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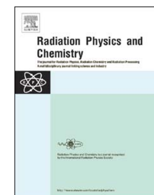

Photon hormesis deactivates alpha-particle induced bystander effects between zebrafish embryos

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ABSTRACT

In the present work, we studied the effects of low-dose X-ray photons on the alpha-particle induced bystander effects between embryos of the zebrafish, *Danio rerio*. The effects on the naive whole embryos were studied through quantification of apoptotic signals (amounts of cells undergoing apoptosis) at 24 h post fertilization (hpf) using vital dye acridine orange staining, followed by counting the stained cells under a fluorescent microscope. We report data showing that embryos at 5 hpf subjected to a 4.4 mGy alpha-particle irradiation could release a stress signal into the medium, which could induce bystander effect in partnered naive embryos sharing the same medium. We also report that the bystander effect was deactivated when the irradiated embryos were subjected to a concomitant irradiation of 10 or 14 mGy of X-rays, but no such deactivation was achieved if the concomitant X-ray dose dropped to 2.5 or 5 mGy. In the present study, the significant drop in the amount of apoptotic signals on the embryos having received 4.4 mGy alpha particles together X-rays irradiation from 2.5 or 5 mGy to 10 or 14 mGy, together with the deactivation of RIBE with concomitant irradiation of 10 or 14 mGy of X-rays supported the participation of photon hormesis with an onset dose between 5 and 10 mGy, which might lead to removal of aberrant cells through early apoptosis or induction of high-fidelity DNA repair. As we found that photons and alpha particles could have opposite biological effects when these were simultaneously irradiated onto living organisms, these ionizing radiations could be viewed as two different environmental stressors, and the resultant effects could be regarded as multiple stressor effects. The present work presented the first study on a multiple stressor effect which occurred on bystander organisms. In other words, this was a non-targeted multiple stressor effect. The photon hormesis could also explain some failed attempts to observe neutron-induced bystander effects in previous studies, as neutron sources invariably emit neutrons with concomitant gamma-ray photons, which is often referred to as gamma-ray contamination.

1. Introduction

The recent Fukushima reactor accident has rekindled immense concerns and interests on radioecological effects of nuclear fallouts. One of the most intriguing phenomena in radioecology is the allelopathy that coordinates a species-level survival response (Mothersill et al., 2007) towards ionizing radiation, which appears to be present at least in aquatic species living in close proximity and sharing the same media.

One essential element contributing to allelopathy was the radiation-induced bystander effect (RIBE) between living organisms (Choi et al., 2015; Mothersill et al., 2006, 2007, 2009; Smith et al., 2011, 2013; Surinov et al., 2005). RIBE was first discovered by Nagasawa and Little (1992) in an *in vitro* study, and the term RIBE was originally used to describe the non-targeted effects where non-irradiated cells responded as if they had themselves been irradiated upon receiving signals from irradiated cells through either partnering or medium transfer (Blyth and Sykes, 2011; Goldberg and Lehnert, 2002). It had been generally accepted that RIBE signals could affect distant or neighboring cells through diffusing soluble molecules from the irradiated cells into the medium conditioning the non-irradiated cells or through cellular gap-junction intercellular communication (Goldberg and Lehnert, 2002; Little, 2006; Morgan and Sowa, 2007; Mothersill and Kadhim, 2012; Mothersill and Seymour, 2001, 2004; Prise and O'Sullivan, 2009; Wang et al., 2015). RIBE was later also confirmed to occur between individuals of mice (Surinov et al., 2005), freshwater rainbow trout

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(Mothersill et al., 2006), zebrafish (*Danio rerio*) and Medaka (*Oryzias latipes*) (Mothersill et al., 2007, 2009), bullfrog tadpoles (*Rana catesbeiana*) (Audette-Stuart and Yankovicha, 2011), and zebrafish (*Danio rerio*) embryos (Choi et al., 2010a, 2012a, 2013; Yum et al., 2009).

Besides ionizing radiations, Asur et al. (2009) suggested chemicals could also induce bystander signaling. Various studies were carried out to investigate the capability of chemicals to induce bystander effect. For instance, Rugo et al. (2005) reported that the progeny of cells exposed to mitomycin C (MMC) could induce genomic instability in unexposed neighboring cells. The ability of chloroethyl nitrosourea, a chemotherapeutic DNA-alkylating agent, to induce the bystander effect through soluble factors in primary melanomas was also demonstrated by Demidem et al. (2006). Moreover, Cogan et al. (2010) showed that a short low dose of Cr (VI) could induce bystander signaling similar to those generated upon exposures to low doses of radiation. More recently, exposures of rat pheochromocytoma (PC12) cells to 10 mM of lead acetate were also found to induce bystander effects in neighboring cells (Guo et al., 2014).

RIBE could be induced by alpha particles (Azzam et al., 1998; Lorimore et al., 1998), X-ray or gamma-ray photons (Lyng et al., 2000; Mothersill and Seymour, 1997; Prise et al., 1998). Interestingly, however, all previous attempts to demonstrate RIBE induced by neutrons failed, including RIBE between cells (Liu et al., 2006; Seth et al., 2014) and RIBE between zebrafish (Wang et al., 2011). It is well established that neutron sources invariably emit neutrons with concomitant gamma-ray photons, which is often referred to as gamma-ray contamination. It has been proposed that such low-dose photon irradiations can help mitigate cellular damages in living organisms induced by other ionizing radiations. Such mitigation will be hereafter referred to as “photon hormesis” in the present work, which can mean gamma-ray hormesis or X-ray hormesis depending on the origin of the photons. The mechanisms underlying photon hormesis included removal of aberrant cells through early apoptosis and induction of high-fidelity DNA repair (Bauer, 2007; Portess et al., 2007; Scott and Di Palma, 2006). Photon hormesis has been proposed as an explanation for the suppression of alpha-particle-induced lung cancers (Scott, 2008; Scott et al., 2008), reduction in the frequency of micronucleated cells in neutron-irradiated human lymphocytes (Rithidech and Scott, 2008), and mitigation of the dose response of zebrafish embryos to neutrons (Ng et al., 2015a). Despite the fascinating proposal of and predictions for photon hormesis, it is indeed quite surprising that there have been no attempts to the best of our knowledge on proving that low-dose photons do mitigate cellular damages in living organisms induced by other ionizing radiations and deactivate RIBE between living organisms. Investigations on these issues formed the objectives of the present work.

In the present research, zebrafish (*Danio rerio*) embryos were chosen as the model for assessing RIBE between living organisms. Zebrafish embryos had been widely employed for examining biological effects of ionizing radiation (Bladen et al., 2005; Choi et al., 2010b, 2010c; Choi and Yu, 2015; Daroczi et al., 2006; Geiger et al., 2006; McAleer et al., 2005; Yum et al., 2007, 2009, 2010). The advantages of this model included its rapid development, high fecundity, and its genomes sharing considerable homology with human genomes (Barbazuk et al., 2000).

We hypothesize that photon hormesis can mitigate cellular damages in living organisms induced by other ionizing radiations, and can thus deactivate alpha-particle induced bystander effects between zebrafish embryos.

2. Materials and methods

2.1. Experimental animals

Adult zebrafish (*Danio rerio*) with mixed gender were kept in 45 L fish tanks with water maintained at 28.5 °C. A light-dark cycle of 14 h of light and 10 h of dark periods was adopted to maintain a good and stable production of embryos. When the photoperiod began, a specially designed plastic collector was introduced onto the bottom of each fish tank to collect the embryos. All embryos were collected within 30 min to ensure the synchronization of their developmental stages. Embryos were then kept in a 28.5 °C incubator until they reached 4 h post fertilization (hpf). Healthy developed embryos were selected under a stereomicroscope (Nikon, Chiyoda-ku, Tokyo, Japan) at the blastula period (i.e., at 4 hpf). Selected embryos were transferred into a clean Petri dish with a thin layer of agarose gel at the bottom and filled with E3 medium (5 mM NaCl, 0.33 mM $MgSO_4$, 0.33 mM $CaCl_2$, 0.17 mM KCl, and 0.1% methylene blue) for dechorionation using a pair of sharp forceps (Dumont, Hatfield, PA, USA).

2.2. Alpha-particle irradiation

An ^{241}Am source with alpha-particle energy of 5.49 MeV under vacuum and an activity of 4.26 kBq was employed in the present study. The setup for alpha-particle irradiation of zebrafish embryos largely followed that devised by Yum et al. (2007). A thin Mylar film (Dupont, Hong Kong) with a thickness of 3.5 µm was used as a biocompatible substrate during the irradiation. The Mylar film was glued to the bottom of a Petri dish, which had a hole with a diameter of 4 mm at the center, using an epoxy (Araldite Rapid, England). With such a setup, the embryos were irradiated with alpha particles coming from below through the support substrate to minimize energy absorption in the medium before the alpha particles could reach and hit the cells of the embryos.

2.3. X-ray irradiation

A self-contained X-ray irradiation system (X-RAD 320, Precision X-Ray (PXi), Connecticut, USA) with voltage and current set at 200 kVp and 2 mA, respectively, was employed in the present study to irradiate the zebrafish embryos. X-ray irradiation was made through a 2.5 mm thick filter made of aluminum, copper and tin. Under such a setting, the dose rate of irradiation was ~15 mGy/min.

2.4. Experimental protocols

In a previous study, Choi et al. (2012b) successfully demonstrated RIBE between zebrafish embryos after some of the 5 hpf embryos were irradiated with 4.4 mGy of alpha particles. As such, a similar experimental setting with the same alpha-particle dose was adopted to induce RIBE between zebrafish embryos. In the present work, low-dose 200 kVp X-ray photons were employed for the photon hormesis to mitigate cellular damages in the zebrafish embryos induced by the alpha particles. Ng et al. (2015a) demonstrated that gamma-ray hormesis became effective in zebrafish embryos when the gamma-ray dose reached between 7 and 10 mGy. Therefore, four X-ray doses, namely, 2.5 mGy (Condition 1), 5 mGy (Condition 2), 10 mGy (Condition 3) and 14 mGy (Condition 4), were employed, expecting that photon hormesis to be effective under Conditions 3 and 4 but not under Conditions 1 and 2. For simplicity, we represented the four conditions by the symbol “*Y*”, where *Y* could take the values of “2.5”, “5”, “10” or “14”, which corresponded to exposures to X-ray doses of 2.5, 5, 10 or 14 mGy, respectively.

For each set of experiment under each condition, dechorionated embryos were divided into 7 groups each having 8–10 embryos. These 7 groups were named as:

(1) AX_Y-*N* group: embryos which first received ~4.4 mGy **A**lpha-particle irradiation and level Y **X**-ray irradiation at 5 hpf, and which were then partnered with **N**on-irradiated embryos immediately after all irradiations;

(2) ***N-AX$_Y$*** group: Non-irradiated embryos which were partnered with embryos in the ***AX$_Y$-N*** group;
(3) ***A-N*** group: embryos which first received ~4.4 mGy Alpha-particle irradiation at 5 hpf, and which were then partnered with Non-irradiated embryos immediately after alpha-particle irradiation;
(4) ***N-A*** group: Non-irradiated embryos which were partnered with embryos in the ***A-N*** group;
(5) ***S-N*** group: Sham irradiated embryos which were partnered with Non-irradiated embryos at 5 hpf;
(6) ***N-S*** group: Non-irradiated embryos which were partnered with Sham irradiated embryos at 5 hpf;
(7) ***Control*** group: embryos without receiving any further treatment,

where embryos in the ***S-N*** group were sham irradiated with both alpha particles and X-ray (i.e., the embryos underwent the same irradiation processes as the ***AX$_Y$-N*** group but in the absence of the alpha-particle source and without switching on the X-ray beam).

In order to allow the embryos in the ***AX$_Y$-N*** group and ***N-AX$_Y$*** group to share the same medium in the same agarose dish during the experiment, two separated shallow regions were dredged on the agarose lining to accommodate the two groups of embryos. A similar setting was also applied to accommodate the embryos in the ***A-N*** group and ***N-A*** group in another dish, and also the ***S-N*** group and ***N-S*** group in a third dish. On the day of each experiment, the embryos were dechorionated at 4 hpf and separated into the seven groups as described above. When the embryos developed into 5 hpf, the embryos in the ***AX$_Y$-N*** group were irradiated with alpha particles (4.4 mGy) and then with X-ray photons (2.5 mGy under Condition 1, 5 mGy under Condition 2, 10 mGy under Condition 3, and 14 mGy under Condition 4). Immediately after irradiation, a glass dropper was used to carefully transfer the ***AX$_Y$-N*** group of embryos into one of the dredged regions on the agarose lining to partner with the ***N-AX$_Y$*** group of embryos which were accommodated in the neighboring dredged region. Under such a setting, the soluble factors communicating the bystander signals, expected to be generated by the ***AX$_Y$-N*** group, could reach the ***N-AX$_Y$*** group. Similarly, the embryos in the ***A-N*** group were irradiated with alpha particles (4.4 mGy) at 5 hpf and then partnered with the ***N-A*** group of embryos in another agarose dish with two separated shallow regions. As a control experiment, one more agarose dish was prepared to accommodate the ***S-N*** group and ***N-S*** group of embryos. Lastly, the ***Control*** group of embryos was accommodated in a new agarose dish to monitor the background signals of the embryos in the corresponding set of experiment. A volume of 3 mL of E3 medium was used in these four agarose dishes and all embryos were incubated in an incubator at 28.5 °C until they reached 24 hpf. The ambient temperatures during the alpha-particle irradiation, X-ray irradiation and also the sham irradiation of embryos were all separately measured. No significant changes in the temperature were observed during all these procedures, and the ambient temperature varied between 25.0 ± 0.5 °C and 26.5 ± 0.5 °C. As such, it was safe to neglect the potential effects induced by changes in temperature. A total of 6 replicates of experiments were carried out under all Conditions 1, 2, 3 and 4 separately. Fig. 1 provides schematic diagrams to illustrate the partnership schemes for all these groups of embryos while Fig. 2 gives a schematic diagram to show the flow of the experiments in the ***AX$_Y$-N***, ***N-AX$_Y$***, ***A-N***, ***N-A***, ***S-N***, ***N-S*** and ***Control*** groups.

2.5. Quantification of apoptosis by vital dye staining

The amounts of cells undergoing apoptosis, hereafter also referred to as the "apoptotic signals", within the whole 24 hpf zebrafish embryos were chosen as the biological endpoint in the present study. Such an endpoint has been widely adopted to quantify the effects of radiation on zebrafish embryos (Bladen et al., 2005; Geiger et al., 2006). When the embryos developed into 24 hpf, they were transferred into a medium with 2 µg/mL of a vital dye acridine orange (AO) (Sigma, St. Louis, MO, USA). Such a vital dye had been commonly employed to quantify the level of apoptosis in zebrafish embryos (Tucker and Lardelli, 2007; Yasuda et al., 2008; Mei et al., 2008). Embryos were kept in the staining solution in dark for 60 min and then washed twice in the culture medium to remove the excess dye. The apoptotic signals in the embryos became visible and countable under a fluorescent microscope as bright green spots. The embryos were anaesthetized using 0.0016 M tricaine (Sigma, St. Louis, MO, USA) before being examined under the fluorescent microscope. For each embryo, three images focusing on different sections of the embryo were captured with a magnification of 40×. The images were then combined into a single image for quantification of the apoptotic cells. A computer program was employed to count the apoptotic signals in each embryo.

2.6. Data analysis

Under each experimental condition (i.e., Conditions 1, 2, 3 and 4 as described in Section 2.4), six replicates of experiments were performed on different days. The number of apoptotic signals on each whole embryo was evaluated as explained above. The statistical significance of the differences between the six groups of embryos, i.e., the ***AX$_Y$-N***, ***N-AX$_Y$***, ***A-N***, ***N-A***, ***S-N*** and ***N-S*** groups, were first assessed by ANOVA. Cases with p values ≤0.05 were considered to correspond to statistically significant differences between at least two of the compared groups. In such cases, post-hoc t-tests were then performed to assess the statistical significance of difference between two particular groups of embryos. In particular, the presence of RIBE was assessed by studying the statistical significance between the ***N-AX$_Y$*** groups and the ***N-S*** groups, and between the ***N-A*** groups and the ***N-S*** groups under Conditions 1, 2, 3 and 4. Cases with p values ≤0.0033 (i.e., 0.05/15, where 15 was the total number of combinations) were considered to correspond to statistically significant differences between the compared groups.

3. Results

3.1. Effects of 2.5 and 5 mGy X-ray on alpha-particle-induced bystander effect

Under Condition 1, embryos in the ***AX$_{2.5}$-N*** group were first irradiated with ~4.4 mGy of alpha particles and 2.5 mGy of X-ray photons at 5 hpf and then partnered with non-irradiated embryos (***N-AX$_{2.5}$*** group). Similarly, under Condition 2, embryos in the ***AX$_5$-N*** group were first irradiated with ~4.4 mGy of alpha particles and 5 mGy of X-ray photons at 5 hpf and then partnered with non-irradiated embryos (***N-AX$_5$*** group). For both Condition 1 and 2, a total of 6 replicate experiments were performed separately on different days. The embryos were stained as described above and representative images of the stained embryos were shown in Fig. 3. The apoptotic signals on each embryo were counted. We denoted the mean number of apoptotic signals for the ***AX$_{2.5}$-N*** (or ***AX$_5$-N***), ***N-AX$_{2.5}$*** (or ***N-AX$_5$***), ***A-N***, ***N-A***, ***S-N***, ***N-S*** and ***Control*** groups as $N_{AX2.5-N}$ (or N_{AX5-N}), $N_{N-AX2.5}$ (or N_{AX5-N}), N_{A-N}, N_{N-A}, N_{S-N}, N_{N-S} and N_{ctrl}, respectively for Condition 1 (or Condition 2). If N_{ctrl} was interpreted as the average background apoptotic signals for embryos in the corresponding set of experiment under each experimental condition, the "net normalized apoptotic signals" for all the ***AX$_Y$-N***, ***N-AX$_Y$***, ***A-N***, ***N-A***, ***S-N*** and ***N-S*** groups could be determined as $N_{AXY-N}{}^{+}=[(N_{AXY-N}-N_{ctrl})/N_{ctrl}]$, $N_{N-AXY}{}^{+}=[(N_{N-AXY}-N_{ctrl})/N_{ctrl}]$, $N_{A-N}{}^{+}=[(N_{A-N}-N_{ctrl})/N_{ctrl}]$, $N_{N-A}{}^{+}=[(N_{N-A}-N_{ctrl})/N_{ctrl}]$, $N_{S-N}{}^{+}=[(N_{S-N}-N_{ctrl})/N_{ctrl}]$ and $N_{N-S}{}^{+}=[(N_{N-S}-N_{ctrl})/N_{ctrl}]$. The results for the 6 sets of experiments were shown in Table 1, in which the results were represented by ($N^{+}\pm SE$) where SE was the standard error.

From Table 1, the average net normalized apoptotic signals of the

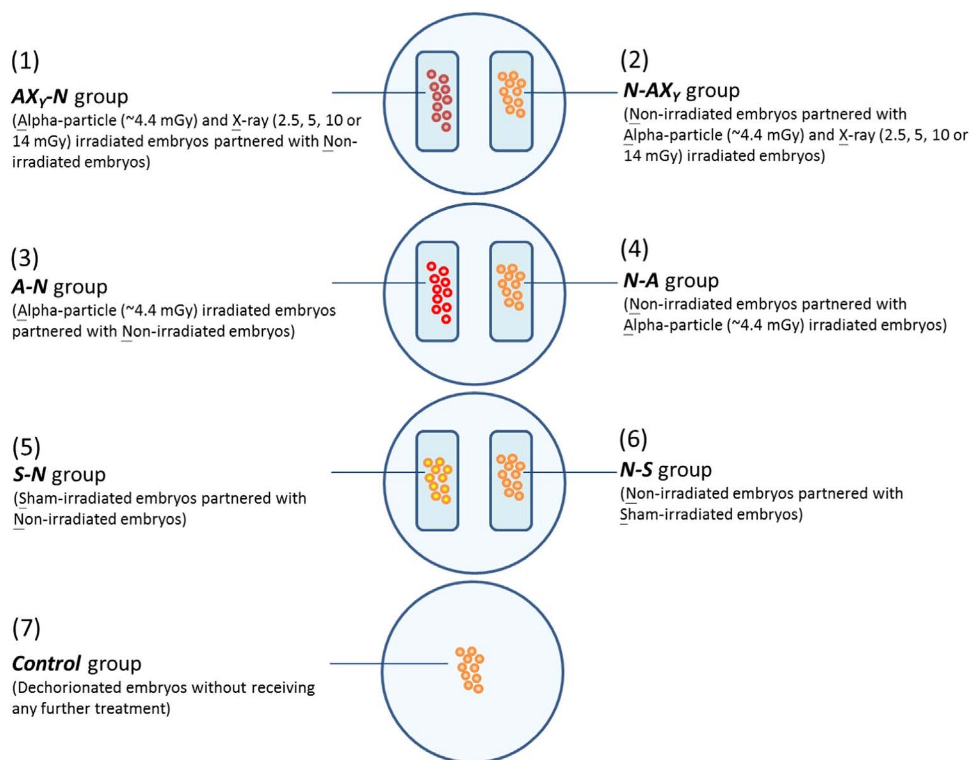

Fig. 1. Schematic diagrams showing the partnering of different groups of embryos. (a) ***AX_Y-N*** group partnered with ***N-AX_Y*** group; (b) ***A-N*** group partnered with ***N-A*** group; (c) ***S-N*** group partnered with ***N-S*** group; (d) ***Control*** group.

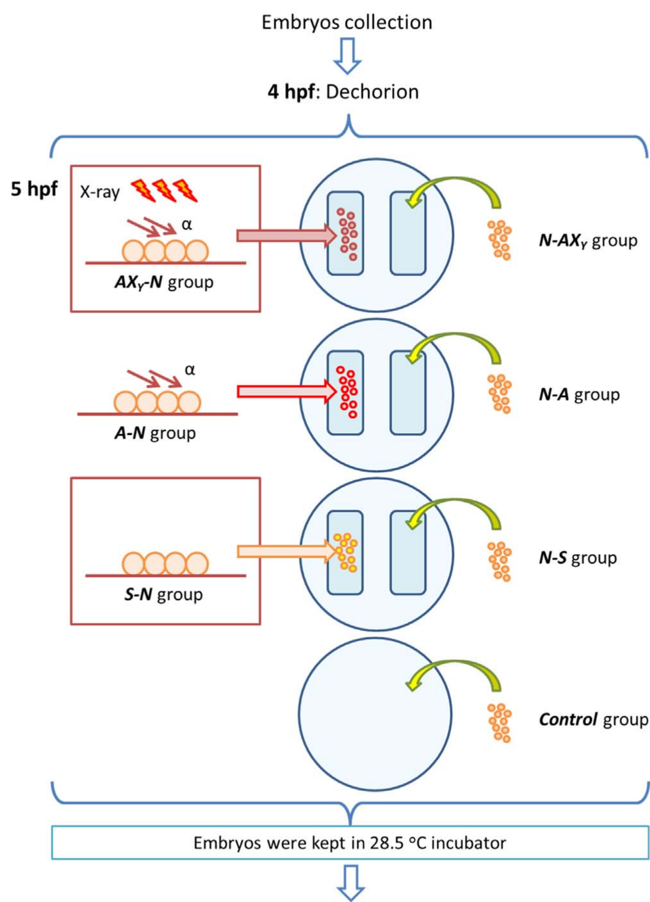

Fig. 2. Schematic diagrams showing the flow of experiments involving the ***AX_Y-N***, ***N-AX_Y***, ***A-N***, ***N-A***, ***S-N***, ***N-S*** and ***Control*** groups of embryos.

embryos in the ***$AX_{2.5}$-N*** and ***A-N*** groups under Condition 1, and in the ***AX_5-N*** and ***A-N*** groups under Condition 2 were all positive, showing that the amounts of apoptotic signals on the embryos in the ***$AX_{2.5}$-N*** and ***A-N*** groups (Condition 1) and in the ***AX_5-N*** and ***A-N*** groups (Condition 2) were higher than those in the corresponding ***Control*** groups. Under Condition 1, the differences among all groups of embryos were first assessed by ANOVA and, if statistically significant ($p \le 0.05$), further assessed by post-hoc *t*-tests between the ***$AX_{2.5}$-N*** and ***S-N*** groups, and between the ***A-N*** and ***S-N*** groups. Similar analysis procedures were applied to the ***$AX_{2.5}$-N***, ***A-N*** and ***S-N*** groups under Condition 2. The results are shown in Table 2. Clearly, both results indicate that exposing the embryos to 4.4 mGy of alpha particles with or without the addition of 2.5 or 5 mGy of X-ray photons would also lead to significant cellular damages in the embryos.

On the other hand, for the six sets of replicates in Table 1 under Condition 1, the positive values of the average net normalized apoptotic signals on the embryos in the ***N-$AX_{2.5}$*** group indicated that the amounts of apoptotic signals on the embryos in the ***N-$AX_{2.5}$*** groups were higher than those in the ***Control*** groups. Similar results were also observed under Condition 2 in Table 1 showing that the amounts of apoptotic signals on the embryos in the ***N-AX_5*** group were also all higher than those in the ***Control*** group. In order to further study whether RIBE could be successfully induced by embryos in the ***$AX_{2.5}$-N*** group and ***A-N*** group under Condition 1, post-hoc *t*-tests were performed between the ***N-$AX_{2.5}$*** and ***N-S*** groups, and between the ***N-A*** and ***N-S*** groups. The same procedures were applied to the ***N-AX_5***, ***N-A*** and ***N-S*** groups under Condition 2. The results are shown in Table 3. For all six sets of experiments under Condition 1, both the ***$AX_{2.5}$-N*** and ***A-N*** groups of embryos succeeded in introducing RIBE on the neighboring non-irradiated embryos. Similarly, both the ***AX_5-N*** and ***A-N*** groups of embryos under Condition 2 also demonstrated RIBE in all six sets of experiments.

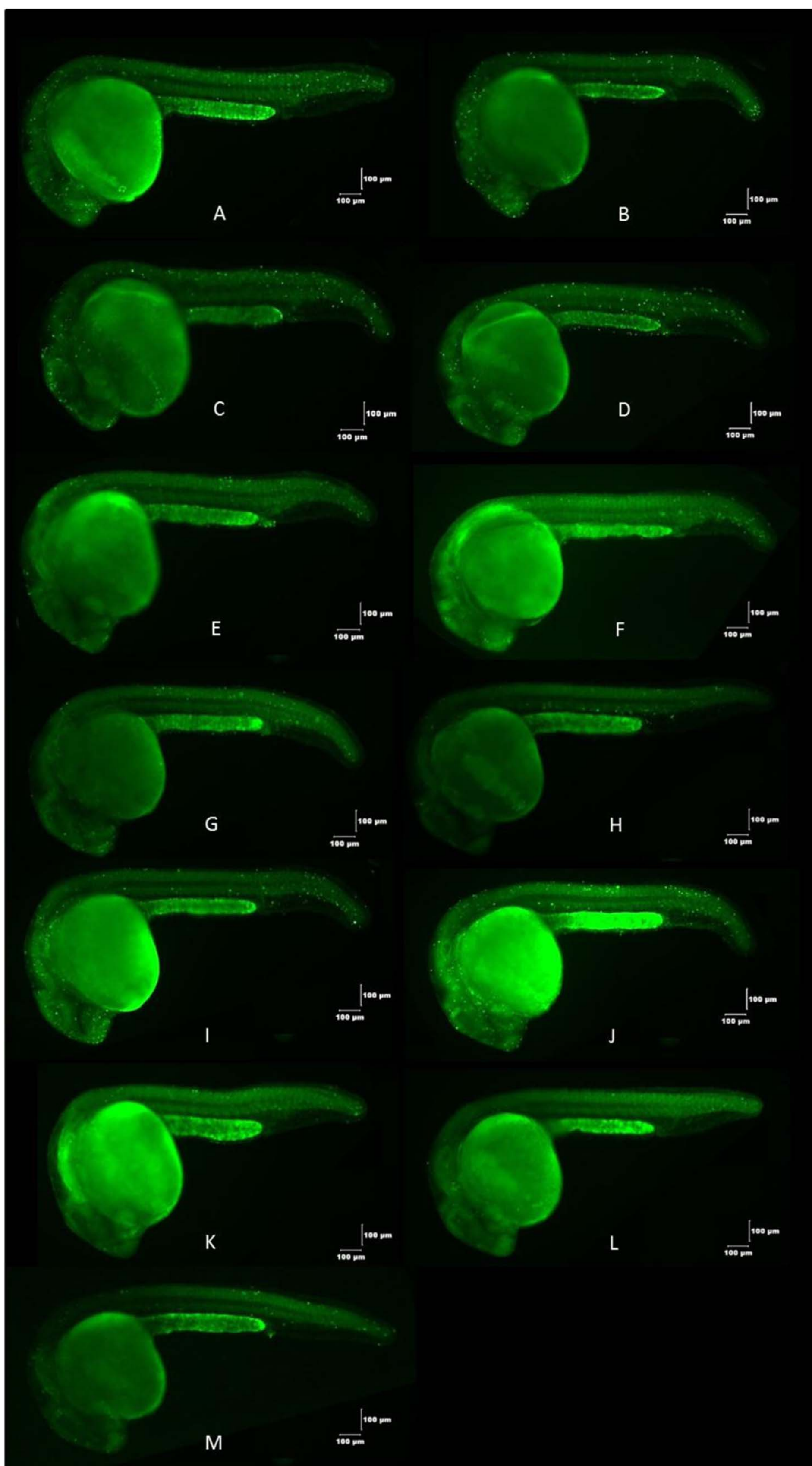

Fig. 3. Representative images of stained embryos in the (A) ***$AX_{2.5}$-N*** group, (B) ***N-$AX_{2.5}$*** group, (C) ***AX_{5}-N*** group, (D) ***N-AX_{5}*** group, (E) ***AX_{10}-N*** group, (F) ***N-AX_{10}*** group, (G) ***AX_{14}-N*** group, (H) ***N-AX_{14}*** group, (I) ***A-N*** group, (J) ***N-A*** group, (K) ***S-N*** group, (L) ***N-S*** group and (M) ***Control*** group, respectively. Images of embryos were captured by a florescent microscope with 40× magnification. For each embryo, a total of 3 images focusing on different sections of the embryos were captured and then combined into one image.

Table 1
The average net normalized apoptotic signals ($N^+ \pm SE$) obtained from embryos in the ***AX_Y-N*** **(**embryos which received ~4.4 mGy Alpha-particle irradiation and *Y* mGy X-ray irradiation at 5 hpf, and which were then partnered with embryos in the ***N-AX_Y*** group**)**, ***N-AX_Y*** (Non-irradiated embryos which were partnered with embryos in the ***AX_Y-N*** group), ***A-N*** **(**embryos which received ~4.4 mGy Alpha-particle irradiation at 5 hpf, and which were then partnered with embryos in the ***N-A*** group**)**, ***N-A*** **(**Non-irradiated embryos which were partnered with embryos in the **A-N** group**)**, ***S-N*** **(**Sham irradiated embryos which were partnered with embryos in the ***N-S*** group at 5 hpf**)**, ***N-S*** (Non-irradiated embryos which were partnered with embryos in the ***S-N*** group at 5 hpf) and ***Control*** groups in six sets of experiments under Conditions 1, 2, 3 and 4. The symbol "*Y*" could take values of "2.5", "5", "10" or "14", which corresponded to exposures to X-ray doses of 2.5 mGy (Condition 1), 5 mGy (Condition 2), 10 mGy (Condition 3) or 14 mGy (Condition 4), respectively.

Condition 1						
	$AX_{2.5}$-N	*N-$AX_{2.5}$*	*A-N*	*N-A*	*S-N*	*N-S*
N^+	1.2 ± 0.1	0.68 ± 0.07	0.80 ± 0.06	0.61 ± 0.05	0.062 ± 0.045	0.095 ± 0.034
n[a]	57	59	57	58	54	57
Condition 2						
	AX_5-N	*N-AX_5*	*A-N*	*N-A*	*S-N*	*N-S*
N^+	1.1 ± 0.1	0.48 ± 0.05	0.71 ± 0.07	0.52 ± 0.07	−0.060 ± 0.033	0.035 ± 0.033
n[a]	57	57	58	60	54	55
Condition 3						
	AX_{10}-N	*N-AX_{10}*	*A-N*	*N-A*	*S-N*	*N-S*
N^+	0.42 ± 0.05	0.046 ± 0.041	0.88 ± 0.07	0.72 ± 0.10	0.070 ± 0.040	0.020 ± 0.039
n[a]	55	56	55	53	56	50
Condition 4						
	AX_{14}-N	*N-AX_{14}*	*A-N*	*N-A*	*S-N*	*N-S*
N^+	0.51 ± 0.07	0.0024 ± 0.0287	0.99 ± 0.10	0.70 ± 0.09	0.061 ± 0.045	−0.011 ± 0.040
n[a]	55	54	55	57	55	53

[a] *n* represents the total number of embryos involved in a particular group.

Table 2
Results from ANOVA on the differences among all groups of embryos under Conditions 1 and 2, and from post-hoc *t*-tests on the differences between the ***AX_Y-N*** **(**embryos which received ~4.4 mGy Alpha-particle irradiation and *Y* mGy X-ray irradiation at 5 hpf, and which were then partnered with embryos in the ***N-AX_Y*** group**)**, the ***A-N*** **(**embryos which received ~4.4 mGy Alpha-particle irradiation at 5 hpf, and which were then partnered with embryos in the ***N-A*** group**)** and the ***S-N*** **(**Sham irradiated embryos which were partnered with embryos in the ***N-S*** group at 5 hpf**)**, under Conditions 1 and 2. The symbol "*Y*" could take values of "2.5" or "5", which corresponded to exposures to X-ray doses of 2.5 and 5 mGy, respectively.

	ANOVA	Post-hoc *t*-tests	
Condition 1	$F(5,336)=59.9$ $p^a=1.7\times10^{-44*}$	***$AX_{2.5}$-N* and *S-N*** $p^b=8.5\times10^{-24\#}$	***A-N* and *S-N*** $p^c=2.1\times10^{-17\#}$
Condition 2	$F(5,335) = 52.2$ $p^a=5.8\times10^{-40*}$	***AX_5-N* and *S-N*** $p^b=7.8\times10^{-22\#}$	***A-N* and *S-N*** $p^c=1.9\times10^{-15\#}$

[a] *p* values obtained from ANOVA.
[b] *p* values obtained using post-hoc *t*-tests for the difference between ***AX_Y-N*** and ***S-N*** groups.
[c] *p* values obtained using post-hoc *t*-tests for the difference between ***A-N*** and ***S-N*** groups.
[*] Cases with $p\leq0.05$ were considered statistically significant.
[#] Cases with $p\leq0.0033$ were considered statistically significant.

3.2. Effects of 10 and 14 mGy X-ray on alpha-particle-induced bystander effect

Under Condition 3, embryos in the ***AX_{10}-N*** group were first irradiated with ~4.4 mGy of alpha particles and 10 mGy of X-ray photons at 5 hpf and then partnered with non-irradiated embryos (***N-AX_{10}*** group). Under Condition 4, embryos in the ***AX_{14}-N*** group were first irradiated with ~4.4 mGy of alpha particles and 14 mGy of X-ray photons at 5 hpf and then partnered with non-irradiated embryos (***N-AX_{14}*** group). The experiments were repeated for 6 times independently under both Conditions 3 and 4. The number of apoptotic signals on each embryo was counted under the fluorescent microscope as described above. Representative images of stained embryos were shown in Fig. 3. The results for the 6 sets of experiments under Conditions 3 and 4 were shown in Table 1, in which the results were represented by ($N \pm SE$) where *SE* was the standard error.

From Table 1, the positive average net normalized apoptotic signals of the embryos in the ***AX_{10}-N*** and ***A-N*** groups under Condition 3, and in the ***AX_{14}-N*** and ***A-N*** groups under Condition 4 showed that the amounts of apoptotic signals on the embryos in the ***AX_{10}-N*** and ***A-N*** groups (Condition 3) and in the ***AX_{14}-N*** and ***A-N*** groups (Condition 4) were higher than those in the corresponding ***Control*** groups. Under both Conditions 3 and 4, the differences among all groups of embryos

Table 3
Results from ANOVA on the differences among all groups of embryos under Conditions 1 and 2, and from post-hoc *t*-tests on the differences between the ***N-AX_Y*** groups **(**Non-irradiated embryos partnered with embryos in ***AX_Y-N*** group, the latter having received ~4.4 mGy Alpha-particle irradiation and level *Y* of X-ray irradiation at 5 hpf), the ***N-A*** groups (Non-irradiated embryos partnered with embryos in ***A-N*** group) and the ***N-S*** groups (Non-irradiated embryos partnered with embryos in ***S-N*** group at 5 hpf), under Conditions 1 and 2. The symbol "*Y*" could take values of "2.5" or "5", which corresponded to exposures to X-ray doses of 2.5 and 5 mGy, respectively.

	ANOVA	Post-hoc *t*-test			
Condition 1	$F(5,336)=59.9$ $p^a=1.7\times10^{-44*}$	***N-$AX_{2.5}$* and *N-S*** $p^b=4.7\times10^{-12\#}$	***RIBE***[d] Yes	***N-A* and *N-S*** $p^c=3.7\times10^{-14\#}$	***RIBE***[d] Yes
Condition 2	$F(5,335)=52.2$ $p^a=5.8\times10^{-40*}$	***N-AX_5* and *N-S*** $p^b=5.7\times10^{-11\#}$	***RIBE***[d] Yes	***N-A* and *N-S*** $p^c=1.2\times10^{-9\#}$	***RIBE***[d] Yes

[a] *p* values obtained from ANOVA.
[b] *p* values obtained using post-hoc *t*-tests on the difference between ***N-AX_Y*** and ***N-S*** groups.
[c] *p* values obtained using post-hoc *t*-tests on the difference between ***N-A*** and ***N-S*** groups.
[d] "Yes": referring to cases with RIBE; "No": referring to cases without RIBE.
[*] Cases with $p\leq0.05$ were considered statistically significant.
[#] Cases with $p\leq0.0033$ were considered statistically significant.

Table 4
Results from ANOVA on the differences among all groups of embryos under Conditions 3 and 4, and from post-hoc *t*-tests on the differences between the ***AX_Y-N*** **(**embryos which received ~4.4 mGy Alpha-particle irradiation and *Y* mGy X-ray irradiation at 5 hpf, and which were then partnered with embryos in the ***N-AX_Y*** group**)**, the ***A-N*** **(**embryos which received ~4.4 mGy Alpha-particle irradiation at 5 hpf, and which were then partnered with embryos in the ***N-A*** group**)** and the ***S-N*** **(Sham irradiated embryos which were partnered with embryos in the** ***N-S*** **group at 5 hpf),** under Conditions 3 and 4. The symbol "*Y*" could take values of "10" or "14", which corresponded to exposures to X-ray doses of 10 and 14 mGy, respectively.

	ANOVA	Post-hoc *t*-tests	
Condition 3	*F* (5,319) =38.3	***AX_{10} -N* and *S-N***	***A-N* and *S-N***
	p^a=1.0×10^{-30*}	p^b=2.7×10$^{-7\#}$	p^c=1.8×10$^{-16\#}$
Condition 4	*F*(5,323)=37.9	***AX_{14} -N* and *S-N***	***A-N* and *S-N***
	p^a=1.5×10^{-30*}	p^b=1.1×10$^{-7\#}$	p^c=3.4×10$^{-12\#}$

[a] *p* values obtained from ANOVA.
[b] *p* values obtained using post-hoc *t*-tests on the difference between ***AX_Y-N*** and ***S-N*** groups.
[c] *p* values obtained using post-hoc *t*-tests on the difference between ***A-N*** and ***S-N*** groups.
[*] Cases with *p*≤0.05 were considered statistically significant.
[#] Cases with *p*≤0.0033 were considered statistically significant.

were first assessed by ANOVA and, if statistically significant (*p*≤0.05), further assessed by post-hoc *t*-tests between the ***AX_{10}-N*** and ***S-N*** groups, and between the ***A-N*** and ***S-N*** groups under Condition 3; between the ***AX_{14}-N*** and ***S-N*** groups, and between the ***A-N*** and ***S-N*** groups under Condition 4. The results are shown in Table 4. The results indicated that irradiating embryos with or without 10 or 14 mGy of X-ray on top of 4.4 mGy of alpha-particle irradiation would also lead to cellular damages in the embryos.

Interestingly, as shown in Table 1, the values of average net normalized apoptotic signals on the embryos in the ***N-AX_{10}*** or ***N-AX_{14}*** groups were small. To further examine if RIBE could be induced successfully by embryos in the ***AX_{10}-N*** group and ***A-N*** group (under Condition 3), or by embryos in the ***AX_{14}-N*** group and ***A-N*** group (under Condition 4), post-hoc *t*-tests were performed between the ***N-AX_Y*** and ***N-S*** groups, and also between the ***N-A*** and ***N-S*** groups to examine the differences between these groups. Cases with *p*≤0.0033 (0.05/15) in the post-hoc *t*-tests were considered statistically significant. The results are shown in Table 5.

Similar to the results obtained under Conditions 1 and 2, the ***A-N*** group of embryos could successfully introduce RIBE to the neighboring non-irradiated embryos in all the six sets of experiments under both Conditions 3 and 4. However, under Condition 3 in Table 5 showed that the embryos in the ***AX_{10}-N*** group failed to trigger RIBE on neighboring non-irradiated embryos. At the same time, the data from the six sets of experiments shown under Condition 4 in Table 5 also consistently showed that the embryos in the ***AX_{14}-N*** group failed to trigger RIBE on neighboring non-irradiated embryos. Such results strongly suggested that an additional 10 or 14 mGy of X-ray photons on embryos already irradiated with 4.4 mGy of alpha particles would deactivate the RIBE.

Table 5
Results from ANOVA on the differences among all groups of embryos under Conditions 3 and 4, and from post-hoc *t*-tests on the differences between the ***N-AX_Y*** groups **(**Non-irradiated embryos partnered with embryos in ***AX_Y-N*** group, the latter having received ~4.4 mGy Alpha-particle irradiation and level *Y* of X-ray irradiation at 5 hpf), the ***N-A*** groups (Non-irradiated embryos partnered with embryos in ***A-N*** group) and the ***N-S*** groups (Non-irradiated embryos partnered with embryos in ***S-N*** group at 5 hpf), under Conditions 3 and 4. The symbol "*Y*" could take values of "10" or "14", which corresponded to exposures to X-ray doses of 10 and 14 mGy, respectively.

	ANOVA	Post-hoc *t*-test			
Condition 3	*F*(5,319)=38.3	***N-AX_{10}* and *N-S***	***RIBE***[d]	***N-A* and *N-S***	***RIBE***[d]
	p^a=1.0×10^{-30*}	p^b=0.32	No	p^c=3.8×10$^{-9\#}$	Yes
Condition 4	*F*(5,323)=37.9	***N-AX_{14}* and *N-S***	***RIBE***[d]	***N-A* and *N-S***	***RIBE***[d]
	p^a=1.5×10^{-30*}	p^b=0.39	No	p^c=8.4×10$^{-11\#}$	Yes

[a] *p* values obtained from ANOVA.
[b] *p* values obtained using post-hoc *t*-tests on the difference between ***N-AX_Y*** and ***N-S*** groups.
[c] *p* values obtained using post-hoc *t*-tests on the difference between ***N-A*** and ***N-S*** groups.
[d] "Yes": referring to cases with RIBE; "No": referring to cases without RIBE.
[*] Cases with *p*≤0.05 were considered statistically significant.
[#] Cases with *p*≤0.0033 were considered statistically significant.

4. Discussion

In the present work, we successfully showed that photon hormesis was operative at a photon dose of 10 and 14 mGy (but not at 2.5 and 5 mGy), which could probably mitigate cellular damages in the zebrafish embryos induced by 4.4 mGy of alpha particles, and which deactivated alpha-particle induced bystander effects between zebrafish embryos. The potential deactivation of RIBE by photon hormesis would bear important consequences on realistic radioecological risk assessments. As explained in the Introduction, RIBE between living organisms is an essential component contributing to allelopathy, so the possible consequences of deactivating the RIBE would deserve more discussion. In a previous study, we demonstrated that zebrafish embryos irradiated by alpha particles released a stress signal into the water to induce radioadaptive response in the naive embryos (Choi et al., 2010a) sharing the same medium, which enabled an effective protection of the population against possible subsequent large radiation exposures. In a subsequent study, we also revealed that the irradiated zebrafish embryos actually also derived benefits from the naive embryos through the rescue effect (Choi et al., 2012b). However, it was believed that derivation of benefits was only a fortuitous by-product rather than a final objective. This was corroborated by the induction of hormetic effect in naive zebrafish embryos by alpha-particle irradiated embryos, from which the irradiated embryos themselves appeared not to have derived additional benefits (Choi et al., 2012a). Following these observations and arguments, it was reasonable to propose that RIBE in the zebrafish embryos was deactivated as a result of photon hormesis because such combination of ionizing radiation (alpha particles + photons) would not pose threat to the population, rather than because the irradiated embryos did not need further help from the naive embryos.

Now that we showed that photons and alpha particles could have opposite biological effects when these were simultaneously irradiated onto living organisms, these ionizing radiations could be viewed as two different environmental stressors, and the resultant effects could be regarded as "multiple stressor effects". In realistic situations, living organisms are exposed to a combination of environmental stressors. These environmental stressors usually include ionizing radiation, heavy metal and environmental impacts (e.g., climate change, habitat loss). Although multiple stressor effects are far from being fully understood, more and more evidence has pointed out that the combined effects can be far more complicated than the simple sum of the effects from individual stressors. The combined effect of alpha particles and photons on a living organism found in the present work was a good example to illustrate this. Moreover, most studies on multiple stressor effect were carried out in the cells or organisms which were themselves subjected to the stressors. The present work presented the first study on a multiple stressor effect which occurred on bystander organisms. In other words, this was a non-targeted multiple stressor effect.

On the other hand, due to the short range of alpha particles and the layered structure of cells in the zebrafish embryos, and due to the small fluence of alpha particles employed, the proportion of cells directly hit by alpha particles was expected to be small. As such, the observed photon hormesis might alternatively be explained by the suppression of propagation of bystander signals from the irradiated cells to non-

irradiated cells within a zebrafish embryo by the X-ray photons. Apparently, more extensive studies would be needed before we can have a better understanding on this newly studied effect.

The onset dose between 5 and 10 mGy for photon hormesis found in the present work agreed with the finding of Ng et al. (2015a) who showed that gamma-ray hormesis became effective in zebrafish embryos when the gamma-ray dose reached between 7–10 mGy, and also agreed with the finding of Ng et al. (2015b) who showed that the upper limit of the neutron dose window for inducing RIBE in zebrafish embryos was between 50 and 70 mGy (with 7–10 mGy of gamma ray contamination). Assuming the onset photon dose as 10 mGy, some non-observations of RIBE induced by neutrons in previous studies could be explained by the photon hormesis. Wang et al. (2011) found that no bystander effect was induced in naïve zebrafish by zebrafish irradiated by neutrons with a dose of ~100 mGy. The gamma-ray contamination was about 16%, i.e., 16 mGy, which was above the onset dose of ~10 mGy. Seth et al. (2014) also observed no bystander effect in normal human lymphoblastoid cell lines irradiated by neutrons with doses of more than 0.5 Gy. The gamma-ray contamination was 5%, i.e., beyond 25 mGy, which was again above the onset dose for photon hormesis. In these two studies, the photon hormesis was operative, which deactivated the RIBE. However, the non-observation of bystander effect induced by neutrons with doses from 1 to 33 mGy in human skin keratinocytes by Liu et al. (2006) could not be explained by the photon hormesis, since the gamma-ray contamination was less than 3% of the neutron dose, i.e., < 1 mGy, and was below the onset dose of ~10 mGy. The failure to observe RIBE in that study was likely due to the insufficient damage level inflicted by neutrons with doses smaller than 33 mGy for induction of bystander effect (Ng et al., 2015b).

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