Influence of orange juice over the genotoxicity induced by alkylation agents: an in vivo analysis

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There is considerable epidemiological evidence indicating an association between diets rich in fresh fruit and vegetables and a decreased incidence of cancers. Methyl methanesulfonate (MMS) and cyclophosphamide (CP) are alkylating agents that differ in their mode of action. MMS is a directly-acting, monofunctional agent, while CP is a bifunctional agent that requires metabolic activation to a reactive metabolite. To evaluate if orange juice could reduce DNA damage induced by these alkylating agents, mice were treated orally (by gavage) with MMS and CP, prior to and after treatment with orange juice. DNA damage was evaluated by the comet assay in peripheral white blood cells. Under these experimental conditions, orange juice reduced the extent of DNA damage caused by both mutagens. For MMS, the antigenotoxic effect of the orange juice was both protective (orange juice pre-treatment) and reparative (orange juice post-treatment); for CP, the effect was reparative only. The components of orange juice can have several biological effects, including acting as targets of toxicants and modulating metabolism/detoxification routes. Considering the different mechanisms of the action of the two drugs, different protective effects are suggested. These results demonstrated the ability of the in vivo comet assay to detect in vivo modulation of MMS and CP mutagenicity by orange juice.

Introduction

Diet represents a major influence on the promotion and progression of cancer. A micronutrient-equilibrated diet can contribute to genomic stability. Deficiencies in vitamins and minerals in the human diet are thought to generate DNA damage by enhancing the occurrence of breaks and oxidative lesions (1–3). Since mutations are key elements in neoplastic processes, there is a considerable amount of epidemiological evidence relating diets rich in fresh fruit and vegetables and a decrease in cancer incidence (2,4).

Methylmethanesulfonate (MMS) and cyclophosphamide (CP) induce neoplastic processes by different mechanisms. MMS and CP alkylate nucleophilic organic macromolecules, including DNA. They can induce depurination and depyrimidination as well as monoadduct formation. CP can also induce DNA-DNA and DNA-protein crosslinks. Both substances have been shown to induce gene mutation (prokaryotes, fungi, insects, plants and mammalian cells), chromosome effects (plants, insects and mammalian cells \textit{in vitro} and \textit{in vivo}), unscheduled DNA synthesis (UDS) (mammalian cells \textit{in vitro} and \textit{in vivo}) and sister chromatid exchange (SCE) (mammalian cells \textit{in vitro} and \textit{in vivo}), as well as other genotoxic effects (http://toxnet.nlm.nih.gov). MMS is a monofunctional sulfur-containing compound commonly used as a solvent and as a catalyst for polymerization, alkylation and esterification reactions (5). It possesses weak mutagenic and carcinogenic activity (6). CP is a widely used and well-documented reference mutagen that expresses its genotoxicity when metabolically activated. The International Agency for Research on Cancer (IARC) has concluded that there is sufficient evidence to classify CP as carcinogenic for animals and humans (7). Since CP needs metabolic activation (8), in vivo studies are the most appropriate method for addressing the complex action of CP.

Orange juice is a complex mixture with, among other things, macro and micronutrients. Its chemopreventative and antimutagenic property is attributed to some vitamins, pro-vitamins and other compounds such as phenolics. However, the same phytochemicals have been characterized as mutagenic (9–11). Most studies conducted to evaluate the biological activity of fruit and vegetable juices and extracts have focused on isolated phytochemicals. Moreover, whole mixtures have been mainly evaluated by \textit{in vitro} test systems. Thus, this work aims to evaluate, in \textit{in vivo}, the effect of orange juice on the genotoxicity of the alkylating agents MMS, a direct acting mutagen, and CP, which requires metabolism to a reactive form, using the comet assay.

Materials and methods

\textbf{Chemical reagents}

Phosphate buffered saline (calcium- and magnesium-free), Tris [tris(hydroxymethyl)aminomethanehydrochloride], disodium ethylenediaminetetra-acetate (EDTA), dimethylsulfoxide (DMSO), ethidium bromide (EtBr), MMS, CP and Triton X-100 were purchased from Sigma (St Louis, MO). Low melting point (LMP) agarose and normal agarose (electrophoresis grade) were obtained from Gibco-BRL (Grand Island, NY). Sodium heparin was purchased from Roche (Brazil) under the commercial name Liquemine\textsuperscript{*}.

\textbf{Animals}

Swiss Webster mice, aged between 5–7 weeks and weighing between 20 and 40 g, were obtained from the Agriculture Ministry, Laboratory of Animal Reference, in Porto Alegre, RS, Brazil. Prior to tests, mice were acclimatized to the laboratory conditions for 7 days (22°C ± 3°C and 60% humidity). During acclimatization and tests, mice received commercial standard mouse cube diet (Nuvilab, CR1, Moinho Nuvipal Ltda., Curitiba, PR, Brazil) and commercial standard water.

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water ad libitum. After acclimatization, they were divided into treatment groups, each containing 3 males and 3 females. All procedures were accomplished according to the international practices for animal use and care under the control of an internal committee of the Universidade Federal do Rio Grande do Sul.

Juice preparation

Juice (in natura) was prepared immediately before the test using Citrus sinensis (Linn.) Osbeck organic oranges (free of agrochemicals). Glass recipients containing the juice were covered to avoid light exposure.

Treatments and test substances

The treatment groups received by gavage 0.1 ml/10 g body wt of: (a) water, (b) juice, (c) MMS and (d) CP. Dose levels of the latter were MMS of 40 mg/kg body wt and CP of 25 mg/kg body wt (Table I). For CP, a dose equivalent to 18.2% of the LD₅₀ dose [25 mg/kg body wt (LD₅₀ = 137 mg/kg body wt) (12)] was used. For MMS, the dose was equal to 13.8% of the LD₅₀ dose [40 mg/kg body wt (LD₅₀ = 290 mg/kg body wt) (13)]. All substances were prepared just before treatment and protected from light. The dose of MMS was selected after a preliminary study (data not shown). The dose of CP was based on the historical data of our laboratory obtained from routine use of the compound. We used the same doses for testing the repair action of vitamin C (14).

Blood sample collection

One or two drops of blood were collected from mouse tail tips by means of a small incision (15). Animals were sampled 24 and/or 48 h after treatment (Table I). Drug administration and blood sampling were performed as described previously (14). Peripheral white blood cells are among the most used cells for genotoxicity studies, mainly with the comet assay. They circulate through the entire body and are easily obtained.

Comet assay

The alkaline comet assay was performed, as described by Singh et al. (16), according to guidelines proposed by Tice et al. (17) with a slight modification developed by Da Silva et al. (18). From each mouse, ~15 μl of blood were sampled and mixed with 7 μl of heparin (anticoagulant). Seven microliters of cell/heparin mixture were then embedded in 93 μl of LMP agarose (0.75 g/100 ml agarose), a cover glass was gently placed over it and the slide placed at 4°C for 5 min to allow gel solidification. The cells were lysed in high salt and detergent solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, (pH 10–10.5), plus 1% Triton X-100 and 10% dimetil sulfoxide-DMSO added just before use) and placed in a horizontal electrophoresis box. Subsequently, the cells were exposed to alkali [300 mM NaOH and 1 mM Na₂EDTA (pH > 13)] for 20 min at 4°C, to allow DNA unwinding. Electrophoresis was performed using an electric current of 300 mA at 25 V (0.9 V/cm) for 15 min at 4°C. After electrophoresis, the slides were neutralized and stained with ethidium bromide. Negative and positive (5 × 10⁻⁵ M MMS, 1-2 h) human blood controls were included in each electrophoresis run. The runs were accepted only when the human blood internal controls showed the appropriate negative and positive responses, respectively.

Table I. Experimental procedures

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Exposure schedule</th>
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<tr>
<td></td>
<td>0 h</td>
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<tr>
<td>Control</td>
<td>Water treatment</td>
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<tr>
<td></td>
<td>1st Blood sampling</td>
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<td></td>
<td>2nd Water treatment</td>
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<tr>
<td>Pre-treatment</td>
<td>Orange juice</td>
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<tr>
<td></td>
<td>1st Blood sampling</td>
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<td></td>
<td>2nd Juice treatment</td>
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<tr>
<td>Post-treatment</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1st Blood sampling</td>
</tr>
<tr>
<td></td>
<td>2nd Orange juice treatment</td>
</tr>
<tr>
<td>Alkylating agents</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1st Blood sampling</td>
</tr>
<tr>
<td></td>
<td>2nd CP treatment</td>
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</tbody>
</table>

Note: Individuals also used as orange juice controls at 24 h.

Microscopic analyses

One hundred cells per animal (two slides of 50 cells each) were analyzed at 200× using a fluorescence microscope equipped with an excitation filter (BP 546/12 nm) and a barrier filter (590 nm). One scorer was used throughout the study and all slides were scored in a blinded way. International guidelines and recommendations for the comet assay consider that visual scoring of ‘comet cells’ is a well-validated evaluation method, since it correlates well with computer-based image analyses (17,19,20). Comet assay parameters were calculated, according to Da Silva et al. (18). The damage index (DI) was calculated for each sample, ranging from 0 (no damage: 100 cells × 0) to 400 (maximum damage: 100 cells × 4), where 0 = no tail and 4 = largest tail (see 18 for pictures of the classes). The damage frequency (DF) was calculated based on the number of cells with damage (classes 1–4).

Statistical analysis

Student’s t-test was used to compare DNA damage values between the different times (24 h versus 48 h, 24 h versus orange juice pre-treatment and 48 h versus orange juice post-treatment). Analysis of Variance (ANOVA) was used to compare DNA damage induced by different substances at the same time (24 or 48 h). A parametric ANOVA was used when data showed normal distribution and were homogeneous in variance. In this case, the Tukey post hoc test was applied for multiple comparisons. When homocedasticity or normality was not present, the Kruskal–Wallis non-parametric ANOVA was used. In this case, Dunn’s post-hoc test was applied to compare groups. Statistical significance was considered at a level of P ≤ 0.05. All statistical analyses were performed independently for the two parameters evaluated.

Results

The internal controls for the comet assay (human blood) demonstrated low damage in the negative control (DI = 0–10) and high damage in the positive control, MMS (DI = 180–300), in agreement with the historical values of our laboratory. In a preliminary experiment, differences in MMS- or CP-induced DNA damage between male and female mice were tested (Figure 1). However, no significant differences in sensitivity of males and females were detected. Thus male and female data were grouped for subsequent analyses.

In the comet assay, little damage was seen in mouse peripheral white blood cells sampled at 24 h for the animals that received only orange juice or water (Table II). The animals that received water showed less DNA damage than the mice that were treated with orange juice, although not significantly (Table II). A slight increase in DNA damage was observed in these groups at 48 h of exposure (Table II). This increase was only significant for the water treatment (for DI, P ≤ 0.001). However, at 48 h, the extent of DNA damage did not differ between mice treated with water and orange juice.

Fig. 1. Damage index (DI) in white blood cells induced by water, orange juice, MMS and CP, as evaluated in male and female mice with the comet assay.
Table II. Detection of DNA damage by the comet assay in white blood cells of mice exposed to water, orange juice, and/or MMS or CP and sampled at 24 h (with and without pre-treatment with orange juice) or 48 h (with and without post-treatment with orange juice)

<table>
<thead>
<tr>
<th>Substances</th>
<th>Single doses (mg/kg body wt)</th>
<th>Schedulea and comet assay parameters</th>
<th>Pre-treatment with orange juiceb</th>
<th>Post-treatment with orange juicec</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>24 h</td>
<td>48 h</td>
<td>24 h</td>
</tr>
<tr>
<td>Water</td>
<td>-</td>
<td>DI ± SD</td>
<td>n</td>
<td>DI ± SD</td>
</tr>
<tr>
<td>Orange juice</td>
<td>-</td>
<td>11.33 ± 2.73</td>
<td>6</td>
<td>19.00 ± 3.29**</td>
</tr>
<tr>
<td>MMS</td>
<td>40.00</td>
<td>22.00 ± 5.36</td>
<td>24</td>
<td>26.67 ± 4.03</td>
</tr>
<tr>
<td>CP</td>
<td>25.00</td>
<td>170.63 ± 20.24**</td>
<td>24</td>
<td>157.67 ± 15.28**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>114.54 ± 39.59**</td>
<td>24</td>
<td>74.83 ± 16.68**</td>
</tr>
</tbody>
</table>

Significance with respect to water and orange juice refers to significance in the same column and was tested using Parametric or non-parametric ANOVA. All other significances refer to the same row and were tested using Student’s t-test.

For more details see Table I.

Group sampled 24 h after treatment with an alkylating agent.

Group sampled 48 h after treatment with an alkylating agent.

Number of individuals obtained from sum of independent experiments.

Significant in relation to 24 h at *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001.

Significant in relation to water at *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001.

Significant in relation to orange juice at *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001.

Significant in relation to 48 h at *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001.

At 24 h, MMS and CP were genotoxic according to both parameters evaluated in comparison to both the water and the orange juice treatments (Table II). A reduction in DNA damage was observed for both MMS and CP at 48 h. However, the reduction was significant only in DI for CP and in DF for MMS (*P ≤ 0.05). Although decreasing in relation to 24 h values, the DNA damage levels for MMS at 48 h remained significantly higher in relation to water (*P ≤ 0.001) and orange juice (for DI and DF, *P ≤ 0.05) treatments. For CP at 48 h, the level of DNA damage remained higher than for water (for DI and DF, *P ≤ 0.05) (Table II).

When the level of DNA damage in white blood cells of mice treated with MMS and sampled at 24 h was compared with mice pre-treated with orange juice, orange juice induced a significant reduction in DNA damage in both evaluated comet assay parameters (*P ≤ 0.001) (Figure 2 and Table II).

Post-treatment with orange juice induced significant reduction in DNA damage in both parameters in white blood cells for both mutagens in mice sampled at 48 h (Figure 2 and Table II).

Discussion

In a previous study, orange juice samples prepared in the same way and of oranges of the same region were mutagenic by the Ames test (11). In the present study, no significant differences in DNA damage between mice treated with water or orange juice were detected by the comet assay. Orange juice is consumed worldwide. The risk for humans consuming orange juice may be low, due to enzymatic activities and pH changes in the digestive tract (11).

The slight DNA damage increase seen at 48 h in the water treated cases might be associated with the stress of manipulation, gavage and blood sampling procedure. It is likely that there is an equal influence in all treatments, since water was the

![Fig. 2. Peripheral white blood cells damage index (DI) modulation induced by orange juice in mice treated with MMS (A) and CP (B), evaluated by comet assay. a: significant in relation to 24 h. b: significant in relation to 48 h. *P ≤ 0.05, **P ≤ 0.01 and ***P ≤ 0.001.](https://academic.oup.com/mutage/article-abstract/20/4/279/1069226)
medium for all substances. Similar results were found in a previous study (11). The decrease in DNA damage (likely due to repair of DNA damage) at 48 h was more pronounced for CP than for MMS.

Vitamin C is an important micronutrient mainly required as a co-factor for enzymes involved in oxi-reduction reactions (3,4,21). It has been studied for its protective action against different diseases (22,23). The mechanisms by which ascorbic acid acts include bio-antimutagenic (24,25) and desmutagenic activities (26). Vitamin C can compete with DNA as a target for alkylation, reducing the genotoxicity of alkylating agents (22). Moreover, vitamin C has a role in the regulation of DNA repair enzymes (27) and high concentrations of vitamin C can also induce apoptotic cell death (28). Vitamin C is not protein-bound and is eliminated with an elimination half-life of 10 h (29).

Phenolic compounds are another constituent of fruit juices. They can protect biological systems in different ways (23,30–32). Phenolic compounds have a dual effect on phase I and phase II enzymes, repressing some enzymes (mainly in phase I) and stimulating others (mainly in phase II) (33). Some flavonoids, like hesperetin, can selectively inhibit human phase I enzymes, repressing some enzymes (mainly in phase II) (33). Some polyphenols, such as limonoids are inducers of the detoxifying enzyme glutathione S-transferase (32). The stimulation of detoxifying enzymes can facilitate the elimination of toxic compounds, significantly affecting the toxic potential of endogenous and exogenous chemicals (32). Moreover, phenolic compounds such as myricetin can stimulate DNA repair pathways, through transcription regulation or mRNA stabilization (35).

The pharmacokinetics of polyphenols is diverse. It depends on the chemical structure of polyphenols (29). Naringenin and hesperitin are among the most prevalent polyphenols in orange juice. They can be detected in urine up to 38 h after administration (36). In liver, polyphenols are subjected to three main types of conjugation after absorption: methylation, sulfation and glucuronidation (37). It is likely that phenolic compounds can be methylated by alkylating agents, instead of the conjugation enzymes, protecting/reducing DNA from alkylation.

MMS can methylate nucleophilic regions of DNA and amino acid molecules, particularly at nitrogen atoms. Methylation of the phosphate groups accounts for a minor percentage of the total methylation by MMS (<1%). MMS’ genotoxicity is mediated by base modifications, which weaken the N-glycosyl bond, leading to depurination/depyrimidination of DNA strands and the appearance of alkali-labile abasic sites (AP sites). The removal of AP sites by AP endonucleases cleaves DNA adjacent to these sites and generates DNA strand breaks in DNA (6,38–40). To a minor extent MMS can also act as a weak oxidative stress inducer, as observed by Horváthová et al. (6), who tested the effect of a synthetic antioxidant (stobadine, SBT) on MMS genotoxicity.

Pre-treatment and post-treatment with orange juice reduced MMS’s genotoxicity about 67 and 40% in DI (61 and 26% in DF), respectively. Thus, orange juice was both preventive and reeptive for MMS. In pre-treatment, phenolic compounds and, to a minor extent, vitamin C (due to the shorter half-life) could have competed as target site for alkylation. With respect to post-treatment, both phenolic compounds and vitamin C could have influenced the kinetics of repair.

CP is absorbed well after oral administration. The parent compound is widely distributed throughout the body with a low degree of plasma protein binding (20%). The half-life of CP is between 6 and 9 h (41). Once activated, CP can, besides monoaadducts, also induce the formation of DNA–DNA and DNA–protein crosslinks (5). CP has the ability to generate free radicals that cause endothelial and epithelial cell damage (41).

Pre-treatment with orange juice slightly reduced the level of DNA damage induced by CP (15 and 13% reduction in DI and DF, respectively), while orange juice post-treatment induced a significantly higher reduction in DNA damage (57 and 71% reduction in DI and DF, respectively). Since CP requires metabolic activation before inducing DNA damage, it is likely that juice components, such as phenolics, alter the rate of metabolism and/or detoxification. In pre-treatment, only the phenolics could have acted as a scavenger, since vitamin C has a short half-life. Despite acting as scavengers, phenolics could have blocked CYP 450 and increased the half-life of the CP. In post-treatment, damage reduction was higher because both compounds could act as reactive species quenchers and DNA repair pathways modulators. Moreover, phenolics could have stimulated phase II enzymes and eliminated CP metabolites. It is important to consider the kind of DNA damage generated by CP, particularly crosslinks. Such lesions can retard the migration of DNA fragments and lead to a wrong evaluation of the extent of DNA damage (5,17,20).

In conclusion, consumption of orange juice can be both protective (MMS and CP) and reparative (MMS and CP) of DNA damage induced in mouse white blood cells by alkylating agents. Such protective effects of orange juice differ depending on the mode of action of the mutagen and may be mediated by, among other things, (1) modulation of phase I and II enzymes; (2) substrate competition for the nucleophilic action of CP and MMS or quenching of CP metabolites and side-products (reactive species); and (3) enhancement of DNA repair. Our results demonstrate the ability of the in vivo comet assay to detect in vivo modulation of MMS and CP mutagenicity by orange juice.

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References


