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Hormetic Effect Induced by Alpha-Particle-Induced Stress Communicated In Vivo between Zebrafish Embryos

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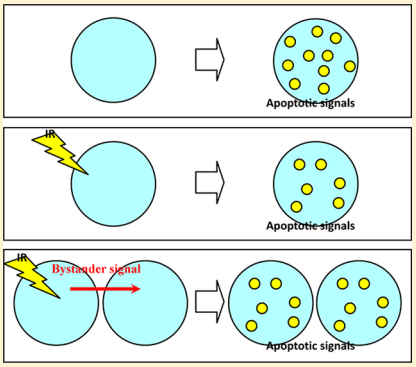
ABSTRACT: We report data showing that embryos of the zebrafish, *Danio rerio*, at 1.5 h post fertilization (hpf) subjected to a low-dose alpha-particle irradiation can release a stress signal into the water, which can be communicated to unirradiated bystander zebrafish embryos sharing the same water medium to induce a hormetic effect in the bystander embryos. Hormetic responses are characterized as biphasic dose−response relationships exhibiting a low-dose stimulation and a high-dose inhibition. The effects on the whole embryos were studied through quantification of apoptotic signals at 24 hpf through staining with the vital dye acridine orange, followed by counting the stained cells under a microscope. The results show that, for low alpha-particle dose, the number of apoptotic signals decreases in the irradiated embryos and also in the unirradiated bystander embryos having partnered with the irradiated embryos. These suggested that alpha-particle-irradiated zebrafish embryos could release a stress signal into the water, which could be communicated to unirradiated bystander zebrafish embryos sharing the same water medium to induce a hormetic effect in the bystander embryos.



INTRODUCTION

Radiation-induced bystander effect (RIBE) in cells describe the phenomenon that unirradiated cells respond as if they have been irradiated, after contacting with the irradiated cells or being exposed to the medium previously conditioning the irradiated cells. RIBE has also been demonstrated to exist between fish in vivo. The McMaster University group showed that X-ray-irradiated freshwater rainbow trout (*Oncorhynchus mykiss, W*) released bystander signals into the water to induce bystander effects in unirradiated naive partners, evidenced through the increased deaths of reporter cells induced by the media from explants from cultured tissues of the naive partners.[1] The work was the first demonstration of RIBE between fish in vivo, although Surinov et al.[2] reported a similar phenomenon earlier between mice, for which case the bystander signal was transmitted through urine. Subsequently, Mothersill et al.[3,4] also demonstrated RIBE between zebrafish (*Danio rerio*) and between Medaka (*Oryzias latipes*) in vivo.

Mothersill et al.[1] suggested that the RIBE was likely an evolutionarily conserved effect which enabled an effective population response. This visionary suggestion has instigated quest for the nature of the benefit brought to the population through RIBE. Recently, our group studied the benefit in terms of induction of radioadaptive response (RAR) by communication of radiation-induced bystander signals.[5] RAR is a low-dose effect, which occurs when a small preceding priming dose decreases the biological effectiveness of a subsequent large challenging dose. Olivieri et al.[6] first reported RAR in peripheral blood lymphocytes. Subsequently, Cai et al.[7] and Wang et al.[8] showed RAR induced in mice in vivo (with the RAR induced within the organisms). More recently, we demonstrated that embryos of the zebrafish, *Danio rerio*, subjected to a low-dose alpha-particle irradiation released a stress signal into the water, which could be communicated to the unirradiated naive zebrafish embryos sharing the same water medium to induce RAR in the naive embryos (with the RAR induced between the embryos).[5] This finding strongly supported the idea that RIBE was designed to enable an effective population response,[1] which in this case was protection of organisms in the population against a subsequent large radiation exposure.

Such a population response is exciting and interesting, but further thought will lead to an equally intriguing question: what if there is no subsequent large radiation exposure? Large radiation exposures are not commonly encountered in the environment, so will there still be benefits to the population brought about by the stress signal released by the irradiated embryos and communicated to the unirradiated naive zebrafish embryos? The present paper is devoted to answering these questions. A radiation effect closely related to the RAR is the "hormetic effect". Hormetic responses are characterized as biphasic dose−response relationships exhibiting a low-dose stimulation and a high-dose inhibition.[9−11] In the present paper, for simplicity, the term "hormetic effect" refers to cases which differ from the RAR in that the hormetic effect occurs

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without the application of a subsequent challenging dose. The more precise way of naming hormesis and adaptive response should be "radiation hormesis" and "radiation conditioning hormesis", respectively.[12]

Evidence has been accumulated that exposures to low doses of oxidants may have a stimulatory effect on cellular processes,[13,14] in contrast to cytotoxic effects of exposures to high doses. On the other hand, in an in vivo study of the effect of low doses of X-ray on zebrafish development, Miyachi et al.[15] observed a significant decrease in time to hatching following exposures of the zebrafish embryos and suggested that such exposures might induce positive effects on physiological functioning. More recently, Yum et al.[16] studied the radiation hormesis in zebrafish embryos at 1.5 h post fertilization (hpf) induced by low-dose alpha particles from an ^{241}Am source with an activity of 4.26 kBq. The number of apoptotic cells revealed in these irradiated embryos at 24 hpf were found to decrease significantly from 0 min irradiation (i.e., the controls) to 1 min irradiation, and then to increase almost linearly to 2, 4, and 8-min irradiation. The trend was a biphasic dose−response pattern which was a characteristic of the hormetic effect.

With our experimental setup and the associated procedures for studying alpha-particle-induced hormetic effect in zebrafish embryos in vivo in place, as well as the affirmative results on the hormetic effect,[16] it is pertinent to explore as a further step the potential benefit of communication of radiation-induced bystander signals to the population in terms of induction of hormetic effect.

In the present work, embryos of the zebrafish, *Danio rerio*, were again employed as the model for studying the hormetic effect induced by alpha-particle-induced stress. *Danio rerio* has become a preferred vertebrate model for studying human disease. The zebrafish and human genomes share considerable homology, including conservation of most DNA repair-related genes.[17] Rapid embryonic development is also an advantage in that the major organ systems become evident within 48 hpf. A growing number of research works have appeared in recent years using the zebrafish or the embryos as a vertebrate model to study the in vivo response to ionizing radiation.[3,18−27]

We hypothesized that hormetic effect would be developed in unirradiated naive embryos of the zebrafish, *Danio rerio*, exposed in vivo to the water shared by alpha-particle irradiated zebrafish embryos.

MATERIALS AND METHODS

Experimental Animals. Adult zebrafish were reared in 45 L glass tanks with water kept at 28 °C and with a 14/10 h light-dark cycle. Embryos were obtained by photoinduced spawning in specially designed embryo collectors.[28] Synchronization of developmental stage of the collected zebrafish embryos was crucial for our experiments. Once the 14 h photoperiod started, the embryo collectors were placed on the bottom of the fish tanks to collect the embryos over a brief period of 15 min to ensure more or less the same developmental stage of the collected embryos. The collected embryos were rinsed with deionized water and then incubated in a 28.5 °C incubator. At 0.75 hpf, healthy developing embryos were selected under a stereomicroscope and were dechorionated in a Petri dish with a layer of agarose as the substrate. The dechorionation step ensured that alpha particles could reach the cells of embryos to provide the radiation dose (see below). All studied embryos, whether they were going to be irradiated or not, were dechorionated to ensure the same conditions.

Exposure Protocol. The experiments have two parts. The objective of the first part was to compare the mean number of apoptotic signals obtained for the sham irradiated samples and embryos directly irradiated for different irradiation periods. This part of the experiment was largely to confirm the results of Yum et al.[16] For each set of experiment, 120 dechorionated embryos were deployed, which were divided into 12 groups each having 10 embryos. The twelve groups of embryos included six irradiated groups RA1, RA2, RA3, RA4, RA5, and RA6 which would be irradiated with alpha particles at 1.5 hpf for 1, 2, 3, 4, 5, and 6 min, respectively, (see Figure 1) using the

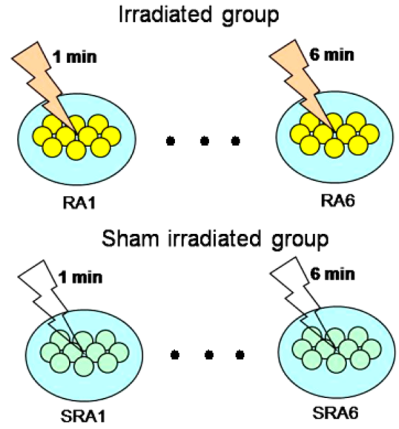

Figure 1. The twelve groups of embryos, namely, the *sham irradiated groups* SRA1, SRA2, SRA3, SRA4, SRA5, and SRA6 which were sham irradiated at 1.5 hpf for 1, 2, 3, 4, 5, and 6 min, respectively; and the *irradiated groups* RA1, RA2, RA3, RA4, RA5, and RA6 which were irradiated with alpha particles from a 4.26 kBq ^{241}Am source at 1.5 hpf for 1, 2, 3, 4, 5, and 6 min, respectively. Only SRA1, SRA6, RA1, and RA6 are shown for clarity.

radioactive source described below; and six sham irradiated group SRA1, SRA2, SRA3, SRA4, SRA5, and SRA6 which would be sham irradiated at 1.5 hpf for 1, 2, 3, 4, 5, and 6 min, respectively. These alpha-particle irradiation conditions overlapped with those previously employed by Yum et al.[16] who successfully demonstrated the presence of hormetic effect in irradiated zebrafish embryos. Four sets of experiments were carried out.

The objective of the second part was to compare the net number of apoptotic signals obtained for the control samples and unirradiated bystander naive embryos partnered with embryos irradiated for different irradiation periods. Here, for each set of experiment, 240 dechorionated embryos were deployed, which were divided into 24 groups. Each group has 10 embryos. These 24 groups of embryos included six irradiated groups RB1, RB2, RB3, RB4, RB5, and RB6 which would be irradiated with alpha particles at 1.5 hpf for 1, 2, 3, 4, 5, and 6 min, respectively; six bystander groups BY1, BY2, BY3, BY4, BY5, and BY6 which were not directly irradiated but were partnered (but separated by a distance of 1 cm) with the irradiated groups RB1, RB2, RB3, RB4, RB5, and RB6, respectively, in the medium within a Petri dish; six sham irradiated groups SRB1, SRB2, SRB3, SRB4, SRB5, and SRB6 which would be sham irradiated at 1.5 hpf for 1, 2, 3, 4, 5, and 6 min, respectively; and six control group C1, C2, C3, C4, C5, and C6, which would be partnered with the sham irradiated groups SRB1, SRB2, SRB3, SRB4, SRB5, and SRB6, respectively. After irradiation, all RB embryos were transferred

from the irradiation dish (with a Mylar film as the support substrate) to the incubation dish (with a thin layer of agarose as the substrate) to be partnered with BY embryos, with no transfer of the medium having conditioned the RB embryos. The partnership of different groups of zebrafish embryos for different experiments is shown in Figure 2. Four sets of experiments were carried out.

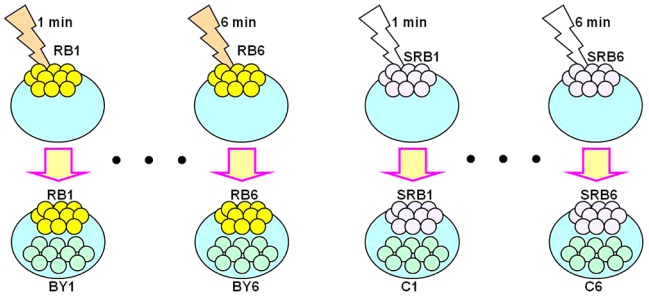

Figure 2. The partnership of different groups of zebrafish embryos for different experiments. Here, for each set of experiment, 240 dechorionated embryos were deployed, which were divided into 24 groups. These 24 groups of embryos included six *irradiated groups* RB1, RB2, RB3, RB4, RB5, and RB6 which would be irradiated with alpha particles at 1.5 hpf for 1, 2, 3, 4, 5, and 6 min, respectively; six *bystander groups* BY1, BY2, BY3, BY4, BY5, and BY6, which were not directly irradiated but were partnered (but separated by a distance of 1 cm) with the irradiated groups RB1, RB2, RB3, RB4, RB5, and RB6, respectively, in the medium within a Petri dish; six *sham irradiated groups* SRB1, SRB2, SRB3, SRB4, SRB5, and SRB6 which would be sham irradiated at 1.5 hpf for 1, 2, 3, 4, 5, and 6 min, respectively; and six *control group* C1, C2, C3, C4, C5, and C6, which would be partnered with the sham irradiated groups SRB1, SRB2, SRB3, SRB4, SRB5, and SRB6, respectively. Each group had 10 embryos. Only the groups corresponding to irradiation for 1 and 6 min are shown for clarity.

Alpha-Particle Irradiation. The setup for alpha-particle irradiation of zebrafish embryos was similar to that devised by Yum et al.[29] The irradiated groups of embryos were irradiated with alpha particles coming from below and across the support substrate. This setup avoided the setback caused by different depths of the medium above the different embryos if the alpha particles were coming from above. Thin Mylar films (Dupont, Hong Kong) with a thickness of 3.5 μm were employed as support substrates for the embryos to minimize energy absorption so that the alpha particles could hit the cells of the embryos with sufficiently large energies. The Mylar films were glued by an epoxy (Araldite Rapid, England) onto the bottom of a ϕ35 mm Petri dish which had a ϕ9 mm hole at the center. The embryos were oriented for the cells to face down toward the Mylar film to facilitate alpha-particle hits on the cells.

At 1.5 hpf, the irradiated groups of embryos were irradiated for 1, 2, 3, 4, 5, and 6 min, respectively, by alpha particles from an ^{241}Am source (with an average α particle energy of 5.49 MeV under vacuum and an activity of 4.26 kBq), which corresponded to absorbed doses of 1.4, 2.8, 4.1, 5.5, 7.0, 8.4 mGy, respectively,[29] which were similar to those employed by Yum et al.[16] The doses were also commensurate with those employed by Salbu et al.,[30] which could be as low as 4 mGy delivered over 5 h.

Vital Dye Staining. Quantification of apoptotic signals has been commonly exploited to show the radiation effect on the whole embryos.[18,22,31,32] In the present work, apoptotic signals in the 24 hpf embryos were quantified through staining with the vital dye acridine orange (AO), as previously suggested.[33] The embryos were stained with 5 μg/mL AO for 45 min, thoroughly washed three times with deionized water, and then anaesthetized using 0.016 M tricaine (Sigma, St. Louis, MO). The apoptotic signals in the embryos at 25 hpf were then counted under a fluorescent microscope. This method was commonly adopted for quantifying apoptosis in zebrafish embryos.[34−36] Our practice was to capture three images on different sections of each embryo under the fluorescent microscope with a magnification of 40×, and then combine them into a single image to facilitate counting of the apoptotic signals with the help of the software MetaMorph Version 7.0r0 (1992−2006 Molecular Devices).

Statistical Analysis. The numbers of apoptotic signals on the whole zebrafish embryos were counted as described above. The data are presented as the average net number of apoptotic signals ± standard error. For each set of experiment, the net number of apoptotic signals for *irradiated group* = number of apoptotic signals for irradiated embryos − average number of apoptotic signals for sham irradiated group; net number of apoptotic signals for *sham irradiated group* = number of apoptotic signals for sham irradiated group − average number of apoptotic signals for sham irradiated group; net number of apoptotic signals for *bystander group* = number of apoptotic signals for bystander group − average number of apoptotic signals for control group; net number of apoptotic signals for *control group* = number of apoptotic signals for control group − average number of apoptotic signals for control group. The development of hormetic effect in unirradiated naive zebrafish embryos was characterized by comparing the net number of apoptotic signals in BY1, BY2, BY3, BY4, BY5, and BY6 groups with the corresponding net number of apoptotic signals in control groups C1, C2, C3, C4, C5, and C6 through the *t*-test. All the analyses were performed after outlier data, if any, were removed. When a group of data was arranged in the descending order, the outliers were defined as values larger than 1.5 times the interquartile range above the 75th percentile or smaller than 1.5 times the interquartile range below the 25th percentile of the group of data, where the interquartile range was defined as

Table 1. Average Net Number (± Standard Error) of Apoptotic Signals (N) Obtained for the RA1, RA2, RA3, RA4, RA5, and RA6 Embryos[a]

	RA1	RA2	RA3	RA4	RA5	RA6
N	−23 ± 5	1 ± 7	8 ± 8	57 ± 11	61 ± 9	118 ± 13
n	39	33	40	34	34	34
p	0.00257	0.498	0.168	1.17×10^{-5}	1.40×10^{-7}	5.42×10^{-11}

[a] n: Number of embryos used in the statistic analyses (n < 40 in some cases due to death of embryos or outliers); p: p values obtained using t-tests for comparison with the corresponding sham irradiated samples. p < 0.05 values are regarded as statistically significant.

Table 2. Average Net Number (± Standard Error) of Apoptotic Signals (N) Obtained for the BY1, BY2, BY3, BY4, BY5, and BY6 embryos[a]

	BY1	BY2	BY3	BY 4	BY 5	BY 6
N	−16 ± 5	−34 ± 6	1 ± 5	41 ± 8	34 ± 8	39 ± 8
n	32	37	28	35	32	30
p	0.0278	0.00014	0.417	5.56×10^{-6}	4.20×10^{-5}	3.30×10^{-5}

[a] n: Number of embryos used in the statistic analyses ($n < 40$ in some cases due to death of embryos or outliers); p: p values obtained using t-tests for comparison with the corresponding control samples. $p < 0.05$ values are regarded as statistically significant.

the difference between the 25th and 75th percentiles of the data. All four sets of data were then combined into one large group of data for analysis. Cases with p values <0.05 corresponded to statistically significant differences between the compared groups.

RESULTS

The average net number of apoptotic signals (N) for the irradiated embryos RA1, RA2, RA3 and RA4, RA5, and RA6 are shown in Table 1. The corresponding p values obtained using t tests for comparison with the corresponding sham irradiated samples are shown. As shown in Table 1, RA1 embryos (having a p value of 0.00257) showed significantly fewer apoptotic signals when compared with the sham irradiated embryos. On the other hand, RA2 and RA3 embryos did not have significantly higher apoptotic signals when compared with the sham irradiated embryos. Finally, the RA4, RA5, and RA6 embryos had significantly more apoptotic signals when compared with the sham irradiated embryos. The trend was a biphasic dose−response pattern which was characteristic of the hormetic effect.

On the other hand, the comparisons between the average net number of apoptotic signals (N) for the control embryos and the BY1, BY2, BY3, BY4, BY5, and BY6 embryos are shown in Table 2. The corresponding p values obtained using t tests for comparison with the control samples are also shown. As shown in Table 2, BY1 and BY2 embryos had significantly fewer apoptotic signals when compared with the control embryos. The BY3 embryos did not have significantly higher apoptotic signals. In contrast, BY4, BY5, and BY6 groups of embryos had significantly more apoptotic signals when compared with the corresponding control embryos. Again, the trend was a biphasic dose−response pattern which was characteristic of the hormetic effect.

DISCUSSION

The present paper demonstrated that zebrafish embryos irradiated by low-dose alpha particles and that unirradiated naive zebrafish embryos partnered with embryos irradiated by low-dose alpha particles could develop a hormetic effect, which had been revealed through quantification of apoptotic signals in the 24 hpf embryos stained with the vital dye acridine orange.

The results of the first part of the experiment showed that the number of apoptotic signals in irradiated zebrafish embryos was significantly decreased below the spontaneous level for small doses (<2.8 mGy) and became significantly beyond the spontaneous level at 5.5 mGy. On the other hand, the number of apoptotic signals was in general also above the spontaneous level at ∼4.1 mGy. The general pattern resembled the well-known biphasic trend for radiation hormesis. The results confirmed the findings of Yum et al.,[16] who studied the radiation effects of low-dose alpha particles on zebrafish embryos and found a significant decrease in the apoptotic signals at 24 hpf when the alpha-particle dose was smaller than 2.8 mGy.

In the second part of the experiment, unirradiated naive embryos allowed to share the same medium with the irradiated embryos showed that their number of apoptotic signals also followed a pattern resembling the biphasic trend for radiation hormesis. The number of apoptotic signals in the unirradiated naive embryos was significantly decreased below the spontaneous level when they were partnered with embryos irradiated with small doses (<2.8 mGy), and were significantly increased above the spontaneous level when they were partnered with embryos irradiated with a larger dose (∼5.5 mGy). On the other hand, the average net number of apoptotic signals did not show a significant difference from the control group for an intermediate dose (∼4.1 mGy). This also demonstrated that alpha-particle irradiated zebrafish embryos communicated stress through the shared medium to their partner unirradiated zebrafish embryos, and that the stress induced a hormetic effect in the partner unirradiated zebrafish embryos. This is the first demonstration of a hormetic effect induced by radiation-induced stress communicated between living organisms. It is remarked here that the stressor agents acting on irradiated and naive embryos were different and could invoke different mechanisms for the hormetic effects, and as such the dose−response curves for the irradiated and naive embryos could have different patterns.

The chemical messengers responsible for the hormetic effect in the bystander naive embryos were not examined. Further investigations on the chemical factors responsible for inducing the hormetic effect in the bystander embryos in vivo, and probably together with investigations on the chemical factors responsible for inducing the radioadaptive response in the bystander embryos in vivo previously revealed by Choi et al.,[5] can help elucidate the mechanisms involved in the hormetic effect (and also RAR) induced by communicated radiation-induced stress. It is noted that the bystander gill proteome of rainbow trout exposed to X-ray is protective and restorative.[37] Salbu et al.[30] remarked that ionizing radiation at natural environmental levels might promote health by stimulating defense and repair mechanisms. In general, the principal mechanism in common between the hormetic effect and the RAR is that low levels of stress activate or upregulate existing cellular and molecular pathways that enhance the ability of the cell and organism to withstand more severe stress.[12]

Hormetic effect has now been demonstrated, in addition to adaptive response as previously shown,[5] to be provoked by a stress induced by a low-dose radiation and communicated in vivo between living organisms. These low-dose radiation effects support the view that radiation-induced stress communicated in vivo between living organisms are actually an allelopathic effect aimed at coordinating a species-level survival response,[3] at least in aquatic species that are close to one another and sharing the same media.

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Notes

The authors declare no competing financial interest.

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