RESEARCH ARTICLE

Flavonoids of *Rosa roxburghii* Tratt Act as Radioprotectors

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Abstract

Background: To study the radioprotective effects of flavonoids from *Rosa roxburghii* Tratt (FRT). Materials and Methods: The radioprotective effects of FRT were investigated by examining cell viability, 30-day survival of mice and the number of colony-forming units in spleen (CFU-S) after total-body 60Co irradiation. Results: The survival rates of irradiated cells gradually increased with increasing concentrations of FRT. The survival rate was the highest at 87% with a concentration of 30 μg/mL. Pretreatment with FRT was needed to realize its radioprotective activity in mice at the dose of 60 mg/kg. With the increasing doses of 30 mg/kg, 60 mg/kg and 120 mg/kg, the numbers of CFU-S increased, and were significantly different compared with the control group. Conclusions: Pretreatment with FRT prior to irradiation resulted in significantly higher cell survival at 24 h after 5 Gy radiation, increased 30-day survival in mice after exposure to a potentially lethal dose of 8 Gy, and resulted in a higher number of CFU-S in mice after exposure to a dose of 6 Gy. These results collectively indicate that FRT is an effective radioprotective agent.

Keywords: FRT - radioprotection - survival rate - AHH-1 - CFU-S

Introduction

Ionizing radiation is any electromagnetic wave or particle capable of producing ions. It can cause immediate chemical alterations in biological tissues that lead to cell damage or death. Radiation therapy is the treatment of choice for a majority of cancer patients. However, radioprotectors are needed to protect normal tissues when ionizing radiation is used to induce irreversible damage in nearby targeted cells. In addition to the importance for cancer treatment patients, radioprotectors are needed to protect other potentially exposed populations, such as workers in the nuclear power industry, space travelers or military personnel facing radiological terrorism from ionizing radiation. Because of their potential importance, researchers put efforts into searching for radioprotectors.

The research on the radiation protection began in the early 20th century, and now, a lot of chemotherapy drugs and biological agents with function of radioprotection have been found, such as WR-2721 (amifostine), superoxide dismutase (SOD), metallic elements chelates (Fe, Zn, Cu etc), potassium iodide and cytokines drugs (G-CSF, IL-6, EPO, KGF etc). With the deepening of the research, it was found that many of them were toxic on the healthy cells, and may trigger other diseases, so people began to look for anti-radiation medicine from natural products.

People have isolated a series of active ingredients from natural anti-radiation substances, such as polysaccharides (Subramanian et al., 2002; Song et al., 2003; Il´in et al., 2004; Wang et al., 2004; Wu et al., 2004; Fan et al., 2005),

flavonoids (Devi et al., 1998; Liu et al., 2002; Zielonka et al., 2003; Wu et al., 2004; Li et al., 2004), polyphenols (Hibasami et al., 2000; Wang et al., 2004; Lv et al., 2004), saponins (Chen et al., 1999; Kim et al., 2001; Li et al., 2001; Kuinar et al., 2003), alkaloids (Liang et al., 2002; Makarchenko and Utkina 2006), and peptides compounds (Zhu and Zhong 2004; Yang 2007), etc. *Rosa roxburghii* Tratt (Figure 1) is the fruit of jiao-si flower or unicuspid jiao-si flower from rosaceae, which was firstly recorded in the "ben-cao-gang-mu-shi-yi". Nutritionist Dengyi Luo found in the study of 170 kinds of fruits and vegetables (Luo, 1987) that *Rosa roxburghii* Tratt Contained flavonoids of 5981~12895 mg every 100g, which was 120~360 times citrus, 11~12 times ginkgo biloba, and was known as "the flavonoids king". FRT were extracted

Figure 1. Rosa Roxburghii Tratt

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out of *Rosa roxburghii* Tratt, and were mainly composed of rutin. It could be a good way to remove a variety of reactive oxygen species, and can inhibit the red blood cell hemolysis and production of MDA in liver tissue, which suggested that it was a good anti-oxidant (Zhang et al., 2005); FRT can very significantly reduce the content of glucose and triglycerides, and elevated levels of insulin in serum. Activities of superoxide dismutase (SOD) and catalase (CAT) in pancreas had a significant increase, but MDA was significantly decreased (Zhang et al., 2004). However, no study has yet reported on the radioprotective activity of it. The present study therefore focused on the use of FRT as a radioprotective agent in experimental cell and animal models.

Materials and Methods

Animals

Male KM mice (4-6 weeks old, weighing l8-22 g) were purchased from the Experimental Animal Center of Xinxiang Medical University. Animals were housed at 10 per cage with ad libitum access to water and food pellets.

Drugs and reagents

Rosa roxburghii Tratt was purchased from Cili Sales Center in Kaifeng (Henan, China; product batch number 11.02.23); "523" was obtained from Institute of Radiation Medicine (Beijing, China).

Cell culture

AHH-1 lymphoblastoid cells were cultured suspendedly in RPMI1640 medium containing 10% fetal bovine serum, 100 U/mL penicillin and 100 U/mL streptomycin at 37°C in a humidified atmosphere of 5% CO_2.

Preparation of FRT (Zhang et al., 2005)

Rosa roxburghii Tratt rough powder was extracted using 10 times the amount of 65% ethanol, and the extract was filtered through four layers of gauze. The entire process was repeated twice. The combined filtrate was centrifuged at 2, 000 rpm for 20 min, and concentrated under reduced pressure. The liquid obtained was vacuum evaporated to produce a powder, which was redissolved in water just before added to resin of HPD 600. Active fractions were collected by 40% ethanol after impurities were removed by water. The liquid obtained was concentrated under reduced pressure, and was vacuum evaporated to produce a powder of FRT.

Irradiation

Cells were irradiated at room temperature with ^{60}Co γ-rays at a dose of 5 Gy. The animals were restrained in holders and exposed to 8 Gy total-body ^{60}Co γ radiation to determine their 30-day survival rate.6 Gy total-body ^{60}Co γ radiation was used to observe nodules of spleen.

Detection of cell toxicity

100 μL/well of AHH-1 cells ($1×10^5$ cells/mL) in logarithmic growth phase were seeded in 96-well plates containing 100 μL/well of drug at a final concentration of 240, 120, 60, 30, 15, 7.5 and 3.75 μg/mL (referred to as the drug groups). The drug groups were compared with control unirradiated cells and with irradiated, untreated cells. CCK-8 solution 10 μL was added to each well at 48 h after drug intervention. The absorbance of each well at 490 nm was determined after a further 4 h, using a multifunctional microplate reader.

Radioprotective effects of different concentrations of FRT in AHH-1 cells

100 μL/well of AHH-1 cells ($1×10^5$ cells/mL) in logarithmic growth phase were seeded in 96-well plates containing 100 μL/well of drug at final drug concentrations of 60, 30, 15, 7.5, 3.75 and 1.875 μg/mL (referred to as the drug groups). These were compared with control unirradiated cells and with irradiated, untreated cells. After 24 h, cells were irradiated with ^{60}Co at a dose of 5 Gy. CCK-8 solution 10 μL was added to each well at 24 h after irradiation. The absorbance of each well was determined after a further 4 h, using a multifunctional microplate reader at 490 nm.

Determination of 30-day survival rate

Mice adapted to the environment were divided into normal (unirradiated), irradiated (untreated), positive control (treated with the known radioprotective agent "523"), and drug treatment groups. All drugs were administered orally. Mice in the normal and irradiation groups were given distilled water, and mice in the treatment groups received the appropriate drugs at the dose of 30 mg/kg, 60 mg/kg and 120 mg/kg (Zhang et al., 2004, Zhang 2005) respectively. All mice were observed for 30 days after irradiation. Experimental design was indicated in Table 1.

Determination of colony-forming unit in spleen (CFU-S)

On the 6th day after irradiation, mice were sacrificed in order to get spleens, which were fixed in Bouin solution (saturated picric acid 75 mL, formaldehyde 25 mL, glacial acetic acid 5 mL) for 24 h. The nodules on the surface of spleen were observed by the use of stereo microscope.

Statistical analysis

Data were expressed as mean±standard deviation (SD). The data were analyzed using one way analysis of variance (ANOVA) on SPSS/PC* (statistical package for social sciences, personal computer) and the group means were compared by Duncan's Multiple Range Test (DMRT). The means of the treated groups were compared with those of the radiation-alone or unirradiated groups. A value of $P < 0.05$ was considered to be statistically significant.

Table 1. Experimental Design

Drug delivery	Time (days)	Positive drug dose (agent "523")	Dose of irradiation (Gy)
Before irradiation	6	24 h; 5 mg/kg	8
After irradiation	6	Within 3 h; 5 mg/kg	8
Before irradiation + after irradiation	6 + 6	24 h; 2.5 mg/kg + within 3 h; 2.5 mg/kg	8

Results

Detection of cell toxicity

The cell toxicities of FRT in AHH-1 cells were tested. The survival rates at 48 h after drug intervention are shown in Figure 2. FRT had no significant cytotoxicity in the range of 3.75-120 µg/mL.

Radioprotective effects of different concentrations of FRT in AHH-1 cells

The effects of different concentrations of FRT on the survival of AHH-1 cells were examined. The survival rates at 24 h after irradiation are shown in Figure 3. The survival rates of the irradiated cells gradually increased with increasing concentrations of FRT. The survival rate was the highest to 87% at a concentration of 30 µg/mL, and this was therefore recommended as the final concentration.

Observation of 30-day survival rate in mice

The 30-day survival rates of mice are shown in Figure 4. Pretreatment with FRT of 30 mg/kg, 60 mg/kg and 120 mg/kg improved the survival rates by 40%, 50% and 50% respectively compared with untreated irradiated animals. Administration of FRT at different doses after irradiation all resulted in a survival rate of 10%, compared with 30% in untreated irradiated animals. FRT of 30 mg/kg, 60 mg/kg, and 120 mg/kg administered before and after irradiation resulted in survival rates of 30%, 20% and 20% compared with 30% in untreated irradiated animals. These results indicate that pretreatment with FRT is needed to realize its radioprotection.

Determination of CFU-S

Experimental results (Table 2, Figure 5) showed that on the 6d after irradiation, CFU-S gradually formed.
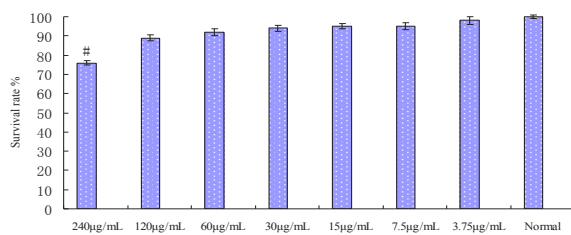

Figure 2. Survival Rates of AHH-1 Cells Treated with FRT for Detection of Cell Toxicity. FRT had no significant cytotoxicity in the range of 3.75-120 µg/mL. (#$p<0.01$ compared with normal)
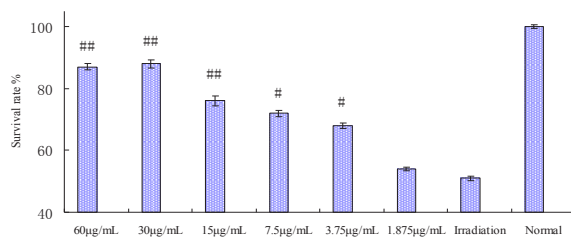

Figure 3. Survival Rates of AHH-1 Cells Treated with FRT of Different Concentrations. The survival rates of the irradiated cells gradually increased with increasing concentrations of FRT. The survival rate was the highest to 87% at a concentration of 30 µg/mL. (#, $p<0.05$ compared with irradiation alone; ##, $p<0.01$ compared with irradiation alone)
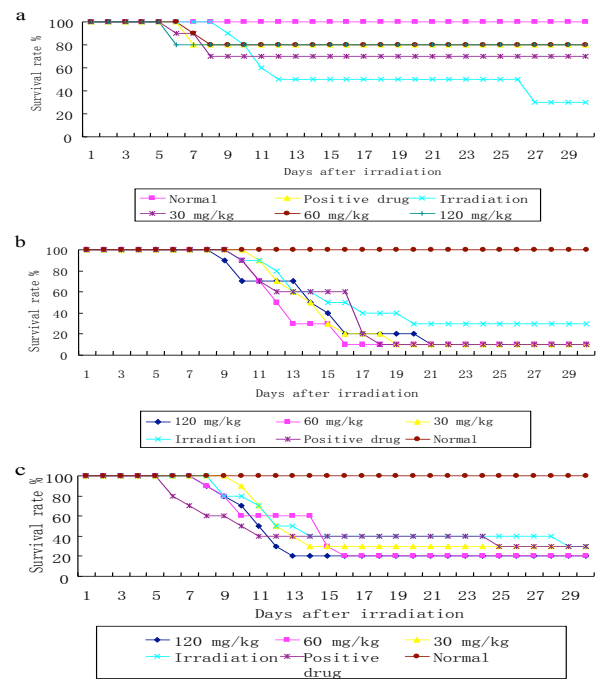

Figure 4. Survival Rates of Mice Treated with FRT. A) Administration of FRT before irradiation; **B)** Administration of FRT after irradiation; **C)** Administration of FRT before and after irradiation. Pretreatment with FRT of 30 mg/kg, 60 mg/kg and 120 mg/kg improved the survival rates by 40%, 50% and 50% respectively compared with untreated irradiated animals. Administration of FRT at different doses after irradiation all resulted in a survival rate of 10%, compared with 30% in untreated irradiated animals

Table 2. Effect of FRT on the Number of CFU-S

Groups	Number of CFU—S
Normal	0
Irradiation	36.3±3.5
Positive drug	72.1±5.1*
30 mg/kg	52.8±2.8*
60 mg/kg	68.4±3.2*
120 mg/kg	69.2±5.0*

With the increasing doses of drugs, the numbers of CFU-S increased, and were significantly different compared with model group. *$p<0.01$ compared with irradiation alone
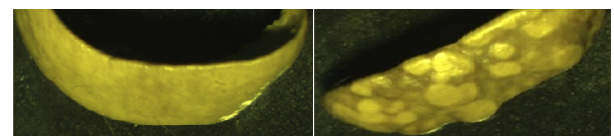

Normal   Drug administration

Figure 5. Effect of FRT on the Number of CFU-S. On the 6d after irradiation, CFU-S gradually formed
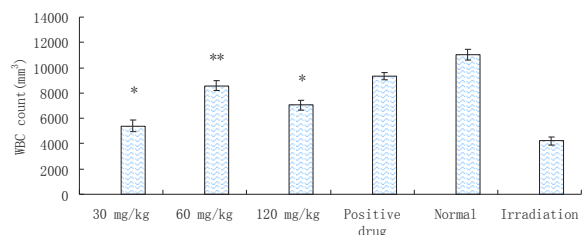

Figure 6. The Effect of FRT on the WBC Count of Radiation-Treated Animals. Radiation significantly lowered the WBC count, and the administration of FRT increased it. The data were presented as mean±S.D. *indicates $p<0.05$; ** $p<0.01$ compared with irradiation

With the increasing doses of low, medium and high, the numbers of CFU-S increased, and were significantly different compared with model group. There is a dose dependent manner.

Discussion

In recent years, it was found that many flavonoids (Devi et al., 1998, Liu et al., 2002, Zielonka et al., 2003, Wu et al., 2004, Li et al., 2004) in plants had effects of anti-radiation. Rosa roxburghii Tratt Contained flavonoids of 5981~12895 mg every 100g, which was 120~360 times citrus, 11~12 times ginkgo biloba, and was known as "the flavonoids king". FRT were extracted out of Rosa roxburghii Tratt and they were a good anti-oxidant. However, no study has yet reported on the radioprotective activity of it. The present study therefore reported the use of FRT as a radioprotective agent in experimental cell and animal models.

Exposure to ionizing radiation significantly decreased cell survival in irradiated cells. FRT at a dose of 30 μg/mL significantly prevented cell damage ($p<0.01$). Similarly, exposure of mice to radiation at 8 Gy also led to lethal damage, and pretreatment with FRT increased the survival rate to 80%, compared with 30% in the untreated irradiation group. FRT elevated the number of CFU-S in mice after exposure to a potentially lethal irradiation. Overall, these results suggest that FRT were a good radioprotector. Further studies are needed to investigate the potent radioprotective mechanism of FRT. The content of total flavonoids of FRT determined by UV spectrophotometry was 80%.

Pretreatment with FRT showed good anti-radiation effect, however, FRT administered after irradiation, before and after irradiation had negative effects on radiation protection. It was may be that the destruction of the structure of biological macromolecules, such as DNA, after radiation damage in mice was not completely repaired (Xu et al., 2011).

Several mechanisms, including a potent antioxidant activity, immune response, and enhanced recovery of bone marrow have been suggested for radioprotection (Malick et al., 1978). The results from the present study suggest that hematopoietic stem cells can be protected from radiation-induced free radical damage by FRT, which was evident in the increased number of radiation-induced spleen colonies (CFU-S) and hematological constituents in peripheral blood in animals of the FRT and radiation combined group (Figure 6). Animals were irradiated at the dose lower than the lethal. The bone marrow and the hematopoietic microenvironment were severely damaged, and the residual hematopoietic stem cells moved to spleen to proliferate and differentiate. Finally, a certain number of spleen colonies were formed on the surface of spleen. Spleen colonies could indirectly reflect ability of proliferation and differentiation of the residual hematopoietic stem cells, and prompt the ability of reconstruction and recovery of bone marrow from radiation injury. Counting of spleen colonies was reliable method for anti-radiation ability of drugs (Chen 2002).

Lymphocytes are the key components of blood, bone marrow, thymus, spleen and other immune organs are very sensitive to radiation. The important reason of immune function suppressed is that lymphocytes are injuried by radiation. It is important that lymphocytes are effectively protected from radiation damage to reduce body damage. So we selected lymphocytes AHH-1 to study anti-radiation drugs.

The ability of ionizing radiations to kill cancer cells through the induction of cell damage makes this an important modality in the therapeutic approach against cancer in humans. But normal human tissues are also affected to the damaging effects of ionizing radiations. The degree of cell damage induced by radiation depends on numerous factors, including the radiation dose, its scheduled administration, the stage of the cell within the cell cycle, the levels of cellular antioxidant defense system, and the availability of oxygen in the tissues (Rajagopalan et al., 2002). Presently we have studied only the hematopoietic recovery by FRT. Even though we have studied the effect of FRT by using mice, it may be effective on other strains of animals also.

Acknowledgements

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