CHAPTER 7

In Vivo Studies of α-Particle Radiation Effects Using Zebrafish Embryos

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Abstract: A brief review on the progress on *in vivo* studies of α-particle radiation effects using zebrafish embryos was given in this chapter. The zebrafish, *Danio rerio*, a small vertebrate from Southeast Asia, has become a preferred model for studying human disease. The main challenges of these α-particle radiobiological experiments included quantification of alpha-particle dose. In particular, specially etched polyallyldiglycol carbonate (PADC) films, which are a kind of solid-state nuclear track detector (SSNTD), were chosen as support substrates for zebrafish embryos. The fabrication procedures were outlined. Methods for quantification of alpha-particle dose were described. Preliminary *in vivo* studies on the radiation effects of alpha particles in zebrafish embryos were briefly reviewed. These included results on low-dose radiation effects of α particles as well as results on alpha-particle-induced bystander effects between zebrafish embryos *in vivo*.

INTRODUCTION

It has been common to study DNA damage responses in vertebrates using *in vitro* cell cultures. However, such experiments cannot be used to study dynamic *in vivo* processes such as temporally and spatially regulated patterns of gene expression [1]. In recent years, the zebrafish, *Danio rerio*, a small vertebrate from Southeast Asia, has become a preferred model for studying human disease, including carcinogenesis. The most important advantage is that the human and zebrafish genomes share considerable homology, including conservation of most DNA repair-related genes [2]. Rapid embryonic development is another advantage so the effects can be assessed within 24 hours post fertilization (hpf).

Recently, there were a number of research works using the zebrafish embryo as an *in vivo* model to study the DNA damage response to ionizing radiation. For example, Bladen *et al.* [3] studied the DNA damage response and Ku80 mRNA function in the zebrafish embryos irradiated with ^{137}Cs gamma rays. McAleer *et al.* [4] evaluated the effects of 250 kVp X-rays in combination with a known radioprotector (free radical scavenger Amifostine) or radiosensitizing agent (tyrosine kinase inhibitor AG1478) with a view to validate zebrafish embryos as a screen for radiation modifiers. McAleer *et al.* [5] also used zebrafish embryos to study radiosensitizing effects of

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flavopiridol in normal tissues exposed to ^{137}Cs gamma rays or 250 kVp X-rays. Daroczi *et al.* [6] evaluated the radioprotective effect of the nanoparticle DF-1, which was a fullerene with antioxidant properties, in zebrafish embryos exposed to ^{137}Cs gamma rays. Geiger *et al.* [1] studied the effects of ^{137}Cs gamma rays and concurrent treatment with Amifostine on the development of the zebrafish embryos. Despite the success of using the zebrafish embryos to study the DNA damage response to ionizing radiation in these studies, these studies only studied energetic photons (X-rays and gamma rays).

Alpha-particle radiobiological experiments are of interest because alpha particles are an ionizing radiation with high linear energy transfer, and alpha particles are emitted from radon and its progeny, which are ubiquitous in our natural environment and constitute the largest natural radiation dose to human and can induce lung cancers. The present chapter will be devoted to the discussion of studying radiation effects of alpha particles *in vivo* in zebrafish embryos.

For these studies, we have two major tasks. The first task concerns the quantification of alpha-particle dose absorbed by the zebrafish embryo cells, which relies on the number of alpha particles actually incident on the embryo cells and the average energy of these alpha particles. The second one concerns the absorption of alpha-particle energies in the chorions of the zebrafish embryos. By using the output results from the SRIM program (www.srim.org), it is noticed that a significant portion of the alpha-particle energy can be absorbed in the fluid enclosed by a chorion. The experimental procedures to deal with these two tasks as well as others will be presented in the following. Preliminary *in vivo* studies on the radiation effects of alpha particles in zebrafish embryos will be briefly reviewed. Brief conclusions will be given at the end.

EXPERIMENTAL PROCEDURES

SUPPORT SUBTRATES FOR ZEBRAFISH EMBRYOS

Solid-state Nuclear Track Detectors as Substrates

As mentioned in the introduction, a task in studying radiation effects of alpha particles *in vivo* in zebrafish embryos is the quantification of alpha-particle dose absorbed by the zebrafish embryo cells. In fact, this is also a task for *in vitro* experiments involving the irradiation of cells with alpha particles. In many of these cases, it is only feasible to control and quantify the alpha-particle energies incident on the cells if the alpha particles pass through the substrate to strike the cells which are in contact with the substrate, instead of passing through the fluid layer above the cells, which has a variable thickness.

As regards the determination of the number and energy of alpha particles actually incident on the embryo cells, there can be two approaches. The first one is to make use of a microbeam facility. Here the hit positions and the energies of the incident ions can be controlled. Another approach is to use a radioactive source to provide the alpha-particle irradiation and to use a solid-state nuclear track detector (SSNTD) as a support substrate to give information on the hit positions as well as the energies of the incident alpha particles. Although the procedures involved in the second approach are a little bit more tedious, they do not involve the expensive and sophisticated equipment associated with the microbeam facility. This second approach will be the focus of the present chapter. The SSNTD employed as the support substrate should be thin enough to allow passage of alpha particles with nominal energies (e.g., those from ^{241}Am source). A review on SSNTDs has been given by Nikezic and Yu [7].

SSNTDs were first used for cell culture substrates by Durante *et al.* [8] who seeded cells on LR 115 SSNTDs, the active layer of which was cellulose nitrate. Irradiation was performed with 3.2 MeV alpha particles from below the substrate. It was possible to measure the number of alpha-particle traversals through the cell nucleus or cytoplasm. The active layer of the LR 115 SSNTDs is below 20 μm so they are thin enough to allow passage of alpha particles with nominal energies. Dorschel *et al.* [9] further investigated the performance of the LR 115 SSNTDs for radiobiological experiments with an experimental setup similar to that of Durante *et al.* [8]. They noticed that the red dye from the LR 115 SSNTD could diffuse into the medium that was conditioning the cells. Because of this, they proposed to use the colorless LR 115 SSNTDs, which could be obtained from the manufacturer of the red LR 115 SSNTDs (DOSIRAD) upon special request. In the application of the red LR 115 SSNTD, a mylar foil between the detector and cell culture should be used. Chan *et al.* [10] further studied the feasibility of colorless LR 115 SSNTDs for alpha-particle radiobiological experiments.

Unfortunately, the relatively poor biocompatibility of the LR115 SSNTDs, together with the diffusion of red dye for the red detectors and the poor quality of tracks on the colorless detectors, have made their applications less straightforward. Another SSNTD which has been extensively explored as support substrates for radiobiological experiments is polyallyldiglycol carbonate (PADC) which is commercially available as the CR-39 detector. PADC films have many advantages. For example, they are transparent, more biocompatible [11] and are not dissolved in the alcohol used for sterilizing the substrate. However, the thinnest commercially available PADC films are ~100 μm thick and are thus not thin enough. According to the SRIM program (http://www.srim.org/), the range of 5 MeV alpha particles in PADC is 28.77 μm. Gaillard *et al.* [12] fabricated ultra-thin PADC films as support substrates for cell cultures but these films were not commercially available. Fabrication of these thin PADC films would require specialized expertise and dedicated equipment, which might not be easily available to all laboratories trying to perform alpha-particle

radiobiological experiments. As a result, it is desirable if methodologies can be explored and devised to fabricate sufficiently thin PADC films from thicker commercially available PADC films, e.g., those with a thickness of 100 μm. The following section will be dedicated to discussions on an established method to fabricate thin PADC films.

Fabrication of Thin PADC Films as Substrates

Chan *et al.* [13] prepared thin PADC films from commercially available CR-39 SSNTDs with a thickness of 100 μm (purchased from Page Mouldings (Pershore) Limited, Worcestershire) by etching them in 1 N NaOH/Ethanol at 40°C to below 20 μm.

For successful preparation of these thin PADC films, the bulk etch characteristics were studied in details. For etching PADC films in NaOH/Ethanol, a layer of precipitate consisting of sodium carbonate always accumulates on the surface of the PADC films [14]. To ensure the most even and the fastest etching, the PADC films were regularly rinsed with distilled water every 2 h during etching in NaOH/Ethanol. The bulk etch rates of PADC films etched in NaOH/Ethanol were found to range from 22 to 75 μm/h for different molarities at 55 °C. Therefore, the desired final thickness of 20 μm could be achieved within as short as ~ 4 h. However, such fast etching rates might result in excessive precipitation of etched products on the detector [14] leading to uneven etching. Finally, Chan *et al.* [13] chose the etchant as 1 N NaOH/Ethanol at 40 °C, for which the bulk etch rate of PADC films was ~10 μm/h. Under such etching conditions, the desired final thickness could be achieved for ~ 8 h. These milder etching conditions also produced thin PADC films which were relatively transparent and less rough for radiobiological experiments which required observations under the optical microscope. Most of the background tracks in PADC films were due to defects present on the surface, and would thus be effectively eliminated on such heavy etching [15].

The thin PADC films were then glued by epoxy to the bottom of petri dishes with a diameter of 5 cm, with a hole of 1 cm diameter drilled at the bottom, to form the cell dishes as shown in Fig. 1. As an example for practical use, the custom-made petri dishes were used for culturing HeLa cervix cancer cells [13]. A PADC cell dish with cell culture inside was irradiated from the bottom with 5 MeV alpha particles under normal incidence as depicted in Fig. 2. After alpha-particle irradiation, the PADC cell dish covered with the lid (with the cell culture inside) was kept floating on the 14 N KOH solution at 37 °C, leaving merely the bottom of the thin PADC film in contact with the etchant until the formation of visible tracks under optical microscope [13]. With the tracks revealed beneath the cell monolayer, the hit positions on the cell could be pinpointed under the optical microscope.

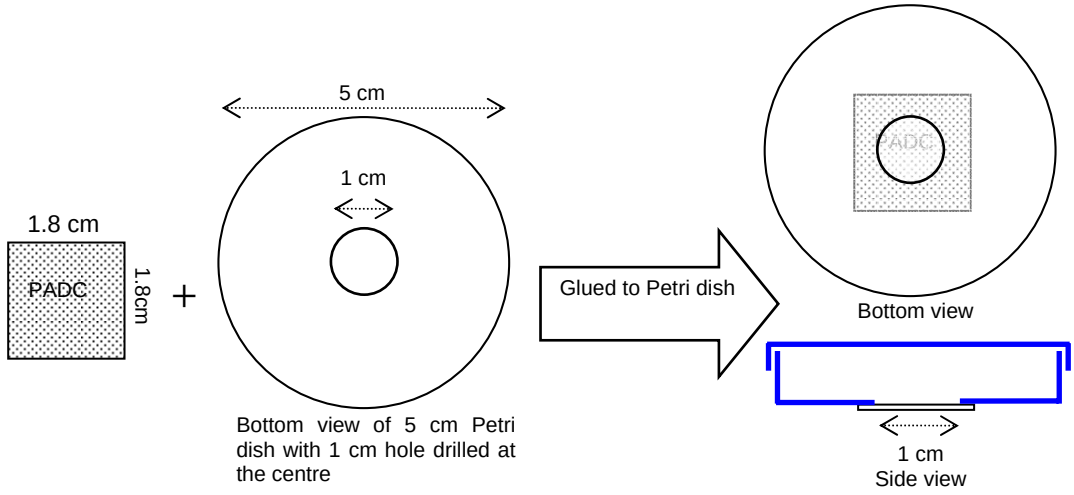

Fig. 1. Preparation of a custom-made PADC-film based holder by gluing a thin PADC film onto the bottom of a petri dish with 5 cm diameter and with a 1 cm hole drilled at the center of the bottom.

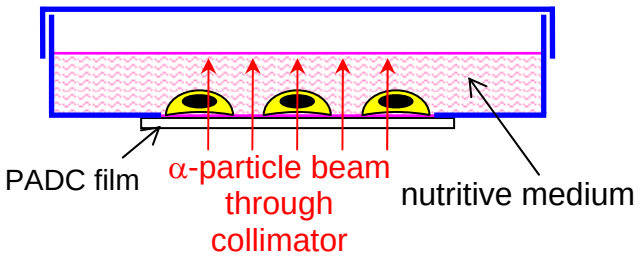

Fig. 2. The irradiation of the cell monolayer through the custom made PADC cell dish.

PADC Films as Support Substrates for Zebrafish Embryos

For alpha-particle irradiation of zebrafish embryos, as is the case for alpha-particle irradiation of cells, it is only feasible to quantify the alpha energies incident on the embryos if the alpha particles pass through the substrate to strike the embryo cells. Yum *et al.* [16] proposed to use PADC films as support substrates to record the positions where the alpha particles hit the embryos as shown in Fig. 3. Similarly, the PADC substrate should be thin enough to allow passage of alpha particles with nominal energies. In the experiments of Yum *et al.* [16], embryos at 4 hpf were irradiated, so it was feasible to orient the cells towards the support substrate. For irradiations at earlier stages, it might only be feasible to orient the embryos in such a way that the yolk and one layer of cells are touching the support substrate (see below).

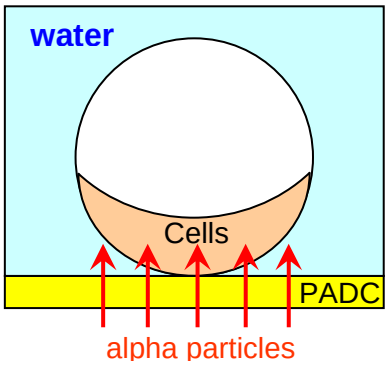

Fig. 3. Schematic diagram showing alpha-particle irradiation of a zebrafish embryo at 4 hpf, with the embryo cells resting on the bottom, and assuming that the alpha particles strike the PADC film vertically [16].

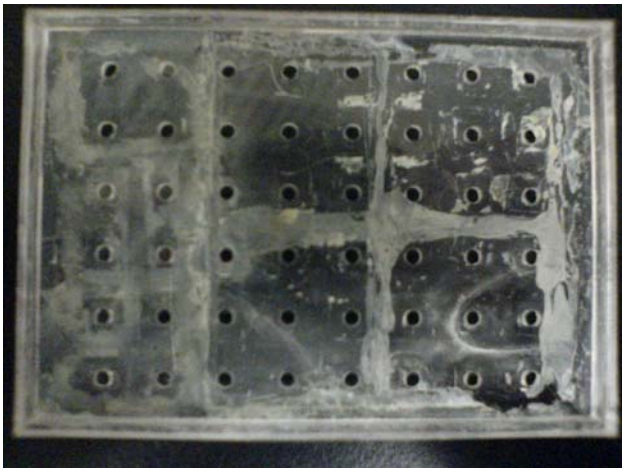

Fig. 4. A custom-made PADC-film based holder for zebrafish embryos [16].

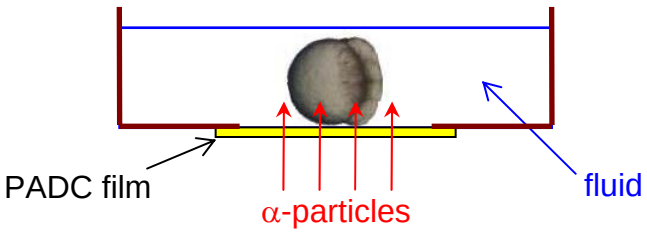

Fig. 5. Schematic diagram showing alpha-particle irradiation of a zebrafish embryo at 1.5 hpf, with the yolk and one layer of cells touching the support substrate, and assuming that the alpha particles strike the PADC film vertically.

Yum *et al.* [16] prepared 16 μm PADC films from commercially available CR-39 films following the procedures described above. These thin PADC films were then glued by an epoxy to the bottom of a custom-made holder made of acrylic resin with 8 × 6 holes drilled at the bottom. The holes had a diameter of 2 mm, and the holes were separated at 8 mm. A photo of the custom-made PADC-film based holder is shown in Fig. 4.

On the other hand, Yum *et al.* [17] irradiated the dechorionated zebrafish embryos at 1.25 hpf, while Yum *et al.* [18,19] made irradiations at 1.5 hpf. At these early stages, for physical stability, the embryos were oriented in such a way that the yolk and one layer of cells were touching the support substrate (see Fig. 5).

DECHORIONATION OF ZEBRAFISH EMBRYOS

The chorions of zebrafish embryos absorbed a significant fraction of the alpha-particle energies, so Yum *et al.* [16] removed the chorions before alpha-particle irradiation. The embryos were placed in a petri-dish lined with a layer of agarose, where their chorions were removed by hand with forceps. Dechorionated embryos were transferred to a petri-dish and were placed into the incubator and allowed to develop to 1.25, 1.5 or 4 hpf for irradiation as required by the experiments.

QUANTIFICATION OF ALPHA-PARTICLE DOSE

Counting of Alpha-particle Tracks

Yum *et al.* [16] captured images of the zebrafish embryos immediately after alpha-particle irradiation, which would be employed to superimpose with the images of the alpha-particle tracks revealed after chemical etching to obtain the alpha-particle hit positions on the embryo cells.

After irradiation and transferal of the embryos, the PADC films were etched in 6.25 N NaOH at 70^{o}C for 3 h. Images of the PADC films with visible alpha-particle tracks were captured with a digital camera attached to a microscope with a magnification of 200×. Images covering different areas of the PADC film were taken and combined to reconstruct an overall image of the PADC film at the bottom of the hole as shown in Fig. 6. The previous captured image of the embryos was then superimposed onto the current image. An example is shown in Fig. 7. However, not all the alpha particles traversing the PADC film could reach the embryo because some would lose all their energies while passing through the water column. By approximating an embryo as a sphere, Yum *et al.* [16] determined the radius of the circular effective area containing alpha-particle tracks which corresponded to the

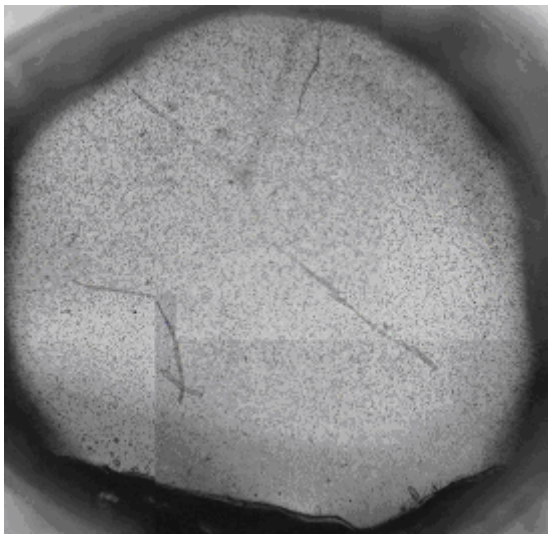

Fig. 6. An overall image of a PADC film at the bottom of the hole with visible alpha-particle tracks, reconstructed from 4 images covering different areas [16].

Fig. 7. Superposition of (a) the image of the PADC film at the bottom of the hole with visible alpha-particle tracks shown in Fig. 6 with (b) the image of embryos [16].

alpha particles that could finally reach the embryos, and the number of these tracks was counted. The effective area was then plotted on the superimposed image of the embryo cells and the alpha particle tracks (as the one shown in Fig. 7) using the freely available image analyzing software called ImageJ (http://rsb.info.nih.gov/ij/).

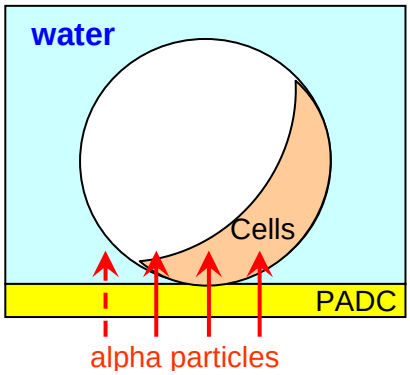

Fig. 8. Schematic diagram showing alpha-particle irradiation of a tilted zebrafish embryo. The alpha particle represented by the dashed arrow does not hit embryo cells and should therefore be discarded when calculating the radiation dose [16].

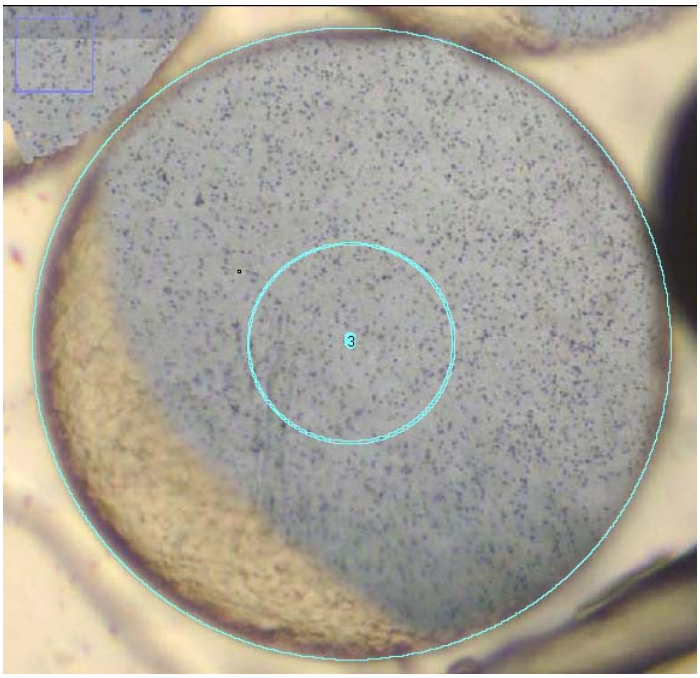

Fig. 9. Outlines of the zebrafish embryo (outer circle) and the effective area (inner circle). The central dot represents the common center of the two circles [16].

In general, however, it was difficult to rotate the embryos so that the embryo cells were exactly resting on the bottom. Instead, these embryos were more likely tilted as shown in Fig. 8. In such cases, a part of the effective area may be void of embryo cells, and only the number of alpha-particle tracks in the intersection between the effective area and the embryo cells should be counted. Fig. 9 shows an example of the effective area drawn on a superimposed image of the embryo cells and the alpha particle tracks using ImageJ. Here, the effective area fell entirely in the area of the

embryo cells, so all the tracks within the effective area should be counted to determine the radiation dose.

Monte Carlo Simulations

If the number of alpha particles incident onto the cells in the embryos is sufficiently large, the absorbed dose can be surrogated by the irradiation duration and estimated through Monte Carlo simulations with acceptable uncertainties. Due to the geometry of the embryos, the alpha particles will have to travel different distances in the water column to reach the embryos after traversing the PADC film. As a result, the alpha particles reach the embryos with different energies, and some may even be stopped in the water column and cannot reach the embryos at all. The energy distribution of alpha particles reaching the embryos is taken care of using Monte Carlo simulations. This approach was adopted by Yum *et al.* [18] in their studies on low-dose radiation effects of α particles.

Integrated Approach

Yum *et al.* [19] adopted this integrated approach, i.e., involving both Monte Carlo simulations and track counts. Considering the distribution of residual energies of alpha particles which could or could not generate visible tracks on the PADC film under the employed etching conditions, they calculated through Monte Carlo simulations the average exit energy (after crossing the PADC film) per one registered alpha-particle track. The images of the etched PADC films with visible alpha-particle tracks and the images of the embryos were superimposed, from which the number of alpha-particle tracks in the areas of the embryo cells was counted. The energy absorbed by the embryos was then given by the product of the number of tracks with the computed average exit energy per one alpha-particle track.

ANALYSES OF APOPTOTIC SIGNALS

In the *in vivo* studies on radiation effects of alpha particles in zebrafish embryos to be described shortly [17-19], after alpha-particle irradiation at 1.25 or 1.5 hpf, the embryos were placed into petri-dishes lined with layers of agarose and returned to the incubator to develop into 24 hpf, which was their chosen endpoint for analyses of apoptosis, a highly regulated biological process during embryonic development. Before 24 hpf, the untreated zebrafish embryos undergo high apoptotic activities as part of the organogenesis processes [20]. The 24 hpf endpoint was also used by Bladen *et al.* [3] who commented that increasing pigmentation after 24 hpf might obscure the signals from the apoptotic cells.

At 24 hpf, the embryos were collected and examined for apoptosis by vital dye staining according to the method described by Chan and Cheng [20]. Briefly, the embryos were transferred to a culture medium containing 5 μg/ml of acridine orange. Embryos were stained for 50 min followed by thorough washing in the culture

medium for three times. Embryos were then anaesthetized with 0.016M tricaine. Images of the stained embryos were captured under a florescent microscope, and the number of apoptotic cells for each embryo was counted.

LOW-DOSE RADIATION EFFECTS OF α-PARTICLE STUDIED USING ZEBRAFISH EMBRYOS IN VIVO

LOW-DOSE RADIATION AND HORMESIS

The "linear, no threshold" (LNT) model is commonly used in the low radiation dose regime. However, more and more evidences are emerging, which challenge the validity of this LNT model. The most studied ionizing radiation at natural environmental levels is the alpha particles emitted from progeny of ^{222}Rn gas, exposures to which are shown to lead to lung cancers. It is well established that elevated levels of radon gas and its progeny in underground mines have posed significant lung-cancer risks for the miners. However, miner exposures were typically 30 times larger than residential exposures, so extrapolation of risk over such a substantial range of exposures remains dubious.

There have been more than twenty case-control studies of the dependence of lung-cancer risk on radon levels in homes. A meta-analysis of seventeen studies suggested a linear dependence [21]. The pooled analysis of seven North American studies [22,23]) found agreement with the LNT model but the 95% CI included the possibility of a threshold. The pooled analysis of 13 European studies [24] produced data which also fitted the LNT model but the 95% CI excluded a threshold. The two poolings of Chinese data [25,26] excluded a threshold. Conversely, in the most recent case-control study, Thompson *et al.* [27] found evidence supporting a hormetic dose-response for radon exposures less than 150 Bqm^{-3}, which deviated significantly from the LNT scenario. Ecological studies of the dependence lacked the individual matching of case-control studies. The studies by Cohen [28,29] were by far the largest and most fully analyzed, also having been criticized and defended. They also found a hormetic response.

Radiation hormesis describes the phenomenon that low doses of ionizing radiation have beneficial effects [30-32]. According to this effect, ionizing radiation at natural environmental levels may promote health. The 2005 report of The Académie des Sciences - Académie nationale de Médecine (French Academy of Sciences - National Academy of Medicine) remarked that 40% of laboratory studies on cell cultures and animals have observed radiation hormesis [33,34], but at the same time also cautioned that occurrence of radiation hormesis in humans was not yet well established [35].

On the cellular level, evidence is accumulating that exposures to low doses of oxidants may have a stimulatory effect on cellular processes [36,37], in contrast to cytotoxic effects of exposures to high doses. In this connection, Miyachi *et al.* [38] attempted to study the effect of low doses of X-ray on zebrafish development. They found a significant decrease in time to hatching following exposures of the zebrafish embryos to 0.025-Gy X-ray irradiation during the cleavage period (1.5 h after fertilization), and also observed that the greatest decrease in this interval after exposures during the blastula period (3.5 h). On the other hand, they also noticed that this radiation-induced effect was eliminated when the dose was increased to 0.15 Gy. They concluded that exposures to low-dose X-rays might induce positive effects on physiological functioning [38].

Inspired by the finding of a hormetic dose-response for low-level radon exposures [27] and the positive effects on physiological functioning of zebrafish embryos induced by low-dose X-rays [38], Yum *et al.* [18] examined the low-dose radiation effects of α particles on zebrafish embryos *in vivo* through the analyses of apoptosis, which is a highly regulated biological process during embryonic development, in the embryos at 24 hpf.

METHODS AND RESULTS

In the experiments of Yum *et al.* [18], about 40 dechorionated embryos were prepared for alpha-particle irradiation each time. The dechorionated embryos were first transferred into a custom-made PADC-film based holder as shown in Fig. 5. The embryos were irradiated with a planar ^{241}Am source (with an activity of 4.259 kBq) from the side of the PADC film at 1.5 hpf. At this developmental stage, the cells have not assumed differentiated cell fates. This time point (1.5 hpf) is also within the cleavage period of embryogenesis (0.7-2.2 h) [39,40]. Walker and Streisinger [41] found that embryos older than 3 h were considerably more resistant to γ-rays, which suggested a possible repair mechanism after the cleavage stages.

Yum *et al.* [18] employed the Monte Carlo approach as outlined above to quantify the alpha-particle dose absorbed by the zebrafish embryos. From the Monte Carlo simulations, the average alpha-particle dose rate on an embryo was 1.4 mGy/min. Their irradiation durations were chosen as 1, 2, 4 and 8 min, and 0 min (as controls), thereby providing alpha-particle doses of 1.4, 2.8, 5.6 and 11.2 mGy.

After alpha-particle irradiation at 1.5 hpf, the embryos were allowed to develop into 24 hpf and then analyzed for apoptosis as described above. The mean numbers of apoptotic cells for zebrafish embryos irradiated for different periods of time are here reproduced in Fig. 10. The mean number decreased significantly from 0-min irradiation (i.e., the controls) to 1-min irradiation, and then increased almost linearly

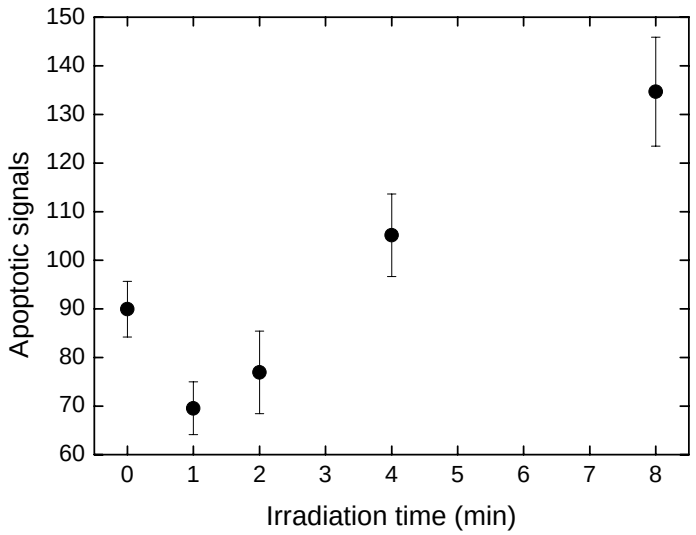

Fig. 10. The relationship between the mean number of apoptotic cells (error bars showing one standard errors) obtained in zebrafish embryos irradiated for different durations [18].

TABLE 1. The *p* values for comparisons between mean numbers of apoptotic cells for zebrafish embryos irradiated for different periods of time, determined using two-tailed t-test. The *p* values smaller than 0.05 are considered statistically significant [18].

	1 min	2 min	4 min	8 min
0 min	0.0117	0.209	0.143	8.16×10^{-4}
1 min		0.469	8.40×10^{-4}	3.39×10^{-6}
2 min			0.0218	1.14×10^{-4}
4 min				3.97×10^{-2}

to 2-min, 4-min and 8-min irradiation. As can be observed in Table 1, the differences were statistically significant ($p < 0.05$) between 0- and 1-min irradiation, between 4-min and 1- or 2-min irradiation, and between 8-min irradiation and all other irradiations. This trend resembled that of a hormetic effect.

These observations were qualitatively in line with the results obtained by Miyachi *et al.* [38] who attempted to study the effect of low doses of X-ray on zebrafish development. They found a significant decrease in time to hatching following exposures of the zebrafish embryos to 0.025-Gy X-ray irradiation during the cleavage period (1.5 hpf), but this radiation-induced effect was eliminated when the dose was increased to 0.15 Gy. It is also interesting to note that Bladen *et al.* [3] found an

absence of ectopic apoptotic cell death in zebrafish embryos irradiated to 0.15 Gy gamma rays at 6 hpf, but a large amount at a 3-fold higher dose, and the authors suggested the existence of a threshold below which radiation-induced cell death did not occur.

As described above, Thompson *et al.* [27] found evidence supporting a hormetic dose-response for radon exposures less than 150 Bqm^{-3}. Incidentally, this radon gas concentration corresponded to an estimated annual absorbed dose in the lungs of 1.2 mGy using nominal values for the equilibrium factor as 0.4 and indoor occupancy factor as 0.7, an effective dose conversion coefficient of 5 mSv/WLM, tissue weighting factor of 0.12 for the lungs and radiation weighting factor of 20 for alpha particles [18].

Finally, the number of apoptotic cells in zebrafish embryos for 4-min irradiation was significantly larger than those corresponding to 1- and 2-min irradiation, and that for 8-min irradiation was significantly larger than those corresponding to 0-, 1- 2- and 4-min irradiation. These results showed that DNA damages during zebrafish embryogenesis could be induced by alpha-particle irradiation, which further justified zebrafish as a model for assessing the effects of alpha-particle radiation.

Yum *et al.* [17] used the same planar ^{241}Am source and extended the above study to irradiation time of 30, 60 and 90s, or to average doses of 0.7, 1.4 and 2.1 mGy, respectively. Zebrafish embryos at 1.25 hpf were irradiated. After alpha-particle irradiation, the embryos were allowed to develop into 24 hpf and then analyzed for apoptosis, as described in section 2.4. The results are reproduced here in Fig. 11. The results showed a nonlinear dose-risk relationship and a reduction of risk at low doses, which did not support the “Linear No Threshold” hypothesis.

ALPHA-PARTICLE-INDUCED BYSTANDER EFFECTS BETWEEN ZEBRAFISH EMBRYOS IN VIVO

BYSTANDER EFFECT

Radiation-induced bystander effects in cells refer to biological effects that the unirradiated cells respond as if they have been irradiated, when they are put in contact with the irradiated cells or in the medium previously holding the irradiated cells [42]. Radiation-induced bystander effects have aroused immense interests because they can involve new biological mechanisms and can have significant implications on radiological protection. For example, low doses of ionizing radiation can become more harmful than previously thought due to the presence of bystander effects [43,44].

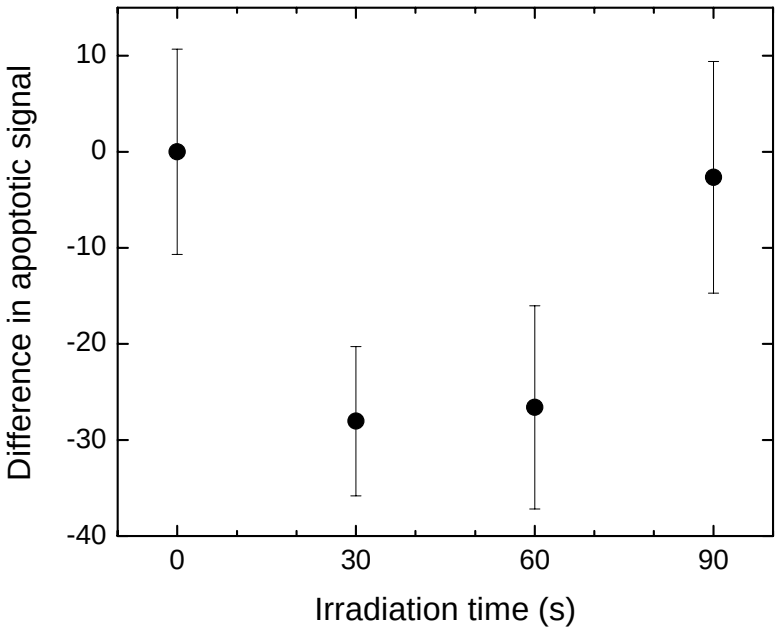

Fig. 11. The mean difference (± SE) in the number of apoptotic cells obtained in embryos irradiated for different time and in the controls [17].

There is a large amount of literature describing or reviewing the radiation-induced bystander effects *in vitro* [45-48]. However, the *in vivo* relevance and/or *in vivo* persistence are always questioned. Furthermore, *in vitro* experiments cannot be used to study allelopathic effects among individual animals or dynamic *in vivo* processes, e.g., temporally and spatially regulated patterns of gene expression. As such, it is always tempting to study the effects through *in vivo* experiments. Recently, there have been researches on bystander effects *in vivo* in mice and in fish [49-52]. In particular, Mothersill *et al.* [51,52]) demonstrated X-ray-induced *in vivo* bystander effects in two non-related freshwater fish, namely, the rainbow trout (*Oncorhynchus mykiss*, W) and the zebrafish (*Danio rerio* L), and suggested that communication signals involved secretion of a chemical messenger into the water, which could then be passed from the "irradiated" fish to the "naive" fish to cause the bystander effects.

With the observations of such X-ray-induced bystander effects, Yum *et al.* [19] studied alpha-particle-induced bystander effects between zebrafish embryos *in vivo*. With the direct assessment of bystander effects in the naive fish themselves, Yum *et al.* [19] also tried to identify the relationship between the absorbed alpha-particle dose and the bystander effect.

BYSTANDER EFFECT

Dechorionated zebrafish embryos accommodated in the holder as described in above were irradiated with alpha particles from a planar ^{241}Am source (with an activity of 4.259 kBq) from the side of the PADC film (air distance = 1 mm) at 1.5 hpf for 4 min (see Fig. 5). After irradiation, the embryos were transferred to an agarose plate, which was then incubated at 37^{o}C with naïve embryos (unirradiated embryos having partnered with the irradiated embryos) in the same plate containing 3 ml medium. The irradiated and naïve embryos were accommodated in two shallow regions dredged in the agarose, separated by a small ridge. This physical setting separated the irradiated and naïve embryos spatially but allowed sharing of the same medium. This enabled subsequent studies on the irradiated and naive embryos separately while at the same time bystander signals, if any, could be transferred from the irradiated embryo to the naive embryos through the water medium. At 24 hpf, the embryos were collected and examined for apoptosis by vital dye staining using acridine orange as previously described. The doses absorbed by embryo cells were determined using the integrated approach as also previously described.

Alpha-particle-induced Bystander Effects Between Zebrafish Embryos *In Vivo*

In this part, 8 irradiated embryos were incubated with 8 naive embryos in the agarose plate. There were two independent experiments. For both experiments, the naive embryos showed significantly more apoptotic signals than the control naive embryos (unirradiated embryos having partnered with the control embryos) ($p < 0.05$). These results gave evidence in supporting the existence of alpha-particle-induced bystander effects between zebrafish embryos *in vivo*, and thus that the bystander factors involved chemical messengers secreted into the water medium [19].

Relationship Between Alpha-particle Absorbed Dose and the Bystander Effect

In this part, 1 irradiated embryo was incubated with 5 naive embryos in the agarose plate. Three sets of experiments were performed on three separate days. The results of Yum *et al.* [19] are reproduced here in Fig. 12. The positive slope is evident. These results supported a general positive correlation between the apoptotic signals in the naive embryos and the alpha-particle dose absorbed by the irradiated embryos. Nevertheless, a different trend in the low-dose region (i.e., < 1 mGy) could not be ruled out because of the relatively smaller number of data points in this region. Previous research also indicated different biological effects of radiation at small doses (see above). Further studies and probably a much larger amount of data for the low dose regime would be necessary before a more definite conclusion on the relationship between alpha-particle absorbed dose and the bystander effect for this low dose regime can be made.

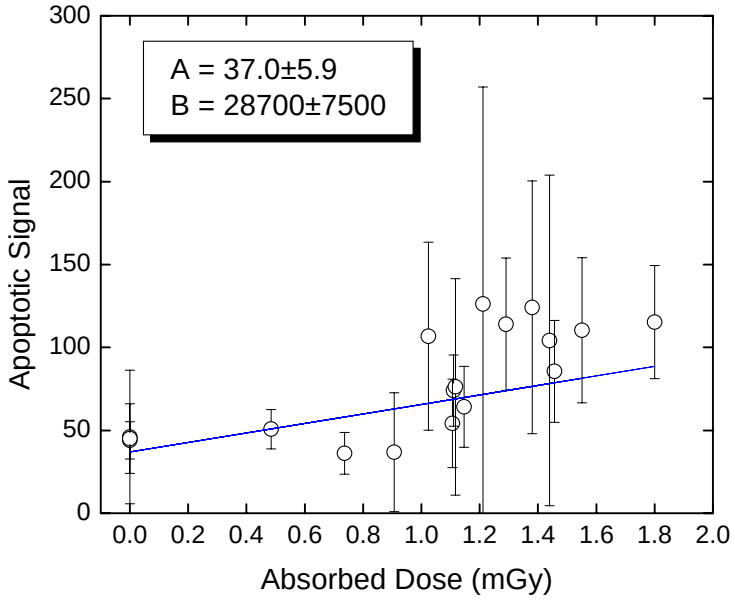

Fig. 12. Results on the relationship between the apoptotic signal in the naive embryos with the alpha-particle dose absorbed by the irradiated embryos. The linear best-fit line is shown with the parameters (A as intercept and B as slope) and the corresponding 95% confidence intervals [19].

CONCLUSIONS

The progress on *in vivo* studies of α-particle radiation effects using zebrafish embryos was briefly reviewed in this chapter. The zebrafish, *Danio rerio*, a small vertebrate from Southeast Asia, has become a preferred model for studying human disease. The main tasks of these α-particle radiobiological experiments included the quantification of alpha-particle dose.

Experimental procedures to deal with these tasks as well as others were presented. First, specially etched polyallyldiglycol carbonate (PADC) films, which are a kind of solid-state nuclear track detector (SSNTD), were chosen as support substrates for zebrafish embryos. The fabrication procedures were outlined. The alpha particles pass through the substrate to strike the embryos which are in contact with the substrate. The chorions of zebrafish embryos would absorb a significant fraction of the alpha-particle energies, so dechorionation is required before the alpha-particle irradiation. As regards the quantification of alpha-particle dose, the number and the energy of alpha particles striking the zebrafish embryos need to be determined. Three methods were described, including the counting of alpha-particle tracks on the etched PADC support substrate, Monte Carlo simulations, and an integrated approach involving

both Monte Carlo simulations as well as track counts. At 24 hpf, the embryos were collected and examined for apoptotic signals by vital dye staining using acridine orange.

Two preliminary *in vivo* studies on the radiation effects of alpha particles in zebrafish embryos were briefly reviewed. Results on low-dose radiation effects of α-particle studied using zebrafish embryos *in vivo* were presented. After alpha-particle irradiation at 1.25 or 1.5 hpf, the embryos were allowed to develop into 24 hpf and then analyzed for apoptosis. The apoptotic signal decreased significantly from no irradiation to irradiations for 30s and 60s, and then increased again. The results showed a nonlinear dose-risk relationship which did not support the "Linear No Threshold" hypothesis, and the trend resembled that of a hormetic effect.

Results on alpha-particle-induced bystander effects between zebrafish embryos *in vivo* were described. The naive embryos showed significantly more apoptotic signals than the control naive embryos (unirradiated embryos having partnered with the control embryos). These results supported the existence of alpha-particle-induced bystander effects between zebrafish embryos *in vivo*. Moreover, a general positive correlation between the apoptotic signals in the naive embryos and the alpha-particle dose absorbed by the irradiated embryos was found.

The experiments and results summarized in this chapter represented only a small start for *in vivo* studies on the radiation effects of alpha particles in zebrafish embryos. The procedures can definitely be improved, including the methodology in irradiation as well as in quantification of the doses. Other biological endpoints are available and should also be explored. Studies on other radiation effects of alpha particles, including targeted or non-targeted, are also imminent.

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