Mutation Research 793–794 (2016) 41–50

Contents lists available at ScienceDirect

Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis

journal homepage: www.elsevier.com/locate/molmut

Community address: www.elsevier.com/locate/mutres

Effects of Ru(CO)3Cl-glycinate on the developmental toxicities induced by X-ray and carbon-ion irradiation in zebrafish embryos

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ARTICLE INFO

Article history:
Received 31 May 2016
Received in revised form
12 September 2016
Accepted 3 November 2016
Available online 5 November 2016

Keywords:
CORM-3
Zebrafish embryos
X-ray irradiation
Carbon-ion irradiation
Oxidative stress
Apoptosis

ABSTRACT

The inhibitory effects of carbon monoxide (CO), generated by $Ru(CO)_3Cl$-glycinate [CO-releasing molecule (CORM-3)], on developmental toxicity in zebrafish embryos induced by ionizing radiation with different linear energy transfer (LET) were studied. Zebrafish embryos at 5 h post-fertilization were irradiated with X-ray (low-LET) and carbon-ion (high-LET) with or without pretreatment of CORM-3 1 h before irradiation. CORM-3 pre-treatment showed a significant inhibitory effect on X-ray irradiation-induced developmental toxicity, but had little effect on carbon-ion irradiation-induced developmental toxicity. X-ray irradiation-induced significant increase in ROS levels and cell apoptosis could be modified by CORM-3 pretreatment. However, embryos exposed to carbon-ion irradiation showed significantly increase of cell apoptosis without obvious ROS generation, which could not be attenuated by CORM-3 pretreatment. CORM-3 could inhibit apoptosis induced by ionizing radiation with low-LET as an effective ROS scavenger. The expression of pro-apoptotic genes increased significantly after X-ray irradiation, but increased expression was reduced markedly when CORM-3 was applied before irradiation. Moreover, the protein levels of P53 and γ-H2AX increased markedly after X-ray irradiation, which could be modified by the presence of CORM-3. The protective effect of CORM-3 on X-ray irradiation occurred mainly by suppressing ROS generation and DNA damage, and thus inhibiting the activation of P53 and the mitochondrial apoptotic pathway, leading to the attenuation of cell apoptosis and consequently alleviating X-ray irradiation-induced developmental toxicity at lethal and sub-lethal levels.

1. Introduction

Ionizing radiation, such as X-ray and γ-ray, can induce the generation of reactive oxygen species (ROS) through the radiolysis of intracellular water. ROS play a critical role in radiation-induced biological injury, such as cellular lethality, lipid peroxidation, chromosomal mutations, protein modification and biological genetic toxicity [1]. However, the presence of oxygen was not necessary for heavy ions to produce their effects [2,3]. Excess ROS can disturb the balance between oxidation and anti-oxidation systems, induce oxidative stress and cause molecular, cellular and tissue injury [4]. Thus, effective protective mechanisms must evolve in organisms to enable adaptation to oxidative environments. The oxidative defense system of organs and mammals plays an important role in confrontation with oxidative environments [5]. Although organisms have their own anti-oxidative defense system, it is pertinent to identify potential chemicals that can protect the cells against ionizing radiation-induced damage [6]. Previous studies reported that antioxidants such as fullerene nanoparticle DF-1 and amifostine could markedly attenuate radiation-induced lethality and morphologic perturbations in zebrafish [7,8]. Our group also reported that oxidative stress induced by ionizing radiation *in vivo* could be mitigated by ferulic acid (FA) [9] and Curcumin [10], a natural phenolic compound, both of which were ROS scavengers.

Recently, various chemical classes of CO-releasing molecules (CORMs) have been used to carry and deliver CO to cells and tissues [11–14]. CORM-3 is a characterized and extensively studied CORM. It is a water-soluble CO-releasing carbonyl complex, containing

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http://dx.doi.org/10.1016/j.mrfmmm.2016.11.001
0027-5107/

ruthenium as a transition metal, that displays fast and controllable CO liberation activated by light [11,15–17]. The toxic effects of CORMs have been assessed in many studies. The results showed that the biological effects of CORMs are dependent on their concentration and the materials used in the studies [18–20]. Our previous study indicated that embryos exposed to CORM-3 below the concentration of 25 μM did not show any toxic signs [21].

A low concentration of CO has been found to be involved in the protection against cell death and oxidative injury, anti-inflammatory and anti-apoptotic effects, control of cell proliferation, tolerance of organ transplantation and neurotransmission [22]. The antioxidant effect of CORMs has been reported in many studies. In HO-1-deficient cells, exogenous CO can suppress the excessive production of ROS and the expression of plasminogen activator inhibitors, causing the preservation of viability [23]. In addition, a net anti-apoptotic effect by pretreatment with low-concentration CO has been observed *in vitro* and *in vivo*. In the endothelial cell model, CO could inhibit TNF-α-induced apoptosis [24]. Rats exposed to CO exhibited a marked decrease of hyperoxia-induced total lung apoptotic index [25]. Similarly, CO could also inhibit hyperoxia-induced lung injury and oxidative damage [26]. In summary, CO has the ability to protect against hyperoxia-induced lethal and inflammatory effects *in vivo* and *in vitro* [16].

Though the exact mechanisms underlying the anti-radiation effect of CO is still under scrutiny, emerging evidence indicates that it may be linked to its antioxidant potential and ability to mediate the apoptosis pathway [16,27]. Zebrafish (*Danio rerio*) embryos are a widely used model for studying DNA damage response to ionizing radiation [7–9,28–30]. One of the most important advantages is that its genome shares considerable homology to human, including conservation of most DNA repair-related genes [31]. The protection of CORMs in a bystander effect induced by irradiation has been identified [6,32–35]. However, there are insufficient studies concerning its protection to irradiated cells *in vivo*. This study was thus carried out to analyze the effect of CORM-3 in alleviating the developmental toxicity, production of oxidative stress, DNA damage and apoptosis in zebrafish embryos exposed to ionizing radiation with different linear energy transfer (LET) and its possible mechanisms of action. The protective effects of CORM-3 at a concentration of 10 μM on X-ray (low-LET) and carbon-ion (high-LET) irradiation were assessed using various biological endpoints at different developmental stages.

2. Materials and methods

2.1. Embryo collection and experimental design

Zebrafish maintenance and embryo collection were accorded to standard operating procedures as described elsewhere [30,36]. Embryos with healthy developing were divided into four groups as follows, (a) the control (C) group, which received no further treatments after rinsed with E3; (b) CORM-3-pretreated (CORM) group, which did not receive irradiation after exposed to 10 μM CORM-3 at 4 h post-fertilization (hpf); (c) irradiation (IR) groups, which received X-ray or $^{12}C^{6+}$ ion irradiation at 5 hpf and (d) CORM-3-pretreatment + irradiation (CORM + IR) group, which received 10 μM CORM-3, which was freshly prepared by dissolving CORM-3 in E3 culture medium, treatment 1 h before X-ray or $^{12}C^{6+}$ ion irradiation. Three repeated experiments were separately carried out.

2.2. CORM-3 treatment and embryo irradiation

$Ru(CO)_3Cl$-glycinate (CORM-3), provided by Pharmacy of Lanzhou University, was synthesized from $[Ru(CO)_3Cl_2]_2$ as described by Clark et al. [15]. Embryos in IR and CORM + IR groups were irradiated using an X-ray machine (Faxitron, Buffalo Grove, Illinois, USA) or the Heavy Ion Research Facility in Lanzhou (HIRFL, Institute of Modern Physics, Chinese Academy of Sciences, Lanzhou, China) at 5 hpf. X-ray irradiation was performed with energy of 100 KeV/u, and dose rate of 1 Gy/min. $^{12}C^{6+}$ ion irradiation was performed with the beam energy of 80 Mev/u, the LET of 70 keV/μm and the dose rate used were 4 Gy/min. The dose of both X-ray and $^{12}C^{6+}$ ion irradiation was 4 Gy. The embryos exposed to the irradiation were placed in the 35-mm polystyrene cell culture dishes with 3 mL E3 culture medium or 10 μM CORM-3. The embryos were washed with E3 culture medium immediately after the irradiation and transferred back to the 100-mm polystyrene cell culture dishes with 20 mL E3 culture medium.

2.3. Developmental toxicity test

According to developmental stage of zebrafish embryos, embryogenesis completes by 72 hpf, and the development of the larva is completed by 120 hpf [37], so the hatching rate and incidence of mortality were calculated at 72 hpf and 120 hpf, respectively according to the criterion described in our previous study [21]. The observations were performed under a dissecting light microscope (Jiangnan Novel Optics, Nanjing, China). In general, the movements of the tail are considered as spontaneous because they are induced by the development of the moto-neurons without any control by the central nervous system at 24 hpf [38]. The larvae have a rich behavioral response and robust response to both light stimulation at 144 hpf [39]. Therefore, the spontaneous movement and larval behavior for visible light test were recorded and analyzed at 24 and 144 hpf, respectively according to the method as described by Wang et al. [40].

2.4. ROS measurement

At 24 hpf, the generation of ROS in the zebrafish embryos from all groups was measured using the fluorescent dye dichlorofluorescein– diacetate (DCFH-DA). Briefly, 40 embryos of each group were washed with PBS (pH 7.4) twice, and incubated with 20 mg/mL DCFH-DA for 1 h in dark at 28 °C. After washing with E3 three times and anaesthetized using 0.01% MS 222 (Sigma, St. Louis, MO, USA), the fluorescence of each embryo was observed and photographed under a fluorescence microscope (Olympus BX51, Tokyo, Japan) at ×40 magnification. At the same time, 30 stained embryos were washed with cold-PBS (pH 7.4) three times and homogenized. The homogenate was centrifuged at 1,000 × *g* at 4 °C for 10 min, and the supernatant was transferred to new tubes for further experimentation. Subsequently, 200 μL of the supernatant was added to a 96-well plate each well. Each group replicates three times. The fluorescence intensity was measured using a microplate reader (TECAN infinite M200, Switzerland) with excitation at 485 nm and emission at 530 nm, respectively.

2.5. Enzyme extraction and biochemical assays

The embryos were homogenized on ice with 500 μL chilled PBS buffer (137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na_2HPO, 2 mmol/L KH_2PO_4, pH 7.4) and then centrifuged at 3000 *g* for 10 min at 4 °C to obtain the supernatants. The concentrations of total protein in the supernatants were measured using the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). The activities of superoxide dismutase (SOD) and catalase (CAT) were assayed using diagnostic reagent kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the specified instructions. The measurements were carried out on a microplate reader (TECAN