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Citrus extract modulates genotoxicity induced by cyclophosphamide in mice bone marrow cells

Seyed Jalal Hosseinimehr and Mohmmad Karami

Abstract

The protective effect of citrus extract was investigated by using the micronucleus assay for anticlastogenic activity in mouse bone marrow cells; liver glutathione (GSH) content was determined against toxicity induced by cyclophosphamide. Mice were orally (gavage) pretreated with solutions of citrus peel extract *(Citrus aurantium* var. *amara)* prepared at three different doses (100, 200 and 400 mg kg^{-1} body weight) for 7 consecutive days. Then mice were injected intraperitoneally on the seventh day with cyclophosphamide (50 mg kg^{-1}) and after 24 h killed for the evaluation of micronucleated polychromatic erythrocytes (MnPCEs) in bone marrow cells. Non-protein thiol levels in liver were estimated in mice injected with citrus extract with or without cyclophosphamide treatment. Administration of citrus extract before cyclophosphamide treatment significantly reduced the frequency of MnPCEs in mice bone marrow compared with the group treated with cyclophosphamide alone ($P < 0.0001$–0.05). Citrus extract at a dose of 400 mg kg^{-1} reduced MnPCEs 2.8 fold against genotoxicity induced by cyclophosphamide. Administration of cyclophosphamide depleted the GSH level in liver. Citrus extract showed excellent scavenging effects on 1,1-diphenyl-2-picryl hydrazyl radical (DPPH) at a concentration of 1.6 mg mL^{-1}. Application of citrus extract 1 h before cyclophosphamide treatment allowed GSH content to reach the normal level. It appeared that citrus extract, particularly flavonoids constituents with antioxidative activity, may return the GSH level to normal in stress conditions and reduces genotoxicity induced by cyclophosphamide in bone marrow cells.

Department of Medicinal Chemistry, Faculty of Pharmacy, Mazandaran University of Medical Sciences, Sari, Iran

Seyed Jalal Hosseinimehr

Department of Toxicology and Pharmacology, Faculty of Pharmacy, Mazandaran University of Medical Sciences, Sari, Iran

Mohmmad Karami

Correspondence: S. J. Hosseinimehr, Department of Medicinal Chemistry, Faculty of Pharmacy, Mazandaran University of Medical Sciences, Sari, Iran. E-mail: sjhosseinim@yahoo.com

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Introduction

Many environmental and industrial chemicals are capable of inducing genotoxic effects in exposed organisms. To deal with attack by toxic chemicals, the human body is equipped with self-defence mechanisms such as detoxification processes through various enzymes. However, increased exposure to hazardous chemicals can lead to mutagenic and carcinogenic events. Epidemiological studies have shown that consumption of vegetables and fruits protects against a variety of diseases, including cancer (Graham 1983; Tiwari 2001). Many fruits, vegetables and foods have been reported to have potential antimutagenic or anticarcinogenic effects (Steinmetz et al 1991a, b; Vijayalaxmi & Venu 1999). Citrus fruits are commonly consumed because they contain a high amount of vitamins, minerals and antioxidant compounds, such as flavonoids. Flavonoids are a family of phenolic compounds that have many biological properties, including hepatoprotective, antithrombotic, antibacterial, antiviral and anticancer activity. These physiological benefits of flavonoids are generally thought to be due to their antioxidant and free radical scavenging properties (Tiwari 2001). The main flavonoid found in the most cultivated citrus species is hesperidin. This compound can account for up to 5% of the dry weight of the leaf and fruit tissue (Betrhow 1998). Citrus flavonoids were reported to decrease capillary fragility and to improve blood flow, and were actually labelled vitamin P. Other therapeutic usages include anticancer and antiulcer (Martin et al 1994; Guthrie et al 1995). Recently we reported that citrus extract protects mice bone marrow cells against gamma irradiation (Hosseinimehr et al 2003). Thus, the importance of evaluating the effects of citrus extract on mutations induced by chemicals is warranted. This study was undertaken to assess the effects of *Citrus aurantium* var. *amara* peel extract against genotoxicity

induced by cyclophosphamide in mouse bone marrow cells using the micronucleus test. We also studied the protective effect of citrus extract on the glutathione (GSH) content of mouse liver which was affected by cyclophosphamide.

Materials and Methods

Chemicals

Cyclophosphamide was obtained from Asta Medica AG (Frankfurt, Germany). Hesperidin and 5,5′-dithio-(bis-2-nitrobenzoic acid) (DTNB) were provide by Sigma and Fluka Chemical Co. (Buchs, Switzerland), respectively. All other chemicals were obtained from Merck company.

Animals

Male Balb/c mice, 25 ± 3 g, were purchased from the Pasteur Institute (Tehran, Iran). Mice were housed in a good condition in the university animal house and given free access to standard mouse pellet and water. All mice were kept under controlled lighting conditions (12-h light–dark) and temperature (22 ± 1°C). Ethical approval for the study was granted by the scientific and ethical committee of Manzandaran University.

Preparation of citrus extract

The ripe fruits of *Citrus aurantium* var. *amara* were collected from Amol in Iran. The peel was dried at room temperature and powdered in a grinder. Aqueous methanol (75%, 750 mL) was added to the powdered peel (100 g) and then stirred for 1 h. The mixture was allowed to stand for 24 h at room temperature. After filtration, methanol was evaporated under reduced pressure at 40°C until the methanol was removed. The remaining aqueous medium was shaken with chloroform (50 mL) to remove lipid-soluble substances. The chloroform phase was discharged, and the aqueous layer was evaporated under reduced pressure. The remaining water extract was freeze-dried. In this way, 25.5 g of extract powder was obtained (25.5% w/w).

Determination of flavonoid

The flavonoid content was determined by the Mg method (Harborne 1998). Citrus extract (100 mg) was placed in a 100-mL volumetric flask and diluted with methanol (dimethyl sulfoxide (DMSO) 5%). Extract solution (50 mL) was added to 10 mL of concentrated HCl and 250 mg of metal Mg. After boiling, a strong cherry-red colour indicated high amounts of flavonoids. After 30 min at room temperature, the absorbance was determined against a blank at 560 nm with a UV-visible spectrophotometer (Shimadzu, Japan). Since the hesperidin content is high in the Citrus family, it was used as a standard for the calibration curve. The flavonoid content was expressed in g of hesperidin/100 g of extract powder by comparison with standard hesperidin-treated samples under the same conditions. The flavonoid content was calculated using the following linear equation based on the calibration curve:

$$Y = 1.3061X - 0.0065,\ r^2 = 0.995 \quad (1)$$

Experimental protocol

For micronucleus assay, mice were divided into 7 groups (Groups 1–7) of five mice each: Group 1, normal control; Group 2, positive control, received a single dose of cyclophosphamide (50 mg kg^{-1}, i.p.) in saline (10 mL kg^{-1}); Group 3, treated with a single intraperitoneal dose of cyclophosphamide, 1 h after the last dose of citrus extract (100 mg kg^{-1} per day for 7 days) in phosphate buffer pH 7.2 (10 mL kg^{-1}) by gavage; Group 4, treated with a single intraperitoneal dose of cyclophosphamide, 1 h after the last dose of citrus extract (200 mg kg^{-1} per day for 7 days) in phosphate buffer pH 7.2 (10 mL kg^{-1}) by gavage; Group 5, treated with a single intraperitoneal dose of cyclophosphamide, 1 h after the last dose of citrus extract (400 mg kg^{-1} per day for 7 days) in phosphate buffer pH 7.2 (10 mL kg^{-1}) by gavage; Group 6, treated with citrus extract (200 mg kg^{-1} per day for 7 days) in phosphate buffer pH 7.2 (10 mL kg^{-1}) by gavage; Group 7, treated with citrus extract (400 mg kg^{-1} per day for 7 days) in phosphate buffer pH 7.2 (10 mL kg^{-1}) by gavage.

For determination of glutathione levels in mouse liver, two concentrations of citrus extract (200 and 400 mg kg^{-1}) were used. Mice were treated with a single dose of citrus extract at 1 h before cyclophosphamide administration. Three mice were used for each experimental end point.

Micronucleus assay

The mouse bone marrow micronucleus test was carried out according to Schmid for evaluation of the chromosomal damage in experimental animals (Schmid 1975; Hosseinimehr et al 2003). The mice were sacrificed by cervical dislocation, 24 h after cyclophosphamide treatment. 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 1500 rev min^{-1} for 10 min at 4°C. The cell pellet was resuspended in a drop of FCS and bone marrow smears were prepared. The slides were coded to avoid observed bias. After 24 h air-drying, smears were stained with May-Grunwald/Giemsa as described by Schmid (1975). For each experimental point, five mice were used and a total of 5000 polychromatic erythrocytes (PCEs) were scored per each experimental point to determine the percentage of micronucleated (Mn)PCEs.

Determination of GSH level

The level of reduced glutathione was determined using the method of Sedlak & Lindsay (1968). The mice were sacrificed by cervical dislocation 4 h after cyclophosphamide treatment. Livers were excised quickly. The liver sample was homogenized with a 0.2 M EDTA solution at a

concentration of 100 mg/1.5 mL. Protein precipitation was carried out with 5% trichloroacetic acid (TCA). The homogenized mixture was then centrifuged at 3500 rev min^{-1} for 15 min. The supernatant was collected for GSH. One millilitre of supernatant was mixed with 2 mL of 0.4 M Tris buffer (pH 8.9) and 0.5 mL of 2 mM 5,5′-dithiobis-2-nitrobenzoic acid (DNTB reagent). The absorbance of the resultant mixture was measured at 412 nm. The reduced form of GSH was used to construct the standard curve for the calculation of GSH level. The final GSH level was expressed as μM of GSH per g liver.

Measurement of free radical scavenging activity

The free radical scavenging capacity of citrus extract was determined as bleaching of the stable 1,1-diphenyl-2-picryl hydrazyl radical (DPPH) (Koleva et al 2002). Different concentrations of citrus extract (0.04–1.6 mg mL^{-1}) were added, at an equal volume, to an ethanolic solution of DPPH (100 μM). After 15 min at room temperature, the absorbance was recorded at 517 nm. The experiment was performed in triplicate. Butylated hydroxytoluene (BHT) was used as an antioxidant standard. Percent of scavenging was calculated using the formula [(Control − Test)/Control] × 100.

Statistical analysis

The data are presented as means ± s.d. One-way analysis of variance and Tukey's HSD test were used for multiple comparisons of data. $P = 0.05$ was accepted to denote significance.

Results

Effects of citrus extract on MnPCEs induced by cyclophosphamide

The flavonoid content was 9.2 ± 0.17 g/100 g in extract powder on the basis of hesperidin as a standard, indicating that there is a high amount of flavonoids in the citrus peel extract.

The effect of cyclophosphamide with or without citrus extract on the induction of MnPCEs in bone marrow cells, 24 h after cyclophosphamide treatment, is shown in Table 1. Mice were treated with 100, 200 or 400 mg kg^{-1} of extract for 7 days before cyclophosphamide treatment. The frequency of micronuclei was increased in all groups of mice treated with cyclophosphamide compared with the control. In mice injected with the extract and cyclophosphamide, the number of MnPCEs was found to be decreased compared with those treated with only cyclophosphamide. Citrus extract at doses of 200 and 400 mg kg^{-1} significantly reduced ($P < 0.0001$–0.05) the frequency of MnPCEs induced by cyclophosphamide treatment. There was a significant difference between the effects of 100, 200 and 400 mg kg^{-1} of citrus extract on the frequency reduction of MnPCE induced by cyclophosphamide. Citrus extract at doses of 200 and 400 mg kg^{-1} cannot induce any genotoxicity and cytotoxicity in bone marrow cells (the PCE/PCE + NCE ratio was not significantly different to control group).

The frequency of MnPCEs was lower in the citrus extract + cyclophosphamide group by a factor of 1.16, 1.5 and 2.8 for the three doses of extract of 100, 200 and 400 mg kg^{-1}, respectively, compared with the cyclophosphamide-treated group (Table 1). This data showed citrus extract to have a suppressive action on cyclophosphamide-induced clastogenic effects.

Effect of citrus extract on GSH content in mice liver

The normal level of non-protein-GSH in liver was about 5.19 μmol GSH equivalents/g tissue (Figure 1). A single

Table 1 Effects of citrus extract (CE) on the formation of cyclophosphamide (CP)-induced micronucleated polychromatic erythrocytes (MnPCEs)

Group	Treatment	MnPCE/PCE (%)
1	Control	0.36 ± 0.09
2	CP	2.50 ± 0.22[a]
3	CP + CE 100 mg kg^{-1}	2.16 ± 0.22
4	CP + CE 200 mg kg^{-1}	1.70 ± 0.32[b,e]
5	CP + CE 400 mg kg^{-1}	0.9 ± 0.23[c,f]
6	CE 200 mg kg^{-1}	0.36 ± 0.11[d]
7	CE 400 mg kg^{-1}	0.33 ± 0.18[d]

Values are means ± s.d. for each group of five mice. [a]$P < 0.0001$ compared with control; [b]$P < 0.05$ compared with CP alone; [c]$P < 0.0001$ compared with CP alone; [d]no significant difference compared with control; [e]$P < 0.05$ compared with group 3; [f]$P < 0.0001$ compared with groups 3 and 4.

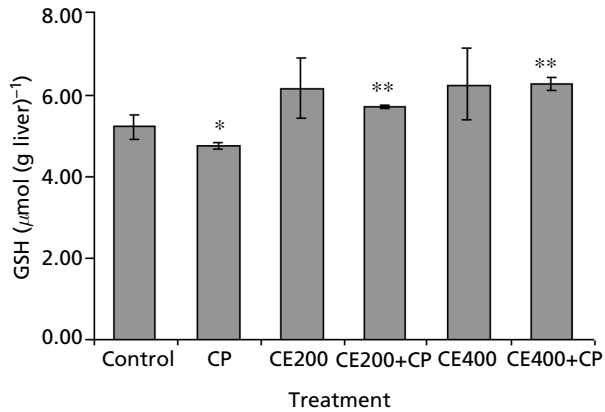

Figure 1 Effect of citrus extract (CE) at doses of 200 and 400 mg kg^{-1} and cyclophosphamide (CP) at dose 50 mg kg^{-1} on liver GSH content in mice. The glutathione content was determined by DTNB reagent at 412 nm. Results are mean ± s.d. obtained from three samples. *$P < 0.05$ compared with control group; **$P < 0.05$ compared with group treated with CP alone.

intraperitoneal dose of cyclophosphamide (50 mg kg^{-1}) administered to mice 4 h before sacrificing resulted in 9% depletion of the GSH level in liver ($P < 0.05$ vs control group). Administration of citrus extract at doses of 200 and 400 mg kg^{-1} increased the hepatic GSH content up to 6.23 μmol (g liver)$^{-1}$. A single intraperitoneal dose of citrus extract administered 1 h before cyclophosphamide treatment caused an increase in GSH content which had been reduced by cyclophosphamide treatment ($P < 0.05$ vs cyclophosphamide-administered group).

Antioxidant activity

The DPPH free radical scavenging method can be used to evaluate the antioxidant activity of specific compounds. An excellent scavenging effect was observed with citrus extract. Scavenging effects of ethanolic extracts were enhanced with increasing concentration; 89.2% was achieved at 1.6 mg mL^{-1} (Figure 2). At lower concentrations, the scavenging effects of BHT were more pronounced than those of citrus extract, while citrus extract displayed a higher antioxidant activity than BHT when used at a higher concentration ($P < 0.001$).

Discussion

We previously reported that citrus extract could protect mice bone marrow cells against gamma irradiation when injected 1 h before exposure (Hosseinimehr et al 2003). The results of this study show the protective effects of citrus extract against genotoxicity caused by cyclophosphamide in mouse bone marrow cells. The protective effect of citrus extract on genotoxicity induced by cyclophosphamide was analysed by determining the frequency of MnPCEs in mouse bone marrow cells at 24 h after cyclophosphamide application and also by estimating the GSH content in mouse liver at 4 h after cyclophosphamide treatment. Cyclophosphamide and its metabolites may be involved in the toxic reactions and cause DNA damage, inducing genotoxic effects in the cells (Krishna et al 1995; Moore et al 1995). In our study, administration of citrus extract for 7 days resulted in a dose-dependent inhibition of micronuclei formation induced by cyclophosphamide in mouse bone marrow cells. Citrus extract significantly reduced the frequency of MnPCEs to show a protective and anticlastogenic effect against the side effects of cyclophosphamide.

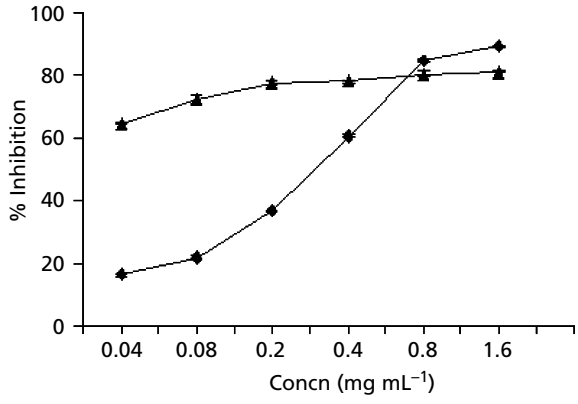

Figure 2 Scavenging effect of different concentrations (0.04–1.6 mg mL^{-1}) of citrus extract (◆) and butylated hydroxytoluene (BHT) (▲) on the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical. The absorbance was recorded at 517 nm after 15 min at room temperature. The experiment was performed in triplicate.

Oxidative damage is one of the many mechanisms leading to cancer and other chronic diseases. A high intake of fruit and vegetables, including citrus fruits, may reduce the risk of cancer (Tiwari 2001; Silalahi 2002). Among the many compounds found in fruit and vegetables are flavonoids. These compounds have been approved as antioxidant agents. Flavonoids, including hesperidin, have strong radical scavenging properties and reduce side effects due to oxidative damage (Tiwari 2001). Flavonoids reduce toxic damage to critical macromolecules such as DNA. Citrus species are extremely rich in flavonones, a class of compounds belonging to the flavonoid family (Proteggente et al 2003). Our results have shown that citrus peel extract contained high amounts of flavonones. Hesperidin, as a flavonone, inhibits chemical-induced tumour promotion and it has potential chemopreventive activity against colon and bladder carcinogenesis (Tanaka et al 1997; Yang et al 1997; Berkarda et al 1998) and it inhibited tumour-promoting activity of the Epstein-Barr virus early antigen (EBV-EA) (Iwase et al 1999). *Citrus unshiu*, which contains hesperidin, has chemopreventive ability against mouse lung tumorigenesis induced by chemical agents (Kohno et al 2001). Although antioxidative effects of flavonoids are proven (Tiwari 2001), other protective mechanisms of these compounds are being investigated. Some flavonoids interact directly with nucleophilic metabolites of polycyclic aromatic hydrocarbons, while others inhibit metabolic activation of promutagens, including benzo(α)pyrene and aflatoxin B_1 (Delaney 2002). In our research we have shown that citrus extract increased the non-protein thiol level in the cells (Figure 1). The intracellular thiol level is accepted to be important in determining the extent of cellular damage induced by stress shock. The intracellular GSH concentration may determine the sensitivity of cells to damage produced by the anticancer drug (Donnerstag et al 1996). WR-2721 and GSH are thiol compounds that protect against the cyto- and genotoxic effects of cyclophosphamide in bone marrow cells with increasing thiol content in the cells (Blasiak et al 2002). The elevation of GSH level by citrus extract can participate in protecting against the genotoxicity induced by cyclophosphamide in bone marrow cells. Flavonoids act either by trapping the initiating radicals or by propagating lipid peroxyl radicals and recycling α-tocopherol. The strong antioxidative activity of citrus extract with elevation of GSH levels in the cells contributed to reduction in the genotoxicity induced by cyclophosphamide.

Conclusions

The aim of this study was to investigate the protective effect of citrus extract against genotoxicity induced by cyclophosphamide in mice bone marrow cells using the micronucleus assay. In this study, citrus extract showed excellent antioxidant activity in a concentration-dependent manner. Citrus extract increased the GSH level in mice liver which had been depleted by cyclophosphamide. Our results showed that citrus extract containing flavonoids significantly reduced the enhancement of MnPCE frequency caused by cyclophosphamide in bone marrow cells. It appeared that citrus extract, particularly its flavonoid constituents with antioxidative activity, may return the GSH level to normal in stress conditions induced by cyclophosphamide. These results showed that citrus peel extract is useful if it is continuously consumed as a food supplement to reduce the genotoxicity induced by hazardous chemical agents.

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