International Journal of Radiation Biology, December 2014; 90(12): 1133–1142

ISSN 0955-3002 print / ISSN 1362-3095 online
DOI: 10.3109/09553002.2014.932031

informa
healthcare

Some properties of the signals involved in unirradiated zebrafish embryos rescuing α-particle irradiated zebrafish embryos

E. Y. Kong[1], V. W. Y. Choi[1], S. H. Cheng[2,3] & K. N. Yu[1,2,3]

Departments of [1]Physics and Materials Science and [2]Biomedical Sciences and [3]State Key Laboratory in Marine Pollution, City University of Hong Kong

Abstract

Purpose: **The in vivo radiation-induced bystander effect (RIBE) and radiation-induced rescue effect (RIRE) induced between embryos of the zebrafish (*Danio rerio*) by alpha-particle irradiation were studied through the number of apoptotic signals revealed at 24 h post fertilization (hpf) through vital dye acridine orange staining.**

Materials and methods: **RIBE and RIRE were verified through the significant increase and decrease in apoptotic signals in the partnered bystander and irradiated embryos, respectively.**

Results: **The medium transfer experiment where irradiated zebrafish embryos were rescued through immersion in the medium previously conditioned by a larger number of irradiated zebrafish embryos showed (a) the involvement of a released stress signal in the induction of RIRE, and (b) RIBE and RIRE signals had the same function. With the help of 500 μM of the specific nitric oxide (NO) scavenger cPTIO (2-(4carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide), NO was confirmed as an essential signaling molecule for inducing both the RIBE and RIRE. On the other hand, the treatment with 20 μM of the carbon monoxide (CO) releasing chemical CORM-3 (tricarbonylchloro(glycinato)ruthenium (II)) suppressed the manifestations of RIBE but did not suppress RIRE.**

Conclusions: **In conclusion, unirradiated zebrafish embryos need NO but not NO-induced damages to rescue α-particle irradiated zebrafish embryos.**

Keywords: Bystander effect, alpha emitters, nitric oxide

Introduction

Many radioecological effects of nuclear fallouts have remained unknown, and these justify more extensive studies, particularly in view of the far-reaching consequences of the recent Fukushima reactor accident. A particularly interesting phenomenon is the allelopathy aimed at coordinating a species-level survival response (Mothersill et al. 2007) against ionizing radiation, which has been found at least in aquatic species that are close to one another and sharing the same media.

One remarkable finding was the discovery of a radiation-induced bystander effect (RIBE) between living organisms (Surinov et al. 2005, Mothersill et al. 2006a, 2007, 2009, Smith et al. 2011, 2013, Choi and Yu 2014). Initially, RIBE was referred to a phenomenon in cells where unirradiated cells responded as if they had themselves been irradiated after receiving signals from irradiated cells. RIBE was first illustrated in an in vitro study (Chinese hamster ovary cells) by Nagasawa and Little (1992) using the frequency of sister chromatid exchanges as the biological endpoint. Two mechanisms underlying RIBE have now been widely accepted, namely, through (a) gap junction intercellular communication in the presence of cell-cell contact, and (b) soluble molecules released by the irradiated cells into the cell culture medium. The soluble molecules involved in RIBE include nitric oxide (NO), reactive oxygen species (ROS), cytokines, transforming growth factor-β1 (TGF-β1), tumor necrosis factor-α (TNF-α), etc. There are many reviews on RIBE (e.g., Mothersill and Seymour 2001, 2004, Goldberg and Lehnert 2002, Little 2006, Morgan and Sowa 2007, Mothersill and Kadhim 2012, Wang et al. 2014). Subsequently, RIBE was also found to occur between organisms. Surinov et al. (2005) discovered that unirradiated mice responded as if they had been irradiated after they were housed together with irradiated mice, and found that the signals for inducing the RIBE were transmitted through urine. Similarly, Mothersill et al. (2006a) also succeeded in demonstrating that X-ray irradiated freshwater rainbow trout (*Oncorhynchus mykiss*, *W*) released bystander signals into the water to induce bystander effects in unirradiated partners. Mothersill et al. (2007, 2009) further demonstrated RIBE between zebrafish (*Danio rerio*) and Medaka (*Oryzias latipes*). Audette-Stuart and Yankovicha (2011) observed adaptive responses in control bullfrog tadpoles (*Rana catesbeiana*) which were partnered with tadpoles that had been previously housed in tritiated water. Our group also demonstrated that alpha-particle irradiated embryos of the zebrafish *Danio rerio* could also release bystander signals into the water to induce bystander effects in unirradiated

Correspondence: Prof. K. N. Yu, Department of Physics and Materials Science, City University of Hong Kong. Tel: + 1 (852) 34427812. Fax: + 1 (852) 34420538. E-mail: peter.yu@cityu.edu.hk

(Received 17 February 2014; revised 3 June 2014; accepted 3 June 2014)

naïve zebrafish embryos (Yum et al. 2009, Choi et al. 2010a, 2012a, 2013).

More recently, another interesting discovery that is highly relevant to radioecology was the radiation-induced rescue effect (RIRE) between living organisms (Choi and Yu 2014). RIRE describes the phenomenon that irradiated cells or irradiated organisms derive benefits from the feedback signals sent from the bystander unirradiated cells or organisms, e.g., the unirradiated cells or organisms help the irradiated cells or organisms mitigate the radiation induced DNA damages. Our group first discovered RIRE in cells in an in vitro study (Chen et al. 2011) involving human primary fibroblast (NHLF) and human cancer cells (HeLa) in a two-cell co-culture system. When the irradiated cells were co-cultured with the bystander cells, there was a significant decrease in the numbers of p53-binding protein 1 (53BP1) foci, micronucleus formation and extent of apoptosis (Chen et al. 2011). Subsequently, our group further reported RIRE in an in vivo study using embryos of the zebrafish *Danio rerio* (Choi et al. 2012b). When the alpha-particle irradiated zebrafish embryos shared the same water medium with unirradiated zebrafish embryos, the number of apoptotic signals in the irradiated embryos quantified at 24 h post fertilization (hpf) became significantly smaller. This hinted that the irradiated zebrafish embryos released a stress signal into the water, which could be communicated to the unirradiated zebrafish embryos, and then these unirradiated zebrafish embryos released a feedback rescue signal back to the irradiated embryos. Furthermore, we had demonstrated that the strength of the rescue effect increased with the number of rescuing bystander unirradiated embryos, while keeping the number of irradiated embryos unchanged (Choi et al. 2012b). Nevertheless, the involvement of a stress signal released in the induction of RIRE has not yet been directly proved.

Radiation-induced stress communication between organisms has appeared to be a universal phenomenon. While the more well-known RIBE represents a one-way communication, the RIRE results described above demonstrated a two-way communication. RIBE can benefit the bystander population, for examples, in terms of induction of radioadaptive response (RAR) and hormetic effect (Choi et al. 2010a, 2012a). RAR occurs when a small preceding priming dose decreases the biological effectiveness of a subsequent large challenging dose. Hormetic responses are characterized as biphasic dose-response relationships exhibiting a low-dose stimulation and a high-dose inhibition. The benefit in the bystander population derived from RIBE strongly suggested an allelopathic effect aimed at coordinating a population response. Confirmation of RIRE further revealed that the irradiated organisms could also derive benefits by communicating the signals of RIBE to the bystander organisms in formulating this population response.

To better understand the RIRE, the underlying mechanisms need to be examined in more detail, including the confirmation of the involvement of a stress signal in the induction of RIRE. In the present work, the involvement of a released stress signal in the induction of RIRE would be studied through medium-transfer experiments. Furthermore, the similarity between the functions of chemical signals for RIBE and RIRE would be demonstrated.

The role played by NO in RIRE will also be explored. NO is an important signaling molecule, which can be generated under inflammatory conditions, carcinogenesis and circulatory shock (Shin et al. 2007). NO was found in various research works (Hei et al. 2008, Prise and O'Sullivan 2009) to be involved in mediating RIBE through the medium. In the present work, the role played by NO will be studied through the treatment using the specific NO scavenger called cPTIO (2-(4carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide).

In relation to studying the involvement of NO as a stress signal in the induction of RIRE, the role played by NO-induced damages in the bystander embryos in RIRE will also be explored. In terms of radiosensitization, the role of NO is still not fully understood. In fact, NO can be both a prooxidant and an antioxidant (Bryan et al. 2012) or, in other words, can be both a radiosensitizer and a radioprotector. Previous researches revealed that it could induce both cytostasis and cytotoxicity to tumor cells and the effects to our body depended on its concentration (Paduch and Kanderfer-Szerszen 2011). Low NO levels would promote tumor cell proliferation. NO could also be oxidized to form peroxynitrite ($ONOO^-$) which would induce apoptosis. On the contrary, a high NO concentration could act as an antioxidant and prevent apoptosis. Given the different possible manifestations of NO in the bystander cells, it is indeed intriguing to study whether the RIRE arises as a response to the NO-induced damages in the bystander embryos, or operates independently of these manifested damages, in order to achieve allelopathy. Li et al. (2006, 2007) reported the protection of PC12 rat cells from the cytotoxicity of NO through the use of low-concentration carbon monoxide (CO). More recently, our group succeeded in mitigating in vitro RIBE by low-concentration CO (Han et al. 2010, Tong et al. 2014), and attributed the mitigation to impairment in the response of bystander cells (Chinese hamster ovary) to NO instead of perturbation in the release of NO by the irradiated cells (Chinese hamster ovary) (Han et al. 2011). Subsequently, our group further demonstrated the mitigation of in vivo RIBE between zebrafish embryos by CO treatment, also in a concentration dependent manner (Choi et al. 2012c). In the present work, the role played by NO-induced damages in RIRE will be studied through the treatment using the CO releasing chemical CORM-3 (Tricarbonylchloro(glycinato) ruthenium [II]).

Thanks to their unique features, embryos of the zebrafish, *Danio rerio*, were again employed as the model for studying RIRE between organisms in the present work. In particular, zebrafish embryos were a well-established model for studying DNA damage response from ionizing radiation (Bladen et al. 2005, McAleer et al. 2005, Daroczi et al. 2006, Geiger et al. 2006, Yum et al. 2007, 2009, 2010, Choi et al. 2010b, 2010c, Choi and Yu 2014). Its rapid embryonic development permitted relatively short experimental turnover time while its optically transparent appearance facilitated relatively convenient analyses with optical inspection and fluorescent-dye staining methods. Incidentally, the human and

zebrafish genomes share considerable homology, including conservation of most DNA repair-related genes (Barbazuk et al. 2000).

We hypothesize that: (1) RIRE involves a stress signal released from the bystander unirradiated embryos, which has similar function to the signal released by the irradiated embryos, and (2) unirradiated zebrafish embryos need the signaling molecule NO, which is essential for mediating RIBE to the unirradiated embryos, but not the NO-induced damages to initiate the process to rescue the α-particle irradiated zebrafish embryos.

Method and materials

Zebrafish maintenance

Approximately 35 adult zebrafish of both genders were kept in a 45-l tank. The water temperature was maintained at 28°C using thermostats. The fish were maintained under a 14/10 h light dark cycle and were fed four times daily with fish food (TetraMin, Melle, Germany) and brine shrimp (Brine Shrimp Direct, Ogden, Utah, USA). To ensure synchronization of the embryonic stages, the embryos were collected using specially designed plastic collectors (Choi et al. 2010c) within 30 min from the start of the light-induced spawning. The collected embryos were incubated at a temperature of 28.5°C until 4 hpf. Healthy developing embryos were then manually selected using a stereomicroscope (Nikon, Chiyoda-ku, Tokyo, Japan), transferred into a Petri dish lined with an agarose (Invitrogen, Life Technologies Corporation, Carlsbad, CA, USA) gel layer and filled with 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 manually dechorionated using a pair of sharp forceps (Dumont, Hatfield, PA, USA) under the stereomicroscope.

Chemicals

CORM-3 was synthesized from the tricarbonyldichlororuthenium(II) dimer (CORM-2 dimer, Sigma Aldrich, St. Louis, MO, USA) (Clark et al. 2003). A 50 mM CORM-3 stock solution was prepared by dissolving it in deionized water and was stored at − 20°C. On the other hand, a 500 mM stock solution of cPTIO (Life Technologies Corporation, Carlsbad, CA, USA) was prepared by dissolving it in denionized water and was then stored at − 20°C. In the present experiments, 20 μM CORM-3 and 500 μM cPTIO solutions were employed, which were prepared by dissolving the CORM-3 stock solution in E3 solution and by dissolving the cPTIO stock solution in deionized water, respectively.

Inactive CORM-3 (iCORM-3) was also required as a negative control to confirm that the effect was due to the released CO but not from CORM-3 itself. It was prepared by first leaving the 20 μM CORM-3 at room temperature for a week to allow complete release of all CO, with the understanding that the half life of CORM-3 to liberate CO in saline was 10.6 h and approximately 1 mole of CO was liberated from 1 mole of CORM-3 (Motterlini et al. 2003), which was then followed by bubbling nitrogen gas through the solution for 10 min to remove the dissolved CO. Choi et al. (2012c) confirmed that the CO released from CORM-3, but not the CORM-3 itself, was responsible for the suppression of RIBE which caused apoptotic signals on the bystander zebrafish embryos.

Alpha-particle irradiation of zebrafish embryos

The alpha-particle irradiation set-up was similar to that devised by Yum et al. (2007) where the dechorionated zebrafish embryos were placed on a biocompatible substrate to allow alpha-particle irradiation from the bottom of the substrate (Figure 1). In the present work, Mylar film (Dupont, Hong Kong, China) with a thickness of 3.5 μm was used as the support substrate. Alpha-particle irradiations of the dechorionated embryos at 5 hpf were performed using an ^{241}Am source (with an alpha-particle energy of 5.49 MeV under vacuum and an activity of 4.26 kBq), which corresponded to an absorbed dose rate of ~ 1.1 mGy/min (Yum et al. 2007). The embryos were oriented in such a way that the alpha particles were incident onto the cells of the embryos. After passing through the Mylar film, the maximum penetration depth of the alpha particles into the embryo cells was 38.2 μm.

Medium transfer experiment protocol

A group of 20 embryos at 5 hpf were irradiated with the ^{241}Am alpha-particle source for 4 min, which were then immediately transferred into a Petri dish containing 2.5 ml of E3 medium, and then placed into the incubator until 29 hpf. The irradiated embryo conditioned medium (IECM) was harvested at 29 hpf. A control experiment was performed in parallel with the same procedures, with the irradiated embryos replaced by sham-irradiated embryos. In this control experiment, the harvested medium was referred to as the sham-irradiated embryo conditioned medium (SECM). While the IECM and SECM were being prepared, a total of 30 dechorionated zebrafish embryos were irradiated at 5 hpf. The 30 embryos were separated into three groups, namely, (a) the control group, (b) the IECM group, and (c) the SECM group. The control group of embryos was placed into 2.5 ml fresh E3 medium, the IECM group into the IECM while the SECM group into the SECM.

Partnering experiment protocol

In the present studies, three separate experiments were performed on zebrafish embryos in different groups, namely, (A) without treatment with chemicals, (B) treated with 500 μM of cPTIO, and (C) treated with 20 μM of CORM-3. From each set of experiment, we aimed at checking both the presence of RIBE and RIRE, so two control experiments

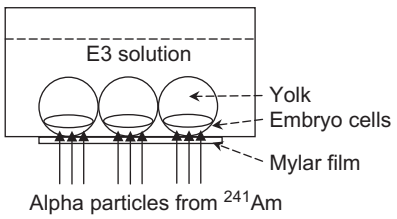

Figure 1. Irradiation of 5 hpf zebrafish embryos with alpha particles through a Mylar film-based holder.

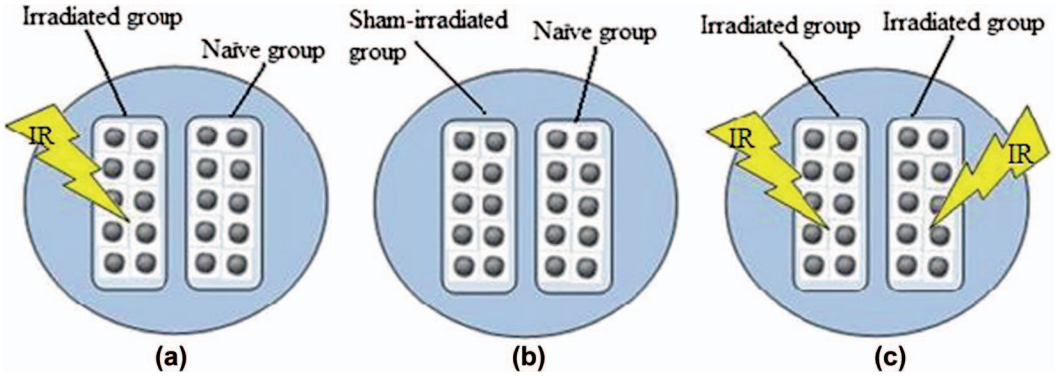

Figure 2. Schematic diagrams to illustrate the set-ups for different experiments: (a) IU group partnered with UI group; (b) RIBE control experiment (for checking the presence of RIBE): SU group partnered with US group; (c) RIRE control experiment (for checking the presence of RIRE): II group partnered with II group. This Figure is reproduced in color in the online version of the *International Journal of Radiation Biology*.

(i.e., the RIBE control experiment and the RIRE control experiment) were involved. For each set of experiment, 70 dechorionated embryos were employed and were divided to six groups, namely,

1. IU group: Irradiated embryos partnered with Unirradiated embryos;
2. UI group: Unirradiated embryos partnered with Irradiated embryos;
3. II group: Irradiated embryos partnered with Irradiated embryos;
4. SU group: Sham irradiated embryos partnered with Unirradiated embryos;
5. US group: Unirradiated embryos partnered with Sham irradiated embryos;
6. Control group: unirradiated embryos after dechorionation.

The II groups had 20 embryos while all the other groups had 10 embryos. Figure 2 gives schematic diagrams to illustrate the setups for different experiments: (a) IU group partnered with UI group; (b) RIBE control experiment (for checking the presence of RIBE): SU group partnered with US group; (c) RIRE control experiment (for checking the presence of RIRE): II group partnered with II group. On each agarose dish, the two groups of embryos were separately accommodated in the two shallow dredged regions to share the same medium (with a volume of 3 ml).

The protocols for the experiments performed on dechorionated zebrafish embryos in different groups (A), (B) and (C) are depicted in the schematic diagram shown in Figure 3, which were similar except for the chemicals used. For groups (B) (cPTIO treatment and group) (C) (CORM-3 treatment), the embryos were treated when they reached 4.5 hpf. When the dechorionated zebrafish embryos reached 5 hpf, the IU group and II group of embryos were irradiated by alpha particles for 4 min, and were then partnered with appropriate groups of embryos, i.e., the IU group was partnered with the UI group, while the II group was separated into two groups with the same number and separately accommodated in the two dredged regions on the same agarose dish. Under this design, the RIBE and RIRE signals, if any, could be mediated between the IU and UI groups, and between the two II groups. Dechorionated embryos in the control group (6) were placed in a Petri dish without further treatment for

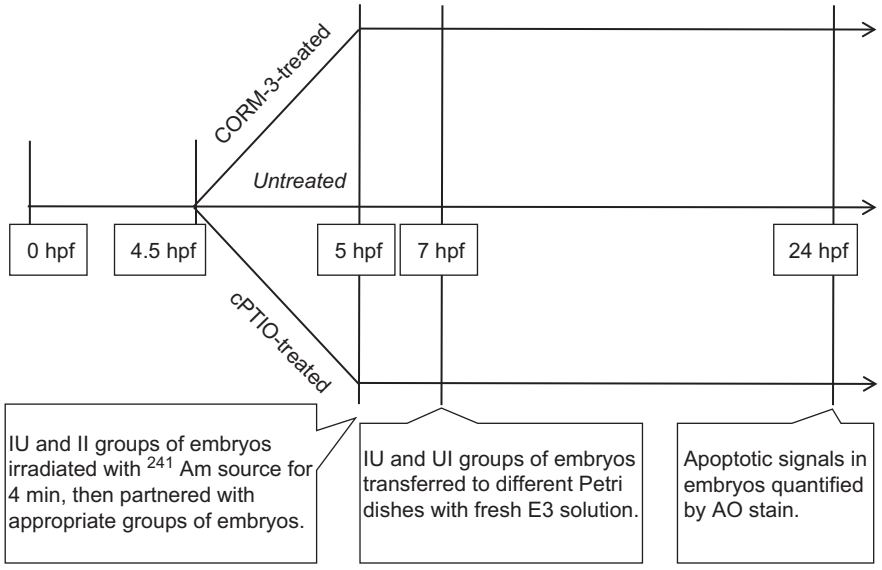

Figure 3. Schematic diagram showing the protocols for the partnering experiments.

monitoring the background apoptotic signals. After partnering, the three Petri dishes housing the partnered embryos were labeled and returned to the incubator at 28.5°C for 2 h. The embryos at 7 hpf from different groups were then relocated into six new labeled Petri dishes with fresh E3 solution until 24 hpf for quantification of apoptosis by vital dye staining as described below.

For the experiments on zebrafish embryos treated with 500 μM of cPTIO, and on zebrafish embryos treated with 20 μM of CORM-3, the three Petri dishes housing the partnered embryos were labeled and returned to the incubator at 28.5°C for partnering for 2 h.

Quantification of apoptosis by vital dye staining

The amount of apoptotic signals were determined when the zebrafish embryos were developed to 24 hpf. Briefly, the embryos were transferred into a culture medium with 2 μg/ml of the vital dye acridine orange (AO) (Sigma, St. Louis, MO, USA) to stain for 45 min in a dark environment to minimize fading of the AO color, and then washed

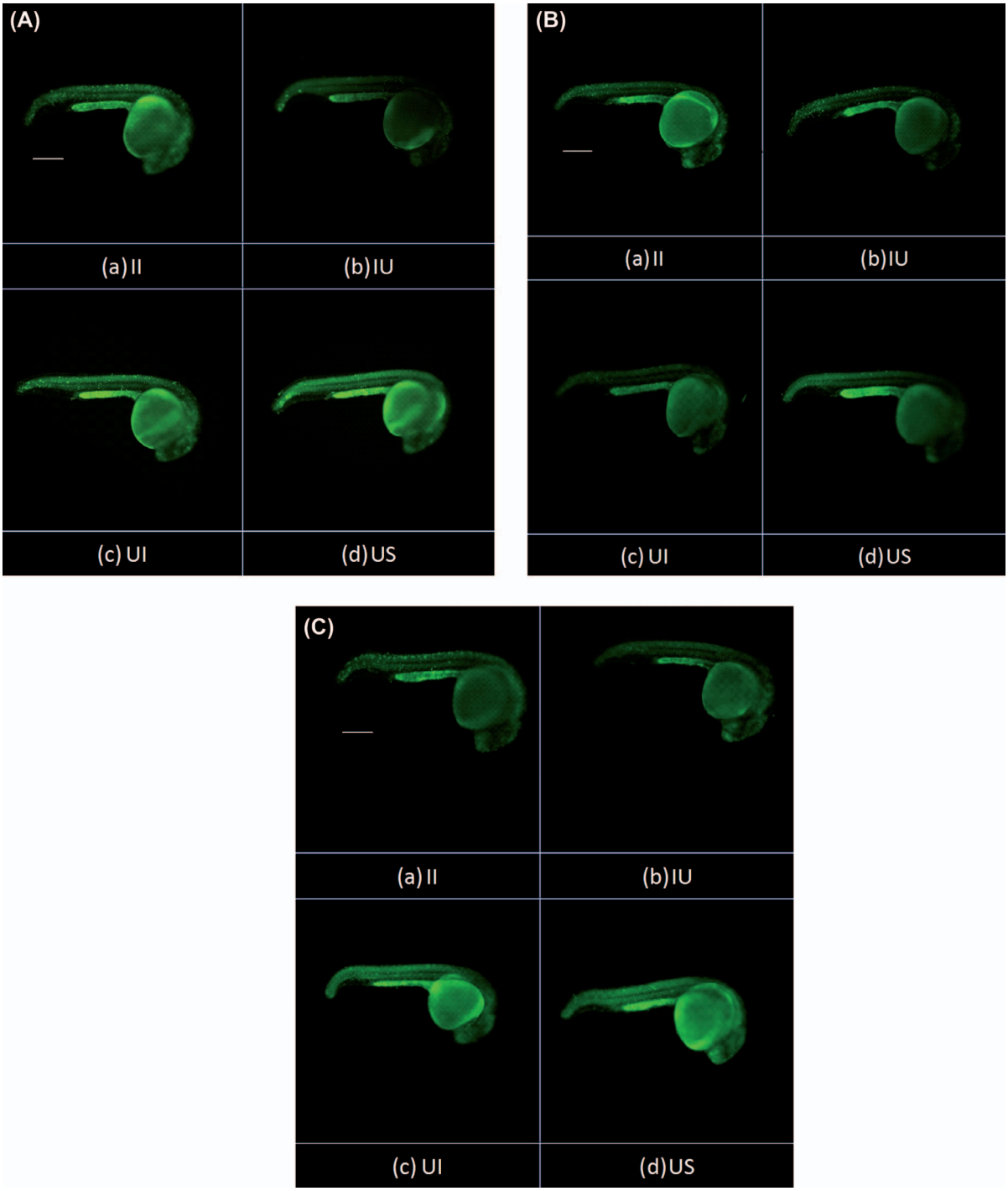

Figure 4. Representative images of apoptotic signals revealed on zebrafish embryos in the (a) II group, (b) IU group, (c) UI group, and (d) US group, all at 25 hpf, for the three experiments, namely, (A) on zebrafish embryos without treatment with chemicals, (B) on zebrafish embryos treated with 500 μM of cPTIO, and (C) on zebrafish embryos treated with 20 μM of CORM-3. Scale bars: 500 μm.

twice using deionized water thoroughly to remove excessive AO. The embryos were then anaesthetized with 0.016 M tricaine (Sigma, St. Louis, MO, USA). The apoptotic signals then appeared as bright green dots under a fluorescent microscope with a magnification of 40×. Three images with focuses on different sections of each anaesthetized embryo were captured using SpotBasic (SPOT 4.7, Diagnostic Instruments Inc., Michigan, USA), which were then combined into a single image for determination of the amount of apoptotic signals.

Statistical analysis

The amount of apoptotic signals on the whole zebrafish embryos were determined as depicted above. All data are shown as the average number of apoptotic signals ± standard error of the mean (SEM). The presence of RIBE was verified by comparing the UI and US groups through the *t*-test, while the presence of the RIRE was verified by comparing between the IU and II groups also through the *t*-test. As regards the medium transfer experiment, the presence of RIRE was verified by comparing between the IECM and SECM groups also through the *t*-test. All data were analyzed after outlier data, if any, were identified through the box plot method and 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, where the interquartile range was defined as the difference between the 25th and 75th percentiles of the data. Statistically significant differences were considered to be present between the compared groups in cases with p values < 0.05.

Results

The biological endpoint employed in the present investigation to characterize the radiation effects was the amount of apoptotic signals determined when the zebrafish embryos were developed to 24 hpf revealed by AO staining. The apoptotic signals appeared as bright green dots under a fluorescent microscope with a magnification of 40×. Figure 4 shows representative images of apoptotic signals revealed on zebrafish embryos in the (a) II group, (b) IU group, (c) UI group, and (d) US group, all at 25 hpf, for the three experiments, namely, (A) on zebrafish embryos without treatment with chemicals, (B) on zebrafish embryos treated with 500 μM of cPTIO, and (C) on zebrafish embryos treated with 20 μM of CORM-3. It was observed that the apoptotic signals were found to be spread through the entire body of the zebrafish embryos.

RIBE and RIRE between zebrafish embryos without treatment

The alpha-particle induced RIBE between zebrafish embryos was previously studied by Yum et al. (2009) and Choi et al. (2010a, 2012a, 2013) with the irradiation applied at 1.5 hpf and 5 hpf, respectively, while alpha-particle induced RIRE between zebrafish embryos was previously studied by Choi et al. (2012b) with the irradiation applied at 5 hpf. In the present study, the presence of RIBE and RIRE mediated between embryos through sharing the same medium was first reconfirmed for 5 hpf zebrafish embryos. A total of six independent experiments were conducted.

The first experiment aimed at showing the presence of RIBE mediated between zebrafish embryos by comparing the differences between the UI and US groups, and the results are given in columns 2–4 in Table I. All the six datasets showed significantly larger average numbers of apoptotic signals in UI embryos ($p < 0.05$), which successfully verified the communication of bystander signals from the IU group to the UI group in the absence of treatment with cPTIO or CORM-3. The second experiment aimed at showing the presence of RIRE on irradiated zebrafish embryos by comparing between the IU and II groups, and the results are given in columns 5–7 in Table I. All the six datasets showed significantly smaller average numbers of apoptotic signals in IU embryos ($p < 0.05$), which successfully confirmed the mitigation of the level of apoptotic signals in the IU embryos with the help of UI embryos in the absence of treatment with cPTIO or CORM-3.

RIBE and RIRE between zebrafish embryos treated with cPTIO

Table II showed the results for treatment with 500 μM of cPTIO. Again, the first experiment aimed at showing the presence of RIBE mediated between zebrafish embryos by

Table I. Mean number of apoptotic signals (± SEM) obtained in four groups of zebrafish embryos (US group, UI group, II group and IU group) from six sets of experiments without any chemical treated. n represented the number of zebrafish embryos in a particular sample. p values obtained using the t-test are also listed below (cases with $p < 0.05$ are asterisked).

Set	US	UI	p	II	IU	p
1	84 ± 2	138 ± 20	0.0136^*	218 ± 19	133 ± 18	$1.78 \times 10^{-3*}$
	($n = 8$)	($n = 10$)		($n = 19$)	($n = 8$)	
2	85 ± 8	138 ± 24	0.0297^*	215 ± 19	154 ± 17	$4.47 \times 10^{-3*}$
	($n = 10$)	($n = 9$)		($n = 19$)	($n = 9$)	
3	61 ± 7	115 ± 10	$1.99 \times 10^{-4*}$	154 ± 13	120 ± 12	0.0373^*
	($n = 9$)	($n = 9$)		($n = 10$)	($n = 7$)	
4	85 ± 14	120 ± 9	0.0130^*	217 ± 21	127 ± 10	$1.61 \times 10^{-4*}$
	($n = 9$)	($n = 9$)		($n = 16$)	($n = 9$)	
5	66 ± 3	101 ± 5	$2.42 \times 10^{-5*}$	118 ± 9	90 ± 4	$6.31 \times 10^{-3*}$
	($n = 8$)	($n = 7$)		($n = 15$)	($n = 8$)	
6	60 ± 6	104 ± 9	$4.00 \times 10^{-4*}$	125 ± 5	89 ± 5	$1.85 \times 10^{-4*}$
	($n = 9$)	($n = 10$)		($n = 17$)	($n = 7$)	

Table II. Mean number of apoptotic signals (± SEM) obtained in four groups of zebrafish embryos (US group, UI group, II group and IU group) from four sets of experiments with treatment of 500 μM of cPTIO. *n* represented the number of zebrafish embryos in a particular sample. *p* values obtained using the *t*-test are also listed below.

Set	US	UI	*p*	II	IU	*p*
1	60 ± 6 ($n=9$)	62 ± 7 ($n=9$)	0.426	141 ± 9 ($n=19$)	148 ± 10 ($n=10$)	0.291
2	75 ± 10 ($n=8$)	75 ± 6 ($n=9$)	0.388	181 ± 15 ($n=16$)	188 ± 18 ($n=9$)	0.378
3	73 ± 5 ($n=9$)	80 ± 7 ($n=9$)	0.198	188 ± 8 ($n=15$)	173 ± 14 ($n=10$)	0.190
4	112 ± 8 ($n=9$)	107 ± 7 ($n=10$)	0.346	247 ± 13 ($n=19$)	236 ± 17 ($n=9$)	0.271

comparing the differences between the UI and US groups, and the results are given in columns 2–4 in Table II. All the four datasets failed to show significantly larger average numbers of apoptotic signals in UI embryos ($p > 0.05$), which meant insignificant RIBE in the presence of cPTIO. The second experiment aimed at showing the presence of RIRE on irradiated zebrafish embryos by comparing between the IU and II groups, and the results are given in columns 5–7 in Table II. Again, all the four datasets failed to show significantly smaller average numbers of apoptotic signals in IU embryos ($p > 0.05$), which meant insignificant RIRE in the presence of cPTIO. One potential argument against this conclusion could be that there had been in fact RIRE but the cPTIO had actually decreased the damages in the II group but not the damages in the IU group. However, such a possibility had been precluded from our results described in the following that treatment with cPTIO would not have significant effects of the II group of embryos.

RIBE and RIRE between zebrafish embryos treated with CORM-3

Table III showed the results for treatment with 20 μM of CORM-3. Again, the first experiment aimed at showing the presence of RIBE mediated between zebrafish embryos by comparing the differences between the UI and US groups, and the results are given in columns 2–4 in Table III. All the four datasets failed to show significantly larger average numbers of apoptotic signals in UI embryos ($p > 0.05$), which meant insignificant RIBE in the presence of CORM-3. And again, the second experiment aimed at showing the presence of RIRE on irradiated zebrafish embryos by comparing between the IU and II groups, and the results are given in columns 5–7 in Table III. All the four datasets showed significantly smaller average numbers of apoptotic signals in IU embryos ($p < 0.05$), which confirmed significant RIRE to mitigate the level of apoptotic signals in the IU embryos even in the presence of CORM-3. A potential argument against this conclusion could be that there had been in fact no RIRE but the CORM-3 had significantly *increased* the damages in the II group but not the damages in the IU group. However, such a possibility had been ruled out from our results described in the following that treatment with CORM-3 actually significantly decreased the damages in the II group of embryos. As such, the significantly fewer apoptotic signals in IU embryos when compared to the already lower level of apoptotic signals in the II embryos still proved the presence of RIRE.

Table III. Mean number of apoptotic signals (± SEM) obtained in four groups of zebrafish embryos (US group, UI group, II group and IU group) from four sets of experiments with treatment of 20 μM of CORM-3. *n* represented the number of zebrafish embryos in a particular sample. *p* values obtained using the *t*-test are also listed below (cases with $p < 0.05$ are asterisked).

Set	US	UI	*p*	II	IU	*p*
1	75 ± 3 ($n=8$)	71 ± 5 ($n=10$)	0.236	114 ± 5 ($n=14$)	92 ± 4 ($n=6$)	9.55×10^{-3}*
2	47 ± 3 ($n=10$)	49 ± 3 ($n=7$)	0.340	91 ± 6 ($n=17$)	69 ± 3 ($n=4$)	3.10×10^{-3}*
3	120 ± 12 ($n=8$)	112 ± 13 ($n=10$)	0.336	157 ± 11 ($n=20$)	122 ± 12 ($n=10$)	0.0215*
4	93 ± 11 ($n=10$)	91 ± 9 ($n=10$)	0.459	192 ± 14 ($n=20$)	133 ± 14 ($n=10$)	3.26×10^{-3}*

Effects of cPTIO or CORM-3 on irradiated embryos (II groups and IU groups)

Tables I–III showed the mean numbers of apoptotic signals obtained in irradiated zebrafish embryos (II and IU groups) in the absence of treatment with cPTIO or CORM-3 (six independent experiments), in the presence of cPTIO (four independent experiments), and in the presence of CORM-3 (four independent experiments), respectively. In order to compare the data for the II and IU groups of embryos for different treatments, the data from different experiments for the same treatment needed to be combined. We first transformed the number of apoptotic signals for the II group of embryos (N_{II}) and for the control group of embryos (N_C) to the normalized net number of apoptotic signals for the II group of embryos (N_{II}*) as $N_{II}^* = [N_{II} - <N_C>]/<N_C>$, where $<N_C>$ was the average apoptotic signals for the corresponding control group of embryos. The numbers of apoptotic signals for the IU group of embryos (N_{IU}) were similarly transformed to the normalized net number of apoptotic signals for the IU group of embryos (N_{IU}*) as $N_{IU}^* = [N_{IU} - <N_C>]/<N_C>$. The statistical significance for differences between the compared groups was obtained through *t*-tests, and *p* values < 0.05 were considered correspond to statistically significant differences. The mean value ± SEM for N_{II}* for (A) no chemical treatment, (B) treatment with cPTIO and (C) treatment with CORM-3 were 1.40 ± 0.08 ($n = 96$), 1.56 ± 0.08 ($n = 69$) and 0.91 ± 0.07 ($n = 71$), respectively. The *p* value for the difference between mean values for (A) and (B) was 0.131 and that for (A) and (C) was 1.27×10^{-6}, which meant insignificant effects of cPTIO but significant effects of CORM-3 on the II group embryos. The mean values ± SEM for N_{IU}* for (D) no chemical treatment, (E) treatment with cPTIO and (F) treatment with CORM-3 were 0.57 ± 0.06 ($n = 47$), 1.54 ± 0.10 ($n = 38$) and 0.44 ± 0.07 ($n = 30$), respectively. The *p* value for the difference between the mean values for (D) and (E) was 2.71×10^{-11} and that for (D) and (F) was 0.16.

Medium transfer experiment

Table IV showed that the mean numbers of apoptotic signals obtained in the 10 irradiated zebrafish embryos in IECM and in SECM (provided by another 20 irradiated zebrafish embryos), respectively. The mean numbers of apoptotic signals in the IECM group of embryos were significantly smaller

Table IV. Mean number of apoptotic signals (± SEM) obtained in two groups of zebrafish embryos (IECM group and SECM group) from four sets of experiments. *n* represented the number of zebrafish embryos in a particular sample. *p* values obtained using the *t*-test are also listed below (cases with $p < 0.05$ are asterisked).

Set	SECM	IECM	*p*
1	256 ± 40 ($n = 8$)	147 ± 20 ($n = 10$)	0.0164*
2	189 ± 12 ($n = 8$)	127 ± 8 ($n = 8$)	4.48×10^{-4}*
3	210 ± 25 ($n = 10$)	121 ± 7 ($n = 9$)	3.08×10^{-3}*
4	186 ± 21 ($n = 10$)	89 ± 6 ($n = 10$)	4.72×10^{-4}*

than those in the SECM. This proved that the signals released by the irradiated embryos into the IECM were capable of rescuing a smaller number of other irradiated embryos when the latter were immersed into the IECM.

Discussion

As a first step, the present work confirmed the presence of both RIBE (acting upon the bystander unirradiated embryos) and RIRE (acting upon the irradiated embryos), through the significant increase and decrease in apoptotic signals in the partnered bystander and irradiated embryos, respectively. The occurrence of alpha-particle induced RIBE in zebrafish embryos was consistent with our previous studies (Yum et al. 2009, Choi et al. 2010a, 2012a, 2013).

The next task was to show the involvement of a released stress signal in the induction of RIRE through medium-transfer experiments. Our results showed that the irradiated embryo condition medium (IECM) provided by 20 irradiated zebrafish embryos was able to mitigate the damages of another 10 irradiated zebrafish embryos subsequently immersed into the IECM. Apart from proving the involvement of a released stress signal in the induction of RIRE, the results also demonstrated that the signals released by the irradiated embryos, i.e., the RIBE signals by definition, were capable of performing functions similar to RIRE on other irradiated embryos, and thus strongly suggested that similarity between RIBE and RIRE signals, but with different concentrations. If this was the situation, an interesting question would be why the rescue was more efficient when the irradiated embryos were (a) partnered with bystander unirradiated embryos, or (b) immersed into an IECM provided by a larger number of irradiated embryos. It is understood that both p53 and nuclear factor -κB (NF-κB) would be induced by many cellular stimuli, with activation of p53 and NF-κB associated with promotion of and protection from apoptosis, respectively. Ghandhi et al. (2008) revealed that the p53 and NF-κB response pathways were activated to dramatically different degrees in irradiated cells and bystander cells (IMR-90 human lung fibroblasts). Activation of the p53 response pathway was minimal in bystander cells, while activation of the NF-κB response pathways was effectively identical in irradiated cells and bystander cells. On the other hand, Webster and Perkins (1999) showed that p53 and NF-κB inhibited each other's ability to stimulate gene expression and that this process was controlled by the relative levels of each transcription factor. As such, if the response of irradiated embryos was dictated by the response of irradiated cells, activation of the p53 response pathway would dominate over the activation of the NF-κB pathway in the absence of RIRE signals, leading to more apoptosis. However, the presence of RIRE signals which were strong enough to activate the NF-κB and to override the p53 pathway could lead to greater survival of the irradiated cells in the irradiated embryos. This conjecture could also explain our previous result that the strength of the rescue effect increased with the number of rescuing bystander unirradiated embryos, while keeping the number of irradiated embryos unchanged (Choi et al. 2012b).

The present work then went on to ascertain the involvement of NO as a stress signal in the RIRE in zebrafish embryos. In particular, treatment with 500 μM of the specific NO scavenger cPTIO on zebrafish embryos was shown to be able to completely suppress both RIBE and RIRE. Choi et al. (2013) also found that cPTIO could suppress the RIBE induced between zebrafish embryos through X-ray irradiation. As such, the suppression of RIBE by cPTIO was independent of the linear energy transfer (LET) of the radiation. It could also be concluded that NO was an essential chemical molecule to induce RIBE and RIRE. On the other hand, the insignificant effects of cPTIO on the irradiated embryos themselves proved that the failure to reduce the apoptotic signals in IU embryos was due to suppression of the RIRE. As such, treatment with cPTIO suppressed RIRE through suppression of RIBE. In conclusion, the bystander zebrafish embryos need NO to initiate their release of the RIRE signals. These also agreed with our results described above that RIBE and RIRE signals actually had the same functions.

The present work then went on to identify the effects of CO on α-particle-induced RIBE and RIRE in zebrafish embryos in vivo. In particular, treatment with 20 μM of CORM-3 on zebrafish embryos was shown to be able to completely suppress the RIBE, which agreed with our previous study (Choi et al. 2012c). In an earlier in vitro study, Han et al. also demonstrated that a low concentration of exogenous CO could protect the bystander cells against the toxicity of NO, which would combine with the superoxide anions to form peroxynitrite ($ONOO^-$), an oxidizing free radical that could cause DNA fragmentation and lipid oxidation (Han et al. 2010) and could then trigger apoptosis. Notably, CO protected the bystander cells instead of moderating the release of NO from the irradiated cells (Chinese hamster ovary) (Han et al. 2010). Interestingly, while the treatment with CORM-3 on zebrafish embryos completely suppressed the manifestations of RIBE in the present experiments, all the four datasets showed significantly smaller average numbers of apoptotic signals in IU embryos ($p < 0.05$). As such, treatment with CORM-3 did not suppress RIRE. In conclusion, the bystander zebrafish embryos do not need NO-induced damages to initiate their release of the RIRE signals. This is in fact a necessary condition for allelopathy to be achieved, given the various possible manifestations of NO in the bystander cells.

In conclusion, unirradiated zebrafish embryos need NO but not NO-induced damages to rescue α-particle irradiated zebrafish embryos. However, there have also been evidences or arguments for physical signals responsible for RIBE, including lights (Mosse et al. 2006), bioelectric or magnetic effects (Mothersill et al. 2006b), and acoustic or electromagnetic effects (Mothersill et al. 2012). These physical signals can also contribute to RIRE, and might even constitute the difference between RIBE and RIRE mechanisms. However, the effects of such physical signals are still far from being fully understood. For example, Mothersill et al. (2012) asserted that it was possible that physical signals for RIBE only operated if chemical transmission was not possible or that the RIBE signal contained both physical and chemical components. More extensive studies and better understanding on the physical signals for RIBE will certainly enlighten us on their involvement in RIRE.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

Audette-Stuart M, Yankovicha T. 2011. Bystander effects in bullfrog tadpoles. Radioprotection 46:S497–497.

Barbazuk WB, Korf I, Kadavi C, Heyen J, Tate S, Wun E, Bedell JA, McPherson JD, Johnson SL. 2000. The syntenic relationship of the zebrafish and human genomes. Genome Res 10:1351–1358.

Bladen CL, Lam WK, Dynan WS, Kozlowski DJ. 2005. DNA damage response and Ku80 function in the vertebrate embryo. Nucleic Acids Res 33:3002–3010.

Bryan OT, Susan JK, Scicinski JJ. 2012. Is the nitric oxide (NO) the last word in radiosensitisation? A review. Transl Oncol 5:66–71.

Chen S, Zhao Y, Han W, Chiu SK, Zhu L, Wu L, Yu KN. 2011. Rescue eÿects in radiobiology: Unirradiated bystander cells assist irradiated cells through intercellular signal feedback. Mutat Res 706:59–64.

Choi VWY, Cheng SH, Yu KN. 2010a. Radioadaptive response induced by alpha-particle-induced stress communicated in vivo between zebrafish embryos. Environ Sci Technol 44:8829–8834.

Choi VWY, Konishi T, Oikawa M, Iso H, Cheng SH, Yu KN. 2010b. Adaptive response in zebrafish embryos induced using microbeam protons as priming dose and x-ray photons as challenging dose. J Radiat Res 51:657–664.

Choi VWY, Lam RKK, Chong EYW, Cheng SH, Yu KN. 2010c. Designing experimental setup and procedures for studying alpha-particle-induced adaptive response in zebrafish embryos in vivo. Nucl Instrum Meth B 268:651–656.

Choi VWY, Cheung ALY, Cheng SH, Yu KN. 2012a. Hormetic effect induced by alpha-particle-induced stress communicated in vivo between zebrafish embryos. Environ Sci Technol 46:11678–11683.

Choi VWY, Ng CYP, Cheng SH, Yu KN. 2012b. α-Particle irradiated zebrafish embryos rescued by bystander unirradiated zebrafish embryos. Environ Sci Technol 46:226–231.

Choi VWY, Wong MYP, Cheng SH, Yu KN. 2012c. Effects of exogenous carbon monoxide on radiation-induced bystander effect in zebrafish embryos in vivo. Appl Radiat Isotop 70:1075–1079.

Choi VWY, Ng CYP, Kobayashi A, Konishi T, Suya N, Ishikawa T, Cheng SH, Yu KN. 2013. Bystander effect between zebrafish embryos in vivo induced by high-dose X-rays. Environ Sci Technol 47:6368–6376.

Choi VWY, Yu KN. 2014. Embryos of the zebrafish Danio rerio in studies of non-targeted effects of ionizing radiation. Cancer Lett (in press) http://dx.doi.org/10.1016/j.canlet.2013.10.020

Clark JE, Naughton P, Shurey S, Green CJ, Johnson TR, Mann BE, Foresti R, Motterlini R. 2003. Cardioprotective actions by a water-soluble carbon monoxide-releasing molecule. Circ Res 93:e2–8.

Daroczi B, Kari G, McAleer MF, Wolf JC, Rodeck U, Dicker AP. 2006. In vivo radioprotection by the fullerene nanoparticle DF-1 as assessed in a zebrafi\sh model. Clin Cancer Res 12:7086–7091.

Geiger GA, Parker SE, Beothy AP, Tucker JA, Mullins MC, Kao GD. 2006. Zebrafi\sh as a "Biosensor"? Effects of ionizing radiation and amifostine on embryonic viability and development. Cancer Res 66:8172–8181.

Ghandhi SA, Yaghoubian B, Amundson SA. 2008. Global gene expression analyses of bystander and alpha particle irradiated normal human lung fibroblasts: Synchronous and differential responses. BMC Med Genom 1:63.

Goldberg Z, Lehnert BE. 2002. Radiation induced effects in unirradiated cells: A review and implications in cancer. Int J Oncol 21:337–349.

Han W, Wu L, Chen S, Yu KN. 2010. Exogenous carbon monoxide protects the bystander Chinese hamster ovary cells in mixed co-culture system after alpha-particle irradiation. Carcinogenesis 31:275–280.

Han W, Yu KN, Wu LJ, Wu YC, Wang HZ. 2011. Mechanism of protection of bystander cells by exogenous carbon monoxide: Impaired response to damage signal of radiation-induced bystander effect. Mut Res 709–710:1–6.

Hei TK, Zhou HN, Ivanov VN, Hong M, Lieberman HB, Brenner DJ, Amundson SA, Geard CR. 2008. Mechanism of radiation-induced bystander effects: A unifying model. J Pharm Pharmacol 60:943–950.

Li MH, Cha YN, Surh YJ. 2006. Carbon monoxide protects PC12 cells from peroxynitrite-induced apoptotic death by preventing the depolarization of mitochondrial transmembrane potential. Biochem Biophys Res Commun 342:984–990.

Li MH, Jang JH, Na HK, Cha YN, Surh YJ. 2007. Carbon monoxide produced by heme oxygenase-1 in response to nitrosative stress induces expression of glutamate-cysteine ligase in PC12 cells via activation of phosphatidylinositol 3-kinase and Nrf2 signaling. J Biol Chem 282:28577–28586.

Little JB. 2006. Cellular radiation eÿects and the bystander response. Mutat Res 597:113–118.

McAleer MF, Davidson C, Davidson WR, Yentzer B, Farber SA, Rodeck U, Dicker AP. 2005. Novel use of zebrafish as a vertebrate model to screen radiation protectors and sensitizers. Int J Radiat Oncol Biol Phys 61:10–13.

Morgan WF, Sowa MB. 2007. Non-targeted bystander effects induced by ionizing radiation. Mutat Res 616:159–164.

Mosse I, Marozik P, Seymour C, Mothersill C. 2006. The effect of melanin on the bystander effect in human keratinocytes. Mutat Res 597:133–137.

Mothersill C, Kadhim M. 2012. Mechanisms involved in the induction of radiation-induced non-targeted effects. Int J Radiat Biol 88:711–712.

Mothersill C, Seymour C. 2001. Radiation induced bystander eÿects: Past history and future directions. Radiat Res 155:759–767.

Mothersill C, Seymour C. 2004. Radiation induced bystander effects – implications for cancer. Nat Rev Cancer 4:158–164.

Mothersill C, Bucking C, Smith RW, Agnihotri N, O'Neill A, Kilemade M, Seymour CB. 2006a. Communication of radiation induced stress or bystander signals between fi\sh in vivo. Environ Sci Technol 40:6859–6864.

Mothersill C, Moran G, McNeill F, Gow MD, Denbeigh J, Prestwich W, Seymour CB. 2006b. A role for bioelectric effects in the induction of bystander signals by ionizing radiation? Dose Response 5:214–229.

Mothersill C, Smith RW, Agnihotri N, Seymour CB. 2007. Characterization of a radiation-induced stress response communicated in vivo between zebrafish. Environ Sci Technol 41: 3382–3387.

Mothersill C, Smith RW, Fazzari J, McNeill F, Prestwich W, Seymour CB. 2012. Evidence for a physical component to the radiation-induced bystander effect? Int J Radiat Biol 88:583–591.

Mothersill C, Smith RW, Hinton TG, Aizawa K, Seymour CB. 2009. Communication of radiation-induced signals in vivo between DNA repair defi\cient and profi\cient medaka (*Oryzias latipes*). Environ Sci Technol 43:3335–3342.

Motterlini R, Mann BE, Rohnson TR, Clark JE, Foresti RF, Green CJ. 2003. Bioactivity and pharmacological actions of carbon monoxide-releasing molecules. Curr Pharm Des 9:2525–2539.

Nagasawa H, Little JB. 1992. Induction of sister chromatid exchanges by extremely low doses of alpha-particles. Cancer Res 52: 6394–6396.

Paduch R, Kanderfer-Szerszen M. 2011. Nitric oxide (NO) and cyclooxygenase-2 (COX-2) cross-talk in co-cultures of tumor spheroids with normal cells. Cancer Microenviron 4:187–198.

Prise KM, O'Sullivan JM. 2009. Radiation-induced bystander signalling in cancer therapy. Nat Rev Cancer 9:351–360.

Shin JI, Lee YK, Kim YM, Hwang JT, Park OJ. 2007. Possible link between NO concentrations and COX-2 expression in systems treated with soy-isoflavones. Ann NY Acad Sci 1095:564–573.

Smith RW, Mothersill C, Hinton T, Seymour CB. 2011. Exposure to low level chronic radiation leads to adaptation to a subsequent acute X-ray dose and communication of modified acute X-ray induced bystander signals in medaka (Japanese rice fish, *Oryzias latipes*). Int J Radiat Biol 87:1011–1022.

Smith RW, Seymour CB, Moccia RD, Hinton TG, Mothersill CE. 2013. The induction of a radiation-induced bystander effect in fish transcends taxonomic group and trophic level. Int J Radiat Biol 89:225–233.

Surinov BP, Isaeva VG, Dukhova NN. 2005. Post-radiation immunosuppressive and attractive volatile secretions: The "bystander eÿect" or allelopathy in groups of animals. Dokl Biol Sci 400:28–30.

Tong L, Yu KN, Bao L, Wu W, Wang H, Han W. 2014. Low concentration of exogenous carbon monoxide protects mammalian cells against proliferation induced by radiation-induced bystander effect. Mut Res 759:9–15.

Wang H, Yu KN, Hou J, Liu Q, Han W. 2014. Radiation-induced bystander effect: Early process and rapid assessment. Cancer Lett (in press) http://dx.doi.org/10.1016/j.canlet.2013.09.031

Webster GA, Perkins ND. 1999. Transcriptional cross talk between NF-κB and p53. Mole Cell Biol 19:3485–3495.

Yum EHW, Ng CKM, Lin ACC, Cheng SH, Yu KN. 2007. Experimental setup for studying the effects of alpha particles on zebrafi\sh embryos. Nucl Instrum Meth B 264:171–176.

Yum EHW, Choi VWY, Nikezic D, Li VWT, Cheng SH, Yu KN. 2009. Alpha-particle-induced bystander eÿects between zebrafish embryos in vivo. Radiat Meas 44:1077–1080.

Yum EHW, Li VWT, Choi VWY, Cheng SH, Yu KN. 2010. Effects of alpha particles on zebrafish embryos. Appl Radiat Isotop 68: 714–717.