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Short Communication

Triphasic Low-dose Response in Zebrafish Embryos Irradiated by Microbeam Protons

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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 irradiate dechorionated zebrafish embryos at the 2-cell stage at 0.75 h post fertilization (hpf) by microbeam protons. Either one or both of the cells of the embryos were irradiated with 10, 20, 40, 50, 80, 100, 160, 200, 300 and 2000 protons each with an energy of 3.37 MeV. The embryos were then returned back 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 a triphasic dose-response for zebrafish embryos with both cells irradiated at the 2-cell stage, namely, (1) increase in apoptotic signals for < 200 protons (< 30 mGy), (2) hormesis to reduce the apoptotic signals below the spontaneous number for 200–400 protons (at doses of 30–60 mGy), and (3) increase in apoptotic signals again for > 600 protons (at doses > 90 mGy). The dose response for zebrafish embryos with only one cell irradiated at the 2-cell stage was also likely a triphasic one, but the apoptotic signals in the first zone (< 200 protons or < 30 mGy) did not have significant differences from those of the background. At the same time, the experimental data were in line with induction of radiation-induced bystander effect as well as rescue effect in the zebrafish embryos, particular in those embryos with unirradiated cells.

INTRODUCTION

It is common to adopt the linear no-threshold (LNT) hypothesis for radiation protection considerations, which assumes that the risk from an ionizing-radiation exposure 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. Data in the low-dose regime are relatively scarce, so 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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Despite the common use of the LNT model, 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, hormesis leads to a dose-response curve with responses at low doses opposite to those at high doses.[3] The typical J-shaped or inverted U-shaped hormetic dose-response curve is biphasic and nonlinear, which does not fit the LNT model. Hormesis will stimulate protective processes at the cellular, molecular, and organismic levels to decrease effects to below the spontaneous levels.

Interestingly, Hooker *et al.*[4] discovered an extra component to the biphasic hormetic dose-response curve, namely a "subhormetic" zone, and the dose-response curve became "triphasic". By using chromosomal inversion frequency as the biological endpoint in the spleen tissue of pKZ1 mice, Hooker *et al.*[4] observed three zones of inversion response with respect to the endogenous inversion frequency, namely, (1) subhormetic zone with increased response at ultra-low doses of 5–10 μGy, (2) hormetic zone with inversion frequency below the spontaneous frequency at low doses from 1–10 mGy, and (3) toxic zone with increased response at high doses of more than 0.1 Gy. In particular, ultra-low dos-

es of radiation form a subhormetic zone which induced inversions which were of similar magnitude to that observed at high doses of more than 0.1 Gy.

These dose responses, particularly those at the low and ultra-low doses, are relevant to environmental exposure to the alpha particles emitted from radon progeny,[5–8] for which the probability that a critical cell having more than one alpha particle crossing its nucleus during its lifetime is negligible. To the best of our knowledge, the study of chromosomal inversion frequency in spleen tissue of pKZ1 by Hooker *et al.*[4] was the only *in vivo* study to demonstrate the triphasic dose-response at low X-ray doses. The first objective of the present paper was to explore whether a similar triphasic dose-response would be present in other *in vivo* models and for a radiation with a different linear energy transfer (LET). On the other hand, Nagasawa and Little[9] observed that irradiation of a population of cells at low doses could trigger radiation-induced bystander effects (RIBE) in non-irradiated cells. The second objective of the present paper was to study whether the present results are in line with induction of RIBE.

Our group has previously explored the feasibility of using zebrafish embryos (*Danio rerio*) as the *in vivo* model to study the effect of low dose radiation, including the hormetic effect, bystander effect and adaptive response.[10–15] An 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.[16] Another advantage is that zebrafish embryos have larger proportions of dividing cells which are radiosensitive, and cellular damages are more likely to translate into damages at the organismic level due to the active morphogenesis in the embryos. As such, it would be interesting to explore whether a triphasic dose-response would be revealed and whether the results would be in line with induction of RIBE in zebrafish embryos at low radiation doses, which form the main objectives of the present work.

In the present work, we studied the effect of low-dose protons on zebrafish embryos by using a microbeam irradiation system (Single-Particle Irradiation System to Cell, acronym as SPICE)[17] at the National Institute of Radiological Sciences (NIRS), Japan. In our recent study, we successfully induced adaptive response in zebrafish embryos through priming doses provided by microbeam protons from SPICE. 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. The energy of the protons would drop a little bit down to 3.37 MeV when they arrived at the target. The number of protons incident onto a cell could be controlled. 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. 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. 18–20).

MATERIALS AND METHODS

Zebrafish embryos

Adult zebrafish (*Danio rerio*) were kindly provided by 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. 14) 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 dechorionated and irradiated at the two-cell stage (at ~0.75 h post fertilization (hpf)).[12] At this developmental stage, the cells have not assumed differentiated cell fates. Zygotic transcription in zebrafish embryos does not start until the midblastula transition (MBT) about 3 h after fertilization. The embryos have synchronous short cell cycles with S and M phases only, i.e., without the G1 and G2 phases, before MBT, while they have full asynchronous cell cycles (e.g., Ref. 21). Walker and Streisinger[22] found that embryos older than 3 h were more resistant to γ-rays, which suggested a possible repair mechanism after cleavage stages.

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).

Irradiation conditions

We made use of protons from the SPICE microbeam facility 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 exposure was delivered by protons when the zebrafish embryos were developed into 0.75 hpf. The dechorionated embryos 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. Either one of the cells (referred to as ×1 cases) or both of the cells (referred to as ×2 cases) of the embryos were irradiated with 10, 20, 40, 50, 80, 100, 160, 200, 300 and 2000 protons each with an energy of 3.37 MeV. The embryos were then returned back 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 number of delivered protons could be used to determine the absorbed dose in the cells of the zebrafish embryos. The absorbed dose for an embryo was then calculated by the relationship $D = E/M$, where E is the total energy of the incident protons and M is the estimated mass of a cell of an embryo at the two-cell stage. The dose conversion was found as 0.15 mGy per proton. Schettino *et al.*[23] studied HRS of cells *in vitro* through irradiating the nuclei of Chinese hamster V79 cells with protons with energies of 1.0 and 3.2 MeV. However, it was established that cytoplasmic irradiation could also induce bystander effects.[24,25] As such, the target for genetic effects of radiation might need to include the cytoplasm of the irradiated cell.

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. The feasibility of apoptosis detection by TUNEL assay was described in our previous work.[15]

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/. Possible outliers were identified and removed before t-test was used to test the statistical significance for differences between samples. A p value smaller than 0.05 was considered to correspond to a statistically significant difference, while a p value larger than 0.05 and smaller than 0.07 was considered to correspond to a marginal difference.

RESULTS

There were no observable effects on the development of the zebrafish as a result of proton irradiation. In particular, the rate of morphological changes for the entire experimental group (rate = 9.6%) was not significantly different from that for the entire control group (rate = 8.9%). Representative images of zebrafish embryos with proton irradiation and without proton irradiation are shown in Fig. 1. The apoptotic signals revealed on the embryos were evenly distributed within the entire body.

Table 1 shows the average net numbers of apoptotic signals on 25 hpf zebrafish embryos with respect to the average numbers of the control samples with sham irradiations. Comparisons of the numbers of apoptotic signals for (×1) and (×2) cases with the control samples were made, and comparisons of the numbers of apoptotic signals between (×1) and (×2) cases were made. The p values are obtained using t-tests. Cases with $p \leq 0.05$ were considered statistically significant. In the present work, cases with $p > 0.05$ and ≤ 0.07 were considered marginally significant.

Since the average numbers of apoptotic signals on control samples with sham irradiations varied among different experiments, normalization of the average net numbers of apoptotic signals with respect to the average numbers of the control samples was necessary to reveal the trends more clearly. Figure 2 shows the relationship between the normalized average numbers of apoptotic signals on 25 hpf zebrafish embryos and the total number of protons irradiated onto the zebrafish embryos at 0.75 hpf at the 2-cell stage. The apoptotic signals from the control groups are caused by spontaneous or endogenous damages, and correspond to the zero equivalent point (ZEP) in describing hormesis.

From Table 1 and Fig. 2, we observed a striking difference

Table 1. The average net numbers (± SD) of apoptotic signals on 25 hpf zebrafish embryos with respect to the average numbers of the control "Ctrl" samples. The experimental groups were irradiated at the 2-cell stage (0.75 hpf), with either one (×1) or both (×2) of the cells having received the indicated numbers of protons with an energy of 3.37 MeV. Comparisons of the numbers of apoptotic signals for (×1) and (×2) cases with the "Ctrl" samples were made, and comparisons of the numbers of apoptotic signals between (×1) and (×2) cases were made. Sample number = number of embryos in the corresponding group. The p values are obtained using t-tests. *cases with $p \leq 0.05$, which are considered statistically significant; #cases with $p > 0.05$ and ≤ 0.07, which are considered marginally significant. The normalized average numbers of apoptotic signals with respect to the average of the control samples are also shown.

Irradiation	Sample number	Average number	s.d.	p value (cf. Ctrl)	p value (×1 vs ×2)	Normalized value
Ctrl	18	0 ± 13	55			
10 × 1	16	28 ± 20	79	0.123		0.242
10 × 2	16	35 ± 19	76	0.069#	0.396	0.352
Ctrl	26	0 ± 3	14			
10 × 1	15	2 ± 5	18	0.397		0.048
10 × 2	22	19 ± 7	34	0.027*	0.062#	0.446
Ctrl	17	–2 ± 3	11			
20 × 1	19	1 ± 3	15	0.30		0.053
Ctrl	14	–2 ± 3	11			
20 × 2	18	4 ± 6	25	0.189	0.315	0.060
Ctrl	31	–1 ± 2	11			
40 × 1	18	0 ± 3	11	0.450		–0.028
Ctrl	14	0 ± 4	16			
40 × 2	16	25 ± 12	47	0.034*	0.027*	0.631
Ctrl	13	0 ± 11	41			
50 × 1	7	38 ± 15	64	0.095		0.429
50 × 2	11	17 ± 17	56	0.210	0.244	0.192
Ctrl	18	0 ± 3	15			
80 × 1	20	0 ± 4	17	0.479		0.152
Ctrl	15	0 ± 2	9			
80 × 2	15	10 ± 5	19	0.050*	0.069#	0.363
Ctrl	16	0 ± 17	67			
100 × 1	22	–3 ± 16	75	0.451		–0.014
100 × 2	14	–35 ± 16	59	0.066#	0.077	–0.220
Ctrl	30	0 ± 2	9			
160 × 1	18	9 ± 3	14	0.017*		0.335
Ctrl	10	0 ± 18	55			
200 × 1	10	–30 ± 16	52	0.116		–0.189
200 × 2	13	–47 ± 12	45	0.022*	0.207	–0.298
Ctrl	11	0 ± 14	48			
300 × 1	8	–2 ± 12	35	0.464		–0.014
300 × 2	10	38 ± 24	76	0.0968	0.0819	0.311
Ctrl	11	0 ± 3	10			
2000 × 1	12	12 ± 5	18	0.029*		0.592
2000 × 2	6	6 ± 2	6	0.0645#	0.153	0.283

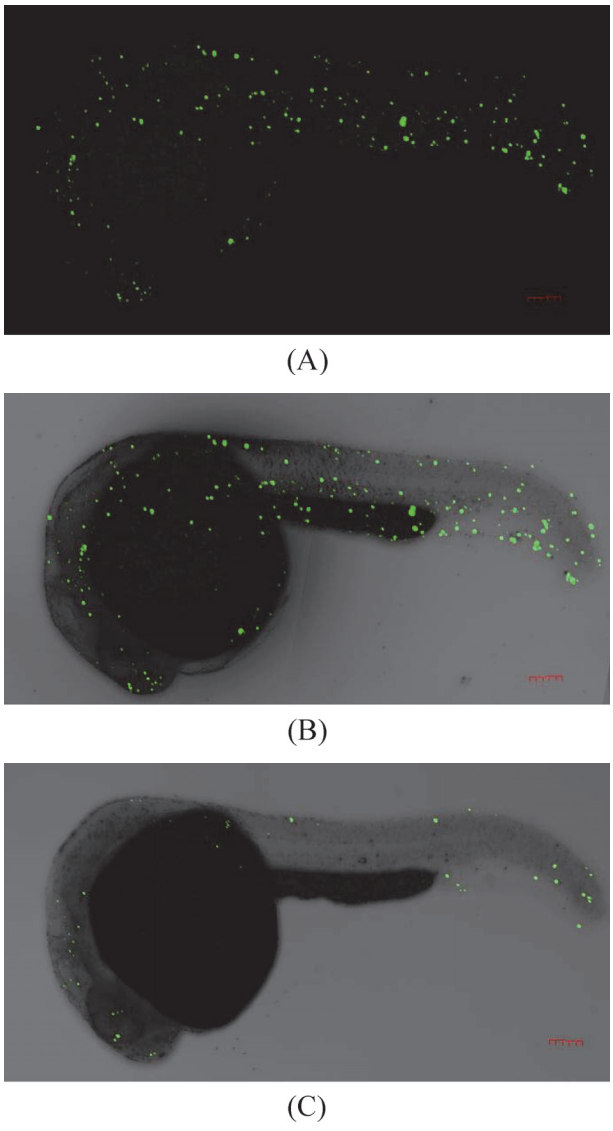

Fig. 1. Apoptotic signals on 25 hpf embryos obtained by TUNEL assay and recorded using a confocal microscope. (A) Fluorescent signals on a representative zebrafish embryo which has been irradiated with 10 protons on one cell at 0.75 hpf. (B) Superposition of fluorescent signals on the bright field image of the same embryo shown in (A). (C) Superposition of fluorescent signals on the bright field image of a non-irradiated embryo. Scale bars = 100 μm.

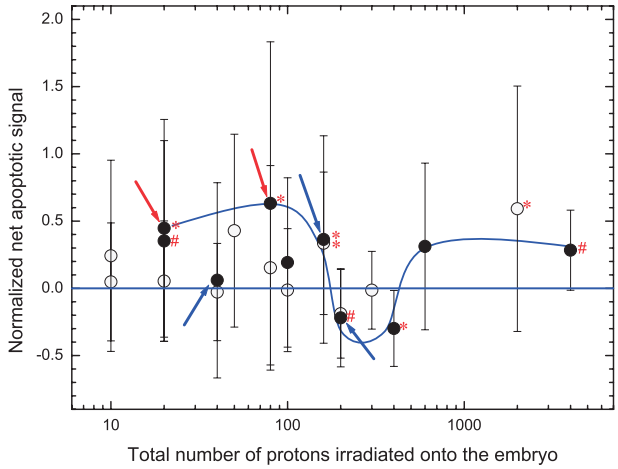

Fig. 2. The relationship between normalized average numbers of apoptotic signals on 25 hpf zebrafish embryos (with respect to the average of the control "Ctrl" samples) and the total number of protons irradiated onto the zebrafish embryos at 0.75 hpf at the 2-cell stage. Error bars represent one standard errors. Open circles: data for irradiation of one cell (×1); closed circles: data for irradiation of two cells (×2). Comparisons between the control samples and the (×1) or (×2) cases: *cases with $p \leq 0.05$, which are considered statistically significant; #cases with $p > 0.05$ and ≤ 0.07, which are considered marginally significant. Comparisons between the (×1) and (×2) cases: red arrows represent cases with $p \leq 0.05$; blue arrows represent cases with $p > 0.05$.

in the dose response of the embryos for the (×1) and (×2) cases. In particular, embryos with only one cell irradiated at the 2-cell stage usually did not produce a response significantly different from the ZEP, except for irradiation with 160 and 2000 protons. In contrast, embryos with both cells irradiated at the 2-cell stage exhibited a response with a more sophisticated pattern. The radiation effect was larger than the ZEP when the total number of irradiated protons was < 200 (with significant differences for a total of 20, 80 and 160 protons), smaller than the ZEP when the total number of irradiated protons was within the range from 200 to 400 protons (with a marginal difference for a total of 200 protons and a significant difference for a total of 400 protons), and becomes larger than the ZEP again when the total number of irradiated protons was > 600 (with a significant difference for a total of 2000 protons). This clearly showed a triphasic dose response and the dose ranges for the subhormetic, hormetic and the toxic zones were < 30 mGy (< 200 protons), 30–60 mGy (200–400 protons) and > 90 mGy (> 600 protons), respectively. The differences were also statistically significant ($p \leq 0.05$) between the responses for the (×1) and (×2) cases for a total of 20 and 80 protons irradiated onto the embryos.

DISCUSSION

Figure 2 shows that embryos with only one cell irradiated at the 2-cell stage in general did not produce a response significantly different from the ZEP, in particular for doses < 24 mGy (< 160 protons). This observation agreed with the findings of Bladen *et al.*,[26] who obtained dose-response curves for buffer-microinjected embryos and Ku80 MO-microinjected embryos irradiated at 6 hpf to 1 to 50 mGy (at 1, 3, 8, 20 and 50 mGy) from a 6 MeV Varian linear accelerator beam. Bladen *et al.*[26] showed no significant increase above background in the number of TUNEL-positive cells in

buffer-microinjected embryos, while a significant linear dose response in Ku80 MO-microinjected embryos. As Ku80 is a protein essential for the nonhomologous end-joining pathway of repairing DNA double-strand breaks (DSBs), their results showed that DNA DSBs were repaired in untreated irradiated embryos even at these low doses. At the same time, the insignificant increase above background in the number of TUNEL-positive cells in buffer-microinjected embryos showed that the repair of DNA DSBs was every effective at these low doses.

In our previous paper, we also reported that zebrafish embryos irradiated at 5 hpf by microbeam protons with as few as 5 protons × 10 irradiation points (i.e., a total of 50 protons) from the SPICE could induce radioadaptive response (RAR) in the embryos at 10 hpf against a 2 Gy challenging exposure of X-ray irradiation.[15] Although the underlying mechanism for RAR in cells is still largely unknown, some research findings suggested that DNA repair might play an important role in inducing RAR.[27–30] This gave further support that DNA repair was operational even for a low dose provided by as few as 50 protons to the embryos.

In the same dose range of < 24 mGy (< 160 protons), the responses of the embryos with both cells irradiated at the 2-cell stage were in general significantly different from the ZEP, and were in general larger than those of the embryos with only one cell irradiated at the 2-cell stage. By making reference to the results of Bladen *et al.*,[26] it became apparent that the repair of DNA DSBs in embryos with both cells irradiated at the 2-cell stage were less efficient when compared to the embryos with only one cell irradiated. This was possible if (but not necessarily a proof that) radiation-induced bystander effect (RIBE) and "rescue effect" were present when only one cell was irradiated (and thus with one unirradiated cell). Recently, Chen *et al.*[31] found that mammalian cells responded to ionization radiation by sending out extracellular signals to affect non-irradiated neighboring cells, which was referred to as RIBE, and then the bystander cells rescued the irradiated cells through intercellular signal feedback, which was referred to as the "rescue effect". In particular, the number of DNA DSBs in the irradiated cells was less than that in the irradiated cells which were not co-cultured with the bystander cells at a statistically significant level. As RIBE was a prerequisite for the rescue effect, the present results being in line with the occurrence of "rescue effect" when only one cell was irradiated were also in line with induction of RIBE in this case.

For embryos with both cells irradiated at the 2-cell stage, when the total number of protons irradiated was between 200 and 400 (dose between ~30 and ~60 mGy), the apoptotic signals dropped below the spontaneous number, i.e., there was hormesis. The occurrence of hormetic effect at low doses agreed qualitatively with the findings of Yum *et al.*[32] who also observed hormetic effect in zebrafish embryos irradiated with α particles at 1.5 hpf. Incidentally, all cells of the zebrafish embryos were irradiated in that work. Hooker *et al.*[4] also observed a hormetic zone in their triphasic dose-response curve for the chromosomal inversion frequency in spleen tissue of pKZ1 mice after single whole-body exposure to X radiation, and proposed that the induced DNA damages triggered a decrease in repair of DSBs in the hormetic zone, which might result in an increase in immediate apoptosis. As such, the cells with spontaneous or endogenous damages were removed from the pool when the damages introduced by the radiation (surrogated by the number of protons or the dose) reached a certain level. This in our case decreased the number of cells in the embryos undergoing apoptosis at 24 hpf. This mechanism of decreasing the repair of DSBs to effect removal of cells with spontaneous or endogenous damages should also be effective in embryos with only one cell irradiated at the 2-cell stage, so we argued that a hormetic zone should also be present in the dose-response curve for these embryos. The irradiation with 200 protons (×1 case) did cause an average signal below the ZEP but without statistical significance. On the other hand, the response significantly above the ZEP corresponding to irradiation with 160 protons (×1) might be attributed to the survival of some cells despite their cumulative damages had led to a stall in the repair of DSBs.

When the dose got progressively higher so that the damages of the irradiated cells had reached the putative threshold to initiate repair of DSBs again, the toxic zone began. In the toxic zone, large amounts of induced DNA damages were repaired.[4] However, some of these were incorrectly repaired, and so more damaged and mis-repaired cells would reach the later stage of the embryonic development, which would undergo apoptosis. As such, the apoptotic signal in the organism at the 24 hpf stage started to increase again.

In conclusion, Fig. 2 shows a triphasic dose response for zebrafish embryos with both cells irradiated at the 2-cell stage, namely, (1) increase in apoptotic signals for < 200 protons (< 30 mGy), (2) hormesis to reduce the apoptotic signals below the spontaneous number for 200–400 protons (at doses of 30–60 mGy), and (3) increase in apoptotic signals again for > 600 protons (at doses > 90 mGy). The dose response for zebrafish embryos with only one cell irradiated at the 2-cell stage is also likely a triphasic one, but the apoptotic signals in the first zone (< 200 protons or < 30 mGy) does not have significant differences from those of the background. The presence of three zones, namely, the sub-hormetic zone, the hormetic zone and the toxic zone were qualitatively consistent with those found by Hooker *et al.*[4] for the spleen tissue of pKZ1 mice after single whole-body exposure to X radiation. For a comparison, the corresponding three zones identified by Hooker *et al.*[4] were in the dose ranges of < 0.05 mGy, 0.05–20 mGy, and > 20 mGy. The spans of the dose ranges were very different for the present work and that of Hooker *et al.*,[4] viz., within 3 orders of

magnitude for the present work and about 4 orders of magnitudes for that of Hooker *et al*..[4] Such a difference can be attributed to the different *in vivo* models, or the different LET values of the radiation employed, or a combination of both. Carefully designed experiments will be further needed to give a clearer picture.

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