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Full Length Research Paper

Lack of modulatory effect of asparagus, tomato, and grape juice on cyclophosphamide-induced genotoxicity in mice

Asita Okorie Asita*, Mann E. Dingann and Sibusisiwe Magama

Department of Biology, National University of Lesotho, P.O. Roma 180 Maseru, Lesotho, South Africa.

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Studies on agents that modulate carcinogen-induced genotoxic effects in experimental animals are used to assess the antimutagenic or anticarcinogenic properties of putative chemopreventive compounds. We investigated the potency of asparagus-, tomato- and red grape-juice to modify the proportion of polychromatic erythrocyte (PCE) and frequency of micronucleated polychromatic erythrocytes (MNPCE) induced by cyclophosphamide (CP) in male NIH mice. Groups of five mice were given the fruit juices (25, 50 or 100%) respectively, *ad libitum*, for 44 days then intraperitoneally (*ip*) injected with 40 mg/kg CP and killed 24 h later for cytological preparations and analysis. The control group animals were injected with CP (positive) or purified water (negative). Each group mean of the proportion of PCE and frequency of MNPCE was compared with the negative and positive control using the Mann-Whitney test. No statistically significant difference was found between the proportion of PCE in any experimental group and the negative control (P<0.05), suggesting that CP treatment alone or CP following pre-treatment with any of the plant juice did not induce erythropoietic cell toxicity. Also, pre-treatment with the plant juices did not modify the frequency of CP-induced MNPCE in this mouse strain using the present route of administration and treatment regime.

Key words: Asparagus, tomato, grape, micronucleus, cyclophosphamide, ant-mutagenicity.

INTRODUCTION

Aging and its degenerative diseases appear to be due in good part to oxidative damage to DNA and other macro-molecules. Four endogenous sources appear to account for most of the oxidants produced by cells: 1) As a consequence of normal aerobic respiration in the mito-chondria, reactive oxygen species (hydrogen peroxide, H_2O_2), and free radicals such as the hydroxyl radical (·OH) and the superoxide anion ($O_2^{\cdot-}$), which are mutagens produced by radiation, are also by-products of normal metabolism. 2) Phagocytic cells destroy bacteria or virus-infected cells with an oxidative burst of nitric oxide (NO), $O_2^{\cdot-}$, H_2O_2, and hypochlorous acid (HClO). 3) Degrading of fatty acids (lipid peroxidation) and other molecules in peroxisomes, gives rise to mutagenic lipid epoxides, lipid hydroperoxides, lipid alkoxyl and peroxyl radicals, and enals (α, ß-unsaturated aldehydes). 4) Cytochrome P450 enzymes induction in animals, pre-vents acute toxic effects from foreign chemicals, but also results in oxidant by-products that damage DNA (Ames et al., 1993).

To protect against oxidative damage animals have many different types of antioxidants defenses, such as vitamin C (ascorbate), vitamin E (tocopherol), and carote-noids, some of which, including β-carotene can be metabolized to vitamin A (retinal), have generated parti-cular interest as anticarcinogens and as defenses against degenerative diseases (Frei and Ames, 1992; Byers and Perry, 1992). Antioxidant enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase are capable of degrading reactive oxygen species (ROS) into inert compounds through a series of chemical reactions (Ames et al., 1981). Fruits and vegetables are the principal source of ascorbate and carotenoids and are

*Corresponding author. E-mail: ao.asita@nul.ls. Tel: +266 22213171. Fax: +266 22 340000.

one source of tocopherol (Ames et al., 1993).

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).

There is an increasing literature on the protective role of dietary tocopherol, ascorbate, and β-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). Supplementation with β-carotene reduces 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; Umegaki et al., 1994). Studies i*n vivo* 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 protects bone marrow cells against the genotoxic effect of mitomycin C (MMC), benzo[*a*]pyrene (BaP) and cyclophosphamide (CP) (Raj and Katz, 1985; Salvadori et al., 1992). Similarly, despite the protection against DNA single-strand breaks induced by BaP in murine forestomach mucosa, no protection was found of β-carotene oral pretreatment against MN induced in bone marrow cells (Lahiri et al., 1993).

In this study, therefore, we have examined the modulatory effects of asparagus (*Asparagus officinalis*), tomato and red grape juices on the incidence of cyclophosphamide (CP) induced micronucleated polychromatic erythrocytes (MPCEs) and the cytotoxicity of cyclophosphamide to erythrocytes in mice bone marrow. Asparagus contains ascorbic acid or "vitamin C", gluta-thione and rutin, which has been reported to be the major antioxidant (Shao et al., 1997; Tsushida et al., 1994). Tomato fruit is a reservoir of diverse antioxidant molecules, such as ascorbic acid, vitamin E, carotenoids, flavonoids and phenolic acids. Lycopene, the carotenoid of interest (in tomato), has the highest antioxidant activity among all dietary antioxidants and has also been shown to induce cell-to-cell communications and modulation of hormones, immune systems and other metabolic pathways (DiMasico et al., 1989). Dietary intake of lycopene is epidemiologically correlated with diminished risk of prostate cancer and it has been found to be superior to α- and β-carotene in inhibiting cell proliferation in various human epithelial cancer cell lines (Giovannucci, 1999). Tomatoes also contain moderate amounts of α-and β-carotene and lutein. β-Carotene is known for its provitamin A activity and lutein for reduced risk of lung cancer (Sies, 1991).

In grapes, resveratrol (3, 5, 4'-trihydroxystilbene) a polyphenolic phytoalexin is found primarily in the skin, and in muscadine grapes also in the seeds (LeBlanc, 2005). Several studies have demonstrated that resveratrol, a flavonoid (stilbene) is an effective antioxidant (Chanvitayapongs et al., 1997). Resveratrol inhibits lipid peroxidation of low-density lipoprotein (LDL), prevents the cytotoxicity of oxidized LDL and protects cells against lipid peroxidation (Chanvitayapongs et al., 1997). It is thought that because resveratrol contains highly hydrophilic and lipophilic properties, it can provide more effective protection than other well-known antioxidants, such as vitamins C and E (Chanvitayapongs et al., 1997).

MATERIALS AND METHODS

Animals

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

Fruit juice processing

Asparagus, tomatoes and red grapes were purchased from Fruits and Vegetables market^{TM} in Maseru, Lesotho.For each fruit, the fruits were washed with clean water, chopped into small pieces and placed in a Philips Comfort HR 1727 blender and blended into a paste. The paste was filtered through ten layers of cheese cloth and named 100% fruit juice. The 100% juice was diluted in 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^{o}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 45 days. A negative control group and a positive control group of five mice each were given water as the sole liquid source, during the 45 days that the experiment lasted. There were five groups in all, each group housed in a (34 x 22 x 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 44th day (i.e. 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 received water treatments, and the positive control group also received *ip* CP treatment. 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 of CP on the 45th day of pretreatment with juice. 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^{O}C using a 21G1/2 needle attached to a 1 mL syringe and pooled in a centrifuge tube. The marrow suspension was centrifuged at 3000 rpm for 5 min. 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 to make a permanent slide and covered with a cover slip. After the slides were dried, they were coded and scored blind using a light microscope (OLYMPUS CXS21).

To evaluate bone marrow toxicity, the ratio of polychromatic erythrocyte (PCE) to total erythrocytes, *i.e.* 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), 2000 PCEs per animal altogether were examined for the presence of micronuclei, which means 10,000 PCEs scored per dose group. Micronucleus frequency (MN º/oo) was calculated as follows: MN º/oo = (number of PCEs containing micronucleus/ total number of PCEs counted) x 1000.

Statistical analysis

The statistical analysis was performed using a non-parametric approach and the SPSS 10.0 statistical program. 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 was 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.

RESULTS AND DISCUSSION

The results of the modulatory effects of the plant 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 water treatment. The proportion of PCE was not statistically different from that of the negative control animals in any of the experimental groups; suggesting that the treatment with CP alone or with CP after pre-treatment with the plants juices did not induce erythropoietic cell toxicity. No modification of the frequency of CP-induced MNPCE was observed in bone marrow smears of mice that were injected with 40 mg/kg body weight of CP following 44 days of the plant juices pre-treatment *ad libitum*, as the sole liquid source and sacrificed 24 h after the injection.

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 bast cells and changes in the incidence of 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).

In this study, following 44 days of water or any of three different doses of asparagus, red grape or tomato juice *ad libitum* as the sole liquid source, intraperitoneal injection of 8-10 week-old male inbred NIH mice with 40 mg/kg CP did not induce erythropoietic cell toxicity. Cyclophosphomide induced clastogenicity was higher in the positive control animals, and in the animals that were pre-treated with the different doses of any of the fruit juices before injection with CP, when compared to the animals not injected with CP (negative control) , to a statistically significant level (Table 1). However, the incidence of micronucleated polychromatic erythrocytes (MNPCEs) in all the groups of mice that were pre-treated with juice before CP injection was not statistically different from that of the positive control group. No modification of the frequency of CP-induced MNPCE was thus observed in bone marrow smears of mice that were injected with 40 mg/kg body weight of CP following 44 days of the fruit juices *ad libitum*, as the sole liquid source and sacrificed 24 h after the injection. From our results (Table 1) we conclude that the selected fruit juices did not protect mouse bone marrow cells against CP-induced lesions.

The fruit juices used in the present study are complex mixtures, containing varying concentrations of all or some vitamin C, vitamin E, various carotenoids, flavonoids, phenolics (quercetin, anacardic acids, tannin) , glutathione, rutin, lutein, lycopene, metals, etc, as biologically active compounds (Shao et al., 1997; Tsushida et al., 1994; DiMasico et al., 1989). Singly, the various vitamins and other components have been shown to inhibit the genotoxicity of different mutagens *in vitro* and *in vivo*. In *in vitro* studies using cultured mammalian cells, β-carotene can inhibit the clastogenic effect of methyl methanesulfonate (MMS), 4-nitroquinoleine-1-oxide and

Table 1. Modulatory effects of plant juices on the incidence of CP-induced frequency of MNPCE and PCEs in total erythrocytes in mouse bone marrow of 8-10 week-old male inbred NIH mice after 45 days pretreatment with plant juice.

Treatment	Dose (%)	MNPCE/1000 PCEs			% PCE/ (PCE + NCE)		
			Comparison of each group with control (MW U-values)			Comparison of each group with control (MW U-values)	
		Individual, group mean and SD	Water-treated	CP-treated	Individual, group mean and SD	Water-treated	CP-treated
Water	100	1;1.5; 1; 1.5; 1.5; (1.3±0.274).	12.5	4	0.434; 0.428; 0.371; 0.494; 0.444; (0.434±0.044).	12.5	10
CP	40 mg/kg bw.	3.5; 7.5; 5; 1; 11.5; (5.7±4.009).	4	12.5	0.36; 0.47; 0.376; 0.491; 0.415; (0.422±0.057).	10	12.5
Asparagus	25	22; 4; 8; 13; 6.5; (10.7±7.120).	0.00*	6	0.219; 0.382; 0.363; 0.367; 0.506; (0.3674±0.1018).	6	9
	50	4.5; 13; 7.5; 7; 4; (7.2± 3.581).	0.00*	9.5	0.382; 0.31; 0.436; 0.47; 0.319; (0.3834±0.0703).	8	8.5
	100	5; 11.5; 6.5; 15; 6; (8.8 ± 4.281).	0.00*	7	0.447; 0.404; 0.368; 0.435; 0.321; (0.395±0.0520)	8	9
Tomato	25	15.5; 7.5; 14; 12; 11; (12±3.062).	0.00*	2.5	0.318; 0.382; 0.43; 0.38; 0.392; (0.3840±0.0403).	5	9
	50	7; 18; 5.5; 18; 12; (12.1±5.899).	0.00*	4	0.488; 0.374; 0.427; 0.46; 0.437; (0.4372±0.0424).	12	11
	100	6.5; 4; 5.5; 6; 6; (5.6±0.962).	0.00*	11	0.442; 0.422; 0.41; 0.37; 0.43; (0.4148±0.0276).	7	12
Grape	25	6; 4.5; 3.5; 8.5; 6.5; (5.8±1.924).	0.00*	11.5	0.415; 0.375; 0.395; 0.319; 0.346; (0.37±0.0383).	3.0	5.5
	50	6; 4.5; 7;6; 15; (7.7±4.177)	0.00*	9	0.523; 0.422; 0.388; 0.454; 0.47; (0.4514±0.0509)	10.0	8.5
	100	37; 8.5; 22.5; 6; 16.5; (18.1±12.427)	0.00*	3	0.544; 0.496; 0.463; 0.446; 0.477; (0.4852±0.0377).	3.0	5

PCE = Polychromatic erythrocytes; NCE = Normochromatic erythrocyte; MNPCE = Micronucleated PCE; CP = Cyclophosphamide; SD = Standard deviation. MW = Mann-Whitney.

* = There is a statistically significant difference between the medians ($P \leq 0.05$, MW U Test) The Null Hypothesis is therefore rejected (U values at n1 = 5 and n2 = 5).

cyclophosphamide (CP), but not that of some phenolic acids, of H_2O_2 or of mitomycin C (MMC) (Salvadori et al., 1993). Studies *in vivo* however, give some contradictory results, perhaps due to the use of different species and administration routes (Gimmler-Luz et al., 1999). Gene mutations induced in rat spleen lymphocytes by the carcinogen N-ethyl-N-nitrosourea (ENU) and chromosome damage induced in Chinese hamster bone marrow by MMS, busulfan and thio-TEPA were reduced by β-carotene, but not the chromosome aberrations produced by CP (Aidoo et al., 1995). Salvadori and associates however, observed a reduction in CP-induced chromosome aberrations in adult mouse bone marrow cells after a 5-day β-carotene pretreatment (Salvadori et al., 1993). In the rat lymphocyte mutation assay (at the hypoxanthine guanine phosphoribosyl transferase (Hprt) locus), treatment of Fischer 344 rats with the dietary antioxidants vitamins C, E, β-carotene and the mineral selenium inhibited the mutant frequency (MF) induced by 7,12-dimethylbenz[a]anthracene (DMBA) or bleomycin (BLM) when the rats were given the antioxidants singly or in a combination 2 weeks prior to mutagen treatment and continued for an additional 4 weeks post-mutagen treatment. However, the degree of inhibitory response was dependent on the type of mutagen and the particular antioxidant while mixtures of antioxidants displayed low inhibitory responses compared to non-mixtures (Khaidakov et al., 2001). A five days pretreatment of 8-10 weeks old male Balb C mice with beta-carotene reduced the frequency cyclophosphamide (CP)-induced chromosomal aberrations in bone marrow cells. However, no direct dose-response relationship was detected, suggesting that beta-carotene might act through different mechanisms at different doses (Salvadori et al., 1992).

Studies with complex fruit- or plant juices show that results obtained depended on the plant species, plant part, treatment regime, end point investigated and the mutagen used. Abraham et al. (1986) found that a single oral dose of 0.15 ml/kg undiluted carrot juice administered immediately after *ip* injection of CP reduced the

induction of MNPCE in adult mice bone marrow (Abraham et al., 1986). No influence of carrot pretreatment was found when rats received the direct acting metabolite of CP, phosphoramide mustard which suggested that carrot juice possibly interferes with enzymatic processes of CP activation (Darroudi et al., 1988). In one *in vitro* assay, grape seed proanthocyanidin extract (GSPE) decreased growth inhibitory effects of the chemotherapeutic agents, idarubicin (Ida) (30 nM) and 4-hydroxyperoxycyclophosphamide (4-HC) (1 microg/ml) on Chang liver cells (Joshi et al., 2000). In studies of the effects of fruit and vegetable homogenates administered orally on the frequencies of MNPCEs induced in bone-marrow cells of male NMRI mice by CP, applied by oral gavage in 0.9% aqueous saline and sacrificed 48 h after CP treatment or benzo[*a*]pyrene (BaP) dissolved in 0.2 ml corn oil and injected *ip*, tomato juice exerted moderate anticlastogenic potency against CP while asparagus juice exerted average anticlastogenic activity. Asparagus juice also exerted moderate anticlastogenic activity against BaP but tomato juice was inactive (Edenharder et al., 1998).

It has been observed that *in 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 the increasing anticlastogenic activity of β-carotene at lower doses and the absence of a protective effect at higher concentrations suggest different mechanisms of β-carotene modulation and a possible alteration of the balance of CP activation/detoxification mechanism of the promutagen (Salvadori et al., 1992). No protection was found of β-carotene oral pretreatment against MN induced in bone marrow cells (Lahiri et al., 1993). The latter results are interpreted as a restriction of β-carotene´s protective activity against BaP damage to the target organ. It is also notable that, during the gastrointestinal digestive process, antioxidants and other functional components could be metabolized or not released from foods, thus affecting the native antioxidant potential of each of the components. Several *in vivo* studies in animals and humans demonstrated a very low intestinal uptake of resveratrol (a major antioxidant in grape) leading to trace amounts in the bloodstream based on extensive metabolism in the gut and liver. Rapid metabolism is also the main reason for the short initial half-life of the primary molecule (Marier et al., 2002). In general, processed foods contain less antioxidants than fresh and uncooked foods, since the

preparation processes may expose the food to oxygen (Henry and Heppell, 2002).

Tomato-, asparagus- and grape-juices have been shown to inhibit the mutagenic effects of cyclophosphamide and/or other mutagens in mice and rats, *in vitro* and/or *in vivo*. The absence of inhibitory effects observed in the present study may be due, possibly to the strain of mice used, the route of administration of juice (*ad libitum* as against oral by gavage) or mutagen (*ip* injection as against oral by gavage) used in the other studies, and treatment regime (long pretreatment as against short pretreatment, post or simultaneous treatment with mutagen). Other possible factors are the effect of the long storage on the stability of the juices and the effect of long pretreatment on a protective mechanism that acts through different mechanisms at different doses and which also gets saturated by antioxidant(s).

Conclusion

In the present study, the juice of asparagus, tomato or red grape had no modulatory effect on the incidence of CP-induced frequency of MNPCE and PCEs in total erythrocytes in the bone marrow of male inbred NIH mice using the present route of administration of juices and CP and treatment regime.

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