Radioprotective effects of hesperidin against genotoxicity induced by γ-irradiation in human lymphocytes

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The radioprotective effect of hesperidin against genotoxicity induced by γ-irradiation has been investigated in vitro in cultured blood lymphocytes from human volunteers. Peripheral blood samples were collected at 0 (10 min before) and at 1, 2 and 3 h after a single oral ingestion of 250 mg hesperidin. At each time point, the whole blood was exposed in vitro to 150 cGy of 60Co γ-irradiation and then the lymphocytes were cultured with mitogenic stimulation to determine the micronuclei in cytokinesis-blocked binucleated cells. For each volunteer, the results showed a significant increase in the incidence of micronuclei after exposure of cells to γ-irradiation as compared to control samples. The lymphocytes in the blood samples collected at 1 h after hesperidin ingestion and exposed in vitro to γ-rays exhibited a significant decrease in the incidence of micronuclei, compared with similarly irradiated lymphocytes from blood samples collected at 0 h. The maximum protection and decrease in frequency of micronuclei (33%) was observed at 1 h after ingestion of hesperidin. These data have important application for the protection of human lymphocytes from the genetic damage and side effects induced by γ-irradiation in patients undergoing radiotherapy.

Introduction

Ionizing radiation passing through living tissues generates free radicals. Interactions of free radicals with DNA can induce DNA damage and lead to mutagenesis and carcinogenesis (1). With respect to potential application of ionizing radiation in medical practices (e.g. radiotherapy and nuclear medicine), the development of effective radiomodifiers is highly desired (2). Amifostine, which belongs to the class of synthetic thiol compounds (3,4), is a powerful radioprotective agent compared to other agents, but this drug has limited use in clinical practice due to its side effects and toxicity. The search for less toxic radiation protectors has spurred interest in the development of natural compounds. We reported that citrus and hawthorn herbal extracts contained high amounts of flavonoids. Flavonoids are a family of polyphenolic compounds found in fruits and vegetables. They have wide-ranging biological properties including antibacterial, antiviral, anticancer, immunostimulant and antioxidant effects (7). Herbal plants have high levels of phenolic compounds that have radioprotective effects (2,6). Recently, we showed the hesperidin, a flavonoid, has powerful protection effects on DNA damage, reducing the frequency of micronuclei induced by γ-irradiation in mice (8). Hesperidin has been reported to have many biological effects including anti-inflammatory, antimicrobial, anticarcinogenic, antioxidant effects and decreasing capillary fragility (9). In combination with a flavon called diosmin, it is used as Daflon® to treat chronic venous insufficiency in Europe (10). This drug is used safely in humans for treatment of chronic diseases. Although many studies have been performed for evaluation of radioprotective effects of natural products in animals, there are few studies that have assessed radioprotection by compounds of natural origin in human volunteers for reducing genetic side effects induced by ionizing radiation.

Thus, the important evaluation effects of hesperidin are warranted on genotoxic effect induced by γ-rays in human volunteers. In continuation of this investigation, the in vivo radioprotective activity of hesperidin has been investigated by in vitro radiation-induced genetic damage in radioprotective of natural origin compounds.

Materials and methods

Human blood samples

This study was performed after obtaining permission from the medical ethics committee of the university. Informed consent was obtained from five healthy, non-smoking human volunteers, males aged between 25 and 35 years. Hesperidin as drug grade was weighed and placed in a gelatin capsule. After overnight fasting, each volunteer was given a single oral dose of 250 mg of hesperidin at 9 a.m. Blood samples were collected in heparinized tubes before (~10 min) and 1, 2 and 3 h after the ingestion of the hesperidin capsule.

γ-Irradiation and cell culture to determine micronuclei

At each of the collection times, for each volunteer, aliquots of heparinized whole blood was divided into two tubes of 1 ml. One tube was the control sample and another tube was irradiated at 37°C with 60Co source (Theratron 780, Canada) with a dose of 150 cGy at a dose rate of 99.81 cGy/min. The samples were kept at 37°C for 1 h. Subsequently, 0.5 ml of each sample (control and irradiated) was added to 4.5 ml of RPMI 1640 culture medium (Gibco), containing 20% fetal calf serum, 100 μl/5 ml phytohemagglutinin (Gibco), 25 μl/5 ml penicillin, 250 μg/5 ml streptomycin and 2 μM glutamine (Sigma) at final concentration. All cultures were set up in duplicate and incubated at 37 ± 1°C in a humidified atmosphere of 5% CO2/95% air. Cytochalasin B (Fluka, final concentration: 30 μl/5 ml) was added after 44 h of culture. At the end of 72 h of incubation, the cells were collected by centrifugation and re-suspended in 0.075 M cold potassium chloride for 8 min at 1000 r.p.m., then fixed immediately in fixative solution (methanol:acetic acid, 6:1) three times. Fixed cells were dropped onto clean microscopic slides, air-dried and stained with Giemsa solution. All slides were coded by an individual other than the score and evaluated at ×100 magnification for the frequency of micronuclei in cytokinesis-blocked binucleated cells with well-preserved cytoplasm (11). Criteria for scoring of micronuclei were diameters between 1/16th and one-third of main nuclei, non-refractile, not linked to main

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nuclei and not overlapping the main nuclei (11). At each blood collection time, for each volunteer, from irradiated and control cultures, a total of 2000 binucleate cells (1000 cells each from duplicate cultures) were examined to record the micronucleus frequency.

**Statistical analysis**

For each volunteer, at each blood collection time, the incidence of radiation-induced micronuclei was recorded. The data were analysed by Student’s t-test.

**Results**

There were no side effects observed after the single oral dose of 250 mg hesperidin. The percentage of micronuclei induced by γ-irradiation on lymphocytes of all five volunteers at 0 (before extract ingestion) and at 1, 2 and 3 h after the ingestion of hesperidin were 12.04 ± 0.27, 8.12 ± 0.77, 11.6 ± 1.32, 12.6 ± 0.55, respectively; the data showed a significant increase in the incidence of micronuclei in irradiated lymphocytes than in the control cells without irradiation. The frequencies of micronuclei found in the hesperidin-treated cell groups were significantly much lower than that of the control group with radiation alone at zero time. Lymphocytes in the blood samples collected at 1 h after the oral ingestion of hesperidin and exposed in vitro to 1.5 Gy γ-radiation exhibited a significant decrease in the incidence of micronuclei as compared with similarly irradiated lymphocytes in the blood collected at 0 h ($ P < 0.001$); the total micronuclei values were 33% fold less in the 1 h after the oral ingestion of hesperidin (Table I). It was usually lower at 1 h as compared with those at 2 and 3 h after the oral dose of hesperidin (Figure 1). We did not observe a time-dependent decrease in the incidence of micronuclei in lymphocytes after ingestion of hesperidin. These data showed that hesperidin is rapidly absorbed and distributed through blood circulation after oral administration. A typical image of a binucleated cell with a micronucleus is shown in Figure 2.

**Discussion**

We previously reported that hesperidin protected mouse bone marrow cells against γ-irradiation, when injected prior to exposure to γ-rays (8). Although many herbal preparations and compounds of plant origin have been evaluated as radioprotective agents in animals, there are few examples of experiments to demonstrate the efficacy of these extracts in human volunteers. With respect to side effects induced by ionizing radiation in patients undergoing radiotherapy, the radioprotectors have an important role for tolerance and increasing survival rate in patients. The results of this study demonstrated the protective effects of hesperidin against genotoxicity induced by γ-irradiation in human lymphocytes. Hesperidin is found mainly in citrus family. It has been reported to have several health beneficial effects, including chemoprotection against carcinogenesis in the colon (12,13) and bladder (10), reducing oxidative stress in rat liver and kidney (14). Vasoprotector and venotonic effects are the most potential use of hesperidin in patients. Hesperidin with

| Table I. The percentages of micronuclei induced in vitro by 150 cGy γ-radiation in cultured blood lymphocytes from human volunteers examined at 0 (10 min before) and at 1, 2 and 3 h after a single oral ingestion of 250 mg hesperidin |
|---|---|---|---|---|
| Volunteer | Time | 0 h | 1 h | 2 h | 3 h |
| Volunteer 1 | Control | 0.4 | 0.5 | 0.6 | 0.5 |
| Radiation | 13.6 | 8.8 | 13.4 | 12.8 |
| Volunteer 2 | Control | 0.2 | 0.4 | 0.3 | 0.3 |
| Radiation | 10.4 | 8.4 | 12 | 12 |
| Volunteer 3 | Control | 0.8 | 0.8 | 0.7 | 1 |
| Radiation | 12.8 | 8.8 | 12 | 13.4 |
| Volunteer 4 | Control | 1.2 | 1 | 1.3 | 1.1 |
| Radiation | 11.8 | 7.2 | 10.4 | 12.2 |
| Volunteer 5 | Control | 0.8 | 0.5 | 0.6 | 0.9 |
| Radiation | 11.6 | 7.4 | 10.2 | 12.6 |

*a1000 binucleate cells were examined in each culture.
diosmin as Daflon is used in treatment of chronic venous insufficiency in Europe (10). Ionizing radiation generates free radical damage in DNA and induces genotoxic effects and death in the cells (1). Free radical scavenging is apparently responsible for inhibitory effect of flavonoids such as rutin, morin, quercetin and genistin on the clastogenic activity induced by γ-irradiation in mice (15). The molecular mechanism of the radioprotective effects of hesperidin is not clear. It has shown antioxidant activity in the cellular oxidative stress associated with neurodegenerative diseases; it attenuated decreases of glutathione peroxidase and glutathione reductase activities and decreased DNA damage in H2O2-induced PC12 cells (16); it also inhibited low-density lipoprotein (LDL) oxidation (17). In this study, we have shown that administration of hesperidin protects human lymphocytes against genotoxicity induced by γ-irradiation. The observations made in all five volunteers in this study clearly demonstrated the reduction in the incidence of micronuclei in their lymphocytes at 1 h after oral ingestion of hesperidin, as compared with the levels at 2 and 3 h. These data suggest that hesperidin acts effectively as a free radical scavenger since radioprotection by radical scavenging would be expected to be concentration dependent. There is a maximum leukocyte protection at 1 h after oral administration.

Melatonin is studied widely as a radioprotective agent in animals and in vitro models. It has low toxicity and showed good antioxidant activity by scavenging free radicals and increasing the activity of some important antioxidant enzymes such as superoxide dismutase. The melatonin chemical structure contributes to its antioxidant activity (18). A similar method to that used here was applied to blood lymphocytes of human for assessment of melatonin as a natural hormone for radioprotection. A single oral dose of 300 mg of melatonin was given to four healthy human volunteers. Peripheral blood samples were collected at different times and exposed in vitro to 1.5 Gy of γ-radiation. A significant decrease in the frequency of micronuclei was observed, as compared with similarly irradiated lymphocytes from the blood sample. Antioxidant activity is the main mechanism of radioprotective effect of melatonin (19). Recently, we showed that the administration of hawthorn extract to healthy humans protects against DNA damage induced by γ-irradiation in cultured lymphocytes. The maximum protection was observed 1 h after ingestion of extract (20). Few studies have been performed that use in vitro/in vivo methods to investigate radioprotective efficacy. Since the study of radioprotective effects in human volunteers is limited due to non-permission to irradiate healthy humans, for experimental research, this method can be used in clinical practice for evaluation of efficacy of safe radioprotective agent in human volunteers. Since hesperidin has been used extensively as a phlebotropic drug, in addition to being safe, this drug could be a useful candidate for protection of occupationally exposed individuals, such as those working in medical practice involving radiotherapy.

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References


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