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Designing experimental setup and procedures for studying alpha-particle-induced adaptive response in zebrafish embryos *in vivo*

V.W.Y. Choi [a], R.K.K. Lam [a], E.Y.W. Chong [a], S.H. Cheng [b], K.N. Yu [a,*]

[a] Department of Physics and Materials Science, City University of Hong Kong, Tat Chee Avenue, Kowloon Tong, Hong Kong
[b] Department of Biology and Chemistry, City University of Hong Kong, Tat Chee Avenue, Kowloon Tong, Hong Kong

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ABSTRACT

The present work was devoted to designing the experimental setup and the associated procedures for alpha-particle-induced adaptive response in zebrafish embryos *in vivo*. Thin PADC films with a thickness of 16 μm were fabricated and employed as support substrates for holding dechorionated zebrafish embryos for alpha-particle irradiation from the bottom through the films. Embryos were collected within 15 min when the light photoperiod began, which were then incubated and dechorionated at 4 h post fertilization (hpf). They were then irradiated at 5 hpf by alpha particles using a planar ^{241}Am source with an activity of 0.1151 μCi for 24 s (priming dose), and subsequently at 10 hpf using the same source for 240 s (challenging dose). The levels of apoptosis in irradiated zebrafish embryos at 24 hpf were quantified through staining with the vital dye acridine orange, followed by counting the stained cells under a florescent microscope. The results revealed the presence of the adaptive response in zebrafish embryos *in vivo*, and demonstrated the feasibility of the adopted experimental setup and procedures.

1. Introduction

Radioadaptive response occurs when a small preceding priming dose decreases the biological effectiveness of a subsequent large challenging dose [1]. Such an adaptive response in cells was first reported by Olivieri et al. [2], who showed that peripheral blood lymphocytes irradiated with tritiated thymidine had fewer chromosomal aberrations when they were subsequently irradiated with 15 Gy of X-rays. This had stimulated immense interests in studying adaptive response, and particularly whether the adaptive response can provide radioprotection to lower cancer rates in the human population (see e.g., Ref. [3]).

Many previous research works have focused on radioadaptive response *in vitro* by examining different biological endpoints in the past decades (e.g., Refs. [4–6]). More recently, the *in vivo* adaptive response was also demonstrated in mice. Cai et al. [7] demonstrated *in vivo* radioadaptive response in mice in terms of decreases in chromosome aberrations. Wang et al. [8] evaluated the adaptive response in mice and found a range of dose rates capable of inducing adaptive response. Although the phenomenon was found to be present *in vivo*, a great variability in the induction of adaptive response was found in mice [9]. Streffer [9] revealed that the induction of adaptive response in mice was not always consistent; it might depend on the dose range, developmental stage of the embryos and the exposure interval. As such, more research will be needed to give a better understanding on adaptive responses *in vivo*.

Traditionally, only radiations with low linear energy transfer (LET), e.g., X-rays and γ-rays, were used as the priming dose to induce adaptive response both *in vitro* and *in vivo*. There were reports that high LET radiation could not induce adaptive response in cell cultures [10,11]. However, there were no similar reports for the *in vivo* case. It is therefore pertinent to study whether high LET radiation can induce adaptive response *in vivo*. In this study, alpha particles will be used both for the priming dose and challenging dose, and the corresponding adaptive response *in vivo* will be explored. Radiation effects of alpha particles are also of immense interest because alpha-particle emitters are ubiquitous in our environment, e.g., they are emitted from naturally occurring radon and its progeny (see e.g., Ref. [12]).

To explore the alpha-particle-induced adaptive response *in vivo*, the choice of a suitable *in vivo* model presented a challenge. In the present work, zebrafish (*Danio rerio*) embryos were employed. Zebrafish is a popular vertebrate model that possesses many advantages for studying human genetic diseases and for environmental toxicity analyses. The most important advantage is that the human and zebrafish genomes share considerable homology, including conservation of most DNA repair-related genes [13]. Moreover, the zebrafish embryos have a rapid developmental process, so analyses can be performed when they develop into 24 h post fertilization (hpf). Time is saved when comparing with other models,

* Corresponding author. Tel.: +852 27887812; fax: +852 27887830.
E-mail address: peter.yu@cityu.edu.hk (K.N. Yu).

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such as mice, used for similar studies. Furthermore, the body of the zebrafish embryos is optically transparent, so we can use a fluorescence dye staining method to characterize the adaptive response.

The objective of the present work is to design the experimental setup and the associated procedures, and then to show the feasibility of demonstrating the alpha-particle-induced adaptive response in zebrafish embryos *in vivo*. In particular, we would explore: (1) the setup for irradiating zebrafish embryos with alpha particles, in particular the use of polyallyldiglycol carbonate (PADC) polymer films as support substrates; (2) the time points for applying the priming dose and the challenging dose; (3) the magnitudes of the applied priming dose and the challenging dose; and (4) a feasible biological endpoint for quantification of the adaptive response. These will be discussed in more detail in Section 2. Preliminary results will be given in Section 3. These results revealed the presence of the adaptive response in zebrafish embryos *in vivo*, and demonstrated the feasibility of the adopted experimental setup and procedures.

2. Designing experimental setup and procedures

2.1. Setup for alpha-particle irradiation

Yum et al. [14] devised an experimental setup to study effects of alpha particles on zebrafish embryos. In the outset, they highlighted challenges in avoiding excessive or variable absorption of the energy of alpha particles before they could actually reach the cells of the embryos. The first one concerned the absorption of energy by the fluid enclosed by the chorions of embryos, so the embryos should be dechorionated before alpha-particle irradiation. The second one concerned the absorption of energy either by the medium bathing the embryos or by the support substrate. Due to the different size, shape and/or orientation of individual embryos, the depth of the medium above the cells of different embryos or even the same embryo could vary to a large extent, so it would be impossible to deliver uniform doses to cells of embryos through alpha-particle irradiation from the top (i.e., from the side of the medium surface), and the resulting doses would also be difficult to characterize. It would be therefore more desirable to irradiate the embryos from the bottom (i.e., from the side of the support substrate) (see Fig. 1). To enable irradiation through the support substrate, it should be sufficiently thin to allow the alpha particles to reach the cells with a sufficiently large energy, and its thickness should also be controlled with a reasonable accuracy so that the delivered alpha-particle energy and dose could also be controlled.

As such, Yum et al. [14] studied the feasibility to use PADC films as the support substrates for the dechorionated zebrafish embryos for alpha-particle irradiation. PADC films are biocompatible [15] and the thickness can be controlled relatively accurately and conveniently through chemical etching [16]. PADC is a solid-state nuclear track detector (SSNTD), which is usually marketed under the name CR-39. A recent review on SSNTDs has been given by Nikezic and Yu [17]. PADC films were also used in the present work as the support substrates for the dechorionated zebrafish embryos. The thinnest PADC films available in the market had a thickness of about 100 μm, so these had to be chemically etched with a controlled rate to achieve the suitable thickness. By using the SRIM program [18], the range of an alpha particle with energy 5.49 MeV (from an ^{241}Am source in vacuum) in PADC could be determined as 33.17 μm. CR-39 detectors purchased from the Page Mouldings (Pershore) Limited (Worcestershire, England), with original thickness of 100 μm were employed in our experiments, and were etched in 0.25 M sodium hydroxide in ethanol [16]. During the etching process, the films were rinsed by distilled water once every two hours. The detectors were finally etched to 16 μm and monitored by using a micrometer (Mitutoyo, Japan) with an accuracy of ±1 μm.

The thin PADC films with a thickness of 16 μm were then glued by an epoxy (Araldite® Rapid, England) onto the bottom of a custom-made holder made of acrylic resin with 8 × 6 holes drilled on it (see Fig. 2). The holes had a diameter of 2 mm, and the holes were separated at 6 mm, and these thin PADC films acted as support substrates for holding zebrafish embryos for alpha-particle irradiation.

Alpha-particle irradiations of the dechorionated embryos were performed with a planar ^{241}Am source (with an alpha-particle energy of 5.49 MeV under vacuum and an activity of 0.1151 μCi) (Fig. 1). The dechorionated embryos were divided into two groups, namely, the adapted group and the control group. They were transferred into the holes and on top of the thin PADC films so that alpha particles could pass through the detector and reached the embryos.

2.2. Time points for applying priming and challenging doses

The time points for applying the priming and challenging doses were important. First we had to decide on the time interval between the application of the priming and challenging doses. The usual protocol of induction of adaptive response in cell culture is to prime cells with a low-dose of low LET radiation, which is then followed with a challenge to a much higher dose after 4 h or more [11]. The adaptive response did not appear right after the application of the priming dose; it took 4–6 h to become fully active [19]. For example, in the study of Yatagai et al. [6] on the radioadaptive response of human lymphoblastoid TK6 cells, the interval between the application of the priming and challenging doses was chosen to be 6 h. Similarly, Kurihara et al. [20] found the adaptive response in a fish cell line to become maximal at 5 h after the adaptive dose

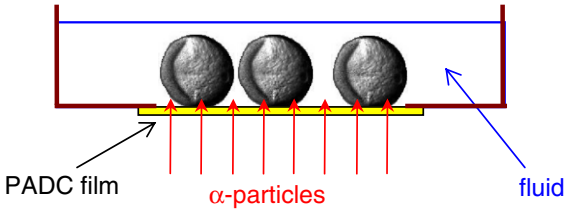

Fig. 1. Irradiation of zebrafish embryos through the PADC-film based holder.

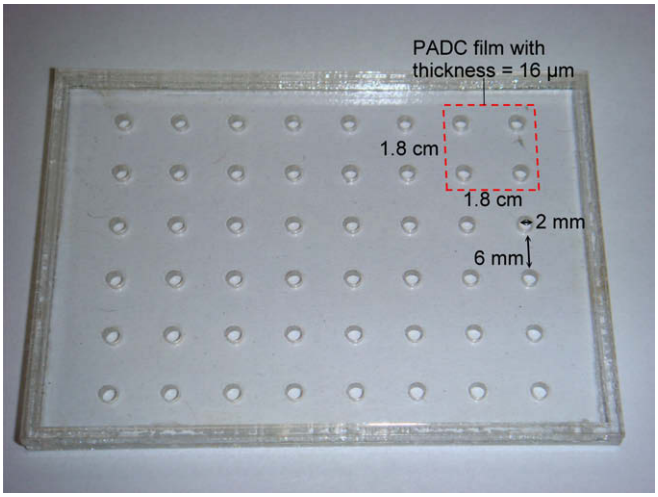

Fig. 2. Custom-made holder for zebrafish embryos with 8 × 6 holes drilled on it. A PADC film with thickness of 16 μm was glued at the bottom of the hole for holding the zebrafish embryos.

and to disappear after 10 h. In the present experiments, a time interval of 5 h was chosen.

Next we had to decide on the time point for applying the priming dose. Although the underlying mechanism for adaptive response in cells is still largely unknown, some research findings suggested that DNA repair might play an important role in inducing adaptive response [5,6,21,22]. Ikushima et al. [5] showed that the rate of rejoining DNA double-strand breaks was higher in adapted cells than in non-adapted cells; Sasaki et al. [22] reported reduction or absence of adaptive-response induction in a repair-deficient cell line. For zebrafish embryos, the DNA repair mechanism starts operating after the cleavage stages (0.7–2.2 hpf) [23]. Hence, the priming exposure in this study was applied to embryos at 5 hpf at the blastula stage (2.2–5.2 hpf), at which stage the DNA repair mechanism should have started. In order to elucidate the importance of the DNA repair mechanism in the alpha-particle-induced adaptive response in zebrafish embryos *in vivo*, we also studied the response when the priming dose was applied to 1 hpf zebrafish embryos, with the challenging dose still applied 5 h later, i.e., at 6 hpf.

Synchronization of the zebrafish embryos was also important in our experiments, which was achieved through a controlled 14:10 h light–dark cycle applied to the fish tanks holding the zebrafish. When the light photoperiod began, a specially designed embryo collector was immersed into the fish tanks and rested on the bottom to collect the embryos. The embryo collector was a rectangular plastic container opened on the top, and with a partition inside to let the embryos but not the adult fish pass through. A layer of plastic fake seaweed on the partition was to attract the adult zebrafish to lay embryos. A schematic diagram of the embryo collector is shown in Fig. 3. The embryos were collected within 15 min to ensure they were at the same developmental stage. The embryos were then incubated at 37 °C in the incubator and allowed to develop into 4 hpf. Healthy developing embryos were selected at 4 hpf under a stereo-microscope; they should be at the sphere stage of the blastula period. Those healthy developing embryos were transferred into a petri dish, which had a layer of agar gel on top of it, for dechorionation.

To sum up, the procedures were as follows. When the light photoperiod began, embryos were collected using a specially designed embryo collector within 15 min to ensure that the embryos were at the same developmental stage. The embryos were then incubated and allowed to develop into 4 hpf. Healthy developing embryos were then dechorionated, which were then irradiated by alpha particles at 5 hpf (priming dose). The control group of embryos was sham irradiated at the same time. Both the adapted group and control group were incubated for a further 5 h, after which they were irradiated again (challenging dose) and then returned back to the incubator for further incubation. In experiments to elucidate the importance of the DNA repair mechanism, the priming and challenging doses were applied at 1 and 6 hpf, respectively, instead.

2.3. Magnitudes of priming and challenging doses

The chosen magnitudes of priming and challenging doses were also important for a successful demonstration of the adaptive response. Ueno et al. [4] demonstrated the adaptive response in human hamster hybrid cells by studying the mutation fraction. A priming dose of 0.04 Gy ^{137}Cs gamma radiation was applied to human hamster hybrid cells, which was followed by a challenging dose of 4 Gy with the same gamma radiation source. The results showed that the mutants' fraction was significantly reduced. Ikushima et al. [5] studied the adaptive response by using Chinese hamster V79 cells. The cells received a priming exposure with 5 cGy of gamma rays and a challenging exposure with 5 or 1.5 Gy gamma rays, which resulted in an increase in the rate of DNA damage repair in the adapted cells. Yatagai et al. [6] investigated the radioadaptive response of human lymphoblastoid TK6 cells. The TK mutation frequency was reduced to about 62% when a challenging dose (2 Gy) of X-rays was applied 6 h after a priming dose (5 cGy) of X-rays. As such, the employed priming dose ranged from 0.01 to 0.03 of the challenging dose. For *in vivo* studies, the survival of mice had been used as an indicator of the presence of radioadaptive response [24,25]. Yonezawa et al. [24] induced adaptive response in mice by a priming dose of 0.5 Gy and a challenging dose of 8.0 Gy with a 2-week interval. Ito et al. [25] applied a mid-lethal challenging dose of X-ray (5.9 Gy) to 8-week-old mice at 1–14 days after a 50 mGy priming dose, and reported an increase in the survival rate of mice compared with the controls. In other words, the employed priming dose ranged from 0.03 to 0.06 of the challenging dose. Enlightened by these employed ratios between the priming and challenging doses, we chose the priming dose to be 0.1 of the challenging dose in the present work.

Yum et al. [14] irradiated dechorionated zebrafish embryos at 4 hpf with alpha particles from a planar ^{241}Am source (with an alpha-particle energy of 5.49 MeV under vacuum and an activity of 0.1151 μCi) for 240 s from the side of the PADC film. Among the 54 irradiated embryos, five abnormal developments were identified while no abnormal case was found in all the 52 control embryos. As such, Yum et al. [14] demonstrated the feasibility to inflict observable radiation effects of alpha particles on dechorionated zebrafish embryos through irradiation for 240 s. We therefore

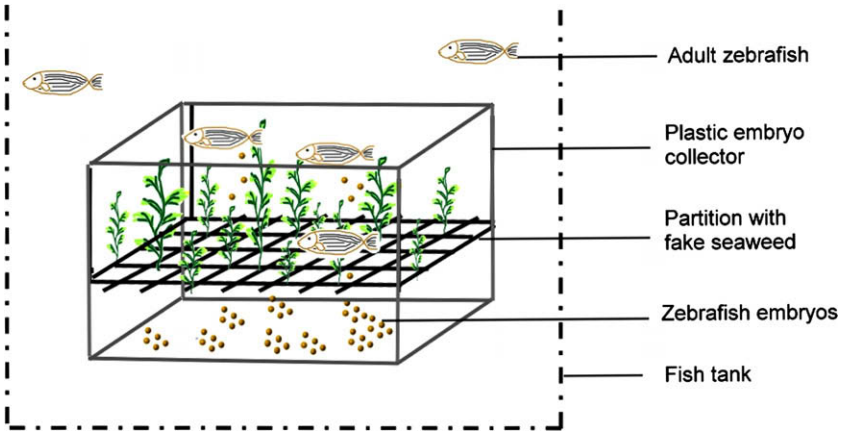

Fig. 3. Schematic diagram of the specially designed embryo collector. Inside the plastic container is a plastic partition which allows the embryos but not the adult fish to pass through. A layer of plastic fake seaweed on the partition is to attract the adult zebrafish to lay embryos.

chose the challenging dose to correspond to the irradiation for 240 s. As we have chosen the priming dose to be 0.1 of the challenging dose, the priming dose corresponded to an irradiation of 24 s.

Yum et al. [14] also revealed the alpha-particle hit positions recorded on PADC support substrates to quantify the number and energy of alpha particles actually incident on the embryo cells, which enabled the calculation of the dose absorbed by the embryo cells. However, the emphasis of the present work was placed on the design of the experimental setup and procedures for studying alpha-particle-induced adaptive response in zebrafish embryos *in vivo*, so we did not spend extra effort in determining the actual dose absorbed by the embryo cells. However, it is noted here that in future experiments where more accurate dose values are needed, determination of the dose through revelation of the alpha-particle hit positions recorded on PADC support substrates will be required. Further refinement can be made through the use of micro-collimators to restrict alpha particles incident on the PADC substrate to those close to normal incidence [26].

2.4. Biological endpoint for quantification of adaptive response

After the application of the challenging dose at 10 hpf, the embryos were returned back to the incubator for further incubation until they developed into 24 hpf, which was our chosen endpoint for more detailed analyses of apoptosis, a highly regulated biological process during embryonic development. Before 24 hpf, the untreated zebrafish embryos undergo high apoptotic activities as part of the organogenesis processes [27]. The 24 hpf endpoint was also used by Bladen et al. [28] who commented that increasing pigmentation after 24 hpf might obscure the signals from the apoptotic cells. A quantitative method for comparative analysis of changes in the frequency of apoptosis in the embryos exposed to different treatments (with or without the priming dose in the present case) would then be required (see Ref. [29]).

In the present study, we have chosen apoptosis as our biological endpoint because it is the simplest and the most rapid way to quantify the effect of alpha particles on the whole zebrafish embryo. Other biological endpoints have also been used by different research groups. For example, survival rates and morphology defects of embryos have been utilized [30–32]. These endpoints are, however, more feasible when the embryos are exposed to large radiation doses (up to 40 Gy when using gamma or X-ray irradiation). Much lower radiation doses have been involved in the present study of radioadaptive response. Another biological endpoint was the hatching time of embryos [23]. Miyachi et al. [23] found that exposure to low-doses of X-ray could decrease hatching time of the embryos and proposed positive effects induced by low-dose X-ray irradiation. However, this endpoint is not suitable for the present study because of the dechorionation required to study the effects of short-range alpha particles. Renal function and neurotoxicity have also been employed [32] but these are only suitable for examining the radiation effects to specific organs. To investigate the effect of radiation on the whole embryos, quantification of the apoptotic signals is widely used [28,30,33,34].

As commented by Tucker and Lardelli [29], the level of apoptosis in zebrafish embryos is commonly quantified through staining with the vital dye acridine orange, followed by counting the stained cells under a microscope. This was one of the two staining methods explored in the present work to quantify the level of apoptosis in zebrafish at 24 hpf. In this method, the embryos were stained for 60 min and washed twice in the culture medium thoroughly. They were then anaesthetized by 0.016 M tricaine (Sigma, St. Louis, MO, USA). For each embryo, two images focusing on different sections of the embryo were captured under a fluorescent microscope, which were then combined into a single image for quantification of apoptotic signals with the help of the software MetaMorph Version 7.0r0 (1992–2006 Molecular Devices).

The other staining method explored in the present work to quantify the level of apoptosis was the TUNEL assay. In this assay, the DNA double strand breaks in the cells were quantified through terminal dUTP transferase-mediated nick end-labeling (TUNEL). Here, the fluorescein labels incorporated in nucleotide polymers were detected and quantified by fluorescence microscopy. The 26 hpf embryos were fixed in 4% paraformaldehyde in PBS with 0.1% Tween 20 at 4 °C for 24 h. The fixed embryos were then dehydrated, and were then rehydrated and treated with 14–22 mg/ml protease kinase for 30 min. After the protease kinase treatment, the embryos were fixed in 4% paraformaldehyde in PBS with 0.1% Tween 20 again. The fixed embryos were immersed in the permeabilisation buffer for 30 min on ice. The TUNEL staining was achieved by using an *in situ* apoptosis detection kit (MK500, Takara Bio. Inc.). The apoptotic cells were labeled by staining the embryos in the mixture of TdT enzyme and labeling safe buffer in the ratio of 1–9. The embryos were then incubated in a 37 °C humidified chamber for 120 min. The embryos were finally washed thoroughly by PBT (phosphate buffered saline in 0.1% Tween 20). The apoptotic signals were captured by a fluorescent microscope.

3. Results and discussion

Here, we first gave our results for applying priming and challenging doses to embryos at 5 and 10 hpf, respectively, and where the levels of apoptosis in zebrafish embryos at 24 hpf were quantified through staining with the vital dye acridine orange. The numbers of apoptotic signals in zebrafish embryos were counted from the combined images. Fig. 4 shows representative apoptotic signals after acridine orange staining of a 25 hpf zebrafish embryos which had received (A) both priming and challenging doses, and (B) only the challenging dose, with each bright fluorescent spot corresponding to an apoptotic signal. Consistent results were obtained in four sets of experiments, as shown in Table 1. Embryos directly irradiated with a challenging dose of alpha particles without a prior exposure to a priming dose showed significantly more apoptotic signals when compared to embryos which received both priming and challenging doses. In these four sets of experiments, two of them showed an adaptive response with a p value very close to 0.05 (i.e., 0.0574 and 0.0509) and the other showed adaptive response with statistical significance (i.e., $p = 0.0037$ and $p = 0.0206$). These results strongly supported the existence of adaptive response in zebrafish embryos *in vivo* induced by alpha particles through the application of a priming exposure and a subsequent challenging exposure. Small variations in the statistical significance were in fact expected. As also commented by Tucker and Lardelli [29], variability in acridine orange staining results could be observed between embryos even for identical treatment, especially when the apoptosis was diffuse throughout the embryo rather than localized.

We also studied the situation in which the priming dose was applied to 1 hpf zebrafish embryos and the challenging dose to 6 hpf zebrafish embryos (i.e., 5 h later). The results showed no adaptive response in this situation. This further confirmed the requirement of DNA repair mechanism on inducing adaptive response. Furthermore, we had also studied the results obtained through staining cells by the TUNEL assay (instead of acridine orange staining) to quantify the level of apoptosis. Fig. 5 shows representative apoptotic signals after staining by the TUNEL assay a 25 hpf zebrafish embryo which had received only the challenging dose, with each bright fluorescent spot corresponding to an apoptotic signal. It was concluded that the quality of the images of zebrafish embryos stained with acridine orange and TUNEL assay,

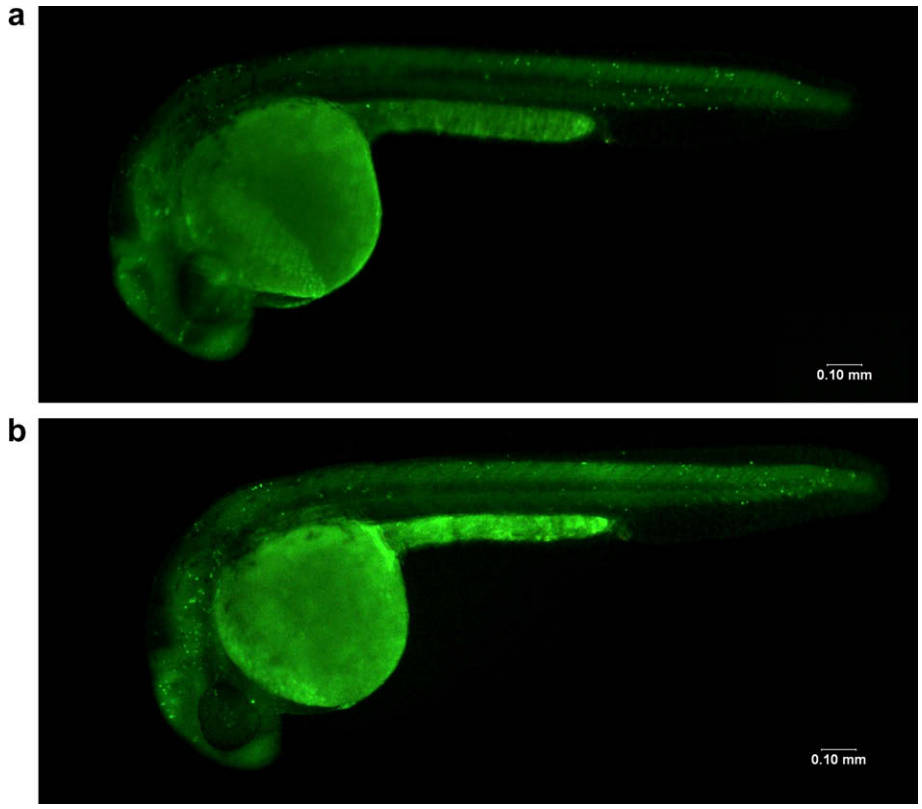

Fig. 4. Apoptotic signals of 25 hpf zebrafish embryos revealed by acridine orange staining: (A) the zebrafish embryo has received a priming dose at 5 hpf and a challenging dose at 10 hpf; (B) the zebrafish embryo has received the challenging dose at 10 hpf only.

Table 1
Number of apoptotic cells (N) obtained for IR (embryos irradiated with both priming and challenging exposures) and control (embryos with only challenging exposure) in four sets of experiments. n: Number of embryos involved in the analyses ($n < 14$ in some cases due to death of embryos); p: p value obtained using t-tests by comparing with the controls (i.e., IR embryos and control embryos).

		IR	Control	p
1	n	14	14	
	N	78	106	0.0574
2	n	14	14	
	N	74	117	0.0037
3	n	13	13	
	N	71	108	0.0206
4	n	12	14	
	N	69	95	0.0509

particularly in terms of the clarity of the fluorescent spots, were comparable. Bearing this in mind, and considering that experimental procedures involved in the TUNEL assay were much more tedious, we chose to use the more rapid and convenient acridine orange staining method to stain the apoptotic cells. Tucker and Lardelli [29] also commented that apoptotic signals in zebrafish embryos were commonly quantified using acridine orange staining. It is noted that the acridine orange staining method was also employed by Yasuda et al. [35] to stain the apoptotic cells in the medaka fish.

As described in the introduction, the objective of the present work was to design the experimental setup and the associated procedures, and then to show the feasibility of demonstrating the alpha-particle-induced adaptive response in zebrafish embryos *in vivo*, which had all been achieved. The current experimental

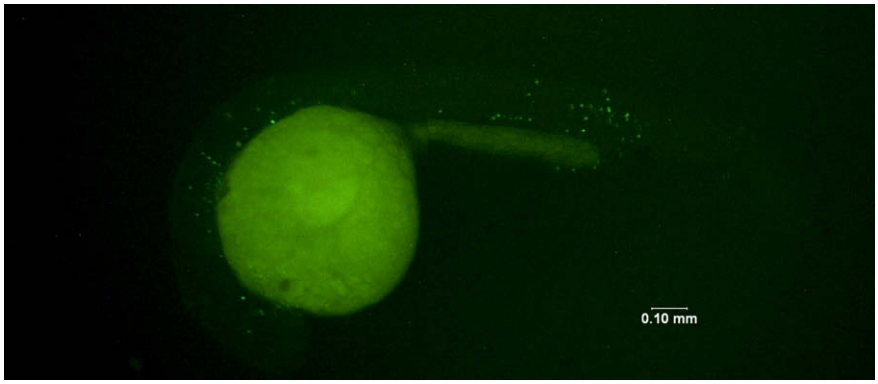

Fig. 5. Apoptotic signals of a 25 hpf zebrafish embryo revealed through TUNEL assay, with the zebrafish embryo having received the challenging dose at 10 hpf only.

setup and the associated procedures could serve as a platform to further study alpha-particle-induced adaptive response in zebrafish embryos *in vivo*, or a starting point to further investigate the potential effects on the adaptive response of the variations in the experimental setup and the procedures themselves, e.g., the change in the time points for applying the priming dose and the challenging dose including the change in the time interval between prime exposure and challenge exposure, the magnitudes of the applied priming dose and the challenging dose and maybe also their dose rates, and the use of a different radiation (e.g., X-ray) for the priming dose (i.e., with alpha-particle irradiation as the challenging dose) or for the challenging dose (i.e., with alpha-particle irradiation as the priming dose).

4. Conclusions

The results of the present work revealed the presence of the adaptive response in zebrafish embryos *in vivo*, and demonstrated the feasibility of the adopted experimental setup and procedures, which are summarized as follows:

(1) setup for irradiating the zebrafish embryos: thin PADC films with a thickness of 16 μm were fabricated and employed as support substrates for holding dechorionated zebrafish embryos for alpha-particle irradiation from the bottom through the films;
(2) time points for applying the priming dose and the challenging dose: embryos were collected within 15 min when the light photoperiod began, which were then incubated, dechorionated at 4 hpf, irradiated by alpha particles at 5 hpf (priming dose) and subsequently at 10 hpf (challenging dose);
(3) magnitudes of the applied priming dose and the challenging dose: the priming and challenging doses were delivered by irradiation of the dechorionated zebrafish embryos for 24 and 240 s, respectively, using a planar ^{241}Am source with an activity of 0.1151 μCi;
(4) feasible biological endpoint for quantification of the adaptive response: the levels of apoptosis in irradiated zebrafish embryos at 24 hpf were quantified through staining with the vital dye acridine orange, followed by counting the stained cells under a florescent microscope.

The current experimental setup and the associated procedures would serve as a platform to further study alpha-particle-induced adaptive response in zebrafish embryos *in vivo*, or a starting point to further investigate the potential effects on the adaptive response of the variations in the experimental setup and the procedures themselves.

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