RADIOBIOLOGICAL STUDIES FOR LOW-LEVEL RA-226

STUDY OF BIOACCUMULATION AND RADIOBIOLOGICAL EFFECTS OF ENVIRONMENTALLY RELEVANT LEVEL OF RADIUM-226

By

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I dedicate this thesis to my parents Xiren Shi and Yingchun Li.
Your endless love and concern give me a warm family. Your unconditional support and help let me go ahead bravely. Without you, it would not be possible for me to be at this point of my life.

ABSTRACT

The primary aim of this thesis is to investigate the bioaccumulation of environmental level of Ra-226 in fish and the induced radiobiological effects. Elevated Ra-226 levels in the environment are of increasing concern because of its high radiotoxicity and long half-life.

One in vivo study analyzed the accumulation of Ra-226 in fathead minnows exposed to environmental level of Ra-226 via ingestion. A second in vitro study focused on the biological effects of chronic low-dose radiation from Ra-226 and the induction of adaptive response.

The main findings of the thesis revealed that the accumulation of Ra-226 in fish was not linear in relation to the dietary Ra-226 activity. The highest concentration factor was obtained from lowest food activity, and uptake was inversely proportional to the dietary Ra-226 concentrations. The fish fed with radioactive food showed some growth suppression compared to control.

The results of the in vitro study indicated that chronic low-dose radiation from Ra-226 had an impact on the clonogenic survival of cells, but had no influence on the proliferation of cells. The reactions of CHSE/F (fish embryonic cells) and HaCaT (human epithelial cells) to chronic radiation from Ra-226 were different. After being cultured in medium containing Ra-226 over multiple generations, CHSE/F cells were sensitized by the radiation, while HaCaT cells were firstly sensitized and after several generations they showed the trend of getting adapted to the radiation. Furthermore, no adaptive response

was induced by long-term low-dose radiation from Ra-226 when cells were subsequently exposed by acute high-dose challenge radiation, except for small adaptive responses at sporadic dose points.

This thesis may provide information about the transfer and influence of low-dose Ra-226. It may motivate other studies to estimate the risks of internal alpha-emitters, to identify the influence of chronic radiation on different species, and to develop radiation protection guidelines.

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Chapter 1

INTRODUCTION

This thesis investigates the bioaccumulation of environmentally relevant levels of Radium-226 (Ra-226) in fish and the induced biological effects in both fish and human cell lines. Ra-226 is a naturally occurring radionuclide in the decay chain of uranium. Due to increasing uranium mining and milling for nuclear power generations, the level of radionuclides in some areas can become elevated, and the influence of these radioactive elements on public health and the environment has attracted increasing attention. Among these radionuclides, Ra-226 is of particular importance because of its long half-life, high radiotoxicity and similar chemical and physiological properties to calcium (Cowart and Burnett, 1994).

Numerous studies demonstrated that Ra-226 levels in water and soil of some natural environments become elevated due to uranium mining, and Ra-226 level in the local plants and animals become high because of bioaccumulation (Giri et al. 2010; Pyle and Clulow, 1998; Brenner et al, 2007). Most of the existing data on the bioaccumulation of Ra-226 in non-human biota come from field studies rather than laboratory experiments. Because the concentration of Ra-226 and the influence of other toxic elements in the natural environment are uncontrollable, information about the uptake and transfer of Ra-226 is limited and not systematic. The first part of this thesis aimed to study Ra-226 bioaccumulation in aquarium fish.

Due to internal radiation from deposited Ra-226, some adverse effects have been reported in plants and animals. These include high embryonic mutation frequency, low germination capacity, inhibited reproduction and decreased biomass (Evseeva et al, 2009; Geras'kin et al., 2011a, b, 2013; Lourenco et al., 2012; Mothersill et al., 2013). A literature search in this area revealed that studies concerning the induced biological effects are rare and are mainly studied at the individual level. Mechanisms underlying these detrimental impacts are complicated and unclear, and are suspected to be related to DNA damage and abnormal gene expression induced by radiation (Lourenco et al., 2012; Olsvik et al, 2012; Mothersill et al., 2013). On the other hand, because irradiation from the elevated levels of Ra-226 in the environment is chronic low-dose irradiation, effects induced by it could be different from effects induced by high-dose radiation, and could involve low-dose radiation phenomena, such as genomic instability (Seymour et al., 1986), bystander effects (Mothersill and Seymour, 1997, 2004; Nagasawa and Little, 1992) and adaptive response (Olivieri et al., 1984; Kadhim et al, 2004; Maguire et al., 2007; Ryan et al., 2009; Bhattacharjee, 1996). The second part of this thesis explores the effects and the mechanisms induced by chronic low-dose radiation from Ra-226 at the cellular level, as well as the possibility of adaptive response induced by such chronic radiation, which could change the subsequent response to a high challenge dose of acute γ-rays from Cesium-137. The results may provide further information about the influence of long-term, low-dose radiation for radiation protection of human and non-human biota in the environment.

Part I consists of work performed to analyze the bioaccumulation of Ra-226 in fathead minnows exposed to environmental levels of Ra-226 via ingestion, see chapter 2. The reason for choosing fathead minnows was because of its wide distribution across North America and its common use as a fish model for toxicology studies because of the small size (Scott and Crossman, 1973). Chapter 2 presents data about the activity of Ra-226 deposited in the body of fish fed with food containing various concentrations of

Ra-226.

Part 2 composes of studies using the fish embryonic cell line CHSE/F and the human epithelial cell line HaCaT to investigate the biological effect of chronic low-dose radiation from Ra-226 and to test whether an adaptive response can be induced by such chronic radiation. This portion of the thesis includes chapter 3 and chapter 4. A published study indicated that *Vicia cracca* grown in sites contaminated by uranium mining and milling waste (mainly Ra-226) showed higher embryonic lethal mutation frequency (Evseeva et al., 2009). The same thing may occur in fish exposed to chronic internal radiation from Ra-226. CHSE/F is a fish cell line derived from Common bluegill embryos and is more sensitive to radiation than other fish cell lines, making it a suitable choice. The reason for choosing HaCaT was because of its common use in our research group for studying bystander effects. These effects are among the phenomena known as "Non-targeted effects" (NTE) associated with low-dose radiation (Mothersill and Seymour, 1997; Fernandez-Palomo et al., 2016; Hanu C et al., 2016). In chapter 3, experiment data about the influence of the chronic low-dose radiation from Ra-226 on clonogenic survival and the growth of the two cell lines are presented. The cells were cultured in medium containing various concentrations of Ra-226 to simulate the environment of internal radiation. In chapter 4, the results of a follow-up study are presented which focused on the influence of the priming chronic 4.78MeV α-radiation from Ra-226 on the reaction of the two cell lines to the subsequent challenge γ-rays radiation from Cs-137.

In summary, the objective of this thesis is to determine the pattern of bioaccumulation of Ra-226 in fish and to examine the biological effects on cells exposed to chronic low-dose radiation from Ra-226. This thesis addresses the following questions:

1. What is the relationship between the accumulation of Ra-226 in fish and it concentration in the food they consume?

Existing studies indicated from field studies in uranium mining areas that the

concentration factor of Ra-226 was higher for fish from low Ra-226 level water or sediments. The reason might be that when the substrate concentrations are low, the fish and the plants are ready to uptake essential elements, and Ra-226, a non-essential element, could simulate an essential one, like Ca (Swanson, 1983, 1985; Pyle and Clulow, 1997a; Madruga et al., 2001). Based on evidence from these literatures, it was hypothesized that the activity of Ra-226 accumulated in fish is non-linear with respect to the Ra-226 concentration in food, and the highest concentration factor will occur to fish fed with food containing the lowest concentration of Ra-226.

2. Can chronic low-dose radiation from Ra-226 affect the clonogenic survival and the growth of CHSE/F and HaCaT cells?

It was hypothesized that the chronic radiation from Ra-226 will result in decreased clonogenic survival for both CHSE/F cell line and HaCaT cell line, and the proliferation of cells will also be depressed.

3. Can the chronic low-dose 4.78MeV α-radiation from Ra-226 induce an adaptive response in CHSE/F and HaCaT cells irradiated by subsequent challenge dose of γ-rays from Cs-137?

Pretreatment of cells with acute low-dose radiation has been shown to result in either a radioresistant response (Gourabi and Mozdarani, 1998) or further cell killing (Cregan et al., 1999; Ryan et al., 2008) after being challenged by the subsequent γ-rays radiation. Therefore it was hypothesized that a chronic pre-exposure to Ra-226 will induce an adaptive response to a subsequent challenge dose of acute γ-rays from a Cs-137 source.

1.1 Radium-226

Radium is a highly reactive element in the 2nd group of the periodic table, also known as the alkaline earth metals. It was first discovered by Marie Curie and Pierre Curie in 1898 (Haynes, 2015). All the isotopes of Radium are radioactive. Among them, Ra-223, Ra-224, Ra-226 and Ra-228 occur naturally in the decay chains of Uranium and

Thorium elements in the earth's crust. Ra-226 is the most abundant radioisotope and is the decay product of Uranium-238. Of the U series radionuclides, Ra-226 is particularly important in studies of uranium mining and milling waste from the point of view of environment protection. The reason is because of its large amount in the waste, high radioactivity, a long half-life and chemical analogy to calcium.

1.1.1 Radioactive decay chain of Ra-226 and radioactivity

The nuclei of radioactive elements are highly unstable and have to undergo radioactive decay to form stable elements by the emission of α or β-particles from the nucleus. Most often the progenies of decay are also radioactive and undergo another nuclear decay until a stable isotope is produced, thus a radioactive decay chain is formed (Cember, 1996; Magill and Galy, 2005). Uranium has three natural radioactive isotopes: Uranium-234, Uranium-235 and Uranium-238. The most abundant isotope in a uranium ore is Uranium-238, accounts for 99.27% (Orloff et al., 2004). Ra-226 is a radionuclide in the decay chain of U-238, and finally ends with the stable isotope Lead-206 (shown in Figure 1.1).

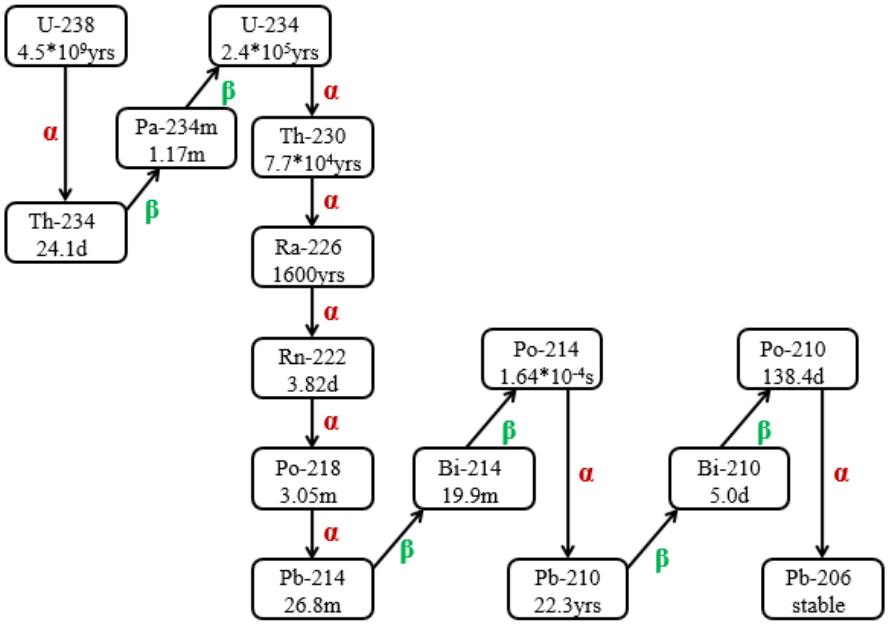

Figure 1.1 The decay chain of Uranium-238.

Radioactivity was first discovered in 1896 by Henri Becquerel who found that invisible radiation from crystals could blacken a photographic plate even placed in the dark by accident (Lawson, 1999). Radioactivity A is the number of decays per unit time, and the unit of it in the International System of Units is Becquerel (Magill and Galy, 2005). The definition of it is:

$$\mathrm{A} = -\frac{dN}{dt} = \lambda N \qquad \text{Equation 1.1}$$

Where N is the total number of particles in the sample, λ is the decay constant (Magill and Galy, 2005).

The number of atoms at any time, N(t) is given by integrating with respect to time (Magill and Galy, 2005)

$$\mathrm{N(t)} = \mathrm{N(0)}e^{-\lambda t} \qquad \text{Equation 1.2}$$

Where N(0) is the initial amount of active substance.

If half of the given nuclides decay, $\mathrm{N(t)} = \frac{\mathrm{N(0)}}{2}$, the amount of time it takes is the half-life, τ (Magill and Galy, 2005). Thus,

$$\tau = \frac{\ln(2)}{\lambda} \qquad \text{Equation 1.3}$$

1.1.2 Large amounts of Ra-226 enter the environment during uranium processing

Uranium is the basic energy mineral used in the present nuclear power plants. In order to meet the world demand for energy, large amounts of uranium are mined every year. Canada was the largest uranium producer for many years until 2009, accounting for about 22% of total world output. McArthur River and Cigar Lake mines in northern Saskatchewan province contain the world's largest deposits of high-grade uranium, shown in Figure 1.2 (Natural Resources Canada, 2016; World Nuclear Association, 2016). After mining, uranium ore is delivered to a nearby mill to extract uranium from the ore. Mining water, mineralized waste rock, and milling tailings associated with the uranium processing contain dissolved radionuclides, radon and heavy metals, which can degrade

the surrounding environment if adequate safety measures are not taken (Tripathi et al., 2008). Tailings could retain approximately 85% of radioactivity of the mined ore and the radioactive elements are primarily thorium-230 and radium-226, along with their decay progenies (Mirka et al., 1996; Canadian Nuclear Safety Commision, 2016). Ra-226 is of most concern because nearly 99% of the Ra-226 in the ore is discarded in the waste during processing and its concentrations may be 100 times the permissible level (Maclaren, 1978; Clulow and Mirka, 1991; ICRP 1978).

Every year, a large amount of Ra-226 is released into the environment from uranium mining. Ra-226 can release into the aquatic system from the leaching of uranium mine tailings and from the release of ore-processing effluents. It was estimated that Ra-226 contained in surface runoff and leachate from uranium mine tailings ranged from 1.4 to 4.3 Bq/L (Swanson 1985; Potential for human exposure). Ra-226 contained in untreated uranium milling effluent could be up to 81kBq/L (Sebesta et al., 1981). Approximately 97 million tons of mine tailings that contain an estimated $2.2*10^9$kBq of Ra-226 had been released to the surface in the western United States (Kaufmann et al., 1976). About 225MBq of Ra-226 were released daily into the rivers from Polish coal mines (Chalupnik et al., 2001). The abandoned and active tailings usually drain into nearby rivers and lakes. During this process, these long half-life radionuclides (the half-life of Ra-226 is about 1600 years, the half-life of Th-230 is about 80 000 years) are easily leached or eroded into surface water systems and need to be monitored for very long time (Lottermoser, 2007). Furthermore, the ability of Ra-226 to form soluble carbonates or chlorides results in enhanced activity of radium in the water, and also makes it possible for radium to migrate in the environment (Pyle and Clulow, 1997). In addition, Ra-226 can also be co-precipitated with Barium sulphate and be deposited in the sediments (Chalupnik et al., 2001). Because the amount of Ra-226 released into the environment is very large and it is one of the most hazardous elements after internal exposure, analysis of Ra-226 in aquatic system and organisms is of great importance for the public health and natural

environment (Burnett and Tai, 1992). Due to this, the US regulations require that the maximum contaminant level for combined Ra-226 and Ra-228 is 5pCi/L (0.185Bq/L) (CFR Title 40, 2016). The maximum acceptable concentration of Ra-226 established in the guideline for Canadian drinking water is 0.5Bq/L (Health Canada, 2009), where the screening level for drinking water established by the World Health Organization is 0.5Bq/L for gross alpha activity (WHO 2008).

As of now, the total amount of Ra-226 released to the environment by water-related discharges every year is unknown. And the concentrations of Ra-226 found in surface and ground water sources have generally been low and been below the established detection limit. But in some uranium mining and milling areas, the concentrations of Ra-226 in the aquatic system and soil were found to be elevated (Clulow et al., 1998; Mirka et al., 1996; Giri et al., 2010; Evseeva et al., 2009). Whether the elevated level of Ra-226 could induce some impacts on public health and natural environment is not clear and this research field is gaining increasing attention.

Figure 1.2 Uranium Resources in Canada (World Nuclear Association, 2016)

1.1.3 High radiotoxicity of Ra-226

From the decay chain of Ra-226 shown in Figure 1.1, it can be observed that α-particles and β-particles are emitted from Ra-226 and its progeny, sometimes accompanied with emissions of γ-rays. All three are types of ionizing radiation-energy in the form of particles or waves are sufficient to remove electrons from atoms (Hall and Giaccia, 2012). The α-particles emitted from the decay chain of Ra-226 have an energy of 2-10 MeV. Such α-particle radiation has a high linear energy transfer (LET), which is defined as the amount of energy an ionizing particle transfers to the material per unit distance (ICRU, Report 16). The range of a α-particle in matter is very short and can cause a high density of ionizations along its path, especially at the end of the range, where very large amount of energy will be deposited. So α-radiation is more effective than low LET radiation at causing biological damage (NRC, 1988). The term "relative biological effect" (RBE) is usually used to compare the biological effect induced by high-LET radiation to that induced by low-LET radiation, such as X-rays. It has been reported that the RBE of α-radiation ranges from 1.6 to 21 depending on the energy of the α-particles and the endpoint used (Thomas et al., 2007; Howell et al., 1994; Franken et al., 2011). The high RBE is thought to be due to the fact that α-radiation can cause more clustered DNA double-strand breaks than sparely ionizing radiation and these breaks are more difficult to repair correctly (Blocher, 1988). The potent ability of α-particles to produce a high proportion of double-strand DNA breaks makes them important in killing cells and carcinogenesis (Uranium mining in Virginia, 2012).

α-particles emitted in the decay of Ra-226 to Rn-222 have a mean energy of 4.78MeV. After decay, its progeny continuously decay in a very short time and several α-particles with other energies are emitted out. Because of the emission of α-particles, an elevated level of Ra-226 has a very high possibility of causing adverse biological effects to human and non-human biota in the environment if the internal exposure takes place through inhalation and ingestion (Uranium mining in Virginia, 2012).

1.1.4 A long physical and biological half-life

Elevated level of Ra-226 in the environment could result in the enrichment of radium in the drinking water and in food for human and non-human species, which would increase the ingestion dose. The physical half-life of Ra-226 is about 1600 years. The biological half-life of Ra-226, which is defined as the time required for the body to eliminate half of an administered dose of the substance by regular physiological processes (Mosby's Medical Dictionary, 2009), has not been measured experimentally in all species, but evidence from some species shows that the biological half-life may be quite long. From data of early time dial workers, the biological half-life of Ra-226 in human body is roughly 30-60 years after first exposure (Rundo et al., 1985). For non-human biota, Johnston et al. (1987) estimated a biological half-life of Ra-226 approximately 9 years in freshwater mussels, and no significant loss of radium occurred during 286 days (Jeffree and Simpson 1986). The long physical and biological half-life of Ra-226 means the ingestion dose will exist in the body for a very long time and this makes the influence of internal radiation of Ra-226 particularly important.

1.1.5 Chemical analogy to calcium

Radium is a member of group II of the periodic table, and thus some of its chemical and biological properties are similar to those of other elements in the same group, such as Ca, Mg and Ba (Cowart and Burnett, 1994). Radium could compete with calcium and be deposited in materials as a substitute for calcium especially when the concentration of available calcium in the environment is low. For example, Ra-226 could become incorporated in the bone matrix in the same way as calcium (Raabe et al., 1983). Thus Ra-226 is a bone-seeking radionuclide, and it can form hot-spots after being absorbed in the body.

1.2 Ra-226 detection method

Methods for the determination of the dose of Ra-226 include the direct detection of

Ra-226 and indirect methods of detecting the progeny of Ra-226. According to the decay chain of Ra-226, the dose of Ra-226 is commonly measured by alpha-spectrometry, gamma-spectrometry, and liquid scintillation counting systems. Measurement using the gamma-rays from Ra-226 daughters ^{214}Bi and ^{214}Pb can reduce the detection limit below 0.1Bq, but the leaking of ^{222}Rn gas reduces the reliability of the analytical results (Hou and Roos, 2008). Alpha-spectrometry is sensitive with high energy resolution (Hancock and Martin, 1991), but has a low counting efficiency of approximately 25% and it is necessary to isolate Ra-226 from other elements (Floeckher, 2011). The counting efficiency for alpha particles of liquid scintillation counting system (LSC) can reach 100%, but separation and liquefaction of samples are required to improve its poor energy resolution. During these processes, a large amount of radioactive material will be lost. A previous study using LSC tried to estimate the activity of Ra-226 in fish samples injected with 10mBq/g, 100mBq/g, 1000mBq/g and 10000mBq/g Ra-226, and it turned out that the amount of Ra-226 was below the minimum detectable activity of LSC (Thompson, 2011). Mass spectrometry is another method for Ra-226 analysis. Its sensitivity is comparable to the radiometric methods, and the detection limits were reported to range from 0.002-1Bq/kg (Hou and Roos, 2008). Mass spectrometry worked in previous study of our group which analyzed the dose of Ra-226 in fathead minnow exposed via ingestion of 10mBq/g to 10000mBq/g Ra-226 for two years. The results showed that accumulation of Ra-226 could be detected at 1 and 6 months, and the activity of Ra-226 was below the detection limit after ingestion for 24 months for fish fed with low activity radioactive food, suggesting depuration or at least non-linear bioaccumulation (Mothersill et al., 2014).

Autoradiography is usually used to determine the spatial distribution of radioactive substances. With autoradiography, particles produced by the radioactive substance will leave tracks on film. Then analyzing the autoradiograph and the histological image can determine the position where the radioisotope deposits. Columnia Resin #39 (CR-39) can

be used as a solid-state nuclear track detector in autoradiography to detect α-particles. It is one of the most sensitive nuclear track detectors and can record all α-particles with energies exceeding 0.5MeV (Nikolaev et al., 2010). With autoradiography using CR-39, the uptake and distribution of various radioisotopes in animals have been determined, such as Uranium-233, americium-241, and Radium-226 in rats (Priest et al., 1982, 1983a, 1983b), Plutonium-239 and Americium-241 in human skeletons (Priest et al., 1992) or Polonium-210 in fish (R&D Technical Report P3-053/TR, 2002). Autoradiography with CR-39 can also be used to quantify the amount of radioisotopes which emit α-particles through analyzing the number of the etch pits on the autoradiograph (Polig et al., 1986; Mori, 2013). Even though autoradiography is time consuming and labor intensive to prepare samples especially when the doses of radiation are low, it is simple and inexpensive and may be a good method to detect extremely low activity of radionuclides emitting α-particles if the exposure time is long enough.

1.3 Deposition of Ra-226

Given the elevated level of Ra-226 in the aquatic systems of the uranium mining and milling areas, the transfer of Ra-226 and the induced influence on the environment are the primary ecological concerns. Elliot Lake city of Canada used to be the leading producer of uranium in the world. The bioaccumulation of Ra-226 in organisms and the impact on the environment in this area have been relatively well studied. The level of Ra-226 in water and sediments, as well as in local plants and animals in this area turned out to be elevated (Clulow and Mirka, 1991; Davé et al., 1985; Wren et al., 1987; Dewit et al., 2002; Clulow et al., 1992, 1998; Clulow, 1986; Mirka et al., 1996). The following literature review is mainly based on studies in this area, supplemented with studies in other uranium processing areas.

1.3.1 Ra-226 level in the aquatic system

Representative studies on the level of Ra-226 in water or sediments of the aquatic environment are summarized in Table1.1 (water) and Table 1.2 (sediment). There's no difference in the concentration of Ra-226 in water between the sea and fresh water systems, but the levels of Ra-226 in sediments of fresh water systems are higher than that of sea.

Table 1.1 Concentrations of Ra-226 in water (Bq/L)

Range	Study site	Reference
0.10-0.13	South Adriatic Sea	Antovic andAntovic, 2011
0.366-95.34	Upper Silesia post-mining areas Poland	Geras'kin et al. 2011b
0.03-0.20	Quirke Lake in Canada	Pyle and Clulow, 1998
0.0043-0.019	Bagjata and Banduhurang mining area, India	Giri et al., 2010
Mean: 0.05	Round Lake of Florida, USA	Brenner et al., 2007

Table 1.2 Concentrations of Ra-226 in sediment (Bq/kg)

Range	Study site	Reference
7.1-9.6[a]	South Adriatic Sea	Antovic andAntovic, 2011
3551-70 025[b]	Upper Silesia post-mining areas Poland	Geras'kin et al. 2011b
155.7-1317.4[b]	Quirke Lake in Canada	Pyle and Clulow, 1998
12-132[a]	Bagjata and Banduhurang mining area,India	Giri et al., 2010
Mean: 445[b]	Round Lake of Florida, USA	Brenner et al., 2007

[a]Units of Bq/kg (wet weight); [b]Units of Bq/kg (dry weight).

1.3.2 Bioaccumulation of Ra-226 in biota

The transfer of Ra-226 in the aquatic system mainly follows the food chain. Aquatic producers, such as rooted aquatic macrophytes can take up radium directly from water and sediment. The consumers, such as fish, mussel and small mammalian animals can take up radium from water and food (Williams, 1982). Through this, Ra-226 can transfer from sediments and water to almost all the organisms in the environment.

The concentration factor (CF) is usually used to indicate the transfer pathway of a given radionuclide from the environment to biota. It is defined as the ratio between the radionuclide's concentrations in connected compartments of a model, such as the animal's tissue and the diet, or the animal's tissue and the environment (ICRP 1978). The CF is calculated using the following equation:

$$\mathrm{CF} = \frac{[\text{radionuclide in tissue}]}{[\text{radionuclide in environment}]} \qquad \text{Equation 1.4}$$

CF is not an indicator of the effects of radionuclide concentration on an organism; but it accesses the transfer of radionuclide by organisms in a simple way. Initially, the simple linear concentration factor model was commonly used by ecologists to describe contaminant flow through ecosystems, and often without any appreciation of its limitations (Williams, 1982; Halbert et al., 1990; Landrum et al., 1992; Linsalata, 1994; McGee et al., 1996). For the linear concentration factor model, there are three assumptions (Williams, 1982): (1) the concentration of radionuclide in the receiving compartment increases linearly as the increase of radionuclide concentrations in the donor compartment. In another world, the predicted concentration factor for the same kind of organism is constant no matter how different the radionuclide concentration in the donor compartment is; (2) the radionuclide between donor and receiving compartments must be in equilibrium; (3) there's only one radionuclide source for biological uptake. Actually, only a few systems studied fit the linear model: water/algae, water/mussels (Jeffree and Simpson, 1986; Bollhöfer et al., 2011), water/insects, water/macrophytes and total diet/human bone (Williams, 1982). In some cases, radionuclide levels in the environment and organisms do not follow the linearity model and a non-linear model is more appropriate. For example, Ra-226 equilibrium was reported to be achieved between the external environment and white sucker fish or lake herring fish, but before the equilibrium non-linear transfer of Ra-226 from environment to fish occurred (Clulow and Pyle 1997, Pyle and Clulow, 1997a, b). Non-linear uptake of Ra-226 was also reported from soil to plant (Simon and Ibrahim, 1987). Elevated levels of Ra-226 have been

reported in leaves, stems and roots of plants from areas near uranium tailings near the city of Elliot Lake, ranging from 41.8 to 1135mBq/g dry weight (Davé et al., 1985; Clulow and Mirka, 1991; Clulow et al., 1992; Mirka et al., 1996). Ra-226 level in fish, lake trout, whitefish and lake herring, from lakes near Elliot Lake city was estimated to be from <20 to 76mBq/g dry weight. The study also found that the burden of Ra-226 in bone of bottom feeding whitefish was at least three times more than that of lake trout in the same location. This may be because the bottom feeding fish ingest radionuclide-rich sediment particles along with food, and this also reflects the greater effectiveness of uptake of Ra-226 in diet item than in water (Clulow et al., 1998; Swanson, 1983). Levels of Ra-226 in small animals, like beaver, otter and muskrat near tailings area of Elliot Lake city were also examined (Clulow and Mirka, 1991; Dewit et al, 2002; Wren et al., 1987; Mirka et al., 1996). As an example, the level of Ra-226 in beaver was reported to be 67.5mBq/g wet weight in bone and 0.8mBq/g wet weight in muscle, the concentration factor was 1.31 for bone/vegetation and 0.02 for muscle/vegetation (Clulow and Mirka, 1991).

1.3.3 Factors affecting the bioaccumulation of Ra-226

Even though numerous studies have demonstrated elevated Ra-226 levels in various biota, precise information concerning the bioaccumulation of Ra-226 is limited. The existing studies indicate that the Ra-226 concentrations in different levels of organisms are quite different. Even within the same level of organisms from the same areas, the variation is large (Hesslein and Slavicek, 1984). This is because of the enormous complexity of the ecosystem and its biodiversity. The bioaccumulation of Ra-226 in organisms is influenced by various factors, including but not limited to the age of the organisms, and the concentration of calcium and other alkaline earth elements in the body and in the surrounding areas.

It has been reported that a significant inverse relationship exists between the Ra-226 level in bone and lake trout fish age (Clulow et al., 1998). High Ra-226 concentrations in

young Carp compared to older fish of the same species have also been observed (Justyn and Havlik, 1990). Mothersill et al. also reported high Ra-226 concentration in younger fathead minnows exposed to environmentally relevant level of Ra-226 via ingestion (Mothersill et al., 2014). The elevated Ra-226 concentration was attributed to the extremely high growth and metabolic rates of the young fish, which make the young fish uptake and absorb more nutrients from the food. Another reason might be the larger demand for calcium by the young fish for the fast growth of their skeleton. On the contrary, similar Ra-226 levels in tissues among various age groups of fish have also been reported. Ra-226 level in bone or muscle of lake whitefish, round whitefish, white suckers, long nose suckers and lake trout taken from lakes near uranium mining areas in Saskatchewan did not vary with age (Swanson 1983, 1985). Clulow and Pyle (1997, 1998) also reported that Ra-226 concentrations in white fish (Clulow et al., 1998), lake herring bones and white sucker tissues did not vary by age. The existing contradictory data might be because of the species diversity and the different physical and chemical characteristics of water and sediments at different sites. The source of the radionuclide Ra-226 might also be a reason. For the lake trout, in which Ra-226 level was inversely related with fish age, there was no significant relationship between bone and water Ra-226 levels. But for white fish in the same location, in which Ra-226 level was not related with fish age, there was significant relationship between bone and water Ra-226 levels (Clulow et al., 1998). For fathead minnows, which absorbed more Ra-226 in younger samples, the Ra-226 they absorbed was from fish food, rather than water (Mothersill et al., 2014).

The uptake and deposition of Ra-226 by the organisms also depends on the concentration of calcium in the surrounding environment. This is due to the biogeochemical similarity of Ra-226 to Ca. Studies on the relationship between uptake of Ra-226 and Ca have been conducted in fish and mussels. Investigations by Rope & Whicker (1985) of Ra-226 accumulation in trout showed that highest Ra-226 concentrations were found in individuals living in water with the highest Ra/Ca ratio.

Bollhöfer et al. (2011) reported that the average Ra-226 activity loads in mussels were strongly positively correlated with the ratio of total Ra-226 activity to the Ca concentrations when they were cultured in water with low Ca concentration. It has also been reported in laboratory experiments exposing mussels to various Ra-226 and Ca or Mg concentrations for relatively short periods of time, that increased Ca and Mg water concentrations reduced the rate of Ra-226 uptake by mussels tissues and competitive inhibition of the uptake of Ra-226 occurred (Jeffree and Simpson, 1986). Accumulation of Ra-226 and Ca in mussels under field condition was consistent with Ca being a metabolic analogue of Ra-226 (Jeffree and Simpson, 1986).

The accumulation of Ra-226 in organisms is also related to the concentrations of calcium and other alkaline metal in the body. It was reported by Jeffree and Smipson (1984) that the concentration of Ra-226 among the mussels tissues was strongly positively correlated with that of Ca, Ba and Mg, and in addition, Ra-226, Ca, Mg and Ba are co-located predominantly in granular deposits throughout the body of mussels. A study with English sole also confirmed the existence of a positive correlation between radium content and calcium concentration in bone tissue (Porntepkasemsan and Nevissi, 1990).

1.3.4 Distribution of Ra-226 in biota

Because radium behaves environmentally and physiologically similar to calcium, Ra-226 tends to compete with calcium and forms some hot spots in certain tissues of organisms, such as bones. The concentration of Ra-226 in bone of fish was reported to be about 3 to 10 times as high as that in muscles (Pyle and Clulow, 1998; Antovic and Antovic, 2011; Clulow et al., 1998). Gut is another area where Ra-226 tends to accumulate. Mean Ra-226 level in the gut of lake trout from McCabe and Quirke Lakes were respectively 126 and 64mBq/g dry weight, while the muscle Ra-226 levels were 3.2 and 2.1mBq/g dry weight respectively (Clulow et al., 1998). In mussels, the

concentration of Ra-226 could vary by more than an order of magnitude between different tissues (Jeffree and Simpson, 1984). In small mammalian animals, the difference in the Ra-226 concentration in different tissues is more obvious. For beavers living near uranium tailings near Elliot Lake of Canada, the concentration of Ra-226 in bone was 67.5mBq/g wet weight while in muscle it was 0.8mBq/g (Clulow and Mirka, 1991). The accumulation of Ra-226 in bone reflects the fact that Ra-226 could be incorporated in the bone matrix in the same way as its analogue Ca (Friedlander and Kennedy, 1962; Raabe et al., 1983). The uneven distribution of Ra-226 also happened in plants. Accumulation of Ra-226 in roots was reported to be higher than in stems and leaves in cattails from uranium tailings near the city of Elliot Lake of Canada (Mirka et al., 1996) and in water-lily grown in Ra-226-labelled laboratory sediment, especially on the surface of the root and rhizomes (Twining, 1993).

1.4 The biological effects of Ra-226

After radium was discovered, it was used in many applications, such as a self-illuminating material in dial clocks and medical diagnoses and radiation therapy (IAEA, 2010). People began to pay attention to the influences in humans caused by chronic, low dose Ra-226 radiation when the damages, especially cancer caused by the absorbed Ra-226 in early radium dial painters were reported (Rowland et al., 1978). After these findings numerous studies were conducted to examine the health risks of Ra-226. Recently, as the Ra-226 level in the environment in mining areas becomes an issue, increasing attention is being paid to the influence of Ra-226 on non-human biota.

1.4.1 Biological effects induced by elevated Ra-226 on human

In man, knowledge of long-term effects of Ra-226 comes almost entirely from watch radium-dial painting workers who accidentally ingested Ra-226 and Ra-228 through their practice of licking the paint brushes to obtain a fine point in the early days. Through

studying them, the health risks and the dose-response of Ra-226 was well studied (Rowland et al, 1978). It was revealed that the incorporated Ra-226 within the human body give rise to two distinct types of malignancies. Bone sarcoma is the most important adverse effect associated with exposure to radium, as well as other bone-seeking radionuclides (Thomas and McNeill, 1982). Carcinomas of the mastoid air cells and paranasal sinuses ("head carcinomas') is another type of malignancy that has been found in the dial painting workers (Rowland et al., 1978). In addition, it was also reported that slightly more breast cancers than expected were found in the dial painters (Stebbings et al., 1983). As to the mechanism for these malignancies, the deposited Ra-226 within the body, especially in bone is confirmed to be the main reason for bone sarcoma, and the accumulation of Rn-222 in the mastoid air cells plays an important role in the induction of the head carcinomas (Rowland et al, 1978; Evans, 1966). However no consistent opinion has been reached about the dose-response relationships. Through considering the two types of malignancies in a population of 496 cases, Evans et al. concluded that a "practical" dose threshold exists, below which the tumor appearance time generally exceeds the life span (Evan et al., 1969). On the contrary, a linear relationship between dose and effect was implied by an Advisory Committee on the Biological Effects of Ionizing Radiations of the National Academy of Sciences (BEIR, 1972). Furthermore, when each type of malignancy was considered separately, the incidence of bone sarcoma could be fitted with a dose-squared-exponential function, and the head carcinomas couldn't be fitted by any function (Rowland et al, 1978). In order to study the radiation damage of Ra-226 better, lots of experiments with animals injected with Ra-226 were conducted. The alpha-particles were found to be efficient at inducing skeletal lesions and osteosarcomas similar to those documented in dial painters. The time of appearance and severity of the radiation damage was demonstrated to be in a dose-related fashion, and the expectancy of death from bone sarcoma increased linearly with increasing amounts of injected radium (Finkel et al., 1969). Histological analysis revealed that features of the

bone sarcomas vary considerably, and fibrosis of cells was involved (Goldman et al., 1969).

Some information about the health risks of Ra-226 comes from the influences of low levels of Ra-226 in drinking water. Researchers found that incidence rates of some cancers, such as lung and breast cancers, in the exposed population, who consumed water with elevated level of Ra-226, were higher than in the control populations (Bean et al, 1982), so as to the leukemia risk (Lyman et al., 1985; Fuortes, 1990). The mortality rates based on deaths coded to malignant neoplasm involving bone also correlated with Ra-226 level in drinking water (Petersen et al, 1966).

1.4.2 Biological effects induced by elevated Ra-226 on non-human biota

Because of the difficulty of field experiments and the perceived lower relevance of laboratory experiments, studies on the impacts of the environmentally relevant Ra-226 on non-human biota are rare. Given the rather low levels of Ra-226 in the contaminated areas, it is uncertain whether toxic effects can be induced on the plants and animals. However several detrimental effects induced by these low levels of radiation from Ra-226 have been reported. The literature review below includes the effects of elevated levels of Ra-226 on plants and animals irrespective of the source of the enhanced Ra-226 level.

1.4.2.1 Effects of elevated level of Ra-226 on plants

The *Allium*-test is a method that is usually used to analyze the genotoxicity and cytotoxicity of contaminated water or soil. It has been reported that frequency of aberrant cells in the first mitosis of root meristem cells of *Allium cepa* L. grown in water samples (0.171Bq/l after 20 times dilution) from underground galleries near mines in the UPPER Silesian Coal Basin, Poland significantly exceeds the level of control group. This means that there is a genotoxic effect of the sampled water. For root meristem cells of *Allium*

*cepa*grown on sediments (7002.5Bq/kg (dry weight) after 10 times dilution) mitotic activity was significantly lower than control, and frequency of aberrant cells was significantly higher, which indicated that there was cytotoxic and genotoxic effects for sediment samples (Geras'kin et al, 2011b). Analysis showed that the main contribution to cytogenetic effect was made by double bridges and lagging chromosomes (Geras'kin et al, 2011b; Oudalova et al., 2009). In this study, it was reported that Ra-226 may be part of the reason for water's genotoxicity, the main reason may be the high concentration of potassium. For the sediment genotoxicity, radionuclides appear to be the main contributors with Ra-226 playing the major role (Geras'kin et al, 2011b). However, in another study even though the radionuclide concentrations didn't exceed the radioactivity guidelines, water samples from a radium production industry storage cell territory still caused a significant increase in chromosome aberration frequency on *Allium* root tips cells as compared to control. In this case the damage may correlate with Zn concentration rather than radioactivity (Evseeva et al., 2003). This reflects the complexity of evaluating the biological effects of radionuclides on the organisms in the natural environment.

Evseeva et al. studied ionizing radiation impacts on natural *Viciacracca* populations growing inthe industrial areas near the Vodny settlement (Komi Republic, Russia), where have been contaminated by uranium mill tailings and radium production wastes. They reported that a major part of the dose at the study site was attributable to internal irradiation and Ra-226 constituted 84.3-99% of the total internal irradiation. For *Viciacracca* populations growing in these sites (Ra-226 concentration in soil 25487Bq/kg dry weight, the absorbed dose: 0.2-0.3Gy), the embryonic lethal mutation frequency was significantly higher and the germination capacity of seeds was significantly lower than those for the control. A significant increase of the frequency of embryonic lethal mutation in legumes and chromosome aberrations in seedlings' root tip cells was seen in *Viciacracca* populations grown in areas where the absorbed dose was 0.4-0.9Gy (Ra-226 concentration in soil 118799Bq/kg dry weight) (Evseeva et al, 2009).

For the pine trees in the same site, where the doses absorbed were mainly delivered by internal irradiation: Ra-226 (65%), survival rate of sprouts significantly decreased at a dose rate of 17mGy/h, while cytogenetic effects and the proportion of abortive seeds increased at 71mGy/h (Geras'kin et al, 2013).

1.4.2.2 Effects of enhanced level of Radium-226 on animals

Through exposing Atlantic cod embryonic cells to environmental relevant concentrations of Ra-226 and quantifying selected genes transcription, Olsvik et al. (2012) found that some gene transcriptional levels were significantly up-regulated even when the Ra-226 concentration was as low as 2.11Bq/L. The low-dose Ra-226 radiation induced oxidative stress and apoptosis in the cod embryonic cells, and the transcriptional induction would in turn trigger the cellular defense against oxidative stress.

Fathead minnows fed with environmental level of Ra-226 exhibited transient growth perturbations. After 6 months of being fed with 10mBq/g Ra-226 contaminated diets, reductions in mean body mass of the fish were found. At 12 months and 24 months, there was a disproportionate increase in body mass relative to fork length. In addition, this low-dose Ra-226 changed the ratios of DNA:RNA, DNA:protein and RNA:protein in the fish body (Mothersill et al., 2013).

Other research about the earthworms grown in α-emitter contaminated soil (Ra-226 concentrations: 1506Bq/kg dry weight) also reported changes in growth. Inhibition of growth and reproduction occurred in earthworms grown in contaminated soil, their biomass significantly decreased, as did cocoon production. After testing the DNA, they found that there was a significant increase of DNA damages even after 1 day of exposure (Lourenco et al., 2012).

Overall, the above researches indicate that plants and animals living in the aquatic system which is contaminated with low dose Ra-226 can absorb and accumulate Ra-226 in their bodies. Ra-226 doesn't distribute evenly in the organisms' bodies and it mainly

deposit in the bone for animals and on the surfaces in the roots and rhizomes of plants. In addition, the chronic, low-dose Ra-226 radiation can cause adverse effects on plants and animals, including the decrease of body mass and reproduction ability in fish and worms (Mothersill et al., 2013; Lourenco et al., 2012), and reduced germination capacity of seeds and the survival rate of sprouts for plants (Evseeva et al, 2009). All in all, studies about the consequences of enhanced level of Ra in aquatic system are not enough, not to mention the biological mechanisms. But this kind of research is really necessary for estimating the risks to the non-human biota to protect the whole ecosystem.

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Chapter 2

QUANTITATIVE ANALYSIS OF ACCUMULATION OF RA-226 IN FATHEAD MINNOW FED WITH ENVIRONMENTAL LEVELS OF RA-226 USING AUTORADIOGRAPHY AND GROWTH INDICES

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The first author, Dr. Mothersill and Dr. Seymour originally designed the research project. Dr. Smith and the first author completed the fish preparation. The first author completed the fish section preparation, autoradiography, data collection and analysis. The first author wrote the manuscript, and Dr. Mothersill helped to revise it. The manuscript was submitted to the International Journal of Radiation Biology on Sep 8Th, 2016. Manuscript ID: TRAB-2016-IJRB-0260.

2.1 Abstract

Purpose: To determine the accumulated activity of Ra-226 in fathead minnows fed with environmentally relevant levels of Ra-226 for five months in water at 20^{o}C, and to evaluate the influence of this level of Ra-226 on the growth of fathead minnows.

Methods: Fathead minnows were continuously fed with fish food containing 10-10000mBq/g Ra-226 for five months. At the end of the experiment, the fish were sacrificed, flash frozen in liquid nitrogen and kept at -20^{o}C. Longitudinal sections of 40μm thickness were cut at the middle of the fish body using a cryostat. The activity of Ra-226 in each section was determined using autoradiography with a nuclear track detector CR-39. According to the weight and the width of the fish, the activity of Ra-226 in the whole fish body could be estimated. In addition, the length and the weight of the fish were measured and the condition factor was calculated to evaluate the growth and fitness of the fish.

Results: There is a positive but non-linear relationship between the accumulated activity of Ra-226 in fish body and the concentration of Ra-226 in fish food. The highest activity of Ra-226 accumulated in fish body was found from fish fed with 10000mBq/g Ra-226 food. This was calculated as 256.42 ± 49.14mBq/g, $p<0.05$, and the calculated dose rate was 6.18 ± 1.18mGy/y. For fish fed with food containing lower concentration of Ra-226 (up to 1000mBq/g), the bioaccumulation of Ra-226 in the body saturated. The Ra-226 concentration factor (CF) for fish was inversely proportional to the Ra-226 activity in food, and the highest CF value was 2.489, obtained from the lowest dietary Ra-226 activity (10mBq/g). In addition, condition factors (K) of fish in all Ra-226-treated groups were significantly lower than those of the controls.

Conclusion: The results show that the bioaccumulation of Ra-226 in fish is not simply related to the dietary Ra-226 activity, and has a saturation value when the dietary activity

is low. In addition, the environmental level of Ra-226 in the fish food has a small adverse effect on the growth and fitness of fathead minnows.

Keywords: Ra-226 bioaccumulation, autoradiography, radium dosimetry, ingestion of radium.

2.2 Introduction

The biological effect induced by Ra-226 in natural aquatic systems is attracting increasing attention because of the elevated level of Ra-226 in uranium mining areas, the long half-life of Ra-226 and its high radiotoxicity. Internally deposited Ra-226 can have a severe detrimental impact if enough activity is absorbed, because α-particles are mainly emitted and they have a high relative biological effect (RBE). In order to assess the effects induced by elevated levels of Ra-226 in the environment, the first important thing is to quantify the bioaccumulation of Ra-226 in organisms.

As mentioned by Mothersill et al. (2014), there is very little known about the bioaccumulation of Ra-226 and the potential biological effects in non-human species in the environment. The existing studies are limited by the complexity and uncertainties in the ecosystem (Mothersill et al., 2014). A literature review found that most of these studies focused on estimating the dose of Ra-226 in plants or animals in uranium mining areas and areas of the natural environment contaminated by the uranium mining industry (Brenner et al., 2007; Antovic and Antovic, 2011; Clulow and Pyle, 1997; Giri et al., 2010). Because of the wide ranges of activities of Ra-226 in different areas and the biodiversity of the environment, the variation in reported bioaccumulation of Ra-226 between different species and different areas is large. The published results are influenced by the presence of other radionuclides and other toxic elements. On the other hand, experiments in laboratory concerning the accumulation of radionuclides are very complicated and limited by the difficulties of long-term maintenance. There is also the problem of the artificiality of laboratory conditions, as well as the need to use relatively

large amounts of radium which poses radiological as well as health and safety issues (Mothersill et al., 2014; Smith et al., 2011). For all these reasons, information about the bioaccumulation of Ra-226 in organisms in the aquatic environment is limited and is not systematic. In order to get systematic and useful data concerning the uptake of Ra-226 and the biological effects in animals in the aquatic environment, a long-term laboratory experiment was set up. In this experiment, fathead minnows were exposed to environmentally relevant activities of Ra-226 from 10mBq/g to 10000mBq/g in fish food, to evaluate the uptake, accumulation and biological effects of Ra-226. The choice of these doses was based on a study conducted by Clulow and Pyle, which reported that the activities of Ra-226 in the gut contents of white suckers captured from contaminated lakes in Northern Ontario ranged from 10-100mBq/g dry weight (Clulow and Pyle, 1997).

Activities of Ra-226 in the environment and in fish are difficult to measure. The relevant levels are at or near detection limits for many techniques. This makes it difficult to analyze and determine dose. Common radiometric approaches used to determine activity of Ra-226 includes alpha-spectrometry, gamma-spectrometry, and liquid scintillation counting systems. Measurement of gamma-rays emitted from Ra-226 daughters ^{214}Bi and ^{214}Pb can reach a detection limit of below 0.1Bq, but leaking of ^{222}Rn gas can reduce the reliability of analytical results (Hou and Roos, 2008). Alpha-spectrometry is sensitive with high energy resolution (Hancock and martin, 1991), but has low counting efficiency of approximately 25% and requires Ra-226 to be isolated from other elements (Floeckher, 2011). The counting efficiency for alpha particles using a liquid scintillation counting system (LSC) can reach 100%, but separation and liquefaction of samples are required to improve its poor energy resolution. During this process, a large amount of radioactive material will be lost. A previous study by our group tried to estimate the activity of Ra-226 in fish samples exposed to 10mBq/g, 100mBq/g, 1000mBq/g and 10000mBq/g Ra-226 by injection with LSC, and it turned out

that the amount of Ra-226 was below the minimum detectable activity of LSC (Thompson, 2011). Mass spectrometry is another method for Ra-226 analysis. Its sensitivity is comparable to that of radiometric methods, and the detection limits were reported to range from 0.002-1Bq/kg (Hou and Roos, 2008). Mass spectrometry was used in a previous study by our group which analyzed the dose of Ra-226 in fathead minnows continuously fed with food containing 10mBq/g to 10000mBq/g Ra-226 for two years. The results showed that accumulation of Ra-226 could be detected at 1 and 6 months, but the activity of Ra-226 was below the detection limit at 24 months for fish fed with lower levels of radioactive food (Mothersill et al., 2014). For these reasons it was decided to partly repeat the earlier study using autoradiography as it can detect extremely low dose of α-particles.

Autoradiography is usually used to determine the spatial distribution of radioactive substances. With autoradiography, particles produced by the radioactive substance will leave tracks on film. Then analyzing the autoradiograph and the histological image can determine the position where the radioisotope deposits. Columnia Resin #39 (CR-39) can be used as a solid-state nuclear track detector in autoradiography to detect α-particles. It is one of the most sensitive nuclear track detectors and can record all α-particles with energies exceeding 0.5MeV (Nikolaev et al., 2010). With autoradiography using CR-39, the uptake and distribution of various radioisotopes by animals have been determined, such as Uranium-233, Americium-241, and Radium-226 in the rat (Priest et al., 1982, 1983a, 1983b), Plutonium-239 and Americium-241 in the human skeleton (Priest et al., 1992), or Polonium-210 in fish (R&D Technical Report P3-053/TR, 2002). Autoradiography with CR-39 can also be used to quantify the amounts of radioisotope present which emit α-particles through analyzing the number of the etch pits on the autoradiograph (Polig et al., 1986; Mori, 2013). Even though autoradiography is time consuming and labor intensive to prepare samples especially when the doses of radiation are low, it is simple and inexpensive and is a good method to detect extremely low

activity of radionuclides emitting α-particles if the exposure time is long enough.

In the current study, autoradiography with CR-39 was used to determine the activity of Ra-226 deposited in fish exposed to environmental levels of Ra-226 via ingestion. The spatial distribution of Ra-226 in the whole body of fish was also studied in order to find out which organs may be at high risk from internal radiation of Ra-226.

Several studies concerning the biological effects of elevated levels of Ra-226 in non-human biota have revealed that some adverse effects can be induced, including increased mutation frequency and mortality in the early stages of life, reduced biomass, and impairment of reproductive ability (Evseeva et al, 2009; Geras'kin et al., 2011, 2013; Lourenco et al., 2012; Mothersill et al., 2013). However these data were not enough to evaluate the influence of the elevated levels of Ra-226 in the environment systematically. In current study, in order to evaluate the influence of chronic radiation from Ra-226 on the growth of fish, we studied physical growth endpoints in fish exposed to low doses of Ra-226. Through analysis of the bioaccumulation of Ra-226 in fish and the study of growth endpoints, this work should provide useful data for evaluating the influence of elevated levels of Ra-226 and help inform radioprotection guidelines for non-human biota in the aquatic environment.

2.3 Materials and methods

2.3.1 Fish husbandry

Fathead minnow (*Pimephales promelas*, Rafinesque, 1820) were chosen as the experimental fish and were supplied by the US Environmental Protection Agency (USEPA, Duluth, MN, USA). The fish were maintained in 20L plastic aquaria, supplied with dechlorinated water from Hamilton, Ontario, Canada, at a flow through rate of not less than 200ml per minute. The temperature of the water was controlled at about 20^{o}C by a heater in every aquarium. Each aquarium was also independently aerated by diaphragm-type air pumps and a ceramic air-stone diffuser. Lighting was controlled to

provide 14h daylight, by means of daylight spectrum fluorescent lighting and 10h darkness.

The fish were divided into five groups: one was the control, in which the fish were fed with normal food; the other four groups were the Ra-226-treated, in which the fish were fed with food containing 10, 100, 1000 or 10000mBq/g Ra-226. Feeding was carried out six days per week until the fish were fed to satiation and ignored any food. Every time after the feeding, the remaining fish food in every group was weighed and recorded. So the amount of food consumed was known. The uneaten food in the aquaria was removed by siphon minutes after feeding to make sure no radioactive food was left in the aquaria. Fish were sampled at 5 months after the start of dietary exposure. The fish were sacrificed and wrapped in aluminum foil and flash frozen in liquid nitrogen, and then stored in -20^{o}C.

2.3.2 Ra-226 dietary

The Ra-226-contaminated diet used in current work was made from commercially available fish food (Silver Cup fish Feed, Nelson and Sons Inc., Murray, UT, USA) and Ra-226, which was supplied as neutralized radium nitrate (Eckert and Ziegler, Valencia, CA, USA). The carrier for the Ra-226 was 10μg/ml Ba (as Ba nitrate). In order to make fish food containing consistent Ra-226, the commercial fish food was first ground to very fine powder, and the appropriate amount of Ra-226 stock solution was mixed thoroughly with water. Then small amounts of the ground food power were mixed completely with the water until the correct amount of food powder had been added. After that, the mixture was extruded through a wide-bore syringe and let dry. The dried mixture was ground to the proper size and stored in a container. In this way, fish food containing 10, 100, 1000 and 10000mBq/g Ra-226 was prepared.

2.3.3 Ra-226 injection

In order to determine the deposition of Ra-226 in fish body, some fish were injected with higher doses of Ra-226. The fish were first weighed, and according to the weight, certain volume of Ra-226 solution was injected into abdominal cavity of the fish with ultra-fine insulin syringe (No. 329466, Becton Dickinson, USA). The final concentration of Ra-226 in fish body was 10Bq/g. After the injection, the fish were returned to the aquaria containing fresh water. The fish were sampled at 28 days after the injection.

2.3.4 Autoradiography

The fish were put on dry ice and taken to the lab to get sectioned in a cryostat at -20^{o}C. In each group, three fish were randomly selected, and three 40 μm longitudinal sections were cut at the middle of the spine of each fish body. Each section was placed on the solid state nuclear track detector CR-39 slide, which was then sandwiched between two glass slides. Two binder clips were used to clamp them together. The exposure time of fish sections to CR-39 detectors was 60 days. During the whole process, the fish sections were kept frozen. Then these CR-39 detectors were etched at 65^{o}C in 6.25M NaOH solution for about 5 hours before being washed and dried. The CR-39 detectors for sections of fish fed with Ra-226 were read and analyzed using a microscope (Olympus Canada, Richmond Hill, Canada), and the tracks on each CR-39 detector were counted. The CR-39 slides for sections of fish injected with Ra-226 were also read as described above and pictures were taken with a camera connected to the microscope. These pictures were put together to rebuild into a whole picture using photoshop.

2.3.5 Dose calculation

The track number (NT) on detectors irradiated by Ra-226 in the fish section is determined by the etching conditions, the concentration of Ra-226 (C_{226}), the detecting efficiency of the detector, the contribution of Ra-226 daughters and the range (R) of α-particles in the fish section (Polig et al., 1986). The concentration of Ra-226 can be

expressed as follows:

$$C_{226} = \frac{N_T}{[\eta_{226}R_{226}+f\eta_d(R_{222}+R_{218}+R_{214}+xR_{210})]\rho St} \quad \text{Equation 2.1}$$

Where C_{226} is the concentration of Ra-226, N_T is the track number on detectors; $\eta_{226}$is the detection efficiency of Ra-226 particles; R_{226}, R_{222}, R_{218}, R_{214}, R_{210} are the ranges of Ra-226, ^{222}Rn, ^{218}Po, ^{214}Po and ^{210}Po in tissue; f is the fractional radon retention in the tissue section, x is the contribution of ^{210}Po; S is the area of the fish section on the CR-39 detector; ρis the density of the fish section; t is the exposing time of CR-39 to the fish section (Polig et al., 1986).

In order to simplify the process of estimating the concentration of Ra-226, we assume that the fractional radon retention in the fish section is 0. So equation 2.1 can be simplified and expressed as follows:

$$C_{226} = \frac{N_T}{\eta_{226}R_{226}\rho St} \quad \text{Equation 2.2}$$

Because the range of α-particle emitted fromRa-226 in fish tissue is about 45μm (Khatib, 2012), and the thickness of the fish sections we used is 40μm, which is smaller than the range, so it is assumed that all α-particles emitted from Ra-226 in the fish sections were able to reach the CR-39 detector. Then equation 2.2 can be expressed as follows:

$$C_{226} = \frac{N_T}{\eta_{226}mt} \quad \text{Equation 2.3}$$

Where m is the mass of the fish section.

According to equation 2.3, the concentration of Ra-226 (C_{226}) in the fish section can be calculated from the track number on the CR-39 detector. In addition, Ra-226 concentrations were assumed to be uniformly distributed in the whole fish body. So the activity of Ra-226 in the examined fish sections was the same as that in the whole fish body. This assumption was necessary as it was impossible to calculate Ra-226 concentrations in every organ.

Using the determined concentration of Ra-226 in the fish (C_{226} in Bqg^{-1}), the dose

rate (D_α) of radiation from Ra-226 can be calculated using the following equation if the α-particle contribution was only considered:

$$D_\alpha = 5.76 \times 10^{-7} C_{226} \times E_\alpha \, Gyh^{-1} (Bqg^{-1})^{-1} \quad \text{Equation 2.4}$$

Where C_{226} is the concentration of Ra-226 in the fish (Bqg^{-1}), E_α is the average energy of α-particles emitting from Ra-226, 4.78 MeV, and 5.76×10^{-7} represents the dose conversion factor.

2.3.6 Concentration factor calculation

According to International Commission on Radiological Protection (ICRP 1978), Fulton's concentration factors (CF) were determined using the following equation:

$$CF = \frac{[\text{radionuclide in tissue}]}{[\text{radionuclide in diet}]} \quad \text{Equation 2.5}$$

Where [radionuclide in tissue] represents the activity concentration of Ra-226 in the whole fish body; [radionuclide in diet] is the activity concentration of Ra-226 in the fish food.

2.3.7 Condition factor

Condition factor (K) is usually used to assess the overall health and robustness of fish, and it is also an indication of sexual maturity (Williams, 2000). It can be calculated with the following equation:

$$K = \frac{\text{Weight (g)}}{\text{Length}^3 \, (\text{cm}^3)} \times 100 \quad \text{Equation 2.6}$$

2.3.8 Statistic Analysis

All data are expressed as means ± SEM (the standard error of the mean).The number of tracks produced by α particles from Ra-226 in fish sections on CR-39 slides was analyzed by two-way Analysis of Variance (ANOVA) method, and Post-hoc testing was performed using Fisher's least significant difference (LSD) test (SPSS22). A confidence

interval of 95% and $p<0.05$ was selected to be statistically significant.

2.4 Results

2.4.1 Activity of Ra-226 in fish fed with Ra-226

The detection efficiency of the CR-39 for Ra-226 (η_{226}) was determined by exposing detectors to known activity of Ra-226. After same etching process, track numbers were measured by counting tracks using the microscope. The detection efficiency was calculated from the track number and the specific activity of Ra-226. The detection efficiency of α-particle from Ra-226 was about 0.23.

Table 2.1 shows the activity concentration of Ra-226 calculated in the whole body of fish for each group, as well as the relative dose rate. The relationship between the activity concentration of Ra-226 detected in the fish for each diet and the dietary activity concentration of Ra-226 is shown in Figure 2.1. As the concentration of Ra-226 increased in the diet, the activity of Ra-226 accumulated in fish body also increased, but they were not in a linear relationship. For fish in the lower Ra-226 concentration groups (the 10mBq/g, 100mBq/g and 1000mBq/g groups), even though the activity concentrations of Ra-226 fed to fish in various groups were orders of magnitude different, the accumulated activity of Ra-226 was almost the same, and significantly higher than that of control, except for 100mBq/g group, which due to high variability did not quite reach statistical significance ($p=0.09$).

Table 2.1 The activity concentration and dose rate of Ra-226 in fish

Group	Concentration of Ra-226 (mBq/g)	Dose rate (mGy/y)
background	13.15 ± 3.41	0.32 ± 0.08
10mBq/g	24.89 ± 3.59*	0.60 ± 0.09*
100mBq/g	29.00 ± 7.80	0.70 ± 0.20
1000mBq/g	32.56 ± 4.40*	0.78 ± 0.11*
10,000mBq/g	256.42 ± 49.14* $^{+}$	6.18 ± 1.18*$^{+}$

Ps: *p<0.05 compared to the activity concentration of Ra-226 in the background; $^{+}$p<0.05 compared to the activity concentration of Ra-226 in fish of other groups; n=9.

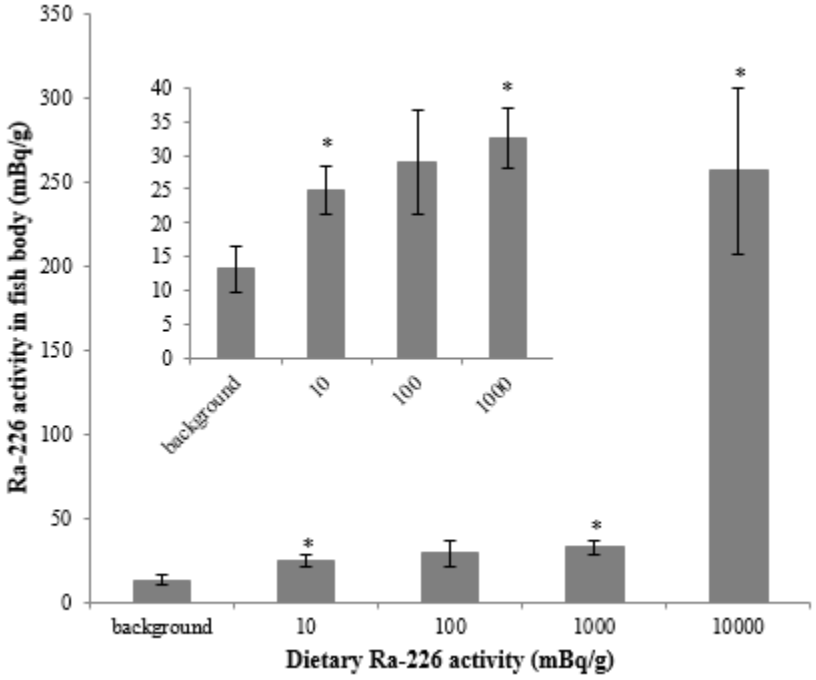

Figure 2.1 Activity of Ra-226 accumulated in the whole body of fathead minnows fed with fish food containing Ra-226 after being cultured in 20 ^{o}C water for 5 months. Error bars represent SEM, n=9. Analysis was performed using independent-samples T-test. A significant change when compared to the background is represented by * (p<0.05). A significant change when compared to the activity concentration of Ra-226 in fish of other groups is represented by $^{+}$p<0.05. The inset expands the scale for the lower activities.

2.4.2 Concentration factors and the uptake percentage

The concentration factor of fish in each group for the 5-month experiment was calculated and is presented in Table 2.2. Figure 2.2 shows the relationship of the Ra-226 concentration factor for each group against the concentration of Ra-226 in the fish food. The maximum concentration factor was found in the group with the lowest dietary Ra-226 concentration, and as the dietary Ra-226 concentration increased, the

concentration factor decreased. The concentration factor was therefore in a negative, non-linear relationship with the activity concentration of Ra-226 in the fish food. By the end of the experiment, the total amount of Ra-226 fed to fish in every group was known. So the uptake percentage of Ra-226 by fish in each group was determined and the data are shown in Table 2.3.

Table 2.2 The Ra-226 concentration factor of fathead minnows in each group

Group	Concentration factor
10mBq/g	2.489 ± 0.359
100mBq/g	0.290 ± 0.078
1000mBq/g	0.032 ± 0.004
10,000mBq/g	0.026 ± 0.005

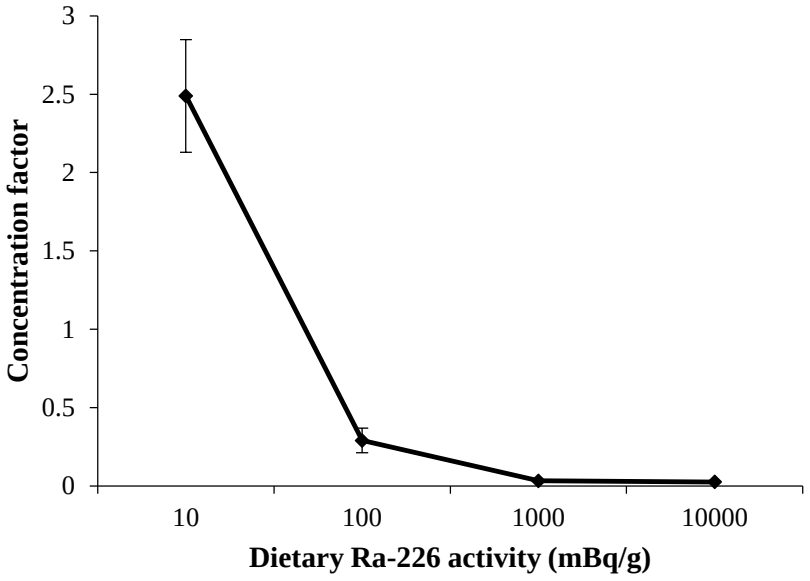

Figure 2.2 Ra-226 concentration factors of fathead minnows fed with fish food containing Ra-226 after being cultured in 20°C water for 5 months. Error bars represent SEM, n=9; the x-axis is in logarithmic scale.

Table 2.3 The uptake percentage of Ra-226 by fathead minnows in each group

Group	Total activity of Ra-226 (Bq) fed to fish	Uptake percentage (%)
10mBq/g	1.84	101.90 ± 14.68
100mBq/g	18.6	14.24 ± 3.83
1000mBq/g	191.5	1.74 ± 0.23
10,000mBq/g	1926.87	0.78 ± 0.15

2.4.3 Distribution of Ra-226 in fish

Because of the low activity of Ra-226 deposited in fish exposed to Ra-226 via ingestion, tracks of Ra-226 on autoradiograph of these fish sections couldn't display the deposited location of Ra-226 in the fish. In order to determine the distribution of Ra-226 in fish body after being absorbed, autoradiography was done with fish sections from fish injected with higher dose of Ra-226. The picture compiled from the autoradiograph of one fish injected with Ra-226 solution is shown in Figure 2.3. The skeleton and the contour of the fish were clearly indicated in the picture, and tracks of α-particles were mainly located in these areas, especially in the area of bones. A small amount of α-particles were distributed evenly in other areas including the muscles and the digestive system.

Figure 2.3 Picture of the autoradiograph of Ra-226 injected fish

2.4.4 Influence of Ra-226 on physical growth of fish

The physical growth indicators, such as the weight, the length and the condition factor (K) of the fish are presented in Table 2.4 and shown in Figure 2.4 to indicate the influence of Ra-226 in food on the physical growth and fitness of fish. The length of fish in Ra-226 treated groups was significantly shorter than that of control fish except for the 1000mBq/g group (p=0.098). The weight of fish fed with food containing Ra-226 was significantly lower than that of control fish except for the 1000mBq/g group (p=0.071). The condition factor of fish in all Ra-226-treated groups was significantly lower than that of control fish.

Table 2.4 Physical growth indicators of fathead minnows fed with food containing Ra-226 for 5 months.

	Control n=17	10mBq/g Ra-226 n=44	100mBq/g Ra-226 n=43	1000mBq/g Ra-226 n=44	10000mBq/g Ra-226 n=34
Length (cm)	5.08 ± 0.16	4.35 ± 0.09*	4.60 ± 0.12*	4.72 ± 0.13	4.34 ± 0.11*
Weight (g)	2.89 ± 0.24	1.71 ± 0.12*	2.13 ± 0.19*	2.32 ± 0.21	1.74 ± 0.15*
Condition factor	2.15 ± 0.08	1.94 ± 0.04*	1.98 ± 0.04*	1.97 ± 0.03*	1.96 ± 0.05*

Ps: *p<0.05 compared to the growth endpoints of control fish.

n, number of fish sampled.

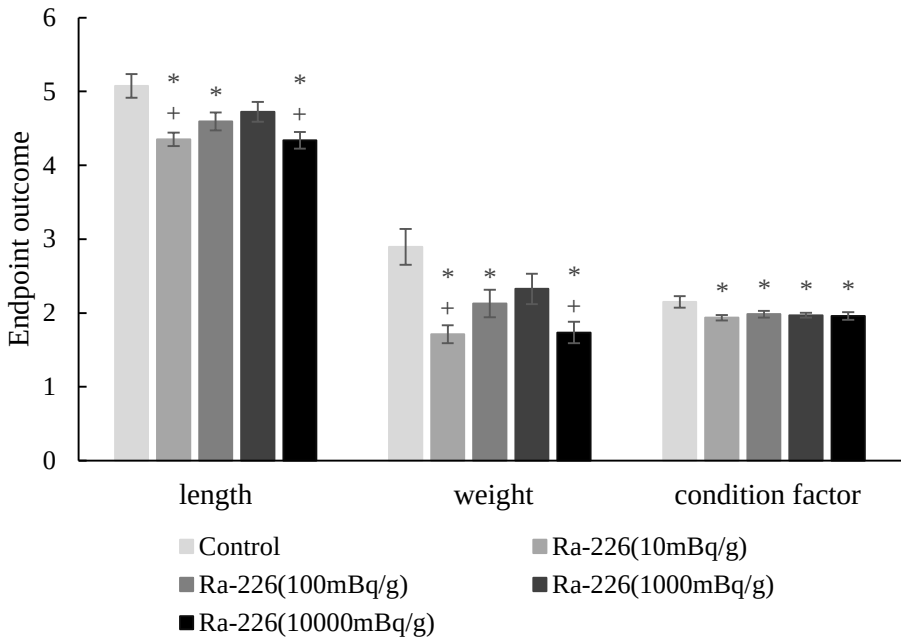

Figure 2.4 Effects of treatment on various growth endpoints of fish. Error bars represent SEM, n>15. Analysis was performed using two-way Analysis of Variance (ANOVA) method, and Post-hoc testing was performed with Fisher's least significant difference (LSD) test. A significant change when compared to the endpoint outcome in Control (0mBq/ml) group is represented by * ($p<0.05$). A significant change when compared to the endpoint outcome in 1000mBq/g group is represented by $^{+}$ ($p=0.05$).

2.5 Discussion

On the autoradiograph from fish injected with 10Bq/g Ra-226 solution, tracks were mainly gathered in the area of skeleton especially the spine and in the outline especially the fins, while tracks in the area of other tissues were sparsely and evenly distributed. This means that Ra-226 was mainly accumulated in the bone and evenly dispersed in the soft tissues of the fish. This finding is in agreement with previous studies about the distribution of Ra-226 in mammalian animals (Priest et al., 1983b; White et al., 1994; Hursh and Lovaas, 1963) and in white sucker fish (Pyle and Clulow, 1998), and confirms that Ra-226 is a bone-seeking radionuclide in fathead minnow. The reason for this is the similar chemical and biological characteristics of Ra-226 and calcium. Ra-226 competes

with calcium to deposit in bone especially when the latter is in short supply. An unexpected outcome was the accumulation of Ra-226 in caudal fins. The track density on the CR-39 detector showed that the activity of Ra-226 in caudal fin was at the same level as it in the bone. This is reasonable because the fins of fathead minnow are composed of rays, which are small bones (Stauffer, 2007). However, a previous study on Ra-226 accumulation in mullet (*Mugilidae*) species from South Adriatic Sea reported that Ra-226 activity concentration in bones was about 14.72Bq/kg, in muscle was about 2.28Bq/kg, while fins showed no radium activity above minimum detectable level (Antovic and Antovic, 2011). The reason might be that in our experiment, the amount of Ra-226 deposited in fish was large because of the increased demand for calcium during the fast growth in temperature-controlled water (20^{o}C), while in their field experiment the Ra-226 level in the fish got from sea was so low that the deposition of Ra-226 in the fins was not obvious.

The activity concentration of Ra-226 accumulated in the fish increased as the dietary Ra-226 activity concentration was increasing. These two were in a positive, non-linear relationship. There seems to be a saturation value for the accumulation of Ra-226 in fish when the concentration of Ra-226 in food was low. The activity of Ra-226 in fish fed with food containing 10mBq/g, 100mBq/g and 1000mBq/g Ra-226 was 24.89 ± 3.59, 29.00 ± 7.80 and 32.56 ± 4.40mBq/g. This means that the fish can accumulate Ra-226 and maintain the level of Ra-226 in their body even when the activity of Ra-226 in the fish food is relatively low.

The concentration factor is a better indicator for the accumulation of Ra-226 by the fish. The highest concentration factor was obtained from fish exposed to the lowest activity of Ra-226 in food. Then as the activity of Ra-226 increased in food, the concentration factor decreased, which indicated the inverse relationship to food activity. This would also suggest a non-linear Ra-226 bioaccumulation in fish, and is in agreement with our previous study which analyzed the activity of Ra-226 in fish exposed to the

same activities of Ra-226 for two years with inorganic mass spectrometry (Mothersill et al., 2014). Non-linear Ra-226 incorporation and greater relative accumulation at lower activities was also reported by Pyle and Clulow (1997) through studying fish samples from uranium mining areas of the city of Elliot Lake in Northern Ontario. The Ra-226 concentration factor obtained from the current work ranged from 0.026 to 2.489, with a mean of 0.71. This is comparable to Ra-226 concentration factors ranging from 0.25 to 1.8 from sediment to fish reported in other studies (Antovic and Antovic, 2011; Pyle and Clulow, 1998; Clulow et al., 1998), but much lower than reported Ra-226 concentration factors from water to fish. As an example, concentration factors for benthic fish varied from 80 to 548 (Clulow et al., 1998; Hosseinia et al., 2008) and for fathead minnow was reported to be 743 (Hesslein and Slavicek, 1984). In our study therefore the CR value is in the range seen from sediment. The fish absorbed Ra-226 from the food directly. This is similar to the situation where fish absorb Ra-226 through ingesting radionuclide-rich sediment along with food (Clulow et al., 1998; Swanson, 1983, 1985). This reflects the greater effectiveness of uptake of Ra-226 in diet item than in water. The concentration factor of Ra-226 found in this experiment (0.026 to 2.489, with a mean of 0.71) is consistent with that found in a previous study by our group using a mass spectrometer to analyze Ra-226 bioaccumulation in fathead minnows fed with Ra-226 (CF of Ra-226 was 0.004-10, with a mean of 1.44) (Mothersill et al., 2014), but the highest CF is a little lower. The reason might be because of the different time point in the growth of fish when the highest CF value obtained. The previous study, which cultured fish in water under ambient conditions (7-15^{o}C), indicated that the highest uptake of Ra-226 occurred in young fish (6 months), and the accumulated Ra-226 was reduced in matured fish body (24 months) (Mothersill et al., 2014). In the current work, some of the fish had matured even though they were maintained in temperature-controlled water (20^{o}C) for only 5 months. We speculate that when the CF of Ra-226 in fish was examined at the end of the current study (5 months), the time point of the highest uptake of Ra-226 by fish was

missed. So the highest CF we obtained here was a little lower than that of the previous work.

Regarding the influence of Ra-226 on the growth and fitness of the fish, the condition factor of fish exposed to Ra-226 via ingestion was significantly lower than that of control fish. The length and the weight were mainly significantly less although there is evidence of a "bell shaped curve" relating both length and weight to activity. Such a relationship could reflect the relatively greater CF seen after the lowest dietary exposure suggesting a non-linearity in the dose effect relationship. Overall from these growth factors for the Ra-226-treated fish compared to the control fish, it can be said that radium seems to reduce fitness in the fathead minnows even though the activity concentration of Ra-226 in the fish food was as low as 10mBq/g. This is consistent with the small transitory deregulation of growth reported in fathead minnows exposed to dietary Ra-226 for 6 and 12 months or in fathead minnows sampled 75 days after injection of Ra-226 (Thompson, 2011; Mothersill et al., 2013).

When the current results are compared with previous studies in this laboratory (Mothersill et al., 2013, 2014) which studied the bioaccumulation of Ra-226 and its influence on fathead minnows cultured in water under ambient conditions (7-15^{o}C), the water temperature was found to have a large effect on the growth of fish and the bioaccumulation of Ra-226. Fathead minnows grow much faster in warm water than in cold water. The average fork length of the 5-month old fathead minnows in the current work was about 4.5cm and the body mass was about 2g, while in the previous study the length and the mass were about 3.5cm and 1g for 12-month old fish, about 5cm and 3g for 24-month old fish (Mothersill et al., 2013). In addition, the fathead minnows in current work began to mature after being cultured for 5 month, while sexual mature fish were only identified at about 18 months in the previous experiments (Mothersill et al., 2013). In order to explain the different rates of growth, "degree-days" were calculated for fathead minnows in both experiments. Degree-days (DD) are a way of incorporating both

temperature and time into one measurement for explaining variation in the growth and development of fish (Chezik et al., 2014). The DD for a single day is calculated as the average of the maximum and minimum temperatures minus the threshold temperature, which is the temperature below which growth is almost zero. If the daily value is added to the total from all the previous days, the total DD will be determined. The threshold temperature for fathead minnows was reported to be about 5.9 ^{o}C (Heath et al., 1994). For fathead minnows in current work, the DD was calculated to be about 2300 ^{o}C days. In previous work, the DD were about 1800 ^{o}C days for 12-month old fathead minnows and 2700 ^{o}C days for 18-month old fathead minnows. So it is reasonable that the physical endpoints and the time to show sexual maturity for fathead minnows in current work (5 months) were comparable to those of fish 12-18 months old in previous work. As to the bioaccumulation of Ra-226, the activity of Ra-226 deposited in fish fed with food containing the highest Ra-226 concentration (10000mBq/g) was 256 ± 49mBq/g in current work, while in previous work the activity of accumulated Ra-226 was about 100mBq/g in 18-month fish in the same group (Mothersill et al., 2014). Because it was reported that the accumulation of Ra-226 reached the peak value for 6-month fish and reduced as time went on (Mothersill et al., 2014), so the level of the deposited Ra-226 in fish in current work was comparable to that of about 18 months old fish in the previous work. If we relate the number of DD with the accumulated amount of Ra-226 in fish, it may mean that the number of DD has a direct relationship with the bioaccumulation of radionuclides.

During the calculation of the concentration of deposited Ra-226, some assumptions were made to simplify the calculations and because of the time consuming nature of autoradiography. These include radon retention and the contribution of the radon progeny. In addition, during the experiment, Ra-226 was assumed to be evenly distributed in the fish body, while actually it was not. Because of this, the dosimetric analysis in this work will provide an estimation of accumulation of Ra-226 in the fish, rather than give a very

accurate result. In the future, the tracks will need to be analyzed in different areas (such as bone areas and soft tissue areas) of the CR-39 detector according to the histological images to improve the accuracy of the result.

2.6 Conclusions

Overall, the results obtained in this work show that the accumulation of Ra-226 in fish is more complicated than previously expected. There is a non-linear relationship between the accumulation of Ra-226 and the dietary Ra-226 concentration and there's a saturation value when the dietary activity is low. In addition, degree-days (DD) is a good measurement for explaining the variety of growth of fish and the bioaccumulation of Ra-226. Fish with the same number of DD showed similar growth condition and similar levels of bioaccumulation of Ra-226. The results also indicate that the Ra-226 in the food can influence the growth and fitness of the fish even when the activity level was as low as 10mBq/g, which may mean that elevated levels of Ra-226 in some areas shouldn't be neglected when considering radiation protection of non-human biota.

2.7 Declaration of interest and acknowledgements

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Chapter 3

THE EFFECTS OF CHRONIC, LOW DOSES OF RA-226 ON CULTURED FISH AND HUMAN CELLS

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The research project was originally designed by Dr. Mothersill, Dr. Seymour and the first author. The first author completed the cell preparation, Ra-226 irradiation, clonogenic assay, data collection and analysis. The first author wrote the manuscript, which was edited by Dr. Mothersill before submission to the journal.

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3.1 Abstract

Purpose: To determine the chronic low-dose radiation effects caused by 4.78MeV α-particle radiation from Ra-226 over multiple cell generations in CHSE/F fish cells and HaCaT human cells.

Methods: CHSE/F cells and HaCaT cells were cultured in medium containing Ra-226 to deliver the chronic low-dose α-particle radiation. Clonogenic assay was used to test the clonogenic survival fractions of cells with or without being exposed to radiation from Ra-226.

Results: The chronic low-dose radiation from Ra-226 does have effects on the clonogenic survival of CHSE/F cells and HaCaT cells. When CHSE/F cells were cultured in Ra-226-medium over 9 passages for about 134 days, the clonogenic surviving fractions for cells irradiated at dose rates ranging from 0.00066 to 0.66mGy/d were significantly lower than that of cells sham irradiated. For HaCaT cells grown in medium containing the same range of Ra-226 activity, the clonogenic surviving fraction decreased at first and reached the lowest value at about 42 days (8 passages). After that, the clonogenic survival began to increase, and was significantly higher than that of control cells by the end of the experimental period.

Conclusion: The chronic, low-dose high LET radiation from Ra-226 can influence the clonogenic survival of irradiated cells. CHSE/F cells were sensitized by the radiation, and HaCaT cells were initially sensitized but later appeared to be adapted. The results could have implications for determining risk from chronic versus acute exposures to radium.

Key words: chronic, low-dose radiation, α particles, Ra-226, surviving fraction.

3.2 Introduction

Radium-226 is a naturally occurring radionuclide in the decay chain of Uranium-238. It can cause severe damage to organisms if it is absorbed into the body because α-particles with an energy of 4.78MeV are mainly emitted from the decay of Ra-226. α-particles with an energy of 2-10MeV have a high linear energy transfer (LET), and is more effective than low LET radiation at causing biological damage and the relative biological effect (RBE) of it ranges from 1.6-21 depending on the endpoint used (Thomas et al., 2007; Howell et al., 1994; Franken et al., 2011). The high RBE is thought to be due to the fact that α-particle radiation can cause more clustered DNA double-strand breaks than sparely ionizing radiation and these breaks are more difficult to repair correctly (Blocher, 1988). In addition, because the chemical and physical properties of radium are similar to those of calcium, radium will compete with calcium after being absorbed and deposit in specific organs to form hot spots of high LET radioactive material (Priest et al., 1983).

Levels of Ra-226 in some areas are higher than the natural level because of human activities, such as uranium mining and milling, exploration and production of oil and gas and the radium production industry. Radium levels in the plants or animals in these areas are higher than normal (Giri et al. 2010; Pyle and Clulow, 1998; Brenner et al, 2007), and some adverse effects can be observed. For *Vicia cracca* populations growing in sites which were contaminated by uranium mill tailings and radium production wastes (Ra-226 constituted 84.3%-99% of the total internal irradiation), even when the mean absorbed dose was 0.2-0.3Gy over about 120 days, the embryonic lethal mutation frequency was significantly higher and the germination capacity of seeds was significantly lower than those of control (Evseeva et al, 2009), so as to pine trees in the same sites (Geras'kin et al., 2011, 2013). The biomass of earthworms living in soil contaminated with α-emitting radionuclides (Radium-226 concentrations: 1506Bq/kg dry weight) was significantly decreased, and their reproduction was inhibited (Lourenco et al., 2012). Studies in our group showed that Fathead minnow fed with the environmental level of Ra-226 exhibited

transient growth perturbations, including the reductions in mean body mass and the disproportionate increase in body mass relative to fork length (Mothersill et al., 2013). The mechanisms underlying biological effects of low level Ra-226 radiation are complicated and unclear. A significant increase of DNA damage in the exposed earthworms (Lourenco et al., 2012) and changed ratios of DNA: RNA, DNA: protein and RNA: protein were found in fish (Mothersill et al., 2013) suggesting growth perturbations. Transcription of some genes was significantly up-regulated in Atlantic cod embryo cells exposed to environmentally relevant levels of Ra-226 and the induced oxidative stress and apoptosis may be trigger factors (Olsvik et al, 2012).

The enhanced level of Ra-226 in organisms in the environment leads to increased exposure to chronic, low-dose rate irradiation, which is important in Environmental Radiation Protection. At present because of the phenomena of bystander effects, adaptive responses and low-dose hypersensitivity, evaluating the risks of chronic low-dose radiation shouldn't be done by extrapolating from data concerning acute often high dose radiation exposure (Seymour and Mothersill, 2000; Joiner et al., 1996; Mothersill and Seymour, 2004; Little, 2006; Morgan and Sowa, 2007; Frankenberg et al., 2006; Miura et al., 2002; Matsumoto et al., 2004; Varès et al., 2011). But studies about the effects induced by low-dose radiation are still rare, not to mention studies about the chronic, low-dose high LET radiation. The current study used fish cells (CHSE/F cell line) and human cells (HaCaT cell line) exposed to low-dose α-particle radiation from Ra-226 in medium for multiple cell-generations could provide a view to understanding the effect of this kind of radiation in a simple system where other confounding stressors were not present.

3.3 Materials and methods

3.3.1 Cell lines

CHSE/F fish cell line and HaCaT human cell line were used in the current study.

CHSE/F (formerly known as CHSE-214) is a cell line derived from embryos of Common bluegill. This cell line has a typical epithelial-like morphology. During these experiments, they had an average plating efficiency of 41.4 ± 2.2% and a doubling time of about 127 hours. This cell line was obtained as a gift from Dr Niels Bols (University of Waterloo, Canada), and was frozen by previous students and stored in liquid nitrogen. The HaCaT cell line is an immortalized, nontransformed human keratinocyte cell line and was originally derived and characterized by Boukamp et al. from human skin keratinocytes (Boukamp et al., 1988). The cell line in our lab was obtained as a gift from Dr. Orla Howe (Dublin, Ireland), and was stored in liquid nitrogen. The average plating efficiency of the cells during these experiments was 51.4 ± 0.8% and the doubling time was about 23.6 hours. Cell cultures of these two cell lines were tested (Plasmo Test rep-pt1, InvivoGen, San Diego, CA) and confirmed to be mycoplasma free prior to use.

3.3.2 Cell culture

CHSE/F cells were cultured in Leibovitz's L-15 medium, supplemented with 12% fetal bovine serum (Invitrogen, Burlington, ON, Canada), 5ml of 100U/mL penicillin and 100μg/ml streptomycin sulphate (Gibco, Burlington, ON), 5ml of 2mM L-Glutamine (Gibco, Burlington, ON) and 25mM Hepes buffer (Gibco, Burlington, ON). Cells stocks were maintained in T75 flasks with 30ml of medium and subculture was routinely performed when cells were 80-100% confluent using a 1:1 solution of 0.125% trypsin and 1mM EDTA at 19 ^{o}C for 8 mins. Cells were grown at 19 ^{o}C in an incubator without CO_2.

For HaCaT cells, RPM1640 medium, supplemented with 10% fetal bovine serum (Invitrogen, Burlington, ON, Canada), 5ml of 100U/mL penicillin and 100μg/ml streptomycin sulphate (Gibco, Burlington, ON), 5ml of 2mM L-Glutamine (Gibco, Burlington, ON), 0.5μg/ml hydrocortisone (Sigma-Aldrich, Oakville, ON), and 25mM Hepes buffer (Gibco, Burlington, ON) was used for all experiments. Cells stocks were

maintained within T75 flasks with 30ml of medium. The subculture of HaCaT cells was routinely performed with a 1:1 solution of 0.25% trypsin and 1mM EDTA at 37ºC for 8 mins. These cells were cultured in an incubator with 5% CO_2 at 37ºC. All experiments were performed in biosafety level 2 laminar flow cabinets.

3.3.3 Ra-226 medium

The radioisotope used in this study is Radium-226, supplied as neutralized radium nitrate by Eckert and Ziegler (Valencia, CA, USA). The carrier for the Ra-226 was 10μg/ml Ba (as Ba nitrate). To prepare stock solutions, 100ml L-15 or RPMI medium was mixed with 1000Bq of Ra-226 solution, so the concentration of Ra-226 in this medium was 10000mBq/ml. The stock Ra-226-medium was filtered into storage tubes. Then serial 1/10 dilutions were made to give final concentrations of medium with 10, 100, 1000 and 10000mBq/ml Ra-226. The amount of Ra-226 in the medium is not sufficient to affect the role of other ingredients (including Calcium) in the medium. Respectively, the estimated concentration of Ba in the medium was about 0.00071μg/l, 0.0071μg/l, 0.071μg/l and 0.71μg/l. According to WHO guidelines for drinking water quality, the guideline value of Barium concentration is 700μg/l and the common concentrations in drinking-water are generally below 100μg/l (WHO guidelines for drinking water quality, 4th edition). Therefore, the barium in the medium can be neglected.

3.3.4 Irradiation

For each cell line, there were five groups of cells: four groups of cells were cultured continuously in medium containing the different radium concentrations and one group of cells was cultured in control medium without radium. All groups of cells were cultured in T25flasks containing 5ml medium in which the concentration of Ra-226 was 0, 10, 100, 1000 or 10000mBq/ml. The amount of radiation energy deposited per unit mass per unit time is known as dose rate. To get the mean absorbed dose rate induced by α-particles

emitted from Ra-226 in the 5ml medium, the radionuclide is assumed to be evenly distributed in the mixture of the medium and the cells, and the density of this mixture is assumed to be equal to water. The mean absorbed dose rate can then be calculated according to the following equation:

$$D_\alpha = C_{Ra\text{-}226} \times V \times E_\alpha / m \ (mBq \cdot MeV/g) \qquad \text{Equation 3.1}$$

$$= 1.38 \times 10^{-5} \times C_{Ra\text{-}226} \times E_\alpha \ (mGy/d)$$

Where D_α is the mean absorbed dose rate of radiation from Ra-226 in medium to the whole mixture. E_α is the average energy of α particles emitting from Ra-226, 4.78MeV. $C_{Ra\text{-}226}$ is the concentration of Ra-226 in the mixture (mBq/ml) and 1.38×10^{-5} is a conversion factor.

After calculation, the mean absorbed dose rate for each group was 0mGy/d, 0.00066mGy/d, 0.0066mGy/d, 0.066mGy/d or 0.66mGy/d respectively. All five groups of cells were treated similarly. At the time of each subculture, a clonogenic assay was done to test the clonogenic survival of cells in each group, and the total number of cells in each flasks were determined to calculate the doubling time of cells during this period.

3.3.5 Clonogenic assay technique

Clonogenic assay technique described by Puck and Marcus (Puck and Marcus, 1956) was used for clonogenic survival analysis. Briefly, cells were detached from the flasks and were resuspended in medium. Then an aliquot of the cell suspension was counted using a Z2 Coulter particle count and size analyzer (Bechman Coulter Electronics, Mississauga Ontario, Canada) to determine the number of viable cells. After that, appropriate number of cells were plated into each flask, and cell cultures were incubated for 4 weeks at 19 °C for CHSE/F cell line or 9 days at 37 °C for HaCaT cell line. Flasks were checked periodically for growth. When colonies in the control flasks were visible to the naked eye, the cells were stained with 20% carbol fuchsin in water (VWR, Bridgeport, NJ, USA) and colonies with 50 cells or more were counted.

3.3.6 Cell population doubling time

The approximate time it takes for CHSE/F cells and HaCaT cells to double in number during every passage was determined through counting the number of cells at the beginning and at the end of each passage. Any changes in the doubling time may indicate possible alternations in the viability of cells. At the time of each subculture, T25 flasks with 5ml of medium were set up for cells in each group, and appropriate number of cells (N_0) was plated into each flask. After a period of time (T hours), when cells were 80-100% confluent, cells were detached from the flasks and were resuspended in medium. Then an aliquot of the cell suspension was counted using a Z2 Coulter particle count and size analyzer (Bechman Coulter Electronics, Mississauga Ontario, Canada) to determine the number of viable cells. Because the volume of the suspension was known, the total number of cells in the flask was determined (N). The doubling time of cells in this passage can be calculated according to the following equation:

$$\text{doubling time} = T \times [\ln(2)/\ln(N/N_0)] \qquad \text{Equation 3.2}$$

3.3.7 Statistical analysis

All data are expressed as means ± SEM (the standard error of the mean).The effect of chronic, low-dose radiation from Ra-226 on the clonogenic survival of CHSE/F cells or HaCaT cells was analyzed by two-way Analysis of Variance (ANOVA) method, and Post-hoc testing was performed using Fisher's least significant difference (LSD) test (SPSS22). A confidence interval of 95% and $p<0.05$ was selected to be statistically significant.

3.4 Results

3.4.1 Radium effects on CHSE/F cell line

3.4.1.1 Radium effects on clonogenic survival of CHSE/F cells

The overall plating efficiency of the CHSE/F cells used in this study was 41.4 ±

2.2%. The plating efficiency of CHSE/F cells in control groups was respectively 33.2 ± 1.8%, 36.9 ± 1.0%, 41.8 ± 3.3%, 44.6 ± 3.3%, 40.4 ± 1.0% and 56.2 ± 1.6% after being cultured in medium for 12, 36, 50, 64, 99 and 134 days. The total mean absorbed dose of radiation that CHSE/F cells exposed over multiple generations in the experiments was shown in Table 3.1. Clonogenic surviving fractions of cells with or without being irradiated by Ra-226 in medium are shown in Figure 3.1 and in Figure 3.2, the percentage of change in clonogenic survival between cells in each Ra-226-medium group and control group is demonstrated. At early time points, Ra-226 didn't induce significant cell killing in CHSE/F cells in each Ra-226-medium group compared with in the control, even though there was the trend towards lower clonogenic surviving fractions for cells in Ra-226-medium group. By 50 days, CHSE/F cells cultured in 0.66mGy/d Ra-226-medium began to show significant cell killing, and the clonogenic surviving fraction was 81.63 ± 4.09%, $p=0.003$. But the sensitizing effect disappeared in the following passages. When cells were cultured in Ra-226-medium for 134 days, significantly decreased clonogenic survival began to appear in most cells. At that time, the clonogenic survival fraction for cells exposed to α-particle radiation at the mean absorbed dose rate of 0.00066mGy/d was about 77.15 ± 2.52%, $p<0.001$, which was 22.85% lower than that of control cells. The clonogenic surviving fractions of cells exposed to 0.066mGy/d and 0.66mGy/d α-particle radiation were 79.43 ± 2.26%, $p=0.001$ and 84.56 ± 3.26%, $p=0.012$.

Table 3.1 The total mean absorbed dose (mGy) of radiation that CHSE/F cells received from Ra-226 over multiple generations

Days / Dose rate	12d	36d	50d	64d	99d	134d
Control (0mGy/d)	0	0	0	0	0	0
0.00066mGy/d	0.00792	0.02376	0.033	0.04224	0.06534	0.08844
0.0066mGy/d	0.0792	0.2376	0.33	0.4224	0.6534	0.8844
0.066mGy/d	0.792	2.376	3.3	4.224	6.534	8.844
0.66mGy/d	7.92	23.76	33	42.24	65.34	88.44

3.4.1.2 Radium effects on CHSE/F cell population doubling time

The average doubling time of CHSE/F cells in control group during the whole experiment was about 127 hours. And for the six selected periods in the experiment, the tested doubling time was respectively 123h, 140h, 149h, 135h, 145h and 110h. The doubling time of CHSE/F cells during every passage was normalized to that of cells during respective passage in the control group to offset the experimental difference, and the result was demonstrated in Figure 3.3. The doubling time of CHSE/F cells grown in medium containing Ra-226 was almost the same as that of cells grown in normal medium except for the period of the 5^{th} passage when cells were cultured in medium from 50 days to 64 days. During this period, the doubling time of cells in the four Ra-226-treated groups was respectively 1.28 ($p=0.008$), 1.39 ($p=0.003$), 1.54 ($p<0.001$) and 1.53 ($p<0.001$) times as long as that of cells in control group. Cell proliferation took longer time for cells cultured in Ra-226-medium during that cell passage.

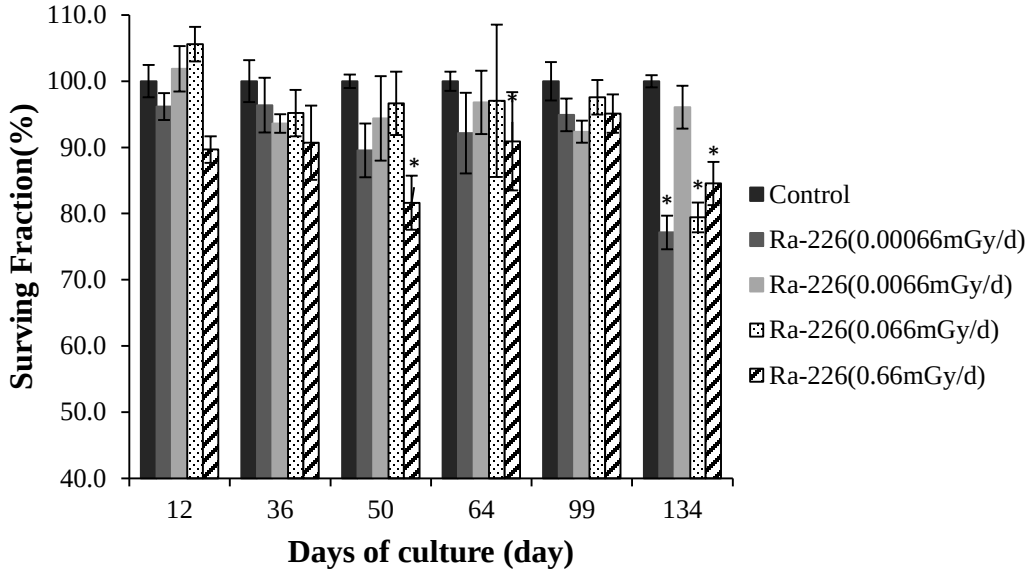

Figure 3.1 The clonogenic surviving fraction of CHSE/F cells cultured in medium with or without Ra-226. Error bars represent SEM, n=6. Analysis was performed using two-way Analysis of Variance (ANOVA) method, and Post-hoc testing was performed with Fisher's least significant difference (LSD) test. A significant change when compared to the respective sample in Control (0mBq/ml) group is represented by * ($p<0.05$).

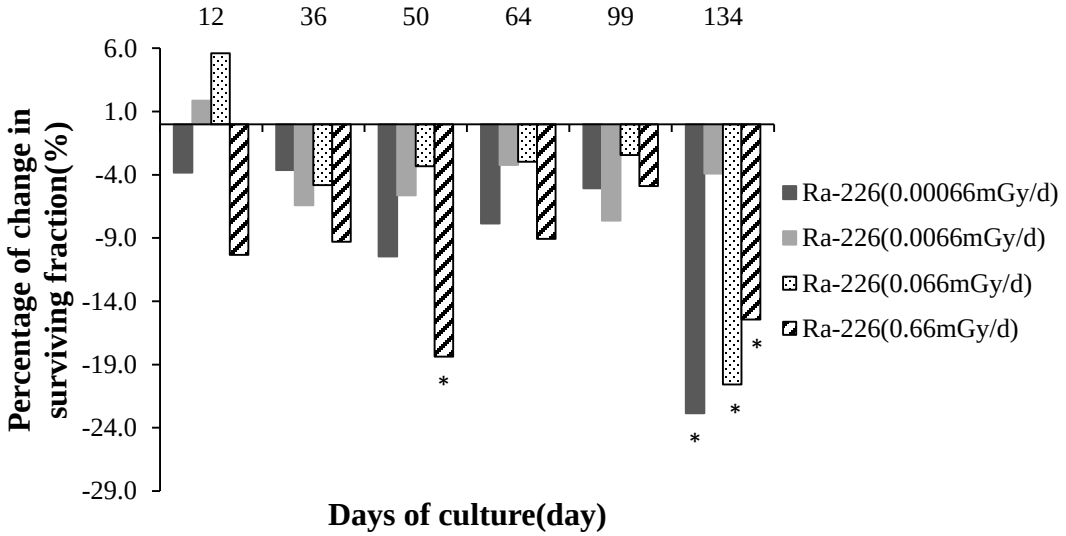

Figure 3.2 Percentage of change in clonogenic surviving fractions of CHSE/F cells

cultured in medium with or without Ra-226, n=6. Analysis was performed using two-way Analysis of Variance (ANOVA) method, and Post-hoc testing was performed with Fisher's least significant difference (LSD) test. A significant change when compared to the respective sample in Control (0mBq/ml) group is represented by * ($p<0.05$).

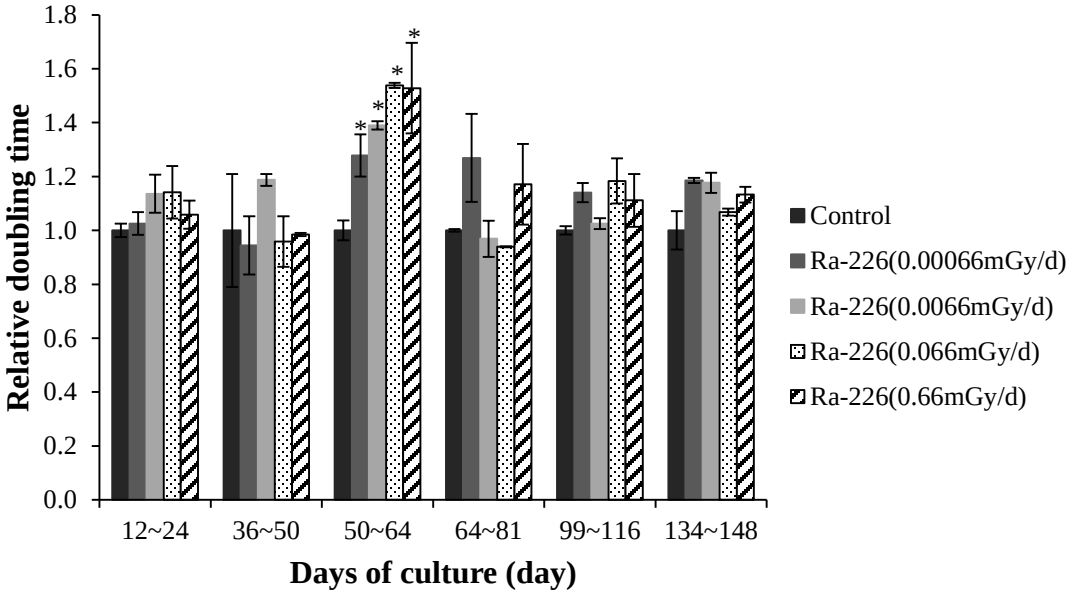

Figure 3.3 The doubling time of CHSE/F cells cultured in medium with or without Ra-226. Error bars represent SEM, n=6. Analysis was performed using two-way Analysis of Variance (ANOVA) method, and Post-hoc testing was performed with Fisher's least significant difference (LSD) test. A significant change compared to Control (0mBq/ml) group is represented by * ($p<0.05$).

3.4.2 Radium effects on HaCaT cell line

3.4.2.1 Radium effects on clonogenic survival of HaCaT cells

The overall plating efficiency of HaCaT cells used in this study was 51.4 ± 0.8%, and at the five selected time points, the plating efficiency of cells in control group was 51.2 ± 1.4%, 51.7 ± 2.6%, 57.1 ± 2.7%, 50.8 ± 1.4% and 48.7 ± 1.4%, which was pretty stable

during the experiment period. The total mean absorbed dose of radiation exposed to HaCaT cells over multiple generations in the experiment was shown in Table 3.2. Figure 3.4 shows the clonogenic surviving fraction of cells with or without exposure to Ra-226 in the experiment. The percentage change in clonogenic surviving fractions for cells in each Ra-226-medium group and the control group is shown in Figure 3.5. The clonogenic survival of HaCaT cells cultured in Ra-226-medium decreased at first and reached the lowest value after being cultured over 8 passages for 42 days. In the next passage the clonogenic survivals began to rise up and kept increasing to the end of the experiment. Decreased clonogenic survival began to happen when HaCaT were cultured in Ra-226-medium for 20 days, except for those grew in medium with the lowest concentration of Ra-226. After being cultured for 42 days, the clonogenic surviving fraction of all cells in Ra-226-medium group reached the lowest value: $87.82 \pm 3.68\%$, $p=0.006$, $87.97 \pm 2.34\%$, $p=0.006$, $85.31 \pm 2.03\%$, $p=0.001$ and $72.95 \pm 2.06\%$, $p<0.001$. Cells irradiated by 0.66mGy/d radiation had the lowest clonogenic survival fraction, which was about 27.05% lower. After that, cells began to show the sign of getting adapted to the low-dose radiation, and their clonogenic survival began to increase. The value was $111.05 \pm 2.80\%$ ($p=0.012$) for cells exposed to 0.00066mGy/d radiation for 54 days and $115.80 \pm 3.572\%$ ($p=0.001$) for cells irradiated by 0.0066mGy/d radiation for 70 days. The increased clonogenic survival also happened to cells irradiated at other dose rates, but didn't reach significance.

Table 3.2 The total mean absorbed dose (mGy) of radiation that HaCaT cells received from Ra-226 over multiple generations

Dose rate \ Days	5d	20d	42d	54d	70d
Control (0mGy/d)	0	0	0	0	0
0.00066mGy/d	0.0033	0.0132	0.02772	0.03564	0.0462
0.0066mGy/d	0.033	0.132	0.2772	0.3564	0.462
0.066mGy/d	0.33	1.32	2.772	3.564	4.62
0.66mGy/d	3.3	13.2	27.2	35.64	46.2

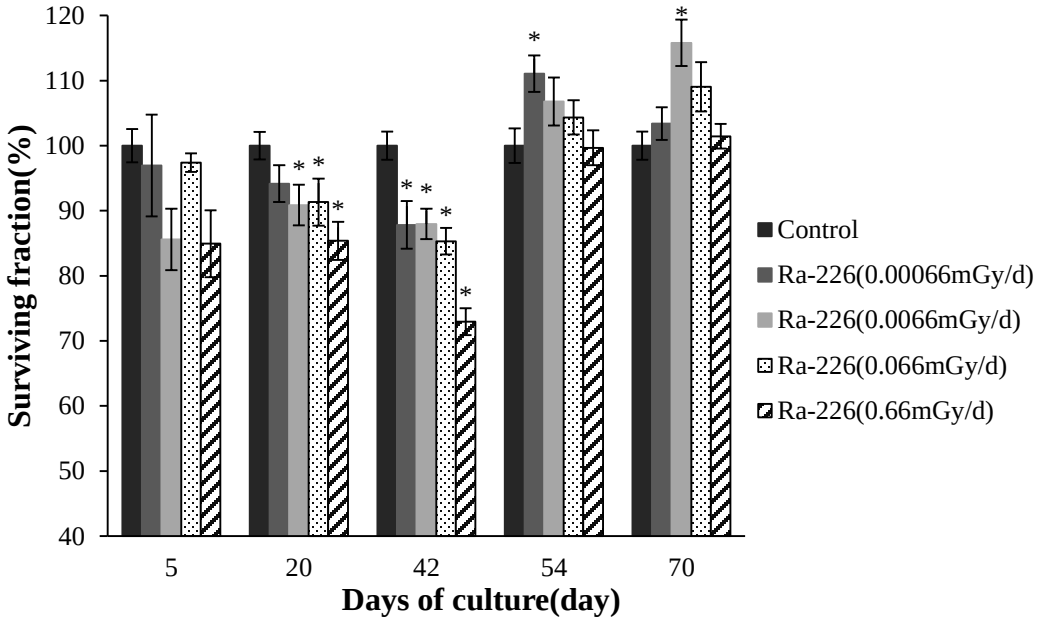

Figure 3.4 The clonogenic survival fractions of HaCaT cells cultured in medium with or without Ra-226. Error bars represent SEM, n=9. Analysis was performed using two-way Analysis of Variance (ANOVA) method, and Post-hoc testing was performed with Fisher's least significant difference (LSD) test. A significant change when compared to the respective sample in Control (0mBq/ml) group is represented by * ($p<0.05$).

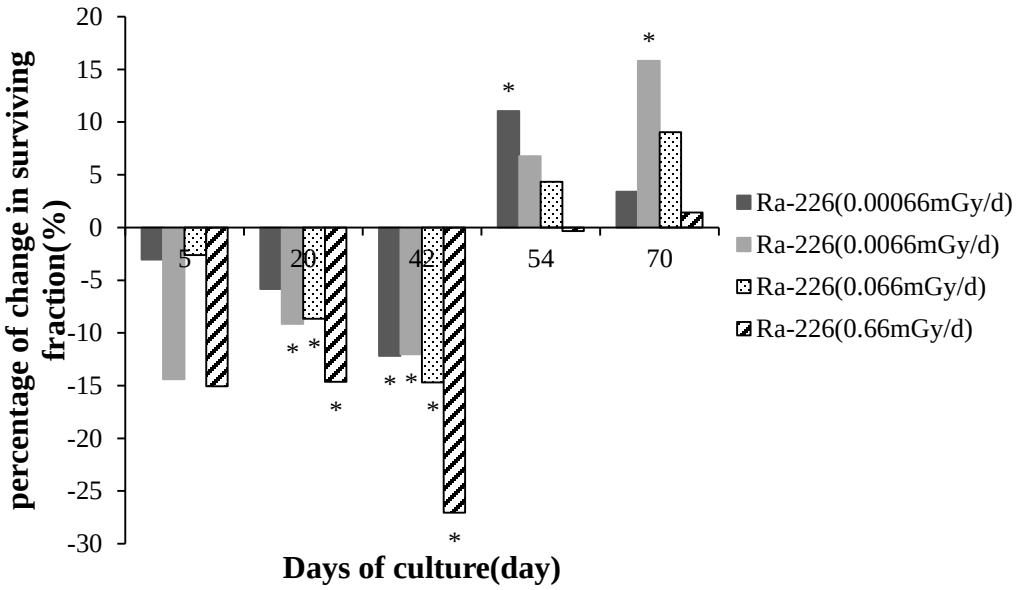

Figure 3.5 The percentage of change in clonogenic surviving fractions of HaCaT cells cultured in medium with or without Ra-226, n=9. Analysis was performed using two-way Analysis of Variance (ANOVA) method, and Post-hoc testing was performed with Fisher's least significant difference (LSD) test. A significant change when compared to the respective sample in Control (0mBq/ml) group is represented by * ($p<0.05$).

3.4.2.2 Radium effects on HaCaT cell population doubling time

The average doubling time of HaCaT cells in control group over the whole experiment was about 23.6 hours, and for the five selected time periods, the doubling time was 19.8h, 21.1h, 25.3h, 26.1h and 24.2h. In order to offset the experimental difference between each cell passage, the doubling time of HaCaT cells during every passage was normalized to that of cells in the control group of that passage, and the result was demonstrated in Figure 3.6. During each passage, the doubling time of HaCaT cells grown in Ra-226-medium was not different from that of cells grown in normal medium. It seems that the chronic radiation from Ra-226 didn't affect the growth rate of HaCaT cells.

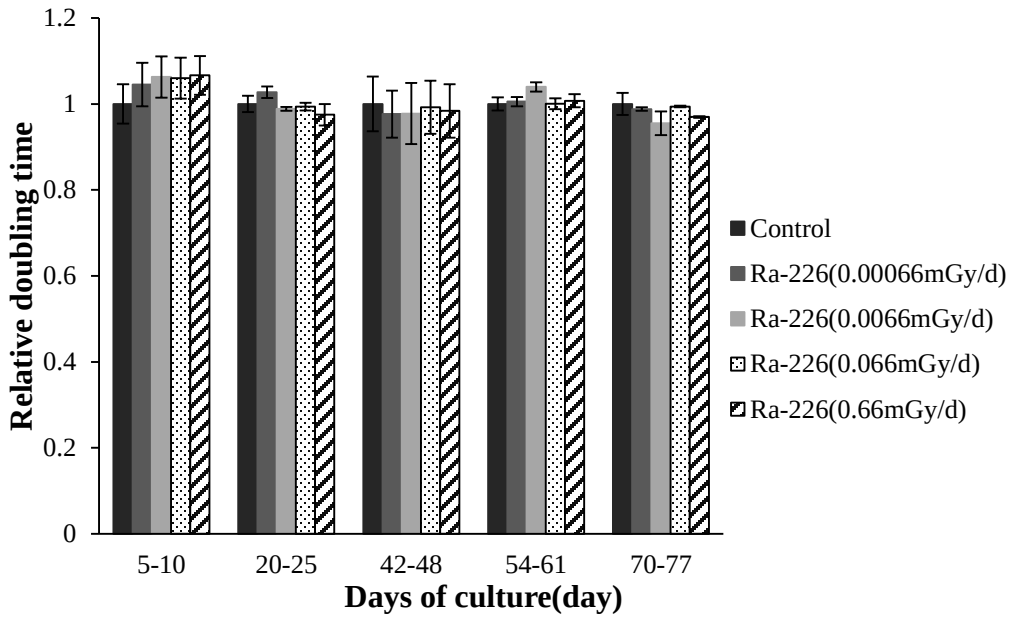

Figure 3.6 The doubling time of HaCaT cells cultured in medium with or without Ra-226. Error bars represent SEM, n=9. Analysis was performed using two-way Analysis of Variance (ANOVA) method, and Post-hoc testing was performed with Fisher's least significant difference (LSD) test.

3.5 Discussion

Generally speaking, the chronic, low-dose radiation from environmental level of Ra-226 may not have obvious influence on the organisms in the natural environment. In our experiment, after being cultured in Ra-226-medium over multiple generations for a long time period, the clonogenic survivals of both CHSE/F cells and HaCaT cells were affected at some time points, but the doubling time of both cell lines was not influenced. So it can be assumed that even though the chronic, low-dose radiation can affect the colonies-forming ability of a single cell, but the impact can be reduced when a large number of cells were present.

For CHSE/F cells, decreased clonogenic survival happened earliest when cells were cultured in 0.66mGy/d Ra-226-medium for 50 days (accumulated mean absorbed dose was 33mGy). When they were cultured in Ra-226-medium for about 134 days, decreased

clonogenic surviving fraction happened to most cells (Figure 3.2). Among the sensitized cells, the lowest mean absorbed dose of radiation they received was 0.0884mGy (concentration of Ra-226 in medium was 10mBq/ml), and the clonogenic survival was 22.85% lower than that of the control. Because the plating efficiency in this time point was higher than other time points, the decreased clonogenic survival of CHSE/F cells in Ra-226-medium group might be partly because of the increased plating efficiency. Compared to CHSE/F cells, the sensitizing reaction of HaCaT cells to the radiation from Ra-226 happened earlier. Cells cultured in Ra-226-medium for about 20 days (4 passages) began to show significantly decreased clonogenic survival except for cells in the lowest Ra-226 concentration group. After being cultured in Ra-226-medium for 42 days, all cells had significantly decreased clonogenic survivals than control cells, and the accumulated mean absorbed dose of radiation received by them was respectively 0.028mGy, 0.28mGy, 2.8mGy and 28mGy. In addition, the sensitizing response of HaCaT cells seemed to be positively relevant to the accumulated dose of radiation in each group. In another word, cells grown in Ra-226-medium for 42 days had lower clonogenic survival than cells cultured in medium containing the same concentration of Ra-226 for 20 days (in Figure 3.5).

The result that low-dose α-radiation have influence on cells' survival is consistent with the gene transcription experiments for Atlantic cod embryo cells, which found that some gene transcriptional levels were changed even when the Ra-226 concentration was as low as 2.11mBq/ml (Olsvik et al, 2012). Chromosome aberration were also reported to be increased in human blood lymphocytes exposed to 0.01, 0.02 and 0.03mGy α-radiation from Radon (Hamza and Mohankumar, 2009), as well as the frequency of sister chromatid exchanges in Chinese hamster ovary (CHO) cells exposed to 0.31mGy α-radiation from ^{238}Pu (Nagasawa and Little, 1992) and in human diploid lung fibroblasts (HFL1) cells exposed to 4-129mGy α-radiation (Deshpande et al., 1996). HPRT point mutations could also be increased by 5mGy α-radiation (Huo et al., 2001). It was reported

that the transmissible genetic instability induced by α-radiation would lead to the occurrence of chromosomal aberrations among progeny cells after many generations of replication (Kadhim et al., 1992). Because the cells we used underwent multiple cell passages in the radioactive Ra-226-medium, we speculate that the low-dose α-radiation induced the genetic instability and aberrations in cells at first, and as time went on, these changes may be developed and expressed over time as cell killing.

But our result that decreased clonogenic survival could be induced by such low mean absorbed doses of α-radiation in cells is different from another study, which found that no evidence of cell killing was observed in CHO cells exposed to α-radiation at doses up to 4.9mGy (Nagasawa and Little, 1992). The possible reason might be the more severe bystander effect induced in our cells. In that study, they irradiated CHO cells from below through the Mylar base dishes by 3.7MeV α-particles from ^{238}Pu for only 0.125 to 2 seconds, and they found that if CHO cells were irradiated for 0.125 second for 0.31mGy, 30% of the cells showed an increased frequency of SCE, even though less than 1% of cell nuclei were actually traversed by an α-particle. This means that communication among cells occurred and DNA damage was not the direct inducement (Nagasawa and Little, 1992). In our work, the cells were directly grown in the radioactive medium and were continuously exposed to α-radiation for multiple passages over dozens of days. We speculate that the bystander effect in our cells might be accumulated over the long time period, and signals from damaged cells and previously-influenced cells can affect more cells as a chain reaction (Mothersill and Seymour, 1998; Seymour and Mothersill, 2000; Lyng et al., 2000).

The bystander effect induced by α-radiation may also be the reason why decreased clonogenic survival happened earlier to HaCaT cells than to CHSE/F cells. HaCaT cells are sensitive to bystander signals, and signals from damaged cells can reduce clonogenic survival in unirradiated cells (Ryan et al., 2009). The adverse influence of α-radiation on cells' clonogenic survival and the cell killing induced by bystander signal might be

additive, so extreme low-dose radiation could induce obvious cell killing in HaCaT cells in current work. On the contrary, CHSE/F cells showed increased cloning efficiency in unirradiated cells treated by irradiated cell conditioned medium (O'Neill-Mehlenbacher et al., 2007). The cell killing caused by α-particle radiation and the increased clonogenic survival induced by bystander effect might offset each other. So for CHSE/F cells, higher doses of radiation were needed to induce significantly decreased clonogenic survival.

The sensitizing response of CHSE/F cells and HaCaT cells to Ra-226 might be because of the apoptosis induced by the low-dose α-radiation. Some apoptotic cells were found in stained CHSE/F cells and HaCaT cells cultured in Ra-226-medium. It was reported that 0.29mGy α-radiation was sufficient to produce an observable increase of apoptosis in cocultured nonirradiated transformed cells (65 hours of coculture) through intercellular induction of apoptosis involving ROS/NOS and TGF-β, and the apoptosis saturated at very low-dose radiation (25mGy for α-particles). In addition, the level of apoptosis continued to increase as cells remained in coculture, and eventually the vast majority of transformed cells were removed by the induced intercellular apoptosis (Portess et al., 2007). Gene expression related to apoptosis, such as bax, bcl-2 and bcl-xl, was also reported to be changed by low-dose α-radiation from ^{222}Rn (0.6 to 8.3mGy) in human breast cancer cells (MCF-7) (Soto et al., 2006). In addition, genes related to cell cycle arrest, DNA replication and repair were differentially expressed in human lung epithelial cells exposed to 0.3 and 0.9Gy of α-particle radiation (Chauhan et al., 2012). So the decreased clonogenic survival in cells in current work could be explained by the induction of apoptosis. The low-dose radiation received by CHSE/F cells and HaCaT cells from Ra-226 in medium might change the microenvironment of the cells and induce higher oxidative stress. Then cell apoptosis was induced through intercellular signal pathways. The cellular defense through killing cells with damaged unrepaired DNA and cells with tumorigenesis possibility could prevent passing mutations onto offspring and decrease the risk of tumorigenesis (Real et al., 2004; Mothersill and Seymour, 2004;

Portess et al., 2007). From this point, the sensitizing effect of Ra-226 on CHSE/F cells and HaCaT cells might be a protective mechanism. In the following work, the apoptosis of cells and genes related with apoptosis and DNA repair will be tested to see whether or not apoptosis is the main reason for the sensitizing effect.

An unexpected result was that after being cultured in Ra-226-medium for 42 days, HaCaT cells showed the sign of getting adapted to the low-dose radiation. The clonogenic surviving fractions of cells in all Ra-226-medium groups began to increase, among which, cells irradiated with 0.00066mGy/d α-radiation for 54 days and cells irradiated with 0.0066mGy/d α-radiation for 70 days had significantly increased clonogenic survival (11.05% higher and 15.80% higher), (shown in Figure 3.5). We suggest that this is a kind of protective adaptive effect, the hormetic response, in which, low-dose radiation can lead to increasing clonogenic survival and cloning efficiency (Wang and Cai, 2000; Redpath et al., 2001; Scott, 2004). There appears to be two types of adaptive protection. One is to remove damaged cells by inducing apoptosis mentioned above (Portess et al., 2007) and terminate differentiation to reduce genomic instability and tumorigenesis. The other one is to prevent and repair DNA damage to keep cells alive and functioning properly (Feinendegen, 2005). The induction of apoptosis usually happens hours to days after acute low-dose low LET radiation, and DNA repair and cell proliferation usually happens days to weeks after the radiation (Feinendegen et al., 2002). The duration of the effectiveness of each mechanism and the time interval between energy deposition events of radiation in the target determine which mechanism of adaptive protection prevails and to what degree damage or protection reach (Feinendegen, 2005). So we speculate that in our experiment, the induction of apoptosis prevailed at first, so the damaged cells and the signal-influenced cells were eliminated, which led to the decreased cell clonogenic survival. As time went on, DNA repair and cell proliferation prevailed, and cell clonogenic survival showed the trend of increase.

In current work, the doubling times of CHSE/F cells and HaCaT cells were not

obviously influenced by the low-dose α-radiation from Ra-226. Only in one time period, when CHSE/F cells were cultured in Ra-226-medium from 50 days to 64 days, their doubling time was significantly longer than that of control cells. Because CHSE/F cells cultured in 0.66mGy/d Ra-226-medium for 50 days had decreased clonogenic survival, this may indicate that the decreased cell clonogenic survival affected the repopulation of cells. No influence of Ra-226 on the doubling time of cells means that the chronic, low-dose radiation couldn't influence the cell proliferation. Cells may get adapted to Ra-226 in the medium, and could keep the normal proliferation. But the colony-forming ability of cells was affected. This might be because of the different number of Ra-226 atoms surrounding each cell. In clonogenic assay, cells were sparsely planted in each flask, and in cell culture for doubling time, much more cells were seeded in each flask. Even though cells in the whole flask received the same mean absorbed dose of radiation, in cell culture for clonogenic survival, the mean absorbed dose for every cell were much higher. Because of the similar chemical and physical characteristics of Ra-226 and calcium, the Ra-226 and calcium in the medium would compete to enter into cells. So more Ra-226 atoms would enter into each cell to form internal radiation when cells were sparsely planted. In addition, a cell is more possible to be hit by α-particles because no other cells available to block the hit. So Ra-226 in medium could have more obvious effect on cells in clonogenic survival analysis than in doubling time analysis. Of course, it is also possible that maybe a separate set of factors determines the population doubling rather than the ability of forming colonies. So further experiments such as analyzing the size of the colonies, measuring the apoptosis and testing the change of calcium flux in the cells are needed to find out the reason.

Overall, the results provide some data about the effects caused by chronic low dose of high LET radiation in the environmentally relevant range. This may be important for the protection of human and non-human biota in the environment. CHSE/F cell line is a fish cell line, which is radiation resistant compared with some human cell lines, so their

reaction to the low dose radiation from Ra-226 may not be very great. The clonogenic survival fraction of HaCaT cells decreased at first and then increased as time of exposure to Ra-226-medium increased suggesting that an adaptive mechanism was induced.

3.6 Conclusions

In this study, the chronic low-dose radiation from Ra-226 does have effects on the clonogenic survival of CHSE/F cells and HaCaT cells, but the influence on the doubling time was not so obvious. When CHSE/F cells were cultured in Ra-226-medium for about 134 days, their clonogenic surviving fractions were significantly lower than that of control cells. CHSE/F cells treated with 0.66mGy/d radiation from Ra-226 in medium for 50 days also had decreased clonogenic survival. The doubling time of CHSE/F cells treated with Ra-226 was almost the same as that of control cells except for the period when cells were cultured in medium containing Ra-226 from 50 days to 64 days. Clonogenic survival of HaCaT cells treated with Ra-226 in medium decreased at first and then increased as the time of culturing in Ra-226-medium increasing. The doubling time of HaCaT cells was not influenced by Ra-226 in the medium. This study could be meaningful for radiation protection of human and non-human biota to the chronic, low-dose high LET radiation in the environment.

3.7 Declaration of interest and acknowledgement

The authors report no conflicts of interest. The authors alone are responsible for the content of the paper.

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Chapter 4

NO ADAPTIVE RESPONSE IS INDUCED BY CHRONIC, LOW-DOSE RADIATION FROM RA-226 IN THE CHSE/F FISH EMBRYONIC CELL LINE AND THE HACAT HUMAN EPITHELIA CELL LINE

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The research project was designed by Dr.Mothersill, Dr.Seymour and the first author. The first author completed the cell preparation, α-irradiation, γ-ray irradiation, clonogenic assay, data collection and analysis. The first author wrote the manuscript, which was edited by Dr.Mothersill before being submitted.

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4.1 Abstract

Purpose: To determine whether chronic low-dose α-particle radiation from Ra-226 over multiple cell generations can lead to an adaptive response in CHSE/F fish embryonic cells or HaCaT human epithelial cells receiving subsequent acute high-dose γ-ray radiation.

Methods: CHSE/F and HaCaT cells were exposed to very low doses of Ra-226 in medium for multiple generations prior to being challenged by a higher dose γ-rays from Cs-137. The clonogenic assay was used to test the clonogenic survival of cells with or without being pretreated by radiation from Ra-226.

Results: In general, pretreatment with chronic irradiation using 4.78MeV α-particles has no significant influence on the reaction of cells to the subsequent challenge dose of γ-rays from Cs-137. Compared to unprimed cells, the change in clonogenic survival of primed cells after receiving challenge radiation is mainly due to the influence of the chronic exposure, and there's little adaptive response induced. However at several dose points, pretreatment of CHSE/F fish cells with chronic radiation resulted in a radiosensitive response to a challenge dose of γ-ray radiation, and pretreatment of HaCaT cells resulted in no effect except for a slightly radioresistant response to the challenge radiation which was not significant.

Conclusion: The results suggest that chronic low-dose radiation is not effective enough to induce adaptive response. There was a difference between human and fish cells and it may be important to consider results from multiple species before making conclusions about effects of chronic or low doses of radiation in the environment. The term "radiosensitive" or "adaptive" make no judgment about whether such responses are ultimately beneficial or harmful.

Key words: α-particle, chronic radiation, Ra-226, adaptive effect, clonogenic survival

fraction.

4.2 Introduction

Radium-226 is a naturally occurring radionuclide in the uranium decay chain. It can cause severe adverse effects if it is absorbed and accumulated in the body of organisms because of its high radiotoxicity and chemical competition for calcium. Due to increasing uranium mining and milling for nuclear power generation, the levels of Ra-226 in some environments can become elevated and radium levels in the local plants or animals can be high (Giri et al. 2010; Pyle and Clulow, 1998; Brenner et al, 2007). Adverse effects observed in plants and animals include high embryonic mutation frequency, low germination capacity, inhibited reproduction and decreased biomass (Evseeva et al, 2009; Geras'kin et al., 2011, 2013; Lourenco et al., 2012; Mothersill et al., 2013). High environmental levels of Ra-226 can also induce abnormal transcription of genes related to apoptosis in Atlantic cod embryo cells (Olsvik et al, 2012). In our previous study, we found that chronic, low-dose radiation from environmentally relevant levels of Ra-226 could affect the colony-forming ability of CHSE/F fish embryonic cells and HaCaT human epithelial cells (Shi et al., 2016). This led to the question of whether radiation from this level of Ra-226 could influence the reaction of cells to subsequent high-dose radiation, i.e. whether an adaptive response can be induced by chronic low-dose pre-exposure.

The adaptive response was first reported in human lymphocytes by Olivieri et al. (1984). It is a process in which biological systems become resistant to a challenge dose of radiation after receiving a very small 'priming' dose, generally below 0.5Gy (Dhattacharjee, 1996; Wiencke, 1986; Gourabi and Mozdarani, 1998; Prise et al., 2003). Typically the adaptive response is a protective mechanism, but in a few reports, the priming radiation could result in increased sensitivity to the challenge radiation (Raaphorst and Boyden, 1999; Cregan et al., 1999; Ryan et al., 2008). The adaptive

response induced by low LET radiation in mammalian systems is well accepted and the mechanisms are becoming clear (Azzam et al., 1994; Preston, 2003, 2005; Park et al., 1999; Filippovich, 1998). There are fewer studies about the adaptive response induced by high LET radiation in mammalian cells. For example, 0.2Gy priming dose of neutrons induced increased survival in Chinese hamster cells V79 subsequently exposed to 1Gy X-ray radiation (Marples and Skov, 1996). A 0.01Gy priming dose of heavy ion radiation induced lower mutation frequencies in TK6 and AHH-1 cells following a subsequent challenge with 1-4Gy high LET radiation (Varès et al., 2011). These studies concerned acute priming radiation rather than the more environmentally relevant chronic low dose exposure. A literature search in the area of adaptive response induced by chronic radiation revealed very few studies. An adaptive response was reported in lymphocytes challenged with 1.5Gy gamma radiation which had been harvested from people exposed to high background radiation (Ghiassi-nejad et al., 2002). Medaka fish exposed to chronic priming gamma radiation prior to challenge dose of acute X-ray radiation also showed an adaptive response (Smith et al., 2011).

The objective in the current study was to determine whether chronic irradiation of 4.78MeV α-particles from radium at doses equivalent to those found in uranium mining areas prior to a challenge dose of γ-rays radiation from Cs-137 could induce adaptive responses in cells. Our previous work indicated that CHSE/F cells irradiated to a range of doses (0.00066 to 0.66mGy/d) of α-radiation from Ra-226 in medium over 9 passages for 134 days had lower clonogenic survival than sham irradiated cells. HaCaT cells initially showed a radiosensitive response reaching the lowest value at about 42 days (8 passages), but then the clonogenic survival began to increase and was higher than that of sham-irradiated cells at 70days (Shi et al., 2016). The doses of the chronic 4.78MeV α-irradiation were selected using these data. Because an adaptive response had been reported in the literature (Ryan et al., 2008) that pre-exposure of CHSE/F cells to 0.1Gy γ-rays from Cobalt-60 sensitized the cells to the subsequent 5Gy challenge dose of γ-rays,

the chronic dose for CHSE/F cells was chosen where cells were exposed to Ra-226 for 185 days, resulting in a cumulative dose of greater than 0.1Gy (122.22mGy) 4.78MeV α-radiation. HaCaT cells were exposed to radioactive medium for 48 days and 77 days to cover the two types of response seen with these cells. The highest cumulative doses of chronic radiation from 4.78MeV α-particles were 31.6mGy and 50.8mGy respectively.

4.3 Materials and methods

4.3.1 Cell culture

CHSE/F fish cell line and HaCaT cell line were used in the current study. CHSE/F is the Common bluegill embryonic cell line and is epithelial in morphology and behavior. The CHSE/F cells in our lab were originally obtained as a gift from Dr.Niels Bols (University of Waterloo, Canada).The average plating efficiency is $41.45 \pm 2.23\%$ and the doubling time is about 102 hours. The CHSE/F cells were cultured in Leibovitz's L-15 medium, supplemented with 12% fetal bovine serum (Invitrogen, Burlington, ON, Canada), 5ml of 100U/mL penicillin and 100μg/ml streptomycin sulphate (Gibco, Burlington, ON), 5ml of 2mM L-Glutamine (Gibco, Burlington, ON) and 25mM Hepes buffer (Gibco, Burlington, ON). The cells were maintained at 19^{o}C in an incubator without CO_2.

HaCaT cell line was originally derived from adult human skin (Boukamp et al., 1988) and is an immortalized human keratinocyte cell. The cell line in our lab was obtained as a gift from Dr.Orla Howe (Dublin, Ireland). It has a plating efficiency of $51.46 \pm 0.80\%$ and a doubling time of 23.6 hours. RPMI-1640 medium was used for experiments with HaCaT cells. It was supplemented with 10% fetal bovine serum (Invitrogen, Burlington, ON, Canada), 5ml of 100U/mL penicillin and 100μg/ml streptomycin sulphate (Gibco, Burlington, ON), 5ml of 2mM L-Glutamine (Gibco, Burlington, ON), 0.5μg/ml hydrocortisone (Sigma-Aldrich, Oakville, ON), and 25mM Hepes buffer (Gibco, Burlington, ON). The cells were grown at 37^{o}C in an incubator with

5% CO_2.

All experiments were performed in biosafety level 2 laminar flow cabinets. Both cell lines were stored in liquid nitrogen and were tested to be mycoplasma free prior to use. Before starting the chronic exposure experiment, cell stocks were maintained inT75 flasks with 30ml of medium and subcultures were performed when cells were 80-100% confluent. The subculture of CHSE/F cells was performed using a 1:1 solution of 0.125% trypsin and 1mM EDTA at 19^{o}C for 8 minutes. The subculture of HaCaT cells was routinely performed with a 1:1 solution of 0.25% trypsin and 1mM EDTA at 37^{o}C for 8 minutes.

4.3.2 Ra-226 medium samples

The radioisotope used in this study is Ra-226, which was supplied as neutralized radium nitrate by Eckert and Ziegler (Valencia, CA, USA). The carrier for the Ra-226 was 10microg/ml Ba (as Ba nitrate). To prepare stock solutions, 100ml L-15 or RPMI medium was mixed with 1000Bq of Ra-226 solution. This was filtered into storage tubes. The concentration of Ra-226 in this stock medium was 10000mBq/ml. Serial 1/10 dilutions were made to give final concentrations of medium with 10, 100, 1000 and 10000mBq/ml Ra-226. The amount of Ba and Ra were not enough to influence the performance of the medium (Shi et al., 2016).

4.3.3 Chronic irradiation using Ra-226 in medium

CHSE/F cells or HaCaT cells were plated into T25 plastic flasks containing 5ml control medium orRa-226medium. For each cell line, there was one control group and four groups of cells cultured continuously in radioactive medium containing 10, 100, 1000 or 10000mBq/ml Ra-226. The mean absorbed dose rate calculated for each group was 0.00066, 0.0066, 0.066 or 0.66mGy/d respectively (Shi et al., 2016). All the experimental CHSE/F cells and HaCaT cells were maintained in T25 plastic flasks

containing 5ml normal medium or radioactive medium. Subculture were performed when cells were 80-100% confluent with the procedure mentioned before. CHSE/F cells were cultured in control or Ra-226-medium over multiple cell generations for 185 days, and the cumulative mean absorbed dose of radiation was 0, 0.122, 1.22, 12.2 and 122mGy. HaCaT cells were cultured for 48 days or 77 days and the cumulative mean absorbed dose was 0.0316, 0.316, 3.16, 31.6mGy or 0.0508, 0.508, 5.08 and 50.8mGy.

4.3.4 Challenge irradiation and clonogenic assay technique

The clonogenic assay technique first described by Puck and Marcus (1956) was used to assess the survival of chronically treated and control cells after they were exposed to challenge dose of γ-rays from Cs-137. Briefly, at the end of the chronic treatment period, cells in each group were removed from the flasks using a 1:1 solution of 0.125% (for CHSE/F cells) or 0.25% (for HaCaT cells) trypsin and 1mM EDTA. After detachment, cells were resuspended in medium. Then a Z2 Coulter particle count and size analyzer (Bechman Coulter Electronics, Mississauga Ontario, Canada) was used to determine the number of viable cells in an aliquot of the cell suspension. Appropriate numbers of cells were plated into flasks adjusted for the challenge dose. CHSE/F cells were incubated at 19^{o}Cfor 6 hours, and HaCaT cells were incubated at 37^{o}Cfor 6 hours.

After the 6 hours period, all cells were exposed at room temperature to the challenge dose of ^{137}Cs gamma radiation using the Taylor source, located at McMaster University, Hamilton, Ontario. The dose rate during the experiments was approximately 0.226Gy/min. CHSE/F cells received acute doses of 0.078, 0.39, 0.78, 1.56 and 3.9Gy. For HaCaT cells, the challenge doses were 0.1, 0.5, 1, 2 and 5Gy.

After irradiation, CHSE/F cells were returned to the incubator at 19^{o}Cand maintained for approximately 28 days, and HaCaT cells were returned to the incubator with 5% CO_2 and maintained at 37^{o}C for approximately 9 days. Flasks were checked periodically for colony formation. When colonies in the sham irradiated (control) flasks

were visible to the naked eye, the cells were stained with 20% carbol fuchsin (VWR, Bridgeport, NJ, USA) and colonies with 50 cells or more were counted.

4.3.5 Statistical analysis

All data are presented as mean ± SEM (the standard error of the mean).The adaptive response in CHSE/F cells or HaCaT cells with or without pretreatment with Ra-226-medium were analyzed using a two-way Analysis of Variance (ANOVA). Post-hoc testing was performed using Fisher's least significant difference (LSD) test (SPSS22). A confidence interval of 95% and $p<0.05$ were selected to be statistically significant.

4.4 Results

4.4.1 Adaptive response in CHSE/F fish cells

The clonogenic survival of CHSE/F cells challenged by acute, high-dose γ-ray radiation from Cs-137 after pretreatment with chronic 4.78MeV α-radiation is shown in Figure 4.1. 0.122mGy chronic radiation had no influence on the clonogenic survival of CHSE/F cells no matter whether they were or were not subsequently exposed to the challenge radiation. However for 1.22mGy and 12.2mGy chronic radiation alone, the colony-forming ability of CHSE/F cells was reduced. Cells pretreated with these doses of chronic radiation had lower clonogenic survival after being irradiated with the challenge dose of radiation (0.078, 0.39, 0.78 or 1.56Gy) when compared with absolute control cells. In addition, even though 122mGy chronic radiation alone had no effect on the survival of CHSE/F, cells pretreated with this dose showed significant lower survival after being challenged by 0.078, 0.39, 0.78, 1.56Gy γ-radiation than non-pretreated cells.

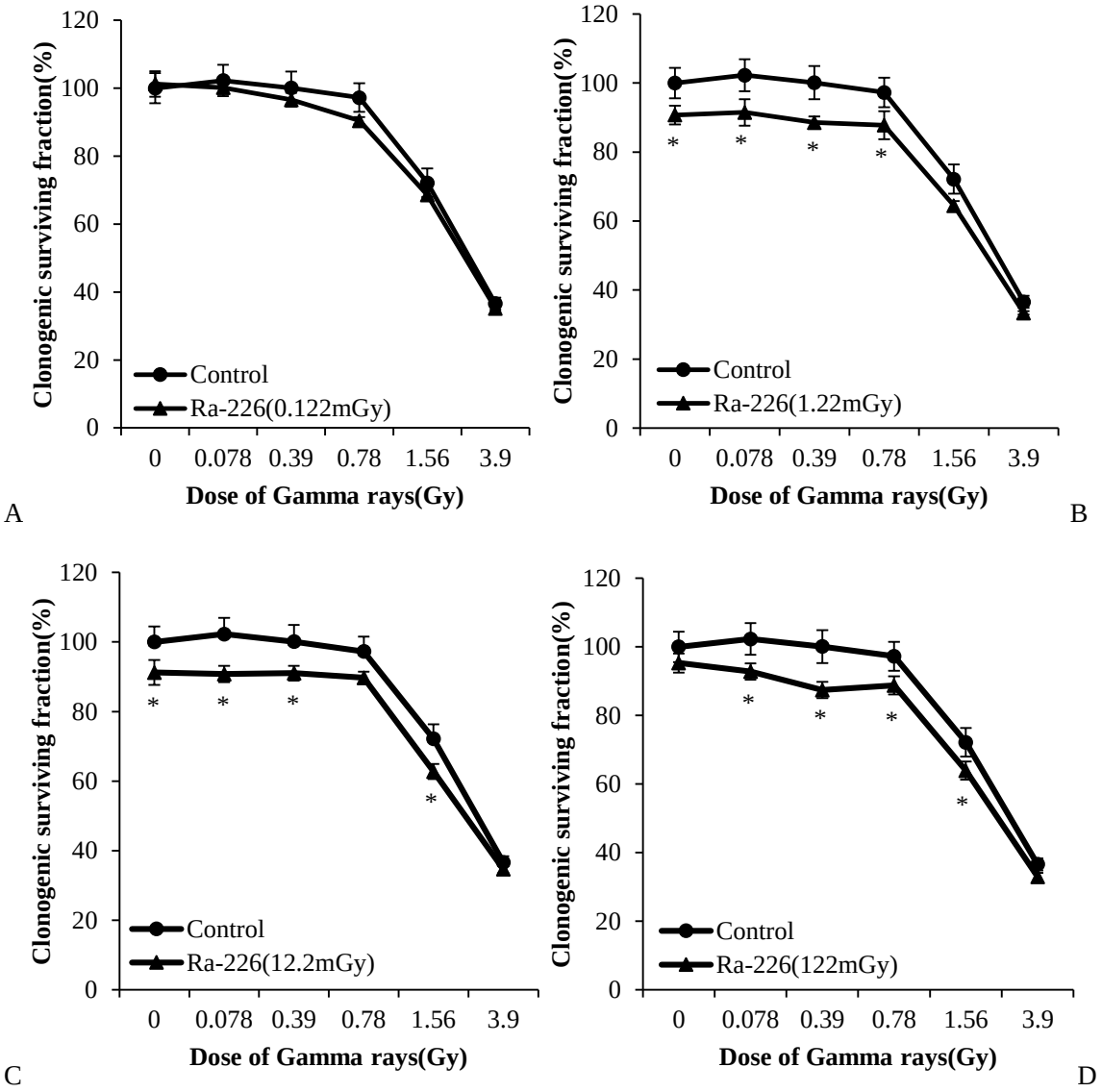


Figure 4.1 Clonogenic survivals for CHSE/F cells with a priming radiation prior to challenge dose of acute γ-rays, compared to control cells (umprimed cells). (A) 0.122mGy priming radiation, (B) 1.22mGy priming radiation, (C) 12.2mGy priming radiation and (D) 122mGy priming radiation. Error bars represent SEM, n=6. Analysis was performed using two-way Analysis of Variance (ANOVA) method, and Post-hoc testing was performed with Fisher's least significant difference (LSD) test. A significant change in survival when compared to unprimed controls is represented by * ($p \leq 0.05$).

In order to determine the relative contribution of the chronic and acute radiation components to the total effect, the combined irradiation effects data were normalized to the corresponding chronic dose effect, and the result is shown in Figure 4.2. Overall, most radiation combinations did not result in significant changes from the controls. However CHSE/F cells primed with 122mGy chronic radiation prior to 0.39Gy challenging radiation had 8.3% lower survival than unprimed cells ($SF_{0+0.39}$=100.1 ± 4.8%, $SF_{122mGy+0.39Gy}$=91.8 ± 2.5%, p=0.04). Decreased clonogenic survival also occurred in cells primed with 0.122mGy chronic radiation prior to 0.78Gy challenge radiation ($SF_{0+0.78Gy}$=97.3 ± 4.2%, $SF_{0.122mGy+0.78Gy}$=89.3 ± 1.0%, p=0.05). Figure 4.3 shows the change in normalized clonogenic survival between cells with or without pretreatment prior to challenge radiation. Most of the chronically pre-exposed cells had lower survival than the control cells.

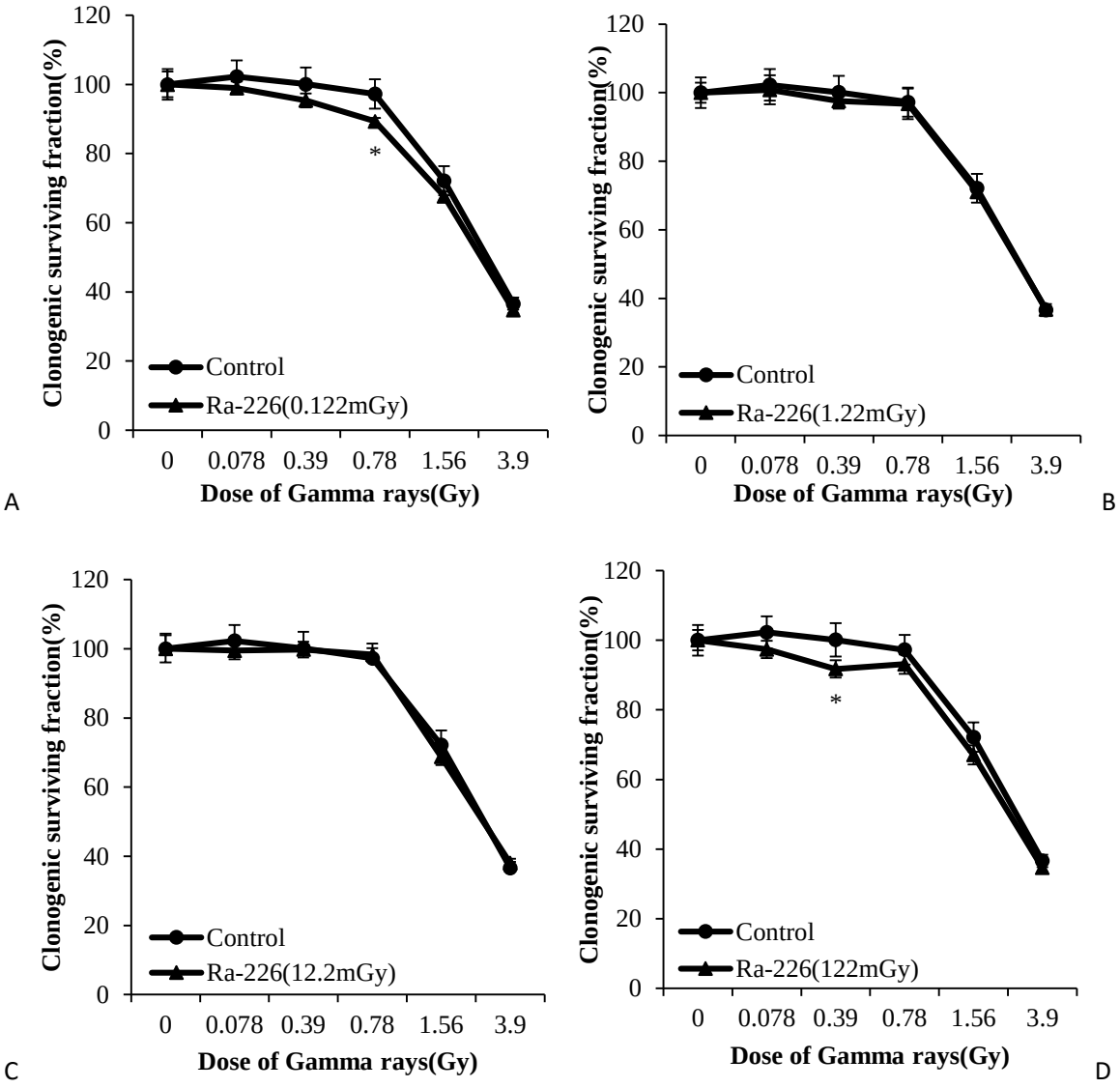


Figure 4.2 Clonogenic survivals (normalized to unchallenged cells in every Ra-226-medium group) for CHSE/F cells with a priming radiation prior to challenge dose of acute γ-rays, compared to control cells (umprimed cells). (A) 0.122mGy priming radiation, (B) 1.22mGy priming radiation, (C) 12.2mGy priming radiation and (D) 122mGy priming radiation. Error bars represent SEM, n=6. Analysis was performed using two-way Analysis of Variance (ANOVA) method, and Post-hoc testing was performed with Fisher's least significant difference (LSD) test. A significant change in survival when compared to unprimed controls is represented by * ($p \leq 0.05$).

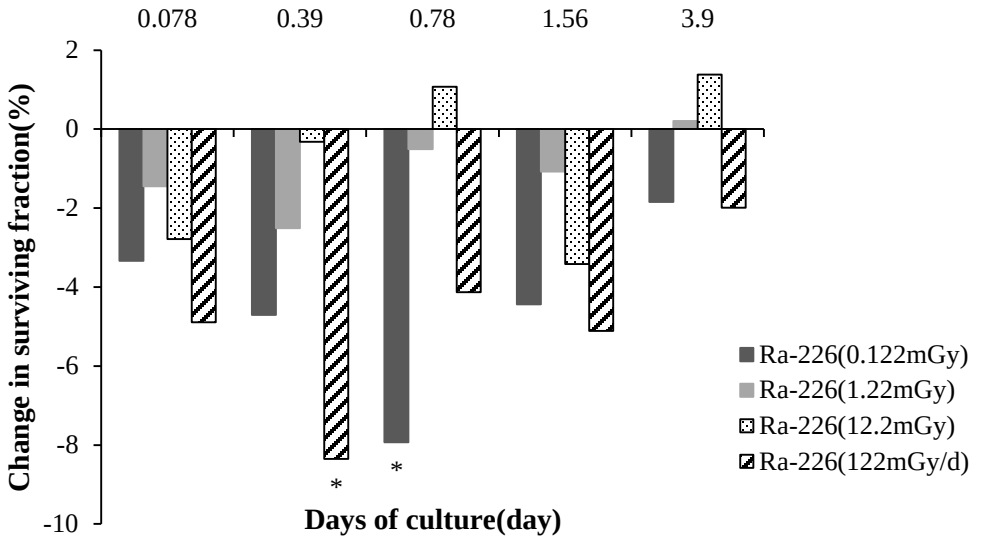


Figure 4.3 The change in normalized clonogenic survivals between CHSE/F cells with or without a priming radiation prior to challenge dose of γ-rays, n=6. A significant change in survival when compared to unprimed controls is represented by * ($p \leq 0.05$).

4.4.2 Adaptive response in HaCaT cells

Figure 4.4 shows the clonogenic survival of HaCaT cells pre-exposed to chronic radiation before being irradiated by challenge dose of γ-rays from Cs-137. For HaCaT cells cultured in medium containing Ra-226 for 48 days, the chronic radiation didn't affect their clonogenic survival, except for cells which received 31.6mGy chronic radiation, where the clonogenic survival was 9.3% lower. For HaCaT cells exposed to acute challenge radiation after being pretreated with the chronic radiation, the clonogenic survivals were not significantly different from cells which were not pretreated. This indicated that the priming chronic radiation had no influence on the reaction of HaCaT cells to the subsequent challenge radiation.

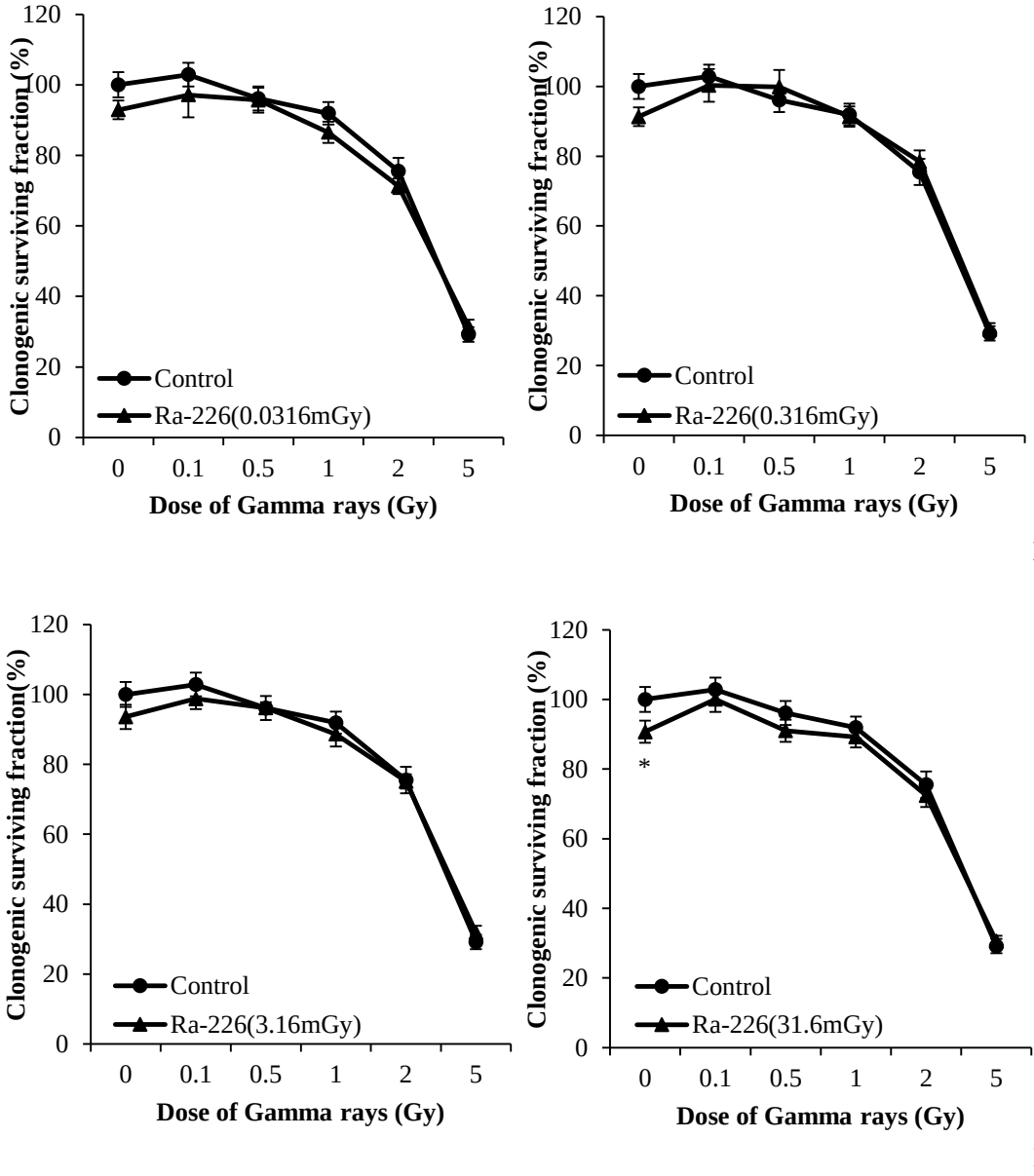


Figure 4.4 Clonogenic survivals for HaCaT cells treated with a priming radiation prior to challenge dose of acute γ-rays, compared to control cells (umprimed cells). (A) 0.0316mGy priming radiation, (B) 0.316mGy priming radiation, (C) 3.16mGy priming radiation and (D) 31.6mGy priming radiation. Error bars represent SEM, n=9. Analysis was performed using two-way Analysis of Variance (ANOVA) method, and Post-hoc testing was performed with Fisher's least significant difference (LSD) test. A significant change in survival when compared to unprimed controls is represented by * ($p \leq 0.05$).

In addition, the combined irradiation effects data were also normalized to the corresponding chronic dose effect to determine the relative contribution of the chronic and acute radiation components to the total effect, and the normalized clonogenic survival of HaCaT cells is shown in Figure 4.5. The figure indicates that again the changes between primed cells and unprimed cells are very small and are mostly not statistically significant, except for several dose points. Cells which received a 0.316mGy priming radiation prior to 0.5Gy or 2Gy challenging radiation had significantly higher clonogenic survival ($SF_{0+0.5Gy}$=96.1%, $SF_{0.316mGy+0.5Gy}$=109.3%, p=0.008; SF_{0+2Gy}=75.5%, $SF_{0.316mGy+2Gy}$=85.9%, p=0.035). Figure 4.6 indicates the changes in normalized clonogenic survival with or without a priming radiation prior to the challenge radiation. It shows that most of the chronically pre-exposed HaCaT cells had higher survival than control cells after the challenge dose irradiation, but without reaching statistical significance.

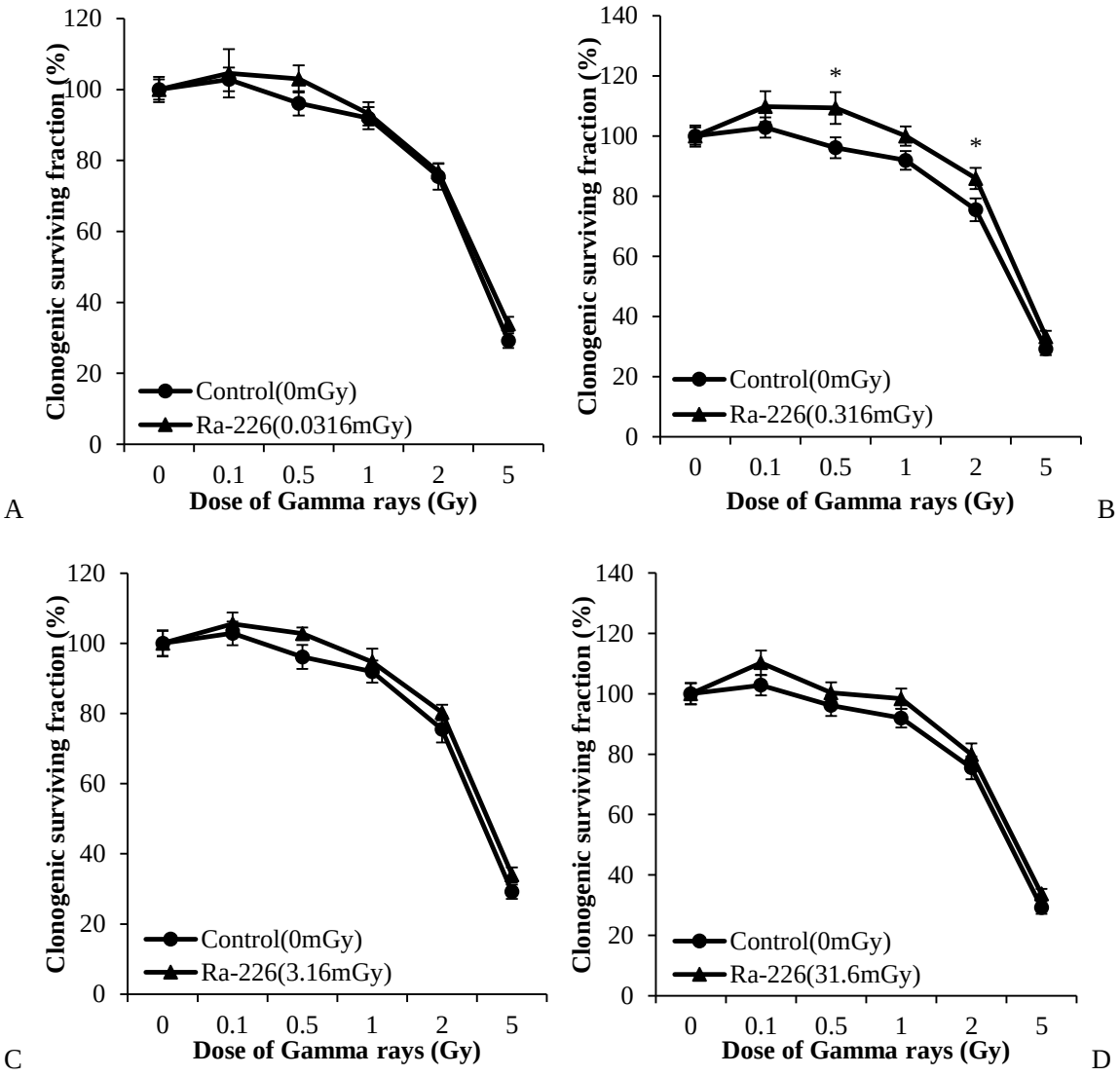


Figure 4.5 Clonogenic survivals (normalized to unchallenged cells in every Ra-226-medium group) for HaCaT cells with a priming radiation prior to challenge dose of acute γ-rays, compared to control cells (umprimed cells). (A) 0.0316mGy priming radiation, (B) 0.316mGy priming radiation, (C) 3.16mGy priming radiation and (D) 31.6mGy priming radiation. Error bars represent SEM, n=9. Analysis was performed using two-way Analysis of Variance (ANOVA) method, and Post-hoc testing was performed with Fisher's least significant difference (LSD) test. A significant change in survival when compared to unprimed controls is represented by * ($p \leq 0.05$).

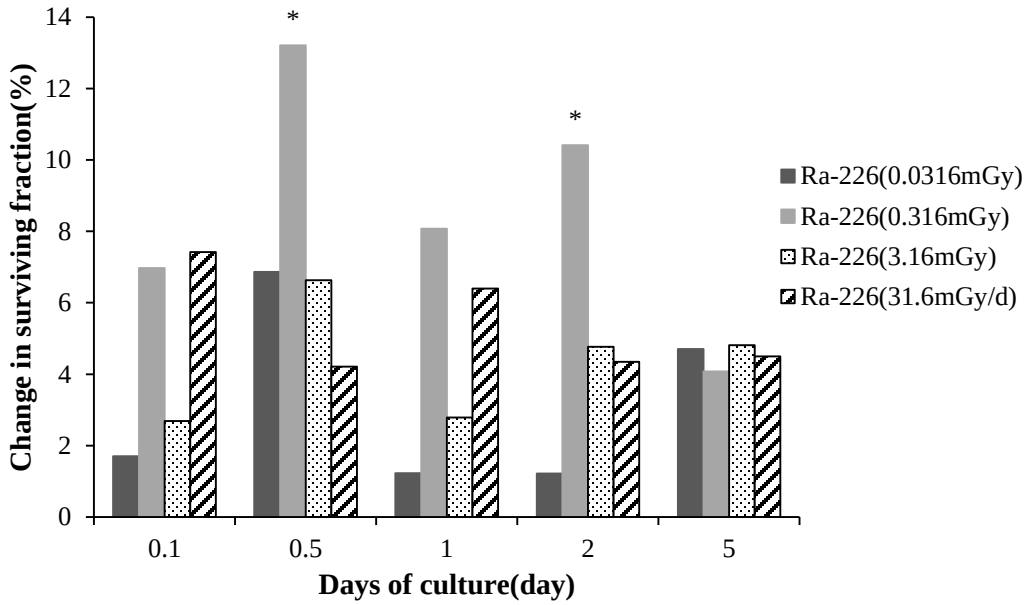
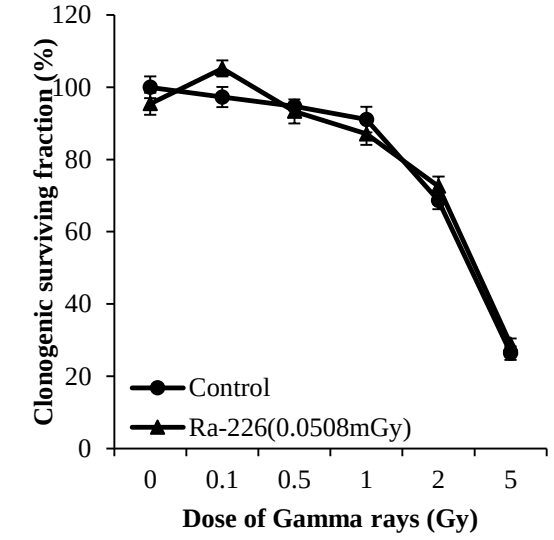


Figure 4.6 The change in normalized clonogenic survivals between HaCaT cells with or without pretreatment by Ra-226-medium for 48 days prior to challenge dose of γ-rays, n=9.A significant change in survival when compared to unprimed controls is represented by * ($p \leq 0.05$).

The clonogenic survival of HaCaT cells receiving longer period of radium pretreatment prior to the challenge dose of γ-rays from Cs-137 is shown in Figure 4.7. The cumulative mean absorbed dose cells received was 0.0508, 0.508, 5.08 and 50.8mGy. The chronic radiation had no significant effect on the clonogenic survival of HaCaT cells no matter whether they were or were not subsequently exposed to the challenge radiation. However cells which received 0.508mGy chronic dose prior to 0.5Gy challenge dose had significantly higher survival than the controls (9.4% higher).

The normalized clonogenic survival of HaCaT cells (normalized to the corresponding chronic dose effect to emphasize the adaptive response) is shown in Figure 4.8, and the changes in the normalized survival are shown in Figure 4.9. Once again the data sets are mainly not significantly different from the controls. However a chronic mean absorbed dose as low as 0.0508mGy could induce protective effect in HaCaT cells

exposed to challenge dose of 0.1Gy. Clonogenic survival increased from 97.4% for unprimed cells to 110.3% for primed cells, p=0.002. Cells receiving a 50.8mGy chronic mean absorbed dose prior to 0.5Gy challenge radiation and cells receiving a 0.0508mGy chronic mean absorbed dose prior to 2Gy challenge radiation also had higher survival than controls, which nearly reached significance (p=0.06 and p=0.08). Figure 4.9 indicates that most of the chronically pre-exposed HaCaT cells had higher survival after the challenge dose than control cells.



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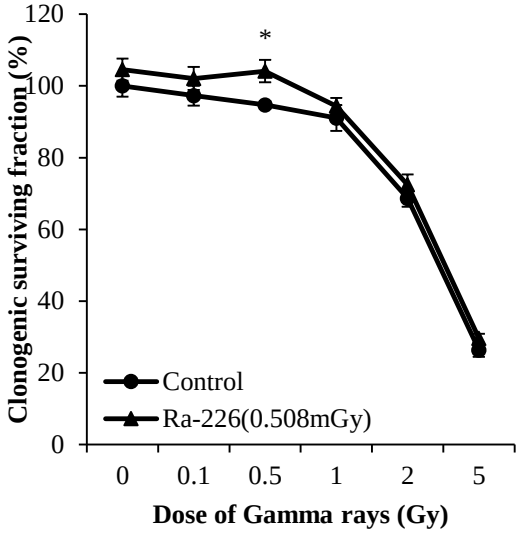

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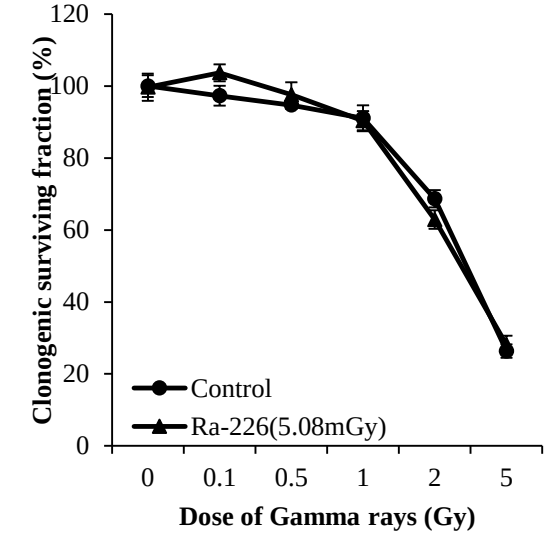

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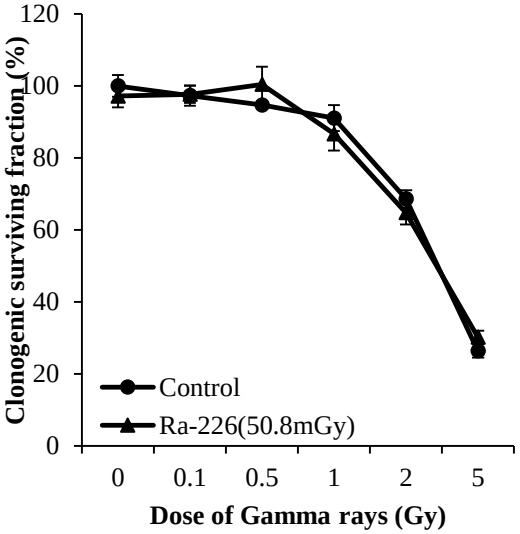

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Figure 4.7 Clonogenic survivals for HaCaT cells with a priming radiation prior to challenge dose of acute γ-rays, compared to control cells (umprimed cells). (A) 0.0508mGy priming radiation, (B) 0.508mGy priming radiation, (C) 5.08mGy priming radiation and (D) 50.8mGy priming radiation. Error bars represent SEM, n=9. Analysis was performed using two-way Analysis of Variance (ANOVA) method, and Post-hoc testing was performed with Fisher's least significant difference (LSD) test. A significant change in survival when compared to unprimed controls is represented by * ($p \leq 0.05$).

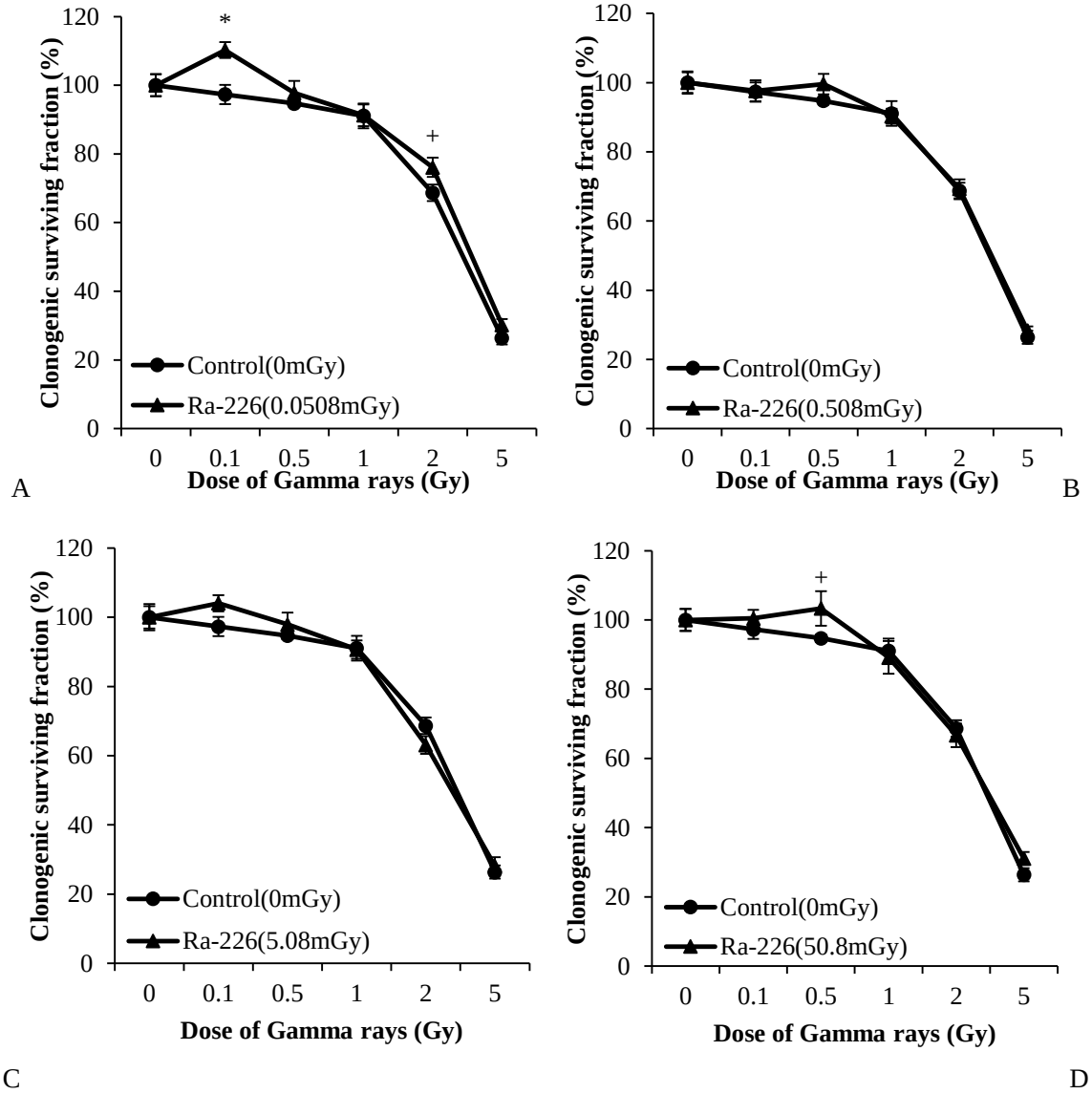


Figure 4.8 Clonogenic survivals (normalized to unchallenged cells in every Ra-226-medium

group) for HaCaT cells with a priming radiation prior to challenge dose of acute γ-rays, compared to control cells (umprimed cells). (A) 0.0508mGy priming radiation, (B) 0.508mGy priming radiation, (C) 5.08mGy priming radiation and (D) 50.8mGy priming radiation. Error bars represent SEM, n=9. Analysis was performed using two-way Analysis of Variance (ANOVA) method, and Post-hoc testing was performed with Fisher's least significant difference (LSD) test. A significant change in survival when compared to unprimed controls is represented by * ($p \leq 0.05$), + ($0.05 < p \leq 0.08$).

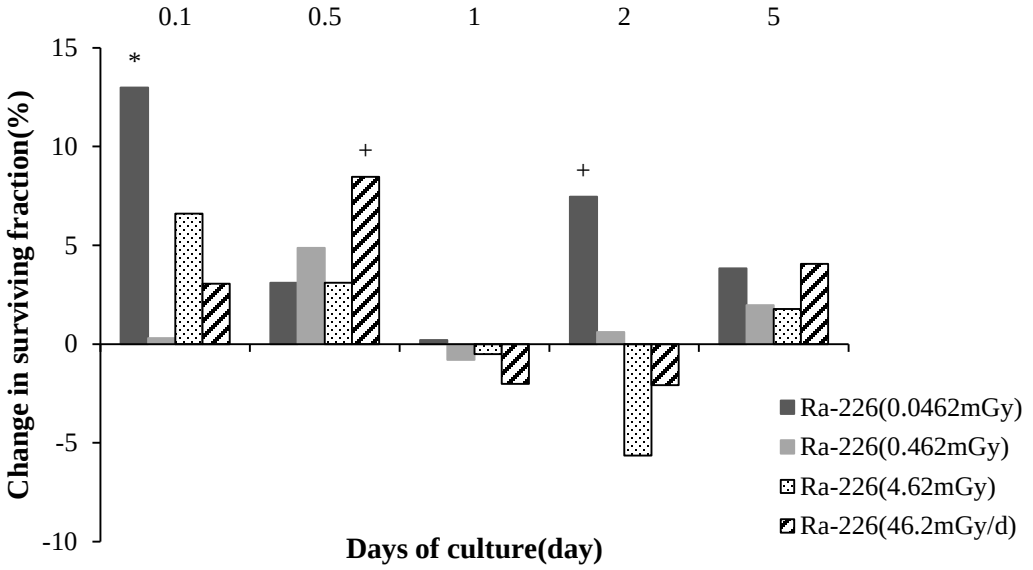


Figure 4.9 The change in clonogenic survivals between HaCaT cells with or without pretreatment by Ra-226-medium for 77 days prior to challenge dose of γ-rays, n=9. A significant change in survival when compared to unprimed controls is represented by * ($p \leq 0.05$), $^{+}$ ($0.05 < p \leq 0.08$).

4.5 Discussion

For the current experiments, the incubation time between priming and challenging radiation was 6 hours. This time interval was chosen based on the available literature concerning the adaptive response and was also based on the doubling time of these two cell lines. In previous adaptive response experiments with mammalian cells, researchers found that 5 hours was the most effective time interval between priming and challenge

radiation at inducing a protective (in terms of cell survival) adaptive response (Cregan et al., 1999; Raaphorst and Boyden, 1999; Ulsh et al., 2004). For CHSE/F cells, because of the long doubling time, Ryan et al. (2008) conducted a time series experiment to find the proper time interval, and the result demonstrated that the adaptive response of the 0.1Gy priming radiation was present from 4-6h, and disappeared at 8h or greater (Ryan et al., 2008). In addition, after being plated into flasks at the end of the priming irradiation, the time interval should be long enough to make sure that all cells are attached to the bottom of the flasks, but not so long that cells would have time to undergo division. The doubling time from the previous experiments was about 127 hours for CHSE/F cells and 24 hours for HaCaT cells (Shi et al., 2016). Therefore in the light of all this information a 6h interval from time of plating to time of challenge dose was chosen.

In the current study, even though the clonogenic survival of CHSE/F cells pretreated with chronic α-radiation from Ra-226 in medium prior to being irradiated with challenge γ-rays radiation from Cs-137 was significantly lower than control cells, it was mainly due to the damaging effect of the chronic radiation. Because after being normalized to the clonogenic survival of cells only treated with chronic radiation, the decreased survival didn't reach statistical significance, even though they have the trend of lower survival than unprimed cells. Only two groups of samples showed significantly decreased survival, which means a small sensitizing response seemed to occur. This is because the cell killing from the priming and challenge doses of radiation are additive. The sensitization induced by low-dose priming radiation has been reported in CHSE/F cells pretreated with a 0.1Gy priming γ-ray radiation from Co-60 prior to 5Gy challenge dose of γ-ray radiation (Ryan et al., 2008) and in human radioresistant cell line Sk-Mel3 (Raaphorst and Boyden, 1999). It is important to point out that decreased survival is not always detrimental at levels of organization higher than the single cell.

CHSE/F fish cells are derived from embryonic tissue, which is sensitive to radiation because of the effective apoptotic machineries. Through apoptosis, damaged DNA will

be removed rather than passing mutations onto offspring (Hall, 2012; Knowles, 1999; Real et al., 2004). Therefore the additive damage and increased sensitivity observed may be a form of protective adaptive response to reduce genomic instability. In Ryan et al.'s research, the sensitizing effect was only seen in CHSE/F cells challenged with 5Gy radiation not in cells challenged with lower doses of radiation (Ryan et al., 2008). However in current study, the increased sensitivity was observed in cells challenged with much lower dose of radiation, such as 0.39Gy and 0.78Gy. A possible explanation for this is that the priming radiation in current study comes from α-particles as well as radon daughters emitted during the decay of Ra-226. Alpha particles have a higher linear energy transfer (LET) and are more effective than low LET radiation in causing biological damages. The relative biological effect (RBE) of α-radiation ranges from 1.6-21 depending on the endpoint (Thomas et al., 2007; Howell et al., 1994; Franken et al., 2011). It is possible that the priming radiation caused sufficient changes, so that only a low challenge dose was needed to produce enough DNA damage to trigger the apoptosis pathway in cells. For high challenge doses of radiation (such as 3.9Gy), the contribution of priming α-radiation to the total cell killing may be too small when compared to the contribution of high challenge doses. So the additive damaging effect was not seen when the dose of challenge radiation was high. There are some contradictory reports of classic adaptive responses in this cell line. A reduction in micronucleus formation and a small increase in survival have been reported in CHSE/F cells pretreated with a 0.5Gy priming dose of gamma radiation prior to receiving a range of challenge doses (Cassidy et al., 2007; Kilemade et al., 2008). The reason for the different observations might be because of the acute high dose of the priming radiation. Taken together with the data in this paper and the data published by Ryan et al, (2008), it appears likely that the dose and dose rate of the priming radiation and the type of the priming radiation can influence the induction of responses to a subsequent challenge dose.

In the case of HaCaT cells, the priming chronic α-radiation from Ra-226 also has no influence on the reaction of cells to the challenge γ-rays radiation from Cs-137, which means that there's no significant adaptive response induced. However several samples showed significantly higher clonogenic survival after being exposed to challenge radiation if they were pretreated for 48 or 77 days in radioactive medium (shown in Figure 4.5 and Figure 4.8). This might be because of a small classic adaptive response induced by the chronic radiation from Ra-226 leading HaCaT cells to become radioresistant to the subsequent high-dose gamma radiation. Ryan et al (2009) reported that the adaptive response could not be induced in HaCaT cells by a 0.1Gy priming γ-rays radiation. The current work confirms that cumulative doses of chronic radiation up to 50.8mGy could not cause a significant adaptive response. Previous work from our group (Shi et al 2016) investigated the influence of chronic radiation from Ra-226 without a subsequent challenge dose on HaCaT cells, and Table 4.1 shows the change in clonogenic survival fractions after cells were cultured in Ra-226-medium over multiple generations. Pretreatment with Ra-226 for 42 days can increase the radiosensitivity of HaCaT cells, while pretreatment with Ra-226 for 70 days can increase the radioresistance of HaCaT cells. In the current study, the adaptive response is randomly observed in pretreated with Ra-226-medium both for 48 day and for 77 days. This means that the adaptive effect is not related to whether the response of cells to the chronic irradiation is positive or negative.

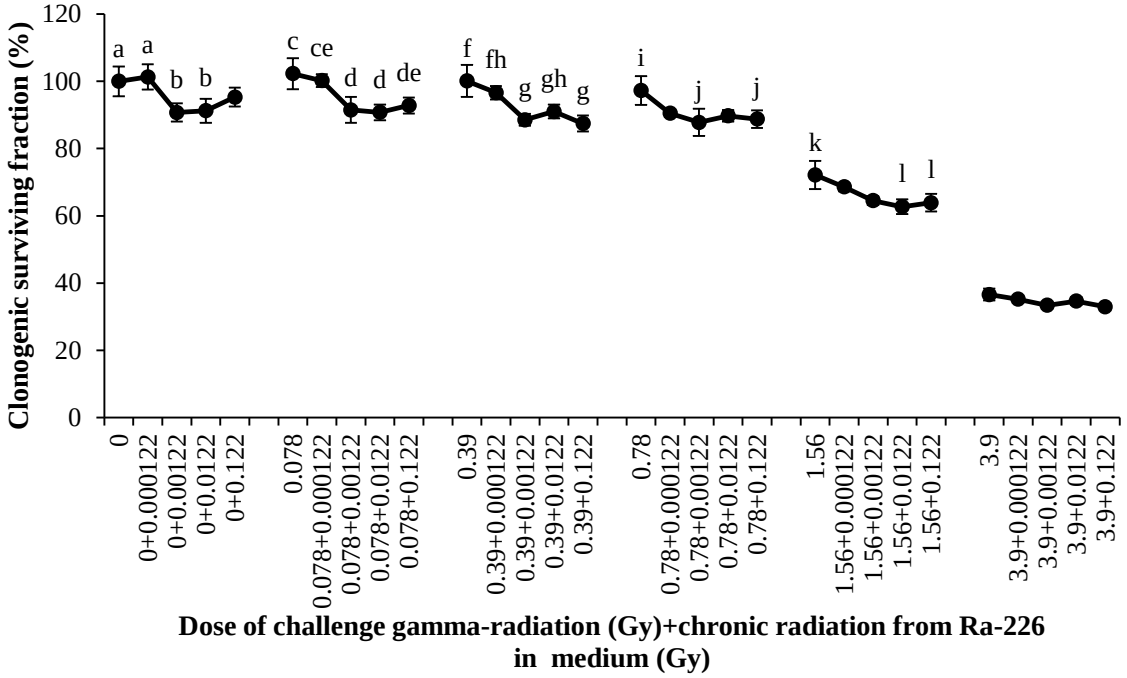
Table 4.1 Change in survival fractions of cells cultured in Ra-226-medium for certain days.

Dose rate (mGy/d) / Days	0.00066	0.0066	0.066	0.66
42	-12.18*	-12.03*	-14.68*	-27.05*
70	6.00	11.48*	2.68	1.74

Note: A significant change when compared to the respective samples grown in medium without Ra-226is represented by *, $p<0.05$, $n=9$.

Overall the results of the current work show that chronic low-dose high LET radiation from Ra-226 couldn't significantly alter the response of the CHSE/F fish embryonic cell line and the HaCaT human epithelial cell line to a subsequent high dose challenge γ-rays radiation from Cs-137. However at several dose points, small changes are seen. The nature of the response differs with the cell line; slightly increased radiosensitivity was seen in the fish CHSE/F cells but slightly increased radioresistance was seen in the human HaCaT cells (shown in Figure 4.10). Both these responses could be protective or harmful at higher levels of organization than the individual cell (Prise et al., 2005, Brenner et al., 2003). In addition, the relationship between the clonogenic survival of cells and the total dose shown in Figure 4.10 indicates that the linear hypothesis is inconsistent with current work. The linear model predicts that the relationship between biological effects and radiation dose is linear, and as the dose increases, the biological effects increase (Hooker et al., 2004). However, for HaCaT cells treated with same challenge dose, survival of cells was higher for the higher priming doses rather than lower. For CHSE/F cells only received 0.39Gy challenge dose, their clonogenic survival was lower than cells pretreated with 0.00122 or 0.0122Gy chronic radiation prior to 0.078Gy challenge dose. Same thing occurred to CHSE/F cells only received 0.78Gy challenge dose. In current study only these two cell lines were tested. The use of cell lines makes the experiments much simpler than using *in vivo* systems, but the environments for the cells in the *in vitro* work are not commonly found in tissues *in vivo*. So in the future work, tissues samples or native cells from primary tissues will be used to study the adaptive effect induced by the environmental level of Ra-226. Other endpoints such as the micronucleus assay, an apoptosis assay and a chromosomal instability assay should be added to determine whether the responses are truly protective or damaging, allowing damaged cells to survive, or die.

A



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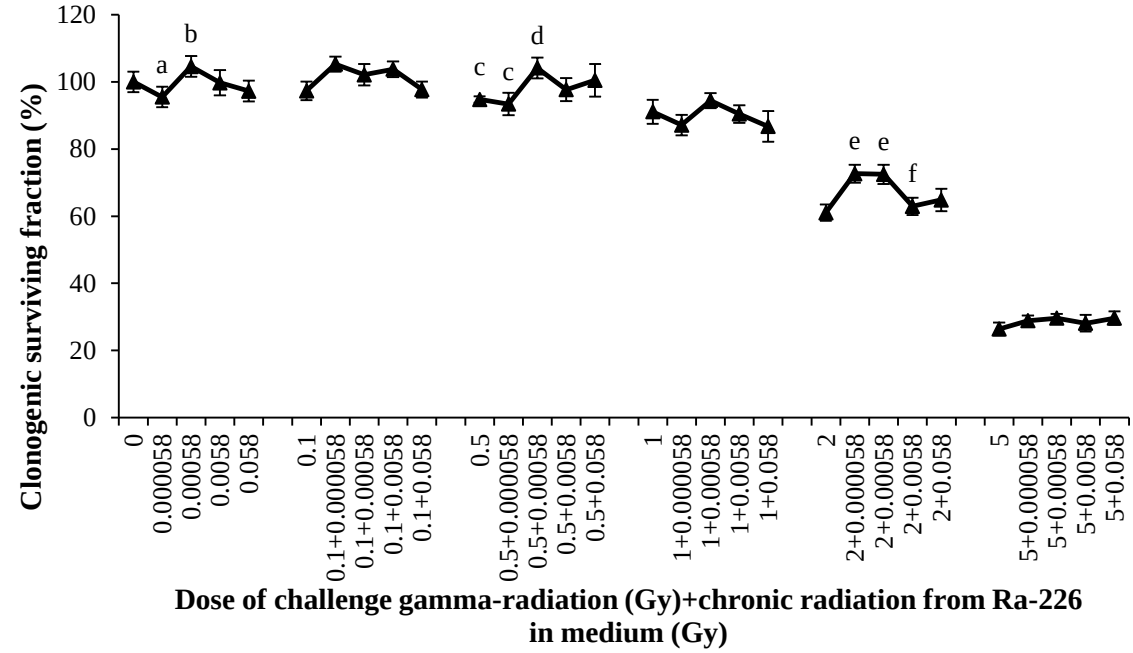

Figure 4.10 Comparison between clonogenic survivals of CHSE/F cells (A) and HaCaT cells (B) irradiated by challenge radiation after being cultured in medium with Ra-226 over multiple generations for 185 days and 77 days. Error bars represent SEM, n≥9. Analysis was performed

using two-way Analysis of Variance (ANOVA) method, and Post-hoc testing was performed with Fisher's least significant difference (LSD) test. The letters indicates statistical similarities and differences within the group for each of the challenge dose.

4.6 Conclusions

In this study, chronic low-dose 4.78MeV α-radiation from Ra-226 was shown to induce small sporadic changes in the response of a fish and a human cell line subsequently exposed to a challenge dose of γ-rays radiation from Cs-137. The aim was to see if a classic adaptive response was induced by the chronic pre-exposure as has been demonstrated for acute exposures. However the response is not obvious and is different in CHSE/F fish embryonic cell line and in HaCaT human epithelial cell line. For CHSE/F cells, priming radiation from Ra-226 sensitized the cells to the subsequent challenge γ-rays irradiation. For HaCaT cells, even if the chronic α-radiation caused a radiosensitive response when given alone, it led to a slightly increased survival of the subsequently challenged cells. It should be pointed out that increased survival while beneficial to the individual cell may be harmful at higher levels of organization as it can mean that damaged cells survive to perpetuate genomic instability. In addition, the survival of cells receiving different priming and challenge dose of radiation implies that the linear model may be inconsistent with current work using chronic low-dose radiation. In terms of the environmental relevance, this study is the first to look at the effects of long-term pre-exposure to chronic relevant doses of alpha particles on the response to a subsequent acute dose. This scenario could occur in the event of an accident occurring against a background of chronic exposure. The fact that the two cell lines responded differently, suggests that effects on different species in the environment may differ. The data also highlight the fact that very low doses and dose rates, considered to have no effect at all, can produce small perturbations in response, and the linear hypothesis is not suitable for the chronic low-dose radiation situation. Overall, since elevated level of

Ra-226 has been found in river and lakes in Uranium mining areas and in other aquatic systems, the result may be meaningful for evaluating the influence of radiation from Ra-226 on non-human biota and humans in the environment. It may also provide useful information for estimating the reaction of biota in nuclear industry areas to the accidental radiation exposure in the event of a nuclear accident.

4.7 Declaration of interest and acknowledge

The authors report no conflicts of interest. The authors alone are responsible for the content of the paper.

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Chapter 5

DISCUSSION, CONCLUSION AND FUTURE WORK

The work presented in this thesis sought to determine the bioaccumulation of Ra-226 in fish and to evaluate the biological effects of the chronic low-dose radiation from environmental level of Ra-226. The purpose was to provide further information for assessing the influence of Ra-226 contaminants from uranium exploration in the environment. In this final chapter, the brief overviews of the papers in this thesis have been presented to discuss the contribution of my work to the field of research. Some limitations of present work and suggestions for future work have also been addressed in this part.

5.1 Bioaccumulation of Ra-226 and the biological effects

In the foreseeable future, the proportion of nuclear power in the energy structure will be increasing to meet the need for carbon neutral energy. Due to the increasing uranium mining and milling for nuclear power generation, the levels of radionuclides in the uranium series, including Ra-226, can become elevated in the environment and in local plants and animals (Giri et al. 2010; Pyle and Clulow, 1998; Brenner et al, 2007). So the influence of these radionuclides on human and non-human biota is inevitable and requires attention. However a literature search revealed that studies investigating the uptake and transfer of these radionuclides are limited, and there are large data gaps for many species.

Also little is known about the biological effects of chronic exposures to internal deposited alpha emitters, including Ra-226 (Evseeva et al, 2009; Geras'kin et al., 2011, 2013; Lourenco et al., 2012; Mothersill et al., 2013). Thus, work in this thesis focuses on these two important points to add information to the very limited literature. The main findings and significances will be summarized in this part.

The whole project was designed to answer the three questions outlined at the beginning of chapter 1, which is listed below with the three hypotheses.

1. What is the relationship between the accumulation of Ra-226 in fish and it concentration in the food they consume?

Hypothesis: The activity of Ra-226 accumulated in fish will have a non-linear relationship with the Ra-226 concentration in food.

Result: The relationship was found to be non-linear with the highest concentration factor obtained from the lowest dietary Ra-226 activity.

2. Can the chronic low-dose radiation from Ra-226 affect the growth of CHSE/F and HaCaT cells?

Hypothesis: Chronic low-dose radiation from Ra-226 will lead to a decrease in the clonogenic survival measured by clonogenic assay technique for both CHSE/F cell line and HaCaT cell line, and furthermore the proliferation of cells will be decreased.

Result: CHSE/F cells had a decreased clonogenic survical during chronic irradiation. Clonogenic survival of HaCaT cells reduced during the early passages in medium containing Ra-226, after that the clonogenic survival showed the trend of increasing. However, the proliferations of both cell lines were not influenced.

3. Can the chronic low-dose 4.78MeV α-radiation from Ra-226 induce an adaptive response in CHSE/F and HaCaT cells irradiated by subsequent challenge dose of γ-rays from Cs-137?

Hypothesis: A chronic pre-exposure to radium will induce an adaptive response to a subsequent challenge dose of γ-rays from Cs-137.

Result: There's no adaptive response induced by the chronic 4.78MeV α-radiation from Ra-226 except for several dose points at which pretreatment of CHSE/F cells with chronic radiation resulted in a radiosensitive response to a subsequent challenge γ-rays radiation from Cs-137.

5.1.1 Part I: Bioaccumulation of Ra-226 in fathead minnows exposed to environmental level of Ra-226 via ingestion

The first part of the thesis sought to find out the relationship between bioaccumulation of Ra-226 and the dietary Ra-226 activity. Numerous studies using field-based approaches have reported the elevated Ra-226 level in plants and animals from aquatic systems with elevated Ra-226 levels (Giri et al. 2010; Pyle and Clulow, 1998; Brenner et al, 2007). However the relationship between accumulation of Ra-226 and Ra-226 activity in the surrounding medium is uncertain. Several field studies examined the relationship, but they were carried out in uranium mining and milling areas making it difficult to separate effects due to other toxic elements (Swanson, 1983, 1985; Clulow and Pyle, 1997; Pyle and Clulow, 1997a, b). Because of the complex multiple stressors in the natural environment and the difficulty of maintaining long-term, low dose alpha-radiation experiments in the laboratory, information in this area is sparse. In this thesis fathead minnows were exposed to diets containing Ra-226 over a wide range of concentrations. The results showed that the accumulation of Ra-226 in fish was non-linear with respect to the dietary Ra-226 concentration. The highest concentration factor obtained from the lowest dietary activity, and CF is inversely proportional to food activity, which means that when the dietary Ra-226 concentration is low, most of the Ra-226 will be absorbed and deposited in the body of fish. In addition, the result also indicated that the Ra-226 in the food could influence the growth of the fish even when the activity level was as low as 10mBq/g. Fish fed with radioactive food showed less mass, shorter length and less condition factor than control. This change may mean that the elevated level of Ra-226 in some areas shouldn't be neglected from the point of

radioprotection of non-human biota. The work in this part may be useful for predicting the bioaccumulation of Ra-226 in fish and other animals.

5.1.2 Part II: The biological effects of environmental level of Ra-226 on cells and the induced adaptive response

The second part of the thesis focuses on questions of whether the chronic low-dose α-radiation from Ra-226 has an impact on the clonogenic survival and the growth of cells, and whether an adaptive response can be induced by this radiation. Using a cell culture model, fish embryonic CHSE/F cells and human keratinocyte HaCaT cells were exposed to long-term treatment with alpha particles from Ra-226 by being cultured continuously in radioactive medium. Cell killing effects including clonogenic survival and the change of proliferation of cells were measured. Adaptive response was also examined by challenging the cultures with an acute high dose of gamma radiation. This result showed that CHSE/F cells had a reduced clonogenic survival during chronic exposure to alpha irradiation. HaCaT cells had a decreased colony-forming ability during the early passages in medium containing radium but, after about 42 population doublings the trend changed to show increasing clonogenic survival. This was higher than that of control by the end of the experiment. This means CHSE/F cells were sensitized by the long-term exposure, and HaCaT cells were initially sensitized but later appeared to adapt. The sensitization of CHSE/F cells and HaCaT cells by low-dose α-radiation fromRa-226 might be related to lethal mutations and genomic instability. Lethal mutations are heritable lethal defects carried in the descendants of cells surviving irradiation, which can result in an enhanced death rate in the progeny of irradiated cells after numerous successful divisions (Seymour et al., 1986; Gorgojo and Little, 1989). Decreased clonogenic survival was reported in survivor colonies of CHO-KI cells formed about 10 generations after being seeded from irradiated flasks (Seymour et al., 1986). Genomic instability is the phenomenon that genomic abnormalities produced by irradiation in cells surviving irradiation can be transmitted to their daughter cells (Kadhim et al., 1992, 1994;

Marder and Morgan, 1993). The transmissible genomic instability could produce some cellular effects detected in the progeny of cells surviving irradiation after many cell cycles, like delayed apoptotic cell death (Kadhim et al., 1995). Apoptosis induced during long-term irradiation could be part of the reason for the sensitization of cells. Apoptosis and related gene expression were shown to be produced in cocultured nonirradiated transformed cells and in low-dose irradiated cells (Portess et al., 2007; Soto et al., 2006).

In addition, no influence of the radiation on the population doubling times of either cell lines was found. This means that these cells could maintain normal proliferation. The possible explanation might be that the available radioactive Ra-226 atoms for each cell in the clonogenic assay are much more than in doubling time experiments. From these results, the initial hypothesis about influence of low-dose α-radiation on the clonogenic survival and proliferation needs to be revised, and cell lines from different species need to be considered before making conclusions about effects of low or chronic doses of radiation.

Regarding the possibility of inducing an adaptive response to a subsequent challenge dose of γ-rays from Cs-137 by chronic 4.78MeV α-radiation exposure from Ra-226, the work indicated that no adaptive response could be induced by the long-term exposure used, except for small sporadic changes in the response of pretreated CHSE/F and HaCaT cells. Among the small sporadic changes, CHSE/F cells which received long-term pre-exposure of 4.78MeV α-radiation prior to a challenge γ-rays radiation from Cs-137 showed slightly lower clonogenic survival than non-pretreated cells, while primed HaCaT cells showed no change in clonogenic survival after being irradiated by challenge dose γ-rays from Cs-137. Thus the initial hypothesis which stated that an adaptive response could be induced needs to be revised, and no significant adaptive response is induced. The result in this part also highlights the importance of considering cell lines from multiple species when evaluating the risks of low or chronic doses of radiation in the environment. The term “radiosensitive” or “adaptive” make no judgment about whether

such responses are ultimately beneficial or harmful at higher levels of organization such as the individual organism or the population.

5.2 Future work

In the first part of the thesis, the dosimetric analysis of Ra-226 deposited in fathead minnows fed with radioactive food was only conducted at the end of the experiment. So no information about the metabolizing process of Ra-226 by the fish was available. However, this information is of considerable importance for evaluating the risks of the deposited Ra-226. On the other hand, bioaccumulation of Ra-226, including uptake and excretion, is complicated and it is not possible to predict it from data only collected at the end. So in future experiments, doses of Ra-226 deposited in fish should be examined at different time points to determine the relationship between the amount of deposited Ra-226 and duration of exposure. The kinetics of bioaccumulation of Ra-226 by fathead minnows could then be determined and modeled. This could then be used to predict the accumulation of Ra-226 by other fish, even by other animals. In addition, since the difficulty of maintaining the laboratory experiment and the limitation of resources, the experiment was not run for a long enough time to determine reproductive effects in the fish. Reproductive impacts are thought to be the most important endpoints to evaluate the influence of long-term radiation (Lourenco et al., 2012). So the reproductive effects should be added to evaluate the influence of chronic exposure to Ra-226 in future work.

In part II of the thesis, the clonogenic survival of CHSE/F fish cell lines and HaCaT human cell lines grown in medium containing Ra-226 was examined, as well as the doubling time that reflects the proliferation rate. Even though the experiments showed that chronic low-dose radiation from Ra-226 could influence the colony-forming ability of both cell lines, and the reaction of the two cell lines were different, mechanisms underlying this phenomenon were not studied in current work. Non-targeted effects such as genomic instability (Seymour et al., 1986; Kadhim et al., 1992, 1994), bystander

effects (Mothersill and Seymour, 1997, 2004; Nagasawa and Little, 1992) and adaptive response (Olivieri et al., 1984; Kadhim et al, 2004; Maguire et al., 2007; Ryan et al., 2009; Bhattacharjee, 1996) are thought to be involved in determining the ultimate effects of low-dose radiation. The different reactions of the two cell lines to low-dose radiation may be associated with these effects, especially genomic instability mediated perhaps by apoptosis and delayed lethal or non-lethal mutations. It has been reported that 0.29mGy α-radiation was sufficient to produce an observable increase of apoptosis in cocultured non-irradiated transformed cells through intercellular induction of apoptosis involving ROS/NOS and TGF-β, and the apoptosis saturated at very low-dose radiation (25mGy for α-particles). In addition, the level of apoptosis continued to increase as cells remained in coculture, and eventually the vast majority of transformed cells were removed by induced intercellular apoptosis (Portess et al., 2007). So in future work, whether non-targeted effects play an important role in the reaction of these two cell lines to the long-term exposure and apoptosis protein expression should be examined to help understanding the mechanism.

In addition, in the current work, *in vitro* experiments with CHSE/F and HaCaT cell lines were applied to study the influence of chronic low-dose α-radiation. The use of cell lines makes the experiments much simpler than using in vivo systems because of the pure populations of cells and the unlimited growth potential. However, the environments for the cells in the *in vitro* work are not commonly found in tissues *in vivo*, where many different populations of cells cooperate with and influence each other. Also while each cell line is derived from a primary tissue, some of their characteristics are modified and are different from original cells of the primary tissue. So in future work, tissue samples or native cells from primary tissues should be considered as a better model for the natural environment.

5.3 Conclusions

This thesis presents the first work investigating the biological effect of long-term exposure to environmentally relevant level of α-radiation from Ra-226 and the influence of this type of radiation on the response of cells to a subsequent acute dose. In addition, a method detecting very low level of Ra-226 deposited in the fish body was developed. The key contributions are:

1. The accumulated amount of Ra-226 has a non-linear relationship with respect to the dietary Ra-226 activity, and a saturation value of accumulation of Ra-226 exists when dietary activity is low. The environmentally relevant level of Ra-226 has a slightly detrimental effect on the growth of fathead minnows.

2. Chronic low-dose α-radiation from environmentally relevant levels of Ra-226 influences the clonogenic survival of irradiated cells, and whether the impact is positive or negative depends on the cell line.

3. Long-term pre-exposure to low-dose 4.78MeV α-radiation does not induce an adaptive response in cells subsequently exposed to a challenge γ-rays radiation from Cs-137, except for small sporadic changes in the response of cells.

Overall, the studies in this thesis may contribute to the knowledge of evaluating the risks from chronic low-dose radiation from radium. Since elevated level of Ra-226 has been found in rivers and lakes in Uranium mining and milling areas and in other aquatic systems (Antovic and Antovic, 2011; Geras'kin et al. 2011; Pyle and Clulow, 1998; Giri et al., 2010; Brenner et al., 2007), but research about the bioaccumulation and effects of the related radionuclides are limited, the information in this thesis may be meaningful for radioprotection of human and non-human biota in the environment. The findings concerning the adaptive response induced by the chronic low-dose α-radiation from Ra-226 in cells subsequently challenged by an acute high-dose γ-rays radiation from Cs-137 provides the first data at this area and is contrary to what might have been predicted. This situation of a challenge exposure coming after long-term pre-exposure could occur in the event of an accident occurring against a background of chronic

exposure. The work may provide useful information for estimating the reaction of biota in the nuclear industry areas to the accidental radiation exposure in the event of a nuclear accident.

5.4 References

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