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α-Particle Irradiated Zebrafish Embryos Rescued by Bystander Unirradiated Zebrafish Embryos

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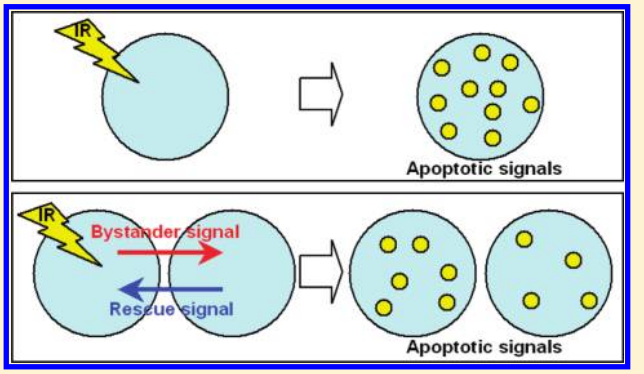
ABSTRACT: We report data demonstrating that zebrafish embryos irradiated by α-particles can release a stress signal into the water, which can be communicated to the unirradiated zebrafish embryos sharing the same water medium, and then these unirradiated zebrafish embryos can release a feedback stress signal back to the irradiated embryos. The effects of radiation on the whole embryos were studied through quantification of apoptotic signals at 24 h post fertilization through staining with the vital dye acridine orange, followed by counting the stained cells under a microscope. We refer to this phenomenon as the "rescue effect", where the unirradiated embryos successfully helped the irradiated embryos mitigate the radiation induced DNA damages. The results showed that the number of apoptotic signals in the irradiated embryos was smaller when they were partnered with bystander unirradiated embryos in the same medium. The results also showed significantly fewer apoptotic signals in the irradiated embryos when the population of bystander embryos increased from 10 to 30, while keeping the population of irradiated embryos at 10. These data suggest that the stress communicated between the unirradiated zebrafish embryos and the irradiated embryos sharing the same medium will help "rescue" the irradiated embryos, and that the strength of the rescue effect depends on the number of rescuing bystander unirradiated embryos.



INTRODUCTION

The recent nuclear reactor accident in Fukushima, Japan has reminded us of the importance of many issues, and understanding the effects of ionizing radiation on living organisms is among the top-priority ones. One recent surprising finding that is highly relevant to radioecology was the discovery of radiation-induced bystander effect (RIBE) between living organisms. RIBE usually refers to the phenomenon that unirradiated cells, which have received signals from irradiated cells, respond as if they have themselves been irradiated. It was first illustrated in in vitro studies by Nagasawa and Little [1] using the frequency of sister chromatid exchanges as the biological end point. There are many excellent reviews on RIBE (see refs 2−6). It has been widely accepted that RIBE is induced mainly through two mechanisms, namely, (1) gap junction intercellular communication (GJIC) in the presence of cell−cell contact, and (2) soluble molecules released by the irradiated cells into the cell culture medium conditioning the nonirradiated cell. The soluble molecules involved in the bystander signaling include reactive oxygen species (ROS), nitric oxide (NO), cytokine, TGF-β1, etc.[7−9]

As regards RIBE between organisms, Surinov et al. [10] reported that unirradiated mice housed together with irradiated mice responded as if they had been irradiated. These unirradiated mice were referred to as bystanders or partners, and the response was referred to as the RIBE. Surinov et al. [10] found that the signals for inducing the RIBE were transmitted through urine. Subsequently, Mothersill et al. [11] showed that freshwater rainbow trout (*Oncorhynchus mykiss*, W) irradiated to 0.5 Gy total-body X-ray dose released bystander signals into the water to induce bystander effects in unirradiated partners. Mothersill et al. [12,13] further demonstrated radiation-induced stress response communicated between zebrafish (*Danio rerio*) and Medaka (*Oryzias latipes*). More recently, our group also demonstrated that dechorionated embryos of the zebrafish *Danio rerio* irradiated with α-particles released bystander signals into the water to induce bystander effects in unirradiated zebrafish embryos.[14]

Now that radiation-induced stress communication between organisms has appeared to be a universal phenomenon, it is natural to explore how such a communication between organisms can benefit the population. Choi et al. [15] studied the benefit in terms of the induction of radioadaptive response (RAR) between zebrafish embryos by communication of such bystander signals. RAR is a kind of low-dose radiation effect, which occurs when a small preceding priming dose decreases the biological effectiveness of a subsequent large challenging dose. Such an adaptive response in cells (in vitro studies) was first reported by Olivieri et al.[16] In the experiments of Choi et al.,[15] dechorionated zebrafish embryos were irradiated and then partnered with two other groups of unirradiated embryos, namely the bystander

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group (no further treatments) and adaptive group (subjected to a further challenging dose) of embryos. The adaptive group of embryos were then separately further irradiated with a challenging dose. Choi et al. [15] showed that the number of apoptotic signals for the adaptive group was smaller than that for the corresponding control group, while that for the bystander group was larger than that for the corresponding control group. These data suggested that the stress communicated between the irradiated zebrafish embryos and those unirradiated embryos sharing the same medium had induced radioadaptive response in the unirradiated embryos.

This finding supported the suggestion of Mothersill et al. [11] that RIBE was likely an evolutionarily conserved mechanism with a final objective to enable an effective population response. One remaining intriguing question was whether the irradiated organisms could derive any benefits by communicating the signals of RIBE to the bystander organisms in formulating this effective population response. Most in vitro studies on RIBE focused on the response of the unirradiated bystander cells. Our group discovered in a recent in vitro study that irradiated cells could actually derive benefit from the feedback signals sent from the bystander cells.[17] By using both human primary fibroblast (NHLF) and cancer cells (HeLa) in a two cell coculture system, a significant decrease in the numbers of 53BP1 foci, micronucleus formation and extent of apoptosis were observed when the irradiated cells were cocultured with the bystander cells.[17] The effect on the irradiated cells was referred to as the "rescue effect". With such an effect identified under in vitro conditions, it is pertinent to study whether such an effect can also be observed between organisms.

In the present work, embryos of the zebrafish, *Danio rerio*, were employed as the model for studying the rescue effect between organisms. In recent years, the zebrafish, *Danio rerio*, a small vertebrate from Southeast Asia, has become a preferred model for studying human disease, including carcinogenesis. The most important advantage is that the human and zebrafish genomes share considerable homology, including conservation of most DNA repair-related genes.[18] Rapid embryonic development, external development of embryos, and their optically transparent body on the first few days have facilitated the characterization of their responses through microscopic inspection and fluorescence dye staining. Numerous research works using the zebrafish embryo as a model to study the DNA damage response to ionizing radiation have emerged (see refs 18−25).

We hypothesized that unirradiated bystander zebrafish embryos exposed to the water shared by α-particle irradiated zebrafish embryos could release a feedback stress signal into the water to rescue the irradiated zebrafish embryos, and that the strength of the rescue effect depends on the number of rescuing unirradiated bystander embryos.

MATERIALS AND METHOD

Zebrafish Maintenance. About 35 adult zebrafish of both genders were kept in a 45 L tank. The water temperature was controlled at 28 °C with the use of thermostats. The fish were fed four times daily with commercial tropical fish food and brine shrimp. The fish were maintained under a 14/10 light dark cycle to ensure a good production of embryos. To synchronize the collected zebrafish embryos, i.e., to ensure the collected embryos were at the same developmental stage, the embryos were collected within 15 min once the 14-h photoperiod began.

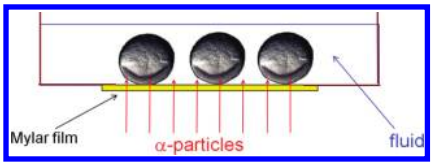

Figure 1. Irradiation of zebrafish embryos with α-particles through a Mylar-film based holder.

The collected embryos were transferred to an incubator with a temperature of 28 °C until 4 h post fertilization (hpf). Healthy developing embryos were then selected under a stereomicroscope and transferred into a Petri dish which had a layer of agarose gel on top of the dish and E3 medium inside (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, 0.1% methylene blue), for further dechorionation.

***α*-Particle Irradiation of Zebrafish Embryos.** The α-particle irradiation setup largely followed that devised by Yum et al. [25] in which the dechorionated zebrafish embryos were placed on top of a biocompatible substrate for α-particle irradiation from the bottom of the substrate (Figure 1). Mylar film (Dupont, Hong Kong) with a thickness of 3.5 μm was used as the support substrate. α-Particle irradiations of the dechorionated embryos at 5 hpf were performed for 4 min using an ^{241}Am source (with an α-particle energy of 5.49 MeV under vacuum and an activity of 4.26 kBq), which corresponded to an absorbed dose of ~4.4 mGy.[25]

Irradiation Protocol. The dechorionated zebrafish embryos 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. For the experiments to investigate the effects of the population size of UI embryos on the strength of the rescue effect, the number of embryos in the UI group was in turn changed to 20 and 30. The UI groups with 10, 20, and 30 embryos were denoted as UI, UI(20), and UI(30), respectively.

The partnership of different groups of zebrafish embryos for different experiments are shown in Figure 2. By definition, (a) IU group is partnered with UI group; (b) SU group is partnered with US group; and (c) II group is partnered with another 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.

When the dechorionated zebrafish embryos were developed into 5 hpf, the IU group and II group of embryos were irradiated by alpha particles for 4 min as described in the previous section. The IU group was then transferred immediately into an agarose plate to partner with unirradiated (UI) embryos, while II group of embryos was separated into two halves to be separately accommodated into the two shallow dredged regions on the same agarose dish (Figure 2c). Under this design, the soluble factors communicating the bystander signal, if any, were expected

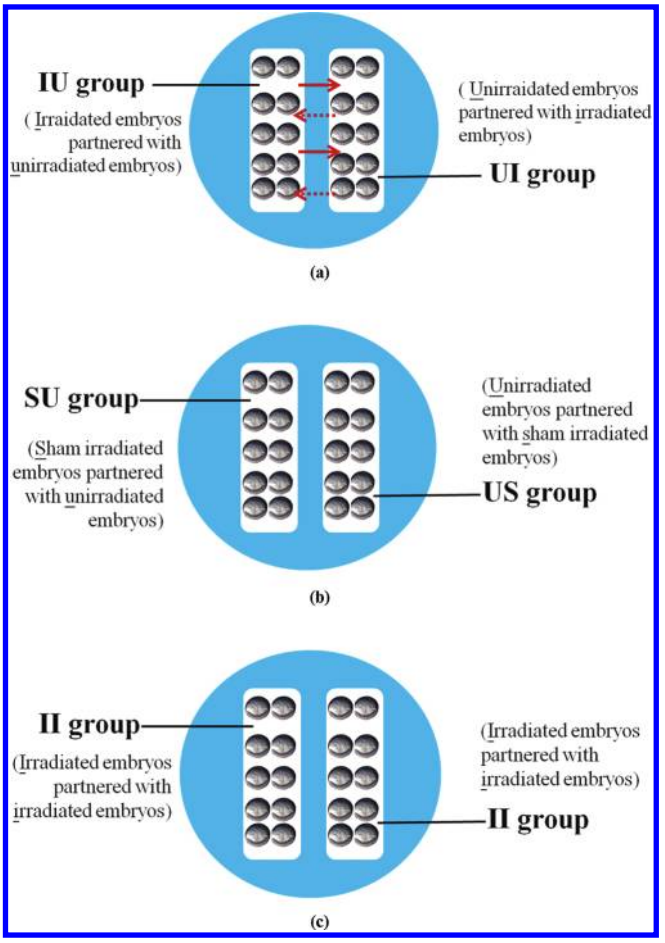

Figure 2. Schematic diagrams to illustrate the partnership of different groups of zebrafish embryos for different experiments. (a) IU group partnered with UI group; (b) SU group partnered with US group; (c) II group partnered with II group.

to be released by the IU group to reach the UI group (schematically represented by the solid arrows in Figure 2a) which shared the same medium (with a volume of 3 mL). Similarly, the rescue signal, if any, was expected to be communicated back from the UI group to the IU group (schematically represented by the dotted arrows in Figure 2a). Another agarose plate was set up by having sham-irradiated (SU) embryos partnered with unirradiated (US) embryos (Figure 2b) as the control experiment. All six groups of embryos were incubated in a 28 °C incubator until they developed to 24 hpf.

Quantification of Apoptosis by Vital Dye Staining. Apoptotic signals were quantified when the zebrafish embryos were developed to 24 hpf according to the method previously described by Choi et al.[15] Briefly, the embryos were transferred into a culture medium containing 2 μg/mL of a vital dye acridine orange (Sigma, St. Louis, MO, U.S.) to stain for 45 min and then washed twice in the culture medium thoroughly. The embryos were then transferred into 0.016 M tricaine (Sigma, St. Louis, MO, U.S.) for anaesthetization. For each embryo, three images with focuses on three different sections of the anaesthetized embryo were captured under a fluorescent microscope with a magnification of 40×, which were then combined into a single image for quantification of apoptotic signals.

Statistical Analysis. The numbers of apoptotic signals on the whole zebrafish embryos were counted as described above. The data are presented as the average number of apoptotic signals ± standard error. The presence of RIBE was characterized by comparing the UI and US groups through the *t* test, while the presence of the rescue effect was characterized by comparing between the IU and II groups through the *t* test. All of the analyses were performed after outlier data, if any, were removed. When a group of data was arranged in the descending order, the outliers were defined as values larger than 1.5 times the interquartile range above the 75th percentile or smaller than 1.5 times the interquartile range below the 25th percentile of the group of data, where the interquartile range was defined as the difference between the 25th and 75th percentiles of the data. Cases with p values ≤ 0.05 corresponded to statistically significant differences between the compared groups.

RESULTS

Bystander Effect between Zebrafish Embryos. The number of apoptotic signals in zebrafish embryos was used as the biological end point to characterize the radiation effects. Representative images of zebrafish embryos at 25 hpf with apoptotic signals revealed by acridine orange staining for the II, IU, UI, and US groups were shown in Figure 3. The α-particle induced bystander effect between zebrafish embryos was previously studied by Yum et al. [14] with the irradiation applied at 1.5 hpf. In the present study, 5 hpf zebrafish embryos were used instead, so the presence of bystander effect communicated between embryos through sharing the same medium had to be reconfirmed. The results are shown in Table 1. Data sets 1 to 4 in Table 1 corresponded to 10 embryos initially in a group (except for the II group, where there were initially 20 embryos), while sets 5 to 9 corresponded to 8 embryos initially in a group. A total of nine independent experiments were conducted.

The first four sets of experiments aimed to show the presence of bystander effect communicated between zebrafish embryos by comparing the differences between the UI and US groups. The data sets 2 to 4 showed that the average numbers of apoptotic signals of UI embryos were significantly larger than those of US embryos ($p < 0.05$). Data set 1 also showed the same trend of increasing apoptotic signals but the difference was not statistically significant. However, there were in general no statistically significant differences between IU and UI embryos in data set 1 ($p = 0.17$), set 2 ($p = 0.42$) and set 4 ($p = 0.29$) (although the p value for set 3 was <0.05). The significant differences between UI and US embryos, and the lack of significant differences between IU and UI embryos strongly supported a successful communication of the bystander signal from IU group to the UI group of embryos through the shared medium, which led to the RIBE.

Rescue Effect between Zebrafish Embryos. The rescue effect on irradiated zebrafish embryos was studied through comparing between the average apoptotic signals of the IU and II groups of embryos. The results are also shown in Table 1. The results showed that the II embryos expressed a higher level of apoptotic signals in all nine sets of experiments with p values <0.05 for data sets 1 to 8, and close to 0.05 for data set 9. These strongly supported the influence of UI embryos on IU embryos, i.e., a rescue effect to have mitigated the level of apoptotic signals in the IU embryos.

Effect of UI Group Size on Strength of Rescue Effect. The rescue effect is brought about by the "feedback signals" sent from the bystander embryos to the irradiated embryos on receiving the bystander signals from the irradiated embryos. It was therefore

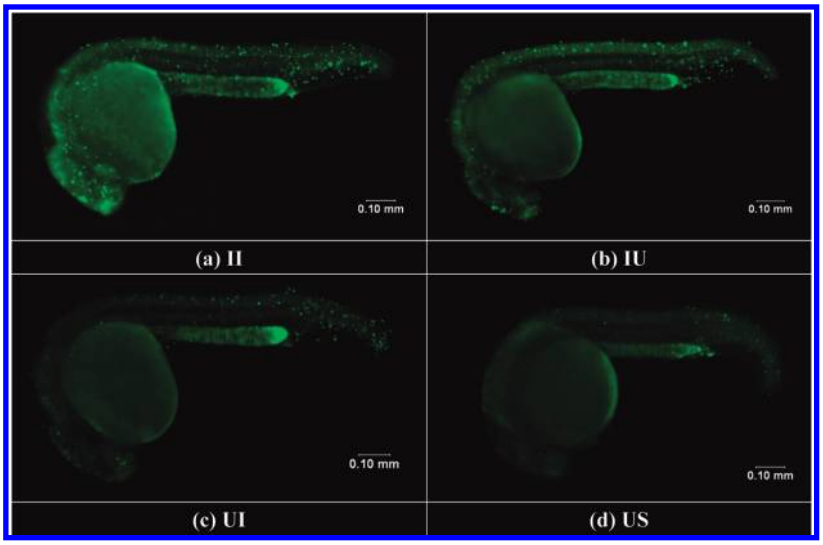

Figure 3. Representative images of zebrafish embryos at 25 hpf with apoptotic signals revealed by acridine orange staining (from experiment set 4). (a) II group: Irradiated embryos partnered with Irradiated embryos; (b) IU group: Irradiated embryos partnered with Unirradiated embryos; (c) UI group: Unirradiated embryos partnered with Irradiated embryos; and (d) US group: Unirradiated embryos partnered with Sham irradiated embryos.

pertinent to study the dependence of the strength of rescue effect on the UI group size. As such, the experiment for comparison between IU and II groups of embryos to show the presence of the rescue effect was repeated by having 20 and 30 embryos in turn in the UI group to provide such information.

The results were shown in Table 2. A total of four sets of independent experiments were carried out. The IU(20) and IU(30) groups corresponded to the irradiated embryos partnered with 20 and 30 unirradiated (UI) embryos, respectively. The results showed that both IU(20) and IU(30) embryos had fewer apoptotic signals when compared with the II group. The p values obtained were all smaller than 0.05 (except the comparison between IU(20) and II embryos in set 2). The data presented in Table 2 showed again the induction of the rescue effect to mitigate the apoptotic signals in the irradiated embryos in IU group when the UI group size was changed.

In order to investigate whether an increase in the UI group size would lead to an increase in the strength of the rescue effect, we calculated the difference in the number of apoptotic signals (D) between the IU and II groups (D = number of apoptotic signals in IU group—number of apoptotic signals in II group) for all 3 UI group size. For each IU group size, the differences D from different sets of experiments were normalized by dividing them with the apoptotic signals for the corresponding control samples, and were then combined into a single set of data. The results are tabulated in Table 3. Negative values of D were obtained for all the three IU group size indicating the presence of the rescue effect, among which IU(30) showed the largest differences. Furthermore, the normalized differences were significantly different between IU(10) and IU(30) (p = 0.018), and between IU(20) and IU(30) (p = 0.032). This implied an increase in the strength of the rescue effect when the size of the IU group size was increased to 30.

DISCUSSION

The present work successfully demonstrated the presence of both the RIBE effect (of the irradiated embryos on the bystander unirradiated embryos) and the rescue effect (of the bystander unirradiated embryos on the irradiated embryos) through quantification of the apoptotic signals in the embryos. The results showed a significant increase in apoptotic signals in bystander embryos while a significantly decrease in apoptotic signals in irradiated embryos partnered with bystander embryos, when compared to the corresponding control groups.

The results were consistent with our previous study that RIBE could be induced by α-particles in zebrafish embryos.[17] Mothersill et al. [11] suggested that RIBE was likely an evolutionarily conserved mechanism with a final objective to enable an effective population response, which was successfully proven in our previous work in that the stress communicated between the irradiated zebrafish embryos and those unirradiated embryos sharing the same medium had induced radioadaptive response in the unirradiated embryos.

Our present results also provided further information to the remaining intriguing question whether the irradiated organisms could derive benefits by communicating the signals of RIBE to the bystander organisms in formulating this effective population response. It was likely that the decrease in the level of apoptosis in the IU group of embryos was due to some kind of soluble factors transmitted from the UI group back to the IU group of embryos. The results were also consistent with our speculation inspired from the rescue effect discovered from in vitro studies.[17]

It is remarked here that rescued cells might not necessarily always lead to a benefit to the organism. For example, Barcellos-Hoff and Brooks [26] considered RIBE and radiation induced genomic instability (RIGI) as positive and negative effects in the complex context of microenvironmental homeostasis in tissues, while Belyakov et al. [27] argued that RIBE could help remove cells with background damage which could contribute to the expression of genomic instability.

When the population size of the bystander embryos increased up to 30 while keeping the population size of the irradiated embryos at 10 embryos, the number of apoptotic signals in the IU(30) group was significantly lower than that of the IU(10) and IU(20) groups. This showed that the strength of the rescue effect depended on the population of bystander embryos (UI group). Incidentally, in vitro studies showed that more irradiated cells

Table 1. Average Number of Apoptotic Signals (N) ± Standard Error Obtained from Different Groups of Embryos (Control, IU, II, UI, US)[a]

set		control	IU	II	UI	US
1	N	100 ± 8	93 ± 10	132 ± 7	127 ± 31	102 ± 6
	n	7	5	7	6	5
	p		0.0068[b]		0.23	
2	N	116 ± 7	134 ± 5	172 ± 14	137 ± 16	97 ± 9
	n	4	6	16	7	8
	p		0.0095[b]		0.028[b]	
3	N	76 ± 11	121 ± 12	167 ± 15	65 ± 4	36 ± 3
	n	10	8	17	7	5
	p		0.014[b]		0.00007[b]	
4	N	97 ± 10	143 ± 15	233 ± 14	133 ± 11	61 ± 4
	n	9	9	19	9	7
	p		0.00011[b]		0.00004[b]	
5	N	133 ± 7	166 ± 14	312 ± 28		
	n	8	7	14		
	p		0.00013[b]			
6	N	88 ± 14	162 ± 14	239 ± 20		
	n	6	6	13		
	p		0.0028[b]			
7	N	109 ± 3	202 ± 7	285 ± 6		
	n	7	5	12		
	p		0.0000015[b]			
8	N	110 ± 10	186 ± 21	244 ± 9		
	n	8	8	9		
	p		0.025[b]			
9	N	134 ± 9	191 ± 10	222 ± 16		
	n	7	7	14		
	p		0.053			

[a] n: number of embryos in a particular group of embryos after removing the outliers. p: p values obtained using t-tests with the corresponding group of embryos (i.e., IU embryos compared with II embryos, and UI embryos compared with US embryos). [b] Cases with $p \leq 0.05$ are considered statistically significant.

Table 2. Average Number of Apoptotic Signals (N) ± Standard Error Obtained from Different Groups of Embryos (Control, IU(20), IU(30), II)[a]

set		control	II	IU(20)	IU(30)
1	N	69 ± 6	227 ± 7	160 ± 20	149 ± 22
	n	8	12	9	8
	p			0.0063[b]	0.0057[b]
2	N	73 ± 5	150 ± 12	135 ± 23	108 ± 14
	n	7	18	9	10
	p			0.28	0.017[b]
3	N	84 ± 9	160 ± 9	132 ± 9	81 ± 4
	n	7	18	8	9
	p			0.026[b]	7.1×10^{-8}[b]
4	N	80 ± 2	210 ± 18	148 ± 18	125 ± 16
	n	8	20	10	7
	p			0.0089[b]	0.0019[b]

[a] n: number of embryos in a particular group of embryos after removing the outliers. p: p values obtained using t-tests with the corresponding group of embryos (i.e., IU embryos compared with II embryos). [b] Cases with $p \leq 0.05$, which are considered statistically significant.

Table 3. Differences (± Standard Error) in Apoptotic Signals between IU and II Groups for Three Different UI Group Size[a]

	10	20	30
difference	−57 ± 9	−44 ± 13	−69 ± 11
normalized difference	−0.61	−0.58	−0.90

[a] The normalized differences were obtained by dividing the differences with the apoptotic signals for the corresponding control samples.

would increase the response of the bystander cells as a result of a transmissible factor from the irradiated cells.[28] If the signals for the rescue effect bear a resemblance to the signals for RIBE, then it would have been expected that a larger number of bystander embryos led to a stronger rescue effect.

To better understand the mechanism of the rescue effect would require identification of the potential soluble factors transmitted from the bystander embryos into the medium. Radiation-induced stress signals included reactive oxygen species (ROS),[29] transforming growth factor-β1 (TGF-β1),[26,30] tumor necrosis factor-α (TNF-α) [31] and nitric oxide.[8] There are still many unclear issues in the interaction between RIBE and rescue effect, e.g., the temporal variation of the rescue signal transmitted from the bystander embryos to the irradiated embryos, and the similarity or difference between the bystander signals transmitted by the irradiated embryos and the rescue signals transmitted to the irradiated embryos.

The results presented in the present work were the first demonstration of RIBE-induced rescue effect between organisms. This further supported the suggestion of Mothersill et al. [11] that the radiation-induced bystander signal was likely an evolutionarily conserved mechanism with a final objective to enable an effective population response. This finding is very interesting and has far-reaching implications. If RIBE is indeed an evolutionarily conserved mechanism, RIBE as well as RIBE-induced rescue effect would be expected to occur between organisms including fish and mammals. The phenomena in fish have been established in the present and previous works, and the signals for inducing the RIBE were transmitted through water. However, RIBE between mice was also established [10] and the signals for inducing the RIBE were transmitted through urine. It is of immediate interest to study whether RIBE-induced rescue effect also occurs between mice. It will be even more pertinent to explore whether such RIBE and RIBE-induced rescue effects occur between human beings. If the answer is no, we might want to know why the evolutionarily conserved mechanism does not apply to human beings or why human beings do not need an effective population response. If the answer is yes, then we might want to know how the signals for inducing the RIBE and RIBE-induced rescue effects can be transmitted, how the RIBE can affect a bystander person, and whether we can exploit the RIBE-induced rescue effect, e.g., as an antidote to radiation-induced injuries.

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