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Alpha radiation exposure decreases apoptotic cells in zebrafish embryos subsequently exposed to the chemical stressor, Cd

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Abstract The aim of this study was to demonstrate that zebrafish embryos subjected to a priming exposure provided by one environmental stressor (low-dose alpha particles) can induce an adaptive response against a subsequent challenging exposure provided by another environmental stressor (heavy metal Cd). The effect thus identified would be an antagonistic multiple stressor effect. The effects of alpha particle radiation and/or Cd on whole embryos were studied through quantification of apoptotic signals at 24 h post-fertilization (hpf). Embryos were stained with the vital dye acridine orange, followed by counting the stained cells. For each set of experiments, 30 dechorionated embryos were divided into three groups, each having ten embryos. The three groups of embryos were referred to as (A) the control group, which received no more further treatments after dechorionation, (B) Cd-treated group, which did not receive any priming exposure and would receive a challenging exposure at 10 hpf and (C) (alpha + Cd)-treated group, which would receive both priming and challenging exposures. We defined the normalized net number of apoptotic signals in the (alpha + Cd)-treated group as N_C* = [(apoptotic signals for (alpha + Cd)-treated group − average apoptotic signals for the corresponding control group)/average apoptotic signals for the corresponding control group] and that in the Cd-treated group as N_B* = [(apoptotic signals for Cd-treated group − average apoptotic signals for the corresponding control group)/ average apoptotic signals for the corresponding control group]. By using the non-parametric Mann–Whitney U statistic, we were able to show that N_C* was significantly smaller than N_B*(p=0.006). These demonstrated an antagonistic multiple stressor effect between ionizing radiation and Cd through the induction of an adaptive response by the ionizing radiation against subsequent exposures to Cd.

Keywords Multiple stressor effect · Zebrafish embryos · Adaptive response · Cadmium · Ionizing radiation · Alpha particles

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Introduction

Living organisms are exposed to a mixture of environmental stressors, and the resultant effects due to such exposures are referred to as multiple stressor effects. Evidence showed that toxicity could be modified by simultaneous or sequential exposures to multiple environmental agents (Carpenter et al. 2002; Hertzberg and Teuschler 2002), and the appraisal of the probability and seriousness of the effects due to these exposures was referred to as cumulative risk assessment (Sexton and Hattis 2007). Sexton and Hattis (2007) gave a comprehensive review on multiple stressor effects and in particular discussed in depth fundamental issues regarding cumulative risk assessment. Multiple stressor effects might not be simply the sum of effects from individual stressors

(Hertzberg and Teuschler 2002; US EPA 2003). The effect can be additive, synergistic or antagonistic.

The overall rationale for our present study and its relevance to living organisms (including human beings) are based on the following three considerations:

(1) Ionizing radiation is ubiquitous in our environment. In particular, there are naturally occurring primordial radionuclides which have been around even before the formation of the solar system, and there are naturally occurring cosmogenic radionuclides which are continually formed in the atmosphere due to cosmic rays.
(2) Cumulating evidences have shown that low-dose ionizing radiation might be beneficial to living organisms, e.g. through the introduction of adaptive response (AR) (Olivieri et al. 1984; Cai et al. 2003; Choi et al. 2010a, b, c).
(3) Existence of multiple stressor effects in general where toxicity could be modified by simultaneous or sequential exposures to multiple environmental agents (Carpenter et al. 2002; Hertzberg and Teuschler 2002; US EPA 2003; Sexton and Hattis 2007; Mothersill et al. 2007a; Salbu et al. 2008).

On the other hand, heavy metals are also ubiquitous in our environment. Cadmium (Cd) is one of the most important heavy metal toxicants in our environment, which causes adverse effects to humans by affecting cell proliferation, differentiation, apoptosis and DNA repair (Hornhardt et al. 2006). However, interaction of radiation with chemical is not generally involved in ecological risk assessment. In recent years, studies gave evidence on the modification of the toxicities of individual stressors by the presence of other stressors at the same time. Additive, synergistic and antagonistic effects had been reported for Cd and gamma rays through in vitro and in vivo studies. Hornhardt et al. (2006) showed an additive effect of Cd and gamma ray exposure on human lymphoblastoid cells through the frequency of micronuclei formation. A synergistic effect between Cd and gamma ray on Wistar rats in terms of their enzyme activities were reported by Salovsky et al. (1993). An in vivo antagonistic effect was demonstrated by Privezentsev et al. (1996), where levels of DNA damage in peripheral blood lymphocytes and splenocytes were decreased after a combined exposure to Cd and gamma rays. Mothersill et al. (2007a) recently reported their work on the multiple stressor effect on the Atlantic salmon (*Salmo salar*, L.) in vivo through exposure to γ-irradiation with and without concomitant exposure to the metals Cd and/or Al, through studying cell deaths in reporter HPV-G cells caused by the media from explants from cultured tissues of the exposed fish (Mothersill et al. 2007a; Salbu et al. 2008). They found different effects (including additive, synergistic and antagonistic effects) between radiation and metal exposure for different tissues (including fin, gill, pronephros or kidney tissues).

At the moment, the groups of radionuclides and heavy metals are separately regulated in ecological risk assessment, which has effectively assumed no interactive effects between radiation and chemicals. Mothersill et al. (2007a) commented that determination of safe levels in the environment or prediction of consequences of acute low-level exposures was not straightforward when multiple stressors act on the concerned organisms. Sexton and Hattis (2007) also highlighted the problems which hampered cumulative risk assessment. As such, it is pertinent to study the multiple stressor effects from simultaneous or sequential exposures to ionizing radiation and Cd. Moreover, the mechanisms of their interactions and the consequence on the exposed organisms have not been extensively studied. It is therefore interesting and relevant to study the multiple stressor effect of radionuclides and heavy metals in terms of the induction of AR by using zebrafish embryos as a vertebrate model, which forms the objective of the present work.

The zebrafish, *Danio rerio*, has become a preferred vertebrate model in recent years for studying human disease. Interestingly, the zebrafish and human genomes share considerable homology, including conservation of most DNA repair-related genes (Barbazuk et al. 2000). Furthermore, embryonic development is rapid so the major organ systems become evident within 48 h post-fertilization (hpf). There have been a growing number of studies using zebrafish or zebrafish embryos as a vertebrate model to study the in vivo response to ionizing radiation (McAleer et al. 2004, 2006; Daroczi et al. 2006; Geiger et al. 2006; Mothersill et al. 2007b).

AR is a low-dose effect, which occurs when a small preceding priming dose decreases the biological effectiveness of a subsequent large challenging dose. Regarding ionizing radiation, such an AR in cells (in vitro studies) was first reported by Olivieri et al. (1984), who showed that peripheral blood lymphocytes irradiated with tritiated thymidine had fewer chromosomal aberrations when they were subsequently irradiated with 15 Gy of X-rays. AR was also shown in mice in vivo. A whole-body exposure of mice using X-radiation was conducted by Cai et al. (2003), who showed that mice with pre-exposure to low doses of radiation had significant decreases in chromosome aberrations. Wang et al. (2004) evaluated the AR in mice and found a range of dose rates capable of inducing AR in mice. Previous works by our group also demonstrated AR in embryos of the zebrafish, *D. rerio*, in vivo (Choi et al. 2010a, b, c). Choi et al. (2010c) demonstrated that AR was successfully induced in zebrafish embryos in vivo with the priming exposure provided by alpha particle irradiation from an ^{241}Am source (with an activity of 0.1151 μCi) for 24 s at

5 hpf and a subsequent challenging exposure provided by alpha particle irradiation from the same source for 240 s at 10 hpf. They proposed that the experimental setup and associated procedures could serve as a platform to further study high-linear energy transfer (LET) radiation-induced adaptive response in zebrafish embryos in vivo. Although the underlying mechanism for AR in cells is still largely unknown, some research findings have suggested that DNA repair might play an important role in inducing AR (Ikushima et al. 1996; Iyer and Lehnert 2002; Sasaki et al. 2002; Yatagai et al. 2008).

We hypothesized that an antagonistic multiple stressor effect of an ionizing radiation and a heavy metal would be induced in embryos of the zebrafish, *D. rerio*, in the form of an AR with an exposure to alpha particles as the priming dose and an exposure to Cd as a challenging dose.

Materials and methods

Experimental animals

Adult zebrafish were reared in glass tanks with water kept at 28 °C using thermostats and with a 14/10 hour light–dark cycle. Custom-made plastic collectors were opened on the top and had a partition with artificial plastic seaweed inside to attract the zebrafish to lay their eggs inside while at the same time separating the eggs from the fish (Choi et al. 2010c). Embryos were obtained by photo-induced spawning. Synchronization of developmental stage of the collected zebrafish embryos was crucial for our experiments. When the 14-h photoperiod started, the embryo collectors were lowered onto the bottom of the fish tanks to collect the embryos over a relatively short period of only 15 min to ensure synchronization of the embryos. The collected embryos were then transferred to and incubated in a 28-°C incubator and allowed to develop. At 4 hpf, healthy developing embryos, which should be at the sphere stage of the blastula period, were selected under a stereomicroscope and were transferred into a Petri dish with a layer of agar gel as the substrate for dechorionation. Chorions were removed directly using a pair of sharp forceps under the stereomicroscope. Alpha particles could reach the cells of embryos only after dechorionation to provide the priming dose (see below). All studied embryos were dechorionated to ensure the same conditions.

Exposure protocol

For each set of experiment, 30 dechorionated embryos were deployed, which were divided into three groups, each having ten embryos. The three groups of embryos were referred to as (A) control group, which received no further treatments after dechorionation, (B) Cd-treated group, which did not receive any priming exposure and would only receive a challenging Cd exposure at 10 hpf (see discussion in the following text) and (C) (alpha + Cd)-treated group, which would receive both priming alpha-radiation and challenging Cd exposures (see discussion in the following text). Fourteen repeated experiments were separately carried out. Analysis of these 14 sets of experimental data would be described in "Data analysis". To facilitate clearer interpretation of these results, we would also need data on zebrafish embryos which had been exposed only to alpha particles from our ^{241}Am source for 24 s at 5 hpf (referred to as alpha-treated group) and which had been sham-irradiated (referred to as sham-irradiated control group). Again, ten dechorionated embryos were employed for each of these two groups. Four sets of experiments were separately conducted.

In the present experiments, the time points for applying the priming and challenging exposures were separated by 5 h as previously suggested (Choi et al. 2010c). Considering that the DNA repair mechanism in zebrafish embryos would only become operative after the cleavage stages (0.7 to 2.2 hpf) (Miyachi et al. 2003), the priming exposure in this study was applied to the embryos at 5 hpf which was within the blastula stage (2.2 to 5.2 hpf).

Priming exposure

The priming exposure using alpha particle irradiation followed the procedures described by Yum et al. (2007). The (alpha + Cd)- and alpha-treated groups of embryos were irradiated with alpha particles from below and across the support substrate to avoid the problem of different depths of the medium above different embryos if the alpha particles were coming from above. Thin Mylar films (Dupont, Hong Kong) with a thickness of 3.5 μm were used as the support substrate to allow the alpha particles to reach the cells with a sufficiently large energy. The Mylar films were glued with epoxy (Araldite® Rapid, England) onto the bottom of a Petri dish which had a diameter of 35 mm and a hole at the centre. The embryos were oriented in such a way that all the cells of the embryos faced down towards the Mylar film to ensure that the alpha particles would be directed towards the cells.

At 5 hpf, as a priming dose to induce the AR, the (alpha + Cd)-treated group and the alpha-treated group of dechorionated embryos were irradiated for 24 s by alpha particles using a planar ^{241}Am source (with an average alpha particle energy of 5.49 MeV under vacuum and an activity of 0.1151 μCi), which corresponded to ~0.18 mGy. The alpha particles could further penetrate into the embryo cells with a range of 38.2 μm after passing through the 3.5-μm Mylar film. The method for determining the dose absorbed by the zebrafish embryos essentially followed that described by Yum et al. (2007). Briefly, the fluence of alpha particles

striking the zebrafish embryos was experimentally determined through counting the number of tracks on polyallyldiglycol-carbonate films (Nikezic and Yu 2004) irradiated by the alpha particle source and then chemically etched. From the fluence, the number of alpha particles N having hit an embryo was found. The effective energy E of alpha particles hitting the embryos was calculated by taking into account the energy loss in different water columns traveled by the alpha particles before they hit the embryos. The dose absorbed by an embryo was then computed as $(N \times E)/M$, where M was the average mass of an embryo.

The Cd-treated group and sham-irradiated control group of embryos were sham-irradiated. The sham-irradiated embryos experienced exactly the same exposure conditions, except that the ^{241}Am source was not used. All groups of embryos were then transferred back to the incubator and allowed to further develop.

Challenging exposure

Cadmium nitrate tetrahydrate (Sigma-Aldrich, MO, USA) was dissolved in deionized water to prepare the 100-μM cadmium nitrate [$Cd(NO_3)_2$] stock solution, which was then stored at room temperature. At 10 hpf, the (alpha + Cd)-treated group and the Cd-treated group of embryos were removed from the medium and transferred to a 100-μM cadmium nitrate solution using a pipette to provide the challenging dose until 24 hpf. After transfer of the (alpha + Cd)-treated group and the Cd-treated group of embryos to the cadmium solution, all groups of embryos were allowed to develop in a 28 °C incubator until 24 hpf for staining and observations. Figure 1 gives a flow diagram for the dose schedules to different groups of zebrafish embryos.

Chan and Cheng (2003) observed significant amounts of apoptotic cells in zebrafish embryos which had been exposed to 100 μM cadmium from 5 to 28 hpf and commented that their data were commensurate with other tissue culture studies using Cd at micromolar ranges (Hader et al. 1996; Bagchi et al. 2000; Alvarez-Barrientos et al. 2001; Chao and Yang 2001; Dong et al. 2001). Chan and Cheng (2003) also examined embryos exposed to 1 μM Cd for 24 h and found 0.67 % of apoptotic cells by flow cytometry assay. With these results and those in the literature, Chan and Cheng (2003) concluded that ectopic induction of apoptosis only occurred for Cd exposures with higher concentrations.

Quantification of apoptotic signals

Quantification of apoptotic signals has been widely adopted to examine the radiation effect on the whole embryos (Bladen et al. 2005, 2007a, b; Geiger et al. 2006). In the current study, apoptotic signals in the 24 hpf embryos were quantified through staining with the vital dye acridine orange (AO) as previously suggested (Choi et al. 2010c). All groups of embryos were separately stained with AO for 60 min, thoroughly washed twice with deionized water and then anaesthetized using 0.016 M tricaine (Sigma, St. Louis, MO, USA). The apoptotic signals of the zebrafish embryos were then counted under a fluorescent microscope. This method is commonly employed to quantify the level of apoptosis in zebrafish embryos (Tucker and Lardelli 2007; Mei et al. 2008; Yasuda et al. 2008). In our experiments, two images on different sections of each embryo were captured under the fluorescent microscope with a magnification of ×40 and were combined into a single image for quantification of apoptotic signals with the help of the software MetaMorph Version 7.0r0 (1992–2006 Molecular Devices).

Data analysis

In the present work, a total of 14 repeated sets of experiments had been carried out on different days, each of which using ten embryos. For each set of experiment, the numbers N_A, N_B and N_C of apoptotic signals for the control group (group A as described in "Exposure protocol"), Cd-treated group (group B) and (alpha + Cd)-treated group (group C) were measured. Since the average numbers of apoptotic signals on the control samples with sham irradiations were different for the 14 sets of experiments, in order to combine the data, we first transformed the data to the normalized net number of apoptotic signals in the Cd-treated group as $N_B^* = [N_B - \langle N_A \rangle]/\langle N_A \rangle$, where $\langle N_A \rangle$ was the average apoptotic signal for the corresponding control group and similarly the normalized net number of apoptotic signals in the (alpha + Cd)-treated group as $N_C^* = [N_C - \langle N_A \rangle]/\langle N_A \rangle$. N_B^* and N_C^* were compared using Mann–Whitney U statistic, and p values< 0.05 were considered as corresponding to significant differences between the mean values.

Results

Figure 2 shows examples of images with apoptotic signals in different groups of zebrafish embryos. In general, the apoptotic signals were distributed over the entire zebrafish embryos. Table 1 shows the results, which summarize the mean ± standard error for the number of apoptotic signals obtained in sham-irradiated control groups and alpha-treated groups of zebrafish embryos from four sets of experiments. It can be observed that, for all four sets of experiments, the apoptotic signals for the alpha-treated group were not significantly larger than those for the sham-irradiated control group. The lack of significant increase in the apoptotic signals was likely due to successful repair of DNA damages, which was in turn likely required for successful induction of adaptive response.

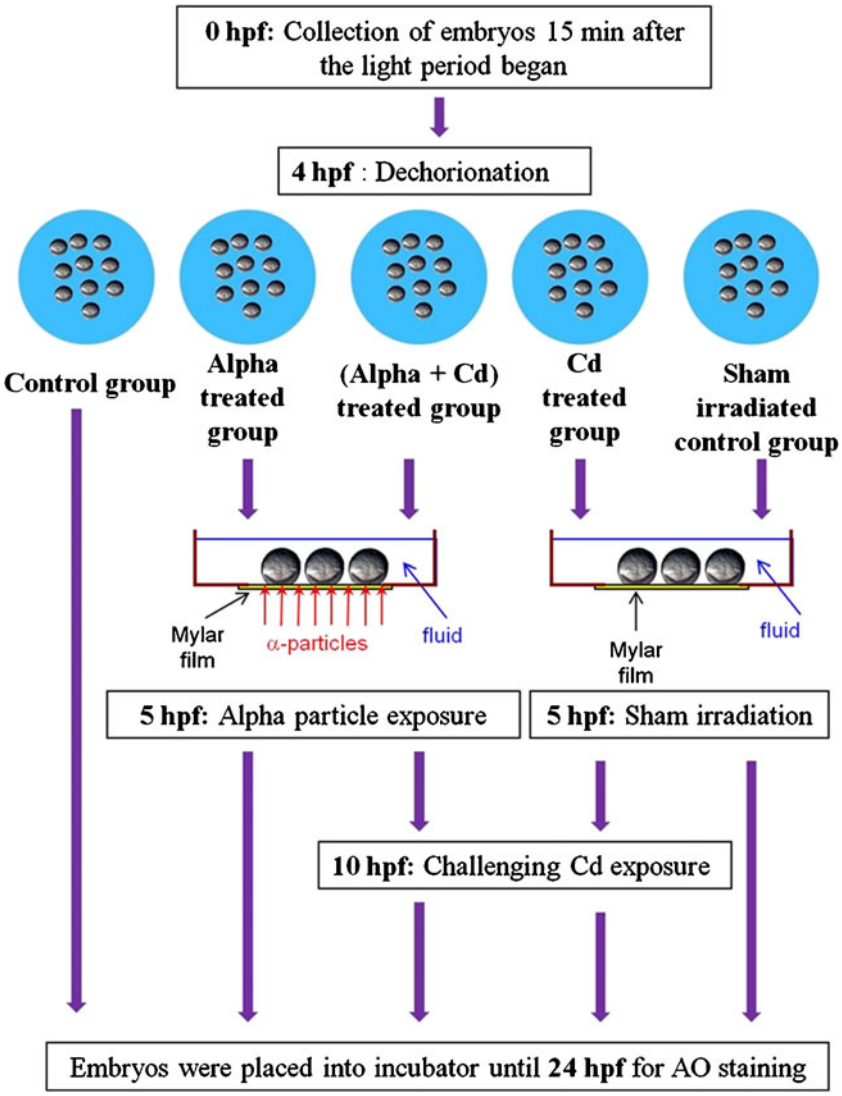

Fig. 1 A flow diagram of the exposure schedules for different groups of zebrafish embryos

Figure 3 shows the distribution of the results on N_B* and N_C*. It can be observed that the distributions are very different, namely, in that of N_C* peaks at a lower value and more skewed toward the smaller values when compared to that of N_B*. The two-sample Kolmogorov–Smirnov test gave a p value of 0.007 (two-tailed) and showed that the distributions of N_B* and N_C* were significantly different.

The mean value ± SE for N_B* and N_C* were 0.53±0.06 (N=117) and 0.34±0.06 (N=103), respectively. The mortality rates were 23 % for the control group, 16 % for the Cd-treated group and 26 % for the (alpha+Cd)-treated group, which were not significantly different. By using the non-parametric Mann–Whitney U statistic, we were able to show that N_C* was significantly smaller than N_B* (p=0.006, two-tailed). For reference, the p value (two-tailed) obtained using the t-test assuming equal variances was 0.029 (with a p value of 0.373 for Levene's test for equality of variances). From these results, we observed that the priming exposure to alpha particles caused a decrease in the normalized net number of apoptotic signals of ($<N_B^* > - < N_C^*>$)/$<N_B^* >$ or (0.53 −0.34)/0.53 or ~ 36 %, with statistical significance. In other words, a prior exposure to low-dose alpha particles could induce an adaptive response against a subsequent challenging exposure provided by the heavy metal Cd, which demonstrated an antagonistic multiple stressor effect.

Discussion

In the present work, the AR against a Cd exposure was studied through quantification of apoptotic signals in developed 24 hpf embryos stained with the vital dye acridine orange. The spatial and temporal patterns of apoptosis in vertebrate embryonic development are tightly regulated events (reviewed in Jacobson et al. (1997)). Exposure to toxicants or genetic mutations can disrupt the regulated

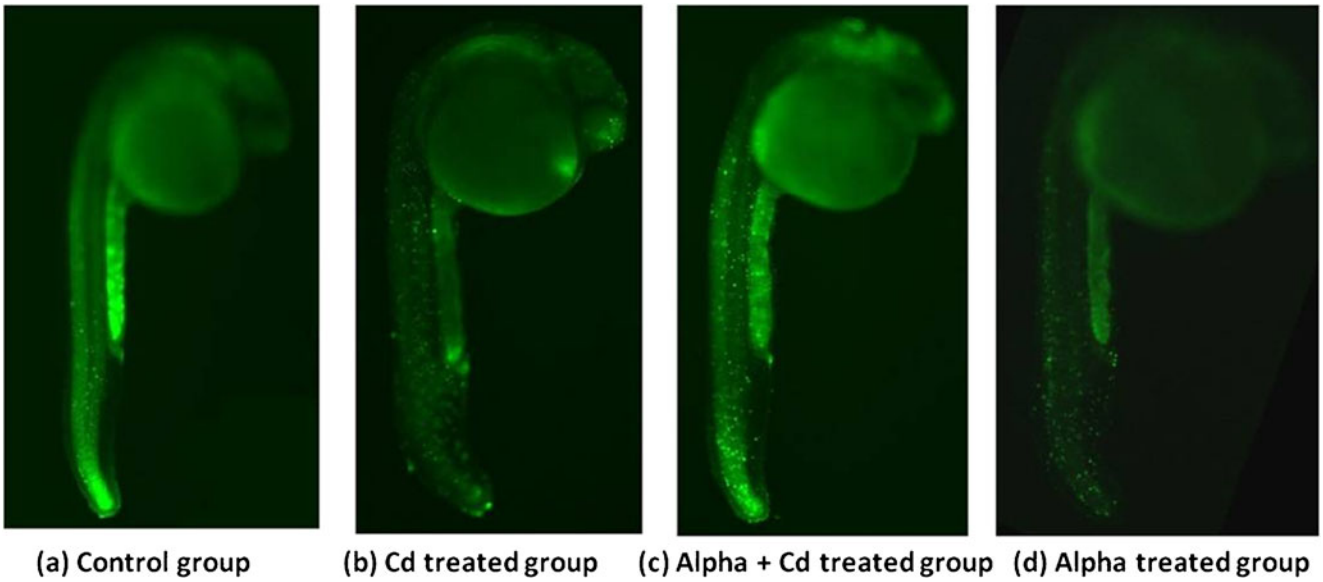

Fig. 2 Apoptotic signals of typical 25 hpf zebrafish embryos revealed by acridine orange staining and captured under a fluorescent microscope (with a magnification of ×40). **a** Control group, embryos which received no further treatments after dechorionation; **b** Cd-treated group, embryos sham-irradiated at 5 hpf and then subjected to a Cd challenging exposure at 10 hpf; **c** (alpha + Cd)-treated group, embryos subjected to an alpha particle priming exposure at 5 hpf and then subjected to a Cd challenging exposure at 10 hpf and **d** alpha treated group, embryos subjected to an alpha particle exposure for 24 s at 5 hpf. The apoptotic signals were found to spread over the entire body of the 24 hpf zebrafish embryos

occurrence of apoptosis, which can lead to developmental abnormalities (Zakeri and Ahuja 1997; Mirkes 2002).

Our group previously designed the experimental setup and the associated procedures for studying alpha particle-induced AR in zebrafish embryos in vivo, which involved counting apoptotic signals in embryos stained with the vital dye acridine orange (Choi et al. 2010c). The results revealed the presence of AR in zebrafish embryos in vivo (Choi et al. 2010c). Subsequently, we also successfully demonstrated the induction of AR in zebrafish embryos in vivo using microbeam protons to provide a very low priming dose and X-ray photons to provide the challenging dose through terminal dUTP transferase-mediated nick end-labeling (TUNEL) assay (Choi et al. 2010b). More recently, we reported that zebrafish embryos irradiated by alpha particles could release a stress signal into the water, which could be communicated to non-irradiated zebrafish embryos that shared the same water medium and thereby inducing AR in these non-irradiated zebrafish embryos (Choi et al. 2010a).

Cd-induced apoptosis has been reviewed in Robertson and Orrenius (2000). The underlying mechanisms are not well understood, but involvement of the caspases enzymatic pathway, suppression of the tumour suppressor gene p53 and protection by the anti-apoptotic gene Bcl-2 were suggested (Meplan et al. 1999; Kim et al. 2000; Biagioli et al. 2001; Ishido et al. 2002). Chan and Cheng (2003) used the induction of ectopic apoptosis in zebrafish embryos to study the developmental toxicity of Cd during their embryonic development.

Our results demonstrated that zebrafish embryos, which had been irradiated by alpha particles, could develop AR against a subsequent Cd exposure. The irradiated embryos having received a priming exposure to alpha particles 5 h before receiving a challenging exposure of Cd had significantly decreased apoptotic signals compared to embryos exposed only to that challenging exposure of Cd. The correlation between the p53-dependent apoptosis with the induction of DNA DSBs had previously been shown by Bladen et al. (2005), who obtained dose–response curves for buffer-microinjected embryos and Ku80 MO-microinjected embryos irradiated at 6 hpf to 1 to 50 mGy (at 1, 3, 8, 20 and 50 mGy) from a 6-MeV Varian linear accelerator beam. Bladen et al.

Table 1 Means ± standard errors of the mean for number of apoptotic signals obtained in sham-irradiated control groups and alpha treated groups of zebrafish embryos from four sets of experiments. The *p* values refer to *t*-tests comparing the 0.18 mGy alpha-treated groups and the corresponding sham-irradiated control groups

Set	Sham-irradiated control group	0.18 mGy alpha-treated group	*p* values
1	41±7 (*n*=9)	41±6 (*n*=9)	0.47
2	34±8 (*n*=8)	48±7 (*n*=10)	0.095
3	42±8 (*n*=8)	39±5 (*n*=9)	0.38
4	54±7 (*n*=10)	55±10 (*n*=8)	0.46

n number of zebrafish embryos in a particular sample

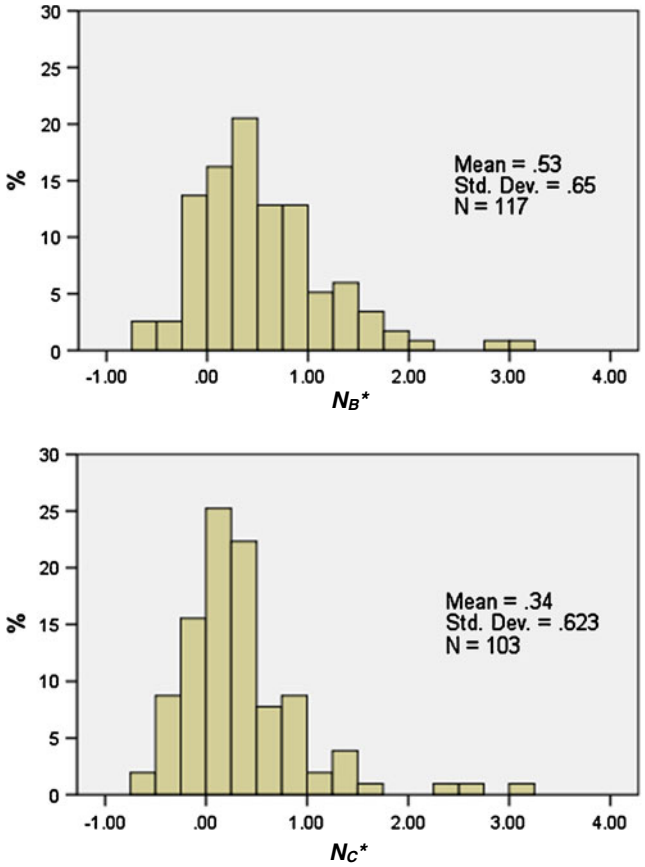

Fig. 3 Distribution of N_B* and N_C*

(2005) showed no significant increase above background in the number of TUNEL-positive cells in buffer-microinjected embryos, while a significant linear dose response in Ku80 MO-microinjected embryos was shown. As Ku80 is a protein essential for the nonhomologous end-joining pathway of repairing DNA DSBs, their results showed that DNA DSBs were repaired in the irradiated wild-type embryos. As such, the reduction in the apoptotic signals in embryos which had received a priming exposure before the challenging was likely a result of more effective repair of DNA DSBs and thus beneficial. In other words, pre-treatment with alpha particle irradiation would decrease the biological effectiveness of Cd on the zebrafish embryos. This demonstrates an AR against a stressor (the heavy metal Cd in this case) induced by a different stressor (the ionizing alpha particle radiation in this case) in living organisms. This also suggests an antagonistic multiple stressor effect between radiations and chemicals. Indeed mechanisms involved in AR response are still largely unknown. Several key findings showed that DNA repair mechanism was involved in the induction of AR (Shadley and Wolff 1987; Zhou et al. 1992; Ikushima et al. 1996; Wojcik et al. 1996). Takahashi (2001) found the level of p53 to be strongly suppressed after a high-dose-rate radiation (1 Gy/min, 5 Gy) when a priming low-dose-rate irradiation of gamma ray (0.001 Gy/min, 1.5 Gy) was applied and concluded that AR was involved in the suppression of p53-mediated apoptosis (Takahashi 2001).

The mortality rates were 23 % for the control group, 16 % for the Cd-treated group and 26 % for the (alpha + Cd)-treated group. The mortality rates obtained in these three situations were close to 20 %. Such mortality rates were close to the lowest values obtainable for untreated dechorionated zebrafish embryos as reported by Henn and Braunbeck (2011). Henn and Braunbeck (2011) studied the mortality rates of dechorionated embryos of the zebrafish (*D. rerio*) and found that the mean mortality rate of untreated zebrafish embryos dechorionated at 4 hpf was about 40 % (with a standard deviation of about 28 %). In other words, our mortality rates were likely due to the dechorionation process itself. As such, we believe that the tests with these mortality rates were still valid.

Related studies of multiple stressor effects were reported by Mothersill et al. (2007a) and Salbu et al. (2008), who found that the multiple stressor effects, in general, varied among different organs and tissues. The major difference from the experimental approaches of the previous studies was that a time gap between the exposures of the two stressors was employed in the present work. A time gap between the priming exposure and the challenging exposure was needed for successful induction of adaptive response (Shadley and Wolff 1987).

The antagonistic multiple stressor effect revealed in the present work supported that the mixture of ionizing radiation and heavy metal appeared to be a priority for studies since the effect was not simply the sum of the effects from individual stressors. The results also reinforced the need for improved cumulative risk assessment. It would also be important to have a better understanding on the mechanism underlying the interactions between radionuclides and heavy metals since mechanistic knowledge will enable quantitative predictions of the resultant effects of such an exposure to a mixture of environmental stressors (Sexton and Hattis 2007).

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