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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