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Full Length Research Paper

Antimutagenic effects of red apple and watermelon juices on cyclophosphamide-induced genotoxicity in mice

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Studies on agents that modulate carcinogen-induced genotoxicity in experimental animals provide end points that can be used to assess the anti-mutagenic properties of putative chemopreventive compounds. Red apple and watermelon juices were assessed for modulation of cyclophosphamide (CP)-induced proportion of polychromatic erythrocyte (PCE) and frequency of micronucleated polychromatic erythrocytes (MNPCE) in mice. Three groups of five mice each were given 100, 50 or 25% concentration of fruit juice *ad libitum* for 7 days; intraperitoneally (*ip*) injected with 40 mg/kg CP and sacrificed 24 h later for preparation of bone marrow smears and analysis. Control group animals drank water before injection with CP (positive) or distilled water (negative). Each group mean of the proportion of PCE and frequency of MNPCE was compared with the group mean of the concurrent negative and positive control using the Mann-Whitney U test. No difference in the proportion of PCE was found between any group and the negative control ($P<0.05$) which suggested that CP treatment alone or after pre-treatment did not induce erythropoietic cell toxicity. Pre-treatment with 100% apple or 25% watermelon juice reduced the frequency of CP-induced MNPCE ($P \leq 0.05$), while pre-treatment with 100% watermelon juice increased the frequency of CP-induced MNPCE.

Key words: Genotoxicity, cyclophosphamide, micronuclei, apple, watermelon.

INTRODUCTION

In respiring cells, a small amount of the consumed oxygen is reduced, yielding a variety of highly reactive chemical entities, collectively called reactive oxygen and nitrogen species (RONS), including nitric oxide (NO), superoxide (O_2^-), hydroxyl radical (•OH), hydrogen peroxide (H_2O_2) and peroxynitrite (ONOO–) (Suzuki et. al., 1997). *In vivo*, some of these RONS play positive roles in cell physiology; however, they may also cause great damage to cell membranes and DNA, leading to cancer, degenerative and other diseases (Ames et al., 1993; Finkel and Holbrook, 2000). Mammalian cells possess elaborate defense mechanisms for free radical detoxification to protect against oxidative damage. Endogenously, antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) are capable of degrading reactive oxygen species into inert compounds through a series of chemical reactions (Ames et al., 1981). In addition to antioxidant enzymes, non-enzymatic molecules of an exogenous nature that are obtained from food, such as α-tocopherol, β-carotene, and ascorbic acid, play important roles in antioxidant defense systems (Halliwell and Gutteridge, 1998). When the balance between ROS production and antioxidant defenses is lost, 'oxidative stress' results through which a series of events deregulates the cellular functions and leads to various pathological conditions including aging, arthritis, asthma, carcinogenesis, diabetes, rheumatism and various neurodegenerative diseases (Gupta and Sharma, 2006).

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Abbreviations: CP, Cyclophosphamide; **PCE,** polychromatic erythrocyte; **MNPCE,** micronucleated polychromatic erythrocytes.

Subsequently, anti-oxidants of plant origin have generated interest as anticarcinogens and as defenses against degenerative diseases (Byers and Perry, 1992). Phenolic compounds act as antioxidants with mechanisms involving both free radical scavenging and metal chelation and have been shown to be more effective antioxidants *in vitro* than vitamins E and C on a molar basis (Rice-Evans et al., 1997). Fruit and vegetables provide the best polypharmacy against the development of a chronic disease, considering that they contain a vast array of antioxidant components such as polyphenols, ascorbate and tocopherol (Ames et al., 1993). Watermelon is rich in carotenoids particularly lycopene, β-cryptoxanthin, beta-carotene and vitamin E (Isabelle et al., 2007; Charoensiri et al., 2009). Apples are a rich source of dietary fibre and phytochemicals, including polyphenol antioxidants, such as procyanidins, quercetin, catechin, phloridzin and chlorogenic acid (Knekt et al., 1996).

A critical factor in mutagenesis is cell division (Ames et al., 1993). When the cell divides, an unrepaired DNA lesion can give rise to a mutation. Thus, an important factor in mutagenesis, and therefore carcinogenesis is the cell division rate in the precursors of tumor cells. Oxidants form one important class of agents that stimulate cell division. This may be related to the stimulation of cell division that occurs during the inflammatory process accompanying wound healing. Antioxidants therefore can decrease mutagenesis, and thus carcinogenesis, in two ways: By decreasing oxidative DNA damage and by decreasing cell division (Ames et al., 1993). However, there is an increasing literature on the protective role of dietary tocopherol, ascorbate, and ß-carotene in lowering the incidence of a wide variety of human cancers (Byers and Perry, 1992). Consumption of naturally occurring compounds can modify the mutagenic and carcinogenic effects of environmental contaminants (Gimmler-Luz et al., 1999). The mutagenic activities of the tetracyclic nitroarenes 3-nitrofluoranthene (3-NFA) and l-nitropyrene (1-NP) in *Salmonella typhimurium* TA98 were reduced by β-carotene, retinol, retinal, retinoic acid, retinol palmitate, riboflavin 5′-phosphate, α-tocopherol, vitamins B_{12}, C, K_1 and K_3, as well as biliverdin, bilirubin, chlorophyll, chlorophyllin and haemin (Tang and Edenharder, 1997). Moreover, apple and watermelon juices have been shown to scavenge hydroxyl (•OH) and superoxide radical ($O_2^{-\bullet}$) radicals and to inhibit lipid peroxidation, and the correlation of these parameters with the ascorbic acid content of the fruit and juices (Leonard et al, 2002).

Furthermore, supplementation with β-carotene reduced the micronucleus (MN) count in epithelial cells of heavy smokers' sputum and in lymphocytes of healthy volunteers exposed to X-rays (Van Poppel et al., 1992). Mutagenic and antimutagenic activities have been correlated with the presence of certain phytochemical substances such as compounds of the flavonoid group (Brown, 1980; Edenharder et al., 1993); and these metabolites could be involved in mutagen deactivation (Horn and Vargas, 2003). *In vivo* studies, however, give some contradictory results, perhaps due to the use of different species and administration routes (Gimmler-Luz et al., 1999). In mice, β-carotene protected bone marrow cells against the genotoxic effect of cyclophosphamide (Salvadori et al., 1992). However, despite the protection against DNA single-strand breaks induced by benzo-[a]pyrene (BaP) in murine for stomach mucosa, no protection was found of β-carotene oral pretreatment against MN induced in bone marrow cells (Lahiri et al., 1993). Studies on agents that modulate carcinogen-induced genotoxic effects in experimental animals provide end points that can be used for assessing the antimutagenic or anticarcinogenic properties of putative chemopreventive compounds and for predicting their protective efficacy in humans (Khaidakov et al., 2001). The micronuclei in young erythrocytes arise primarily from dislocated chromosomes from disturbed mitotic spindle or chromosome fragments that are not incorporated into the daughter nuclei at the time of cell division in the erythropoietic blast cells and changes in the incidence of micronucleated polychromatic erythrocytes (MNPCE) are considered to reflect chromosomal damage (Salamone and Heddle, 1983).

Cyclophosphamide (CP) is a promutagen that is first oxidized by the microsomal cytochrome P450-linked enzyme to be further converted into its biologically reactive ultimate metabolites, acrolein, phosphoramide mustard and nornitrogen mustard. Phosphoramide mustard alkylates DNA (Mohn and Ellenberger, 1976). The purpose of this study was to investigate the modulatory effects of red apple (*Malus domestica*) and watermelon (*Citrullus lanatus*) juices on the incidence of cyclophosphamide (CP)-induced micronucleated polychromatic erythrocytes (MNPCEs) and the cytotoxicity of cyclophosphamide to erythrocytes in mice bone marrow. Both fruits are widely consumed.

MATERIALS AND METHODS

All animals used for this study were about 8 to 10 week-old male inbred NIH mice. The original stock of mice was purchased from the Animal Unit of the University of Free State (Bloemfontein, Republic of South Africa), from which we bred our own colony that was housed with food (pelleted horse feeds (Voernet (PTY) LTD Republic of South Africa)) and tap water *ad libitum* in the animal house of the Biology Department of the National University of Lesotho.

Fruit juice processing

Watermelon and Red apple were purchased from Fruits and Vegetables Market™ in Maseru, Lesotho. For apples, the fruits were washed with clean water, cut open and the seeds removed. The flesh together with the back was chopped into small pieces. While for watermelon, the fruits were washed with clean water, the back was peeled off, the seeds were removed and the fruit was chopped into small pieces. The pieces of each fruit were placed in a Philips Comfort HR 1727 blender and blended into a paste. The paste was then filtered through ten layers of cheese cloth and named 100% fruit juice. The 100% juice was further diluted with drinking water at 1:1 or 1:3 (v/v) and named 50 and 25% juice

respectively. The juices were stored frozen in a deep freezer at -15°C.

Mice were randomly distributed into cages; five mice per cage and tail marked. Three groups of five male mice per group were given 100, 50 or 25% juice, respectively as the sole liquid source *ad libitum,* for 7 days. A negative control group and a positive control group of five mice each were given water as the sole liquid source during the 7 days that the experiment lasted. For each experiment, there were five groups in all, each group housed in a (34 × 22 × 18 cm) cage. All mice were allowed free access to pellet horse cubes. The juices, water and food pellets were changed daily.

Micronucleus test

To evaluate the modulatory action of the juices on cyclophosphamide (CP)-induced clastogenicity and toxicity, the juices-pretreated groups and the positive control group received on the 6th day (that is 24 h before sacrifice), a single dose of 40 mg/kg intraperitoneal (*ip*) injection of CP (Fluka Biochemika, Germany) dissolved in purified water BP (MEDICOLAB, Republic of South Africa). The negative control group animals were intraperitoneally (*ip*) injected with distilled water. The *ip* administrations were done at a rate of 10 ml/kg body weight.

Slide preparation

The mice were sacrificed by cervical dislocation 24 h after *ip* injection on the 7th day of pretreatment with juice or water, in the case of the control groups. Both femoral bone marrows were dissected from each animal and slide preparations done based on the technique developed by Schmid (1975). Briefly, the bone marrow from both femurs of each animal was flushed out from the marrow canal with 1 ml of pre-filtered newborn calf serum (SIGMA, USA) at 37°C using a 21G1/2 needle attached to a 1 ml syringe and pooled in a centrifuge tube. The marrow suspension was centrifuged at 1560 × *g* for 5 min using an IEC CL2 table-top centrifuge. The supernatant was discarded to leave a small drop in which the pellet was re-suspended by vortexing. One drop of the suspension was smeared on a glass microscope slide, air dried and fixed in methanol for 10 min. The preparations were air dried again after which they were stained with 10% Giemsa at pH 6.8 for 10 min. The smear was rinsed in 4 changes of buffered (6.8) purified water, air dried and euparal added and covered with a cover slip to make the slides permanent. After the slides were dried, they were coded and scored blind using the 100X objective lens with oil immersion of a light microscope (OLYMPUS CXS21). The most representative of the erythrocytes of each category were photographed using a Zeiss PrimoStar microscope mounted with Canon camera model, Power Shot A640.

To evaluate bone marrow toxicity, the ratio of polychromatic erythrocyte (PCE) to total erythrocytes, that is PCE and normochromatic erythrocytes (NCE) was calculated by counting a total of 1000 erythrocytes per animal using these slides; PCE/(PCE + NCE). To determine the frequency of micronucleated polychromatic erythrocytes (MNPCE), all together 2000 PCEs per animal were examined for the presence of micronuclei, which means 10,000 PCEs were scored per dose group. Micronucleus frequency per thousand (MN º/oo) PCEs was calculated as follows: MN º/oo = (number of PCEs containing micronucleus/total number of PCEs counted) × 1000.

Statistical analysis

The statistical analysis was performed using a non-parametric approach and the SPSS (version 10.0) software. To determine the modulatory effect of the plant juices on CP-induced frequency of MNPCE and toxicity, the three dose groups of each plant juice (100, 50 and 25%) and the negative control group were separately compared with the concurrent positive control group, in the incidences of MNPCE/1000 PCEs and the PCE/(PCE + NCE), ratio were determined by the Mann-Whitney U test for 2 independent samples. A statistical difference in the medians of the positive control and a test group was recorded when the calculated value of U was smaller than the tabulated critical value at a significance level of 0.05 ($p < 0.05$).

RESULTS

The results of the modulatory effects of the red apple and watermelon juices on CP-induced *in vivo* mouse bone marrow clastogenicity and toxicity are presented in Table 1. The intraperitoneal injection of mice with a single dose of 40 mg/kg body weight of CP induced a significant increase in the frequency of MNPCE 24 h after injection when compared with animals that received *ip* injection of distilled water. However, no statistically significant difference was found in the proportion of PCE in any of the experimental groups compared with the negative control ($P<0.05$), suggesting that CP treatment alone or following pre-treatment with the fruit juices did not induce erythropoietic cell toxicity.

Moreover, all three doses of apple juice and the 50 and 25% doses of watermelon juice reduced the frequency of CP-induced MNPCE (Figure 1). However, the reductions achieved the level of statistical significance ($P\leq 0.05$) only for 100% apple juice and 25% watermelon juice. Pre-treatment with 100% watermelon juice before the *i.p* injection with CP increased the frequency of CP-induced MNPCE by about 55.88%, which was not statistically significant ($P>0.05$).

DISCUSSION

The results of the present study are summarized in Table 1. The proportion of PCE in any of the experimental groups was not statistically different from that of the negative control group animals, which suggested that intraperitoneal injection of CP alone or following pre-treatment with red apple or watermelon juice did not induce erythropoietic cell toxicity. The observation of non-induction of erythropoietic cell toxicity in mice after intraperitoneal injection of 40 mg/kg body weight of CP was similar to that of Gimmler-Luz et al. (1999). The fruit juices that were used in the present study were complex mixtures, containing varying concentrations of all or some of vitamin C, vitamin E, various carotenoids, flavonoids, phenolics (quercetin, anacardic acids and tannin), glutathione, rutin, lutein, lycopene, metals etc, as biologically active compounds (Knekt et al., 1996; Isabelle et al., 2007; Charoensiri et al., 2009). Singly, some of the different vitamins and other components have been shown to inhibit the genotoxicity of different mutagens *in vitro* and *in vivo*.

Table 1. Summary table of the modulatory effects of fruit juices on the incidence of CP-induced frequency of MNPCE and frequency of PCEs in total erythrocytes in mouse bone marrow of 8-10 week-old males inbred NIH mice after 7 days pretreatment with fruit juices.

Treatment	Dose	MNPCE/1000 PCEs				%(PCE/ (PCE + NCE))		
		Individual, group mean and SD	% Change of CP-Induced MNPCE	Comparison of each group with control (Mann-Whitney U-values)		Individual, group mean and SD	Comparison of each group with control (Mann-Whitney U-values)	
				Negative control (water treated)	Positive control (CP-treated)		Negative control (water treated)	Positive control (CP-treated)
Water	100%	1; 0.5; 2; 0.5; 0.5; (0.9±0.652).		12.5	0*	0.393; 0.443; 0.482; 0.495; 0.475; (0.458±0.041)	12.5	7
CP	40 mg/kg (BW)	15; 24; 9; 12; 8; (13.6±6.43)	0.00	0*	12.5	0.37; 0.518; 0.439; 0.439; 0.139; (0.382±0.145)	7	12.5
Red apple	100%	1.5; 2.5; 4.5; 9.0; 2.0 (3.9± 3.07)	71.32↓	2*	1.5*	0.379; 0.347; 0.527; 0.461; 0.447 (0.4323 ± 0.071)	9	11
	50%	3.5; 7; 7; 6.5; 6.5; (6.1± 1.475).	55.15↓	0.00*	12	0.373; 0.432; 0.466; 0.504; 0.456; (0.446± 0.0484).	10	9
	25%	8.5; 3.0; 3.5; 4.5; 7.0; (5.3± 2.3611).	61.03↓	0.00*	8.5	0.512; 0.398; 0.447; 0.422; 0.491; (0.454±0.047)	12	9
Watermelon	100%	27;18; 21; 18;22; (21.2±3.70)	55.88↑	0.00*	4	0.25; 0.607; 0.481; 0.452; 0.481; (0.455±0.128).	12	7
	50%	4; 5; 4.5; 8; 3; (4.9± 1.884).	63.97↓	0.00*	8	0.402; 0.486; 0.307; 0.398; 0.305; (0.3802±0.0758).	6	10
	25%	3.5; 4.5; 4.5; 6.5; 1.5; (4.1±1.817).	69.85↓	1.00*	0*	0.446; 0.471; 0.408; 0.377; 0.403; (0.421±0.037)	7	11

PCE = Polychromatic erythrocytes; NCE = Normochromatic erythrocyte; MNPCE = Micronucleated PCE; CP = Cyclophosphamide. * = There is a statistically significant difference between the medians ($P \leq 0.05$, Mann – Whitney U Test). The Null Hypothesis is therefore rejected. (U values at n1 = 5 and n2 = 5); ↑=%Increase in CP-Induced MNPCE; ↓ =% reduction in CP-Induced MNPCE).

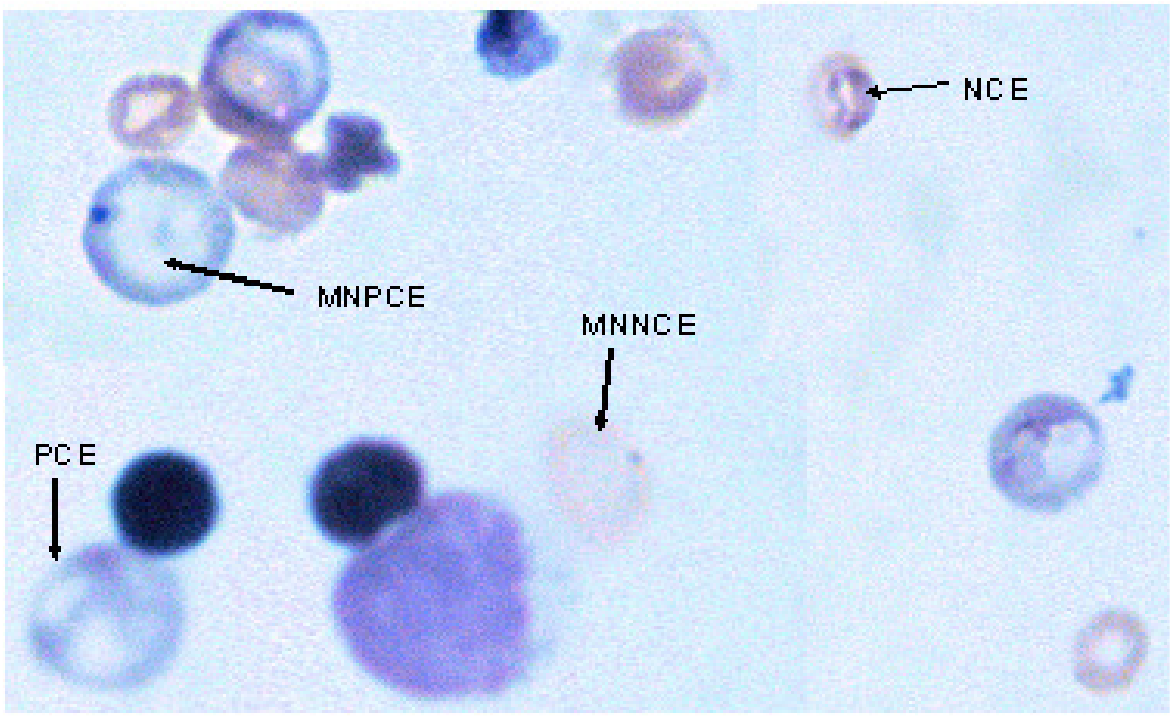

Figure 1. Mice bone marrow smear to show micronucleated and non-micronucleated polychromatic and normochromatic erythrocytes following treatment of male NIH mice with cyclophosphamide.

The frequency of MNPCE of the positive control group that received only CP injection and the experimental groups, that received CP injection after pre-treatment with red apple or watermelon juices were statistically higher than those of the negative control group ($p<0.05$). In a similar study, CP induced a significantly higher frequency of chromosome aberrations in adult mouse bone marrow compared to the negative control (Gimmler-Luz et al., 1999). Pretreatment of the mice with the fruit juices reduced the frequency of CP-induced MNPCE, which achieved the level of statistical significance ($P\leq0.05$) for 100% apple juice and 25% watermelon juice only. Salvadori et al. (1993) also observed a reduction in CP-induced chromosome aberrations in adult mouse bone marrow cells after a 5-day β-carotene pretreatment. Moreover, in studies using cultured mammalian cells, β-carotene inhibited the clastogenic effect of methyl methanesulfonate (MMS), 4-nitroquinoleine-1-oxide and cyclophosphamide (CP), but not that of some phenolic acids, of H_2O_2 or of mitomycin C (MMC) (Salvadori et al., 1993). In the *in vitro* Salmonella/reversion assay with TA98 and TA100, the mutagenicity of *N*-nitrosopiperidine (NPIP) was inhibited markedly (54%) by apple extract (Ikken et al., 1998).

Watermelon has been shown in the *in vitro* HPRT-mutagenicity test to strongly reduce the genotoxic activity of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) or 2-acetylaminofluorene (AAF) (Edenharder et al., 2002). It has been proposed that the reduction of MNPCE frequencies by fruits and vegetables could be due either to causation of a cell cycle delay in the bone marrow cells of animals which might influence the total number of micronuclei generated such that the recorded frequency of micronucleated erythrocytes would depend on the number of cells undergoing mitosis. Secondly, the reduction of MNPCE frequencies could be caused by the suppression of micronucleus induction (Edenharder et al., 2003). Pre-treatment with 100% watermelon juice before the *i.p* injection with CP increased the frequency of CP-induced MNPCE by about 55.88%. The increases, which suggested a synergistic effect was however not statistically significant ($P>0.05$). Some compounds have also been shown to exert antigenotoxic as well as genotoxic effects depending on the concentration (Edenharder et al., 2003). This principle well known for quercetin has, however, so far not been described in the mouse bone marrow micronucleus assay. The flavonoids quercetin and its glucoside isoquercitrin administered orally in doses of 0.03 mmol/kg body weight simultaneously with intraperitoneally given BaP, reduced the number of micronuclei in polychromatic erythrocytes of the bone marrow of mice by 73 and 33%. Ten-fold higher concentrations, however, reversed the effects with a particular strong increase observed with isoquercitrin (+109%) and quercetin (+16%) (Edenharder et al., 2003).

It has been observed that i*n vivo* studies give contradictory results, perhaps due to the use of different animal species and administration routes (Gimmler-Luz et al., 1999). The degree of inhibitory response too depends on

the mutagen/antioxidant combination and on the treatment regime (pre, post or simultaneous) (Aidoo et al., 1995). Finally, the reduction in the damage induced by CP also depends on the dose of antioxidant. For instance in mice, Salvadori et al. (1992) observed an increase in the anticlastogenic activity of ß-carotene at lower doses and an absence of a protective effect at higher concentrations and interpreted the observation to mean different mechanisms of ß-carotene modulation and a possible alteration of the balance of CP activation/detoxification mechanism of the promutagen.

Conclusion

Cyclophosphamide treatment of mice, alone or after pre-treatment with red apple or watermelon juices did not induce erythropoietic cell toxicity. Pretreatment of the mice with the fruit juices reduced the frequency of CP-induced MNPCE which achieved the level of statistical significance ($P \leq 0.05$) for 100% apple juice and 25% watermelon juice. Moreover, pre-treatment with 100% watermelon juice before the *i.p* injection with CP increased the frequency of CP-induced MNPCE by about 55.88%, which was not statistically significant ($P>0.05$). These results demonstrate the presence of natural antigenotoxins in red apple and watermelon. A study to determine the products in the juices that modified the genotoxic effects of CP and their mechanisms of action is necessary.

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