IOP PUBLISHING JOURNAL OF RADIOLOGICAL PROTECTION

J. Radiol. Prot. **33** (2013) 101–112 doi:10.1088/0952-4746/33/1/101

Adaptive response to ionising radiation induced by cadmium in zebrafish embryos

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Received 23 February 2012, in final form 2 May 2012, accepted for publication 20 August 2012
Published 7 January 2013
Online at stacks.iop.org/JRP/33/101

Abstract

An adaptive response is a biological response where the exposure of cells or animals to a low priming exposure induces mechanisms that protect the cells or animals against the detrimental effects of a subsequent larger challenging exposure. In realistic environmental situations, living organisms can be exposed to a mixture of stressors, and the resultant effects due to such exposures are referred to as multiple stressor effects. In the present work we demonstrated, via quantification of apoptosis in the embryos, that embryos of the zebrafish (*Danio rerio*) subjected to a priming exposure provided by one environmental stressor (cadmium in micromolar concentrations) could undergo an adaptive response against a subsequent challenging exposure provided by another environmental stressor (alpha particles). We concluded that zebrafish embryos treated with 1 to 10 μM Cd at 5 h postfertilisation (hpf) for both 1 and 5 h could undergo an adaptive response against subsequent ∼4.4 mGy alpha-particle irradiation at 10 hpf, which could be interpreted as an antagonistic multiple stressor effect between Cd and ionising radiation. The zebrafish has become a popular vertebrate model for studying the *in vivo* response to ionising radiation. As such, our results suggested that multiple stressor effects should be carefully considered for human radiation risk assessment since the risk may be perturbed by another environmental stressor such as a heavy metal.

(Some figures may appear in colour only in the online journal)

1. Introduction

In realistic situations, living organisms are exposed to a mixture of environmental stressors. The resultant effects due to such exposures are referred to as multiple stressor effects.

0952-4746/13/010101+12$33.00

The multiple stressor effects of ionising radiation and chemicals have recently started to generate research interest. Radionuclides and heavy metals are separately regulated nowadays, effectively assuming no interactive effects between radiation and chemicals. However, it has been shown that simultaneous or sequential exposures to multiple environmental agents could modify the toxicities of individual stressors (Carpenter *et al* 2002, Hertzberg and Teuschler 2002), and the consequence could be different from a simple sum of the effects (i.e. an additive effect) of individual stressors (Hertzberg and Teuschler 2002, US EPA 2003). Other forms of multiple stressor effects, including synergistic or antagonistic effects, are also possible. However, relatively few works have been performed on the form of the multiple stressor effects, and very little is known about the underlying mechanisms.

Cadmium (Cd) is an important heavy-metal toxin in our environment, and has adverse effects on humans by affecting cell proliferation, differentiation, apoptosis and DNA repair (Hornhardt *et al* 2006). On the other hand, ionising radiation arising from natural or artificial radionuclides is also ubiquitous in our environment. As such, it is pertinent to study the multiple stressor effects from simultaneous or sequential exposures to Cd and ionising radiation. Additive, synergistic and antagonistic effects had been reported for Cd and gamma rays through *in vitro* and *in vivo* studies (Salovsky *et al* 1993, Privezentsev *et al* 1996, Hornhardt *et al* 2006, Mothersill *et al* 2007a, Salbu *et al* 2008). The multiple stressor effects of Cd and gamma rays on Wistar rats were studied through their enzyme activities, where a synergistic increase was reported (Salovsky *et al* 1993). Another study on human lymphoblastoid cells through the frequency of micronucleus formation showed an additive effect of Cd and gamma-ray exposure (Hornhardt *et al* 2006). An *in vivo* antagonistic effect was reported by Privezentsev *et al* (1996). Levels of DNA damage in peripheral blood lymphocytes and splenocytes were decreased after combined exposure to Cd and gamma rays. More recently, the McMaster University group studied the multiple stressor effect on the Atlantic salmon (*Salmo salar*, L.) *in vivo* though exposure to γ-irradiation together with Cd or Al or both metals (Mothersill *et al* 2007a, Salbu *et al* 2008). The multiple stressor effect of radiation and metal exposure was found to be different for different tissues including fin, gill, pronephros and kidney. The effects could be additive, synergistic or antagonistic. The authors concluded that the responses varied among the different tissues.

The present paper studied the multiple stressor effect from Cd and alpha-particle exposures in terms of the adaptive response (AR) induced by a small preceding exposure to Cd against a large exposure to alpha particles. The AR is the phenomenon in which a small priming dose decreases the biological effectiveness of a subsequent large challenging dose. An AR for ionising radiation in cells was first reported by Olivieri *et al* (1984). An AR was also shown in mice *in vivo* by Cai *et al* (2003) and Wang *et al* (2004), and in embryos of the zebrafish (*Danio rerio*) *in vivo* by our group (Choi *et al* 2010a, 2010b, 2010c). The zebrafish and human genomes share considerable homology, including conservation of most DNA repair-related genes (Barbazuk *et al* 2000). Zebrafish adults or embryos have become a popular vertebrate model for studying the *in vivo* response to ionising radiation (McAleer *et al* 2004, 2006, Daroczi *et al* 2006, Geiger *et al* 2006, Mothersill *et al* 2007b).

Our group was successful in showing the induction of AR in zebrafish embryos *in vivo* using alpha particles, by counting the apoptotic signals in the embryos stained with the vital dye acridine orange (AO) (Choi *et al* 2010c). We were subsequently also successful in demonstrating the induction of AR in zebrafish embryos *in vivo* using microbeam protons, through a terminal dUTP transferase-mediated nick end-labelling (TUNEL) assay (Choi *et al* 2010b). More recently, we reported that zebrafish embryos irradiated by alpha particles could also induce an AR in unirradiated zebrafish embryos sharing the same water medium (Choi *et al* 2010a).

We hypothesised that an antagonistic multiple stressor effect between ionising radiation and a heavy metal would be induced in zebrafish embryos in the form of an AR, with an exposure to micromolar concentrations of Cd as the priming dose and an exposure to ∼4.4 mGy alpha particles as the challenging dose.

2. Materials and method

2.1. Experimental animals

Adult zebrafish were kept in a 45 l glass tank with water maintained at 28 °C and with a 14/10 h light–dark cycle. Spawning of embryos was induced at the beginning of the 14 h light period, and the embryos were collected in specially designed plastic collectors (Choi *et al* 2010c) over a relatively short period of only 15 min to ensure synchronisation of the embryos. Synchronisation of the developmental stage of the collected embryos was crucial for our experiments. This ensured the same developmental stage for the embryos when they were subjected to the priming and challenging exposures and when they were stained. The collected embryos were then transferred to a Petri dish 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 incubated at 28 °C for development. At 4 h postfertilisation (hpf), healthy developing embryos were picked under an optical microscope and transferred into a Petri dish with a layer of agar gel as the substrate for dechorionation. All studied embryos, whether or not they were going to receive the challenging dose of alpha particles, were dechorionated to ensure the same conditions.

2.2. Chemicals

Cadmium nitrate tetrahydrate (Sigma-Aldrich, MO, USA) was dissolved in deionised water to prepare the 100 μM cadmium nitrate [$Cd(NO_3)_2$] stock solution, which was then stored at room temperature. The same stock solution was used for all experiments. Different concentrations of cadmium nitrate solution (namely, 1, 5, 10 μM) employed for the present experiments were prepared by dissolving the stock solution in deionised water.

2.3. Exposure protocol

For each set of experiment, 75 dechorionated embryos were deployed; these were divided into three groups. Group A was the control group; this group did not receive any priming exposure after dechorionation and would be sham irradiated at 10 hpf. Group B was the adaptive control group, which did not receive any priming exposure, and would receive a challenging exposure at 10 hpf (see discussion below). Group C was the adaptive group (with three subgroups), and would receive a priming exposure provided by the Cd solution (with a Cd concentration of 1, 5 or 10 μM) and then a challenging exposure at 10 hpf. The control group, adaptive control group and each subgroup of the adaptive group (each corresponding to a particular Cd concentration) had 15 dechorionated embryos.

2.4. Priming exposure

At 5 hpf, only the adaptive group of embryos was removed from the medium using a pipette and transferred to cadmium nitrate solutions with concentrations of 1, 5 and 10 μM to provide the priming dose. Since research findings suggested that DNA repair might play an important role in inducing an AR (Ikushima *et al* 1996, Iyer and Lehnert 2002, Sasaki *et al* 2002, Yatagai *et al* 2008), we chose 5 hpf embryos, which was within the blastula stage (2.2–5.2 hpf), as the time

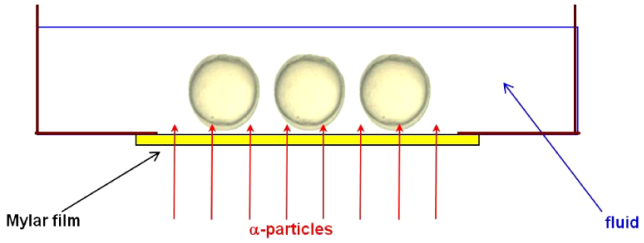

Figure 1. Irradiation of 10 hpf zebrafish embryos through the Mylar film based holder.

point for priming exposure with the consideration that the DNA repair mechanism of zebrafish embryos would be operative after the cleavage stages (0.7–2.2 hpf) (Miyachi *et al* 2003). The time point of 5 hpf for priming exposure on zebrafish embryos was previously suggested by Choi *et al* (2010c). In the present work, we tried to examine the effects of the duration of priming exposure, so we also compared the AR induced by exposing the zebrafish embryos at 5 hpf to the Cd priming dose for 1 h (until 6 hpf) and for 5 h (until 10 hpf).

2.5. Challenging exposure

The setup for alpha-particle irradiation to provide the challenging dose largely followed that devised by Yum *et al* (2007), and is shown in figure 1. Both the adaptive group and the adaptive control group of embryos were irradiated with alpha particles coming 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, and were glued by an epoxy (Araldite® Rapid, UK) onto the bottom of a Petri dish which had a diameter of 35 mm and a hole at the centre.

Dechorionated embryos from both the adaptive group and the adaptive control group were irradiated for 240 s by alpha particles from 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 ~4.4 mGy (Choi *et al* 2012). The control group of embryos was sham irradiated to ensure the same conditions as the adaptive control group and the adaptive group, except that the ^{241}Am source was not involved. All three groups of embryos were then transferred back to the incubator and allowed to develop further under a temperature of 28 °C. Figure 2 shows the schedules for different groups of zebrafish embryos.

2.6. Vital dye staining

Quantification of apoptotic signals has been commonly practised for studying the effect of radiation on whole embryos (Bladen *et al* 2005, 2007a, 2007b, Geiger *et al* 2006). In the present work, apoptotic signals in the 24 hpf embryos were quantified through staining with the vital dye AO as previously suggested (Choi *et al* 2010c). Different groups and subgroups of embryos were separately stained with AO for 60 min, thoroughly washed twice with deionised water, and then anaesthetised 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 has been commonly adopted to determine the level of apoptosis in zebrafish embryos (Tucker and Lardelli 2007, Mei *et al* 2008, Yasuda *et al* 2008). In our procedures, three 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.

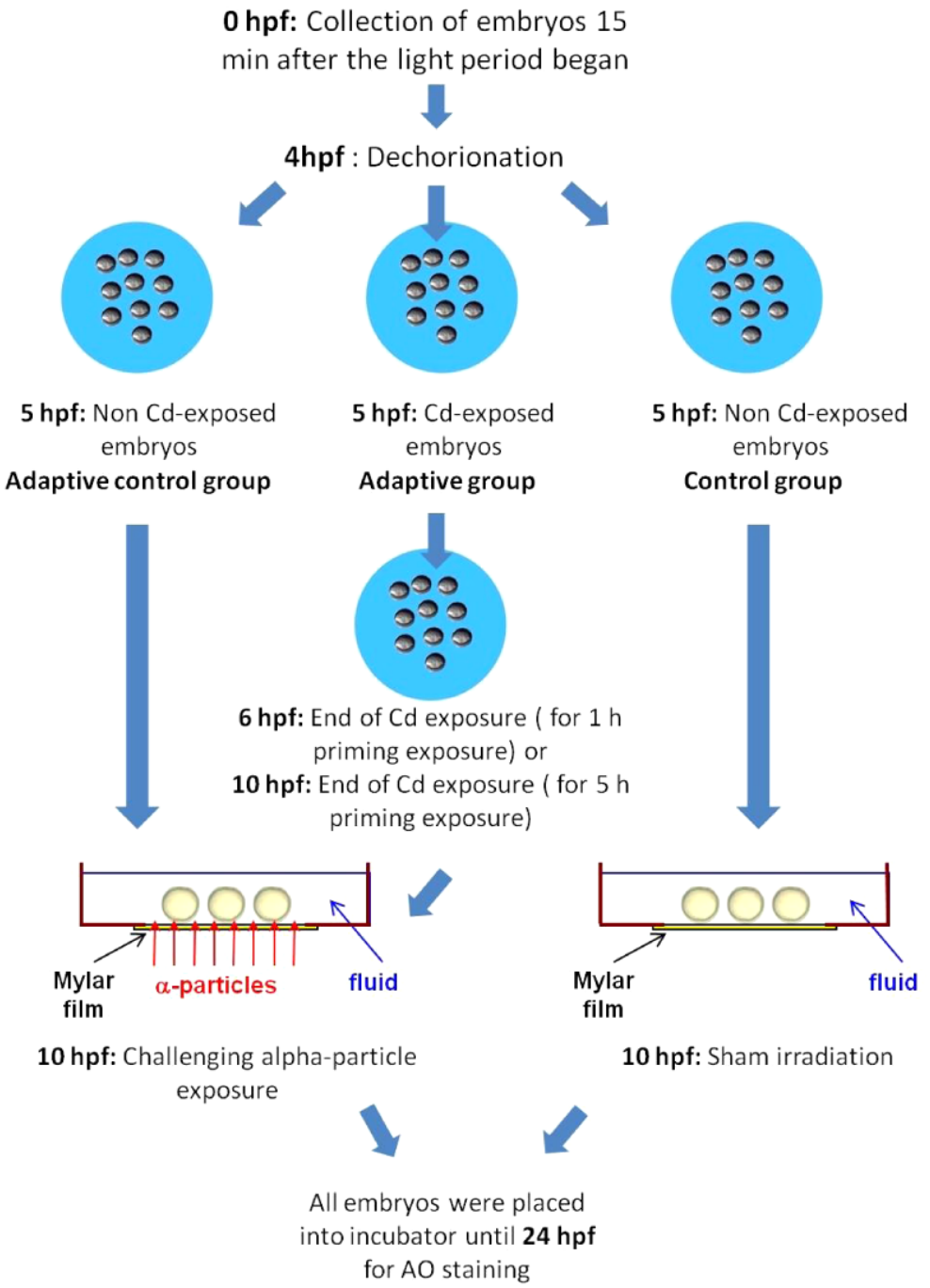

Figure 2. A flow diagram showing the exposure schedules for different groups of zebrafish embryos.

2.7. Data analysis

For each set of experiments, the number N_B of apoptotic signals for the adaptive control group (group B in section 2.3) was compared with the corresponding number N_C of apoptotic signals

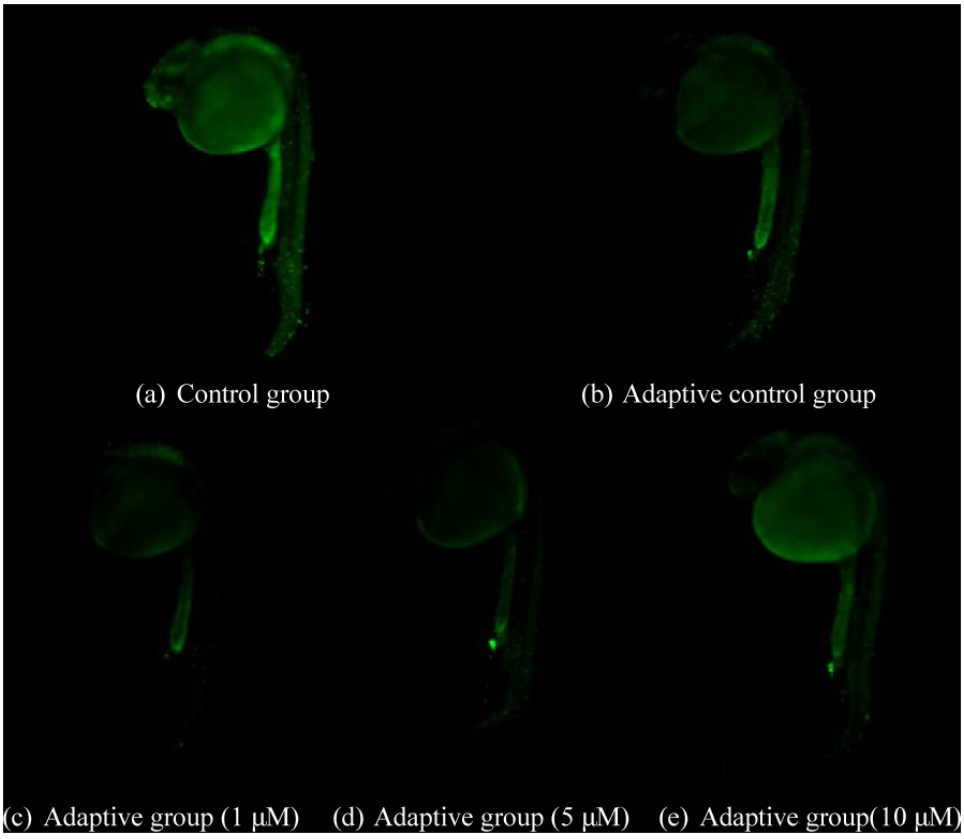

Figure 3. Apoptotic signals in typical 25 hpf zebrafish embryos revealed by acridine orange staining and captured under a fluorescent microscope (with a magnification of 40×). (a) Control group: embryos sham irradiated at 10 hpf. (b) Adaptive control group: embryos irradiated at 10 hpf. (c) Adaptive group 1: embryos exposed to 1 μM Cd at 5 hpf and then to alpha particles at 10 hpf. (d) Adaptive group 2: embryos exposed to 5 μM Cd at 5 hpf and then to alpha particles at 10 hpf. (e) Adaptive group 3: embryos exposed to 10 μM Cd at 5 hpf and then to alpha particles at 10 hpf.

for the adaptive group (group C) and the corresponding number N_A of apoptotic signals for the control group (group A) using *t*-tests. Differences with p values smaller than 0.05 were considered statistically significant.

We defined $D_A = (N_B - N_C)$, which was used to identify the presence of an AR induced by Cd exposure against subsequent alpha-particle irradiation, and defined $D_B = (N_B - N_A)$ to ascertain that alpha-particle irradiation alone caused significant damage to the zebrafish embryos. For cases with significant values of D_A and D_B ($p < 0.05$), by considering N_A as the background signal, we also determined the percentage decrease (ΔD) in the damage with both the priming and challenging exposures compared to that with the challenging exposure alone, i.e. $\Delta D = [(N_B - N_A) - (N_C - N_A)]/(N_B - N_A) = (N_B - N_C)/(N_B - N_A) = D_A/D_B$.

3. Results

Figure 3 shows the apoptotic signals revealed by AO staining of typical 25 hpf zebrafish embryos in (a) the control group, (b) the adaptive control group, (c) the adaptive group and

Table 1. Mean (± SE) number of apoptotic signals obtained in three treatment groups of zebrafish embryos (control group, adaptive control group and adaptive group) from 15 sets of experiments. The duration of priming exposure for the adaptive groups in experimental sets 1–10 was 1 h, while that for the adaptive groups in experimental sets 11–15 was 5 h. n : number of zebrafish embryos in a particular sample. The t-test p values are presented in table 2.

Set	Control	Adaptive control	Adaptive (1 μM)	Adaptive (5 μM)	Adaptive (10 μM)
1	77 ± 6	195 ± 25	115 ± 15	113 ± 11	110 ± 11
	($n = 14$)	($n = 12$)	($n = 11$)	($n = 13$)	($n = 15$)
2	96 ± 6	135 ± 5	92 ± 7	97 ± 5	96 ± 4
	($n = 14$)	($n = 10$)	($n = 10$)	($n = 10$)	($n = 8$)
3	95 ± 10	169 ± 13	119 ± 12	118 ± 10	118 ± 9
	($n = 12$)	($n = 11$)	($n = 10$)	($n = 10$)	($n = 11$)
4	64 ± 13	138 ± 10	56 ± 3	66 ± 8	97 ± 7
	($n = 8$)	($n = 8$)	($n = 8$)	($n = 11$)	($n = 9$)
5	103 ± 13	182 ± 14	88 ± 15	109 ± 7	137 ± 19
	($n = 11$)	($n = 12$)	($n = 8$)	($n = 9$)	($n = 10$)
6	81 ± 14	149 ± 19	87 ± 10	81 ± 12	97 ± 3
	($n = 9$)	($n = 8$)	($n = 9$)	($n = 9$)	($n = 10$)
7	103 ± 11	185 ± 18	124 ± 12	145 ± 9	140 ± 10
	($n = 12$)	($n = 10$)	($n = 13$)	($n = 15$)	($n = 15$)
8	80 ± 8	116 ± 13	92 ± 13	73 ± 9	76 ± 6
	($n = 13$)	($n = 12$)	($n = 9$)	($n = 8$)	($n = 8$)
9	102 ± 16	152 ± 14	101 ± 6	140 ± 13	93 ± 13
	($n = 12$)	($n = 11$)	($n = 8$)	($n = 8$)	($n = 10$)
10	42 ± 5	117 ± 19	70 ± 8	76 ± 6	80 ± 11
	($n = 12$)	($n = 9$)	($n = 9$)	($n = 9$)	($n = 9$)
11	102 ± 7	232 ± 26	148 ± 12	NA	135 ± 10
	($n = 9$)	($n = 9$)	($n = 10$)		($n = 10$)
12	89 ± 4	224 ± 18	126 ± 8	NA	115 ± 9
	($n = 9$)	($n = 10$)	($n = 10$)		($n = 10$)
13	90 ± 9	231 ± 10	171 ± 9	NA	162 ± 11
	($n = 9$)	($n = 10$)	($n = 9$)		($n = 10$)
14	85 ± 7	244 ± 33	153 ± 10	NA	161 ± 24
	($n = 10$)	($n = 10$)	($n = 9$)		($n = 10$)
15	81 ± 8	202 ± 11	133 ± 11	NA	173 ± 10
	($n = 10$)	($n = 10$)	($n = 10$)		($n = 10$)

in the subgroup corresponding to a priming dose provided by 1 μM Cd, (d) the adaptive group and in the subgroup corresponding to a priming dose provided by 5 μM Cd, and (e) adaptive group and in the subgroup corresponding to a priming dose provided by 10 μM Cd. Both the adaptive control group and the adaptive group of embryos showed more apoptotic signals than the control group, while the adaptive groups showed fewer apoptotic signals than the adaptive control group.

A total of 15 repeated sets of experiments were carried out on different days. The (mean ± standard error (SE)) for the number of apoptotic signals obtained in the different groups are summarised in table 1. The results for experimental sets 1–10 were obtained with a 1 h priming exposure time, while those for sets 11–15 were obtained with a 5 h priming exposure time. The number (n) of embryos in each sample was also shown, and some samples had $n < 15$ due to mortality.

Table 2 shows the values of ($D_{\mathrm{A}} \pm$ SE) and ($D_B \pm$ SE) together with their p values obtained using t-tests. We also calculated $\Delta D = D_{\mathrm{A}}/D_{\mathrm{B}}$ for those cases with significant values of D_{A} and D_{B} ($p < 0.05$ to obtain the percentage decrease in the damage with both the priming and challenging exposures compared to that with the challenging exposure alone.

Table 2. The p values for comparisons of zebrafish groups in table 1. D_A = apoptotic signals for adaptive control—apoptotic signals for adaptive group; D_B = apoptotic signals for adaptive control—apoptotic signals for control group; $\Delta D = D_A/D_B$ determined only for those cases with significant values of D_A and D_B ($p < 0.05$); p: t-test p values (cases with $p < 0.05$ are asterisked).

	D_A				ΔD(%)		
Set	1 μM	5 μM	10 μM	D_B	1 μM	5 μM	10 μM
Set 1.	80 ± 29	81 ± 26	85 ± 25	118 ± 24	68	69	72
p	0.006*	0.004*	0.003*	0.0003*			
Set 2.	43 ± 8	38 ± 7	39 ± 7	39 ± 9	110	96	99
p	5.2×10^{-5}*	1.3×10^{-5}*	7.5×10^{-6}*	3.6×10^{-5}*			
Set 3.	50 ± 17	51 ± 16	51 ± 15	95 ± 16	68	69	69
p	0.005*	0.002*	0.002*	0.0001*			
Set 4.	82 ± 10	72 ± 12	41 ± 12	74 ± 16	110	97	56
p	1.5×10^{-5}*	1.7×10^{-5}*	0.002*	0.0003*			
Set 5.	94 ± 21	73 ± 18	45 ± 23	79 ± 19	119	92	56
p	0.0001*	0.0001*	0.039*	0.0003*			
Set 6.	62 ± 21	68 ± 22	51 ± 17	68 ± 23	90	100	75
p	0.009*	0.006*	0.016*	0.006*			
Set 7	61 ± 21	40 ± 19	45 ± 19	82 ± 21	NA	49	55
p	0.104	0.005*	0.011*	0.001*			
Set 8.	24 ± 19	44 ± 16	40 ± 17	36 ± 15	NA	122	113
p	0.103	0.003*	0.007*	0.014*			
Set 9.	52 ± 19	13 ± 20	59 ± 20	51 ± 22	101	NA	116
p	0.003*	0.262	0.004*	0.014*			
Set 10.	48 ± 21	41 ± 20	38 ± 22	75 ± 17	63	55	NA
p	0.022*	0.033*	0.055	0.002*			
Set 11.	85 ± 27	NA	97 ± 27	130 ± 27	65	NA	74
p	0.006*	NA	0.003*	0.0004*			
Set 12.	98 ± 20	NA	109 ± 20	89 ± 19	73	NA	81
p	0.0001*	NA	4.9×10^{-5}*	1.2×10^{-5}*			
Set 13.	55 ± 14	NA	69 ± 15	90 ± 14	39	NA	49
p	0.0005*	NA	0.0001*	7.1×10^{-9}*			
Set 14.	90 ± 36	NA	82 ± 41	158 ± 34	57	NA	52
p	0.013*	NA	0.031*	0.0005*			
Set 15.	69 ± 16	NA	29 ± 15	81 ± 14	57	NA	24
p	0.0002*	NA	0.033*	8.0×10^{-8}*			

As shown in table 2, all 15 sets of experiments showed a significantly positive value of D_B ($p < 0.05$); this implied that significant damage to the 10 hpf zebrafish embryos could be inflicted by 4.4 mGy alpha-particle irradiation alone. Among the experiments which involved 1 h Cd exposure as the priming exposure (i.e. sets 1–10), six sets (sets 1–6) showed significant positive values of D_A ($p < 0.05$) for all three Cd concentrations used. No significant values of D_A were obtained in set 7 (1 μM), set 8 (1 μM), set 9 (5 μM) or set 10 (10 μM); however, those values of D_A were still all positive. The insignificant results were probably due to the large spread in the corresponding datasets.

The values for ΔD from those significantly positive D_A in sets 1–10 ranged from 49 to 119%. The mean values of ΔD for Cd concentrations of 1, 5 and 10 μM were separately calculated from the corresponding ΔD, and the results are shown in figure 4. The mean value of ΔD was the highest when the priming dose was provided by 1 μM Cd. However, no significant differences ($p < 0.05$) were found among the mean values of ΔD for the priming dose provided by different Cd concentrations for 1 h exposure.

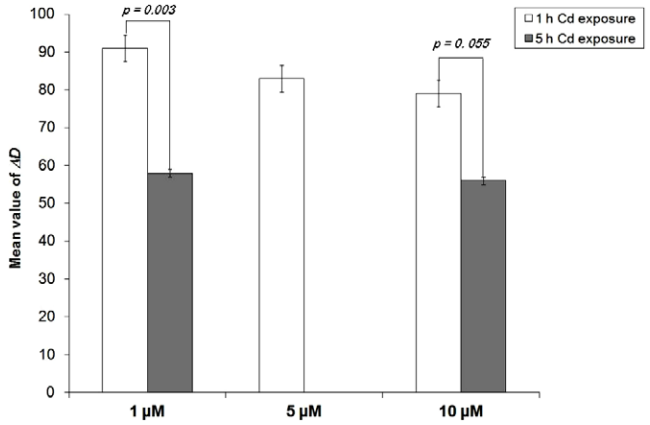

Figure 4. Comparison between the mean values of ΔD (%) for priming exposures provided by Cd concentrations of 1 μM (exposure time = 1 and 5 h), 5 μM (exposure time = 1 h) 10 μM (exposure time = 1 and 5 h).

In order to study the effects of length of exposure to Cd on AR induction, we changed the duration of Cd exposure from 1 to 5 h (corresponding to sets 11–15). All five sets showed significantly positive values of D_A for a priming dose provided by Cd concentrations of 1 and 10 μM ($p < 0.05$), with values of ΔD ranging from 24 to 74%. The mean values of ΔD for the priming dose provided by 5 h exposure and with Cd concentrations of 1 and 10 μM are shown in figure 4. The mean values of ΔD for both concentrations were very similar with no significant difference.

From the above experimental results, we concluded that zebrafish embryos treated with 1–10 μM Cd at 5 hpf for exposure times of both 1 and 5 h could induce an AR against a subsequent 4.4 mGy alpha-particle irradiation applied at 10 hpf. No significant variations in the strength of adaptation were found when the embryos were treated with 1–10 μM Cd.

4. Discussion

This paper demonstrated that zebrafish embryos exposed to Cd in micromolar concentrations could develop an AR against a subsequent alpha-particle irradiation, which was revealed by apoptotic signals at 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 toxins or genetic mutations can disrupt the regulated occurrence of apoptosis, which can lead to developmental abnormalities (Zakeri and Ahuja 1997, Mirkes 2002).

In the present study, Cd in micromolar concentrations was chosen to provide the priming exposure for the embryos. The effect of such Cd concentrations on zebrafish embryos was studied by Chan and Cheng (2003) who concluded that ectopic apoptosis was only induced for Cd exposures at higher concentrations. Significant numbers of apoptotic cells in zebrafish embryos which had been exposed to 100 μM Cd from 5 to 28 hpf were observed by Chan and Cheng (2003), while only 0.67% of apoptotic cells were found in zebrafish embryos exposed to 1 μM Cd for 24 h. Their data were commensurate with other tissue culture studies using Cd at micromolar ranges. Cd-induced apoptosis was reviewed by Robertson and Orrenius (2000). The underlying mechanisms are not well understood, but involvement of the caspases enzymatic

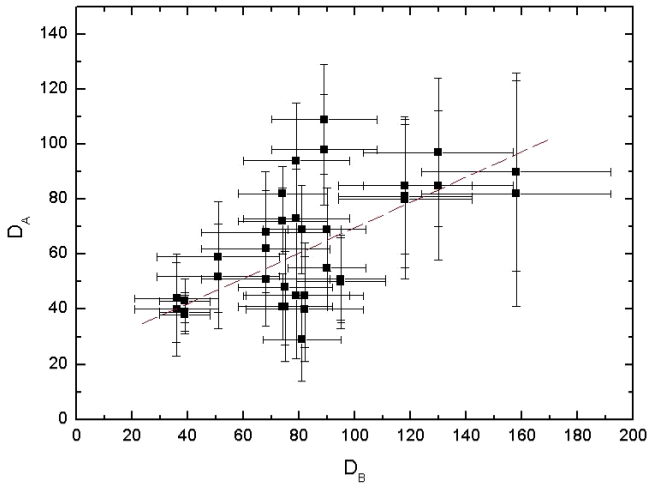

Figure 5. Relationship between D_A (apoptotic signals for adaptive control–apoptotic signals for adaptive group) and D_B (apoptotic signals for adaptive control–apoptotic signals for control group).

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).

The strength of AR was studied in the present work through the percentage decrease in damage with both the priming and challenging exposures compared to that with the challenging exposure alone, given by $\Delta D = D_A/D_B$, for those cases with significant values of D_A and D_B ($p < 0.05$). All 15 sets of experiments displayed positive values of ΔD. Pre-treatment with Cd at concentrations ranging from 1 to 10 μM would therefore decrease the biological effect of subsequent alpha-particle irradiation on the zebrafish embryos. However, the strength of the AR did not increase with the Cd concentration employed for the priming exposure. No correlation was found between the strength of the AR and the Cd concentration, which ranged from 1 to 10 μM for the priming exposure, for exposure durations of both 1 and 5 h. On the other hand, for the priming exposure provided by 1 μM Cd, the mean value of ΔD for an exposure duration of 1 h was significantly larger than that for 5 h ($p = 0.003$), as shown in figure 4. Similarly, as shown in figure 4, regarding the priming exposure provided by 10 μM Cd, the mean value of ΔD for the exposure duration of 1 h was also larger than that for 5 h and the difference was very close to significant ($p = 0.055$). The stronger AR induced in embryos with a shorter exposure duration might be explained by the longer time interval between the priming and challenging exposures. Shadley *et al* (1987) suggested that an AR was not induced immediately after the provision of the priming dose, and would take 4–6 h to become fully active. A 5 h interval was used to successfully induce an AR in zebrafish embryos in our previous studies (Choi *et al* 2010a, 2010b, 2010c).

Furthermore, we investigated the relationship between D_A and D_B by plotting D_A against D_B as shown in figure 5. A significant positive correlation was obtained between the two parameters ($p < 0.0001$), which suggested that the 'magnitude' of the AR represented by D_A depended on the 'magnitude' of damage caused by the corresponding challenging exposure represented by D_B.

The present results demonstrated an AR induced by a stressor (the heavy metal Cd in this case) against a different stressor (ionising alpha-particle radiation in this case) in a vertebrate

model. This also meant an antagonistic multiple stressor effect between an ionising radiation and a chemical, which supported the findings of Mothersill *et al* (2007a) and Salbu *et al* (2008). Further studies on the mechanisms involved in the induction of an AR between different stressors or in the antagonistic multiple stressor effect in general will be pertinent. These findings are also very interesting and relevant to environmental protection.

The zebrafish has been established as a popular vertebrate model for studying the *in vivo* response to ionising radiation. The results in the present paper showed that the radiation risk could be perturbed by another environmental stressor such as a heavy metal, and as such a realistic human radiation risk assessment should in general take into account multiple stressor effects. This has far reaching consequences for radiation protection.

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