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Neutron induced bystander effect among zebrafish embryos

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HIGHLIGHTS

- Reported first-ever observation of neutron induced bystander effect (NIBE).
- Studied NIBE using zebrafish (*Danio rerio*) embryos as the *in vivo* model.
- Observed a neutron-dose window (20–50 mGy) which could induce NIBE.
- Explained the dose window by the amount of neutron-induced damages.

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ABSTRACT

The present paper reported the first-ever observation of neutron induced bystander effect (NIBE) using zebrafish (*Danio rerio*) embryos as the *in vivo* model. The neutron exposure in the present work was provided by the Neutron exposure Accelerator System for Biological Effect Experiments (NASBEE) facility at the National Institute of Radiological Sciences (NIRS), Chiba, Japan. Two different strategies were employed to induce NIBE, namely, through directly partnering and through medium transfer. Both results agreed with a neutron-dose window (20–50 mGy) which could induce NIBE. The lower dose limit corresponded to the threshold amount of neutron-induced damages to trigger significant bystander signals, while the upper limit corresponded to the onset of gamma-ray hormesis which could mitigate the neutron-induced damages and thereby suppress the bystander signals. Failures to observe NIBE in previous studies were due to using neutron doses outside the dose-window. Strategies to enhance the chance of observing NIBE included (1) use of a mono-energetic high-energy (e.g., between 100 keV and 2 MeV) neutron source, and (2) use of a neutron source with a small gamma-ray contamination. It appeared that the NASBEE facility used in the present study fulfilled both conditions, and was thus ideal for triggering NIBE.

1. Introduction

Neutrons are an indirectly ionizing radiation. For the general public, cosmic radiation constitutes the largest exposure to neutrons. Moreover, nuclear reactor workers, airline crew members, astronauts, medical doctors and patients involved in clinical radiotherapy can be subjected to larger neutron exposures. While the effects of neutrons on directly irradiated cells or organisms have been extensively studied, there were only very few studies on radiation induced bystander effects (RIBEs) due to neutrons (Liu et al., 2006; Wang et al., 2011; Seth et al., 2014). For simplicity, RIBEs due to neutrons are also referred to as neutron induced bystander effect (NIBE) in the present work.

RIBE in cells generally describes the phenomenon that non-irradiated cells respond as if they have themselves been irradiated upon receiving signals from directly irradiated cells, either through partnering or medium transfer (e.g., Blyth and Sykes, 2011). RIBE was first discovered by Nagasawa and Little (1992) who demonstrated a significant increase in the occurrence of sister

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chromatid exchanges in Chinese hamster ovary cells upon irradiation to a low dose of alpha particles. Generally speaking, RIBE signals could affect neighboring or distant cells either through cellular gap-junction intercellular communication or through diffusion in the medium (e.g., Little, 2006; Morgan and Sowa, 2007; Prise and O'Sullivan, 2009).

While it has been well established that bystander effects could be induced by gamma radiation and alpha-particle radiation (Azzam et al., 1998; Lorimore et al., 1998; Mothersill and Seymour, 1997; Prise et al., 1998), it was intriguing that all previous *in vitro* or *in vivo* studies failed to observe NIBE (Liu et al., 2006; Seth et al., 2014; Wang et al., 2011). The present work aimed to study the NIBE using zebrafish (*Danio rerio*) embryos as the *in vivo* model. Zebrafish embryos have been widely used for studying biological effects related to ionizing radiation (e.g., Bladen et al., 2005; Daroczi et al., 2006; Geiger et al., 2006; Kong et al., 2014; Mothersill et al., 2007; Yum et al., 2007; Choi et al., 2010a, 2012a,b; Choi and Yu, 2015) due to its fecundity, rapid development and the fact that zebrafish and human genomes share considerable homology, including conservation of most DNA repair-related genes (Barbazuk et al., 2000).

We hypothesized that only neutron doses within a certain range (the dose-window) could lead to NIBE and that failures in previous attempts to observe NIBE were due to using neutron doses outside the dose-window. We also proposed explanations for the occurrence of such a dose-window in terms of the various phenomena recently identified by Ng et al. (2015) from the neutron-dose response of zebrafish embryos, including neutron hormesis and gamma-ray hormesis. In particular, hormetic responses are biphasic dose–response relationships characterized by a low-dose stimulation and a high-dose inhibition (Calabrese and Baldwin, 2002; Calabrese and Linda, 2003; Calabrese, 2008). We also suggested strategies to enhance the chance of observing NIBE in future.

2. Materials and methods

2.1. Neutron irradiation facility

In the present work, the neutron exposures were provided by the Neutron exposure Accelerator System for Biological Effect Experiments (NASBEE) facility at the National Institute of Radiological Sciences (NIRS), Chiba, Japan (Suda et al., 2009). NASBEE is a coaxial Tandetron™ accelerator (High Voltage Engineering Europa B.V., Amersfoort, Netherlands) with a multi-cusp ion source, which provides relatively monochromatic neutrons with energies up to 2 MeV. Neutrons are generated by bombarding deuterons with an energy of 4 MeV onto the Be target. In the current study, neutrons with an energy of 2 MeV at a dose rate 220 mGy/h was employed. The same dose rate was used throughout the present work. A shutter was installed at the beam port to shield the gamma rays from striking the samples so as to maintain a low level of gamma-ray contamination in the neutron beam, which was 14% at the present dose rate (Suda et al., 2009). It is noted that Ng et al. (2015) employed the same NASBEE facility with the same neutron energy and dose rate in their studies.

2.2. Experimental animals

Adult zebrafish (*Danio rerio*) were kindly provided by the RIKEN Brain Science Institute, JAPAN (courtesy Prof. Hitoshi Okamoto). Fish of both genders were mixed and reared in a 45 L-water filled glass tanks in a laboratory where the ambient environment was kept at 28 °C. A 14/10 h light-dark cycle was adopted to maintain a good production of embryos. When the photo-induced spawning began, a special collector was placed on the bottom inside each tank to collect the embryos (Choi et al., 2010b). To ensure synchronization of their developmental stages, all embryos were collected within a brief period of 15–30 min after the lights were switched on. These collected embryos were rinsed with the E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, 0.1% methylene blue) and then incubated at 28 °C until 4 h post fertilization (hpf). Fertilized and healthy developing embryos were selected under a stereomicroscope (Model SZH, Olympus Co., Shinjyuku-ku, Tokyo, Japan) at 4 hpf and were transferred into a new Petri dish lined with a thin layer of agarose (Invitrogen, Life Technologies Corporation, Carlsbad, CA, USA) gel for dechorionation (Choi et al., 2013).

2.3. Experimental setup

2.3.1. Bystander effect induced through partnering

The first objective in the present study was to investigate if neutron-irradiated zebrafish embryos could induce bystander effect on partnered non-irradiated zebrafish embryos. On the day of each experiment, embryos were collected and then dechorionated at 4 hpf as described above. These embryos were then divided into 5 groups, namely:

(1) ***I–N*** group: **I**rradiated embryos partnered with **N**on-irradiated embryos;
(2) ***N–I*** group: **N**on-irradiated embryos partnered with **I**rradiated embryos;
(3) ***S–N*** group: **S**ham irradiated embryos partnered with **N**on-irradiated embryos;
(4) ***N–S*** group: **N**on-irradiated embryos partnered with **S**ham irradiated embryos; and
(5) ***Control*** group: Dechorionated embryos without receiving any further treatment.

To allow the zebrafish embryos in the ***I–N*** group and the ***N–I*** group to simultaneously share the same medium in the same agarose dish, two separated shallow regions were dredged on the agarose lining to accommodate the two groups of embryos. Two different neutron doses, namely, 50 and 100 mGy, were employed in this part of study.

When the dechorionated zebrafish embryos were developed into 5 hpf, the embryos in the ***I–N*** group were placed within the uniform-dose irradiation field of the NASBEE facility with a diameter of 26 cm ($\pm$ 2%) and irradiated to a neutron dose of either 50 or 100 mGy. Immediately after irradiation, the ***I–N*** group embryos was transferred into one of the dredged regions on the agarose lining to partner with the ***N–I*** group embryos which were accommodated in the other dredged region. With this design, the soluble factors, if any, communicating the bystander signals were expected to be released by the ***I–N*** group to reach the ***N–I*** group. Similarly, as the control experiment, another agarose dish was prepared to accommodate sham-irradiated embryos (***S–N***) in one dredged region partnering with non-irradiated embryos (***N–S***) in the other dredged region. A volume of 3 ml of E3 medium was used in each agarose dish. All five groups of embryos were incubated at 28 °C until they reached 25 hpf. Fig. 1 shows schematic diagrams to illustrate the partnership of ***I–N***, ***N–I***, ***S–N*** and ***N–S*** groups of embryos.

2.3.2. Bystander effect induced through medium transfer

In this part of our study, we further investigated if bystander effect could be induced in non-irradiated zebrafish embryos immersed into the medium which had previously been conditioned by the neutron-irradiated zebrafish embryos. Fig. 2 shows the procedures for studying the NIBE on zebrafish embryos through

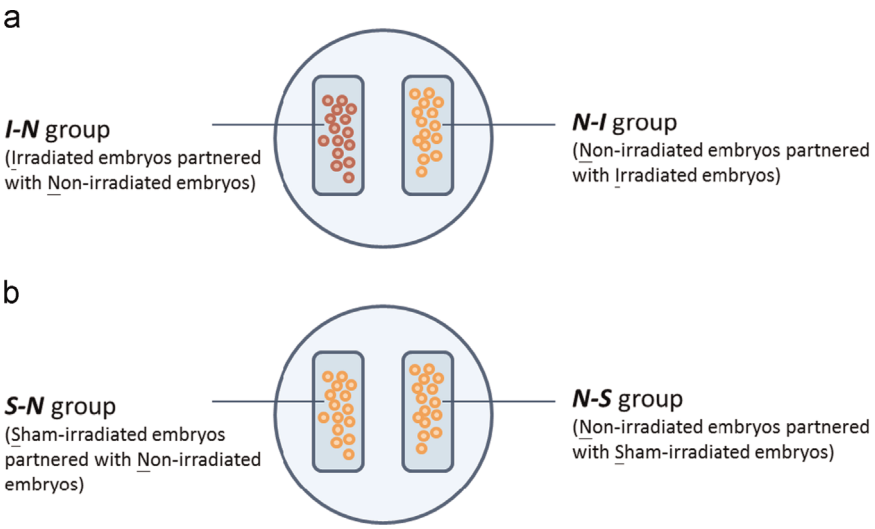

Fig. 1. Schematic diagrams showing the partnership of different groups of embryos: (a) ***I–N*** group partnered with ***N–I*** group and (b) ***S–N*** group partnered with ***N–S*** group.

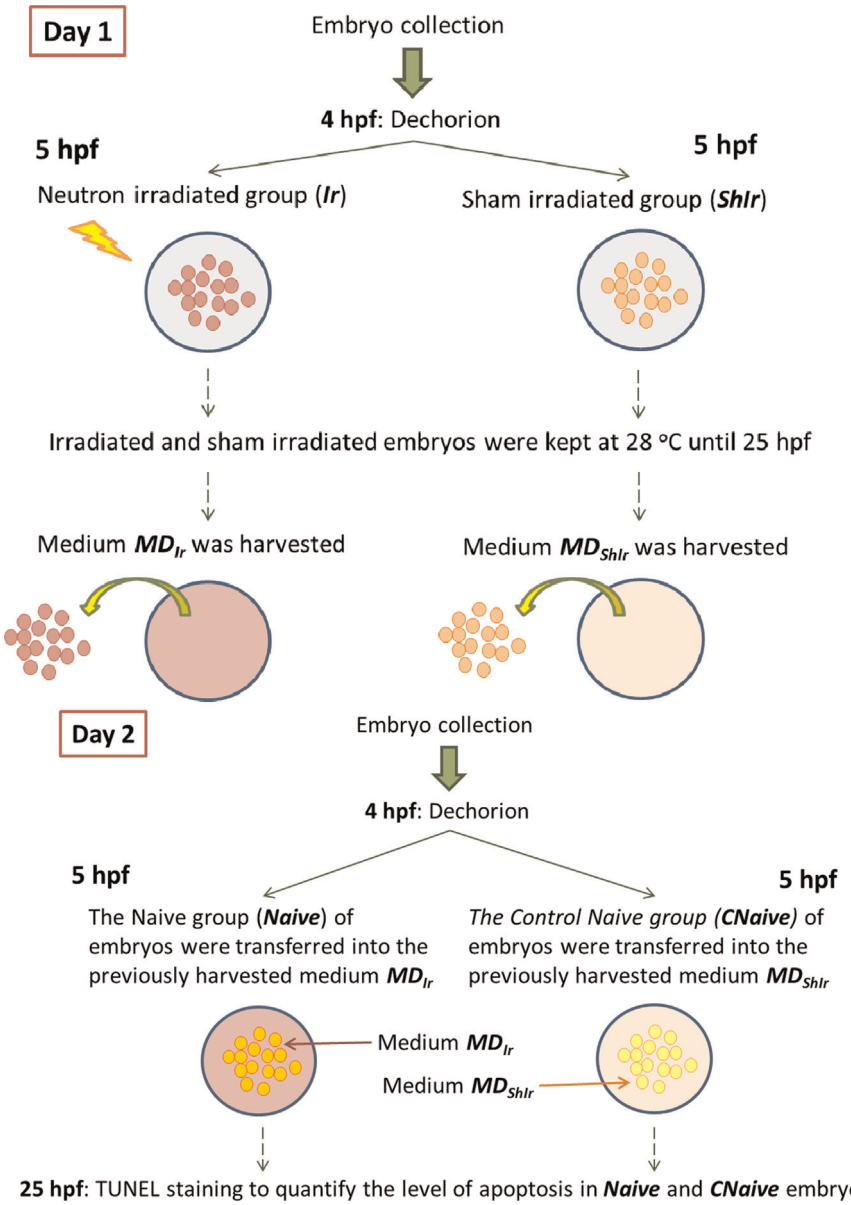

Fig. 2. Experimental scheme showing the steps for studying NIBE on zebrafish embryos through medium transfer.

medium transfer. For each set of experiment, the embryos were collected on two consecutive days. On the first day (Day 1), the embryos were collected and then dechorionated at 4 hpf. These embryos were then divided into two groups, namely: (1) the neutron-irradiated (***Ir***) group and; (2) the sham-irradiated (***ShIr***) group:

(1) Neutron-irradiated group (Ir): dechorionated embryos exposed to the desired neutron doses at 5 hpf; and
(2) Sham-irradiated group (ShIr): dechorionated embryos sham-irradiated at 5 hpf.

All embryos in the ***Ir*** group were placed within the uniform dose irradiation field of the NASBEE facility. A total of 9 neutron doses, namely, 1, 5, 10, 15, 20, 35, 50, 70 or 200 mGy, were employed in this part of the study. The embryos in the ***Ir*** group were irradiated with different neutron doses while the *ShIr* group of embryos was used as controls to account for the corresponding background apoptotic signals. All embryos were accommodated in separated wells in a 6-well cell culture dish with a thin layer of agarose lining the bottom. The embryos were kept in 3 ml of the E3 medium. After irradiation, the embryos were transferred to the 28 °C incubator until they reached 25 hpf. At 25 hpf, all embryos in the ***Ir*** and ***ShIr*** groups were removed from their media and the media were then separately harvested. The medium which had previously been conditioned by the *Ir* group of embryos for 19 h was referred to as MD_{Ir}, while the medium which had previously been conditioned by the *ShIr* group of embryos for 19 h was referred to as MD_{ShIr}.

On the second day (Day 2), new embryos were collected in the same way as those collected on Day 1. They were dechorionated at 4 hpf and divided into three groups. The first group of embryos was referred to as the Control group (C) in which the embryos were dechorionated without receiving any further treatment. The other two groups of embryos were transferred to the conditioned media MD_{Ir} and MD_{ShIr}, and were referred to as the Naive group (Naive) and the Control Naive group (CNaive), respectively. In other words, a total of three groups of embryos were prepared:

(1) Control group (C): embryos dechorionated at 4 hpf without receiving any further treatment;
(2) Naive group (Naive): dechorionated embryos transferred into the previously collected medium MD_{Ir} at 5 hpf; and
(3) Control Naive group (CNaive): dechorionated embryos transferred into the previously collected medium MD_{ShIr} at 5 hpf.

All embryos were then kept in the 28 °C incubator until they developed into 25 hpf.

2.4. TUNEL assay

In the present work, the amount of apoptotic cells within the whole embryos was chosen as the biological endpoint. The terminal dUTP transferase-mediated nick end-labeling (TUNEL) assay was employed to detect apoptotic cells on the 25 hpf embryos. When the embryos reached 25 hpf, they were fixed in 4% paraformaldehyde (162– 16,065, Wako Pure Chemical Industries. Ltd., Chuo-Ku, Osaka, Japan) in phosphate buffered saline (PBS) (21–040, Mediatech, Inc., A corning Subsidiary, 9345 Discovery Blvd. Manassas, VA 20109, USA) with 0.1% Tween 20 at room temperature for 5 h. The fixed embryos were dehydrated and then rehydrated with methanol. After that, the embryos were treated with 20 μg/ml protease kinase (Wako Pure Chemical Industries Ltd., Osaka, Japan) for 10 min before fixing with 4% paraformaldehyde in PBS with 0.1% Tween 20 again. TUNEL staining was performed by an *in situ* apoptosis detection kit (MK500, Takara Bio. Inc., Otsu, Shiga, Japan). Briefly, the fixed embryos were treated with permeabilization buffer on ice for 30 min, followed by staining in the mixture of labeling-safe buffer containing Fluorescein labeled-2′-Deoxyuridine, 5′-Triphosphate, FITC-dUTP and Terminal Deoxynucleotidyl Transferase (TdT) enzyme in the ratio of 9–1. The staining process was performed in dark and inside a 37 °C humidified chamber for 110 min. Lastly, the stained embryos were washed thoroughly with PBS with 0.1% Tween 20 several times to remove the excessive stains. The apoptotic signals within each whole stained embryo could then be studied under a fluorescent microscope. Images of embryos were captured by a confocal laser microscope (FV-1000, Olympus Corporation, Tokyo, Japan) with 4× objective lens (NA:0.16, UPLSAPO 4×, Olympus Corporation, Tokyo, Japan). For each embryo, a total of 15–25 sliced images (2.12×2.12 mm^2, 2.06 μm/pixel) with 25 μm intervals were captured. These images were finally combined into one for further analysis.

2.5. Data analysis

The combined image obtained using the confocal microscope was converted into a binary image to be analyzed using ImageJ, which was a free software obtained from the website http://rsb.info.nih.gov/ij/, to determine the number of apoptotic signals throughout each 25 hpf stained embryo. The number of apoptotic cells was evaluated by the "Analyze particle" function in ImageJ. Student's *t*-test was employed to assess the statistical significance of the differences between samples. A p value smaller than 0.05 was considered to correspond to a statistically significant difference.

3. Results

3.1. Bystander effect induced through partnering

In this part of the study, NIBE was induced through partnering the ***N–I*** group of embryos with the ***I–N*** group of embryos for 19 h. Two different neutron doses, namely, 50 and 100 mGy, were employed for different experiments. The apoptotic signals on a total of 204 embryos were examined. The mean number of apoptotic signals (N) for the ***I–N***, ***N–I***, ***S–N***, ***N–S*** and ***control*** groups were determined as N_{I-N}, N_{N-I}, N_{S-N}, N_{N-S} and N_C, respectively, where N_C was the average background apoptotic signal for embryos in the corresponding set of experiment. The results were recorded as shown in Tables 1 and 2.

From Tables 1 and 2, it was found that the amounts of apoptotic signals on the embryos in the ***I–N*** groups for both investigated neutron doses (50 and 100 mGy) were significantly higher than

Table 1
The average number of apoptotic signals (N ± SEM) obtained from embryos in the ***I–N*** group (Irradiated embryos partnered with Non-irradiated embryos), ***N–I*** group (Non-irradiated embryos partnered with Irradiated embryos), ***S–N*** group (Sham irradiated embryos partnered with Non-irradiated embryos), ***N–S*** group (Non-irradiated embryos partnered with Sham irradiated embryos) and Control group. Embryos in the ***I–N*** and ***S–N*** groups were irradiated or sham irradiated with 50 mGy of neutrons.

	I–N group	***N–I*** group	***S–N*** group	***N–S*** group	*Control*
N	108 ± 8 1.84×10^{-10}[a,*]	40 ± 4 0.032[b,*]	27 ± 3	30 ± 3	31 ± 4

[a] p value obtained using Student's *t*-test on the difference between the ***I–N*** and *Control* groups of embryos.
[b] p value obtained using Student's *t*-test on the difference between the ***N–I*** and ***N–S*** groups of embryos.
* Cases with $p \leq 0.05$ are considered as statistically significant.

Table 2
The average number of apoptotic signals ($N \pm$ SEM) obtained from embryos in the ***I–N*** group (**I**rradiated embryos partnered with **N**on-irradiated embryos), ***N–I*** group (**N**on-irradiated embryos partnered with **I**rradiated embryos), ***S–N*** group (**S**ham irradiated embryos partnered with **N**on-irradiated embryos), ***N–S*** group (**N**on-irradiated embryos partnered with **S**ham irradiated embryos) and ***Control*** group. Embryos in the ***I–N*** and ***S–N*** groups were irradiated or sham irradiated with 100 mGy of neutrons.

	I–N group	***N–I*** group	***S–N*** group	***N–S*** group	*Control*
N	94 ± 8	29 ± 3	24 ± 2	26 ± 1	31 ± 3
	8.07×10^{-9}[a,*]	0.200[b]			

[a] *p* value obtained using Student's *t*-test on the difference between the ***I–N*** and *Control* groups of embryos.
[b] *p* value obtained using Student's *t*-test on the difference between the ***N–I*** and ***N–S*** groups of embryos.
* Cases with $p \leq 0.05$ are considered as statistically significant.

those in the corresponding control groups, which confirmed that irradiating embryos with neutron doses of 50 and 100 mGy led to damaging effects in terms of increased amounts apoptotic signals, and which agreed with the findings of Ng et al. (2015). From Table 1, when embryos of the ***I–N*** group were irradiated with 50 mGy of neutrons, a significant difference between the ***N–I*** and ***N–S*** groups of embryos was observed. This indicated that neutron-irradiated (***I–N***) embryos would induce a bystander effect on the partnered (***N–I***) embryos when they simultaneously shared the same medium. This was the first-ever observation of NIBE. On the other hand, from Table 2, when the embryos in the ***I–N*** group were irradiated with 100 mGy of neutrons, no significant differences were found between the ***N–I*** and ***N–S*** groups of embryos, which suggested that no bystander effect was induced.

3.2. Bystander effect induced through medium transfer

In this part of the study, the media previously conditioned by the embryos that had been irradiated with different neutron doses were harvested 19 h post irradiation, into which non-irradiated embryos were immersed for a further 19 h to investigate whether bystander effect could be induced in these non-irradiated embryos.

A total of nine different neutron doses (viz., 1, 5, 10, 15, 20, 35, 50, 70 and 200 mGy) were employed. The numbers of apoptotic signals on a total of 580 embryos were counted. The mean numbers of apoptotic signals (N) for the ***C***, ***Naive*** and ***CNaive*** groups for each set of experiment were determined as N_C, N_{Naive} and N_{CNaive}, respectively. The net apoptotic signals for all the ***Naive*** and ***CNaive*** groups within the same batch of embryos could be calculated as $N_{Naive}{}^{N} = (N_{Naive} - N_C)$ and $N_{CNaive}{}^{N} = (N_{CNaive} - N_C)$ by considering N_C as the average number of background apoptotic signals for the embryos in the corresponding set of experiment. Therefore, the net normalized apoptotic signals for these groups of embryos were evaluated as $N_{Naive}{}^{N*} = [(N_{Naive} - N_C)/N_C]$ and $N_{CNaive}{}^{N*} = [(N_{CNaive} - N_C)/N_C]$. The net normalized results were shown in Table 3.

From Table 3, significant differences between the ***Naive*** and ***Control Naive*** groups of embryos in some of the cases were observed, which demonstrated that neutron-irradiated embryos could induce bystander effect on the naive embryos through medium transfer. However, such an effect only occurred when the ***Ir*** group of embryos was exposed to neutrons with doses between 20 and 50 mGy. This was the second important observation of NIBE.

4. Discussion

In the first part of the present study, it was found that bystander effect could be successfully induced in non-irradiated (***N–I***) embryos when they were partnered with irradiated (***I–N***) embryos which simultaneously shared the same medium, but the induction was successful only when the irradiated embryos were irradiated with 50 mGy of neutrons and unsuccessful when the irradiated embryos were irradiated with 100 mGy of neutrons. In the second part of the present study, it was found that bystander effect could be successfully induced in the naive (***CNaive***) embryos when they were introduced into the medium which had been conditioned by the (***Ir***) embryos irradiated with neutrons, but only for a certain neutron-dose range (20–50 mGy). These were first-ever observations of NIBE to the best of our knowledge. All previous studies reported no NIBE, including studies between cells *in vitro* (Liu et al., 2006; Seth et al., 2014) and studies between zebrafish *in vivo* (Wang et al., 2011). Moreover, the strategies employed in these two parts of the study to induce NIBE were different, namely, through directly partnering and through medium transfer. It was remarkable that both results agreed with a neutron-dose window (20–50 mGy) which could induce NIBE. In the following, we would first explore the reasons for the occurrence of such a neutron-dose window, and would then explore the potential reasons why previous studies failed to observe NIBE. Finally, suggestions on strategies to enhance the chance of observing NIBE in future would be provided.

The reasons behind the non-induction of bystander effect when the neutron doses received by the irradiated embryos were < 20 mGy could be two-fold, namely (1) the induction of neutron hormesis, and (2) the presence of a threshold dose for induction of bystander effect. Recently, Ng et al. (2015) studied the dose response of zebrafish embryos to neutron doses from 0.6 to 100 mGy generated by NASBEE using the same dose rate (i.e., 220 mGy/h) as that used in the present study. The authors proposed that neutron doses between 0.6 and 5 mGy led to neutron hormetic effects as evidenced by reductions in the amounts of apoptotic signals. As a result of neutron hormesis, the irradiated (***Ir***) groups of embryos

Table 3
The average net normalized apoptotic signals expressed as ($N^{N*} \pm$ SEM) obtained from embryos in the ***Naive*** groups (where dechorionated embryos were transferred into the previously collected medium $\boldsymbol{MD_{Ir}}$ at 5 hpf) and the ***CNaive*** groups (where dechorionated embryos were transferred into the previously collected medium $\boldsymbol{MD_{ShIr}}$ at 5 hpf). The medium which had previously been conditioned by the ***Neutron-irradiated (Ir)*** group of embryos for 19 h was referred to as $\boldsymbol{MD_{Ir}}$, while the medium which had previously been conditioned by the ***Sham-irradiated (ShIr)*** group of embryos for 19 h was referred to as $\boldsymbol{MD_{ShIr}}$.

	Neutron doses (mGy) for the corresponding *Ir* groups of embryos								
	1	5	10	15	20	35	50	70	200
$N_{Naive}{}^{N*}$	0.119 ± 0.098	0.089 ± 0.118	0.203 ± 0.171	0.346 ± 0.097	0.962 ± 0.195	0.792 ± 0.118	0.372 ± 0.192	-0.222 ± 0.074	0.018 ± 0.125
$N_{CNaive}{}^{N*}$	0.135 ± 0.185	-0.048 ± 0.078	-0.019 ± 0.081	0.174 ± 0.120	0.213 ± 0.116	0.477 ± 0.125	0.010 ± 0.087	-0.119 ± 0.122	-0.031 ± 0.104
D [a]	−0.016	0.137	0.222	0.171	**0.748**	**0.316**	**0.363**	−0.103	0.049
p value[b]	0.470	0.168	0.123	0.136	**0.001***	**0.036***	**0.047***	0.240	0.379

[a] D = mean apoptotic signal for **Naive group** (***Naive***) – mean apoptotic signal for **Control Naive** (***CNaive***) group
[b] *p* value obtained using Student *t*-test to compared the *Naive* and *CNaive* groups of embryos.
* Cases with $p \leq 0.05$ were considered to correspond to statistically significant differences.

irradiated with neutron doses ≤ 5 mGy (i.e., 1 and 5 mGy) in the present work were not expected to induce bystander effect on the ***Naive*** embryos, as a significant generation of bystander signals would rely on the extent of damages inflicted on the irradiated cells. As regards the threshold dose for induction of bystander effect, a threshold gamma-ray dose between 2 and 3 mGy on human skin cell line for inducting RIBE was reported (Liu et al., 2006). It would therefore be anticipated that there was also a threshold damage level and thus a threshold dose value below which no NIBE could be induced. The present results thus suggested the lower threshold dose value for neutrons to induce bystander effect on zebrafish embryos was between 15 and 20 mGy.

On the other hand, the non-induction of bystander effect when the neutron doses received by the irradiated embryos were >50 mGy was likely due to a phenomenon known as gamma-ray hormesis. Neutron radiation is always contaminated by gamma radiation and low-dose gamma rays could lead to hormesis response. Removal of aberrant cells through early apoptosis and induction of high-fidelity DNA repair were proposed as the underlying mechanisms of the gamma-ray hormesis (Portess et al., 2007; Bauer, 2007; Scott and Di Palma, 2006). In fact, it was also suggested that the lung cancers induced by alpha-particle irradiation could be suppressed by a small gamma-ray dose (Scott, 2008; Scott et al., 2008). Rithidech and Scott (2008) firstly demonstrated that the reduction in the frequency of micronucleated cells in neutron-irradiated human lymphocytes was due to gamma-ray hormesis. In their experiments, mono-energetic neutron sources with energies 0.22, 0.44, 1.5, 5.9 and 13.7 MeV were used to irradiate human lymphocytes with total absorbed doses of 10, 50 and 100 mGy. The associated gamma-ray doses were estimated to be 1%, 1%, 2%, 6%, and 6% for the five neutron energies, respectively. Ng et al. (2015) also demonstrated that gamma-ray hormesis was operative on zebrafish embryos when the neutron dose was increased to above 50 mGy with 7 mGy of gamma ray contamination. The gamma-ray hormesis was expected to mitigate the neutron-induced damages to below the threshold, which led to non-induction of bystander effect when the neutron doses received by the irradiated embryos were >50 mGy.

Taken together, the occurrence of a neutron-dose window which could induce NIBE was due to the requirement of neutron-induced damages to exceed the threshold level. When the neutron dose was too low (lower than $\sim$15–20 mGy), the damage level was inadequate. When the neutron dose was too high such that the associated gamma-ray dose (higher than $\sim$7 mGy) could trigger the gamma-ray hormesis to mitigate neutron-induced damages, the damage level again became inadequate.

As described earlier, previous studies reported that neutron irradiations could not induce RIBE between cells *in vitro* (Liu et al., 2006; Seth et al., 2014) or between zebrafish *in vivo* (Wang et al., 2011). Liu et al. (2006) employed neutrons produced through the ^{7}Li(p,n)^{7}Be reaction using 2.30 MeV protons, which generated neutrons with a broad spectrum up to 600 keV, together with gamma rays with an energy of 0.478 MeV produced through the competing ^{7}Li(p,p′) reaction. Liu et al. (2006) found no induction of bystander effect in human skin keratinocytes (human papillomavirus line G-HPV-G) from these neutrons with doses from 1 to 33 mGy. Here, the contribution from gamma rays was less than 3% of the neutron dose, i.e., <0.03–0.99 mGy, and was below the threshold of $\sim$7 mGy for induction of gamma-ray hormesis as described above. According to the results for zebrafish embryos from Ng et al. (2015), a certain part of this dose range (viz., 10–33 mGy) should have inflicted damages to the irradiated cells. As such, the non-induction of RIBE in the human skin keratinocytes for neutron irradiation doses from 1 to 33 mGy was likely due to that these doses were below the threshold for NIBE induction. The lower threshold gamma-ray dose (between 2 and 3 mGy) for inducing RIBE compared to that for neutrons (>33 mGy) might be explained by the different cellular recognition of DNA damages inflicted by neutrons and photons, as well as the different subsequent repair processes (Seth et al., 2014). Seth et al. (2014) also proposed that some critical bystander signaling pathways might not be activated because neutrons generated fewer oxidative damages and free radicals when compared to photons (Joiner and Van der Kogel, 2009). Using the same neutron source, Wang et al. (2011) also found no induction of bystander effect in the zebrafish having received signals from irradiated zebrafish when the neutron dose was $\sim$100 mGy. Here, the contribution from gamma rays was about 16%, i.e., 16 mGy, which was already above the threshold dose ($\sim$7 mGy) for inducing gamma-ray hormesis which was expected to have mitigated the neutron-induced damages to below the threshold for NIBE. Seth et al. (2014) also did not observe RIBE in normal human lymphoblastoid cell lines irradiated with 0.5, 1, 1.5, 2, 3 or 4 Gy neutrons with an average energy 17 MeV generated by bombarding near-monoenergetic 50.5 MeV protons at a Be target. The gamma-ray contamination was 5%, which was again above the threshold dose ($\sim$7 mGy) for inducing gamma-ray hormesis.

One special feature of the source employed by Liu et al. (2006) and Wang et al. (2011) was its broad spectrum up to 600 keV so it was possible that a certain amount of neutron doses received by the irradiated cells was contributed by low-energy neutrons which caused lesser damages. As a result, these irradiated cells had damages below the threshold levels and thus would not contribute to a successful induction of NIBE. Although it can be controversial to apply the Relative Biological Effectiveness (RBE) values or radiation weighting factors (W_R) for humans to non-human biota, the different W_R for neutrons with different energies (viz., 5 for <10 keV; 10 for 10–100 keV; 20 for >100 keV–2 MeV; 10 for >2 MeV–20 MeV; 5 for >20 MeV) do hint at different damages caused by neutrons with different energies. The use of a mono-energetic neutron source, such as the one used in the present work, which had an average energy of 2 MeV and higher than 600 keV, helped inflict sufficient damages to the cells with the same neutron dose or, in other words, helped decrease the lower threshold neutron dose (i.e., <33 mGy) for inducing NIBE. On the other hand, using a neutron source with a smaller gamma-ray contamination could help widen the energy window for NIBE through raising the upper threshold neutron dose for inducing gamma-ray hormesis. Implementation of these two measures could likely widen the neutron-dose window, which might enhance the chance of observing NIBE in future.

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References

Azzam, E.I., de Toledo, S.M., Gooding, T., Little, J.B., 1998. Intercellular communication is involved the bystander regulation of gene expression in human cells

exposed to very low fluences of alpha particles. Radiat. Res. 150, 497–504.
Barbazuk, W.B., Korf, I., Kadavi, C., Heyen, J., Tate, S., Wun, E., Bedell, J.A., McPherson, J.D., Johnson, S.L., 2000. The syntenic relationship of the zebrafish and human genomes. Genome Res. 10, 1351–1358.
Bauer, G., 2007. Low dose radiation and intercellular induction of apoptosis: potential implications for the control of oncogenesis. Int. J. Radiat. Biol. 83, 873–888.
Bladen, C.L., Lam, W.K., Dynan, W.S., Kozlowski, D.J., 2005. DNA damage response and Ku80 function in the vertebrate embryo. Nucleic Acids Res. 33, 3002–3010.
Blyth, B.J., Sykes, P.J., 2011. Radiation-induced bystander effects: what are they, and how relevent are they to human radiation exposures. Radiat. Res. 176, 139–157.
Calabrese, E.J., Baldwin, L.A., 2002. Defining hormesis. Hum. Exp. Toxicol. 21, 91–97.
Calabrese, E.J., Linda, A.B., 2003. Toxicology rethinks its central belief. Nature 421, 691–692.
Calabrese, E.J., 2008. Hormesis: why it is important to toxicology and toxicologists. Environ. Toxicol. Chem. 27, 1451–1474.
Choi, V.W.Y., Cheng, S.H., Yu, K.N., 2010a. Radioadaptive response induced by alpha-particle-induced stress communicated in vivo between zebrafish embryos. Environ. Sci. Technol. 44, 8829–8834.
Choi, V.W.Y., Lam, R.K.K., Chong, E.Y.W., Cheng, S.H., Yu, K.N., 2010b. Designing experimental setup and procedures for studying alpha-particle-induced adaptive response in zebrafish embryos *in vivo*. Nucl. Instrum. Methods B 268, 651–656.
Choi, V.W.Y., Ng, C.Y.P., Cheng, S.H., Yu, K.N., 2012a. α-Particle irradiated zebrafish embryos rescued by bystander unirradiated zebrafish embryos. Environ. Sci. Technol. 46, 226–231.
Choi, V.W.Y., Cheung, A.L.Y., Cheng, S.H., Yu, K.N., 2012b. Hormetic effect induced by alpha-particle-induced stress communicated in vivo between zebrafish embryos. Environ. Sci. Technol. 46, 11678–11683.
Choi, V.W.Y., Ng, C.Y.P., Kobayashi, A., Konishi, T., Suya, N., Ishikawa, T., Cheng, S.H., Yu, K.N., 2013. Bystander effect between zebrafish embryos *in Vivo* induced by high-dose X-rays. Environ. Sci. Technol. 47, 6368–6376.
Choi, V.W.Y., Yu, K.N., 2015. Embryos of the zebrafish Danio rerio in studies of non-targeted effects of ionizing radiation. Cancer Lett. 356, 91–104.
Daroczi, B., Kari, G., McAleer, M.F., Wolf, J.C., Rodeck, U., Dicker, A.P., 2006. *In vivo* radioprotection by the fullerene nanoparticle DF-1 as assessed in a zebrafish model. Clin. Cancer Res. 12, 7086–7091.
Geiger, G.A., Parker, S.E., Beothy, A.P., Tucker, J.A., Mullins, M.C., Kao, G.D., 2006. Zebrafish as a "biosensor"? Effects of ionzing radiation and amifostine on embryonic viability and development. Cancer Res. 66, 8172–8181.
Joiner, M.C., Van der Kogel, A., 2009. Basic Clinical Radiobiology. Macmillan Publishing Solutions, London, UK.
Kong, E.Y., Choi, V.W.Y., Cheng, S.H., Yu, K.N., 2014. Some properties of the signals involved in unirradiated zebrafish embryos rescuing α-particle irradiated zebrafish embryos. Int. J. Radiat. Biol. 90, 1133–1142.
Little, J.B., 2006. Cellular radiation effects and the bystander response. Mutat. Res. 597, 113–118.
Liu, Z., Mothersill, C.E., McNeill, F.E., Lyng, F.M., Byun, S.H., Seymour, C.B., Prestwich, W.V., 2006. A dose threshold for a medium transfer bystander effect for a human skin cell line. Radiat. Res. 166, 19–23.
Lorimore, S.A., Kadhim, M.A., Pocock, D.A., Papworth, D., Stevens, D.L., Goodhead, D. T., Wright, E.G., 1998. Chromosomal instability in the descendants of unirradiated surviving cells after alpha-particle irradiation. Proc. Natl. Acad. Sci. USA 95, 5730–5733.
Morgan, W.F., Sowa, M.B., 2007. Non-targeted bystander effects induced by ionizing radiation. Mutat. Res. 616, 159–164.
Mothersill, C., Seymour, C.B., 1997. Medium from irradiated human epithelial cells but not human fibroblasts reduces the clonogenic survival of unirradiated cells. Int. J. Radiat. Biol. 71, 421–427.
Mothersill, C., Smith, R.W., Agnihotri, N., Seymour, C.B., 2007. Characterization of a radiation-induced stress response communicated in vivo between zebrafish. Environ. Sci. Technol. 41, 3382–3387.
Nagasawa, H., Little, J.B., 1992. Induction of sister chromatid exchanges by extremely low doses of alpha-particles. Cancer Res. 52, 6394–6396.
Ng, C.Y.P., Kong, E.Y., Konishi, T., Kobayashi, A., Suya, N., Cheng, S.H., Yu, K.N., 2015. Low-dose neutron dose response of zebrafish embryos: neutron hormesis and gamma-ray hormesis. Radiat. Phys. Chem. 114, 12–17.
Prise, K.M., Belyakov, O.V., Folkard, M., Michael, B.D., 1998. Studies of bystander effects in human fibroblasts using a charged particle microbeam. Int. J. Radiat. Biol. 74, 793–798.
Prise, K.M., O'Sullivan, J.M., 2009. Radiation-induced bystander signalling in cancer therapy. Nat. Rev. Cancer 9, 351–360.
Portess, D.I., Bauer, G., Hill, M.A., O'Neill, P., 2007. Low-dose irradiation of non-transformed cells stimulates the selective removal of precancerous cells via intercellular induction of apoptosis. Cancer Res. 67, 1246–1253.
Rithidech, K.N., Scott, B.R., 2008. Evidence for radiation hormesis after *in vitro* exposure of human lymphocytes to low doses of ionizing radiation. Dose Response 6, 252–271.
Seth, I., Schwartz, J.L., Stewart, R.D., Emery, R., Joiner, M.C., Tucker, J.D., 2014. Neutron exposures in human cells: bystander effect and relative biological effectiveness. PLoS One 9, e98947. http://dx.doi.org/10.1371/journal.pone.0098947.
Scott, B.R., 2008. Low-dose-radiation stimulated natural chemical and biological protection against lung cancer. Dose Response 6, 299–318.
Scott, B.R., Sanders, C.L., Mitchel, R.E.J., Boreham, D.R., 2008. CT scans may reduce rather than increase the risk of cancer. J. Am. Phys. Surg. 13, 8–11.
Scott, B.R., Di Palma, J., 2006. Sparsely ionizing diagnostic and natural background radiation are likely preventing cancer and other genomic-instability associated diseases. Dose Response 5, 230–255.
Suda, M., Hagihara, T., Suya, N., Hamano, T., Takada, M., Konishi, T., Maeda, T., Ohmachi, Y., Kakinuma, S., Ariyoshi, K., Shimada, Y., Imaseki, H., 2009. Specifications of a neutron exposure accelerator system for biological effects experiments (NASBEE) in NIRS. Radiat. Phys. Chem. 78, 1216–1219.
Wang, C., Smith, R.W., Duhig, J., Prestwich, W.V., Byun, S.H., McNeill, F.E., Seymour, C.B., Mothersill, C.E., 2011. Neutrons do not produce a bystander effect in zebrafish irradiated *in vivo*. Int. J. Radiat. Biol. 87, 964–973.
Yum, E.H.W., Ng, C.K.M., Lin, A.C.C., Cheng, S.H., Yu, K.N., 2007. Experimental setup for studying the effects of alpha particles on zebrafish embryos. Nucl. Instrum. Methods B 264, 171–176.