The multiple stressor effect in zebrafish embryos from simultaneous exposure to ionising radiation and cadmium

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Abstract
Living organisms are exposed to a mixture of environmental stressors, and the resultant effects are referred to as multiple stressor effects. In the present work, we studied the multiple stressor effect in embryos of the zebrafish (Danio rerio) from simultaneous exposure to ionising radiation (alpha particles) and cadmium through quantification of apoptotic signals at 24 h postfertilisation (hpf) revealed by vital dye acridine orange staining. For each set of experiments, 32–40 dechorionated embryos were deployed, which were divided into four groups each having 8–10 embryos. The four groups of embryos were referred to as (1) the control group (C), which received no further treatments after dechorionation; (2) the Cd-dosed and irradiated group (CdIr), which was exposed to 100 \( \mu \)M Cd from 5 to 24 hpf, and also received about 4.4 mGy from alpha particles at 5 hpf; (3) the irradiated group (Ir), which received about 4.4 mGy from alpha particles at 5 hpf; and (4) the Cd-dosed group (Cd), which was exposed to 100 \( \mu \)M Cd from 5 to 24 hpf. In general, the CdIr, Ir and Cd groups had more apoptotic signals than the C group. Within the 12 sets of experimental results, two showed significant synergistic effects, one showed a weakly synergistic effect and nine showed additive effects. The multiple stressor effect of 100 \( \mu \)M Cd with \( \sim \)4.4 mGy alpha-particle radiation resulted in an additive or synergistic effect, but no antagonistic effect. The failure to identify significant synergistic effects for some sets of data, and thus their subsequent classification as additive effects, might be a result of the relatively small magnitude of the synergistic effects. The results 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 the multiple stressor effects.

(Some figures may appear in colour only in the online journal)
1. 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. Multiple stressor effects might not be simply the sum of effects from individual stressors [1, 2]. Heavy metals like cadmium (Cd) are ubiquitous in our environment. Cd exposure has been related to human prostate, pancreatic and renal cancers [3–5]. While there are already many research works on the effect of multiple stressors, relatively few of them have been on the multiple stressor effects of simultaneous exposure to ionising radiation and Cd, the study of which formed the objective of the present work.

The present work used embryos of the zebrafish, *Danio rerio*, as a vertebrate model to study multiple stressor effects. The zebrafish and human genomes share considerable homology, including conservation of most DNA repair-related genes [6]. There has been a growing number of research works using zebrafish or their embryos as a vertebrate model to study the *in vivo* response to ionising radiation [7–17]. In the present work, exposure to ionising radiation was achieved by alpha-particle irradiation because (1) alpha particles due to their large linear energy transfer will be most effective in causing DNA double-strand breaks, which are considered the most relevant lesions for mutations and carcinogenesis, and (2) natural and artificial radionuclides with alpha-particle emission are common in our environment. Alpha-particle irradiation was employed together with exposure to Cd with a concentration of 100 µM, and quantification of apoptotic signals was used as the biological endpoint.

2. Materials and method

2.1. Experimental animals

Adult zebrafish were kept in glass tanks with water controlled at 28 °C. To maintain a good production of embryos, a 14/10 h light–dark period was adopted. A plastic embryo collector, as described by Choi *et al* [12], was used to collect the embryos. Once the 14 h photoperiod started, photo-induced spawning commenced, and the embryo collector was lowered onto the bottom of the each glass tank to collect the embryos. Collection of embryos lasted a relatively short period of only 15–30 min to ensure synchronisation of the developmental stage of the collected embryos. The collected embryos were then immediately transferred to and incubated in a 28°C incubator.

2.2. Preparation of embryos

The embryos had to be dechorionated to avoid excessive loss of energy before the alpha particles reached the cells. At 4 h postfertilisation (hpf), healthily developing embryos were selected under a stereo-microscope and transferred into a Petri dish with E3 solution (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, 0.33 mM MgSO₄, 0.1% methylene blue) and with a layer of biocompatible agarose as the substrate for dechorionation. All studied embryos, including those which were not going to receive the radiation dose, were dechorionated to ensure uniform conditions.

2.3. Exposure protocol

For each set of experiments, 32–40 dechorionated embryos were employed. These embryos were separated into four groups, each having 8–10 embryos, and accommodated in four separate Petri dishes, referred to as:
(1) the control group (C): embryos were dechorionated without receiving any further treatment;
(2) the Cd-dosed and irradiated group (CdIr): embryos were exposed to 100 µM Cd from 5 to 24 hpf, and also received about 4.4 mGy from alpha particles at 5 hpf;
(3) the irradiated group (Ir): embryos received about 4.4 mGy from alpha particles at 5 hpf;
(4) the Cd-dosed group (Cd): embryos were exposed to 100 µM Cd from 5 to 24 hpf.

Considering that the DNA repair mechanism in zebrafish embryos would only become operative after the cleavage stages (0.7–2.2 hpf) [18], the embryos were exposed to Cd and/or alpha-particle radiation at 5 hpf which was within the blastula stage (2.2–5.2 hpf). The embryos in the CdIr group were exposed to 100 µM Cd (cadmium nitrate tetrahydrate, Cd(NO₃)₂·4H₂O) and ∼4.4 mGy of alpha-particle radiation, while those in the Ir and Cd groups were exposed to ∼4.4 mGy of alpha-particle irradiation and 100 µM Cd, respectively.

2.4. Alpha-radiation exposure

The setup for alpha-particle irradiation in the present experiment largely followed that designed by Yum et al [10]. A biocompatible substrate, Mylar film (Dupont, Hong Kong) with a thickness of 3.5 µm was used as the support substrate during irradiation. The irradiation dish consisted of a Mylar film glued to the bottom of a Petri dish which had a diameter of 35 mm and a hole at the centre using an epoxy (Araldite® Rapid, UK).

The 5 hpf embryos in the CdIr and Ir groups were placed on the substrate in the irradiation dish and irradiated with alpha particles for 4 min using an ²⁴¹Am 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 of ∼4.4 mGy. To avoid the problem of having different travelling depths of alpha particles in the medium before reaching the embryos, these two groups of embryos were irradiated with alpha particles coming from below through the supporting substrate instead of coming from above. 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. Figure 1 shows a side-view schematic diagram of the irradiation setup [10]. On the other hand, the embryos in the Cd group experienced exactly the same treatment, except the use of the ²⁴¹Am source. After sham-irradiation and irradiation for the Cd and CdIr groups, respectively, they were ready for further Cd exposure.

2.5. Cadmium exposure

A 100 µM cadmium nitrate stock solution was prepared by dissolving cadmium nitrate tetrahydrate (Sigma-Aldrich, MO, USA) in de-ionised water, and was then stored at room temperature. The same stock solution was used for all experiments in the present project. It
was established that significant amounts of apoptotic cells were observed in zebrafish embryos after exposure to 100 µM Cd from 5 to 28 hpf [19], which was commensurate with other tissue-culture studies using micromolar Cd concentrations [20–24]. Chan and Cheng [19] also found 0.67% of apoptotic cells by flow cytometry assay for embryos which were exposed to 1 µM Cd for 24 h. With reference to these results and to the literature, they concluded that ectopic induction of apoptosis only occurred for Cd exposures with higher concentrations [19]. As such, 100 µM Cd has been chosen for the present study. A volume of 2 ml of cadmium nitrate solution, which was sufficient to cover all embryos accommodated in each dish, was prepared in two new Petri dishes. Immediately after the CdIr and Cd groups of embryos were irradiated and sham irradiated, respectively, they were removed from the medium and transferred to 100 µM cadmium nitrate solution using a glass dropper to provide the Cd dose until 24 hpf. All the four groups of embryos (C, CdIr, Ir and Cd) were then incubated at 28 °C in an incubator until they developed to 24 hpf.

2.6. Quantification of apoptosis by vital dye staining

Quantification of apoptosis, which is the biological endpoint chosen for the current study, has been widely adopted to examine the radiation effect on whole embryos [8, 25]. The vital dye acridine orange (AO) (Sigma, St Louis, MO, US) was used to stain the apoptotic cells in the 24 hpf embryos as previously suggested by Choi et al [12]. Briefly speaking, the four groups of embryos were transferred into a culture medium containing 5 µg ml⁻¹ of AO to stain in the dark for 60 min and then thoroughly washed twice in the culture medium. The embryos were then anaesthetised by transferring into 0.016 M tricaine (Sigma, St Louis, MO, US). The apoptotic signals of the embryos were finally counted under a fluorescent microscope. Vital dye staining has been commonly employed to quantify the level of apoptosis in zebrafish embryos [26–28]. For each embryo, three images with focus on different sections of the embryo were captured under the fluorescent microscope with a magnification of 40×. These images were then combined into one signal image for quantification of the apoptotic signals. A computer program ‘Particle Counting 2.0’ (developed by Zhang) was used to count the apoptotic signals in an embryo.

2.7. Statistical analysis

In the current study, 12 repeated sets of experiments with 32–40 zebrafish embryos each were carried out on different days. The number of apoptotic signals on each entire zebrafish embryo was counted as described above. After rearranging a group of data in a descending order, values lying within the range of 1.5 times the interquartile range above the 75th percentile and below the 25th percentile of the group of data were preserved while other values were referred as ‘outliers’ and were excluded from further data analysis. Here, the interquartile range was defined as the difference between the 25th and 75th percentiles of the data.

We denoted the mean numbers of apoptotic signals for the C, CdIr, Ir and Cd groups as $N_C$, $N_{CdIr}$, $N_Ir$ and $N_{Cd}$, respectively. If $N_C$ was interpreted as the average background apoptotic signal for the embryos in the corresponding set of experiments, the net apoptotic signals for the CdIr, Ir and Cd groups could be determined as $N_{CdIr}^\ast = (N_{CdIr} - N_C)$, $N_{Ir}^\ast = (N_{Ir} - N_C)$ and $N_{Cd}^\ast = (N_{Cd} - N_C)$, respectively.

The multiple stressor effect was compared with the effects from individual stressors. Two methods were employed to construct the expected mean number of apoptotic signals considering the effects contributed by individual stressors as described in section 3 below. Student’s $t$-test was performed between these constructed groups and the CdIr group, with $p$ values < 0.05 representing statistically significant differences.
3. Results

In the present study, the number of apoptotic signals present in the 25 hpf zebrafish embryos was used as the biological endpoint to characterise the effects of Cd and radiation exposure. Representative images of zebrafish embryos with apoptotic signals revealed by vital dye staining using AO for groups C, CdIr, Ir and Cd are shown in figure 2.

A total of 12 repeated sets of experiments with 32–40 zebrafish embryos each was carried out on different days. The mean numbers of apoptotic signals $N$ obtained for the groups C, CdIr, Ir and Cd, namely $N_C$, $N_{CdIr}$, $N_{Ir}$ and $N_{Cd}$, respectively, are shown in table 1. In general, $N_{CdIr}$, $N_{Ir}$ and $N_{Cd}$ were larger than $N_C$. In all 12 sets of experiments, the $N_{Cd}$ values were significantly larger than the $N_C$ values, which agreed with the findings of Chan and Cheng [19]. In 11 out of the 12 sets of experiments, the $N_{CdIr}$ values were significantly larger than both the $N_{Ir}$ and $N_{Cd}$ values.

The multiple stressor effect was compared with the effects from individual stressors. Table 2 shows the effects on zebrafish embryos of the combined action of 4.4 mGy alpha-particle irradiation and 100 µM Cd. Two methods were employed to construct the expected mean number of apoptotic signals considering the effects contributed by individual stressors:

1. addition of $N_{Ir}^*$ to each embryo in the Cd group, to form the Cd(Ir) group (case 1);
2. addition of $N_{Cd}^*$ to each embryo in the Ir group, to form the Ir(Cd) group (case 2).

By definition, the mean numbers of apoptotic signals obtained for these two groups, namely $N_{Cd(Ir)}$ and $N_{Ir(Cd)}$, were equal. Student’s $t$-tests were performed to see whether the Cd(Ir) or Ir(Cd) groups were significantly different ($p < 0.05$) from the CdIr group. If $p < 0.05$
Table 1. The average number of apoptotic signals \( N \) (±SE) obtained for different groups of embryos (C, CdIr, Ir, Cd). \( n \) is the number of zebrafish embryos in a particular sample after removal of outliers. The \( p \) values for the Ir and Cd groups correspond to comparisons between these groups and the CdIr group using the \( t \)-test.

<table>
<thead>
<tr>
<th>Set</th>
<th>C</th>
<th>CdIr</th>
<th>Ir</th>
<th>Cd</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>( N )</td>
<td>95 ± 2</td>
<td>263 ± 3</td>
<td>177 ± 3</td>
</tr>
<tr>
<td>2</td>
<td>( N )</td>
<td>76 ± 3</td>
<td>167 ± 3</td>
<td>135 ± 3</td>
</tr>
<tr>
<td>3</td>
<td>( N )</td>
<td>81 ± 2</td>
<td>237 ± 5</td>
<td>162 ± 2</td>
</tr>
<tr>
<td>4</td>
<td>( N )</td>
<td>91 ± 2</td>
<td>223 ± 2</td>
<td>157 ± 4</td>
</tr>
<tr>
<td>5</td>
<td>( N )</td>
<td>81 ± 2</td>
<td>202 ± 3</td>
<td>156 ± 4</td>
</tr>
<tr>
<td>6</td>
<td>( N )</td>
<td>138 ± 5</td>
<td>371 ± 4</td>
<td>261 ± 1</td>
</tr>
<tr>
<td>7</td>
<td>( N )</td>
<td>52 ± 3</td>
<td>172 ± 5</td>
<td>95 ± 3</td>
</tr>
<tr>
<td>8</td>
<td>( N )</td>
<td>67 ± 2</td>
<td>209 ± 2</td>
<td>166 ± 3</td>
</tr>
<tr>
<td>9</td>
<td>( N )</td>
<td>95 ± 2</td>
<td>184 ± 3</td>
<td>144 ± 1</td>
</tr>
<tr>
<td>10</td>
<td>( N )</td>
<td>87 ± 2</td>
<td>165 ± 1</td>
<td>142 ± 2</td>
</tr>
<tr>
<td>11</td>
<td>( N )</td>
<td>59 ± 2</td>
<td>153 ± 9</td>
<td>115 ± 4</td>
</tr>
<tr>
<td>12</td>
<td>( N )</td>
<td>75 ± 1</td>
<td>185 ± 2</td>
<td>143 ± 1</td>
</tr>
</tbody>
</table>

\( n \) is the number of zebrafish embryos in a particular sample after removal of outliers. The \( p \) values for the Ir and Cd groups correspond to comparisons between these groups and the CdIr group using the \( t \)-test.

\( a \) Cases with \( p \) values < 0.05 are considered to be statistically significant.

for both cases 1 and 2, we would conclude on either a synergistic effect if \( (N_{Ir}^* < N_{CdIr}^* - N_{Cd}^*) \) or \( (N_{Cd}^* < N_{CdIr}^* - N_{Ir}^*) \), or an antagonistic effect if \( (N_{Ir}^* > N_{CdIr}^* - N_{Cd}^*) \) or \( (N_{Cd}^* > N_{CdIr}^* - N_{Ir}^*) \). If \( p \geq 0.05 \) for both cases 1 and 2 but \( p < 0.1 \) for at least one of the cases 1 and 2, we would conclude on a weakly synergistic effect or a weakly antagonistic effect (non-significant). The remaining cases were considered to display an additive effect.

Within the 12 sets of experimental results shown in Table 2, two showed significant synergistic effects, one showed a weakly synergistic effect (non-significant) and nine showed additive effects. These results indicate that the multiple stressor effect of 100 \( \mu \)M Cd with \( \sim 4.4 \) mGy alpha-particle radiation results in an additive or synergistic effect, but no antagonistic effect.
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Table 2. Expected values ($N_{Cd}^*_{Ir}$) and observed values ($N_{Cd,Ir}$ or $N_{Ir,Cd}$, which are equal) for the combined effects of Cd and alpha-particle irradiation on the zebrafish embryos at 25 hpf, indicating whether the interactions are additive, synergistic or antagonistic.

<table>
<thead>
<tr>
<th>Set</th>
<th>Expected</th>
<th>Observed</th>
<th>$p$ (case 1$^a$)</th>
<th>$p$ (case 2$^b$)</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>168 ± 5</td>
<td>140 ± 11</td>
<td>0.11</td>
<td>0.088</td>
<td>Weakly synergistic</td>
</tr>
<tr>
<td>2</td>
<td>91 ± 6</td>
<td>78 ± 10</td>
<td>0.17</td>
<td>0.23</td>
<td>Additive</td>
</tr>
<tr>
<td>3</td>
<td>156 ± 7</td>
<td>160 ± 10</td>
<td>0.45</td>
<td>0.45</td>
<td>Additive</td>
</tr>
<tr>
<td>4</td>
<td>132 ± 4</td>
<td>129 ± 11</td>
<td>0.41</td>
<td>0.44</td>
<td>Additive</td>
</tr>
<tr>
<td>5</td>
<td>121 ± 5</td>
<td>145 ± 12</td>
<td>0.13</td>
<td>0.15</td>
<td>Additive</td>
</tr>
<tr>
<td>6</td>
<td>233 ± 9</td>
<td>208 ± 17</td>
<td>0.25</td>
<td>0.16</td>
<td>Additive</td>
</tr>
<tr>
<td>7</td>
<td>120 ± 8</td>
<td>104 ± 12</td>
<td>0.26</td>
<td>0.26</td>
<td>Additive</td>
</tr>
<tr>
<td>8</td>
<td>142 ± 4</td>
<td>115 ± 9</td>
<td>0.0049</td>
<td>0.034</td>
<td>Significantly synergistic</td>
</tr>
<tr>
<td>9</td>
<td>89 ± 5</td>
<td>100 ± 8</td>
<td>0.26</td>
<td>0.19</td>
<td>Additive</td>
</tr>
<tr>
<td>10</td>
<td>78 ± 3</td>
<td>83 ± 10</td>
<td>0.35</td>
<td>0.23</td>
<td>Additive</td>
</tr>
<tr>
<td>11</td>
<td>94 ± 11</td>
<td>76 ± 10</td>
<td>0.34</td>
<td>0.35</td>
<td>Additive</td>
</tr>
<tr>
<td>12</td>
<td>110 ± 3</td>
<td>83 ± 4</td>
<td>0.0032</td>
<td>0.0039</td>
<td>Significantly synergistic</td>
</tr>
</tbody>
</table>

$^a$ By comparing the CdIr and Cd(Ir) groups, the latter constructed by adding $N_{Ir}^*$ to each embryo in the Cd group.

$^b$ By comparing the CdIr and Ir(Cd) groups, the latter constructed by adding $N_{Cd}^*$ to each embryo in the Ir group.

4. Discussion

Both Cd and ionising radiation are well known gene mutagens [29, 30]. Since simultaneous exposure to these two stressors is common in natural as well as occupational environments, the multiple stressor effect of Cd with ionising radiation has generated considerable interest in recent years. The present study revealed additive and synergistic effects in zebrafish embryos for simultaneous exposure to Cd and alpha-particle irradiation. Nevertheless, the failure to identify significant synergistic effects for some sets of data, and thus their subsequent classification as additive effects, might be a result of the relatively small magnitude of the synergistic effects.

Cd-induced apoptosis has been reviewed by Robertson and Orrenius et al [31]; however, the underlying mechanisms are still not well known. Possible mechanisms such as involvement of the caspases enzymatic pathway, suppression of the tumour suppressor gene p53 and protection by the anti-apoptotic gene Bcl-2 have been suggested [32–35]. The induction of apoptotic cell death by Cd has also been demonstrated in some other mammalian and fish cell lines [36]. Besides, Cd has an impact on genomic stability by inhibiting various DNA repair enzymes [37, 38]. There is experimental evidence indicating that Cd interferes with many DNA repair pathways, such as mismatch repair (MMR), non-homologous end-joining (NHEJ) and homologous recombination (HR) repair of double-strand breaks and base excision repair [39–41]. Cd has also been found to inhibit the repair of oxidative DNA damage in different mammalian cells both in vivo and in vitro [42, 37]. As such, the yield of DNA damage in zebrafish embryos due to alpha-particle irradiation might be increased by suppression of the efficiency of DNA repair systems by Cd [43, 44], which would likely lead to a synergistic effect.

To conclude, the combined action of 4.4 mGy alpha-particle irradiation and 100 µM Cd in general resulted in an additive and synergistic effect, while the additive effect was likely a manifestation of the weakly synergistic effect. 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 show that the radiation risk can 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 the multiple stressor effects. This has far reaching consequences in radiation protection.

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