Radioprotective Effects of Citrus Extract Against γ-Irradiation in Mouse Bone Marrow Cells

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The radioprotective effects of citrus extract were investigated by using the micronucleus test for anti-clastogenic and cell proliferation activity. A single intraperitoneal (i.p.) injection of citrus extract (Citrus aurantium var. amara) at 250, 500, 1,000 mg/kg body weight 1 h prior to γ-ray irradiation (1.5 Gy) reduced the frequencies of micronucleated polychromatic erythrocytes (MnPCEs) and normochromatic erythrocytes (MnNCEs). All three doses of citrus extract significantly reduced the frequencies of MnPCEs and MnNCEs in mice bone marrow compared to non-drug-treated irradiated control (p < 0.005–0.05). The optimum dose for protection in mouse was 250 mg/kg to protect mice bone marrow 2.2-fold against the side effects of γ-irradiation with respect to the non-drug-treated irradiated control. The flavonoids were contained in citrus extract, probably to show protective activity, and reduced the clastogenic effect of radiation on mice bone marrow. Therefore fruits and vegetables contain flavonoids to be useful as protective effects under such stress conditions as irradiation.

INTRODUCTION

With respect to radiation damage to humans, it is important to seek possible radioprotectants to modify the normal response of biological systems to radiation-induced toxicity or lethality.1 It has been known that 5-hydroxytryptamine (5-HT), s-2-(3-amino-propylamino)ethyl phosphorothioic acid (WR-2721)2 and prostaglandin3 have protective action against irradiation. Using an in vivo electron spin resonance study, Miura Y. et al. recently showed that WR-2721 has powerful radioprotective effects compared to 5-HT and Trolox.5 However, its toxicity has limited its application in medicine or in hazardous radiation environments.4 For this reasons, the search for less-toxic radiation radioprotectants has spurred interest in the development of different compounds. Epidemiological studies have shown that the consumption of vegetables and fruits protects against a variety of diseases, including coronary heart disease, stroke and cancer.6 Flavonoids are a family of polyphenolic compounds found ubiquitously in fruits and vegetables as well as in food products and beverages derived from plants, such as tea, olive oil and citrus.7,8 As evidenced in the most recent literature, flavonoids have been reviewed for their wide biological properties, including hepatoprotective, antithrombotic, antibacterial, antiviral, anticancer and immunostimulant activities.9 These physiological benefits of flavonoids are generally thought to be due to their antioxidant and free-radical scavenging properties.10 As phenolic compounds, flavonoids can scavenge free hydroxyl and peroxy radicals; on the other hand, as metal-chelating agents, they may extract irons and, by this depress the superoxide-driven fenton reaction, which is currently considered to be the most important route to active oxygen radicals.11,12 Flavonoids reduce the frequency of micronucleated reticulocyte (MNRETs) in mice exposed to γ-rays.13,14 It is known that γ-rays generate hydroxyl radicals in organisms and induce cellular DNA damage, which leads to mutation and chromosomal aberrations.15 The main flavonoids found in most cultivated citrus species are flavanone glycosides, such as hesperidin and naringin. These compounds can account for up to 5% of the dry weight of the leaf and fruit tissue.16 Citrus flavonoids were reported to decrease capillary fragility and to improve blood flow, and were actually labeled “Vitamin P.” Other therapeutic usages are anticancer17,18 and antiulcer.19 Flavonoids such as hesperidin exhibit strong antioxidant activity.20

In continuation of this line of investigation, the in vivo radioprotective activity of citrus aurantium var. amara extract was investigated by using γ-rays as an oxidative DNA damaging agent, and evaluating any reduction in the frequency of micronu-
cleated polychromatic erythrocytes (MnPCEs) and normochromatic erythrocytes (MnNCEs) in mouse bone marrow exposed to γ-rays. The radioprotective effects of citrus extract were studied with the micronuclei method as a reliable method for the detection of chromosomal damage by chemical agents and radiation.21–24)

**MATERIALS AND METHOD**

**Preparation of citrus extract**

The ripe fruit of *citrus aurantium var.amara* collected in the area of Amol, Iran, and is consumed as a fruit in Iran. Dried peels of the plant (100 g) were extracted at room temperature with 75% aqueous ethanol (750 ml) for 24 h. After filtration, the solvent was evaporated under reduced pressure at 40°C until the ethanol was removed. The aqueous medium was shaken with chloroform to remove liposoluble substances, and the aqueous layer was evaporated under reduced pressure. In this way, 18 g of extract as a powder was obtained (18% w/w).

**Diagnostic test of flavonoid**

The presence of flavonoids in citrus extract was estimated by the Mg method.25) Two milliliters of aqueous ethanol extract solution was added to 0.5 ml of concentrated HCl and 20 mg of metal Mg. After boiling, 1 ml of amyl alcohol was added and shacked. A strong cherry-red color indicated high amounts of flavonoid in the citrus extract.

**Animals**

Male BALB/c mice weighing 25 ± 3 g were purchased from the Pasteur Institute (Tehran, Iran). Mice were housed in good conditions in the university animal house and given standard mouse pellets and water *ad Libitum*. All of the mice were kept under a controlled lighting condition (light:dark, 12:12 h) and temperature (22 ± 1°C).

**Irradiation**

Whole-body irradiation was performed with a cobalt-60 γ-radiation source (Teratron 780, Canada). Mice were placed in ventilated Plexiglas cages and irradiated in groups of five mice simultaneously. The source-to-skin distance was 80 cm with a dose rate of 74 cGy/min at room temperature (23 ± 2°C). The mice were irradiated with a total dose of 1.5 Gy γ-rays.

**Pretreatment of mice with citrus extract**

All solutions were freshly prepared before to eating of the animals. The citrus extract was dissolved in steril-distilled water. Three test doses (250, 500 and 1,000 mg/kg) of freshly prepared *citrus aurantium* were administered to the experimental animals intraperitoneally (i.p.) 1 h before gamma irradiation. The control animals received the same volume of steril-distilled water.

**Micronucleus assay**

The mouse bone marrow micronucleus test was carried out according to Schmid to evaluate of the chromosomal damage in experimental animals.21) The animals were sacrificed by cervical dislocation 24 h after irradiation. The bone marrow from both femurs was flushed in the form of a fine suspension into a centrifuge tube containing fetal calf serum (FCS). The cells were dispersed by gentle pipetting and collected by centrifuge at 1,500 rpm for 10 min at 4°C. A cell pellet was resuspended in a drop of FCS, and bone-marrow smears were prepared. The slides were coded to avoid any observed bias. After 24 h of air-drying, the smear were stained with May-Grunwald/Giemsa, as described by Schmid with this method, polychromatic erythrocytes (PCEs) stain reddish-blue and normochromatic erythrocytes (NCEs) stain orange, while nuclear material is dark purple.25) For each experimental point five mice were used, and a total of 5,000 PCEs were scored per each experimental point to determine the percentage of micronucleated polychromatic erythrocytes (MnPCEs), micronucleated normochromatic erythrocytes (MnNCEs) and the ratio of PCE to (PCE+NCE). The ratio of PCE to (PCE+NCE) was determined for each experimental group to assess the radiation effects with or without citrus extract on bone-marrow proliferation.26,27)

**Statistical analysis**

The data are presented as means ± SD. The significance of any intergroup differences in the number of micronucleated PCE and NCE, as well as the ratio of PCE to (PCE+NCE) was statistically evaluated by the Mann-Whitney U-test.

**RESULTS**

**Effects of γ-irradiation on mice bone marrow**

A diagnostic test with Mg powder and concentrated HCl gave an intense cherry-red color, which indicated high amounts of flavonoids in the citrus extract.

The results obtained in experiments with γ-irradiation are summarized in Table 1. The untreated control group showed 1.35% and 1.23% MnPCEs and MnNCEs, respectively. The frequency of PCE and NCE with Mn increased after exposure to 1.5 Gy of γ-ray. Statistically significant increase in MnPCE and MnNCE were observed in the group that received only 1.5 Gy irradiation (p < 0.01). This data showed that γ-irradiation increased the frequency of Mn in mice bone marrow. The determination of the ratio of PCE/PCE+NCE in the γ-irradiated mice showed a pronounced cytotoxicity effect of radiation on bone-marrow proliferation (Table 1). The ratio of PCE/PCE+NCE reduced after exposure to 1.5 Gy of γ-irradiation, but this reduced ratio was not significantly lower that of the untreated control group (p > 0.05) (Table 1).

**Effects of citrus extract on radiation-induced micronuclei**

Similar to the group that received radiation alone, groups of mice were treated with 250, 500 and 1,000 mg/kg of citrus extract 1 h prior to irradiation. The frequencies of MnPCE and MnNCE found in the latter groups were significantly much
lower than that of the treated group with radiation alone. The total MnPCE values were 2.1, 1.8 and 2.5-fold less in the 250, 500 and 1,000 mg/kg citrus group after being exposed to 1.5 Gy of γ-rays, respectively, than that in the respective irradiated control (Table 1). Although all three doses were effective in significantly reducing (p < 0.005–0.05) the frequency of MnPCEs induced by 1.5 Gy radiation, there was no significant difference between the effects of various doses of citrus extract. With a further increase in the citrus dose to 1,000 mg/kg, there was no significant difference with other doses of citrus extract on the frequency of MnPCE induced by γ-irradiation. An analysis of variance performed on data from MnPCEs showed no significant difference for a dose of 250 mg/kg to citrus treated the group + irradiation and the unirradiated control group. This data showed a suppressive effect of citrus extract on radiation-induced-clastogenic effects.

The frequency of MnNCEs was significantly lower in the citrus + irradiated group, when compared to the irradiated-alone group at all three doses of citrus extract by factors of 2.1, 2.4 and 1.6 for 250, 500 and 1,000 mg/kg extract (Table 1).

The treatment of mice with citrus extract arrested the radiation-induced decline in the PCE/PCE+NCE ratio (Table 1), and this increase in the PCE/PCE+NCE ratio in the citrus extract + irradiated groups was higher than that of the irradiated-alone group, though differences were not statistically significant.

### DISCUSSION

We previously reported that synthetic 2-iminothiazolidine-thiol containing compounds protect mice against a lethal dose of γ-irradiation. Difficulties were encountered when administering aminothiols to humans which caused adverse toxic effects, such as hypotension, nausea, vomiting and allergy. Thus, the use of these agents is limited because of their toxicities. It is quite possible that antioxidant phytochemicals consumed in human diet might provide a variable degree of radioprotection. Natural antioxidants, including flavonoids, may play a role in scavenging free radicals, such as hydroxyl radicals with γ-rays, in mice. There is a possibility that a pretreatment with flavonoids induces a tolerable function against oxidative stress. The frequency of Mn in PCE and NCE has been significantly increased by γ-ray irradiation. We have also confirmed that 1.5 Gy γ-rays increased the MnPCE and MnNCE in mice bone marrow (p < 0.005). These results show that the Mn test is a reliable effective alternative for the evaluation of clastogenic effects of irradiation.

Table 1 shows the influence of citrus extract on the frequencies of MnPCEs and MnNCEs in bone marrow-irradiated animals, permitting a study of the potential toxicity of treatment vs their anti-clastogenic activity. The mice with to eat of citrus extract prior to irradiation showed very low levels of MnPCEs and MnNCEs generation with respect to the irradiated-alone group. The citrus significantly reduced the frequency of Mn, and showed radioprotective and anticlastogenic effects against the side effects of γ-irradiation on mice bone marrow. The radioprotective effect and, consequently, the anticlastogenic activity of different doses of citrus extract were established according to the reduction in MnPCEs and MnNCEs in animals after irradiation and a comparison with the level observed in control irradiated animals. This gave a percentage value that reflected the degree of protection offered by each dosage of treatment against gamma irradiation. Although various doses of citrus significantly protect mice bone marrow (p < 0.05), statistically there is no significant difference between different doses. The optimum dose for protection in mouse is 250 mg/kg body weight; the higher dose of citrus extract showed no significant anticlastogenic or cell proliferation compared to that for a dose of 250 mg/kg (p > 0.05). An important color test in an alcoholic solution is reduction with magnesium powder and concentrated HCl; flavonoids give a cherry-red color. A citrus extract alcoholic solution had an intense cherry-red color, indicating high amounts of flavonoid in citrus extract. This result confirms other studies that flavonoids are widely presented in citrus. It is quite possible that antioxidant phytochemicals consumed in human diet might provide a variable degree of radioprotection. Natural antioxidants, including flavonoids, may play a role in scavenging free radicals, such as hydroxyl radicals with γ-rays, in mice. There is a possibility that a pretreatment with flavonoids induces a tolerable function against oxidative stress. The frequency of Mn in PCE and NCE has been significantly increased by γ-ray irradiation. We have also confirmed that 1.5 Gy γ-rays increased the MnPCE and MnNCE in mice bone marrow (p < 0.005). These results show that the Mn test is a reliable effective alternative for the evaluation of clastogenic effects of irradiation.

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can protect mice bone marrow from the side effects of total-body γ-irradiation when it is injected prior to γ-ray exposure. Under oxidative stress condition, when the endogenous antioxidant systems are defective or insufficient, an exogenous agent with a strong radical scavenging capacity must be used. Fruits and vegetables contain flavonoids used prior γ-irradiation; they may reduce the interaction of free-radical induced γ-irradiation with critical macromolecules, such as DNA, and protect cells against the side effects of γ-irradiation on cells.\(^{39}\) In conclusion, our results demonstrate that citrus extract gives significant protection to mice bone marrow against the clastogenic effect of gamma irradiation. It is important that fruits and vegetables contain flavonoids that can protect human organs from the side effects of oxidative stress. In our research, we showed that crude citrus extract is useful if consumed as supplement food to reduce the side effects of irradiation in hazardous radiation environments.

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**REFERENCES**


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