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# Effects of exogenous carbon monoxide on radiation-induced bystander effect in zebrafish embryos *in vivo*

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ABSTRACT

In the present work, the influence of a low concentration of exogenous carbon monoxide (CO) liberated from tricarbonylchloro(glycinato)ruthenium (II) (CORM-3) on the radiation induced bystander effect (RIBE) *in vivo* between embryos of the zebrafish was studied. RIBE was assessed through the number of apoptotic signals revealed on embryos at 25 h post fertilization (hpf). A significant attenuation of apoptosis on the bystander embryos induced by RIBE in a CO concentration dependent manner was observed.



## 1. Introduction

In contrast to the linear-no-threshed (LNT) model widely accepted for radiation protection saying that biological effects caused by ionizing radiation are linearly proportional to the absorbed dose, evidence accumulated in the past decades showed that the dose-response of radiation in the low-dose regime deviated from the LNT model. A notable example causing such a deviation is the radiation induced bystander effect (RIBE) which amplifies the biological effect of radiation by eliciting damages in non-targeted unirradiated cells. RIBE was first demonstrated by Nagasawa and Little (1992). There are a number of reviews describing the RIBE both *in vitro* and *in vivo* (e.g., Mothersill and Seymour, 2001; 2004; Goldberg and Lehnert, 2002; Little, 2006; Morgan and Sowa, 2007). The RIBE could affect the effectiveness of radiotherapy in that the bystander signal released from the irradiated tumor tissue to the normal surrounding tissue could increase the cancer risk.

Until now, the mechanism underlying RIBE is still poorly understood. However, reactive oxygen species (ROS) and nitric oxide (NO) acted as important signals to mediate the transduction of RIBE (Hei et al., 2008; Prise and O'Sullivan, 2009; Chen et al., 2009; Han et al., 2010). It is pertinent to identify potential chemicals that can protect the cells from the damage of RIBE. Konopacka and Rzeszowska-Wolny (2005) reported that antioxidants such as Vitamin C and E could reduce the frequency of micronuclei in bystander cells in a concentration dependent manner. Our group also reported that RIBE *in vitro* could be mitigated by epigallocatechin gallate (a major component of the polyphenolic fraction of green tea) (Law and Yu, 2009; Law et al., 2010) and Magnolol (a traditional Chinese medicine) (Wong et al., 2009, 2010), both of which were ROS scavengers. The more recent observation by Chen et al. (2010) of the "rescue effect", where unirradiated bystander cells assisted irradiated cells through intercellular signal feedback further complicated the situation.

More recently, our group successfully used a carbon-monoxide (CO) releasing molecule as a pharmaceutical agent to release a low dose of exogenous carbon monoxide (CO) to attenuate the effect on bystander cells (Han et al., 2010). By dissolving tricarbonyldichlororuthenium (II) dimer (CORM 2) in a dose range of 5–20 μM into the medium of a mixed co-cultured CHO-K1 cell, both the levels of DNA double strand breaks (DSBs) and the frequencies of micronucleus formation in the bystander cell population were significantly suppressed.

A low concentration of CO had been found to be involved in many defense mechanisms in biological and physiological situations. A number of research works demonstrated the beneficial effects on animals exposed to low concentrations of CO through inhaling CO or using CO releasing molecules (CORM), which encompassed anti-inflammatory effects, anti-apoptotic effect, control of cell proliferation and neurotransmission (Wu and Wang, 2005).

With the successful *in vitro* demonstration of the beneficial effects of CO against RIBE, it is interesting to study the effects in *in vivo* conditions, which form the objective of the present work. In the present study, embryos of the zebrafish (*Danio rerio*) was employed as the vertebrate model. The effects of exogenous CO

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on the bystander embryos were examined. Zebrafish embryos were a well-established model for studying DNA damage response from ionizing radiation (Mcaleer et al., 2004; Bladen et al., 2005; Daroczi et al., 2006; Geiger et al., 2006; Yum et al., 2007, 2009, 2010; Choi et al., 2010a, 2010b, 2010c). One of the most important advantages is that the human and zebrafish genomes share considerable homology, including conservation of most DNA repair-related genes (Barbazuk et al., 2000). In the present paper, zebrafish embryos were treated by tricarbonylchloro(glycinato)ruthenium (II) (CORM-3) with concentrations ranging from 5 to 20 μM. Induction of RIBE was assessed through the number of apoptotic signals revealed on the 25 hpf embryos.

## 2. Materials and methods

### 2.1. Zebrafish

Adult zebrafish were kept in a 45 L glass tank with water maintained at 28 °C using thermostats. The fish were maintained under a 14/10 h light dark cycle. Spawning of embryos was induced at the beginning of the 14 h light period. The developmental stages of embryos were synchronized by keeping the collection period of embryos to 15 min after the light period began. The collected embryos were then transferred to a Petri dish with normal saline as the medium and incubated at 28 °C for development.

### 2.2. Chemicals

Tricarbonylchloro(glycinato)ruthenium (II) (CORM-3) was synthesized from commercially available tricarbonyldichlororuthenium(II) dimer (CORM-2 dimer, Sigma Aldrich) as described by Clark et al. (2003). CORM-3 was stored in a closed vial at −20 °C. A stock solution (50 mM) was prepared by dissolving CORM-3 in distilled water. CORM-3 is soluble in water and avoids the potentially toxic organic solvents such as dimethyl sulfoxide (DMSO) required to dissolve CORM-2 (Motterlini et al., 2003). Different concentrations of CORM-3 (namely, 5, 10, 20 μM) were prepared by dissolving the stock solution in normal saline 1 h before irradiation. Inactive CORM-3 (iCORM-3) was prepared by leaving the 20 μM CORM-3 at room temperature for a week, in which case effectively all CO should have been released, since the half life of CORM-3 to liberate CO in saline was 10.6 h and approximately 1 mol of CO was liberated from 1 mol of CORM-3 (Motterlini et al., 2003). Finally, nitrogen gas was bubbled through the iCORM-3 solution for 10 min to remove the dissolved CO in the solution. The use of iCORM-3 served as a negative control to confirm that the effect actually came from the CO released from CORM-3, and not from CORM-3 itself.

### 2.3. Alpha-particle irradiation of zebrafish embryos

Healthy developing zebrafish embryos were selected for dechorionation at 3 hpf. The dechorionated zebrafish embryos were divided into five groups, viz., bystander group, bystander control group, irradiated group, sham irradiated group, and control group. The bystander group of embryos were unirradiated embryos which were partnered with the irradiated group of embryos, and these two groups of embryos shared the same medium in the same Petri dish. The bystander control group of embryos were unirradiated embryos which were partnered with sham-irradiated group of embryos, and the two groups also shared the same medium in the same Petri dish. The internal bottom of these Petri dishes was lined with agarose with two shallow dredged regions to accommodate the different groups of embryos. The experimental setup for studying RIBE between zebrafish embryos was previously designed by Yum et al. (2009). Finally, the control group of embryos were dechorionated embryos which did not receive further treatment.

The bystander group of embryos together with the irradiated group of embryos were treated by CORM-3 with concentrations of 0, 5, 10 and 20 μM from 4 to 24 hpf. Alpha-particle irradiation was applied to the irradiated group of embryos at 5 hpf for 4 min using an $^{241}$Am source (with an alpha-particle energy of 5.49 MeV under vacuum and an activity of 0.1151 μCi), which corresponded to an absorbed dose of ~4.4 mGy. Fig. 1 shows the setup for alpha-particle irradiation of zebrafish embryos. Mylar film was used as the substrate for holding the zebrafish embryos. The embryos were oriented in such a way that the alpha particles were incident onto the cells of the embryos. The irradiated embryos were then partnered with the bystander group of embryos as described above. All embryos were incubated in a 28 °C incubator until they developed into 24 hpf.

### 2.4. Vital dye staining

Apoptosis was the biological endpoint assessed in the present study. Quantification of apoptotic signals was facilitated through staining with vital dye acridine orange when the embryos were developed to 24 hpf. This method was widely adopted to examine the radiation effect on the whole embryos (Bladen et al., 2005, 2007; Geiger et al., 2006). The embryos were stained for 45 min and washed twice thoroughly with deionized water. Embryos were then transferred to 0.016 M tricaine (Sigma, St. Louis, MO, USA) for anesthetization. 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.

## 3. Results

### 3.1. Induction of RIBE on zebrafish embryos

The present experiment aimed to investigate the influence of CO released from CORM-3 on bystander embryos. The number of apoptotic signals was revealed through acridine orange staining. Fig. 2(a) shows a representative image of apoptotic signals revealed on a 25 hpf zebrafish embryo having partnered with irradiated embryos without CORM-3 treatment; Fig. 2(b) shows one having partnered with sham-irradiated embryos without CORM-3 treatment; while Fig. 2(c) shows one having partnered with irradiated embryos and having treated with 20 μM CORM-3. A bright green spot corresponded to an apoptotic signal. The signals were found to be spread through the entire body of the zebrafish embryos.

Fig. 3 shows the results summarizing the effect of CO with different concentrations on bystander embryos (viz. treated with

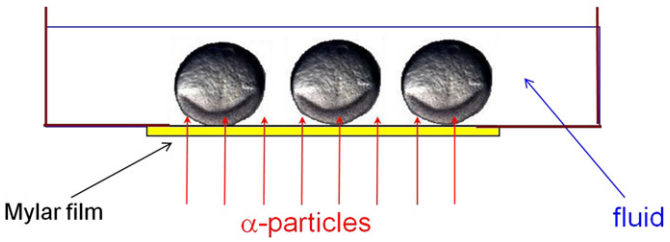


**Fig. 1.** Irradiation of 5 hpf zebrafish embryos with alpha particles through a Mylar film based holder.

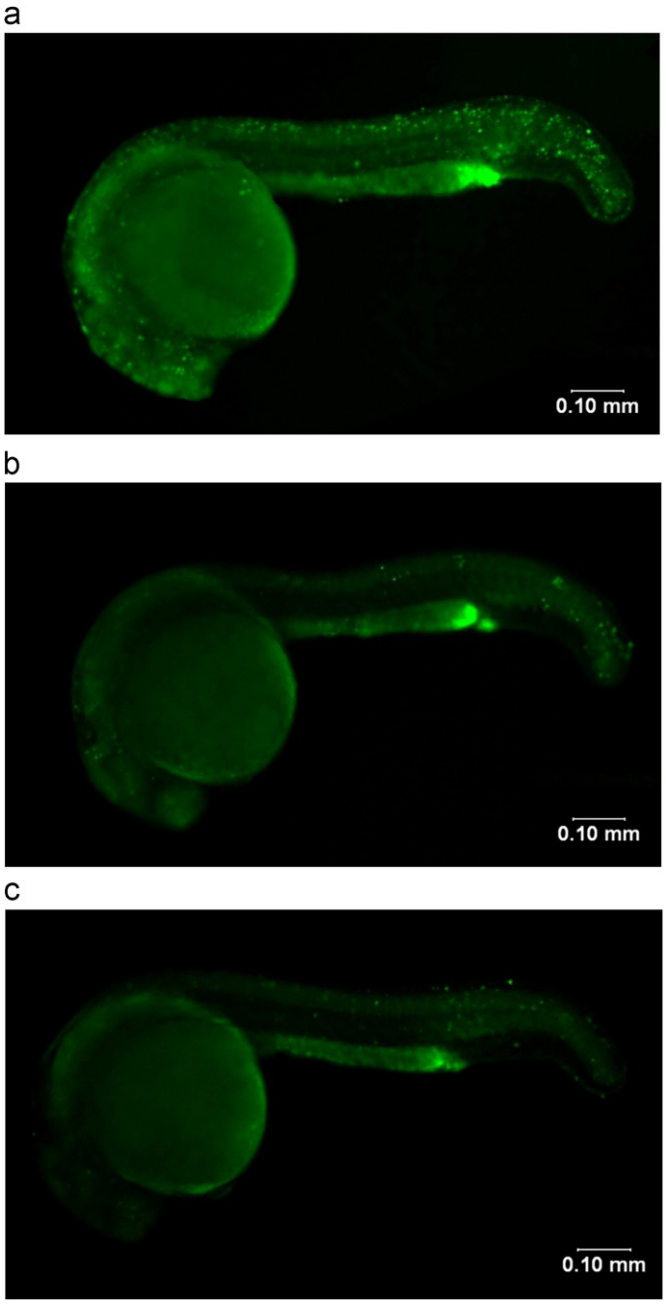


**Fig. 2.** Representative images of apoptotic signals on 25 hpf zebrafish embryos revealed by acridine orange staining with magnification of 40×: (a) embryo having partnered with irradiated embryos without CORM-3 treatment; (b) embryo having partnered with sham-irradiated embryos without CORM-3 treatment; (c) embryo having partnered with irradiated embryos and having treated with 20 μM CORM-3. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

0, 5, 10 and 20 μM of CORM-3). The data were pooled from four independent experiments, and all data had been subtracted by the mean number of apoptotic signals of the corresponding control groups. The bystander embryos without CORM-3 treatment had the highest apoptotic signals. On the other hand, the mean apoptotic signals of bystander embryos treated with CORM-3 were all lower than that without CORM-3 treatment, with the values decreasing progressively with increasing concentrations of CORM-3.

The differences between the mean apoptotic signals (±standard error) of different groups of embryos were shown in Table 1. We defined $D_A$ as (mean apoptotic signal of bystander group − mean apoptotic signal of bystander control group). A large $D_A$ suggested an intense RIBE. The bystander group of embryos without CORM-3 treatment had the largest $D_A$ value while increasing concentrations of CORM-3 lead to decreasing $D_A$ values. The statistical significance of the results was revealed by the $p$ values from $t$-tests. Differences with $p$ values smaller than 0.05 were considered statistically significant. The results suggested that only the bystander group of embryos treated with 0 μM and 5 μM of CORM-3 had mean apoptotic signals significantly higher than that of the bystander control group of embryos. This difference implied that RIBE could only be induced in these two groups of embryos. In other words, RIBE was entirely suppressed by treating the embryos with 10 and 20 μM of CORM-3.

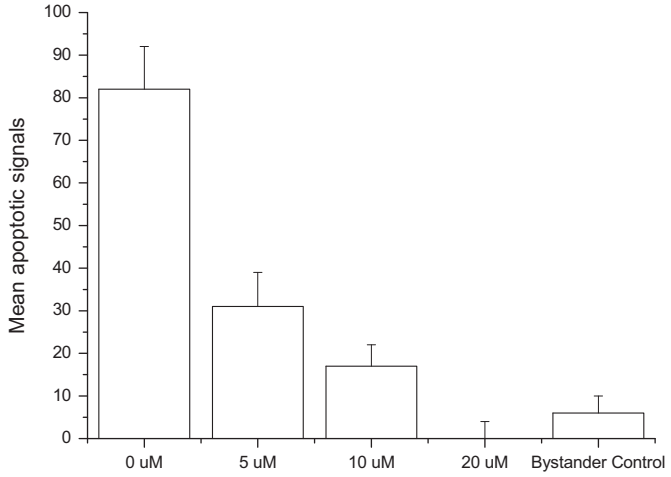


**Fig. 3.** Effect of CO with different concentrations on bystander embryos. The mean numbers of apoptotic signals are shown. Error bars represent one standard error. Data were pooled from four independent experiments. Quantitative analyses of the results are given in Table 1.

**Table 1**
Comparisons among different groups of zebrafish embryos and the corresponding $p$ values[a].

| | $D_A$ | $p$ | $D_B$ | $p$ |
|---|---|---|---|---|
| 0 μM | $76 \pm 13$ | $2 \times 10^{-7}$* | / | / |
| 5 μM | $24 \pm 10$ | $7.6 \times 10^{-3}$* | $-52 \pm 15$ | $3.5 \times 10^{-4}$* |
| 10 μM | $11 \pm 7$ | 0.077 | $-65 \pm 14$ | $3.8 \times 10^{-6}$* |
| 20 μM | $-7 \pm 7$ | 0.16 | $-83 \pm 14$ | $3.1 \times 10^{-8}$* |

[a] $D_A$ = mean apoptotic signal of bystander group − mean apoptotic signal of bystander control group; $D_B$ = mean apoptotic signal of bystander group of embryos with CORM-3 treatment − mean apoptotic signal of bystander group of embryos without CORM-3 treatment.

* Cases with $p \leq 0.05$ are considered statistically significant.

### 3.2. CO (CORM-3) decreased apoptotic signals on bystander group of embryos

We further studied the level of suppression of apoptotic signals through $D_B$, which was defined as (mean apoptotic signal of bystander group of embryos with CORM-3 treatment − mean apoptotic signal of bystander group of embryos without CORM-3 treatment). Negative values of $D_B$ were obtained for all three concentrations of CORM-3, with $p < 0.05$. As such, the mean apoptotic signals of bystander embryos treated with 5, 10 and 20 μM CORM-3 were significantly decreased. Treatment by CORM-3 with a concentration of 20 μM resulted in the strongest suppression and the obtained apoptotic signals were almost

comparable to the "background" level of the bystander control embryos.

### 3.3. Role of CO liberated by CORM-3

In order to confirm that the protective effect on the bystander embryos actually came from CO and not from CORM-3 itself, iCORM-3 was synthesized as a negative control. Fig. 4 shows the effect of iCORM-3 on RIBE. The mean apoptotic signal on bystander embryos treated with iCORM-3 was significantly larger than that on bystander control embryos. In other words, RIBE was induced in the presence of iCORM-3. When comparing the mean apoptotic signal of embryos treated by iCORM-3 with that of the bystander embryos without chemical treatment, no significant difference was observed ($p=0.26$). These results confirmed that the CO released from CORM-3, but not the CORM-3 itself, was central to the suppression of RIBE which caused apoptotic signals on the bystander zebrafish embryos.

## 4. Discussion

This paper demonstrated that CO suppressed RIBE between zebrafish embryos *in vivo*, the latter being able to cause apoptotic signals on the bystander zebrafish embryos, in a CO-concentration dependent manner. Bystander embryos treated with 5, 10 and 20 μM of CORM-3 showed a significant decrease in the apoptotic signals when compared with the bystander embryos without CORM-3 treatment. However, only 10 and 20 μM of CORM-3 entirely suppressed the RIBE. CORM-3 with a concentration of 5 μM was not strong enough to entirely suppress the RIBE. The present results agreed with our previous studies on the influence of CO on RIBE through a mixed co-cultured CHO-K1 cell *in vitro* (Han et al., 2010, 2011).

Previous RIBE studies suggested that both gap junction intercellular communication (Azzam et al., 2001) and the medium which had conditioned the irradiated cells (Mothersill and Seymour, 1997) could induce RIBE in unirradiated cells. A recent study indicated that nitric oxide (NO) and transforming growth factor-β1 (TGF-β1) were detected in the medium conditioning irradiated T98G cells which were responsible for the damages on non-irradiated cells caused in a medium transfer experiment (Shao et al., 2008). Identification of potential factors involved in RIBE can enlighten us on possible ways to control RIBE. The use of low-concentration CO to protect cells from the cytotoxicity of NO was previously reported by Li et al. (2006, 2007). More recently, Han et al. (2010) demonstrated the mitigation of *in vitro* RIBE by low-concentration CO in a concentration dependent manner. Subsequently, Han et al. (2011) found that the mitigation was initiated by impairing the response of bystander cells to NO rather than by affecting the release of NO by the irradiated cells. In the present work, RIBE induced between zebrafish embryos was still significant when 5 μM of CORM-3 was added into the medium, which was likely due to the inadequate impairment of the response of bystander embryos to NO. On the other hand, CO might also suppress RIBE through inhibition of the cyclooxygenase-2 (COX-2) signaling pathway (Alcaraz et al., 2003) and attenuation of activation of nuclear factor-kappaB (NF-κB) (Cepinskas et al., 2008), which could also contribute to the induction of RIBE (Hei et al., 2008). Further investigations will be needed to provide a better understanding on the mechanisms involved in the inhibitory effect of CO on RIBE in zebrafish embryos *in vivo*.

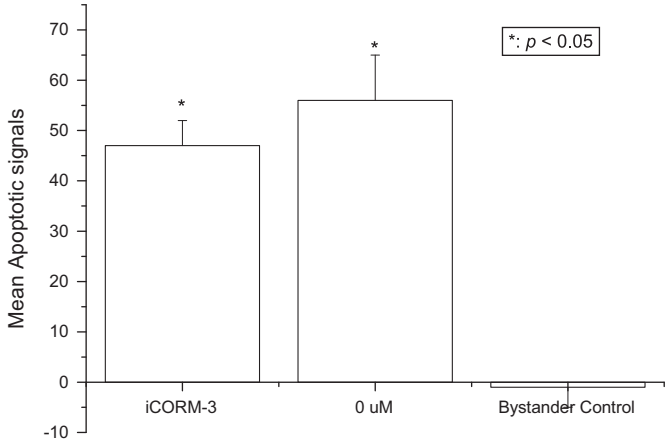


**Fig. 4.** Effect of iCORM-3 on RIBE. Data were pooled from three independent experiments. The mean number of apoptotic signals are shown. Error bars represent one standard error. Comparisons were made with the mean number of apoptotic signals for the bystander control group, and differences with $p<0.05$ are considered statistically significant and asterisked.

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