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Adaptive Response in Zebrafish Embryos Induced Using Microbeam Protons as Priming Dose and X-ray Photons as Challenging Dose

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Microbeam/Protons/Adaptive response/*Danio rerio*/Zebrafish embryos.

In the studies reported here, a high-linear-energy-transfer (high-LET)-radiation dose was used to induce adaptive response in zebrafish embryos *in vivo*. Microbeam protons were used to provide the priming dose and X-ray photons were employed to provide the challenging dose. The microbeam irradiation system (Single-Particle Irradiation System to Cell, acronym as SPICE) at the National Institute of Radiological Sciences (NIRS), Japan, was employed to control and accurately quantify the number of protons at very low doses, viz., about 100 μGy. The embryos were dechorionated at 4 h post fertilization (hpf) and irradiated at 5 hpf by microbeam protons. For each embryo, ten irradiation points were arbitrarily chosen without overlapping with one another. To each irradiation point, 5, 10 or 20 protons each with an energy of 3.4 MeV were delivered. The embryos were returned back to the incubator until 10 hpf to further receive the challenging exposure, which was achieved using 2 Gy of X-ray irradiation, and then again returned to the incubator until 24 hpf for analyses. The levels of apoptosis in zebrafish embryos at 25 hpf were quantified through terminal dUTP transferase-mediated nick end-labeling (TUNEL) assay, with the apoptotic signals captured by a confocal microscope. The results revealed that 5 to 20 protons delivered at 10 points each on the embryos, or equivalently 110 to 430 μGy, could induce radioadaptive response in the zebrafish embryos *in vivo*.

INTRODUCTION

For radiation protection purposes, prediction of risk from an ionizing-radiation exposure is enabled by adopting the linear no-threshold (LNT) hypothesis which states that the risk is linearly proportional to the dose normalized by the radiation weighting factor, and which assumes no threshold dose value below which no radiation risk is expected. Moreover, as data in the low-dose regime are relatively scarce, the detrimental effect from exposure to low-dose radiation is commonly extrapolated from data obtained in the high-dose regime by using the LNT model (e.g., ref. 1)).

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Although this model is commonly adopted for radiation protection purposes, there is a considerable amount of evidence showing that organisms may exhibit different responses to a low dose exposure from that to a high dose exposure.[2)] For example, Mothersill *et al.* reported that a small preceding priming dose actually decreased the biological effectiveness of a subsequent large challenging dose to an organism, which was described as a radioadaptive response.[3)] Such radioadaptive responses pose problems for the LNT model which implies that an addition of a dose will lead to an addition in the risk.

The radioadaptive response was first discovered by Olivieri *et al.*[4)] who showed that peripheral blood lymphocytes irradiated with tritiated thymidine had fewer chromosomal aberrations when they were subsequently irradiated with 15 Gy of X-ray. A lot of research works were since then carried out to study radioadaptive response *in vitro* using various biological end points such as survival of cells,[5)] neoplastic transformation,[6)] chromosomal aberrations and micronucleus formation,[7,8)] as well as mutation frequency.[9)]

Although radioadaptive response has been shown in *in vitro* studies, these results do not automatically imply the same response in *in vivo* situations. Therefore, researches on

in vivo radioadaptive response are necessary. Mice as an animal experimental model were used for most *in vivo* studies to examine the induction of radioadaptive response, e.g., using their survival rate as a biological endpoint.[1,10,11] Our group has explored the feasibility of using zebrafish embryos (*Danio rerio*) as the *in vivo* model to study the effect of low dose radiation.[12–15] The main advantage of using zebrafish embryos as the *in vivo* model is that the human and zebrafish genomes share considerable homology, including conservation of most DNA repair-related genes. Recently, we designed the experimental setup and the associated procedures for alpha-particle-induced adaptive response in zebrafish embryos *in vivo*.[16] A planar ^{241}Am source was used for alpha-particle irradiation. The conditions of induction of radioadaptive response, in particular the time interval between the priming and the challenging doses as well as the time point for irradiation of zebrafish embryos, were explored. The results suggested that irradiation of the zebrafish embryos at 5 hour post fertilization (hpf) and 10 hpf (embryos at 5 hpf and 10 hpf were at the developmental stage of 5 hours and 10 hours, respectively, after fertilization) with 5 h interval time was feasible in inducing radioadaptive responses.[16] The experimental setup and the associated procedures could serve as a platform to further study high-linear-energy-transfer (high-LET)-radiation-induced adaptive response in zebrafish embryos *in vivo*.

One interesting and critical parameter which deserves more detailed studies is the magnitude of the applied priming dose needed to induce the adaptive response. Iyer and Lehnert[17] found that supernatants conditioning normal human lung fibroblasts (HFL-1) irradiated with 1 cGy of particles could increase the radioresistance of bystander unirradiated HFL-1 cells. On the other hand, Broome *et al.*[18] reported an apparent limit or threshold dose between 0.1 and 1 mGy for low-LET radiations such as γ-rays from ^{60}Co for inducing adaption. It appears that the priming dose of high-LET-radiation needed to induce adaptive response would be very low, probably a few hundreds of μGy. To be able to control and accurately quantify such low doses of the high-LET-radiation, it seems necessary to switch from the radioactive source to microbeam facilities.[19]

In the present study, a microbeam irradiation system (Single-Particle Irradiation System to Cell, acronym as SPICE)[20] at the National Institute of Radiological Sciences (NIRS), Japan, was employed for the priming irradiation. The use of microbeam irradiation techniques provides a unique opportunity to control precisely the number of particles traversing individual cells and localization of dose within the cells. Therefore, there has been growing interest in the use of micobeams in radiation biology, and many groups in the world are showing advancement in their system developments and radiation biological studies (e.g., refs. 19,21,22)). The SPICE was originally designed for radiobiological studies, such as *in vitro* experimental strategies for investigating the cellular basis of hazards associated with occupational and environmental exposure to low dose radiation. This microbeam system is capable of delivering a desired number of 3.4 MeV protons within a beam diameter of 2 μm to individual cell nucleus. In this work, we made use of the SPICE to irradiate the zebrafish embryos with a control of the irradiation spots. Protons with an initial energy of 3.4 MeV would first travel through a Si_3N_4 exit window with a thickness of 100 nm, and then through a 2.5 μm Mylar film with less than 50 μm air gap between the exit window and Mylar film before the protons finally reached the target. The energy of the protons would drop a little bit down to 3.37 MeV when they arrived at the target. The protons with energy of 3.37 MeV have an LET of about 11.0 keV/μm, so they can be classified as a high-LET radiation. The main objectives of the present study were two fold, namely (1) to design the experimental setup for the *in vivo* irradiation of zebrafish embryos by using SPICE microbeam; and (2) to study the small number of protons or equivalently the high-LET-radiation dose required to induce a radioadaptive response.

MATERIALS AND METHODS

Zebrafish embryos

Adult zebrafish with ages between 7 and 10 months were kindly provided as gifts from RIKEN Brain Science Institute, JAPAN (courtesy Prof. Hitoshi Okamoto). The fish were kept in an indoor environment with an ambient temperature of 28°C. A 14/10 hour light-dark cycle was adopted in order to maintain a good production of embryos. Once the 14-h photoperiod began, a plastic container housing a plastic filter mounted with artificial seaweed (see ref. 16)) was placed at the bottom of each tank to collect the embryos for a short period lasting only 15 to 30 min to ensure more-or-less synchronization of the embryos. The collected embryos were then incubated in a 28°C incubator for development and dechorionated at 4 h post fertilization (hpf).[16]

Preparation of embryo dish for irradiation

A specially designed dish consisting of a Si_3N_4 plate (7.5 × 7.5 mm frame with a thickness of 200 μm thick, and with a 3 mm × 3 mm hole area at the centre, Silson Ltd., Northwood, England) and a steel ring with 33 mm diameter was fabricated for embryo irradiation. A Mylar film with thickness of 2.5 μm (Chemplex Industries, Inc., Florida) was stretched across the steel rings and formed a substrate for the embryos. In order to restrict the movement of the embryos, the Si_3N_4 plate was attached to the centre of Mylar film by Vaseline (Wako Pure Chemical Industries Ltd., Osaka, Japan). Figure 1 shows the specially designed dish for embryo irradiation, with a Mylar film as the substrate to which a rectangular frame is attached.

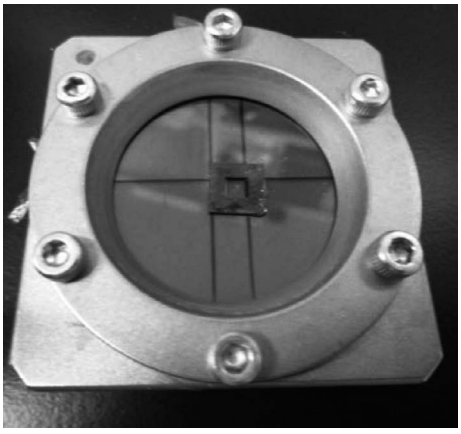

Fig. 1. Specially designed dish for embryo irradiation, with a Mylar film as the substrate for the embryos and a rectangular frame attached to the centre of the Mylar film by Vaseline to restrict the movement of the embryos.

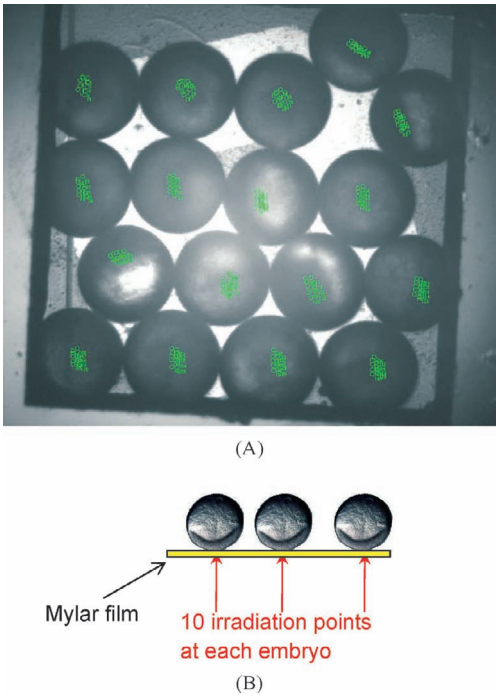

Fig. 2. (A) Photograph showing the top view of 17 zebrafish embryos at 5 hpf placed in the rectangular frame on the specially designed embryo irradiation dish (the latter also shown in Fig. 1). The 10 irradiation points on each embryo are shown in green color. (B) A side-view schematic diagram showing the irradiation points.

Irradiation conditions

The priming exposure was delivered by protons from the SPICE microbeam facility when the zebrafish embryos were developed into 5 hpf. About 15 to 17 dechorionated embryos, which were placed in the embryo irradiation dish with all the embryo cells oriented towards the substrate. The cells of the embryos were the targets. The cells of the embryos were first orientated towards the Mylar film. Figure 2A shows the top view of the embryos. In order to reduce the absorption of energy by the medium bathing the embryos, the 10 irradiation points were chosen to be around the centre (indicated by the green circles in Fig. 2A) without overlapping with one another. A side-view schematic diagram showing the irradiation points is shown in Fig. 2B. Five protons each with energy of 3.37 MeV were delivered to each irradiation point. After the priming exposure, the embryos were returned back to the incubator until 10 hpf to further receive the challenging exposure, which was achieved using 2 Gy of X-ray irradiation. The embryos were then again returned to the incubator until 24 hpf for analyses. The control experiment was performed by sham irradiating the 5 hpf embryos followed by a 2 Gy X-ray irradiation when they had developed into 10 hpf. The experiment was then repeated with priming exposures using 10 and 20 protons, instead of 5 protons. For X-ray irradiation, an X-ray generator (TITAN, Shimazu Corporation, Kyoto, Japan) set at 200 kVp and 20 mA was employed, and irradiation was made through a copper and aluminum filter with a thickness of 0.5 mm producing an effective energy of approximately 83 keV. The dishes holding the embryos were set at 580 mm away from the X-ray target, which received X-ray doses at a dose rate of about 1 Gy/min.

The number of delivered protons can be used to determine the absorbed dose for the zebrafish embryo. The average mass of the zebrafish embryos at the irradiation with the priming exposure, i.e., at 5 hpf, should be measured. A total of 105 zebrafish embryos at 5 hpf were collected. The embryos were first soaped in 3% glutaraldehyde for 18 min, and this mild fixation toughened the surface of the embryos by cross-linking the proteins. This step could prevent the dechorionated embryos from rupturing when the surrounding water was removed. Hagedorn *et al.*[23] suggested that glutaraldehyde satisfactorily preserved lipids and proteins, and reported that a minimum fixation time of 18 min was needed to produce a gentle mechanical contact with the surface of the embryos without hardening the entire tissue. As such, a fixation time of 18 min was chosen for the 5 hpf embryos in the present study. After mild fixation, the embryos were transferred onto a glass coverslip and the surrounding water was removed using a dropper. The 105 embryos were then weighted together by an electronic balance (Sartorius, CP225D), and the average mass of a 5-hpf embryo was measured as 255 μg. This was slightly larger than the average mass of a 1.5-hpf embryo measured as 222 μg.[14] Using the average mass of 255 μg for a 5-hpf embryo, the absorbed doses corresponding to 5, 10 and 20 protons delivered to ten points each on the embryos were 106, 212 and 424 μGy, respectively. The absorbed dose was calculated by the relationship $D = E/M$, where D is the absorbed dose for an embryo, E is the energy of the protons and M is the average

mass of an embryo.

TUNEL assay

Apoptosis was the biological endpoint chosen for the present study. To detect the apoptotic cells in the embryos, terminal dUTP transferase-mediated nick end-labeling (TUNEL) assay was employed. The 25 hpf embryos were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) with 0.1% Tween 20 at room temperature for 5 h. The fixed embryos were then dehydrated, and were then rehydrated and treated with 60 µg/ml protease kinase (Wako Pure Chemical Industries Ltd., Osaka, Japan) for 10 min. After the protease kinase treatment, the embryos were fixed in 4% paraformaldehyde in PBS with 0.1% Tween 20 again. The TUNEL staining was achieved by using an *in situ* apoptosis detection kit (MK500, Takara Bio. Inc., Japan). The fixed embryos were immersed in the permeabilization buffer for 30 min on ice. The apoptotic cells were labeled by staining the embryos in the mixture of Terminal Deoxynucleotidyl Transferase (TdT) enzyme and labeling safe buffer containing Fluorescein labled-2'-Deoxyuridine, 5'-Triphosphate, FITC-dUTP in the ratio of 1 to 9. The embryos were then incubated in a 37°C humidified chamber for 120 min. The embryos were finally washed thoroughly by PBS in 0.1% Tween 20. The apoptotic signals were captured by a confocal laser microscope (FV-1000, Olympus Corporation, Tokyo) with 4× objective lens (NA:0.16, UPLSAPO 4X, Olympus Corporation, Tokyo). For each embryo, a total of 15 to 20 sliced images (2.12 × 2.12 mm, 2.06 µm/pixel) were captured with 25 µm intervals from top to bottom of the embryo.

Statistical analysis

The number of apoptotic signals on each 25 hpf embryo after TUNEL assay was counted using the ImageJ software freely obtainable from the website http://rsb.info.nih.gov/ij/. The image obtained from the confocal microscope was first converted into a binary image. The number of apoptotic signals was then obtained using the "Analyze particle" function in ImageJ. Possible outliners were identified and removed before t-test was used to test the statistical significance for differences between samples. A p value less than 0.05 was considered to correspond to a statistically significant difference.

RESULTS

In the present study, we employed the TUNEL assay to quantify the apoptotic cells present in the 25 hpf zebrafish embryos. The feasibility of apoptosis detection by TUNEL assay on zebrafish embryos was studied using 5 hpf embryos irradiated by X-ray photons (with doses up to 3 Gy). The embryos were dechorionated after X-ray irradiation at about 24 hpf. The results are shown in Fig. 3. It is noted that the number of apoptotic signals increased with the X-ray dose

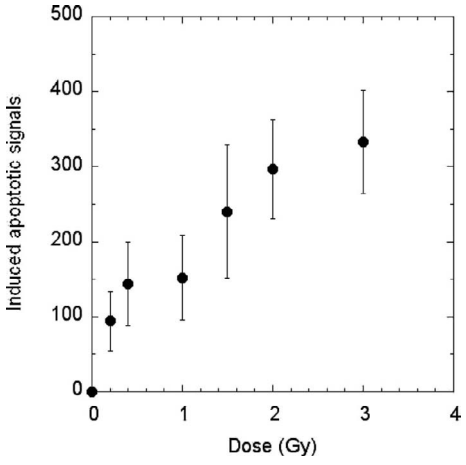

Fig. 3. Relationship between the number of apoptotic signals on 5 hpf zebrafish embryos obtained by TUNEL assay with the X-ray dose. The error bars represent the standard deviations obtained from 20 embryos.

up to 3 Gy, which demonstrated the successful use of TUNEL assay on zebrafish embryos to detect apoptotic signals. Figure 4A shows a confocal-microscopic fluorescent image of a 25 hpf embryo which has received both the priming and challenging exposures. The fluorescent signals shown in Fig. 4A were superimposed onto the corresponding bright field image of the same embryo, which are shown in Fig. 4B. A similarly superimposed image for a non-irradiated 25 hpf embryo is shown in Fig. 4C. Each green spot corresponds to an apoptotic signal. Apoptotic signals were observed throughout the body both in irradiated and non-irradiated embryos. The number of green spots was counted with the help of the ImageJ software. Table 1 shows the statistical results. Seven sets of experiments were conducted by delivering 5, 10 and 20 protons at the chosen spots on the zebrafish embryos as the priming exposure. Sample size in Table 1 represents the number of embryos employed in each group of experiments. It is the sum of (1) the number of embryos which had received both the priming and challenging exposures and (2) the number of embryos which had received only the challenging exposure (i.e., sample size = number of embryos in experimental group + number of embryos in control group). All the embryos in these seven sets of experiments received the same dose for the challenging exposure (2 Gy) delivered by X-ray photons. The average number of apoptotic signals (N) from the experimental group (which had received both priming and challenging exposures) was compared with the average number of apoptotic signals (n) from the control group (which received only the challenging exposure). 'N' in Table 1 refers to the average number of apoptotic signals obtained from the 25 hpf embryos which had received both the priming dose at 5 hpf and challenging dose at 10 hpf. It is the average number of apoptotic signals per embryo obtained from the whole

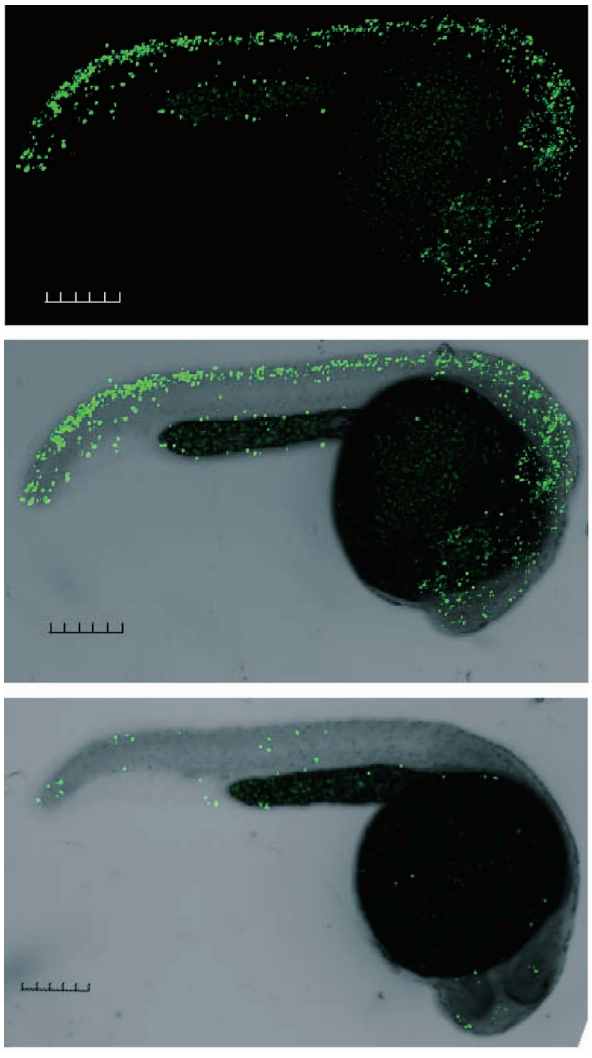

Fig. 4. Apoptotic signals on 25 hpf embryos obtained by TUNEL assay and recorded using a confocal microscope: (A) fluorescent signals of a zebrafish embryo which has received a priming dose at 5 hpf and a challenging dose at 10 hpf; (B) superposition of fluorescent signals on the bright field image of the same embryo shown in (A); (C) superposition of fluorescent signals on the bright field image of a non-irradiated embryo. Scale bars = 200 μm.

Table 1. The average number of apoptotic signals (N) from the experimental group (which had received both priming and challenging exposures) and the average number of apoptotic signals (n) from the control group (which received only the challenging exposure) on 25 hpf zebrafish embryos exposed to various priming doses followed by a 2 Gy X-ray with a 5 h interval time. Sample size = number of embryos in experimental group + number of embryos in control group. $D = (n - N)$, and magnitude of adaptation is defined as (D/n). The p values are obtained using t-tests.

Group	Priming exposure	Sample size	N	n	D	Magnitude of adaptation (%)	p value
A	5 protons	58	397	460	63	13.7	0.050*
B		62	245	310	65	21.0	0.000903*
C	10 protons	52	373	410	37	9.02	0.0305*
D		35	381	435	54	12.4	0.0709
E		19	464	504	39	7.74	0.177
F	20 protons	40	284	341	57	16.7	0.0350*
G		47	260	310	51	16.5	0.00807*

*cases with $p \leq 0.05$, which are considered statistically significant

experimental group of experiments. On the other hand, 'n' refers to the average number of apoptotic signals obtained from the 25 hpf embryos which had only received the challenging dose at 10 hpf. It is the average number of apoptotic signals per embryo obtained from the whole control group of experiments. The differences in the number of apoptotic signals were obtained ($D = n - N$). A positive value of D shows that the number of apoptotic signals on the embryo has decreased after receiving both the priming and challenging doses, when compared to receiving the challenging dose only. The significance in the difference has been revealed using the statistical t-test. From Table 1, all the seven sets of experiments show a positive D value. Among those, five of them had a $p \leq 0.05$. These strongly support the existence of radioadaptive response induced by 5 to 20 protons, or equivalently 106 to 424 μGy, as the priming exposure.

The present work used zebrafish embryos as an *in vivo* animal model to study the radioadaptive response induced using protons from the SPICE microbeam facility at NIRS. With the microbeam as the radiation source, we could control to deliver a small number of protons, and thus a small radiation dose, to the targets. As expected, the stability of the beam intensity played an important role in delivering the desired number of protons to the targets. In fact, a number of factors might affect the stability of the beam intensity, e.g., stability of the accelerator, timing of the shutter opening, and speed of the beam shutter, etc. In the general use of the SPICE, the scintillation counter, which is installed above the cell dish, sends a signal to the beam shutter to close it as soon as the preset number of protons is reached. However, in the present work, the 3.4 MeV protons could not reach the detector because of the size of the embryos which had a diameter of approximately 800 μm. To overcome this, the desired number of protons was achieved through control of the shutter opening time, in the order of a few ms in general. The number of protons was measured between irradiations of the embryos, i.e., without the cell dish set on the irradiation stage. Separations between two consecutive measurements of the number of protons were never longer than 15 min. In fact, it was not straightforward to have exactly the same number of protons as that expected from the beam exit, which inevitably constituted a source of error. These errors

Table 2. Error measurements of the numbers of protons received by the embryos.

Group	Expected number of protons	True average number of proton per shot	S.E.
A	5	5.6	2.20
B	5	5.4	1.58
C	10	11.1	2.80
D	10	11.1	2.80
E	10	11.3	4.00
F	20	20.2	5.03
G	20	20.2	5.03

were determined and are shown in Table 2. The actual numbers of protons received by the embryos were ± 25% to 39% deviated from the expected values. This error was acceptable as the average number of protons remained close to our expected values.

DISCUSSION

For all the data obtained in Table 1, the corresponding challenging dose was 2 Gy from X-ray photons irradiated at 10 hpf. The average number of apoptotic signals per embryo (n) obtained from the whole control group ranged from about 300 to 500 as shown in Table 1. On the other hand, the average number of apoptotic signals for 25 hpf embryos irradiated by 2 Gy X-ray photons at 5 hpf ranged from about 250 to 350 as shown in Fig. 3. Although both the data shown in Fig. 3 and Table 1 were the apoptotic signals obtained from the embryos irradiated with 2 Gy of X-ray photons, the embryos were irradiated at different developmental stages. Since embryos at different developmental stages might have different radiosensitivity, the number of apoptotic signals in Fig. 3 and Table 1 cannot be directly compared. Furthermore, the time points at which the embryos were dechorionated were different for the experiments corresponding to the data shown in Fig. 3 and Table 1. For Table 1, the embryos were dechorionated before 5 hpf. On the other hand, for Fig. 3, the embryos were dechorionated at 24 hpf. The time point at which the embryos were dechorionated is also likely to affect the number of apoptotic signals revealed at 25 hpf. The main objective of Fig. 3 was to demonstrate the feasibility of TUNEL assay in the detection of apoptosis.

In fact, the delivered radiation dose in the present experiment might not necessarily lead to malformations. Otherwise, it would be much more straightforward to determine the radiation effects based on the differences in the observed numbers of malformations. Nonetheless, we did actually monitor the malformations of the embryos. Only 2.2% of the embryos from the entire experimental group and 2.5% of the embryos from the entire control group showed morphological changes, which were mainly developed on the curvature of the tail. As such the rate of morphological changes in the experimental group was about the same as that in control group. Therefore, apoptosis was chosen as the biological endpoint for the present study.

In this study, radioadaptive response was found to be induced by 5, 10 and 20 protons as the priming exposure followed by a 2 Gy X-ray challenging exposure. For experiments involving 5 and 20 protons, the adapted embryos (groups A, B, F and G) showed decreased number of apoptotic signals with statistical significance ($p < 0.05$), ranging from 13.7 to 21%. Three sets of experiments were performed using 10 protons as the priming dose (groups C, D and E). Here, only one group (group C) showed a statistically significant difference between the adapted samples and the controls. Nevertheless, although groups D and E did not give significant differences, the adapted embryos still showed a decreased number of apoptotic signals when compared to the control embryos. The relatively smaller sample size might be the reason behind the insufficient significances.

For the induction of the radioadaptive response, several parameters are critical. The time interval between the priming and challenging exposures as well as the time point for applying radiation in the experiment employed here were the same as those in our previous feasibility study which used alpha particles both for the priming and challenging exposures.[16] Briefly speaking, we chose to apply the priming and the challenging exposures at 5 hpf and 10 hpf, respectively, i.e., with a 5 h interval time. We had to apply the priming dose to the embryos after cleavage stages (0.7 to 2.2 hpf), which was important because the DNA repair system started to operate after the cleavage stage and some researchers[9,24,25] suggested that the DNA repair system played an important role in the induction of radioadaptive response. Furthermore, the *in vitro* experiment of Kurihara *et al.*[7] involving a fish cell line showed the adaptive response to be maximum at 5 h after the priming exposure. As such, we also chose 5 h as the interval time between our priming and challenging doses.

It has been common to employ only one type of radiation for both the priming exposure and the challenging exposures in the study of radioadpative responses. In particular, low LET radiation has been more commonly used. Nevertheless, high-LET radiations have also been shown to induce adaptive response in mammalian cells.[17,26,27] Moreover, as Choi *et al.*[16] commented, radiation effects of high-LET radiations are also of immense interest, e.g., alpha-particle emitters are ubiquitous in our environment and are emitted from naturally occurring radon and its progeny (see e.g., ref. 28)). In the present work, protons with LET of about 11 keV/μm which are also a kind of high-LET radiation were used to provide the priming dose. On the other hand, X-ray photons which are a kind of low-LET radiation were used to provide the challenging dose. X-ray photons were employed for the

challenging dose to minimize the effects from the uncertain numbers of protons received by the embryos as described above, so the challenging dose would be easier to characterize.

As a result, two different types of ionizing radiations with very different LETs were used (namely, protons for the priming exposure while X-ray photons for the challenging exposure) for the induction of radioadaptive response. Our present results showed that zebrafish embryos pre-exposed by a low dose of high-LET radiation (protons) followed by a high dose of low-LET radiation (X-ray photons) could also demonstrate the induction of radioadaptive response. This result suggested that the concerned cells were not only adapted to the specific type of radiation identical to that for the priming exposure. Until now, the mechanism involved in the radiation induced adaptive response was not fully understood. The DNA repair system and cell cycle regulation systems governed by the ATM-p53 signal transduction pathway was considered to be the most important mediators of the radioadaptive response.[29] Furthermore, studies of Matsumoto *et al.*[30] and Takahashi *et al.*[31] suggested that NO, among others, also played an important role in initiating the radioadaptive response.

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