Universidade Federal do Rio Grande do Sul
Centro de Biotecnologia do Estado do Rio Grande do Sul
Programa de Pós-Graduação em Biologia Celular e Molecular

SUCO DE LARANJA E VITAMINA C:
EFEITO SOBRE A ESTABILIDADE GENÔMICA

Tese de Doutorado

Silvia Isabel Rech Franke

Porto Alegre, maio de 2006

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Tese submetida ao Programa de Pós-Graduação em Biologia Celular e Molecular da UFRGS como parte dos requisitos para a obtenção do Grau de Doutor

Orientador: João Antonio Pêgas Henriques
Co-orientador: Bernardo Erdtmann

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Ao GENOTOX que subsidiu as despesas desta tese.

APRESENTAÇÃO

Em 1999, tive a oportunidade de visitar o Centro de Biotecnologia da UFRGS (CBIOT), como etapa inicial do "Programa de Apoio Financeiro a Profissionais com Potencial para a Docência em Novos Cursos" da Universidade de Santa Cruz do Sul (UNISC), visando à qualificação dos docentes da UNISC junto ao CBIOT. Na ocasião estava envolvida na implantação do Curso de Nutrição da UNISC. A participação no referido programa pressupunha a proposição de um tema de pesquisa vinculado às linhas de pesquisa do CBIOT.

Minha proposta envolvia a avaliação do efeito cumulativo de pesticidas em alimentos cultivados em solos utilizados também para o plantio de fumo na região de Santa Cruz do Sul. Após conhecer os Professores João Antonio Pêgas Henriques e Bernardo Erdtmann, fiquei fascinada pelo tema da genotoxicidade relacionada aos alimentos.

Após algumas negociações e idas-e-vindas a Santa Cruz do Sul, iniciei o Mestrado, avaliando os potenciais antioxidante *in vitro* e mutagênico no teste de Ames de diferentes sucos de laranja (quanto ao conteúdo de vitamina C e de fenólicos totais e ao processamento e armazenamento). Este trabalho foi finalizado em setembro de 2001 e publicado posteriormente com alguns acréscimos sob o título "Study of the antioxidant and mutagenic activity of different orange juices" (Food Chemistry, 88, 45-55, 2004). Tal trabalho foi reconhecido como uma contribuição relevante na área da saúde pela *Sociedad Iberoamericana de Informa cion Cientifica (SIIC)* e foi reeditado no site da organização (http://www.siicsalud.com/dato/dat041/05203017.htm).

O suco espremido manualmente e não-processado (*in natura*) foi o que apresentou mais respostas positivas quanto à mutagenicidade nas bactérias. Este resultado instigou-nos a testar, em mamíferos, o efeito genotóxico e antigenotóxico deste suco, bem como da vitamina C, um composto fortemente presente no suco de laranja. Este foi o tema da presente tese, que foi desenvolvida nas dependências da UFRGS, no GENOTOX - Laboratório de Genotoxicidade do Centro de Biotecnologia do Estado do Rio Grande do Sul, no Laboratório de Citogenética Animal e Evolução do Departamento de Genética, bem como no Laboratório de Implantação Iônica do Instituto de Física.

Esta tese está dividida em 7 partes: 1) introdução, onde o suco de laranja e a vitamina C, bem como o tipo de dano no DNA induzido pelas substâncias teste e os respectivos mecanimos de defesa, assim como as metodologias e os compostos testados, são descritos de forma sucinta; 2) objetivos; 3) parte experimental, onde os resultados da pesquisa estão

apresentados em três artigos já publicados; 4) discussão geral; 5) perspectivas; 6) referências; e 7) anexos.

Os anexos estão divididos em 5 partes: a) artigo publicado com os dados do meu mestrado, que instigou o tema desta tese; b) artigo publicado com os dados do meu mestrado reeditado em Espanhol e publicado pela SIIC; c) revisão bibliográfica focada nos efeitos biológicos do suco de laranja como mistura complexa, dando ênfase aos efeitos antimutagênicos, anticarcinogênicos, vasoprotetores e relacionados à interação com drogas e nutrientes; d) revisão bibliográfica visando compilar a concentração média e os efeitos biológicos dos principais componentes bioativos do suco de laranja, bem como, descrever a influência do processamento sobre a composição do suco de laranja; e e) *Curriculum vitae* da autora.

RESUMO

Título: suco de laranja e vitamina C: efeito sobre a estabilidade genômica

Existem evidências crescentes indicando a associação entre dietas ricas em frutas e vegetais e a diminuição da incidência de câncer. O suco de laranja (OJ) pode ser incluído entre os alimentos com potencial quimioprotetor e seu estudo é muito relevante pelo amplo consumo desta bebida. O OJ possui vários nutrientes e compostos bioativos com atividades antioxidante, antimutagênica, anticarcinogênica e antiaterogênica, entre outras. A vitamina C (Vit C) é um dos nutrientes mais abundantes no OJ, e o único nutriente que pode ser provido em quantidade superior à recomendação diária por uma única porção de 200 mL de OJ. A Vit C, a exemplo de outros componentes do OJ, pode ser tanto benéfica quanto maléfica para os sistemas biológicos, dependendo do contexto metabólico. Neste sentido, vários nutrientes presentes no OJ têm sido identificados como mutagênicos ou carcinogênicos, especialmente quando administrados de forma isolada. Este estudo utilizou o ensaio Cometa alcalino em sangue de camundongos (*in vivo*) para avaliar: 1) a genotoxicidade do OJ e da Vit C; 2) a genotoxicidade do $FeSO_4$ e do $CuSO_4$: 3) o efeito modulador do OJ e da Vit C sobre a genotoxicidade do $FeSO_4$ e $CuSO_4$, bem como do metilmetanosulfonato (MMS) e da ciclofosfamida (CP). A versão alcalina do ensaio Cometa foi utilizada para avaliar o dano no DNA em células brancas do sangue periférico de camundongos. Adicionalmente, os níveis de cobre e ferro no sangue e no fígado dos camundongos tratados com metais e OJ foram avaliados pela metodologia de PIXE (*Particle-Induced X-ray Emission*). Grupos com pelo menos 6 camundongos (metade de cada sexo) foram tratados por *gavage* com uma ou duas doses de água (controle), CP, MMS, $FeSO_4$ ou $CuSO_4$. OJ (0.1 mL/Kg) foi administrado tanto antes (pré-tratamento) quanto após a administração das substâncias-teste (pós-tratamento). A Vit C (1 e 30 mg/Kg) foi administrada apenas no pós-tratamento. O dano no DNA foi avaliado 24 e 48 h após o início do tratamento. Após 24 h, o OJ induziu um suave aumento no dano no DNA, enquanto a Vit C foi genotóxica (30 mg/Kg > 1 mg/Kg). O tratamento duplo com Vit C (a 0 e a 24 h) induziu uma resposta genotóxica cumulativa a 48 h, que foi mais intensa para a dose maior. O $FeSO_4$ e o $CuSO_4$ foram genotóxicos após 24 h, mas tiveram seu dano efetivamente reparado após 48 h do tratamento. O pré-tratamento com OJ reduziu a genotoxicidade do $FeSO_4$ e do $CuSO_4$ (efeito preventivo). O pós-tratamento com OJ também reduziu a genotoxicidade do $CuSO_4$ (efeito reparador). O OJ mostrou tanto efeito preventivo quanto reparador sobre a genotoxicidade do MMS. O OJ teve apenas efeito reparador sobre a CP. Ambas doses de Vit C aumentaram os danos no DNA causados pelo $FeSO_4$ e pelo $CuSO_4$. Os danos no DNA gerados pelo MMS foram reduzidos significativamente pela dose menor, mas não pela dose maior de Vit C. Para a CP, o dano no DNA não foi afetado pelo pós-tratamento com nenhuma das doses de Vit C. O PIXE indicou uma correlação positiva entre os danos no DNA e os níveis hepáticos de ferro. Por outro lado, uma correlação negativa entre os níveis de cobre no sangue total e os danos no DNA foi observada. Uma correlação negativa entre o nível hepático de ferro e o nível de cobre no sangue total também foi observada para os tratamentos com $FeSO_4$ ou $CuSO_4$. Estes resultados apontam uma interação dinâmica entre a genotoxicidade e a deposição tecidual do ferro e do cobre. A Vit C e os demais componentes do OJ têm diversos efeitos biológicos, incluindo: 1) a ação como alvo de ataque de alquilação, 2) influência na metabolização/detoxificação de drogas, e 3) efeito na homeostasia e reparo do DNA. Além disso, a Vit C e os metais de transição, especialmente cobre e ferro, presentes no OJ podem induzir estresse oxidativo; contudo, estes últimos agem simultaneamente nos sistema de defesa antioxidante. Estudos adicionais com outros esquemas de tratamento são necessários para melhor entendimento do impacto da mistura complexa OJ e de seus componentes na estabilidade genômica. Com certeza, o consumo de OJ fresco ou

processado e armazenado de forma a preservar o seu potencial biológico é um alimento sugerido como uma das porções de uma dieta equilibrada (contendo pelo menos 5 porções de frutas e vegetais), recomendada para uma vida saudável e longeva.

ABSTRACT

Title: Orange juice and vitamin C: effects in genome stability

Evidence indicates an association between diets rich in fresh fruit and vegetables and a decreased incidence of cancers. Orange juice (OJ) is a food with chemoprotective potential highly relevant for study due to its widespread consumption. OJ is composed of several nutrients and bioactive compounds with antioxidant, antimutagenic, anticarcinogenic and antiathrerogenic activities. Vitamina C (Vit C) is the most abundant nutrient in OJ, and the only one that can be provided in amounts higher that the daily recommended intake by a single portion of OJ (200 mL). Vit C, like other components of OJ, can be either benefical or noxious for biological system, depending of their metabolic context. Indeed, some components of juices have been identified as mutagenic or carcinogenic when isolated. In this study we tested by comet assay in mice *in vivo*: 1) the genotoxicity of orange juice (OJ) and vitamin C (Vit C); 2) the genotoxicity of $FeSO_4$ and $CuSO_4$: 3) the modulator effect of orange juice and Vit C over genotoxicity of $FeSO_4$ and $CuSO_4$, as well as over methyl methanesulfonate (MMS) and cyclophosphamide (CP). We used the alkaline version of the comet assay to assess DNA damage in peripheral white blood cells of mice. Moreover, the levels of iron and copper in the whole blood and liver of the mice treated with this metals were evaluated by PIXE (Particle-Induced X-ray Emission). Groups of at least 6 mice (half of each gender) were orally given a single dose of either water (control), CP, MMS, $FeSO_4$ or $CuSO_4$. OJ (0.1 mL/Kg) was given either before (pre-treatment) or after (post-treatment) administration of the test substances. Vit C (1 and 30 mg/Kg) was only administered after treatment (post-treatment). DNA damage was evaluated 24 and 48 h after the beginning of the treatment. After 24 h, OJ induced a slight increase in DNA damage and Vit C was genotoxic (30 mg/Kg > 1 mg/Kg). Double treatment with Vit C (at 0 and 24 h) induced a cumulative genotoxic response at 48 h, more intense for the higher dose. $FeSO_4$ and $CuSO_4$ were genotoxic after 24 h and significant DNA damage repair was observed after 48 h of treatment. OJ pre-treatment reduced the genotoxicity of $FeSO_4$ (preventive effect). OJ had a preventive effect over the genotoxicity of $CuSO_4$. OJ post-treatment also reduced the genotoxicity of $CuSO_4$ (restorative effect). OJ had both protective and reparative effects over MMS. OJ had only a reparative effect over CP. Both doses of Vit C enhanced DNA damage caused by $FeSO_4$ and $CuSO_4$. DNA damage caused by MMS was significantly reduced by the lower dose, but not by the higher dose of Vit C. For CP, the DNA damage was not affected by the post-treatment with any of the doses of Vit C. PIXE analysis indicated a positive correlation between DNA damage and the hepatic levels of iron and a negative correlation between whole blood copper and DNA damage. A negative correlation between hepatic iron and whole blood copper content was also seen in the treatment with both ferrous and cupric sulfates. These results point a dynamic interaction between the genotoxicity and the tecidual fate of iron and copper. Vit C and the other components of OJ have several biological effects, including: 1) action as targets for toxicants; 2) influence in drug metabolization/detoxification; and 3) effect in DNA repair and homeostasis. Moreover, Vit C and transition metals, particularly copper and iron, can induce oxidative stress; however, they can also play roles in antioxidant defense system, being a DNA repair modulators. Further data from other treatment schedules are needed to shed light upon the beneficial/noxious effects of OJ as a complex mixture, as well as of its compounds in genomic stability. Indeed, one glass of fresh or adequate processed and stored OJ can be among the options for the daily 5 portions (or even more) of fruits and vegetables recommended for a health and longevity.

Key words: genotoxicity, oxidative stress, alkylating agents, copper, iron, comet assay.

LISTA DE FIGURAS

LISTA DE TABELAS

ABREVIATURAS

Abreviatura/ estrutura química	Termo	Abreviatura/ estrutura química	Termo
OJ	Suco de laranja	NAD	Nicotinamida adenina dinucleotídeo
Vit C	Vitamina C	FAD	Flavina adenina dinucleotídeo
SOJ	Suco de laranja azedo	GSSG	Glutationa oxidada
MMS	Metilmetanosulfonato	G6PD	Glicose-6-fosfato desidrogenase
CP	Ciclofosfamida	CYP	Citocromo oxidase
$FeSO_4$	Sulfato ferroso	PARP-1	Poli(ADP-ribose) polimerase-1
$CuSO_4$	Sulfato cúprico	MTHFR	Metil tetrahidrofolato redutase
ROS	Espécies reativas de oxigênio	hOGG1	8-oxoguanina-DNA glicosilase 1
FR	Radicais livres	DNA poli β	DNA polimerase β
RS	Espécies reativas	AGT	O^6-alquilguanina-DNA alquiltransferase
RNS	Espécies reativas de nitrogênio	8-OHdG	8-hidroxi-deoxiguanosina
$O_2^{\bullet-}$	Ânion superóxido	N7G	Nitrogênio 7 da Guanina
$^1O_2$	Oxigênio singleto	N3A	Nitrogênio 3 da Adenina
$NO^\bullet$	Óxido nítrico	Sítio AP	Sítio apurínico/apirimidínico (abásico)
$ONOO^-$	Peroxinitrito	BER	Reparo por excisão de bases
H_2O_2	Peróxido de hidrogênio	NER	Reparo por excisão de nucleotídeos
$OH^\bullet$	Radical hidroxila	MMR	*Mismatch repair* (reparo por emparelhamento incorreto)
SOD	Superóxido dismutase	HR	Reparo por Recombinação Homóloga
CAT	Catalase	NHEJ	Reparo por recombinação não homóloga *(Nonhomologous End Joining)*
GPx	Glutationa peroxidase	PIXE	Particle-Induced X-ray Emission
GR	Glutationa redutase	Fenton-like	Reações do tipo Fenton
GSH	Glutationa (reduzida)		
B1	Tiamina		
B2	Riboflavina		
B3	Niacina		
B5	Ácido pantotênico		
B6	Piridoxina		
B9	Folato/ácido fólico		

SUMÁRIO

1 INTRODUÇÃO

1.1 O suco de laranja e a vitamina C

O consumo mundial de sucos naturais está aumentando como conseqüência da busca por um estilo de vida mais saudável. O setor de produção de suco, particularmente o do suco de laranja (OJ), está se expandindo em vários países. As empresas brasileiras são responsáveis por aproximadamente 70% das laranjas plantadas ou processadas globalmente.

No Brasil o controle de qualidade dos sucos de frutas é feito: a) por análise das características organolépticas, b) pela ausência de microrganismos patogênicos, c) pela ausência de substâncias nocivas, e d) pela regulamentação sobre aditivos (BRASIL, 1974). Portanto, não considera os efeitos biológicos das substâncias naturais presentes nos sucos. Desse modo, é muito importante direcionar as pesquisas para os efeitos de substâncias amplamente consumidas.

O OJ é uma mistura complexa contendo quantidades consideráveis de vitaminas e minerais, bem como de outros compostos bioativos. A composição do OJ é bastante variável em conseqüência de vários fatores, tais como varietal, condições climáticas e grau de maturidade das laranjas, e/ou forma de processamento, envasamento e estocagem. Uma porção de 200 mL de OJ, em média, contém alta concentração [> 13% da Ingestão Dietética de Referência (DRI – *Dietary Reference Intake*)] de Vit C, folato (B9), sendo uma fonte-chave destes nutrientes. Esta mesma porção pode também ser uma boa ou excelente fonte (>5-13% da DRI) de riboflavina (B2), niacina (B3), ácido pantotênico (B5) e piridoxina (B6), bem como de magnésio, cobre e potássio. Adicionalmente, contém quantidades regulares (>4-5 % da DRI) de carotenóides pró-vitamina A e ferro. O OJ também contém altas quantidades de flavonóides, bem como quantidades consideráveis de limoneno e *p*-coumarina. As

vitaminas, os minerais e os outros compostos bioativos do OJ podem desempenhar vários efeitos biológicos, afetando o estresse oxidativo e a estabilidade genômica (para revisão ver ANEXO D).

O OJ, como mistura complexa, também tem efeitos sobre o estresse oxidativo, estabilidade genômica, câncer, doenças cardiovasculares (para revisão ver ANEXO C).

Assim como o consumo de OJ vem aumentando, a busca desenfreada pela longevidade vem gerando uma mudança nas atitudes frente ao consumo de antioxidantes. A Vit C está entre os antioxidantes mais consumidos, tanto na forma adicionada aos alimentos quanto como suplemento/medicamento (Halliwell, 2001).

A Vit C deve ser provida pela dieta para os humanos, pois estes, os demais primatas e outras espécies (como porcos da Índia) não sintetizam o composto. Nos humanos, a última enzima na rota de biossíntese da Vit C (1-gulonolactona oxidase) não é funcional (WHO, 2001).

A Vit C é um micronutriente importante requerido principalmente como um co-fator de enzimas envolvidas em reações de oxi-redução (Fenech & Ferguson, 2001; Halliwell, 2001; Edenharder et al., 2003). Esta vitamina C atua como doadora de elétrons em oito enzimas, envolvidas principalmente na biossíntese de colágeno e carnitina (WHO, 2001).

Por ser hidrosolúvel, a Vit C não é transportada por proteínas. Após ser administrada por via oral, ela é eliminada com uma meia-vida de 10 h (Schwedhelm et al., 2003). A meia vida-curta está associada a doenças. O escorbuto é uma patologia classicamente relacionada à deficiência de Vit C. A anemia também está relacionada à falta de Vit C, pela atuação do composto na absorção do ferro inorgânico. Apesar do escorbuto ser facilmente reversível num período curto com a ingestão de apenas 10 mg/dia de Vit C, a deficiência de Vit C continua sendo um problema especialmente em países mais pobres. Contudo, não se pode desconsiderar a relação inversa entre ao nível plasmático de Vit C e a prevalência de doenças infecciosas, comum nestes países (WHO, 2001).

A Vit C tem sido estudada por sua ação protetora contra diferentes doenças (Vijayalaxmi & Venu, 1999; Edenharder et al., 2002). Os mecanismos pelos quais a Vit C atua incluem atividades antioxidantes (Halliwell & Guterridge, 2000), bio-antimutagênicas e/ou desmutagênicas (Sram et al., 1983; Kojima et al., 1992; Guha & Khuda-Bukhsh, 2002). Atividade antimutagênica é a capacidade de diminuição da freqüência da fixação de mutações, enquanto a atividade desmutagênica diz respeito à redução/inativação da mutagenicidade de um dado composto (Kuroda et al., 2001).

O mecanismo para a atividade genoprotetora da Vit C pode estar ligado a capacidade do composto de competir com o DNA como um alvo de alquilação, reduzindo a genotoxicidade de agentes alquilantes (Vijayalaxmi & Venu, 1999), bem como interceptar radicais livres (FR). A Vit C pode quelar metais, impedindo que os mesmos gerem espécies reativas (Halliwell & Guterridge, 2000). A Vit C também tem um papel na regulação das enzimas de reparo do DNA (Cooke et al., 1998) e pode induzir apoptose quando em altas concentrações (Sakagami et al., 2000).

A capacidade redutora da Vit C protege outros compostos da oxidação. A hidroxila presente no carbono 3 da molécula pode ser liberada facilmente, explicando grande parte das propriedades fisiológicas da Vit C (Spada & Silva, 2004) (Figura 1).Em ambiente oxidante, a Vit C pode apresentar uma atividade fortemente pró-oxidante. Em condições fisiológicas, onde o ambiente químico é altamente complexo e suscetível a variações, ainda existem dúvidas sobre o resultado do balanço entre a atividade antioxidante (interceptação/quelação) e pró-oxidante (redução de metais de transição) da Vit C.
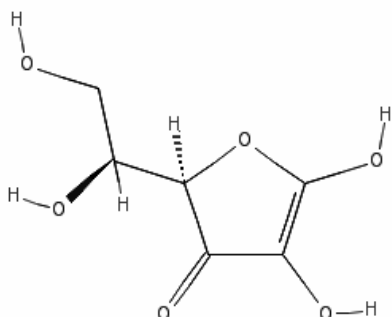
Figura 1. Vitamina C.

Embora o limite superior de ingestão (UL – *upper level*) de Vit C seja de aproximadamente 1,8 g/dia para adultos, a ingestão de 1 g/dia de Vit C já é suficiente para causar distúrbios gastrointestinais, pois metade desta dose não é absorvida. A ingesta de quantidades mais elevadas de Vit C, tais como 5-10 g/dia, pode causar rapidamente hiperoxaluria e conseqüentes cálculos renais em humanos, embora o risco de cálculo já seja significativo para ingestas com mais de 1 g/dia. O potencial pró-oxidante da Vit C em humanos é evidenciada pela resposta hemolítica apresentada por indivíduos portadores de deficiência na enzima glicose-6-fosfato desidrogenase, enzima envolvida na proteção contra estresse oxidativo nas hemácias (WHO, 2001).

1.2 Danos no DNA e a estabilidade genômica

O homem permanece continuamente exposto a vários riscos de genotoxicidade, devido à incontestável existência de substâncias genotóxicas capazes de produzir alterações no material genético de células germinativas ou somáticas. Os agentes que induzem mutações podem ser divididos em mutágenos físicos (radiações ionizantes e não ionizantes) e químicos (elementos, substâncias e misturas químicas, como metais, agentes alquilantes e alimentos) (Saffi & Henriques, 2003).

Os danos no DNA que não são oportunamente reparados ou erroneamente corrigidos, provocam mudanças na seqüência de bases do DNA, resultando freqüentemente na eliminação ou alteração de genes responsáveis pelo controle da divisão e da diferenciação celular (Choy, 1996; Jackson & Loeb, 2001; Goldman & Shields, 2003).

A instabilidade genômica resulta do acúmulo de mutações, especialmente das que afetam a manutenção da homeostasia da célula nos processos de reparo, replicação e recombinação do DNA, bem como na divisão celular (Fenech, 2005b).

Existe uma correlação entre a mutagenicidade e a carcinogenicidade, pois muitos compostos potencialmente mutagênicos podem ser carcinogênicos, indutores de câncer em animais e humanos, participando como ativadores e/ou iniciadores de carcinogenicidade (Ames, 1989; Sarasin, 2003). As alterações no DNA representam a primeira etapa da carcinogênese e estão envolvidas na mutagênese e no envelhecimento do DNA (Wang et al., 1998; Sarasin, 2003).

Os principais tipos de alterações no DNA incluem: quebras de cadeia simples ou dupla, deleções ou modificações de base, ponte de DNA-DNA ou DNA-proteína e pontes intracadeias de DNA (Saffi & Henriques, 2003). Essas alterações são geradoras de substituições de bases, erros de leitura, deleções, inserções ou translocações na seqüência de DNA e, ainda, podem causar modificações maiores como as aberrações cromossômicas estruturais (MacPhee, 1998a, 1998b; Goldman & Shields, 2003; Saffi & Henriques, 2003).

A oxidação e a metilação estão incluídos nos processos endógenos que causam lesões significativas no DNA (Halliwell & Guterridge, 2000; Brozmanova et al., 2001a; Risom et al., 2005).

A oxidação e a metilação serão enfocados a seguir, com vistas a explicar o modo de ação das substâncias-teste empregadas neste trabalho (metais de transição e agentes alquilantes).

1.2.1 Estresse oxidativo e danos no DNA, nas proteínas e nos lipídios

Danos oxidativos no DNA são decorrentes do estresse oxidativo. O estresse oxidativo ocorre quando há um desbalanço entre a formação e a eliminação de espécies reativas de oxigênio (ROS). As ROS são geradas como subprodutos do metabolismo oxidativo que foi um marco na evolução, pois gerou um aumento da eficiência metabólica. Por outro lado, também aumentou a instabilidade dos sistemas biológicos, pois 2-5 % de todo o oxigênio metabolizado é convertido em ROS. As ROS são as principais responsáveis pelo nível basal de mutações (para revisão, ver Halliwell & Guterridge, 2000).

Estima-se que um indivíduo padrão sofra cerca de 10.000–20.000 ataques de ROS e outros FR por célula por dia como parte do metabolismo oxidativo normal. Para um atleta em treinamento intenso, estes ataques podem aumentar cerca de 50 % (Valko et al., 2004).

Os danos no DNA resultantes de ataques de ROS incluem diversas modificações oxidativas, incluindo quebras de cadeia e oxidação nas bases e nas pentoses, bem como pontes DNA-proteína. O 8-hidroxi-deoxiguanosina (8-OHdG) é o principal produto da oxidação de bases (Cerda & Weitzman, 1997; Dizdaroglu et al., 2002; Goldman & Shields, 2003; Saffi & Henriques, 2003; Risom et al., 2005).

As ROS incluem um vasto número de moléculas quimicamente derivadas do oxigênio. As mais importantes ROS são o radical ânion superóxido ($O_2^{\bullet-}$), o peróxido de hidrogênio (H_2O_2), o radical hidroxila ($OH^{\bullet}$) e o oxigênio singleto ($^1O_2$) (Figura 2)(Imai & Nakagawa, 2003; Saffi & Henriques, 2003; Slupphaug et al., 2003).
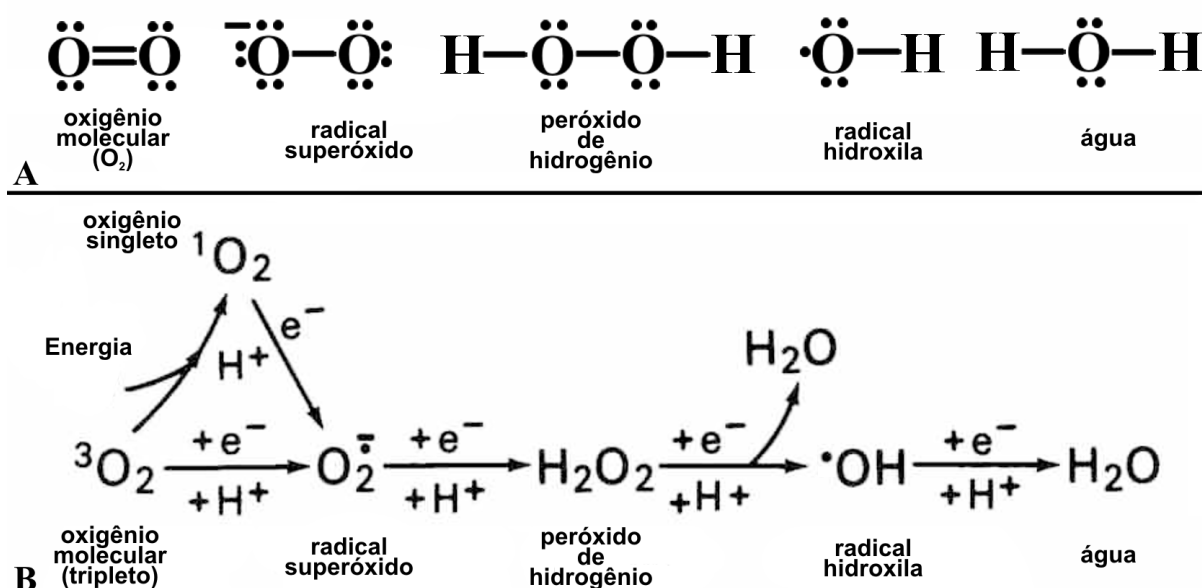
Figura 2. Principais espécies reativas de oxigênio: A) estrutura de Lewis e B) reações de formação (adaptado de Slupphaug et al., 2003).

O $O_2^{\bullet-}$ e o H_2O_2 não são capazes de atacar o DNA diretamente. O $OH^{\bullet}$ e o $^1O_2$, atacam diretamente o DNA. O $OH^{\bullet}$ pode causar modificações nas quatro bases do DNA, além de causar quebras de cadeia e sítios abásicos - apurínicos/apirimidínicos (AP). Já o $^1O_2$, pode causar ciclo-adição aos carbonos de ligação dupla do anel imidazol, gerar sítios AP e quebras simples em posições adjacentes às guaninas. Apesar do $OH^{\bullet}$ ser a ROS mais reativa, ela é a de meia-vida mais curta (Picada et al., 2003; Saffi & Henriques, 2003). O óxido nítrico ($NO^{\bullet}$) também é considerado uma ER (Figura 3).

Figura 3. Estrutura de Lewis do óxido nítrico.

A maior parte dos $O_2^{\bullet-}$ é gerado nas mitocôndrias como um sub-produto da cadeia de transporte de elétrons, sendo o DNA mitocondrial seu alvo primário. Uma quantidade menor de $O_2^{\bullet-}$ é formada no retículo endoplasmático pela atividade do citocromo P450 (CYP450). O $O_2^{\bullet-}$ pode dar origem ao $OH^{\bullet}$ pela reação de Haber-Weiss (seção 1.2.2, Figura 3). O $OH^{\bullet}$ causa danos às biomoléculas, especialmente quando a reação ocorre nas mesmas ou na sua proximidade (Imai & Nakagawa, 2003; Picada et al., 2003; Slupphaug et al., 2003).

Cerca de 80 % de todo H_2O_2 é produzido pelos microssomos/peroxissomos hepáticos envolvidos no metabolismo lipídico. O restante é formado a partir da dismutação do $O_2^{\bullet-}$. A toxicidade do H_2O_2 está associada principalmente ao seu papel intermediário na formação do $OH^{\bullet}$. A maior parte do $OH^{\bullet}$ é gerada pela decomposição do H_2O_2 mediada por metais (Imai & Nakagawa, 2003; Picada et al., 2003; Slupphaug et al., 2003).

O $NO^{\bullet}$ tem uma meia vida longa e alta reatividade com outras ROS (Dedon & Tannenbaum, 2004). A reação do $NO^{\bullet}$ com $O_2^{\bullet-}$ gera peroxinitrito ($ONOO^-$), agente oxidante como o $OH^{\bullet}$ (Picada et al., 2003; Dedon & Tannenbaum, 2004).

Os efeitos das ROS não dependem somente de seus níveis, sendo influenciados também: pelo ambiente químico (p.ex. pela concentração substâncias que reagem com as ROS e pelo nível de defesas antioxidantes, tanto enzimáticas como não-enzimáticas); e pela distância entre o local de geração das ROS e o sítio alvo dos mesmos, considerando-se o

potencial de difusão e a meia-vida das RS e dos FR (Halliwell & Guterridge, 2000; Linder, 2001). Os metais de transição são os principais catalizadores da formação de ROS.

A modificação oxidativa de proteínas por ROS também está associada à origem ou à progressão de várias doenças e desordens fisiológicas. A oxidação de proteínas e aminoácidos por ROS pode gerar modificações na cadeia lateral de aminoácidos, rompimento de ligações peptídicas e a formação de pontes proteína-proteína (Cerda & Weitzman, 1997). Tais alterações estruturais podem causar a inativação parcial ou total da função da proteína. As ROS também estimulam as vias de transdução de sinais, afetando a regulação gênica (Halliwell & Guterridge, 2000). A acumulação de proteínas danificadas, bem como alterações no perfil da expressão gênica, diminuem a eficiência homeostática, ao longo do tempo, aumentando as alterações no DNA.

A exemplo das proteínas, os fosfolipídios de membrana celular são suscetíveis à oxidação, devido aos seus ácidos poliinsaturados (PUFA). A abstração de um átomo de hidrogênio de um PUFA ou da cadeia lateral de um PUFA por um RL gera radicais centrados no carbono. Após rearranjos, esses são transformados em radicais peroxil. Este processo é bem conhecido e chamado de peroxidação lipídica. Hidroperóxidos lipídicos causam alterações reversíveis às membranas e são uma fonte de aldeídos altamente reativos. Os aldeídos disparam reações em cadeia, causando danos às proteínas e a ácidos nuclêicos (Terao & Piskula, 1999).

1.2.2 Metais de transição, estresse oxidativo e estabilidade genômica

Os metais de transição são ubíquos no ambiente e fundamentais à manutenção da vida, apesar de terem potencial mutagênico e carcinogênico. A mutagenicidade de metais pode ocorrer por vários mecanismos, tanto diretos quanto indiretos. Os mecanismos diretos incluem: 1) interação com diferentes bases do DNA, alterando o pareamento das bases ou disponibilidade do substrato para replicação de DNA ou transcrição de RNA; 2) interação de íons metais com DNA polimerases, diminuindo a fidelidade da síntese de DNA; e 3) interação íons metais com as ligações fosfodiésteres do DNA, alterando a estrutura do DNA e a formação de pontes DNA-proteína (Tkeshelashvili et al., 1991). Já o mecanismo indireto, pode ocorrer pela formação de OH$^\bullet$ pela reação do tipo Fenton (*Fenton-like*) *in situ* no DNA (Meneghini, 1997); ou pela formação de ROS e RNS envolvendo o $O_2^{\bullet-}$, OH$^\bullet$, NO$^\bullet$ e H_2O_2,

entre outras substâncias. O mecanismo indireto, geralmente, envolve múltiplas etapas (De Freitas & Meneghini, 2001; Linder, 2001; Valko et al., 2005; Franke et al., 2006).

A evidência mais convincente da ligação entre danos oxidativos gerados por metais e a carcinogênese provém da "mutagenicidade" das modificações de base no DNA, que são resultado do estresse oxidativo mediado por metais. Além disso, a influência de certos metais no reparo de DNA [tais como Cu (Guecheva et al., 2001) e Pb (Valko et al., 2005)] e em rotas de sinalização celular está bem caracterizada e de fato, tal modificação está relacionada à carcinogenicidade. A formação de FR metal-induzida por Fe e Cu é mais reconhecida do que para os metais altamente tóxicos e carcinogênicos (p.ex. Cr, Ni) (Valko et al., 2005).

A maior parte do $OH^{\bullet}$ é gerada pela decomposição do H_2O_2 mediada por metais, no caso da reação de Fenton (quando catalisada por Fe) ou *Fenton-like* (quando catalisada por outros metais, como Cu). O $OH^{\bullet}$ pode reagir de três formas com outras moléculas: abstraindo prótons, transferindo elétrons ou adicionando-se a outras moléculas. A partir disso, ocorre uma reação em cadeia, uma vez que outras moléculas tornam-se reativas, embora com menos energia (Halliwell & Guterridge, 2000).

A reação de Fenton consiste na decomposição de H_2O_2 mediada por sais de ferro que gera $OH^{\bullet}$. A adição de um agente redutor, como o ascorbato, leva a um ciclo que aumenta o dano às biomoléculas (McNaught & Wilkinson, 1997). A reação de Fenton pode ser representada pela seguinte reação (Figura 4). A reação de Fenton pode ser mediada por outros metais como o cobre (*Fenton-like*).

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + OH^{\bullet}$$

Figura 4. Reação de Fenton.

A reação de Haber-Weiss consiste de duas etapas (Figura 5), sendo a segunda reação uma fonte potencial de $OH^{\bullet}$. Acredita-se que os complexos contendo Fe(III) podem catalisar esta reação, primeiro sendo reduzido a Fe(II) por $O_2^{\bullet-}$ e então reoxidado por H_2O_2. A exemplo das reações *Fenton-like*, a reação de Haber-Weiss também pode ser catalisada por outros metais (McNaught & Wilkinson, 1997).

$$H_2O_2 + OH^{\bullet} \xrightarrow{Fe} H_2O + O_2 + H^+$$
$$H_2O_2 + O_2^- \xrightarrow{Fe} O_2 + OH^- + OH^{\bullet}$$
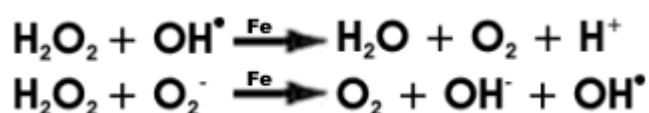
Figura 5. Reação de Haber-Weiss

Os íons metálicos também podem reagir com o ascorbato (Vit C) para produzir $^1O_2$ a partir de oxigênio tripleto normal ($^3O_2$) (Figura 6) (McNaught & Wilkinson, 1997).

$$Cu^{2+} + \text{vitamina C} + {}^3O_2 \longrightarrow {}^1O_2$$

Figura 6. Reação de formação do oxigênio singleto mediada por vitamina C.

1.2.2.1 Ferro e cobre

O cobre e o ferro são micronutrientes com inúmeros papéis metabólicos. O cobre, por exemplo, é consituinte da superóxido dismutase Cu/Zn e da ceruloplasmina. O ferro também é essencial, participando de inúmeras enzimas envolvidas desde o transporte de oxigênio, metabolismo oxidativo, defesas antioxidantes, síntese de ácidos nucléicos, até a metabolização de xenobióticos (De Freitas & Meneghini, 2001; Guecheva et al., 2001).

Se por um lado o cobre e o ferro são essenciais, por outro lado a exposição a quantidades excessivas desses metais pode danificar células e órgãos pela participação destes em reações que geram ROS. Muitos estudos mostram que compostos contendo ferro apresentam genotoxicidade e mutagencidade frente a diferentes sistemas biológicos *in vitro* (Heidelberger et al., 1983; Tucker et al., 1993; Abalea et al., 1999; Dunkel et al., 1999). Da mesma forma, compostos contendo cobre também são genotóxicos e mutagênicos em diferentes sistemas biológicos tanto *in vitro* quanto *in vivo* (Heidelberger et al., 1983; Ma et al., 1998; Guecheva et al., 2001). Contudo, a resposta pode variar dependendo do organismo e do sistema teste. O que pode ser explicado pela interação dos metais tanto entre si (i.e. competição no transporte) quanto com os sistemas biológicos, que é complexa, especialmente no que diz respeito ao impacto na carcinogenicidade e genotoxicidade (Rojas et al., 1999).

O ferro é rapidamente absorvido quando administrado sobre a forma de tablete (meia-vida de absorção de 0,54 h). O ferro administrado dessa forma tem um pico sérico 2,4 h após a administração e tem uma meia-vida de eliminação no sangue de cerca de 9,5 h (Farheen et al., 2002). O cobre também tem uma absorção rápida e intensa (1-3 h após a administração) (Earl et al., 1954; Bissig et al., 2005; Uriu-Adams et al., 2005) e a sua meia-vida plasmática

ainda é controversa, tendo sido associada a um período de até quatro semanas (Dekaban et al., 1975).

1.2.3 Alquilação do DNA

O metilmetanosulfonato (MMS) e a ciclofosfamida (CP) são agentes alquilantes utilizados em estudos de mutagênese e na quimioterapia. O MMS é um agente alquilante monofuncional de ação direta, considerado um mutágeno fraco. Por outro lado, a CP é um agente alquilante bifuncional de ação indireta, e, portanto, sua genotoxicidade é mediada por seus metabólitos, que também geram estresse oxidativo. A meia-vida plasmática da CP é de aproximadamente 6-9 h (Horvathova et al., 1998; Brozmanova et al., 2001a; Brozmanova et al., 2001b; Saffi & Henriques, 2003; Souliotis et al., 2003; Boiteux & Guillet, 2004; Matalon et al., 2004; Jenkins et al., 2005; NIH, 2006).

Tanto o MMS como a CP alquilam moléculas nucleofílicas como o DNA. Todas as bases do DNA possuem sítios suscetíveis à alquilação, especificamente no nitrogênio (N) e oxigênio (O). As bases púricas como a adenina (nas posições N1, N3 e N7) e a guanina (nas posições N2, N3 e N7 e O6) são mais suscetíveis à alquilação do que as bases pirimídicas [citosina (N3, N4 e O2) e timina (N3, O2 e O4)]. Destes, o N7 da guanina (N7G) e o N3 da adenina (N3A) são os sítios mais sucetíveis à alquilação (para revisão, ver Saffi & Henriques, 2003).

O MMS e a CP induzem substancialmente alquilações em N7G. Contudo, diferentemente do MMS, a CP também pode induzir pontes do tipo DNA-DNA e DNA-proteína, bem como adutos nos fosfatos. O MMS induz cerca de 83 % das alquilação em N7G, 11 % em N3A e apenas 0, 3% em O6-guanina (alquilações em outras posições de O não parecem ocorrer) (Jenkins et al., 2005). Das alquilações induzidas por CP, 67% são monoadutos fosfotriésteres, 26% são monoadutos em N7G e 7% são pontes (diadutos) que ligam duas guaninas através da posição N7G (Souliotis et al., 2003).

Enquanto a O6-alquilguanina causa transição de GC para AT, as N-alquilpurinas (N7G e N3A) não causam erros de emparelhamento durante a replicação. Contudo, as N-alquilpurinas podem levar à formação espontânea de sítios AP, devido ao enfraquecimento das ligações glicosídicas do DNA. Os sítios AP podem ser mutagênicos pela reincorporação de uma base errada. Além disso, adutos derivados do tipo anel-aberto (ring-opened) em N7G

podem inibir a replicação e constituem uma ameaça adicional de mutagênese (Jenkins et al., 2005).

Os fosfotriésteres são derivados da reação de agentes alquilantes com os fosfatos do DNA. A formação de monadutos metilfosfodiésteres, provavelmente tem pouco efeito pró-mutagênico, embora ainda se careça de evidências experimentais conclusivas sobre a mutagenicidade destas lesões. Por outro lado, adutos nos fosfatos formados por grupamentos grandes (*bulky*) podem induzir distorções significativas na conformação do DNA. Como conseqüência, levando ao emparelhamento incorreto durante a replicação do DNA, bem como impedindo a ligação de proteína ao DNA por neutralizar a carga negativa do esqueleto açúcar-fosfato do DNA, o que pode contribuir para quebra de cadeia de DNA (Singh et al., 1997; Voitkun et al., 1998).

As pontes intercadeias destorcem amplamente a estrutura do DNA e têm conseqüências catastróficas para a célula se não forem adequadamente reparadas, pois previnem a ocorrência da replicação e da transcrição, dentre outros processos (Saffi & Henriques, 2003; Drablos et al., 2004).

1.3   Modulação do estresse oxidativo e da estabilidade genômica: o papel do suco de laranja e da vitamina C

Os efeitos antimutagênicos e possivelmente anticarcinogênicos relacionados à ingestão de dietas ricas em Vit C e compostos fenólicos, bem como a Vit C de forma isolada, vêm sendo amplamente estudados. Esses efeitos têm sido relacionados à capacidade de proteção contra os danos ao material genético.

Há evidências epidemiológicas associando dietas ricas em vegetais e a redução da incidência de cardiopatias, doenças neurodegenerativas e câncer. Os efeitos protetores têm sido atribuídos, principalmente, à natureza complexa da composição das frutas (combinação de carotenóides, compostos fenólicos e vitaminas) (Wang et al., 1996; Kabasakalis, 2000; Halliwell, 2001). Entretanto, apesar das propriedades benéficas atribuídas aos compostos naturais, diversos trabalhos relatam efeitos mutagênicos ou carcinogênicos em sucos (Patrineli et al., 1996a; Ames & Gold, 1998; Yoshino et al., 1999; Franke et al., 2004).

Várias vezes os efeitos carcinogênicos ou genotóxicos podem ser mediados pela interação de componentes dos sucos com metais de transição ou com os sub-produtos de auto-oxidação (*browning*), por ação enzimática (polifenoloxidases), bem como por reações não-

enzimáticas (Reações de Maillard) (Patrineli et al., 1996a; Patrineli et al., 1996b; Vercet et al., 2001; Franke et al., 2004).

Alguns compostos fenólicos podem interagir com enzimas de metabolização, afetando a toxicidade de compostos (Doostdar et al., 2000). Além disso, a Vit C e os flavonóides podem agir como um pró-oxidante por sua capacidade redutora, em reações de Fenton e Fenton-*like* com metais de transição; mas também pode agir como *scavenger* (Vinson, 1998; Sakagami et al., 2000; Edenharder & Grunhage, 2003).

Resultados controversos e confusos associam o consumo de Vit C e a estabilidade genômica (Ames, 2001; Halliwell, 2001). Uma abordagem interessante é testar se a genotoxicidade de mutágenos de referência pode ser modulada pela Vit C. Além disso, uma vez que a Vit C pode interagir com metais e levar à formação de ROS, é importante testar a ação desta substância sobre a genotoxicidade de compostos metálicos.

1.3.1 Efeito na metabolização dos xenobióticos

Pelo menos três etapas podem ser enumeradas na descrição da interação dos xenobióticos com os sistemas biológicos: 1) absorção, transporte e eliminação; 2) metabolização; e 3) interação com o sítio-alvo. A absorção, o transporte e a eliminação podem ser espontâneos, ou mediados pelo metabolismo. Da mesma forma, os xenobióticos podem sofrer metabolização ou não antes de exercerem seus efeitos biológicos. A metabolização, em geral, tem efeito direto sobre a interação com o sítio-alvo, bem como com o transporte e a eliminação dos xenobióticos (Gaspar, 2003; Guecheva & Henriques, 2003; Henriques & Prá, 2005).

A metabolização envolve duas fases típicas, a fase I e a fase II. Atualmente, uma terceira fase vem sendo proposta como etapa do metabolismo de xenobióticos. As enzimas atuantes nas três fases são, via de regra, bastante polimórficas (Gaspar, 2003; Guecheva & Henriques, 2003; Henriques & Prá, 2005).

As reações da fase I incluem oxidação, redução e hidrólise, introduzindo grupamentos funcionais, tais como hidroxilas, na molécula do xenobiótico. A fase I é mediada principalmente (86%) pela ação das citocromo P450 monooxigenases (CYP). As CYPs são uma família ampla de enzimas dividida em várias classes de acordo com a substância metabolizada, apesar de muitas vezes duas ou mais classes serem capazes de metabolizar a mesma substância. O genótipo e a expressão gênica das CYPs apresentam grande

variabilidade interindividual (Evans & McLeod, 2003; Gaspar, 2003; Guecheva & Henriques, 2003; Henriques & Prá, 2005).

Na fase I, muitos compostos são convertidos a metabólitos altamente reativos, no fenômeno denominado bio-ativação. Já as enzimas da fase II reduzem a toxicidade pela conjugação dos metabólitos da fase I com substratos endógenos, como glutationa (GSH), sulfato ou glicose. Assim, os metabólitos tornam-se hidrofílicos e passíveis de excreção (Guecheva & Henriques, 2003) (Evans & McLeod, 2003). A fase II parece ser efetiva na quimioprevenção, fato mostrado pela incidência aumentada de vários tipos de câncer em camundongos com fatores de transcrição de enzimas de fase II disruptos (Moon et al., 2006).

Embora o suco afete a metabolização de várias drogas, os efeitos do OJ nas CYPs ainda está pouco caracterizado. Dessa forma, enquanto o suco de laranja azedo (SOJ - sour orange juice, obtido de *Citrus auratium*) inibe a CY3A4 (Malhotra et al., 2001; Di Marco et al., 2002; Mouly et al., 2005), o OJ (doce, obtido de *Citrus cinensis)* parece não afetar essa enzima (Bailey et al., 1991; Hashimoto et al., 1998; Takanaga et al., 2000; Tian et al., 2002). Os mesmos flavonóides estão presentes no OJ e no SOJ, embora em quantidades diferentes. Esses flavonóides têm sido reconhecidos como os responsáveis pela modulação das CYPs. A heperidina/hesperitina inibe CYP1A1 e CYP1B1 *in vitro*. Já a naringina/naringenina inibe CYP1A2 e CYP19 *in vitro* e CYP3A4, *in vivo* (Moon et al., 2006) (para revisão, ver ANEXOS C e D).

A família CYP1 está constituída pelos membros 1A1 e 1A2 e 1B1, que são capazes de ativar pró-carcinógenos. A CYP19, ou aromatase, é uma enzima fundamental para a síntese de estrógenos e sua expressão aumentada é comum em tumores mamários. As CYP3A4 estão envolvidas no metabolismo de cerca de 50 % dos agentes terapêuticos, bem como na ativação de substâncias tóxicas e carcinogênicas (Moon et al., 2006). A atividade inibitória do OJ ou de seus componentes sobre as CYPs parece ser um mecanismo efetivo da quimioproteção do alimento.

Quanto a fase II, o OJ inibe as sulfotransferases e promove o aumento da biodisponibilidade de drogas (Nishimuta et al., 2005). Contudo, a naringina/naringenina estimula a UDP-glucuronidação *in vitro*, embora seja um inbidor específico para a UGT1A1, também *in vitro* (Moon et al., 2006). A ação das sulfotranferases media a inativação tanto de xenobióticos quanto de compostos endógenos. A UDP-glucuroniltransferase 1 (UGT1) cataliza a glucuronidação hepática de xenobióticos, catabólitos do ferro heme, bem como estrógenos de catecol e flavonóides (Moon et al., 2006) (para revisão, ver ANEXOS C e D).

Enquanto o OJ parece não influenciar a atividade de fosfoglicoproteínas (P-gp), que catalizam o efluxo dos quimioterápicos das células mutantes quando em baixas concentrações, tanto em ratos como em humanos, ele pode inibir transportadores de influxo, como OATP [(*organic anion-transporting polypeptides* (OATP) (Ikegawa et al., 2000; Kamath et al., 2005; Lim & Lim, 2006)]. Desse modo, o OJ pode bloquear a entrada de compostos tóxicos nas células e estimular o efluxo dos mesmos quando em altas concentrações (para revisão, ver ANEXOS C e D).

1.3.2 Efeito no estresse oxidativo: as defesas antioxidantes enzimáticas e não-enzimáticas

As células possuem defesas eficientes contra danos oxidativos. A defesa antioxidante é exercida tanto por antioxidantes não enzimáticos, como por antioxidantes enzimáticos (Franke et al., 2003). As enzimas do sistema de defesa antioxidante são representadas principalmente pela catalase (CAT), pela superóxido dismutase (SOD) e pela glutationa peroxidase (GPx). As defesas antioxidantes enzimáticas procedem de maneira orquestrada para eliminar os sub-produtos do metabolismo oxidativo (Figura 7).

Os antioxidantes não enzimáticos podem ser sintetizados endogenamente ou serem absorvidos como parte da dieta ou por suplementação (para revisão, ver Halliwell & Guterridge, 2000). Os antioxidantes não-enzimáticos incluem minerais tais como selênio (Rayman, 2005) e zinco (Valko et al., 2005), moléculas orgânicas, tais como vitaminas (A e carotenóides, C e E) (Ames et al., 2005), um açúcar-álcool (mannitol) (Gillbe et al., 1996), um ácido graxo (ácido lipôico) (Vasdev et al., 2005), bem como um aminoácido (cisteína) e um peptídeo (GSH) (Sen & Packer, 2000). Os bioflavonóides, o ácido úrico (Glantzounis et al., 2005), a bilirrubina (Sedlak & Snyder, 2004) e a melanina (Wang et al., 2005) são também antioxidantes. O potencial antioxidante de muitos antioxidantes-não-enzimáticos é atribuído às hidroxilas (para revisão, ver Halliwell & Guterridge, 2000).

$$4 O_2^{\cdot -} \xrightarrow[+4H^+]{SOD} 2 O_2$$

catalase → $2 H_2O + O_2$

$2 H_2O_2$ — ou

GSH peroxidase

GSH redutase

4 GSH → 2 GSSG → 4 H_2O

NADP+ / NADPH
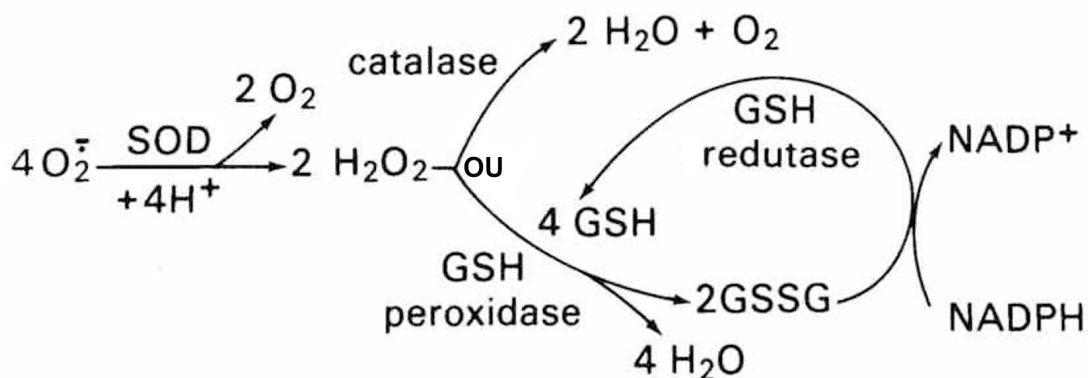
Figura 7. Principais defesas antioxidantes enzimáticas. Adaptado de Proctor & Reynolds (1984)

O potencial antioxidante do ácido lipóico, bem como da cisteína e da GSH é devido ao grupamento tiol. Os tióis são uma classe de derivados orgânicos caracterizados pela presença de resíduos sulfidril. Em sistemas biológicos, os tióis têm várias funções, incluindo um papel central na coordenação da rede de defesa (Sen & Packer, 2000). Os antioxidantes-não-enzimáticos podem ser tanto hidrofóbicos como hidrofílicos. Os hidrofóbicos, tais como os carotenóides e as vitaminas A e E, estão localizados na bicamada lipídica das membranas, enquanto a Vit C e os bio-flavonóides devem estar concentrados na superfície das membranas, bem como espalhados dentro e fora das células (Fang, 2004).

Dos antioxidantes presentes no OJ, os compostos fenólicos e a Vit C podem atuar: de forma preventiva, reduzindo a formação de ROS por quelar os metais de transição; de forma interceptativa, capturando ou estabilizando as ROS. Além disso, as vitaminas do complexo B e os carotenóides podem atuar interceptando ROS, além de atuar de outras formas, ainda pouco caracterizadas. Os carotenóides, por exemplo, podem estabilizar o $^1O_2$, extinguindo a sua energia (*quencher*) (Picada et al., 2003).

Os antioxidantes enzimáticos são representados por duas classes principais de enzimas, as superóxido dismutases (SOD) e as peroxidases (GPx e CAT). Tais enzimas são responsáveis pela conversão em espécies menos reativas ou pela neutralização das ROS. As enzimas $NO^{\cdot}$ redutase, S-nitrosoglutationa redutase e peroxinitrito redutase estão envolvidas na defesa bacteriana contra RNS (Slupphaug et al., 2003). É provável que tais enzimas sejam responsáveis pela resposta fisiológica em organismos mais complexos. Os $O_2^{\cdot -}$ são eliminados pela SOD, que a partir de dois $O_2^{\cdot -}$ e dois prótons gera H_2O_2 (menos reativo) e oxigênio. A atividade da SOD está presente tanto na mitocôndria como no núcleo e no espaço extracelular

(Mates et al., 1999). Há três diferentes formas de SOD em humanos: SOD Cu/Zn (citosólica), SOD-Mn (mitocondrial) e a SOD Fe (extracelular) (Mates et al., 1999).

Os sistemas celulares primários de defesa antioxidante enzimáticos contra H_2O_2 e hidroperóxidos lipídicos são o ciclo redox glutationa, o ciclo tioredoxina e a CAT. O ciclo redox glutationa oxida GSH a GSSG para converter H_2O_2 a H_2O (Figura 8).

As peroxidases são enzimas que utilizam uma variedade de redutores celulares para inativar peróxidos. Além de agirem sobre H_2O_2, também neutralizam peróxidos orgânicos, como alquil-hidro-peróxidos. Em mamíferos, as principais peroxidases são as GPx, que em associação com outras enzimas, previnem a interação do $O_2^{\cdot-}$, H_2O_2 e íons metálicos, especialmente nas mitocôndrias de mamíferos, onde não apresentam catalase. A GPx é uma seleno-enzima, cuja ação é baseada na oxidação da GSH a glutationa oxidada correspondente (GSSG) (Figura 8). A razão GSH/GSSG em células normais é alta, pois existe um mecanismo para reduzir GSSG novamente à GSH, realizado pela enzima glutationa redutase (GR) (Figura 9). O NADPH necessário para essa reação é provido por uma série de sistemas enzimáticos, incluindo a via das pentoses fosfato. Nesta via, a enzima glicose-6-fosfato desidrogenase (G6PD) é a responsável pela manutenção do NADPH (Borella & Varela, 2004).

Glutationa peroxidase

$$H_2O_2 + 2GSH \text{-------------------------> } GSSG + 2H_2O$$

Figura 8. Ação da glutationa peroxidase

Glutationa redutase

$$GSSG + NADPH + H^+ \text{-----------------------> } 2GSH + NADP^+$$
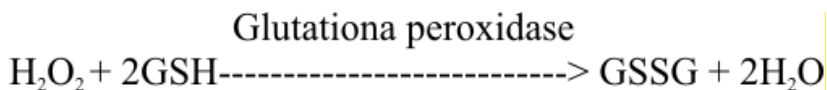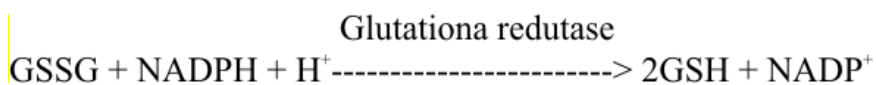
Figura 9. Redução da glutationa oxidada

Cabe lembrar que as GPx não atuam diretamente sobre peróxidos esterificados em moléculas de lipídios, que necessitam ser lisadas por lípases para a ação de GPx e que a GPx regenera o ascorbato a partir de dehidroascorbato (Borella & Varela, 2004). Este último aspecto ilustra a função da GPx no ciclo tioredoxina.

O ciclo da tioredoxina ou ciclo redox do tiol desempenha um papel chave na rede de defesas antioxidantes (Figura 10). Neste ciclo, tanto a GSH quanto o ácido lipóico são coordenados por equivalentes redutores celulares para gerar as suas formas reduzidas (GSH e

DHLA). A atividade do lipoato em aumentar o conteúdo celular de GSH é mediada pela redução de cistina para cisteína, o precursor da GSH (Sen & Packer, 2000).
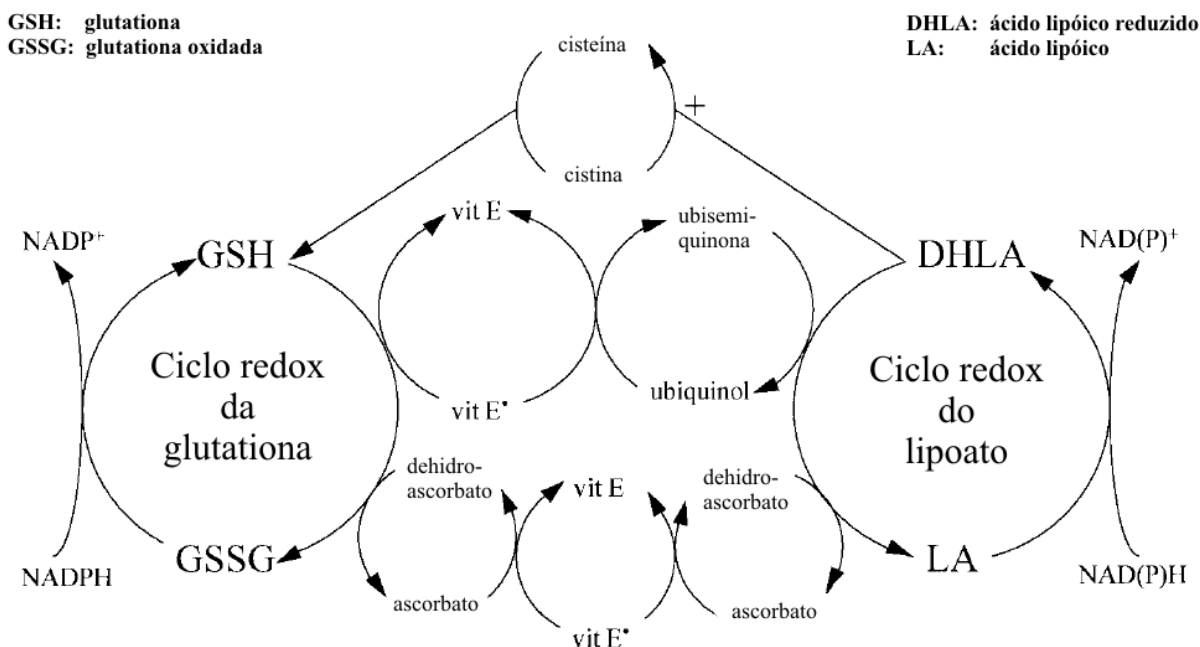
Figura 10. Ciclo redox do tiol. Adaptado de Sen & Packer (2000)

A CAT é a segunda enzima atuante na decomposição do H_2O_2, uma vez que está presente principalmente nos peroxissomas. Ela apresenta íon férrico no sítio ativo e converte duas moléculas de H_2O_2, cada uma em uma molécula de H_2O e uma de O_2 (Chelikani et al., 2004). Há três classes de proteínas com atividade de CAT: uma monofuncional que contém subunidade heme, uma bifuncional, também contendo heme e uma não-heme, contendo manganês (Jans et al., 2005).

A presença de micronutrientes como Fe e Cu no OJ, que participam dos sítios ativos de enzimas envolvidas no sistema de defesas antioxidante enzimático, poderiam atribuir ao mesmo apenas efeito benéfico. Contudo, estes mesmos micronutrientes podem induzir estresse oxidativo (Fridovich, 1998; Picada et al., 2003).

1.3.3 Efeito no reparo de DNA: mecanismos de reparo de danos oxidativos e de lesões induzidas por agentes alquilantes

As lesões no DNA podem ser corrigidas por diversas vias. A sobrecarga momentânea de uma via pode disparar outros processos de reparo, e uma densidade local de lesões pode

impedir um trabalho bem coordenado dos sistemas de reparação. Além disso, os próprios danos podem ser indutores de certas funções de reparação (Saffi & Henriques, 2003). A seguir, as vias de reparo preferenciais para os metais e os agentes alquilantes serão exploradas. Contudo, se dará ênfase à via de excisão de bases (BER), uma vez que essa via é preferencial para reparar danos oxidativos e de alquilação; e, os componentes do OJ podem modulá-la.

O dano oxidativo, induzido por agentes oxidantes endógenos ou exógenos (p.ex. metais e ROS), é reparado por BER, o principal "guardião" contra o dano oxidativo (Risom et al., 2005). As modificações nas bases ou açúcares dos nucleotídeos, bem como a presença de sítios AP, ativam o BER. Esse ocorre em duas etapas, uma de excisão e uma de pós-excisão (Figura 11). A clivagem da ligação N-glicosídica (açúcar-base) por uma DNA glicosilase (p.ex. 8-oxoguanina-DNA glicosilase 1–hOGG1) é a primeira etapa do BER. A clivagem da ligação fosfodiéster por uma 5'-AP endonuclease ou uma 3'-AP liase é a segunda etapa do BER. No caso de um sítio AP, o primeiro passo do BER é a clivagem da ligação fosfodiéster, pois a ligação N-glicosídica já está rompida. Em seguida, o açúcar correspondente à base excisada é também excisado por uma atividade 5'fosfodiesterase ou 3'diesterase. Estes passos constituem a etapa de excisão. A etapa de pós-excisão envolve a adição do(s) nucleotídeo(s) faltante(s) por uma DNA polimerase (DNA poli β) e a re-ligação da cadeia por uma DNA ligase. A etapa de pós-excisão pode ocorrer por duas vias distintas em mamíferos, uma via curta e uma via longa, dependendo do número de nucleotídeos reparados (Boiteux & Guillet, 2004; Rosa et al., 2004).
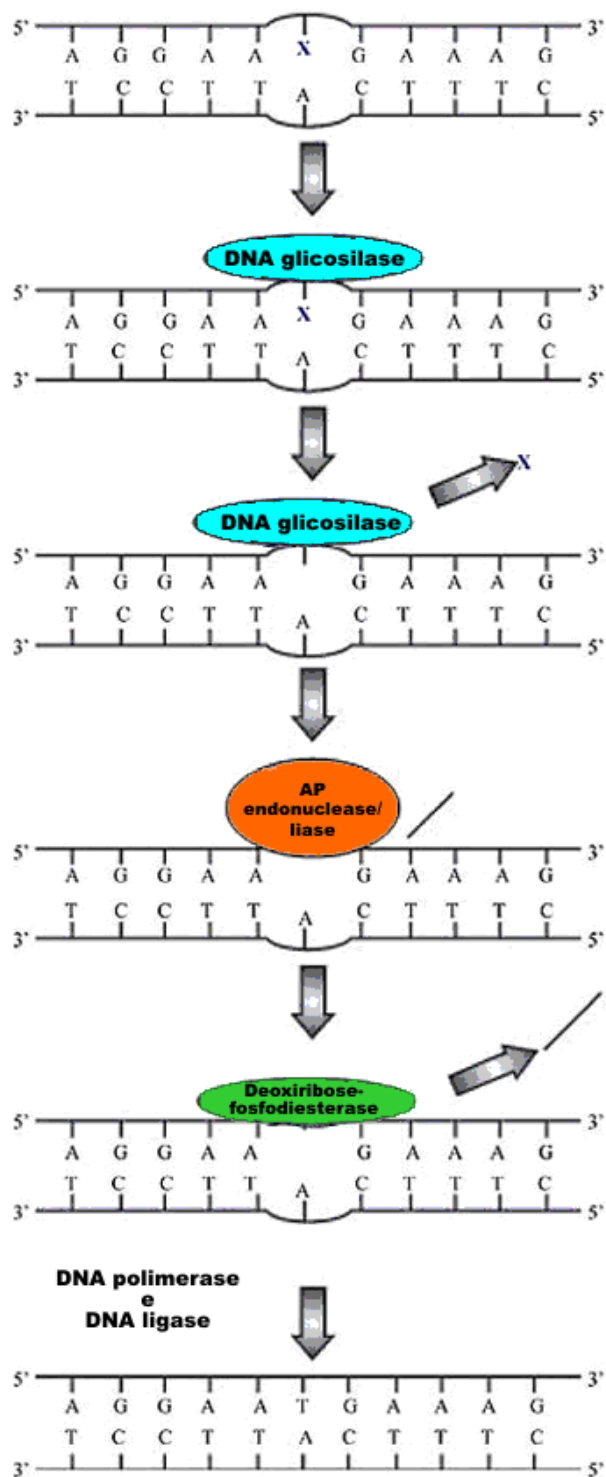
Figura 11. Reparo por excisão de bases (BER)

As alquilações no N e O das bases de DNA são reparadas principalmente BER, e por reversão de dano *in situ* (reparo direto de bases), e, em menor extensão, por reparo por excisão de nucleotídeos (NER) (Figura 12) (Drablos et al., 2004). As alquilações no N são reparadas tanto diretamente, por demetilases oxidativas de DNA (homólogos humanos das

proteínas alquiltransferases AlkB de *Escherichia coli)*, quanto por BER. Já, a alquilação no O é reparada tanto diretamente, por reversão de dano *in situ*, mediada pela enzima O^6-alquilguanina-7 DNA alquiltransferase (AGT), quanto por BER/NER (Figura 12) (Drablos et al., 2004).
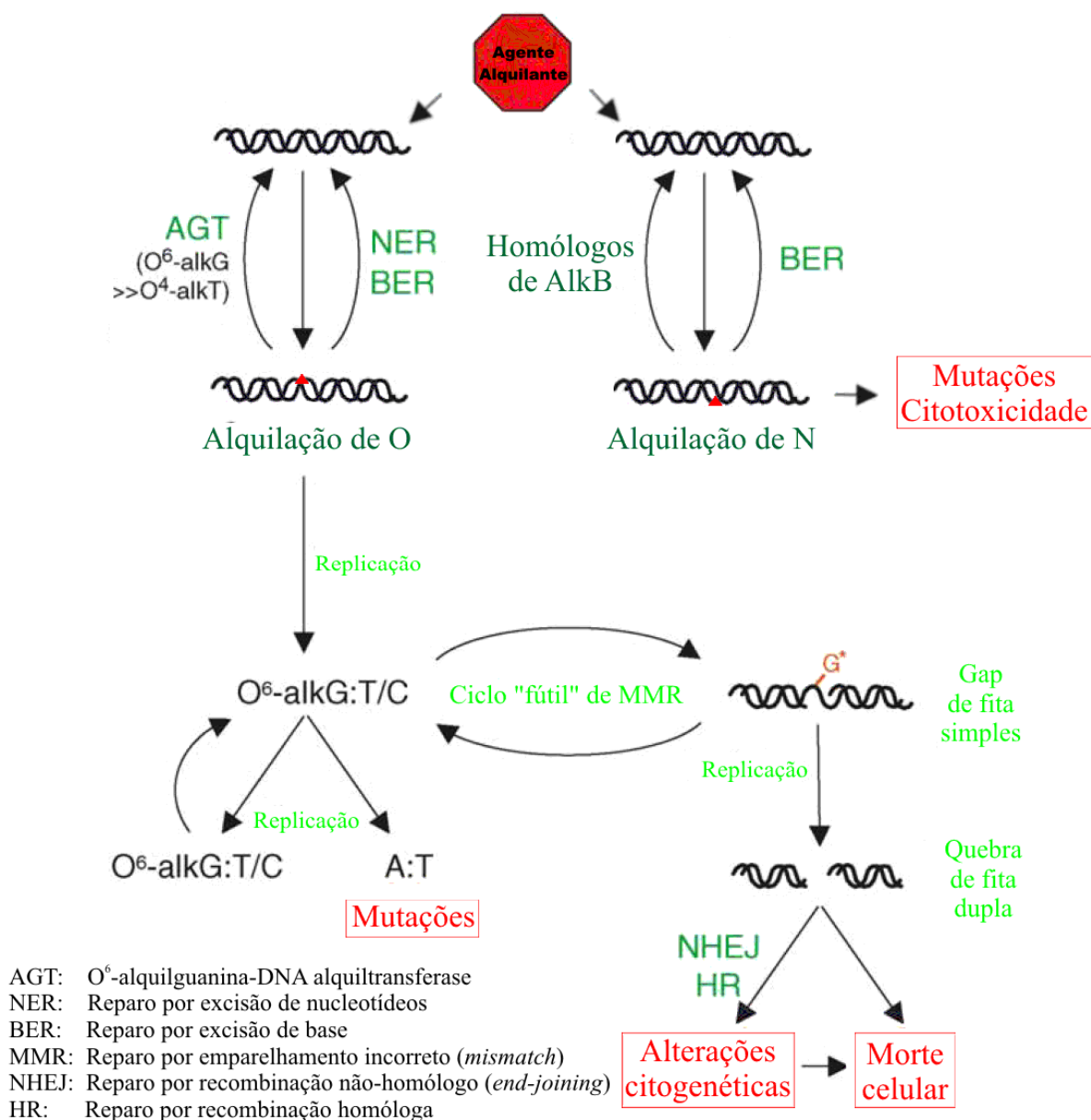
Figura 12. Reparo de sítios reativos para alquilação no DNA. As N-alquilações são reparadas tanto diretamente por demetilases ou por BER. As O-alquilações são reparadas por AGT ou por NER/BER. Se as lesões O6-alkG:T não forem reparadas até a replicação, elas podem ser reconhecidas por MMR, induzindo "ciclos fúteis de reparo", levando a formação de quebras duplas de cadeia de DNA, alterações citogenéticas e morte celular. Adaptado de Drablos et al. (2004).

O envolvimento de ambas cadeias de DNA nas pontes torna o reparo destas lesões difícil. Por isso, as células, provavelmente, utilizam fatores de diversas rotas de reparo de maneira coordenada, incluindo enzimas do NER, bem como enzimas da via de recombinação homóloga (Saffi & Henriques, 2003; Drablos et al., 2004).

Como as 3NA (Saffi & Henriques, 2003), as pontes intercadeias no DNA (McHugh et al., 2001) induzem reparo recombinacional envolvendo terminações de DNA com regiões homólogas (HR) e não homólogas (*Nonhomologous End Joining* - NHEJ), sendo esta rota de reparo possivelmente comum na resposta das células aos danos no DNA induzidos tanto por CP quanto por MMS. Convém ressaltar que o reparo recombinacional do tipo NHEJ leva a perda de informações, portanto é mutagênico.

Quando as alquilações não são adequadamente reparadas, elas podem induzir lesões secundárias no DNA. Por exemplo, as alquilações O6-alkG:T, que promovem o emparelhamento incorreto do DNA, quando não reparadas até a replicação podem ser reconhecidas pelo sistema MMR (*mismatch repair)* e induzir um "ciclo fútil de reparo". Como conseqüência, estas lesões podem gerar quebras duplas de cadeia de DNA, e conseguintes alterações citogenéticas de maior ou menor toxicidade para as células (Drablos et al., 2004). As quebras geradas, podem, então, ser processadas por sistemas de reparo específicos para quebras de cadeia dupla no DNA, como os reparos por recombinação homóloga (HR) e não homóloga (NHEJ) (Drablos et al., 2004) . As pontes também induzem alterações secundárias no DNA (Drablos et al., 2004).

Existem evidências do estímulo da via BER pela naringinina (Gao et al., 2006) e pela Vit C (Cooke et al., 1998). O tratamento por 24 h com naringinina aumentou significativamente a expressão de hOGG1 e DNA poli β em células derivadas de tumores de próstata tratadas com sulfato ferroso (Gao et al., 2006). Além disso, a Vit C em doses de até 500 mg/dia estimulou a excreção urinária de 8-OHdG. Este aumento da excreção de 8-OHdG tem sido interpretado como um estímulo potencial de reparo (Cooke et al., 1998).

Outros nutrientes presentes no OJ, como o folato, a piridoxina, riboflavina, a niacina e o magnésio têm papéis importantes no metabolismo e no reparo de DNA (Fenech et al., 2005). O folato (B9) na forma de 5-metil tetrahidrofolato previne a incorporação do uracil no DNA, bem como mantém o padrão de metilação do DNA (Fenech et al., 2005). Essa substância é gerada a partir da redução de 5,10-metileno tetrahidrofolato folato pela enzima metil tetrahidrofolato redutase (MTHFR), que utiliza a riboflavina (B2) como cofator (Powers, 2005). A piridoxina (B6) regenera 5,10-metileno tetrahidrofolato (Wei et al., 2005) e também parece promover o reparo por excisão (Shimoi et al., 1992). A niacina (B3) está

envolvida no reparo por excisão de bases por atuar como substrato da poli(ADP-ribose) polimerase-1 (PARP-1) (Hageman & Stierum, 2001). O magnésio atua como cofator das DNA polimerases e participa de BER e NER e do reparo por emparelhamento incorreto (MMR) (Ames et al., 2005; Fenech et al., 2005).

1.3.4 Modulação por competição/interceptação de substâncias

Embora se atribua a quimioproteção principalmente ao potencial antioxidante, outros mecanismos de proteção também podem ocorrer. O caráter nucleofílico de alguns compostos presentes no OJ pode atuar como agentes preventivos contra a mutagênese e carcinogênese. A Vit C, pode competir com as bases do DNA como alvo de ataque para ação de agentes alquilantes. Argumenta-se que a Vit C pode proteger contra ataques eletrofílicos ao DNA pela interceptação de agentes reativos. Como a Vit C pode ser alquilada, ela compete efetivamente como alvo de ataque com o DNA frente a agentes alquilantes (Edgar, 1974; Vijayalaxmi & Venu, 1999). Os retinóides também podem proteger os sítios nucleofílicos do DNA (De Flora, 1998). Assim como a Vit C, os flavonóides também podem formar complexos com compostos tóxicos, inibindo a reatividade dos mesmos com os sistemas biológicos (Wang et al., 1996; De Flora, 1998; Middleton et al., 2000).

1.3.5 Modulação por regulação do ciclo celular

O ciclo celular eucariótico é composto de uma cadeia de eventos que garantem a eliminação de mutações, a replicação do material genético e a segregação cromossômica adequada. A parada no ciclo celular é uma estratégia adotada pelas células para aumentar o tempo para reparar as lesões no DNA, reduzindo a letalidade e as conseqüências genéticas das mesmas (Saffi & Henriques, 2003).

A regulação do ciclo celular é efetuada por uma cascata de eventos relacionados a pontos de checagem (*checkpoint*). Os *checkpoints* são mecanismos vigilantes que monitoram processos metabólicos chaves e a integridade genômica durante o ciclo celular e atuam para atrasar ou parar a progressão do ciclo celular em resposta a defeitos ou danos (Hartwell & Weinert, 1989). Os flavonóides, os retinóides e o limoneno são inibidores efetivos da carcinogênese, por inibirem a progressão do ciclo celular, inibirem onconogenes, protegerem

a comunicação intercelular e/ou induzirem apoptose (So et al., 1996; De Flora, 1998; Guthrie & Carroll, 1998). No caso dos retinóides, a inibição da tirosina quinase pode estar relacionada à quimioproteção (De Flora, 1998). A inibição da tirosina quinase, a qual torna-se hiperfosforilada, causa parada ou atraso no ciclo celular que permite o *checkpoint* para reparo de danos no DNA, gerando um efeito quimiopreventivo (Booher et al., 1993).

Altas concentrações de Vit C também podem induzir a morte celular apoptótica (Sakagami et al., 2000). Além disso, a nicina, outro componente do OJ, tem papel na expressão e ativação do gene p53 (Hageman & Stierum, 2001). O gene p53 garante o funcionamento da via de apoptose, reduzindo a probabilidade de eventos promotores de processos tumorais (Saffi & Henriques, 2003).

1.4 Substâncias, organismos-teste e metodologias empregados neste estudo

Neste estudo serão empregados dois agentes alquilantes, dois compostos metálicos, uma mistura complexa (OJ) e uma vitamina isolada (Vit C) presente nesta mistura. Serão utilizadas duas metodologias, uma para avaliar a genotoxidade (ensaio Cometa) e uma para avaliar o nível de metais (PIXE) tanto no OJ como em dois tecidos de camundongos (fígado e sangue).

1.4.1 Substâncias-teste

Os agentes alquilantes metilmetanosulfonato (MMS) e ciclofosfamida (CP) e os metais cobre e ferro como sulfatos ($FeSO_4$ e $CuSO_4$, respectivamente) foram empregados neste estudo como indutores de genotoxicidade. Adicionalmente, o OJ e a Vit C foram avaliados quanto a sua genotoxicidade e, também, quanto à capacidade de modular a genotoxicidade gerada por MMS, CP, $FeSO_4$ e $CuSO_4$. O critério de seleção dos agentes alquilantes se baseia na alta prevalência dos danos gerados por estes compostos. Além disso, utilizou-se a CP, por ser dependente da ação diversa de seus metabólitos, em comparação ao MMS, que tem ação direta sobre o DNA. Optou-se pelo uso de Cu e Fe pela sua ubiqüidade ambiental e presença em alimentos enriquecidos, em suplementos alimentares ou em medicamentos; pelo seu papel biológico; e pela interação dos mesmos com as vitaminas, especialmente com a Vit C. Durante muito tempo a Vit C foi considerada como o principal antioxidante exógeno aos humanos. Contudo, vários estudos têm reconhecido que ela trabalha

em conjunto com outras substâncias antioxidantes. Desse modo, estudos relacionando o papel da Vit C *per se* e como parte de misturas são necessários para o melhor entendimento da real participação da mesma na atividade antioxidante, antigenotóxica e anticarcinogênica.

1.4.2 Organismos testes

De acordo com os *guidelines* descrito por Hartmann et al. (2003) e atualizado por Collins (2004), os roedores, como os camundongos, são organismos testes preferenciais para o ensaio Cometa *in vivo*.

1.4.3 Ensaio Cometa

O ensaio Cometa, conhecido como SGCE (*Single Cell Gel Electroforesis*), tem sido amplamente utilizado pela comunidade científica como um teste rápido *in vivo* e *in vitro* para detectar genotoxicidade como conseqüência de danos ao DNA. O teste Cometa é utilizado amplamente em genética médica, genética toxicológica e ecotoxicológica, em diagnósticos e tratamentos médicos, medicina ambiental, ocupacional, biomonitoramento ambiental, dentre outras aplicações (Da Silva et al., 2000).

Em condições alcalinas, o ensaio Cometa detecta quebras simples e dupla, além de sítios álcali-lábeis. Dentre as vantagens do teste, destacam-se: sensibilidade para detecção de baixos níveis de danos ao DNA; pequeno número de células; flexibilidade; economia; facilidade de aplicação; habilidade para conduzir estudos usando um pequeno número de amostras; rapidez (curto período de tempo para o experimento); pode ser usado em humanos e em estudos ambientais, e para avaliar reparo de DNA (Singh et al., 1988; Fairbairn et al., 1995; Tice et al., 2000; Hartmann et al., 2003; Collins, 2004).

As células englobadas em gel sobre uma lâmina são submetidas a uma corrente elétrica, que faz migrar para fora do núcleo os segmentos de DNA livres, resultantes de quebras. Após a eletroforese, as células que apresentam um núcleo redondo são identificadas como normais, sem dano reconhecível no DNA. Por outro lado, as células lesadas são identificadas visualmente por uma espécie de cauda, como de um cometa, formada pelos fragmentos de DNA. Estes fragmentos podem se apresentar em diferentes tamanhos, e ainda estar associados ao núcleo por uma cadeia simples. Para alguns autores o tamanho da cauda é

proporcional ao dano que foi causado, mas somente é de consenso que a visualização do "Cometa", significa dano ao nível do DNA, podendo ser quebra simples, duplas e/ou lesões álcali-lábeis (Singh et al., 1988; Fairbairn et al., 1995; Tice et al., 2000; Hartmann et al., 2003; Collins, 2004). Os danos no DNA, avaliados pelo ensaio Cometa, são classificados de acordo com a migração do DNA em 4 classes, podendo ser de zero (nenhum dano) até 4 (dano máximo). Além disso, o ensaio Cometa permite a visualização de células apoptóticas (Figura 13)(Franke et al., 2005a; 2005b; Prá et al., 2005; Franke et al., 2006)**.**
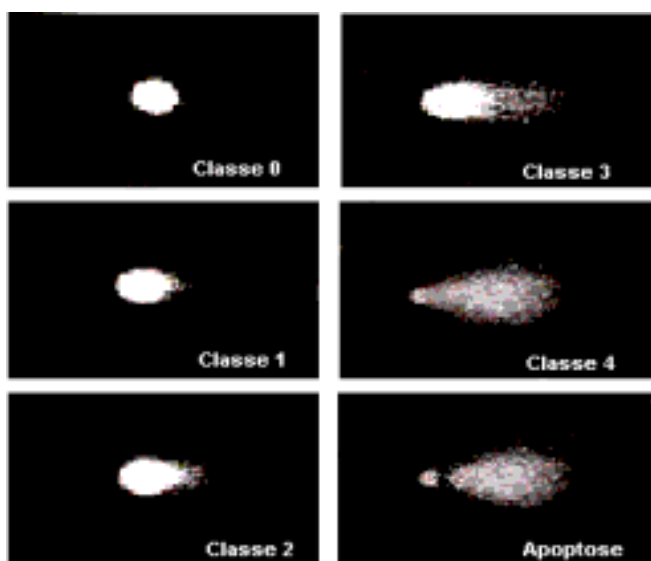
Figura 13. Classes de dano no DNA das células como visualizado no ensaio Cometa.

A identificação do dano no DNA pode ser feita por diferentes maneiras, uma forma é medir o comprimento do DNA migrante com a ajuda de uma ocular de medidas. Outra forma é, após classificar visualmente em diferentes classes as células com dano, se obter um valor arbitrário que expresse o dano geral que uma população de células sofreu (Speit et al., 1996). O dano geral, chamado de índice de dano (ID), é obtido pela soma das classes, podendo este valor ser de zero (100 células com danos classe zero) até 400 (dano máximo: 100 células com classe de dano 4).

1.4.4 PIXE

A técnica PIXE (*Particle-Induced X-ray Emission*) baseia-se, como o próprio nome indica, na produção de raios-X característicos induzidos pela passagem de um íon qualquer na

vizinhança de um átomo constituinte do material em estudo (Johansson et al., 1995; Yoneama & Dias, 2004).

Nesse processo de interação íon-matéria, elétrons de camadas internas dos átomos do material podem ser emitidos, deixando uma vacância nessa particular camada. Neste caso, o átomo, como um todo, encontra-se em um estado excitado, com o excesso de energia correspondente à energia transferida pelo íon incidente. Imediatamente, o átomo excitado procura restabelecer seu estado natural através de um reordenamento de seus elétrons de suas diversas camadas. Em particular, elétrons de camadas mais externas poderão preencher as vacâncias deixadas por elétrons emitidos de camadas mais internas. Nesse processo de transição eletrônica entre camadas, pode haver a emissão de um raio-X do átomo em questão. Uma vez que a energia do raio-X emitido depende da diferença de energia das camadas eletrônicas envolvidas na transição, e levando-se em conta que essa energia é particular para cada elemento da tabela periódica, então esse raio-X é dito característico por representar, univocamente, um determinado elemento da tabela periódica (Yoneama & Dias, 2004).

A metodologia PIXE é uma metodologia relativamente rápida e altamente sensível para detectar a concentração elementar de uma faixa ampla de elementos químicos em diversos tipos de amostras tais como, proteínas (Follmer *et al.*, 2002) e organismos inteiros (Kern *et al.*, 2004).

Para a análise por PIXE convencional no vácuo, as amostras devem estar na forma sólida, homogêneas (para possibilitar uma análise representativa do material investigado) e não devem apresentar rugosidades (a superfície da amostra deve ser plana). Amostras líquidas como sangue, bem como amostras de órgãos de animais, devem ser compactadas em alvos (pastilhas). O processamento de secagem das amostras pode ser feito tanto por liofilização quanto por aquecimento em estufa/forno. O sangue e os tecidos sólidos são geralmente mantidos em *ultra-freezer* a –80°C, posteriormente liofilizadas, homogenizadas e transformadas em pastilhas por prensagem e mantidas em dessecador.

O Laboratório de Implantação Iônica do IF-UFRGS conta com um acelerador Tandetron que possui uma linha PIXE convencional a vácuo. Esse acelerador eletrostático possui uma tensão de terminal de 3 MV positivos. Nesta configuração, as amostras são irradiadas com feixes de prótons de 2 MeV. A câmara de reações é mantida em vácuo da ordem de 10^{-6} milibar por meio de uma bomba turbo-molecular. Internamente, a câmara de reações possui um filamento de tungstênio que opera a 4,5 V localizada próxima ao suporte de alvos. Esse filamento funciona como um canhão de elétrons, jogando estes em amostras que são isolantes. A finalidade deste sistema é descarregar amostras isolantes, diminuindo

assim a radiação de fundo gerada por elétrons secundários acelerados a altos potenciais. As amostras a serem estudadas são carregadas em um suporte com capacidade para 10 alvos (suporte para amostras com 2 cm de diâmetro) e 15 alvos (suporte para amostras com 1 cm de diâmetro). Esse suporte é montado em uma pré-câmara, que é conectada à câmara de reações. Esse sistema permite o carregamento das amostras sem quebra do vácuo dentro da câmara principal (Yoneama & Dias, 2004). As pastilhas dispostas nos suportes e colocadas na câmara de reação são irradiadas pelo feixe de prótons (2,0 MeV e 1 nA). Os raios-X característicos induzidos pelas reações são detectados por dois detectores, um de germânio hiperpuro (resolução de energia de 175 eV a 5,9 keV) e outro de silício-lítio (155 eV a 5,9 keV) (Figura 14 e Figura 15).
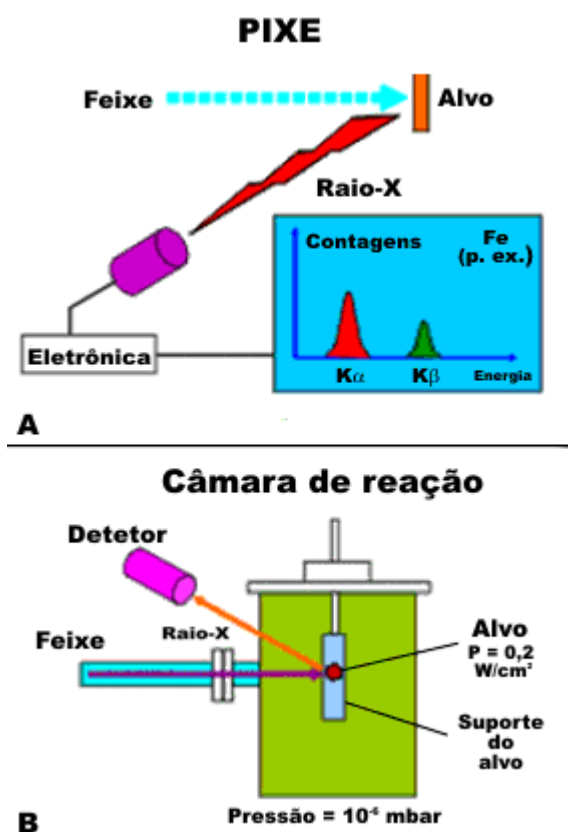
Figura 14. Particle-Induced X-ray Emission (PIXE): A) representação esquemática da metodologia e B) disposição da amostra na câmara de reação. Adaptado de figura cedida pelo Dr. Johnny Ferraz Dias.

Particle-Induced X-ray Emission

ÁTOMO X

protons

	K_β	K_α
Se X = Fe	7,06 KeV	6,41 KeV
Se X = Cu	8,91 KeV	8,05 KeV
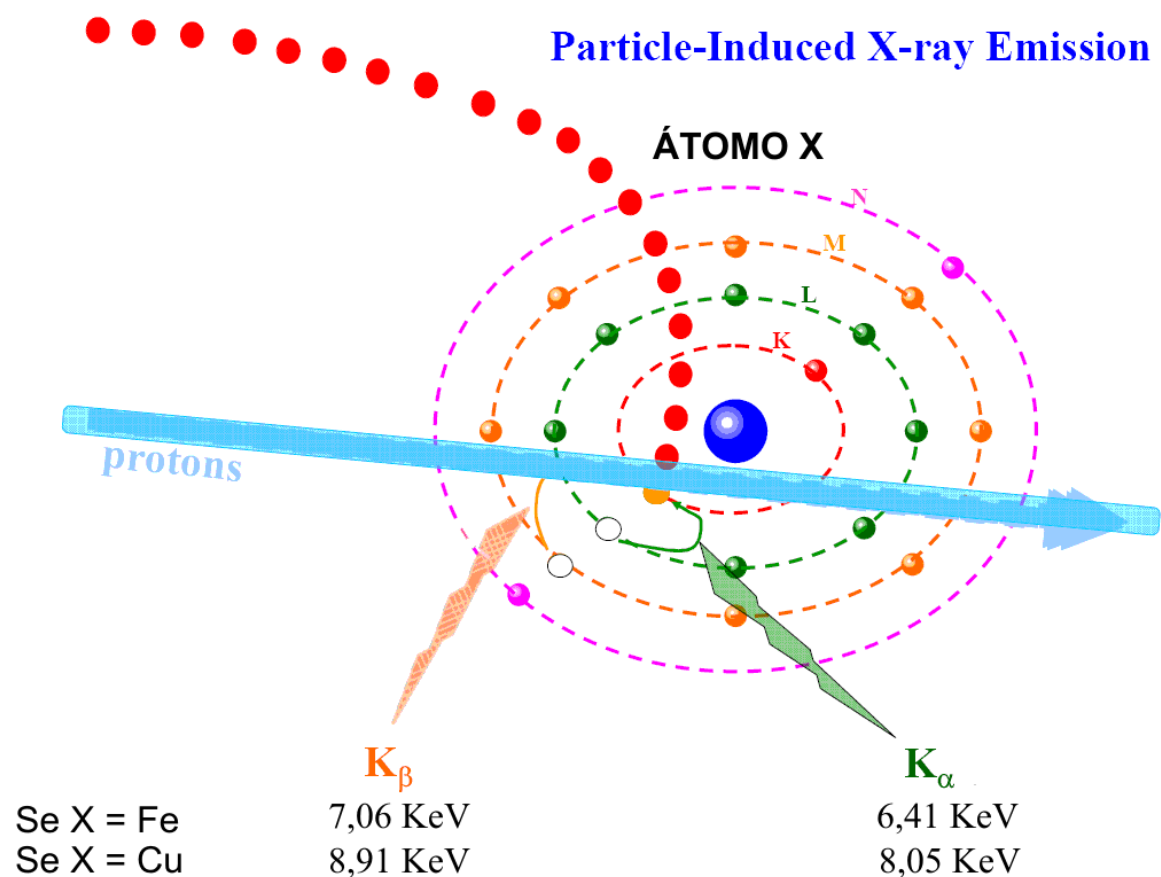
Figura 15. Princípio físico da metodologia de Particle-Induced X-ray Emission (PIXE). Adaptado de figura cedida pelo Dr. Johnny Ferraz Dias.

Os espectros de raios-X são analisados utilizando o código GUPIX, desenvolvido na Universidade de Guelph (Maxwell et al., 1989; Maxwell et al., 1995; Campbell et al., 2000). Através da técnica de padronização, responsável pela análise quantitativa dos elementos, as áreas dos picos dos raios-x são convertidas em concentrações elementares utilizando-se o programa GUPIX. Uma amostra padrão de fígado bovino da NIST (material de referência 1577b) é utilizada para o procedimento de padronização para análise das amostras como sangue e fígado.

2   OBJETIVOS

2.1   Geral

Avaliar, *in vivo*, a genotoxicidade e a possível capacidade moduladora do OJ e de duas doses de Vit C sobre a genotoxicidade de compostos metálicos e agentes alquilantes, utilizando o ensaio Cometa.

2.2   Específicos

- Avaliar a genotoxicidade do OJ em camundongos pelo teste Cometa.
- Avaliar a genotoxicidade da Vit C em camundongos pelo teste Cometa.
- Determinar a genotoxicidade do ferro e do cobre pela aplicação do teste Cometa e em células sangüíneas de camundongos.
- Determinar se o pré e o pós-tratamento com OJ afetam a genotoxicidade dos compostos metálicos e dos agentes alquilantes pelo ensaio Cometa.
- Determinar se o pós-tratamento com Vit C pode modular a genotoxicidade dos compostos metálicos pelo teste Cometa em camundongos.
- Quantificar os níveis séricos e hepáticos de ferro e cobre nos camundongos submetidos aos tratamentos, por Particle-Induced X-ray Emission analysis (PIXE).

3   MATERIAIS E MÉTODOS, RESULTADOS E DISCUSSÕES (ARTIGOS)

3.1 Capítulo 1: "Influence of orange juice over the genotoxicity induced by alkylating agents: an *in vivo* analysis"

Franke, S.I.R., Prá, D., Erdtmann, B., Henriques, J.A.P., Da Silva, J.

Apesar da existência de estudos sobre a genotoxicidade/antigenotoxicidade do OJ em procariotos e *ex vivo* em humanos, há poucos estudos *in vivo* em mamíferos. Neste sentido, o primeiro objetivo deste estudo foi avaliar a genotoxicidade para mamíferos *in vivo*, tendo em vista as respostas positivas observadas no nosso estudo prévio com bactérias. O segundo objetivo foi verificar a influência do OJ na genotoxicidade induzida pelos dois agentes alquilantes, levando-se em consideração as diferenças quanto ao modo de ação dessas duas drogas. Para tanto, testamos o efeito do OJ tanto antes quanto após o tratamento com uma única dose dos agentes alquilantes.

Com base nos resultados, procurou-se propor possíveis mecanismos de defesa/reparo, contra a genotoxicidade de agentes alquilantes, mediados pelo OJ. Como os seres humanos são expostos a agentes alquilantes no dia-a-dia, os resultados deste estudo podem servir ao entendimento dos aspectos genoprotetores dos alimentos.

Influence of orange juice over the genotoxicity induced by alkylating 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 metabolization/detoxification routes. Considering the different mechanisms of the action of the two drugs, different protective effects are suggested. These results demonstated 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 neoplasic 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 neoplasic processes by different mechanisms.

MMS and CP alkylate nucleophilic organic macromolecules, including DNA. They can induce depurination and depyrimidation 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 *in vitro* and *in vivo*), unscheduled DNA synthesis (UDS) (mammalian cells *in vitro* and *in vivo*) and sister chromatid exchange (SCE) (mammalian cells *in vitro* and *in vivo*), as well as other genotoxic effects (http://toxnet.nlm.nh.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 *in vitro* test systems. Thus, this work aims to evaluate, *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

Chemical reagents

Phosphate buffered saline (calcium- and magnesium-free), Tris [tris (hydroxymethyl) aminomethanehydrochloride], disodium ethylenediamine-tetra-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®.

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

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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 LD_{50} dose [25 mg/kg body wt (LD_{50} = 137 mg/kg body wt (12)] was used. For MMS, the dose was equal to 13.8% of the LD_{50} dose [40 mg/kg body wt (LD_{50} = 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). The resulting mixture was spread over a pre-coated microscope slide (1.5 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 Na_2EDTA (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.

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 $P \leq 0.05$. 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, $P \leq 0.001$). However, at 48 h, the extent of DNA damage did not differ between mice treated with water and orange juice.

Table I. Experimental procedures

Procedure	Exposure schedule		
	0 h	24 h	48 h
Control	Water treatment	1st Blood sampling 2nd Water treatment	Blood sampling
	Orange juice treatment	1st Blood sampling 2nd Juice treatment	Blood sampling
Pre-treatment	Orange juice treatment	1st Blood sampling[a] 2nd Treatment: 1. MMS 2. CP	Blood sampling
Post-treatment	Treatment: 1. MMS 2. CP	1st Blood sampling[b] 2nd Orange juice treatment	Blood sampling
Alkylating agents	Treatment: 1. MMS 2. CP	1st Blood sampling – –	Blood sampling

[a]Individuals also used as orange juice controls at 24 h.
[b]Individuals also used as MMS and CP at 24 h.

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)

Substances	Single doses (mg/kg body wt)	Schedule[a] and comet assay parameters							
		24 h		48 h		Pre-treatment with orange juice[b]		Post-treatment with orange juice[c]	
		Damage index (DI)							
		DI $\pm$ SD	n[d]	DI $\pm$ SD	n	DI $\pm$ SD	n	DI $\pm$ SD	n
Water	–	11.33 $\pm$ 2.73	6	19.00 $\pm$ 3.29[e]***	6	–	–	–	–
Orange juice	–	22.00 $\pm$ 5.36	24	26.67 $\pm$ 4.03	6	–	–	–	–
MMS	40.00	170.63 $\pm$ 20.21[f]***, [g]***	24	157.67 $\pm$ 15.28[f]***, [g]*	6	56.17 $\pm$ 42.60[e]***	6	95.17 $\pm$ 16.34[h]***	6
CP	25.00	114.54 $\pm$ 39.59[f]***, [g]***	24	74.83 $\pm$ 16.68[e]*, [f]*	6	97.83 $\pm$ 12.38	6	32.33 $\pm$ 20.16[h]**	6
		Damage frequency (DF)							
		DF $\pm$ SD	n	DF $\pm$ SD	n	DF $\pm$ SD	n	DF $\pm$ SD	n
Water	–	9.33 $\pm$ 2.34	6	11.17 $\pm$ 3.66	6	–	–	–	–
Orange juice	–	17.71 $\pm$ 3.93	24	20.50 $\pm$ 3.33	6	–	–	–	–
MMS	40.00	98.75 $\pm$ 2.71[f]***, [g]***	24	87.00 $\pm$ 8.74[e]*, [f]***,[g]*	6	38.50 $\pm$ 21.99[e]***	6	64.33 $\pm$ 11.59[h]**	6
CP	25.00	73.96 $\pm$ 22.04[f]**, [g]***	24	62.33 $\pm$ 15.27[f]*	6	64.50 $\pm$ 16.54	6	28.67 $\pm$ 18.12[h]**	6

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.
[a]For more details see Table I.
[b]Group sampled 24 h after treatment with an alkylating agent.
[c]Group sampled 48 h after treatment with an alkylating agent.
[d]n, Number of individuals obtained from sum of independent experiments.
[e]Significant in relation to 24 h at *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.
[f]Significant in relation to water at *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.
[g]Significant in relation to orange juice at *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.
[h]Significant in relation to 48 h at *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.

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 ($P \leq 0.05$). Although decreasing in relation to 24 h values, the DNA damage levels for MMS at 48 h remained significantly higher in relation to water ($P \leq 0.001$) and orange juice (for DI and DF, $P \leq 0.05$) treatments. For CP at 48 h, the level of DNA damage remained higher than for water (for DI and DF, $P \leq 0.05$) (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 ($P \leq 0.001$) (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
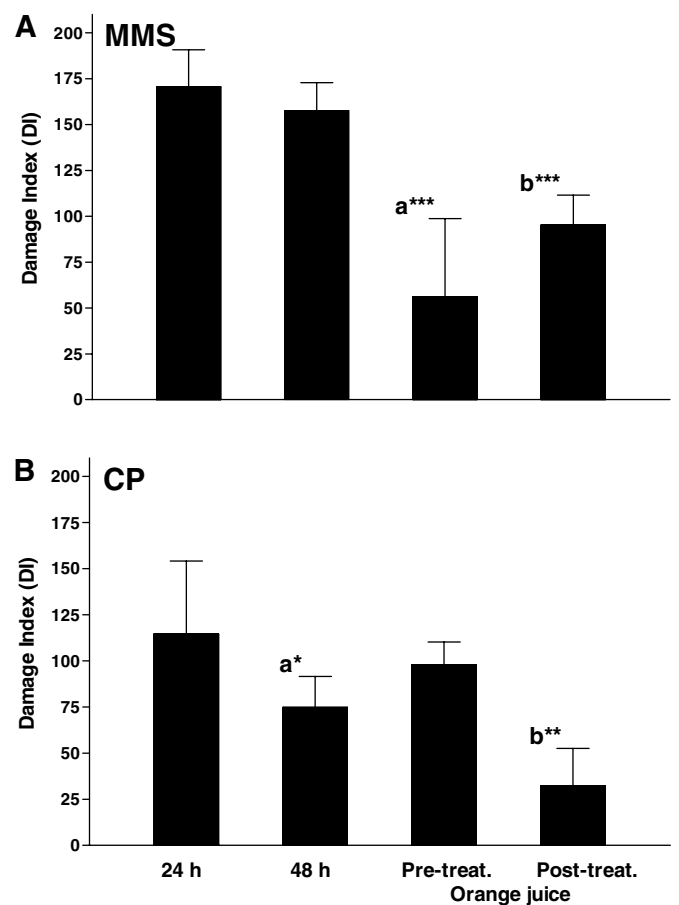
Fig. 2. Peripheral white blood cells damage index (DI) modulation induced by orange juice in mice treated with MMS (**A**) and CP (**B**), evaluated by comet assay. a: significant in relation to 24 h. b: significant in relation to 48 h. *$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.001$.

281

34

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 flavonids, like hesperetin, can selectively inhibit human Cytochrome P450 (34), reducing the absorption/elimination of toxic compounds. Other phenolic compounds, such as limonoids are inducers of the detoxifying enzyme gluthatione *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*-glycosylic 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 reparative 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 monoadducts, 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 metabolization 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 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 demonstate the ability of the *in vivo* comet assay to detect *in vivo* modulation of MMS and CP mutagenicity by orange juice.

Acknowledgements

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3.2 Capítulo 2: "Influence of orange juice in the levels and in the genotoxicity of iron and copper"

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É conhecido que as reações redox mediadas ou facilitadas por metais de transição e agentes redutores, ambos compostos presentes no OJ, podem gerar ROS capazes de danificar o DNA. Contudo, também é conhecido que sucos cítricos podem proteger contra os danos oxidativos. Em nossos estudos prévios *in vitro*, o OJ mostrou forte atividade antioxidante, não obstante tendo sido pró-oxidante em alguns sistemas. Baseado nestes aspectos, decidiu-se avaliar *in vivo* o efeito do OJ no dano oxidativo induzido pelo cobre e ferro. Para tanto, testou-se o efeito do OJ tanto antes quanto após o tratamento com uma dose de ferro ou cobre de baixa toxicidade para camundongos (cerca de 10 % do LD_{50} a 48 h).

Surpreendentemente, os resultados apontaram um efeito diferencial do OJ para os dois metais. Ademais, servem para melhorar a compreensão dos efeitos da interação dos alimentos ricos em compostos antioxidantes e os metais ingeridos como parte da dieta normal ou através de suplementos.

Influence of orange juice in the levels and in the genotoxicity of iron and copper

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Abstract

World consumption of natural juices is increasing as a consequence of the human search for a healthier life. The juice production industry, especially for orange juice, is expanding in several countries and particularly in Brazil. Despite scientific data reporting beneficial properties derived from juice consumption, some components of juices have been identified as mutagenic or carcinogenic. Carcinogenic or genotoxic effects may be mediated by the interaction of juice components with transition metals or by sub-products of juice auto-oxidation. In this study, the mutagenic potential of orange juice and two metallic agents used in dietary supplementation, $FeSO_4$ and $CuSO_4$, were investigated using the comet assay in mouse blood cells (in vivo). Both metal compounds were genotoxic for eukaryotic cells after 24 h treatment at the doses used. Significant damage repair was observed after 48 h of treatment with the same compounds. Orange juice had a modulating effect on the action of metallic sulfates. In the case of iron treatment, the presence of the orange juice had a preventive, but not restorative, effect. On the other hand, in the case of copper treatment, the effects were both preventive and restorative. PIXE (particle induced X-ray emission) analysis indicated a positive correlation between DNA damage and the hepatic levels of iron and a negative correlation between whole blood copper and DNA damage. A negative correlation between hepatic iron and whole blood copper content was also seen in the treatment with both ferrous and cupric sulfates.
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Keywords: Orange juice; Genotoxicity; Ferrous and cupric sulfates; Comet assay; Mice; DNA repair; PIXE

1. Introduction

There is considerable evidence revealing an association between diets rich in fresh fruit and vegetables and a decreasing incidence of cardiovascular and neurodegenera-

tive diseases and cancer. These protective effects have been attributed mainly to compounds naturally present in juices such as phenolic compounds, carotenoids and vitamin C (Wang et al., 1996; Kabasakalis et al., 2000; Halliwell, 2001). World consumption of natural juices is increasing as a consequence of the human search for a healthier life. The juice production industry, especially that of orange juice, is thus expanding in several countries. Brazilian enterprises are responsible for about 70% of the global orange industry and there has been a clear increase during recent years. Since most natural foodstuffs are not

evaluated for their safety prior to consumption, it is very important to study them, particularly in large markets like Brazil.

Orange juice is a complex mixture composed of, among other substances, proteins, carbohydrates, lipids, vitamins (C > B complex > A), metals (mainly Fe), carotenoids, phenolic compounds and fibers, which may exert various biological effects (Franco, 1999; Wang et al., 1996; Fenech, 2005). Sanchez-Moreno et al. (2003) showed that drinking two glasses of orange juice (500 mL/d: containing 250 mg of vitamin C) increased vitamin C concentrations in plasma and reduced the concentrations of markers of oxidative stress. The authors also state that further attention should be paid to the evaluation of the health-promoting properties of vitamin C in fruit.

Despite scientific data reporting beneficial properties derived from juice consumption (for example, antimutagenic or anticarcinogenic effects), some compounds also present in juices have been identified as being themselves mutagenic or carcinogenic (Patrineli et al., 1996; Ames, 1998; Franke et al., 2004). The carcinogenic or genotoxic effects may be mediated by the interaction of juice components with transition metals or by sub-products of juice auto-oxidation. For example, vitamin C can act as a pro-oxidant, because of its reducing ability, through Fenton and Fenton-like reactions. Moreover, it can also act as a scavenger. Besides acting as metal chelators, some phenolic compounds can interact with enzymes, repressing their activity, diminishing oxidative damage by reducing superoxide anion levels by inhibiting cytocrome oxidase C and, on the other hand, increasing DNA damage by acting as a toposisomerase poison. Carotenoids and phenolic compounds can also scavenge reactive oxygen species (ROS). In a previous study we observed that vitamin C is genotoxic, especially at higher doses. Mice treated with either ferrous sulfate or cupric sulfate and post-treated with vitamin C showed increased DNA damage in comparison to those treated with only ferrous sulfate or cupric sulfate, respectively (Franke et al., 2005a).

Although many of the juice components have already been evaluated individually for their genotoxicity, it is important to test the effect of whole juice, a complex mixture, in different biological systems and subsystems. We have previously tested the mutagenicity of orange juice samples processed in different ways using the Ames test. Mutagenicity was present particularly for fresh *in natura* orange juice in bacteria (Franke et al., 2004). These results led us to test the genotoxicity of fresh *in natura* orange juice in mammals and to evaluate the interaction of whole juice with substances containing transition metals. In a previous study, we observed that orange juice reduced the genotoxicity of cyclophosphamide in post-treatment as well as of methyl methanesulfonate either in pre- or post-treatment in mice, as evaluated by the comet assay. Despite this protective effect, orange juice induces a mild increase in DNA damage in mice in vivo (Franke et al., 2005b). Our results are in agreement with Riso et al. (2005), who showed that

orange juice increases lymphocyte DNA resistance to oxidative stress.

While some metals are essential for human nutrition, others are found as contaminants in foodstuffs. One feature of the normal human diet is the simultaneous presence of both essential and toxic metals (Rojas et al., 1999). Metal ions can induce DNA damage by two mechanisms. They can generate DNA damage directly or induce formation of ROS, leading to DNA damage indirectly probably via Fenton-like reactions (Linder, 2001; De Freitas and Meneghini, 2001). Oxidative stress can also damage enzymes. There is growing body of evidence that proteins might be early targets of reactive oxygen species, and that the altered proteins can in turn damage other biomolecules (Kruszewski, 2003). Thus an increase in DNA damage can occur by release of metals from proteins that contain them.

It has already been reported that iron compounds are mutagenic in mammalian culture cells, as detected by syrian hamster embryo cell transformation/viral enhancement assay (Heidelberger et al., 1983), sister chromatin exchange (SCE) in hamster cells (Tucker et al., 1993), base tautomerization in rat hepatocyte cultures (Abalea et al., 1999), and genetic alterations in the mouse lymphoma assay (Dunkel et al., 1999). Iron is also rapidly absorbed after administration as tablets (absorption half-life of 0.54 h). Iron administered by this route peaks 2.4 h after administration and has a relatively short elimination half-life in blood of about 9.5 h (Farheen et al., 2002). Transferrin accepts iron from intestinal absorptive cells and serves as the mechanism for delivering iron to other organs. One of the major roles of transferrin is to bind iron so that it is unavailable to facilitate free radical formation (Conrad and Umbreit, 2002), as an important strategy of antioxidant defense. In this sense, the iron bound to biomolecules (i.e., transferases) would not participate in $OH^{\cdot-}$ formation.

In the same way, the genotoxicity of copper compounds has been reported in mammals in in vitro assays. Indeed, positive results were reported for the syrian hamster embryo cell transformation/viral enhancement assay (Heidelberger et al., 1983) and for HL-60 cells (Ma et al., 1998). Guecheva et al. (2001) showed the genotoxicity of $CuSO_4$ using the comet assay, as well as the inhibitory effect of copper on DNA repair in planarians. Many studies show that metal interactions are complex, especially with regard to their impact on carcinogenicity and genotoxicity (Rojas et al., 1999). Despite a general agreement about the rapid and high absorption of copper (maximum levels of copper occur within 1–3 h) following oral administration; (Earl et al., 1954; Uriu-Adams et al., 2005; Bissig et al., 2005), the elimination time has been little studied. Early work reported a biological half-life of up to 4 weeks (Dekaban et al., 1975).

The aim of this study was to investigate the mutagenic potential of orange juice and two metallic agents used in dietary supplementation, $FeSO_4$ and $CuSO_4$, using the comet assay in mouse blood cells (in vivo). The potential of orange juice to modulate the effects of sulfates was also studied. The PIXE (particle induced X-ray emission)

technique was employed to study the iron and copper levels in blood and hepatic tissues of the mice used in this study.

2. Materials and methods

2.1. Chemicals

Phosphate buffered saline (calcium- and magnesium-free), Tris-(tris-(hydroxymethyl)-aminomethanehydrochloride), disodium ethylenedia-mine-tetra-acetate (EDTA), dimethylsulfoxide (DMSO), ethidium bromide (EtBr), copper sulfate ($CuSO_4$), and triton X-100 were purchased from Sigma (St. Louis, MO, USA). Ferrous sulfate ($FeSO_4$) was obtained as a commercial medicine for anemia treatment—Sulfato Ferroso: Xarope Heptahidratado® (300 mg/mL $FeSO_4 \cdot 8\ H_2O$) from Ducto (Brazil). Low (LMP) and normal (NMP—electrophoresis grade) melting point agarose were obtained from Gibco-BRL (Grand Island, NY, USA). Heparin sodium was bought from Roche (Brazil) under the commercial name Liquemine®. Methyl methanesulfonate (MMS) (CAS 66-27-3) was purchased from Sigma (St. Louis, MO, USA).

2.2. Animals

Swiss Webster mice, aged between 5 and 7 weeks and weighting, on average, 24.0 ± 0.7 for females and 28.7 ± 1.6 for males, thus showing a minimal variation between treatment groups, were obtained from the Agriculture Ministry, Laboratory of Animal Reference, Porto Alegre, RS, Brazil. As soon as mice arrived in the laboratory, they were identified and separated according to sex and treatment group. The mice of each treatment group were placed in two boxes, one for males and one for females. They were acclimatized to laboratory conditions for seven days ($22 \pm 3\ °C$ and 60% humidity), receiving a commercial standard mouse cube diet (Nuvilab, CR1, Moinho Nuvital Ltd., Curitiba, PR, Brazil) and water ad libitum.

2.3. Treatment and test substances

Table 1 shows the experimental procedures, including treatment protocols and blood sampling schedules. Groups, composed of a minimum of three males and three females (except for one group), received by *gavage* 0.1 mL/10 g body weight (b.w.) of: (a) the control substances [water and MMS (40 mg/kg b.w.)]; (b) orange juice (juice *in natura* was prepared from

Citrus sinensis (Linn.) Osbeck organic oranges, free of agrochemicals); (c) ferrous sulfate [(33.23 Fe mg/kg), 10.86% mice oral LD_{50} ($LD_{50} = 306$ mg Fe/kg b.w. (Budavari et al., 1996)]; and (d) cupric sulfate [(8.50 Cu mg/kg), 11.14% mice oral LD_{50} ($LD_{50} = 66–82$ mg Cu/kg b.w. (Canton et al., 1989)]. All substances were prepared immediately before use and were kept covered to avoid light exposure.

The amount of orange juice administered to the mice (0.1 mL orange juice to 10 g body weight) would correspond to approximately 700 mL when we consider a typical 70 kg human male. Riso et al. (2005) tested a dose of 600 mL orange juice per day for 21 days in humans (16–23.3 kg/m^2). Mice have a much higher metabolic rate than humans. We observed that mice could eat daily up to 1/4 of their body weight (data not shown). Thus, a single dose of 0.1 mL of orange juice per 10 g is reasonable.

We compared the DNA damage between genders and treatment groups in a preliminary test (Table 2). As we found no significant differences between genders for any substance, we grouped males and females together for testing the modulator effect of orange juice on the genotoxicity of either ferrous or cupric sulfates.

2.4. Blood and liver sampling

For the comet assay, blood samples were obtained from mouse tail tips (about 15 μL), by means of a small incision, and mixed with heparin (7 μL).

For PIXE analysis, the whole blood and livers of the treated animals were collected at the same time as blood samples were collected for the comet assay. Animals were submitted rigorously to the same experimental conditions as those used for the comet assay tests. The samples were deep frozen, lyophilized, homogenized and finally pressed into pellets.

2.5. Comet assay

The alkaline comet assay was performed as described by Tice et al. (2000) with further modifications suggested by Hartmann et al. (2003) and Da Silva et al. (2000). Blood cells (7 μL) were embedded in 93 μL of LMP agarose (0.75%) and this mixture (cell/agarose) added to a pre-coated microscope slide with NMP agarose (1.5%). After solidification, the slides were placed in lysis buffer (2.5 M NaCl, 100 mM EDTA and 10 mM Tris, pH 10.00–10.50, with freshly added 1% Triton X-100 and 10% DMSO) for a minimum of 1 h. Subsequently, the slides were incubated in alkali buffer (300 mM NaOH, 1 mM Na_2EDTA, pH > 13) for 20 min at 4 °C, to allow DNA unwinding. The DNA was electrophoresed for 15 min (4 °C)

Table 1
Experimental procedures: treatment protocols and blood sampling schedules

Group	Exposure and sampling schedule		
	0 h	24 h	48 h
Water	Treatment: • Water	1. Blood sampling 2. Treatment: Water	Blood sampling
Orange juice	Treatment: • Orange juice	1. Blood sampling[a] 2. Treatment: Orange juice	Blood sampling
Metallic agents	Treatment: • $FeSO_4$ • $CuSO_4$	1. Blood sampling[b]	Blood sampling

Pre-treatment with orange juice	Treatment:
	• Orange juice
		1. Blood sampling[a]
		2. Treatment:
		  • $FeSO_4$
		  • $CuSO_4$
			Blood sampling

Post-treatment with orange juice	Treatment: • $FeSO_4$ • $CuSO_4$	1. Blood sampling[b] 2. Treatment: Orange juice	Blood sampling

[a] Individuals also used as orange juice controls at 24 h.
[b] Individuals used as substance controls at 4 h.

Table 2
Preliminary test by comet assay showing mean and standard deviation (SD) of damage index in mouse blood cells exposed to orange juice, $FeSO_4$ and $CuSO_4$ in vivo for 24 h

Treatment group	Dose (mg/kg b.w.)	n[a]	Gender	Average weight		Comet assay: mean damage index ± SD	
				Per gender	Per group	Per gender	Per group
Water	–	5	Male	29.2 ± 2.6	26.7 ± 3.3	11.6 ± 3.0	12.5 ± 4.0
		5	Female	24.2 ± 1.5		13.4 ± 4.9	
Orange juice	–	5	Male	29.4 ± 2.5	27.0 ± 3.3	21.6 ± 3.3	21.3 ± 4.1[b***]
		5	Female	24.6 ± 2.1		21.0 ± 5.2	
Ferrous sulfate	33.23 Fe	5	Male	30.0 ± 1.9	26.8 ± 4.0	112.2 ± 16.7	109.5 ± 18.3[b***,c***]
		5	Female	23.6 ± 2.6		106.8 ± 21.3	
Cupric sulfate	8.50 Cu	5	Male	26.4 ± 1.8	25.0 ± 2.6	148.0 ± 7.8	139.2 ± 17.8[b***,c***]
		5	Female	23.6 ± 2.0		130.4 ± 21.4	

The results were evaluated according to gender and treatment by two-way ANOVA. Tuckey post-hoc test was used to compare damage among treatment groups.
[a] Number of individuals in a single experiment.
[b] Significant in relation to water at [*]$P < 0.01$; [***]$P < 0.001$.
[c] Significant in relation to orange juice at [***]$P < 0.001$.

at 300 mA and 25 V (0.90 V/cm). After electrophoresis, the slides were neutralized (0.4 M Tris, pH 7.5) and stained with ethidium bromide (2 µg/mL). In order to ensure adequate electrophoresis conditions and efficiency, positive (blood cells treated with 8×10^{-5} M MMS for 1.5 h) and negative human blood controls were included in each electrophoretic run.

Images of 100 randomly selected cells per animal (50 cells per replicate slide) were analyzed at 200× magnification 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. The analyses of damaged nuclei were carried out according to two parameters. The first one was the damage index (DI), which was determined visually by the categorization of comets into five classes according to DNA migration, from 0 (no tails) to 4 (maximally long tails). DI was obtained by the sum of the individual cell classes, ranging from 0 (no damage: 100 cells × 0) to 400 (maximum damage: 100 cells × 4). The second parameter used was the damage frequency (DF in%), which was calculated from the number of cells with tail versus those without. Non-detectable cell nuclei (head and tail clearly separated) were also observed, but not evaluated.

2.6. PIXE analysis

In order to obtain the whole blood and hepatic levels of iron and copper, the PIXE technique was employed (Johansson et al., 1995). This non-destructive technique has several advantages such as high sensitivity and a relatively quick analysis, providing concentrations for a broad range of elements. PIXE has become a common choice for elemental analysis in environmental studies (Teixeira et al., 2004) and has been successfully applied to studies related to metal concentrations in proteins (Follmer et al., 2002) and eukaryotic organisms (Kern et al., 2004).

The pellets were mounted in a target holder inside a reaction chamber for the PIXE experiments, which were carried out at the Ion Implantation Laboratory of the Physics Institute (UFRGS). The reaction chamber was kept at a pressure above 10^{-3} Pa throughout the experiments. A beam of 2.0 MeV protons with an average current of about 1 nA was used to irradiate the targets. The characteristic X-rays induced in the reactions were detected by two detectors. One was a high purity germanium detector, with an energy resolution of about 175 eV at 5.9 keV. The other detector was a lithium-doped silicon detector, with an energy resolution of about 155 eV at 5.9 keV. The spectra were analyzed using the GUPIX code developed at the University of Guelph (Maxwell et al., 1989, 1995; Campbell et al., 2000). The standardization procedure was carried out using a bovine liver standard from NIST (reference material 1577b). In this way, element concentrations were obtained from the raw data.

2.7. Statistical analysis

Two-way ANOVA was used to compare DNA damage between genders and treatment groups only in the preliminary test. The Tuckey post-hoc test was used to compare treatment groups. One-way ANOVA was used to compare DNA damage between treatment groups either within 24 h or within 48 h groups. Parametric ANOVA was used for those cases where the data exhibited homoscedasticity and normality. A logarithmic (Ln) transformation was applied to obtain homoscedasticity when data were not homogenous in regard to variance. When this attempt was not efficient, the Kruskal–Wallis non-parametric ANOVA was used with Dunn's correction to compare groups pairwise. Student's t-test was used to compare damage either between 24 h and 48 h, between 24 h and pre-treatment or between 48 h and post-treatment groups.

Correlation analysis was used to test the association between the average values of DNA damage and the average iron and copper content in whole blood and livers.

Significance was considered at a level of $P \leqslant 0.05$, excepting when Student's t-test was used. In this case, significance was considered at $P \leqslant 0.017$ due to the Bonferroni adjustment for multiple comparisons.

3. Results

3.1. Genotoxicity of the test compounds

In all experiments, negative (DI = 0–5) and positive human controls (blood cells treated with 8×10^{-5} M MMS for 1.5 h) (DI = 180–300) for each electrophoretic run demonstrated negative and positive results, respectively, thus validating the electrophoretic conditions of the tests. Table 2 shows the results of a preliminary experiment to evaluate the genotoxicity of the substances. Animals were treated with a single dose of the substances 24 h prior to blood sampling. Only DI was considered for analysis. Metal compounds were genotoxic when compared to water and orange juice ($CuSO_4 > FeSO_4 >$ orange juice > water). Orange juice was genotoxic in relation to water ($P < 0.001$). No difference between genders was observed (Table 2); hence the levels of DNA damage of males and females of each treatment group were clustered for further analyses. The doses used in this work were based on the results of this preliminary experiment.

41

3.2. Modulator effect of orange juice

In the main experiment, there was a similar increase of DNA damage for $FeSO_4$ and $CuSO_4$ groups at 24 h in comparison to either water or orange juice groups (Table 3). In this test, the animals treated with orange juice did not show an increase in DNA damage in relation to those treated with water. The positive control animals, treated with MMS, showed a significant increase in DNA damage in relation to the water control ($DI = 171 \pm 20$, $DF = 99 \pm 3$ at 24 h; and $DI = 158 \pm 15$, $DF = 87 \pm 9$ at 48 h; data not shown).

As assessed by DI, the DNA damage for water was higher at 48 h than at 24 h. The DNA damage at 48 h was lower than at 24 h for ferrous sulfate, as assessed both by DI and DF, and for cupric sulfate (only by DI) (Table 3 and Fig. 1).

Mice pre-treated with orange juice and treated with ferrous or cupric sulfate showed less DNA damage than the groups not so pre-treated.

Mice treated with ferrous sulfate and post-treated with orange juice showed more DNA damage than the group treated for 48 h with ferrous sulfate. Mice treated with cupric sulfate and post-treated with orange juice showed less DNA damage than the group treated for 48 h with cupric sulfate. Results for post-treatment for both substances were not statistically significant. However, they are at the edge of significance when applying the Bonferroni adjustment (Fig. 1 and Table 3).

3.3. Levels of iron and copper in whole blood and liver

Table 4 shows the levels of iron and copper in whole blood and liver in mice submitted to the treatments, as evaluated by PIXE analysis. At 24 h and 48 h, the levels of iron in whole blood were lower in mice treated with orange juice, ferrous sulfate and cupric sulfate than in mice treated with water. Levels of iron in whole blood in mice pre-treated with orange juice and treated with ferrous or cupric sulfate were higher than the group treated for 24 h with either ferrous sulfate or cupric sulfate, respectively, although they were lower than the water treatment level. Mice treated with ferrous sulfate and post-treated with orange juice showed a higher level of iron in whole blood than the group treated for 48 h with ferrous sulfate. Mice treated with cupric sulfate and post-treated with orange juice showed a lower level of iron in whole blood than the group treated for 48 h with cupric sulfate. Results of levels of iron in whole blood were not statistically tested, as mentioned in Section 2.

At 24 h and 48 h, the levels of iron in the liver did not differ significantly between treatment groups. The level of iron was lower at 48 h than at 24 h for orange juice and cupric sulfate treatment groups. Mice treated with ferrous sulfate and post-treated with orange juice showed a higher level of iron in the liver than the group treated for 48 h with ferrous sulfate.

At 24 h, the level of copper in whole blood was similar in mice treated with water or orange juice, but was lower in

Table 3
Detection of DNA damage in mouse blood cells exposed to water, orange juice and metal compounds in vivo for 24 l (with and without pre-treatment with orange juice) and for 48 h (with and without post-treatment with orange juice)

Treatment group	Single doses (mg/kg b.w.)	Schedule[a] and comet assay parameters							
		24 h		48 h		Pre-treatment with orange juice[b]		Post-treatment with orange juice[c]	
Damage index (DI)									
		n[d]	DI ± SD	n	DI ± SD	n	DI ± SD	n	DI ± SD
Water	–	5[e]	11.40 ± 3.05	5	19.00 ± 3.67[f**]	–	–	–	–
Orange juice	–	36	21.00 ± 8.00	8	25.12 ± 4.52	–	–	–	–
Ferrous sulfate	33.23 Fe	24	114.96 ± 42.72[g***,h****]	6	30.67 ± 32.06[f***]	6	65.50 ± 18.38[f**]	6	79.83 ± 28.24
Cupric sulfate	8.50 Cu	24	132.63 ± 36.68[g***,h****]	6	84.50 ± 18.71[f*,g**]	6	63.17 ± 20.15[f***]	6	55.50 ± 23.45
Damage frequency (DF)									
		n	DF ± SD	n	DF ± SD	n	DF ± SD	n	DF ± SD
Water	–	5	9.40 ± 2.61	5	11.20 ± 4.09	–	–	–	–
Orange juice	–	36	17.50 ± 6.07	8	20.87 ± 3.94	–	–	–	–
Ferrous sulfate	33.23 Fe	24	79.92 ± 18.36[g***,h****]	6	28.50 ± 27.05[f***]	6	49.83 ± 9.15[f**]	6	65.00 ± 21.44
Cupric sulfate	8.50 Cu	24	86.71 ± 18.83[g***,h****]	6	67.83 ± 15.18[g***]	6	39.66 ± 15.72[f***]	6	42.33 ± 16.88

Significant in relation to 24 h refers to the same row, and was evaluated using Student's *t*-test. Significant in relation to either water or orange juice refers to the same column, and was evaluated using analyses of variance.
[a] For more details see Table 1.
[b] Group exposed to metal compounds over 24 h.
[c] Group exposed to metal compounds over 48 h.
[d] Number of individuals obtained from independent test performed simultaneously. Half were male and half were female.
[e] One mouse died. Date of male and female were pooled since they did not differ in any group.
[f] Significant in relation to 24 h at $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.
[g] Significant in relation to water at $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.
[h] Significant in relation to orange juice at $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.
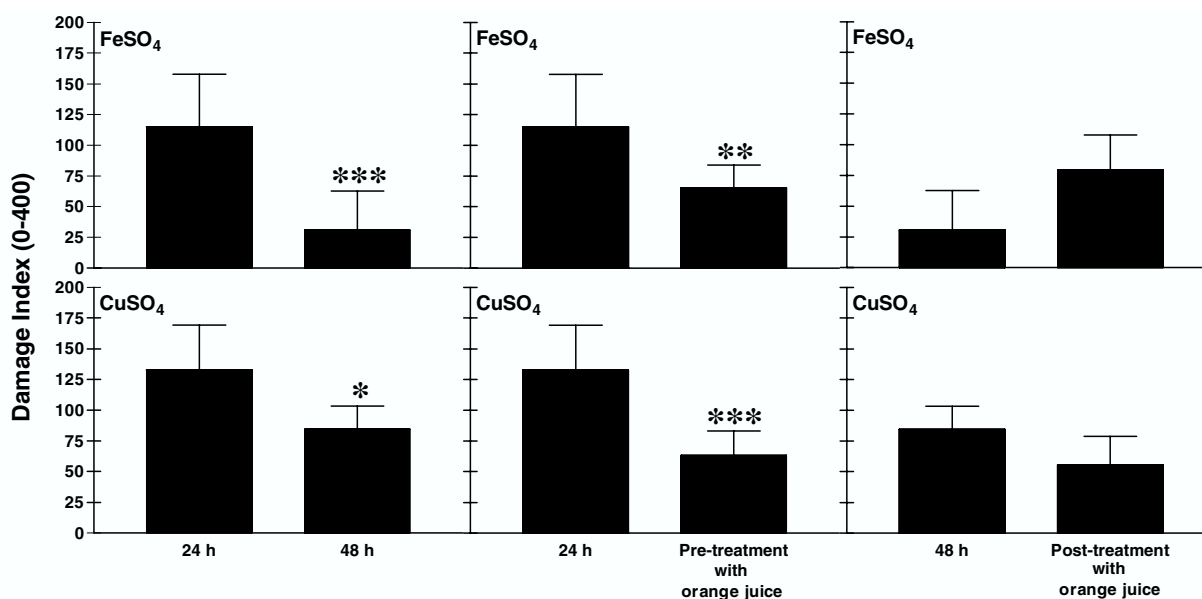
Fig. 1. Comparisons between the mean damage index values for mouse blood cells. Significant at $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. (A and B) Comparison between damage at 24 and 48 h. (C and D) Comparison between damage at 24 h and pre-treatment with orange juice. (E and F) Comparison between damage at 48 h and post-treatment with orange juice.

Table 4
Total content of iron and copper in blood and liver tissues of mice evaluated by PIXE analysis

Treatment group	Single doses (mg/kg b.w.)	Schedule			
		24 h	48 h	Pre-treatment with orange juice	Post-treatment with orange juice
		Average level $\pm$ SE[c]			
Fe levels (whole blood dry weight)					
Water	–	1423 ± 72	1317 ± 4	–	–
Orange juice	–	633 ± 100	1114 ± 1	–	–
Ferrous sulfate	33.23 Fe	1034 ± 15	1209 ± 37	1175 ± 4	1424 ± 286
Cupric sulfate	8.50 Cu	887 ± 20	1268 ± 18	1137 ± 33	1100 ± 7
Fe levels (liver dry weight)					
Water	–	315 ± 79	207 ± 33	–	–
Orange juice	–	569 ± 112	$209 \pm 19^{a**}$	–	–
Ferrous sulfate	33.23 Fe	634 ± 157	189 ± 19	263 ± 58	$342 \pm 15^{b**}$
Cupric sulfate	8.50 Cu	320 ± 48	$239 \pm 22^{a**}$	401 ± 179	220 ± 34
Cu levels (whole blood dry weight)					
Water	–	5.7 ± 3.3	[d]	–	–
Orange juice	–	5.6 ± 1.2	1.8 ± 1.0	–	–
Ferrous sulfate	33.23 Fe	3.6 ± 0.3	2.7 ± 1.6	[d]	1.9 ± 1.1
Cupric sulfate	8.50 Cu	2.8 ± 1.6	5.1 ± 2.9	[d]	[d]
Cu levels (liver dry weight)					
Water	–	20.2 ± 2.0	16.9 ± 1.2	–	–
Orange juice	–	27.5 ± 3.5	15.3 ± 0.9	–	–
Ferrous sulfate	33.23 Fe	21.8 ± 4.2	15.9 ± 0.8	17.8 ± 2.2	14.6 ± 2.0
Cupric sulfate	8.50 Cu	26.8 ± 6.4	16.3 ± 2.6	19.9 ± 3.0	$22.1 \pm 1.1^{b**}$

All significances refer to the same row, and were tested using the Student's t-test.
[a] Significant in relation to 24 h at $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.
[b] Significant in relation to 48 h at $^*P < 0.05$; $^{**}P < 0.01$.
[c] n: 6 individual per group.
[d] Values below detection levels.

mice treated with ferrous or cupric sulfate. At 48 h, the level of copper in whole blood was below the detection level in mice treated with water and the highest for mice treated with cupric sulfate. Mice treated with ferrous sulfate and post-treated with orange juice showed a higher level of copper in whole blood than the group treated for 48 h with

ferrous sulfate. The levels of copper in whole blood were not statistically tested, as mentioned in Section 2.
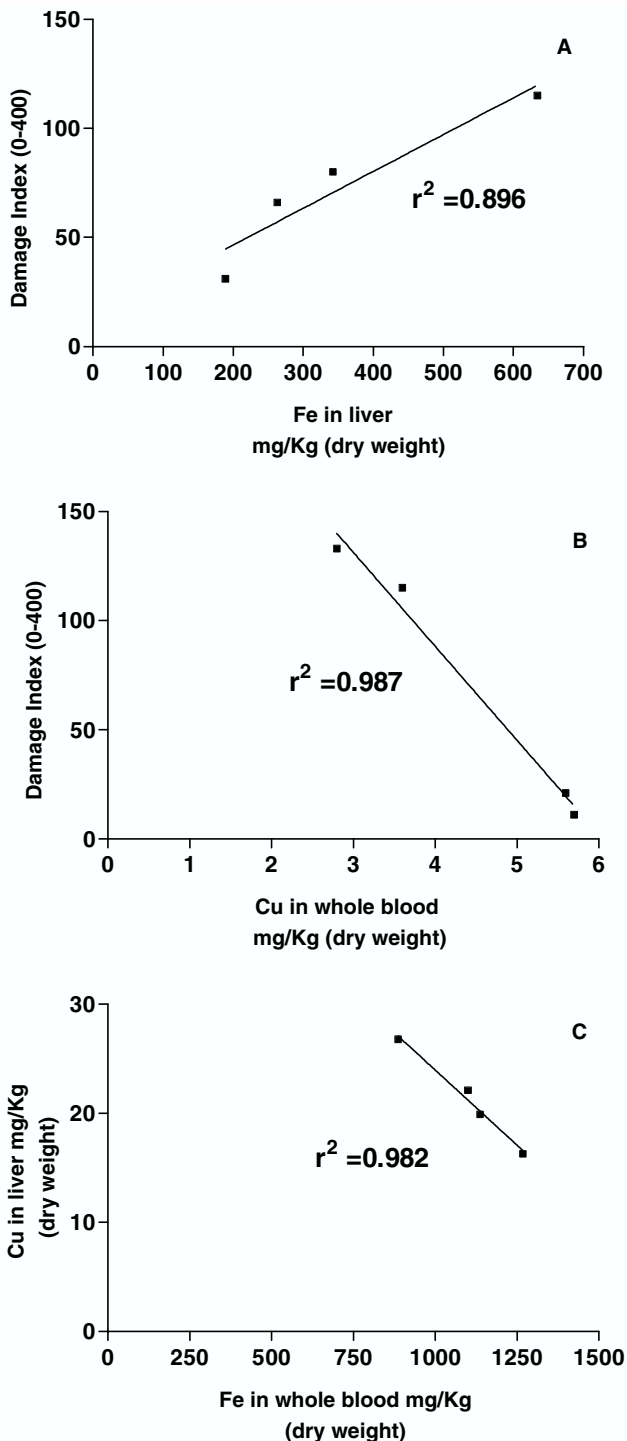
Fig. 2. Correlation between genotoxicity and iron and copper levels in whole blood and liver. (A) Correlation between the levels of DNA damage in the blood and the hepatic levels of iron at 24 h, 48 h, pre-treatment and post-treatment in ferrous sulfate group. (B) Correlation between the levels of DNA damage for water, orange juice, ferrous sulfate and cupric sulfate and the levels of copper in whole blood at 24 h. (C) Correlation between the levels of iron in whole blood and the hepatic levels of copper at 24 h, 48 h, pre-treatment and post-treatment in cupric sulfate group.

At 24 h and 48 h, the levels of copper in the liver did not differ significantly between treatment groups. Mice treated with cupric sulfate and post-treated with orange juice showed a higher level of copper in the liver than the group treated for 48 h with cupric sulfate. A positive correlation ($r = 0.95$ and $p = 0.052$) was observed between the levels of DNA damage in the blood and the hepatic levels of iron at 24 h, 48 h, pre-treatment and post-treatment in the ferrous sulfate group (Fig. 2A). There was a negative correlation ($r = -0.99$ and $p \leqslant 0.01$) between the levels of DNA damage for water, orange juice, ferrous sulfate and cupric sulfate and the levels of copper in the whole blood at 24 h (Fig. 2B). A negative correlation ($r = -0.99$ and $p \leqslant 0.01$) between the levels of iron in the whole blood and the hepatic levels of copper at 24 h, 48 h, pre-treatment and post-treatment in cupric sulfate group was also detected (Fig. 2C).

4. Discussion

4.1. Levels of iron and copper in orange juice and in the diet

A small quantity of iron (4.00 mg/L) and copper (0.61 mg/L) was detected in our orange juice samples using PIXE. The iron content of the juice accounted for about 0.12% of the iron contained in the $FeSO_4$ treatment dose. The copper content of the orange juice accounted for about 0.07% of the copper contained in the $CuSO_4$ treatment dose. The amount of iron and copper contained in the mouse cube diet was 50 and 10 mg/kg, respectively, according to the producer. The mouse cube diet consumption was 270–300 g/kg of mouse b.w. Thus, each kilogram of mouse ingested about 13.5–15 mg Fe and 2.7–3.0 mg Cu. A low amount of the minerals present in the diet is absorbed. The allowance of iron is about 5–10% for vegetable foodstuffs. However, less is known about the absorption and homeostasis of copper (Linder, 2001). Based on the low amount of metals in the mouse diet and in orange juice, it is likely that the dietary amounts and orange juice treatments were a minor influence with respect to the treatment doses and the administered form (i.e., in solution, which is more readily absorbed). In any case, the metals ingested in the diet will have influenced all groups equally.

4.2. Genotoxicity of the treatments

In this study, orange juice increased DNA damage in comparison to the water treated group as evaluated by the comet assay. Franke et al. (2004) detected a similar effect with the Ames test. Such effects can be attributed to several components of orange juice as well as to its acidity (pH ≈ 3.5). It is possible that low pH can reduce metabolic rate and thus depress antioxidant defenses. Low pH values can also be important in the solubility of toxic compounds (Vidal et al., 2002; Rajaguru et al., 2002). For water and juice treatments, the damage index increased

44

between the first and second day. We suggest that this is a stress response due to animal handling and also a response to cumulative acidity damage.

For humans, the recommended dietary allowance (RDA), according to WHO, is 0.9 mg/day (it used to be 1–3 mg/day before 2001) for copper and a mean of 8–18 mg/day for iron, depending on gender (Food Nutrition Board, 2001). The RDA is about 10–20 fold higher for iron than for copper. Conversely, the oral LD_{50} to mice is only 4.2 times higher for iron than for copper. The tested doses corresponded to about 11% of the LD_{50} for both $FeSO_4$ and $CuSO_4$ and were genotoxic 24 h after treatment. DNA damage reduction was more effective at 48 h for $FeSO_4$ than for $CuSO_4$. DNA damage produced by both $FeSO_4$ and by juice at 48 h converged to a similar level. This effect was not observed for $CuSO_4$, indicating a higher susceptibility to high levels of Cu and confirming the lower RDA for copper. Extremely low amounts of free copper are found within organisms, because copper binds to proteins (i.e., albumin and transcuprein) with a very high affinity as soon as it enters the blood plasma (Linder, 2001). It is likely that the tested dose saturated these transporters and resulted in free copper in the blood or induced the release of metals from metal-containing proteins. For example, the inactivation of ceruloplasmin (ferroxidase) interferes in the availability of Fe^{2+} ions, resulting in additional DNA damage (Linder, 2001).

Despite the DNA damage at 24 h, it is not clear whether iron was free or bound in blood. Although it is not likely to exist free iron in biological systems, it is well known that the presence of trace amounts of "free" iron (i.e., weakly bound) is involved in the generation of oxidative stress. The critical factor for the hazard associated with iron seems to be the availability and abundance of cellular labile iron pool (LIP) that constitutes a crossroad of metabolic pathways of iron-containing compounds and is midway between the cellular need of iron, its uptake and storage (Kruszewski, 2003). The levels of iron are rapidly cleared as they enter the blood. Beshara et al. (2003) noticed that levels of an iron complex in blood reduce significantly as early as 1 h after a single venous injection with the half-live being between about 3 and 7 h. It is likely that the DNA damage was generated early after exposure as a consequence of the iron peak in the blood and inside the cells. DNA damage repair seems to occur after the clearance of the metal to the liver or other tissues.

4.3. Levels of iron and copper in whole blood and liver in the treatments

In our study, mice treated with water showed the highest levels of iron and copper in whole blood and the lowest levels of copper and iron in the liver at 24 h. Roughead and Hunt (2000) noticed that iron absorption in human erythrocytes was higher in the placebo group in comparison to a group supplemented with 50 mg Fe as ferrous sulfate for 12 weeks. It is likely that a similar effect occur for both copper and iron, based on the present results.

In our study, mice treated with orange juice showed a high level of either iron or copper in the liver at 24 h, similar to the group treated with $FeSO_4$ or $CuSO_4$, respectively. Premkumar and Bowlus (2003) reported that vitamin C enhances hepatic levels of iron. Vitamin C can reduce the intestinal absorption of copper; however, and can facilitate its entrance into the cells (Harris and Percival, 1991). The high levels of iron in the liver of mice treated with orange juice may have been influenced by the amount of vitamin C present in the orange juice. The acidity of the juice could also have increased body levels (as represented by the hepatic level) of iron and copper, by increasing their absorption in the gut. The tested orange juice samples contain about 56 mg of vitamin C per 100 mL (Franke et al., 2004).

The level of iron in whole blood in the $FeSO_4$ group was lower than that in the group that received water. Ajioka et al. (2002) suggested that mice down regulate iron absorption and that iron loading normally leads to decreased absorption by the enterocyte. When transferrin saturation levels are high, newly absorbed non-transferrin bound iron is rapidly cleared by the liver. In our study, as expected, the hepatic level of iron was the highest in the group treated with iron (at 24 h). However, at 48 h all groups showed similar levels of iron in either whole blood or liver to the mice treated with water. Possibly, a single dose of iron does not represent a hazard to the organisms at 48 h.

The group treated with $CuSO_4$ showed a low level of copper in whole blood and a high level of copper in the liver at 24 h. At 48 h, the group treated with $CuSO_4$ showed the highest level of copper in the whole blood, but a level similar to all treatments in the liver. Thus, while the level of copper in the whole blood increased, the level in the liver decreased. Accumulation of excess copper in liver and other tissues is not accompanied by marked or predictable increases in copper within the blood (Linder, 2001). This assumption seems to be valid at 24 h, but not at 48 h. In a study using radioisotopes in rodents, Uriu-Adams et al. (2005) noticed that the main destination of copper after 24 h of administration was, in decreasing order, kidney, liver, blood, skin, bone, muscle and intestine. Uriu-Adams et al. (2005) also noticed that the level of copper almost doubled in erythrocytes at 48 h. A general increase in copper levels could be expected for the whole blood. In fact, this increase can be observed in our study. Thus, the high damage at 48 h observed for copper treatment could be related to an increase in the copper content in the white blood cells, the target of the comet assay in our study.

4.4. Correlation between genotoxicity and levels of iron and copper in whole blood and liver induced by the treatments

The positive correlation between the levels of DNA damage in the blood and the hepatic levels of iron at 24 h, 48 h, pre-treatment and post-treatment in ferrous sulfate group indicates the clearance of iron from the blood,

possibly to the liver as well as to other tissues such as spleen and bone marrow. In fact, Beshara et al. (2003) have shown a much higher uptake of a radioactive iron–sucrose complex by the bone marrow in relation to the liver, blood and spleen uptake.

The negative correlation between the levels of DNA damage for water, orange juice, ferrous sulfate and cupric sulfate and the levels of copper in the whole blood at 24 h indicates that copper is cleared form the whole blood to the liver and other tissues up to 24 h after exposure, possibly as a response to the oxidative stress. Damage seems to occur early after exposure. DNA damage declines in parallel to the mobilization of copper to the liver (between exposure and 24 h) and from the liver to other tissues between 24 and 48 h (mainly to muscles and skin and a minor amount to the blood). However, DNA damage reduction is lower possibly because copper levels in blood cells can increase between 24 and 48 h, as noticed by Bissig et al. (2005). These authors detected an almost two fold increase in copper in erythrocytes within 24–48 h after a single dose of copper. Copper accumulation is likely to occur also in white blood cells, the target of the comet assay.

The negative correlation between the levels of iron in the whole blood and the hepatic levels of copper at 24 h, 48 h, pre-treatment and post-treatment in the cupric sulfate group indicates a relation between iron and copper homeostasis. The accumulation of copper down regulated DMT1 Divalent Metal Transporter #1), the main responsible for intestinal non-heme Fe that appears to have an additional function in Cu transport in intestinal cells (Arredondo et al., 2004). Moreover, the accumulation of excess copper in the liver and other tissues is not accompanied by marked or predictable increases in copper within the blood (Linder, 2001).

4.5. Modulator effect of orange juice on genotoxicity of iron and copper and on levels of iron and copper in whole blood and liver

Pre-treatment with orange juice protected the cells against the DNA damage generated by ferrous sulfates, despite an increase in the levels of iron in the whole blood compared with the levels of iron at 24 h. Juice components such as vitamin C, carotenoids and phenolics could have protected the cells by diverse mechanisms leading to the preventive effect on DNA damage, as discussed by Franke et al. (2005b). The complexes formed between juice components and iron could be more easily absorbed than iron itself (Coelho, 1995a). Despite the preventive effect of pre-treatment with orange juice on sulfate damage, post-treatment with orange juice hampered the reduction of DNA damage in the group treated with $FeSO_4$, being only preventive for iron exposure. The low damage at 48 h without post-treatment (repair), shows what seems to be a perfect homeostatic regulation of iron overload.

Pre- and post-treatment with orange juice reduced the genotoxicity of copper as well as the levels of copper in

the whole blood to levels undetectable by PIXE. It is possible that the complexes formed between juice components and copper were not helpful, and in fact neutral (Linder, 2001); in addition, vitamin C in high doses can reduce copper absorption at the gastrointestinal level (Harris and Percival, 1991). There is also a negative interaction between vitamin C and copper in tissue distribution (Coelho, 1995a,b). These facts could explain the damage reduction in both pre- and post-treatment with orange juice. However, it should be mentioned that this interaction of metals and orange juice must be examined carefully, since treatments were not administered at the same time and there is a lack of knowledge about the kinetics of copper. Moreover, more extensive tracer studies would be necessary to shed light upon the kinetic of iron and copper.

4.6. Conclusion

Orange juice exerted protective modulatory effects over DNA damage generated by iron and copper sulfates. Orange juice induced a slight increase in DNA damage. Despite this, we do not discourage orange juice consumption, since the various compounds present in the juice have both beneficial and noxious effects. The precise balance of these properties, as well as the interactions among the multiple components of juice, result in a real protective effect (Halliwell, 2001). For this reason, various authors recommend daily ingestion of at least five portions of fruit and vegetables to prevent diseases (Halliwell, 2001; Ames, 1989), rather than micronutrient supplementation.

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3.3 Capítulo 3: "Possible repair action of Vitamin C on DNA damage induced by methyl methanesulfonate, cyclophosphamide, FeSO₄ and CuSO₄ in mouse blood cells *in vivo*"

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Atribui-se a Vit C um alto potencial antioxidante e antigenotóxico, sendo ela um dos componentes mais abundantes no OJ. Considerando estas características da Vit C e os resultados dos Capítulos 1 e 2, onde se observou que o OJ afetava a genotoxicidade tanto dos metais quanto dos mutágenos, decidiu-se avaliar o efeito do pós-tratamento com a Vit C sobre a genotoxicidade dos dois alquilantes (Capítulo 1) e do Fe e Cu (Capítulo 2). Foram selecionadas duas doses de Vit C, uma equivalendo a cerca de uma vez a DRI e a outra, a aproximadamente 30 vezes a DRI (segundo a RDA anterior a 2000, que preconizava a ingestão diária de 60 mg para adultos de ambos os sexos). Coincidentemente, a dose maior também corresponde a uma vez a UL para humanos, segundo a DRI de 2000. Este estudo considerou a capacidade de síntese de Vit C pelos camundongos, procurando simular o excesso de Vit C que poderia ocorrer em humanos que tem uma dieta adequada em relação à DRI de Vit C, e mesmo assim usam suplementos.

ERRATA: Para o correto entendimento da tabela 1, as letras "e", "f", "g" e "h" devem ser substituídas por "g", "h", "i" e "e", respectivamente, no **corpo** desta tabela. A **legenda** da tabela está correta.

Possible repair action of Vitamin C on DNA damage induced by methyl methanesulfonate, cyclophosphamide, FeSO$_4$ and CuSO$_4$ in mouse blood cells in vivo

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Abstract

Interaction between Vitamin C (VitC) and transition metals can induce the formation of reactive oxygen species (ROS). VitC may also act as an ROS scavenger and as a metal chelant. To examine these possibilities, we tested in vivo the effect of two doses of VitC (1 and 30 mg/kg of mouse body weight) on the genotoxicity of known mutagens and transition metals. We used the alkaline version of the comet assay to assess DNA damage in peripheral white blood cells of mice. Animals were orally given either water (control), cyclophosphamide (CP), methyl methanesulfonate (MMS), cupric sulfate or ferrous sulfate. A single treatment with each VitC dose was administered after treatment with the mutagens or the metal sulfates. Both doses of VitC enhanced DNA damage caused by the metal sulfates. DNA damage caused by MMS was significantly reduced by the lower dose, but not by the higher dose of VitC. For CP, neither post-treatment dose of VitC affected the DNA damage level. These results indicate a modulatory role of Vitamin C in the genotoxicity/repair effect of these compounds. Single treatment with either dose of VitC showed genotoxic effects after 24 h but not after 48 h, indicating repair. Double treatment with VitC (at 0 and 24 h) induced a cumulative genotoxic response at 48 h, more intense for the higher dose. The results suggest that VitC can be either genotoxic or a repair stimulant, since the alkaline version of the comet assay does not differentiate "effective" strand breaks

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from those generated as an intermediate step in excision repair (incomplete excision repair sites). Further data is needed to shed light upon the beneficial/noxious effects of VitC.

1. Introduction

Over the course of evolution, many animals have lost the ability to synthesize specific substances that nevertheless continue to play critical roles in their metabolism [1,2]. Vitamins are among the substances that are required in micronutrient amounts in the diet. Vitamin deficiencies in the human diet are generally thought to lead to DNA damage. Humans cannot produce ascorbic acid (VitC) for themselves; however, they need this nutrient for health.

VitC, found in fresh fruits and vegetables, is an important micronutrient, mainly required as a co-factor for enzymes involved in oxi-reduction reactions [1,3–6]. It has been studied for its protective action against different diseases [4,5,7]. The mechanisms by which ascorbic acid acts include bio-antimutagenic [8,9] and desmutagenic activities [10] as well as regulation of DNA-repair enzymes [11,12]. Some studies have shown that VitC can display ambiguous genotoxic effects [13], acting as a pro-oxidant by reaction with some metals [1,6,14]. Many of the results reported are based on in vitro studies.

VitC has been insufficiently studied for its ability to interact, either directly or indirectly, with mutagens, especially in view of the controversial results of its consumption on genome stabilization [1,3]. The aim of the present work was to investigate the genotoxic effect of VitC associated with direct and indirect mutagens, as well as with metallic agents, in cells of mice in vivo, in order to improve our understanding of the role of dietary antimutagens and anticarcinogens in humans.

2. Materials and methods

2.1. Chemical reagents

Phosphate buffered saline (calcium- and magnesium-free), Tris (tris(hydroxymethyl) aminomethane-hydrochloride), disodium ethylenediamine-tetra-ace-

tate (EDTA), dimethylsulfoxide (DMSO), ethidium bromide (EtBr), methyl methanesulfonate (MMS) (CAS 66-27-3), cyclophosphamide (CP) (50-18-0), copper sulfate ($CuSO_4$) (CAS 7758-98-7), ascorbic acid (VitC) (CAS 50-81-7) and Triton X-100 were purchased from Sigma (St. Louis, MO, USA). Ferrous sulfate ($FeSO_4$) (CAS 7782-63-0) was obtained from Ducto (Brazil). Low melting point (LMP) agarose and normal agarose (electrophoresis grade) were obtained from Gibco-BRL (Grand Island, NY, USA). Heparin sodium was bought from Roche (Brazil) under the commercial name Liquemine®.

2.2. Animals

CF1 male mice, aged 5–7 weeks and weighing from 27 to 32 g, were obtained from the State Foundation for Production and Research in Health (FEPPS), Porto Alegre, RS, Brazil. The mice were acclimatized to laboratory conditions ($22 \pm 3\,^\circ$C and 60% humidity) for 7 days, during which they received a commercial standard mouse cube diet (Nuvilab, CR1, Moinho Nuvipal Ltda., Curitiba, PR, Brazil) and water ad libitum. After acclimatization, the mice were clustered and identified as control and test groups. All procedures were carried out 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.

2.3. Treatments and blood sampling

Seven groups were dosed by gavage with a single dose of: (a) water; (b) CP (25 mg/kg weight body); (c) MMS (40 mg/kg w.b.); (d) $FeSO_4$ (33.23 mg Fe/kg w.b.); (e) $CuSO_4$ (8.25 mg Cu/kg w.b.); (f) VitC1 (1 mg/kg w.b.), (g) VitC30 (30 mg/kg w.b.). For CP, a dose equivalent to 18.2% of the LD_{50} dose [15] was used. For VitC1 and VitC30, the animals were dosed once (24 h) and twice (24 and 48 h), those not treated at 48 h being used to assess repair. For MMS, the dose was equal to 13.80% of the LD_{50} dose [16]. The dose used

for iron was equal to 10.86% mouse oral LD_{50} [17] and for copper was equal to 11.14% mouse oral LD_{50} [18]. The doses of VitC were based on the ratio of mouse to human recommended dietary allowance (RDA) per kilogram per day (medium human weight of 60 kg): VitC30, equal to 30 times the RDA (30 mg/kg); VitC1, equal to the RDA (1 mg/kg). VitC30 corresponded to the human dietary upper intake level (UL). Eight groups received post-treatment with VitC. A single treatment with each VitC dose was administered 24 h after the treatment with either CP, MMS, $CuSO_4$ or $FeSO_4$. A double treatment with either dose of Vitamin C was also tested. All substances were prepared just before treatment and protected from light. There were at least six animals per treatment group. Blood was obtained 24 or 48 h after the beginning of the experiment from mouse tail tips (about 15 μL) by means of a small incision and immediately mixed with heparin sodium (7 μL).

2.4. Comet assay

The alkaline version of the comet assay was performed according to guidelines proposed by Tice et al. [19], with the slight modification developed by Da Silva et al. [20]. Seven microliters of blood cells/heparin mixture were embedded in 93 μL of LMP agarose (0.75 g/100 mL) and the resulting mixture was spread over a pre-coated microscope slide (1.5 g/100 mL agarose). A cover glass was then gently placed over the slide and it was placed at 4 °C for 5 min to allow gel solidification. The cells were lysed in high salt and detergent and placed in a horizontal electrophoresis box. They were then exposed to alkali (300 mM NaOH, 1 mM Na_2EDTA, pH > 13) for 20 min at 4 °C, to allow DNA unwinding. Electrophoresis was performed at 300 mA and 25 V (0.90 V/cm) for 15 min at 4 °C. The slides were then neutralized (Tris 0.4 M, pH 7.5) and stained with ethidium bromide (20 μg/mL). In order to ensure adequate electrophoresis conditions and efficiency, negative and positive internal controls (human blood) were included in each experiment. Test slides were scored only when internal controls showed clearly positive and negative appearances.

One hundred cells per individual (50 cells per replicate slide) were analyzed at 200× magnification using a fluorescence microscope equipped with an excitation filter (BP 546/12 nm) and a barrier filter (590 nm). Three parameters were used to assess damage: (i) dam-

age index (DI), determined visually by the categorization of comets into five classes according to DNA migration, from 0 (no tails) to 4 (maximally long tails). The damage index (DI) was obtained by the sum of the individual cell classes, ranging from 0 (no damage: 100 cells × 0) to 400 (maximum damage: 100 cells × 4); (ii) image length (IL) (nuclear regions plus tail), evaluated using an eyepiece calibrated in micrometers. From all individual cell lengths a mean image length index was calculated for each animal; (iii) damage frequency (DF in %), based on the number of cells with tails versus those without.

International guidelines and recommendations for the comet assay consider that visual scoring of comets is a well-validated evaluation method. It has a high correlation with computer-based image analysis [19,21]. The damage index is based on the length of migration and on the amount of DNA in the tail and is considered a sensitive measure of DNA. According to [19], the damage frequency, or the proportion of cells that show tails after electrophoresis, is less informative than the damage index (DI), because it does not consider the extent of the DNA damage in the cells. Image length or migration length gives information only about the size of DNA fragments and is largely dependent upon electrophoresis conditions (i.e. voltage and duration). Thus damage index (DI) is emphasized in our analyses. The other parameters, damage frequency (DF) and image length (IL), although considered in the analysis, were used only as complementary DNA damage parameters.

2.5. Statistical analysis

Student's t-test was used to compare the DNA damage at 24 and 48 h (24 h versus post-treatment with VitC). The one-way ANOVA was used to rank the DNA damage within the same exposure time (24, 48 h, post-treatment with VitC30 and post-treatment with VitC1). The parametric ANOVA with Tukey's post-hoc test was used when data presented homoscedasticity and normality. If data were not homogenous in regard to variance, the Kruskal–Wallis test with Dunn's post-hoc test was used. The SPSS statistical package was used to run the Student's t-tests and the parametric ANOVA. The GraphPad Prism (Graph-Pad Software, San Diego, CA) was used for the Kruskal–Wallis test. Significance was considered to be at $P < 05$. Values are expressed as mean ± S.D.

52

3. Results

3.1. Genotoxicity of the tested compounds

The internal controls of the comet assay (human blood) showed low damage in the negative control (DI = 0–10) and high damage in the positive control—MMS (DI = 180–300), thus validating the test conditions.

Significant damage increase was observed between 24 and 48 h (double treatment) for the water treated control group ($P \leq 0.001$) (Fig. 1).

The single (24 h) and double (24 and 48 h) treatments with both doses of VitC were genotoxic when compared to the respective control groups ($P \leq 0.001$) (Fig. 1). The higher dose (VitC30) induced significantly more DNA damage than the lower dose (VitC1) for both single and double treatments ($P \leq 0.001$). However, only VitC30 increased damage significantly when comparing single and double treatments ($P \leq 0.001$) (Fig. 1).

Table 1 shows that the DNA damage at 48 h (repair) after a single treatment, as assessed by DI, was significantly lower than at 24 h ($P \leq 0.05$) for both VitC1 and VitC30.

All drugs (CP, MMS, $FeSO_4$ and $CuSO_4$) significantly induced more DNA damage in relation to water 24 h after treatment, except in DF for CP and IL for $FeSO_4$ (Table 1). At 48 h, all compounds apart from

MMS reduced damage in relation to 24 h, but this was significant only for the metals. All substances except $FeSO_4$ were genotoxic at 48 h. Results are shown in Table 1 and Fig. 2 shows the genotoxicity of the compounds as evaluated by the damage class frequency.

When damage index was the parameter, all drugs induced significantly more DNA damage than VitC1 at 24 h. For VitC30, this was only significant for MMS and $FeSO_4$; other comparisons can be seen in Table 1.

3.2. Modulator effect of Vitamin C

Post-treatment with VitC1 reduced the DNA damage caused by MMS at 48 h, but increased it for the other drugs. This increase was slight and not significant for CP, but significant for the metals. Post-treatment with VitC30 significantly increased the damage only for the metals. The damage decrease was very slight for MMS and moderate but not significant for CP.

The damage observed in post-treatment with VitC1 after $CuSO_4$ was significantly higher than that caused by post-treatment with VitC1 itself (Table 1). Significant differences were only observed in the modulatory action of the doses of VitC for MMS.

4. Discussion

4.1. Dietary allowance of Vitamin C

Mice do not depend primarily on dietary intake of VitC. Unlike primates, they synthesize ascorbic acid [1,2]. By giving ascorbic acid to mice, we demonstrated the effect of an extra dose of an endogenously present nutrient. The suggested dietary allowances for several micronutrients, such as ascorbic acid, have been updated constantly during recent decades. Until the beginning of the 2000s, the recommended dietary allowance (RDA) most commonly indicated for young human adults was 60 mg per day [3,22]. At that time, higher levels were suggested for people subject to stress and for smokers [1]. Recently the levels were increased to 75 and 90 mg per day for females and males, respectively, at the same stage of life [23]. We considered 60 mg of ascorbic acid as the standard for our study. The VitC1 dose was equal to the RDA while VitC30 was equal to 30 times the RDA. Although there is considerable evidence supporting the antiox-

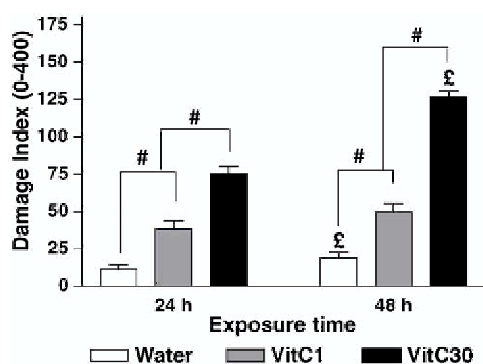Fig. 1. Genotoxicity induced by single (at 24 h) and double treatment (at 48 h) with water or Vitamin C. £Significant when comparing DNA damage between 24 and 48 h, for the same substance at $P \leq 0.001$, as tested by Student's *t*-test. #Significant when comparing DNA damage at the same time for the different substances at $P \leq 0.001$, as tested by one-way ANOVA. Damage index (DI) ± S.D. $N = 6$ mice per group.

Table 1
DNA damage modulation induced by post-treatment with two doses of Vitamin C, evaluated by the comet assay

Substances	Single doses (mg/kg b.w.)	Exposure time						
		24 h		Repair (48 h)		Post-treatment (VitC1) (48 h)[a]		Post-treatment (VitC30) (48 h)[b]	
		DI ± S.D.	n^c	DI ± S.D.	n	DI ± S.D.	n	DI ± S.D.	n
Water	#	11.50 ± 2.74	6	$19.17 \pm 3.31^{d***}$	6	–	–	–	
CP	25.00	$109.00 \pm 40.39^{e*}$	18	$74.83 \pm 16.68^{e*}$	6	100.17 ± 44.21	6	106.33 ± 44.56	6
MMS	40.00	$169.28 \pm 13.45^{e***,f***,g*}$	18	$157.67 \pm 15.28^{e***}$	6	$68.50 \pm 34.62^{h***,i***}$	6	146.83 ± 14.16	6
Ferrous sulfate	33.23 Fe	$109.67 \pm 26.58^{e*,f***,g**}$	18	$30.67 \pm 32.06^{d***}$	6	$119.83 \pm 28.01^{h***}$	6	$137.17 \pm 31.11^{h***}$	6
Cupric sulfate	8.50 Cu	$127.89 \pm 41.19^{e**,f**}$	18	$84.50 \pm 18.71^{d**,e*}$	6	$141.33 \pm 10.67^{h***,f**}$	6	$144.67 \pm 19.61^{h***}$	6
Vitamin C (VitC1)	1.00	38.33 ± 13.40	6	$24.40 \pm 3.85^{d*}$	6	$49.50 \pm 14.43^{h**}$	6	–	–
Vitamin C (VitC30)	30.00	75.33 ± 10.95	6	$48.20 \pm 4.66^{d**}$	6	–		$126.50 \pm 9.79^{d***,h***}$	6

Substances	Single doses (mg/kg b.w.)	Exposure time						
		24 h		Repair (48 h)		Post-treatment (VitC1) (48 h)		Post-treatment (VitC30) (48 h)	
		DF ± S.D.	n	DF ± S.D.	n	DF ± S.D.	n	DF ± S.D.	n
Water	#	9.50 ± 2.35	6	11.33 ± 3.67	6	–	–	–	–
CP	25.00	73.67 ± 25.07	18	$62.33 \pm 15.27^{e*}$	6	68.17 ± 21.88	6	70.83 ± 21.85	6
MMS	40.00	$98.44 \pm 3.05^{e***,f***,g**}$	18	$87.00 \pm 8.74^{d*,e*}$	6	$55.67 \pm 26.86^{i**}$	6	93.67 ± 6.65	6
Ferrous sulfate	33.23 Fe	$78.39 \pm 21.62^{e*}$	18	$18.17 \pm 9.77^{d***}$	6	$82.67 \pm 19.88^{h***,f**}$	6	$93.67 \pm 14.56^{h***}$	6
Cupric sulfate	8.50 Cu	$84.00 \pm 21.95^{e**,f*}$	18	$67.83 \pm 15.18^{e**}$	6	$93.17 \pm 7.65^{h*,f***}$	6	$95.67 \pm 3.01^{h*}$	6
Vitamin C (VitC1)	1.00	26.33 ± 10.33	6	24.20 ± 3.90	6	$39.33 \pm 13.59^{h*}$	6	–	–
Vitamin C (VitC30)	30.00	64.50 ± 9.73	6	$46.80 \pm 3.56^{d**}$	6	–	–	$93.33 \pm 4.55^{d***,h***}$	6

Substances	Single doses (mg/kg b.w.)	Exposure time						
		24 h		Repair (48 h)		Post-treatment (VitC1) (48 h)		Post-treatment (VitC30) (48 h)	
		IL ± S.D.	n	IL ± S.D.	n	IL ± S.D.	n	IL ± S.D.	n
Water	#	19.24 ± 1.97	6	$22.09 \pm 1.06^{d**}$	6	–	–	–	–
CP	25.00	$28.14 \pm 3.87^{e**}$	18	$25.73 \pm 1.09^{d*,e*}$	6	25.40 ± 3.46	6	25.44 ± 4.19	6
MMS	40.00	$31.19 \pm 1.56^{e***,f***,g**}$	18	$31.80 \pm 1.57^{e***}$	6	$24.45 \pm 2.32^{h***,i***}$	6	29.28 ± 1.00	6
Ferrous sulfate	33.23 Fe	26.64 ± 1.90	18	$22.99 \pm 2.02^{d***}$	6	$27.17 \pm 2.00^{h**}$	6	$28.53 \pm 2.16^{h***}$	6
Cupric sulfate	8.50 Cu	$29.08 \pm 4.02^{e***,f*}$	18	$26.08 \pm 0.92^{d**,e*}$	6	$28.20 \pm 1.85^{f**}$	6	$29.38 \pm 1.90^{h**}$	6
Vitamin C (VitC1)	1.00	22.75 ± 0.93	6	22.07 ± 0.75	6	22.25 ± 1.22	6	–	–
Vitamin C (VitC30)	30.00	24.70 ± 1.37	6	$24.94 \pm 0.34^{e*}$	6	–	–	$28.46 \pm 1.38^{d***,h**}$	6

Damage index (DI), damage frequency (%) (DF), image length (μm) (IL). #: mice were treated twice with water. The significance in relation to water is 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.

[a] n: number of individuals.

[b] Group pre-exposed to substances and then exposed to a single dose of Vitamin C (VitC1).

[c] Group pre-exposed to substances and then exposed to a single dose of Vitamin C (VitC30).

[d] Significant in relation to 24 h at $^*P \le 0.05$; $^{**}P \le 0.01$; $^{***}P \le 0.001$.

[e] Significant in relation to 48 h at $^*P \le 0.05$; $^{**}P \le 0.01$; $^{***}P \le 0.001$.

[f] Significant difference between the doses of the post-treatment at $^*P \le 0.05$; $^{**}P \le 0.01$; $^{***}P \le 0.001$.

[g] Significant in relation to water at $^*P \le 0.05$; $^{**}P \le 0.01$; $^{***}P \le 0.001$.

[h] Significant in relation to VitC1 at $^*P \le 0.05$; $^{**}P \le 0.01$; $^{***}P \le 0.001$.

[i] Significant in relation to VitC30 at $^*P \le 0.05$; $^{**}P \le 0.01$; $^{***}P \le 0.001$.
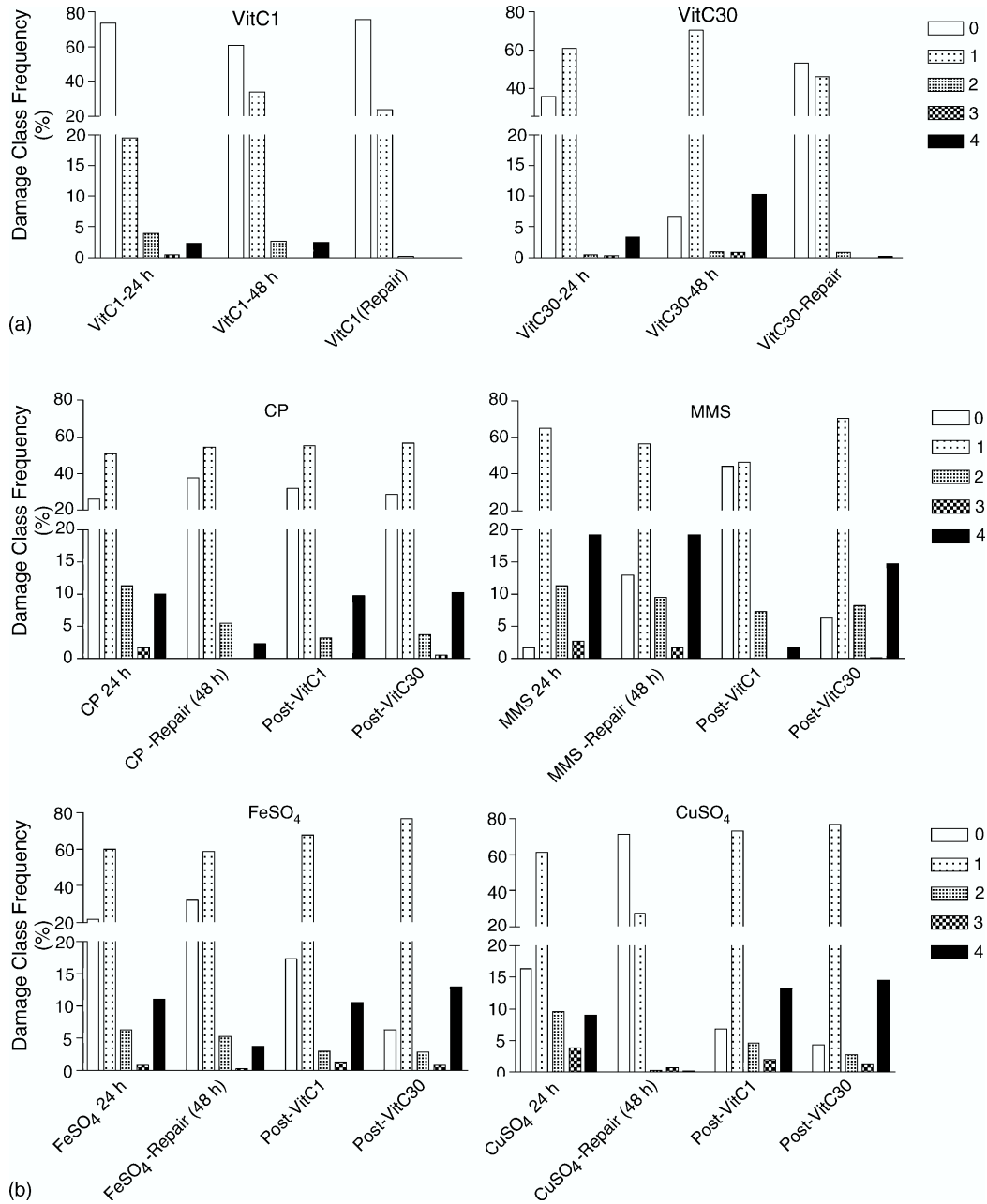
Fig. 2. Genotoxicity of the tested compounds, as evaluated by damage class frequency: (a) VitC1 and VitC30; (b) CP, MMS, FeSO$_4$ and CuSO$_4$.

idant role of ascorbic acid, there is a tolerable upper intake level (UL). The dose of 30 mg/kg is similar to the amount present in some commercial supplements available in markets worldwide and corresponds to the UL [23].

4.2. Genotoxicity of the tested substances

In our experiments with the comet assay in mouse blood cells, all drugs, including VitC, were genotoxic at 24 h. At 48 h, at least one parameter indicated repair.

The DNA damage increase between 24 and 48 h for the water treatment might be associated to the stress associated with manipulation, gavage and blood sampling of the mice as well as to noise in the animal facility during the procedures. As water was the vehicle for all substances, the DNA damage, whether or not associated to water, would have been influenced equally in all treatment groups.

The dose and frequency of administration of the drugs are important aspects to consider when evaluating the biological response to chemical stressors. Two different phenomena can arise from oxidative stress, cell adaptation and cell death [24]. For VitC1, animals seemed to show adaptation and a slight damage increase between 24 and 48 h (repair). For VitC30, the DNA damage increased significantly over this time. With extra days of treatment, it might be expected that the blood cells of the mice treated with VitC30 would die due to the accumulation of DNA lesions. As reviewed by Park et al. [25], L-ascorbic acid can induce apoptosis in several tumor cell lineages. This suggests that chronic usage of high levels of VitC could lead to deleterious effects. Many authors are concerned with VitC deficiency; however, our experiments showed that vitamin supplementation could be dangerous for organisms with normal levels of VitC, whether this can be produced by synthesis or dietary intake.

4.3. Modulator effect of Vitamin C

4.3.1. Alkylating agents

CP is an indirect bifunctional alkylating agent. Its genotoxicity is mainly mediated by its metabolites. CP can also generate reactive species, in a second mechanism for damaging DNA [26]. The results of post-treatment with VitC do not indicate a repair effect for CP. Conversely, they indicate a non-significant additive effect (i.e. sum of the damage generated by CP and VitC). In a previous study, it was observed that post-treatment with orange juice reduced the DNA damage induced by CP (data not shown). This indicates varying effects following administration of VitC in isolation and in combination with other nutrients (e.g. phenolic compounds). One mechanism of genotoxicity for ascorbic acid, a strong reducing agent, is enhancement of the conversion of CP into genotoxic metabolites. It can also contribute to oxidative stress, via endogenous iron and the Fenton reaction [25], causing an imbalance

in cell defenses and repair ability. Vijayalaxmi and Venu [7] showed that 10, 30 and 60 mg/kg mouse b.w. of VitC reduced the frequency of micronuclei (MN) generated by CP at 24 h. While MN are the result of chromosomal material loss and, consequently, mutations, DNA strand breaks are early events which can lead to mutations. The alkaline version of the comet assay simultaneously detects different kinds of DNA damage, such as cross-links, strand breaks, alkali-labile and incomplete excision repair events. The use of only one electrophoretic condition does not differentiate the kind of damage. Thus, it is possible to detect an increase in DNA strand breaks as a consequence of a stimulus in DNA repair. Indeed, if VitC is a repair stimulant, a positive response in the comet assay would be observed after treatment with CP. One could argue that the DNA damage increase associated with excision repair events, detected in our experiments, would result in a decrease in MN frequency, as detected by Vijayalaxmi and Venu [7].

For CP, the IL was similar at 48 h and after post-treatment with both doses of Vitamin C. Conversely, the DI increased after post-treatments. Fig. 2b shows a reduction in the frequency of cells in class 2 and an increase of cells in class 4 when compared with 48 h. One can explain this in four ways: (a) Vitamin C intervention reduced repair and the DNA damage thus increased; (b) Vitamin C stimulated repair and while DI increased, IL remained at the same level; (c) Vitamin C stimulated the metabolism of CP, resulting in a longer exposure time and, thus, cytotoxicity; (d) Vitamin C acted as a target for CP metabolites as well as reactive species, reducing the reactivity of CP. Further analyses using repair enzymes or different pH values would be interesting in order to address this issue.

MMS is a direct acting monofunctional alkylating agent that has low ability to break the DNA strands directly. Its action is mediated by base tautomerization. The animals treated with MMS and post-treated with VitC showed various results: while VitC1 reduced DNA damage significantly, VitC30 apparently had no effect. This indicates that lower doses of VitC may be more beneficial than high doses. It is possible that the imbalance generated by higher doses of VitC do not allow for the cell adaptation that occurs at lower doses. The IL for MMS was similar after post-treatment at 48 h, except for VitC1, which reduced DNA damage. The increase in the frequency of cells

in class 0 in the VitC1 post-treatment, when compared with 48 h, indicates repair activity induced by Vitamin C (Fig. 2b). Another possible mechanism is protection against MMS by the reduction of its ability to alkylate DNA. Indeed, the auto alkylation of ascorbic acid can prevent or reduce the cellular alkylation of macromolecules [7]. However, taking into account that MMS has non-metabolism-dependent genotoxicity, its antigenotoxicity could be mediated mostly by competition. Horváthová et al. [27] mentioned that some synthetic antioxidants can act against mutagen reactivity, although in a still unexplained fashion. The prevalence of high damage at 48 h showed that, indeed, low does of VitC reduce MMS action and/or stimulate repair (e.g., by induction of DNA strand breaks during repair steps).

4.3.2. Metal sulfates

The administered form of iron (Fe^{2+}) is that more easily absorbed in the mammalian small intestine [28,29] and which can directly break DNA at specific nucleotide sequences, as well as participating in oxidative stress reactions [30]. Our data suggest that post-treatment with both doses of VitC induces a considerable increase in DNA damage generated by $FeSO_4$, in relation to damage at 48 h. This increase was slightly higher for post-treatment with VitC30 than for VitC1. A co-genotoxicity effect seems to have occurred for both doses. Low damage at 48 h agrees with previous data observed by Franke et al. [28] for post-treatment with orange juice (data not shown). This result reinforces the idea that VitC, either in isolation or as complex mixtures, prejudices repair. In contrast to what would be expected from post-treatment with VitC, damage was not reduced by the ROS scavenging ability of ascorbic acid over hydroxyl radicals generated by ferrous ions. Considering the low concentration of VitC1, at least some beneficial effect would be expected, but it did not occur.

There is no doubt that exposure to excess copper can damage cells and organs. The chemistry of copper makes it an ideal participant in redox reactions, as it easily cycles between the cuprous and cupric state. However, it is not known if it is likely to occur in vivo, and if so under what conditions. Our experiments showed that VitC post-treatment of animals treated with copper increased DNA damage at both doses. Thus a co-genotoxicity effect was observed in a similar manner for both metals. Low damage at 48 h in animals treated only with copper indicated that the repair was much

more effective than in post-treatment with VitC. Post-treatment with VitC at both doses, therefore, probably reduced oxygen to H_2O_2 and Cu^{2+} to Cu^+ reactions. Presumably the Cu^+ then reacts with H_2O_2 to produce hydroxyl radicals [31]. It has been shown that mixtures of VitC and Cu^{2+} cause DNA fragmentation, DNA-repair synthesis and chromosome aberrations, including chromatid breaks and exchanges [32,33], indicating that ascorbic acid breaks DNA when hydroxyl radicals are produced in the presence of oxygen, a reaction that is stimulated by Cu^{2+} ions. One explanation is that ascorbic acid, erythorbic acid and D-isoascorbic acid enhance DNA single strand breaks in the presence of Cu^{2+}. It is likely that the enediol group of ascorbic acid has an essential role in the breakage of nucleic acids and that Cu^{2+} increases this effect [32]. Yoshino et al. [34] found that the interaction of copper with VitC led to five times more DNA damage than the same interaction with iron. We found about 20% increase in damage for copper for a similar dose: about 11% of the LD_{50}.

According to Fenech and Ferguson [6], Halliwell states that the protective effect of VitC supplementation in vivo is weak in those who are not deficient in this micronutrient. Additionally, the authors advise that although the VitC pro-oxidant role has not been proved, the possible deleterious effect of VitC supplements must be taken into consideration. Vitamin C supplements should not substitute diets rich in fruit and vegetables. Orange juice is as a strong antioxidant [35] and can reduce the genotoxicity of all substances tested in the present study (data not shown). Supplementation with VitC, sometimes at high doses, has been recommended by enthusiastic defenders such as the Nobel Prize winner, Linus Pauling. Scientific evaluations of the effects of VitC have produced controversial results, even showing deleterious action. In this study, VitC induced DNA damage and enhanced the genotoxicity of metal sulfates. Since DNA damage generated by both doses of VitC can be easily repaired and the lowest dose significantly reduced the genotoxicity of MMS, further data is still needed to shed light upon the beneficial/noxious effects of VitC.

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4 DISCUSSÃO GERAL

Há evidências epidemiológicas crescentes associando dietas ricas em frutas e vegetais frescos e a redução da incidência de doenças associadas ao envelhecimento e a perturbação da estabilidade genômica, como cânceres, doenças cardiovasculares e neurodegenerativas . As deficiências em vitaminas e minerais na dieta humana são geradoras de instabilidade genômica pelo aumento da ocorrência de quebras e lesões oxidativas no DNA. Portanto, estas deficiências devem ser evitadas e o OJ pode contribuir com componentes-chave para a estabilidade genômica. Apesar de serem considerados benéficos em concentrações adequadas, as vitaminas, os minerais e outros componentes bioativos das frutas e vegetais têm sido apontados como mutagênicos ou carcinogênicos, especialmente quando administrados em excesso ou fora de matrizes alimentares (Halliwell & Guterridge, 2000; Ames, 2001; Fenech & Ferguson, 2001; Ames et al., 2005; Fenech, 2005a; Fenech et al., 2005; Halliwell et al., 2005).

O OJ pode ser incluído entre os alimentos com potencial quimioprotetor e seu estudo é relevante pelo amplo consumo deste suco. O OJ possui vários nutrientes e compostos bioativos com atividades antioxidante, antimutagênica, anticarcinogênica e antiaterogênica, entre outras. O OJ contém quantidades significativas de vitamina C, folato, tiamina, riboflavina e compostos fenólicos. Além disso, o OJ contém quantidades apreciáveis de carotenóides (pró e não-pró-vitamina A), niacina, ácido pantotênico, piridoxina, magnésio, potássio, cobre, ferro e fósforo. Estas substâncias podem influenciar o *status* oxidativo e a estabilidade genômica de forma direta ou indireta (ANEXO C).

Nesse sentido, os resultados de um estudo anterior (ANEXOS A e B), avaliando o potencial antioxidante *in vitro* e mutagênico em *Salmonella* de diferentes OJs quanto ao conteúdo de Vit C e de fenólico(s) totais e ao processamento e armazenamento, evidenciaram a necessidade de investigar os efeitos do OJ em mamíferos. Observou-se que o OJ espremido

manualmente e não-processado (*in natura*) foi o que apresentou mais respostas positivas quanto à mutagenicidade em bactérias. Com base neste resultado utilizamos o OJ *in natura* para: 1) avaliar se o OJ poderia ser genotóxico em mamíferos (camundongos); 2) avaliar se e em que extensão o OJ poderia modular a genotoxicidade de agentes alquilantes, bem como do cobre e do ferro, dois metais de transição.

A Vit C é a vitamina mais abundante no OJ e um copo de 200 mL OJ (uma porção) pode prover uma quantidade de Vit C superior à preconizada pelas diretrizes alimentares – DRI (IOM, 2000). A Vit C tem sido foco de muitos estudos ao longo das últimas décadas quanto ao seu potencial anti/pro-oxidante (Halliwell, 2001; Johnston et al., 2003; Kang et al., 2005). Por esse motivo, a capacidade modulatória da genotoxicidade dos agentes alquilantes e dos metais, pela vit C, também foi avaliada neste trabalho.

O PIXE foi utilizado como uma medida auxiliar para quantificar os níveis sangüíneos e hepáticos de ferro e cobre nos animais tratados com estes metais.

Adicionalmente, foram compiladas duas revisões bibliográficas abordando os efeitos biológicos e a composição do OJ, para melhor entendimento dos efeitos observados.

Os resultados da parte experimental, apresentados na seção 3 em camundongos (ensaio Cometa), diferiram dos previamente observados em bactérias (*Salmonella*/microssoma – Anexos A e B). Em bactérias, o OJ *in natura* apresentou respostas positivas frente a várias linhagens de *S. typhimurium* com e sem metabolização. Entretanto, em camundongos tratados com OJ foi observado apenas um incremento no índice de danos em relação ao controle negativo a 24 h. Portanto, pelo ensaio Cometa o OJ não foi genotóxico em camundongos, conforme o teste estatístico. Essa tendência se repetiu tanto no experimento com os agentes alquilantes (Capítulo 1), quanto no experimento com os metais (Capítulo 2). A diferença não significativa observada para a genotoxicidade do OJ a 24 h poderia estar relacionada ao maior desvio observado no grupo tratado com OJ. A maior variabilidade intragrupo quanto ao índice de danos do grupo OJ poderia ser interpretada como decorente de variações metabólicas entre os camundongos, fato comum na resposta a fármacos e misturas complexas.

Conforme avaliado pelo teste de *Salmonella*/microsoma, o OJ foi considerado mutagênico, aumentando a taxa de mutação (número de revertentes) até 5,6 vezes (de forma dose-dependente). Já no ensaio Cometa em camundongos, observou-se, um aumento na quantidade de danos primários no DNA, porém não significativo (que podem dar origem a mutações). Portanto, o OJ foi considerado não genotóxico em camundongos nas condições testadas. Cabe ressaltar que nos mamíferos o OJ é submetido a uma maior atividade

enzimática e química (pH), especialmente no trato gastrintestinal. Além disso, o *end-point* avaliado em bactérias (mutações) é diferente do avaliado nos camundongos (dano do DNA).

É importante mencionar que o OJ possui características favoráveis à indução de genotoxicidade. O pH do OJ pode aumentar a biodisponibilidade de metais, que podem gerar danos no DNA via estresse oxidativo. Este estresse pode ser intensificado pela presença tanto de flavonóides quanto de Vit C, que podem reduzir os metais, facilitando a participação dos mesmos em reações oxidativas. Um exemplo deste fato é a geração de $^1O_2$ pela reação da Vit C com metais, bem de outras ROS geradas pela reação de Fenton e *Fenton-like*. Outra justificativa para a genotoxicidade do OJ é o efeito do mesmo sobre a xantina oxidase. Enquanto compostos específicos do suco, como as flavanonas (hesperidina e naringinina e seus glicosídeos - Anexo D, Tabela 3), podem inibir a formação de $O_2^{\bullet-}$, pela xantina oxidase C, o OJ como mistura complexa estimula as xantina oxidases (Dew et al., 2005). Estas estão envolvidas na conversão de hipoxantina a ácido úrico e a sua expressão tem sido ligada a doenças degenerativas. Em determinadas condições fisiológicas, a xantina oxidase processa O_2 ao invés de NAD^+ e gera $O_2^{\bullet-}$ e $NO^{\bullet}$ (Glantzounis et al., 2005).

Embora não existam evidências da genotoxicidade e da mutagenicidade do OJ em humanos e em outros mamíferos (ANEXO C), um único estudo relacionou o risco de melanoma e a ingestão de vitamina C, a partir de alimentos como o OJ (Feskanich et al., 2003).

O BER é um dos mecanismos de reparo mais utilizados para o reparo de danos oxidativos e alquilação e pode ser estimulado pelos componentes do suco, como a Vit C, a naringinina e vitaminas do complexo B (ANEXO D). O BER, a exemplo de outros sistemas de reparo, envolve a clivagem e a ressíntese da porção de DNA danificada. Desse modo, a estrutura do DNA fica rompida até que a DNA polimerase resintetize a cadeia e esta seja religada. O ensaio Cometa detecta principalmente quebras no DNA, que podem ser resultado de genotoxicidade ou da atividade de reparo. Portanto, um aumento de genotoxicidade detectado pelo ensaio Cometa também pode indicar um aumento na atividade de reparo, uma vez que as quebras detectadas podem ser provenientes de excisão das cadeias de DNA pelas enzimas de reparo (Tice et al., 2000). A genotoxicidade do OJ também pode ser interpretada como um aumento da atividade de reparo sobre os danos basais ao DNA das células.

Assim como nos animais tratados com suco, um incremento menor, contudo significativo, na genotoxicidade foi observado entre 24 e 48 h para o grupo controle em todos os experimentos (Capítulos 1-3). O estresse associado à manipulação dos animais durante os experimentos, bem como lesões gastrintestinais geradas pela *gavage* poderiam gerar respostas

inflamatórias ligadas ao aumento do estresse oxidativo. De qualquer forma, esse aumento é inerente a todos os tratamentos. Além disso, a resposta às lesões e aos danos no DNA também pode sofrer influência das diferenças interindividuais. Cabe ressaltar que a presença de metais na água de beber dos animais também poderia afetar a resposta, uma vez que a água foi provida pelo sistema municipal de abastecimento da cidade de Porto Alegre. Contudo, água destilada foi utilizada para diluir as substâncias-teste e para tratar os animais do controle negativo. Ressalta-se, novamente, que a influência da água foi inerente a todos os tratamentos.

Neste estudo, camundongos foram utilizados para avaliar o efeito genotóxico e antigenotóxico da Vit C. A intenção foi simular indivíduos que têm níveis adequados de Vit C na dieta diária e que fazem uso abusivo de suplementos dessa vitamina.

Neste estudo, as doses foram calculadas em função da recomendação dietética anterior a 2000 (60 mg/dia tanto para homens quanto para mulheres adultos). Para fins de discussão, as recomendações de 2000 – DRI_{2000} [82,5 mg/dia, média da recomendação para homens (90 mg/dia) e mulheres (75 mg/dia)] serão utilizadas (IOM, 2000).

A Vit C foi genotóxica de maneira dose-dependente 24 h após o tratamento para ambas doses [1 (VitC1) e 30 (VitC30) mg/Kg] (Capítulo 3). Quando os animais foram tratados duas vezes, a 24 e a 48 h (duplo-tratamento), a genotoxicidade aumentou significativamente apenas para a dose maior (30 mg/Kg). A genotoxicidade induzida por ambas doses de Vit C foi efetivamente reparada após 48 h de uma única administração (reduzindo cerca de 35% em relação a 24 h). É importante mencionar que o índice de dano no DNA dos animais tratados uma única vez com VitC1 foi similar ao dos controles negativos a 48 h, mostrando reparo efetivo nestas condições. O efeito pró-oxidante ou estimulador do reparo poderiam estar associados ao aumento dos danos no DNA detectados pelo ensaio Cometa.

Enquanto o duplo-tratamento (0 e 24 h) tanto com o OJ quanto com VitC1 manteve o dano entre 24 e 48 h, o tratamento duplo com VitC30 causou um incremento considerável (~70 %) na genotoxicidade entre 24 e 48 h. A dose de suco de 0,1 mL/10 g (10 mL/Kg) de peso de camundongo corresponde a 600 mL para um humano de 60 Kg. Utilizando o conteúdo médio de Vit C calculado na Tabela 1 do ANEXO D, uma porção de 600 mL de OJ representa uma entrada de 303 mg de Vit C. Esse valor corresponde a aproximadamente 3,7 vezes a DRI_{2000} de Vit C. Desse modo, a quantidade de Vit C presente no suco ministrado aos camundongos foi intermediária às doses de Vit C utilizadas no estudo (1 e 30 mg/Kg), demonstrando que quando presente no suco, uma dose maior que a RDA de Vit C, como parte

de uma mistura complexa (OJ), parece não ser genotóxica em animais que tem síntese endógena dessa vitamina. Por outro lado, a dose maior de Vit C [equivalente à UL para humanos (IOM, 2000)] quando administrada *per se* foi fortemente genotóxica. Estudos adicionais, utilizando diferentes tempos e doses, são necessários para esclarecer se poderia ocorrer uma adaptação à Vit C a longo prazo. Nossos estudos avaliando o efeito do tratamento por uma semana com as mesmas doses de Vit C indicam que a vitamina C foi tanto mutagênica quanto genotóxica (dados não apresentados).

Tanto o OJ quanto a Vit C foram antigenotóxicos contra o MMS. A Vit C foi testada apenas no pós-tratamento e foi altamente antigenotóxica para o MMS (~60 % de redução no índice de dano) apenas na dose menor. Já o OJ, administrado tanto antes quanto após o tratamento com MMS, reduziu o dano no pré (~70 % de redução no índice de dano) e pós-tratamento (~40 % de redução no índice de dano), reforçando a idéia de que prevenir o dano é melhor do que remediá-lo, pelo menos para alimentos como o OJ. Neste sentido, parece que a a Vit C e os flavonóides do OJ são alvos de alquilação efetivos (pré-tratamento) e agentes estimulante do reparo (pós-tratamento) (Edgar, 1974; Wang et al., 1996; Cooke et al., 1998; De Flora, 1998; Vijayalaxmi & Venu, 1999; Middleton et al., 2000; Gao et al., 2006). A Vit C, quando administrada como substância única, parece ser benéfica apenas para a dose menor; e, doses maiores devem ser vistas com cautela quanto a seu efeito reparador.

Os resultados permitiram observar que quantidades de Vit C entre ~0,7 (VitC1) e ~4,7 (dose presente no suco) vezes a DRI_{2000} estimularam o reparo em camundongos. A quantidade de Vit C de ~22 vezes a DRI_{2000} (dose maior de Vit C) mostrou-se ineficiente no estímulo ao reparo do DNA. A dose de Vit C contida no OJ (385 mg) mostrou-se menos efetiva na redução de dano ao DNA gerado pelo MMS do que a dose menor de Vit C. Há evidências de que a dose ótima para estimular o reparo de danos no DNA induzida pela dose testada de MMS em camundongos (que sintetizam Vit C) esteja entre ~0,7 e ~3,7 vezes a DRI_{2000} para Vit C. Em humanos (que não sintetizam Vit C) o tratamento a longo prazo com doses entre ~3 a ~6 vezes a DRI_{2000} (250-500 mg/dia) de Vit C estimulou a excreção urinária de 8-OHdG, como revisado por Cooke et al. (1998). Esse aumento da excreção tem sido interpretado como um estímulo do reparo de DNA.

Se de fato a Vit C pode estimular o reparo, a DRI poderia ser aumentada para a população em geral (submetida a poluição e a dietas deficientes em micronutrientes), e não apenas para fumantes ou indivíduos com altos níveis de estresse, como tem sido preconizado pelas diretrizes alimentares (IOM, 2000). A ingestão de 5 porções diárias de frutas e vegetais frescos, recomendada como prevenção às doenças crônico-degenerativas, pode fornecer cerca

de 500 mg/dia de Vit C, destoando da DRI_{2000} (75-90 mg/dia para mulheres e homens, respectivamente).

O OJ, mas não a Vit C, apresentou efeito reparador sobre a CP (Capítulos 1 e 3), o que sugere a importância de outras substâncias presentes no OJ na modulação da genotoxicidade induzida por CP. Os compostos fenólicos, os carotenóides, bem como o folato, a riboflavina e outras vitaminas do complexo B já foram associadas à estabilidade genômica e ao estresse oxidativo (Fenech, 2005a; Fenech et al., 2005). A atividade destes compostos justifica as diferenças entre o OJ e a Vit C quanto ao potencial antigenotóxico (Tabelas 1-3 e anexo D).

Tabela 1. Efeito das substâncias bioativas presentes no suco de laranja sobre a estabilidade genômica: vitaminas

| Vitamina | Contribuição para a estabilidade genômica | |
	Atividade antioxidante	Homeostasia do DNA
B1	Relacionadas à presença de grupamento sulfidrila (quelam ROS e metais)	Envolvida na síntese de pentoses e na regulação do ciclo celular (p53)
B2	Papel como FAD	Cofator de MTHFR
B3	Papel como NAD	Substrato de PARP-1
B5	Papel na síntese de cisteamina e reciclagem de GSH	Desconhecida
B6	Capturadora de AGE	Regeneradora do substrato da MTHFR; estimuladora do reparo por excisão
B9	Capturadora fraca de ROS	Substrato da MTHFR
C	Anti (quela ROS e metais) e pró-oxidante (agente redutor)	Leva à apoptose em altas concentrações; estimula BER

NAD: nicotinamida adenina dinucleotídeo; FAD: flavina adenina dinucleotídeo; GSH: glutationa; AGE: produtos finais avançados de glicação; MTHFR: metileno tetrahidrofolato redutase; PARP-1: Poli(ADP-ribose) polimerase-1; BER: reparo por excisão de bases
Fonte: ANEXO D

Tabela 2. Efeito das substâncias bioativas presentes no suco de laranja sobre a estabilidade genômica: minerais

Mineral	Contribuição para a estabilidade genômica	
	Atividade antioxidante	Homeostasia do DNA
Cu	Anti (componente das ferroxidases e da SOD Cu/Zn) e pró-oxidante (metal de transição)	Influência desconhecida
Mg	Influência desconhecida	Cofator de diversas enzimas, incluindo as DNA polimerases (atua na síntese e no reparo do DNA)
K	Níveis celulares aumentados têm sido relacionados à diminuição do estresse oxidativo	Substrato dos canais de membrana (envolvidos na sinalização e no controle da progressão celular)
P	Influência desconhecida	Compõe a estrutura do DNA e está envolvido na sinalização e no controle da progressão celular
Fe	Anti (componente das CATs e da SOD Fe) e pró-oxidante (metal de transição)	Influência desconhecida

SOD: superóxido dismutase
Fonte: ANEXO D

Tabela 3. Efeito das substâncias bioativas presentes no suco de laranja sobre a estabilidade genômica: outros compostos

Substância bioativa	Contribuição para a estabilidade genômica	
	Atividade antioxidante	Homeostasia do DNA
Carotenóides	Forte antioxidantes (caputadores de ROS e metais)	Inibidores da proliferação de células tumorais
D-limoneno	Forte antioxidante (pouco caracterizado)	Inibidores da proliferação de células tumorais
Compostos fenólicos	Capturador fraco de ROS e forte quelante de metais	Diversas atividades relacionadas ao metabolismo de substâncias, ao controle do ciclo celular e a mimetização de estrógenos

ROS: espécies reativas de oxigênio
Fonte: ANEXO D

Os compostos fenólicos estão entre os componentes mais abundante no OJ, e a maioria dos estudos aponta grande responsabilidade destes compostos na antigenotoxicidade e no potencial antioxidante do OJ (Anexo D). Os numerosos mecanismos de ação dos fenólicos poderiam ter contribuído para a antigenotoxicidade do OJ frente à CP. A hesperidina, a narirutina e as formas agliconadas destes compostos (hesperitina e naringenina, respectivamente) têm capacidade de inibir algumas enzimas de fase 1 (CYPs). A modulação

das CYPs envolvidas na metabolização de CP poderia ter reduzido a genotoxicidade da CP no pré-tratamento com o suco. Outro mecanismo de ação dos fenólicos é a atuação como alvo de alquilação ou como capturadores de FR. Apesar destas atividades, a modulação da genotoxicidade da CP, pelo pré-tratamento com OJ, pode ter sido reduzida por dois motivos: pela incapacidade dos fenólicos ou do OJ de modularem as principais enzimas envolvidas na metabolização da CP [CYP3A4, CYP3A5, CYP2C9 (Watters et al., 2003; Matalon et al., 2004)] (para revisão, ver ANEXOS C e D); e pela maior reatividade da CP. A CP possui dois sítios de alquilação, e, portanto, a quantidade dos componentes do suco pode não ter sido suficiente para competir com o DNA como alvo de alquilação da CP, como parece ter ocorrido para o MMS que possui apenas um sítio de alquilação.

Ao se comparar a atividade do OJ e da Vit C sobre o reparo da CP, é necessário considerar o tipo de dano gerado pela CP. Tanto o MMS como a CP alquilam moléculas nucleofílicas como o DNA. Diferentemente do MMS, que somente alquila o N e O das bases, a CP também induz pontes do tipo DNA-DNA e DNA-proteína. As lesões induzidas pela CP são, portanto, complexas e requerem vários mecanismos de reparo. Os adutos podem ser reparados por reversão de dano *in situ* (alquiltransferases) e por BER, principalmente. Há apenas evidências do efeito da Vit C (Cooke et al., 1998) e da naringinina sobre BER (Gao et al., 2006).

As pontes intercadeias e DNA-proteína, que podem ser geradas apenas pela CP, requerem NER e fatores de recombinação homóloga para serem reparados. A recombinação homóloga também parece ser o mecanismo para o reparo de 3NA, aproximadamente 11 % das lesões, bem como para lesões O6-guanina (menos de 0,5 % das lesões) induzidas por MMS (Saffi & Henriques, 2003; Drablos et al., 2004). Como as pontes intercadeias geradas pela CP envolvem outras rotas de reparo além do BER (como NER e recombinação homóloga), e os nossos resultados (Capítulos 1 e 3) apontam efeito reparador do OJ sobre a CP, é possível que algum componente do OJ tenha uma influência de forma ainda desconhecida sobre estas rotas de reparo. Além disso, tanto a CP quanto os seus metabólitos (p.ex. acroleína) induzem estresse oxidativo, e estão implicados na depleção de GSH e na inativação de genes relacionados à resposta antioxidante (Kehrer & Biswal, 2000). Neste sentido, o magnésio atua como cofator das DNA polimerases e participa de BER e NER e do reparo por MMR (Ames et al., 2005; Fenech et al., 2005). A deficiência de folato, por sua vez, afeta BER, NER, MMR, bem como o reparo de quebras de cadeia dupla, indicando um papel do folato nestas vias de reparo (Winterbourn et al., 2000; Wei et al., 2003). Como anteriormente citado, os trabalhos de Cooke et al. (1998) apontam a influência da vitamina C em BER, pelo aumento

da excreção urinária de 8-OHdG. Portanto, a presença do magnésio, folato e de Vit C no suco pode ter auxiliado o sistema de reparo frente aos danos gerados pela CP. Além disso, cabe ressaltar que outros componentes derivados do OJ, como a vanilina, um composto derivado dos ácidos hidroxicinâmicos também podem induzir o reparo recombinacional (Sinigaglia et al., 2004).

O ensaio Cometa tende a subavaliar as pontes intercadeias geradas pela CP, uma vez que as pontes tendem a dificultar a migração do DNA (Vrzoc & Petras, 1997; Tice et al., 2000; Collins, 2004). Existem alguns protocolos do ensaio Cometa utilizando enzimas (endonucleases para pontes DNA-DNA e proteinases para pontes DNA-proteína) para avaliar especificamente pontes no DNA (Tice et al., 2000). O uso destas técnicas poderia ser empregado para melhorar a compreensão da dinâmica das lesões induzidas pela CP.

O pós-tratamento com Vit C pode ter tido dois efeitos sobre a genotoxicidade da CP: a) induziu quebras no DNA por facilitar a reparação das pontes; e b) não afetou o reparo do DNA e foi co-genotóxica com a CP. A primeira hipótese é suportada pela manutenção de dano nas duas doses de Vit C. A efetividade do reparo da genotoxicidade da CP pelo pós-tratamento com OJ aponta a sinergia da Vit C com os demais componentes do suco na reparação do dano. A interação dos fenólicos com a Vit C pode ter um efeito sinergístico sobre o reparo de DNA e sobre a estabilidade destes compostos. Quanto a este último aspecto, é bem sabido que a Vit C e os fenólicos estabilizam-se mutuamente, aumentando a estabilidade destes compostos e, portanto, garantindo o efeito biológico dos mesmos (Middleton et al., 2000).

O pré-tratamento com OJ reduziu a genotoxicidade tanto do Fe quanto do Cu (Capítulos 2). Tanto a Vit C quanto o OJ têm sido prescritos para aumentar a absorção de Fe no tratamento das anemias. Pelos resultados dos experimentos pode-se observar que mesmo com um nível maior de metais nas células por um aumento na absorção, especialmente do Fe da dieta, o efeito antioxidante do OJ prevalece sobre o efeito pró-oxidante para os dois metais testados. Os compostos fenólicos podem ter atuado como capturadores de metais, indisponibilizado os mesmos para que a Vit C atue como pró-oxidante. Além destes, os outros antioxidantes como os carotenóides, a riboflavina e a niacina e a piridoxina podem ter atuado protegendo contra o dano oxidativo (ANEXO D, Tabela 1A-C).

O pós-tratamento tanto com Vit C quanto com OJ foi pró-oxidante para o Fe (Capítulos 3 e 2, respectivamente). Já, o pós-tratamento com OJ reduziu a genotoxicidade do Cu (Capítulo 2). É importante mencionar que quando o Fe é disponibilizado antes do agente redutor, o tratamento tanto com Vit C quanto com OJ tem um claro papel pró-oxidante. A

homeostasia refinada do Fe acaba sendo extremamente eficiente para capturar o ferro e deixá-lo imobilizado na célula, como pode ser visto pelo reparo eficiente após 48 h de uma única administração do elemento. O Fe tem três destinos dentro das células: 1) ser estocado na ferritina; b) ser associado a proteínas, principalmente como heme ou grupamentos Fe-S; c) ser armazenado na reserva lábil de Fe (LIP – *labile iron pool*), sendo ligado a moléculas de baixa afinidade (Anderson et al., 2005). Desse modo, uma parcela do Fe imobilizado como LIP ou na parcela mais externa da ferritina pode atuar na geração de ROS. Isto parece ocorrer, quando os animais são tratados com uma única dose de ferro e são pós-tratados com um agente redutor, que pode liberar o Fe estocado e/ou reagir com ele para formar OH$^{\bullet}$ pelas reações de Fenton e Haber-Weiss (Kruszewski, 2003; Arredondo & Nunez, 2005).

Os sistemas biológicos parecem apresentar uma resposta bifásica para a concentração de Cu. O transporte aumenta tanto na deficiência quanto no excesso de cobre intracelular. A relação entre a quantidade na dieta e a quantidade absorvida é maior para o Cu do que para o Fe. Além disso, o transporte do Cu é mais intenso do que a do Fe, uma vez que ocorre uma grande ciclagem de Cu entre o trato gastrintestinal e os órgãos excretores (Arredondo & Nunez, 2005). O suco parece acelerar a retirada do Cu do organismo (Capítulo 2), o que pode justificar a diferença do efeito do pós-tratamento com OJ nos animais tratados com Fe ou Cu.

O OJ é muito mais do que uma boa fonte de vit C e compostos fenólicos. O OJ é fonte de vitaminas do complexo B, minerais, bem como de outros compostos bioativos que desempenham papel na estabilidade genômica. Apesar de algumas evidências de mutagenicidade do OJ em bactérias (ANEXOS A e B), este estudo e os dados da literatura (ANEXO C) não indicam mutagenicidade ou genotoxicidade para o OJ em mamíferos. Contudo, deve-se considerar que muitos compostos presentes no OJ podem ter efeitos deletérios se o OJ for utilizado em excesso na dieta diária.

O OJ é um alimento de origem vegetal, rico em carboidratos e pobre em fibras. Portanto, a ingestão de grandes quantidades em curto período de tempo eleva a glicemia de forma abrupta e deve ser vista com parcimônia. O OJ não deve ser utilizado como substituto de refeições, principalmente para crianças, por ser um alimento pobre em cálcio. A qualidade do OJ pode ser largamente influenciada pela adição de cálcio. Vale lembrar que a adição de cálcio e vitaminas (principalmente D) é comum nos Estados Unidos. E, portanto, o OJ é um bom veículo de suplementação.

A maior parte do OJ consumido mundialmente é processada. O processamento pode tanto preservar ou aumentar a concentração/biodisponibilidade dos componentes do OJ, quanto depreciar a qualidade do OJ e/ou quantidade dos componentes do suco. Além disso,

estabilizantes, corantes e outros aditivos alimentares são freqüentemente reportados nos rótulos dos sucos. Por isso, a análise nutricional do OJ é chave para a seleção adequada de um OJ com potencial benéfico para a saúde. A adição de micronutrientes é uma forma de corrigir as perdas ocorridas no processamento.

Considerando o exposto, pode-se concluir que o consumo de OJ *in natura* protege e auxilia no reparo de danos no DNA, de forma melhor do que a Vit C *per se*. Contudo, não se deve "cair na euforia" de acreditar que a busca pela longevidade possa ser alcançada pelo consumo de um único alimento. O consumo de OJ fresco ou processado e armazenado de forma a preservar o seu potencial biológico é um alimento sugerido como uma das porções de uma dieta equilibrada (contendo pelo menos cinco porções de frutas e vegetais), recomendada para uma vida saudável e longeva.

5 PERSPECTIVAS

A antigenotoxicidade do OJ e genotoxicidade do ferro e do cobre, mostradas nesse estudo, são um indicativo de que novas pesquisas avaliando os efeitos do OJ, bem como do cobre e do ferro são de grande relevância à saúde humana. Neste sentido, a avaliação do efeito do ferro e do cobre e de compostos bioativos do OJ, como a hesperidina, pela utilização de outras condições e sistemas-teste se faz necessária. Nesta perspectiva é apresentada uma lista de experimentos, incluindo alguns que estão sendo executados e outras que estão em fase de planejamento (Tabela 4).

Tabela 4. Perspectivas para pesquisas adicionais à tese.

Item	Objetivo a avaliar	Substância(s)	Sistema teste	Local de execução
1	Potencial antigenotóxico do pré-tratamento com Vit C	MMS, CP, $FeSO_4$, $CuSO_4$ e Vit C	Micronúcleos e Cometa *in vivo* em camundongos	CBiot/IF-UFRGS
2	Potencial mutagênico, genotóxico e de acumulação subcrônicos (7 dias)	$FeSO_4$, $CuSO_4$ e Vit C	Micronúcleos, Cometa e PIXE *in vivo* em sangue e fígado de camundongos	CBiot/IF-UFRGS
3	Composição de minerais da ração de camundongos	Rações de camundongo	PIXE e absorção atômica	CBiot/IF-UFRGS ALAC
4	Influência da ração na genotoxicidade (sangue) e mutagenicidade (medula)	Rações de camundongo e $FeSO_4$	Micronúcleos e Cometa *in vivo* em camundongos	CBiot-UFRGS

5	Comportamento, genotoxicidade, acumulação e antigenotoxicidade	FeSO$_4$, desferal e Vit C	Cometa e PIXE no sangue em diferentes regiões do cérebro de ratos; testes de comportamento	CBiot/IF-UFRGS Fisiologia-PUCRS
6	Potencial genotóxico, antigenotóxico e antiproliferativo da hesperidina *in vivo* e *in vitro*	Hesperidina, H$_2$O$_2$ e MMS	Cometa em células V79 e no sangue de camundongos	CBiot-UFRGS
7	Relação entre a dieta, a estabilidade genômica e o estresse oxidativo em escolares	-	Cometa e dano oxidativo em lipídios e proteínas em sangue de crianças	UNISC
8	Genotoxicidade da Vit C em peixes com e sem síntese do nutriente	Mutágenos, metais e Vit C	Micronúcleos e Cometa *in vivo* em peixes	CBiot-UFRGS INPA

Os experimentos descritos nos ítens 2-6 foram iniciados em paralelo aos experimentos apresentados na presente tese. O experimento descrito no item 7 é um projeto de pesquisa submetido à UNISC e órgãos de fomento com início previsto para o 2º semestre de 2006. Já o experimento descrito no item 8, é uma proposta a ser executada de forma colaborativa entre o INPA e o CBiot-UFRGS.

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7   ANEXOS

7.1 ANEXO A: "Study of the antioxidant and mutagenic activity of different orange juices"

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Study of antioxidant and mutagenic activity of different orange juices

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Abstract

The mutagenic effects of *in natura* and processed (fresh and frozen) orange juices were evaluated by the *Salmonella*/microsome assay (Ames Test). Antioxidant potential was determined by deoxyribose degradation, as well as by the nitro blue tetrazolium reduction test. The juices inhibited degradation of deoxyribose and were able to trap the superoxide anion. At concentrations up to 1% they acted as pro-oxidants in lipid peroxidation in a concentration-dependent manner. However, this activity was less effective at 10% juice concentration. The sweetened processed juice was not able to decrease peroxidation nor to trap superoxide anions. Significant correlation between total phenolics and the inhibition of deoxyribose degradation was observed; also, a significant correlation between lipid peroxidation and total phenolics was found. Vitamin C was a pro-oxidant in tests employing transition metals. Mutagenicity was observed in the Ames test, particularly for fresh, *in natura*, juice samples. The highest responses were observed in strains TA97a and TA98. Fresh processed juice, which had the best antioxidant potential, was not mutagenic in any of the strains tested. Positive results for mutagenesis in TA97a, with metabolization, were correlated with total phenolics and vitamin C.
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Keywords: Orange juice; Mutagenicity; Ames test; Antioxidant and pro-oxidant activity; Vitamin C; Phenolic compounds

1. Introduction

Most free radical reactions involve the reduction of molecular oxygen, leading to the formation of reactive oxygen species (ROS), including superoxide anion and hydroxyl radical. These oxygen species can cause oxidative damage to several cell components and may thus play an important role in various pathological conditions. The inflicted damage may contribute to aging and to degenerative diseases such as brain dysfunction, cataracts, cancers, and cardiovascular diseases (Ko, Cheng, Lin, & Teng, 1998). Therefore, natural antioxidants present in orange juice can neutralize free radicals, due to their ability to act as free radical scavengers and/or as metal chelators (Aruoma, 1999; Wang, Cao, & Prior, 1996). However, many antioxidants have also been identified as natural mutagens and carcinogens, apart from their role as natural antimutagens and anticarcinogens (Ames, 1983; Middleton, Kandaswami, & Theoharides, 2000; Stavric, 1994).

Vitamin C and phenolic compounds are important antioxidants present in orange juice (Wang et al., 1996). They may act as antioxidants and as radical scavengers. The metal-binding ability of phenolic compounds has been shown to inhibit formation of hydroxyl radicals in the Fenton reaction by complexing ferrous ions. However, vitamin C and phenolic compounds, in the presence of Cu (II) and Fe (III), can also cause DNA degradation through generation of ROS, i.e. hydroxyl radicals (Ferguson, 2001; Stadler, Turesky, Müller, Markovic, & Leong-Morgenthaler, 1994; Wang et al., 1996). These radicals can either interact directly with

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84

DNA or may facilitate redox cycling, interacting with molecular oxygen to produce oxidative stress-generating and genotoxic ROS, that may cause DNA damage (Patrineli, Clifford, & Ionnides, 1996b).

The aim of this study was to investigate the mutagenic/genotoxic effects of *in natura* and processed, fresh and frozen orange juices by using the *Salmonella*/microsome assay (Ames Test), as well as to investigate their antioxidant properties by in vitro biochemical analysis. Also, the presence of organophosphorus and carbamate pesticides in the juices was determined, as these substances have been associated with mutagenicity in the Ames test (Hübner et al., 1997; Sierra-Torres, Cajas-Salazar, Hoyos, & Zuela Whorton, 1998). Finally, we also measured the amounts of vitamin C and of total phenolic compounds in all orange juice samples.

2. Materials and methods

2.1. Oranges and processed orange juices

The *in natura* oranges, cultivated without synthetic pesticides (organic), were obtained from the Cooperativa Ecológica Coolméia Ltda of Porto Alegre, RS, Brazil. Processed orange juices were bought at supermarkets, observing the expiry dates and keeping the same trademarks throughout the tests.

2.2. Sample preparation

Organic fresh and Organic frozen juices were prepared by washing the oranges, dipping them in 70% ethanol and flaming them at the Bunsen burner for about 5 s for sterilization. The oranges were then cut in half with a sterilized knife. Sterile crusher and sieve were employed for the preparation of the orange juice. An 1 ml aliquot was used immediately for the Ames test; the remaining juice was frozen at −20 °C and labeled "Org.frozen". The freshly processed orange juice samples were utilized immediately or frozen at −20 °C.

Fresh orange juices were of 4 types:
Org.fresh: organic *in natura* fresh orange juice.
Proc.fresh1: freshly processed orange juice that needs refrigeration, with a shelf life of 30 days (Tetra Pak).
Proc.fresh2: freshly processed orange juice that does not need refrigeration (stored at room temperature), with a usual shelf life of 365 days, sweetened (Tetra Pak).
Proc.fresh3: freshly processed orange juice that needs refrigeration, with a usual shelf life of 20 days (plastic package). Frozen orange juices were of the same classes and have their name changed in that fresh is substituted by frozen, e.g., *Org. frozen*, *Proc.frozen1* (Tetra Pak), *Proc.frozen2* (Tetra Pak), and *Proc.frozen3* (plastic package).

2.3. Microsome fraction

The post-microsomal fraction S9 was prepared from livers of Sprague–Dawley rats pre-treated with polychlorinated biphenyl mixture (Aroclor 1254), purchased from Molecular Toxicology Inc. (Moltox™). The S9 metabolic activation mixture was prepared according to Maron and Ames (1983) and Mortelmans and Zeiger (2000). All reagents were from Sigma.

2.4. Salmonella/microsome assay (Ames test)

Mutagenicity was determined in the pre-incubation procedure (Maron & Ames, 1983), using various concentrations of juices, with *Salmonella typhimurium* strains TA98, TA97a, TA100, and TA102, kindly provided by Dr. Bruce Ames (Dept. of Biochemistry, University of California, USA) (Levin, Hollstein, Christman, Schiwiers, & Ames, 1984), with or without S9 mix. The mixture, consisting of the juice samples to be tested, 500 µl of S9 mix (in test with metabolization) and 100 µl of the bacterial suspension (1–2×10^9 cells/ml) was pre-incubated for 20 min at 37 °C without shaking. Then, 2000 µl of top agar (0.55% agar, 0.55% NaCl, 50 µM L-histidine, and 50 µM biotin, at pH 7.4, 45 °C) were added to the test tube and poured onto minimal agar (1.5% agar, Vogel-Bonner E medium, containing 2% glucose). All assays were carried out in triplicate.

After incubation for 48 h, colonies (His$^+$ revertants) were counted and the results were expressed as mutagenic index (MI = # of His$^+$ induced in the sample/# of spontaneous His$^+$ in the negative control).

Negative (appropriate solvent) and positive (10 µg sodium azide per plate for strain TA100; 5 µg 4-nitroquinoline 1-oxide per plate for strains TA98, TA97a, and TA102) controls were included in each assay. Aflatoxin B1 (10 µg per plate) was used as positive control for the metabolic activation for all strains.

A compound was considered positive for mutagenicity when (a) the number of revertants was at least double the spontaneous yield (MI $\geqslant$ 2), (b) a significant response for analysis of variance ($p \leqslant 0.05$) was found, and (c) a corresponding positive dose–response curve ($p \leqslant 0.01$) was present, as evaluated by the Salmonel software (Myers, Adams, Kier, Rao, Shaw, & Williams, 1991).

2.5. Determination of total phenolics, vitamin C, and pesticides

The total amount of phenolic compounds in the orange juices was determined according to the Folin-Ciocalteu procedure (Singleton & Rossi, 1965). Atleast 2 orange juice samples (100 µl each) were introduced into test tubes; 250 µl of Folin-Ciocalteu's reagent and 500 µl

of sodium carbonate were added and the tubes vigorously shaken. After standing for 40 min, absorption at 750 nm was measured. The total phenolics content was derived by comparison with a gallic acid standard curve (Sigma Co., 0.2–1 mg/ml gallic acid) and expressed as µg/ml gallic acid equivalents (GAE).

Vitamin C (ascorbic acid) was determined according to the Kabasakalis, Siopidou, and Moshatou (2000) at the University of Santa Cruz, RS (UNISC/Brazil). Organophosphorus and carbamate pesticides in the juice samples were determined as methyl parathion-equivalent activity, which causes inhibition of the enzyme acetylcholinesterase (AChE), as previously described by Bastos, Cunha, and Lima (1991) and Lima, Cunha Bastos, and Cunha Bastos (1996). A calibration curve of methyl parathion (Folidol 600® – Bayer) was used to express AChE activity in ppm of methyl parathion.

2.6. Deoxyribose assay

ROS used for the detection of oxidative damage to deoxyribose were generated according to Nishida, Yoshizawa, and Akamatsu (1991). Oxidative products, caused by the formation of thiobarbituric acid-reactive substances (TBARS), were assayed as described by Halliwell and Gutteridge (1981). Assays were in triplicate, in a final volume of 1.2 ml. 120 µl of the different concentrations of orange juices were added to every test tube before adding hydrogen peroxide. The absorbance of each assay was measured against a specific blank, with the same juice concentration as used in the assay. In this system, mannitol (1mM) was used as the positive control.

2.7. Superoxide anion scavenging activity

The phenazine-methosulfate(PM)-NADH method (Robak & Gryglewski, 1988) was used for the generation of $O_2^{\cdot-}$. The test tubes contained 12 µM PM, 100 µM NADH, and 100 µM NBT in 0.1 M phosphate buffer (K_2HPO_4–KH_2PO_4) at pH 7.8. After 2 min of incubation at room temperature, 100 µl of HCl 0.1 M were added to stop the reaction. The spectrophotometric measurement was at 560 nm against blank samples, in the absence of PM. Different concentrations of orange juices were added to the test tubes before adding PM. Superoxide dismutase (SOD) (100 U/ml) enzyme was used as a positive control.

2.8. Oxidation of methyl linoleate

Protecting activity against oxidative damage caused to methyl linoleate (MeLo) was measured by a modified method of Osawa, Katsuzaki, Hagiwara, Hagiwara, and Shibamoto (1992). Oxidative damage was induced by $CuSO_4$ (5 mM). The tubes contained phosphate buffer (0.01 M, pH 7.4), MeLo (14 mM), 10% SDS and $CuSO_4$ (5 mM). 120 µl of the different concentrations of orange juices were added to the test tube containing the previously added reaction mixture. The tubes were incubated for 20 h at 37 °C, with agitation. Then, 1 ml of 1% thiobarbituric acid (TBA) and 1 ml of 2.8% trichloroacetic acid (TCA) were added and this mixture was further incubated for 15 min at 100 °C for development of pigmentation. The reaction was stopped by chilling in ice for 5 min. After centrifugation, the absorbance of the clear supernatant was measured at 532 nm, against blank samples not containing MeLo and SDS. The absorbance of each assay was measured against a specific blank, containing with same juice concentration as used in the assay. Betahydroxytoluene (10 mM) was used as a positive control.

2.9. Statistical analysis

Spearman's (r_s) correlation coefficient was calculated with the SPSS/PC© programme/version 98. Differences at the $p \leqslant 0.05$ level were considered significant. Results obtained for the antioxidant effect of juice were analyzed by the ANOVA (analysis of variance) statistics programme. Difference between each juice and its control (100% oxidation) was evaluated by the Tukey test and differences at the $p \leqslant 0.01$ level were considered significant. The results for the antioxidant effect and for oxidation of MeLo, caused by different juice samples (fresh and frozen) at the same dilution, were evaluated by Student's t test and the level of significance considered was $p \leqslant 0.01$. Differences between fresh and frozen forms of the same juice were evaluated by t test and considered significant at the $p \leqslant 0.01$ level.

3. Results

The frozen and fresh forms of the same juice showed similar concentrations of total phenolic compounds except for *Proc.frozen1* and *Proc.fresh1* juices ($p \leqslant 0.01$, Table 1). The vitamin C concentrations of *Proc.frozen1* and *Proc.frozen3* juices were nearly 50% lower than that found in the respective processed fresh *Proc.fresh1* and *Proc.fresh3* juices. All other juices had no significant variation of vitamin C between fresh and frozen forms (Table 1). No preservatives, such as sorbic and benzoic acids, or sulfites were detected in the fresh juice samples (data not shown). Only *Proc.fresh1* had traces of methyl parathion-equivalent (<0.02 ppm, Table 1).

As shown in Fig. 1, all juices inhibited oxidative damage to deoxyribose in a dose-dependent manner. A similar protection was seen in the positive mannitol control (1 mM). At a juice concentration of 10%, the protective effect was in the order of 65–85%, and at 1% juice concentration it ranged from 60% to 83%. No

Table 1
Chemical analysis of *in natura*, processed, fresh and frozen orange juices

Juices	Vitamin C (mg/100 ml)	Total phenolics (μg/ml GAE[a])	Pesticides (ppm of methyl parathion-equivalents)
Org.frozen	65.2	598 ± 31.4	ND
Org.fresh	56.4	580 ± 132	ND
Proc.frozen1	16.7	$557 \pm 4.73^*$	ND
Proc.fresh1	45.8	$439 \pm 4.35^*$	<0.02
Proc.frozen2	42.3	550 ± 7.00	ND
Proc.fresh2	47.6	548 ± 3.96	ND
Proc.frozen3	26.4	534 ± 61.61	ND
Proc.fresh3	53.7	581 ± 10.8	ND

[a] Corresponds to the phenolic concentration expressed in μg/ml equivalent to gallic acid.

$^*p < 0.01$ (test t); ND, not detected.

significant differences were observed between frozen and fresh juices, except between *Proc.fresh1* and *Proc.frozen1*. The *Proc. fresh1* juice gave the highest protection against oxidative damages to deoxyribose when compared to its frozen *Proc.frozen1* form at concentrations of 1% and 0.1% (Fig. 1(b)).

For the NBT reduction assay, which evaluates the $O_2^{\cdot-}$ scavenging ability, our results show that, with the exception of *Proc.fresh2* (Fig. 2(c)), all tested juices inhibited NBT reduction of 42–70% at the highest concentration tested (10%) (Figs. 2(a), (b), and (d)). Fig. 2 shows significant differences in the $O_2^{\cdot-}$ trapping ability between fresh and frozen samples (Figs. 2(b) and (d)) at the 10% level.

Up to a concentration of 1%, all juices caused oxidative damage to MeLo, showing a significant increase in lipid peroxidation (Fig. 3). However, at 10% concentration, *Org.fresh* and *Org.frozen in natura* juices, as well as the processed ones in the fresh forms (*Proc.fresh1* and *Proc.fresh3*), caused lower lipid peroxidation than the 1% samples (Figs. 3(a), (b) and (d)), though this reduction of MeLo peroxidation did not reach the level of the positive control [(−76%) 10 mM BHT]. Clearly, lipid peroxide increased significantly at all juice concentrations. The extent of peroxidation varied with fresh juice more effective in Fig. 3(b) and frozen was more effective in Figs. 3(a) and (d).

Spearman's (r_s) correlation coefficient showed that the total phenolic compounds of the juice samples were correlated to the lipid peroxidation level ($r_s = 0.857$). However, no significant correlation was detected in relation to vitamin C ($r_s = 0.690$). The concentration of phenolic compounds showed correlation with the antioxidant activity in the deoxyribose degradation ($r_s = -0.857$) and no correlation with NBT-reduction ($r_s = 0.143$) assays (data not shown).

As shown in Table 2, *Org.fresh*, *Org.frozen.*, and *Proc.frozen1* juices, both in the presence and absence of metabolic activation, had mutagenic activity in strain
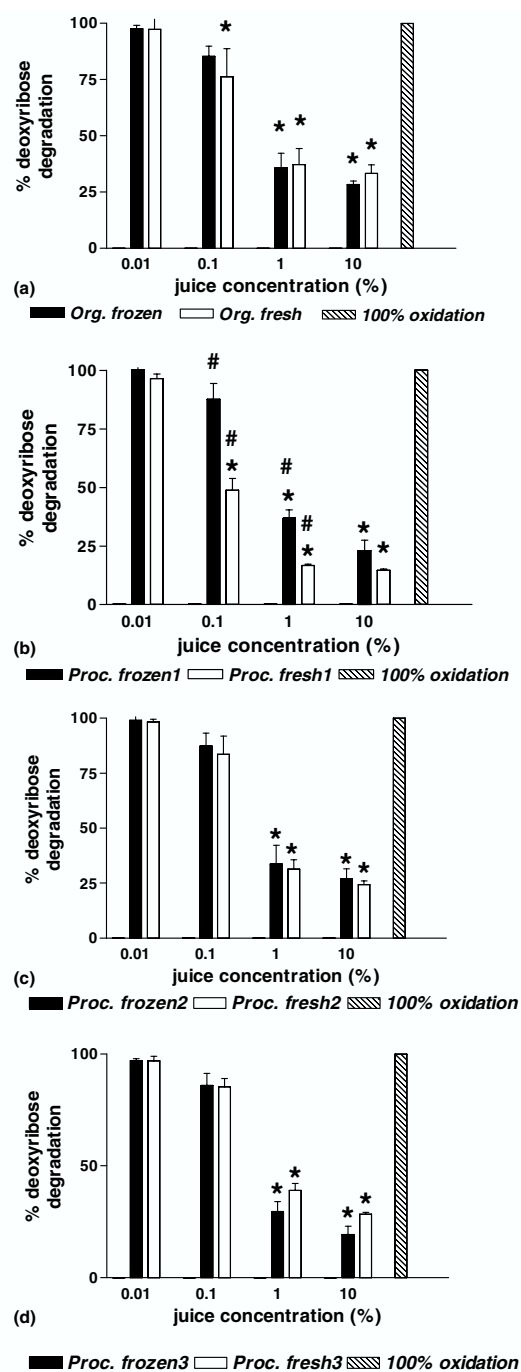
Fig. 1. Effect of in natura (a) and processed (b–d) orange juices on the degradation deoxyribose (Fe^{3+}–NTA–H_2O_2 system). Mannitol (1 mM), positive control, inhibited around 71% of deoxyribose degradation. $^*p < 0.001$ (ANOVA/Tukey) – difference of each juice in relation to its control (100% oxidation); $^\#p < 0.01$ (Test t, Student) – difference between fresh and frozen juices at the same concentration.

TA98, which detects frameshift mutations (Maron & Ames, 1983). The *Proc.frozen2* and *Proc.fresh2* juices were mutagenic in the absence of metabolic activation in strain TA98. However, *Proc.frozen3* juice required metabolization in order to cause frameshift mutation in
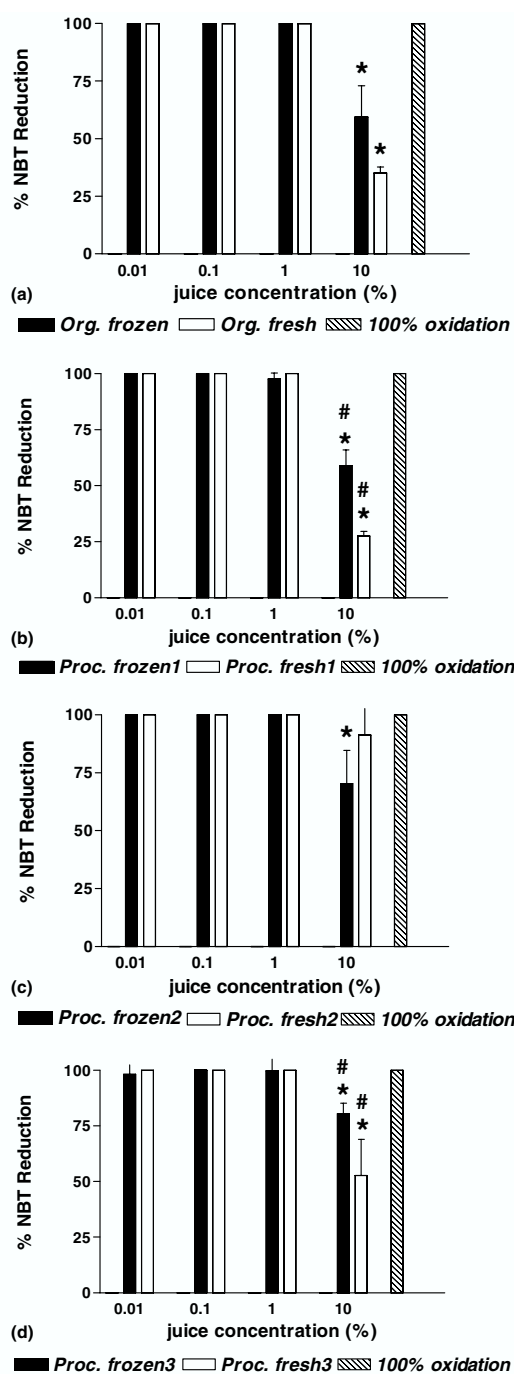
Fig. 2. Effect of *in natura* (a) and processed (b–d) orange juices on the NBT reduction (phenazine–NADH methosulfate system). SOD (100 U/ml) positive control, inhibited around 75% the NBT-reduction. *$p < 0.001$ (ANOVA/Tukey) – difference of each juice in relation to its control (100% oxidation); #$p < 0.01$ (Test t, Student) – difference between fresh and frozen juices in relation to the same concentration.

Fig. 3. Effect of *in natura* (a) and processed (b–d) orange juices on CuSO$_4$-induced lipid peroxidation. BHT (10 mM), positive control, inhibited around 76% the peroxidation. *$p < 0.001$ (ANOVA/Tukey) – difference of each juice in relation to its control (100% oxidation); #$p < 0.01$ (Test t, Student) – difference between fresh and frozen juices in relation to the same concentration.

TA98 (Tables 2 and 4). The *Org.fresh* juice was mutagenic in strain TA97a, both in the presence and absence of metabolic activation (Tables 2 and 4). The *Proc.-fresh3* and *Proc.frozen1* juices were able to induce frameshift mutation only in the absence of metabolic activation (Tables 2 and 4). In strain TA100, only the *Org.fresh* juice, both in the presence and absence of metabolization, was able to induce base-pair substitutions (Tables 3 and 4). When orange juices were tested in TA102, which detects oxidative and alkylating mutagens

Table 2
Induction of His$^+$ revertants in *S. typhimurium* TA97a and TA98 by fresh and frozen orange juices, without (−S9) and with (+S9) metabolic activation

Spontaneous revertants		*Salmonella typhimurium* strains							
		TA98				TA97a			
		−S9		+S9		−S9		+S9	
		15 ± 6		26 ± 14		104 ± 16		115 ± 18	
Juices	Conc. (μl)	(a)	IM^b	(a)	IM^b	(a)	IM^b	(a)	IM^b
Org.fresh	0*	15 ± 5		12 ± 4		61 ± 27		75 ± 4	
	100	24 ± 4	1.57	25 ± 7	2.03	65 ± 10	1.05	91 ± 7	1.21
	500	24 ± 12	1.54	28 ± 8	**2.30**	86 ± 6	1.40	104 ± 3	1.38
	2000	52 ± 9	**3.41**	52 ± 6	**4.19**	144 ± 21	**2.34**	151 ± 13	**2.00**
Org.frozen	0	14 ± 6		20 ± 4		77 ± 12		94 ± 14	
	100	18 ± 1	1.31	23 ± 6	1.15	68 ± 24	0.99	101 ± 11	1.08
	500	18 ± 6	1.31	24 ± 3	1.20	60 ± 27	0.79	103 ± 3	1.10
	2000	78 ± 20	**5.57**	87 ± 27	**4.37**	99 ± 21	1.30	143 ± 22	1.52
Proc.fresh1	0	22 ± 2		26 ± 5		61 ± 27		68 ± 29	
	100	16 ± 9	0.70	24 ± 12	0.91			76 ± 10	1.11
	500	15 ± 5	0.67	35 ± 16	1.33			72 ± 9	1.06
	2000	13 ± 3	0.58	14 ± 7	0.53			50 ± 25	0.70
Proc.frozen1	0	11 ± 3		19 ± 5		122 ± 4		115 ± 12	
	50							95 ± 5	0.82
	75							94 ± 13	0.81
	100	10 ± 1	0.96	19 ± 3	1.02	106 ± 9	0.87	81 ± 2	0.70
	500	49 ± 2	**4.57**	38 ± 17	1.50	137 ± 7	1.12	104 ± 11	0.90
	2000	54 ± 13	**5.00**	35 ± 18	**2.44**	265 ± 35	**2.18**		
Proc.fresh2	0	4 ± 1		23 ± 7		113 ± 4		105 ± 13	
	100	6 ± 3	1.38	28 ± 2	1.23	107 ± 28	0.95	107 ± 20	1.02
	200			39 ± 7	1.71				
	300			23 ± 9	1.01				
	400			34 ± 6	1.55				
	500	10 ± 5	**2.23**			91 ± 3	0.81	106 ± 8	1.01
	1000								
	1500								
	2000	21 ± 3	**4.85**			62 ± 15	0.55	122 ± 3	1.16
Proc.frozen2	0	10 ± 1		51 ± 7		143 ± 13		214 ± 8	
	100	12 ± 6	0.83	20 ± 3	1.13	140 ± 24	0.98	176 ± 35	0.82
	500	14 ± 1	1.40	13 ± 9	0.77	189 ± 27	1.32	178 ± 23	0.83
	1000					194 ± 12	1.36		
	1500								
	2000	27 ± 4	**2.70**	12 ± 4	0.70	214 ± 4	1.50	153 ± 9	0.72
Proc.fresh3	0	15 ± 4		16 ± 4		8 ± 6		61 ± 16	
	25					73 ± 7	9.16		
	50					19 ± 27	3.16		
	75					42 ± 9	**5.20**		
	100	9 ± 4	0.56	23 ± 5	1.42	41 ± 4	**5.12**	52 ± 17	0.86
	500	17 ± 5	1.09	20 ± 4	1.23			94 ± 6	1.55
	2000	17 ± 8	1.09	22 ± 15	1.38			77 ± 21	1.27
Proc.frozen3	0	13 ± 4		13 ± 2		94 ± 11		238 ± 6	
	100	11 ± 2	0.84	18 ± 6	1.37	64 ± 11	0.68	238 ± 54	1.00
	500	6 ± 1	0.50	26 ± 1	**1.98**	65 ± 10	0.70	218 ± 12	0.92
	2000	13 ± 2	1.00	32 ± 1	**2.40**	114 ± 20	1.22	132 ± 19	0.55
4NQO	5 μg	246 ± 135				647 ± 325			
AFB$_1$	10 μg			323 ± 200				562 ± 186	

(a): Number of revertants. His+/plate.

^{b} Mutagenic index: no. of His$^+$ induced in the sample/no. of spontaneous His$^+$ in the negative control.

* O: Negative control: distilled water; positive control: (−S9) 4-nitroquinoline 1-oxide (4NQO; 5 μg/plate) for both strains; (+S9) aflatoxin B$_1$(10 μg/ plate) for all strains. Results in bold: positive mutagenics (ANOVA, $p \leqslant 0.05$; dose–response curve, $p \leqslant 0.01$; MI $\geqslant 2$).

and active forms of oxygen (Levin et al., 1984), no positive response was observed, regardless of microsome activation (Tables 3 and 4).

The observed mutagenicity might be due to histidine residues in some of the juice samples which would, by allowing growth of the auxotrophic test bacteria on the

Table 3
Induction of His$^+$ revertants in *S. typhimurium* TA100 and TA102 by fresh and frozen orange juices, without (−S9) and with (+S9) metabolic activation

Spontaneous revertants		*Salmonella typhimurium* strains							
		TA100				TA102			
		−S9		+S9		−S9		+S9	
		133 ± 27		121 ± 26		241 ± 51		282 ± 52	
Juices	Conc. (µl)	(a)	IM^b	(a)	IM^b	(a)	IM^b	(a)	IM^b
Org.fresh	0*	138 ± 20		118 ± 13		224 ± 42		271 ± 13	
	100	155 ± 13	1.12	127 ± 5	1.07	181 ± 21	0.81	257 ± 8	0.95
	500	177 ± 1	1.28	167 ± 22	1.41	193 ± 6	0.86	253 ± 41	0.94
	2000	312 ± 28	**2.26**	280 ± 7	**2.37**	239 ± 54	1.07	383 ± 81	1.41
Org.frozen	0	77 ± 13		94 ± 14		14 ± 6		20 ± 4	
	100	102 ± 22	1.04	159 ± 68	1.03	266 ± 74	0.92	258 ± 24	0.89
	500	105 ± 13	1.07	118 ± 8	0.77	202 ± 14	0.70	227 ± 37	0.79
	2000	98 ± 19	1.00	255 ± 62	1.65	307 ± 28	1.06	273 ± 21	0.94
Proc.fresh1	0	145 ± 24		119 ± 14		258 ± 14		195 ± 23	
	100	115 ± 45	0.80	109 ± 8	0.92	172 ± 28	0.66	202 ± 7	1.04
	500	99 ± 8	0.68	153 ± 27	1.29	174 ± 38	0.67	227 ± 30	1.16
	2000	131 ± 61	0.90	131 ± 30	1.11	47 ± 30	0.18	216 ± 0	0.94
Proc.frozen1	0	165 ± 38		159 ± 32		271 ± 8		271 ± 20	
	50	131 ± 24	0.79						
	75	135 ± 17	0.81						
	100	139 ± 15	0.84	213 ± 14	1.34	281 ± 16	1.04	253 ± 45	0.94
	500	137 ± 16	0.83	223 ± 40	1.41	373 ± 19	1.38	251 ± 27	0.93
	2000			182 ± 57	1.15	381 ± 6	1.41	256 ± 50	0.95
Proc.fresh2	0	162 ± 8		105 ± 29		167 ± 8		238 ± 29	
	100			251 ± 50	1.05	162 ± 2	0.97	251 ± 50	1.05
	500					130 ± 4	0.78	279 ± 44	1.17
	1000	119 ± 18	0.74	88 ± 25	0.84				
	1500	145 ± 24	0.89	152 ± 29	1.45				
	2000	163 ± 26	1.00	164 ± 4	1.56	115 ± 23	0.69	155 ± 19	0.65
	2500	157 ± 3	0.96						
Proc.frozen2	0	152 ± 19		153 ± 18		270 ± 19		317 ± 21	
	50	150 ± 7	0.99						
	75	127 ± 10	0.84						
	100	119 ± 12	0.78			215 ± 4	0.79	311 ± 6	0.98
	500	116 ± 11	0.76			229 ± 58	0.85	276 ± 18	0.87
	1000			155 ± 25	1.01				
	1500			203 ± 22	1.32				
	2000					177 ± 69	0.65	264 ± 48	0.84
	2500			227 ± 38	1.48				
Proc.fresh3	0	117 ± 9		145 ± 10		237 ± 42		251 ± 8	
	100	90 ± 16	0.77	119 ± 20	0.82	227 ± 29	0.96	244 ± 29	0.97
	500	91 ± 17	0.78	126 ± 9	0.87	156 ± 4	0.66	258 ± 62	1.03
	2000	87 ± 22	0.75	108 ± 35	0.75	243 ± 40	1.02	136 ± 28	0.54
Proc.frozen3	0	146 ± 6		170 ± 28		219 ± 26		241 ± 37	
	25	137 ± 86	0.94						
	50	121 ± 8	0.83						
	75	111 ± 24	0.76						
	100	131 ± 48	0.90	123 ± 41	0.72	200 ± 17	0.82	259 ± 17	1.07
	500	110 ± 15	0.75	123 ± 22	0.72	245 ± 14	1.01	256 ± 21	1.06
	2000			270 ± 25	1.58	263 ± 50	1.08	237 ± 38	0.98
4NQO	5 µg					1219 ± 786			
Sodium azide	10 µg	721 ± 250							
AFB$_1$	10 µg			769 ± 223				765 ± 211	

(a): Number of Revertants. His$^+$/plate.

^{b} Mutagenic index: no. of His$^+$ induced in the sample/no. of spontaneous His$^+$ in the negative control.

* O: Negative control: distilled water; positive control: (−S9) 4-nitroquinoline 1-oxide (4NQO; 5 µg/plate) for TA102 strain and sodium azide (10 µg/plate) for TA100; (+S9) aflatoxin B1 (AFB1: 10 µg/plate) for all strains. Results in bold: positive mutagenics (ANOVA, $p \leqslant 0.05$; dose–response curve, $p \leqslant 0.01$; MI $\geqslant 2$).

Table 4
Summary of the mutagenic activity responses of processed and *in natura* orange juices, for *Salmonella typhimurium* strains without (−S9) and with (+S9) metabolic activation

Juices	*Salmonella typhimurium* Strains							
	TA 98		TA 97a		TA 100		TA 102	
	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9
Org.frozen.	+	+	−	−	−	−	−	−
Org.fresh	+	+	+	+	+	+	−	−
Proc.frozen1	+	+	+	−	−	−	−	−
Proc.fresh1	−	−	−	−	−	−	−	−
Proc.frozen2	+	−	−	−	−	−	−	−
Proc.fresh2	+	−	−	−	−	−	−	−
Proc.frozen3	−	+	−	−	−	−	−	−
Proc.fresh3	−	−	+	−	−	−	−	−

Mutagenic response: + (positive), − (negative).

Table 5
Verification of mutagenicity of *Org.fresh* orange juice

Incubation in presence of orange juice	0 h		3.5 h	
	cfu/ml	His$^+$/10^7 survivors	cfu/ml	His$^+$/10^7 survivors
Control growth	2.9×10^7	2.94 ± 1.01 (17)[a]	9.5×10^7	2.42 ± 0.88 (46)
Org.fresh (+S9) (2000 µl)	3.6×10^7	6.28 ± 3.26 (45)	3.0×10^7	42.3 ± 6.19 (255)

Mutagenicity of orange juice (*Org.fresh*) was determined using *S. typhimurium* strain TA98 (with metabolization). Orange juice, bacteria and buffer were pre-incubated for 0 (control) or 3.5 h in a shaking air-bath at 37 °C. Subsequently, the bacteria were washed with saline buffer, re-suspended in soft agar and plated on agar plates. Results are presented as means ± SD of duplicates. This study was repeated and generated similar data.
[a] Numbers in parentheses represent the sum of His$^+$ revertants of two plates.

plates, enhance the number of spontaneous his revertants. This possibility was examined for a mutagenic juice sample. Test bacteria were exposed to the juice and either plated immediately or plated after 3.5 h incubation in the presence of the orange juice (Table 5). By determining the survival (c.f.u.) after each treatment (before plating), we could establish that (1) 10% orange juice did not support growth of the bacteria during the 3.5 h exposure (in fact there was a 20% decrease in c.f.u.) and (2) that the juice was mutagenic for strain TA98 (Table 5).

Spearman's correlation coefficient (r_s) shows a strong correlation of phenolic compounds with juice mutagenicity for strains TA97a when metabolically activated ($r_s = 0.833$). The amount of juice-contained vitamin C was also correlated with juice mutagenicity in strain TA97a ($r_s = 0.810$) (data not shown). For all other strains, no correlation between the amount of juice-contained phenolic compounds and of vitamin C was found.

4. Discussion

A positive effect is frequently attributed to the ingestion of fruits and their juices with regard to the prevention of many diseases. It is believed that this protection is partially due to the natural antioxidant substances present in these foods. On the other hand, several antioxidant substances related to natural anti-mutagenicity or anti-carcinogenicity have simultaneously been identified as mutagenic or carcinogenic (Ames & Gold, 1998; Yoshimo, Haneda, Naruse, & Murakami, 1999). Fruits and their juices contain a number of flavonoids and other phenolic compounds. Orange juice is also rich in β-carotene and ascorbic acid and is, therefore, a natural supplier of several antioxidants (Kabasakalis et al., 2000; Wang et al., 1996).

This study shows that orange juices indeed have antioxidant potential, since they could inhibit the hydroxyl radical-caused degradation of deoxyribose (Fig. 1) and also could decrease lipid peroxidation [though only at high concentration (Fig. 3)]. Also, most juices were able to scavenge $O_2^{\cdot-}$ (Fig. 2). Phenolic compounds and vitamin C were identified as possible antioxidants in orange juice (Table 1). Phenolic compounds are able to scavenge radicals and to chelate metals (Halliwell & Gutteridge, 2000; Sanchez-Moreno, Larrauri, & Saura-Calixto, 1999), while vitamin C can play a pro-oxidant role in the presence of transition metals [Fenton reaction (Halliwell, 2001; Sies, Stahl, & Sundquist, 1992)]. These compounds can also act as antioxidants, because of their ability to trap superoxide anions (Sies et al., 1992; Stadler et al., 1994). Depending on the concentrations of

phenolic compounds and of transition metals, a complex can be formed that facilitates the redox process (Khan, Ahmad, & Hadi, 2000; Rice-Evans, Miller, & Paganga, 1997). In general, the phenolic compounds at low concentrations show antioxidant behaviour, while presenting pro-oxidant behaviour at higher concentrations; upon further increasing of their concentration they again show antioxidant behaviour. This always depends on the type (position and number of hydroxyl in the molecule) and the concentration of the phenolic compound, as well on that of the transition metal (Galato, Giacomelli, Ckless, & Spinelli, 1999; Khan et al., 2000). Thus it is possible that, at concentrations up to 1%, the orange juices caused a significant increase of lipid peroxidation, i.e. acted as pro-oxidants while, at 10%, they had antioxidant properties; this effect was most pronounced in fresh juice samples (Fig. 3). Also we wish to point out that the same amount of copper was employed in these tests, whereas the juice concentrations varied by a factor of 10.

The sample with the highest antioxidant effect – *Proc.fresh1* (Figs. 1(b),2(b),3(b)) had a lower concentration of phenolic compounds (Table 1), especially when compared to its frozen form (*Proc.frozen1*). These differences in antioxidant potential may partially be attributed to qualitative variations of the phenolic compounds, as the structural differences may modify the antioxidant potential of phenolic acids (Galato, Ckless, Susin, Giacomelli, Ribeiro-do-Vale, & Spinelli, 2001). Alteration of one or more hydroxyls, or oxidation of the phenolic compounds, may have occurred during the freezing/thawing process, thus leading to a decreased antioxidant potential.

Interestingly, *Proc.fresh2* juice was unable either to trap superoxide anion (Fig. 2(c)) or to decrease the lipid peroxidation at 10% (as compared to 1% concentration) (Fig. 3(c)). This juice is sugar-sweetened, suggesting that the presence of carbohydrates can interfere with the lipid peroxidation assay.

The different results observed for the antioxidant potential of the juices are probably due to various factors, namely climate, soil, fruit variety, degree of maturation (Wang et al., 1996) and to the techniques employed for juice preparation. Juice sample *Proc.-fresh1* shows the highest antioxidant activity (Figs. 1–3) and also has no mutagenic potential in the *Salmonella/*microsome test. All other juices, at least in one batch, induced frameshift mutation in TA98 or TA97a. The strongest positive results for mutagenesis were found for *Org.fresh* juice (Table 4). While the different mutagenicity of juices may be explained by the intrinsic differences inherent in the fruits from which they were derived, by variations in processing methods, and to their varying shelf life (Tassara, 1998; Wang et al., 1996), the high mutagenicity of *Org.fresh* juice is not expected to stem from components of the orange peel, as

might be expected in industrially processed juices. Amount and quality of phenolic compounds can partially contribute to juice mutagenicity. Moreover, the oxidation of phenolic compounds can generate ROS, partially responsible for the observed mutagenicity (Patrineli, Clifford, Walker, & Ionnides, 1996a; Patrineli et al., 1996b). Thus the mutagenicity of some fresh, frozen and processed *in natura* orange juices may be caused by polyphenol oxidase-generated quinones that are already present in the fruits prior to juice production (Patrineli et al., 1996a, 1996b). These quinones can be converted to semiquinone radicals by loss of one electron, and can directly interact with DNA or facilitate redox cycling. Thus, ROS would be generated in the presence of molecular oxygen, leading to oxidative stress and to DNA damage (Patrineli et al., 1996a, 1996b; Yoshimo et al., 1999).

Processed and *in natura* orange juices contain the phenol quercitin (Hertog, Hollman, & De Putte, 1993). This may explain the induced mutations found in strains TA98 and TA100 (Tables 2 and 3). Caffeic acid is present in citrus fruits and could be another juice component responsible for the detected mutagenicity (Rice-Evans et al., 1997). However, although this monophenolic compound is clastogenic in Chinese hamster ovary (CHO) cells, no mutagenic activity has been observed in strain TA98 (Vargas, Motta, Leitão, & Henriques, 1990).

In our samples, vitamin C alone could not be responsible for the mutagenicity as a pro-oxidant due to the intrinsic presence of a transitional metal in the oranges, since the samples with similar amounts of this vitamin showed different mutagenic responses (see Tables 1 and 4). Therefore, the differences of composition of the oranges from which the juices derived should be held responsible for the different results of mutagenicity. We did not find any food preservatives, e.g., benzoic and sorbic acid or bisulfite (data not shown), and organophosphorous or carbamate pesticides were not detected. However, the influence of pesticides containing transition metals, such as molecular copper, could, in the presence of vitamin C, induce hydroxyl radicals and thus induce mutagenesis (Guecheva, Henriques, & Erdtmann, 2001).

By means of the correlation coefficient of Spearman (r_s), one can clearly see that the mutagenic effect for TA97a, with metabolization ($r_s = 0.833$), is directly related to the presence of total phenolics and also to vitamin C ($r_s = 0.810$) in the juices (data not shown).

In summary, our results show that the analyzed orange juices have antioxidant and mutagenic potential; phenolic compounds and vitamin C can cooperate to enhance the mutagenic activity. However, other antioxidants present in oranges, such as vitamin E, carotenoids and minerals (e.g. selenium), as well as the synergistic effects among all antioxidants (combinations

of different compounds) can increase the total antioxidant potential of the juices, explaining the results observed in the biochemical assays (Halliwell, 2001; Sanchez-Moreno et al., 1999). This strong antioxidant potential of orange juice (Halliwell, 2001; Wang et al., 1996) must be overcome by a solid mutagenic activity in order to induce the observed bacterial mutations. Clearly, it would be interesting to confirm these results using eukaryotic mutagenic test systems (Mavournin, Blakey, Cimino, Salamone, & Heddle, 1990; Tice et al., 2000; Wolf & Peter, 1997).

In spite of the fact that some of the analyzed orange juices had mutagenic activity (Table 2), the risk for humans consuming orange juice may be low, due to enzymatic activities and pH changes in the digestive tract. Also, antimutagenic acting juice ingredients and detoxificants, e.g. carotenoids and some vitamins (Ames, 1989; Halliwell, 2001; Middleton et al., 2000), may prevent damage there.

Acknowledgements

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7.2 ANEXO B: "El jugo de naranja tiene potencial antioxidante y mutagenico"

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! El JUGO DE NARANJA TIENE POTENCIAL ANTIOXIDANTE Y MUTAGENICO

Santa Cruz do Sul, Brasil

La actividad mutagénica detectada en diversos tipos de jugo de naranja no implicaría un riesgo para la salud del ser humano debido a las actividades enzimáticas y a los cambios del pH que tienen lugar en el sistema digestivo

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Título en castellano:

Estudio de la Actividad Antioxidante y Mutagénica de Diferentes Jugos de Naranja

*Extensión del Resumen-*SIIC *en castellano:* 2.41 páginas impresas en papel A4

Introducción
La mayoría de las reacciones de radicales libres comprenden la reducción del oxígeno molecular con formación de especies reactivas de oxígeno (ERO), como el anión superóxido y el radical hidroxilo. Las ERO pueden producir lesión oxidativa en varios componentes celulares y, por lo tanto, desempeñar un papel importante en varias patologías. Este daño puede contribuir al envejecimiento y desarrollo de enfermedades degenerativas tales como disfunción cerebral, cataratas, neoplasias y patologías cardiovasculares. Los antioxidantes naturales presentes en el jugo de naranja pueden neutralizar a los radicales libres debido a su capacidad de secuestro de éstos, o como quelante de metales o ambos. Sin embargo, muchos antioxidantes también han sido identificados como mutágenos y carcinógenos naturales, aparte de su desempeño como antimutágenos y anticarcinógenos.
La vitamina C y los compuestos fenólicos son antioxidantes importantes que se hallan en el jugo de naranja. La capacidad de unión a metales de los compuestos fenólicos inhibe la formación de radicales hidroxilos. Sin embargo, la vitamina C y los compuestos fenólicos pueden, en presencia de Cu (II) y Fe (III), producir la degradación del ADN mediante la generación de ERO. Estos radicales pueden actuar directamente en el ADN o facilitar el ciclo de oxidorreducción, interactuando con el oxígeno molecular para producir ERO genotóxicas. En la

96

presente experiencia los autores investigaron los efectos mutagénicos y genotóxicos de los jugos de naranja naturales y procesados mediante el ensayo de *Salmonella*/microsoma Ames y analizaron sus propiedades antioxidantes. También determinaron la presencia de pesticidas organofosforados y carbamato, ya que estas sustancias se asocian con mutagenicidad. Por último, midieron la cantidad de vitamina C y de compuestos fenólicos en todas las muestras.

Métodos

Las naranjas *in natura*, cultivadas sin pesticidas sintéticos provinieron de una cooperativa ecológica, mientras que los jugos procesados fueron adquiridos en supermercados. Los jugos congelados y frescos orgánicos fueron preparados después del lavado y esterilización de las frutas. Una alícuota de 1 ml fue utilizada inmediatamente para la prueba Ames, mientras que el resto fue congelado a -20ºC y clasificado como "Org. congelado". La muestra de jugo procesado fresco se empleó de inmediato o fue congelada a -20 ºC. Los jugos naturales fueron clasificados como "Org. fresco" (jugo fresco *in natura* orgánico), "Proc. fresco 1" (jugo procesado fresco que requiere refrigeración, con una duración de 30 días en Tetra Pack), "Proc. fresco 2" (jugo procesado fresco que no requiere refrigeración, con una duración de 365 días en Tetra Pack) y "Proc. fresco 3" (requiere refrigeración, con una duración de 20 días en envase plástico).

La mutagenicidad fue determinada en el procedimiento de preincubación, utilizando varias concentraciones de jugos, con cepas de *Salmonella typhimurium* TA98, TA97a, TA100 y TA102. Después de la incubación durante 48 horas, se contaron las colonias y los resultados fueron expresados como índice mutagénico. Como control positivo de la activación metabólica de todas las colonias se utilizó aflatoxina B1. Por otra parte, la cantidad de compuestos fenólicos en los jugos de naranja se determinó de acuerdo al procedimiento de Folin-Ciocalteu. La vitamina C fue valorada por el método de Kabasakalis, Siopidou y Moshatou. La presencia de pesticidas en las muestras de jugo se estableció como la actividad equivalente del metilparatión, que produce inhibición de la acetilcolinesterasa.

En otro orden, las ERO para la detección de daño oxidativo a la desoxirribosa fueron generadas de acuerdo a Nishida, Yoshizawa y Akamatsu. El ensayo de la reducción del nitroazul de tetrazolio (NBT) permitió cuantificar la producción del anión superóxido. Los productos oxidantes, producidos por la formación de sustancias reactivas al ácido tiobarbitúrico (TBARS) fueron evaluados de acuerdo con la descripción de Halliwel y Gutteridge. Los autores también determinaron la actividad protectora contra el daño oxidativo ocasionado por el linoleato de metilo (MeLo). El análisis estadístico comprendió la valoración del efecto antioxidante y mutagénico de los jugos, las diferencias entre cada jugo y su control (100% de oxidación) y las diferencias entre las formas fresca y congelada del mismo jugo.

Resultados

Las formas frescas y congeladas del mismo jugo presentaron concentraciones similares de compuestos fenólicos, con excepción de los jugos Proc. congelado 1 y Proc. fresco 1. Las concentraciones de vitamina C de los jugos Proc congelado 1 y Proc. congelado 3 fueron 50% menores que las encontradas en el Proc. fresco 1 y Proc. fresco 3. Sólo el jugo Proc. fresco 1 presentó trazas de equivalente de metilparatión. Por otra parte, todos los jugos inhibieron el daño oxidativo a la desoxirribosa en forma dependiente de la dosis. Similar protección se observó en el control con manitol. Con una concentración del 10%, el efecto protector varió

entre el 65% y 85%, mientras que a una concentración del 1% los valores se encontraron entre el 60% y 83%. Sólo se observaron diferencias entre los jugos Proc. fresco 1 y Proc. congelado 1, ya que el primero otorgó la mayor protección contra el daño oxidativo en las concentraciones del 1% y 0.1%.

En otro orden, todos los jugos, con excepción de la muestra de Proc. fresco 2, inhibieron la reducción del NBT del 42% al 70% con la mayor concentración utilizada (10%). Con concentraciones de hasta el 1%, todos los jugos produjeron lesión oxidativa al MeLo, con aumento significativo de la peroxidación lipídica. Sin embargo, con concentraciones del 10%, los jugos Org. fresco y Org. congelado *in natura*, así como los jugos Proc. fresco 1 y 3 produjeron menor peroxidación lipídica. La cantidad de compuestos fenólicos estuvo en correlación con el nivel de peroxidación lipídica, sin relación con la vitamina C. Las concentraciones de compuestos fenólicos presentaron conexión con la actividad antioxidante en la degradación de la desoxirribosa, no así con la reducción de NBT. Los jugos Org. fresco, Org. congelado y Proc. congelado 1, en presencia y ausencia de activación metabólica, presentaron actividad mutagénica en la cepa TA98, que detecta mutaciones del marco de lectura. Los jugos Proc. congelado 2 y Proc. fresco 2 fueron mutagénicos en ausencia de activación metabólica en la cepa TA98. Sin embargo, el jugo Proc. congelado 3 requirió la metabolización para producir la mutación del marco de lectura en la cepa TA98. El jugo Org. fresco fue mutagénico en la cepa TA97a, en presencia y ausencia de activación metabólica. Los jugos Proc. fresco 3 y Proc congelado 1 indujeron mutación del marco de lectura sólo en ausencia de activación metabólica. En la cepa TA100, sólo el jugo Org. fresco, en ausencia y presencia de activación metabólica, pudo producir sustitución de bases. Con la cepa TA102, que detecta mutágenos oxidantes y alquilantes y formas activas de oxígeno, no se observó ninguna respuesta positiva, independientemente de la activación del microsoma. Los autores comprobaron una correlación importante de los compuestos fenólicos con la mutagenicidad del jugo para las cepas TA97a. La cantidad de contenido de vitamina C también estuvo asociada con la mutagenicidad del jugo en la cepa TA97a.

Conclusión

Los presentes hallazgos indican que los jugos de naranja analizados tienen potencial antioxidante y mutagénico; los compuestos fenólicos y la vitamina C pueden cooperar en la actividad mutagénica. Sin embargo, otros antioxidantes presentes en las naranjas, como la vitamina E, los carotenoides y ciertos minerales, así como los efectos sinérgicos entre los antioxidantes pueden aumentar el potencial antioxidante total de los jugos. Este importante potencial antioxidante debe ser superado por una actividad mutagénica sólida para poder inducir las mutaciones bacterianas observadas. A pesar de que algunas muestras analizadas evidenciaron actividad mutagénica, el riesgo para los seres humanos sería bajo, debido a las actividades enzimáticos y a las variaciones del pH en el sistema digestivo. Además, ingredientes con actividad antimutagénica y destoxificante también evitarían este riesgo. Los autores estiman que sería interesante confirmar los resultados descritos en sistemas eucarióticos.

(←atrás)

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7.3 ANEXO C "Review: Orange juice, genomic stability and oxidative stress. Part I: Effect of the whole juice"

Franke, S.I.R., Prá, D., Henriques, J.A.P.

Review:

Orange juice, genomic stability and oxidative stress. Part I: Effect of the whole juice

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Abstract

Orange juice (OJ) is a complex mixture composed of, among other substances, proteins, carbohydrates, lipids, vitamins [in decreasing order, C , B complex (including folate), and A], carotenoids (xanthophylls, cryptoxanthins, carotenes), metals (mainly Fe and Cu), phenolic compounds (hesperidin and hesperitin, naringenin and narirutin) and fibers, which may exert either beneficial or deleterious biological effects. This article reviews the reported effects of OJ on genomic stability and oxidative stress. OJ can vary greatly in composition, but it is generally a good antioxidant and has antigenotoxic/antimutagenic, anticarcinogenic and atheroprotective effects both in vivo and in vitro. It also interacts strongly with drugs and micronutrients, can reduce stone formation and esophageal alkali injury and can protect against bacterial urinary tract infections and the common cold. It can, however, induce hyperkalemia, allergy and cavities and diarrhea when taken in excess. Excessive intake of any food, even the healthiest, can lead to oxidative status imbalance. The true protective effect of food is mediated by the equilibrium of the diet and not by a single food or a single compound in a food. Clinical studies mapping the effect of supplementation with preventive antioxidants have shown surprisingly little or no effect on cancer incidence. However, the epidemiological evidence associating diets rich in fresh fruit and vegetables with a decreased incidence of cardiovascular and neurodegenerative diseases and cancer has been reported.

Key-words: DNA damage and repair, (anti)mutagenic and (anti)carcinogenic effects, atheroprotective effect, drug interaction, vitamin C and complex B vitamins.

Contents

Abreviations

OJ: General or sweet orange juice

SOJ: sour orange juice

LDL: low-density lipoprotein-cholesterol

HDL: high-density lipoprotein-cholesterol

1   Orange juice

Oranges have been cultivated since ancient times; they are possibly a hybrid between pomelo (*Citrus maxima*) and tangerine (*Citrus reticulata*). They are widely grown in warm climates worldwide; Brazil and US produce about 50% of the total world production. The flavors of oranges vary from sweet to sour. The fruit is commonly peeled and eaten fresh, or squeezed for its juice. There are two kinds of orange; *Citrus sinensis* is called sweet orange and *Citrus aurantium* is called sour orange (other names include bitter orange, bigarade orange and Seville orange) (Morton, 1987; Wikipedia, 2006).

Sweet orange oil is a by-product of the juice industry produced by pressing the peel. It is used as a flavoring for food and drink and for its fragrance. Sweet orange oil consists of about 90% *D*-limonene, a solvent used in various household chemicals. Sour oranges are also used for their essential oil and in herbal medicine, as well as for making marmalade and for orange-flavored liqueurs (Morton, 1987; Wikipedia, 2006).

The composition of oranges is variable, depending on varietal differences as well as on climatic conditions, soil composition, light and pathogen exposure and maturation state. These aspects influence the color and flavor of the orange. Red pulp oranges are rich in anthocyanins.

Orange juice (OJ) is complex mixture, composed of, among other substances, proteins, carbohydrates, lipids, [in decreasing order, C , B complex (including folate), and A], carotenoids (xanthophylls, cryptoxanthins, carotenes), metals (mainly Fe and Cu), phenolic compounds (hesperidin and hespiritin, naringenin and narirutin) and fibers, which may exert various biological effects (Patrineli *et al.*, 1996b; Ames & Gold, 1998; Yoshino *et al.*, 1999; Franke *et al.*, 2004; Franke *et al.*, 2005a). Despite scientific data

reporting beneficial properties derived from the consumption of juice (for example, antimutagenic or anticarcinogenic effects), some compounds also present in juices have been identified as being mutagenic or carcinogenic (Ames, 1983; 1989; Patrineli *et al.*, 1996a; Patrineli *et al.*, 1996b; Fenech *et al.*, 2005; Franke *et al.*, 2006). The carcinogenic or genotoxic effects may be mediated by the interaction of juice components with transition metals or by sub-products of juice auto-oxidation.

The purpose of this review is to discuss the reported effects of OJ on genomic stability and oxidative stress.

2   Genotoxic, antigenotoxic and antimutagenic effects

As early as 1982, Mazaki *et al.* (1982) tested the mutagenicity of citrus fruit juice extracted with ether or chloroform, and observed mutagenic activity as indicated by the *Salmonella*/microsome assay (Ames test).

Ekasari *et al.* (1989) showed that heated OJ, as well as the acid hydrolysate, was mutagenic and cytotoxic for *Salmonella typhimurium* TA 100 without S-9-mix, after neutralization to pH 7.4. Although heating, normally utilized in processing OJ, is insufficient to release mutagenic flavonol as aglycones from their glycosides (kaempherol and quercetin), other intermediary products can lead to mutagenicity and cytotoxicity. Ekasari *et al.* (1990) also detected mutagenicity, using *Salmonella*/microsome assay, in three fractions of heated OJ, when pH was adjusted to 7.4.

Franke *et al.* (2004) evaluated the mutagenic activity of frozen and fresh forms of *in natura* and processed OJ (pH not adjusted) using the *Salmonella*/microsome assay. Only fresh *in natura* OJ was mutagenic, with or without S9 mix, for *Salmonella typhimurium* TA 100, which detects base-pair substitutions. This result agrees with those

of Ekasari *et al.* (1989), in spite of differences in OJ processing. Franke *et al.* (2004) also showed that fresh and frozen *in natura* OJ was mutagenic for TA98, both in the presence and absence of metabolic activation. The same occurred for fresh *in natura* OJ for TA 97a. Frozen and sweetened OJ samples were mutagenic for either TA 97a or TA 98, which detect frame-shift mutations. Positive results with TA97a in the presence of S9 mix were correlated with total phenolics and vitamin C content. No mutagenicity was detected when testing TA 102, which is sensitive to oxidative and alkylating damage. Only an unsweetened, unfrozen and processed OJ sample was not mutagenic for any of the strains tested; this also had the best antioxidant potential (Franke *et al.*, 2004).

As early as 1989, Bala & Grover (1989) showed that 10 citrus fruit juices reduced the mutagenicity induced by N-nitro-o-phenylenediamine (NPD) for TA 97a and by sodium azide for TA 100 strains using the *Salmonella*/microsome assay. The co-treatment of OJ with either NPD or sodium azide enhanced the inhibitory effect. The authors also observed that ascorbic acid or citric acid or combined ascorbic and citric acids showed antimutagenic activity.

He & Campbell (1990) evaluated the effects of OJ (containing cryptoxanthin) and other carotenoids on aflatoxin B1 (AFB1)-induced mutagenesis in *S. typhimurium* TA 100 and TA 98. OJ inhibited AFB1-induced mutagenesis in both test strains, being the most potent inhibitor, at least an order of magnitude more potent than the other tested carotenoids.

Higashimoto *et al.* (1998) used the *Salmonella*/microsome assay with strain TA100 in the absence of S9 mix to test the antimutagenicity of 9 citrus juices on 1-methyl- 1,2,3,4-tetrahydro-beta-carboline-3-carboxy acid (MTCCA), a mutagen

precursor of soy sauce that give rise to mutagens after treatment with nitrite and alcohols. OJ presented the strongest antimutagenic effect, reducing the mutagenicity of MTCAA about 75% at 100 μL/2 mL. Conversely, Oshawa *et al.* (2003) found no suppression of DNA migration in the liver or stomach of mice orally treated with OJ just before a simultaneous oral dose of morpholine and NaNO$_2$.

Riso *et al.* (2005) showed that lymphocytes of individuals supplemented with blood OJ (600 mL x 21 days) had increased resistance to H$_2$O$_2$ mediated DNA damage, as evaluated by the Comet assay. Franke *et al.* (2005a) observed a non-significant decrease in the 8-hydroxydeoxyguanosine/deoxyguanosine ratio in healthy volunteers supplemented for 3 weeks with 3 daily glasses of OJ.

Franke *et al.* (2005b) evaluated the effect of OJ on alkylating agents [methyl methanesulfonate (MMS) and cyclophosphamide (CP)] in mice blood cells *in vivo,* using the comet assay. OJ reduced the DNA damage induced by MMS and CP. Pre-treatment with OJ only protected against DNA damage caused by MMS. Post-treatment with OJ was reparative for both alkylating agents. (Franke *et al.*, 2006) also evaluated the modulatory action of OJ on the genotoxicity of iron and copper. For iron, OJ reduced DNA damage only when mice were pre-treated with OJ and then treated with iron. However, it reduced DNA damage generated by copper either when administered before or after copper treatment.

Hosseinimehr *et al.* (2003) evaluated the radioprotective effects of sour orange extract using the micronucleus test for anti-clastogenic and cell proliferation activity. A single intraperitoneal injection 1 h prior to gamma-ray irradiation reduced the frequencies of micronucleated polychromatic and normochromatic erythrocytes. These authors

suggest that fruits and vegetables contain flavonoids that have protective effects under such stress conditions as irradiation. Hosseinimehr & Karami (2005) evaluated the protective effect of sour orange peel extract against cyclophosphamide by using the micronucleus assay for anticlastogenic activity in mouse bone marrow cells. Citrus extract at 400 mg/kg reduced micronucleated polychromatic erythrocytes 2.8 fold against genotoxicity induced by cyclophosphamide.

Physiological conditions related to climate, soil, the maturation and the varietal of orange, as well as variations in processing methods and shelf life, can explain the differences in genotoxic/antigenotoxic potential of OJ. As concluded in a previous study, the risk for humans consuming OJ is low, based in the metabolic differences between bacteria and mammals (Franke *et al.*, 2004). OJ was genotoxic in some cases to bacteria but not to mammals. It is important to mention that OJ can induce a slight non-significant transient increase in DNA damage in blood cell of mice treated with a high dose (equivalent to about 600 mL in humans), as detected by Franke *et al.* (2005b; 2006).

The cited studies showing antigenotoxicity and antimutagenicity against different genotoxins in bacteria, mice and humans reinforce the beneficial effect of OJ consumption.

3   Anticarcinogenic effects

Several studies associate the consumption of bioactive compounds with anticarcinogenesis. OJ is one of the most consumed foods rich in bioactive compounds. It has been associated with reduction in risk of laryngeal, colon and mammary cancer, particularly due to the content of carotenoids and flavonoids (Crosignani *et al.*, 1996; Miyagi *et al.*, 2000; Slattery *et al.*, 2000). OJ is also related to the prevention of skin

cancer. In 2000, the first study that evaluated the anticarcinogenic effect of the consumption OJ containing peel was performed. This effect was attributed to the bioavailability of *D*-limonene in OJ peel (Hakim *et al.*, 2000).

So *et al.* (1996) tested the ability of OJ concentrate to inhibit development of mammary tumors induced by 7,12-dimethylbenz[a]anthracene in female Sprague-Dawley rats. Rats given OJ had a lower tumor burden than controls.

The administration of orange oil 1h prior to treatment with nythomethylurea inhibited pulmonary adenoma formation and the occurrence of forestomach tumors in mice (Wattenberg & Coccia, 1991). Orange oil was found to prevent rat mammary carcinomas induced by the direct-acting carcinogen nitrosomethylurea (Maltzman *et al.*, 1989).

Feskanich *et al.* (2003), studying two Nurse´s Health Study Cohorts, found an unexpected association between the increased risk of melanoma and higher intake of food vitamin C, particularly OJ. The authors describe this association as random, but also state that the association was strongest among the higher risk sun-sensitive women. It is unlikely that vitamin C was responsible for the association, since supplements of vitamin C were not associated to a higher risk of melanoma. OJ contains appreciable quantities of furocoumarins such as psoralens which are substances that cause interstrand cross-links and monoadditions in DNA when photoactivated by UVA (Brendel & Henriques, 2001; Brendel *et al.*, 2003).

Miyagi *et al.* (2000) observed that feeding single-strength, pasteurized OJ inhibited azoxymethane-induced colon cancer in male Fischer 344 rats by 22%.

When administered to rats in place of drinking water, double strength OJ was more effective than double strength grapefruit juice in inhibiting mammary tumorigenesis induced by DMBA in female Sprague-Dawley rats. This activity was attributed mainly to hesperitin, as well as to other compounds that have anti-cancer activity and that may act synergistically with hesperitin. This synergism may be due to the fact that the compounds are exerting their inhibitory effects by different mechanisms (Guthrie & Carroll, 1998).

The anticarcinogenic effect of OJ and orange oil can be attributed, among other aspects, to modulation of metabolizing enzymes (phase I and II), mainly flavonoids, as well as to regulation of the cell cycle (So *et al.*, 1996; Guthrie & Carroll, 1998) (Rowland, 1999). In the case of orange oil, the activity of *D*-limonene in inhibiting the post-translational farnesylation of Ras ocoprotein can be related to carcinogenesis inhibition and blocking (Rowland, 1999).

4   Antioxidant, pro-oxidant and antiatherogenic effects

Most studies show antioxidant effects for OJ. However, some studies suggest a pro-oxidant role (Table 1).

Antioxidants are substances that, when present in low concentration compared with the substrate to be oxidized, can delay or prevent autoxidation or free radical-mediated oxidation (Vinson, 1998). Vitamin C and phenolic compounds are important antioxidants in OJ (Wang *et al.*, 1996) which act as scavengers. The metal-binding ability of phenolic compounds has been shown to inhibit the formation of hydroxyl radicals in the Fenton reaction by complexing ferrous ions. Franke *et al.* (2004) showed that all OJs tested inhibited oxidative damage to deoxyribose in a dose-dependent manner, similar to the protection seen for a positive control (mannitol). Conversely, all OJs showed a

significant increase in lipid peroxidation. When reacting with Cu^+, OJ in low doses (up to 1%) was pro-oxidant, while at higher doses it was less pro-oxidant (up to 10 %), even though this reduction of peroxidation did not reach the level of the positive control (betahydroxytoluene) (Franke *et al.*, 2004).

In conclusion, the oxidant/antioxidant role of OJ is likely to depend on its concentration and composition, as well as the oxidant used in the test system (Table 1).

The oxidative theory of atherogenesis links oxidative stress and an increase in risk of developing coronary heart disease. According to this theory, oxidized lipoproteins have an important role in the formation and progression of atheromas (Vinson, 1998). Thus, the reduction in the risk of coronary heart disease depends either on the oxidative stress or on the lipid profile, particularly with respect to the level of potentially oxidizable LDL (Granot & Kohen, 2004; Stocker & Keaney, 2004).

Keevil *et al.* (2000) tested the effect of different commercial juices on ex vivo human platelet activity. OJ did not inhibit human platelet aggregation. This result agrees with that of (Osman *et al.*, 1998), who showed OJ does not inhibit platelet aggregation in dogs and monkeys. Conversely, OJ prolonged epinephrine/collagen-induced clotting time in an ex vivo platelet model (Polagruto *et al.*, 2003).

Kurowska *et al.* (2000a) replaced drinking water with OJ for rabbits with high serum low-density lipoprotein-cholesterol (LDL). After 3 weeks, they observed a significant decrease in the level of cholesterol excretion as well as in the levels of cholesterol in the serum and liver of the rabbits. The same authors (Kurowska *et al.*, 2000b), determined the effect of OJ consumption (sequential introduction of 1, 2, or 3 cups of 250 mL each for 4 wk) on blood lipids of human subjects with moderate

hypercholesterolemia. 750 mL, but not 250 or 500 mL, OJ daily increased high-density lipoprotein-cholesterol (HDL), triacylglycerol, folate and vitamin C concentrations and decreased the LDL/HDL ratio. Gorinstein *et al.* (2004) observed an improvement in plasma lipid metabolism induced by OJ in rats fed diets supplemented with cholesterol. Conversely, Franke *et al.* (2005a) observed a decrease in the LDL/HDL ratio, though not for total cholesterol, in healthy volunteers supplemented for 3 weeks with 3 daily glasses of OJ. Harats *et al.* (1998) showed a decrease in total cholesterol and LDL after one month of OJ ingestion (equivalent to 50 mg of vitamin C). Devaraj *et al.* (2004) suggested that OJ fortified with plant sterols would control LDL and could easily be incorporated into changes in therapeutic lifestyle as a dietary regimen.

OJ inhibited atherosclerosis and lowered cholesterol and triglycerides in a hamster model of atherosclerosis (Vinson *et al.*, 2002). Supplementation with a combination of orange and carrot juice decreased copper oxidized-LDL in habitual smokers (Abbey *et al.*, 1995). This result was confirmed *in vitro* by Vinson (2002).

In contrast, sour (Seville) orange (*Citrus auratium*) extract can induce weight gain, blood pressure increase and cardiac dysfunctions in hypertensive subjects, possibly due to its synephrine, octopamine and furocumarin content. Indeed, SOJ should be avoided by individuals with severe hypertension, glaucoma, or by those taking monoamine oxidase inhibitors. Conversely, SOJ consumption seems to be safe for normotensive subjects (Penzak *et al.*, 2001).

Hesperitin can play a major role in optimizing the lipid profile. Hesperitin and its derivatives appear to have potent plasma lipid-lowering effect by inhibing 3-hydroxy-3-

methyl-glutaryl-CoA reductase and acylCoA:cholesterol acyltransferase, two key enzymes of cholesterol biosynthesis in high-cholesterol-fed rats (Choi *et al.*, 2004).

5   Other effects

5.1   Drug interactions

The interaction of OJ with drugs has been much studied. OJ has been widely prescribed for increasing the absorption of several compounds (eg. iron), owing to its acidity or influence on gastric emptying (Odou *et al.*, 2001). However, OJ can interfere in drug absorption and metabolism, largely depending on its composition. For example, it has been shown to interfere in the absorption of acetyl salicylic acid (Odou *et al.*, 2001), of β-adrenergic blocking agents (Lilja *et al.*, 2004; Lilja *et al.*, 2005; Nishimuta *et al.*, 2005), of an antihistamine (Kamath *et al.*, 2005), of a HIV-1 protease inhibitor (Penzak *et al.*, 2002), of a chemotherapeutic agent (Ikegawa *et al.*, 2000; Honda *et al.*, 2004) and of a hypoglycemic drug (Satoh *et al.*, 2005).

OJ can either stimulate or reduce drug absorption. It can block the inflow of compounds into the cells or modify the efflux of compounds from the interior of the cell to the extra-cellular space. OJ and its components in isolation can interact with metabolizing enzymes [e.g. cytochrome P450 3A4 (CYP3A4) and sulfotransferase isoforms] and transporters involved in influx [organic anion-transporting polypeptides (OATP)] and efflux [P-glycoproteins (P-gp) and multiple drug resistance protein (MDR-2)] of compounds. Due to this intricate influence of OJ on drug absorption and metabolism, it is recommended that patients avoid citrus juice while taking medication and that healthcare providers advise against citrus juice intake in this setting until any interactions with subject drugs can be clarified in clinical studies (Saito *et al.*, 2005).

Whereas OJ at 5 % seems not to influence P-gp activity either in rats or humans, it inhibits OATP. Thus, OJ inhibits OATP over P-gp (Kamath *et al.*, 2005), even though at 30% OJ (Lim & Lim, 2006) and its components (Ikegawa *et al.*, 2000) in isolation inhibit P-gp-mediated efflux of chemotherapeutic drugs.

The activity of OJ on CYP3A4 is still controversial. Sweet OJ seems not to influence CYP3A4 activity (Bailey *et al.*, 1991; Hashimoto *et al.*, 1998; Takanaga *et al.*, 2000; Tian *et al.*, 2002). SOJ seems to be an inhibitor of CYP3A (Malhotra *et al.*, 2001; Di Marco *et al.*, 2002; Mouly *et al.*, 2005).

OJ at 10 % also inhibited the sulfation of a $\beta2$-agonist by recombinant human sulfotransferases isoforms 1A1 and 1A3, indicating a possible action in increasing the bioavailability of the compound (Nishimuta *et al.*, 2005).

5.2   Nutrient interactions

OJ greatly enhances aluminium absorption and should not therefore be taken in conjunction with aluminium-containing antacid preparations (Fairweather-Tait et al., 1994). OJ significantly improved calcium bioavailability, independent of its pH (Mehansho et al., 1989; Griffin et al., 2002). It seems to increase iron absorption by 85 % (Hallberg & Rossander, 1982), although whether citrate or ascorbate is the major enhancer of Fe absorption and diffusion is controversial (Ballot et al., 1987; Hazell & Johnson, 1987). OJ increases selenium absorption (Robinson et al., 1985), but reduces Zn absorption (Flanagan et al., 1985). Glycine absorption is also markedly enhanced by OJ (El-Shobaki et al., 1977).

5.3 General effects

OJ contains low to moderate amounts of phytoestrogens, although this is poorly discussed in relation to other foods rich in phytoestrogens (Horn-Ross et al., 2000). Indeed, hesperidin and vitamin C, the major bioactive components of OJ, are prescribed for the treatment of hot flushes (Philp, 2003).

OJ can reduce the risk of kidney stone formation (Hesse et al., 1993; Campoy Martinez et al., 1994; Honow et al., 2003). The daily consumption of 500 mL fresh OJ increased urinary citrate concentrations from 0.35 to around 1.21 mg/mL and pH rose from 7.24 to 8.2 (Suller et al., 2005). OJ reduced acute esophageal alkali injury in an *ex-vivo* study (Homan *et al.*, 1995).

Embil *et al.* (1976) observed that the consumption of 300 mL OJ did not significantly alter urinary pH, while 1500 mL changed urinary pH by an average of one unit. A change of this magnitude could cause a clinically significant variation in the overall excretion pattern of acidic or basic drugs. OJ, like other juices, inhibits bacterial adherence to bladder cells, most likely because of its fructose content, preventing urinary tract infections (Zafriri *et al.*, 1989)

OJ in excessive amounts can induce hiperkalemia, especially in renally compromised inpatients (Fan & Leehey, 1996; Berk *et al.*, 2004).

OJ may induce severe food allergy in sensitive individuals (Zhu *et al.*, 1989), but according to Yap *et al.* (1990), the ingestion of fresh OJ per se did not heighten nonspecific bronchial hyperreactivity.

Baird *et al.* (1979) evaluated the effects of the consumption of 1 glass of natural OJ and synthetic OJ (80 mg of vitamin C), or placebo per 72 days on the prevention of

the common cold in 362 normal young adult volunteers. They observed a 14 to 21% reduction in total cold symptoms. Results with both natural and synthetic OJ with a physiological content of ascorbic acid were similar to those obtained using higher doses of ascorbic acid.

OJ was found to stimulate salivary secretion, to decrease the salivary pH immediately after consumption and to decrease the redox potential of whole saliva. This can be caries-promoting in the oral cavity in persons with low salivary flow rate who consume the juice regularly (Tenovuo & Rekola, 1977).

6   Conclusions

OJ can be widely variable in composition, depending on physiological conditions (related to climate, soil and maturation) and genetic characteristics (varietal) of the oranges, as well as on variations in processing methods and time and packing mode. In general, OJ is a good antioxidant and has antigenotoxic and antimutagenic, anticarcinogenic and atheroprotective effects, both in vivo and in vitro. Moreover, it markedly interacts with drugs and micronutrients, can reduce kidney stone formation, esophageal alkali injury and can protect against bacterial urinary tract infections and the symptoms of common cold. Conversely, OJ can induce hyperkalemia, allergy, tooth cavities and diarrhea on excessive intake.

OJ has been used as a source of various micronutrients with or without supplementation. While fruit juice is a healthy, low-fat, nutritious beverage, there is increasing concern about the excessive intake of fruit juices among children. The use of OJ as a substitute for meals can lead to malnutrition and decreased stature in children (Dennison *et al.*, 1997; Dennison *et al.*, 1999). OJ must be considered as a part of the

116

recommended daily ingestion of at least five (maybe nine) portions of fruit and vegetables to prevent diseases (Wang *et al.*, 1996; McCall & Frei, 1999). Excessive intake of any food, even for the healthiest, can lead to oxidative status unbalance. The true protective effect of feeding is mediated by the equilibrium of the diet and not by a single food or a single food component. Indeed, clinical studies mapping the effect of supplementation with preventive antioxidants have shown surprisingly little or no effect on cancer incidence (Correa *et al.*, 1998; Donaldson, 2004). However, the epidemiological evidence associating diets rich in fresh fruit and vegetables with a decreasing incidence of cardiovascular and neurodegenerative diseases and cancer has been reported (Wang *et al.*, 1996; Kabasakalis, 2000; Halliwell, 2001).

7   Acknowledgements

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Table 1. Review of the antioxidant effects of orange juice in different test systems

Orange derivative	Concentration	Test system	Result	Putative agent	Source
OJ	1-2 mL per 24 weeks	Rats fed or not on a high cholesterol diet (DPPH of plasma)	Increase in plasma total antioxidant potential	Total polyphenols and flavonoids	(Gorinstein *et al.*, 2004)
OJ VitC suppl	Various concentrations	In vitro TAA	Positive - phenolic compound had a protective role over vitamin C	Hesperidin, narinrutin and vitamin C	(Miller & Rice-Evans, 1997)
SOJ peel extract	Maximal effect at 1.6 mg/mL	In vitro DPPH	Excellent scavenging effects	Flavonoids	(Hosseinimehr & Karami, 2005)
OJ	Various concentrations	In vitro inhibition of lipoprotein oxidation by Cu	Inhibitory effect - Cardiovascular protective	Polyphenols were more effective than vitamin C	(Vinson *et al.*, 2002)
OJ	2 month OJ supplementation	In vitro inhibition of lipoprotein (of OJ treated subjects) oxidation by Cu	In vitro reduction of the susceptibility of lipoprotein to oxidation	Interaction of vitamin C and E. Obs.The main source of vitamin C was not OJ	(Harats *et al.*, 1998)
OJ	3 weeks OJ supplementation x 250 mL daily	In vitro inhibition of lipoprotein (of OJ treated habitual cigarette smokers) oxidation by Cu	Reduction of copper-oxidized LDL despite a diet rich in polyunsaturated fatty acids.	Vitamin C Obs. Subjects also received 300 mL of carrot juice daily	(Abbey *et al.*, 1995)
5 varieties of OJ	Various concentrations	DPPH, TAA, LP-LUV and NO tests	Evident antioxidant effect in all samples	Total phenols and anthocyanins, while vitamin C had a minor role	(Rapisarda *et al.*, 1999)
OJ versus Vitamin C	2 weeks treatment with 1 or 2 glasses of OJ or ~70 mg vitamin C	TBARS in plasma	Similar reduction in the level of TBARS for 1 glass of OJ and ~70 mg of vitamin C.	Vitamin C, in spite of the complex nature of OJ. Obs. One glass was more effective than 2 glasses of OJ.	(Johnston *et al.*, 2003)
Reconstituted versus chilled and fresh versus stored (8 days at 4oC) OJ.	2 hour postprandial intake of 1 glass of SOJ	TBARS in plasma	Reductive (reconstituted) or neutral (chilled) effect for OJ at day one and increasing effect in TBARS levels for both juices at day 8.	Negative correlation of TBARS with vitamin C level, indicating that the loss of ascorbic acid in refrigerated juice may impact postprandial oxidative stress.	(Johnston & Hale, 2005)

OJ	Various concentrations	XO activity in vitro	XO promoter activity – risk of increasing hypouremia and increasing global oxidative stress	Anthocyanidins and other flavonoids Obs. Further studies are needed in vivo	(Dew et al., 2005)
Frozen and in natura processed or fresh OJ	Various concentrations	Deoxyribose degradation, NBT reduction assays, TBARS	Antioxidant or pro-oxidant effect depending on test system and concentration	Positive correlation between total phenolic content and the inhibition of deoxyribose degradation and lipid peroxidation. Vitamin C content did not correlate with any chemical parameter	(Franke et al., 2004)
OJ processed by high pressure	14 days treatment x 500 mL. Healthy volunteers	Measurement of metabolic products related to oxidative stress	Reduction in the level of 8-epiPGF$_{2a}$ (isoprostane resulting from oxidation related to cardiovascular conditions) and PGE$_2$ (primary product of arachidonic acid metabolism related to impaired immune function).	Inverse correlation between levels of vitamin C and 8-epiPGF$_2$	(Sanchez-Moreno et al., 2003)
Blood OJ	21 days x 600 mL Healthy volunteers	Plasma antioxidant capacity, TBARS and DNA damage resistance evaluation by the Comet assay ex vivo	No effect on plasma antioxidant capacity or TBARS; increase in resistance to DNA damage mediated by oxidative stress.	Increase in plasma vitamin C, cyanidin-3-glucoside, β-cryptoxanthin and zeaxanthin	(Riso et al., 2005)
OJ	3 weeks x 3 glasses (3x236 mL) Healthy volunteers	TBARS and measurement of DNA oxidation (dG ratio)	Non-significant 16-29% decrease in dG ratio in blood cells and no effect in TBARS	Higher plasma level of vitamin C (59%), folate (46%), carotenoid (23%) and flavanone (800%)	(Franke et al., 2005a)
12 Blood OJ samples differently processed	In vitro relation between antioxidant potential and anthocyanin content during OJ decomposition	BR system, DMPD, ABTS, DPPH, and FRAP	Positive antioxidant activity related to the content of anthocyanins.	Reduction of anthocyanins, typical of commercial long-shelf life juices, leads to a notable loss of antioxidant power.	(Fiore et al., 2005)

XO: xanthine oxidase, NBT: nitro blue tetrazolium; TBARS: thiobarbituric acid reactive substances; dG ratio: 8- hydroxydeoxyguanosine/ deoxyguanosine; DPPH test: sequester of 1,1-diphenyl-2-picryl hydrazyl radical; TAA test: total antioxidant status; LP-LUV test- linoleate peroxidation in LA/DPPC LUVs; NO test - scavaging activity against nitric oxide; BR system: mixture that measures Briggs-Rauscher reaction; DMPD: (N,N-dimethyl-p-phenylenediamine dihydrochloride; ABTS: 2-29-azinobis-(3-ethylenbenzothiazoline-6-sulfonic acid) diammonium; FRAP: ferric reducing antioxidant power.

7.4 ANEXO D: "Review: Orange juice, genomic stability and oxidative stress. Part II: Role of the main constituents"

Franke, S.I.R., Prá, D., Henriques, J.A.P.

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Review:

Orange juice, genomic stability and oxidative stress. Part II: Role of main constituents

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Abstract

Orange juice (OJ) is one of the most consumed beverages worldwide. It can be an important constituent of healthy and balanced diets characterized as high in antioxidant potential and, thus, with genome stabilization properties. OJ is high in vitamins, minerals and other bioactive compounds, which are involved in its biological function. The purpose of this review is to discuss the influence of the main nutrients of OJ on oxidative stress and genome stability. Topics covered are: a) the average composition of OJ; its main constituents in relation to daily reference intakes (DRI) of vitamins and minerals, where these are available; b) the antioxidant and genome stabilizing role of the main constituents of OJ; c) the effect of OJ processing and storage on its constituents. One portion of OJ can provide more than 13 % of the DRI of vitamin C, folate and thiamin and between >5-13% of the DRI of riboflavin, niacin, pantothenic acid, pyridoxine, magnesium, copper and potassium. It can provide minor amounts (>4-5 % of the DRI) of pro-vitamin A carotenoids and iron. OJ is also rich in phenolic compounds, such as hesperdin and narirutin and their aglycones, and in phenolic acids, as well as *D*-limonene. Vitamin C is a potent antioxidant because of its metal chelating, oxidant scavenging and DNA repair modulation effects. B complex vitamins, including folate (B9), have a myriad of functions related to antioxidation and DNA homeostasis. Phenolic compounds are antioxidant, metal scavengers, DNA repair modulators, antiproliferative and antiangiogenic agents. Carotenoids and the simple terpene *D*-limonene have controversial roles in carcinogenesis, although they are generally considered as anticarcinogens and antioxidants. Copper and iron are related to antioxidant defenses, as well as to the oxidative metabolism and immune system. Magnesium is involved in DNA synthesis. Phosphorus has a role in signal transduction and energy carriage and its deficiency has been linked to aging-like symptoms. Potassium is important in maintaining membrane potential and fundamental for muscle contraction and neurotransmission; impairments in this nutrient have been linked to

carcinogenesis. OJ compounds can be affected by processing techniques, such as pasteurization, freezing and concentration. Since these techniques are widely used in the OJ industry, more studies to address this issue are needed. One glass of fresh or adequately processed and stored OJ is one of the options for the daily 5 portions (or even more) of fruit and vegetables recommended for a healthy life.

Key-words: Enzymatic and non enzymatic antioxidants, DNA damage and repair, (anti)mutagenic and (anti)carcinogenic effects, bio-flavonoids and carotenoids, vitamin C and complex B vitamins.

Contents

1   Orange juice

OJ is probably the most consumed and the best known of fruit juices. It is among those foods regarded as part of a healthy and balanced diet. Numerous studies have associated fruit consumption with genome stability and protection against oxidative stress. These effects are probably due to synergism between the various components of fruits (Ames, 2001; Ames & Wakimoto, 2002; Fenech, 2005; Fenech *et al.*, 2005; Franke *et al.*, 2006).

One must keep in mind that many fruit juices are submitted to different processes in order to preserve them. Processes like pasteurization, freezing and high-pressure extraction have been developed to attend the growing demand for fruit juices.

OJ contains significant amounts of vitamin C, folate, thiamin, riboflavin and phenolic compounds. It also contains appreciable amounts of carotenoids (pro and non-pro-vitamin A), niacin, panthothenic acid, pyridoxine, magnesium, potassium, copper, iron and phosphorus. Such substances can directly or indirectly influence oxidative state and genome stability. Other compounds, such as the terpene *D*-limonene and the phenolic acid *p*-coumarin, are also present in OJ and also have roles in oxidative stress and DNA homeostasis.

The composition of orange is widely variable, depending on varietal differences, as well as on climatic conditions, soil composition, light and pathogen exposure and maturation state. These aspects influence the color and flavor of the oranges. For example, the color of red pulp oranges is mostly due to anthocyanins. However, it has also been reported that carotenoids, such as cryptoxanthins, can be responsible for this coloration (Mouly *et al.*, 1999; Lee, 2001).

OJ composition does not only reflect the chemical composition of the oranges. It is considerably influenced by the extraction process, packaging and shelf time before consumption. In addition, the various components of oranges are not homogenously distributed within the fruits, they are compartmentalized. For example, the hydrophobic

carotenoids and phenolic compounds tend to be located in the peel and pulp rather than in the aqueous fraction. Indeed, Vitamin C and the B complex vitamins, which are hydrophilic, are easily extracted, while the yield of hydrophobic compounds can be optimized with further extraction (such as high pressure) (Pupin *et al.*, 1998; Mouly *et al.*, 1999; Rapisarda *et al.*, 1999; Gil-Izquierdo *et al.*, 2001; Gil-Izquierdo *et al.*, 2002; Franke *et al.*, 2004; Vanamala *et al.*, 2006).

The purpose of this review is to discuss the influence of the main nutrients of OJ on oxidative stress and genome stability. To define the main nutrients of OJ, we initially calculated the percentage of daily energy provided by the intake of a standard 200 mL serving of OJ in relation to the average daily Estimated Energy Requirement (EER) of male and female adults over two life stages (19-30 and 31-50 years). To calculate EER, we considered a Body Mass Index (BMI) of 21 for females and 22 for males, together with a height of 1.65 m, low physical activity and average age for each life cycle (IOM, 2005). The energy provided by the intake of one standard (200 mL) serving of OJ was 98.13 ± 19.96 Kcal/day, which represented 4.4% of the calculated average daily EER (amount of energy). This was then used to evaluate the density of nutrients in one standard (200 mL) serving of OJ in relation to the respective Dietary Reference Intake (DRI) for each nutrient. We include in this review all nutrients that can provide at least 4% of the respective DRI in a standard 200 mL serving of OJ (Table 1). If a given nutrient provides between 4.0 and 4.4% (regarded as one energy density unit) of its DRI in one standard (200 mL) OJ portion, then OJ was considered as a regular source of this nutrient. If the nutrient density is between one and two times, two and three times or more than three times the energy value, OJ was considered a good, excellent or key source of it, respectively (Figure 1).

We also include in the present review other bioactive compounds, such as non pro-vitamin A carotenoids and phenolics, which are not considered as nutrients and do not have DRIs (Tables 2 and 3).

In vivo effects of compounds are influenced by the amounts consumed, by the quantities absorbed or metabolized, as well as by the plasma and/or tissue concentrations (Prior, 2003). Due to the complexity of this theme, we focus on the level of the compounds present in OJ and overlook the absorption, metabolism, and the tissue concentrations of the nutrients. Since OJ compounds can be affected by processing techniques, we discuss the influence of pasteurization, freezing and concentration, techniques widely used in the OJ industry, and consider how these alterations can impact the biological role of OJ.

2   Main components of orange juice: biological role

2.1   *Vitamin A, carotenoids and D-limonene*

2.1.1   Vitamin A

Retinoic acid (RA) is a key signalling molecule that regulates the patterning and development of the body plan and many organ systems during embryogenesis. RA is an important regulator of steady-state hematopoiesis in fetal as well as in adult life. Indeed, hematopoietic defects caused by a deficiency of vitamin A are still a serious problem in the developing world (Evans, 2005). Vitamin A is present as retinol activity equivalent (RAE) in OJ (see item 2.1.3). The vitamin A content of the standard 200 mL OJ portion is 34.8 µg RAE (as calculated in Table 2), which is equivalent to 4.4 % of the DRI (Table 1). Thus, OJ is considered as a regular source of vitamin A (Figure 1).

2.1.2   Carotenoids

60% of the total carotenoids in oranges is in the peel (Mouly *et al.*, 1999). In a 2002 review, Nishino *et al.* (2002) state that, of more than 600 carotenoids identified to date, about 40 carotenoids are found in our daily foods. They also state that only 14 carotenoids, with some of their metabolites, are present in human plasma, as a result of selective uptake in digestive tract and tissues. Carotenoids are tightly bound to macromolecules, in particular proteins and lipids. For instance, less lipophilic carotenoids, such as lutein, are released into an aqueous environment more readily than β-carotene (Sanchez-Moreno *et al.*, 2005). Carotenoids can be lost by oxidation, depending on the structure of the molecule, the action of light and heating, as well as by the action of oxidants such as peroxides. The oxidation can be inhibited by vitamin C or other antioxidants intrinsic or extrinsic to OJ (Sanchez-Moreno *et al.*, 2003).

Early findings showed very low levels of carotenoids in OJ (Holden *et al.*, 1999). More recently, Mouly *et al.* (1999), using 18 standards in HPLC analysis, showed that OJ has

about 4,790-21,100 μg carotenoids per liter (~1,000-4,000 μg/200 mL), depending on the extraction process and more influenced by the origin then by the variety. In the same study, pro-vitamin A carotenoids corresponded to about 20% of the total carotenoids. Sanchez-Moreno *et al.* (2003; 2005), using 6 standards in HPLC analysis, detected about 1000-1500 μg of total carotenoids in 100 mL of OJ, about 30 % being pro-vitamin A carotenoids. Lutein and zeaxanthin accounted for the additional 70%. When pooling the data obtained by Mouley *et al.* (1999) on carotenoid profile of OJ from oranges of 5 countries, it can be seen that lutein, isolutein and zeaxanthin together account for only about 22% of the total carotenoids. Indeed, other carotenoids, such as mutatoxantin A and B, auroxanthin A and cis-violaxanthin, are present in high proportions in OJ, accounting for 9.5 and 12%, 8.2 and 11.3 %, respectively. Surprisingly, Lee (2001) found large amounts of red-colored lycopene as the major carotenoid in a red navel cultivar; this is not present in standard navel oranges or other major sweet orange cultivars.

The stability of carotenoids is similar to that of vitamin A in that they are sensitive to oxygen, light and acid media (Vercet *et al.*, 2001).

2.1.3   Pro-vitamin A carotenoids

α and β-carotene and α and β-cryptoxanthin are converted to vitamin A and are considered together as RAE, according to a FAO/WHO standard; RAE=μg β-carotene/12 + [(μg α-carotene + μg α- cryptoxanthin + μg β- cryptoxanthin)/24 (FAO/WHO, 1988). Pro-vitamin A carotenoids represent about 30% of the total carotenoids present in OJ (Table 2).

2.1.4   α and β-carotene

Initially, β-carotene was the most extensively studied, since β-carotene has the highest pro-vitamin A activity among carotenoids in vegetables and fruits. Later, it was shown that the unconverted form of carotenoids could also have a protective effect against cancer, without being converted to vitamin A (Nishino *et al.*, 2002).

Most carotenoids are only slightly absorbed (as low as 2% in some cases). Conversely, between 60 and 75 % of the β-carotene in a high bioavailable diet is converted to vitamin A and a minor extent (15 %) is absorbed intact. Besides the central enzyme-mediated cleavage that produces vitamin A, a central cleavage can occur, giving rise to deleterious sub-products, such as aldehydes and epoxide derivatives. β-carotenes and their derivatives are LDL-cholesterol-transported, being presumably susceptible to oxidation (IOM, 2000).

β-carotene supplementation significantly increases the incidence of lung cancer in humans, indicating that β-carotene supplementation could be dangerous, especially among people who currently smoke more than 20 cigarettes per day and drink more than 11 g/day of ethanol. Conversely, α-carotene can induce G1-arrest in the cell cycle and has a higher suppressor activity than β-carotene for tumorogenesis in mouse skin, lung, liver and colon (Nishino *et al.*, 2002). Carotene deficient diets have been related to a general increase in oxidation susceptibility markers (IOM, 2000). The Institute of Medicine (IOM) panel on DRI (IOM, 2000) states that, although most studies have tested high doses of β-carotene obtained from food alone, these studies do not show an increase in oxidative or antioxidant markers on supplementation. Results seem more effective in subjects with a higher oxidative stress background (IOM, 2000).

β–carotene is an efficient quencher of singlet oxygen and free radicals. It can reduce DNA and chromosomal damage generated by various agents (Cozzi *et al.*, 1997). It is interesting to notice that, while low intake can significantly increase genome stability, high intake of β-carotene can induce a marginal increase in genome instability (Fenech *et al.*, 2005).

2.1.5 α– and β-Cryptoxanthin

The main contributor to the color of oranges is β-cryptoxanthin (Mouly *et al.*, 1999). It accounts for more than 50 % of the total pro-vitamin A carotenoids in OJ (Table 2).

Cryptoxanthin is distributed in our daily foodstuffs and is one of the major carotenoids detectable in human blood. β-cryptoxanthin seems to be a promising carotenoid, since it showed the strongest inhibitory effects on the Epstein-Barr virus activation activity of 12-O-tetradecanoylphorbol-13-acetate (TPA) in Raji cells, in comparison to 50 other carotenoids (Tsushima *et al.*, 1995; Nishino *et al.*, 2002). *In vivo*, β-cryptoxanthin showed anti-tumor promoting activity in mouse skin, but not in rat colon (Nishino *et al.*, 2002).

β-cryptoxanthin is one of the major carotenoids detectable in human blood (Nishino *et al.*, 2002) and a modest increase in its intake, equivalent to one glass of freshly squeezed orange juice per day, is associated with a reduced risk of developing inflammatory disorders such as rheumatoid arthritis (Fenech & Ferguson, 2001; Halliwell, 2001; Edenharder et al., 2002).

2.1.6 Non-pro-vitamin A carotenoids

2.1.7 Lutein and zeaxanthin

Lutein is the dihydroxy-form of α-carotene, and is distributed among a variety of fruit and vegetables, including oranges. Lutein shows anti-tumor promoting activity in the lung and skin of mice and the colon of rats (Nishino *et al.*, 2002). It is predominantly transported by HDL-cholesterol and to a lesser extent by LDL-cholesterol, sited in the outer region of the molecule due to its lower hydrophobicity (IOM, 2000).

Zeaxanthin is the dihydroxy-form of β-carotene, and is distributed in our daily foods. Zeaxanthin was found to suppress TPA-induced expression of the early antigen of Epstein–Barr virus in Raji cells, to inhibit TPA-enhanced ^{32}Pi-incorporation into phospholipids in cultured cells, and to suppress spontaneous carcinogenesis in male mice (Nishino *et al.*, 2002).

2.1.8 Cis-violaxanthin, mutatoxanthin and auroxanthin

Despite the high level of cis-violaxanthin, mutatoxanthin and auroxanthin in OJ (Table 2), data on the biological role of these compounds are still lacking. Cis-violaxanthin seems to be involved in the formation of the plant hormone abscisic acid (ABA), which plays a crucial role in the adaptation of plants to various environmental stresses and in several physiological processes, such as seed maturation and dormancy and fruit development, or senescence (Rodrigo *et al.*, 2006).

2.1.9 Remarks

Which is the most beneficial carotenoid is still undecided. In fact, is likely that carotenoids have synergistic effects. Nishino *et al.* (2002) found that multicarotenoids (a mixture of various carotenoids, such as β-carotene, α-carotene, lutein, lycopene, etc.) showed potent anti-carcinogenic activity. Anlasik *et al.* (2005) observed that healthy subjects aged 65 years and older, consuming high levels of fruit and vegetables daily, had significantly higher plasma levels of retinol, tocopherols and carotenoids. They also showed that malondialdehyde (MDA) levels were inversely correlated with vitamin A and α-carotene, as well as that protein carbonyls in Imunoglobulin G were inversely correlated with γ-tocopherol. Thus, a higher intake of retinol, tocopherols and carotenoids, mediated by a higher intake of fruit and vegetables, seems to be important for the prevention of age-related diseases.

There is no DRI for carotenoids and their supplementation for healthy subjects with normal intakes of carotenoids is not recommended (IOM, 2000).

Freezing preserves pro-vitamin A carotenoids and long term storage at -40°C has been show to substantially increase the amount of these compounds in OJ (Cortes *et al.*, 2005). Heat treatment may lead to significant loss of lutein and, to a minor extent, of pro-vitamin A carotenoids (Sanchez-Moreno *et al.*, 2005). It is generally thought that heating increases the

bioavailability of carotenoids and cooking vegetables rich in carotenoids can help to attain the vitamin A DRI.

It is important to mention that, while pasteurization has a mild effect on carotenoid content, sieving can remove large amounts of vesicle-bound liposoluble carotenoids, which tend to settle on the cellulose fragments (Mouly *et al.*, 1999). High pressure processing could be a way of increasing the amount of carotenoids in sieved juice; Sanchéz-Moreno *et al.* (2005) showed that high pressure extraction can significantly increase the amount of carotenoids in OJ. However, in this case it was observed that vitamin C is not proportionally increased and can be lost during storage. Lower levels of vitamin C offer less protection to carotenoids from oxidation and the unbalanced increase of carotenoids and vitamin C can result in further oxidation (Sanchez-Moreno *et al.*, 2003).

2.1.10 *D*-limonene

There are several volatile compounds responsible for OJ flavor. These include: ethyl butanoate, *D*-limonene, linalool, alpha-pinene, geranial, neral and alpha-terpineol (Pattison *et al.*, 2005).

D-limonene (p-mentha-1,8-diene) is the most studied of these compounds, since it is the most abundant in orange oil, comprising about 90% (Del Toro-Arreola *et al.*, 2005). It is likely that OJ obtained from squeezing of whole oranges would contain larger amounts of D-*D*-limonene, since it is the major constituent of orange peel. Juice extracted by an orange press contains about 100 (blood and bitter OJ) to 300 mg/L (sweet OJ) *D*-limonene, the major part (60-90%) being the volatile compounds (Moufida & Marzouk, 2003).

D-limonene was found to cause tumors, at high doses only, in the kidney of the male rat, in association with hyaline droplet nephropathy (Hard & Whysner, 1994); this led to the compound being considered as a carcinogen. Further evidence, however, showed that *D*-limonene was not genotoxic to colon, liver, kidney, bladder, lung, brain, or bone marrow of

either mice or rats (Sekihashi *et al.*, 2002), nor was it mutagenic to male Big Blue^{TM} rats (Turner *et al.*, 2001).

In 1999, *D*-limonene was reclassified from possibly carcinogenic to humans (group 2B) to not classifiable as to carcinogenicity for humans (group 3) by the International Agency for Research on Cancer (WHO, 1993; WHO, 1999).

Indeed, *D*-limonene has shown chemopreventive and therapeutic activity against a wide variety of experimental tumors, such as lung neoplasms, mammary cancer, and pancreatic and prostatic tumors (Crowell, 1999; Del Toro-Arreola *et al.*, 2005). Citrus peel consumption (the major source of dietary *D*-limonene) is not uncommon and is inversely related to the degree of skin cancer risk (Hakim *et al.*, 2000). However, it is important to consider the numerous compounds in citrus peel, such as psoralens, that have cancer promotion potential (Brendel & Henriques, 2001; Brendel *et al.*, 2003).

D-limonene is antioxidant, for example inhibiting ferric-ion-stimulated lipid peroxidation in rat brain homogenates(Youdim *et al.*, 2002; Grassmann, 2005).

D-limonene is a relatively stable terpene, but easily oxidizable, and OJ processing can particularly interfere in *D*-limonene stability, due to its easy oxidation. Moreover, it can be readily absorbed in high amounts by low-density polyethylene (LDPE), but not by polycarbonate (PC) and polyethylene terephthalate (PET), materials utilized in food packaging (Van Willige *et al.*, 2003). Pasteurization by continuous ohmic heating does not seem to influence *D*-limonene stability (Leizerson & Shimoni, 2005).

2.2   B Complex vitamins

Deficiencies in B series vitamins and folic acid (B9), which are essential for DNA synthesis and repair, are among the key causative factors for diabetes and organ damage (Wu & Ren, 2006).

2.2.1 B1 (thiamin)

Thiamin (B1) is the second most abundant vitamin in OJ. OJ can be a better source of vitamin B1 than many foods known as good sources, such as whole wheat bread (Dreher *et al.*, 2003). A 200 mL portion of OJ can supply 18.5% of the DRI for B1, OJ being a key source of this vitamin (Table 1 and Fig. 1).

B1 in its diphosphate form is an important coenzyme for transketolase (TK) and pyruvate dehydrogenase, among others, which are important in the biosynthesis of a number of cell constituents, including neurotransmitters, and for the production of reducing equivalents used in oxidative stress defenses (e.g. maintenance of NADPH levels), as well as for the synthesis of pentoses that are used as nucleic acid precursors(Singleton & Martin, 2001; Shangari *et al.*, 2005)

There is growing evidence for the role of B1 in preventing several types of cancers, as well as neurodegeneration (Gibson & Zhang, 2002; Lee *et al.*, 2005). B1 deficiency may pose a cancer risk, owing to the deficiency of thiamin-dependent enzymes involved in glycolysis and an increase of reactive intermediates in carbohydrate metabolism-oxaldehydes, generated by the spontaneous rearrangement of triose phosphates (Bruce *et al.*, 2003). Hyperglycemia can increase the accumulation of α-oxoaldehydes.

B1 and derivatives, at high doses, can increase the conversion of triose phosphates to ribose-5-phosphate, and strongly inhibit the development of oxidative stress, protein glycation and/or inflammatory responses, either *in vivo* or *in vitro* (Babaei-Jadidi *et al.*, 2003; Nandi *et al.*, 2005; Wu & Ren, 2006). TB1 diphosphate and thiamin can modulate p53 activity, affecting apoptosis (McLure *et al.*, 2004). Moreover, B1 transporter gene expression and exogenous B1 modulate the expression of genes involved in drug and prostaglandin metabolism in breast cancer cells (Liu *et al.*, 2004). The sulfhydryl group of B1 can act as a chelant or an antioxidant.

On the other hand, some authors claim that thiamin can stimulate tumor cell survival, proliferation and chemotherapy resistance, by the stimulation of TK synthesis leading to a high rate of nucleic acid ribose synthesis (Cascante *et al.*, 2000; Lee *et al.*, 2005). Thus, excess B1 supplementation in common food products may contribute to the increased cancer rates of the Western world and anti-B1 therapy should be used (Boros *et al.*, 1998; Boros, 2000). In fact, McLure *et al.* (2004) have shown that thiamin reduces p53 induced apoptosis in irradiated murine thymocytes.

B1 is relatively unaffected by light and stable to oxidation, but it is among the least stable vitamins in solution at neutral pH (Vercet *et al.*, 2001). B1 decomposition leads to the formation of meat flavors by thermal degradation of B1, as well as through a Maillard reaction involving cysteine and various sugars. One of the most significant B1 thermal degradation products is 2-methyl-3-furanthiol (MFT) (Dreher *et al.*, 2003). This compound and other Maillard reaction products seem to quench reactive oxygen species and inhibit LDL-oxidation (El-massry *et al.*, 2003).

2.2.2   B2 (riboflavin)

A standard 200 mL portion of OJ can provide about 10% of the DRI for riboflavin (B2) and OJ is an excellent source of it (Table 1 and Fig. 1). B2, in eukaryotes, is converted to flavin adenine dinucleotide (FAD). FAD is a coenzyme for glutathione reductase and other enzymes. Flavin coenzymes are widely distributed in the body and are involved in carbohydrate, fat, amino acid and vitamin metabolism. Oxidative folding (formation of disulfide bonds) in the endoplasmic reticulum (ER) before the secretion of proteins, such as apolipoprotein B-100 (apoB), depends on flavoproteins in eukaryotes. Furthermore, B2 influences epithelial integrity, by modulating tissue flavin concentrations, the rates of prostaglandin biosynthesis and glutathione metabolism (Manthey *et al.*, 2005; Siassi & Ghadirian, 2005; Manthey *et al.*, 2006).

B2 deficiency causes ER stress and activation of unfolded protein response and decreases protein secretion in HepG2 cells, possibly interfering with lipid homeostasis *in vivo*, in the case of apoB secretion. B2 deficiency is also associated with decreased activity of glutathione reductase and with decreased concentrations of reduced glutathione in human cells, as well as with an increase in B2-deficient cells arrested in G1 phase of the cell cycle. Moreover, oxidative stress caused by B2 deficiency is associated with increased expression of clusters of genes that play a role in cell stress and apoptosis (Manthey *et al.*, 2005; Siassi & Ghadirian, 2005; Manthey *et al.*, 2006).

B2 deficiency may predispose esophagus squamous carcinogenesis by altering gastrointestinal function. A high intake of B2 can generate genome instability (Fenech *et al.*, 2005) and has been associated with increased gastric cancer risk, although it may reduce the frequency of micronucleated cells and carcinogen DNA adducts and the associated esophageal adenocarcinoma (Manthey *et al.*, 2005; Siassi & Ghadirian, 2005; Manthey *et al.*, 2006). The precise role of B2 in the development of this tumor at different stages of initiation, promotion and progression is controversial. It is plausible to hypothesize that B2 deficiency enhances the risk of tumor development at initiation and promotion stages, while reducing tumor growth at the progression stage (Siassi & Ghadirian, 2005).

B2 has received much less attention than other vitamins, but there is increasing interest in the well-established role that flavins play in folate metabolism and the possible synergy of a protective effect between these 2 vitamins. B2, as FAD, is a cofactor for 5,10 methylene tetra hydro folate reductase (MTHFR) and there is evidently some interaction between B2 status, folate status, and plasma homocysteine (Powers, 2005).

B2 and UV light in combination selectively enhance damage to guanine bases in DNA, making the damage less likely to be repaired by normal repair pathways in host cells (Kumar *et al.*, 2004).

B2 is a heat stable vitamin, but it is very sensitive to oxidation and mainly to degradation by light. Its oxidation causes not only a loss in the nutritive value of dairy products but also off-flavors (Vercet *et al.*, 2001).

2.2.3 B3 (niacin)

A standard 200 mL portion of OJ can provide about 5 % of the DRI for niacin and thus OJ juice is a good source of niacin (Table 1 and Fig. 1). Niacin (B3) is the common name for two compounds: nicotinic acid and nicotinamide. Niacin is a water-soluble vitamin that participates in many metabolic functions. It is a precursor for nicotinamide adenine dinucleotide (NAD), which is required for DNA synthesis, as well as for the activity of the enzyme poly(ADP-ribose) polymerase-1 (PARP-1; EC 2.4.2.30), for which NAD is the sole substrate (Hageman & Stierum, 2001).Thus, B3 has beneficial effects, as a redox agent in energy metabolism and as an antioxidant. It also influences DNA repair and genome stability, as well as the immune and nervous systems (Ikeda *et al.*, 2003; Kirkland, 2003).

The action of NAD in protecting biological systems against oxidative stress and DNA damage can be related to an up-regulating of the stress response enzymes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and glucose 6-phosphate dehydrogenase (G6PD), among others. These enzymes are involved in the generation of the antioxidant reduced pyridinenucleotides, NADPH and NADH, from NAD (Yan *et al.*, 1999; Crowley *et al.*, 2000).

PARP-1, strongly activated by DNA strand breaks during the cell genotoxic stress response, is involved in base excision repair, and plays a role in p53 expression and activation; hence it is thought to be important for genome stability. In addition to being a precursor for NAD, niacin has also been reported to possess oxygen radical scavenging activity comparable to that of ascorbic acid (Hageman & Stierum, 2001). Finally, NAD and

NADP are required for the synthesis of cyclic ADP-ribose and nicotinic acid adenine dinucleotide, two mediators of intracellular calcium signaling pathways (Kirkland, 2003).

B3 has an impact on cancer risk, according to data obtained from animal models of leukemogenesis and skin cancer, as well as epidemiological data from human populations, and on side effects of chemotherapy in cancer patients (Kirkland, 2003; Hirakawa *et al.*, 2005). *In vitro* and animal studies indicate that B3 deficiency increases genome instability and the risk of certain tumors, especially in combination with genotoxic and oxidative stress (Crowley *et al.*, 2000).

While pellagra (a disturbance with nervous, gastrointestinal and dermatological effects) is the consequence of severe B3 deficiency, excessive intake of this vitamin in supplements is associated with hepatic toxicity, nausea and vomiting, as well as flushing (IOM, 1998).

Both the beneficial effect and the noxious effect of B3 on cancer can be related to PARP-1. On the one hand, B3 replenishes NAD, avoiding the cell death related to PARP induced draining of the NAD+ pool, which may compromise the further repair of DNA and mitochondrial respiration (i.e affecting electron transport, redox state and membrane potential) (Crowley *et al.*, 2000). On the other hand, excessive levels of nicotinamide in the body may result in inhibition of PARP-1 and increased genome instability. More studies are therefore needed to define an optimal level of B3 to avoid genome stability and tumorigenesis (Hageman & Stierum, 2001). B3 has a role in the maintenance of telomere length. Low intake of nicotinic acid is related to increased genome instability (Fenech *et al.*, 2005).

Tryptophan can be converted to B3 in a vitamin B6-dependent reaction, as well as, with low efficiency, by enteric bacteria. B3 seems to be stable and is used as a preservative for drugs and foods (IOM, 1998), it is more stable than thiamin and B2. It is resistant to heating and light (Mahan & Escott-Stump, 1996).

2.2.4 B5 (pantothenic acid)

A standard 200 mL portion of OJ can provide about 7 % of the DRI for B5, and is a good

source of it (Table 1 and Fig. 1). Pantothenic acid is a water-soluble vitamin important for the

metabolism of carbohydrates, proteins and lipids. It is part of coenzyme A, acting in the fatty

acid oxidation and citric acid cycles. Pantothenic acid, as pantothenate, is present in food

mostly as CoA, which cannot be directly absorbed through enterocytes, whereas pantothenate

freely diffuses across the epithelial barrier. Thus, one might speculate that conversion of CoA

into pantothenate requires an extracellular, membrane-bound pantetheinase capable of

recycling pantothenate in the gut. Pantetheinase is a ubiquitous enzyme, which has been

shown *in vitro* to recycle pantothenic acid and to produce cysteamine, a potent anti-oxidant.

Membrane-bound pantetheinase is the main source of cysteamine in tissues under

physiological conditions (Pitari *et al.*, 2000).

Pantethine, pantothenate, and phosphopantothenate can partially prevent lipid

peroxidation in hepatic and heart tissue. They have beneficial effects on wound healing as

well as inflamation, in the latter case by blocking the release of myeloperoxidase from

neutrophils. Pantothenate and some related compounds protect Ehrlich ascites tumor cells

against lipid peroxidation, but not due to scavenging of free radicals. In fact, these compounds

exerted only a negligible effect on peroxidation and it is likely that some metabolic products

of pantothenic acid or its derivatives, rather than the original compounds, were effective

(Slyshenkov *et al.*, 1996).

There are two possible explanations for the antioxidant potential of panthotenic acid:

the antioxidant potential of cysteamine and maybe gluthathione generated by pantetheinase,

and the enrichment of cells with CoA, with resulting changes in metabolism. As evaluated by

Slyshenkov *et al.* (1996), the preincubation of cells with pantothenic acid and some of its

related compounds greatly increased the cell level of CoA. According to the authors, this is

evidence that CoA may facilitate removal of lipid peroxides by increasing metabolism of fatty acids and promote repair of the plasma membrane by activating phospholipid biosynthesis. It can only be speculated that elevated levels of CoA may increase the supply of a glutathione precursor, i.e., glutamate, by accelerating the tricarboxylic acid cycle (Slyshenkov *et al.*, 1995).

High intake of pantothenic acid is associated with increased genome instability, as measured by micronucleus frequency. Only one study suggested a marginal promoting effect induced by calcium pantothenate in the Balb/c-3T3 assay (Fenech *et al.*, 2005).

Pantothenic acid is easily destroyed by heat in acidic or alkaline conditions, but is stable in neutral solution (Mahan & Escott-Stump, 1996). Substantial losses of vitamin B5 can occur after freezing (Pallaoro, 1997).

2.2.5 **B6 (pyridoxine, pyridoxamine, etc.)**

A standard 200 mL portion of OJ can provide about 7% of the DRI for vitamin B6 and is a good source of it (Table 1 and Fig. 1). There are several structural isomers (vitamers) of vitamin B6 [e.g. pyridoxamine (PM) and pyridoxine] that share a general enzymatic mechanism. PM is a transient intermediate in enzymatic transamination reactions. Dietary PM is consumed mostly in the form of PM-5'- phosphate, which is hydrolyzed to PM by intestinal phosphatases and further metabolized in the liver and, to a lesser degree, in intestinal tissues (Voziyan & Hudson, 2005)**.**

PM is a promising pharmacological agent for protecting against the progressive tissue damage that occurs in diabetes and other diseases, because it is a potent inhibitor of the formation of advanced glycation end products (AGEs), deleterious protein modifications. There are three likely mechanisms to explain the inhibitory action of vitamin B6 on AGE formation: a) blockage of oxidative degradation of the Amadori intermediates of the Maillard reaction; b) scavenging of toxic carbonyl products of glucose and lipid degradation; and c)

trapping of reactive oxygen species (Voziyan & Hudson, 2005). High levels of vitamin B6 can suppress the growth of animal or human cancer cells *in vitro*, suppress nitric oxide, and supress angiogenesis (Anand, 2005; Wei *et al.*, 2005).

At normal levels, vitamin B6 seems to be related to the regeneration of 5,10-methylene tetrahydrofolate, which is involved in avoiding the misincorporation of uracil in place of thymine in DNA. Thus, deficiency of vitamin B6 can induce genome instability. The anticarcinogenic/antimutagenic property of vitamin B6 may be related to this aspect, as well as to its role in alternative pathways (e.g. as a co-factor in amino acid metabolism, lipid metabolism, and nervous system function) (Wei *et al.*, 2005). Beside these above mechanisms, pyrodoxal and pyridoxal 5'-phosphate seem to promote DNA excision repair directly or indirectly and to decrease the amount of unrepaired DNA damage, reducing mutations in *E. coli* and micronuclei in mouse peripheral blood cells (Shimoi *et al.*, 1992). However, one should recall that pyridoxine, like riboflavin, can generate cytotoxic photoproducts after UV-irradiation (Sato *et al.*, 1993).

Vitamin B6 is quite stable to heat but is sensitive to air, UV light and alkali. Freezing can substantially reduce (36-55 %) vitamin B6 content (Mahan & Escott-Stump, 1996).

2.2.6 B9 (folic acid)

Folate, a water-soluble vitamin, includes naturally occurring food folate and synthetic folic acid in supplements and fortified foods. OJ can be a good source of folate, given the instability of the compound in cooking. A standard 200 mL portion of OJ can provide about 14% of the DRI for folate and is, indeed, a key source of it (Table 1 and Fig. 1).

Inadequate folate status may result in hyperhomocysteinemia, a significant risk factor for atherosclerotic vascular disease, as well as changes in DNA that may result in pro-carcinogenic effects and increased risk of cognitive dysfunction. Folate status may be negatively influenced by inadequate intake, genetic polymorphisms and interactions with

various drugs. Folate-rich foods include OJ, dark green leafy vegetables, asparagus, strawberries and legumes. These foods are also excellent sources of other health-promoting nutrients associated with reduction in risk of chronic disease (Brevik *et al.*, 2005)**.** Thus, plasma folate concentration may be a useful biomarker for the intake of fruit and vegetables in populations consuming unfortified food products. The association can be attenuated by, and should be corrected for, individual intake of folic acid supplements (Clifford *et al.*, 1991).

Folate coenzymes participate in biochemical processes involving single carbon group transfers, including the metabolism of amino acids and synthesis of purines and pyrimidines that are incorporated into DNA and RNA (Rampersaud *et al.*, 2003).

Folate has a key role in genome stability and DNA homeostasis. Folate is important for the maintenance of telomere length and for the prevention of uracil incorporation into DNA, as well as for the maintenance methylation of cytosines adjacent to guanine sequences (CpG) in DNA (Fenech, 2005).

FA is a poor antioxidant. The more FA is reduced, the higher its antioxidant activity becomes: THF and 5-MTHF are the folates with the most prominent antioxidant activity. The antioxidant pharmacophore of THF and 5-MTHF, i.e. 4-hydroxy-2,5,6-triaminopyrimidine, resides in the pterin moiety. It is suggested that an electron donating effect of the 5 amino group is of major importance in the antioxidant activity of 4-hydroxy-2,5,6-triaminopyrimidine. A similar electron donating effect is probably involved in the antioxidant activity of THF and 5-MTHF (Rezk *et al.*, 2003).

Folate in food is very unstable and considerable losses occur during short-term storage and cooking. Folate decomposition has been related to the widespread under-provision of folate common in human populations. However, food folate bioavailability and the factors that affect it are poorly understood (McNulty & Pentieva, 2004). The consumption of whole OJ with minor processing after extraction could be a better way to optimize the intake of

folate, since folate from OJ is as available as in beef liver and folic acid supplements (Gorinstein *et al.*, 2004).

2.3   Vitamin C

A standard 200 mL portion of OJ can provide more than 100% of the DRI for vitamin C and is a key source of it (Table 1 and Fig. 1).

Vitamin C is an important micronutrient, mainly required as a co-factor for enzymes involved in oxi-reduction reactions (Lunec *et al.*, 2002). Vit C acts as an electron donor for 8 human enzymes, involved mainly in the biosynthesis of collagen and carnitine (WHO, 2001). It has an increasing involvement in supporting intracellular redox status by maintaining important sulphydryl compounds, such as glutathione, in a reduced state (Vijayalaxmi & Venu, 1999; Edenharder *et al.*, 2002). It has been studied for its protective action against various diseases (Kojima *et al.*, 1992; Guha & Khuda-Bukhsh, 2002).

Vit C is antioxidant (Halliwell & Guterridge, 2000), bio-antimutagenic and/or desmutagenic (Sram *et al.*, 1983; Kojima *et al.*, 1992; Vijayalaxmi & Venu, 1999; Guha & Khuda-Bukhsh, 2002). Antimutagenic activity is related to a reduction in the fixation of mutations, while desmutagenic activity means the reduction/inactivation of the mutagenicity of a given substance (Kuroda *et al.*, 2001). According to Wang *et al.* (1996), only about 15% of OJ antioxidant potential is due to vitamin C.

Vitamin C can compete with DNA as the target for alkylation, reducing the genotoxicity of alkylating agents (Sakagami *et al.*, 2000), as well as scavenging free radicals. Vit C can also chelate metals, blocking the generation of free radicals (Halliwell & Guterridge, 2000). Moreover, it has a role in the regulation of DNA repair enzymes (Schwedhelm *et al.*, 2003). High concentrations of vitamin C induce apoptotic cell death (Cooke *et al.*, 1998).

It is important to note that vitamin C at high doses can function as a pro-oxidant, generating oxygen radicals by itself (Cozzi *et al.*, 1997).

Vitamin C is not protein-bound and is eliminated with a half-life of 10 hours (Middleton *et al.*, 2000; Tassaneeyakul *et al.*, 2000; Ferguson, 2001; Edenharder *et al.*, 2002; Kelly *et al.*, 2003). It is quite resistant to heat treatment at low pH values, but is sensitive to oxidation (Vercet *et al.*, 2001). Pasteurization has a mild influence on vitamin C content (Gil-Izquierdo *et al.*, 2002); however, storage seems to be a major issue. Leizerson & Shimoni (2005) showed no influence of heating on the degradation rate of ascorbic acid. They showed that the content of vitamin C was reduced by about 55% in 100 days of storage at 4°C. Franke *et al.* (2004) showed a similar reduction of vitamin C content (-20°C) when OJ was frozen for 9 months. This is important, since freezing is a technique widely used in the industry to preserve OJ, when production exceeds the market demand (Sanchez-Moreno *et al.*, 2005). It is interesting to bear in mind that pulp suffers a loss of 58% in vitamin C due to pasteurization. However, pulp contributes only 10 % to the juice (Gil-Izquierdo *et al.*, 2002). High pressure extraction does not improve the vitamin C level in OJ (Sanchez-Moreno *et al.*, 2005). Gil-Izquierdo *et al.* (2002) showed that commercially squeezed OJ contains 25 % more vitamin C than domestically squeezed, due to the processing of the whole fruit, particularly the solid portion, in the former.

The reducing potential of vit C protects other substances from oxidation. The hydroxyl groups in C3 can be easily liberated, explaining much of its activity (Spada & Silva, 2004). In oxidizing environments it can, however, be very pro-oxidant. Indeed, there is no consensus as to whether it will act as an antioxidant (intercepting/quenching) or as a pro-oxidant (reducing transition metals and forming ROS), owing to the very complex physiological chemistry of mutation.

In general, vitamin C is tolerable up to 1 gram per day, for avoiding gastrointestinal distress and kidney stone formation (WHO, 2001). The upper level for preventing stones formation is 1.8 g/day and it is not easy to achieve this value solely by the consumption of OJ. However, there are risks attached to such high consumption of OJ in the context of a diet rich in fresh fruits and vegetables. Supplementation/fortification with vit C can worsen this situation.

2.4 Phenolic compounds

Phenolic compounds are another constituent of OJ. Flavonoids are naturally occurring polyphenols synthesized by all vascular plants that can protect biological systems in different ways (Lee *et al.*, 2003a; McPhail *et al.*, 2003; Wilmsen *et al.*, 2005). The phamacokinetics of the few polyphenols so far assessed are as diverse as their chemical structures (Manach *et al.*, 2004).

Citrus flavonoids, including those of OJ, have diverse pharmacological activities: a) antioxidant, b) lipid profile optimizer (by lowering lipids and cholesterol levels), c) anticarcinogenic and d) anti-inflammatory. Two phenolic compounds are thought to be responsible for much of the biological activity of citrus juices, hesperidin and narirutin, together with their aglycones, hesperitin and naringenin. Hesperidin is the main flavonoid in oranges, while narirutin is predominant in sour oranges, although it is the main flavonoid in grapefruit.

Phenolic compounds have a dual effect on phase I and phase II enzymes, on the one hand repressing some enzymes (mainly in phase I) and on the other stimulating them (mainly in phase II) (Doostdar *et al.*, 2000). One could relate this potential to the partial protection of smokers ingesting dietary phenolics, probably flavonoids, against the harmful effects of tobacco carcinogens on bladder mucosal cells (Malaveille *et al.*, 1996).

While previous studies (Hertog *et al.*, 1993) have shown the presence of quercitin in OJ, Vanamala *et al.* (2006) did not detect it in 26 commercially available OJs in the United States. In the latter study, it was found that hesperidin accounted for 84% of the total flavonoids, in agreement with other studies (Pupin *et al.*, 1998; Mouly *et al.*, 1999). The second most abundant flavonoid is narirutin, which accounts for about 12-15 of total flavonoids (Pupin *et al.*, 1998; Mouly *et al.*, 1999; Vanamala *et al.*, 2006), while the third is dydimin, accounting for less than 5 % of the total (Vanamala *et al.*, 2006) (Table 3).

Flavanones tend to precipitate at low pH and low temperature, leading to an increase in the proportion of flavanones in the vapor during processing (Sanchez-Moreno *et al.*, 2005). Gil-Izquierdo *et al.* (2001) compared the loss of flavonoids in the soluble fraction and the vapor fraction (pulp) and showed differential loss. Didymin was the most labile flavanone, losing about 52% in the soluble fraction compared to the levels before concentration, while hesperidin and naringenin showed only a slight decrease (Vanamala *et al.*, 2006). Thus, didymin showed losses of only 1% in OJ pasteurizing, being the more stable compound (Vanamala *et al.*, 2006).

Commercially squeezed OJ provides more flavanones than domestically squeezed, hesperidin being 4 times higher in commercial OJ, probably due to the commercial squeezing method (Gil-Izquierdo *et al.*, 2002).

In a study comparing two groups of commercially available OJ in the United States, it was observed that the content of hesperidin varies more in pasteurized not-from-concentrate (NFC) OJ than in made-from-concentrate (MFC) OJ. Moreover, an increase of 45 %, 63 % and 44% was observed for, respectively, hesperidin, narirutin and total flavonoid in MFC above that in NFC (Vanamala *et al.*, 2006). These results are in agreement with Gil-Izquerdo *et al.* (2002), who showed a 2-fold increase in flavanones in commercial squeezing. On the other hand, freezing can reduce flavonones by about 23, 39 and 43 % for, respectively,

156

didymin, hesperidin and narirutin (Gil-Izquierdo *et al.*, 2002). Gil-Izquierdo *et al.* (2002)

showed a decrease in total phenolics, but Franke *et al.* (2004) tested the effect of freezing,

storage (90 days at -20°C) and thawing in the levels of total phenolics in OJ and observed

similar levels in the frozen and fresh form of the same OJ, a slightly higher level being

observed for the frozen samples. It is interesting to mention differences in the processes used

by the two studies. Gil-Izquierdo *et al.* (2002) froze the samples at -40°C and re-pasteurized

them after thawing, while Franke *et al.* (2004) maintained the samples at -20°C and analyzed

the level of phenols immediately after thawing in the refrigerator (4°C). Further studies are

still necessary. However, it is likely that freezing and thawing lead to the alteration of one or

more hydroxyls or oxidation of the phenolic compounds, generating either qualitative or

quantitative alterations in the phenolic content.

Data on the biovailability of flavanones in plasma are scarce and plasma metabolites

have not yet been identified, although undefined monoglucuronides of hesperitin, accounting

for about 9% of the intake, appear to be the most abundant metabolites of OJ in plasma.

Microbial metabolites of naringenin and hesperitin, either *in vivo* (urine of rats), or *in vitro*

test systems, are *p*-hydroxyphenylpropioinc acid, *p*-coumaric acid and *p*-hydroxybenzoic acid

(Manach *et al.*, 2005).

2.4.1 Hesperidin and hespiritin

Hesperitin is the aglycone of the flavanone hesperidin. Both compounds have antioxidant

(Garg *et al.*, 2001) and anticarcinogenic (Berkarda *et al.*, 1998; Kohno *et al.*, 2001), anti-

inflammatory (Emim *et al.*, 1994; Crespo *et al.*, 1999; Rotelli *et al.*, 2003), analgesic (Galati

et al., 1994) anti-allergic (Matsuda *et al.*, 1991; Lee *et al.*, 2004), hypolipidemic and

vasoprotective (Monforte *et al.*, 1995; Lee *et al.*, 2003b; Kurowska & Manthey, 2004) and

hormonal (Zierau *et al.*, 2004) activity. Hesperidin also has tranquilizing and sleeping

intensifier properties (Fernandez *et al.*, 2005). Thus, hesperidin and hesperitin are used as supplements and in medicine.

Long term studies showed that methyl hesperidin was not carcinogenic (Kurata *et al.*, 1990), nor did it induce effects on body and organ weight, food and water consumption, hematology, clinical chemistry, or on gross and histopathological examination of the major organs (Kawabe et al., 1993).

Hesperidin and hesperitin are weaker antioxidants than other phenolics present in foods. In comparison to other flavonoids and non-flavonoid antioxidants, hesperidin is inactive or only moderately active (Berkarda *et al.*, 1998). Hesperidin did not appreciably suppress the autoxidation of linoleic acid (Wang & Zheng, 1992) and had a low inhibitory activity (about 10 %) on superoxide anion radical genesis (at 10^{-4} and 5×10^{-4}M), as well as on non-enzymatic lipid peroxidation (at 10^{-3}M). Indeed, hesperidin seems to have low antiradical and very weak anti-H_2O_2 activities (Sroka et al., 2005) and does not affect hydroxyl radical formation when the ascorbic acid-Fe^{3+}-EDTA system is used (Suarez *et al.*, 1998).

Conversely, Wilmsen *et al.* (2005) showed that hesperidin has considerable antioxidant activity by sequestering 1,1-diphenyl-2-picrylhydrazyl (DPPH). Okamura *et al.*(1994), using a ferric thiocyanate method, evaluated the antioxidant activities of hesperidin and three other flavonoid glucuronides of luteolin isolated from an extract of *Rosmarinus officinalis* (rosemary) leaves, and detected that hesperidin showed the greatest antioxidant activity. Hesperitin, like other flavonoids, was found to reduce ferrylmyoglobin, MbFe(IV)=O, to metmyoglobin, MbFe(III), possibly due to the presence of only one hydroxyl group in the B-ring of the molecule (Jorgensen & Skibsted, 1998). In fact, hesperidin and other flavonoids function as antioxidants mainly by chelating iron ions and by scavenging peroxyl radicals, whereas their OH radical scavenging effect is much less important (Deng *et al.*, 1997).

Hesperidin and hesperitin present anti-inflammatory activity, shown by their ability to suppress nitric oxide formation, as well as inducible nitric oxide synthase (iNOS) and COX-2 gene expression and prostaglandin E_2 synthesis. Moreover, hesperitin, but not hesperidin, induced heme oxygenase 1 (HO-1) protein expression, in the presence or absence of lipopolysaccharide (LPS). These responses link the anti-inflammatory and the antioxidant activity of the compounds (Sakata *et al.*, 2003; Lin *et al.*, 2005; Saito *et al.*, 2005).

Removal of rutinose at position C7 of hesperidin by enzymatic digestion with hesperidinase produced inhibitory activity on LPS-induced NO production, according to the production of the aglycones and corroborated by high-performance liquid chromatography. Indeed, rutinose at C7 is a negative moiety in flavonoid inhibition of LPS-induced NO production (Lin et al., 2005).

Despite being a weak antioxidant *in vitro*, there are several pieces of evidence for a strong antioxidant effect *in vivo*. Wilmsen *et al.* (2005) evaluated the antioxidant activity of hesperidin *in vivo* using the eukaryotic cells of superoxide-dismutase proficient and deficient strains of *Saccharomyces cerevisiae*. Their results clearly demonstrated that hesperidin protected against the damaging effects induced by paraquat (methylviologen; 1,1'-dimethyl-4,4'-bipyridinium dichloride) and hydrogen peroxide.

Miyake *et al.* (1998) investigated the antioxidant effect of 28 day diets supplemented with 0.2 % of either crude flavonoids, eriocitrin or hesperidin, prepared from lemon juice, in streptozotocin-induced diabetic rats. After 28 days treatment, the level of thiobarbituric acid-reactive substance in the serum, liver, and kidney of diabetic rats, as well as of 8-hydroxydeoxyguanosine (produced from deoxyguanosine owing to oxidative stress) in the urine, significantly decreased for all treatments.

Subcutaneous application of hesperidin did not inhibit 7,12-dimethyl benz(a)anthracene-induced tumor initiation but did inhibit 12-O-tetradecanoyl-13-phorbol

acetate-induced tumor promotion (two polycyclic aromatic hydrocarbons) in the skin of CD-1 mice (Berkarda et al., 1998). Intraperitonial introduction of hesperidin can substantially suppress CCl_4-induced hepatosis in Wistar rats, by decreasing the formation of nitric oxide radicals (Timoshin et al., 2005). Hesperidin may play an important role in the protection of populations chronically exposed to arsenic, since it reduced As(III)-induced acute toxicity in the liver and kidneys of mice (das Neves *et al.*, 2004).

Hesperidin was anticarcinogenic in the initiation phase of 4-nitroquinoline 1-oxide (4-NQO)-induced tongue carcinomas in male rats (Kohno *et al.*, 2001). Either alone (1000 ppm), or in combination with diosmin (900 ppm diosmin + 100 ppm hesperdin), it inhibited the development of oesophageal, colonic or oral cancer in male rats induced with N-methyl-N-amylnitrosamine (MNAN), azoxymethane or 4-nitroquinoline 1-oxide (4-NQO) (Kohno *et al.*, 2001). Hesperidin either singly (1000 ppm) or in combination with diosmin (4,900 ppm diosmin + 100 ppm) inhibited the development of N-butyl-N-(4-hydroxybutyl)nitrosamine (OH-BBN)-induced urinary-bladder carcinogenesis in male ICR mice (Yang *et al.*, 1997). The chemoprotective effect is accompanied by a reduction in translation and replication of DNA, as evaluated by the frequency of silver-stained nucleolar-organizer-region-associated proteins (AgNORs) and the 5-bromodeoxyuridine (BrdU)-labeling index, respectively (Yang *et al.*, 1997; 2001).

Hesperetin and hesperidin were among the most potent compounds, completely inhibiting neoplastic transformation induced by 3-methylcholanthrene in C3H 10T1/2 murine fibroblasts when applied at 1.0 μM after exposure to the carcinogen. This activity was related to the vicinal diphenol structure in ring 'B' and a saturated 'C' ring present in the molecule (Franke *et al.*, 1998).

Hirose *et al.* (1999) evaluated the effect of diets supplemented with 0.5% hesperidin and other phenolic compounds on 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx)-

induced hepatocarcinogenesis in male F344 rats. They observed that, while hesperidin induced an insignificant increase (2-3%) and green tea chatechin had a slight and non-significant decrease (27-35%), all other phenolic compounds mildly to severely increased (27-127%) the number and area of preneoplasic lesions (glutatione *S*-transferase placental form foci) induced by MeIQx, showing significant co-carcinogenic effects.

Hesperidin was only weakly antimutagenic against benzo[a]pyrene, but not against 2-aminofluorene and nitroquinoline N-oxide, as shown by the *Salmonella*/microsome assay (Calomme *et al.*, 1996). Conversely, hesperetin and hesperidin, either in isolation or as inclusion complexes with (2-hydroxypropyl)-β-cyclodextrin, reduced DNA damage generated in freshly collected whole blood incubated with benzo[a]pyrene (BaP). Reduced DNA damage in stressed cells was observed with all antioxidant compounds tested and it was more pronounced with complexed flavanones than with free compounds. The highest dose provided effective protection even with free flavanones (Tommasini et al., 2005).

Hesperidin was dose-dependent antimutagenic (0.1-1 mg/2 mL) against 1-methyl-1,2,3,4-tetrahydro-beta-carboline-3-carboxy acid (MTCCA), a mutagen precursor of soy sauce, in the *Salmonella*/microsome assay with strain TA100 in the presence of S9 mix (Higashimoto et al., 1998).

Hesperitin can selectively inhibit human cytochrome P450 (Kelly *et al.*, 2003), reducing the absorption/elimination of toxic compounds. Heperidin/hesperitin inibits CYP1A1 and CYP1B1 *in vitro*. The CYP1 family is thought to be responsible for the activation of pro-carcinogens (Moon et al., 2006).

The content of hesperitin is not modified either by pasteurization or by freezing (Sanchez-Moreno *et al.*, 2005).

2.4.2   Narirutin, naringin and naringenin

Naringenin is the aglycone of the flavonone naringin. Naringin is the bittering compound in oranges and the application of naringinase has been used for industrial debittering of OJ (Puri *et al.*, 1996).

Naringenin lowers blood lipids (Santos *et al.*, 1999), blocks Herpes virus replication (Lyu *et al.*, 2005) and has hormonal (Szaefer *et al.*, 2004) and immunomodulatory activities (Lyu & Park, 2005).

Naringenin is reported to have anti-proliferative effects in many cancer cell lines by antioxidant action, as well as by kinase and glucose uptake inhibition mechanisms and by its hormonal action, which induces apoptosis (Totta *et al.*, 2004). However, it also inhibits apoptosis (Choi *et al.*, 2004).

Naringenin is cardio and nephroprotective as well as protecting against ethanol-induced gastric lesions (Motilva *et al.*, 1994; Badary *et al.*, 2005; Mojzisova *et al.*, 2006). It can be cytotoxic to tumor cells (Shen *et al.*, 2004). It is a strong mutagen in *Salmonella typhimurium,* although it is also a strong antimutagen in the same system (Edenharder & Grunhage, 2003). Naringenin protected DNA from UV-induced damage in template plasmid DNA in an *in vitro* run-off transcription assay (Kootstra, 1994).

Naringenin was cytotoxic, genotoxic and pro-oxidant in human lymphocytes *ex vivo*. It also inhibited the activity of glutathione reductase (Yen *et al.*, 2003). It induced a pro-oxidant-like concentration-dependent peroxidation of nuclear membrane lipids, concurrent with DNA strand breaks, which was generally not affected by antioxidants (Sahu & Gray, 1997).

However, early findings have shown naringenin can protect against lipid oxidation by modulating cyclo-oxygenase and lipoxygenase (Baumann *et al.*, 1980). A more recent report shows that naringenin has a low antioxidant potential, being a weak free radical scavenger

162

(Edenharder & Grunhage, 2003). Thus, its antimutagenic potential cannot be explained by radical scavenging effects and must be caused by some other characteristics, as yet unknown (Edenharder & Grunhage, 2003).

Naringin has exceptional antimutagenic activity; less than half the equimolar amount can reduce the mutagenic potency of the direct-acting carcinogen N-methyl-N'-nitro-N-nitrosoguanidine by 50% in *Salmonella typhimurium* strain TA100 NR. Indeed, it attenuates human cytochrome P450-mediated activation of aflatoxin B1 to the exo-8,9-epoxide (Guengerich *et al.*, 1994).

Naringenin also suppressed the mutagenicity of a nitrosamine in humanized (CYP competent) *Salmonella typhimurium* strain, because of its capacity to modulate CYP activity, depending on its concentration (Kang *et al.*, 2004). Naringenin inhibited the mutagenicity of the heterocyclic amines MeIQx and Glu-P-1 in *S. typhimurium* TA98 with S9 fraction (Bear & Teel, 2000). In such cases, the antimutagenic action could be mediated by preventing passage of the carcinogen into bacterial cells, or by altering some cellular processes (Francis *et al.*, 1989).

Gao *et al.* (2006) have shown that naringenin stimulates mRNA expression of 8-oxoguanine-DNA glycosylase 1 (hOGG1), apurinic/apyrimidic endonuclease and DNA polymerase β, three main DNA repair genes of the base excision repair (BER) pathway, in prostate cancer cells. This increase in the expression of BER enzymes, together with the reduction in the 8-hydroxydeoxyguanosine(8-OH-dG) to deoxyguanosine (dG) ratio following oxidative damage observed by the authors, suggests the role of naringenin in DNA repair modulation.

Naringenin inhibits CYP1A2 and CYP19 *in vitro* and CYP3A4 *in vivo* (Moon et al., 2006). While the CYP1 family and CYP3A4 are involved in the activation of procarcinogens, CYP19 is commonly overexpressed in mammary tumors (Moon et al., 2006). The several

targets of naringenin suggest that it has a widespread role in blocking procarcinogen activation.

Naringenin is involved in stimulation of the overall UDP-glucuronidation *in vitro*, although it is specifically inhibitory for UGT1A1, also *in vitro* (Moon et al., 2006). Glucoronidation is responsible for reducing the toxicity of toxic substances, either exogenous or endogenous. UDP-glucuroniltransferase 1 (UGT1) is responsible for the hepatic glucoronidation of strogens, catechols, heme catabolites and flavonoids (Moon et al., 2006).

In general, pasteurization and freezing diminish naringenin content in OJ, as there is a lower release of naringenin during extraction (Sanchez-Moreno *et al.*, 2005).

2.4.3 Anthocyanins

Although there are a few controversial studies in favor of carotenoids, the red coloration in sweet orange (*Citrus sinensis*) is mostly attributed to the presence of anthocyanins such as cyanidin-3-glucoside (Lee, 2001). Only blood OJ contains appreciable amounts of anthocyanins, principally as cyanidin-3-glucoside (Rapisarda *et al.*, 1999). Indeed, anthocyanin content depends on the cultivar and the stage of maturation of the fruit.

Like other flavonoids, anthocyanins and anthocyanidins (the aglycone form) have antioxidant properties (Prior, 2003). They have been indicated as the main factor influencing antioxidant effectiveness of fresh pigmented juices, being potent scavengers of free radicals and reactive oxygen species. Cyanidin is also an excellent scavenger of nitric oxide radicals (Rapisarda *et al.*, 1999). In addition, anthocyanins are benefical for the treatment of diabetic retinopathy, as well as various microcirculation diseases. They also show anti-neoplastic, anti-inflammatory, and hepatoprotective effects. They are regarded as the main components of antioxidant activity as evaluated in different *in vitro* systems (Ghiselli *et al.*, 1998).

Cyanidin-3-glucoside is the most abundant anthocyanin in red OJ. It has a high antioxidant activity against lipid peroxidation and had a higher oxygen radical absorbance

capacity than other anthocyanins or Trolox. Cyanidin glycosides tend to have high antioxidant properties, probably because of the free hydroxyl groups on the 3' and 4' positions of cyanidin (Rapisarda *et al.*, 1999).

Cyanidin glycosides spontaneously degrade at a pH higher than that at which they may convert to the chalcone. This is similar to the fate of the compound at physiological pH. Indeed, although anthocyanins can have antioxidant effects in cell culture and other *in vitro* systems at relatively high concentrations, it is not clear whether sufficient tissue concentrations can be reached *in vivo* to produce antioxidant effects (Prior, 2003).

Degradation of anthocyanins by the microflora occurs to a much more limited extent than with other flavonoids. The absorption of anthocyanins is low in comparison to other polyphenols, since they are rapidly absorbed, but easily excreted (Manach *et al.*, 2005).

2.4.4 Phenolic acids - hydroxycinnamic acids

Most hydroxicinnamic acids (HCA) are present in oranges in bound forms that can be liberated by hydrolysis as ferrulic, synaptic, coumaric and caffeic acids, in increasing order. These phenolic acids have been suggested to influence toxicological, nutritional, color, sensory and antioxidant properties. In addition, they are directly involved in the biosynthesis of coumarins, flavonoids and lignin. The peel contains the majority of HCA when compared with other parts of the fruit. Thus, the extraction process can affect the amount of HCA transferred into the juice. HCA level was not affected by the harvest date, but a trend towards an increase in bound sinaptic and coumaric acids occurred (Naim *et al.*, 1992). This effect can be related to biochemical changes that occur when oranges reach their maximum size and growth is terminated.

Vinyl phenols, such as 4-vinyl guaiacol (PVG), can be produced from HCA during processing and storage and they affect acceptability because of the old-fruit flavor produced (Naim et al., 1992). Guaiacol seems to be produced in OJ by bacteria, intrinsic to oranges, that

survive pausterization (Gocmen et al., 2005). Guaicol is cytotoxic but not genotoxic in human pulp fibroblasts *in vitro* (Chang et al., 2000). It is clastogenic and induces unscheduled DNA synthesis and morphological transformation in Syrian hamster embryo cells. It is also a major constituent of creosote that induces mutations in different strains of *Salmonella typhimurium* after exogenous metabolic activation (Hikiba et al., 2005). One important sub-product of PVG is vanillin (Naim et al., 1992). Vanillin displays antioxidant and antimicrobial properties and hence has the potential for use as a food preservative, being biostatic against bacteria, yeasts and moulds. There is some evidence for the antimutagenic effects of vanillin, for example in suppressing chromosomal damage induced by methotrexate in the Chinese hamster V79 cell line (Walton et al., 2003). In addition, vaniloids such as capsaicin can modulate carcinogenesis in various model systems, apparently by triggering apoptosis in tumor cells via mitochondrial decay. They can also be useful in managing certain types of neurogenic pain and inflammation via their interactions with specific receptors (Hail, 2003). Conversely, Sinigaglia *et al.* (2004) point out that vanillin can either increase or decrease the genotoxicity of chemical agents, depending on the nature of both the agent and the genetic event measured. For instance, they showed, using the somatic mutation and recombination test (SMART) in *Drosophila melanogaster*, that vanillin increases the mitotic recombination induced by ethyl methanesulfonate (a monofunctional alkylating agent) and bleomicin (an intercalating agent). This increase in DNA damage may, however, be linked to an increase in recombinational DNA repair systems (Sinigaglia et al., 2004).

The various compounds generated from HCA show that they are labile. However, caffeic acid derivatives and p-coumaric acid derivatives in the whole juice seem not to be affected by pasteurization, nor by concentration processes. Conversely, freezing can cause a decrease of about 30 % in these hydroxycinnamic derivatives (Gil-Izquierdo et al., 2002).

Chlorogenic acid formation, resulting from the esterification of caffeic acids, can markedly reduce the absorption of caffeic acid. Several metabolites, such as ferrulic acid and vanillic acid, were reported after ingestion of chlorogenic or caffeic acid. An underestimation of the absorption of anthocyanins can occur due to lack of identification of all their metabolites (Manach *et al.*, 2005).

2.5   Fibers

In general, vegetable foodstuffs are good sources of dietary fibers and have beneficial physiological effects, attenuating cholesterol and glucose blood. However, OJ is, in general, a low source of dietary fibers (about 1% of daily adequate intake (AI) per 200 mL of OJ) (Table 1). However, the fiber content of OJ can be increased by the method of preparation (e.g. not stewing, or whole fruit squeezing).

Orange processing in the United States produces about 7 million tons of peel byproduct solids annually, and these peels are a source of several bioactive compounds (Manthey & Grohmann, 2001). Citrus peel has been reported to be a good source of pectin and dietary fiber in general, with an equilibrated proportion of soluble and insoluble fractions; thus fiber products are widely consumed and the industry produces fibre concentrates worldwide (Larrauri *et al.*, 1996). Pectin is a potent chemoprotective agent against food derived mutagens (Higashimoto *et al.*, 1998).

Dietary fiber primarily consists of plant cell walls that vary tremendously in structure and composition and, thus, have diverse effects on the potential for carcinogenesis (Papas *et al.*, 2004). Many studies have concentrated on fiber isolates, resulting in findings that have ignored fiber as a component of fruits, vegetables, nuts, cereals, and legumes in the general diet. The principle actions of fiber are to alter the nature of the contents of the gastrointestinal tract and to modify the absorption of other nutrients and chemicals (Eastwood & Kritchevsky, 2005). However, there is no agreement as to how and to what extent fibers can protect against

oxidative stress and genomic instability. It is important to mention that terpenes and phenolics are associated with the citrus fibers, thus acting synergistically for their beneficial chemopreventive role.

2.6 Minerals

Copper, magnesium, potassium, phosphorus and iron are present in significant amounts in OJ. A standard 200 mL serving of OJ can supply 8.9% of the DRI for copper, being an excellent source of it. The same portion of OJ can supply 7.4, 6.7 and 4.9% of the RDI for magnesium, potassium and phosphorus, respectively, and is a good sorce of these minerals. Moreover, a 200 mL portion of OJ is a regular source of iron (4% of the RDI) (Table 1 and Fig. 1). OJ contains other minerals in relatively minor quantities that will not be discussed in terms of oxidative stress and genomic stability.

2.6.1 Copper

Copper is essential for the function of most living organisms. The relative ease with which copper can be converted between different redox states, such as oxidized Cu(II) and reduced Cu(I), has been exploited by living organisms (Bertinato & L'Abbe, 2004). Indeed, most of the cellular Cu is associated with enzymes, where it is required for catalytic activity, correct folding, and stability (Kubat & Prohaska, 1996). These enzymes include Cu/Zn superoxide dismutase (antioxidant defense), cytochrome c oxidase (mitochondrial respiration), lysyl oxidase (development of connective tissue), tyrosinase (melanin biosynthesis), ceruloplasmin (iron homeostasis), hephaestin (intestinal iron efflux), dopamine β-hydroxylase (catecholamine production), and peptidylglycine β-amidating mono-oxygenase (peptide hormone processing) (Linder, 2001; Bertinato & L'Abbe, 2004). Thus, Cu is involved in a variety of biological reactions, from energy metabolism to iron metabolism, free radical eradication and neurological function (Valko *et al.*, 2005).

Cu interacts intimately with iron, participating in its absorption, maintenance in the oxidized state, and in hemoglobin synthesis. Copper accumulation can lead to nervous and hepatic dysfunction. Copper homeostasis is tightly regulated to avoid free copper within the cells and there are a variety of copper chaperones designed to insure that the metal is sequestered from interaction with cell membranes, proteins, or DNA, where it can result in oxidative damage. Greatly elevated levels of copper have been shown in cancer tissues, including Wilson's and Menkes' diseases, pathologies characterized by copper accumulation due to different metabolic deficiencies (Daniel *et al.*, 2004).

In spite of its role in antioxidant defense (in Cu/Zn superoxide dismutase) and in the reoxidation of iron within the cells (i.e. in ceruloplasmin and hepaestin), Cu is a potent cytotoxin when allowed to accumulate in excess of cell requirements, because of its special redox chemistry. Like Fe, Cu readily participates in reactions that result in the production of ROS, including hydroxyl radicals. In addition to the generation of ROS, Cu may manifest its toxicity by displacing other metal cofactors from their natural ligands in key cell signaling proteins. For example, the replacement of Zn(II) by Cu(II) in the zinc-finger DNA binding domain, as well as induction of the release of metals from metalloenzymes, potentially interferes in homeostasis (Pena *et al.*, 1999; Franke *et al.*, 2006).

2.6.2 Magnesium

Magnesium has an important role in mitochondria. Mg is present as a complex with ATP in the mitochondria and the cell and is a component of membranes and nucleic acids. Mg ions are a required cofactor in the mitochondrial electron transport chain complex subunits, methylenetetrahydrofolate dehydrogenase 2 and pyruvate dehydrogenase phosphatase. Magnesium is required as a cofactor for a variety of DNA polymerases, in nucleotide excision repair, base excision repair and mismatch repair. It is also essential for microtubule polymerization and chromosome segregation (Ames *et al.*, 2005; Fenech, 2005).

Mg deficiency is associated with decreased antioxidant defenses, as well as with reduced fidelity of DNA replication, reduced DNA repair and chromosome segregation errors (Ames *et al.*, 2005; Fenech, 2005). It is also associated with hyperlipidemia, hypertension, diabetes and obesity, since Mg has a key role in lipid metabolism (Inoue, 2005) and changes the expression of genes involved in the anti-oxidative response, regulation of cell cycle and growth and apoptosis, as well as cell-cell and cell-matrix interactions in mouse lung (Nasulewicz *et al.*, 2004). Symptoms of severe and moderate magnesium deficiency include secondary calcium and potassium deficiencies, neuromuscular distress and increased risk of diabetes (Ames *et al.*, 2005). Moderate magnesium deficiency is quite common, because the source is mainly green leafy vegetables (Ames *et al.*, 2005).

2.6.3   Potassium

OJ (~ 13 mmol K per 8 fluid ounces) is a foodstuff particularly high in potassium (K) (greater than 6.4 mmol K per serving), being as good a source as bananas (18.5 mmol K each), the most frequently quoted dietary source of K (Evans & Greenberg, 2005). K is the principal intracellular cation that contributes to osmotic and bioenergetic balance (Ames et al., 2005). It is thus essential for many body functions, including the muscular and nervous systems (Evans & Greenberg, 2005).

K disorders are the most common electrolyte abnormality identified in clinical practice. Hypo- and hyperkalemia have similar symptoms, impairing the cardiac, neuromuscular, and gastrointestinal systems. Hyperkalemia is less common but more serious, being in general more likely to be lethal (Schaefer & Wolford, 2005).

While decreased levels of K can cause hyperpolarization of the resting membrane potential, disrupting cardiac and other muscular functions, hyperkalemia has been implicated in the depolarization of electrically active cell membranes, including cardiac and skeletal muscle. Moreover, neuromuscular manifestations of hyperkalemia include diarrhea,

abdominal pain, myalgias, and flaccid paralysis of the extremities. The potentially deadly effect of hyperkalemia on cardiac muscle is of greatest concern (Evans & Greenberg, 2005).

Although the relation between K and genome stability or oxidative stress has not been fully elucidated, there is some evidence for a protective role of K. Indeed, high K diets have vasculoprotective properties mediated by natriuresis and vasodilatation, as well as by inhibitory/decreasing effects over proliferation of vascular smooth muscle cells, platelet aggregation, thrombosis, hypertension-induced endothelial dysfunction and macrophage adherence to the vascular wall. Increases in K levels also inhibit oxygen free radical formation from vascular endothelial cells and macrophages (Coca et al., 2005).

There is growing evidence linking impaired membrane ion channels, particularly those driven by K, and the development of cancer (Kunzelmann, 2005). Plasma membrane (K^+) channels, along with other ion channels, belong to the fundamental equipment of any living cell and are required for cell proliferation. Proliferation studies show that inhibition of K^+ channel expression, or channel blockade by more or less specific inhibitors, reduces cell proliferation. Such channels regulate cell proliferation dynamics by affecting membrane voltage, Ca^{2+} signaling, cytosol pH and cell volume. It is interesting to mention that apoptosis is generally related to a general decrease in the concentration of potassium (Kunzelmann, 2005).

2.6.4 Phosphorus

Phosphorus is a nonmetal important to several biological processes, particularly as phosphate; when linked to oxygen, P is highly reactive. Phosphate is an essential nutrient for all living systems. It is used in the storage and transport of energy, in signal transduction (particularly cell cycle regulation) and in the structure of molecules such as nucleic acids and phospholipids. Since phosphate cannot be synthesized, the need for this nutrient is met by ingestion of phosphate in the diet, both naturally and as added phosphate. Inorganic phosphate

salts have been used as food ingredients for decades and are considered 'Generally Recognized As Safe' (GRAS) by the US Food and Drug Administration (FDA) (Weiner *et al.*, 2001).

Since little soluble inorganic phosphate (Pi) is directly available in many natural ecosystems, organisms use several adaptive mechanisms, including the constitutive expression of phosphatases. Phosphatases catalyze the hydrolysis of a wide variety of phosphate esters to release Pi (Tasaki *et al.*, 2006). Despite the role of phosphate as a fertilizer, it can also be highly toxic, as organophosphorated compounds. Inorganic phosphates *per se* exhibit low acute oral toxicities. Chronic studies in mammals have demonstrated that the kidney is a target organ at high doses of phosphate, causing increased bone demineralization and release of calcium as part of a physiological regulatory mechanism. Excessive phosphate and calcium loads result in nephrocalcinosis and other renal effects. Inorganic phosphates cannot produce teratogenic effects and the phosphate ion is not genotoxic or mutagenic in standard test systems (Weiner *et al.*, 2001).

The homeostasis of phosphorus, as well as calcium, is considered to be modulated by vitamin D, and phosphorus restriction is reported to improve calcium utilization. The correct ratio of calcium to phosphorus in the diet is also important for mineral mobilization and bone mineralization (Takeda *et al.*, 2004).

Phosphate retention, or hyperphosphataemia, has been identified as playing a major role in the progression of renal failure, hyperparathyroidism, ostedistrophy and metastatic calcification of blood vessels and soft tissue. The Klotho mouse bears a mutation in a key phosphate regulating gene, which shows a human age-like syndrome. It has been shown that a low phosphate diet can modulate the phenotypes of Klotho mice. It is important to mention that the expression of Klotho RNA was greatly reduced in kidneys of all chronic renal failure (CRF) patients. Dietary P(i) restriction induced Klotho expression, which enhanced the

beneficial effect of P(i) restriction in patients with CRF and/or on haemodialysis (Miyamoto *et al.*, 2003).

Phosphorus intake has increased in both western and eastern regions of the world, possibly due to the increase of soft drink consumption. Since the restriction of phosphorus intake seems to be important under low calcium intake high life quality, further studies on the amount and the source of phosphorus intake are important, particularly in regard to the amount of phosphorus from processed foods, imported foods, and phosphorus-containing food additives (Takeda *et al.*, 2004).

2.6.5   Iron

Iron is a transition metal present in numerous biomolecules, such as hemoglobin and myoglobin, as well as several enzymes. It is a key component of the cytochromes involved in energy metabolism and drug metabolism. It is also a key component of the immune and antioxidant systems (De Freitas & Meneghini, 2001).

Both iron deficiency and iron excess have been linked to genome instability. While iron deficiency is related to impairment in oxidative/antioxidant metabolism, excess iron has been particularly linked to oxidative stress and hepatic carcinogenesis, possibly by its pro-oxidant role through Fenton and Haber-Weiss chemistries.

Iron deficiency is the most widespread micronutrient deficiency. According to WHO, anemia affects more than 20 % of the world population, about 46 % of 5- to 14-year-old children and 48% of pregnant women. The symptoms of iron deficiency range from weakness and reduction in performance to developmental defects of the nervous system when anemia is present in early childhood (Beard, 2001).

According to the seminal work of Bruce Ames' group (Ames, 1998; Ames, 2001; Atamna *et al.*, 2001; Atamna *et al.*, 2002; Ames *et al.*, 2005), heme deficiency can cause mitochondrial decay, oxidative stress and oxidative damage. This may be, for example,

through impaired energy production caused by corrupting complex IV of oxidative phophorylation, apparently because of a lack of heme-*a*.

Iron overload is a less frequent condition, and involves defects in iron absorption and transport, as well as the secondary outcome of several diseases, collectively referred to as hemochromatosis (Valko et al., 2005; Beutler, 2006). High iron content has been associated with several pathological conditions, including liver and heart disease, cancer , neurodegenerative disorders, diabetes, hormonal abnormalities, and immune system abnormalities (Valko et al., 2005).

3   Conclusion

Supplementation by natural antioxidants contained in OJ, achieved through a balanced diet, could be more effective and also more economical in protecting the body against oxidative damage than supplementation by an individual antioxidant.

A wide variability in the metabolism of OJ components is likely to occur within the population. In spite of the difference in background diet, subjects might have differences in gastrointestinal composition and activity, as well as in metabolizing enzymes and transporters (Manach *et al.*, 2005).

Whole, fresh, home made OJ, readily consumed, without pasteurization, is a good source of vitamin C and folate (one of the rare sources in the diet). On the other hand, OJ industrial processing saves time and effort and, when controlled, can increase the bioavailability of pro-vitamin A carotenoids and hesperitin, among other nutrients. The use of the whole fruit for OJ extraction is another feature in favor of industrial processing.

For both home made or industrially processed OJ, the storage time should be minimized, in order to avoid losses in nutritional value. Freezing is preferred for long time storage. Maintaining the pulp would be another approach to optimize nutrient content, since many hydrophobic components tend to be in the cloud fraction.

OJ can be used as a vehicle for supplementation. However, one should always bear in mind that it cannot be used as a substitute for major meals.

The packing system should be carefully evaluated, some materials can adsorb nutrients and light, which can change the chemical structure of several compounds. However, even if nutritional value is reduced, it is better to consume fruit juices than other drinks with low nutrient value and high-energy content.

OJ is much more than a good source of vitamin C. It has a useful amount of B complex vitamins, together with minerals such as copper and magnesium, which have been

shown to have a central role in antioxidant defenses, DNA homeostasis and genome stability. The interaction of the multiple components of OJ is likely to be responsible for its biological role. This idea is supported by the fact that, while clinical studies on the effect of supplementation with preventive antioxidants have shown surprisingly little or no effect on cancer incidence (Correa *et al.*, 1998; Donaldson, 2004), the epidemiological evidence shows that diets rich in fresh fruit and vegetables have significant beneficial effects (Wang *et al.*, 1996; Kabasakalis, 2000; Halliwell, 2001). Indeed, one glass of fresh or adequately processed and stored OJ is one of the options for the daily 5 portions (or even more) of fruit and vegetables recommended for a healthy life.

4   Acknowledgements

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Legend to Figure

Figure 1. Density (percentage of Recommended Dietary Allowance) of the main nutrients in a standard (200 mL) serving of orange juice. OJ: Orange juice; DRI: Daily Reference Intake. RAE: Equivalents of retinol.
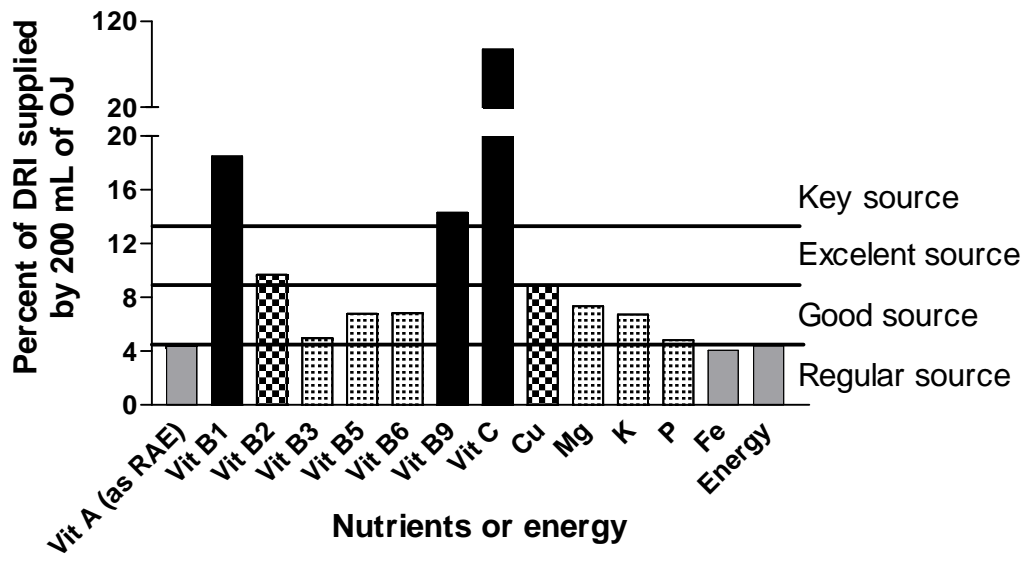
Figure 1.

Table 1. Nutritional composition of a standard (200 mL) portion of orange juice in terms of the contribution to the Dietary Reference Intakes (DRI) for the most abundant nutrients in the juice

Nutrient	Dietary Reference Intake (DRI)[a]					Upper Level (UL)[a]	Orange juice contribution[*] (% of average DRI)	Orange juice composition Content per serving (200 mL) Average ± SD	Source
	Gender (Age)				Average DRI				
	Male (19-30)	Female (19-30)	Male (31-50)	Female (31-50)					
Vitamin A (µg/d)	**900**[¢]	**900**[¢]	**700**[¢]	**700**[¢]	**800**[¢]	3000[¢]	4.4[**]	34.80[**]	b
Vitamin B1 (mg/d)	**1.2**	**1.2**	**1.1**	**1.1**	**1.15**	ND	18.5	0.21 ± 0.11	c-f
Vitamin B2 (mg/d)	**1.3**	**1.1**	**1.3**	**1.1**	**1.15**	ND	9.7	0.11 ± 0.11	c-f
Vitamin B3 (mg/d)	**16**	**14**	**16**	**14**	**15**	35	5.0	0.75 ± 0.10	c-f
Vitamin B5 (mg/d)	5	5	5	5	5	ND	6.8	0.34 ± 0.06	e,f
Vitamin B6 (mg/d)	**1.3**	**1.3**	**1.3**	**1.3**	**1.3**	100	6.9	0.09 ± 0.01	e,f
Vitamin B9 (µg/d)	**400**	**400**	**400**	**400**	**400**	1000	14.3	57.33 ± 24.11	d-f
Vitamin C (mg/d)	**90**	**75**	**90**	**75**	**82.5**	2000	122.6	101.12 ± 14.72	c-g
Copper (µg/d)	**900**	**900**	**900**	**900**	**900**	10000	8.9	80.00 ± 8.00	e-g
Magnesium (mg/d)	**400**	**310**	**420**	**320**	**362.5**	350[£]	7.4	26.67 ± 9.87	e-g
Potassium (g/d)	4.7	4.7	4.7	4.7	4.7	ND	6.7	0.32 ± 0.12	c-g
Phosphorus (mg/d)	**700**	**700**	**700**	**700**	**700**	4000	4.9	33.92 ± 6.51	c-g
Iron (mg/d)	**8**	**18**	**8**	**18**	**13**	45	4.0	0.53 ± 0.16	c-g
Dietary fibers (g/d)	38	25	38	25	31.5	ND	0.9	0.27 ± 0.12	c-f
Carbohydrates (g/d)	**130**	**130**	**130**	**130**	**130**	ND	16.8	21.85 ± 2.93	c-f
Energy (Kcal/d)[§]	2474	2126	2322	2015	2234	ND	4.4	98.13 ± 19.96	c-f

DRI: Goals for daily nutrient intake by healthy individuals used for planning and assessing diets. UL: The maximal level of daily nutrient intake that is likely to pose no risk of adverse effects; ND: Not determined; SD: Standard deviation.

Bold type: Recommended Dietary Allowance (RDA); Plain type: adequate intakes (AI).

[*] For a standard portion of 200 mL; [**] Retinol Activity Equivalents (RAE) as described in IOM (2001); [¢] Referred to vitamin A only.

[£] For pharmaceutical agents only; [§] Based on the Estimated Energy Requirement (EER) considering a Body Mass Index (BMI) of 21 for females and 22 for Males, a height of 1.65 m, low physical activity and average age for each life cycle (IOM, 2005).

[a] IOM (1997; 1998; 2000; 2001; 2004; 2005); [b] Table 2; [c] Morton (1987); [d] Franco (1999); [e] USDA (2005); [f] Møller et al. (2005); [g] Unpublished data from the authors.

Table 2. Content of carotenoids in a standard (200 mL) serving of orange juice

Carotenoid	Content per serving (μg/200 mL) Average ± SD	Source
Pro-vitamin A Carotenoids		
α-carotene	79.01 ± 64.19	[a-c, e, g]
β-carotene	91.3 ± 57.41	[a-e, g]
α-cryptoxanthin	177.14 ± 103.14	[a-c]
β-cryptoxanthin	396.19 ± 167.22	[a-g]
RAE	**34.80**	
Other relevant carotenoids*		
Lutein	608.13 ± 574.05	[a-e]
cis-violaxanthin	531.76 ± 462.72	[a,d,e]
Zeaxanthin	456.35 ± 288.24	[b-g]
ζ-Carotene	150.2 ± 127.56	[a,e]
Phytofluene	35.33 ± 9.43	[a,d]

SD: Standard deviation

RAE: Retinol Activity Equivalents based on FAO/WHO (1988) = [β-carotene / 12 + (α-carotene + α-cryptoxanthin + β-cryptoxanthin) / 24].

* High amounts of other carotenoids such as mutatoxanthin and auroxanthin have been reported by Mouly *et al.* (1999).

[a] Mouly *et al.* (1999); [b] Sanchez-Moreno *et al.* (2003); [c] Sanchez-Moreno *et al.* (2005); [d] Dhuique-Mayer *et al.* (2005); [e] Gama & Sylos (2005); [f] Schlatterer & Breithaupt (2005); [g] USDA (2005).

Table 3. Content of phenolics in a standard (200 mL) serving of orange juice

Phenolic[*]	Content per serving (mg/200 mL) Average ± SD	Source
Flavanones	117.50[**]	
Hesperidin	69.07 ± 15.28	a,d,f,h
Hesperitin	23.34 ± 2.46	e,g
Narirutin	13.42 ± 4.81	a,d,f,h
Naringenin	5.77 ± 1.74	e,g
Didymin	5.94 ± 4.24	d,h
Flavones	12.38 ± 4.29	c,d
Hydroxicinnamic acids	11.14 ± 6.72	b-d

SD: Standard deviation

[*] Trace amounts of the flavonone eriodictyol and flavonols have also been reported by USDA (2003). ** Calculated by summing the average content per serving of all flavonones.

[a] Pupin *et al.* (1998);[b] Rapisarda *et al.* (1999);[c] Gil-Izquierdo *et al.* (2001);[d] Gil-Izquierdo *et al.* (2002);[e] USDA (2003);[f] Dhuique-Mayer *et al.* (2005);[g] Sanchez-Moreno *et al.* (2005);[h] Vanamala *et al.* (2006).

7.5 ANEXO E: *Curriculum Vitae* **- Silvia Isabel Rech Franke**

Silvia Isabel Rech Franke

CURRICULUM VITAE

SANTA CRUZ DO SUL
2006

CURRICULUM VITAE
Maio, 2006

1 DADOS PESSOAIS

Nome: Silvia Isabel Rech Franke
Filiação: Claudino Aloisio Rech e Tharsilla Rech
Nascimento: 19/03/1964, Santa Cruz do Sul/RS - Brasil
Carteira de identidade: 6007004911 / SSP / RS / 16/10/1998
CPF: 47300671004

Endereço profissional: Universidade de Santa Cruz do Sul, Departamento de Educação Física e Saúde, Curso de Nutrição.
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96815900 SANTA CRUZ DO SUL, RS - Brasil - Caixa Postal: 188
Telefone: (51) 37177300 Ramal: 7386 Fax: 37171855
E-mail: silviafr@unisc.br

Endereço residencial: Avenida Senador Alberto Pasqüaline, 688
Verena
96820050 SANTA CRUZ DO SUL, RS - Brasil
Telefone: (51) 37171262 Fax: 37171262
E-mail: silviafr@unisc.br

2 FORMAÇÃO ACADÊMICA/TITULAÇÃO

1999 - 2001   Mestrado em Biologia Celular e Molecular.
Universidade Federal do Rio Grande do Sul, UFRGS, Rio Grande do Sul, Brasil.
Título: O estudo da resposta mutagênica em procariontes e do potencial antioxidante in vitro de sucos de laranja in natura e industrializados.. Ano de obtenção: 2001.
Orientador: João Antonio Pêgas Henriques.
1987 - 1987   Especialização em Administração e Serviço de Nutrição. (Carga horária: 410h)
Instituto Metodista de Educação e Cultura, IMEC, Rio Grande do Sul, Brasil.
1983 - 1986   Graduação em Nutrição.
Universidade do Vale do Rio dos Sinos, UNISINOS, Rio Grande do Sul, Brasil.
2001   Doutorado em Biologia Celular e Molecular.
Universidade Federal do Rio Grande do Sul, UFRGS, Rio Grande do Sul, Brasil.
Título: Suco de laranja e vitamina C: efeito sobre a estabilidade genômica.
Orientador: João Antonio Pêgas Henriques.

3 FORMAÇÃO COMPLEMENTAR

2005 - 2005   Normas Técnicas de Elaboração de Referências e Cit.
Universidade de Santa Cruz do Sul, UNISC, Rio Grande do Sul, Brasil.
2005 - 2005   Projeto de Pesquisa a Elaboração de Um Problema e.
Universidade de Santa Cruz do Sul, UNISC, Rio Grande do Sul, Brasil.
2005 - 2005   A Arte da Docência no Ensino Superior.
Universidade de Santa Cruz do Sul, UNISC, Rio Grande do Sul, Brasil.
2005 - 2005   O Processo de Ensino Aprendizagem e Avaliação Part.
Universidade de Santa Cruz do Sul, UNISC, Rio Grande do Sul, Brasil.
2005 - 2005   Mediação de Conflitos no Ambiente Universitário.
Universidade de Santa Cruz do Sul, UNISC, Rio Grande do Sul, Brasil.
2005 - 2005   Comitê de Ética Médica Bioética Ética Em Pesquisa. (Carga horária: 3h)

	Universidade de Santa Cruz do Sul, UNISC, Rio Grande do Sul, Brasil.
2005 - 2005	Histórico da Bioética Ética Em Pesquisa e Principa. (Carga horária: 3h)
	Universidade de Santa Cruz do Sul, UNISC, Rio Grande do Sul, Brasil.
2005 - 2005	Formação Moral Educação Moral e Ensino da Ética. (Carga horária: 3h)
	Universidade de Santa Cruz do Sul, UNISC, Rio Grande do Sul, Brasil.
2005 - 2005	Ensaios Bioquímicos de Estresse Oxidativo. (Carga horária: 20h)
	Universidade de Caxias do Sul, UCS, Rio Grande do Sul, Brasil.
2004 - 2004	As Novas Recomendações Dietéticas Dris e a Prát.... (Carga horária: 16h)
	Universidade do Vale do Rio dos Sinos, UNISINOS, Rio Grande do Sul, Brasil.
2004 - 2004	Genética e Conservação. (Carga horária: 8h)
	Universidade Luterana do Brasil, ULBRA, Rio Grande do Sul, Brasil.
2003 - 2003	Como As Vitraminas e Minerais Podem Contribuir .... (Carga horária: 4h)
	Sociedade Brasileira de Mutagênese, Carciongenese e Teratogênese Ambiental, SBMCTA, Paraná, Brasil.
2000 - 2000	Guias Alimentares A Pirâmide e a Avaliação do ..... (Carga horária: 12h)
	Associação Gaúcha de Nutricionistas, AGAN, Rio Grande do Sul, Brasil.
2000 - 2000	Nutrição de Microelementos Vitaminas e Minereiais. (Carga horária: 18h)
	Universidade do Vale do Rio dos Sinos, UNISINOS, Rio Grande do Sul, Brasil.
2000 - 2000	Curso de Formação Em Medicina Biomolecular e Radic. (Carga horária: 64h)
	Sociedade Gaúcha de Medicina Biomolecular e Radicais Livres, SOCIEDADE GAÚCHA, Rio Grande do Sul, Brasil.
1998 - 1998	Formação de Auditores Internos. (Carga horária: 24h)
	Supremo Consultoria, SUPREMO CONSULTO, Rio Grande do Sul, Brasil.
1998 - 1998	Extensão universitária em Administração da Qualidade Total. (Carga horária: 40h)
	Supremo Consultoria, SUPREMO CONSULTO, Rio Grande do Sul, Brasil.
1998 - 1998	Formação de Multiplicadores. (Carga horária: 16h)
	Supremo Consultoria, SUPREMO CONSULTO, Rio Grande do Sul, Brasil.
1996 - 1996	Como Evitar Toxinfecções Alimentares. (Carga horária: 12h)
	Associação Comercial e Industrial de Santa Cruz do Sul, ACI-SCRUZ, Rio Grande do Sul, Brasil.
1992 - 1992	Curso de Qualidade no Atendimento ao Cliente.
	Refeições Puras Rid Limitada, PURAS, Rio Grande do Sul, Brasil.
1992 - 1992	Curso de Preparação Para Instrutores do Programa...
	Refeições Puras Rid Limitada, PURAS, Rio Grande do Sul, Brasil.
1992 - 1992	Programa de Desenvolvimento Gerencial .....
	Refeições Puras Rid Limitada, PURAS, Rio Grande do Sul, Brasil.
1992 - 1992	Curso de Higiene na Manipulação dos Alimentos.
	Refeições Puras Rid Limitada, PURAS, Rio Grande do Sul, Brasil.
1992 - 1992	Curso de Higiene na Manipulação dos Alimentos.
	Refeições Puras Rid Limitada, PURAS, Rio Grande do Sul, Brasil.
1986 - 1986	Extensão universitária em Extensão Universitária - Nutrição em Pediatria. (Carga horária: 40h)
	Associação Gaúcha de Nutricionistas, AGAN, Rio Grande do Sul, Brasil.

4 ATUAÇÃO PROFISSIONAL

Universidade Federal do Rio Grande do Sul - UFRGS

Vínculo institucional
1999 - 2001   Vínculo: Aluna de Mestrado, Enquadramento funcional: Aluna de mestrado, Carga horária: 1.
2001 - Atual   Vínculo: Aluna de Doutorado, Enquadramento funcional: Aluna de Doutorado.

Atividades
6/1999 - 6/2001

Participação em projeto
1. Estudo da Resposta Mutagênica em Procariontes e do Potencial Antioxidante in vitro de Sucos de Laranja in natura e Industrializados.

3/2001 - 3/2001

Disciplinas ministradas
1. O estudo da resposta mutagênica em procariontes e do potencial antioxidante in vitro em suco de laranja naturais e industrializados.

6/2004 - Atual

Participação em projeto
1. Avaliação do estresse oxidativo e da capacidade de reparo do DNA em atletas.

7/2001 - Atual

Participação em projeto
1. Avaliação da possível ação moduladora da genotoxicidade e da mutagenicidade de suco de laranja e de vitamina C em camundongos in vivo.

Universidade de Santa Cruz do Sul - UNISC

Vínculo institucional
1998 - Atual   Vínculo: Professor, Enquadramento funcional: Professor, Carga horária: 30, Regime: Dedicação exclusiva.

Outras informações
Disciplinas de Nutriçao Normal, Nutriçao Básica e Estágio Supervisionado de Alimentação Instuitucional do Curso de Nutrição da Universidade de Santa Cruz do Sul- UNISC - RS

Atividades
8/2005 - 8/2005

Disciplinas ministradas
1. Seminários Avançados: Genética e Nutrição.

02/2003 - 08/2003

Disciplinas ministradas
1. Estágio Supervisionado em Alimentação Institucional.

3/1999 - 9/2001

Linhas de pesquisa
1. Potencial mutagênico e antioxidante.
2. Radicais livres e danos oxidativos.

11/1998 - 3/2001

Cargos ou funções
1. Coordenador de Curso de Graduação de Nutrição.

9/2000 - 10/2000

Atividades de extensão realizadas
1. Comissão Organizadora do Curso de Bioquímica Nutricional.

8/2000 - 8/2000

Atividades de extensão realizadas
1. Comissão Organizadora da Semana Acadêmica do Curso de Nutrição.

7/2000 - 7/2000

Atividades de extensão realizadas
1. Palestra sobre Alimentação do Trabalhador no Curso e Pós-Graduação em Enfermagem (UNISC).

5/2000 - 5/2000

Atividades de extensão realizadas
1. Palestra.

8/1999 - 9/1999

Atividades de extensão realizadas
1. Comissão Organizadora do I Seminário Regional sobre Fitoterapia.

7/1999 - 7/1999

Atividades de extensão realizadas
1. Concurso Público para Docentes da Disciplina de Nutrição Normal - APROVADA.

12/1998 - 12/1998

Atividades de extensão realizadas
1. Palestra.

11/1998 - 11/1998

Atividades de extensão realizadas
1. Palestra.

3/2006 - Atual

Cargos ou funções
1. Sub-chefe de departamento.

7/2005 - Atual

Disciplinas ministradas
1. Nutrição e Dietética I.
2. Alimentação Institucional I.
3. Nutrição.

7/2005 - Atual

Disciplinas ministradas
1. Metodologia de Pesquisa Científica.

3/2005 - Atual

Participação em projeto
1. Jogo, exercíco e estilo de vida na saúde, cultura e desenvolvimento humano.

2/2005 - Atual

Disciplinas ministradas
1. Nutrição e Dietética II.
2. Estágio Supervisionado em Alimentação Institucional.

7/2004 - Atual

Disciplinas ministradas
1. Estágio de Escola de Educação Básica.

03/2004 - Atual

Cargos ou funções
1. Coordenador Geral do Curso de Pós-graduação em Nutrição Clínica: Ênfase nas Doenças Crônico-Não-Transmissíveis.

10/2001 - Atual

Linhas de pesquisa
1. Potencial mutagênico e antioxidante.
2. Radicais livres e danos oxidativos.

02/2000 - Atual

Disciplinas ministradas
1. Nutrição Básica.

11/1998 - Atual

Disciplinas ministradas
1. Nutriçao Normal.

Universidade Federal de Santa Catarina - UFSC

Vínculo institucional
2000 - 2000   Vínculo: Estagiário, Enquadramento funcional: Aluna de Mestrado, Carga horária: 8.

Atividades
7/2000 - 7/2000

Estágios realizados

1. Determinação Antioxidante in vitro de amostras de suco de laranja industrializadas e natural.

Petróleo Brasileiro S/A - PETROBRAS

Vínculo institucional

1986 - 1986   Vínculo: Estagiário, Enquadramento funcional: Voluntário, Carga horária: 20.

Atividades

7/1986 - 12/1986

Estágios realizados
1. Estágio de Estudante de Nível Superior.

Serviço Social da Industria - SESI

Vínculo institucional

1986 - 1986   Vínculo: Estágio voluntário, Carga horária: 20.

Atividades

9/1986 - 12/1986

Estágios realizados
1. Estágio de Complementação Educacional.

Universidade do Vale do Rio dos Sinos - UNISINOS

Vínculo institucional

1985 - 1985   Vínculo: Bolsista Iniciação Cien.CNPq, Enquadramento funcional: Aluno de Graduação, Carga horária: 20, Regime: Dedicação exclusiva.

Atividades

3/1985 - 7/1985

Atividades realizadas
1. Bolsa CNPq.

5 LINHAS DE PESQUISA

1   Potencial mutagênico e antioxidante.
               Palavras-chave: Vitamina C; Sucos de laranja; Radicais Livres; Fenólicos Totais; Mutagenicidade.

2   Potencial mutagênico e antioxidante.

3   Radicais livres e danos oxidativos.
               Palavras-chave: Modulação Mutagênica; Genotoxicidade; Atividade Antioxidante; Vitamina C.

4   Radicais livres e danos oxidativos.

6 PROJETOS DE PESQUISA

2005 - Atual   Jogo, exercíco e estilo de vida na saúde, cultura e desenvolvimento humano.
                 Descrição: ..
                 Situação: Em andamento; Natureza: Extensão.
                 Alunos envolvidos: Graduação (1).
                 Integrantes: Silvia Isabel Rech Franke (Responsável); Danusia Puntel.

2004 - Atual   Avaliação do estresse oxidativo e da capacidade de reparo do DNA em atletas.
                 Descrição: ..
                 Situação: Em andamento; Natureza: Pesquisa.
                 Integrantes: Silvia Isabel Rech Franke (Responsável); Bernardo Erdtmann; Carlos Mantese; Daniel Prá; Janaína P Jaeger; João Antonio Pêgas Henriques.

2001 - Atual	Avaliação da possível ação moduladora da genotoxicidade e da mutagenicidade de suco de laranja e de vitamina C em camundongos in vivo.

Descrição: ..
Situação: Em andamento; Natureza: Pesquisa.
Integrantes: João Antonio Pêgas Henriques (Responsável); Silvia Isabel Rech Franke; Bernardo Erdtmann; Daniel Prá; Juliana da Silva.

1999 - 2001	Estudo da Resposta Mutagênica em Procariontes e do Potencial Antioxidante in vitro de Sucos de Laranja in natura e Industrializados.

Descrição: ..
Situação: Concluído; Natureza: Pesquisa.
Integrantes: Silvia Isabel Rech Franke (Responsável); Bernardo Erdtmann; Gabriel Rubensam; Jaqueline Deos Silveira; João Antonio Pêgas Henriques; Karina Ckless; Martin Brendel; Ribeiro do Valle.

7 ÁREAS DE ATUAÇÃO

1	Ciências da Saúde, Nutrição.
2	Genética, Mutagenese.
3	Biologia Molecular, Radicais Livres.
4	Nutrição, Bioquímica da Nutrição.
5	Mutagenese, Antimutagenese.
6	Farmácia, Análise Toxicológica.

8 IDIOMAS

Compreende:	Alemão (Razoavelmente), Espanhol (Bem), Inglês (Bem).
Fala:	Espanhol (Razoavelmente), Inglês (Razoavelmente).
Lê:	Espanhol (Bem), Inglês (Bem).
Escreve:	Espanhol (Pouco), Inglês (Razoavelmente).

9 PRÊMIOS E TÍTULOS

2005	Mejor presentacion en la Sesión de Posters del Area Genotoxicidad - Mutagénesis, ATA/ALAMCTA.
2004	Reconhecimento por contribuição relevante na área [artigo intitulado 'Study of antioxidant and mutagenic activity of different orange juices (Food Chemistry 88, 45-55)]., Sociedad Iberoamericana de Información Científica (SIIC).
1999	Membro nato da comissão de ensino do Conselho Regional de Nutricionistas, Conselho Regional de Nutricionistas 2ª Região.

10 PRODUÇÃO CIENTÍFICA, TECNOLÓGICA E ARTÍSTICA/CULTURAL

10.1 PRODUÇÃO BIBLIOGRÁFICA

10.1.1 Trabalhos completos em anais de eventos

1   PRÁ, Daniel; GUECHEVA, Temenouga N; FRANKE, Silvia Isabel Rech; KNAKIEVICZ, Tanise; ERDTMANN, Bernardo; HENRIQUES, João Antonio Pêgas. Toxicidade e gentoxicidade do sulfato de cobre em planárias de água doce e camundongos. In: VIII CONGRESSO BRASILEIRO DE ECOTOXICOLOGIA/ SOCIEDADE BRASILEIRA DE ECOTOXICOLOGIA - SETAC BRASIL, Florianópolis. 2005.

10.1.2 Resumos simples em anais de eventos

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11   WOLSCHIK, Scheila. **Relação entre a deficiência de micronutrientes com possíveis danos no DNA e as atuais DRIs.** 2005. Monografia (Aperfeiçoamento/Especialização em Especialização Em Nutrição Clínica) - Universidade de Santa Cruz do Sul. Orientador: Silvia Isabel Rech Franke.

10.3.2 Graduação

1   FUMACO, Bianca Gioda. **Câncer de mama: a influência da alimentação e do estilo de mama.** 2005. Trabalho de Conclusão de Curso (Graduação em Nutrição) - Universidade de Santa Cruz do Sul. Orientador: Silvia Isabel Rech Franke.

2   KURZ, Francine. **Os aditivos encontrados nos produtos alimentícios e os possíveis efeitos adversos à saúde humana.** 2005. Trabalho de Conclusão de Curso (Graduação em Nutrição) - Universidade de Santa Cruz do Sul. Orientador: Silvia Isabel Rech Franke.

3   GIANESSINI, Camila Bigliardi. **Perfil antropométrico e alimentar de escolares do ensino fundamental de uma escola particular do município de Encantado, RS.** 2005. Trabalho de Conclusão de Curso (Graduação em Nutrição) - Universidade de Santa Cruz do Sul. Orientador: Silvia Isabel Rech Franke.

4   CEOLIN, Rosana. **A importância da mudança no etilo de vida para o diabético tipo 2.** 2004. Trabalho de Conclusão de Curso (Graduação em Nutrição) - Universidade de Santa Cruz do Sul. Orientador: Silvia Isabel Rech Franke.

5   CALLIERO, Elenara. **A influência do aleitamento materno e do desmame precoce no desenvolvimento infantil.** 2004. Trabalho de Conclusão de Curso (Graduação em Nutrição) - Universidade de Santa Cruz do Sul. Orientador: Silvia Isabel Rech Franke.

6   SEHN, Eliana. **Biodisponibilidade de cálcio e ferro.** 2004. Trabalho de Conclusão de Curso (Graduação em Nutrição) - Universidade de Santa Cruz do Sul. Orientador: Silvia Isabel Rech Franke.

7   FUERSTENAU, Sabrina. **Perfil alimentar e antropométrico de adolescentes de uma escola particular no município de Santa Cruz do Sul.** 2004. Trabalho de Conclusão de Curso (Graduação em Nutrição) - Universidade de Santa Cruz do Sul. Orientador: Silvia Isabel Rech Franke.

8   FAGUNDES, Francine. **A influência dos suplementos nutricionais em diferentes patologias: riscos e benefícios para a saúde.** 2003. Trabalho de Conclusão de Curso (Graduação em Nutrição) - Universidade de Santa Cruz do Sul. Orientador: Silvia Isabel Rech Franke.

10.3.3 Iniciação científica

1   PUNTEL, Danusia. **Perfil nutricional e o risco às doenças cardiovasculares em crianças e adolescentes das escolas de Santa Cruz do Sul, RS.** 2005. Iniciação científica (Graduando em Nutrição) - Universidade de Santa Cruz do Sul, Universidade de Santa Cruz do Sul. Orientador: Silvia Isabel Rech Franke.

10.3.4 Orientações de outra natureza

1   LIRA, Glória Rodrigo. **Treinamento Teórico e Prático sobre o Ensaio Cometa.** 2005. Orientação de outra natureza, Universidad Mayor de San Andres. Orientador: Silvia Isabel Rech Franke.

2   SOTILLO, Wendy Soria. **Treinamento Teórico e Prático sobre o Ensaio Cometa.** 2005. Orientação de outra natureza, Universidad Mayor de San Andres. Orientador: Silvia Isabel Rech Franke.

10.4 DEMAIS TRABALHOS

1   FRANKE, Silvia Isabel Rech. **Docente da Disciplina de Nutriçao Basica do Curso de Nutriçao da Universidade de Santa Cruz do Sul.** 2002. (Docencia em Curso de Graduaçao de Nutriçao).

2   FRANKE, Silvia Isabel Rech. **Coordenador de Curso de Graduaço de Nutriçao.** 2001. (Coordenaçao do Curso de Graduaçao de Nutriçao).

3   FRANKE, Silvia Isabel Rech. **Docente da Disciplina Nutriçao Basica do Curso de Nutriçao da Universidade de Santa Cruz do Sul.** 2001. (Docencia em Curso de Graduaçao de Nutriçao).

4   FRANKE, Silvia Isabel Rech. **O estudo da resposta mutagenica em procariontes e do potencial antioxidante in vitro de suco de laranja in natura e industrializados.** 2001. (Seminario).

5   FRANKE, Silvia Isabel Rech. **Coordenador de Curso de Graduaçao de Nutriçao.** 2000. (Coordenaçao de Curso de Graduaçao de Nutriçao).

6   FRANKE, Silvia Isabel Rech. **Curso de Bioquimica Nutricional.** 2000. (Comissao Organizadora de Seminario).

7   FRANKE, Silvia Isabel Rech. **Determinaçao antioxidante in vitro de amostras de sucos de laranja industrializadas e natural.** 2000. (estagio).

8   FRANKE, Silvia Isabel Rech. **Docente da Disciplina de Nutriçao Basica do Curso de Nutriçao da Universidade de Santa Cruz do Sul.** 2000. (Docencia em Curso de Graduaçao de Nutriçao).

9   FRANKE, Silvia Isabel Rech. **Nutriçao e Bem-estar.** 2000. (Palestra).

10   FRANKE, Silvia Isabel Rech. **Programa de Alimentaçao do Trabalhador (PAT).** 2000. (Palestras para Pos-graduaçao em Enfermagem).

11   FRANKE, Silvia Isabel Rech. **Semana Academica do Curso de Nutriçao da Universidade de Santa Cruz do Sul.** 2000. (Comissao Organizadora de Semana Academica).

12   FRANKE, Silvia Isabel Rech. **Coordenador de Curso de Graduaçao de Nutriçao.** 1999. (Coordenaçao de Curso de Graduaçao de Nutriçao).

13   FRANKE, Silvia Isabel Rech. **Docente da Disciplina Nutriçao Normal do Curso de Nutriçao.** 1999. (Docencia em Curso de Graduaçao de Nutriçao).

14   FRANKE, Silvia Isabel Rech. **I Seminario Regional sobre Fitoterapia.** 1999. (Comissao Organizadora de Seminario).

15   FRANKE, Silvia Isabel Rech. **Coordenador de Curso Graduaçao de Nutriçao.** 1998. (Coordenador de Curso de Graduaçao).

16   FRANKE, Silvia Isabel Rech. **Educaçao Nutricional.** 1998. (Palestra).

17   FRANKE, Silvia Isabel Rech. **Orientaçao Nutricional para Grupo de Diabeticos do SIS (Sistema Integrado de Saude).** 1998. (Palestra).

18   FRANKE, Silvia Isabel Rech. **Bolsista do CNPq.** 1985. (Bolsista).

11 DADOS COMPLEMENTARES

11.1 PARTICIPAÇÃO EM BANCAS EXAMINADORAS

11.1.1 Trabalhos de Conclusão de Curso de Graduação

1   FRANKE, Silvia Isabel Rech; OLIVEIRA, Mari Silvia de; GRANADA, Graziele. Participação em banca de Renata Herzog. **Análise de nitratos e nitritos em alimentos, sob o aspecto toxicológico.** 2005. Trabalho de Conclusão de Curso (Graduação em Nutrição) - Universidade de Santa Cruz do Sul.

2   FRANKE, Silvia Isabel Rech; POLL, Fabiana; GRANADA, Graziele. Participação em banca de Raquel Rodrigues. **Doenças de Parkinson e Alzheimer e suas complicações no estado nutricional do idoso.** 2005. Trabalho de Conclusão de Curso (Graduação em Nutrição) - Universidade de Santa Cruz do Sul.

3   FRANKE, Silvia Isabel Rech; MIRAGLIA, Fernanda; POLL, Fabiana. Participação em banca de Mileine Mussio. **Perfil alimentar e gasto energético de atletas de elite do atletismo, competidores das provas de 800m e 1500m da Universidade de Santa Cruz do SUl.** 2005. Trabalho de Conclusão de Curso (Graduação em Nutrição) - Universidade de Santa Cruz do Sul.

4   FRANKE, Silvia Isabel Rech; CUNHA, Ana Zoé Shiling da. Participação em banca de Daniela Bertolo. **Reinternação por gastrite: fatores nutricionais predisponentes.** 2004. Trabalho de Conclusão de Curso (Graduação em Nutrição) - Universidade de Santa Cruz do Sul.

5   FRANKE, Silvia Isabel Rech; HERRMANN, Stela Maris. Participação em banca de Micaela Eliza Kettermann. **A influência da alimentação nas doenças cardiovasculares.** 2003. Trabalho de Conclusão de Curso (Graduação em Nutrição) - Universidade de Santa Cruz do Sul.

6   FRANKE, Silvia Isabel Rech; FEOLI, Ana Maria. Participação em banca de Francine Fagundes. **A influência dos suplementos nutricionais em diferentes patologias: riscos e benefícios.** 2003. Trabalho de Conclusão de Curso (Graduação em Nutrição) - Universidade de Santa Cruz do Sul.

7   FRANKE, Silvia Isabel Rech; FEOLI, Ana Maria. Participação em banca de Michele Milleto Troitinho. **Os efeitos da utilização de isoflavonas de soja na menopausa.** 2003. Trabalho de Conclusão de Curso (Graduação em Nutrição) - Universidade de Santa Cruz do Sul.

8   FRANKE, Silvia Isabel Rech; RAMOS, Renata; BERTI, Simone. Participação em banca de Patrícia Fernandes Boesel. **A Influência de Radicais Livres nas Cardiopatias.** 2002. Trabalho de Conclusão de Curso (Graduação em Nutrição) - Universidade de Santa Cruz do Sul.

11.2 PARTICIPAÇÃO EM BANCAS DE COMISSÕES JULGADORAS

11.2.1 Concurso público

1   **Membro da Banca de Concurso Público para Docentes, para a disciplina 'Introdução à Dietoterapia'.** 2006., Universidade de Santa Cruz do Sul.

2   **Membro de Banca de Concurso Publico para Docente da Disciplina de Introdução à dietoterapia.** 2005., Universidade de Santa Cruz do Sul.

3   **Presidente de Banca de Concurso Publico para Docente da Disciplina de Alimentaçao Institucional II do Curso de Nutriçao da Universidade de Santa Cruz do Sul.** 2001., Universidade de Santa Cruz do Sul.

4   **Suplente de Banca Examinadora de Concurso para Professor da Disciplina de Tecnica Dietetica I do Curso de Nutriçao da Universidade de Santa Cruz do Sul.** 2000., Universidade de Santa Cruz do Sul.

11.3 PARTICIPAÇÃO EM EVENTOS

1   **4th International Workshop on Genotoxicity Testing (IWGT).** 2005. (Participação em eventos/Encontro).

2   **The 9th International Conference on Environmental Mutagens, and the 36th Annual Meeting of the Environmental Mutagen Society.** 2005. (Participação em eventos/Congresso).

3   **VII Congresso Brasileiro de Mutagênese, Carcinogênese e Teratogênese Ambiental.** 2005. (Participação em eventos/Congresso).

4   **50° Congresso Brasileiro de Genética.** 2004. (Participação em eventos/Congresso).

5   **VIII Congresso Brasieliro de Ecotoxicologia.** 2004. (Participação em eventos/Congresso).

6   **XIV Encontro de Geneticistas do Rio Grande do Sul.** 2004. (Participação em eventos/Encontro).

7   **4th International Conference on Environmental Mutagens in Human Populations - 4th ICEMHP e 6° Congreso da Sociedade Brasileira de Mutagênese Carcinogênese e Teratogênese Ambiental SBMCTA.** 2003. (Participação em eventos/Congresso).

8   **Comet Assay Workshop.** 2002. (Participação em eventos/Outra).

9   **VII Congresso Brasileiro de Ecotoxicologia e os Novos Desafios no Monitoramento Ambiental, V Reuniao da SETAC Latino Americana.** 2002. (Participação em eventos/Congresso).

10   **XV Jornada de Nutriçao.** 2002. (Participação em eventos/Outra).

11   **Nutrição Clínica: Interações Nutricionais.** 2000. (Participação em eventos/Outra).

12   **Nutrição no Esporte: Estratégias para o Aumento do Desempenho.** 2000. (Participação em eventos/Outra).

13   **Curso de Atualização e Prática Clínica à Nível de Consultório.** 1999. (Participação em eventos/Outra).

14   **Diagnóstico de microorganismos usando técnicas de biologia molecular.** 1999. (Participação em eventos/Outra).

15   **I Seminário regional Sobre Fitoterapia. Universidade de Santa Cruz do Sul-UNISC.** 1999. (Participação em eventos/Seminário).

16   **1o Seminário de Garantia de Qualidade em Alimentação.** 1998. (Participação em eventos/Seminário).

17   **Workshop Avaliação - Nível 2 do SEBRAE/RS.** 1998. (Participação em eventos/Simpósio).

18   **Workshop GAS - Nível 1 do SEBRAE/RS.** 1998. (Participação em eventos/Simpósio).

19   **Tetraciclo em Pediatria.** 1997. (Participação em eventos/Encontro).

20   **IX Jornada de Nutrição do Hospital de Clínicas de Porto Alegre.** 1996. (Participação em eventos/Encontro).

21   **IV Jornada de Nutrição do Hospital de Clínicas de Porto Alegre.** 1991. (Participação em eventos/Encontro).

22   **Seminário de Nutricionistas.** 1987. (Participação em eventos/Seminário).

23   **Seminário de Nutricionistas - Cozinheiros-Chefe e Cozinheiros-líder.** 1987. (Participação em eventos/Seminário).

24   **III Enconto Estadual de Nutrição.** 1986. (Participação em eventos/Encontro).

25   **II Encontro Estadual de Nutrição.** 1985. (Participação em eventos/Encontro).

11.4 ORIENTAÇÕES EM ANDAMENTO

11.4.1 Graduação

1   KESSLER, Deise. **A influência da propaganda nos hábitos alimentares dos adolescentes.** Início:2006. Trabalho de Conclusão de Curso (Graduação em Nutrição) - Universidade de Santa Cruz do Sul. (Orientador).

2   NEUMANN, Mariele. **Avaliação do consumo de gordura trans de escolares de Santa Cruz do Sul.**
     Início:2006. Trabalho de Conclusão de Curso (Graduação em Nutrição) - Universidade de Santa
     Cruz do Sul. (Orientador).

3   MULLER, Amanda S. **Avaliação do estado nutricional e dos hábitos alimentares de idosos praticantes
     de hidroginástica.** Início:2006. Trabalho de Conclusão de Curso (Graduação em Nutrição) -
     Universidade de Santa Cruz do Sul. (Orientador).

4   GRIEBEL, Rosali Izolde. **O papel do licopeno sobre o desenvolvimento e progressão do Câncer de
     próstata.** Início:2006. Trabalho de Conclusão de Curso (Graduação em Nutrição) - Universidade
     de Santa Cruz do Sul. (Orientador).

5   LAKUS, Ethiene Schirmann. **Transtornos alimentares em adolescentes.** Início:2006. Trabalho de
     Conclusão de Curso (Graduação em Nutrição) - Universidade de Santa Cruz do Sul. (Orientador).

12 INDICADORES DE PRODUÇÃO

Produção bibliográfica

    Artigos publicados em periódicos - 17
        Completos - 5
        Resumos - 12

    Trabalhos em eventos - 29
        Completos - 1
        Resumos - 27
        Resumo expandido - 1

    Livros e capítulos - 1
        Capítulos de livros publicados - 1

    Textos em jornais ou revistas (magazines) - 2
        Jornais de notícias - 2

Produção artística/cultural

Orientações concluídas

    Aperfeiçoamento/Especialização - 11

    Graduação - 8

    Iniciação científica - 1

    Orientações de outra natureza - 2

Demais trabalhos

Dados complementares

    Participação em bancas examinadoras - 8

    Participação em bancas de comissões julgadoras - 4

    Participação em eventos - 25

    Orientações em andamento - 5