exposed to the challenging dose, all the embryos were returned to the incubator again until 25 hpf, for further analysis. For the control experiment, the embryos in the control (***C***) group were first sham irradiated with neutrons at 5 hpf, and then irradiated with 2 Gy of X-rays together with the ***AR*** group embryos at 10 hpf. The experiment was repeated for all desired neutron priming doses. Figure 1 shows the procedures for the experiments.

Irradiation protocols for studies on alpha-particle–induced RAR

In this part of the study, an alpha-particle dose of 0.88 mGy, with or without a supplementary X-ray dose of 5 or 10 mGy, was used as the priming dose, while an X-ray dose of 2 Gy applied 5 h after the priming dose was used as the challenging dose. All the X-ray doses were provided by 200 kVp X-ray photons (X-RAD 320, Precision X-Ray (PXi), Connecticut, USA) in this part of the study. The supplementary X-ray doses of 5 or 10 mGy were chosen to be commensurate with the gamma-ray contamination in the neutron beams from NASBEE, as described in the Discussion section below. Briefly, with 14% gamma-ray contamination in the NASBEE facility, the gamma dose amounted to 3.5 and 7 mGy for neutron doses of 25 and 50 mGy, respectively.

When the embryos developed into 4 hpf, they were dechorionated and then separated into the ***AX$_Y$***, ***A***, ***Control*** and ***D*** groups in four

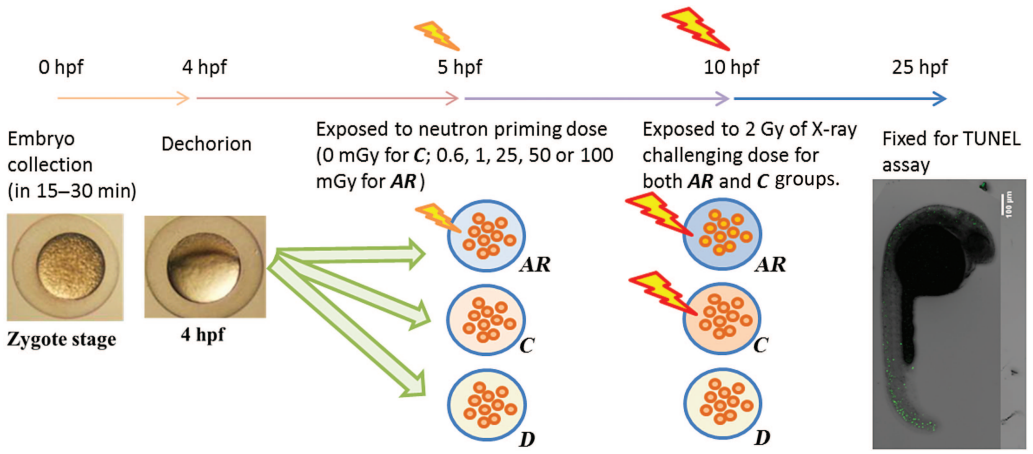

Fig. 1. Schematic diagram showing the procedures for studying the radioadaptive response induced in zebrafish embryos that have been dechorionated at 4 hpf using a neutron priming dose and an X-ray challenging dose. *AR*: adaptive group, in which the dechorionated embryos received both the neutron priming dose and the X-ray challenging dose; *C*: adaptive control group, in which the dechorionated embryos were exposed to the X-ray challenging dose alone, without receiving a prior priming dose; *D*: dechorionated control group, in which the dechorionated embryos did not receive any radiation dose.

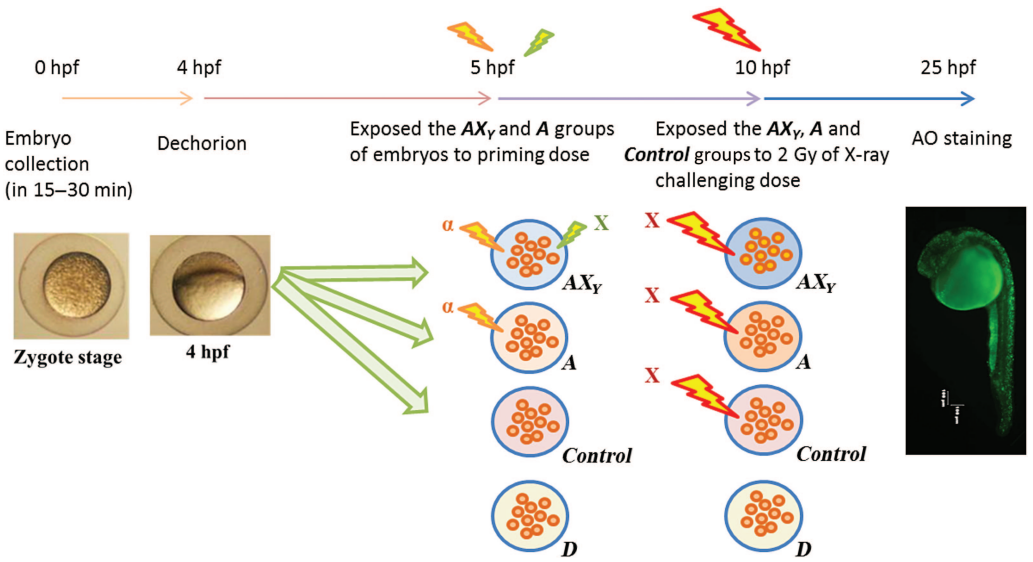

Fig. 2. Schematic diagram showing the procedures for studying the effect of X-ray photons on the radioadaptive response induced by alpha particles in zebrafish embryos that have been dechorionated at 4 hpf using a priming dose provided by (alpha particles) or (alpha particles + low-dose X-ray photons) and an X-ray challenging dose. *AX_Y* group: in which the dechorionated embryos received both the priming dose provided by (~0.88 mGy alpha-particle irradiation + level-Y X-ray irradiation) and the 2 Gy X-ray challenging dose, where level-Y was either 5 or 10 mGy; *A* group: in which the dechorionated embryos received both the priming dose provided by ~0.88 mGy alpha-particle irradiation and the 2 Gy X-ray challenging dose; *Control* group: in which the dechorionated embryos were exposed to the X-ray challenging dose alone, without receiving a prior priming dose; *D* group: in which the dechorionated embryos did not receive any radiation dose.

Petri dishes, each having a thin layer of agarose. For each set of experiments, a total of 40 dechorionated embryos were deployed, which were divided into the four groups, each having 10 embryos. A volume of 3 ml of E3 medium was used in each of these agarose dishes. At 5 hpf, the embryos in the *A* group were transferred into the irradiation dish and irradiated with ~0.88 mGy of alpha particles, while those in the AX_Y group were irradiated with ~0.88 mGy of alpha particles immediately followed by X-ray photons (5 or 10 mGy). After this, all embryos were returned to the 28°C incubator for further development. At 10 hpf, those in the AX_Y, *A* and *Control* groups were exposed to 2 Gy of X-ray photons, then returned to the incubator again until they reached 25 hpf. The dechorionated control (*D*) group did not further receive any radiation dose for monitoring purposes. Figure 2 shows the procedures for the experiments.

TUNEL assay

For the studies on neutron-induced RAR, the responses of embryos to X-rays, (neutrons + X-rays) or to no irradiation were assessed through quantification of the number of apoptotic signals within the whole embryos. The terminal dUTP transferase-mediated nick end-labeling (TUNEL) assay [35, 49, 54, 55] was employed to determine the numbers of apoptotic signals in the embryos. Briefly, at 25 hpf, the embryos were fixed for 5 h at room temperature with 4% paraformaldehyde in phosphate-buffered saline (PBS) with 0.1% Tween 20. The fixed embryos were dehydrated, and were then rehydrated with methanol before a 10-min treatment of 20 μg/ml protease kinase (PK) (Wako Pure Chemical Industries Ltd, Osaka, Japan). TUNEL staining was performed by using an *in situ* apoptosis detection kit (MK500, Takara Bio. Inc., Japan). Before applying the TUNEL stain, PK-treated embryos were first fixed once again in 4% paraformaldehyde in PBS with 0.1% Tween 20 for 2 h, and were then immersed on ice in the permeabilization buffer for 30 min. The apoptotic cells in each embryo were labeled by staining in a mixture containing terminal deoxynucleotidyl transferase (TdT) enzyme and labeling safe buffer containing fluorescein labeled-2′-deoxyuridine, 5′-triphosphate, FITC-dUTP in the ratio of 1 to 9 inside a humidified chamber at 37°C. This staining process lasted 110 min. The stained embryos were eventually rinsed five times thoroughly by PBS in 0.1% Tween 20. The apoptotic signals became visible under a fluorescent microscope. For each embryo, 15–25 sliced images (2.12 × 2.12 mm, 2.06 μm/pixel) were captured with 25-μm intervals from top to bottom by a confocal laser microscope (FV-1000, Olympus Corporation, Tokyo) with ×4 objective lens (NA:0.16, UPLSAPO 4X, Olympus Corporation, Tokyo). These images were then combined into a single image for further analysis. The number of apoptotic signals within each whole embryo was counted using ImageJ software (freely obtainable from the website http://rsb.info.nih.gov/ij/). After converting the captured image into a binary image, the number of apoptotic signals was determined using the 'Analyze particle' function in ImageJ.

Acridine orange staining

For the studies on alpha-particle–induced RAR, the numbers of apoptotic signals within the total 25 hpf zebrafish embryos were determined through staining using acridine orange (AO) (Sigma, St Louis, MO, USA) and counting under the fluorescent microscope (see [47]). At 25 hpf, the embryos were stained in a medium containing 2 μg/ml of AO for 1 h in the dark and then rinsed with the culture medium twice to remove the excess dye. After anesthetizing the embryos with 0.0016 M tricaine (Sigma, St Louis, MO, USA), the stained embryos were examined under a fluorescent microscope. The apoptotic cells in the embryos became visible as bright green spots under the fluorescent microscope. For each embryo, three images focusing on different parts of the embryo were captured and were then combined into a single image for quantification of the apoptotic cells with the help of a computer program 'Particle Counting 2.0' (developed by J. Zhang).

Data analysis

The number of apoptotic signals within each whole embryo was counted as described above. The statistical significance of the difference between two samples was assessed through Student's *t*-test, and a P value ≤ 0.05 was considered to correspond to a statistically significant difference.

RESULTS

Neutron-induced RAR

Figure 3 shows representative combined images of stained 25-hpf embryos after they had either received both the priming and challenging exposures (***AR*** group), only the challenging dose (***C*** group) or no irradiation dose at all (***D*** group). Each green spot represented an apoptotic signal, and the number of apoptotic signals throughout the whole embryo was quantified using the ImageJ software.

The results are summarized in Table 1. The average number of apoptotic signals for the ***AR***, ***C*** and ***D*** groups were denoted as N_{AR}, N_C and N_D, respectively. A total of five different neutron doses (0.6, 1, 25, 50 and 100 mGy) were employed in the present study as the priming doses for studying the protective effects of neutron irradiation against the challenging dose of X-rays applied 5 h later. All the embryos in the ***AR*** and ***C*** groups were exposed to the same challenging dose of 2 Gy, which was delivered by X-ray photons. The total number (n) of embryos employed in each set of experiments (i.e. the sample size) is also shown in Table 1.

When considering the mean number of apoptotic signals in the ***D*** group (N_D) as the average background apoptotic signals for the embryos in the corresponding set of experiments, the net apoptotic signals for the ***AR*** and ***C*** groups could be written as $N_{AR}^{Net} = (N_{AR} - N_D)$ and $N_C^{Net} = (N_C - N_D)$, respectively. As such, the normalized net apoptotic signals for these groups of the embryos could be expressed as $N_{AR}{}^{\#} = (N_{AR}^{Net}/N_D)$ and $N_C{}^{\#} = (N_C^{Net}/N_D)$. To compare the ***AR*** and ***C*** groups in each set of experiments, the differences in the numbers of apoptotic signals ($Diff = N_C{}^{\#} - N_{AR}{}^{\#}$) between these two groups were calculated. A positive value of *Diff* meant a reduction in the number of apoptotic signals in the 25-hpf embryos after receiving the neutron priming dose and a subsequent X-ray challenging dose, when compared with those embryos receiving only the X-ray challenging dose, without the preceding neutron priming dose. The differences were assessed using Student's *t*-tests, and cases with $P \leq 0.05$ were considered statistically significant. In other words, a positive *Diff* value with $P \leq 0.05$ indicated the occurrence of RAR. From Table 1, positive *Diff* values occurred only in both or only one of the two sets of experiments when the priming neutron doses were 0.6 or 50 mGy, respectively. None of the experiments had $P \leq 0.05$. The present data suggested no RAR was induced in zebrafish embryos by neutron priming doses of 0.6, 1, 25, 50 or 100 mGy.

Alpha-particle-induced RAR

The results are shown in Tables 2 and 3 for supplementary priming X-ray doses of 5 or 10 mGy, respectively. All experiments (with supplementary priming X-ray doses of 5 or 10 mGy) were performed three times independently on separate days. Tables 2 and 3 consistently revealed that the zebrafish embryos that had received a priming alpha-particle dose of 0.88 mGy at 5 hpf developed RAR (all P values ≤ 0.05 when compared the results in the ***A*** and ***Control*** groups), while those zebrafish embryos that had received priming doses of [0.88 mGy of alpha particles + (5 or 10) mGy of X-ray photons] at 5 hpf did not develop RAR (all P values > 0.05 when comparing the results in the $\boldsymbol{AX_5}$ or $\boldsymbol{AX_{10}}$ groups with the corresponding ***Control*** groups). In other words, adding 5 or 10 mGy of X-ray photons to the alpha-particle priming dose of 0.88 mGy at 5 hpf would disable the RAR induced by the alpha-particle priming dose alone.

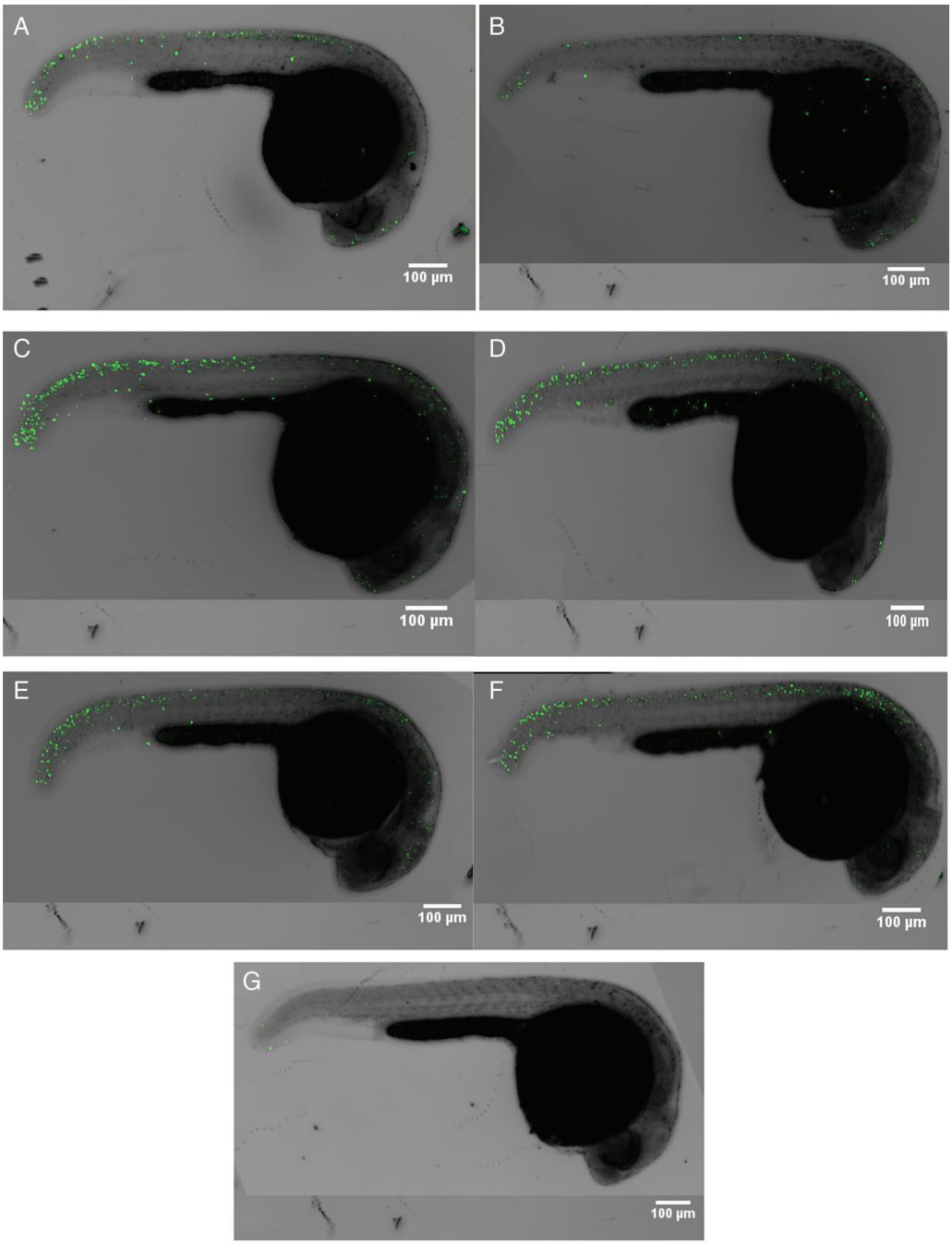

Fig. 3. Representative images of stained embryos. (A) to (E): embryos from *AR* groups after first receiving a neutron priming dose of (A) 0.6 mGy, (B) 1 mGy, (C) 25 mGy, (D) 50 mGy and (E) 100 mGy, and then an X-ray challenging dose of 2 Gy; (F): a *C* group embryo after receiving an X-ray challenging dose of 2 Gy only; (G): a *D* group embryo without receiving any radiation dose. Images of embryos were captured by a confocal laser microscope with ×4 objective lens. A total of 15 to 25 sliced images with 25 µm intervals were captured for each embryo, which were then combined from top to bottom to generate the final image.

Specifically, we found that X-ray photons with a dose of 5 or 10 mGy were capable of disabling the RAR induced by a priming dose of 0.88 mGy of alpha particles delivered to 5-hpf zebrafish embryos against a challenging dose of 2 Gy of X-ray photons delivered to the embryos at 10 hpf (same challenging dose as that employed in the current study).

DISCUSSION

In the present study, the effect of a small neutron priming dose on the response of zebrafish embryos to a subsequent large X-ray challenging dose was studied. Five different neutron doses (0.6, 1, 25, 50 and 100 mGy) were employed as the priming dose, and 2 Gy of X-rays was employed as the challenging dose. The number of

Table 1. The mean number of normalized net apoptotic signal ($N \pm SE$) for the adaptive (AR) group of embryos that had received both priming dose at 5 hpf and challenging dose at 10 hpf

Priming dose (mGy)		$N_{AR}^{\#}$	n	*Diff*	P
0.6	Set 1	1.72 ± 0.31	36	0.30	0.25
	Set 2	1.70 ± 0.40	32	0.31	0.28
1	Set 1	3.35 ± 0.27	36	−0.49	0.12
	Set 2	3.58 ± 0.37	31	−0.25	0.30
25	Set 1	6.65 ± 0.44	39	−1.03	0.07
	Set 2	5.77 ± 0.60	36	−0.09	0.46
50	Set 1	7.01 ± 0.79	42	−1.39	0.08
	Set 2	6.04 ± 0.43	37	0.34	0.33
100	Set 1	2.64 ± 0.26	34	−0.42	0.18
	Set 2	1.67 ± 0.19	35	−0.16	0.33

The P values were obtained using t-tests to compare between the adaptive (**AR**) group of embryos with the corresponding adaptive control (**C**) group of embryos, the latter having received only the challenging dose at 10 hpf. n = sample size, *Diff* = difference in the amounts of apoptotic signals between the AR and C groups of embryos ($N_C^{\#} - N_{AR}^{\#}$).

Table 2. The average number of apoptotic signals ($N \pm SE$) obtained from the embryos in the AX_5, A and Control groups in the three sets of experiments, where the embryos in the AX_5 group were irradiated with a priming dose of 0.88 mGy of alpha particles and 5 mGy of X-rays at 5 hpf

		N_{AX5}	N_A	N_{ctrl}
1	N	311 ± 12	212 ± 24	294 ± 15
	p^a	0.20	0.0089*	
2	N	297 ± 14	176 ± 8	292 ± 6
	p^a	0.38	5.0×10^{-10}*	
3	N	298 ± 14	213 ± 9	283 ± 8
	p^a	0.19	1.5×10^{-5}*	

[a]P values obtained using Student's t-test for assessing differences from the ***Control*** groups of embryos. *Cases with $P \leq 0.05$ were considered statistically significant.

apoptotic signals within the embryos was chosen as the biological endpoint to study the RAR. In the present work, no RAR was observed among the 10 sets of experiments conducted, which indicated that none of the studied neutron doses could induce RAR in the zebrafish embryos. The results were intriguing because RAR was previously successfully induced in zebrafish embryos by alpha particles and protons. Choi *et al.* [36] demonstrated that zebrafish embryos irradiated at 5 hpf by alpha particles from a ^{241}Am source (~0.44 mGy) developed a RAR against alpha particles from the same source (~4.4 mGy) delivered at 10 hpf. On the other hand, Choi *et al.* [35] revealed that 5–20 3.4-MeV protons delivered at 10 points on each zebrafish embryo at 5 hpf, or equivalently 0.11–0.43 mGy, could induce a RAR against 2 Gy of X-ray irradiation delivered at 10 hpf. Although recoiled protons contribute to many of the ionization

Table 3. The average number of apoptotic signals ($N \pm SE$) obtained from embryos in the AX_{10}, A and Control groups in three sets of experiments, in which the embryos in the AX_{10} group were irradiated with a priming dose of 0.88 mGy of alpha particles and 10 mGy of X-rays at 5 hpf

		N_{AX10}	N_A	N_{ctrl}
1	N	317 ± 17	259 ± 6	329 ± 8
	p^a	0.26	3.3×10^{-6}*	
2	N	321 ± 21	307 ± 13	349 ± 16
	p^a	0.16	0.028*	
3	N	258 ± 8	180 ± 7	275 ± 16
	p^a	0.18	0.00013*	

[a]P values obtained using Student's t-test for assessing differences from the ***Control*** groups of embryos. *Cases with $P \leq 0.05$ were considered statistically significant.

events associated with neutrons, generation of these protons through interaction of the neutrons with the embryos has a stochastic nature. The energy of the recoiled proton E_p, the energy of the neutron E_n and the recoil angle θ are related by $E_p = E_n \cos^2(90° - \theta)$ [56]. The protons recoiling with a larger angle have smaller energies and shorter ranges in the embryos. Moreover, even for neutrons with the same energy, interactions can take place at different depths in the embryos [57]. Therefore, it would be difficult to directly compare the damages caused by the mono-energetic (3.4-MeV) protons with that of the recoiled protons generated by the neutrons.

Non-induction of a RAR in embryos having received 0.6 and 1 mGy of neutron priming dose was less surprising because such neutron doses induced hormetic effects. In our previous study on the neutron dose response of zebrafish embryos through the induction of apoptotic signals [49], it was found that embryos subjected to single neutron doses of 0.6 and 1 mGy (also delivered using NASBEE) displayed neutron hormetic effects when compared with those not receiving any neutron doses. With the contribution of hormesis, the number of damaged cells could be maintained below the threshold, and thus a RAR would not be enabled. It was well established that RAR induction depends on the magnitude of the priming dose. In particular, it has been proposed that a RAR can only be induced when the dose of priming radiation reaches a certain level [58]. In other words, RAR mechanisms could not be triggered at very low acute priming doses, where cells cannot detect damage efficiently. It has been suggested that for the occurrence of a RAR, the inflicted damage should be large enough to be recognized by cellular sensing systems and transduced to a response that lasts long enough for some effector molecules to mitigate the potentially harmful damages induced by the subsequent challenging dose [59]. In relation to this, Choi *et al.* [53] studied the RAR in zebrafish embryos induced by 3.4-MeV protons, and found that at least 200 protons were needed for the RAR induction.

In the same study of the neutron dose response of zebrafish embryos through the induction of apoptotic signals [49], it was also shown that with 14% contamination of gamma rays, gamma-ray hormesis appears to become fully operative in embryos that have subjected to single neutron doses >50 mGy [49]. Therefore, non-induction of a RAR in embryos that have received 100 mGy of

neutron priming dose was also expected because such a neutron dose induced gamma-induced hormetic effects [49]. However, from the study of Ng *et al.* [49], embryos subjected to single neutron doses of 25 and 50 mGy exhibited larger numbers of apoptotic signals when compared with those not receiving any neutron doses, and did not show signs of neutron-induced hormetic effects or fully operative gamma-ray–induced hormetic effects. It was understood that with 14% contamination of gamma rays, the gamma dose amounted to 3.5 and 7 mGy for neutron doses of 25 and 50 mGy, respectively, so there might still be some effects from the gamma rays, although gamma-ray hormesis did not appear to be fully operative. As long as the neutron-induced damages were reduced to below the "threshold" amount (e.g. to synthesize *de novo* proteins for RAR as discussed in the following paragraph), significant RAR would not be triggered.

Until now, the mechanism involved in RAR was not fully understood. RAR was associated with DNA damage repair. For instance, DNA repair protein DIR1 and a base excision repair endonuclease APE1 were reported to be involved in RAR [60–62]. DNA-PK, TP53 and ATM, which were involved in DNA damage recognition and signaling, were also shown to be involved in RAR [63–65], and the ATM-p53 signal transduction pathway governing the DNA repair system and the cell cycle regulation system were considered the most important mediators of the radioadaptive response [64, 65]. Interestingly, the requirement of *de novo* protein synthesis was implied in RAR induction [66, 67]. The requirement of *de novo* proteins for RAR could explain the non-induction of RAR when the neutron-induced damages were below the "threshold" number, as described above. In relation, Choi *et al.* [68] examined the effects of the CO liberator tricarbonylchloro(glycinato)ruthenium (II) (CORM-3) on the RAR in zebrafish embryos against 2 Gy of X-ray irradiation. Here, the RAR was induced by introducing the zebrafish embryos into medium that had been conditioned by other 5-hpf zebrafish embryos previously irradiated with 30 3.4-MeV protons. Choi *et al.* [68] showed that transfer of irradiated embryos into media with CORM-3 within 3 h after priming exposure disabled RAR, while transfer at 5 h did not. This was explained by *de novo* synthesis of factors, and thus a RAR in <5 h after the priming exposure (this would be disabled if the bystander cells were protected by CO).

The current experimental results demonstrating the suppression of RAR induced by low-dose alpha particles with supplementary low-dose X-ray photons strongly supported the proposal that gamma-ray contamination in the neutron beams led to non-induction of RAR for neutron doses of 25 or 50 mGy. With 14% gamma-ray contamination for the NASBEE facility, the corresponding gamma-ray doses were 3.5 and 7 mGy, respectively. The supplementary X-ray doses of 5 or 10 mGy were chosen to be commensurate with these gamma-ray doses. The suppression of RAR by such doses of high-energy photons suggested that in a neutron irradiation, the gamma rays had important effects, even when gamma-ray hormesis was not fully operative.

In conclusion, neutrons in general could not induce a RAR in zebrafish embryos against X-rays, which was likely due to neutron hormesis and gamma-ray hormesis mitigating the neutron-induced damages. The one subject (out of eight subjects) who developed neutron-induced RAR in the study of Gajendiran *et al.* [26] was likely an outlier. It was well established that the development of RAR varied with individuals [69].

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