UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FERRO E ESTABILIDADE GENÔMICA: UMA ANÁLISE NUTRIGENÔMICA DOS EFEITOS DA DEFICIÊNCIA E DA SOBRECARGA

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“Toda a nossa ciência, comparada com a realidade, é primitiva e infantil – e, no entanto, é a coisa mais preciosa que temos”.

Albert Einstein (1879-1955)

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APRESENTAÇÃO

Embora Paracelso, o pai da toxicologia, tenha cunhado a frase “o que faz o veneno é a dose” no século 16, muito antes já se faziam menções aos efeitos adversos à saúde tanto do excesso quanto da carência de certos elementos, substâncias ou compostos químicos. Esse conceito é válido também para os macronutrientes (proteínas, carboidratos e lipídios) e para os micronutrientes (vitaminas e a grande maioria dos minerais), que obedecem à Regra de Bertrand: padrão descrito na observação de que o crescimento de plantas era ótimo apenas para uma faixa ideal de um dado nutriente. Isso decorre do fato de que os nutrientes podem ser deletérios quando em excesso e simultaneamente fundamentais à homeostasia, afetando direta e indiretamente a expressão e estrutura do genoma (p.ex. interagindo com fatores de transcrição, sendo substratos ou co-fatores de enzimas ou atuando nas redes de defesa antioxidantes). O ferro é um dos micronutrientes para o qual se dispõe de algumas informações do metabolismo e dos efeitos gerais da carência e do excesso. Cabe ressaltar que a carência de ferro é a deficiência de micronutriente mais comum mundialmente. Não obstante, pouco é sabido sobre o impacto dessas situações na estabilidade genômica e, por conseguinte, no risco de câncer.

Essa tese objetiva avaliar o efeito do ferro sobre a estabilidade genômica em modelos murinos e grupos populacionais humanos (crianças com alta vulnerabilidade sócio-econômica: deficiência e pacientes de anemia falciforme: excesso), testar estratégias para reduzir a toxicidade do ferro para a memória e para o DNA (pela administração de alimentos, vitaminas e medicamentos), e, finalmente, determinar qual a dose ideal de ferro para a manutenção da estabilidade genômica em células em cultura.

A tese segue as normas do Programa de Pós Graduação em Genética e Biologia Molecular da Universidade do Rio Grande do Sul, de acordo com a estrutura clássica para trabalhos científicos (introdução, objetivos, materiais e métodos, resultados, discussão, conclusões, perspectivas e anexos). Não obstante, o trabalho apresenta uma inovação - a seção resultados trás, de acordo com os objetivos, uma introdução e os resultados de publicações ou manuscritos, todos incluídos como anexo. Esse formato foi escolhido por três razões: a) aumentar a fluidez do texto principal da tese; b) reduzir o tamanho do corpo

principal do trabalho; c) livrar o leitor de dados contidos nos artigos que não são diretamente relacionadas ao tema da pesquisa.

Na introdução faz-se uma revisão detalhada de aspectos metabólicos e fisiológicos do ferro, com ênfase à influência do metal na estabilidade genômica em excesso e carência, bem como de estratégias para aumentar a biodisponibilidade e reduzir o risco de instabilidade genômica da suplementação com ferro. A seção objetivos apresenta estes em tópicos que serão retomados nas seções resultados e discussão. A seção metodologia descreve de forma mais abrangente as principais metodologias empregadas, dando menor ênfase aos protocolos experimentais (detalhados nos artigos, em anexo). Em seguida, os resultados são apresentados de forma condensada (com considerações introdutórias, metodológicas e de resultados propriamente ditos), destacando as informações mais relevantes da pesquisa. Uma discussão geral finaliza o corpo principal da tese e precede uma seção de conclusões, e outra de perspectivas. Em anexo, são apresentados 12 artigos científicos.

RESUMO

A deficiência de ferro, quando severa denominada de anemia, é a deficiência nutricional mais comum globalmente, em especial nos países em desenvolvimento. A deficiência de ferro há muito tempo tem sido relacionada à diminuição da atividade do sistema imunológico e da capacidade de trabalho. Mais recentemente, o excesso de ferro tem sido associado ao aumento do risco de doenças crônico-degenerativas e de câncer. Existem evidências inconclusivas de que a carência de ferro poderia elevar o nível de danos no DNA. Mais ainda, com base nesse conceito pode-se pensar na hipótese de que a carência de ferro eleve a suscetibilidade ao câncer, especialmente nos tecidos que sofrem efeitos mais severos da carência de ferro. Portanto, os **primeiros objetivos** deste trabalho foram: (a) avaliar experimentalmente quais as repercussões da deficiência de ferro na estabilidade genômica em crianças e adolescentes; e (b) revisar a literatura acerca da associação entre a deficiência de ferro e o risco de câncer no trato gastrintestinal. Outro aspecto que não pode ser negligenciado é o fato de que o ferro é extremamente pró-oxidante nos sistemas biológicos, como no caso da anemia falciforme, e que a toxicidade de sua sobrecarga endógena precisa ser avaliada. Com efeito, o **segundo objetivo** deste trabalho foi avaliar tais efeitos em humanos com anemia falciforme (sobrecarga endógena) e em camundongos suplementados com ferro (sobrecarga exógena). A resposta ao problema global de carência de ferro está centrada no tratamento com doses profiláticas de ferro e a um incremento contínuo da oferta de ferro na dieta (p.ex. programas de suplementação nas farinhas e suplementos nutricionais), o que tem impacto ainda desconhecido sobre a estabilidade genômica. Daí emerge o **terceiro objetivo** desse estudo: avaliar se a vitamina C, o suco de laranja ou uma dieta rica em antioxidantes poderia diminuir a toxicidade ao DNA gerada pela suplementação com ferro em camundongos. Soma-se a esse objetivo, avaliar se o composto quelante de ferro desferoxamida ou a vitamina C (vitamina chave na biodisponibilização do ferro não heme) administrados na fase adulta podem melhorar os efeitos de perda de memória e dano no DNA induzidos por um tratamento com ferro no período perinatal em ratos, uma vez que o mal de Parkinson e Alzheimer têm progressão lenta e emergência tardia e estão associados ao acúmulo de ferro no cérebro. O **quarto objetivo** do trabalho foi definir a concentração ideal de ferro para células em cultura, em paralelo a avaliação de marcadores nucleares e mitocondriais de estabilidade genômica e

da análise da expressão de genes ferro-dependentes envolvidos no reparo de DNA. Em resumo, o objetivo deste trabalho foi avaliar o efeito para a instabilidade genômica da deficiência e do excesso de ferro em modelos murinos e grupos populacionais humanos (crianças com alta vulnerabilidade: deficiência; pacientes de anemia falciforme: excesso), testar estratégias para reduzir a toxicidade do ferro para a memória e para o DNA (pela administração de alimentos, vitaminas e medicamentos), e, finalmente, determinar qual a dose ideal de ferro para manter a estabilidade genômica de células em cultura. Para tal, as metodologias principais empregadas foram: ensaios cometa neutro e alcalino, para avaliação de danos primários ao DNA; teste de micronúcleos em medula óssea de camundongos e em células em cultura citocinese-bloqueadas para determinação de clastogenênese/aneugênese; *Particle Induced X-ray Emission* (PIXE) para avaliação do nível de ferro nos tecidos; *polimerase chain-reaction* quantitativo em tempo real (QRTPCR) para avaliação do comprimento de telômeros e do número de cópias do genoma mitocondrial e nível de expressão de genes de reparo de DNA. Adicionalmente, testes de memória foram utilizados em ratos e avaliações do consumo de nutrientes foram empregados nas crianças e adolescentes com carência de ferro. Os resultados da pesquisa apontam um aumento de danos no DNA, tanto em situações de excesso como de deficiência de ferro. Para o caso da deficiência de ferro, observou-se em crianças e adolescentes avaliados um padrão geral de má-alimentação, marcado pela carência de várias vitaminas, especialmente ácido fólico e niacina e incidência de verminoses. A revisão bibliográfica apontou evidências preliminares do aumento do risco de câncer no trato intestinal em função de baixa ingestão de ferro, bem como uma quantidade de ferro ideal para minimizar este risco (20 mg/dia Fe, valor maior do que as recomendações nutricionais atuais para humanos). Os mecanismos associados a esse aumento de risco incluem desequilíbrio nas respostas imunes contra células malignas, no metabolismo de compostos tóxicos, bem como na regulação redox e na biossíntese e reparo do DNA. Quanto às estratégias para reduzir a toxicidade do ferro, os resultados indicam que a vitamina C, comumente associada à suplementação com ferro, pode aumentar a toxicidade de ferro e é inefetiva para reduzir o déficit de memória induzido desse metal. O suco de laranja, uma alternativa saudável à vitamina C no aumento da biodisponibilidade do ferro não heme, parece ser mais adequada para reduzir a toxicidade do composto, da mesma forma que a dieta rica em antioxidantes testada. O quelante de ferro mais empregado

globalmente (desferoxamida) foi efetivo em reverter o déficit de memória e os danos induzidos pelo ferro. Quanto à definição da dose ótima de ferro para o cultivo celular, evidenciou-se que a suplementação com ferro a longo-prazo, tanto na forma de sulfato ou como ferro ligado à transferrina pode contribuir no aumento da estabilidade genômica. Esses dados provêm de por metodologias de vanguarda, como comprimento de telômeros e estabilidade mitocondrial acessados por QTRTPCR, e indicam que a concentração ideal de ferro se situa numa faixa bastante estreita (6-20 µM Fe no meio de cultura). A análise da expressão de genes de reparo, executada em paralelo aos experimentos de suplementação com ferro nas células, revelou um novo mecanismo de controle pós-trascricional de genes dos genes WRN e MUTYH, que codificam, respectivamente, para uma DNA helicase e uma DNA glicosilase, ambas contendo ferro. Os resultados ora apresentados visam fornecer subsídios para manter a estabilidade genômica, reduzir o risco de câncer e maximizar a saúde dos indivíduos estudados, sendo aplicáveis globalmente pela da universalidade do cenário de desequilíbrio nutricional de ferro. Os avanços da pesquisa contribuem à incorporação do conceito de estabilidade genômica às recomendações nutricionais, que tradicionalmente sugerem níveis mínimos de nutrientes para diminuir a incidência de doenças associadas à deficiência e não visam deficiências nutricionais sutis e crônicas. Os resultados também contribuem a nutrigenômica: área incipiente de interface entre a genética e a nutrição, que aborda a interação entre os genes e os alimentos e, de uma forma particular, em estudar como os nutrientes afetam o genoma humano. Considerando a possibilidade de efeitos adversos do tratamento de ferro em indivíduos debilitados do ponto de vista bioquímico (p.ex. falta de antioxidantes) e as sugestões de associação entre o aumento da ingestão de ferro e acréscimo na incidência de câncer nos países de primeiro mundo, faz-se necessária uma definição parcimoniosa da melhor estratégia de suplementação/tratamento com ferro, no intuito de evitar instabilidade genômica em indivíduos com alimentação depauperada em nutrientes fundamentais à estabilidade genômica. Os resultados dos estudos in vitro desenvolvidos neste trabalho auxiliam no desenvolvimento de meios de cultura fisiológicos, lançam luzes sobre o problema da definição dos níveis ótimos de ferro para maximização da estabilidade genômica (p.ex. para células-tronco e protocolos de transplante) e evidenciam um novo mecanismo de controle do ferro sobre a expressão de genes de reparo de DNA.

ABSTRACT

Iron deficiency, named anemia when severe, is the far most common micronutrient deficiency worldwide, particularly among citizens of developing countries. Iron deficiency has been linked for a long time to immune system impairment and reduction in work capacity. Iron excess has been also associated, lately, with increased risk of chronic degenerative diseases and cancer. There are inconclusive evidences iron deficiency could increase the DNA damage level. Based in this concept, one could hypothesize iron deficiency increases cancer susceptibility, especially in tissues that suffer higher effect of iron deprivation. Therefore, the first aims of this research were: (a) to evaluate experimentally the repercussions of iron deficiency over genome stability and cancer risk in gastrointestinal tract; and (b) to review the literature towards the potential association between iron deficiency and gastrointestinal cancer risk increase. Another aspect that deserves attention is the fact of iron been extremely pro-oxidant in biological systems, as in sickle cell disease. Indeed, the second aim of this research was to evaluate such effects in humans with sickle cell disease (endogenous overload) and in mice supplemented with iron (exogenous overload). The answer to the global issue of iron deficiency is centered in treatment with prophylactic or curative iron doses and there is a general increase in dietary iron levels (e.g. flour supplementation programs and nutritional supplements) which has and not fully elucidated impact over genomic stability. Form this issue, emerges the third aim of this research: to evaluate if vitamin C, orange juice or a diet rich in antioxidants could reduce iron genotoxicity associated to iron supplementation. Summed to this objective, is the evaluation if the iron chelator desferoxamide and vitamin C (vitamin has a key role in iron bioavailability) administered in adulthood could ameliorate the memory impairment and DNA damage increase induced by iron treatment during neonatal period; particularly given the fact Parkinson and Alzheimer diseases have slow progression and are associated to selective accumulation of iron in brain. The fourth objective of the research was to define the ideal concentration of iron to cell cultures, in parallel to the evaluation of nuclear and mitochondrial markers of genomic stability as well as of the analyses of expression of iron-dependent genes involved in DNA synthesis and repair. All objectives

can be summarized, as to evaluate the effect over genomic stability of iron deficiency and overload in murine models and human populations (children and adolescents with low socioeconomic background: deficiency and sickle cell disease: overload), to test strategies to reduced iron toxicity to memory and DNA (through the administration of foods, vitamins and medicines, and, finally, to determine the ideal dose for genome maintenance in cell cultures. The main methods used were: neutral and alkaline comet assay, to evaluate primary DNA damages; micronucleus test in mice bone marrow and cytokinesis-block cell cultures, to determine clastogenenicity/aneugenicity; Particle Induces X-ray Emission (PIXE), to determine iron level in tissues; and quantitative polymerase chain-reaction (QRTPCR) to evaluate telomere length, mtDNA copy numbers and mRNA level of DNA repair genes. Additionally, memory tests and nutrient consumption evaluations were used. Results suggested an increase in DNA damage either for iron deficiency or overload. Regarding iron deficiency, children and adolescents with low socioeconomic status evaluated had a poor nutritional profile, marked by nutritional deficiency, particularly of folic acid and a high prevalence of parasitosis. In the review article regarding the association between iron deficiency and cancer risk increase, the review of a few experimental studies in murine animals and some epidemiological studies in humans indicated preliminary evidences towards an association between iron deficiency and increased cancer risk. Moreover, the mechanism by which iron deficiency could induce early onset and progression of tumors in gastrointestinal tract was discussed, including: impairment of iron-dependent metabolic functions (e.g. depressed immune defenses against malignant cells, reduced metabolization of toxic compounds, and unbalance in antioxidant defenses and DNA biosynthesis and repair). Regarding the strategies to reduce iron supplementation-associated toxicity, results suggest vitamin C was ineffective to reduce iron genotoxicity, could increase iron toxicity and was not able to repair iron-induced memory impairment. On the other hand, orange juice, a healthy alternative to vitamin C, could reduce iron toxicity, similarly to the also tested diet rich in antioxidants. Desferoxamide, the most used iron chelator worldwide, was effective to reverse iron induced memory deficit and DNA damages induced by iron; however, its clinical usage is difficult. Regarding the definition of the optimal dose for genomic stability for cells in culture, we observed an increase in cell proliferation and genomic stability (comet assay, telomere length and micronucleus) either for iron sulfate or holotransferrin, however in a

very narrow range. The analyses of expression of iron-related genes showed a new post-transcriptional regulation mechanism for iron-containing DNA helicase WRN and DNA glycosylase MUTYH. The results herein presented aim to provide subsides to maintain genomic stability, reduce cancer risk and to maximize the health, being widely applicable in face to the universality of iron deficiency. The research advances can contribute to the incorporation of the concept of genomic stability to nutritional recommendations; which traditionally are based in minimal levels of nutrients to diminish the incidence of diseases (e.g. anemia for iron) and are not focused in subtle nutrient deficiencies that can have chronic effects. Results also contribute to nutrigenomics: incipient area of interface between nutrition and genetics, which aims to evaluate how nutrients influence human genome. Considering the potential noxious outcomes of iron treatment in nutrient deprived individuals (i.e. with low antioxidant defenses or shortage of B vitamins), and the increase of dietary iron content, there is an urgency for the definition of the best supplementation strategy to prevent genomic stability. In vitro studies to define the adequate iron concentration can be useful for the definition of the optimal levels of iron to maximize genomic stability that could also be applied in improving cell culture protocols, for example to ex vivo culture of bone marrow previous to grafting after radiotherapy. In vivo studies also shed light upon a new mechanism for iron-dependent DNA repair regulation.

LISTA DE FIGURAS

LISTA DE TABELAS

LISTA DE ABREVIATURAS E SÍMBOLOS

8-oxoG	8-oxo-7,8-dihydro-2'-deoxyguanosine
AF	anemia falciforme
CBMN	teste de micronúcleos em células citocinese-bloqueadas
DCytB	*duodenal brushborder ferrireductase*
DFO	Desferoxamida
DMT1	*divalent metal transporter 1*
DNA	ácido deoxirribonucléico
DRI	*dietary reccomended intake*
ERO	espécies reativas de oxigênio
FDA	*Food and Drug Administration*
Fe-NTA	nitriloacetato férrico
GSHPx-1	gene da glutationa peroxidase 1
HH	hemocromatose hereditária
HO·	radical hidroxila
hTf	holotransferrina
HU	Hidroxiuréia
IMC	índice de massa muscular
IREs	elementos regulatórios de ferro
LIP	labile iron pool
LTM	treinamento de memória de longo prazo
MHC	*major histocompatibility complex*
MN	Micronúcleos
NBUD	botões nucleares (*nuclear buds*)
NPB	pontes nucleoplásmicas
NTBI	ferro não ligado à transferrina
O_2-	radical superóxido
PCR	polimerase chain reaction
PIXE	*particle-induced X-ray emission*
QRTPCR	reação em cadeia de polimerase quantitativa em tempo real
R24	recordatório 24 horas
RD	registro diário
RR	ribonucleotídeo redutase
SI	total de ferro no soro (*serum iron*)
SL	suco de laranja
STM	teste de memória de curto prazo
TfR1	receptor de trasferrina 1
TfR2	receptor de trasferrina 2
TIBC	capacidade total de ligação do ferro
UL	upper level

SUMÁRIO

1 INTRODUÇÃO

1.1 O ferro como um dos principais micronutrientes

O ferro compõe grande parte da terra, formando o cerne do planeta e tem uso amplo em diversos materiais. O ferro é um micronutriente fundamental para o crescimento e a manutenção de virtualmente todos os seres vivos (Valko et al., 2005). Nos organismos superiores, ele possui a principal função associada ao transporte e armazenamento de oxigênio, localizando-se na hemoglobina nos eritrócitos e na mioglobina dos tecidos musculares, respectivamente. O ferro também possui outras funções não menos importantes, compondo enzimas do metabolismo oxidativo (p.ex. citocromo C oxidase, succinato dehidrogenase e aconitase, que são envolvidas no ciclo de Krebs e na fosforilação oxidativa) e dos sistemas imunológico (p.ex. mieloperoxidase), nervoso (p.ex. tirosina hidroxilase) e antioxidante (p.ex. catalase). (Cook & Lynch, 1986; Imai & Nakagawa, 2003; Ortiz et al., 2004; Arredondo & Nunez, 2005). Mais ainda, o ferro está envolvido na síntese de catecolamidas (p.ex. adrenalina), no sistema de metabolização (família do citocromo P450), na sinalização dos níveis de oxigênio do organismo (óxido nítrico sintase), bem como em uma enzima muito importante para a síntese de DNA, a enzima ribonucleotídeo redutase (Atamna et al., 2001). Recentemente, o papel do ferro em diversas enzimas de reparo do DNA vem sendo descrito, com no caso de alquiltransferases (Begley & Samson, 2003; Mishina & He, 2006), glicosilases (Boal et al., 2007) e helicases (Rudolf et al., 2006; Pugh et al., 2008).

Dentro do corpo, o ferro pode ocorrer na forma de hemoproteínas ou como ferro não-heme, em grupamentos ferro-enxofre, ferro-oxigênio ou como ferro firmemente preso às proteínas (Papanikolaou & Pantopoulos, 2005). Quando o ferro está livre, ele representa um risco muito grande para a vida, pois a mesma propriedade que o faz ser útil quando preso às proteínas (a capacidade de oxidar substratos), passa a agir contra o organismo, gerando radicais livres. Dessa forma, evoluíram nas células diversos mecanismos para inibir a ocorrência de ferro livre, mesmo que em quantidades muito pequenas.

A proteção contra o excesso de nutrientes se dá, tipicamente em três etapas, na absorção, no uso e na excreção. Paradoxalmente, para o ferro a excreção é um processo pouco eficiente e se ele entrar em grande quantidade se depositará no organismo, gerando doenças. Sendo assim, o metabolismo do ferro é controlado principalmente pela absorção e uso e, secundariamente na estocagem (Kruszewski, 2003). Dessas, a absorção é a mais importante,

como evidenciado pelo prognóstico das doenças associadas ao acúmulo de ferro, veja-se como exemplo, a hemocromatose hereditária (primária); condição onde, tipicamente defeitos no metabolismo absortivo do ferro levam a conseqüências severas, como aumento do risco de diversos tipos de câncer, doenças cardiovasculares e envelhecimento precoce. Interessantemente, a hemocromatose secundária, associada ao acúmulo de ferro pelo estilo de vida ou por algumas doenças, também pode estar associada ao risco dessas doenças (Beutler, 2006). Contudo, referências a esse fato são escassas na literatura.

Duas proteínas são importantes para evitar a sobrecarga e garantir o metabolismo perfeito do ferro, a transferrina e a ferritina. Enquanto a função da transferrina é transportar virtualmente todo o ferro livre entre os locais de absorção e armazenamento e utilização, a ferritina tem a função de armazenar o ferro não utilizado. A ferritina existe principalmente no citoplasma, mas também pode ocorrer no núcleo celular e no exterior das células. Em todos esses locais, ela armazena o ferro, impedindo que o mesmo participe de reações de estresse oxidativo (Conrad & Umbreit, 2002; Lee & Jacobs, 2004; Surguladze et al., 2004; Papanikolaou & Pantopoulos, 2005). Mesmo com esse elaborado mecanismo de estocagem e transporte, entre 3-5% do conteúdo celular de ferro existe na forma lábil (*labile iron pool* – LIP), isso é, ligada fracamente a substâncias como fosfatos, nucleotídeos, hidroxilas, aminas e grupos sulfidrila. O LIP é elástico e aumenta bastante em situações de sobrecarga de ferro (Arredondo & Nunez, 2005).

A recomendação nutricional humana (*Dietary Recommended Intake* –DRI) para o ferro varia de 7 mg por dia em crianças de 1-3 anos de idade até 27 mg por dia para mulheres grávidas de qualquer idade. Além disso, indica um aumento de ingestão durante a adolescência para 11 e 15 mg por dia para homens e mulheres respectivamente. Para as mulheres, após a menarca, a recomendação passa a ser de 18 mg por dia, só diminuindo após a menopausa. Nesse ciclo da vida a recomendação de ferro para homens e mulheres se torna idêntica novamente, sendo de 8 mg por dia. O nível de consumo máximo tolerável (*Upper Level* – UL) para adultos saudáveis é de 45 mg de ferro por dia, com base em distúrbios gastrintestinais que podem ocorrer em decorrência de doses maiores (IOM, 2001).

O ferro ocorre na dieta tanto na forma inorgânica (sulfatos, óxidos ou íons livres), quanto na forma heme. Os alimentos de origem animal são os principais contribuintes (66 % do total em países consumidores de carne) para o ferro da dieta, por apresentarem cerca de 50% de seu ferro na forma heme, estando o fígado entre as principais fontes. Cereais integrais como aveia e gérmen de trigo bem como coco são fontes de ferro de média biodisponibilidade, enquanto feijões e espinafre têm grandes quantidades de ferro de baixa

biodisponibilidade (USDA, 2005). Uma vez que atualmente vários alimentos são enriquecidos com ferro e muitas pessoas consomem suplementos alimentares, esses aspectos também devem ser considerados nos cálculos da ingestão do nutriente. Quanto à biodisponibilidade, essa pode variar em função do composto usado na fortificação ou suplementação (Gibson, 2007).

O ferro heme tem alta biodisponibilidade (20-30%), por ser absorvido intacto e por ser mais solúvel e ter poucas interações no trato gastrintestinal, enquanto o ferro inorgânico tem baixa disponibilidade (5-15%) e pode ser amplamente influenciado pela composição da dieta (Tabela 1) (Arredondo & Nunez, 2005; Singh et al., 2006). Pela proporção variável entre ferro heme e não-heme na dieta e pela ampla influência da dieta na absorção do último, salienta-se que a biodisponibilidade do ferro deve ser calculada em termos de cada refeição.

A densidade de ferro no corpo (60 ppm para homens e 55 ppm para mulheres) é controlada pelo balanço entre a absorção e a perda de ferro pela excreção e descamação dos epitélios. Com efeito, um homem adulto (70 kg) possui cerca de 4 gramas de ferro em seu corpo (Conrad & Umbreit, 2002).

Utilizando os valores de necessidades nutricionais e de biodisponibilidade pode-se fazer algumas ilações acerca das quantidades de ferro absorvidas diariamente. Avaliando-se o caso de um homem que ingere 8 mg de ferro em um dia típico (consumindo carne e vegetais), sendo 2 mg de ferro heme (supondo que 200 g de carne contendo 5 mg de ferro, sendo 40% na forma heme) e o restante de ferro não heme; considerando-se 23 e 8% de absorção para ferro heme e não-heme, respectivamente; conclui-se a absorção de 0,46 mg de ferro na forma heme e 0,48 mg na forma inorgânica, ou 0,94 mg de ferro no total. Quanto às perdas, estima-se que um homem adulto perca ferro predominantemente por descamação do trato gastrintestinal, pele e trato urinário (0,6 mg, 0,3 mg, e <0,1 mg por 24 hr, respectivamente)(Baynes & Bothwell, 1990). O produto da absorção e da perda é próximo da nulidade, mostrando manutenção entre a entrada e a saída de ferro.

Um fluxo menstrual mediano (20-30 ml) eleva a necessidade basal diária média de uma mulher de 55 kg de cerca de 0,8 mg/dia para 1,3 mg/dia de ferro. Como a perda de ferro no pico de menstruação é de 1,6 mg/dia para 95% das mulheres, a necessidade nesses dias atinge 2,4 mg de ferro (Baynes & Bothwell, 1990). Com vistas a suplantar este pico de perda (aproximadamente 2,5 vezes o masculino) a DRI de ferro para mulheres em fase reprodutiva é de 18 mg ferro/dia, ou 2,25 vezes a masculina, 8 mg/dia ferro). O uso de contraceptivos que modulam o fluxo menstrual tem influência direta nas necessidades de ferro. Durante a gravidez, uma mulher de 55 kg necessita um aporte de 1 g de ferro (230 mg para perda basal,

450 mg para o aumento no hematócrito, 270-300 mg para o feto e 50-90 mg para os produtos de concepção); concentrados no segundo e terceiro trimestres (5-6 mg/dia). Apesar de parte do ferro ser reduzida pela acomodação do hematócrito após o parto, muito é perdido por sangramentos no periparto. A amenorréia resguarda a perda adicional via lactação. Um aspecto que merece destaque é o fato de que a carência de ferro reduz o peso ao nascer, além de causar problemas de desenvolvimento (Baynes & Bothwell, 1990); especialmente em países de terceiro mundo.

Cerca de 2/3 do ferro corporal está associado à hemoglobina e às hemácias. Essas últimas apresentam meia-vida de cerca de 120 dias e, por isso, grande parte do estoque corporal de ferro é constantemente reciclado para ser utilizado na síntese de novas moléculas de hemoglobina. Incrivelmente, cerca de 20 mg por dia de ferro, o equivalente a mais de 20 vezes o que é absorvido por dia (para o exemplo apresentado anteriormente), passa diariamente por esse processo de reciclagem (Conrad & Umbreit, 2002) conduzido pelos macrófagos do sistema reticuloendotelial (Anderson et al., 2007). Dos outros 1/3 do ferro contidos no corpo humano, 80% estão em outras proteínas que não a hemoglobina (p.ex. citocromos e principalmente mioglobina muscular) e os 20% restantes são estocados em macrófagos ou hepatócitos (Anderson et al., 2007) ou estão localizados no LIP das diferentes células do organismo.

Como já mencionado, os adultos podem absorver até 2 mg de ferro por dia (2 mg para mulheres e 1 mg dia para homens, geralmente) a partir de dietas contendo 10-20 mg de ferro (Arredondo & Nunez, 2005). O ferro é geralmente absorvido rapidamente quando administrado como pílula, tendo meia-vida de absorção de cerca de 5 horas, pico sérico em 2,4 horas, meia-vida de eliminação de 9,4 horas (Farheen et al., 2002). Diferentemente de outros minerais, o excesso de ferro é lentamente excretado (cerca de 0,025 a 0,05% da reserva de ferro por dia) na ausência de hemorragia e hemosiderinúria, e a excreção tem menor importância na homeostasia do ferro (Conrad & Umbreit, 2002).

Tanto o ferro heme, quanto o inorgânico são absorvidos na membrana apical dos enterócitos intestinais. A absorção do ferro heme é pouco entendida (Papanikolaou & Pantopoulos, 2005), mas sabe-se que ocorre a liberação do grupamento heme a partir da hemoglobina por enzimas do lúmen para que o heme possa entrar nas células como metaloporfirina. No interior das células, o ferro é liberado do heme pela ação da enzima heme oxigenase, de modo que esse seja metabolizado de forma idêntica ao ferro inorgânico. Os mecanismos de absorção do ferro inorgânico estão mais elucidados O ferro inorgânico existe principalmente como Fe^{3+} na dieta e precisa ser reduzido à forma $Fe^{2+,}$ o que pode ser

desempenhado por componentes ácidos da própria dieta (p.ex. vitamina C) ou pela enzima citocromo ferriredutase duodonenal das *brushborders* –(*duodenal brushborder ferriredurase* - DcytB){a Em seguida, o fero entra nos enterócitos por meio do transportador divalente de metais (*divalent metal transporter* 1 - DMT1, também conhecido como DCT1 ou nramp2) (Arredondo & Nunez, 2005).

Tabela 1. Sumário dos nutrientes e antinutrientes que interagem com o metabolismo do ferro**.**

Nutriente	Efeito	Mecanismo	Referências
Vitamina C	Aumento da absorção do ferro	Redução do ferro (solúvel em pH 2 e 3,5 parcialmente solúvel a pH 8,0)	(Arredondo & Nunez, 2005)
	Aumento da absorção do ferro de refeições que contenham inibidores do mesmo	Formação de quelato ferro-Vitamina C	(Teucher et al., 2004; Gibson, 2007)
Ácidos orgânicos (cítrico, láctico, acético, propiônico, fórmico).*	Aumento da absorção do ferro	Formação de quelato ou redução do ferro.	(Gibson, 2007)
Proteína (dependendo da composição de aminoácidos)	Aumento da absorção de ferro	Possivelmente pela liberação de peptídeos que contém aminoácidos cisteína que ligam ferro (são chamados fator carne)	(Gibson, 2007)
	Redução da absorção do ferro	Possivelmente pela presença de caseína em laticínios	(Gibson, 2007)
Mucinas, aminoácidos, açúcar, aminas e amidas.	Aumento da absorção de ferro	Quelantes. A formação de quelato dessas substâncias com o ferro inorgânico, previne a sua precipitação em pH maior que 3, quando entram no duodeno	(Arredondo & Nunez, 2005; Singh et al., 2006)
*Compostos fenólicos (fitatos, taninos e outros), carbonatos, fosfatos e oxalatos.***	Diminuição da absorção do ferro	Formação de complexo com o ferro inorgânico	(Arredondo & Nunez, 2005; Singh et al., 2006)
Cálcio (principalmente o leite)	Inibição aguda da entrada de ferro heme e não heme	Mecanismo não totalmente elucidado.	(Singh et al., 2006; Gibson, 2007)
Iodo	O iodo combinado com ferro reverte o volume da tireóide para um estado normal. A deficiência mútua de iodo e ferro é comum em 20-38% das crianças de populações dos paises em desenvolvimento	Mecanismo não totalmente elucidado. A suplementação combinada de ferro e iodo é muito mais efetiva para reverter a perda de volume da tireóide	(Zimmermann, 2006);
Vitamina A	Aumento da concentração de hemoglobina	Mecanismo não conhecido que possivelmente está relacionado à eritropoiese	(Singh et al., 2006)

	em crianças anêmicas e em mulheres gestantes	e mobilização do ferro dos depósitos. È ainda controverso se a vitamina A pode ou não aumentar a biodisponibilidade do ferro nos alimentos.	
Folato (vitamina B9)	Melhora da eritropoiese	Na deficiência de folato ocorre o aumento dos níveis de ferro sangüíneos devido ao bloqueio da eritropoiese. Na deficiência de ferro o metabolismo do folato também é alterado	(Oconnor, 1991; Juarez-Vazquez et al., 2002)
Folato + ferro	Aceleram a reversão da anemia por deficiência de ferro em gestantes***	Desbloqueio da eritropoiese e aumento da síntese de DNA	(Thompson et al., 2001; Juarez-Vazquez et al., 2002)
Riboflavin (vitamina B2)	Alteração na absorção, mobilização e utilização dos estoques de ferro para a síntese de hemoglobina na medula óssea.	A deficiência de vitamina B2 aumenta as perdas gastrintestinais de ferro endógeno. A adição de vitamina B2 na suplementação de ferro-folato melhora a eritropoiese pelo aumento do nível de hemoglobina.	(Powers, 1995)
Cobalamina (vitamina B12)	A deficiência de B12 tem sido ligada à anemia megaloblástica, pancitopenia e contagem elevada de plaquetas, o que é típico em idosos e vegetarianos. O tratamento com a vitamina melhora a eritropoiese e a metilação do DNA.	Papel chave na eritropoiese na síntese de hemoglobina e na metilação do DNA.	(Kwok et al., 2002; O'Leary et al., 2005; Maamar et al., 2006; Nusier & El-Dwairi, 2007).
Zinco	Redução da absorção do ferro.	Interação negativa mútua. A interação negativa é dependente da forma como o zinco e o ferro são administrados. O zinco pode reduzir a absorção do ferro especialmente quando é administrado em jejum e concomitantemente ao ferro na concentração molar Zn:Fe de 5:1 (28% de redução da absorção do ferro) até a concentração de 20:1 (40% de redução na absorção de ferro)	(Teucher et al., 2004)
Cobre	Estresse oxidativo e envelhecimento	As enzimas ceruloplasmina (ferroxidase) e citocromo oxidase, dependentes de cobre, podem apresentar diminuição em sua atividade pela deficiência de cobre. A diminuição da atividade da ceruloplasmina e citocromo oxidase tem sido associada ao estresse oxidativo e envelhecimento. A absorção do cobre pode ser prejudicada pelos níveis de ferro na dieta e nos suplementos.	(Haschke et al., 1986; Kim et al., 2001; Ramirez-Cardenas et al., 2005).

* encontrados em vegetais, em molho de soja, cereais ou produtos do leite; ** encontrados em chás, café, cacau, vinho tinto, cereais, legumes e leguminosas; *** as gestantes em estágios mais avençados de anemia são as que têm maiores benefícios com o tratamento de folato e ferro

Uma vez no citoplasma o ferro tem quatro destinos: a) ser utilizado para a síntese de proteínas com grupamentos heme, grupamentos Fe-S ou ferro em outros arranjos moleculares; b) ser estocado na ferritina; ou c) se associar ao LIP, estando preso a ligantes com fraca afinidade por ele; d) ser exportado para a corrente sangüínea, em direção a locais de uso ou de armazenamento (fígado). No caso de ser exportado, o Fe^{2+} precisa ser reoxidado antes de deixar os enterócitos pela membrana lateral. A enzima ferroportina 1 (também conhecida como IREG1 ou MTP1), em associação com a ferroxidase hepaestina que é presa na membrana basolateral dos enterócitos, controla a saída do ferro. Durante a sua saída das células, o ferro se associa à transferrina, que pode aceitar até dois íons de ferro por molécula. Interessantemente, a ceruloplasmina, uma ferroxidase solúvel cujo co-fator é cobre, também pode participar desse processo (Papanikolaou & Pantopoulos, 2005).

A transferrina carregada com ferro segue então pela corrente sangüínea até o fígado ou até os tecidos de uso, onde encontra os receptores de transferrina 1 (TfR1) e sofre endocitose conjuntamente com seu receptor, sendo depositada em endossomos. O pH ~ 5,5 dos endossomos desprende a transferrina de seu receptor, bem como o ferro da transferrina. O ferro é exportado, então, para fora da membrana endossomal, por ação de DMT1 (Papanikolaou & Pantopoulos, 2005). A expressão do TfR1, em conjunto com o seu homólogo receptor de transferrina 2 (TfR2) (encontrado apenas em hepatócitos e células hematopoiéticas) aumenta a entrada de ferro através desse mecanismo e é um marcador da necessidade fisiológica de ferro (Anderson et al., 2007).

Sabe-se pouco sobre o transporte do ferro dentro das células (Papanikolaou & Pantopoulos, 2005). O metabolismo celular de ferro é principalmente regulado pelas proteínas regulatórias de ferro 1 e 2 (IRP1 E IRP2). A IRP1 é aconitase, proteína citosólica com efeito dual, que atua tanto no ciclo de Krebs como na ligação a mRNA de genes do metabolismo do ferro; controlando pós-transcricionalmente sua expressão. Exemplos nesse sentido são o bloqueio da tradução do mRNA da ferritina e a estabilização dos receptores de transferrina, através da ligação a elementos regulatórios de ferro (IREs) em situações de falta do elemento (Lobmayr et al., 2005).

Avanços recentes no entendimento da regulação sistêmica do metabolismo do ferro têm mostrado que duas moléculas têm papel importante: HFE, um *major histocompatibility complex* (MHC) de classe I não-típico, que é ubiquamente expresso em níveis baixos e expresso em grandes quantidades no fígado, e tem capacidade de afetar a expressão de TfR1 e, portanto, controlar a tomada de ferro pelos tecidos; e hepcidina, um pequeno peptídeo antimicrobiano sensível a citocinas, que é sintetizado nos hepatócitos e que

quando secretado na circulação catalisa o efluxo de ferro a partir de macrófagos, enterócitos intestinais, possivelmente por bloquear ferroportina1. HFE e TfR1e 2 parecem agir antes da hepcidina na regulação do metabolismo do ferro. Uma terceira molécula, hemojuvelina, a proteína codificada pelo gene HFE2 parece agir no mesmo nível que HFE e TfR1 no metabolismo sistêmico de ferro. A transferrina carregada com duas moléculas de ferro também parece agir com um elemento regulador a montante (Anderson et al., 2007).

A faixa de referência para o conteúdo total de ferro no soro [*serum iron* (SI)] é de 9-36 μM, sendo de cerca de 20 μM em indivíduos saudáveis (Annanmaki et al., 2007). Em função de sua reatividade e por ser um nutriente limitante ao crescimento de microrganismos patogênicos, existem quantidades mínimas de ferro livre no sangue. A vasta maioria do ferro no soro está ligada à transferrina, outra parte à ferritina no soro e uma reduzidíssima quantidade é detectada como ferro não ligado à transferrina (NTBI).

A quantidade típica de transferrina no sangue humano é de 1,75–3,13 g/L, ou cerca de 2,5 g/l (30 μM) para indivíduos saudáveis (Annanmaki et al., 2007). Levando em consideração o valor normal de transferrina (30 μM) e sabendo que 1 M de transferrina pode comportar até 2 M de ferro, pode-se concluir que o soro de um sujeito hipotético poderia teoricamente acomodar uma quantidade de até 60 μM de Fe. Na verdade esse conceito dá origem a um parâmetro indireto do status de ferro: a capacidade total de ligação a ferro do soro (TIBC), que varia na faixa de 60 μM de ferro. Lembrando-se que o soro de um indivíduo típico apresenta apenas 20 μM de ferro do TIBC (60 μM) ocupados por moléculas de ferro, pode-se lançar mão de um outro conceito: o da saturação de transferrina (TS); cerca de 1/3 nesse exemplo. A mediana da saturação de transferrina varia entre 22 e 28%, de acordo com o sexo e a idade (Koziol et al, 2001).

O NTBI de indivíduos saudáveis (SI=20 μM, TIBC=60 μM e TS=33%) é geralmente menor que 1 μM (ou <5% SI). Por outro lado, o NTBI de pacientes com hemocromatose hereditária (HH) (SI> 35 μM, TS≈100%) pode ser tão elevado como 10 μM (Evans et al., 2008). TS superior a 60% é um marcador de risco geral aumentado de câncer e mortalidade (Mainous et al., 2005). Outro parâmetro hematológico que aparece aumentado na HH é a ferritina sérica (SF). Enquanto a ferritina sérica mediana varia entre 2,5-4,0 nM (mulheres, aumentando com a idade) e 5,5 nM (homens), homens com HH podem apresentar SF >100 nM (McLaren et al, 2008). Como cada molécula de ferritina pode armazenar 4500 moléculas de ferro, um indivíduo com 100 nM SF poderia armazenar

cerca de 200 mg de ferro em 8 litros de sangue, equivalendo à quantidade a ser absorvida ao longo de 200 dias por um homem saudável.

Um aspecto interessante é que embora tenha sido hipotetizado até então que o NTBI só ocorreria após a capacidade da TS ser suplantada, evidências experimentais e clínicas sugerem que o NTBI possa aumentar substancialmente em algumas situações patofisiológicas (Lee & Jacobs, 2004), bem como após o consumo de doses medicamentosas de ferro (Dresow et al., 2007). Dresow et al (2007) mostraram um aumento dose-relacionado no NTBI até valores de 9 μM Fe 1–4 h após a administração de preparações farmacêuticas de ferro (complexos diferentes) entre 10 e 100 mg Fe; não ocorrendo em nenhum dos tratamentos TS maior do que 76% (para as preparações contendo 100 mg Fe).

1.2 Instabilidade genômica, anemia e câncer: efeitos da carência e excesso de ferro

1.2.1 Deficiência de ferro

Em geral os efeitos da deficiência de ferro são mencionados apenas em situações severas, como no caso da anemia. Faz-se isso pela avaliação dos níveis de hemoglobina na circulação, sendo difícil determinar apenas a partir desse dado qual a real concentração de ferro nos tecidos, o que pode ter defeitos severos na saúde humana (Cook & Lynch, 1986). Cabe lembrar, que os tecidos podem estar depauperados de ferro, mesmo quando o nível de hemoglobina na circulação é normal. A anemia e a deficiência de ferro têm efeitos severos no organismo, dos quais se pode citar:

i) Imunidade e infecção: a falta de ferro reduz a atividade bactericida dos neutrófilos, possivelmente devido à repressão da atividade da mieloperoxidase (Sagone & Balcerzak, 1970). A carência de ferro também reduz o número e a proliferação dos linfócitos T (Arredondo & Nunez, 2005); portanto, aumentando a suscetibilidade a infecções comuns. A remoção de ferro do sangue é um mecanismo pelo qual o organismo diminui o risco de infecção (Bullen et al., 2005; Vyoral & Petrak, 2005). Desse modo, a literatura remete a uma discussão vigorosa acerca do risco de que a terapia com ferro pode aumentar a suscetibilidade a infecções (Cook & Lynch, 1986;

Oppenheimer, 2001; Gera & Sachdev, 2002). Apesar disso, a terapia com ferro parece reduzir o risco de infecções, por melhorar a eficiência do sistema imune quando esse está deprimido por falta de ferro (Cook & Lynch, 1986).

ii) Função gastrintestinal: os tecidos do trato gastrintestinal apresentam diversas alterações em função da falta de ferro, incluindo glosite, estomatite, redes esofágicas, gastrite crônica e má absorção. A gastrite atrófica é a conseqüência mais severa da carência de ferro, por reduzir ainda mais a absorção de federo devido à diminuição da acidez gástrica (Jacobs, 1971; Jacobs, 1982).

iii) Função neurológica: a estrutura e funcionamento do cérebro podem apresentar defeitos consideráveis em resposta à deficiência de ferro, especialmente nas fases de mais intenso desenvolvimento quando a formação de mielina é defectiva (Ortiz et al., 2004; Arredondo & Nunez, 2005).

iv) Outros efeitos: diversas alterações funcionais nas unhas e nos tecidos orais são associadas à deficiência de ferro e os indivíduos podem também apresentar desejo de ingerir materiais não alimentares (IOM, 2001). Crianças com deficiência de federo mostram alterações funcionais na função renal (Ozcay et al., 2003) e a termorregulação é prejudicada por um efeito da carência de ferro na tireóide (Zimmermann, 2006). Outro efeito é uma diminuição na capacidade de trabalho dos indivíduos, que causa perdas econômicas para os países e reduz a remuneração dos indivíduos; em um círculo vicioso, pois o padrão alimentar se depaupera progressivamente.

Tanto humanos quanto modelos animais geralmente apresentam aumento de estresse oxidativo e alterações no nível de enzimas antioxidantes em conseqüência da deficiência de ferro. A maioria dos estudos mostra uma redução no metabolismo da glutationa peroxidase e da catalase, bem como efeitos variáveis na glutationa redutase, superóxido dismutase e glicose 6-fosfato desidrogenase (Tabela 2). Aslan e colaboradores (2007) reportaram uma diminuição na atividade das enzimas paraoxanase e ariltransferase no soro de indivíduos anêmicos. Sabe-se que o estresse oxidativo e o nível de ferro têm efeito sobre a expressão da glutationa peroxidase (gene GSHPx-1) (Fuchs, 1997). Mais ainda, os estudos mostram que a atividade das enzimas pode ser revertida pela correção dos níveis de ferro nos organismos (Macdougall, 1972; Isler et al., 2002; Kurtoglu et al., 2003).

O trabalho pioneiro de Atamna e colaboradores (Atamna et al., 2001; Atamna et al., 2002; Atamna et al., 2002) acerca dos efeitos moleculares da deficiência de ferro em termos da estabilidade genômica trouxeram esse tema, um tanto negligenciado pela

ciência, ao foco do interesse científico. Esses pesquisadores apontam que a deficiência na formação de heme leva à redução da produção mitocondrial de energia, por disfunções no complexo IV mitocondrial, e que estes efeitos são associados à instabilidade das mitocôndrias, ao aumento do estresse oxidativo e a aceleração do processo de envelhecimento. Sendo assim, nota-se a relação entre alterações nas enzimas envolvidas no metabolismo energético, no ciclo de Krebs e na cadeia transportadora de elétrons, as quais executam esse processo. Confirmando essa hipótese, outras enzimas do metabolismo oxidativo, que contém grupamentos Fe-S, aconitase e succinato desidrogenase, parecem ser prejudicadas pela deficiência de ferro (Chen et al., 2005; Liew & Shaw, 2005).

Tabela 2. Resumo dos estudos que avaliaram o efeito da deficiência de ferro sobre o estresse oxidativo e as principais enzimas antioxidantes em humanos e roedores.

Tecido/organismo	**Estresse oxidativo**	**Enzimas antioxidantes**					**Referência**
		SOD	**GSH-Px**	**GSSG-R**	**CAT**	**G-6-PD**	
Soro humano	↑LOOH	NA	NA	NA	NA	NA	(Aslan, Kosecik et al. 2007)
Eritrócitos humanos	↑MDA	↓	↓	NA	↓	NA	(Kurtoglu, Ugur et al. 2003)
Eritrócitos humanos	NA	↓	-	NA	NA	NA	(Isler, Delibas et al. 2002)
Eritrócitos humanos	NA	-	↓	NA	-	NA	(Tekin, Yavuzer et al 2001)
Eritrócitos humanos	↑MDA	↓	↓	-	↑	↓	(Kumerova, Lece et al. 1998)
Mucosa intestinal de ratos	↓MDA, ↓proteínas carboniladas	-	↓	NA	↓	NA	(Srigiridhar & Nair 1998)
Fígado de ratos	↓MDA	-	↑	NA	-	NA	(Rao & Jagadeesan, 1996)
Eritrócitos humanos	↑GSH; -MHb; -MDA	NA	NA	NA	NA	NA	(Bartal, Mazor et al. 1993)
Eritrócitos humanos	↑MDA	↑	NA	-	NA	NA	(Acharya, Punchard et al. 1991)
Eritrócitos de ratos	↑MDA após incubação com H_2O_2	↑	NA	NA	NA	NA	(Jansson, Perkkio et al. 1985)
Pulmões de fetos	NA	-	-	NA	↓	-	(Tanswell and Freeman 1984)

de rato

Eritrócitos de ratos	NA	NA	↓	NA	↓	NA	(Lee, Layman et al 1981)
Fígado de ratos	NA	-	↓	-	↓	NA	
Fígado de ratos	NA	NA	NA	NA	↑	NA	(Burk, Nishiki et al 1978)
Eritrócitos humanos	NA	NA	↓	NA	NA	NA	(Perona, Cellerino et al 1977)
Eritrócitos humanos	NA	NA	↓	NA	NA	NA	(Cellerino, Guidi et al 1976)
Eritrócitos de Coelho	NA	NA	↓	↑	NA	NA	(Rodvien, Gillum et al 1974)
Eritrócitos humanos	NA	NA	↓	NA	NA	NA	(Hopkins and Tudhope 1973
Eritrócitos humanos	NA	NA	↓	NA	↓	NA	(Macdougall 1972)

SOD: superóxido dismutase; GSH-Px: glutationa peroxidase; GSSG-R: glutationa redutase; CAT: catalase; G-6-PD: glicose 6-fosfato desidrogenase; ↑: aumento; -: manutenção, ↓: diminuição; NA: não avaliado; LOOH: lipídios peroxidados; MDA: malondialdeído; GSH: glutationa; MHb: metahemoglobina.

1.2.2 Sobrecarga de ferro

O envenenamento acidental com ferro está entre as causas mais comuns de morte por envenenamento em crianças menores de 6 anos nos Estados Unidos da América. Doses entre 20 e 60 mg/kg de ferro causam irritação intestinal, enquanto doses maiores causam toxicidade sistêmica (IOM, 2001). Doses agudas de 10-20 mg Fe/kg são consideradas não-tóxicas para humanos (Schumann, 2001). Interessantemente, quantidades corporais elevadas de ferro têm sido relacionadas a várias condições patológicas, incluindo doenças hepáticas e cardíacas, câncer, doenças neurodegenerativas (p.ex. Alzheimer), diabetes, anormalidades hormonais e anormalidades do sistema imune (Valko et al., 2005). Nesse sentido, alguns estudos sustentam que estoques corporais elevados de ferro, avaliados pela TS, estão altamente associados com um aumento no risco geral de mortalidade, mesmo quando se controla esse risco por fatores de risco comuns que poderiam estar associados com o aumento na ingestão de ferro (p.ex. ingestão elevada de suplementos de ferro ou de grandes quantidades de carne vermelha) (Mainous et al., 2005).

O termo hemocromatose se refere a todas as condições associadas ao aumento nos estoques corporais de ferro, ocorrendo, na maioria dos casos de hemocromatose, elevações significativas e permanentes na transferrina plasmática, que não estão relacionadas a

períodos pós a ingestão de ferro. Portanto, indicando que as anormalidades no transporte de ferro não estão apenas limitadas às células do cólon, mas incluem àquelas de diversos outros tecidos (Beutler, 2006). A HH é designada como primária por não estar relacionada a qualquer outra patologia no que diz respeito ao acúmulo de ferro. A HH é caracterizada por degeneração progressiva, iniciada por defeitos nos macrófagos e posteriormente, por volta da quarta década de vida, observa-se um acúmulo excessiva e disfunções funcionais no fígado, pâncreas, pituitária e outros órgãos endócrinos, bem como coração, articulações e derme (Papanikolaou & Pantopoulos, 2005; Beutler, 2006). Com efeito, tais complicações podem ser associadas àquelas vistas nas doenças crônico degenerativas, típicas do processo de envelhecimento, como artrite, diabetes, além de disfunções hepáticas, câncer e parada cardiovascular. A HH tem prevalência de 1:300 entre indivíduos do norte da Europa. A doença de manifestação na idade adulta está geralmente associada a mutações nos genes TfR2 e HFE, possivelmente por defeitos no sistema da hepcidina. A HH juvenil é relacionada a mutações no gene HAMP, possivelmente associada a defeitos na rota regulatória hepcidina-hemojuvelina. Outras formas de HH, relacionadas a outros *loci*, também são conhecidas [para revisão, ver Papanikolaou e Pantapoulos (2005)].

A hemocromatose secundária pode se originar de transfusões sanguíneas repetitivas e de condições associadas a deficiências na eritropoiese, como talassemia e anemias sideroplásicas ou hemolíticas, síndromes mielodisplásicas e deficiências de enzimas eritrocitárias (p.ex. piruvato quinase). A eritropoiese inefetiva resulta no desequilíbrio do mecanismo homeostático do ferro e na conseqüente reabsorção de altos níveis de ferro via sistema gastrintestinal (Papanikolaou & Pantopoulos, 2005; Beutler, 2006). As talassemias e a anemia falciforme (AF) são hemoglobinopatias relativamente comuns relacionadas à acumulação hepática de ferro e anormalidade no órgão que podem elevar o risco de hepatocarcinomas (Borgna-Pignatti et al., 2004; Mueller et al., 2006; Jastaniah et al., 2007). Por um lado, indivíduos com talassemia têm eritropoiese inefetiva, que provavelmente é associada a altos níveis de apoptose dos precursores das hemácias na medula óssea, bem como a uma diminuição na meia-vida das hemácias (Birgens & Ljung, 2007). Por outro lado, os pacientes de AF sofrem vaso-oclusões dolorosas e síndrome aguda do peito, derivadas do bloqueio de pequenos capilares pelas hemácias em forma de foice, o que leva à liberação de hemoglobina no sangue (Steinberg, 1999). Ambas as doenças são oxidativas, inflamatórias e estão associadas a níveis elevados de ferro heme pró-oxidante na circulação. De fato, o ferro heme pode danificar as biomoléculas (proteínas, lipídios e DNA) pela geração de espécies reativas de oxigênio (ERO), que são

associados a várias doenças (Kumar & Bandyopadhyay, 2005). O dano no DNA de pacientes com talassemia é confirmado, (Anderson et al., 2000), mas faltam estudos em pacientes de AF.

No cérebro, o ferro tem sido associado à etiologia de diversas doenças degenerativas, como as doenças de Parkinson (Berg et al., 2001) e Alzheimer (Fredriksson et al., 1999). Com efeito, a suplementação no período neonatal em camundongos, quando a barreira hematoencefálica ainda não está totalmente formada, induz acumulação de ferro em determinadas regiões do cérebro e defeitos na capacidade de memória (Fredriksson et al., 1999; Fredriksson et al., 2000; Schroder et al., 2001). Embora o mecanismo do defeito na memória esteja longe de ser totalmente elucidado, animais com sobrecarga de ferro apresentam aumento duradouro na peroxidação lipídica e carbonilação protéica (Dal-Pizzol et al., 2001; de Lima et al., 2005) e talvez a dano no DNA.

A toxicidade do ferro é bastante descrita na literatura [ver (Meneghini, 1997; Valko et al., 2005; Papanikolaou et al., 2006)]. A ciclagem entre Fe^{+2} (doador de elétrons) e Fe^{+3} (aceptor de elétrons), por um lado faz do ferro um participante ideal para diversas reações biológicas, mas por outro lado o caracteriza como uma substância altamente tóxica em condições oxidativas. Enquanto o Fe^{+3} é menos reativo do que Fe^{+2} (a forma prioritária para o armazenamento e o tráfico do elemento nos sistemas biológicos), ambos os íons podem reagir em reações em cascata de oxidação. Por exemplo, na reação de Fenton, o Fe^{+2} reage com peróxido de hidrogênio (H_2O_2) e se transforma em Fe^{+3} e gera em conseqüência o radical hidroxila ($HO^{\cdot}$), a ERO mais reativa de todas. Já na reação de Haber-Weiss, tanto Fe^{+2} como Fe^{+3} podem catalisar a formação do $HO^{\cdot}$ a partir de H_2O_2 e/ou do radical superóxido (O_2^{-}). É importante lembrar que o ferro está sempre apto a reagir com moléculas que contenham oxigênio, mesmo quando o ferro e/ou o oxigênio estejam livres ou complexados com ligantes fracos ou até mesmo dentro de grandes biomoléculas, como lipídios, proteínas, açúcares ou DNA e RNA (Halliwell & Guterridge, 2000).

Há evidências de que o ferro induz peroxidação lipídica em animais, como observado no cólon e no cécum de ratos, que também apresentaram redução no nível de algumas enzimas antioxidantes na região (Huang, 2003). Estudos com idosos apontam associação entre os níveis plasmáticos de ferro e a peroxidação lipídica (Lasheras et al., 2003). O ferro (frequentemente como complexo) sem sombra de dúvida é um catalisador efetivo do processo de peroxidação lipídica, contudo por mecanismos longe de serem completamente entendidos (Cheng & Li, 2007). No que diz respeito ao dano no DNA,

compostos inorgânicos contendo ferro mostraram toxicidade ao material genético no ensaio de transformação viral em embriões de hâmster (Heidelberger et al., 1983), troca de cromátides irmãs em células de hâmster (Tucker et al., 1993), tautomerização de bases em culturas de hepatócitos de ratos (Abalea et al., 1999). Nossos resultados também confirmam a toxicidade ao DNA em leucócitos (Franke et al., 2005) e na medula óssea (Prá et al., 2007) de ratos. O ferro heme também é um importante catalisador de oxidação, que induz pontes entre proteínas e danos ao DNA (Kumar & Bandyopadhyay, 2005), como exemplificado pela hemoglobina e hemina (Glei et al., 2006).

Reservas corporais aumentadas de ferro, medidas pela ST, estão associadas a um aumento no risco geral de morte e uma associação fraca com o risco geral de câncer (Mainous et al., 2005). Humanos com HH apresentam maior incidência de carcinomas hepatocelulares (Mueller et al., 2006) e outros tipos de câncer, como de esôfago, melanomas de pele e leucemia mielóide aguda (Papanikolaou & Pantopoulos, 2005). Estudos com animais confirmam a carcinogenicidade do ferro, após injeções intraperitoneais repetidas de diversos compostos contendo ferro, tanto em ratos como em camundongos (Toyokuni, 1996). Além disso, diversos estudos mostraram uma maior incidência de diferentes tipos de câncer entre trabalhadores envolvidos na mineração, siderurgia e metalurgia de ferro em diversos países, como Coréia (Ahn et al., 2006) e Alemanha (Adzersen et al., 2003). O asbesto, um material que contém cerca de 30% (em peso) de ferro, e magnetita (Fe_3O_4) são carcinógenos conhecidos por meio de poeira (Garry et al., 2004; Valko et al., 2005). Uma revisão de Nelson (2001), evidencia que ¾ da maioria dos grandes estudos suportam a associação do ferro no risco de neoplasias colorretaisUma análise mais detalhada dos dados levantados por Nelson (2001) mostra que a relação entre o nível de ferro e o risco de câncer é complexa, com alguns estudos mostrando relações inversas entre as reservas corporais de ferro e o risco de câncer; o que poderia ser interpretado como uma associação entre a deficiência de ferro e um aumento no risco de câncer.

O estresse oxidativo crônico tanto em lipídios, proteínas e DNA *in situ* (Meneghini, 1997) é o mecanismo proposto para a carcinogenicidade. Evidências crescentes sugerem que o ferro é capaz de induzir danos específicos em gene supressores de tumor codificados pelas quinases ciclina-dependentes p15 e p16, e particularmente o gene p53 gene, possivelmente pela formação de 4-hidroxi-2-nonenal (Hussain et al., 2000; Huang, 2003; Petersen, 2005). No fígado, o acúmulo de ferro leva à fibrogênese (Papanikolaou & Pantopoulos, 2005) e a um ambiente celular pró-inflamatório (Petersen, 2005). Um estudo

recente apontou que o ferro afeta a expressão de genes envolvidos no transporte de ferro e na adesão celular em células cancerosas de cólon. Essa última evidência suporta que o ferro pode ter um papel metastático devido à desagregação celular (Brookes et al., 2006). O ferro pode potencializar outros fatores de risco de câncer como o consumo de álcool (Ioannou et al., 2007), tabagismo (Zhou et al., 2005), hormônios (Liehr & Jones, 2001; Kabat et al., 2007); bem como pode ter efeito sinergístico ou antagonístico com a vitamina C, dependendo dos níveis da mesma (Deneo-Pellegrini et al., 1999; Mainous et al., 2005).

Outros mecanismos de toxicidade gastrintestinal do ferro têm sido propostos para explicar a carcinogenicidade nessa região. Valko e Morris (2001) propõem que os ácidos biliares (ácido deoxicólico), as vitaminas K, complexos contendo Fe^{2+} e oxigênio interagem na indução de efeitos oncogenéticos no cólon, pela geração de radicais livres. Lee e colaboradores (2004) sugeriram um aumento no risco de câncer induzido por ferro entre indivíduos que ingerem suplementos nutricionais de ferro e grandes quantidades de substratos fermentáveis (p.ex. fibras dietéticas e amido resistente). Essa hipótese está fundamentada nas evidências de aumento de câncer de cólon concentração-dependente entre mulheres que consumiam quantidades acima da mediana de substratos fermentáveis, visto que estes podem aumentar a absorção de ferro por acidificar o intestino grosso.

A sobrecarga de ferro tem sido relacionada também a progressão de tumores, por exemplo, na mucosa gástrica (Wu et al., 1996) e pele. Animais experimentais com sobrecarga de ferro apresentam taxas mais elevadas de conversão de papilomas benignos em carcinomas, possivelmente pela indução de radicais livres (MacPhee, 1998; Bhasin et al., 2002; Bhasin et al., 2004). Há ainda evidências que a sobrecarga de ferro suprime a anti-tumorogênica do sistema imunológico (Huang, 2003). De fato, diversos estudos abordam a possibilidade do uso da quelação de ferro como uma estratégia terapêutica em protocolos de quimioterapia (Buss et al., 2004).

1.3 Estratégias para aumentar a biodisponibilidade e reduzir o risco da suplementação com ferro

É bastante provável que a suplementação com ferro em doses elevadas gere estresse oxidativo em diferentes tecidos e órgãos. Reduzir o risco associado à suplementação é fundamental. A primeira estratégia é a adoção de uma alimentação balanceada e rica em antioxidantes. Se na realidade isso acontecesse, a anemia não se desenvolveria, porque tal

alimentação forneceria os níveis adequados de ferro e de outros nutrientes para a síntese de hemoglobina. Assim sendo, devemos pensar em alternativas para tratar esses pacientes. Quatro classes de compostos têm sido estudadas para reduzir os efeitos desfavoráveis da sobrecarga de ferro, compostos fenólicos (flavonóides), vitaminas e quelantes de ferro.

Os compostos fenólicos, presentes em diversos alimentos como frutas e seus derivados (p.ex. sucos e vinho) também podem reduzir a toxicidade do ferro. Esses compostos têm a capacidade de atuar como antioxidantes, além de capturar o ferro livre no sangue e nos tecidos moles, indisponibilizando esse para reações de estresse oxidativo. Interessantemente, alguns flavonóides como a miricetina são capazes de ativar o reparo de DNA, facilitando a remoção de bases danificadas geradas pelo tratamento de células com ferro (Abalea et al., 1998).

Diversos estudos mostram que a vitamina E pode reduzir a toxidade do ferro para diferentes tecidos e órgãos. A suplementação com vitamina E parece inibir a peroxidação lipídica, a apoptose, a formação de bases oxidadas no DNA e o desenvolvimento de câncer em ratos tratados com nitriloacetato férrico (Fe-NTA), um modelo de câncer renal (Zhang et al., 1997). A vitamina E também parece melhorar a toxicidade para o trato gastrintestinal de suplementos de ferro. A colite ulcerativa e as outras doenças do colón irritável demandam a suplementação com ferro, mas essa é geralmente de grande toxicidade para os pacientes (Carrier et al., 2002). A vitamina E também parece reduzir o nível da proteína *ras-p21* em ratos, uma proteína oncogênica que tem níveis aumentados durante o desenvolvimento de câncer de cólon em ratos e em pacientes com câncer avançado (Earl et al., 1954). Por outro lado, um estudo avaliando o efeito da vitamina E sobre o estresse oxidativo gerado por diferentes oxidantes em células de ratos, mostrou que o composto é pouco efetivo para reduzir o dano ao DNA, inclusive induzido pela sobrecarga de ferro in vivo, conforme avaliado pelo teste de micronúcleos em células binucleadas (Record & Jannes, 2000).

A vitamina C é um micronutriente importante, requerido como co-fator para enzimas envolvidas em reações de oxi-redução e para a manutenção do estado redox da glutationa (Ames, 2001; Fenech & Ferguson, 2001; Halliwell, 2001; Edenharder et al., 2002; Edenharder et al., 2003). A vitamina C tem sido estudada por sua ação potencial contra diversas doenças (Vijayalaxmi & Venu, 1999; Edenharder et al., 2002; Edenharder et al., 2003). No que diz respeito ao ferro, a vitamina C tem papel importante em sua absorção (Davidsson, 2003), bem como influencia a maturação e o funcionamento dos tecidos nervosos, extremamente dependentes de ferro (Bourre, 2006). Apesar do efeito

antioxidante da vitamina C, sua interação com o ferro pode levar a efeitos nocivos nos organismos; uma vez que a vitamina C reduz o ferro e o torna mais ativo em reações oxidativas (Oikawa & Kawanishi, 1998; Valko et al., 2004). Alguns estudos mostram que a vitamina C tem efeitos ambíguos sobre o material genético (Genetox-TOXNET, 2008), com alguns estudos mostrando que o composto pode reduzir o estresse oxidativo de lipídios (Chen et al., 2000) e o dano ao DNA (Premkumar & Bowlus, 2003) em situações de sobrecarga de ferro, e em outros estudos mostrando efeitos pró-oxidantes da vitamina C, pela sua reação com metais (Stadler et al., 1994; Fenech & Ferguson, 2001; Halliwell, 2001). No que diz respeito à memória, alguns estudos apontam que a vitamina C melhora a memória tanto em modelos animais como em humanos (Bourre, 2006).

A desferoxamida (DFO) é o quelante de ferro mais utilizado globalmente, sendo capaz de reduzir a toxicidade ao DNA por agentes oxidantes (Barbouti et al., 2001; Shackelford et al., 2003; Farombi, 2006), reduzir a instabilidade genômica de células com sobrecarga primária de ferro tratadas com agentes oxidantes (Shackelford et al., 2006). Também é capaz de reduzir o estresse oxidativo e a apoptose em oligodendrócitos em cultura submetidos a insultos isquêmicos (Hemdan & Almazan, 2006), proteger contra os efeitos da hemorragia intracerebral por reduzir o edema e déficits neuronais (Nakamura et al., 2004) e até melhorar a performance da memória espacial em ratos submetidos a lesões cerebrais (Long et al., 1996). Contudo, o composto pode ser pouco efetivo em reduzir a mutagenicidade de outros agentes (Edenharder & Grunhage, 2003) e existem diversos relatos de sua toxicidade. A DFO reduz a viabilidade de linhagens celulares derivadas de neuroblastomas e astrocitomas, possivelmente via geração de radicais hidroxila (Lee & Wurster, 1995). Em pacientes anêmicos dependentes de transfusão, a DFO induz neurotoxicidade acústica e visual (Freedman et al., 1988). Portanto, é provável que a DFO tenha um efeito bifásico antioxidante/pró-oxidante, dependendo da dose. Algo que é comum na relação entre reações catalisadas pelo ferro e a ação de agentes redutores fortes de alta mobilidade como a desferoxamida (Borg & Schaich, 1986). A DFO também pode reduzir o *pool* de deoxiribonucleotídeos necessário à síntese e reparo de DNA, por bloqueio da ribonucleotídeoredutase via quelação do ferro (Dayani et al., 2004). Portanto, especula-se que o tratamento a longo prazo tenha potencial de bloquear o ciclo celular, particularmente entre as células que tenham maiores necessidade de ferro, como as do cérebro em desenvolvimento (Fredriksson et al., 1999). Em relação a seu uso clínico, a desferoxamida tem algumas desvantagens (Chaston & Richardson, 2003), incluindo a necessidade de aplicação subcutânea lenta e dolorosa (Giardina & Grady, 1995; Giardina

& Grady, 2001); a potencialidade de contaminação com toxinas bacterianas, durante a sua extração a partir desses organismos (Cianciulli et al., 1996); baixa lipofilicidade, não atingindo sítios-alvos de sobrecarga de ferro como as mitocôndrias (Richardson et al., 2001; Bergeron et al., 2006); e o bloqueio da absorção de ferro pelas células (Richardson et al., 1994). Interessantemente, as vantagens parecem superar as desvantagens do uso da DFO.

2 OBJETIVOS

O ferro parece ter um papel dual na estabilidade genômica e no risco de câncer. Avaliar a estabilidade genômica em diferentes status de ferro é imperativo, da mesma forma que estratégias para aumentar a estabilidade genômica em situações de excesso de ferro devem ser avaliadas.

2.1 Objetivo geral

Avaliar tanto a toxicidade para o material genético da deficiência como do excesso de ferro em grupos populacionais humanos (crianças com alta vulnerabilidade sócio-econômica: deficiência; pacientes com anemia falciforme: excesso), testar estratégias para reduzir a toxicidade do ferro para a memória e para o DNA (pela administração de alimentos, vitaminas e medicamentos), e, finalmente, determinar qual a dose ideal de ferro para a manutenção da estabilidade genômica em células em cultura.

2.2 Objetivo específico 1: efeitos da carência de ferro sobre a estabilidade genômica e risco de câncer

Avaliar experimentalmente quais as repercussões da deficiência de ferro na estabilidade genômica em crianças e adolescentes de alta vulnerabilidade social atendidas por uma unidade do Programa da Saúde da Família (PSF) de Santa Cruz do Sul, RS; e revisar a literatura acerca da associação entre a deficiência de ferro e o risco de câncer no trato gastrintestinal.

2.3 Objetivo específico 2: Efeito do excesso de ferro sobre a estabilidade genômica

Avaliar a toxicidade ao DNA gerada tanto por sobrecarga tanto endógena, em humanos com anemia falciforme (uma doença típica de indivíduos afro-descententes), quanto exógena, em animais suplementados com ferro.

2.4 Objetivo específico 3: estratégias para reduzir a genotoxicidade e a neurotoxicidade do ferro

Avaliar se a vitamina C, o suco de laranja ou uma dieta rica em antioxidantes podem diminuir a toxicidade ao DNA gerada pela suplementação com ferro; e avaliar se o composto quelante de ferro desferoxamida ou a vitamina C podem amenizar os efeitos de perda de memória e os danos ao DNA induzidos pelo ferro.

2.5 Objetivo específico 4: determinação da dose ideal de ferro para a manutenção da estabilidade genômica de células em cultura

Definir a concentração ideal de ferro para células em um contexto de cultivo celular a partir da avaliação do dano ao DNA e do comprimento dos telômeros como conseqüência de diferentes concentrações de ferro no meio de cultura; ampliando os conhecimentos sobre o nível ótimo de ferro para reduzir a senescência celular, problema comum em transplantes onde é necessário cultivar as células ex-vivo.

3 MATERIAIS E MÉTODOS

3.1 Organismos, tratamentos e metodologias

Tabela 3. Sumário dos organismos/sistemas experimentais ou teóricos, tratamentos e metodologias empregados nos experimentos, de acordo com os objetivos.

Objetivo	Organismo/sistema experimental ou teórico	Tratamento	Metodologia
1	Crianças e adolescentes de alta vulnerabilidade com potencial carência de ferro	Sem tratamento	Avaliação do consumo de nutrientes Avaliação do histórico familiar Ensaio cometa em leucócitos
	Diferentes organismos – busca da associação entre a deficiência de ferro e o risco de câncer no trato gastrintestinal	Não se aplica	Revisão bibliográfica
2	Pacientes de anemia falciforme	Hidroxiuréia	Avaliação de parâmetros clínicos Ensaio cometa em leucócitos Ensaio de micronúcleos em leucócitos binucleadas
	Camundongos	Suplementação com ferro	Ensaio cometa em leucócitos Ensaio de micronúcleos em medula óssea
3	Camundongos tratados com vitamina C	Suplementação com ferro	Ensaio cometa em leucócitos
	Camundongos tratados com suco de laranja	Suplementação com ferro	Ensaio cometa em leucócitos
	Camundongos mantidos em dieta rica em antioxidantes	Suplementação com ferro	Ensaio cometa em leucócitos Ensaio de micronúcleos em medula óssea
4	Linhagem WIL2-NS de células linfoblastóides	Diferentes concentrações de ferro no meio de cultura	Ensaio cometa Ensaio de micronúcleos em células binucleadas Avaliação do comprimento de telômeros, da abundância de mtDNA e da expressão de genes de reparo

3.2 Avaliação da genotoxicidade e da mutagenicidade: o ensaio cometa e o teste de micronúcleos

3.2.1 O ensaio cometa

O ensaio cometa consiste na micro-eletroforese de células individualizadas suspensas em uma fina camada de agarose. Embora as bases bioquímicas do ensaio tenham sido estabelecidas em meados da década de 1970, o ensaio cometa se difundiu após a publicação da versão alcalina do ensaio em 1988. O ensaio cometa é hoje um método padrão para avaliar danos no DNA. Nesse sentido, o ensaio cometa vem sendo aplicado: 1) na determinação da genotoxicidade de agentes químicos, físicos e biológicos; 2) no estudo de mecanismos de genotoxicidade; 3) no estudo de reparação de DNA; 4) no biomonitoramento humano e ambiental; e 5) na epidemiologia molecular (Cotelle & Férard, 1999; http://www.cometassay.com, 2005).

As bases bioquímicas do ensaio cometa surgiram a partir do trabalho de Cook e colaboradores (1976), como um método para investigar as estruturas nucleares de células lisadas com detergentes aniônicos e alta concentração de cloreto de sódio. No final da mesma década, foi publicada a primeira avaliação do dano no DNA em células individuais de mamíferos (Rydberg & Johanson, 1978). Na década de 1980, o método foi proposto como uma técnica para quantificar danos ao DNA de células individualizadas (Ostling & Johanson, 1984). A versão alcalina do ensaio foi desenvolvida logo em seguida, por Singh e colaboradores (1988). Embora não tenham usado o termo “cometa”, Ostling e Johanson descreveram as estruturas emanadas de dentro dos núcleos (“caudas”) com superenrolamento relaxado de DNA similar ao “modelo de nucleóides” de Cook e colaboradores (para revisão, ver Collins, 2004).

Na versão alcalina do ensaio cometa, as células são inicialmente lisadas por detergentes e altas concentrações salinas, desnaturados com hidróxido de sódio e submetidas à eletroforese. O ensaio cometa alcalino envolve pelo menos sete etapas após a obtenção de uma suspensão celular/nuclear: 1) confecção de lâminas de microscopia; 2) lise das células para expor o DNA nuclear; 3) desnaturação alcalina do DNA; 4) eletroforese; 5) neutralização; 6) coloração; e 7) análise. O objetivo principal da confecção das lâminas é a obtenção de géis regulares, uniformes e com poucos resíduos. Os géis devem ser também resistentes aos procedimentos experimentais subseqüentes e apresentar cometas claramente visíveis. A etapa de lise é responsável pela remoção das membranas,

do citoplasma e do nucleoplasma. Como resultado da lise, é obtido um halo de DNA embebidos em agarose a partir de cada célula. Cada halo reflete a estrutura bioquímica superenrolada do DNA nuclear da célula que o originou. As lesões no DNA alteram a compactação do DNA e deixam a molécula sensível à ação de álcalis. O objetivo do desenovelamento alcalino é produzir DNA fita-simples e expressar lesões álcali-labeis como quebras simples. Já a eletroforese tem a função de mobilizar o DNA para o lado e para formar os cometas. Essa etapa é a base do ensaio cometa, pois é nela que se manifestam o grau de relaxamento e os fragmentos de DNA. Na neutralização, o pH do gel é reduzido pela lavagem sucessiva com tampão neutro para diminuir a coloração inespecífica (para revisão, ver Tice et al., 2000).

Embora os primeiros trabalhos com o ensaio cometa tenham utilizado predominantemente corantes fluorescentes com afinidade por DNA, os cometas também podem ser corados com nitrato de prata para análise. As lâminas devem ser codificadas para análise de modo que o analisador desconheça o código. A análise pode ser feita em microscópio comum ou de fluorescência (para corantes fluorescentes) na magnificação desejada pelo pesquisador (usualmente 200-400 vezes). Os métodos utilizados para quantificar a migração do DNA variam quase tanto quanto o número de pesquisadores que utilizam a técnica (para revisão, ver Collins, 2004).

Variantes metodológicas do ensaio cometa estão atualmente disponíveis. Nesse sentido, a simples variação do pH do tampão de desnaturação e eletroforese permite detectar desde prioritariamente quebras duplas até quebras simples, duplas e sítios alcali-lábeis simultaneamente. Adicionalmente, várias enzimas específicas de lesão podem ser empregadas para converter diferentes tipos de alterações em nucleotídeos em quebras, portanto, revelando vários tipos de danos ao DNA. Algumas variantes metodológicas, como a incorporação de bromodeoxiuridina para detectar replicação de DNA e a hibridização in situ com sondas de DNA (FISH-Comet) para localizar segmentos de DNA, também são utilizadas (para revisão, ver Collins, 2004).

As vantagens do ensaio cometa são: 1) sensibilidade para detectar pequena quantidade de danos no DNA; 2) a necessidade de um pequeno número de células por amostra; 3) flexibilidade; 4) custos reduzidos para execução; 5) facilidade de desenvolvimento; 6) a capacidade de utilizar poucos volumes de substâncias-teste para conduzir estudos; e 7) o período relativamente curto para executar um teste completo. As desvantagens do ensaio cometa incluem: 1) a sensibilidade excessiva decorrente das interferências técnico-metodológicas; 2) a natureza primária das lesões detectadas; 3) a

dificuldade de interpretação dos resultados; e 3) a variabilidade intra-testes devido ao número amostral reduzido de células (para revisão, ver Tice et al., 2000).

3.2.2 O teste de micronúcleos

Durante as décadas de 1930 e 1940 vários autores descreveram a ocorrência de corpúsculos menores que o núcleo da célula em decorrência da exposição à radiação ionizante em diferentes organismos (para revisão ver Evans, 1997). Após a descoberta de que essas estruturas continham material genético, elas foram denominadas de micronúcleos (MN) e se passou a teorizar que continham parte do genoma das células que haviam sido perdidos do núcleo durante a divisão celular. Hoje se sabe que o MN é geralmente resultado de cromossomo(s) não unido(s) ao fuso mitótico, ou fragmento(s) cromossômico(s) sem centrômero(s). Desta forma, todos os processos (físicos, químicos ou biológicos) que interferem no processo de ligação do cromossomo às microfibrilas do fuso (aneugênicos) e aqueles que quebram os cromossomos (clastogênicos) podem induzir à perda de material genético. Esses são, portanto, genotóxicos ou mutagênicos (Mavournin et al., 1990; Hayashi et al., 2000). Incialmente a análise de MN passou a ser utilizada em paralelo à análise de aberrações cromossômicas, técnica laboriosa que verifica a estrutura individual de todos os cromossomos celulares (em um minuto é possível analisar 150 células com MN contra 0,7 aberrações por célula metafásica). Atualmente, o teste de MN é internacionalmente aceito como parte da bateria de testes para a avaliação do potencial mutagênico de substâncias conhecidas e para o registro de novos produtos químicos (Evans, 1997).

Apesar da existência de diversas variações metodológicas para o teste de MN, duas técnicas são amplamente utilizadas para o teste de MN, a análise em células hematopoiéticas anucleadas e a análise em células binucleadas.

O teste de MN in vivo em células hematopoiéticas de roedores foi desenvolvido simultaneamente por Schmid e Heddle no início da década de 1970 (para revisão ver Evans, 1997). Esse teste consiste na observação dos precursores medulares das hemácias, células que não possuem núcleos, na busca de MN que permanecem na célula quando o núcleo é eliminado. Como vantagem, a técnica é de fácil execução e sensibilidade, visto que é possível analisar apenas células que sofreram mitose recente (MacGregor et al., 1987; Heddle, 1990; Maluf, 2004). Uma limitação do teste em medula é que ele é restrito a

animais de laboratório, pois a punção medular é inviável como procedimento rotineiro em humanos.

O teste de MN em células bloqueadas em citocinese/binucleadas (CBMN) é um teste relativamente novo e robusto que pode ser desenvolvido a partir de punções venosas e células em cultura. Essa técnica se constitui no cultivo de leucócitos em cultura celular, seguido de um bloqueio da citocinese pelo tratamento com citocalasina. Com efeito, são produzidas células binucleadas, um marcador perfeito da sincronicidade das células, que garante uma elevada sensibilidade para o teste (Fenech, 1993; Fenech, 1997; Fenech, 2000). A partir da análise simultânea da ocorrência de toxicidade celular, pela contagem de células em apoptose e necrose o teste evoluiu em um ensaio "citoma" para avaliar em paralelo a instabilidade cromossômica, a disfunção mitótica e a morte celular (Fenech, 2006). O teste também permite avaliar diferentes tipos de danos no DNA: micronúcleos (MN), botões nucleares - *buds* (NBUD) e pontes nucleoplásmicas (NPB). Enquanto MN indica dano no DNA originado da perda completa ou quebra de cromossomos (efeitos clastogênicos e aneugênicos), NPB indica rearranjos cromossômicos e NBUD indica amplificação de genes. Além disso, a freqüência de MN também fornece uma medida indireta da hipometilação do genoma (Kimura et al., 2004), pois a hipometilação causa a perda de cromossomos e o nível de MN é diretamente correlacionado com o nível de hipometilação (Fenech et al., 1998).

3.3 Determinações dos níveis de metais em amostras biológicas: o método 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. Nesse 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, homogeneizadas e transformadas em pastilhas por prensagem e mantidas em dissecador.

O Laboratório de Implantação Iônica do Instituto de Física 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. Nessa 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 esses em amostras que são isolantes. A finalidade desse 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).

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.

3.4 Reação em cadeia de polimerase em tempo real: uma ferramenta robusta para a avaliação da estabilidade genômica e expressão de RNA

A reação em cadeia de polimerase (PCR) é uma técnica que permite replicar (amplificar) uma determinada seqüência de DNA (p.ex. um gene ou seqüências teloméricas) milhões ou até bilhões de vezes. Após ser amplificado, cada produto pode ser analisado quanto a seu tamanho ou composição, duas características potencialmente alteráveis pelas mutações. O PCR tradicional pressupõe que os produtos sejam visualizados em géis ou processados em um seqüenciador de DNA. Por outro lado, o PCR quantitativo em tempo real (QRTPCR) permite avaliar automaticamente a quantidade/qualidade do DNA na amostra, a partir da determinação precisa do número de moléculas amplificadas. De fato, dois fatores reduzem a eficiência da amplificação, a quantidade de distorções na estrutura (p.ex. nucleotídeos oxidados ou pontes) e a quantidade de moléculas do DNA de interesse em relação ao DNA total da amostra. Desse modo, pode-se comparar as quantidades de produtos de amplificação de segmentos de DNA de interesse, obtidos de quantidade iguais de DNA e provenientes de células com tratamentos diversos. Se por ventura for observada uma redução no número de moléculas amplificadas, pode-se concluir, depois das normalizações estatísticas adequadas, que a quantidade/qualidade do segmento de DNA na amostra é menor.

Uma das aplicações do QRTPCR é a medição do comprimento dos telômeros, sabendo-se que quanto menos amplificação ocorrer menores são os telômeros. Lembrando: telômeros são seqüências de DNA que se situam, usualmente, nas extremidades dos cromossomos e servem como protetores mecânicos do DNA durante os ciclos de divisão

celular e, de forma ainda pouco compreendida, protegem o DNA contra oxidação (Passos et al., 2007). O QRTPCR também é indicado na avaliação da quantidade/qualidade do DNA mitocondrial, que, a saber, é particularmente suscetível ao dano oxidativo, pois a organela armazena porção significativa do ferro celular e é desprovida de defesas antioxidantes tão proficiente como as nucleares (Atamna et al., 2002). A amplificação de um gene cópia única nuclear deve ser utilizada como referência da quantidade de DNA, tanto na análise de telômeros como de cópias do genoma mitocondrial.

Três procedimentos são executados para a avaliação quanti/qualitativa do DNA por RTQPCR, extração de DNA, reação de QRTPCR e cálculos matemáticos. A avaliação qualitativa requer a preservação do DNA desde o seu isolamento até o final da reação de PCR. Um protocolo padrão nesse sentido consiste no isolamento de DNA total, utilizando o kit "DNeasy Tissue Kit" (Qiagen, Valencia, CA), observando o protocolo padrão e as recomendações de Lu e colaboradores (2004) e Atamna e colaboradores (2000), lise à baixa temperatura, purgância de oxigênio dos reagentes e adição de ditiothreirol. Outro aspecto fundamental ao RTQPCR é a manutenção de uma quantidade de DNA idêntica entre as amostras. Para tanto, o DNA deve ser quantificado em triplicatas, preferencialmente por espectrofotômetro de gota (NanoDrop Technologies, Delaware, EUA), diluído para uma concentração padrão e utilizado para o RTPCR tão logo possível.

A maior vantagem do QRTPCR é a possibilidade da análise quantitativa da expressão gênica, pela avaliação da abundância relativa das moléculas de mRNA correspondentes ao gene de interesse. Tecnicamente, o procedimento é bastante similar ao da quantificação relativa de moléculas de DNA, à exceção do fato de que um procedimento específico deve ser utilizado para extrair o RNA (molécula altamente instável) e esse precisa ser convertido por transcrição reversa em cDNA para análise.

A medida dos telômeros empregada nessa tese foi desenvolvida segundo o método de Cawthon (2002), seguindo os ajustes de O'Callaghan e colaboradores (2008). O conteúdo de DNA mitocondrial foi avaliado segundo o método descrito por Wang e colaboradores (2007). Todas as amostras foram processadas em um *ABI 7300 Sequence Detection System* equipado com o *software SDS Ver. 1.9* (Applied Biosystems [AB], Foster City, CA). Cada amostra foi analisada em duplicata. O gene mitocondrial altamente conservado *citocromo b* foi utilizado como marcador do conteúdo de mtDNA, segundo descrito por Wang (2007). Um gene nuclear de cópia única, *36B4* que codifica a fosfoproteína ribosomal P0, é utilizado como um controle para a amplificação de cada amostra amplificada (Cawthon, 2002). Cada reação de 20µl é composta por 20ng DNA,

1xSYBR Green master mix (AB), mais 100nM de cada um dos prímers. Os primers foram os seguintes, para os telômeros: F (CGG TTT GTT TGG GTT TGG GTT TGG GTT TGG GTT TGG GTT) e R (GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT TAC CCT); para o gene *36B4*: F (CAG CAA GTG GGA AGG TGT AAT CC) e R (CCC ATT CTA TCA TCA ACG GGT ACA A); e para o gene *citocromo b*: F (TAT CCG CCA TCC CAT ACA TT) e R (GGT GAT TCC TAG GGG GTT GT). As condições de amplificação são: 10 minutos a 95^{o}C, seguido de 40 ciclos de 95^{o}C por 15 segundos, 60^{o}C por 1 minuto, para todas as seqüências de DNA (Cawthon, 2002; Callicott & Womack, 2006; Wang et al., 2007).

O procedimento para análise da expressão gênica utilizado pode ser resumido pelo que se segue: O RNA total foi extraído com o uso do Trizol Reagent® (Invitrogen, Carlsbad, CA), de acordo com as instruções do fabricante. O cDNA foi sintetizado a partir de 1,3 µg total RNA, pelo uso do High-Capacity cDNA Archive Kit® (ABI, Foster City, CA), seguindo as instruções do fabricante. A expresão de mRNA foi medida utilizando-se TaqMan® assays (ABI, Foster City , CA). Os genes de interesse foram marcados com VIC e *18S RNA* foi utilizado como controle endógeno (marcado com FAM) em todas as reações. Os cDNA (2 µL) foram aliquotados em uma reação final de 20 µL por poço, que continha também TaqMan® universal PCR master mix e os ensaios para cada gene de interesse e controle endógeno. Um ABI 7300 Sequence Detection System equipadoc com o software SDS Versão 1.9 (ABI, Foster City, CA) foi utilizado, utilizando o perfil térmico padrão: 50◦C por 2 min e 95 ◦C por 10 min para a ativação da enzima, seguido de desnaturação a 95 ◦C por 15 s, anelamento e alongamento a 60 ◦C por 1 min, com total de 40 ciclos.

O cálculo da quantidade relativa de produto de amplificação nas amostras foi executado ou por curva de acordo com amostra padrão (*standard*) em diluições seriados ou pelo método comparativo $\Delta\Delta C_T$. Para esse método, a menor concentração de ferro é utilizada como parâmetro, isto é, a média dos valores ΔC_T dos controles foi calculada e definida como 1 para a determinação comparativa (comparador). Resumidamente, um valor ΔC_T é calculado para cada amostra testada, em valor único ou duplicata pela equação: $C_T$produto - C_T*gene de referência*. O valor $-\Delta\Delta C_T$ é calculado pela diferença de ΔC_T entre o valor amostral e do comparador. O valor relativo de expressão é mostrado como $2^{-\Delta\Delta CT}$.

3.5 Outras metodologias

3.5.1 Avaliação do nível de calorias, macro e micronutrientes na dieta

Dada a grande influência da dieta na estabilidade genômica, avaliar a ingestão de nutrientes em grupos populacionais e comparar esses valores com as recomendações nutricionais se faz fundamental para garantir a prevenção das doenças para as quais as recomendações nutricionais foram sugeridas. Por outro lado, como a dieta é um fator chave para a estabilidade genômica, novas recomendações dietéticas podem ser projetadas com vistas à otimização do metabolismo (Ames, 2003) e otimização da estabilidade genômica (Fenech, 2001; Bullen et al., 2005).

Os métodos recordatório 24 horas (R24) e registro diário (RD) são os métodos preferenciais para a determinação da ingestão atual de nutrientes, se baseiam na memória recente e permitem conhecer o consumo detalhado dos grupos de estudo. Sua aplicação pode ser repetida diversas vezes ao longo de um longo período de tempo, capturando variações sazonais e diárias. Não se deve esquecer que todos os métodos de avaliação da dieta não são livres de erros, que são intrínsecos a qualquer método de avaliação nutricional. Trata-se de entrevista pessoal em profundidade (presencial ou pelo telefone) conduzida por um entrevistador treinado. É possível ainda que o indivíduo preencha o seu próprio R24h, assim como ocorre no RD; listando os alimentos e as bebidas que consumiu. Dá-se preferência pelo contato pessoal, para manter o diálogo, a fim de obter mais informações, diminuir a abstenção e facilitar a interpretação das medidas caseiras. A informação obtida pelo método depende da habilidade do entrevistado em recordar de forma precisa sua alimentação, variando em função do sexo, idade e nível de escolaridade (Fisberg et al., 2005). Por esse fato, algumas vezes, como de crianças e idosos, pode ser necessário recrutar uma pessoa de convívio próximo para relatar a informação.

3.5.2 Teste de memória

A memória é um excelente marcador de alterações fisiológicas e muitas das substâncias que agridem o DNA, simultaneamente afetam o cérebro (Laranjinha & Ledo, 2007). Além disso, a memória tende a se degenerar com a idade, em associação com o acúmulo de ferro em determinadas regiões do cérebro (Atamna & Frey, 2007).

Para avaliar a memória em roedores, utilizou-se um aparato de campo aberto (45 x 40 x 60 cm) feito em *polywood* (material inodoro) com vidro frontal e coberto com serragem. No primeiro dia, os ratos foram submetidos a uma sessão de ambientação em campo aberto vazio por 5 min. No dia seguinte, os ratos receberam um treinamento de 5 minutos, quando foram expostos a 2 objetos idênticos (A1 e A2) posicionados em dois cantos adjacentes, 9 cm das paredes. No teste de memória de curto prazo (STM) (90 min após a sessão de treinamento), foi permitido aos ratos explorar o campo aberto por 5 min na presença dos 2 objetos: o objeto familiar A e um novo objeto (B). Os ratos foram colocados no mesmo local da sessão de treinamento. No treinamento de memória de longo prazo (LTM) (24 horas após a sessão de treinamento), se permite aos ratos explorar o campo aberto por 5 min, na presença de 2 objetos: o objeto familar A e um terceiro objeto (C). Todos os objetos tinham texturas, cores e tamanhos similares, mas formas distintas. A exploração dos objetos foi medida por 2 cronômetros, para registrar o tempo gasto explorando os objetos durante a sessão experimental. A exploração foi registrada pelo ato de cheirar ou tocar o objeto com o nariz. Sentar no objeto não foi considerado exploração. Um índice de reconhecimento foi calculado para cada animal, sendo expresso pela razão TB/(TA+TB) [TA= tempo gasto explorando o objeto A; TB= tempo gasto explorando o objeto B] (Schroder et al., 2003; de Lima et al., 2005; de Lima et al., 2005; de Lima et al., 2005).

4 RESULTADOS

4.1 Efeitos da carência de ferro sobre a estabilidade genômica e risco de câncer

4.1.1 Efeito da deficiência de ferro na estabilidade genômica: um estudo em crianças e adolescentes de alta vulnerabilidade social de Santa Cruz do Sul, RS (ANEXO A)

A anemia afeta grande parte da população de mulheres e crianças, particularmente nos países em desenvolvimento (Tolentino & Friedman, 2007). Embora haja uma visão de que a origem da anemia seja ferropriva, sabe-se que o ácido fólico e outras vitaminas do complexo B também influenciam a síntese de hemoglobina e a maturação das hemácias. Como o ferro atua em diferentes rotas metabólicas associadas à proteção, síntese e reparo do DNA (Atamna, 2004) e a anemia aumenta o nível de estresse oxidativo (Aslan et al., 2007), a carência de ferro poderia levar a um acúmulo de danos no DNA. Outro aspecto destacável é a suplementação das farinhas com ferro, prática adotada no Brasil desde 2006 para prevenir a anemia ferropriva. Contudo, existem poucas informações acerca da repercussão da suplementação na saúde de nossas populações. O excesso de ferro tem efeitos negativos, particularmente quando associado à deficiência de vitaminas do complexo B. A ingestão de vitaminas do complexo B, proveniente de uma alimentação equilibrada e rica em alimentos integrais pouco processados e com carnes, é limitada nas famílias de baixa renda. Além disso, uma dieta com essas características é dificilmente consumida por crianças e adolescentes por questões de preferências alimentares. O objetivo desse trabalho foi avaliar o dano no DNA e o consumo de nutrientes, buscando associação entre esses parâmetros, em crianças e adolescentes de alta vulnerabilidade de um bairro popular de Santa Cruz do Sul, RS. O nível de danos primários no DNA foi avaliado pelo ensaio cometa em leucócitos e a ingestão de macro e micronutrientes, parâmetros hematológicos e antropométricos foram avaliados numa amostra de 30 crianças e adolescentes.

Os resultados indicaram baixa prevalência de anemia, de acordo com o nível de hemoglobina que foi elevado para a amostra (12,93 ± 0,81 g/dl). Observou-se um incremento no dano no DNA quando a amostra foi estratificada pela presença ou não de deficiência na maturação das hemácias e na presença de alteração da contagem de células brancas (Fig. 1). A anemia microcítica é habitualmente associada a deficiências de vitaminas do complexo B, como o ácido fólico. Quanto à alimentação, observou-se mediana elevada de ingestão de ferro (13,92 mg/dia) em relação aos níveis preconizados pela DRI. Por outro lado, a mediana da ingestão de ácido fólico foi de 125,6 mg/dia, valor notoriamente abaixo da recomendação nutricional desse nutriente (200 mg/dia para crianças de 6 a 8 anos; 300 mg/dia para crianças de 9 a 13 anos; 400 mg/dia para adolescentes de 14 a 17 anos) (IOM, 1998). Observou-se alta prevalência de eosinofilia entre as crianças, o que, geralmente, indica verminose.

Idade, índice de massa corporal (IMC) e a ingestão de folato foram correlacionados negativamente com o nível de danos primários ao DNA. Uma menor ingestão de niacina e magnésio e uma ingestão intermediária de sódio foram associadas a um menor nível de danos primários ao DNA. A ingestão de energia (calorias totais) e o consumo de diversos nutrientes foram correlacionados negativamente com o número de leucócitos (Tabela 2 e Fig. 2, ANEXO A).

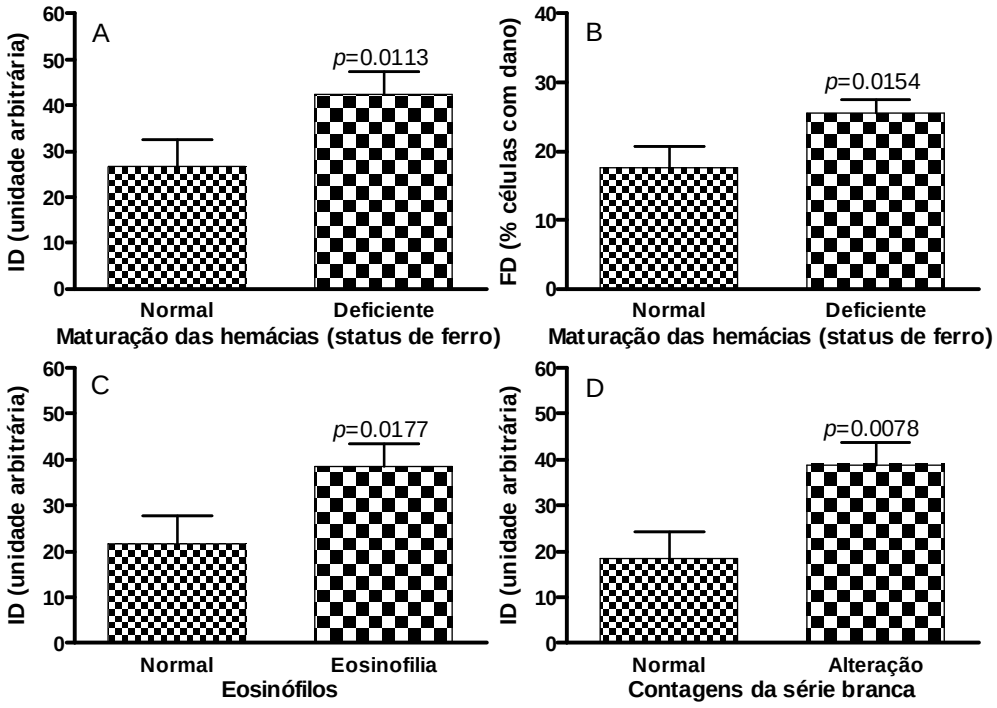

Figura 1. Dano primário no DNA de crianças de alta vulnerabilidade social de acordo com parâmetros hematológicos. Em relação à deficiência na maturação das hemácias (A: ID e B: FD), eosinofilia (B) ou alteração em pelo menos uma contagem da série branca (D). *p*: nível de significância estatística de acordo com o teste U de Mann-Wittney. ID: índice de dano; FD: freqüência de dano.

4.1.2 Revisão da literatura sobre a possível relação entre a deficiência de ferro e o risco de câncer no sistema gastrointestinal (ANEXO B)

A deficiência de ferro pode ter diversos efeitos fisiológicas, incluindo redução da imunidade (Jacobs, 1982), diminuição das defesas antioxidantes e aumento do estresse oxidativo (Isler et al., 2002) e de danos no DNA mitocondrial (Atamna et al., 2001) e nuclear (Aslan et al., 2006). Uma vez que tais efeitos estão envolvidos na patofisiologia do câncer, pode-se levantar a hipótese de que a carência de ferro tenha efeito carcinogênico. Com base nesse conceito, decidiu-se revisar a literatura acerca da associação entre a deficiência de ferro e o risco de câncer no trato gastrintestinal, local chave para a homeostasia do ferro (Anderson & Frazer, 2005).

Existem na literatura alguns estudos epidemiológicos em humanos indicando evidências preliminares da associação da deficiência de ferro e o aumento do risco de câncer em diferentes partes do trato gastrintestinal. Contudo, a grande maioria dos estudos negligencia tal associação e dá ênfase ao risco associado ao excesso de câncer. Alguns testes em modelos murinos de indução de câncer apontam que animais ferro-deficientes apresentam origem precoce e progressão acelerada de tumores em relação a animais controle. Da mesma forma, evidências clínicas apontam melhor prognose após cirurgia de remoção de tumores em indivíduos com status de ferro normal em relação aos com depleção desse nutriente. Soma-se a isso, o reconhecimento do papel chave do ferro como componente de diversas enzimas. De fato, o ferro atua diretamente na síntese e reparo de DNA (tem papel catalítico na ribonucleotídeo redutase e nas DNA helicases e glicosilases), no sistema de defesa antioxidante (co-fator da catalase), na metabolização de compostos (centro de ferro heme nas citocromo P450), no *oxidative burst* de macrófagos (pigmento heme nas mieloperoxidases), na sinalização celular e detecção de flutuações na tensão de oxigênio (papel catalítico nas óxido nítrico sintases), bem como no disparo de apoptose (papel catalítico chave nas citocromo C). Todas essas funções metabólicas ferro-dependentes podem ser relacionadas direta ou indiretamente à proteção em manutenção do

genoma (p.e.x. desequilíbrio nas respostas imunes contra células malignas, no metabolismo de compostos tóxicos, bem como na regulação redox e na biossíntese e reparo do DNA). Interessantemente, não existe até o momento qualquer estudo avaliando a capacidade de reparo de DNA em células deficientes de ferro, tampouco algum estudo epidemiológico especificamente desenhado para avaliar o risco de câncer em indivíduos com deficiência de ferro (para revisão, ver ANEXO B).

A revisão de diversos estudos epidemiológicos mostrou que as evidências ligando a deficiência de ferro e o risco de câncer gastrintestinal são moderadas, ou classe **II-2-boas** de acordo com o *Current methods of the U.S. Preventive Services Task Force* (Harris et al., 2001). A classe II-2 é atribuída de acordo com a hierarquia de desenho do estudo para evidências proveniente de estudos de coorte ou caso-controle bem desenhados, preferencialmente de mais de um centro ou grupo de pesquisa. Já a nomenclatura **boa** se refere às evidências que sejam provenientes de estudos que apresentem resultados consistentes, sejam bem desenhados, conduzidos em populações representativas e avaliem os efeitos diretamente nos desfechos de saúde de interesse.

4.2 Efeito do excesso de ferro sobre a estabilidade genômica

4.2.1 Toxicidade ao DNA na anemia falciforme: uma doença de sobrecarga endógena de ferro típica de indivíduos afro-descendentes (ANEXOS C e D)

A AF é uma hemoglobinopatia hereditária caracterizada pela deformação na estrutura das hemácias, que leva essas ao formato de foice (Buchanan et al., 2004). A AF está associada à sobrecarga endógena de ferro devido ao rompimento das hemácias. A AF é relativamente comum em indivíduos de origem africana, pois o alelo que confere a hemoglobinopatia protege as hemácias contra o parasita da malária. A malária é bastante freqüente em populações africanas. A AF ocorre quando o alelo AF está em homozigose. Os indivíduos homozigotos apresentam cardiopatologias, associadas à vaso-oclusão dolorosa e *advanced chest syndrome* (ACS) e ao acúmulo de ferro (Makani et al., 2007).

O tratamento dos indivíduos com AF se faz mandatória pela severidade da patologia. A hidroxiuréia (HU) é a droga consensual para o manejo da AF. O uso da HU é aprovado pelo *Food and Drug Administration* (FDA) dos EUA, apesar da droga ser um quimioterápico com potencial de aumentar o risco de câncer. A HU induz um efeito

mielosupressor (pancitopenia) por um mecanismo ainda desconhecido, que provavelmente envolva um bloqueio na síntese de DNA por afetar a enzima ribonucleotídeo redutase (RR). A RR contém ferro como co-fator e converte ribonucleotídos em deoxiribonucleotídeos (Leanza et al., 2008). Embora se saiba que a talassemia, outra hemoglobinopatia, esteja associada a um aumento no nível de estresse oxidativo e dano no DNA (Anderson et al., 2000), esse cenário para a AF é pouco caracterizado. O acúmulo de dano no DNA na AF pode ser uma situação mais complexa, pois os indivíduos, além de apresentarem um acúmulo de estresse oxidativo pela sobrecarga de ferro, podem também estar sofrendo a genotoxicidade da HU. Para tanto, caracterizar esse aspecto é fundamental.

Nesse sentido, realizaram-se dois estudos com o objetivo de caracterizar os danos primários (potencialmente transitórios) e permanentes ao DNA, avaliados pelo ensaio cometa e pelo CBMN, respectivamente, em pacientes de AF de diferentes idades tratados com HU. A dose média e final de HU, o tempo de tratamento com HU, a idade, o IMC, e a etnia dos pacientes foram otimizados para comparar os níveis de dano ao DNA.

O primeiro estudo **(ANEXO C)** objetivou a avaliação do dano primário ao DNA, utilizando o ensaio cometa alcalino (pH>13) em leucócitos do sangue periférico de 28 pacientes de AF tratados com HU (13 homens; 23,1±2,4 anos; 14,3% fumantes) e 28 controles saldareis (15 homens; 24,1±2,5 anos; 14,3% fumantes), pareados por sexo, idade e consumo de tabaco. Os resultados desse estudo apontaram maior dano ao DNA entre os indivíduos com AF tratados com HU do que entre os controles (18,7±2,5 ID versus 14,3±2,5 ID; $p=0,02$). Não houve diferenças quanto ao gênero, idade ou consumo de tabaco. A dose média de HU foi positivamente correlacionada com o índice de dano (Fig. 2.A) e os indivíduos que receberam dose média de >20 mg/kg HU apresentaram significativamente mais danos primários no DNA que aqueles que receberam menor dose HU (24,9±5,5 ID versus 14,6±1,8 ID; $p<0,05$). Da mesma forma, aqueles tratados por ≥42 meses apresentaram significativamente mais dano ao DNA do que aqueles tratados por menos tempo (23,1±4,2 ID versus 13.6±1.9 ID; $p<0,05$). O índice de dano também foi inversamente correlacionado com o IMC no grupo tratado com HU (Fig. 2 B).

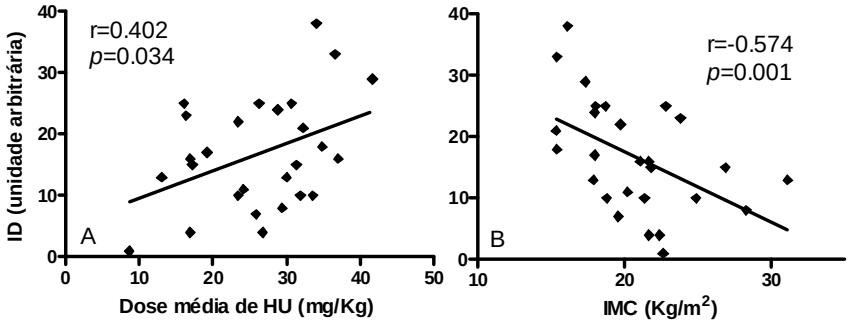

Figura 2. Relação entre a dose de hidroxiuréia (HU) e o nível de dano primário no DNA de pacientes de anemia falciforme tratados com HU (AFHU). Correlação paramétrica (Pearson) entre o índice de dano no DNA (ID), avaliado pelo ensaio cometa, e a dose média de hidroxiuréia (HU) (A) ou índice de massa corporal (IMC) (B) em AFHU.

O segundo estudo **(ANEXO D)** objetivou avaliar o nível de lesões permanentes no DNA (micronúcleos - quebra e perda de cromossomos, NBUD – amplificação gênica e NPB – ligações cruzadas entre cromossomos) em linfócitos do sangue periférico pelo ensaio CBMN em 35 indivíduos com AF tratados com HU (42,9% homens; 26,3 ± 14,42 anos; 11,8% fumantes) e 34 controles saudáveis (50% homens; 26,6 ± 14,3 anos; 11,8% fumantes), pareados por gênero, idade e tabagismo. Os resultados desse estudo apontaram aumento da freqüência de MN (*P*=0,032) entre os indivíduos com AF tratados com HU, mas não de NBUD ou NPB, em relação ao grupo controle (Fig. 3 A). Houve uma tendência de redução da freqüência de MN ao longo do tempo de tratamento, bem como uma associação dose-resposta entre a dose final de HU e a freqüência de MN (Fig. 3 B e C).

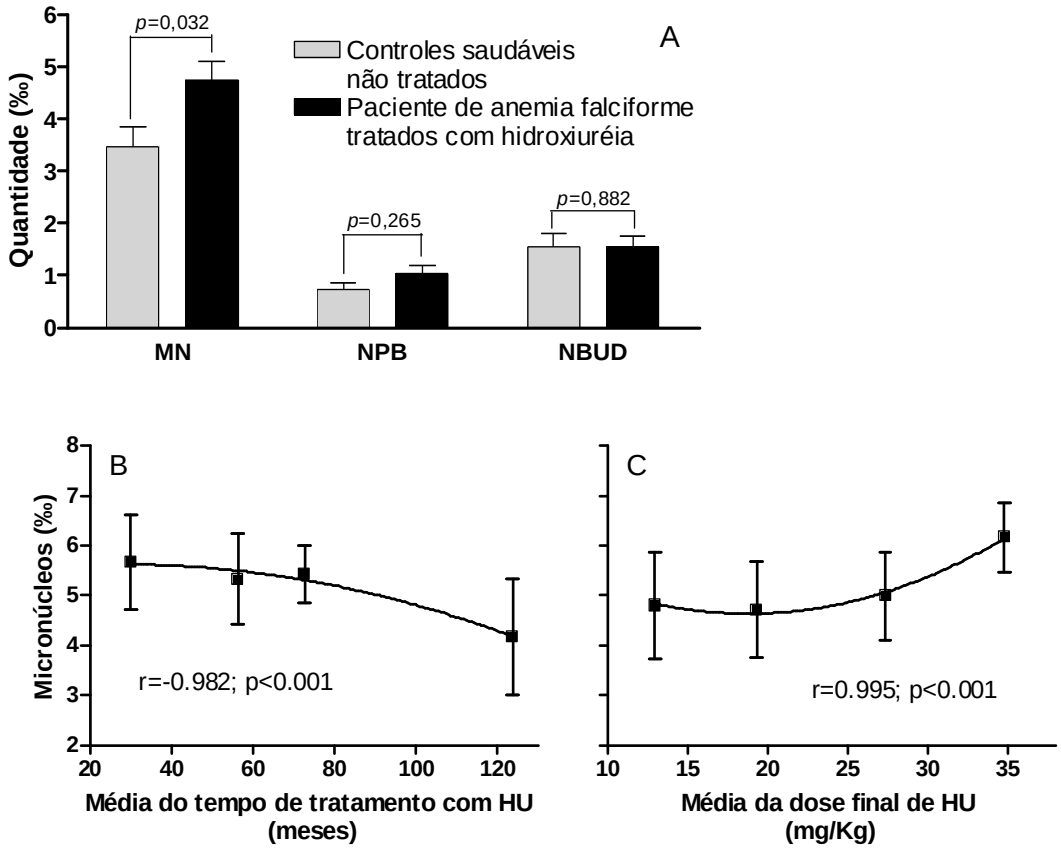

Figura 3. Efeito clastogênico/aneugênico em pacientes de anemia falciforme tratados com hidroxiuréia (AFHU). A) Quantidade de micronúcleos (MN), pontes nucleoplásmicas (NPB) e botões nucleares (NBUD) em controles saudáveis não tratados (n=34) e pacientes com anemia falciforme (AF) tratados com hidroxiuréia (HU) (n=35). p: nível de significância segundo o teste U de Mann-Wittney. Quantidade de micronúcleos de acordo com a média do tempo de tratamento com HU (A) e de acordo com a dose final média de HU (B) em uma amostra de 25 pacientes de AF tratados com HU. r e p: coeficiente de correlação e nível de significância de acordo com uma curva polinomial de segunda ordem.

4.2.2 Toxicidade ao DNA da suplementação com ferro: estudos em vivo com camundongos (ANEXOS E, F e G)

O ferro pode catalizar a formação de ERO quando reage com moléculas contendo oxigênio. Na verdade, o ferro parece ser o mais importante canalizador da formação de ERO nos sistemas biológicos (Fang et al., 2002). Além disso, tem-se atribuído uma importância crescente das ERO no envelhecimento, em doenças degenerativas (p.ex.

doenças cardiovasculares e diabete mellitus), bem como no câncer (Mates & Sanchez-Jimenez, 1999). O principal fator etiológico do câncer são as alterações no DNA. As ERO são responsáveis pela ampla maioria das alterações no DNA das células, originando cerca de 10.000 lesões no DNA de cada célula por dia (Loft & Poulsen, 1996).

Considerando o potencial do ferro em induzir ERO, que poderiam lesar o DNA, foram realizados dois estudos para avaliar o efeito de uma suplementação com grande quantidade de ferro (correspondendo a 50 vezes a UL para humanos) sobre o DNA de camundongos. No primeiro estudo, avaliou-se o dano primário ao DNA de leucócitos 24 e 48 horas após uma única dose de sulfato ferroso (o suplemento de ferro mais utilizado mundialmente) pelo ensaio cometa. No segundo estudo, o dano primário ao DNA e a freqüência de MN na medula óssea foram avaliados após 6 tratamentos diários consecutivos com o mesmo composto. O nível de ferro acumulado nos tecidos sangüíneo e hepático também foi avaliado por PIXE. Animais tratados com água destilada foram utilizados como controles.

No primeiro e segundos estudos (**ANEXOS E e F**), o ferro foi genotóxico tanto a 24 horas como a 48 horas após o tratamento, contudo o dano reduziu-de significativamente a 48 horas. Isso possivelmente está associado à atividade de reparo de DNA. Foi observada uma correlação positiva entre o dano no DNA e o nível hepático de ferro. Além disso, indicou uma correlação negativa entre o nível hepático de ferro (Fig. 1.A, ANEXO F).

Um terceiro estudo (**ANEXO G**) executado com metodologia similar, porém por 6 dias subseqüentes de tratamento, indicou que o ferro induz tanto aumento no nível de danos primários no DNA no ensaio cometa e alcalino (Fig. 4.A e B, respectivamente), como da freqüência de MN nos animais (Fig. 4. D). Interessantemente, apesar de não ter-se observado diferença no ensaio cometa, o ferro não aumentou a freqüência de MN entre os machos. O tratamento com ferro também induziu um aumento significativo nos níveis hepáticos de ferro (Fig. 4. C).

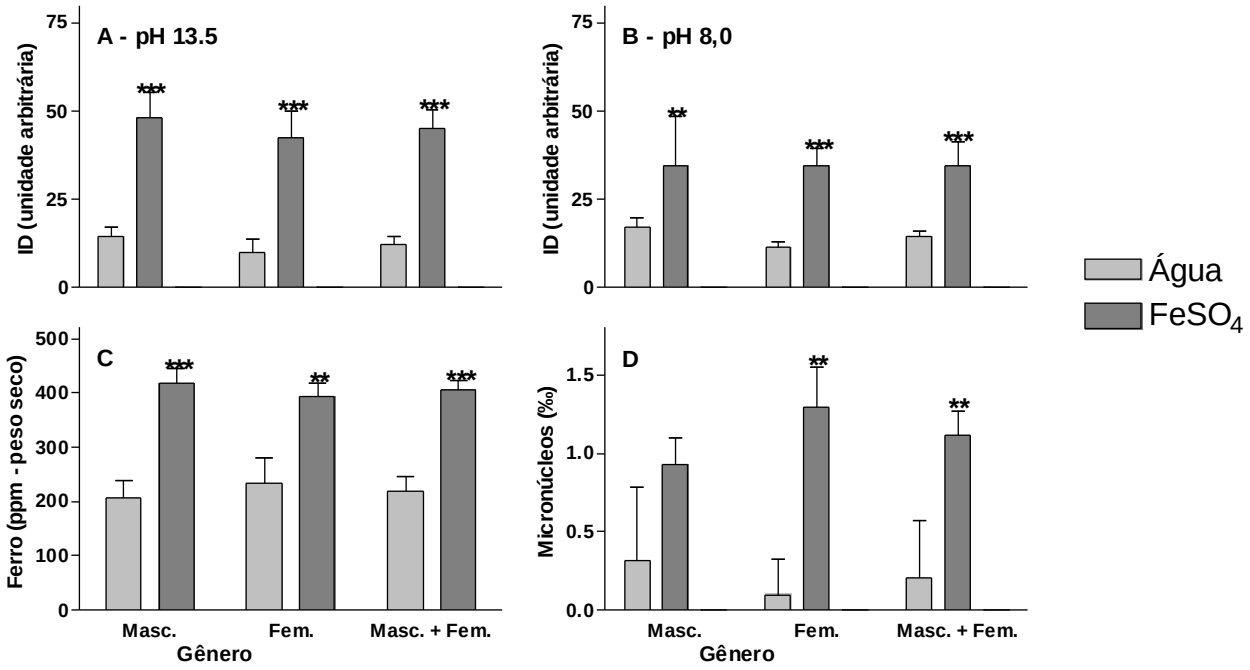

Figura 4. Danos primários no DNA, acumulação hepática de ferro e freqüência de micronúcleos em camundongos tratados com ferro por 6 dias. Danos no DNA (A: pH>13; B: pH=8,0), acumulação hepática de ferro (C) e quantidade de micronúcleos na medula óssea (D) em camundongos tratados com ferro por 6 dias. O teste de ANOVA de 2-vias mostrou que o dano no DNA diferiu significativamente apenas entre os tratamentos. Os asteriscos indicam diferenças significativas em relação ao grupo controle (água) utilizando o teste post-hoc de Dunn, sendo * $p<0.05$, ** $p<0.01$ or *** $p<0.001$. Média ± erro padrão (n= 5 camundongos para cada gênero).

4.3 Estratégias para reduzir a neurotoxicidade e a genotoxicidade do ferro

4.3.1 Efeito da vitamina C no dano ao DNA induzido pelo ferro: um estudo em camundongos (ANEXO E)

A vitamina C é um composto altamente antioxidante, contudo com algumas evidências de que seu acúmulo possa ser deletério (Halliwell, 2001). A vitamina C tem a capacidade de reduzir o ferro no trato gastrintestinal e por isso sua co-suplementação com ferro é uma estratégia para aumentar a biodisponibilidade do ferro (Teucher et al., 2004).

Pouco ou nada se sabe sobre os efeitos no DNA do tratamento com a vitamina C imediatamente antes, durante ou após a suplementação com ferro. Com efeito, nesse estudo objetivou-se avaliar em camundongos qual seria o efeito no DNA se tratasse os animais com uma dose aguda de sulfato ferroso e após 24 horas fossem administradas diferentes doses de vitamina C.

Foram selecionadas duas doses de vitamina C, uma equivalendo a cerca de uma vez a DRI para humanos e a outra a aproximadamente 30 vezes a DRI (segundo a RDA anterior a 2000, que preconizava a ingestão diária de 60 mg/dia 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 Vitamina C pelos camundongos, procurando simular o excesso de Vitamina C que poderia ocorrer em humanos que têm uma dieta adequada em relação à DRI de Vitamina C, e mesmo assim usam suplementos. No tempo 0 h, 3 grupos de camundongos receberam uma dose de sulfato ferroso e 3 grupos foram tratados com água destilada. A 24 h, 1 grupo pré-tratado com sulfato ferroso e 1 grupo pré-tratado com água, foram tratados com a dose menor de vitamina C, a dose maior de vitamina C ou água destilada (controles). Adicionalmente, 1 grupo de camundongos foi tratado a 0 e 24 horas com cada uma das doses de vitamina C. O ensaio cometa alcalino foi utilizado para avaliar o nível de dano no DNA 24 horas e 48 horas após o início do tratamento.

Ambas as doses de vitamina C aumentaram o nível de dano primário no DNA (Fig. 5). O tratamento duplo (0 e 24 h) com vitamina C induziu um acúmulo de danos no DNA a 48 h, especialmente para a dose maior (Fig. 1, ANEXO E). Quando administradas aos animais pré-tratados com sulfato ferroso, ambas as doses de vitamina C também aumentaram os danos ao DNA gerados pelo sulfato ferroso (Fig. 5). Como o ensaio cometa mede danos primários ao DNA que podem ser reparados, ou podem ser gerados como intermediários durante o reparo do DNA, o resultado do estudo indica que a vitamina C pode ser tóxica para organismos que apresentam níveis adequados dela, e mais ainda que ela possa afetar a genotoxicidade e a capacidade de reparo do DNA (talvez estimulando essa) nos organismos após o tratamento com sulfato ferroso.

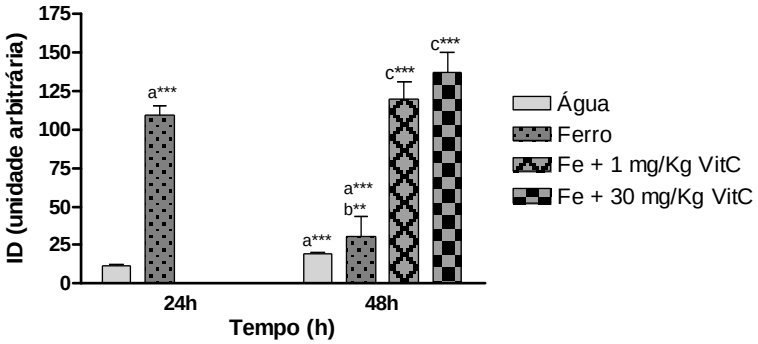

Figura 5. Efeito da vitamina C sobre o dano no DNA induzido pelo ferro em leucócitos de camundongos. a: em relação ao grupo água a 24 h; b: em relação ao grupo ferro 24 h; c: em relação a grupo ferro 48 h no nível ** p<0,01 ou ***p<0,001, segundo o teste ANOVA. Valores apresentados como média ± erro padrão (n= pelo menos 5 indivíduos por grupo).

4.3.2 Efeito do suco de laranja no dano ao DNA induzido pelo ferro: um estudo em vivo com camundongos (ANEXO F)

Sabe-se que os sucos cítricos podem proteger contra os danos oxidativos, pela presença de antioxidantes tais como a vitamina C e os compostos fenólicos (Franke et al., 2005). A administração conjunta de ferro com suco de laranja (SL) pode aumentar a biodisponibilidade do elemento (Ballot et al., 1987). Baseado nesses aspectos decidiu-se avaliar *in vivo* o efeito do SL no dano oxidativo induzido pelo ferro em camundongos.

O SL (0,1 mL por 100g de camundongo, correspondendo a 600 mL para um humano de 60 kg) foi adminstrado 24 horas antes ou 24 horas após o tratamento com sulfato ferroso (33,3 mg/kg, igual a mesma dose utilizada nos estudos anteriores e equivalendo a cerca de 10 % do LD_{50} a 48 h para camundongos). O ensaio cometa alcalino foi utilizado para avaliar o dano ao DNA no final do tratamento, 24 horas após o tratamento com SL (para os animais pré-tratados com sulfato ferroso) e 24 horas após o tratamento com sulfato ferroso (para os animais pré-tratados com SL). O sangue periférico coletado na cauda dos animais foi utilizado para o ensaio cometa. O sangue total e o fígado dos animais foram liofilizados e processados, de acordo com o procedimento padrão (ver metodologia), para determinação do nível de ferro por PIXE.

O SL quando administrado antes, mas não após o tratamento com sulfato ferroso parece reduzir o dano induzido por esse oxidante (Fig. 6). Portanto, parece que o SL pode prevenir, mas não melhorar o reparo dos danos induzidos pelo ferro. Observou-se uma

correlação positiva (r=0,95; *p*=0,052) entre os danos no DNA e os níveis hepáticos de ferro.

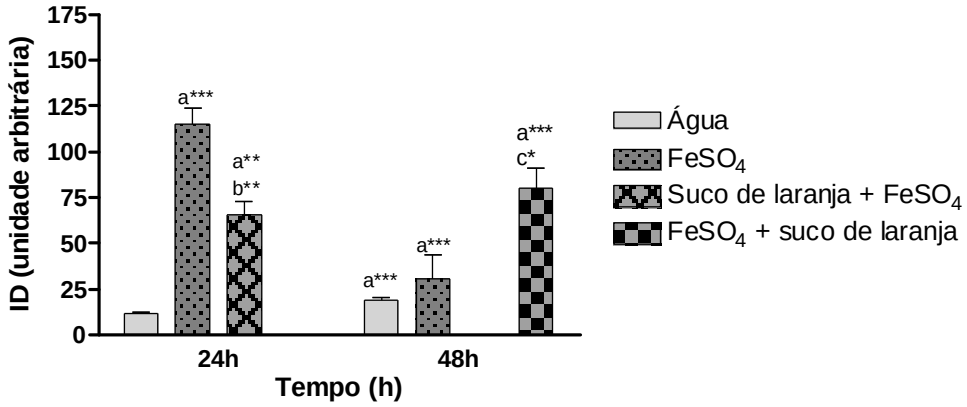

Figura 6. Efeito do suco de laranja (pré ou pós-tratamento) sobre o dano no DNA induzido pelo ferro em leucócitos de camundongos. a: em relação ao grupo água a 24; b: em relação ao grupo ferro 24 horas; c: em relação a grupo ferro 48 horas no nível *p<0,05, ** p<0,01 ou ***p<0,001, segundo o teste ANOVA. Valores apresentados como média ± erro padrão (n= pelo menos 5 indivíduos por grupo).

4.3.3 Efeito de uma dieta rica em compostos antioxidantes sobre o dano no DNA induzido pelo ferro: um estudo em vivo com camundongos (ANEXO H)

Apesar de ser essencial o ferro em quantidades elevadas e em ambientes pró-oxidantes pode induzir danos ao DNA, mutações e até câncer (Toyokuni, 1996). Contudo, há falta de conhecimento sobre o potencial da dieta em reduzir a toxicidade do ferro. Portanto, objetivou-se avaliar a influência de uma dieta padrão e uma dieta rica em grãos antioxidantes sobre os danos no DNA e a quantidade de MN induzidos pelo ferro em camundongos tratados por gavagem durante 6 dias com $FeSO_4$ (33,23 mg de Fe/kg), pelo ensaio cometa em leucócitos e a freqüência de MN na medula óssea. O nível de ferro no fígado e no sangue dos animais bem como na dieta, em conjunto com outros metais foram avaliados por diferentes metodologias.

Os resultados indicam um nível similar no sangue total e um nível hepático aumentado de ferro nos camundongos tratados com ferro, independentemente da dieta. A dieta padrão possuía cerca de 4 vezes mais ferro do que a ração rica em grãos antioxidantes (Tabela 1, ANEXO H). O ferro induziu aumento de dano no DNA dos leucócitos apenas nos animais mantidos na dieta padrão, que também apresentaram um aumento na

freqüência basal de dano no DNA (Fig. 7). O tratamento com ferro induziu um aumento na freqüência de MN tanto nos camundongos mantidos na dieta padrão como na dieta rica em antioxidantes e a freqüência de micronúcleos apresentou correlação positiva com o nível hepático de ferro (Fig. 8). Em relação à composição da dieta, o conteúdo de diversos metais foi significativamente maior na dieta padrão do que na dieta rica em grãos (Tabela 1, ANEXO H). É possível que a maior quantidade de antioxidantes na dieta rica em grãos tenha contribuído na redução dos danos primários no DNA, contudo sem afetar a freqüência de MN induzida pelo ferro.

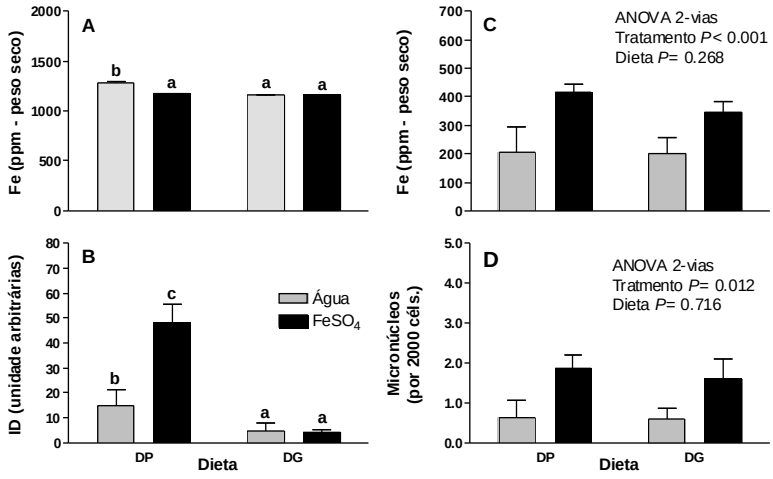

Figura 7. Níveis corporais de ferro, danos primários no DNA e freqüência de micronúcleos em camundongos mantidos em duas dietas e tratados com ferro por 6 dias. Níveis de ferro (A) e dano no DNA (B) no sangue e nível de ferro no fígado (C) e de micronúcleos na medula óssea (D) de camundongos mantidos em uma dieta padrão (DP) ou rica em grãos antioxidantes (DG) e tratados com ferro. Letras diferentes indicam o ranking dos valores segundo diferença significativa para $P<0.05$ pelo teste ANOVA. Valores apresentados como média ± erro padrão (n= pelo menos 5 indivíduos por grupo).

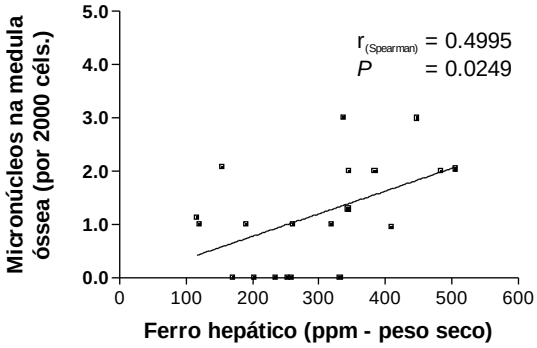

Figura 8. Correlação não paramétrica (Spearmann) entre o nível de ferro no fígado e a freqüência de micronúcleos na medula óssea de camundongos mantidos em dieta rica ou pobre em antioxidantes e tratados com ferro ou água.

4.3.4 Efeito da vitamina C sobre a perda de capacidade de memorização e os danos no DNA induzidos pelo ferro: um estudo in vivo com ratos (ANEXO I)

Assim como para os outros órgãos e tecidos, o ferro é tanto fundamental quanto tóxico em grandes quantidades para o cérebro. O ferro é essencial à síntese de neurotransmissores, às enzimas mitocondriais (muito ativas no cérebro) e, especialmente à formação de melanina (Beard, 2001); mas, por outro lado, a acumulação de ferro em determinadas áreas do cérebro vem sendo implicada na etiologia de doenças neurodegenerativas como o mal de Parkinson e de Alzheimer (Berg et al., 2001).

A administração de ferro para camundongos e ratos no período neonatal, quando a barreira hematoencefálica ainda não está consolidada, leva a um acúmulo de ferro em pontos particulares do cérebro (p.ex. substância nigra), gerando estresse oxidativo e déficit na capacidade de memorização (Fredriksson et al., 1999). Algumas drogas (p.ex. inibidores de monoamina-oxidases e quelantes de ferro), quando administradas na idade adulta dos animais, podem reverter a perda de memória associada à sobrecarga de ferro no período neonatal (de Lima et al., 2005; 2007). Alguns estudos apontam uma associação entre a capacidade cognitiva e o nível sérico de vitamina C e outros estudos apontam que o tratamento com vitamina C pode melhorar a diminuição da capacidade de memorização associada ao envelhecimento (Li & Schellhorn, 2007). A vitamina C é um antioxidante que desempenha papéis chave na absorção de ferro, não obstante tendo capacidade de gerar ERO quando interage com o ferro (Halliwell, 2001). Portanto, o objetivo desse estudo foi avaliar o efeito da vitamina C, em ratos com sobrecarga de ferro, sobre: a) o déficit de memória; b) o nível de dano no DNA de neurônios e leucócitos; c) o nível de ferro no

sangue e no cérebro. Os ratos foram tratados oralmente com veículo ou 10,0 mg/kg de ferro nos dias 12-14 após seu nascimento. Na fase adulta, os animais foram tratados com duas doses de vitamina C por 15 dias (as mesmas doses utilizadas no estudo anterior, equivalentes a 1 vez e 30 vezes a recomendação nutricional da vitamina para humanos adultos). Antes e após a última administração de vitamina C, os ratos foram submetidos a um teste de reconhecimento de objetos. No final do experimento, os tecidos cerebrais e sangüíneos foram avaliados quanto aos níveis de danos no DNA, pelo ensaio cometa, e nível de ferro, pelo método de PIXE.

Os resultados indicaram que a vitamina C não foi capaz de reverter o déficit de memória induzido pelo tratamento neonatal com ferro (Fig. 1, ANEXO I). Também indicaram que o tratamento com ferro não induziu um aumento significativo de danos no DNA dos leucócitos. Contudo, a vitamina C induziu um aumento no nível de danos no DNA nas células da substância nigra, independentemente do tratamento com ferro (Fig. 9). Em relação ao nível de ferro, observou-se um aumento não significativo no sangue total e uma correlação negativa entre a quantidade sangüínea de ferro e o dano no DNA das células da substância nigra (Fig. 10).

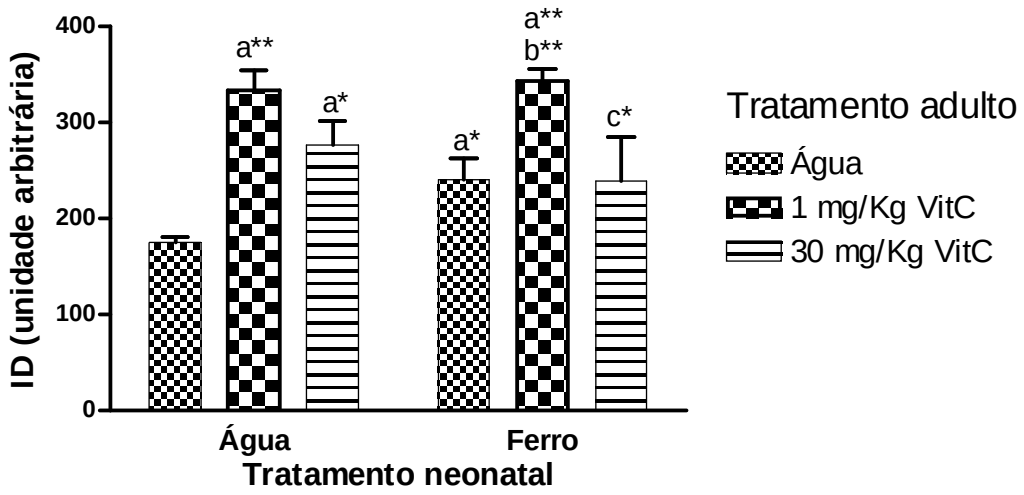

Figura 9. Dano no DNA em células da substância nigra de ratos tratados com ferro no período neonatal e com vitamina C na idade adulta. a: em relação ao grupo água-água; b: em relação ao grupo Ferro-água e c: em relação ao grupo Ferro-30 mg;kgVitC a ** $P < 0,01$ e *$P < 0,05$. ANOVA 2-vias seguida do teste de Dunn. Valores apresentados como média ± erro padrão (n=5).

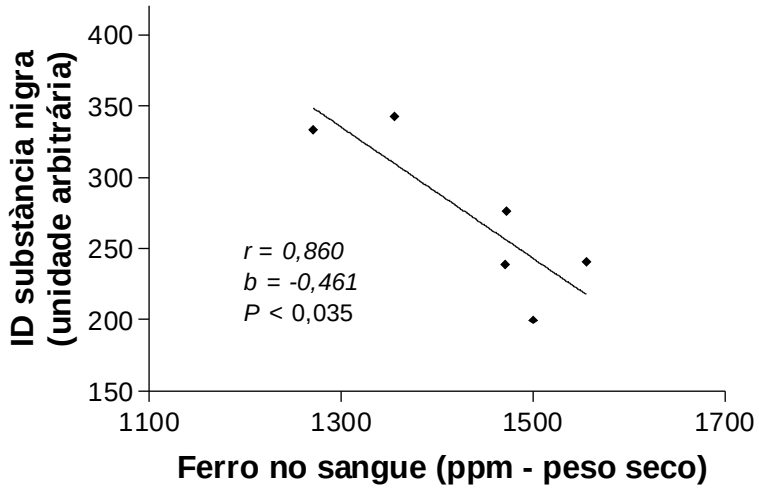

Figura 10. Correlação paramétrica (Pearson) entre o nível de ferro no sangue e o nível de dano no DNA de células da substância nigra de ratos tratados com ferro na idade neonatal.

4.3.5 Efeito do quelante de ferro desferoxamida sobre a perda de capacidade de memorização e os danos no DNA induzidos pelo ferro: um estudo in vivo com ratos (ANEXO J)

Há fortes evidências que o ferro, quando administrado no período neonal em ratos e camundongos, induza déficit na capacidade de memorização na vida adulta, que estão associados a aumento no nível de estresse oxidativo (Dal-Pizzol et al., 2001; de Lima et al., 2005). A DFO, o principal composto quelante de ferro utilizado no tratamento da hemocromatose primária e secundária, apresenta um potencial para reduzir o dano no DNA. O objetivo desse estudo foi avaliar o efeito da DFO no déficit de memória, danos no DNA e nível de ferro no sangue e no cérebro no modelo de sobrecarga de federo em ratos.

Ratos machos foram tratados oralmente com água destilada (controle) ou ferro nos dias 12-14 após seu nascimento e DFO intraperitonealmente (30 ou 300 mg/kg) ou veículo (solução salina) por 15 dias quando adultos. Antes e após a última administração de DFO, os ratos foram submetidos a um teste de reconhecimento de objetos. No final do experimento, os tecidos cerebrais e sangüíneos foram dissecados para determinação dos níveis de danos no DNA, pelo ensaio cometa, e de ferro, pelo método de PIXE.

Os animais que receberam a dose maior de DFO (300 mg/kg) mostraram memória de reconhecimento normal, sugerindo que esse composto pode reverter o déficit de memória associado ao acúmulo de ferro. Os ratos tratados com ferro mostraram maior

nível de dano no sangue, mas não no córtex cerebral. A mesma dose de DFO (300 mg/kg) reduziu o nível de dando no DNA tanto no sangue como no córtex dos ratos, independentemente se esses foram tratados ou não com ferro. O tratamento com ferro aumentou o nível de federo no córtex dos ratos e a dose maior de DFO (300 mg/kg) bloqueou esse aumento (Fig. 11). A desferoxamida parece ter um papel em aumentar a estabilidade genômica, tanto por quelar o federo ou por outros mecanismos ainda desconhecidos, que também podem contribuir no efeito do composto sobre o déficit de memória induzido pela sobrecarga de ferro.

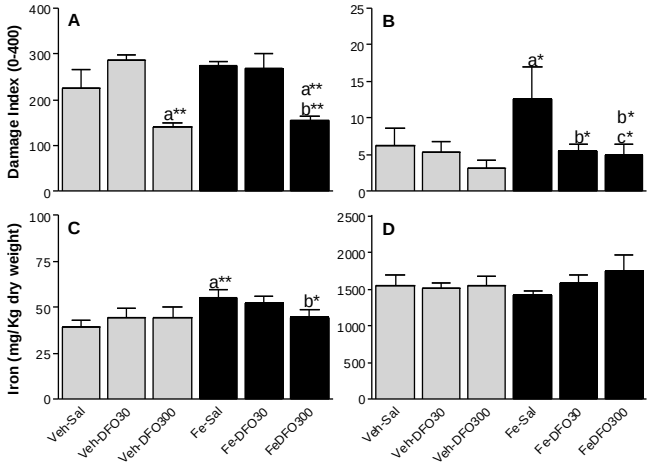

Figura 11. Dano primário no DNA de células sangüíneas e cerebrais e níveis corporais de ferro em ratos tratados com ferro na idade perinatal e com desferoxamida na idade adulta. Dano no DNA no sangue (A) e no córtex (B) e nível de ferro no sangue (C) e no córtex. a: em relação ao grupo Veh-Sal; b: em relação ao grupo Fe-DFO30; c: em relação ao grupo Veh-DFO300 ao nível de significância * $P < 0.05$ ou ** $P < 0.01$ de acordo com o teste ANOVA. Valores apresentados como média ± erro padrão (n≥4). Veh: veículo: Sal: salina; DFO30: desferoxamida 30 mg/kg; DFO300: desferoxamida 300 mg/kg; Fe: ferro.

4.4 Determinação da dose ideal de ferro para a manutenção da estabilidade genômica de células em cultura

4.4.1 Determinação da concentração de ferro para a viabilidade e estabilidade genômica de células humanas em cultura (ANEXO K)

Sabe-se que o ferro participa da estabilidade do material genético, representando risco tanto em situações de deficiência quanto de excesso. O excesso de ferro gera ERO

que podem induzir danos oxidativos no DNA (De Freitas & Meneghini, 2001). A deficiência, por outro lado, pode reduzir as defesas antioxidantes (Isler et al., 2002) e possivelmente o reparo de DNA das células. Enquanto o citoplasma e o núcleo celular possuem sistemas de defesa antioxidante e de reparo de DNA mais complexos, as mitocôndrias são mais suscetíveis aos radicais livres, particularmente pela proximidade de seu DNA à cadeia transportadora de elétrons (Santos et al., 2004). Além disso, o DNA nuclear possui uma grande densidade de segmentos capazes de servir de alvo à oxidação, impedindo que as seqüências gênicas sofram alterações. Entre essas seqüências destacam-se os telômeros, seqüências de repetições curtas que se situam nas extremidades dos cromossomos e diminuem seu comprimento ao longo do envelhecimento das células (Henle et al., 1999). Interessantemente, o comprimento dos telômeros é um marcador da senescência celular e, células com telômeros muito curtos não conseguem passar pelo processo de divisão celular (Lansdorp et al., 1996). Algumas evidências sugerem o papel tanto do excesso de ferro quanto da deficiência em aumentar a instabilidade mitocondrial, acelerando o envelhecimento celular (Walter et al., 2002). Por outro lado, não se sabe qual o provável efeito da deficiência e do excesso de ferro sobre o comprimento dos telômeros. Portanto, hipotetiza-se que células cultivadas em concentrações adequadas de ferro irão apresentar telômeros mais longos do que àquelas cultivadas com níveis reduzidos ou níveis aumentados de ferro.

Os resultados ora apresentados dizem respeito aos experimentos utilizando holo-transferrina (hTf) como fonte de ferro, visto que a transferrina é o transportador preferencial de ferro no sangue e armazena a vasta maioria do metal (ANEXO K). As células foram expostas por 14 dias a 6 concentrações dessa molécula (0,25-10.5 μM hTf, ou 2,5-23 μM Fe, visto que cada molécula de 1M hTf corresponde a 2M Fe e o *background* de ferro no meio era 2 μM Fe). O ensaio cometa e o ensaio CBMN foram executados ao final do período de tratamento. Adicionalmente, o comprimento dos telômeros e a quantidade de mtDNA foram avaliados por QRTPC. Conforme esperado, o comprimento dos telômeros apresentou um perfil de hipérbole, tendo comprimento máximo na concentração de 1,7 μM hTf (5,4 μM Fe) e um decréscimo de aproximadamente 50% na concentração máxima (23 μM Fe, que é próxima ao limite máximo de ferro sérico correspondente a 23 μM Fe (van Tits et al., 2007) (Fig. 12 E). Para a quantidade de mtDNA, observou-se curva similar, com pico na concentração de 0,7 μM hTf (3,4 μM Fe) e redução pronunciada na ordem de 50% a partir da concentração de 5,4 μM Fe (Fig. 12 F). Houve redução dose dependente na freqüência de MN e na extensão de

danos primários ao DNA no ensaio cometa (contudo não significativamente) (Fig. 12 A e B). O conjunto dessas informações, somadas ao fato de que não houve variação na viabilidade celular nas diferentes concentrações de hTf testadas, conforme o teste de exclusão de azul de Trypan, e considerando que a proliferação máxima ocorreu na concentração de hTf equivalente a 10 µM Fe (Fig 1, ANEXO K), sugere-se que as células linfoblastóides necessitam pelo menos 6 µM ferro com vistas ao aumento de estabilidade genômica. Interessantemente, essa concentração é extremamente lógica do ponto de vista clínico; já que o limite de diagnóstico de anemia pelo ferro sérico é de 5 µM Fe (Goldblatt et al., 2001).

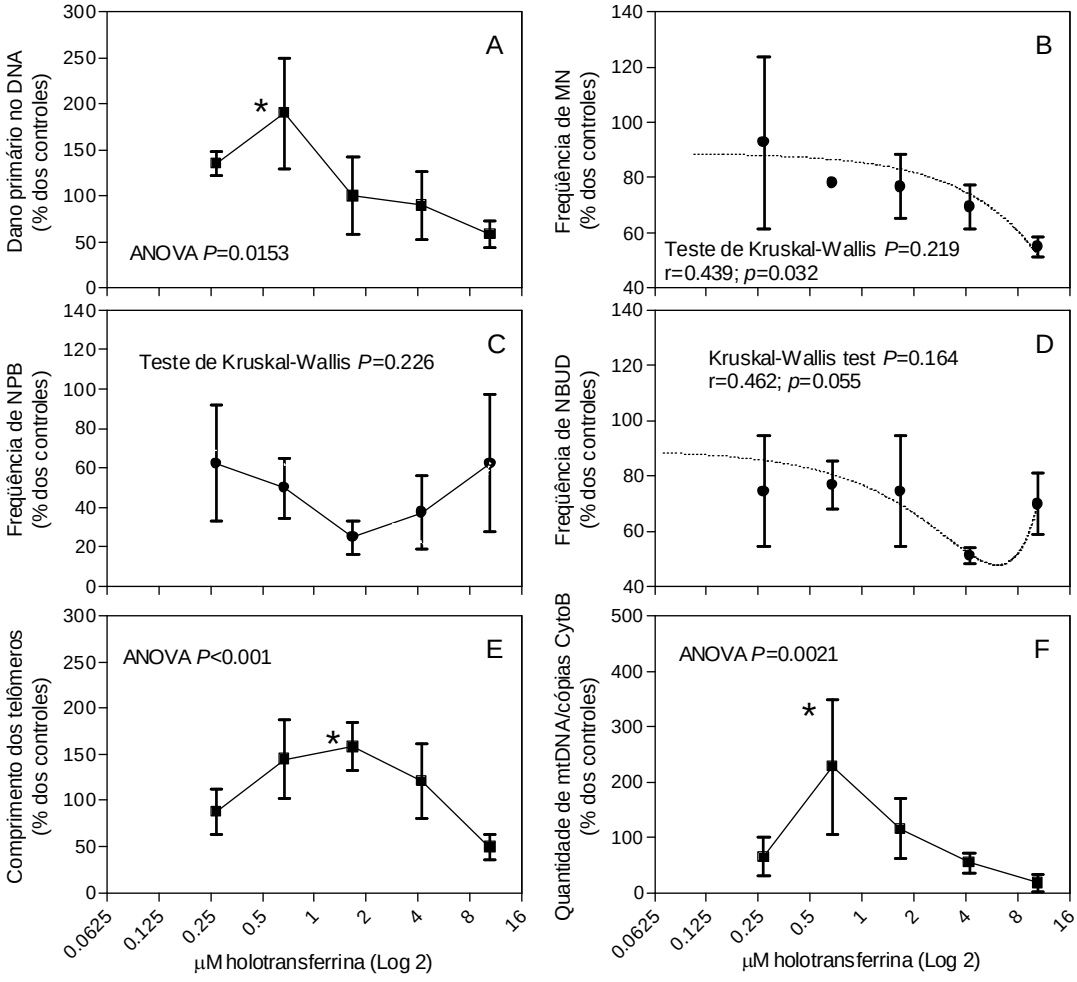

Figura 12. Marcadores de estabilidade genômica em células WIL2-NS suplementadas por 14 dias com diferentes concentrações de ferro (como holotransferina). A) Nível de dano primário no DNA, (B) freqüência de micronúcleos – MN, (C) freqüência de pontes

nucleoplásmicas (NPB), (D) freqüência de botões nucleares – NBUD, (E) comprimento médio dos telômeros, e (F) quantidades de cópias de mtDNA. Valores expressos em relação aos controles (não tratados com transferrina). * $p<0,05$ de acordo com Teste de Dunnet (ANOVA) ou Teste de Dunn (Teste de Kruskal-Wallis). As linhas tracejadas indicam correlações polinomiais de ordem 2.

4.4.2 Efeito do ferro sobre a expressão de genes de reparo de DNA (ANEXO L)

Estudos recentes apontam que o ferro, além de fazer parte da RR (Strand et al., 2004), também desempenha papel importante para o funcionamento de diversas enzimas de reparo, incluindo DNA helicases e glicosilases (Brzoska et al., 2006). Com efeito, decidiu-se selecionar 2 genes típicos do metabolismo de ferro e um conjunto de genes de reparo para avaliar o efeito da suplementação de ferro no meio de cultura sobre seu nível de expressão.

Os resultados ora apresentados dizem respeito aos genes WRN e MUTYH. WRN é um gene defectivo na síndrome de Werner, doença progeróide adulta marcada por doenças crônico-degenerativas como aterosclerose, osteosporose, catarata e câncer. O gene WRN codifica uma DNA helicase pertencente simultaneamente à subfamília das RecQ e à subfamília das DEAH (Asp-Glu-Ala-His), que possui atividade intrínseca de helicase 3´-5´e exonuclease 3´-5´ e um cluster ferro-enxofre. WRN atua no reparo de quebras duplas, recruta a maquinaria de reparo para essas lesões, bem como interage diretamente com a proteína TP53. Com efeito, os *knock outs* de WRN apresentam desregulação da vigilância de DSBs e apoptose. *Single nucleotide polymorphism* (SNIPS) em WRN estão associados ao aumento do risco de linfoma do tipo não-Hodkin e câncer de mama familial, bem como hipersensibilidade à hidroquinona (Galvan et al., 2008). O gene MUTYH codifica para uma DNA glicosilase que atua em BER removendo guaninas mal-incorporadas em posição oposta a 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxoG). A proteína MUTYH possui sítios de interação com AP endonuclease, PCNA e RPA e um cluster ferro-enxofre (Cheadle & Sampson, 2007). O cluster ferro-enxofre está envolvido no reconhecimento de Adeninas mal incorporadas à 8-oxoG e possivelmente tem papel catalítico (Brzoska et al., 2006). Mais de 30 polimorfismos germinativos no gene MUTYH são associados à polipose associada a MUTYH (MAP), uma desordem recessiva caracterizada pela ocorrência de múltiplos adenomas e carcinomas colorretais (Cheadle & Sampson, 2007).

A metodologia empregada neste estudo foi a mesma do ANEXO K. A expressão dos genes de interesse WRN e MUTYH e dos genes de absorção de ferro-ligado à transferrina (receptor de transferrina - TFRC e gene da cadeia leve da ferritina - FTL), bem como diferentes marcadores de estabilidade genômica foram avaliados. Adicionalmente, uma análise in silico foi utilizada para identificar motivos pós-transcricionais de regulação dos mRNAs dos genes de interesse. Os resultados apontaram um aumento saturável no nível de mRNA de TFRC nas concentrações 0,7-10,5 μM hTf (3,4-23 μM Fe), nenhum efeito no nível de FTL, exceto na concentração máxima (Fig. 1, ANEXO L). Para o gene WRN observou-se uma diminuição progressiva no nível de mRNA até o máximo de 4 μM hTf (10 μM Fe), com uma tendência ao aumento a partir da concentração máxima (Fig. 13 A). Observou-se uma curva tipo "U" descendente e ascendente para o nível de expressão do gene MUTYH, com nível mínimo na concentração 1,7 μM hTf (5,4 μM Fe) e aumento significativo na máxima (Fig. 13 B).

Os níveis de expressão de WRN apresentaram concordância com o resultado do ensaio de MN e cometa – danos primários no DNA (descendência em relação à concentração máxima) (Fig 13 C e D). Concordância similar foi observada para o gene MUTYH, cuja curva em "U" no nível de mRNA foi diametralmente oposta àquela do comprimento dos telômeros e de danos primários no DNA (Fig. 13 E e F)

Os resultados *in silico*, apontaram uma novidade: a presença de IRE nos mRNAs dos genes WRN e MUTYH (Fig. 14), indicando mecanismo de regulação pós-transcricional ortólogo aquele já bem caracterizado para TFRC e FTL (Pinero et al., 2001). Os dados observados no experimento com hTf mostraram concordância com os resultados observados em um experimento com sulfato ferroso desenvolvido em paralelo (dados não apresentados na tese).

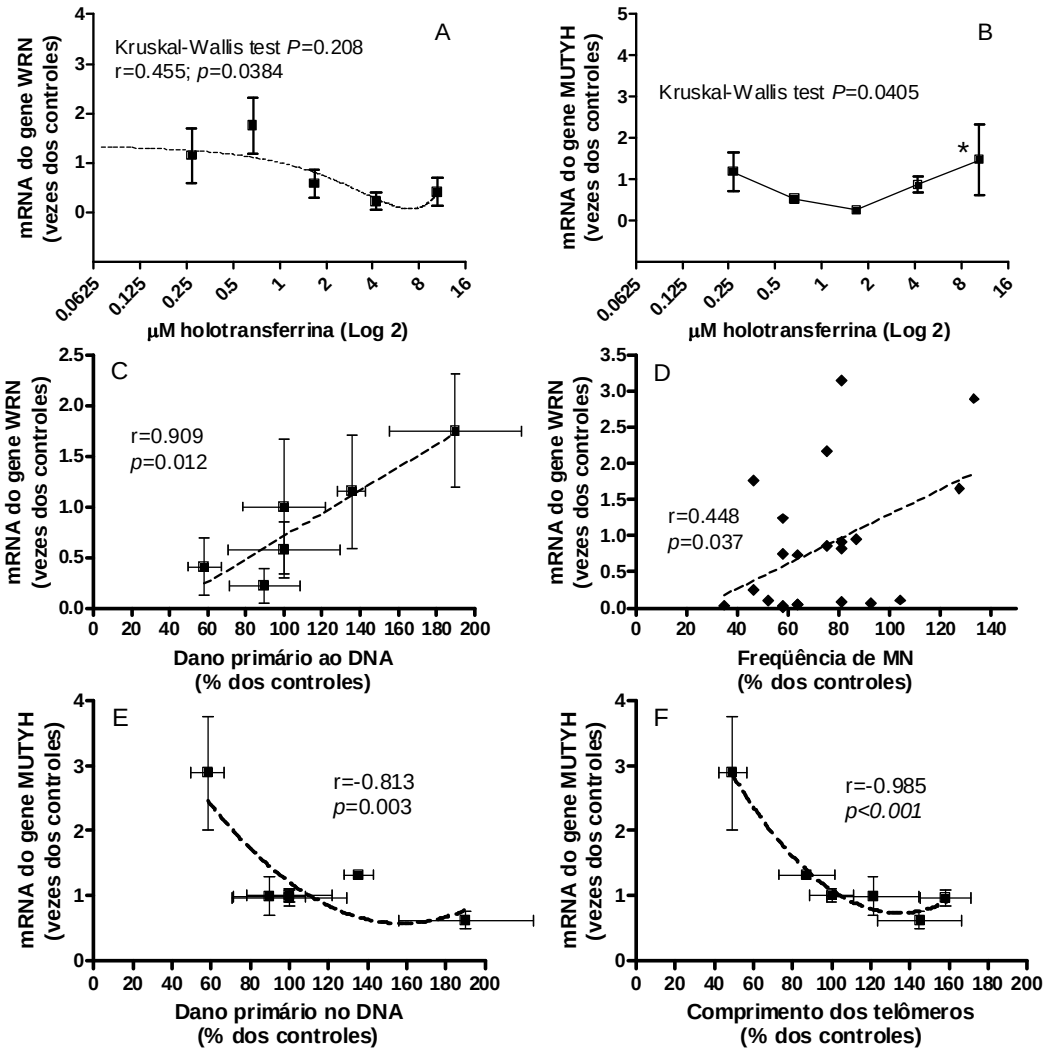

Figura 13. Nível de mRNA e correlação entre marcadores de estabilidade genômica e o nível de mRA dos genes WRN e MUTYH em células WIL2-NS suplementadas por 14 dias com diferentes concentrações de ferro (como holotransferina). A) Nível de mRNA do gene WRN, (B) nível de mRNA do gene MUTYH, (C) correlação linear entre o nível de dano primário no DNA e o nível de mRNA do gene WRN, (D) correlação linear entre a freqüência de micronúcleos – MN – e o nível de mRNA do gene WRN, (E) correlação polinomial de ordem 2 entre o nível de dano primário no DNA e o nível de mRNA do gene MUTYH, e (F) correlação polinomial de ordem 2 entre o nível de dano primário no DNA e o nível de mRNA do gene MUTYH. Valores expressos em relação aos controles (não tratados com transferrina).* p<0,05 para o teste de Dunn.

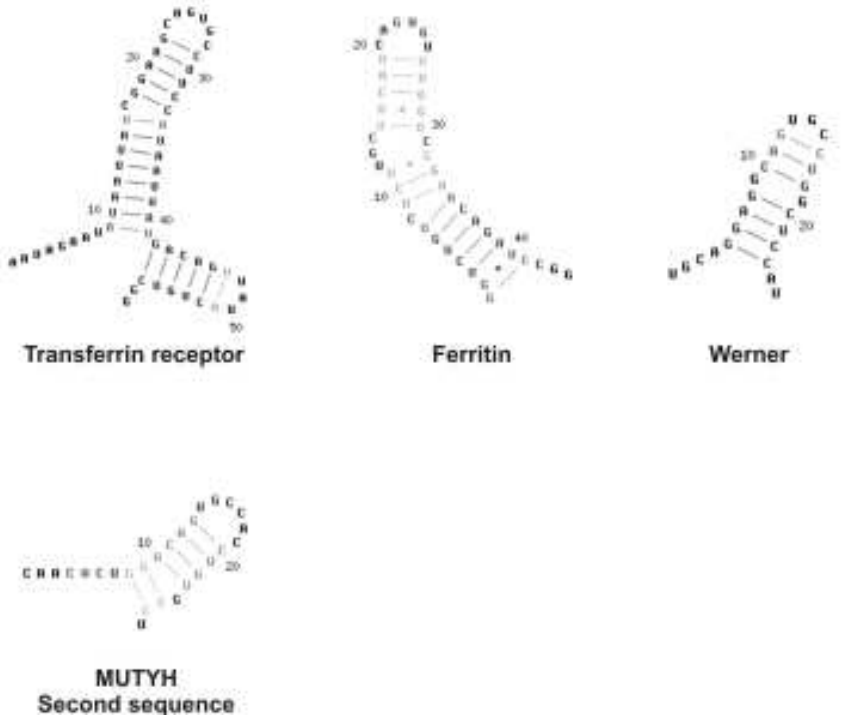

Figura 14. Motivos regulatórios do tipo “Iron responsive elements” (IRE) no mRNA dos genes do receptor de transferrina (TFRC), ferritina (FTL), Werner (WRN) e MUTYH.

5 DISCUSSÃO

5.1 Efeitos da carência de ferro sobre a estabilidade genômica e risco de câncer

Os resultados do presente trabalho mostram pela primeira vez um aumento no nível de dano de DNA em crianças com deficiência sutil de ferro, segundo detectado pela maturação deficiente dos eritrócitos. Indivíduos anêmicos apresentam aumento no estresse oxidativo e redução da capacidade antioxidante (Macdougall, 1972; Tekin et al., 2001; Aslan et al., 2006). Adultos com anemia severa também apresentaram aumento de danos no DNA (Aslan et al., 2006). Interessantemente, os indivíduos com eosinofilia apresentaram significativamente mais dano no DNA de seus leucócitos do que os indivíduos com contagem normal de eosinófilos. Isso concorda com o conceito de que um micro-ambiente infeccioso esteja associado a um aumento no nível de estresse oxidativo e do risco de câncer (Jackson & Loeb, 2001). Esse cenário foi, possivelmente, piorado pelo baixo consumo de micronutrientes com papel chave na manutenção do DNA, conforme amplamente discutido por Fenech (Fenech et al., 1998; Fenech, 2001; Fenech & Ferguson, 2001; Fenech, 2005; Fenech et al., 2005) e Ames (1989; 2001; 2003; 2005). Nesse sentido, a associação entre deficiência nutricional e inflamação possivelmente tem um efeito singergístico, e requer atenção em estudos futuros.

Apesar de saber-se que o estresse oxidativo e o dano ao DNA estão associados ao risco de câncer e diversas outras doenças (Halliwell & Guterridge, 2000), inexistia na literatura uma revisão consistente da associação entre deficiência de ferro e o aumento do risco de câncer. A revisão apresentada no ANEXO B traz evidências primárias para a associação entre risco de câncer gastrintestinal e deficiência de ferro. Muitos dos estudos sumarizados na revisão mostram que o ferro tem um efeito dual sobre o risco de câncer, sendo carcinogênico tanto em situações de deficiência como na sobrecarga. De fato, curvas dose-resposta do tipo "U" entre o consumo de ferro e marcadores de risco de câncer, seguindo a regra de Bertrand (1912), foram observadas por de Walter et al (2002) – dano ao mtDNA, Bird et al (1996) – risco de pólipos intestinais e Cross et al (2006) – tumores coloretais. De acordo com os dados dos últimos dois autores (Fig. 1, ANEXO B) foi possível hipotetizar que o consumo de 20 mg/dia ferro seria o nível

próximo do ideal para reduzir o risco de câncer colorretal. Tal ingestão é levemente maior que a DRI para mulheres em idade reprodutiva (18 mg/dia ferro), mas acima da sugerida para homens no mesmo ciclo de vida (8 mg/dia ferro); definidas com o objetivo da prevenção de anemia (IOM, 2001). Ademais, está abaixo da UL (45 mg/dia ferro) para o nutriente (IOM, 2001), e é muito menor do que o nível de ferro ingerido na dieta paleolítica (85 mg/dia ferro) (Benzie, 2003).

5.2 Efeito do excesso de ferro sobre a estabilidade genômica

Como esperado, os experimentos com camundongos mostraram uma alta toxicidade ao material genético do tratamento com ferro, possivelmente pela capacidade do composto gerar estresse oxidativo (De Freitas & Meneghini, 2001). No estudo com tratamento único de ferro, observou-se um aumento de danos primários no DNA (ensaio cometa) 24 horas após o tratamento, que se reduziu 48 h após o tratamento. Isso indica que as células têm capacidade de reparar os danos efetivamente após exposições agudas, mesmo para a dose elevada empregada. Os resultados do estudo com 6 exposições consecutivas da mesma dose mostrou, por outro lado, que o organismo continua sofrendo com as doses elevadas de ferro, contradizendo o conceito de que o organismo poderia regular a entrada de ferro. No 7º dia após a exposição foi observado um aumento nos danos primários no DNA em leucócitos, e um aumento na freqüência de MN na medula óssea, sítio-alvo para a acumulação de ferro (Beshara et al., 2003). Dessa forma sugerem-se evidências experimentais de um possível efeito indutor de leucemia por doses elevadas de ferro. De fato, um aumento da incidência de leucemia em indivíduos com sobrecarga de ferro já foi reportada em estudos epidemiológicos e clínicos (Beutler, 2006).

A dose de ferro administrada aos camundongos (33 mg Fe/kg/dia) foi elevada em relação àquela rotineiramente administrada para indivíduos anêmicos (entre 1 e 5 mg Fe/kg/dia), portanto suscitando a hipótese dos efeitos diferirem significativamente em doses menores.

Uma preocupação com a suplementação com ferro diz respeito ao risco de teratogênese. Embora a suplementação com ferro seja rotineira e sustentada para os últimos 4 ou 5 meses de gestação pela Organização Mundial da Saúde (OMS), devido à alta prevalência de anemia em países em desenvolvimento, a suplementação suscita controvérsias, pela falta de evidências sólidas acerca da conduta e da definição clara dos benefícios do tratamento. Preocupantemente, algumas mulheres ingerem doses

significativamente maiores (até 180 mg Fe/dia) do que aquelas recomendadas pela OMS (25-30 mg Fe/dia). Ainda existem evidências não confirmadas do potencial teratogênico do ferro administrado no primeiro trimestre da gravidez (do Carmo & Nitrini, 2004).

Os dados de Walter et al (2002) e Knutson et al (2000) sugerem que a administração de suplementos contendo ferro a cada três dias em relação à suplementação diárias induz menos danos ao DNA e estresse oxidativo. Esses resultados têm grande concordância com a hipótese ora sustentada, que os sistemas biológicos reparam rapidamente danos ao DNA induzidos por exposições agudas a ferro.

No que diz respeito à AF, observamos que os indivíduos acometidos pela patologia e tratados com HU apresentaram aumento de danos primários no DNA e MN em relação a controles saudáveis não tratados com a droga. A dose média e o tempo de tratamento com HU mostraram associação com o nível de danos primários ao DNA e freqüência de MN. Houve ainda uma correlação negativa entre o nível de danos primários no DNA e o IMC. Portanto, parece prudente a recomendação do monitoramento estrito desses sujeitos com vistas a adequar a dose do medicamento para maximizar a eficiência terapêutica da droga e reduzir o risco de dano ao DNA de forma conjunta. Ressalta-se mais uma vez que a HU garante a sobrevida desses indivíduos, e o tratamento não deve ser interrompido para minimizar o sofrimento dos pacientes (Steinberg et al., 2003).

Os efeitos colaterais da HU parecem envolver a formação de radicais livres, além da redução do *pool* de nucleotídeos para a replicação e o reparo de DNA pelo bloqueio da enzima ribonucleotídeo redutase (Leanza et al., 2008). Considerando que uma alimentação equilibrada e rica em antioxidantes e a prática de atividade física podem minimizar o efeito do estresse oxidativo. Portanto, ressalta-se a importância de melhorar esses aspectos entre os pacientes (Urso & Clarkson, 2003).

5.3 Estratégias para reduzir a genotoxicidade e a neurotoxicidade do ferro

Com já mencionado as evidências epidemiológicas associando dietas ricas em frutas e vegetais e a diminuição na incidência de doenças neurodegenerativas e câncer têm sido descritas amplamente na literatura (Correa et al., 1998; Donaldson, 2004). Esse efeito protetor é atribuído principalmente a compostos naturalmente presentes nos alimentos, como compostos fenólicos, carotenóides e vitamina C (Wang et al., 1996; Kabasakalis, 2000; Halliwell, 2001). Além disso, acredita-se que o efeito protetor dos alimentos decorre da interação entre os componentes dos mesmos (Halliwell, 2001). Portanto, vários autores

recomendam a ingestão diária de pelo menos 5 porções de frutas e vegetais para prevenir doenças (Ames, 1989; Halliwell, 2001). O mecanismo de proteção contra o câncer e o envelhecimento pode ser atribuído à capacidade desses compostos em proteger contra o ataque dos radicais livres (p.ex. vitaminas A, E e C e os flavonóides (Crystal, 1991; Ames et al., 1993; Sies, 1997; Ames, 2001; Halliwell, 2001; Urso & Clarkson, 2003) bem como participar em rotas metabólicas importantes para a manutenção da estabilidade do patrimônio genético [p.ex. ferro (Walter et al., 2002) e ácido fólico (Ames, 2001)]. Ademais, muitos desses compostos podem modular a transcrição por interagirem com receptores de fatores de transcrição; afetando a atividade de rotas metabólicas, por alterarem a concentração de substratos ou intermediários, quando envolvidas em rotas metabólicas primárias ou secundárias; e/ou afetando rotas de sinalização (Kaput & Rodriguez, 2004)

Os resultados desse trabalho, utilizando diferentes estratégias para reduzir a toxicidade do ferro, mostraram efeitos diferentes, dependendo do alimento, vitamina ou medicamento testado. A vitamina C, apesar de ser administrada em paralelo com o ferro para aumentar a biodisponibilidade desse (Gibson, 2007), foi capaz de induzir danos no DNA no sangue de camundongos e no cérebro de ratos, sendo também incapaz de reverter déficit de memória induzido em ratos tratados com ferro no período neonatal. O SL e a ração rica em grãos foram capazes de minimizar parcialmente o nível de danos no DNA induzidos pelo tratamento com ferro. Até o momento, não se dispõe de dados acerca do efeito desses dois tratamentos sobre o déficit de memória induzido pelo ferro. O medicamento quelante de ferro DFO teve destaque, pois reverteu o déficit de memória e reduziu os danos no DNA de células do cérebro e do sangue observados nesses animais. Interessantemente, a droga reduziu o dano no DNA de forma similar tanto para os animais tratados ou não com ferro, mostrando um efeito do composto em reduzir o dano basal das células cerebrais desses animais.

O pré-tratamento com SL reduziu a genotoxicidade do ferro. Tanto a vitamina C quanto o SL 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 ferro da dieta, o efeito antioxidante do SL prevalece sobre o efeito pró-oxidante. Os compostos fenólicos podem ter atuado com capturadores de metais, indisponibilizando os mesmos para que a vitamina C atuasse como pró-oxidante, ou até como modulador do reparo de DNA (Abalea et al., 1999). Além desses, os outros antioxidantes ou nutrientes do SL que tem papel na

homeostasia do DNA como os flavonóides, a riboflavina, a niacina, a piridoxina e o magnésio podem ter atuado protegendo contra o dano oxidativo e participando do metabolismo do DNA (Fenech, 2005; Fenech et al., 2005; Gao et al., 2006). O pós-tratamento tanto com vitamina C quanto com SL foi pró-oxidante para o ferro. É importante mencionar que quando o Fe é disponibilizado antes do agente redutor, o tratamento tanto com vitamina C quanto com SL tem um claro papel pró-oxidante. A grande adaptação do organismo ao dano ao DNA induzido pelo ferro parece ser uma hipótese interessante, visto pelo reparo eficiente após 48 h de uma única administração do elemento. O ferro 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 no LIP, 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 ERO. Isso 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). Interessantemente, o tratamento a longo prazo com doses entre 250 e 500 mg/dia de vitamina C vem sendo associado a um estímulo na excreção urinária de 8-OHdG, conforme revisado por Cooke e colaboradores (1998). Esse aumento da excreção tem sido interpretado como um estímulo do reparo de DNA. Sendo assim, estudos de interação entre vitamina C e ferro sobre a estabilidade genômica são relevantes. Esse tema é importante visto que 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 vitamina C, sendo muito maior do que a DRI para o nutriente (25 mg/dia vitamina C para crianças de 6-8 anos; 45 a 75 mg/dia vitamina C para crianças maiores e adolescentes); nível similar de ingestão de vitamina C similar ao da dieta paleolítica (Benzie, 2003).

A dieta rica em antioxidantes empregada no estudo, que contêm muitos dos compostos presentes no SL, foi efetiva em reduzir a incidência de danos primários ao DNA das células sangüíneas dos animais tratados com ferro. Não obstante, foi inefetiva para reduzir a freqüência de MN na medula óssea desses. A dieta parece ter um papel importante na redução dos danos ao DNA, mas não é suficiente para reduzir os danos permanentes ao DNA em órgãos-alvo para a acumulação de ferro. Como a dose de ferro empregada no estudo é bastante superior àquela empregada em situações de suplementação com ferro em humanos, fica a pergunta se doses menores de ferro podem induzir lesões

permanentes no DNA; e qual seria a influência de uma dieta rica em antioxidantes nessa situação. Independentemente de saberem-se os riscos reais da suplementação com ferro, sugere-se que os indivíduos suplementados adotem uma dieta saudável a longo-prazo. Essa dieta, além de prevenir a toxicidade do ferro, também poderá contribuir para a saúde, reduzir o risco de doenças crônico-degenerativas ou do restabelecimento da anemia, bem como poderia até atenuar o processo de envelhecimento.

Os efeitos da DFO na reversão do déficit de memória, dos níveis de ferro e da taxa basal de dano no DNA se constituem em resultados relevantes dessa pesquisa. Ressaltando que o acúmulo de ferro está associado às doenças neurodegerativas e ao câncer (Fredriksson et al., 1999), a identificação de que a DFO pode reduzir os efeitos da sobrecarga de ferro, tendo um efeito benéfico sobre a estabilidade genômica em paralelo é útil para diversas decisões clínicas. Uma ressalva são os possíveis riscos do tratamento a longo-prazo com a desferoxamida e as dificuldades associadas ao seu manejo clínico (p.ex. meia-vida curta e método cutâneo de administração)(Freedman et al., 1988).

5.4 Determinação da dose ideal de ferro para a manutenção da estabilidade genômica de células em cultura

A saúde ideal e a longevidade máxima requerem harmonia metabólica, para as quais a ingestão adequada de micronutrientes é essencial. As recomendações nutricionais reconhecem esse princípio, contudo, na maioria dos casos, ainda estão centradas primariamente na prevenção de patologias graves classicamente imputadas seja à falta como ao excesso de nutrientes. Para o ferro, as recomendações nutricionais visam prevenir a anemia; num extremo visam proteger contra anemia (extremo da carência) e em outro extremo buscam evitar o desconforto gastrintestinal associado ao excesso de ferro; que está associado à indução de estresse oxidativo (Halliwell et al., 2000). Há um consenso na área de nutrigenômica de que é urgente definir as doses ideais de nutrientes para otimizar o metabolismo, reconhecendo que as variações metabólicas e genéticas podem influenciar as necessidades individuais. Esse conceito tem sido bastante divulgado por Bruce Ames (Ames, 2003) e Michael Fenech (Fenech, 2005), que vêm realizando pesquisas sistemáticas para explorar o efeito de diferentes nutrientes em reduzir os danos no DNA. As faixas metabólicas nas quais os nutrientes otimizam o metabolismo parecem ser mais estreitas do que as das recomendações nutricionais, como confirmado por inúmeros estudos epidemiológicos e experimentais, que observam variações na incidência de danos

no DNA, câncer ou outras doenças como conseqüências de variações sutis na ingestão de nutrientes (Fenech et al., 2005).

Cabe lembrar que os dados da quantidade ideal de micronutrientes muitas vezes emergem de estudos em células em cultura mantidas em meios não fisiológicos; que precisam ser aprimorados com vistas a simular diferentes compartimentos do organismo. A partir de então poder-se-á utilizar esses sistemas como marcadores precisos das necessidades nutricionais; por exemplo: ao reconhecerem-se as variações na absorção dos nutrientes. Desta forma, poder-se-á avançar no sentido de ligar uma determinada ingestão de nutrientes com a estabilidade genômica e prevenção de doenças. Até lá, teremos que continuar empreendendo esforços nos sistemas celulares menos fisiológicos e confiar nos estudos experimentais e epidemiológicos que, em sua maioria, apresentam muitas variações entre os indivíduos, devido ao estilo de vida e da dificuldade de precisar a variabilidade entre a ingestão e o nível metabólico de nutrientes (mesmo quando se usam sistemas bioquímicos de detecção de nutrientes nos tecidos).

O ferro traz inúmeras dificuldades para se determinar a faixa fisiológica ideal de sua ingestão para reduzir a instabilidade genômica em sistemas celulares em cultura. Parece que ao se tratar células com formas inorgânicas de ferro geram-se quantidades significativas de estresse oxidativo, pois as células em ambiente fisiológico adequado são expostas a quantidades mínimas de ferro livre (esse fica quase totalmente preso à quelantes). Uma alternativa para contornar esse problema é tratar as células com ferro ligado à transferrina. Os resultados utilizando hTf apresentados no presente trabalho indicam evidências inovadoras acerca da faixa ideal de ferro para maximizar a estabilidade genômica de células linfoblastóides em cultura. Segundo os resultados, foi possível maximizar o comprimento dos telômeros e minimizar a extensão de danos primários ao DNA e a freqüência de MN. Entretanto, com a maximização do comprimento dos telômeros ocorrendo em uma concentração de hTf menor do que àquela que minimizou os danos primários no DNA e a freqüência de MN. Esse fato sustenta a idéia de que os telômeros atuem como *hotspots* para a formação de 8-OHdG (Henle et al., 1999), protegendo o genoma contra o efeito pró-oxidante do ferro. Quanto ao efeito sobre a quantidade relativa de mtDNA, os dados podem ser interpretados com base na idéia de que a ocorrência de um efeito proliferativo nas mitocôndrias pode estar associado a doses menores de ferro, uma tendência à acomodação no número de organelas em função de um ganho de eficiência e um posterior decréscimo devido à toxicidade associada ao estresse oxidativo. Contudo, esses dados devem ser vistos com cautela já que a amplificação

poderia ser aumentada à medida que a qualidade do DNA é superior (menos lesões) (Chen et al., 2007). Segundo experimentos em curso com um kit de *clean up* de DNA, que homogeneíza a eficiência de amplificação, tem-se observado pouca influência de lesões sobre a amplificação para as concentrações de hTf testadas.

Interessantemente, a redução do comprimento dos telômeros é uma limitação para vários protocolos de transplante, que precisam cultivar as células ex vivo (Kobari et al., 2000). Como o ferro não está presente em quantidades ótimas em meio de cultura, a definição de condições de cultura é fundamental para maximizar a eficiência de transplantes. Portanto, definir a dose ideal de ferro para culturas celulares é imperativa para otimizar as condições de cultivo e lançar as primeiras luzes no sentido da definição da quantidade de ferro com vistas a reduzir a instabilidade genômica.

Os resultados da análise da expressão dos genes de reparo WRN e MUTYH e dos genes de metabolismo da hTf TFRC e FTL apontam associação notória entre a dose de ferro e o nível de mRNA dos genes. Como esperado, apenas para a dose maior de hTf foi verificado um aumento da expressão do nível de FTL, indicando necessidade de estocagem do ferro, possivelmente associada à toxicidade. Isso está em consonância com o fato dessa concentração correspondente a 23 μM Fe ser bastante próxima a 25 μM Fe, limite máximo de ferro no soro em pessoas saudáveis (van Tits et al., 2007). O acréscimo saturável no nível de mRNA de FTRC também aponta que a entrada de hTf atingiu o nível máximo no modelo de estudo avaliado. O papel do gene WRN no reparo de quebras duplas (Galvan et al., 2008) foi associado ao decréscimo do nível de dano no DNA e da freqüência de MN, mínimos na maior concentração testada. Uma vez que essa concentração está controversamente próxima ao limite fisiológico de ferro no sangue para pessoas saudáveis, que induz aumento do nível de mRNA da transferrina e está simultaneamente associada à uma redução na freqüência de MN. Portanto, estudos adicionais de estabilidade genômica nessa faixa de concentração são sugeridos. A forte associação entre o nível de mRNA do gene MUTYH e o comprimento dos telômeros, levanta a hipótese de um circuito regulatório entre lesões induzidas nos telômeros e a expressão de MUTYH, algo não discutido na literatura. Outra descoberta relevante é a presença de IRE nos mRNAs, indicando regulação pós-trascricional para os genes WRN e MUTYH, a exemplo dos genes TFRC e FTL. Com efeito, a análise da expressão das primeiras proteínas deve ser executada com vistas ao entendimento do papel do ferro nesse novo mecanismo de regulação desses genes de reparo.

Testes adicionais, utilizando formas fisiológicas de ferro (p.ex. transferrina e ferritina sérica), bem como células com necessidades fisiológicas maiores do que os leucócitos (p.ex. enterócitos e hepatócitos) também estão sendo desenvolvidos.

A definição da dose ótima de ferro para células em cultura auxiliará, na definição dos níveis de ferro para a preparação de meios de cultura fisiológicos, em alguns avanços no entendimento de como diferentes níveis de ferro influenciam marcadores celulares da estabilidade genômica. Além disso, a otimização da estabilidade genômica e o aumento do comprimento dos telômeros de células linfocitárias pode diretamente contribuir para os protocolos de transplante, que se deparam com uma grande senescência ao cultivar células para depois transplantá-las (Kobari et al., 2000).

6 CONCLUSÕES

Diante dos resultados apresentados avaliando-se o efeito da carência e do excesso de ferro sobre a estabilidade genômica e genes do metabolismo do ferro e de reparo do DNA em humanos, ratos e camundongos e células em cultura, conclui-se o que se segue:

- A deficiência leve de ferro, detectada por parâmetros de maturação das hemácias, leva ao acúmulo de danos primários ao DNA em crianças e adolescentes, possivelmente de forma sinergística com infecção por parasitas. As variações na composição das subfrações de células brancas parecem estar associadas à nutrição e têm influência potencial no perfil de dano no DNA desses indivíduos.
- O consumo de ácido fólico apresenta correlação negativa com o nível de dano no DNA. O maior consumo de niacina e magnésio, bem como o consumo intermediário de sódio estão associados à diminuição do nível de DNA nos mesmos sujeitos.
- Existem evidências clínicas, experimentais e epidemiológicas, essas últimas de grau II-2-boas, de que a deficiência de ferro possa estar associada a um risco aumentado de câncer no trato gastrintestinal.
- A ingestão de 20 mg/dia de ferro parece estar associada à diminuição de lesões pré-neoplásicas (pólipos) e câncer na região colorretal.
- A HU foi genotóxica e mutagênica de forma dose e tempo-dependente em pacientes de AF. Portanto, o manejo clínico estrito da condição deve ser adotado com vistas à melhoria do padrão dietético (o IMC mostrou associação negativa com o ID) e minimização da dose de HU, com manutenção da eficiência terapêutica.
- O ferro induziu danos primários e MN no DNA em camundongos; contudo, o reparo de DNA foi efetivo a 24 horas após administração de uma única dose de ferro e é provável que a suplementação de ferro em dias alternados (p.ex. a cada três dias) possa reduzir o risco de instabilidade genômica associado à suplementação.
- A vitamina C foi genotóxica per se no sangue periférico de camundongos, mas não no de ratos. A interação da vitamina C parece ser pró-oxidante; contudo, pode também estar associada ao estímulo do reparo de DNA.

- O tratamento com vitamina C foi inefetivo para reverter na fase adulta o déficit de memória induzido pelo tratamento neonatal de ratos com sobrecarga de ferro.
- O tratamento com SL protegeu os leucócitos de camundongos contra genotoxicidade induzida pelo ferro.
- Uma dieta rica em antioxidantes reduziu drasticamente o nível de danos primários ao DNA de leucócitos, mas teve pouco efeito sobre a freqüência de MN em células de medula óssea de camundongos tratados com uma dose elevada de ferro.
- A DFO reduziu a genotoxicidade no cérebro de ratos adultos, independente do tratamento perinatal ou não com ferro.
- O nível de ferro no meio de cultura RPMI 1640 é muito reduzido em relação ao nível sérico humano do metal.
- A suplementação do meio de cultura com hTf tem um efeito bastante positivo sobre a estabilidade genômica de células linfoblastóides humanas, conforme evidenciado pelo comprimento de telômeros e pela freqüência de MN em células bloqueadas em citocinas.
- Uma concentração de ferro > 6 μM estabilizou o genoma destas células, com concentrações maiores estando associadas à erosão dos telômeros; contudo, sem aumento na freqüência de MN até a dose de 23 6 μM Fe. Essas concentrações apresentam uma correspondência marcante com os níveis mínimos e máximos da faixa normal de ferro no soro, de respectivamente 5 μM (anemia) e 25 μM (sobrecarga).
- A expressão dos genes do metabolismo do ferro comprovam a absorção de hTF de forma saturável e da ferritina apenas para a dose maior (ponto do início do excesso de ferro intercelular).
- Os genes WRN e MUTYH, codificantes para proteínas que contém ferro em regiões catalíticas, responderam ao tratamento de ferro, de forma lógica com os marcadores de estabilidade genômica avaliados e segundo os modelos de regulação compartilhados por genes de metabolismo do ferro.
- Interessantemente, os genes WRN e MUTYH possuem regiões IRE em seus mRNAs, evidenciando um novo mecanismo de regulação pós-transcricional para esses genes.

7 PERSPECTIVAS

São perspectivas desse trabalho, que podem ser desenvolvidas por nosso grupo de pesquisa ou por outros pesquisadores:

- Analisar os experimentos referentes à suplementação com sulfato ferroso, avaliação da estabilidade genômica e da expressão de genes de reparo, executados, mas não apresentados na tese.
- Desenvolver estudos adicionais, focando o nível ideal de ferro com vistas à diminuição do risco de câncer para o trato gastrintestinal e, em outros sistemas; particularmente nas populações de países em desenvolvimento, onde a carência de ferro é mais comum. Estudos acerca de efeitos teratogênicos associados à deficiência gestacional de ferro também podem ser desenvolvidos.
- Avaliar se a sobrecarga de ferro pode aumentar o risco de leucemia em experimentos epidemiológicos controlados.
- Avaliar o efeito sobre o DNA de doses de ferro entre 0,5e 3 UL.
- Avaliar o efeito da vitamina C em doses intermediárias àquelas testadas no estudo.
- Para os indivíduos com AF tratados com HU, fazer uma análise criteriosa de cada indivíduo, sugerir a suplementação com antioxidantes no sentido de minimizar as lesões no DNA; sejam essas intrínsecas a própria AF ou geradas pelo tratamento com HU. Monitorar o nível de ferro sérico nesses pacientes também seria importante, com vistas a definir o efeito da HU na toxicidade intrínseca da doença.
- Comparar os efeitos da desferoxamida com outros quelantes de ferro.
- Avaliar o possível efeito da suplementação do meio de cultura com diferentes compostos contendo ferro em mutantes de genes de reparo com mutações que afetem a ligação do ferro às proteínas correspondentes, enfatizando a estabilidade do DNA mitocondrial nesses estudos.
- Elucidar os mecanismos de regulação do reparo de DNA modulados pelo ferro.

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9 ANEXOS

9.1 ANEXO A: “Deficient red cell maturation and inflammation are associated to DNA damage increase in undernourished children and adolescents”

Manuscrito a ser submetido ao periódico *Mutation Research.*

Deficient red cell maturation and inflammation are associated to DNA damage increase in undernourished children and adolescents

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Abstract
This study aimed to evaluate DNA damage level and its association with hematological parameters and nutrient intake in a sample of healthy children and adolescents with low socioeconomic status. The level of DNA damage was evaluated by the alkaline comet assay, a full automated blood count was also obtained using an automate hematology analyzer and the intake of calories, fibers and nutrients was measured using a food frequency questionnaire. Only one out of the 30 individuals evaluated was anemic according to hemoglobin levels. Notwithstanding, about half of the sampled subjects showed deficient red cell maturation/altered iron status (iron deficiency) as evaluated by reduced erythrocyte volume (microcytosis) and had about two-fold more DNA damage ($p \approx 0.01$) than those with normal erythrocytes indexes. About 70% of the individuals showed eosinophilia, a marker of parasitosis, having also higher DNA damage than those with normal counts ($p \approx 0.01$). Age, body mass index and the intake of folate were negatively correlated with DNA damage level. Lower intake of niacin was also associated to a higher DNA damage level. The intake of energy and several nutrients was negatively correlated to the leukocytes number, indicating the undernourishment is associated DNA damage and inflammation.
Key words: DNA damage, anemia, inflammation, micronutrients.

Introduction

Iron deficiency (ID) is the most prevalent micronutrient deficiency worldwide, particularly among poor children and women. Anemia, a hallmark of high ID, affects as much as about 50 % of the 5- to 14-year-old children and of the pregnant women [1]. It is well know for a long time that ID can irreversibly affect brain development [2], impairs immunity [3] as well as increases oxidative stress and decreases antioxidant defenses [4,5]. More recently, it has been shown that both

ID and moderate iron excess induce mitochondrial DNA damage and functional deterioration of mitochondria, resulting in a “U” shaped dose-response curve known as Bertrand’s Rule [6]. Oxidative stress mediated by mitochondrial uncoupling is associated with DNA damage and aging and has been detected in most neurodegenerative diseases [7]. ID has also been shown to increase nuclear DNA damage in adults, as observed by an increase in DNA damage in anemic subjects [8]. Further studies are still needed in order to evaluate the impact of iron deficiency on genome maintenance.

There are four major non-exclusive causes for iron deficiency which are reduced iron intake or absorption, excessive blood loss, inefficient erythropoiesis and chronic inflammation. Pour nutrition or gastrointestinal distress are the major causes of reduced iron bioavailability. Inefficient erythropoiesis can arise either by low heme synthesis or low DNA metabolism due to folate and/or B12 deficiency or both conditions combined. Indeed, the several nutrients that influence iron absorption and utilization as well as those involved in heme and DNA biosynthetic pathways may play major roles in iron deficiency and normal red cell maturation. Typically, a reduction of erythrocytes volume (microcytosis) is linked to insufficient levels of hemoglobin and the opposite (cell increase or macrocytosis) is linked to defects in DNA synthesis [9,10]. Anemia of inflammation is a common finding in patients with chronic inflammation (infections) which is associated to iron depletion without evident alterations in hemoglobin content or red blood size [11]. The impact of anemia of inflammation over genomic stability is poorly characterized. Therefore, in this paper we aim to evaluate the whole blood DNA damage level and its association with hematological and dietary parameters in a sample of young individuals from a poor community typically affected by parasitosis.

Material and methods

We evaluated the extent of DNA damage in whole blood sampled from 30 healthy children (61.3% males) aged 11.5 ± 2.5 years enrolled within the Brazilian “Family Health Program – PSF” in a poor neighborhood of Santa Cruz do Sul, RS. The study protocol was approved by the internal human experimentation ethics committees of UNISC, and the relatives of all participants gave written informed consent.

Peripheral blood samples from all individuals were collected in the same morning. Blood samples were immediately processed as follows: a subsample was mixed with heparin for the comet assay, another subsample was mixed with EDTA for hematological analysis, and the rest of the blood was centrifuged for 10 minutes at 3000 rpm to obtain plasma samples for fasten glucose evaluation. The comet assay and the hematological analysis were carried out immediately. Individuals were asked to be fasten and in rest for the 12 hours previously to blood sampling. Normal glucose levels confirmed fasting for all individuals (data not shown).

The standard protocol of the comet assay with silver staining and visual scoring, as described elsewhere, was used (for details see [12]). Hundred cells were evaluated per individual (50 in each replicate slide). Cells were examined visually to determine DNA damage scores ranging from 0 (no migration) to 4 (maximal migration) based in tail intensity (size and shape). Two DNA damage parameters were calculated accordingly: the damage index (DI) - for 100 cells ranged from 0 (all cells with no migration) to 400 (all cells with maximal migration); and the damage frequency (DF) as a percentage of damaged cells. All slides were

processed, coded, mixed and evaluated together by a single analyzer. Slides were examined using a standard light microscope at 200 times magnification.

The full automated blood count was analyzed in triplicates using an Abbott Cell Dyn 3000 Hematology Analyzer (Abbott Diagnostics, Mountainview, CA), in accordance with the manufacturer's protocol and classified based in hematological reference ranges [13-16], as described in Table 1.

Table 1. Limits of normal age- and gender-related hematological parameters for children and adolescents.

Hematological parameter (unit)	**Age**	5.00-7.99	8.00-11.99	12.00-14.99	15.00-17.99	Reference
Red cells	**Sex**					
Hemoglobin - Hb (g/dL)	Females	109	110	115	117	[13]*
	Males			120	123	
Red cells count - RBC ($x10^{12}$/L)	Females	3.1	3.8	3.9	3.9	
	Males			4.1	4.2	
Mean cell volume - MCV (fL)	Females	74	76	77	78	
	Males			77	79	
	Males and females	83				[14]*
Mean cell hemoglobin - MCH (pg)	Females	25	26	26	26	
	Males			26	27	[13]*
Red blood cell distribution width-RDW (coefficient of variation %)	Males and females	14.5				[15]**
Mean corpuscular hemoglobin concentration - MCHC (g/dL)	Males and females	32				[13]*
White blood cells* **	Age	6.00-13.00		13-01-18.00		
	Sex					
Neutrophil granulocytes ($x10^9$/L)	Males and females	1.8-8.0		1.8-10.0		[16]***
Eosinophil granulocytes ($x10^9$/L)		0-0.6				
Basophil granulocytes ($x10^9$/L)		0-0.2				
Lymphocytes ($x10^9$/L)		1.2-6.0		1.0-5.0		
Monocytes ($x10^9$/L)		0.15-1.3		0.08-1.2		

* Lower limit; ** upper limit; ***normal range.

Individuals were measured and weighted and the body mass index-for-age (Kg/m^2) was calculated and classified according to standard procedure described by the World Health Organization [17].

The habitual diet was evaluated in three non-consecutive days by a validated questionnaire containing open questions about the usual intake of foods in each meal [18]. The type of food, its main ingredients, the way of preparation, the brand as well as the addition of salt, sugar, sauces, peels and if the product was "sugar-free"/"low calorie" were considered for every food. Home measurements were

presented during the interview to aid in the interpretation of the amount of the foods ingested.

Individuals were stratified to categories (subsamples) according to hematological values and percentiles of nutrient intake. The Student *t* test or one-way ANOVA with the Bonferroni post-hoc test was used to compare DNA damage within subsamples and the Pearson correlation test was used to evaluate associations between DNA damage, hematological and dietary variables. An univariate model was used to evaluate the influence of variables as a whole over DNA damage. Dietary data was processed with the *Virtual Nutri* 7.0 (São Paulo, SP, Brazil), statistical analysis was performed with the Statistical Package for Social Sciences – SPSS 11.0 (Chicago, IL, USA) and graphs were plotted with the GraphPad Prism 4.0 (San Diego, CA, USA) The level of significance was $P<0.05$.

Results

The body mass index-for-age classification [17] showed 19.4% of the studies subjects were underweight or light underweight, 61.3% had normal weight and 16.1% were overweight or obese (only one individual was obese).

Table 2 presents the hematological characterization of the studied sample according to reference values. The hemoglobin level was normal in the vast majority of the sample (only one individual was classified as anemic). However, 33.3% of the individuals showed reduced mean corpuscular volume (MCV) and 12.9% showed increased red cell distribution width (RDW) or reduced mean corpuscular hemoglobin (MCH). Regarding white blood cells, 66.6% of individuals had eosinophilia and 10% altered monocyte counts.

Table 2. Main hematological characteristics of sample of children and adolescents from Santa Cruz do Sul, RS, Brazil low socioeconomic status.

Hematological parameter (unit)	Average level	Individuals below or above the reference range* (%)
Red cells		
Hemoglobin (g/dL)	12.93 ± 0.81	3.2 below
Red cells count ($x10^{12}$/L)	4.76 ± 0.29	0
Mean cell volume - MCV (fL)	83.76 ± 3.09	3.2 below 33.3 below
Mean cell hemoglobin - MCH (pg)	27.23 ± 1.18	12.9 below
Red blood cell distribution width- RDW (coefficient of variation %)	13.64 ± 0.68	12.9 above
Mean corpuscular hemoglobin concentration - MCHC (g/dL)	32.48 ± 0.33	3.2 below
White blood cells		
Neutrophil granulocytes ($x10^9$/L)	5.27 ± 1.75	3.2 above
Lymphocytes ($x10^9$/L)	3.11 ± 0.81	0
Monocytes ($x10^9$/L)	0.85 ± 0.36	9.7 above
Eosinophil granulocytes ($x10^9$/L)	1.03 ± 0.89	67.7 above
Basophil granulocytes ($x10^9$/L)	0.09 ± 0.03	0

* Reference ranges, as defined in Table 1.

No major significant differences between males and females for DNA damage (Table 3) and individuals were pooled for further analyses. Since all red cells parameters are relevant to the diagnosis of both anemia and iron deficiency [13], individuals were ascertained into two groups: control group (normal range) and ID individuals (change in at least one red cell parameter). ID individuals showed significantly higher DNA damage than the control group, when the level of DNA damage was compared between control and ID individuals either as evaluated by comet assay (Fig. 1A,B). There was no significant correlation between any red cell parameter and DNA damage.

Table 3. DNA damage level according to gender, age and body mass index (BMI) in a sample of children and adolescents from Santa Cruz do Sul, RS, Brazil with low socioeconomic status.

		Female	Male	
Gender	DI	28.6 ± 19.9 (12)	36.6 ± 24.2 (18)	0.349
	DF	17.4 ± 10.7 (12)	23.0 ± 12.5 (18)	0.216
		≤11 years	≥12 years	
Age	DI	40.3 ± 21.5 (15)	26.5 ± 22.2 (15)	0.096
	DF	23.4 ± 9.8 (15)	18.1 ± 13.6 (15)	0.234
		≤17 Kg/m^2	>17 Kg/m^2	
BMI	DI	41.6 ± 22.1 (14)	26.3 ± 21.1 (16)	0.062
	DF	24.4 ± 10.1 (14)	17.6 ± 12.8 (16)	0.118

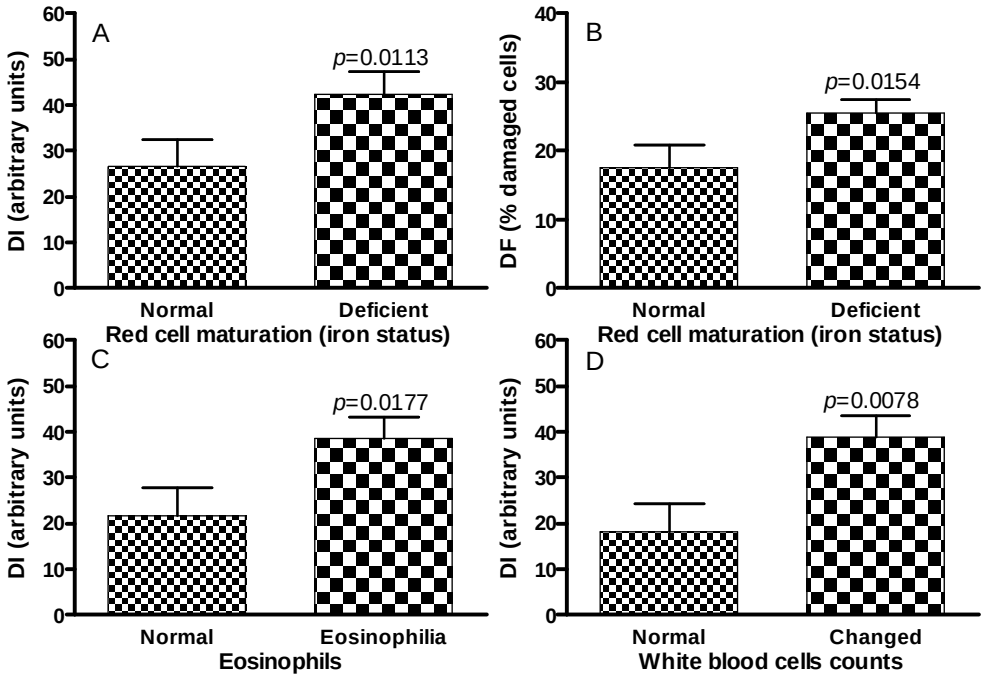

Figure 1. DNA damage level according to red cell maturation deficiency/iron deficiency (A, DI e B, DF) and eosinophilia (C) or high count in at least one white blood cell parameter (D) in a sample of children and adolescents from Santa Cruz do Sul, RS, Brazil with low socioeconomic status. *p* = significance according to the Student's t test. DI: damage index; DF=damage frequency.

Individuals with eosinophilia showed significantly higher DNA damage than individuals with normal levels of eosinophils (Fig.1C). Since any alteration in white blood cell parameter show generally either impaired immunity or inflammation, the sample was also a stratified in two groups: control group (normal values for all white cell parameters) and individuals with increase in at least one white cells parameter. In agreement, the latter individuals showed higher DNA damage than controls (Fig. 1D). The study also showed a positive correlation between DNA damage and the percentage of basophils and eosinophils (Fig. 2 A,B), a negative correlation between DNA damage and the percentage of neutrophils (Fig. 2C) as well as a negative correlation between the percentage of basophils and neutrophils (Fig. 2D).

DNA damage level was negatively correlated with age, body mass index, total calories and folate intake (Fig. 2E-H). Negative correlations between number of lymphocytes and intake of several micronutrients, fibers, total lipids, and proteins were observed (Table 4). Individuals with at least one alteration in white blood counts had significantly lower intake of magnesium (208.4 ± 92.6 versus 154.5 ± 44.7 mg day, p=0.035), fibre (18.3 ± 11.6 versus 12.3 ± 4.4, p=0.042) and borderline lower intake of carbohydrates (338.9 ± 195.0 versus 228.9 ± 131.8 g day, p=0.077) compared to the control group.

Individuals were stratified to 2 and 3 groups based in percentiles of nutrient intake to look for associations to DNA damage levels. Those with lower niacin or carbohydrates intakes had significantly higher DNA damage. Individuals with moderate intake of sodium showed lower DNA damage. Although folate intake correlated negatively with DNA damage, the difference in DNA damage between those with lower or higher intakes of folate was non-significant (Table 5). Based in significant values in Figure 1 and Table 5, we constructed a univariate model to evaluate the relationship between DNA damage, ID, eosinophilia and nutrient intake. According to the model, ID (p=0,014), eosinophilia (p=0,004), niacin intake (p=0,004) and sodium intake (p=0,089) significantly affected DNA damage conjointly as evaluated by DF (R^2=0,844; p=0,005).

Table 4. Correlation between the intake of several nutrients and the number of lymphocytes in sample of children and adolescents from Santa Cruz do Sul, RS, Brazil with low socioeconomic status.

Nutrient	Pearson's correlation coefficient (r)	Level of significance (*p*)
Vitamin B1	-0,579	0.001
Vitamin B2	-0.426	0.017
Vitamin B3	-0.507	0.004
Vitamin B6	-0.399	0.026
Vitamin E	-0.450	0.011
Potassium	-0.492	0.005
Sodium	-0.460	0.009
Phosphorus	-0.477	0.002
Selenium	-0.565	0.001
Fibers	-0.436	0.014
Total lipids	-0.377	0.009
Proteins	-0.535	0.002

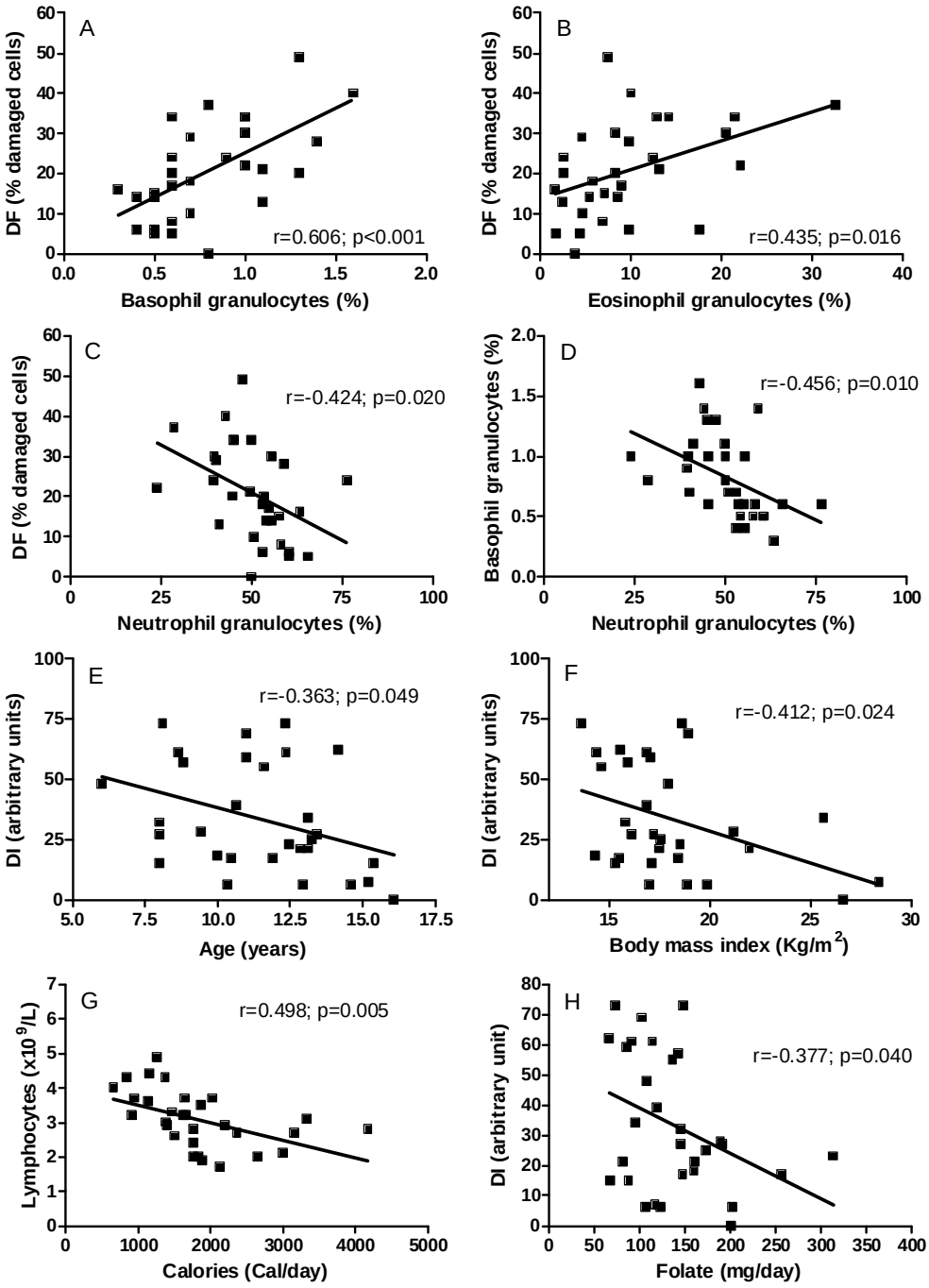

Figure 2. Correlations between DNA damage levels and hematological and nutritional parameters in a sample of children and adolescents from Santa Cruz do Sul, RS, Brazil with low socioeconomic status. Correlation between the DNA damage and the percentage of basophils (A), eosinophils (B) and neutrophil granulocytes (C); correlation between the percent of neutrophils and basophil granulocytes (D); correlation between DNA damage, age (E) and body mass index - BMI (F); correlation between total Calories and lymphocytes number (G); and correlation between the intake of folate and the DNA damage level (H). r and p, correlation coefficient and level of significance, respectively, according to Pearson's correlation analysis.

Table 5. DNA damage level according to nutrient intake level in a sample of children and adolescents from Santa Cruz do Sul, RS, Brazil with low socioeconomic status.

Nutrient	DNA damage parameter	Nutrient intake level (n)			*p*
		Low	Medium	High	
Niacin (B3)	DI	42.5 ±22.4 (14)	---	25.4±20.2 (16)	0.036
	DF	25.±12.7 (14)	---	16.5±9.8 (16)	0.034
Folate (B9)	DI	40.7 ±24.2 (14)	---	27.0±19.6 (16)	0.098
	DF	24.7±13.7 (14)	---	17.3±9.3 (16)	0.091
Carbohydrates	DI	41.6±21.4 (15)	---	25.2±21.13 (15)	0.045
	DF	25.7±11.7 (15)	---	15.9±10.4 (15)	0.022
Sodium	DF	28.0±13.9 (9)	15.1±7.9 (11)	20.5±11.4 (10)	0.050

DI: damage index; DF: damage frequency; n: number of individuals; *p*: level of significance according to t test or ANOVA.

Discussion

ID is likely to increase DNA damage by different mechanisms:

I) Impairment of enzymes involved in DNA damage and repair. For example, iron depletion inhibits the activity of ribonucleotide reductase (RR), a Fe-containing enzyme that converts ribonucleotides into deoxinucleotides, blocking DNA synthesis and arresting cell cycle progression [19-21]. Iron is also present in several DNA repair enzymes, such as DNA glycosylases [22], alkyltransferases [23] and helicases [23] and its deficiency might affect them. Notwithstanding, there are as yet no studies evaluating the effect of ID on DNA repair enzyme activity.

II) Impairment of antioxidant enzymes – iron is a co-factor for catalase and there are numerous evidences of a positive correlation between hemoglobin and catalase levels [24] as well as many studies show reductions in catalase activity in ID both in humans and lab animals [5,25-27].

III) Impairment of the metabolization P450 enzymes - ID has been shown to reduce the cytochrome P450 complex activity in gut of iron deprived rats. As much as 50% of all heme can be used in the assembling of P450 [28].

IV) Impaired energy production – iron is a critical component of oxidative phosphorylation complexes. It has been shown that heme deficiency corrupts mitochondrial complex IV, apparently, causing mitochondrial decay, oxidative stress, and oxidative damage [7,29].

VI) Impaired nitric oxide synthase (NOS) activity - ID can suppress NOS activity as observed for gut cells [30]. As a consequence, immune cells reduce the tumorocidal activity of macrophages which secrete nitric oxide to inhibit iron containing enzymes, DNA synthesis, mitochondrial respiration and citric acid enzymes of microbes and tumor cells [31].

Heme iron has higher bioavailability than inorganic iron, however account for a smaller amount of total iron intake; particularly among individuals with lower intake of meat. Moreover, inorganic iron absorption is modulated by many compounds [32]. Organic acids (e.g. citric and ascorbic acid), cysteine-rich proteins, mucins, sugars, amines and amides increase iron absorption, while some protein derived from dairy products (e.g. casein), phenolic compounds, carbonates, phosphates, and possibly calcium can reduce iron absorption [3,32-34]. Despite of their low socio-economical background, the studied individuals reported a good intake of meat. The low prevalence of anemia as evaluated by the hemoglobin level

can be justified by this fact. Moreover, Brazil has adopted a mandatory program for iron/folate supplementation to wheat and corn flour that may have also contributed to a higher iron intake. Further studies evaluating the impacts of iron fortification are needed. Notwithstanding, the intake of fruits was low within the sample, as noticed by the low vitamin C intake level (44.7 ±38.5 mg/day, median=36.4 mg/day) according to the Dietary Reference intakes to the life cycles of the subjects [35]. In this situation, even an adequate intake of iron (13.8 ± 4.5 mg/day) would be of medium bioavailability, since vitamin C is amongst the main iron absorption enhancers in human diet [36].

Anemia of inflammation is marked by hypoferremia resulted from reticuloendotelial sequestration of iron and interruption of intestinal iron absorption mediated by an increase in the major regulatory hormone hepcidin as a response to the cytokine IL-6 [37], without evident alterations in hemoglobin content or red blood size [11]. The mild microcytosis (as compared to more stringent parameters than those of the WHO, see table 1) observed in the present study is more likely to be related to reduced bioavailability or metabolism of iron resulting in heme deficiency, anemia of inflammation, or both conditions combined. However, we can not make further judgments of its cause without further evaluating the body iron status of the studied individuals that we did not judge fundamental in designing the study, since hemoglobin is the worldwide standard measure for monitoring iron status and serum markers of iron status show low correlation with body iron or iron intake [38].

It was observed lower intake of magnesium among individuals with higher DNA damage. Moreover folate was negatively correlated to DNA damage and those with a higher intake of niacin, and intermediate intake of sodium had lower DNA damage. It is well known that genomic instability increase is often related to deficiency in the intake of other nutrients than iron, particularly of B vitamins. Indeed, a nutritionally balanced diet is needed for iron homeostasis. Several vitamins and minerals are involved in iron absorption, intracellular metabolism and erithropoiesis. In one hand, zinc [34,39] and copper [40] share much of their metabolism with iron and can compete in different levels. In the other hand, deficiencies of the following nutrients can induce defects in iron utilization and erytropoiesis: vitamin A [32], riboflavin-B2 [41,42], iodine [43], folic acid-B9 [44,45], cobalamin-B12 [46-48]. Specific enzymes of the heme biosynthetic pathway directly depend on adequate tissue levels of B6, zinc, and flavin. Additionally, heme biosynthesis depends on micronutrients important for producing succinyl-CoA from the Krebs (TCA) cycle, including: biotin, lipoic acid, and pantothenic acid [49]. Genomic stability also depends on adequate levels of other nutrients. B9 (folate), B2, B12 and pyridoxine (B6) are essential for the methylation of DNA, synthesis of dTMP from dUMP and efficient recycling of folate; and zinc is required as a cofactor for Cu/Zn superoxide dismutase, endonuclease IV, function of p53, Fapy glycosylase and in Zn finger proteins such as PARP. Niacin is required as substrate for poly(ADP-ribose) polymerase (PARP) which is involved in cleavage and rejoining of DNA and telomere length maintenance [50]. Magnesium is the second most abundant element in biological systems whose deficiency has been linked to increased oxidative stress and aging. It acts in more than 300 enzymes, and particularly in those related to chromatin assembly and DNA synthesis and repair [51].

It was observed a negative correlation between BMI and DNA damage, and inviduals consuming more carbohydrates showed lower DNA damage.

Undernourishment is likely to be linked to a general reduction in intake of energy as well as of many micronutrients and DNA damage from micronutrient deficiency is possibly a major cause of cancer [52]. Ortiz et al [53] observed a two-fold increase in micronuclei in the spleen lymphocytes of experimentally malnourished rats during lactation. In another study using the comet assay, Cortes et al [54] observed that malnourished rats at weaning (21 days of age) had higher DNA damage in spleen, peripheral blood, and bone marrow cells, with some cell types showing more susceptible to DNA damage. Lymphocytes of malnourished children also showed a decreased capacity to repair hydrogen peroxide-induced DNA damage compared to that of well-nourished controls[55]. Moreover, Neri et al [56] support in a recent review children may have higher primary DNA damages than adults after exposure to environmental pollutants. In agreement, there are evidences leanness may increase oxidative stress, particularly in smokers, and is associated to increased risk of some forms of cancers, such as lung cancer [57].

Inflammation is also a major inducer of DNA damage and cancer [58]. As seen in the present study, Betancourt et al [53] also using the comet assay observed a two-fold increase in DNA damage in blood cells of undernourished children with severe bacterial infection in relation to well-nourished and also severely infected children. Interestingly, infections are generally associated to hematological alterations, even as inflammation anemia. Mechanisms of iron subrogation during infection are conserved metabolic strategies through evolution to protect against proliferation of microbes, since iron is a limiting nutrient for microbes [59].

Infection and malnutrition are two factors that might interact synergistically in increasing DNA damage and cancer risk, possibly through immunity impairment. Undernourishment and ID/inefficient erythropoiesis are linked to impaired immunity [60]. The negative correlations observed in the present study between the number of leukocytes and the intake of energy and several nutrients confirms this concept. Although impaired immunity has been linked to cancer risk increase [61], further studies must be conducted. As observed in the present study, changes in the composition of the subpopulation of leukocytes are likely to be both associated to DNA damage and immune function. Other interesting results refer to the positive association between the DNA damage level and the percent of basophil granulocytes, even without basophilia (basophil granulocytes accounting for less than 2% of the total leukocytes); and the negative correlation between neutrophil granulocytes and either DNA damage or basophil granulocytes percentage (Fig. 2A,C,D). This aspect is fairly addressed in literature and needs further clarification. Given the relative change in the subpopulation of leukocytes, particularly of eosinophil granulocytes that contribute substantially to the oxidative burst [62], we expect differences in the comet assay response due to this fact. Lymphocytes have about 3-fold more DNA damage background and are much more susceptible to hydrogen peroxide and gamma-radiation [63,64]. Among lymphocytes´subpopulations, despite some controversies T cells seem to have higher DNA damage background due to longer lifespan and B cells tend to enter in apoptosis firsts due to reduced repair capacity [65,66]. Despite a general lack of studies in granulocytes, it has been recently shown that eosinophils have an efficient DNA repair capacity and could be multifunctional lymphocytes involved in inflammatory response [67]. Indeed, the results of our study must be considered as to realistically reflect the whole blood DNA damage level, without evaluating the impact in each leukocyte subpopulation. Further studies with cellular

subpopulations, specially granulocytes and lymphocytes, should be conducted to check the impact of ID and inflammation over genomic stability.

The present results suggest an association between erythrocytes volume (microcytosis), a common marker of ID due to insufficient heme synthesis, and changes in the leukocytes profiles and increased DNA damage in a sample of poor children and adolescent. Moreover, results also indicate a potential inverse association between lymphocytes number and the intake of energy and several nutrients and associations between a lower intake of niacin and folate and an increase in DNA damage. Given the potential high incidence of parasitosis, the possible adequate intake of iron as confirmed by the high hemoglobin values and the dietary data and the complex interplay of various nutrients in erythropoiesis (DNA and heme synthesis) further studies are needed to clarify the nature of the DNA damage increase observed.

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9.2 ANEXO B: “Review: A possible link between iron deficiency and gastrointestinal carcinogenesis”

Manuscrito no prelo no periódico *Nutrition and Cancer*.

Review

A possible link between iron deficiency and gastrointestinal carcinogenesis

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Abstract

There are definitive evidences that iron overload induces oxidative stress and DNA damage which enhance carcinogenic risk. However, other evidences suggest that iron deficiency and anemia also increase oxidative stress and DNA damage, which might increase carcinogenesis risk, especially in the gastrointestinal tract (GI). The aim of this review is to provide essential baseline information for the accurate interpretation of future research on iron deficiency and increased GI cancer risk. Based in clinical, epidemiological and experimental evidences, we discuss how iron deficiency could contribute to cancer risk increase through the impairment of several iron-dependent metabolic functions that are related directly or indirectly in genome protection and maintenance (e.g. impaired immune responses against cancer-initiated cells, metabolism of toxic compounds as well as redox regulation of DNA biosynthesis and repair). A few epidemiological studies indicate increased risk of GI tumors among individuals with low iron intakes or low body iron stores and in vivo data from rodent cancer models indicate early progression of GI tumors in iron deficiency. Given the preliminary but consistent evidence relating iron deficiency and cancer risk and the fact that iron deficiency affects about 1/3 of the world population, further studies are needed to define the extent of iron deficiency that might increase GI cancer risk.

Key-words: iron, cancer risk, colorectal cancer, DNA/mutagenesis/adducts /repair, ROS

Iron overload and carcinogenesis

The subcutaneous injection of Fe-nitriloacetic acid is a well-characterized renal murine cancer-induction system (Toyokuni 1996). Iron containing compounds, such as furnace fumes (Ahn, Park et al. 2006), asbestos (Valko, Morris et al. 2005), magnetite (Garry, Nesslany et al. 2004) and iron salts induce oxidative stress as their main carcinogenic activity (Franke, Prá et al. 2006; Prá, Franke et al. 2007).

A meta-analyses showed a significant weak association between heme iron, the main source of iron in meat, and the risk of GI cancer (Norat, Lukanova et al. 2002), depending of the population studied. For example, although several studies interpret an increase in intestinal cancer using heme as a marker of iron intake [e.g. (Lee, Anderson et al. 2004; Senesse, Meance et al. 2004; Lee, Anderson et al. 2005)], a recent cohort study of 49654

Canadian women did not find any association between the intake of iron, heme iron, or iron from meat with risk of cancer in GI tract (Kabat, Miller et al. 2007). Heme iron is more bioavailable than inorganic iron (IOM 2001) and is also genotoxic (Glei, Klenow et al. 2006). It must be noted, however that when eating meat, significant levels of saturated fat, preservatives and/or cooking by-products are being eaten together (Gray and Morton 1981) with heme iron, some of which have known carcinogenic potential (Hatch, Knize et al. 1988). Indeed, it is difficult to estimate what is the independent contribution of each genotoxin in cooked meat to cancer risk.

Iron overload, as measured by high serum ferritin (SF) and/or transferrin saturation (TS), is strongly associated to a general increase in mortality, and in a weaker and controversial extent to cancer risk increase (Mainous, Gill et al. 2005). Some reviews showed TS > 60% substantially increased the risk of GI cancer (Knekt, Reunanen et al. 1994; Herrinton, Friedman et al. 1995; Nelson 2001) in a synergistic way with alcohol (Kabat, Miller et al. 2007) and fat consumption (Deneo-Pellegrini, De Stefani et al. 1999; Kuvibidila, Gauthier et al. 2004). Conversely, one study in 35-60 years subjects involved in a double-blind, placebo-controlled, primary prevention trial evaluating the effect of antioxidant supplementation on chronic diseases in France., country with low intakes of iron supplements, showed no association between iron intake, body iron status and GI cancer (Hercberg, Estaquio et al. 2005). Another case-control study with Californian old subjects involved in a volunteer endoscopy screening study looking for intestinal polyps incidence according to iron consumption and boy iron status, showed that when those with iron supplementation were excluded from analysis higher iron intakes were not linked to increased odds ratio for polyps (Bird, Witte et al. 1996).

Iron deficiency and body iron status

Iron deficiency (ID) is the most prevalent global micronutrient deficiency, particularly among poor children and women (Umbreit 2005). Anemia, a hallmark of high iron deficiency affects about 50 % of the 5- to 14-year-old children and of pregnant women (Beard 2001). It is well known for a long time that ID can irreversibly affect brain development (Ortiz, Pasquini et al. 2004), impair immunity (Arredondo and Nunez 2005) as well as to increase oxidative stress and to decrease antioxidant defenses (Macdougall 1972; Acharya, Punchard et al. 1991; Knutson, Walter et al. 2000).

As reviewed by Trost et al (2006), ID can be viewed as a continuum: iron depletion, iron-deficient erythropoiesis (IDE), and iron deficiency anemia (IDA). In iron depletion, body iron stores are reduced, but functional and transport iron remain normal, leaving little or no reserves if the body requires more iron. In IDE, both storage and transport iron are decreased. Red blood cell production is diminished, resulting in insufficient iron for growth and function. Finally, in IDA, storage, transport, and functional iron are severely decreased and can lead to impaired function of multiple organ systems (Trost, Bergfeld et al. 2006). The adult current Dietary Reference Intake (DRI) of iron ranges from 8 mg/day (males and post-menopausal woman), 18 mg/day (fertile woman) to 27 mg/day iron (pregnancy), as the adequate level in order to prevent IDA (IOM 2001).

Transferrin (Tf) is the principal iron transporter in serum. Tf molecules can bind two molecules of iron each and constitute together with serum ferritin (SF) – which can store up to 4,500 molecules of iron – the vast majority of serum iron (SI)(Umbreit 2005). The serum of a median healthy male has typically around 30 μM of Tf, which about 28%(Koziol, Ho et al. 2001) is saturated by iron (SI≈18 μM(Wu, Sempos et al. 2004)), 5.5 nM Ft (McLaren, Gordeuk et al. 2008) and virtually zero (far less than 1μM) non-transferrin bound iron (NTBI) (van Tits, Jacobs et al. 2007). However, the best marker of body iron status is still

controversial, particularly because it has been very difficult to unravel how iron metabolism works (Beutler 2007). The gold standard would be bone marrow sample staining, but this is impractical for population studies. SF shows the highest correlation with bone marrow staining, however it can suffer large fluctuations during infections. SI and total iron binding capacity (TIBC), measured indirectly by the unsaturated binding capacity of serum, are used to calculate transferring saturation - TS (TS= SI/TIBC) (WHO 1996). ID is generally associated to a reduction in SI, and an increase in transferrin levels, as seen by higher TIBC and lower TS levels among individuals with marked anemia (Herrinton, Friedman et al. 1995; WHO 1996). TIBC correlates inversely with total iron status specifically in people with normal or depleted iron status (Sempos 2001). Indeed, although these parameters are not as reliable as the gold standard bone marrow staining, they are routinely used in evaluating iron status (Nelson 2001).

Aims, scope, methods, and structure of the review

In this review we will not discuss further the relation between iron overload and carcinogenesis. Conversely, our aim is to discuss the suggestive evidences linking ID and GI cancer, which exist in scientific literature and are reported many times as inconsistencies between iron overload and cancer risk.

Primary studies were identified in Pubmed and Web of Sciences using the combination of the following terms: cancer, tumor, DNA damage, oxidative stress, iron, iron deficiency, anemia, transferrin, ferritin, gastrointestinal tract, mouth, esophagus, stomach, colon, rectum, colorectum. Additional studies were identified by cross-referencing primary studies.

We will discuss initially the clinical evidences linking upper GI tract carcinogenesis and ID. In the next section we will discuss epidemiological studies linking stomach and colorectal cancer and ID. In the following section, we will present evidences from rat models of carcinogenesis with ID. Later, we will discuss how the impairment of several iron-dependent metabolic functions might be related mechanistically to genomic instability, a fundamental cause of cancer. Finally, we will weight the evidences linking ID and increased GI cancer risk.

Clinical and epidemiological evidences linking ID and upper GI carcinogenesis: the Paterson-Kelly Syndrome

The role of ID as a carcinogenic condition was first suggested at the beginning of 20th century associated to sideropenic dysphagia by Paterson (1919) and Kelly (1919). Sideropenic dysphagia was first described in the literature as early as 1893 and was later named Paterson-Kelly and Plummer-Vinson syndrome in honor of the physicians who further described it clinically (Kim, Kim et al. 2005). Paterson-Kelly syndrome (PKS) is generally associated with nail defects, lips and tongue abnormalities, stomatitis, chronic gastritis, malabsorption and esophageal webs (Jacobs 1971; Jacobs 1982). PKS was very common in the northern parts of the world and endemic in some parts of rural Sweden (Larsson, Sandstron et al. 1975), but reduced its incidence as a consequence of improved nutrition; being restricted today to middle aged and elderly women, and also children (Benjelloun, Essakhraoui et al. 1999; Mansell, Jani et al. 1999). Although, the link between ID, PKS and cancer risk might be regarded as controversial, the effective amelioration of PKS with iron treatment (Jessner, Vogelsang et al. 2003) and the higher incidence of cancer in the condition demand further attention towards this issue. Notwithstanding, given the rarity of the syndrome in endemic regions of iron deficiency, it is likely that other dietary or environmental factors are involved (Hoffman and Jaffe 1995).

Anatomic studies in ID individuals and lab animals have shown fibrosis, epithelial atrophy, and even epithelial hyperplasia in association to chronic inflammation; which are typical feature of PKS (Larsson, Sandstron et al. 1975; Vitale, Broitman et al. 1977) and of submucous fibrosis - an oral precancerous condition common in iron deficient individuals (Pillai and Burde 2004). Chronic inflammation is strongly associated with increased cancers risk in several organs, such as the GI tract, liver, and lungs (Dedon and Tannenbaum 2004). In parallel to inflammation, a decrease in esophageal motility has been shown in anemic individuals (Miranda and Dantas 2003), possibly due to mitochondrial function impairment and/or a decrease of neuronal nitric oxide synthetase activity (Miranda and Dantas 2003). Accordingly, ID and heme biosynthesis impairment increase mtDNA damage and functional deterioration of mitochondria, through a not yet fully elucidated mechanism (Atamna, Liu et al. 2001; Walter, Knutson et al. 2002). Indeed, mitochondrial impairment has been linked to cancer, aging and most neurodegenerative diseases (Atamna 2004).

Regarding PKS carcinogenicity, while some authors detect no carcinogenicity (Mohandas, Swaroop et al. 1991), other associate it to a cancer increase in GI tract (Gore 1997; Kim, Kim et al. 2005). From the epidemiological point of view, it is estimated 4 - 15% of individuals with PKS develop carcinomas of the upper alimentary system, according to the analyses of 4 follow up studies regarding the association between PKS syndrome and carcinoma of the upper alimentary tract (Chisholm 1974). Moreover, the analyses of Swedish Cancer Registry and Hospital statistical data (Larsson, Sandstron et al. 1975) showed nutritional improvement along the 20th century reduced the incidence of PKS, which influenced the pattern of hypopharyngeal cancer particularly among women in northern Sweden.

According to the case-control studies summarized in Table 1, there are also evidences that individuals with higher intakes of iron have lower risk for developing upper GI carcinomas (Zhang, Kurtz et al. 1997), as well as of oral precancerous lesions associated to smoking (Gupta, Hebert et al. 1999; Hebert, Gupta et al. 2002).

Epidemiological studies relating GI cancer risk in stomach and colorectum

According to two cohort studies, low body stores of iron, as measured either by SF (Akiba, Neriishi et al. 1991) or transferrin and ferritin combined (Nomura, Chyou et al. 1992), have been associated to an increase in the incidence of stomach cancer in Japanese and Japanese-descendant Hawaiians (Table 1). The same negative association was observed between colorectal cancer and TS (Herrinton, Friedman et al. 1995) in Californian males of a cohort study or SF levels (Kato, Dnistrian et al. 1999) in New Yorker woman of case-control nested study.. In another cohort study with Finish, it was shown that individuals with higher TIBC, i.e. reduced body iron stores, were more likely to develop rectal cancers (Knekt, Reunanen et al. 1994).Moreover, other cohort study with American detected men and post-menopausal woman (Ioannou, Rockey et al. 2002) had 5 and 31 times more risk of developing gastrointestinal cancer if they were, respectively, ID or anemic 2 years before cancer diagnostics. In agreement, other case-control studies of Harrison et al (1997) showed higher intakes of iron can be associated with lowered incidence of intestinal adenocarcinomas (Table 1). In the same direction, Broitman et al (1981) showed ID individuals had extremely high incidence (90%) of preneoplasic stomach lesions not associated to bleeding (about 25, 50 and 20% of superficial gastritis, chronic atrophic gastritis and gastric athrophy with intestinal metaplasia, respectively) after histological analyses of 104 hypochromic anemic Colombian patients attending to a clinic due to abdominal symptoms without cancer or peptic ulcer (as evaluated by gastroscopy).

One very interesting case-control study with elective endoscopy in 50-75 years old Californians by Bird et al (1996) showed that either low or high intakes of iron can increase the odds ratio of colorectal polyps, in a typical "U" shaped curve, although reducing the risk of higher intakes when individuals with supplementation were excluded from analyses. In another case-control study aiming to evaluated the anticarcinogenic potential of antioxidants in 50-69 years old male Finish, Cross et al (2006) showed the risk of colorectal carcinoma decreased in dose dependent manner with increasing dietary iron intake. Figure 1 summarizes data of odds ratio of both studies according to the iron intake and indicates an interesting "U" shaped curve for odds ratio. As a generalization, one would expect a daily intake of about 20 mg/day iron would reduce the risk to polyps and colorectal cancer to its minimum. This value is a higher than the current DRI for older adults with is of 8 mg/day iron and lower than the upper level (UL) of 45 mg/day iron (IOM 2001). Moreover, it is closer to the 87 mg/day iron found in Paleolithic diet, which also had much a much higher antioxidants` concentration (Benzie 2003). Low body iron stores seem to be also related to the increased risk of cancer in other tissues then the GI tract. For example, Ali et al (2003) showed that high serum levels of ferritin and nitrite but not total SI could be associated with a decreased risk of renal cancer, although not statistically significant in the study; and Kuvibidila et al (2004) measured serum ferritin, SI, TIBC, and TS in serum samples of prostate cancer patients and controls and observed that prostate cancer patients had significantly lower mean concentrations of serum ferritin and TS than the controls, suggesting that elevated body iron stores are less common in men with prostate cancer compared to those without this malignancy.

Table 1. Epidemiological evidences linking iron deficiency and increased gastrointestinal (GI) cancer risk.

GI region / evidence	Study characteristics and population	Author
Upper dietary tract and esophagus		
Strong dose-response relationship between increased intake of iron and decreased risk of adenocarcinomas of the esophagus and gastric cardia (ACEGC) and individuals with high dietary intakes of iron were less likely to develop cancer of the larynx and esophagus (OR= 0.5; CI 95% 0.3-0.9)	Case-control study based in endoscopy diagnostic and data of a self-administered Health Habits and History Questionnaire responded 1-2 years preceding the exam in New York, conducted in 95 incident cases with the pathological diagnosis of ACEGC, 67 patients with adenocarcinomas of the distal stomach, and 132 cancer free controls.	(Zhang, Kurtz et al. 1996; Zhang, Kurtz et al. 1997)
2.5 times increased risk of precancerous oral lesions among women in the lower quartile of iron intake in relation to the 2nd and 3rd quartiles among smokers	Case control study in India with 226 cases of precancerous oral lesions and equal number of controls (all smokers)	(Gupta, Hebert et al. 1999)
Protective linear effect of iron intake against precancerous oral lesions among smokers	Case control study in India with 485 cases of precancerous oral lesions and 487 controls of 19 rural cities of India (all smokers)	(Hebert, Gupta et al. 2002)
Stomach		
Individuals with lower serum ferritin, but not transferrin, had three fold more stomach cancers than those in the higher quartile of ferritin level	Case control study in a cohort of 20,000 atomic bomb survivals from Hiroshima and Nagasaki, including 233 stomach cancer cases and matched controls whose serum was collected and frozen at least 5 years before cancer diagnostics	(Akiba, Neriishi et al. 1991)
Inverse association between stomach cancer incidence and serum ferritin or transferrin	Case-control study in Hawaiian males of Japanese origin, including 121 cases and 121 controls whose sera was collected about 20 years and thawed after diagnosis	(Nomura, Chyou et al. 1992)
Higher dietary intake of iron and other nutrients was associated with a lower incidence of gastric adenocarcinoma	Case-control study based in endoscopy diagnostic and data of a self-administered Health Habits and History Questionnaire responded 1-2 years preceding the exam in New York, conducted in 134 cases and 131 controls	(Harrison, Zhang et al. 1997)
Colorectum		
Higher risk of rectal cancer among women with high TIBC (reduced iron stores)	Cohort study with14 years follow up in 41,276 Finish	(Knekt, Reunanen et al. 1994)
Negative association between TS and the risk of colon and rectal carcinoma in men	Cohort study with 20 years follow-up in 2,150 Californians aged 20-84 years.	(Herrinton, Friedman et al. 1995)

Either individuals consuming low (<11.6 mg/day) or high (>27.3/mg day) iron diets had higher risk of colorectal polyps in comparison to those consuming adequate amounts of iron, in a "U"-shaped curve trend	Case-control study with 965 Californian men and women aged 50-75 years voluntarily subjected to sigmoidoscopy evaluation	(Bird, Witte et al. 1996)
Significant inverse association between serum ferritin and the risk of colorectal cancer	Nested case-control study with average follow-up of 4.7 year in 105 incident cases of colorectal cancer and 523 individually matched control woman of New York City	(Kato, Dnistrian et al. 1999)
The proportion of individuals diagnosed with GI malignancy was 31 and 5 times higher among men and post-menopausal women anemic and with iron deficiency, respectively	Cohort with 2 years follow up in 9024 Americans	(Ioannou, Rockey et al. 2002)
Increasing iron intake, serum ferritin, serum iron and TS reduced the odds ratio for colon but not for rectal cancer in a trend like manner	Nested case-control study within the α-tocopherol, β-carotene cancer prevention (ATBC) study in male Finish smokers aging 50-69 years, including 130 colorectal cancer cases and 260 matched controls whose serum was collected and frozen at least 5 years before diagnostics	(Cross, Gunter et al. 2006)

PKS: Paterson-Kelly syndrome; TIBC: total iron binding capacity; TS: transferrin saturation.

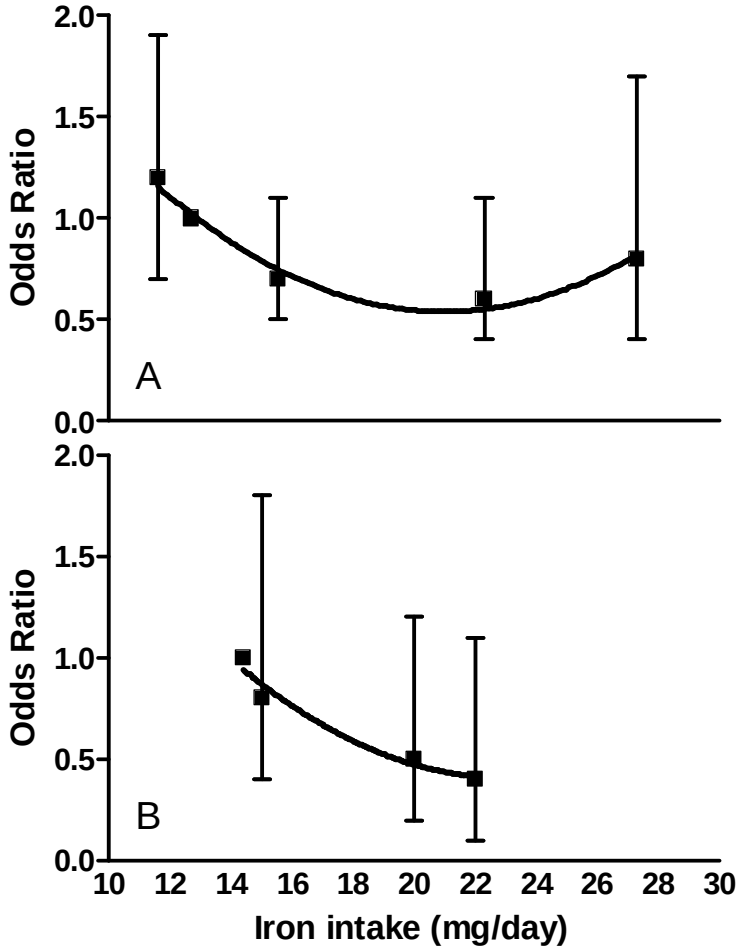

Figure 1. Odds ratio for colorectal polips (A) and colorectal cancer (B) according to iron intake. A) based in data of a case-control study of elderly healthy Californian voluntarily subjects screened by flexible sigmoidoscopy (Bird, Witte et al. 1996). B) based in data of a nested case-control study with Finish male smokers (Cross et al, 2006). The lower intake was set as the upper cut point for the first quartile/quintile, the higher intake was set as the lower cut point for the last quartile/quintile and the intermediate intake were ser as the average of the lower and upper cut points for each quartile/quintile if intake.

Abnormal iron levels also seem to affect the prognosis of GI cancers patients. Lorenzi et al (2006) evaluated the survival of 97 colorectal patients who underwent curative or palliatives surgeries in a 5 year follow up study. They observed that those with lower or higher serum ferritin levels had a shorter survival than patients with normal levels. Toward the same direction, Tseng et al (1997) follow up for 4 years the relation between dietary iron and the recurrence of colorectal adenomas in 419 controls and 247 cases (at least one adenoma removed within 3 months between the enrollment) and found dietary iron was inversely related with adenoma risk, however being significant only for the higher intake. The odds ratio was of 0.32 for the higher intake (media intake of 20.9 mg/day) in relation to the lower intake (11 mg/day). Anemia as evaluated by hemoglobin level is common in about 25-45% of colorectal cancer cases, which could be due to colorectal bleeding, a common manifestation in colorectal cancer. Anemia can impair colon cancer survival due to nutritional or immune deficiency (Lorenzi, Lorenzi et al. 2006) and the evaluation of body iron has been suggested as a screening method for GI cancer (Rai and Hemingway 2005). Increased serum ferritin

levels can also affect about 30% of colon cancer patients, being related to ferritin overexpresion by tumors. This condition can possibly impair colon cancer survival prognosis due to the noxious effects associated to iron accumulation (Lorenzi, Lorenzi et al. 2006).

Two major confounding factors may interfere in defining the carcinogenic potential of iron deficiency in GI. First of al, iron intake insufficiency is likely to be associated to the lack of several other nutrient, or to a general undernourishment pattern; which might act synergistically in promoting cancer. Secondly, GI bleeding is linked to GI cancer and sometimes a major cause of anemia. Notwithstanding, most studies report that GI bleeding is detected only in a small percentage of elder anemic subjects (Joosten, Ghesquiere et al. 1999).

Experimental evidences using rodent cancer models relating ID and increased GI cancer risk

Experimental evidences also support that iron deficiency mediated either by blood loss (venisection), iron depleted diet or both combined accelerates the emergence of oral/liver tumors (Vitale, Broitman et al. 1977; Prime, Macdonald et al. 1983) and increase the incidence of colonic/duodenal tumors (Jagadeesan, Rao et al. 1994) in rats exposed to model carcinogens in relation to iron sufficient counterparts (Table 2). In spite of the fact that blood loss could be related to other physiological effects (such as lower oxygen tension) than only iron deficiency, such data supports a potential role for iron deficiency in enhancing the susceptibility to carcinogens. Notwithstanding, further animal studies might be developed to evaluate if the background incidence of tumors would increase as a consequence of iron deficiency. Given the physiological differences between humans and murine animals and the higher level of evidence of epidemiological studies, population studies should be also planned to evaluate the effect of iron deficiency in carcinogenesis.

General physiological effects of ID related to increased carcinogenesis risk

Table 3 summarizes molecules and metabolic function impaired by ID and evidences linking such impairment to genomic instability and cancer.

Atamna et al (2001; 2002) have shown that heme deficiency and the consequent imbalance in iron-containing mitochondrial enzymes lead to DNA damage, oxidative stress and mitochondrial decay an iron accumulation which are known hallmarks of ageing. As an example, iron is a key component in cytochrome C oxidase. This enzyme, despite playing a key role in oxidative metabolism, is an important intermediate in apoptosis cascade (Riedl and Salvesen 2007). Indeed, it has been shown ID impairs apoptosis in blood cells (Kempe, Lang et al. 2005; Berrak, Angaji et al. 2007).

Table 2. Experimental evidences linking iron deficiency and increase in gastrointestinal (GI) cancer risk.

Evidence	Study characteristics and model	Author
Iron deficient rats showed a change in the site of tumors from colon to liver and much earlier emergence of liver tumors (126 versus 245 days) than iron replete rats	Assessment of tumors in liver, GI, kidney, lungs and ear channels in Lewis male rats treated subcutaneously with dimehylhydrazine fed normal or low iron diets	(Vitale, Broitman et al. 1977)
Similar incidence but earlier development (183 versus 229 days) of oral squamous cell carcinomas in anemic rats in comparison to non anemic ones. Variation in tumor distribution, with iron deficiency being associate to lingual abnormalities	Assessment of oral tumors in Charles River rats fed normal diet or fed a low iron diet and repeated venisectioned whose plate was painted 4-Nitroquinilone-N-oxide	(Prime, Macdonald et al. 1983)
Significant higher overall incidence of proximal GI tumors (colonic and duodenal) in the iron-deficient (66%) than in the control group (46%) as well of evidences of altered hepatic cells	Assessment of the incidence of GI tumors in Fischer 344 rats fed diets with different iron levels treated with dimethylhydrazine by gastric intubation	(Jagadeesan, Rao et al. 1994)

Table 3. Summary of the molecules and metabolic function impaired by iron deficiency, and evidences linking such impairment to genomic instability and cancer.

Molecule impaired	Metabolic function impaired	Enzyme impaired	Evidence linking enzymatic impairment and cancer	Evidence linking iron deficiency and enzymatic impairment
DNA	DNA synthesis	Ribonucleotide reductase	Ribonucleotide reductase inhibitors are leukemogenic (Lofvenberg, Nordenson et al. 1990)	Anemic mothers have increase risk of leukemia in their offspring (Thompson, Gerald et al. 2001; Wen, Shu et al. 2002; Schuz, Weihkopf et al. 2007).
	DNA repair	DNA glicosilase (BER system)	Defects in BER are associated to cancer (Fortini, Pascucci et al. 2003)	? BER repair in iron deficient cells?
		MMR system	MMR defects are associated to cancer (Slupphaug, Kavli et al. 2003)	? MMR repair in iron deficient cells?
Cythochromes	Metabolization of xenobiotics	P450 family	Polymorphisms in P450 interfere in cancer risk (Perentesis 2001)	Anemic individuals have lower P450 (Dhur, Galan et al. 1989)
	Oxidative metabolism	Mitochondrial complex IV	Complex IV deficiency leads to genomic instability and aging (Atamna, Liu et al. 2001)	Iron deficiency inhibits complex IV assembly (Atamna, Liu et al. 2001)
		Cytochrome C oxidase	Cytochrome C decrease leads to apoptosis inhibition and is associated to GI carcinogenesis (Payne, Holubec et al. 2005)	Iron deficiency inhibits cytochrome C assembly (Atamna, Liu et al. 2001)
	Trigger apoptosis			
Peroxidases	Kill pathogens	MPO	MPO deficiency increases the susceptibility of infection and malignant tumors.(Lanza 1998)	Anemic individuals have impaired immunity (Oppenheimer 2001)
	Neutralize ROI	CAT	Polymorphisms in CAT gene are associated to increase in oxidative stress and cancer risk (Ahn, Nowell et al. 2006)	Anemic individuals has lower CAT levels (Macdougall 1972; Kurtoglu, Ugur et al. 2003)
	Trigger apoptosis		CAT overexpresion triggers apoptosis (Bai and Cederbaum 2000)	Iron deficient children have decreased apoptosis rate (Berrak, Angaji et al. 2007)
Oxygen sensing molecules	Oxygen sensing and iron regulation	NOS	Association of polymorphism of NOS and gastric adenocarcinoma risk (Tatemichi, Sawa et al. 2005)	Iron deficiency lead to NOS inhibition (Goldblatt, Choi et al. 2001)

BER: base excision repair; MMR: mismatch repair; MPO: mieloperoxidase; ROI: reactive oxygen species; CAT: catalase; NOS: nitric oxide synthase.

Several authors have also shown that iron deficiency causes oxidative damage, due to abnormal assembly of mitochondrial electron transport chain (Atamna, Walter et al. 2002). In the same direction, Walter et al (2002) showed that either iron deficiency or excess impaired liver mitochondrial metabolism and increased mtDNA damage and iron deficiency increased levels of oxidants in polymorphonuclear-leukocytes. As another in vivo evidence that iron impairs genomic stability, Aslan et al (2006) have shown increased DNA damage and lowered antioxidant defenses in leukocytes of anemic women. These evidences confirm the role of iron in DNA synthesis and repair, as well as in other cellular functions related to genomic stability.

Iron is a functional part of ribonucleotide reductase (RR) (Strand, Karlsen et al. 2004), the fundamental enzyme that converts ribonucleotides to deoxynucleotides. It is well known that inhibitors of RR, such as hydroxyurea, have leukemogenic potential (Al-Jam'a, Al-Dabbous et al. 2002; Le Fevre, Boitier et al. 2007). Interestingly, there are evidences of a higher leukemia risk in children whose mothers were anemic during pregnancy and iron supplementation in association with folic acid during pregnancy substantially reduces the leukemogenic risk (Thompson, Gerald et al. 2001; Wen, Shu et al. 2002; Schuz, Weihkopf et al. 2007). More recently, the role of iron as a not fully understood functional part of DNA repair glycosylases has also been described (Brzoska, Meczynska et al. 2006). DNA glycosylases are responsible for the excision of DNA lesions in the Base Excision Repair system (BER), which repairs a wide range of base lesions, including 8-oxoguanine. Defects in BER are described in several pro-carcinogenic conditions (Fortini, Pascucci et al. 2003). Iron is also present in alkyltransferases (Mishina and He 2006) and seems to play a fundamental role in XPD family DNA helicases (Pugh, Honda et al. 2008). Moreover, defects in the iron-containing DNA glycosidase MUTYH are directly associated with colorectal cancer (David, O'Shea et al. 2007). Additionally, it has been observed that iron chelators and hypoxia have been also linked to a down regulation of some genes of mismatch repair system (MMR), enzymes whose defects have been linked to hereditary nonpolyposis colon carcinoma (Mihaylova, Bindra et al. 2003) as well having been associated to sporadic colorectal cancer risk in some populations (Schafmayer, Buch et al. 2007). Nevertheless, there are no studies to date evaluating the effect of iron deficiency in DNA repair enzymes.

Regarding oxidative stress, the impairment of antioxidant defenses and the increase of oxidized lipids or proteins or susceptibility to oxidative damage is fully reported in literature for a long time (Macdougall 1972; Jansson, Perkkio et al. 1985; Kurtoglu, Ugur et al. 2003). Moreover, the harm of antioxidant depression can be extremely noxious in GI, particularly because GI is exposed to pro-oxidant to a grater degree then other body tissues (Halliwell, Zhao et al. 2000). Myeloperoxidases (MPO) are cytotoxic heme-containing enzymes secreted by phagocytes (Thukkani, Albert et al. 2003) that have multiple targets, such as multicellular worms or parasites, bacteria, viruses, and host cells (Wu, Samoszuk et al. 2000; Dedon and Tannenbaum 2004). There are increasing evidences that MPO deficiency increases the susceptibility of infection and malignant tumors. Since MPO deficiency can occur as a consequence of iron deficiency (Lanza 1998), one can establish the link between iron deficiency induced MPO impairment and genomic instability. Similarly, each catalase (CAT) molecule, an enzyme required for the neutralization of hydrogen peroxide which is a noxious by-product of oxygen metabolism, contains four porphyrin heme groups. CAT activity seems to be dose-related to body iron status, as measured by hemoglobin content (Goth and Vitai 1996). Interestingly, CAT overexpression might be associated with blockage of cell division progression, or even the induction of

apoptosis of DNA damaged cells (Chen, Liang et al. 2004). Recent studies have linked iron containing enzymes in oxygen sensing, among other reason by its presence in nitric oxide synthase (Brzoska, Meczynska et al. 2006). Indeed, iron is also a co-factor of nitric oxide synthethase (NOS). It has been shown that iron deficiency can suppresses ileal NOS activity (Goldblatt, Choi et al. 2001). The blockage of nitric oxide production, which also occurs in iron overload, reduces the tumorocidal activity of macrophages which secrete nitric oxide to inhibit iron containing enzymes, DNA synthesis, mitochondrial respiration and citric acid cycle enzymes of microbes and tumor cells (Harhaji, Vuckovic et al. 2004).

Finally, the enzymes of the cytochrome P450 superfamily are also heme containing involved in the metabolism of the majority of xenobiotic compounds. In rats the concentration of cytochrome P450 is higher in duodenal area and in the most mature villous cells (Jacobs 1982). In humans, intestinal P450 expression seems to be more prominent than in the others parts of GI tract, being maximal in the upper part and reducing slightly in proceeding from the duodenum to the jejunum and then acutely towards the ileum. This intestinal region has a significant xenobiotic absorptive function. CYP metabolism might serve as a barrier to the systemic uptake of xenobiotics, including drugs, by facilitating excretion to the lumen of the intestine or by bioactivation of the xenobiotics, with consequent binding to enterocyte macromolecules and are removed fast with the sloughed-off enterocytes (Ding and Kaminsky 2003). Iron deprivation substantially reduce P450 activity in the intestine of the iron-deficient subjects (Dhur, Galan et al. 1989). Rao and Jagandeesan (1995) have also studied the effect of iron deprivation over xenobiotic metabolism in rat and observed a decrease of several phase I (activating) and phase II (conjugating) enzymes, but not of P450 in a differential pattern, depending if in the liver or in extra-hepatic tissues (kidney, lung and GI).

Weight of evidence of the relation between ID and GI cancer risk increase

The summarized studies suggest a potential link between ID and increased GI cancer risk (Tables 1 and 2). Many of these studies have shown that iron has a dual effect, being carcinogenic either in overload or deficiency situation. In fact, a "U" shaped dose-response pattern named Bertrand's rule is typical for most macro and micronutrients (Bertrand 1912; Raubenheimer, Lee et al. 2005), Data of Walter et al (2002), Bird et al (1996) and Cross et al (2006)(Fig. 1) support the potential "U" shaped curve for, respectively, DNA damage, preneoplasic lesions and colorectal cancer according to iron intake. This trend agrees with the concept that only a definite physiological range of most nutrients will favor health and genomic stability (Fenech 2001; Ames 2003; Fenech 2005; Fenech, Baghurst et al. 2005). According to data of Fig 1, the daily consumption of around 20 mg/day of iron would be beneficial to reduce colorectal cancer. This amount is above the current DRI (8-24 mg for adults), below the UL (45 mg/day) (IOM 2001) and much lower to the level of iron ingested in Paleolithic diet (85 mg/day) (Benzie 2003). Therefore, further studies focusing in such levels of intake are suggested.

In spite of the clinical evidences and the animal studies addressing the relation between ID and GI cancer risk, even though ID is the far most micronutrient deficiency, there is a lack of well controlled studies evaluating if there is a adequate amount of iron that can minimize cancer risk. For this reason, in this review we intend to provide essential baseline information for the accurate interpretation of future research on iron deficiency and cancer risk increase. The evidence posed by the epidemiological studies herein summarized (Table 1) can be classified as **II-2-good** according to the "Current methods of the U.S. Preventive Services Task Force" (Harris, Helfand et al. 2001). Regarding "hierarchy of research design", class II-2 is attributed since it is defined as "well-designed cohort or case-control analytic studies, preferably from more than one center or research group". Regarding internal validity, evidence can be classified as **good,** since they of the studies show consistent results are well designed and conducted in representative populations and evaluate the effect directly into health outcomes.

The summarized evidence can be also classified as **Class 1** according to the "hierarchy of robustness", as defined by the "Joint Panel of the World Research Fund and the American Institute for Cancer Research (2007)"; that rank human and animal experimental studies in evaluating the role of dietary and physical activity in the risk/prevention of human cancer. Class 1 refers to: a) in vivo data from controlled human feeding studies, b) data from genetically modified models of human diseases, and c) in vivo studies using rodent cancer models designed to investigate modifiers of the cancer process (World Cancer Research / American Institute for Cancer Research 2007).

There are not strong evidences of an increase in GI cancer incidence in iron deficient populations, possibly because no systematic effort was performed, in spite of some research in India (Gupta, Hebert et al. 1999; Hebert, Gupta et al. 2002) and Colombia (Broitman, Velez et al. 1981). Moreover, the potential reverse causality is a critical issue in evaluating the relation between ID and GI cancer risk. For example, it is not known to what extent cancer derived GI bleeding could arise from reduced body iron stores as a result of bleeding.

ID surely correlate to the deficiency of other nutrients, diet and lifestyle have complex interaction in cancer risk and there are evidences that malnourishment impairs immunity and DNA repair capacity (Ortiz, Cortes et al. 1995; González, Nájera et al. 2002). Vegetarians, that tend to show nutrients unbalance, do not have higher GI cancer incidence than omnivorous (Key, Appleby et al. 2006). While in one hand vegetarians and vegans tend to have lower body stores of iron, zinc and vitamin B12 due to lower intake of animal products, on the other hand they have a generally healthier diet and lifestyle than non-vegetarians and a higher intake of cancer-protecting phytonutrients (Hunt 2003; Key, Appleby et al. 2006). Indeed, evidencing the complex interplay between diet components and lifestyle in cancer risk/prevention; involving mechanisms still far away from being elucidated. For iron one additional problem is the fact of defining the best body stores marker, and considering the interplay of the nutrient with other micronutrients and genetic polymorphisms and nutritional peculiarities of populations under evaluation (e.g. the extent of iron supplements consumption). Moreover, given the fact of different nutritional demand from various organs must also complicate the evaluation.

The also summarized growing evidences that ID may hamper key physiological roles related to genomic maintenance, apoptosis and immune response against malignant cells advocate in the opposite direction; leading to the concept that ID cells might not be able to synthesize and repair DNA properly. Moreover, cells can be impaired in P450 for metabolizing toxic compounds, in phagocyting malignant cells, in detoxifying reactive oxygen intermediates, in triggering apoptosis, as well as not responding properly to oxygen tension fluctuations (Table 3). Other mechanisms regarding cancer risk and nutrient deficiency might be investigated. One suggestion towards this end would be to evaluate the DNA repair capacity of iron deficient cells/individuals.

Given the preliminary but consistent evidence relating iron deficiency and GI cancer risk and the fact that iron deficiency affects about 1/3 of the world population, we suggest that the carcinogenic risk of iron deficiency and anemia should be further monitored in epidemiological studies designated to specifically evaluate this condition.

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9.3 ANEXO C: “DNA damage in blood leukocytes of individuals with sickle cell disease treated with hydroxyurea”

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DNA damage in blood leukocytes of individuals with sickle cell disease treated with hydroxyurea

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Abstract

Hydroxyurea (HU) plays an important role in the treatment of patients with sickle cell disease (SCD). Although HU has been associated with an increased risk of leukemia in some patients with myeloproliferative disorders, the mutagenic and carcinogenic potential of HU has not been established. This study investigated levels of DNA damage using the alkaline (pH > 13) comet assay to analyze peripheral blood leukocytes sampled from 28 patients with SCD treated with HU (SCHU) and from 28 normal individuals. The damage index (DI) in the SCHU group was significantly higher than in controls ($p < 0.05$). Gender, smoking or age were not associated with DNA damage in controls or SCHU individuals. In the group of SCHU individuals, mean HU dose and DI were positively correlated, and individuals who received a mean dose of >20 mg/kg HU (DI = 24.9 ± 5.5) showed significantly more DNA damage than those who received ≤20 mg/kg HU (DI = 14.6 ± 1.8) ($p < 0.05$). Individuals treated for ≥42 months (DI = 23.1 ± 4.2) showed significantly greater DNA damage than those treated for <42 months (13.6 ± 1.9) ($p < 0.05$). DI was inversely correlated with body mass index in the SCHU group.

Keywords: Genotoxicity; Comet assay; Hydroxyurea; Sickle cell disease

1. Introduction

Sickle cell disease (SCD) is an inherited blood disorder caused by a single gene mutation with clinical manifestations that vary in severity and may range from rare painful vaso-occlusion crises to life-threatening acute chest syndrome [1].

Hydroxyurea (HU) inhibits ribonucleotide diphosphate reductase, the enzyme that converts ribonucleotides into deoxyribonucleotides, which are essential for DNA synthesis and repair. HU is a cytotoxic and antineoplastic agent that specifically affects the S phase and interrupts the cell cycle in the G2 and S phases [2].

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This mechanism has led to clinical applications in a variety of contagious diseases, neoplasias and hematological disorders [3–6]. Although HU has been experimentally shown to have clastogenic [7,8], teratogenic [9,10], and mutagenic effects [11], its most common short-term toxicity manifestation is myelosuppression, a transient and reversible side effect affecting granulocyte progenitors [12]. Several studies have reported that patients with myeloproliferative disorders, which are known preleukemic conditions, underwent leukemic transformation when on HU therapy [13–15]. Larger studies have documented an increased risk of leukemia in patients with myeloproliferative disorders receiving HU therapy [16,17].

A landmark clinical trial showed that HU reduced the severity of SCD, with a 50% reduction in the frequency of hospitalizations for painful crises [18]. HU is approved by the U.S. Food and Drug Adminstration (FDA) for adults with SCD, and clinical trials with children support its clinical use for selected patients [19–23]. So far, no malignancies have been reported in adults with SCD enrolled in the Multicenter Study of Hydroxyurea trial [24], or in the 226 adults with SCD treated at the Medical College of Georgia and followed for 15 years [25]. However, case reports have described acute leukemia in three patients with sickle cell disease receiving HU therapy, two of whom developed leukemia after 6 and 8 years of treatment [26–29]. Although the risk of developing leukemia or any other cancer is impossible to evaluate based on case reports, future large cohort studies with patients on treatment since early in life may eventually show an increased cancer risk for patients on long-term HU therapy. Until such studies are conducted, only surrogate markers of DNA damage can be evaluated.

This study evaluated the levels of DNA damage in the peripheral blood of SCD individuals treated with HU and in healthy age-matched control subjects using the comet assay to investigate possible secondary consequences, such as the development of neoplasia as a result of increased genomic instability.

2. Materials and methods

2.1. Patients and blood sampling

We evaluated the extent of DNA migration in blood cells sampled from 28 (13 males) SCD patients (two with SC hemoglobinopathy, one with *S*-β-thalassemia, and 25 with SS hemoglobinopathy), aged 2–59 years (23.1 ± 2.4 years), who were receiving a median oral HU dose of 27.8 mg/(kg day) (27.0 ± 1.7) for 0.8–13.4 years (56.9 ± 8.7 months). The control group was composed of 28 healthy individuals (15 males) aged 5–59 (24.1 ± 2.5) years. In both groups, 14.3% of the participants were smokers.

Patients with SCD on HU therapy for at least 6 months were enrolled after signing an informed consent term in accordance with the guidelines of the Ethics Committee at Hospital de Clinicas de Porto Alegre (HCPA), RS, Brazil. Ten-milliliter samples of heparinized peripheral blood were collected, immediately protected from light, and stored at 4 °C for evaluation. Given the severity of SCD, all individuals seen in our hematological services receive treatment, so that comparisons between SCD patients receiving and others not receiving HU treatment were not possible. Therefore, healthy individuals selected at HCPA and matched by age, gender and smoking habit were used as controls.

2.2. Comet assay

The standard alkaline protocol for comet assay was used as reported by Singh et al. [30]. The assay was performed in accordance with general guidelines for in vivo use of the comet assay [31,32].

Briefly, blood samples were mixed with low melting point agarose and spread onto glass slides pre-coated with agarose, and coverslips were gently placed over their content. Once the samples solidified, coverslips were removed and the slides were soaked in freshly made, chilled lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10.2, to which 1% Triton X-100 and 10% DMSO had been added) for 1–2 days under refrigeration. Excess liquid was blotted away from each slide's back and edges; the slides were then transferred to an electrophoresis tank, and an alkaline solution (300 mM NaOH, 1 mM EDTA, pH > 13) was added. The slides were exposed to the alkaline solution for 20 min to allow for DNA unwinding and for the expression of alkali-labile sites as single strand breaks. DNA was then electrophoresed for 20 min (25 V; 300 mA; 0.9 V/cm). Slides were removed from the electrophoresis tank, cleaned, washed three times (5 min each time) with neutralization buffer (0.4 M Tris, pH 7.5), washed three times with distilled water, and allowed to air dry. All steps of the assay, from blood storage to the end of electrophoresis, were conducted under dim light.

Slides were then fixed and silver-stained according to Nadin et al. [33]. For evaluation of DNA damage, 100 cells per subject were analyzed at 200× magnification under a light microscope. Cells were assessed visually and received scores from 0 (no migration) to 4 (maximal migration) according to tail intensity (size and shape). Therefore, the total score (damage index, or DI) for 100 cells ranged from 0 (all cells with no migration) to 400 (all cells with maximal migration). Slides from patients and controls were processed, coded, mixed and evaluated together [34].

2.3. Statistical analyses

Two complete univariate analyses were conducted to test the effect of all variables on the DI detected in the comet

assay. In the first univariate analysis, individuals were clustered according to gender, age, and smoking habit, so as to compare DNA damage between SCHU individuals and controls. The second univariate analysis was conducted within the SCHU group to evaluate the effect of the following variables on DI: ethnic group, body mass index [BMI: weight(kg)/height(m^2)], average dose of HU per weight, and HU treatment length.

In both analyses, DI data were ln-transformed, and age, BMI, average HU dose, and treatment length were separated into two categories according to their median. The nonparametric Mann–Whitney *U*-test was used to analyze DI raw data and to compare variables that resulted significant in the univariate models. This comparison was also used to test the differences between SCHU individuals and controls and within SCHU individuals. The intraindividual concordance between ln-transformed DI and age, BMI, average HU dose, or HU treatment length were evaluated using nonparametric correlation analysis (Spearman test). The Statistical Package for the Social Sciences (SPSS) 10.0 for Windows (Chicago, IL, USA) was used for all data analyses, and graphs were built with the GraphPad Prism 4.0 for Windows (San Diego, CA, USA). The level of significance was 0.05 for all tests, and data were presented as mean ± S.E. of the mean.

3. Results

Tables 1 and 2 show individual DI values and the number of cells found in each damage class for the SCHU and control groups, respectively. Greater DNA migration ($p = 0.020$) was found in the study group ($DI = 18.7 \pm 2.5$) when compared to the control group ($DI = 14.3 \pm 2.5$). No effect of gender, smoking habit or age was found in the analysis using a general linear model (Table 3). Fig. 1 shows the distribution of the number of cells found in each damage class for both patients and controls. Young individuals (≤20 years) from both the SCHU (particularly) and the control groups had a greater DI than older ones (≥21 years), but the difference was not significant (Table 3).

Table 1
Individual DI values and number of cells found in each damage class in the SCHU group

Age/sex	Smoker	SCGE	Damage class			
			1	2	3	4
25/M	N	10	8	1	0	0
18/M	N	17	13	2	0	0
19/F	Y	25	19	3	0	0
11/F	N	33	19	6	1	0
12/M	N	29	29	0	0	0
16/M	N	24	5	6	1	1
22/M	N	22	14	4	0	0
9/F	N	25	7	9	0	0
37/F	Y	16	12	2	0	0
45/F	N	10	5	2	1	1
44/F	N	23	23	0	0	0
32/F	N	10	10	0	0	0
15/M	N	13	13	0	0	0
14/F	N	10	8	1	0	0
15/M	N	4	2	1	0	0
31/F	N	8	2	1	0	1
18/M	N	7	5	1	0	0
2/F	N	70	20	22	2	0
28/F	N	1	1	0	0	0
29/F	Y	15	2	5	1	0
59/F	N	13	9	2	0	0
21/M	N	11	5	3	0	0
19/M	N	38	8	7	4	1
10/M	N	21	2	5	3	0
29/M	N	4	1	0	1	0
22/F	N	15	3	2	0	2
18/F	Y	18	12	3	0	0
40/M	N	27	3	6	4	0
∑			261	96	21	10

SCGE: single cell gel electrophoresis.

Table 2
Individual DI values and number of cells found in each damage class in controls

Age/sex	Smoker	SCGE	Damage class			
			1	2	3	4
25/M	N	7	7	0	0	0
19/M	N	12	3	3	1	0
20/F	Y	3	3	0	0	0
11/F	N	4	2	1	0	0
12/M	N	3	1	1	0	0
22/F	Y	6	6	0	0	0
8/F	N	56	4	4	11	2
37/F	N	17	1	4	0	2
45/M	N	2	2	0	0	0
43/M	N	24	14	3	0	1
33/F	N	8	4	2	0	0
12/M	N	48	11	5	5	3
11/M	N	18	6	1	2	1
49/F	N	12	8	2	0	0
7/F	N	90	29	24	1	0
31/M	N	39	23	8	0	0
52/F	N	7	7	0	0	0
21/M	N	3	3	0	0	0
42/M	Y	10	10	0	0	0
2/M	N	22	2	5	3	1
24/F	N	18	5	5	1	0
26/F	Y	23	17	3	0	0
59/F	N	3	3	0	0	0
24/F	Y	42	18	12	0	0
54/F	N	7	7	0	0	0
27/F	N	3	3	0	0	0
20/F	N	5	3	1	0	0
41/F	N	13	2	2	1	1
∑			204	86	25	11

SCGE: single cell gel electrophoresis.

Table 3
DNA damage according to gender, smoking habit and age evaluated using the comet assay in controls and patients with SCHU

	Controls		SCHU		
	DI ± S.E.	*n*	DI ± S.E.	*n*	*p*
Gender					
Male	15.3 ± 3.4	15	17.3 ± 2.9	13	0.44
Female	13.0 ± 4.0	13	19.9 ± 4.1	15	
Smoking					
No	14.9 ± 2.9	24	18.7 ± 3.0	24	0.72
Yes	10.5 ± 4.4	4	18.5 ± 2.3	4	
Age					
≤20 years	15.6 ± 4.4	14	23.9 ± 4.4	14	0.89
≥21 years	12.9 ± 2.7	14	13.5 ± 1.8	14	
Total	14.3 ± 2.5	28	18.7 ± 2.5	28	0.020[a]

SCHU: patients with sickle cell disease treated with hydroxyurea; DI: damage index; S.E.: standard error of the mean; *n*: number of individuals; *p*: statistical significance according to the univariate linear model (overall $p = 0.464$).
[a] Statistical significance according to Mann–Whitney *U*-test.

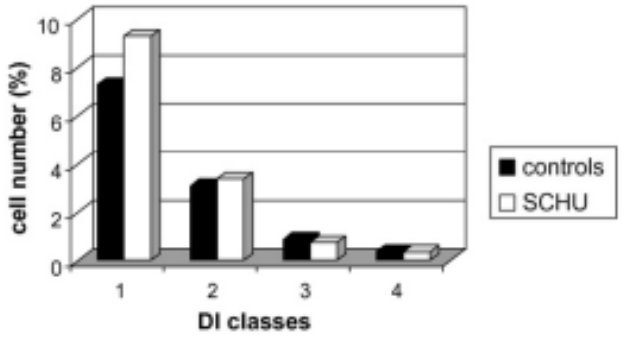

Fig. 1. Distribution of the number of cells found for each damage class in patients and controls.

Within SCHU individuals, no influence of ethnic group was observed on DI ($p = 0.105$). However, DI was significantly influenced by BMI ($p = 0.005$), mean dose of HU ($p = 0.038$), and length of HU treatment ($p = 0.016$); in addition, the overall analysis was significant ($r = 0.808$; $p = 0.001$) (Table 4).

DI was inversely correlated with BMI ($r = -0.57$; $p < 0.001$) in individuals treated with HU, and individuals with BMI ≤20 kg/m^2 (DI = 25.1 ± 4.1) had a significantly greater DI than those with BMI >20 kg/m^2 (DI = 12.2 ± 1.8) (Fig. 2, Table 4).

There was a significant positive correlation ($r = 0.402$; $p < 0.05$) between mean HU dose and DI (Fig. 2),

Table 4
DNA damage according to ethnic group, BMI, mean HU dose and length of HU treatment evaluated using the comet assay in patients with SCHU

	DNA damage		
	n	DI ± S.E.	*p*
Ethnic group			
Black	19	19.3 ± 2.0	
Mulatto and white	9	17.3 ± 7.0	0.105
BMI			
≤20 kg/m^2	14	25.1 ± 4.1	
>20 kg/m^2	14	12.2 ± 1.8	0.005
Mean dose of HU			
<27.8 mg/kg HU	14	13.8 ± 2.2	
>27.8 mg/kg HU	14	23.6 ± 4.3	0.038
Length of HU treatment			
<40 months	14	24.0 ± 4.4	
≥40 months	14	13.4 ± 2.5	0.016

BMI: body mass index; HU: hydroxyurea; SCHU: sickle cell disease treated with hydroxyurea; DI: damage index; S.E.: standard error of the mean; *n*: number of individuals; *p*: statistical significance comparing DI between groups according to the univariate linear model (overall $p = 0.001$).

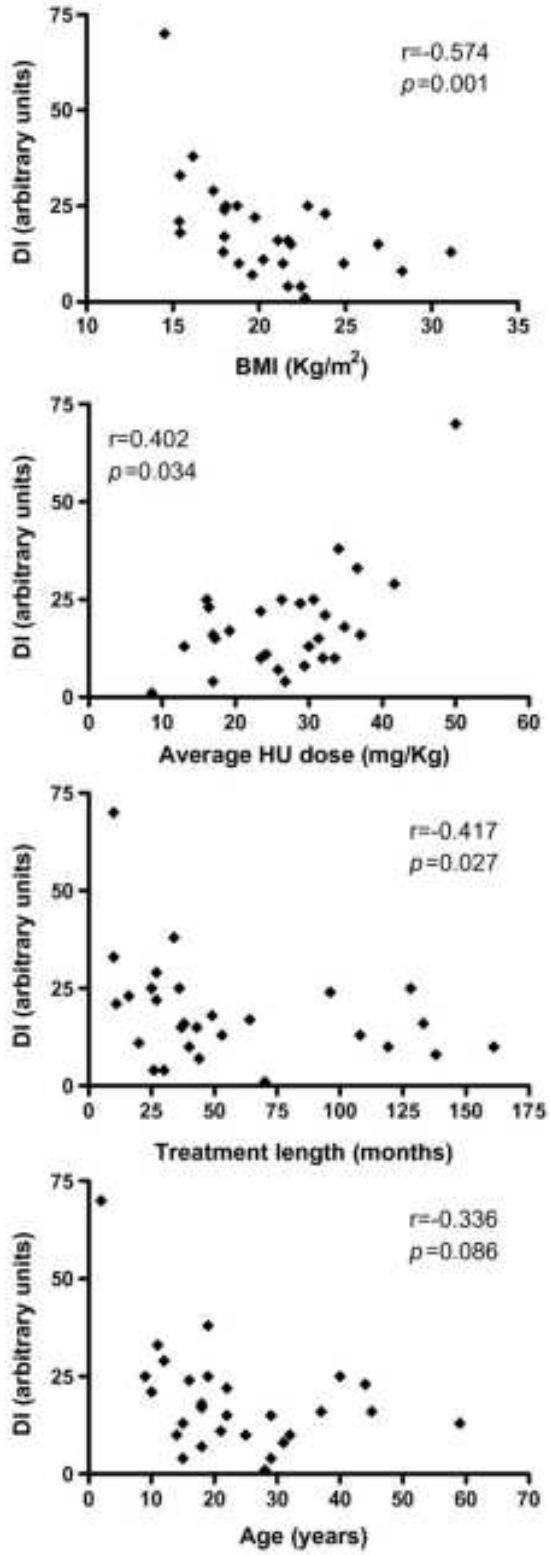

Fig. 2. Association between DNA damage (DI) and BMI, mean HU dose, length of HU treatment and age in SCHU patients, as evaluated by the Spearman correlation test. DI: damage index; BMI: body mass index; HU: hydroxyurea; SCHU: individuals with sickle cell disease treated with hydroxyurea.

and a significant negative association ($r = -0.388$; $p = 0.041$) between age and mean HU dose (mg/kg) in the SCHU group. Individuals who received a mean HU dose >27.8 mg/kg showed a significantly higher DI (DI = 23.6 ± 4.3; $p < 0.05$) than those who received ≤27.8 mg/kg (DI = 13.8 ± 2.2) (Table 2). No correlation was found between DI and the total HU dose, regardless of patient weight ($r = -0.22$; $p > 0.05$).

Interestingly, there was also a negative correlation between treatment length and DI ($r = -0.41$; $p < 0.05$), and individuals treated for ≥40 months (DI = 13.4 ± 2.5) had significantly lower DI ($p < 0.05$) than those treated for <40 months (24.0 ± 4.4) (Fig. 2; Table 4). However, there was a positive association ($r = 0.53$; $p = 0.004$) between HU treatment length and age.

4. Discussion

The clinical efficacy of HU therapy, as well as its low toxicity and the ease of oral administration, makes it an attractive therapeutic option for patients with myeloproliferative disorders and SCD. However, although the literature has not reported harmful effects associated with HU therapy, it may have long-term mutagenic or carcinogenic effects.

The results of this study showed significantly greater levels of DNA migration, and thus DNA damage, in individuals treated with HU when compared to controls. When comparing the distribution of damage classes in patients and controls (Fig. 1), it is possible to observe that the difference is primarily caused by an increased number of cells in damage class 1, reflecting a homogeneously distributed increase in the number of slightly damaged cells rather than a low number of highly damaged cells. DNA damage was dose-dependent, and individuals treated with >27.8 mg/kg HU presented about twice more DNA damage than those treated with lower doses. On the other hand, there was a negative association between treatment length and the level of DNA damage: individuals treated for longer periods (≥42 months) showed about half the DNA damage of patients treated for shorter times. Individuals treated for shorter periods tended to be younger, to receive higher doses of HU and, consequently, to have a lower BMI.

One possible mechanism of HU toxicity is the reduction of intracellular dNTP pools through the inhibition of diphosphate reductase, which impairs the repair of DNA damage due to a lack of nucleotides for DNA polymerases. Hence, DNA damage accumulates, potentially leading to carcinogenesis and favoring leukemic transformation [35]. However, the oxidative and inflammatory nature of SCD, the condition for which patients

are treated with HU, should also be considered. SCD is associated with elevated levels of redox-active iron in heme form, released from intracellular heme proteins derived from hemolyzed erythrocytes. Free heme damages lipids, proteins, and DNA through the generation of reactive oxygen species, which are implicated in numerous diseases [36]. Lymphocytes from thalassemic patients, who share several clinical features with SCD patients, were reported to have higher levels of DNA damage [37]. Since untreated SCD individuals are not available in health care services, we were not able to design an in vivo study to address only the effect of HU, independently of SCD condition.

Khayat et al. [38] assessed the genotoxicity of HU in short-term cultures of lymphocytes from SCD patients. HU was not cytotoxic or genotoxic at the concentrations tested in the G2 phase of the cell cycle. The same authors [39] followed patients treated with HU to investigate possible mutagenic effects using cytogenetic methods (mitotic index and chromosome aberrations) at 2-month intervals for 1 year, and did not detect any genotoxic or cytotoxic effect. De Lima et al. [40], on the other hand, evaluated and compared the cytotoxicity of HU as well as its ability to induce chromosomal damage (clastogenicity) by scoring chromosome aberrations and the mitotic index. In their study, in vitro short-term cultures of lymphocytes were exposed to several concentrations of HU, and the effects were analyzed at various cell cycle phases. There was a significant increase in cytotoxicity at G1 and G1/S, as well as in the G2 phase of the cell cycle. However, HU did not significantly increase the frequency of chromosome aberrations. There was an S-dependent cytotoxic effect of HU, which should be expected because of the known activity of HU in inhibiting diphosphate reductase. Because of the potential cytotoxicity of HU, individuals submitted to treatment should be constantly monitored [39], specifically in regard to cytotoxicity endpoints.

Greater DNA damage was seen in individuals $\leq$20 years old, both in the control and in the SCHU groups, but the difference was not significant; this result may be associated with greater baseline DNA damage. Data comparing the levels of DNA damage in children and adolescents with those in adults are scarce in the literature. A comprehensive literature review and meta-analysis conducted by Neri et al. [41] concluded that, although there is evidence of higher baseline levels of chromosome aberrations, sister chromatid exchanges and micronuclei in adults, children may be more susceptible than adults, as shown by the greater genotoxic damage (micronuclei and DNA single strand breaks) that has been found in the fetal tissue when compared to the maternal tissue of rodents following transplacental exposure to benzo(*a*)pyrene [41]. Interestingly, they also observed an increase in micronuclei and sister chromatid exchanges in children exposed to arsenic in drinking water and to environmental pollutants released from a chemical dump, respectively. Nevertheless, the authors concluded that, due to lack of data, any meaningful conclusion about the greater susceptibility of children is precluded.

In the alkaline (pH > 13) version of the comet assay developed by Singh et al. [30], increased DNA migration can be associated with incomplete excision repair sites [31], which are generated as an intermediate step during the action of different DNA repair systems and typically formed after exposure to some genotoxins [42]. Therefore, an increase in DNA migration may be expected as a consequence of HU treatment, particularly in younger individuals, who seem to have more active DNA repair systems than older adults [43,44].

Interestingly, a negative correlation was found between BMI and DNA damage in SCHU patients. Such correlation persisted even when extreme outliers were removed from the sample assessment, which could be excessively influencing the result. Although BMI is usually positively correlated with DNA damage levels [45–47], it may be an indicator of general health among patients in the SCHU group, and may indicate that individuals more prone to gain weight are healthier and thus have better DNA homeostasis. It is important to point out that age was associated with BMI as children were the leanest individuals in the SCHU group, and that only the oldest individuals in the SCHU group had a BMI greater than 30 kg/m^2 and were classified as obese [48].

In conclusion, we found greater levels of DNA damage in the SCHU group when compared to controls, and DNA damage in the SCHU group was positively associated with mean HU dose and negatively associated with age, treatment length and BMI. Our results and other positive findings suggest that the use of HU may have an impact on the levels of DNA damage in blood leukocytes. As HU has been increasingly used in children with SCD, which may mean a life-long exposure to this potentially mutagenic drug, additional studies should be conducted, and constant surveillance for malignant or pre-malignant conditions should be performed.

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9.4 **ANEXO D: "**Length of treatment and dose as a determinant of mutagenicity in sickle cell disease patients treated with hydroxyurea"

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Length of treatment and dose as determinants of mutagenicity in sickle cell disease patients treated with hydroxyurea

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ABSTRACT

Hydroxyurea (HU) is an antineoplastic drug widely used in the clinical management of patients with sickle cell disease (SCD), and many questions related with its use remain unresolved. Given the severity of SCD, HU benefits, although not thoroughly confirmed, seem to outweigh its potential carcinogenicity. This study aimed to assess the genotoxicity associated with HU dose and treatment length by evaluating mutagenicity in patients with SCD treated with HU (SCHU) using the cytokinesis-block micronucleus assay (CBMN) in white cells. The study was conducted with 35 individuals in the SCHU group and 34 controls matched according to age, sex and smoking habit. CBMN results showed an increase ($p = 0.032$) in the number of micronuclei (MN), but not of nucleoplasmic bridges (NPB) or nuclear buds (NBUD) in the SCHU group. The increased frequency of MN in the SCHU group was significantly correlated with treatment length and final HU dose, which confirms that patients with SCD treated with HU should be carefully monitored to reduce the risk of carcinogenicity.

1. Introduction

Hydroxyurea (HU), also known as hydroxycarbamide, is an S-phase-specific and non-DNA hypomethylating chemotherapeutic agent (Borgna-Pignatti, 2007). Its use in adult patients with sickle cell disease (SCD) is approved by the Food and Drug Administration (FDA), and clinical trials carried out with children have recommended its clinical use for patients with major clinical manifestations (Obene-Frempong and Smith-Whitley, 1997; Rogers, 1997; Kinney et al., 1999; Ferster et al., 2001; Koren et al., 1999). Observational studies involving both adults and children support the use of HU to reduce the complications of SCD (including pain, hospitalizations, blood transfusions, and acute chest syndrome) and decrease mortality. Data are limited regarding the effectiveness of HU treatment for SCD, and although the drug is currently underutilized, it seems to be highly effective (Brawley et al., 2008).

SCD is an inherited blood disorder with a single gene mutation, but its clinical manifestations, such as painful vascular occlusion and acute chest syndrome, vary in severity from one individual to another (Steinberg, 1999).

HU increases the synthesis of globins (Atweh and Loukopoulos, 2001), particularly fetal hemoglobin (HbF). It also kills dividing cells, so that primitive erythroid progenitors, more committed to HbF synthesis, can develop (Borgna-Pignatti, 2007). Moreover, HU reduces ineffective erythropoiesis (Loukopoulos et al., 1998) and induces well-being (Atweh and Loukopoulos, 2001). Although treatment efficacy may decrease over time (Mancuso et al., 2006), a landmark clinical trial showed that HU reduced in about 50% the frequency of hospitalizations due to painful crises in SCD patients (Charache et al., 1995).

On the other hand, HU seems to have clastogenic (Gebhart, 1981; Oppenheim and Fishbein, 1965), teratogenic (Murphy and Chaube, 1964; Aliverti et al., 1980) and, in some settings, mutagenic effects (Ziegler-Skylakakis et al., 1985), and the safety of long-term HU therapy has not been definitely established. A few in vivo studies have reported that the use of HU for sickle cell anemia led to acute myeloid leukemia, but a review found a low frequency of such findings and a long latency in patients treated with HU alone (Atweh and Loukopoulos, 2001). Accordingly, previous studies have shown that the most common short-term toxic effect of HU is transient and reversible myelosuppression, particularly of granulocytes (Griffith, 1964). A key target of HU within cells is ribonucleotide diphosphate reductase (RR), the enzyme that converts ribonucleotides into deoxyribonucleotides, the building blocks for DNA synthesis and repair. As a result of this process, HU generates free

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radicals, which explains its associated toxicity (Przybyszewski and Kasperczyk, 2006).

According to the most recent National Institutes of Health Consensus Development Conference Statement: Hydroxyurea Treatment for Sickle Cell Disease (Brawley et al., 2008), several issues about the use of HU in SCD patients remain unresolved, including concerns on the part of patients and health practitioners about the overall safety and effectiveness of the drug, as well as a lack of expertise in the treatment of patients with SCD. The NIH consensus suggests the performance of future research towards providing information that will ensure the most appropriate application of HU therapy for SCD, with the adoption of a surveillance system and the development of additional efficacy studies aimed at defining mechanisms of action, establishing pharmacological markers for HU treatment, and further evaluating HU safety and cost-effectiveness. Appropriate studies are needed to provide more information about developmental and reproductive adverse effects, carcinogenic risks, and long-term clinical outcomes (Brawley et al., 2008).

Therefore, in the present study, we evaluated mutagenicity in the white blood cells of individuals with SCD treated with HU using the cytokinesis-block micronucleus assay (CBMN) as a function of HU dose and treatment length.

2. Materials and methods

Eligible subjects were patients with sickle cell anemia (hemoglobinopathy SS) receiving HU therapy for at least 6 months. Patients were enrolled after signing an informed consent term, in accordance with the guidelines of the Ethics Committee of Hospital de Clínicas de Porto Alegre, RS, Brazil.

We evaluated 35 patients with SCD (SCHU group, 15 males and 20 females) aged 4–59 years (mean 26.3 years) receiving HU orally at 8.7–28.5 mg/kg/day (median 26.5 mg/kg/day) for 16–161 months (median 49 months). The control group was composed of 34 healthy individuals (17 males and 17 females) aged 2–59 years (mean 26.6 years). The individuals in the SCHU group were compared with controls matched for age, sex and smoking habit. In addition to the comparison between the SCHU and the control groups, 25 out of the 35 SCHU patients who had all relevant clinical information available, i.e., body mass index (BMI), ethnicity, HU dose and treatment length, were further assessed regarding the effect of treatment length and final HU dose over micronuclei (MN) frequency.

Peripheral blood samples were collected from patients and controls at rest, early in the morning on Mondays, just before the daily dose of HU, and CBMN was performed. For the CBMN assay, an aliquot of blood (0.5 ml) was added to 5 ml of RPMI 1640 medium supplemented with 20% fetal calf serum and 0.2% phytohemagglutinin. The flasks were cultured at 37 °C for 44 h, after which 4.5 mg/ml cytochalasin B (Cyt B, Sigma) was added according to the method described by Fenech and Morley (1985) and revised by Fenech (1993). The cell suspension was fixed in 3:1 methanol:acetic acid with no hypotonic treatment, and dropped onto clean slides. The slides were stained with Giemsa. One thousand binucleate cells on coded, unidentified slides from each individual were scored for micronuclei, nucleoplasmic bridges (NPB) (dicentric bridges between two daughter nuclei) (Fenech, 1997), and nuclear buds (NBUD) (amplified DNA) (Fenech and Crott, 2002).

Slides from patients and controls were processed, coded, mixed and evaluated together. Differences between values for controls and individuals in the SCHU group were analyzed using the Mann–Whitney test. Associations between MN frequency on the one hand and treatment length and final HU dose on the other were assessed by second-order polynomial curves. Treatment length and final HU dose were clustered according to quartiles and expressed as mean values for plotting graphs. The Statistical Package for Social Science (SPSS) 10.0 was used for data analysis and the Graphpad Prism was used to plot graphs. Frequencies of MN, NPB and NBUD were expressed as absolute numbers found in the 1000 cells analyzed. Other data were expressed as means (±standard deviations). The level of significance was of $p < 0.05$.

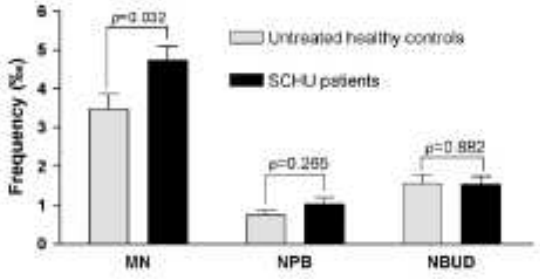

Fig. 1. Frequency of micronucleus (MN), nucleoplasmic bridges (NPB) and nuclear buds (NBUD) in a sample of patients with sickle cell disease treated with hydroxyurea (SCHU) and untreated healthy controls. p = significance according to one-tail Mann–Whitney U-test.

3. Results

Mean frequencies of MN, NPB and NBUD in healthy controls and in SCHU individuals are shown in Fig. 1. The MN frequency in the SCHU group was significantly higher than in the control group (4.74 ± 2.11 versus 3.47 ± 2.26, $p = 0.032$). The frequency of NPB and NBUD did not differ between the SCHU and the control groups, although NPB frequency was slightly greater than in the SCHU group.

Smoking did not affect the frequency of any parameter (data not shown). Average age and the male-to-female ratio were similar in the two groups. In addition, no sex or age differences were found in the frequency of MN, NPB or NBUD. On the other hand, when data of the SCHU and the control groups were pooled together, positive correlations were found between the frequency of MN and NPB ($r = 0.299$, $p = 0.013$) and between MN and NBUD ($r = 0.296$, $p = 0.013$).

Fig. 2 shows the effect of treatment length and final HU dose over MN frequency in 25 patients from the SCHU group. Treatment length was strongly associated with a stepwise decrease in MN frequency, while final HU dose showed a dose–response effect over MN frequency, with higher doses (about 35 mg/kg HU) inducing about 1.5-fold more MN than lower doses (about 20 mg/kg HU). Sex, ethnicity and BMI did not influence MN frequency.

4. Discussion

According to the newest National Institutes of Health Consensus Development Conference Statement: Hydroxyurea Treatment

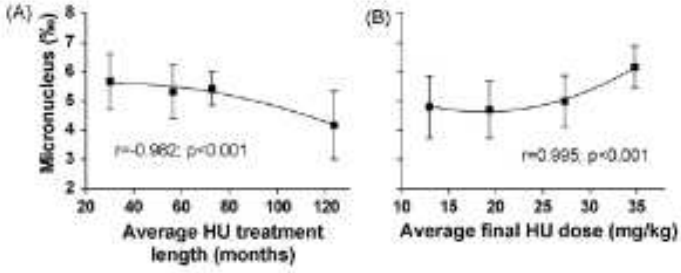

Fig. 2. Effect of treatment length (A) and final hydroxyurea dose (B) over micronucleus (MN) frequency in a sample of patients with sickle cell disease treated with hydroxyurea. r = correlation coefficient; p = level of significance of second-order polynomial correlations.

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for Sickle Cell Disease (Brawley et al., 2008), one major study randomly assigned nearly 300 adults with SCD who had more than three severe painful crises or episodes per year to either hydroxyurea or placebo. The study was interrupted early due to the fact HU clearly reduced the number and severity of pain episodes in patients with SCD, and consequently the use of placebo was considered unethical. The decision was reinforced by the fact that, in addition to reducing the severity of clinical symptoms associated with SCD, HU had already been shown to increase life expectancy among treated patients (Steinberg et al., 2003).

The clinical efficacy of HU therapy, its low toxicity and ease of administration (orally) make it an attractive therapeutic option for patients with myeloproliferative diseases and SCD. However, the potential mutagenic and carcinogenic effects of long-term therapy with HU may pose serious risks. The results of this study showed a greater frequency of MN among individuals with SCD treated with HU than among controls.

One possible mechanism of HU toxicity is the reduction of intracellular dNTP pools resulting from the inhibition of RR, which impairs DNA repair mechanisms due to the lack of nucleotides for DNA polymerase. Hence, DNA instability potentially accumulates and leads to carcinogenesis, mainly by leukemic transformation (Li and Kaminskas, 1987). On the other hand, the oxidative and inflammatory nature of SCD, the disease for which patients are treated with HU, should also be taken into consideration. SCD is associated with elevated levels of redox-active iron contained in heme and released from intracellular heme proteins derived from hemolyzed erythrocytes. Free heme damages lipids, proteins, and DNA through the generation of reactive oxygen species, which are implicated in several diseases (Kumar and Bandyopadhyay, 2005). Indeed, lymphocytes from patients with thalassemia, who share several clinical features with patients with SCD, seem to have high levels of DNA damage, with much higher background levels of damage and induced damage (Anderson et al., 2000).

In a previous study (Friedrisch et al., 2008), we used the alkaline comet assay to evaluate the level of primary DNA damage in peripheral blood leukocytes sampled from 28 SCHU patients and 28 untreated individuals. Patients receiving a mean dose of >20 mg/kg HU showed a 1.7-fold increase in DNA damage than those receiving lower doses (damage index = 24.9 ± 5.5 versus 14.6 ± 1.8; $p < 0.05$). In addition, individuals treated for longer than 42 months showed significantly greater DNA damage than those treated for <42 months (damage index = 23.1 ± 4.2 versus 13.6 ± 1.9; $p < 0.05$). Damage index was inversely correlated with BMI in the SCHU group.

Khayat et al. (2004) evaluated the genotoxicity of HU in short-term cultures of lymphocytes of patients with SCD. HU was not cytotoxic or genotoxic at the concentrations tested in the G2 phase of the cell cycle. In another study, Khayat et al. (2006) monitored patients under treatment with HU for possible mutagenic effects using cytogenetic tests (mitotic index and chromosome aberrations) for one year at two-month intervals, and they reported that cytotoxic effects were not evident. In another in vitro study, de Lima et al. (2003) tested short-term cultures of lymphocytes and several concentrations of HU at various cell cycle phases. They found no significant effects on mitotic index or significant increases in chromosome aberrations, but did find S-dependent cytotoxicity.

Le Fevre et al. (2007) found a dose-dependent increase of MN and consistent G1 arrest in association with the down-regulation of the G1–S transition genes *CKS2*, *UBE2C* and *CDCA8* as a response to treatment with HU. Accordingly, Hanft et al. (2000) found low mutagenic and carcinogenic effects and suggested that the potential for carcinogenicity in in vivo HU therapy is low. Nevertheless, the authors recommended that young SCD patients treated with HU should be monitored serially for increases in DNA mutations. They also observed that, although treated and untreated adults with SCD had similar levels of mutation, the number of mutations increased with treatment length. Our data did not show any correlation between mutagenicity and age or sex.

In our study, MN frequency in the SCHU group was slightly but significantly greater than in the control group ($p = 0.038$). HU dose was also positively associated with an increase in MN frequency, whereas the association with treatment length showed an opposite tendency. Indeed, HU dose should be the minimum necessary to achieve clinical benefits, maybe up to 20 mg HU/kg.

The findings reinforce that patients treated with HU must be closely monitored because of the oxidative nature of SCD, as widely recommended in the literature. In addition, since HU has been increasingly used in children with SCD, which may mean a lifelong exposure to this potentially mutagenic drug, follow-up studies should be conducted to establish the long-term safety of HU treatments, and constant surveillance for malignant or pre-malignant conditions should be performed.

Conflict of interest

None declared.

Acknowledgements

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9.5 ANEXO E: "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*"

Artigo publicado no volume 583, páginas 75-85 (2005) do periódico *Mutation Research.*

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.

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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.

Keywords: Genotoxicity; MMS; Cyclophosphamide; Ferrous and cupric sulfates; Comet assay; DNA repair

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-acetate (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 ± 3 °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) damage 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.

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.

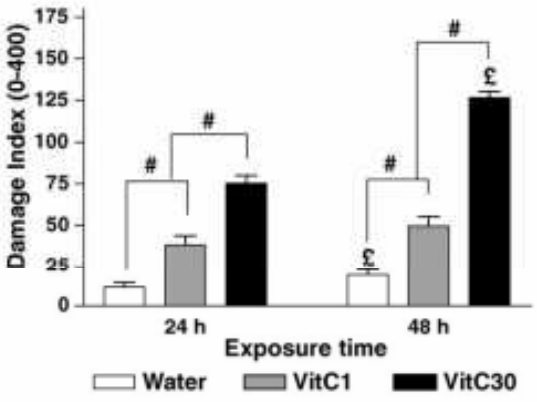

Fig. 1. Genotoxicity induced by single (at 24 h) and double treatment (at 48 h) with water or Vitamin C. £Significant when comparing DNA damage between 24 and 48 h, for the same substance at $P \leq 0.001$, as tested by Student's *t*-test. #Significant when comparing DNA damage at the same time for the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

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-

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 ± 3.31[d***]	6	–	–	–	
CP	25.00	109.00 ± 40.39[e*]	18	74.83 ± 16.68[e*]	6	100.17 ± 44.21	6	106.33 ± 44.56	6
MMS	40.00	169.28 ± 13.45[e***,f***,g*]	18	157.67 ± 15.28[e***]	6	68.50 ± 34.62[h***,i***]	6	146.83 ± 14.16	6
Ferrous sulfate	33.23 Fe	109.67 ± 26.58[e*,f***,g**]	18	30.67 ± 32.06[d***]	6	119.83 ± 28.01[h***]	6	137.17 ± 31.11[h***]	6
Cupric sulfate	8.50 Cu	127.89 ± 41.19[e**,f**]	18	84.50 ± 18.71[d**,e*]	6	141.33 ± 10.67[h***,f**]	6	144.67 ± 19.61[h***]	6
Vitamin C (VitC1)	1.00	38.33 ± 13.40	6	24.40 ± 3.85[d*]	6	49.50 ± 14.43[h**]	6	–	–
Vitamin C (VitC30)	30.00	75.33 ± 10.95	6	48.20 ± 4.66[d**]	6	–		126.50 ± 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 ± 15.27[e*]	6	68.17 ± 21.88	6	70.83 ± 21.85	6
MMS	40.00	98.44 ± 3.05[e***,f***,g**]	18	87.00 ± 8.74[d*,e***]	6	55.67 ± 26.86[i**]	6	93.67 ± 6.65	6
Ferrous sulfate	33.23 Fe	78.39 ± 21.62[e*]	18	18.17 ± 9.77[d***]	6	82.67 ± 19.88[h***,f**]	6	93.67 ± 14.56[h***]	6
Cupric sulfate	8.50 Cu	84.00 ± 21.95[e**,f*]	18	67.83 ± 15.18[e**]	6	93.17 ± 7.65[h*,f***]	6	95.67 ± 3.01[h*]	6
Vitamin C (VitC1)	1.00	26.33 ± 10.33	6	24.20 ± 3.90	6	39.33 ± 13.59[h*]	6	–	–
Vitamin C (VitC30)	30.00	64.50 ± 9.73	6	46.80 ± 3.56[d**]	6	–	–	93.33 ± 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 ± 1.06[d**]	6	–	–	–	–
CP	25.00	28.14 ± 3.87[e**]	18	25.73 ± 1.09[d*,e***]	6	25.40 ± 3.46	6	25.44 ± 4.19	6
MMS	40.00	31.19 ± 1.56[e***,f***,g**]	18	31.80 ± 1.57[e***]	6	24.45 ± 2.32[h***,i***]	6	29.28 ± 1.00	6
Ferrous sulfate	33.23 Fe	26.64 ± 1.90	18	22.99 ± 2.02[d***]	6	27.17 ± 2.00[h**]	6	28.53 ± 2.16[h***]	6
Cupric sulfate	8.50 Cu	29.08 ± 4.02[e***,f*]	18	26.08 ± 0.92[d**,e***]	6	28.20 ± 1.85[f**]	6	29.38 ± 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 ± 0.34[e*]	6	–	–	28.46 ± 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 $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.
[e] Significant in relation to 48 h at $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.
[f] Significant difference between the doses of the post-treatment at $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.
[g] Significant in relation to water at $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.
[h] Significant in relation to VitC1 at $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.
[i] Significant in relation to VitC30 at $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.

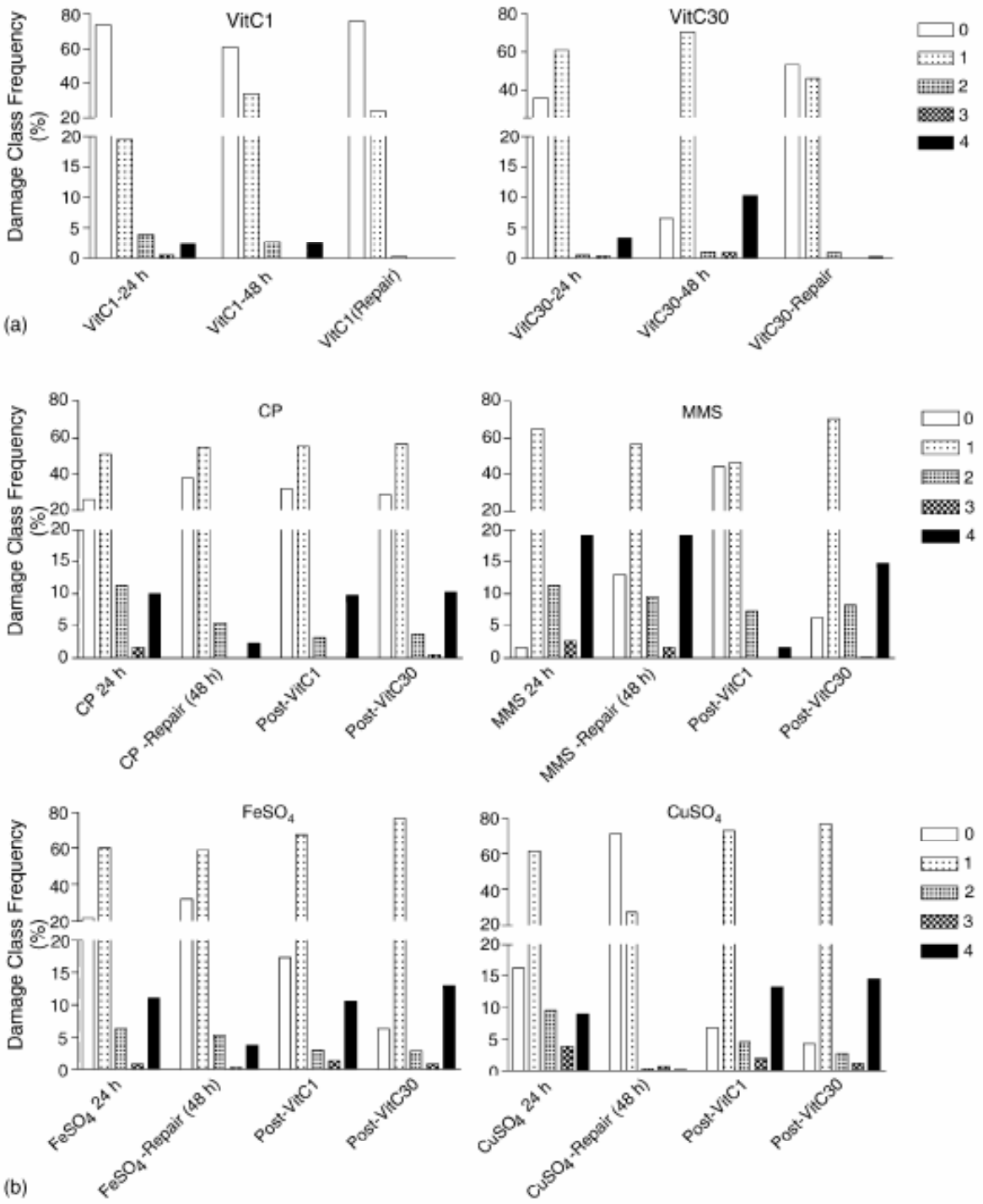

Fig. 2. Genotoxicity of the tested compounds, as evaluated by damage class frequency: (a) VitC1 and VitC30; (b) CP, MMS, $FeSO_4$ and $CuSO_4$.

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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9.6 ANEXO F “Influence of orange juice in the levels and in the genotoxicity of iron and copper”

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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.

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 neurodegenerative 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

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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 ethylenediamine-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 ± 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.

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 h (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 $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

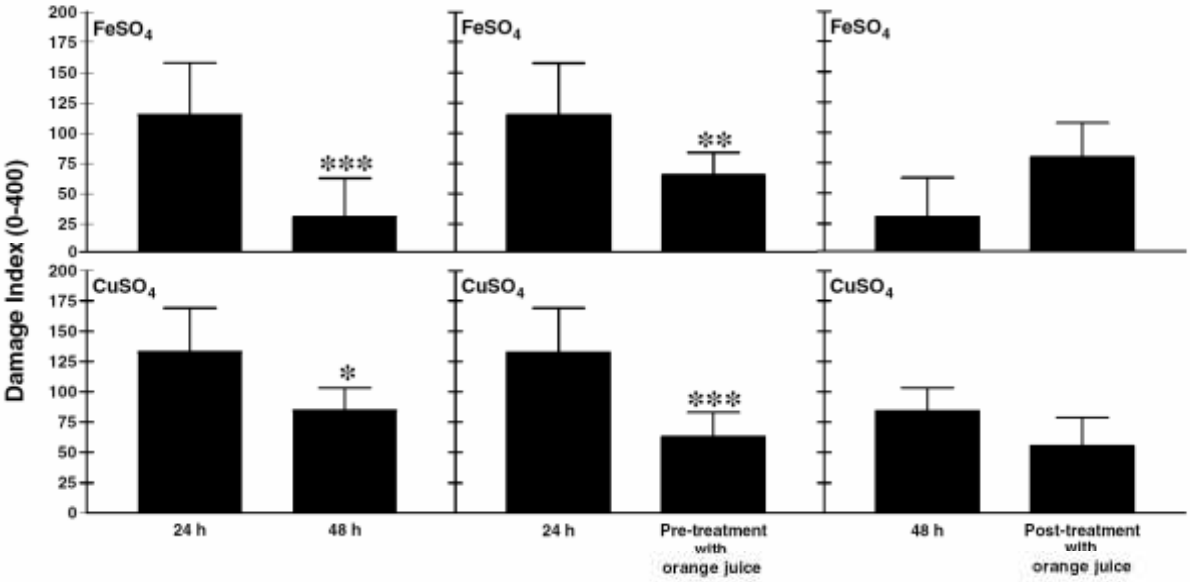

Fig. 1. Comparisons between the mean damage index values for mouse blood cells. Significant at $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. (A and B) Comparison between damage at 24 and 48 h. (C and D) Comparison between damage at 24 h and pre-treatment with orange juice. (E and F) Comparison 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 ± 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 ± 19[a**]	–	–
Ferrous sulfate	33.23 Fe	634 ± 157	189 ± 19	263 ± 58	342 ± 15[b***]
Cupric sulfate	8.50 Cu	320 ± 48	239 ± 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 ± 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.

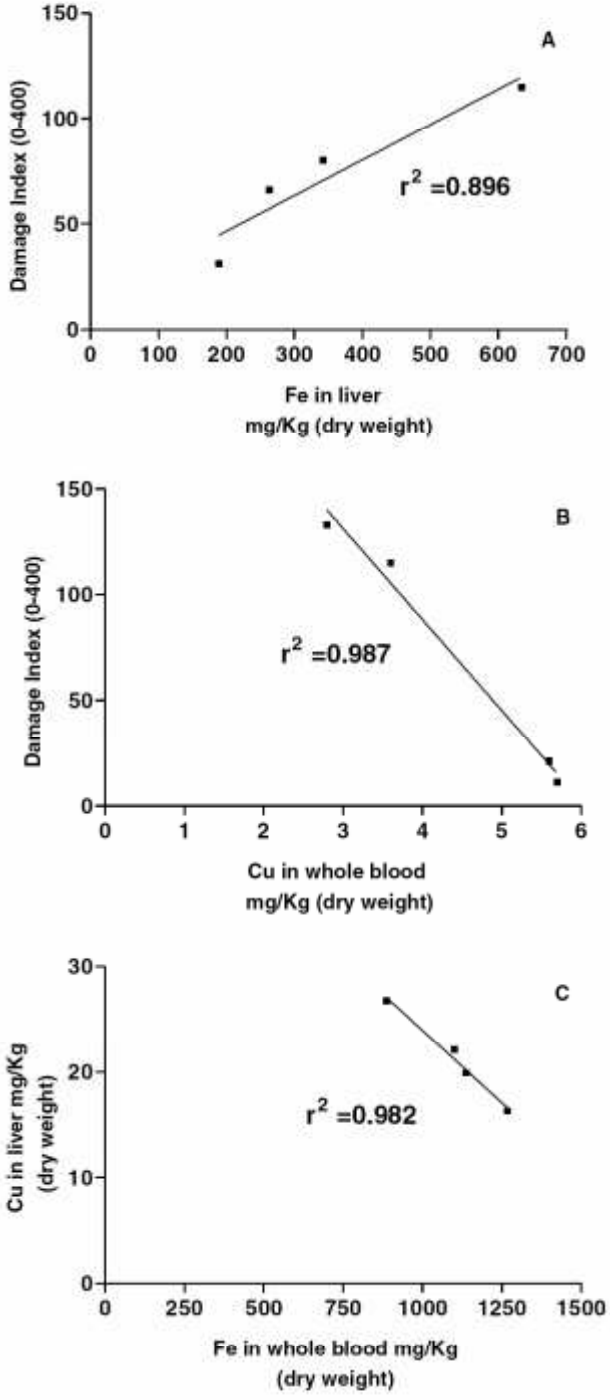

Fig. 2. Correlation between genotoxicity and iron and copper levels in whole blood and liver. (A) Correlation between the levels of DNA damage in the blood and the hepatic levels of iron at 24 h, 48 h, pre-treatment and post-treatment in ferrous sulfate group. (B) Correlation between the levels of DNA 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

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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9.7 ANEXO G “Genotoxicity and mutagenicity of iron and copper in mice”

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Genotoxicity and mutagenicity of iron and copper in mice

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Abstract The toxicity of trace metals is still incompletely understood. We have previously shown that a single oral dose of iron or copper induces genotoxic effects in mice in vivo, as detected by single cell gel electrophoresis (comet assay). Here, we report the effect of these metals on subchronic exposure. Mice were gavaged for six consecutive days with either water, 33.2 mg/kg iron, or 8.5 mg/kg copper. On the 7th day, the neutral and alkaline comet assays in whole blood and the bone marrow micronucleus (MN) test were used as genotoxicity and mutagenicity endpoints, respectively. Particle induced X-ray emission was used to determine liver levels of the metals. Females showed a slightly lower DNA damage background, but there was no significant difference between genders for any endpoint. Iron and copper were genotoxic and mutagenic. While copper was more genotoxic in the neutral version, iron was more genotoxic in the alkaline version of the comet assay. Copper induced the highest mutagenicity as evaluated by the MN test. Iron was not mutagenic to male mice. Iron is thought to induce more oxidative lesions than copper, which are primarily detected in the alkaline comet assay. Treatment with iron, but not with copper, induced a significant increase in the hepatic level of the respective metal, reflecting different excretion strategies.

Keywords Transition metals · Ferrous sulfate · Cupric sulfate · Comet assay · Micronucleus test

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Introduction

Lack of specific information makes it difficult to reach firm conclusions on the hazards of dietary

metals (Rojas et al. 1999), specially for iron and copper, which are among the most important.

Iron is a micronutrient required for almost all organisms because of its key role in biological systems, including oxygen transportation, oxidative metabolism (i.e., in several enzymes of the tricarboxylic acid cycle and oxidative phosphorylation), DNA homeostasis (ribonucleotide reductase), antioxidant defenses (peroxidases) and immune system function (myeloperoxidases). However, iron can be toxic because of its role in oxidative stress (De Freitas and Meneghini 2001).

Iron deficiency is a worldwide problem (Beard 2001) and is, in general, more frequent than its excess. Iron overload 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). Moreover, iron can damage biomolecules mainly through Fenton and Haber-Weiss chemistry, leading to the production of hydroxyl radicals and other reactive oxygen species (ROS) (Halliwell and Guterridge 2000). Iron compounds have been reported to be mutagenic in mammalian culture cells, as detected by Syrian hamster embryo cell transformation/viral enhancement assay (Heidelberger et al. 1983), base tautomerization in rat hepatocyte cultures (Abalea et al. 1999) and genetic alterations in the mouse lymphoma assay (Dunkel et al. 1999). However, negative results have also been reported, including from recombinational assay in DNA repair-deficient *Bacillus subtilis* (Leifer et al. 1981), sister chromatic exchange in hamster cell culture (Tucker et al. 1993), sex-linked recessive lethal gene mutation in *Drosophila melanogaster* (Lee et al. 1983), gene mutation in *Glycine max* (Vig 1982) and tryptophan reverse gene mutation in *Escherichia coli* (Brusick et al. 1980).

Copper is also an essential metal that can be hazardous at high levels. It is required as a co-factor for many enzymes that catalyze oxidation/reduction reactions, including those of the electron transport (cytochrome c oxidase) antioxidant enzymes (Cu/Zn superoxide dismutase and ceruloplasmin), melanin and collagen biosynthetic pathways (tyrosinase and lysyl oxidase, respectively) and hormones (e.g., dopamine-monooxygenase and a-amidating monooxygenase). The accumulation of large amounts of copper in cells and organs can be destructive. Destruction is thought to be due to ROS whose formation is catalyzed by free copper ions (or certain complexes) that might occur when the ability of the cells to store excess copper in benign form has been exceeded (Linder 2001). The genotoxicity of copper compounds has been reported mainly in vitro. For instance, 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). We have shown in vivo genotoxicity for freshwater planarian, using the comet assay (Guecheva et al. 2001). In the same study, copper inhibited DNA repair. However, negative results have been observed in the recombinational assay in DNA repair-deficient *B. subtilis* (Leifer et al. 1981), *G. max* gene mutation (Vig 1982) and mitotic recombination or gene conversion in *Saccharomyces cerevisiae* (Zimmermann et al. 1984). An in vivo study by Saleha Banu et al (2004) in mice showed that, although copper genotoxicity demonstrated a clear dose-dependent response pattern, it gradually decreased from 48 h post-treatment, returning to the control levels 2 weeks after treatment. Given the conflicting results, the question is whether the toxicity of copper in vitro and in theory is likely to occur in vivo, and, if so, under what conditions (Linder 2001).

Since the genotoxicity of iron and copper is controversial, further clarification is needed in order to address the potential toxicity of these compounds. In a previous study, we observed genotoxicity after acute exposure (24 and 48 h) of mice to iron and copper, as evaluated by the alkaline version of the comet assay (Franke et al. 2006). The aim of the present work was to evaluate the genotoxicity and the mutagenicity of these metals in mice in vivo with a subchronic exposure schedule of 7 days. Particle induced X-ray emission (PIXE) was used to evaluate the iron and copper content of the livers of the treated animals.

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, cyclophosphamide (CP) (CAS 50-18-0), copper sulfate ($CuSO_4$, purity ≥ 98%) (CAS 7758-98-7), Triton X-100 and bovine calf serum were purchased from Sigma. Ferrous sulfate ($FeSO_4$, purity ≥ 99%) (CAS 7782-63-0) was obtained from Ducto (Brazil). Low melting point (LMP) agarose and normal agarose (electrophoresis grade) were obtained from Gibco-BRL. Heparin sodium was bought from Roche (Brazil) under the commercial name Liquemine®.

Animals

CF1 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 ± 3°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 in groups according to sex 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.

Treatments and tissue sampling

Three groups of ten mice (five males and five females) were used in the experiments. Each group was gavaged (0.1 ml/10 g of body weight) with a single daily dose of either water, $FeSO_4$ (33.23 mg Fe/kg body weight) or $CuSO_4$ (8.25 mg Cu/kg body weight) for six consecutive days. In addition, one group of five male mice was gavaged with CP (25 mg/kg body weight) on the 5th day of exposure, for comet assay. The dose used for iron, copper and cyclophosphamide was equal to 10.86% (Budavari et al. 1996), 11.14% (Canton et al. 1989) and 18.2% (Lewis 1996) of the mice oral LD_{50}, respectively. All substances were prepared just before treatment and protected from light. For the comet assay, blood (about 15 µl) was obtained on the 7th day after the beginning of the experiment by means of a small incision on mouse tail tips and immediately mixed with heparin sodium (7 µl). On the same day, femur bones were dissected to prepare bone marrow smears for the micronucleus (MN) test and livers were removed for PIXE analysis.

Comet assay

The neutral comet assay was adapted from Singh et al. (2003). The alkaline version of the comet assay was performed according to Franke et al. (2005a, b, 2006). Slides were prepared and cells were lysed rigorously in the same manner for neutral and alkaline versions. Briefly, an aliquot of blood cells/heparin mixture were embedded in LMP agarose and spread (final concentration 0.7% w/v) over a pre-coated microscope slide and a cover glass was gently placed over the slide. The slides were placed at 4°C for 5 min to allow gel solidification, the coverslips were removed and the slides were put 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 24 h, to allow cell lysis. Slides were removed from lysis buffer, cleaned and immediately placed in a horizontal electrophoresis box. Slides were exposed to either an equilibration solution (500 mM NaCl, 100 mM Tris, 1 mM Na_2EDTA, pH 8.0) or an alkaline solution (300 mM NaOH, 1 mM Na_2EDTA, pH > 13) for 20 min at 4°C, for neutral and alkaline comet assays respectively. Electrophoresis was performed in the same solutions at 250 mA (neutral) or 300 mA (alkaline) 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 silver stained after washing, fixing and rehydration (Nadin et al. 2001).

One hundred cells per individual (50 cells per replicate slide) were scored at 400× magnification using a conventional light microscope. Cells were visually divided 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). 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 (Tice et al. 2000; Hartmann et al. 2003).

Micronucleus test

Bone marrow preparations were made by using fetal calf serum; two smears were prepared from each mouse. Slides were stained in 5% Giemsa and coded for "blind" analysis. To avoid false negative results and as a measure of toxicity of the compounds to bone marrow, the polychromatic erythrocyte (PCE)/normochromatic erythrocyte ratio was scored in 1,000 cells. Data are presented as the frequency of micronuclei per 1,000 PCE. Historical lab data for mice treated with 25 mg/kg CP for 48 h were used as the positive control (MacGregor et al. 1987; da Silva et al. 2002).

Particle induced X-ray emission analysis

Quantification of Fe and Cu in the liver of treated animals was carried out using PIXE. This technique has a truly multielemental capability; that is, all elements with atomic number higher than 11 can be simultaneously detected in a single measurement on the same target (Johansson et al. 1995). The sensitivity is very good and varies smoothly as a function of atomic number. It is important to note that PIXE sensitivity depends on the sample being analyzed. Typically, sensitivity is of the order of a few parts per million. The analysis is relatively fast and the measuring time is a few minutes. Since this technique is non-destructive, it preserves the original samples, allowing extra measurements if required. Sample preparation in its solid form (for a variety of samples) does not require either sophisticated handling or chemical treatment, thus reducing drastically any chance of contamination. Nowadays, PIXE is widely used to characterize a variety of materials, including biological, geological and environmental samples (Kern et al. 2004; Braga et al. 2005; Franke et al. 2006).

For PIXE analysis the liver samples were deep frozen (–80°C), lyophilized and pelleted, as described by Franke et al. (2006). Measurements were carried out at the Ion Implantation Laboratory of the Physics Institute of the Federal University of Rio Grande do Sul. A 3 mV Tandetron accelerator provided a 2 MeV proton beam with an average current of 2 nA for the experiments. Details of the experimental set-up are described in Dias et al. (2002). The characteristic X-rays induced by the proton beam were detected with a lithium doped silicon detector with an energy resolution of 155 eV at 5.9 keV, which was positioned at an angle of 45°C with respect to the beam direction. The data was analyzed using the GUPIX code (Maxwell et al. 1989, 1995; Campbell et al. 2000) The standardization procedure was carried out using a bovine standard from NIST (SRM–1577b).

Quantitative PIXE analysis of a sample in a thick target approximation (pellets) requires a knowledge of its matrix composition. Therefore, another ion beam technique, Rutherford Backscattering Spectroscopy (Chu et al. 1978), was employed to obtain this information for the liver and bovine standard used to calibrate the PIXE analysis. The matrix composition consisted of approximately C (70%), O (15%) and H (15%).

Statistical analysis

Two-way ANOVA was used to compare the genotoxicity/mutagencity and the levels of iron and copper according to sex and treatment. One-way ANOVA with Bonferroni's multiple comparison test was used to compare the genotoxicity/mutagenicity within sexes. When data did not show homoscedasticity, data were Ln transformed. For the MN test, the Kruskal–Wallis with Dunn's post-hoc test was used to compare the mutagenicity within sexes. Student's *t*-test was used to compare the levels of iron and copper within sexes. Prior to analyses, homoscedasticity was tested by Bartlett's test and parametric or non parametric tests were used accordingly. The GraphPad Prism (Graph-Pad Software, San Diego, CA, USA) was used for statistical analyses. Significance was considered to be at $p<0.05$. Values are expressed as mean ± standard error.

Results and discussion

The group of male mice treated orally with 25 mg/kg CP showed DI values of 83.74 ± 25.95 and 98.51 ± 28.82 in the neutral and the alkaline comet assays, respectively. Laboratory data showed averages in the MN test of 12.25 ± 4.35 MN‰ for males and 11.25 ± 4.45 MN‰ for females after a single intraperitoneal dose of 25 mg/kg CP 48 h before death. These values show the validity of the experiments.

Iron and copper were generally genotoxic and mutagenic to both male and female mice in the treatment scheme. As for previous lab data, females always showed lower DNA damage than males, although not significantly. Data for genotoxicity, mutagenicity and hepatic iron and copper levels are presented for each sex as well as jointly, since there was no significant difference between sexes (Figs. 1, 2).

Copper induced more DNA damage than iron in the neutral comet assay and the MN test [about 58.6% (43.5 ± 4.5 versus 38.3 ± 6.5) and 13.5% (1.76 ± 0.31 versus 1.11 ± 0.16) higher, respectively]. On the other hand, the alkaline version of the comet assay was more sensitive to detect iron genotoxicity (Fig. 1). These data are in disagreement with a previous acute experiment (24 h after exposure) using alkaline conditions, which showed 20% more DNA damage for copper at the same dose, of similar toxicity (about 11% of the LD_{50}) (Franke et al. 2006). It seems that DNA damage can be different after chronic or acute exposure.

While the neutral comet assay is known to detect primarily double strand breaks (DSB), the alkaline version is sensitive to single strand breaks (SSB), excision repair sites (ERS) and alkali labile sites (ALS) (Tice et al. 2000). Both iron and copper seem to induce DSB at a considerable level. Double lesions consist of two modifications of the DNA in close proximity. There is general agreement that most double lesions arise from adjacent independent lesions. Nevertheless, some studies support the idea that these lesions can be generated by a single free radical-initiating event, often involving guanine (Box et al. 2001). The results indicate that iron may have induced more SSB and ALS than copper, since there was 41.5% more DNA damage caused by iron (45.3 ± 5.0) than by copper (32.0 ± 4.2) in the alkaline comet assay. Continuous treatment with iron probably led to an accumulation of oxidative lesions in DNA that act as ALS or ERS. Oxidative lesions and more specifically 8-OHdG [one of the most prevalent lesions induced by iron containing substances (Abalea et al. 1999)] are removed from the damaged DNA by a repair enzyme called hOGG1, one of the DNA glycosylases (Nakano et al. 2003). The action of these enzymes leads to excision of 8-OHdG and, therefore, to ERS and ALS.

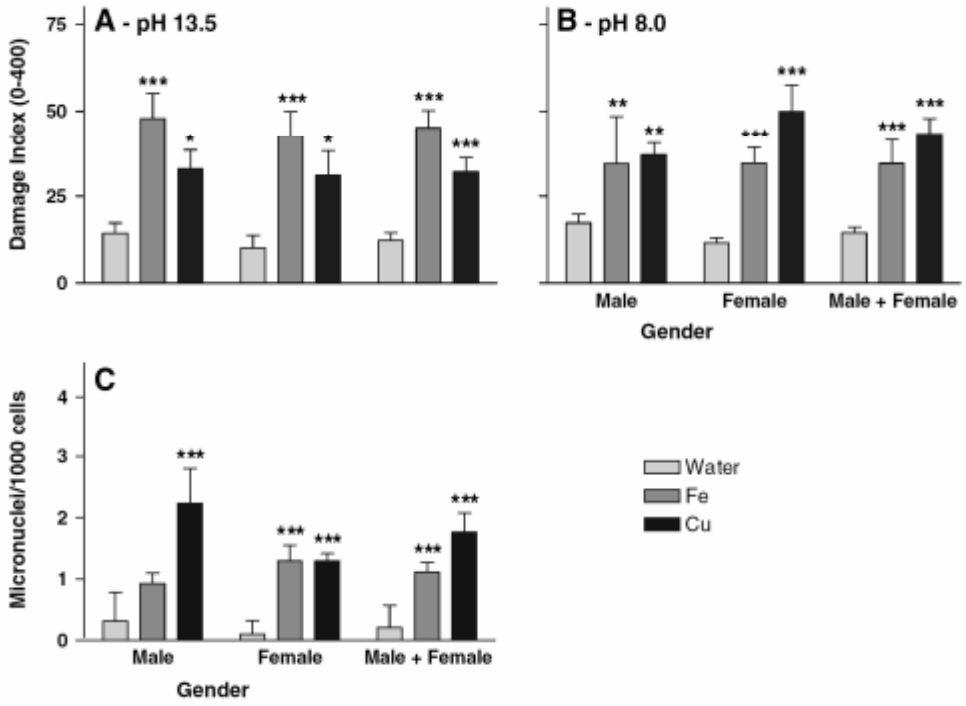

Fig. 1 Genotoxicity and mutagenicity of iron and copper to male and female mice, as evaluated by the Comet assay and the micronucleus test. (**A**) Comet assay under alkaline conditions (pH >13.5). (**B**) Comet assay under modified neutral conditions (pH = 8.0). (**C**) Bone marrow micronucleus test. Two-way ANOVA showed that DNA damage significantly differed between the treatments and controls and did not differ between genders for either the comet assay (alkaline and neutral) or the micronucleus test. *Asterisks* indicate significant differences from the group treated with water (control) using Dunn's post-hoc test at $^{*}p < 0.05$, $^{**}p < 0.01$ or $^{***}p < 0.001$. Averages ± standard errors. $N = 5$ mice of each sex

Copper is more reactive in vitro than iron (Cai et al. 1995; Oikawa and Kawanishi 1998), possibly because of the ready cycling between cuprous and cupric states, which share the same genotoxicity (Tkeshelashvili et al. 1991). This can be enhanced by interaction with oxidants. For instance, Yoshino et al. (1999) showed that the interaction of copper with vitamin C led to five times more DNA damage than that with iron. On the other hand, Lloyd et al. (1998) showed that Fe(II) induced significantly more 8-OHdG than Cu(II) in vitro. While hydroxyl radicals are thought to be major intermediaries in iron induced base oxidation (Tkeshelashvili et al. 1991), copper compounds oxidize DNA in complexes formed with other molecules (Yamamoto and Kawanishi 1989; Fujimoto et al. 2005), usually produced within the biological system (Linder 2001).

DNA adducts formed from oxidative reactions mediated by metals may also be involved in the mutagenicity of transition metals. They can be formed by inter or intrastrand depurinations that have aromatic adduct-like characteristics (Lloyd et al. 1997). The results of a comparative study by Lloyd et al (1997), testing the induction of cross link-like lesions and strand breaks by several metals in vitro, showed that, while the copper(II)/hydrogen peroxide system produced by far the highest yield of bulky lesions and strand breakage, the iron(II)/hydrogen peroxide system induced a low proportion of DNA bulky lesions and an intermediate level of DNA strand breaks. The copper system also generated significant levels of bulky lesions in DNA that were unique to this system. Given the lack of correlation between DNA strand breaks and bulky adducts, the authors concluded that it was likely that the lesions arise via two different mechanisms, perhaps involving the association of transition metal ions with different regions of the double helix and different moieties of individual nucleotides (Lloyd et al. 1997). DNA cross-linking can be detected by the Comet assay, as demonstrated by retardation in the rate of DNA migration (Tice et al. 2000) and thus a reduction in DNA damage as evaluated by this method. Therefore, it is possible that cross-links induced by copper cause a detrimental reduction in DNA migration. However, further studies are needed to confirm this.

Although copper and iron share some common features in transport, most of their metabolism and storage is different. While iron is needed in much higher amounts and is stored within the cells either in ferritin and in the labile iron pool (Kruszewski 2003), an intracellular pool of free copper is almost non-detectable (Rae et al. 1999; Linder 2001). Thus, as expected, the hepatic level of iron was much higher in mice treated with iron than in controls. In the case of copper, the same trend was not seen, except for females treated with the metal, which showed a significantly higher hepatic level of copper (Fig. 2). This can be explained by the fact that excess copper is not stored in the liver. Analysis of the data of Uriu-Adams et al. (2005), who gavaged rats with a single dose of ^{67}Cu, revealed minor fluctuations in retention of the isotope in the liver up to 5 days after treatment. Conversely, a progressive increase in the percentage of retained ^{67}Cu was noticed in muscle, skin and

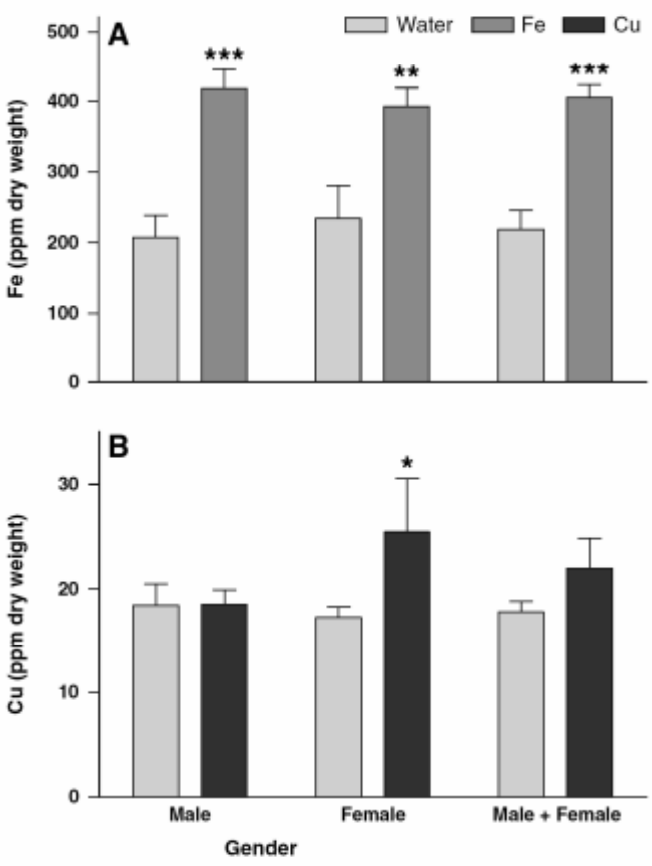

Fig. 2 Hepatic level of iron (**A**) and copper (**B**) of mice treated with iron and copper, respectively. Two-way ANOVA showed that the level of the metals differed significantly between the treatments and controls and did not differ between genders. *Asterisks* indicate significant differences from the group treated with water (control) using Student's *t*-test test at $^{*}p < 0.05$, $^{**}p < 0.01$ or $^{***}p < 0.001$. Averages ± standard errors. $N = 5$ mice of each sex

bone. These tissues accounted for about 50% of all ^{67}Cu retained 5 days after treatment (Uriu-Adams et al. 2005). With respect to iron, the liver is the key storage organ and metabolism is through hepatocytes (major storage site) and mastocyte-like Kupfer cells (major recycler of iron derived from senescent red blood cells and storage in overload situations) (Anderson and Frazer 2005).

The Recommended Dietary Allowance (RDA), or the amount supposed to meet the nutritional requirement of 97.5% of the healthy population (Schumann 2001), is of 900 μg/day copper for adult men and women. For iron, the RDA is of 8 mg/day for all age groups of men and postmenopausal women and of 18 mg/day for premenopausal women. The tolerable upper intake level (UL) for adults is of 10 mg/day copper, based on protection from liver damage as the critical adverse effect, and of 45 mg/day iron, based on gastrointestinal distress as an adverse effect (Institute of medicine, IOM 2001). If we extrapolate the doses administered to mice in this study to a standard 65 kg human subject, the doses would account to about 0.54 g/day of copper and 2.16 g/day of iron, corresponding to about 50 times the UL for either copper or iron.

There are few studies addressing the harm of copper to human subjects as well as its average consumption as supplement. It has been shown that the daily drink of about 5 mg/day copper can lead to gastrointestinal distress and there were no liver-related adverse effects from daily consumption of 10–12 mg/day of copper in foods. Notwithstanding, it was shown that the chronic consumption of 30 mg/day copper as supplements can lead to liver failure (Institute of medicine, IOM 2001).

Accidental iron overdose is the most common cause of poisoning deaths in children under 6 years of age in the United State. Poisoning symptoms occur with doses between 20 and 60 mg/kg of iron, with the low end of the range associated primarily with gastrointestinal irritation while systemic toxicity occurs at the high end (Institute of medicine, IOM 2001) and dose between 10 and 20 mg Fe/kg are regarded as non toxic to humans (Schumann 2001). The dose used in this study was in the lower limit of this range. Regarding the dietary source of iron, Troppmann et al (2002) showed that adult Canadian individuals users of multivitamin supplementation have significantly higher iron intake than non users in all age group either females or males (40–85% more), although the average intake of iron in this group was lower than the UL.

The current results show significant genotoxicity for iron and copper at the high doses tested. The ingestion of trace nutrients is increasing as a result of the consumption of enriched foods, as well as of multiple vitamin–mineral supplement tablets and the consumption of high level of nutrients as supplements in not unusual (Troppmann et al. 2002). Many ideas about the physiology of metals have being questioned and there is growing evidence that copper and iron might be associated to DNA through in situ reactions, leading to genome damage (Tkeshelashvili et al. 1991; Meneghini 1997). Recent studies have even shown that phytochemicals can mobilize copper stores, leading to genotoxic effects (Azmi et al. 2006). In another had, iron accumulation and deficiency has been linked to several types of cancer (Ahmed 2004), as well as to cardiovascular (Cabantchik et al. 2005), neurodegenerative (Fredriksson et al. 1999) and other diseases (Oppenheimer 2001; Vyoral and Petrak 2005). Further studies on the physiology of copper are still needed before we can make more definitive statements. We need to know, for example, whether similar effects would be observed at lower doses than those tested. It is essential to elucidate the correct dosages of iron and copper to improve health without leading to noxious effects under various metabolic situations.

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9.8 ANEXO H “An antioxidants rich diet can suppress the genotoxicity but not the mutagenicity of iron in mice”

Manuscrito submetido ao periódico *Biometals.*

An antioxidant-rich diet can suppress the primary DNA damage but not the clastogenicity/aneugenicity induced by iron in mice

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Short running title: Effect of Diet over iron genotoxicity and clastogenicity

Abstract
Despite being essential, iron at high amounts and, particularly, in high oxidative situations, can be genotoxic, mutagenic and carcinogenic. Moreover, there is a general lack of information regarding the potential role of diet in reducing iron toxicity. Therefore, the aim of this study is to address the possible influence of a high iron standard diet (SD) and a lab prepared antioxidant-rich grain-based diet (GD) over iron primary DNA damage and clastogenicity/aneugenicity in mice orally treated for 6 days with $FeSO_4$ (33.23 mg of Fe/Kg), by the comet assay in leukocytes and the frequency bone marrow micronuclei (MN), respectively. The level of iron in mice livers and blood as well as the level of iron and other metals in diets were also evaluated by different methodologies. Results indicated similar levels of iron in whole blood and higher hepatic level in mice treated with iron irrespectively of the diet they were fed. SD diet had 4 times more iron than GD. Fe induced DNA damage increase only in mice fed SD, which also showed higher DNA damage background. Iron treatment induced MN in either SD or GD fed mice and the MN frequency was positively associated to the hepatic level of iron. Regarding the diets composition, the content of several metals was significantly higher in SD than in GD, having SD about 4-fold more Fe than GD. It is likely that the higher content of antioxidants present in GD could have accounted for reducing the primary DNA damage of iron, but not its clastogenicity/aneugenicity.
Key words: ferrous sulfate; liver; bone marrow; iron accumulation; diet; DNA damage.

Introduction
Many of the cancers common in the Western world are thought to be related to dietary habits. Among the known risk factors, many will act through increasing the probability of mutation which arise through the reaction of metals (Ferguson et al. 2004). Endogenous or acquired by diet, antioxidants are able to neutralize the toxicity of metals by scavenging and/or blocking the oxidative cascades triggered by them (Halliwell and Guterridge 2000). There are numerous evidences linking adequate serum level of vitamins and minerals with lower level of mutations and cancer incidence. Moreover, it is generally accepted that diets poor in antioxidants can modulate the rhythm of tumor progression in mice tumor's models (Salganik et al. 2000).

Some nutrients present a linear dose/frequency of consumption relation associated to a decrease in the risk of cancer. On the other hand, for other nutrients a typical "U" shaped curve is observed, with low and high levels being noxious particularly for individuals exposed to pollutants (Nyberg et al. 2003). This seems to be the case for iron. Iron is one of the most abundant elements in universe and the most abundant element, by weight, in earth, being a

micronutrient required for almost all organisms that plays key roles in biological systems (Valko et al. 2005). It is the key component of hemoglobin and has main roles in immune and nervous system as well as for the oxidative and antioxidant metabolism (Ortiz et al. 2004; Arredondo and Nunez 2005). Despite being essential, Fe can be toxic particularly due to its participation in oxidative stress reactions (Halliwell and Guterridge 2000). In previous studies, Fe was found to be genotoxic to mice blood cells as evaluated by the alkaline version of the comet assay (Franke et al. 2005). We also observed that whole compounds with known oxidant/antioxidant potential (i.e. orange juice) or micronutrients in isolation (i.e. vitamin C) could increase the genotoxicity of test compounds and a general agreement among the level of metals (i.e. Fe and Cu) in blood and DNA damage was detected (Franke et al. 2006).

In spite of the fact that metals and antioxidants, among other substances, can influence the genotoxicity of test compounds, there is a lack of data relating diet quality and the response of genotoxicity tests. Therefore, we aimed to evaluate the effect of two diets over the genotoxicty and mutagenicity of iron. To achieve this goal, we orally treated two groups of mice for 7 days with $FeSO_4$, being each group fed with a high iron standard diet (SD) and a lab prepared antioxidant-rich grain-based diet (GD). At the end of the treatment the level of DNA damage in whole blood was evaluated by the comet assay as a genotoxicity endpoint and the frequency of micronucleus (MN) was evaluated in bone marrow as the mutagenicity endpoint. Moreover, the accumulation of iron in the liver and whole blood of the mice was evaluated by Particle Induced X-ray Emission (PIXE). Additionally, the level of iron in diets was evaluated by PIXE, Atomic Absorption Spectrophotometric after Dry Ashing (AASDA) and Inductively Coupled Plasma-Optical Emission Spectrometry (ICPOES).

Materials and methods

Determination of metals in diets and mice tissues

In order to obtain the metal composition of the diets as well as the whole blood and hepatic levels of iron, the PIXE (Johansson et al. 1995) technique was employed as described by Franke *et al*. (2006). AASDA and ICP-OES were used to obtain other measures of iron composition in the diets. AASDA was performed in accordance with the guidelines of AOAC INTERNATIONAL (2000). Briefly, the samples were burned at 270^{o}C, digested in HCl and eluted to the equipment and the metal determination was accomplished by an external calibration curve with iron standard solutions. ACP-OES was in accordance with AOAC INTERNATIONAL (2000). Briefly, the samples were dried at 75^{o}C and extracted by using nitric-percloric solution and eluted to the equipment.

Mice diets and iron treatment

A commercial heavy iron standard mouse cube diet (Nuvilab, CR1, Moinho Nuvipal Ltda., Curitiba, PR, Brazil), named as SD, was used as heavy iron/low antioxidant diet. Another mouse cube diet, named as GD, was prepared from a mixture of grains composed by sunflowers seeds, corn, soybeans, peanuts, and flaxseed and used as low iron/high antioxidants diet. Grains were triturated to a fine powder and then pressed into cubic pellets. Table 1 shows the levels of metals in the diets according to PIXE analysis. The content of all metals was significantly higher in SD than GD, excepting for strontium (Sr) which was not detected in GD. Remarkable levels of titanium (Ti) were observed in both diets while low levels of Sr were found only in GD. Figure 1 shows the comparison of the levels of Fe and Cu obtained by PIXE, AASDA and ICP-OES analytical techniques. According to PIXE data, the level of Fe was about 8 times higher in SD than in GD (see table 1). AASDA showed lower levels of Fe than PIXE, but 7 fold more Fe in SD than in GD (860 ± 268 and 121 ± 8 mg/Kg Fe, respectively) while ICP-OES showed about 70% more Fe in SD than in GD (1000.00 ± 0.2) versus 581 ± 47). In average, SD presented about 4 fold more Fe than GD (1148 ± 222) and 304 ± 141 mg/Kg Fe, respectively). AASDA and ICP-OES data showed very similar

levels of Cu than PIXE for SD (16.3 ± 2.3, 21.5 ± 0.7 and 17.4 ± 4.5 mg/Kg Cu, respectively) and GD (7.6 ± 0.4, 14.0 ± 0.1 and 10.2 ± 2.5 mg/Kg Cu, respectively). Two-Way ANOVA showed the content of Fe and Cu differed between diets (P<0.001), but not between methodologies (P>0.07). The content of protein in diets was of 22.7 ± 0.4 and 17.4 ± 0.9 g/100 g for SD and GD, respectively.

Table 1. Level of transition metals in standard diet (SD) and grains diet (GD), as evaluated by Particle Induced X-ray Emission (PIXE)

Element	mg/Kg*	
	SD	GD
Ti	64 ± 2	21.4 ± 2.3
Mn	89.6 ± 3.5	46.5 ± 2.9
Fe	1585 ± 96	211 ± 5
Cu	17.4 ± 2.3	10.2 ± 2.5
Zn	172 ± 23	54.7 ± 5.4
Sr	ND	8.9 ± 5.6

Average ± SE; ND = below detection level

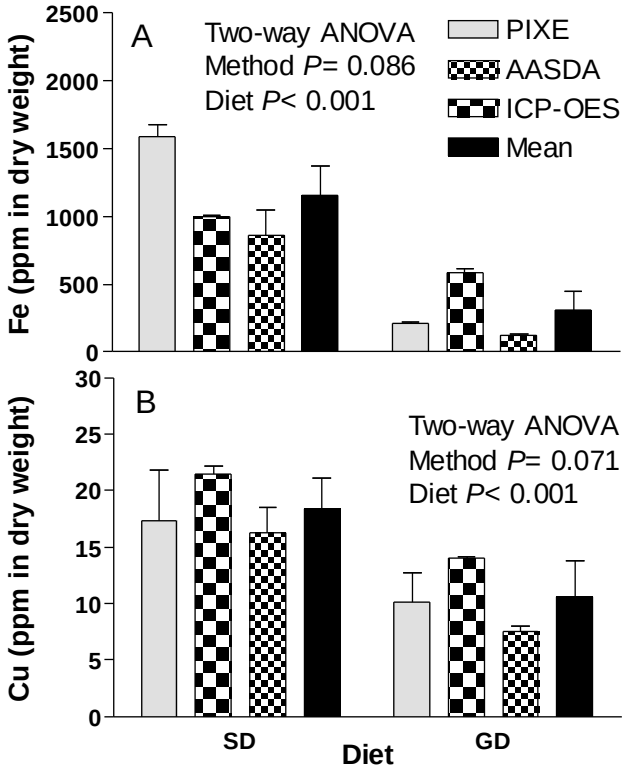

Figure 1. Level of iron (A) and copper (B) in standard diet (SD) and grains diet (GD), as evaluated by Particle Induced X-ray Emission (PIXE), Atomic Absorption Spectrophotometry after Dry Ashing (AASDA) and Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES).

CF1 male mice, aged 5-7 weeks and weighing from 27 to 32 grams, were obtained from the State Foundation for Production and Research in Health (FEPPS), Porto Alegre, RS, Brazil. As soon as mice arrived to the laboratory they were separated into five groups of 5

male mice. The mice were acclimatized to laboratory conditions (22º ± 3ºC and 60% humidity) for seven days. During this period, 2 groups received SD (water and $FeSO_4$ groups) and 2 groups received GD (water and $FeSO_4$ groups). All groups received cube diets and water *ad libitum*. 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, Brazil. Fe (33.23 mg Fe/Kg body weight as $FeSO_4.7H_2O$), CAS 7782-63-0, Ducto, Brazil) or distilled water were administered by gavage (0.1 ml per 10 grams of body weight) with a single dose for 6 days. Fe dose was equal to 10.86 % mouse oral LD_{50} (Budavari et al. 1996), being used in previous studies (Franke et al. 2005; 2006). All substances were prepared just before treatment and protected from light.

Tissue sampling

For comet assay, Blood was obtained at 7 days after the beginning of the experiment from mouse tail tips (about 15 μL) by means of a small incision and immediately mixed with 7 μL sodium heparin (Liquemine®, Roche, Brasil). PIXE was used to evaluate the level of iron in whole blood and livers of the treated animal. For obtaining the samples, mice were deceased by decapitation and the whole blood and liver were dissected. Whole blood and liver samples were deep freezed (-80C), lyophilized and pelleted as described by Franke et al. (2006).

Comet Assay

The alkaline version of the comet assay was performed as described by Franke et al. (2005a; 2005b; 2006) and in accordance the guidelines for conducing the Comet assay in vivo (Tice et al. 2000; Hartmann et al. 2003; Burlinson et al. 2007). Briefly, an aliquot of blood cells/heparin mixture were embedded in LMP agarose (final concentration of 0.7 % w/v; Gibco, US) and spread over a pre-coated microscope slide and a cover glass was gently placed over the slide. The slides were placed at 4°C for 5 min to allow gel solidification, the coverslips were removed and the slides were put in lyses buffer (2.5M NaCl, 100 mM EDTA and 10 mM Tris, pH 10.00–10.50, with freshly added 1% Triton X-100 and 10% DMSO; Sigma, US) for 24 hours, to allow cell desegregation. Slides were removed from lyses buffered, cleaned and immediately placed in a horizontal electrophoresis box. They were then exposed to alkali (300 mM NaOH, 1mM Na_2EDTA, pH > 13) for 20 min at 4° C, to allow DNA unwinding. Electrophoresis was performed at 300 mA and 25V (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 silver staining (Nadin et al. 2001). Briefly, slides were washed and fixed, rehydrated and stained. 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 scored at 400 X magnification using a conventional light microscope. Cells were visually ascertained 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 x 0) to 400 (maximum damage: 100 cells x 4). 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 (Tice et al. 2000; Hartmann et al. 2003).

Micronucleus test

Bone marrow preparations were made by using fetal calf serum and two smears from each mouse were made. Slides were stained in 5% Giemsa (Merck, US) and coded for a "blind" analysis. To avoid false negative results and as a measure of toxicity of the compounds on bone marrow, the polychromatic erythrocytes (PCE) and normochromatic erythrocytes (NCE) ratio was scored in 1000 cells. Data are presented as the frequency of micronuclei per 2000

PCE (MacGregor et al. 1987; Da Silva et al. 2002). The historical data of the laboratory of mice treated with 25 mg/kg cyclophosphamide was used as positive control.

Statistical analysis

The Two-Way ANOVA was used to asses simultaneously the effect of the treatment (water or $FeSO_4$) and the diet (SD or GD) on the DNA damage and micronuclei frequency as well as on the level of iron in the blood and in the livers of the mice. The Neuman-Keuls test was used to compare ranked means between the different treatments. Prior to analyses, homoscedasticity was tested by Bartlett´s test and Ln transformation was applied when needed. In order to identify correlations between the variables we used Pearson or Spearman correlation equations. The GraphPad Prism (GraphPad Software, San Diego, CA) was used for statistical analyses. Significance was considered to be at $P < 0.05$. Values are expressed as mean ± standard errors of the mean.

Results

The level of iron in whole blood was generally similar between mice treated with iron or water (controls), however, it was higher (P <0.001) in controls fed SD (Fig. 2A). Mice fed SD showed a slightly higher (non significant) hepatic level of iron than those fed GD (Fig. 3A), but the hepatic levels of iron was about 2-fold higher (P <0.001) in those treated with iron than in controls independently of the diet.

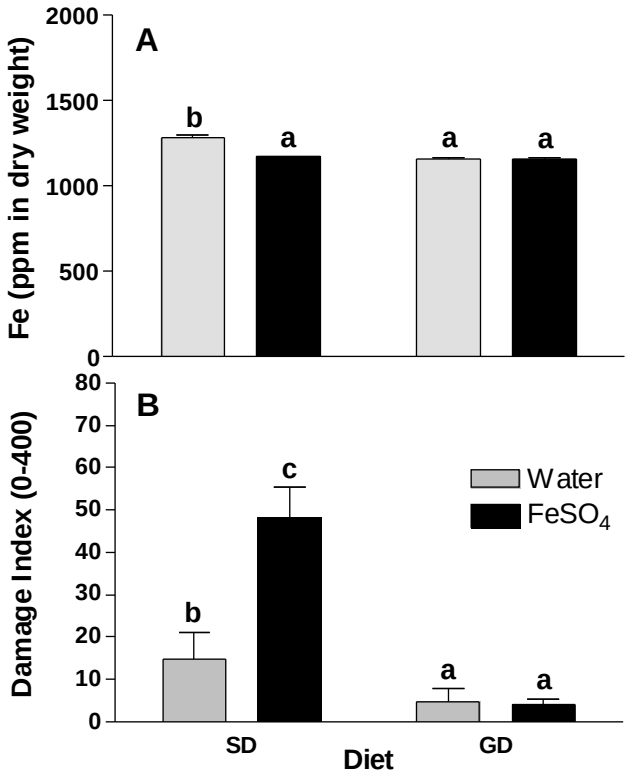

Figure 2. Whole blood level of iron as evaluated by Particle Induce X-ray Emission - PIXE (A) and DNA damage as evaluated by the alkaline version of the comet assay in blood cells (B) of mice maintained under in standard diet (SD) and antioxidant rich grains diet (GD) and treated with iron ($FeSO_4$) or water. Different letters indicate the rank of the averages at P<0.05 according to Two-Way ANOVA followed by Neuman-Keuls post-hoc test.

Figure 2B shows the whole blood DNA damage induced by Fe in mice maintained under SD or GD as evaluated by the alkaline version of the comet assay. $FeSO_4$ treatment was genotoxic only for mice maintained under SD. Moreover, control mice fed SD showed a higher DNA damage background ($P < 0.001$).

Figure 3B shows the frequency of bone marrow MN per 2000 cells induced by Fe in mice maintained in the SD or GD diets. Iron treatment increased MN frequency in mice fed either SD or GD ($P= 0.012$). No significant difference was observed in terms of the mitotic index (data not shown). The positive control of our laboratory for male mice is of 12.25 ± 4.35 MN ‰. Interestingly, the hepatic levels of Fe and the micronuclei frequency were significantly correlated when all mice tested were pooled (r= 0.5; P <0.05) (Fig. 4).

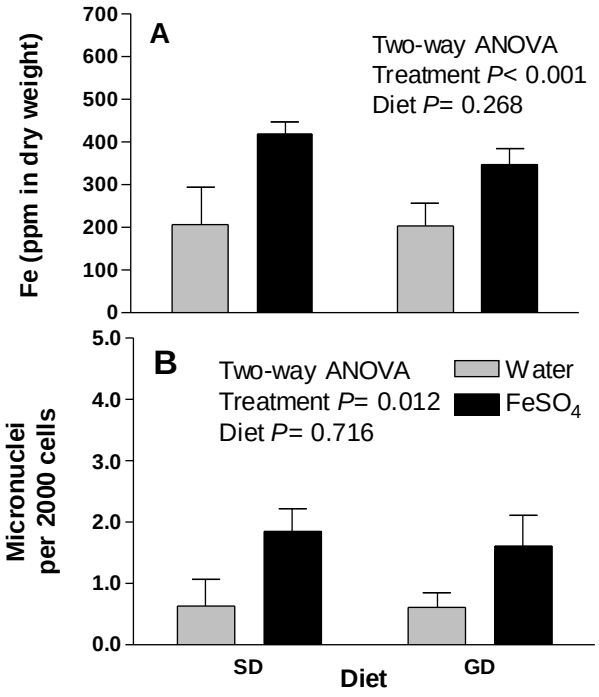

Figure 3. Hepatic iron level as evaluated by Particle Induce X-ray Emission – PIXE (A) and frequency of bone marrow micronuclei (B) of mice maintained under in standard diet (SD) and grains diet (GD) and treated with iron ($FeSO_4$) or water.

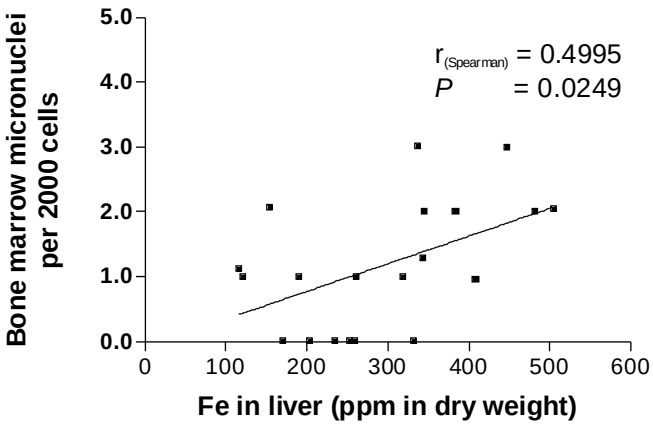

Figure 4. Correlation between the level of iron in the liver and the frequency of bone marrow micronuclei of mice maintained under SD and GD diets and treated with iron ($FeSO_4$) or water.

Discussion

The protein contents observed in diets was in agreement with the level reported by Demigné et al. (2006) of 21.4 and 14.3 g/100g for a westernized and a standard diet, respectively. Moreover, they agree with those of the "AIN" (American Institute of Nutrition) diets AIN-73 and 93, which are among the best standard diets for rodents (Reeves et al. 1993). In this sense, mice fed SD or GD did not differ in food intake (data not shown).

In agreement with our previous result (Franke et al. 2006) as well to Rouhead and Hunt (2000), it was observed in the present study a lower level of Fe in blood of Fe treated mice than in controls; which has been considered as a result of the active clearance induced after the intake of Fe (Franke et al. 2006). Liver is the key organ for iron metabolism and storage (Anderson and Frazer 2005). Indeed, hepatic levels of iron level increased in the organ of iron treated mice irrespectively to the diet, indicating a similar clearance rhythm for iron in both groups.

Iron compounds generate reactive oxygen species (ROS) through Fenton and Haber-Weiss chemistries (Halliwell and Guterridge 2000). Iron compound can induce DNA strand breaks (Franke et al. 2005; Franke et al. 2006), micronucleus (Prá et al. 2007), sister chromatic exchange (Tucker et al. 1993), base tautomerization (Abalea et al. 1999; Dunkel et al. 1999), as well as viral enhancement (Heidelberger et al. 1983). There are well characterized murine models of iron-induced carcinogenesis(Toyokuni 1996), however iron carcinogenicity to humans needs further classification (Huang 2003; Hercberg et al. 2005).

Although there is a lack of studies addressing the effects of antioxidant rich diets over iron toxicity, there are inconclusive results evaluating four classes of compound against iron-induced oxidative stress and genomic instability, namely the iron chelator desferoxamide (Shackelford et al. 2003; Shackelford et al. 2006) and phenolic compounds (Abalea et al. 1999; Halliwell et al. 2005) as well as the antioxidant vitamins C (Chen et al. 2000; Premkumar and Bowlus 2003; Franke et al. 2005; Kang et al. 2005) and E (Record and Jannes 2000; Carrier et al. 2002; Winklhofer-Roob et al. 2003). In the present study, GD was able to inhibit the primary DNA damage induced by iron to leukocytes but not the frequency of bone marrow micronuclei, in agreement with the concept that the level of DNA lesion detected by the comet assay is negatively correlated to the intake of fruits and antioxidants (Hofer et al. 2006). There are evidences of antioxidant and antigenotoxic properties of all components of GD (Table 2). Conversely, GD was not able to reduce the increase in MN frequency induced by Fe treatment. Premkumar & Bowlus (2003) observed a significant reduction in bone marrow micronucleus in mice treated with 300 mg/kg Fe as $FeSO_4$ by the co-administration of 15 g/kg diet vitamin C during a 3 weeks period. Notwithstanding, according to Halliwell (2001), there are no compelling evidence to date that ascorbate supplements can decrease the levels of oxidative DNA damage in vivo, there are sounding evidences that the co-administration of iron and vitamin C induces oxidative stress (Halliwell et al. 2000; Lachili et al. 2001), as well as that high levels of this nutrient induce apoptosis (Sakagami et al. 2000), possibly through major influences in iron metabolism (Kang et al. 2005). The upper level of vitamin C to humans is of 1,800 mg/day (IOM 2000), which would correspond to 30 mg/ Kg to a 60 Kg individual and almost virtually ingested only through diet. In fact, our result support that a dose of 30mg/Kg of vitamin can induces high acute DNA damage increase in mice, as evaluated by the comet assay (Franke et al. 2005). Regarding vitamin E, in agreement with a non protective effect agains iron-induced micronuclei, a study of Record ad Jannes (2000) observed that the supplementation with the alpha -tocopherol has no apparent

protection against oxidative genotoxicity, as judged by the formation of micronuclei in rat splenocytes subjected to oxidative stress ex vivo.

Table 2. Reported protective effects against oxidative stress and DNA damage of grains diet (GD) components.

Food	Examples of main protective components	Reported protective effects (Reference)
Flaxseed	α-linolenic acid, secoisolariciresinol diglucoside (lignan), matairesinol and pinorecinol	counteracts the toxicity of CCl_4 by the modulation of hepatic enzymes (Rajesha et al. 2006)
Peanut	Dihydroquercetin, luteolin and/or ethyl protocatechuate	Antioxidant (Huang et al. 2003) and antimutagenic(Yen and Duh 1996) effects
Sunflower	High content of α-linoleic acid in oil	Controversial effects, in one hand quenching radicals (Ramadan and Moersel 2006), but in the other hand generating a marginal increase in oxidative DNA-damage (Eder et al. 2006)
Soy	Several isoflavones, mainly genistein	Controversial cardiovascular protection and reduction of cancer incidence, through blockage of initiation and progression (Sarkar and Li 2003; Sirtori et al. 2005)
Corn	Greater total phenolic content than wheat, oats, and rice	Antioxidant (Li et al. 2007) as well as antimutagenic properties (Pedreschi and Cisneros-Zevallos 2006), depending largely on grain composition; with greater total antioxidant activity than wheat, oats, and rice (Adom and Liu 2002)

It is also important to highlight that GD was prepared without any thermal treatment, just before the feeding of the animals. This approach could have accounted for a higher preservation of several micronutrients and other bioactive compounds. Vitamins, such as folate (McNulty and Pentieva 2004) an panthotenic acid (Mahan and Escott-Stump 1996), are poorly stable under typical conditions of cooking which can substantially reduce the amount ingested, limiting their health effects. Storage is another limiting factor for vitamin availability, since many vitamins like pyridoxine (Mahan and Escott-Stump 1996), riboflavin, vitamin C (Vercet et al. 2001) and folate (McNulty and Pentieva 2004) which are easily oxidized under normal environmental conditions, can be lost along time.

The increase in MN frequency for mice fed either GD or SD give rise to an interesting aspect and in spite of its antigenotoxic property, GD was not able to affect the mutagenicity of iron. It is likely that GD only protects the DNA of the blood cells due to a higher transportation of iron to visceral organs. Therefore, GD seems not to play the same role in blocking the formation of micronuclei in bone marrow as it does in blood white cells. Bone marrow is a hotspot for iron accumulation, presenting a higher influx rate compared with the liver (the key organ of iron metabolism) after the injected of radio-labeled iron (Beshara et al. 1999). The hepatic level of iron seems to be a good predictor of iron toxicity, given the positive correlation between MN frequency and hepatic iron level seen in this study. We have observed a similar correlation between iron hepatic level and the DNA damage as evaluated by the comet assay in white blood cells in a previous study (Franke et al. 2006).

The amounts of Ti and Sr found in GD are within the expected level for foodstuff (István and Jones Jr. 1997). The levels of Mn, Cu and Zn in SD and GD diets and Fe in GD diet were close to the levels of AIN diets (Reeves et al. 1993) or the standard and westernized diets used by Demigné et al. (2006). Although the level of Fe in SD was high, being in average five times higher than in GD, it does not seem to have any considerable influence in iron toxicity. Indeed, Roughhead et al. (1999) have showed that dietary Fe intakes 10-fold greater than required did not induce noteworthy oxidative stress increase in female rats, although it was a weak predictor of liver thiobarbituric acid reactive substances (TBARS).

In conclusion, even in the high dose tested, an antioxidant-rich diet can suppress the primary DNA damage induced by iron in leukocytes induced iron as evaluated by the comet assay, but does not seem to interfere with its clastogenicity/aneugenicity, as evaluated by MN in bone marrow cells. It is interesting to highlight that bone marrow is one key target sites for iron metabolism and that a high antioxidant diet can not suppress iron toxicity in this site. Therefore, the relation between the level of iron and antioxidants in diet must be further evaluated when addressing iron toxicity, particularly because excess iron intake is likely carcinogenic and iron bioavailability in diet has been increasing in parallel to the rise in the incidence of some cancers (Crawford 1998).

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9.9 ANEXO I: “Vitamin C does not protect against recognition memory impairment induced by neonatal iron treatment and is genotoxic to rat brain”

Manuscrito a ser submetido ao periódico Neuroscience

Vitamin C does not ameliorate recognition memory impairment induced by neonatal iron treatment and is genotoxic to male rat brain

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Abstract

Iron (Fe) can be either essential or harmful to brain, since its accumulation has been implicated in the pathogenesis of neurodegenerative disorders such as Parkinson's disease. When Fe is administered to mice and rats at the neonatal period it accumulates in particular spots of the brain (e.g. substantia nigra) where it triggers oxidative stress as well as induces memory impairment. Iron-induced memory impairment can be reverted with some drugs such as monoamine oxidase (MAO) inhibitors or desferoxamine (an iron chelator agent). Vitamin C (vitC) plays key roles in iron transport and metabolism, has a controversial effect on genomic stability and is suggested as a protector against memory impairment. The aim of this study was to evaluate, in rats treated with Fe in neonatal period, the effect of vitC over: a) memory impairment; b) DNA damage in brain and blood; c) the level of iron in blood. Given the fact that neurodegeneration takes place during adulthood as a consequence of long term iron burden, we designed the study as follows: rats were treated with vehicle or 10.0 mg/kg of Fe^{++} at postnatal days 12 to 14 – the peak of iron uptake by brain – and, then, with two doses of vitC in the adulthood (1 mg/Kg, equivalent to the human daily recommended intake – DRI and 30 mg/Kg, equals to the human upper level - UL). After the last administration of vitC, the rats were submitted to a novel object recognition task and the blood and brain tissues were dissected for evaluating DNA damage, by using the comet assay, and iron level, by using the Particle Induced X-ray Emission (PIXE). Results indicated that vitC was not able to protect against iron-induced recognition memory deficits. It was also observed that Fe treatment did not induce an increase in DNA damage in rat white blood cells and that vitC treatment induced an increase in DNA damage irrespectively to Fe treatment in substantia nigra cells.
Key word: substantia nigra; cortex; recognition memory; DNA damage; comet assay; iron level; ascorbic acid.

1. Introduction

VitC is a water-soluble glucose derivative antioxidant vitamin involved in oxi-reduction reactions(Halliwell 2001), including quenching of free-radicals, collagen synthesis, cellular

differentiation {Li, 2007 #71}, as well as elaboration and functioning of nervous tissue (Bourre 2006). The consumption of foods rich in vitC is associated with decreased risk of cardiovascular disease, of many types of cancer and possibly of neurodegenerative disease (Vijayalaxmi and Venu 1999; Halliwell 2001; Edenharder, Sager et al. 2002; Edenharder, Krieg et al. 2003). Notwithstanding, several studies have shown that vitC can display ambiguous genotoxic effects (GENE-TOX 2007), acting as a pro-oxidant by reaction with some metals (Stadler, Tursky et al. 1994; Fenech and Ferguson 2001; Halliwell 2001). Indeed, vitamin C has a key role over iron absorption and transport, including within brain (Davidsson 2003){Bourre, 2006 #20}. vitC reduces iron, making it prone to be a strong oxidant (Oikawa and Kawanishi 1998; Valko, Izakovic et al. 2004).

Iron treatment within 12-14 days of life causes a selective iron accumulation in brain regions, especially in the basal ganglia, which was associated with behavioral changes in adult mice (Fredriksson, Schroder et al. 1999; Fredriksson, Schroder et al. 2000) and rats (Schroder, Fredriksson et al. 2001); possibly through the induction of long lasting lipid peroxidation and protein carbonylation in substantia nigra (Dal-Pizzol, Klamt et al. 2001). Iron genotoxicity is well characterized, mainly through the generation or reactive oxygen species by Fenton and Haber-Weiss chemistry [for review see {Pra, 2007 #79}]. We have also provided some evidences that vitC migh enhance iron genotoxicity {Franke, 2005 #51}.

Recent reviews enthusiastically support the view that vitC can ameliorate ageing-related degenerative diseases (Li and Schellhorn 2007){Li, 2007 #72}. However, there are few studies showing that vitC could improving memory either in animal and human models {e.g. (Parle and Dhingra 2003; Bourre 2006)]. Memory impairment amelioration by vitC treatment could be expected since other bioactive compound can mediate this effect. In this sense, it has shown that neonatal iron-induced memory deficits can be reverted by the adulthood treatment with selegiline [an monoamine oxidase (MAO) inhibitor] (de Lima, Laranja et al. 2005) and desferoxamine [an iron chelator an antioxidant] (de Lima, Presti-Torres et al. 2007), indicating that the blockage of oxidative stress can ameliorate iron burden. In agreement, there are growing evidence that neurodegeneration and cognitive decline are conditions marked by an age-related increase in oxidative damage (e.g. by accumulation of iron) and decrease in antioxidant levels (Droge and Schipper 2007). Indeed, the supplementation with antioxidant compound during adulthood is a consensual approach toward blocking the progression of neurodegenerative diseases, such as VitC for Alzheimer's Disease (Frank and Gupta 2005).

The aim of this study was to evaluate the effect of vitC treatment during adulthood in male rats overloaded with iron in neonatal period, using the following biomarkers: a) memory impairment; b) DNA damage in brain and blood; c) the level of iron in brain and blood. The experimental protocol followed our consensual treatment scheme (de Lima, Presti-Torres et al. 2007). Initially newborn male rats were treated with vehicle or 10.0 mg/kg of Fe^{++} at postnatal days 12 to 14 with and, then, treated with vitC [1 mg/Kg, equivalent to the human daily recommended intake – DRI and 30 mg/Kg, equals to the human upper level – UL (Franke et al, 2005) for 21 days starting at the age of 60 months. After the last administration of vitC, the rats were submitted to a novel object recognition task and the blood and brain tissues were dissected for evaluating DNA damage by the use of the comet assay and iron level by PIXE.

2. Material and methods

2.1. Animals

Pregnant Wistar rats were obtained from Fundação Estadual de Pesquisa e Produção em Saúde, Porto Alegre, RS, Brazil. After birth, each litter was adjusted within 48h to eight rat pups and to contain offspring of both genders in about equal proportions. Each pup was

kept together with its mother in a plastic cage with sawdust bedding in a room temperature of 22 ± 1°C and a 12/12h light/dark cycle. At the age of 4 weeks, pups were weaned and the males were selected and raised in groups of three to five rats. For postnatal treatments, animals were supplied with standardized pellet food and tap water ad libitum. All experimental procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the Brazilian Society for Neuroscience and Behavior (SBNeC) recommendations for animal care.

2.2. Treatment and test substances

Rats received vehicle or 10.0 mg/kg of Fe++ (Ferromyn® - AB Hässle, Göteborg, Sweden) orally via a metallic gastric tube at postnatal days 12 to 14 – the peak of iron uptake by brain (de Lima, Presti-Torres et al. 2007). Both groups were further divided in three experimental groups receiving orally vehicle or VitC diluted in saline solution (Novartis, SP, Brazil) at the doses of 1 or 30 mg/Kg. VitC was given three times per week for 21 days, starting when the animals reached the age of 2 months. Animals were trained in a novel object recognition task 24h after the last administration of VitC. The dose of iron and the treatment times follow our consensual treatment scheme (de Lima, Presti-Torres et al. 2007). The doses of VitC were based on the ratio of rats to the human recommended dietary allowance (RDA before 2000) 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) previously evaluated (Franke, Pra et al. 2005).

2.3. Object Recognition

It was used an open field apparatus (45 x 40 x 60 cm) made of polywood with a frontal glass wall with sawdust covering its floor. On the first day, rats were submitted to a habituation session during which they were placed in the empty open field for 5 min. On the following day, rats were given one 5-min training trial in which they were exposed to two identical objects (A1 and A2). The objects were positioned in two adjacent corners, 9 cm from the walls. On the short-term memory (STM) testing trial (90 min after the training session), rats were allowed to explore the open field for 5 minutes in the presence of two objects: the familiar object A and a novel object B. These were placed in the same locations as in the training session. On the long-term memory (LTM) testing trial (24 hours after the training session), rats were allowed to explore the open field for 5 minutes in the presence of two objects: the familiar object A and a third novel object C. All objects presented similar textures, colors, and sizes, but distinctive shapes. Object exploration was measured using two stopwatches to record the time spent exploring the objects during the experimental sessions. Exploration was defined as follows: sniffing or touching the object with the nose. Sitting on the object was not considered as exploration. A recognition index calculated for each animal was expressed by the ratio TB/(TA+TB) [TA= time spent exploring the object A; TB= time spent exploring the object B] (Schroder, O'Dell et al. 2003; de Lima, Laranja et al. 2005; de Lima, Polydoro et al. 2005; de Lima, Laranja et al. 2005). A single analyzer performed all analyzes “blinded” to the treatment groups.

2.4 Tissue sampling

After all behavioral proceedings, rats were decapitated within a few minutes for obtaining the samples. The blood was collected immediately after the death of the animal directly from the neck. The brain was surgically extracted and a portion of the substantia nigra was dissected, using standard anatomic landmarks. For comet assay, the blood and brain samples were immediately transferred to the laboratory for processing. For PIXE analyses, 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 the Comet assay guidelines (Tice, Agurell et al. 2000; Hartmann, Agurell et al. 2003; Burlinson, Tice et al. 2007). Whole

blood was used for mounting the comet assay microscope slide. For the brain samples, a small piece of tissue was dismantled by using a forceps in PBS. An aliquot of this cell suspension was used for mounting the slides. For either blood or substantia nigra, 7 μL of the sample were embedded in 93 μL of low melting point - LMP agarose (0.75 %) and this mixture (cell/agarose) added to a pre-coated microscope slide with normal melting point - NMP agarose (1.5 %). The slides were placed at 4°C for 5 min to allow gel solidification, the coverslips were removed and the slides were put in lyses buffer (2.5M NaCl, 100 mM EDTA and 10 mM Tris, pH 10.00–10.50, with freshly added 1% Triton X-100 and 10% DMSO) for 24 hours, to allow cell desegregation. Slides were removed form lyses buffered, cleaned and immediately placed in a horizontal electrophoresis box. They were then exposed to alkali (300 mM NaOH, 1mM Na_2EDTA, pH > 13) for 20 min at 4° C, to allow DNA unwinding. Electrophoresis was performed at 300 mA and 25V (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 silver staining (Nadin, Vargas-Roig et al. 2001). Briefly, slides were washed and fixed, rehydrated and stained. 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 scored at 400 X magnification using a conventional light microscope. Cells were visually ascertained 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 x 0) to 400 (maximum damage: 100 cells x 4). 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 (Tice, Agurell et al. 2000; Hartmann, Agurell et al. 2003).

2.6. PIXE analysis

In order to obtain the whole blood levels of iron, the PIXE (Johansson, Campbell et al. 1995) technique was employed. 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, Streck et al. 2004) and has been successfully applied to studies related to metal concentrations in proteins (Follmer, Carlini et al. 2002) and eukaryotic organisms (Kern, Bonatto et al. 2004; Viau, Yoneama et al. 2006).

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, Campbell et al. 1989; Maxwell, Teesdale et al. 1995; Campbell, Hopman 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

For object recognition, comparisons among groups were performed with a Kruskal-Wallis analysis of variance followed by Mann-Whitney U tests when necessary. Comparisons between sessions within the same group were performed with a Wilcoxon test. Statistical

comparison of total time exploring both objects during training session was made using one way analysis of variance (ANOVA). For comet assay and PIXE analyses, the One-Way ANOVA was used to compare DNA damage between treatment groups either. Parametric ANOVA was used for those cases where the data exhibited homoscedasticity (Bartlet Test). Log-transformed (Ln) data were applied to homogenize the variance. When this attempt was not efficient, the Kruskal-Wallis non-parametric ANOVA was used with Dunn's correction to compare groups pairwise. Correlation analysis was used to test the association between the average values of DNA damage and the average metal content in whole blood. Significance was considered at a level of $P \leq 0.05$.

3. Results

Results for object recognition task are presented in Figure 1. Statistical comparison of recognition indexes showed that groups treated neonatally with iron and receiving vehicle in the adulthood showed long-term recognition memory deficits. Short-term retention was not affected by iron. Comparisons in recognition indexes between training and the long-term memory trial within each group indicated that animals given iron showed no significant difference between training and retention test performances, suggesting that these animals had a complete memory blockade revealed by the lack of preference towards the novel object in the long-term retention test trial. The vitC treatment (at both doses) had no effect on iron-induced recognition memory deficits, since the rats treated with iron and vitC presented recognition indices comparable to those seen in the Fe-Veh group. Moreover, recognition indices of iron-treated rats that received vitC were significantly lower than the control group (veh-veh). VitC by itself had no effect on recognition memory in adult rats, as revealed by comparisons between the groups given vehicle and vitC and the respective control group treated with vehicle in the neonatal period and vehicle in the adulthood. In addition, there were no significant differences among groups in the total time exploring both objects during acquisition (training session), indicating that the treatments with iron and/or VitC did not affect sensorimotor parameters such as locomotion, motivation and anxiety (overall mean ± SE time exploring both objects during the training trial was 31,86 ± 1,83).

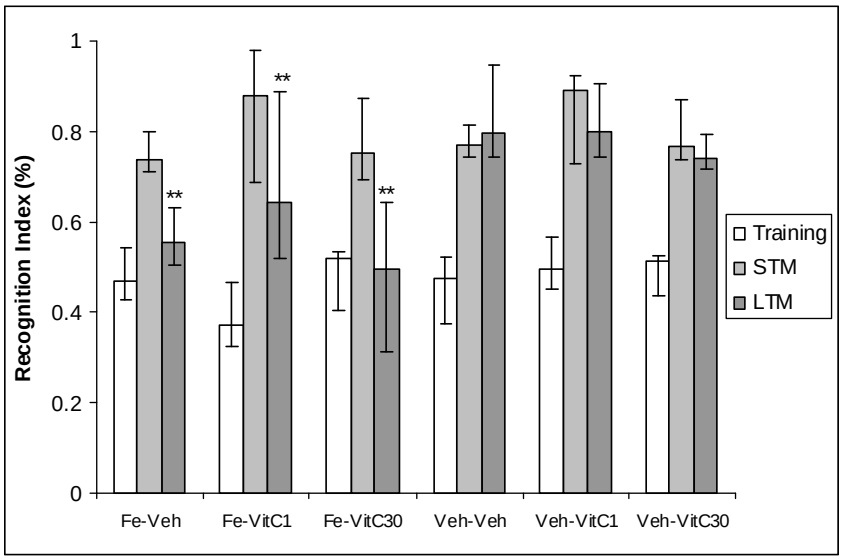

Figure 1. Effect of vitamin C on iron-induced recognition memory deficits. Short-term (90 min after training) and long-term retention tests (24 h after training) were conducted in rats treated with iron in the neonatal period and VitC in the adulthood. Behavioral testing was carried out when animals were 3 months old. The proportion of the total exploration time that the animal spent investigating the novel object was the "Recognition Index" expressed by the ratio TB/(TA+TB), TA = time spent exploring the familiar object and TB = time spent

exploring the novel object. Data expressed as median [interquartile ranges], n = 8-9 per group. Differences between vehicle- and iron-treated groups are indicated: **p < 0.01. Fe: iron; Veh: vehicle; VitC1: vitamin C at 1 mg/Kg; VitC30: Vitamin C at 30 mg/Kg.

Table 1 presents the levels of DNA damage and iron in the blood of treated rats. Low magnitude of differences regarding iron and DNA damage levels were observed between treatment groups.

Table 1. DNA damage in blood white cells and Fe level in whole blood of rats overloaded with iron in perinatal period and treated with vitamin C in adulthood.

Group	Damage Index ± SEM	Fe (mg/Kg dry weight) ± SEM
Fe-Veh	7.11 ± 2.23	1556 ± 177
Fe-VitC1	6.07 ± 1.22	1356 ± 30
Fe-VitC30	7.54 ± 3.05	1471 ± 64
Veh-Veh	5.32 ± 0.88	1500 ± 96
Veh-VitC1	6.35 ± 1.10	1271 ± 54
Veh-VitC30	4.65 ± 1.39	1472 ± 141

SEM: standard error of the mean based in 4 or 5 rats. Fe: iron; Veh: vehicle; VitC1: vitamin C at 1 mg/Kg; VitC30: Vitamin C at 30 mg/Kg.

Table 2 presents the levels of DNA damage in substantia nigra. Iron treatment in neonatal period induced a significantly increased DNA damage in the substantia nigra of the treated rats ($P<0.05$) which was not reversed by adulthood treatment with any dose of vitC. On the contrary, the lower dose of VitC (1 mg/Kg) induced a significant DNA damage increase either when rats were treated or not with iron in neonatal period ($P<0.01$). The adulthood treatment with the highest dose of Vit C (30 mg/Kg) increased the DNA damage ($P<0.05$) only among controls (Veh-VitC30) but not among rats treated with iron in neonatal period (Fe-VitC30).

Table 2. DNA damage in substantia nigra of rats overloaded with iron in perinatal period and treated with vitamin C in adulthood.

Group	Damage Index ± SEM
Fe-Veh	240.6 ± 22.6a*
Fe-VitC1	342.6 ± 13.3 a**,b**
Fe-VitC30	239 ± 45 c*
Veh-Veh	175 ± 6
Veh-VitC1	333.4 ± 20.3 a**
Veh-VitC30	276 ± 25 a*

SEM: standard error of the mean based in 4 or 5 rats. Fe: iron; Veh: vehicle; VitC1: vitamin C at 1 mg/Kg; VitC30: Vitamin C at 30 mg/Kg. a: in relation to Veh-Veh; b: in relation to Fe-Veh and c: in relation to Fe-VitC1 at ** $P < 0.01$ or *$P < 0.05$.

It was seen a negative correlation regarding the average values of the treatment groups between the whole blood level of iron and the DNA damage level in substantia nigra (Fig. 2).

4. Discussion

The brain nerve endings contain the highest concentrations of vitC in the human body (after the suprarenal glands) (Bourre 2006), being 10 fold higher in concentration than in blood (Parle and Dhingra 2003). The synthesis of cathecolamines take place in VitC rich sites (Bourre 2006). VitC is also required for the transformation of dopamine into noradrenaline

(Bourre 2006). Surprisingly, vitC does not penetrate blood-brain barrier, but its oxidized form dehydroascorbic acid ready enters the brain by facilitated transport (Parle and Dhingra 2003).

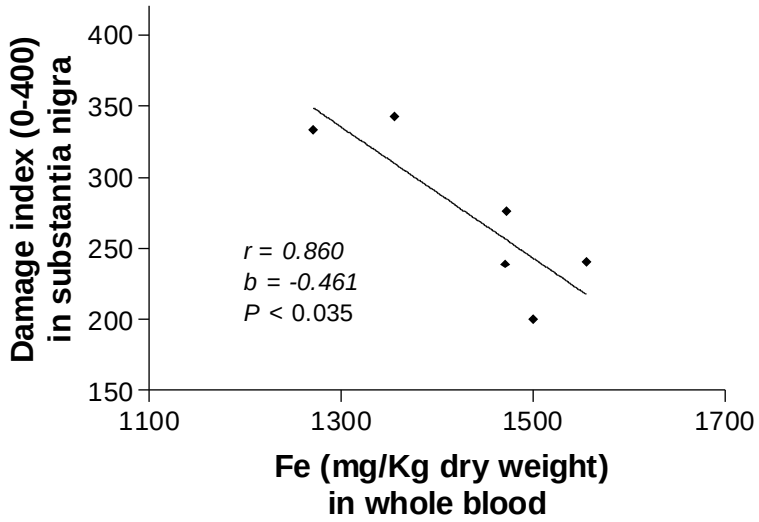

Figure 2. Correlation between the level of iron in the whole blood and the level of DNA damage in the substantia nigra of rats overloaded with iron in neonatal period.

The present findings are in agreement with our previous reports indicating that the same iron neonatal treatment induces severe long-term recognition memory impairment, without affecting short-term memory, in adult rats (de Lima, Polydoro et al. 2005; de Lima, Laranja et al. 2005). In the present study, we have investigated the possibility that vitC given in the adulthood would be able to ameliorate the deleterious effects of iron on cognition. Results have indicated that vitC was not able to reverse the iron-induced recognition memory deficits in rats.

There is one report of a positive correlation between the level of vitC in plasma and serum and intelligence quotient in adolescents (Southorn and Powis 1988). It was also reported vitC had an anti-stress effect in rats (Bourre 2006) and reduced severe cognitive impairment in elder Australian subjects (Paleologos, Cumming et al. 1998). A study by Parle and Dhingra (2003) has shown that vitC treatment (60 and 120 mg/kg) for 3 or 8 consecutive days was able to improve age-induced memory impairment and could protect young animals from scopolamine- and diazepam-induced impairment of memory; possibly due to vitC antioxidant properties. In another study, Han et al (2007) investigated the effect of co-treatment with 100 mg/Kg vitC in the offspring of rats treated with lead during pregnancy and lactation and observed attenuated histopatological signals of attenuation of apoptosis in the developing hippocampus and also spares hippocampal CA1, CA3 and dentate gyrus neurons in pups as well as reduced lead level in blood of dams. It is interesting to mention that the vitC dose used in these studies (>60 mg/Kg) were much higher than the doses used in the present study (1 and 30 mg/Kg).

In agreement with our results, Coskoweun et al. (2005) have shown that chronically exercised rats supplemented with vitC during the training period showed significantly elevated thiobarbituric acid reactive substances (TBARS) levels in brain. They, then, concluded that “vitC supplementation may not protect brain tissue against exercise-induced oxidative damage, in such circumstances this water-soluble antioxidant behaves as a pro-oxidant”. This remark seems to be valid for the present study, in which vitC induced DNA damage increase independently of Fe neonatal treatment.

Although vitC has been consumed as an antioxidant with a supposed myriad of health effects, including cold prevention and treatment, its biological role has been discussed elsewhere (Podmore, Griffiths et al. 1998; Carr and Frei 1999; Halliwell 2001; Traxer, Pearle et al. 2003). Some studies have shown that the consumption of foods rich in vitC decreased the levels of oxidative DNA damage in vivo, whereas vitC consumption alone did not (Halliwell, Rafter et al. 2005). Other have even indicated vitC as a potential risk of cancer in humans (Feskanich, Willett et al. 2003), despite this compound had been considered non-carcinogenic to mice or rat (Elmore 2005). In a general sense, like most antioxidants, VitC can also acts as a pro-oxidant (Podmore, Griffiths et al. 1998), particularly when interacting with metals. VitC presents an ambiguous effect in terms of genotoxicity and antigenotoxicity. We observed that while VitC can be genotoxic, it could act as a DNA repair stimulator in mice white blood cells in vivo (Franke, Pra et al. 2005). Similarly, it was also shown that vitC is bio-antimutagenic (Kojima, Konishi et al. 1992; Guha and Khuda-Bukhsh 2002) against the alkylating agent methymethanesulfonate and desmutagenic activities, against chromosome aberrations, per example in coal-tar workers (Sram, Dobias et al. 1983). In the same direction, Blasiak and Kovalik (2000) observed that VitC could act against the DNA-damaging effect of hexavalent chromium in isolated peripheral blood lymphocytes and Premkumar et al (2003) showed that vitC reduced the frequency of iron induced micronuclei in bone marrow cells of mice. In agreement, experimental data (Cooke, Evans et al. 1998; Lunec, Holloway et al. 2002; Tarng, Liu et al. 2004) suggest that VitC can stimulate DNA repair excision systems. Conversely, Krishnaja and Sharma (Krishnaja and Sharma 2003) have shown that vitC potentiated mitomicin C-induced chromosome damage in human lymphocytes in vitro, possibly through metal-involved reactions. Moreover, Osawa et al (Ohsawa, Nakagawa et al. 2003) have shown that while VitC can suppress the mutagenicty of nitrogenous compounds in some organs such as liver, it can lead to increase DNA damage in organs such as the stomach through the generation of reactive intermediates. The result of the present study provides novel evidences of a non beneficial role of vitamin C, particularly in the dose and the endpoint evaluated.

Adulthood treatment with vitC had not a major influence either in whole blood iron level or leukocytes DNA damage. Given the long period elapsed and the short extent of iron treatment and the fact of blood not being a key target for iron accumulation {Pra, 2007 #79}, the low variability between groups can be justified. In a study evaluating the genotoxicity of the same doses of vitC to mice, we observed higher DNA damage 24 hs after a single vitC administration with a trend for no genotoxicity at later times{Franke, 2005 #51}. Results of the current study show almost none DNA damage after 21 days of administration of the same doses of vitC every three days, suggesting adaptation.

The theme of sites of accumulation, transportation and efflux of brain iron is both scarce and controversial in literature. It is known that subcellular iron is bound to various proteins, including ferritin which is present in high levels in oligodendrocytes, microglia and astrocytes (Berg, Gerlach et al. 2001). Regarding efflux, Dwork and coworkers (1990) suggest that all iron acquired by the brain during neonatal period remains in the brain without possibly returning to plasma sites. As opposing, Zhang and Pardridge {, 2001 #1132} have shown that iron can be exported in vivo from the brain to blood through the reverse-transcytosis of transferrin, in a saturable process. In agreement, transferrin expression in adult mice brain seems to be related to the maintenance of the level of the intracellular concentration and distribution of some cations (e.g., zinc, copper, and manganese), involved in transcriptional activation during oligodendrocytes differentiation and myelin maintenance (Sow, Lamant et al. 2006).

Interestingly, at the molecular level, vitC mobilizes Fe from the ferritin crystal core in vitro, and in vivo facilitates the enzymatic incorporation of iron into protoporphyrin for heme

formation (Tarng, Hung et al. 2004). The negative correlation between DNA damage in substatia nigra and whole blood iron concentration (Fig. 2) gives us a glimpse of a potential role of vitC in mediating iron release from brain towards other tissues, such as blood. The release of iron from ferritin, which contributes to free radical production, has been shown in the pathogenesis of Parkinson's disease (Berg, Gerlach et al. 2001).

The present finding shows, for the first time, that iron given neonatally induces an increase in DNA damage in the substantia nigra in rats. Moreover, it shows no effect of VitC on memory impairment for the same doses tested. We have previously demonstrated that iron selectively accumulates in the basal ganglia and substantia nigra in mice and rats (Fredriksson, Schroder et al. 1999; Fredriksson, Schroder et al. 2000; Schroder, Fredriksson et al. 2001) and increases oxidative damage in these brain regions (Dal-Pizzol, Klamt et al. 2001; de Lima, Polydoro et al. 2005). Supporting the role of VitC to catalyze the efflux of Fe from brain or other tissues, it was observed in the present study a negative correlation between the average DNA damage in substantia nigra and the average levels of iron in the whole blood, which possibly indicates that neonatal administered-iron can be mobilized back to blood in later ages. Further studies are still needed in this sense, because of the number of different cell types in the brain and the heterogeneous distribution of iron in the brain and body, the layers of complexity of the brain are too many to understand the mechanism underlying iron redistribution (Pinero, Li et al. 2001) and efflux.

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9.10 ANEXO J: “Reversion of iron-induced recognition memory deficits by desferroxamine is associated to the reduction of iron level and DNA damage”

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Reversion of iron-induced recognition memory deficits by desferroxamine is associated to the reduction of iron level and DNA damage

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Abstract
We have previously reported that desferoxamine (DFO), a metal chelating agent, can suppress iron induced memory deficits, which is possibly linked to oxidative stress. The aim of this paper is to address whether DFO reduces the DNA damage level and the tissular accumulation of iron in rat blood and brain. Male rats were treated with vehicle or 10.0 mg/kg of Fe^{++} at postnatal days 12 to 14 and with DFO (30 or 300 mg/kg) in the adulthood. After the last administration of DFO, blood and brain tissues were dissected for evaluating DNA damage, by using the comet assay, and iron level, by using the Particle Induced X-ray Emission (PIXE). Iron-treated rats showed higher DNA damage than controls in blood, but not in brain cortex. DFO at 300 mg/Kg reduced the level of DNA damage either in blood or in brain cortex, irrespectively of iron treatment. Iron treatment increased the level of iron in cortex and DFO at 300 mg/Kg reduced it. DFO seems to stabilize genome, either by its metal chelator activity or through other unknown mechanisms, which can contribute to DFO role in reversing recognition memory deficits associated with iron accumulation.

Key word: cortex, oxidative stress, DNA damage, comet assay, iron level.

1. Introduction

In spite of its essentiality for several key biological functions such as immune and nervous system[1], iron overload can have devastating effects [2]. In brain, iron is fundamental in neuronal development (e.g for myelinaton) [3], but is also involved in the etiology of several neurodegenerative diseases, such as Parkinson's [4] and Alzheimer's diseases [5]. Schröder et al [5-7] have demonstrated that iron supplementation in the neonatal period induces a selective iron accumulation in brain which was associated with behavioral changes in adult mice and rats. In another study by de Lima et al [8] it was demonstrated that iron given in the neonatal period induced recognition memory impairment, possibly by inducing oxidative damage in the brain. Indeed, neonatal iron supplementation induced lipid peroxidation and protein carbonylation in brain [9], in agreement with its genotoxic and mutagenic effects

already reported by us and others in different test systems both in vitro [10-12] and in vivo [13,14].

Iron load in the early stages of life and, most importantly, the iron-induced deficits can be reverted by selegiline [an monoamine oxidase (MAO) inhibitor] [15] and by desferoxamide (DFO) [16]. DFO is an iron chelator with widespread usage for the last three decades in the treatment of systemic iron-load diseases [16]. DFO has been studied regarding its antioxidant potential in a myriad of test systems, showing antioxidant activity in erythrocytes [17], hepatocytes [18] and brain cells [19] in vitro and in reumathoid artrites subjects [20], for example. Conversely, DFO can show high toxicity both in vivo [21] and in vitro [22], even with positive results for mutagenicity in the mouse lymphoma assay [23], but no substitute compound with similar clinical efficacy owing to toxicity, a lack of efficacy, or no patent protection [16]. DFO has also been proposed as a cancer chemotherapy adjuvant, because it diminishes the intracellular iron pool level and shows antiproliferative effect over several tumoral cells lines [23].

Considering the striking evidences linking iron accumulation and neurodegeneration and the widespread and controversial usage of DFO, this research aims to evaluate the effect of DFO over the DNA damage and iron accumulation in brain and blood. In order to do that, we treated rats with vehicle or 10.0 mg/kg of Fe^{++} at postnatal days 12 to 14 and with DFO in the adulthood. After the last administration of DFO, blood and brain tissues were dissected for evaluating DNA damage by the use of the comet assay and iron level by PIXE.

2. Material and methods

2.1. Animals

Pregnant Wistar rats were obtained from Fundação Estadual de Pesquisa e Produção em Saúde, Porto Alegre, RS, Brazil. After birth, each litter was adjusted within 48h to eight rat pups and to contain offspring of both genders in about equal proportions. Each pup was kept together with its mother in a plastic cage with sawdust bedding in a room temperature of 22 ± 1°C and a 12/12h light/dark cycle. At the age of 4 weeks, pups were weaned and the males were selected and raised in groups of three to five rats. For postnatal treatments, animals were supplied with standardized pellet food and tap water ad libitum. All experimental procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the Brazilian Society for Neuroscience and Behavior (SBNeC) recommendations for animal care.

2.2. Treatment and test substances

Rats received vehicle or 10.0 mg/kg of Fe^{++} (Ferromyn® - AB Hässle, Göteborg, Sweden) orally via a metallic gastric tube at postnatal days 12 to 14. Both groups were further divided in three experimental groups receiving orally vehicle or DFO diluted in saline solution (Novartis, SP, Brazil) at the doses of 30 or 300 mg/Kg. DFO was given three times per week for 21 days, starting when the animals reached the age of 2 months.

2.3. Tissue sampling

At the end of the treatment, rats were quickly decapitated for obtaining the samples. The blood was collected immediately after the death of the animal directly from the neck. The brain was surgically extracted and a portion of the cortex was dissected. For comet assay, the blood and brain samples were immediately transferred to the laboratory for processing. For PIXE analyses, the samples were deep frozen, lyophilized, homogenized and finally pressed into pellets.

The comet assay and PIXE analyses were conducted separately due to sample volume be insufficient for running both methodologies with the same sample. All treatment and handling procedures were rigorously at the same manner for both methods.

2.4. Comet Assay

The alkaline comet assay was performed as described by Tice et al. [24] with further modifications suggested by Hartmann et al [25] and Da Silva et al [26]. Whole blood was used for mounting the comet assay microscope slide. For the brain samples, a small piece of tissue was dismantled by using a forceps in PBS. An aliquot o this cell suspension was used for mounting the slides. For either blood or cortex cells, 7 μL of the sample 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 %). The slides were placed at 4°C for 5 min to allow gel solidification, the coverslips were removed and the slides were put in lyses buffer (2.5M NaCl, 100 mM EDTA and 10 mM Tris, pH 10.00–10.50, with freshly added 1% Triton X-100 and 10% DMSO) for 24 hours, to allow cell desegregation. Slides were removed form lyses buffered, cleaned and immediately placed in a horizontal electrophoresis box. They were then exposed to alkali (300 mM NaOH, 1mM Na2EDTA, pH > 13) for 20 min at 4° C, to allow DNA unwinding. Electrophoresis was performed at 300 mA and 25V (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 silver staining [27]. Briefly, slides were washed and fixed, rehydrated and stained. 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 scored at 400 X magnification using a conventional light microscope. Cells were visually ascertained 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 x 0) to 400 (maximum damage: 100 cells x 4). 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 [24,25].

2.5. PIXE analysis

In order to obtain the whole blood and hepatic levels of iron and copper, the PIXE [28] technique was employed. 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 [29] and has been successfully applied to studies related to metal concentrations in proteins [30] and eukaryotic organisms [31].

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 [32-34]. 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.

2.6. Statistical analysis

The One-Way ANOVA was used to compare DNA damage between treatment 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. Significance was considered at a level of $P \leq 0.05$.

3. Results

Figure 1 presents the levels of DNA damage and Fe in the blood of treated rats. Rats treated in the neonatal period with iron showed higher DNA damage than controls and DFO treatment significantly reduced DNA damage induced by iron. Moreover, DFO also induced a dose dependent decrease among rats treated with vehicle in neonatal period (Fig. 1A). Regarding iron level in whole blood, DFO induced dose dependent increase in its level, although not significantly (Fig.1B).

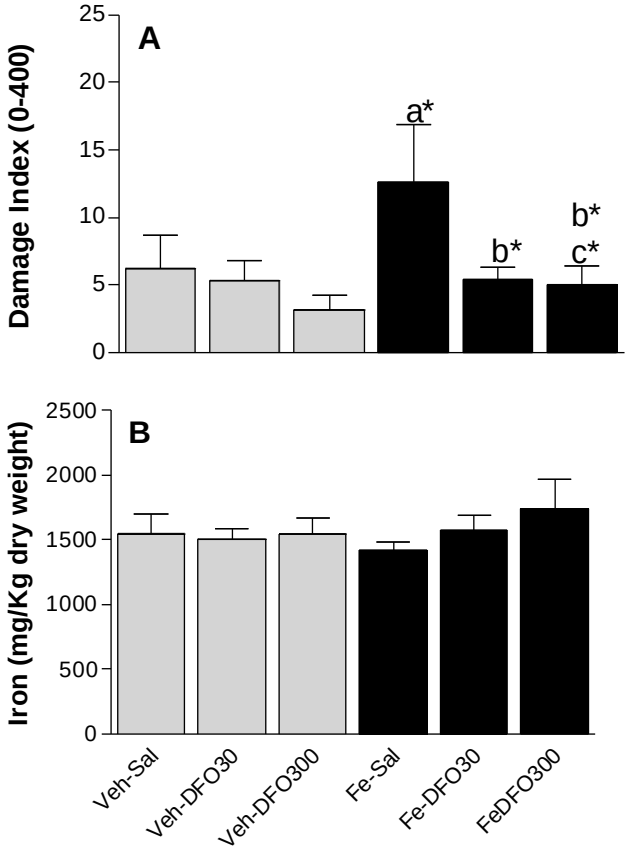

Figure 1. DNA damage in blood white cells (A) and Fe level in whole blood (B) of rats overloaded with iron in perinatal period and treated with desferal in adulthood. a: in relation to Veh-Sal; b: in relation to Fe-DFO30; c: in relation to Veh-DFO300 at * $P < 0.05$. Veh: vehicle: Sal: saline; DFO30: desferoxamine 30 mg/Kg; DFO300: desferoxamine 300 mg/Kg; Fe: iron.

Figure 2 presents the level of DNA damage and iron level in the cortex of treated rats. Iron treatment in neonatal period did not induced DNA damage increase in the cortex of the treated rats. Conversely, the higher DFO dose reduced the DNA damage, irrespectively to iron treatment (Fig 2A). Regarding to the level of Fe, iron treatment induced a significant increase in the level of iron in the cortex of the rats and the higher dose of DFO significantly reduced it (Fig 2B).

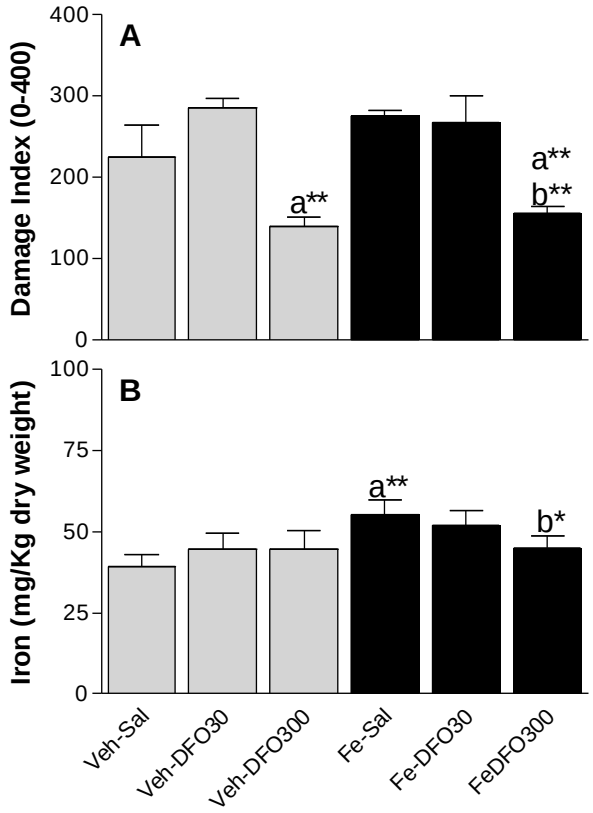

Figure 2. DNA damage (A) and Fe level (B) in cortex of rats overloaded with iron in perinatal period and treated with desferal in adulthood. a: in relation to Veh-Sal; b: in relation to Fe-DFO30 at * $P < 0.05$ or ** $P < 0.01$. Veh: vehicle: Sal: saline; DFO30: desferoxamine 30 mg/Kg; DFO300: desferoxamine 300 mg/Kg; Fe: iron.

4. Discussion

According to the present results, DFO was able to reduce iron induced DNA damage in blood, in agreement with several studies found in literature. For example, DFO increased the plating efficiency (up to 0.15mM DFO and 3mM at long and short term, respectively) in the colony forming-efficiency assay [35] and reduced the frequency of chromosome aberration (about 5 nM DFO) induced by tert-butyl hydroperoxide (BHP)[36] in *Ataxia-telangiectasia* (A-T) cells. A-T cells present increased genomic instability and transferin receptor activity, a receptor induced by oxidative stress that leads to a higher iron import [36]. DFO was not genotoxic to and inhibited DNA damage in a time- and dose-dependent manner in cultured Jurkat cells exposed to low rates of continuously generated hydrogen peroxide by the glucose/glucose oxidase system as evaluated by the comet assay [37]. DFO at 100 and 250 μmol/L significantly decreased chloroquine (CQ), a 4-aminoquinoline antimalarial drug, induced strand breaks and base oxidation in rat liver cells, as evaluated by the alkaline comet assay [38]. Conversely, DFO (up to 0.5 mM /plate) was low effective in reducing the mutagenicity induced by (BHP) or cumene hydroperoxide (CHP) in *Salmonella typhimurium* TA102 [39].

The higher dose of DFO (300 mg/Kg) was able to reduce the background DNA damage either among iron or vehicle treated rats. The same dose was able to repress iron-induced memory deficit in vivo [16]. Therefore, it is likely that DFO plays a role in ameliorating iron homeostasis in brain. DFO has been shown to protect cultured oligodendrocytes progenitors against oxidative stress and apoptosis generated by ischaemic

insults [40]. Ischemic insults can be mediated by metals or rounds of hypoxia–ischaemia and lead to the release of various toxic neurotransmitters, such as dopamine [40]. DFO was also shown to protect against intracerebral hemorrhage by reducing brain edema and neurological deficits, which can be generated by iron accumulation and oxidative stress in the brain [41]. Moreover in brain in vivo, DFO can improve spatial memory performance following experimental brain injury in rats [42].

Even though the present study have showed frank positive results regarding DFO effect over DNA damage level both in blood and in brain, one must recall that DFO there are several reports of DFO toxicity in literature. For instance, DFO was show to decrease the viability of human neuronal tumor cells (SK-N-MC neuroblastoma and U-373 MG astrocytoma) in a dose-dependent manner, not by the chelating activity, but possibly due to the production of hydroxyl radicals [22]. In vivo, high DFO doses induce visual and auditory neurotoxicity in patients with transfusion-dependent anemia [21]. The antiproliferative effects both *in vitro* and *in vivo of DFO* through the decreasing the intracellular iron pool needed for DNA synthesis [43] is another point of concern, and, therefore, long term effects derived of DFO suppression of cellular normal cell cycle are likely to occur, particularly among cells rich in iron or that require high uptakes, such as the developing brain [5]. Regarding clinical usage, DFO has a range of considerable disadvantages (for review see [44]). It is expensive, orally ineffective and need long lasting daily subcutaneous administration [45,46]; with potential for producing allergic reactions in some individuals, because it is recovered form bacterial cultures and can contains fermentation products derived from the purification process [47]. DFO has low lipophilicity, therefore not reaching some cellular target sites of iron accumulation, such as mitochondria [48,49]. It is also low effective to inhibit Fe uptake in vitro, needing long incubation for preventing radiolabeled-Fe uptake from transferrin by cells [50].

It is likely that DFO has a biphasic antioxidant/prooxidant as a function of dose which may be common with iron-catalyzed oxidizing reactions when mobile strong reducing agents are present [51]. In the present study it was observed an increase in whole blood iron level, although not significantly, indicating that blood is possibly behaving at least as a transitory organ for iron during its mobilization. Regarding the brain, as expected, the level of iron in the cortex was increased by iron treatment and DFO reduced the level of iron either in both doses, significantly only for the higher dose. According to Dwork and coworkers [52], iron acquired by the brain during the neonatal period remains in the brain without possibly returning to plasma sites. Although the increase in iron level in blood can not be associated to iron derived from brain, since iron can be mobilized from other sites within the body, the mobilization of iron through the blood-brain barrier is a controversial theme and needs further clarification.

In conclusion, the present results give further evidence about the role of DFO in reducing the background DNA damage in brain and the iron-induced oxidative burden which can be associated to the physiological cognitive decline observed in normal aging and possibly in neurodegenerative disorders. Besides, it indicates the potential of DFO and other metal chelators, although DFO was not overcame by any synthetic alternatives in clinical efficacy [44], in the endeavor against the neurodegenerative disorders associated to oxidative stress and iron homeostasis unbalance.

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9.11 ANEXO K “Iron transferrin increases genomic stability in beta-lymphoblastoid cell cultures”

Mansucrito a ser submetido ao periódico Blood

Iron transferrin increases genomic stability in beta-lymphoblastoid cell cultures

Short tile: iron and genomic stability in vitro

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Abstract

Recent evidences suggest that iron deficiency may reduce genomic maintenance and impair key metabolic routes linked to DNA synthesis and repair, apoptosis and redox metabolism; however the definition of the adequate physiological levels of iron to maximize genomic stability is far away to by accomplished. In this report we describe the genomic stability effect of the supplementation of beta-lymphoblastoid with holotransferrin in order to provide physiological levels of iron (up to 21 µM iron or 10.5 µM holotrasferrin). The lower concentrations of holotransferrin (up to 0.67 µM) increased primary DNA damage and induced a minor decrease in micronuclei frequency. The higher doses of holotransferrin (1.68-10.5 µM) induced a concentration-dependent decrease in both primary DNA damage and micronuclei. Intermediate concentrations of holotransferrin (0.67 and 1.9 µM) increased telomere length and mtDNA copy number and substantially reduced the frequency of dicentric chromosomes. Considering the iron background of 2 µM of our medium (5% fetal bovine serum), we envisage a final concentration >6 µM iron to maximize genomic stability in our test system. Nothwitstanding, this level could be increased since we observed lower MN frequency at the serum iron physiological level (21 µM iron) and anemia cut-off is around 10 µM iron.

Key words: anemia, genomic instability, cell culture, DNA damage

Introduction

There are growing evidences that iron deficiency (ID) can increase DNA damage in nuclei[1] and mitochondria[2], contributing to mitochondrial decay as well as to accelerated aging [3,4]. Recent studies implicated iron as a essential cofactor of DNA repair enzymes, including DNA alkyltransferases [5], DNA glycosilases [6] and several DNA helicases [7,8]. Iron is also a co-factor of ribonucleotide reductase[9], a critical enzyme for ribonucleotide pool maintenance. Moreover, iron deficiency has been showed to accelerate the onset of cancers in murine models[10-12] and to decrease the survival after tumor surgeries in humans [13]. Iron deficiency is known for a long time as a driver in antioxidant defenses reduction in parallel to an increase in oxidative stress[14]. Interestingly, iron overload shares much of the outcomes of iron deficiency [2] and has been more often addressed in literature; mainly due to the pro-oxidant role of iron in biological systems [15].

Transferrin (Tf) is the principal iron transporter in serum. Tf molecules can bind two molecules of iron each and constitute together with serum ferritin (Ft) – which can store up to 4,500 molecules of iron – the vast majority of serum iron (SI)[16]. The serum of a median healthy male has typically 30 µM of Tf, which about 28%[17] saturated by iron (SI≈18 µM[18]),

5.5 nM Ft[19] and virtually zero (far less than 1μM) non-transferrin bound iron (NTBI) [20]. The level of iron in standard cellular mediums is below the physiological serum levels. For example, RPMI 1640 contains no iron in its formulation [21] and the single source for iron for cells cultured in this medium comes from the supplementation with bovine serum (BS), typically around 5-10% of the final volume of the culture medium. Such levels correspond to 1.8-3.6 μM iron from 36 μM iron in BS (unpublished data).

There is a lack of in vitro studies regarding the impact of suboptimal versus physiological iron concentrations over genomic stability. In this report we describe the genomic stability effect of Tf supplementation (14 days) under physiological levels in beta-lymphoblastoid as evaluated by cell proliferation/viability, telomere length, mtDNA copy number, single strand break (comet assay), and chromosome lost and breaks (cytokinesis-block micronuclei).

Material and methods

The procedure described by Umegaki and Fenech [22] with slight modifications was used in the study. Briefly, WIL2-NS [non secreting human B lymphoblastoid cell line isolated from the spleen of a Caucasian male with hereditary spherocytosis (American Type Culture Collection no. CRL8155) which has been reported to have a high mutational response to X-irradiation] were seeded in RPMI 1640 without L-Glutamine (Sigma, US) culture medium [21], containing, 1% antibiotics solution (Penicillin [5000IU/mL]/ Streptomycin [5mg/mL] - Thermo Trace, Australia), 1% 200 mM L-glutamine (Sigma,US) as well as 5% dialized FBS (Thermo Trace, Australia) a at a initial cell density of $2.5x10^5$ cells/mL. Holo-transferrin – hTf (Sigma, US) at concentrations ranging 0.3-10.5 μM was added to the medium and cells were grown for 14 days, being subcultured on days, 4, 7 and 12. Cells were electronically counted using a Coulter Counter (Fullerton, CA) when cells were subcultured. Viability was accessed by trypan blue (Sigma, US) at the end of the treatment. Iron concentration in the complete medium (supplemented ore control) and in FBS was measured by Atomic Absorption Spectrophotometry (Varian SpectrAA-400; Varian, Inc., Palo Alto, CA). All assays were performed in quaduplicates, i.e. for each dose tested, four cell suspensions were treated separately and each of these cell suspensions provided a separate culture for each assay. The results shown, unless otherwise indicated, represent the means ± SE for these quadruplicate experiments.

Real Time-PCR methods

DNA and RNA purification

Total DNA and RNA were extracted using Trizol Reagent® (Invitrogen, Carlsbad, CA), according to manufacturer's instructions. The fraction containing DNA was isolated using the silica-gel-membrane based DNeasy Tissue Kit (Qiagen) as described by Lu et al [23]. To reduce oxidative DNA damage [24], all buffers were purged with nitrogen and supplemented with 50 μM phenyl-tert-butyl nitrone (Sigma), proteinase K treatment was accomplished at 37°C for 3 hours, and 1 mM DTT (dithiothreitol) was added to DNA solution at the end of the extraction [23]. DNA and RNA were quantified in triplicate with a NanoDrop spectrophotometer (NanoDrop Technologies, Delaware, USA) and used as soon as possible for PCR

Telomere length and mtDNA copy number

Quantitative Real-Time PCR amplification (QRTmPCR) was used to measure telomere length and mtDNA copy number, according to O'Callaghan et al [25] and Wang et al [26], respectively. All samples were run on an ABI 7300 Sequence Detection System with the SDS Ver. 1.9 software (Applied Biosystems [AB], Foster City, CA). Each sample was analysed in duplicate. The highly conserved mitochondrial gene *cytochrome b* was used as a marker of mtDNA content, as described by Wang [26]. A nuclear single copy gene, *36B4* which encodes the acidic ribosomal phosphoprotein P0, was used as a control for amplification for every

sample performed, as described in Cawthon [27]. Each 20µl reaction was performed as follows: 20ng DNA, 1xSYBR Green master mix (AB) plus 100nM of forward and 100 nM of reverse primers. For telomere: forward (CGG TTT GTT TGG GTT TGG GTT TGG GTT TGG GTT TGG GTT) and reverse (GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT TAC CCT); for *36B4*: forward (CAG CAA GTG GGA AGG TGT AAT CC) and reverse (CCC ATT CTA TCA TCA ACG GGT ACA A); and for *cytochrome b*: forward (TAT CCG CCA TCC CAT ACA TT) and reverse (GGT GAT TCC TAG GGG GTT GT). Cycling conditions were: 10 min at 95°C, followed by 40 cycles of 95°C for 15 sec, 60°C for all amplicons.

Relative telomere length or mtDNA copy number were calculated using the comparative $\Delta\Delta C_T$ method. For this method, the endogenous control was used as the comparator (i.e. ΔC_T values of controls were averaged and set as 1 for comparative expression determination). Briefly, a ΔC_T value was calculated for each individual tested by C_T*target* - C_T*36B4*. -$\Delta\Delta C_T$ was calculated by the difference of ΔC_T between experimental and comparator. The relative expression value is shown as $2^{-\Delta\Delta CT}$.

Comet assay

For comet assay, one aliquot of cell culture was immediately used for preparing the slide of each cell culture. The preparation of the slides and the further steps of the comet assay were developed according to standard protocols [28] in agreement to the International Guidelines for conducting the assay [29]. Briefly, leukocytes were embedded in low melting point agarose and this mixture was added to a pre-coated microscope slide. After solidification, the slides were placed in lysis buffer for at least 1 h. Subsequently, the slides were incubated for 20 min in alkali buffer (pH<13) to allow DNA unwinding and, then, electrophoresed for 20 min (4°C) at 300 mA and 20 V (0.8 V/cm). After electrophoresis, the slides were neutralized and stained with propidium iodide. All reagents used were PA grade supplied by Australian or American companies. Images of 100 randomly selected cells per cell culture (50 cells per replicate slide) were analyzed at 200 X magnification using an optical microscope. The slides were coded and scored using established scoring methods and criteria and the same scorer in all experiments [30,31]. The analyses of damaged nuclei were carried out according to two parameters, the frequency of damaged nuclei and the extent of the DNA damage. Non-detectable cell nuclei (head and tail clearly separated) were not considered in analyses.

Cytokinesis block micronucleus (CBMN)

For CBMN assay, the cells were incubated for a further 24 h in the presence of 0.6 mg/mL cytochalasin-B (Sigma, US). The slides were prepared by cytocentrifugation (Shandon; Southern Products, Cheshire, U.K.) Before cytocentrifugation, DMSO (Sigma, US) was added at a final concentration of 5% to minimize clumping of the cells and thus optimize recognition of cytoplasmic boundaries. The slides were air dried for 10 min and then fixed and stained using Diff-Quik (LabAids, Brisbane, Australia). The slides were coded and scored by a single individual which was aware of the treatment group of the samples, using established scoring methods and criteria [32] based in the HUman Micro-Nucleus (HUMN) project [33]. Accordingly, binucleated cells (BNC; 1000 cells) were scored for the presence of micronuclei (MNi), nuclear buds (NBUD) and nucleoplasmic bridges (NPB). The level of mononucleated, binucleated, multinucleated, apoptotic and necrotic cells was determined by scoring 500 cells.

Statistical analyses

One-Way ANOVA followed by Dunnet post-tests, Kruskal-Wallis test followed by Dunn post-tests or Two-Way Repeated Measured followed by Bonferroni post-tests were used to compare cell proliferation, DNA damage or gene expression end-points. Linear of polynomial curve fitting were used to evaluate dose response correlations. The software Graph Prism 4.0 was used for statistical analyses and to plot graphs. The level of significance was of $P<0.05$. Values are expressed as average ± standard error of the mean. All values were normalized according to the average value in control to express relative differences.

Results

Figure 1A summarizes the results of cell proliferation in days 3, 7, 12 and 14 of hTf treatment. In spite of slightly difference regarding day 14, the dose-response was generally constant between the analyzed days, with a "U" parabola-like trend with maximum proliferation (110-180% of control) in the concentration of 4.2 µM hTf (8.5 µM Fe). Cell viability, as measured by Trypan blue exclusion at day 14 (Fig. 1B) showed a consistent non-significant decrease in viability in all hTf concentrations.

The lower concentrations of holotransferrin (up to 0.67 µM) increased primary DNA damage and induced a minor decrease in MN frequency. The higher doses of holotransferrin (1.68-10.5 µM) induced a concentration-dependent decrease in both primary DNA damage and micronuclei, although not significantly. There was a "U" parabola-like trend, significant for NPB, but not for NBuds (Fig. 2).

Figure 3 shows the effect of hTf concentration over telomere length and mtDNA copy number. Telomere length showed a "U" parabola-like trend with average telomere length increasing in concentrations ranging 1.5-4.2 µM hTf (significantly only at 2.5 µM hTf) and substantially decreasing at 10.5 µM hTf (2-fold, but not significant). On the other hand, mtDNA copy number only increased in concentrations ranging 1.5-2.5 µM hTf (significantly only at 2.5 µM hTf), and decreased in all other concentrations (about one fold in 1.2 and 4.2 µM hTf and 2-fold in 10.5 µM hTf, although not significantly).

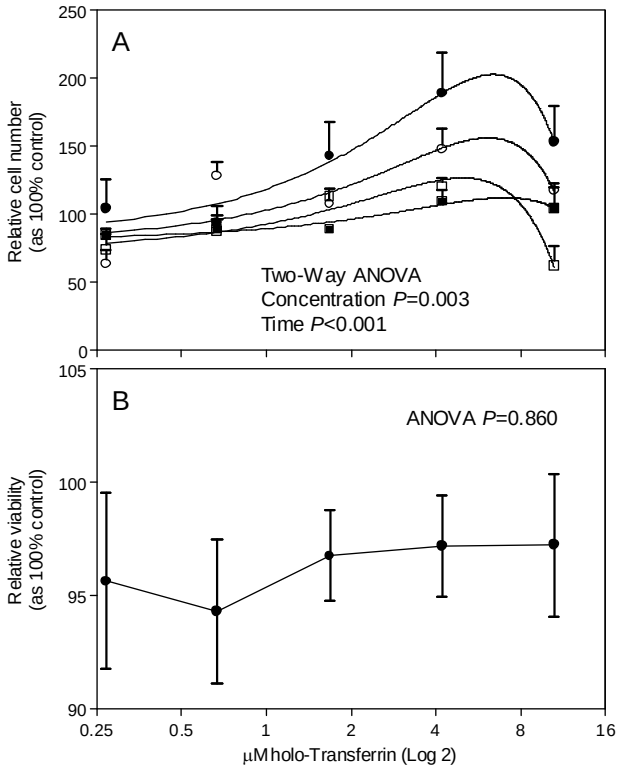

Figure 1. Cell proliferation and viability in response to increasing physiological concentrations of holo-transferrin along 14 days of beta-lymphoblastoid cells' culture. A) relative cell number and second-order curve fits for dose response are shown for days 3 (○, r=0.599; p=0.008), 7 (□, r=0.670; p<0.001), 12 (●, r=0.638; p=0.015) and 14 (■, r=0.365; p=0.022) of treatment with different concentrations of holo-transferrin. B) relative cell viability according to Trypan Blue exclusion assay. Values relative to control cells.

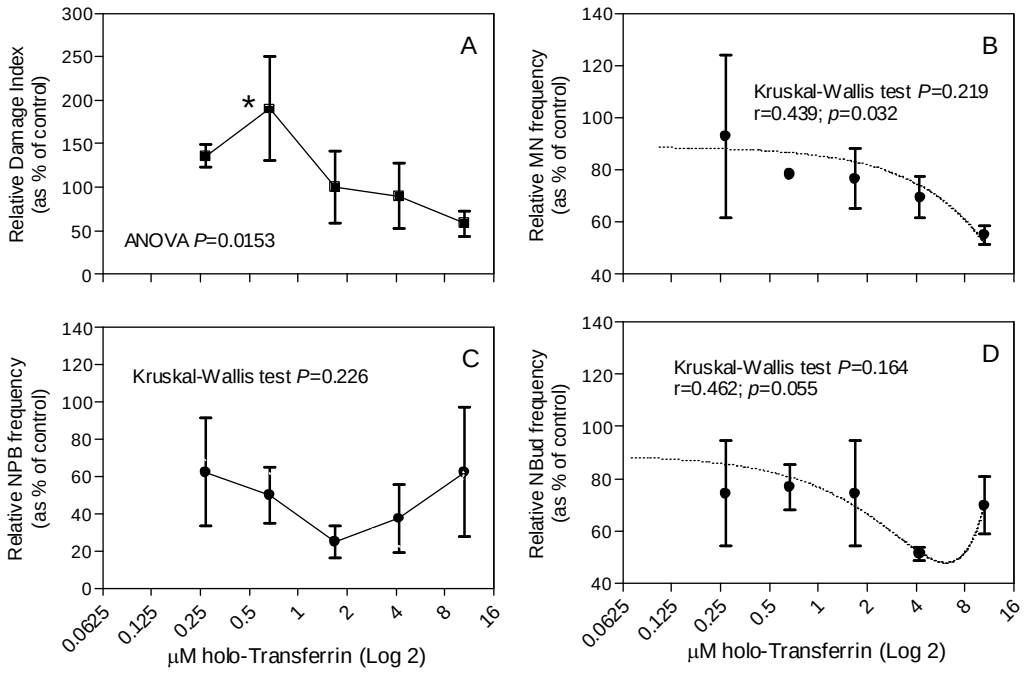

Figure 2. DNA damage as evaluated by Comet and Cytome-Micronucleus assays in response to increasing physiological concentrations of holo-transferrin in beta lymphoblastoid cells' cultures. A) relative Damage Index (DI) in Comet Assay; B) relative Micronucleus (MN) frequency; C) relative Nucleoplasmic bridges (NB) frequency; D) relative Nuclear Buds (NBuds) frequency. Values relative to control cells. * p<0,05 in relation to control cells.

Discussion

Like many others nutrient deficiency, iron deficiency is a continuum from a mild deficiency, that initially has a subtle effect in some physiological functions, starts to hamper erythropoieisis, and then, in the extreme situation, induces severe changes in key physiological changes; in the clinical condition named anemia[34]. ID is the far most common micronutrient deficiency worldwide whose effect over cancer risk and genomic stability has been overlooked in literature, particularly due the role of iron in antioxidant and DNA synthesis and repair enzymes.

We observed the maximal cell proliferation in 4.2 µM hTf. Given the fact that each hTf molecule can provide 2 iron atoms and there were 2 µM iron as the background level in our culture medium (5% fetal bovine serum), there was around 10 µM iron in cell culture medium in this concentration. Anemic individuals have typically SI around 5 µM [35] and the median SI of healthy individuals is around 18 µM [18]. ID individual, whose SI is in between this values show reduction in the proliferative capacity of lymphocytes[36]. Conversely, the 10.5 µM hTf (23 µM Fe, considering the iron background) is closer to the upper SI iron level of 25 µM, the upper border for normal SI values[20] and started to inhibit cell proliferation.

Conventional cell cultures have extremely low levels of iron, making the addition of iron supplementation to culture mediums a very throughput system to shed light upon the precise amount of iron to improve genomic stability. Through this system our data indicate hTf supplementation can increase genomic stability in 14 days cultures of beta-lymphoblastoid cells.

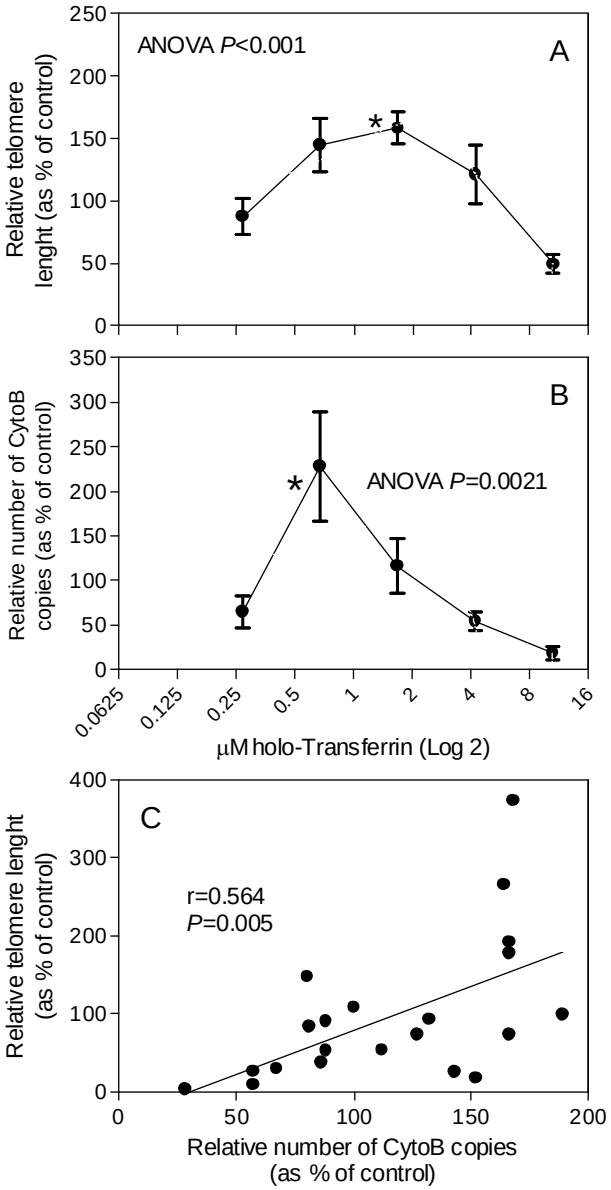

Figure 3. Telomere length and mtDNA copy number are correlated in response to increasing physiological concentrations of holo-transferrin in beta lymphoblastoid cells' cultures. A) relative telomere length; B) relative mtDNA copy number (mtDNA/nDNA ratio; C) Pearson's correlation between telomere length and mtDNA copy number. Values relative to control cells. * p<0,05 in relation to control cells.

In agreement with a possible apoptosis increase (decrease in cell viability) induced by iron hTf supplementation, Berrak et al[37] recently reported iron deficient young subjects have delayed apoptosis reversible by iron supplementation. On the contrary, high iron doses would be expected to decrease apoptosis, by inducing cell cycle abnormalities through a not yet fully characterized mechanism [38,39]. In spite of a mild increase in NPB and NBuds and a decrease in cell number at the higher hTf at the concentration of 10.5 µM hTf concentration, a decrease in MN frequency and comet assay does not support toxicity for this concentration. In agreement, some studies give evidence of low or no genotoxicity for doses in the same range as the tested at least at the short term. Knobel et al[40] observed almost no DNA damage increase in two primary colon cells exposed to 10µM Fe-EDTA for 30 minutes, as evaluated by the comet

assay. In another study, Park et al[41] also using the comet assay, observed a slightly significant increase of single strand breaks, but not of endonuclease-sensitive sites, Fpg-sensitive sites or in the percentage of TP53 migration in human leukocytes treated with 1 or 10µM Fe-NTA for 30 minutes.

One expected effect of long-term iron supplementation at low doses to iron deficient cells would be an increase in the expression and/or activity of iron-containing proteins involved in DNA synthesis and repair and antioxidant defenses. An increase in DNA repair efficiency would be measured by a faster turnover of DNA lesion. In agreement with this hypothesis, a large portion of the DNA damage detected by comet assay can be related to incomplete excision repair sites[42]. Indeed, one could explain the increase in DNA damage at lower iron doses with this mechanism. Notwithstanding, further studies must be conducted to define the kinetics of DNA repair in terms of iron concentration under low iron doses both in vitro and in vivo.

Telomere shortening is an important risk factor for cancer and accelerated ageing and one of the most used indicators of cellular senescence [43-46]. Present results show 0.67-4.2 µM hTf were associated to increased telomere length, while the higher concentration (10.5 µM hTf) induced a substantial decrease in telomere length. In one hand, there are convincing evidences that telomere shortening rates can be diminished if oxidative stress levels by using free radical scavengers, enzymatic or non-enzymatic antioxidants or low ambient oxygen concentrations [47], supporting the concept that the proper iron concentration could counteract telomere attrition generated by its deprivation. There are few studies evaluating the impact of iron over telomere length. Liu et al [48] showed an increase in telomere length of both cancerous and normal hepatocytes cultured for 1 month in medium supplemented with 20 µM ferric chloride. This difference could be explained by the fact that hepatocytes demand more iron than lymphocytes and can surpass telomere loss by increasing telomerase activity under iron-loading conditions [49]. Regarding the demand or iron to lymphocytes, our unpublished data with inorganic iron supplementation in long term WIL2-NS cultures also support telomere increase for doses below 10 µM iron.

On the other hand, telomere primary sequence is considered highly sensitive to oxidation, which could explain why the concentration of 10.5 µM hTf started to generate telomere erosion. A purine nucleotide followed by three or more G residues, RGGG, and purine nucleotides flanking a TG combination, RTGR, are known to be unusually sensitive to nicking via in situ Fe^{+2}-mediated Fenton reaction. The sequence RGGG is constitutes the core of the consensus mammalian telomere sequence $(TTAGGG)_x$ and the sequence RTGR sites in promoters of iron or oxidative stress-regulated genes [50]. Henle et al [51] have shown that telomere-like DNA sequences are much more prone (7-fold) to be cleaved by Fe_{2+}/H_2O_2-mediated oxidations and suggested that telomere sequences might surrogate other DNA sequences as iron-driven oxidation targets. Moreover, repair of SSB is significantly less efficient in telomeres as compared to the bulk of the genome [52] and there are several reports of decreased telomerase activity in cells submitted to oxidative stress [47]. The apparently disagreement between telomere erosion and the decrease of primary DNA damage level and MN frequency seen for the higher hTf concentrations could be explained based in the higher susceptibility of telomere DNA to oxidation than general nuclear DNA.

Mitochondrial dysfunction has been proposed as a major mechanistic link between stress-dependent and telomere-dependent physiological ageing processes in cells. Moreover, it has been is hypothesized telomeres could act as a sensors of mitochondrial DNA damage, making the replicative lifespan submitted to a network of processes involving mitochondrial dysfunction, oxidative stress and telomere shortening[47]. The high correlation between telomere length and mtDNA copy number (Fig. 3C) supports this hypothesis. Iron metabolism/oxygen consumption might play a key role in such network Iron concentration in

culture medium has been shown to modulate mitochondrial oxygen consumption[53] and mitochondrial inhibition has been also shown to decrease iron uptake though down-regulation of key iron metabolism genes (excepting in neurons)[54].

Regarding mtDNA stability, it has not been possible to discern whether mtDNA damage caused mitochondrial dysfunction or was a consequence of it. It could well be that other factors such as the regulation of mitochondrial turnover or mitochondrial fusion and fission in addition to extra-mitochondrial ones could be important [47]. Two lines of defense protects mitochondria: i) surveillance of protein quality control; ii) constant fusion and fission events that grant the dynamic nature off the mitochondrial population of a cell [55]. The hepatocytes of iron-deficient rats showed enlarged, rounded, and electron-lucent mitochondria which have the same number, occupy an increased proportion of total cytoplasm and sometimes are transversed by double membranes[56]. Similarly, treatment of Chang cells with the iron chelator desferoxamide induced elongated giant mitochondria, senescent arrest, increased intracellular ROS level and decrease mtDNA content, in association to the downregulation to the mitochondrial fission protein Fis1 [57]. Iron repletion in iron-deficient rats leads to a partial reversal in mitochondrial configuration, with some normal and some abnormal within 2 days of iron treatment, which could attributed to either division or changes in membranes composition [56]. The increase in mtDNA/nDNA ratio seem in present results could be associated to a proliferative effect in mitochondria which could no compensate their functionality and then generated increased ROS as seen by the increase in primary DNA damage in parallel to mtDNA. In agreement with a non toxic mtDNA toxicity regarding the tested iron concentrations, Itoh et al [58] did not show mtDNA damage at concentrations of 10 µM Fe, but did show at $\geq$100µM Fe in HTC rat hepatoma cell culture treated for 3 hours. Conversely, Oexle et al [53] did not observe changes in total mtDNA content after 24 hours treatment with neither iron nor DFO obviously altered the amount of mitochondrial DNA, suggesting changes in enzyme activity following iron perturbation cannot be relate to varying numbers of mitochondria. Therefore, a specific mtDNA stability end-point should be developed in order to better understand the relationship between iron replenishing, mitochondrial proliferation and DNA damage. The use of a short segment of the mitochondrial cytochrome *b* to quantify absolute mtDNA copy number per diploid nuclear genome leads to values in the same range (of the order of thousands) as those originally observed (e.g. Bogenhagen & Clayton[59]) in cultured mammalian cells [60]. Moreover, RT PCR seems to be more accurate the southern blotting to measure the mtDNA/nDNA ratio [61]. It has been suggested that PCR amplification efficiency can be altered by conformational changes in supercoiled DNA which are typical of mtDNA [62]. Notwithstanding, our PCR protocol starts with a 95^{o}C denaturation for 10 minutes, which would linearize all DNA

In this paper we showed lymphocytes genomic stability could be increased when cell cultures had > 6 µM iron and telomere length started to erode at concentrations around 23 µM iron. These values correspond, respectively, to the lower (anemia) [35] and upper level (overload)[57] for SI in humans. We expect our data can be useful to direct future research regarding iron deficiency and genomic stability. We also expect it can be useful for setting the optimal amount of nutrients in physiological mediums to increase cell viability and reduce the risk of genomic instability, often associated to malignant transformation. One direct application is to improve cell culture conditions of cellular therapies that include cell culture ex vivo previously to transplantation and stem cells maintenance.

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Authorship and Conflict of Interest Statements
Authors declare no conflict interests.

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9.12 ANEXO L “Short report: WRN and MUTYH mRNAs have iron responsive elements and are modulated by iron status”

Manuscrito a ser submetido ao periódico DNA repair

Short report: WRN and MUTYH mRNAs have iron responsive elements and are modulated by iron status

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Abstract
Iron overload induces oxidative stress and is carcinogenic. On the other hand, there are growing evidences that iron deficiency may impair antioxidant defenses and DNA repair. Iron has been known for a long time as co-factor of ribonucleotide reductase, an enzyme that converts ribonucleotides in deoxiribonucleotides and has a key role in maintaining nucleotide pool for DNA synthesis and repair. Recently, iron has been also show to have fundamental roles in DNA repair enzymes, including XPD family DNA helicases, MUTYH and hNTH1 glycosilases and alkyltransferases. However, there is a lack regarding the relation between iron concentration and the expression of these enzymes. In this report we examined WRN and MUTYH mRNA level in WIL2-NS cells grown at physiological concentrations of holotransferrin (hTf) for 14 days. Two genes involved in iron metabolism were also evaluated, transferrin receptor (TFRC) and ferritin light chain (FTL) together with different markers of DNA damage/repair. Results showed that WRN and MUTYH mRNAs can be modulated by iron content in medium in correlation with genomic stability endpoints. Moreover, in silico analysis showed either WRN or MUTYH, similarly to TFRC and FTL, contain typical post-transcriptional iron regulatory elements (IRE) and could have their expression post-transcriptionally modulated by iron.
Key words: iron, DNA repair, glycosilases, helicases, genomic stability

1. Introduction

Iron is a functional part of ribonucleotide reductase (RR) [1]. More recently, the role of iron as a not fully understood functional part of DNA repair glycosylases has also been described [2]. DNA glycosylases are responsible for the excision of DNA lesions in the Base Excision Repair system (BER), which repairs a wide range of base lesions, including 8-oxoguanine. Defects in BER are described in several pro-carcinogenic conditions [3]. Iron is also present in alkyltransferases [4] and seems to play a fundamental role in XPD family DNA helicases [5]. Moreover, defects in the iron-containing DNA glycosidase MUTYH are directly associated with colorectal cancer [6]. Additionally, it has been observed that iron chelators and hypoxia have been also linked to a down regulation of some genes of mismatch repair system (MMR), enzymes whose defects have been linked to hereditary nonpolyposis colon carcinoma [7] as well having been associated to

sporadic colorectal cancer risk in some populations [8]. Nevertheless, there are no studies to date evaluating the effect of iron deficiency in DNA repair enzymes.

WRN gene encodes a RecQ DNA helicase with exonuclease activity, with a causative linkage to Werner syndrome (WS), an autosomal recessive genetic disorder marked by premature aging and hypermutator phenotype [9]. WRN is a housekeeping gene that maps to 8p11-p12 and is composed of 35 exons that transcribes into a mRNA with 5,189 nucleotides. WRN helicase, such as other helicases, might play a key role in providing DNA single-stranded templates for replication, recombination and repair [9]. Most of WRN polymorphisms in WS patients generate truncated DNA helicase molecules lacking the nuclear localization signals in the C-proximal region, rendering WRN products unable to transport to the nucleus, explaining a common phenotype for the disease [9]. WRN polymorphisms in non-WS patients have been associated to increased benzene-induced hemotoxicity and hydroquinone hypersensibility as well as increased type B non-Hodkin lymphoma and familial breast cancer risk [10,11]

MUTYH gene encodes a A/G-specific adenine DNA glycosylase. Germline mutations in MUTYH predispose to MUTYH-associated polyposis (MAP), an autosomal recessive genetic disorder characterized by multiple adenomas and carcinomas [12]. MUTYH maps to 1p32-34 has 7·1 kb in length, and has 16 exons that encode a 535-aminoacid protein with 41% homology to the shorter *E coli* MutY protein and has multiple transcript variants encoding different isoforms [13]. The enzyme excises adenine bases from the DNA backbone at sites where adenine is inappropriately paired. The protein is localized to the nucleus and mitochondria and there is limited biochemical data available on MUTYH. A recent mutation spectrum analyses survey showed 30 truncated variants and 52 missense variants, with Y165C and G382D together accounting for approximately 73% of all mutations reported [12].

Both iron deficiency/anemia and iron overload have been implicated in increased oxidative stress and DNA damage. However, studies evaluating the effect of iron deficiency over DNA repair genes are scarce. To further understand the impact of iron concentration over DNA repair genes expression, we examined WRN and MUTYH expression in WIL2-NS cells grown at 6 physiological concentrations of holotransferrin (hTf) for 14 days (0.25-10.5 μM hTf, or 2.5-23 μM Fe, considering 1M hTf: 2M Fe and iron background was about 2 μM Fe). Two genes involved in iron metabolism were also evaluated, transferrin receptor (TFRC) and ferritin light chain (FTL) together with different markers of DNA damage/repair.

2. Material and methods

2.1. Cell culture, iron treatment, cell counting and viability

The procedure described by Umegaki and Fenech [14] with slight modifications was used in the study. Briefly, WIL2-NS [non secreting human B lymphoblastoid cell line isolated from the spleen of a Caucasian male with hereditary spherocytosis (American Type Culture Collection no. CRL8155) which has been reported to have a high mutational response to X-irradiation] were seeded in RPMI 1640 without L-Glutamine (Sigma, US) culture medium [15], containing, 1% antibiotics solution (Penicillin [5000IU/mL]/ Streptomycin [5mg/mL] - Thermo Trace, Australia), 1% 200 mM L-glutamine (Sigma,US) as well as 5% dialized FBS (Thermo Trace, Australia) a at a initial cell density of $2.5x10^5$ cells/mL. Holo-transferrin – hTf (Sigma, US) at concentrations ranging 0.3-10.5 μM was added to the medium and cells were grown for 14 days, being subcultured on days, 4, 7 and 12. Iron concentration in the complete medium (supplemented ore control) and in FBS was measured by Atomic Absorption Spectrophotometry (Varian SpectrAA-400; Varian, Inc., Palo Alto, CA). All assays were performed in quaduplicates, i.e. for each dose tested, four cell suspensions were treated separately and each of these cell suspensions provided a separate culture for each

assay. The results shown, unless otherwise indicated, represent the means ± SE for these quadruplicate experiments.

2.2. Comet assay

For comet assay, one aliquot of cell culture was immediately used for preparing the slide of each cell culture. The preparation of the slides and the further steps of the comet assay were developed according to standard protocols [16] in agreement to the International Guidelines for conducting the assay [17]. Briefly, leukocytes were embedded in low melting point agarose and this mixture was added to a pre-coated microscope slide. After solidification, the slides were placed in lysis buffer for at least 1 h. Subsequently, the slides were incubated for 20 min in alkali buffer (pH<13) to allow DNA unwinding and, then, electrophoresed for 20 min (4°C) at 300 mA and 20 V (0.8 V/cm). After electrophoresis, the slides were neutralized and stained with propidium iodide. All reagents used were PA grade. Images of 100 randomly selected cells per cell culture (50 cells per replicate slide) were analyzed at 200 X magnification using an optical microscope. The slides were coded and scored using established scoring methods and criteria and the same scorer in all experiments [18,19]. The analyses of damaged nuclei were carried out according to two parameters, the frequency of damaged nuclei and the extent of the DNA damage. Non-detectable cell nuclei (head and tail clearly separated) were not considered in analyses.

2.3. Cytokinesis block micronucleus (CBMN)

For CBMN assay, the cells were incubated for a further 24 h in the presence of 0.6 mg/mL cytochalasin-B (Sigma, US). The slides were prepared by cytocentrifugation (Shandon; Southern Products, Cheshire, U.K.) Before cytocentrifugation, DMSO (Sigma, US) was added at a final concentration of 5% to minimize clumping of the cells and thus optimize recognition of cytoplasmic boundaries. The slides were air dried for 10 min and then fixed and stained using Diff-Quik (LabAids, Brisbane, Australia). The slides were coded and scored by a single individual which was aware of the treatment group of the samples, using established scoring methods and criteria [20] based in the HUman Micro-Nucleus (HUMN) project [21]. Accordingly, binucleated cells (BNC; 1000 cells) were scored for the presence of micronuclei (MN), nucleoplasmic bridges (NPB) and nuclear buds (NBud). The level of mononucleated, binucleated, multinucleated, apoptotic and necrotic cells was determined by scoring 500 cells.

2.4. Real Time-PCR methods

2.4.1. DNA and RNA purification

Total DNA and RNA were extracted using Trizol Reagent® (Invitrogen, Carlsbad, CA), according to manufacturer's instructions. The fraction containing DNA was isolated using the silica-gel-membrane based DNeasy Tissue Kit (Qiagen) as described by Lu et al [22]. To reduce oxidative DNA damage [23], all buffers were purged with nitrogen and supplemented with 50 µM phenyl-tert-butyl nitrone (Sigma), proteinase K treatment was accomplished at 37^{o}C for 3 hours, and 1 mM DTT (dithiothreitol) was added to DNA solution at the end of the extraction [22]. DNA and RNA were quantified in triplicate with a NanoDrop spectrophotometer (NanoDrop Technologies, Delaware, USA) and used as soon as possible for PCR

2.4.2. Telomere length and mtDNA copy number

Quantitative Real-Time PCR amplification (QRTmPCR) was used to measure telomere length and mtDNA copy number, according to O'Callaghan et al [24] and Wang et al [25], respectively. All samples were run on an ABI 7300 Sequence Detection System with the SDS Ver. 1.9 software (Applied Biosystems [AB], Foster City, CA). Each sample was analysed in duplicate. The highly conserved mitochondrial gene *cytochrome b* was used as a marker of mtDNA content, as described by Wang [25]. A nuclear single copy gene, *36B4* which encodes the acidic ribosomal phosphoprotein P0, was used as a control for amplification for every sample performed, as described in Cawthon [26]. Each 20µl reaction was performed as

follows: 20ng DNA, 1xSYBR Green master mix (AB) plus 100nM of forward and 100 nM of reverse primers. For telomere: forward (CGG TTT GTT TGG GTT TGG GTT TGG GTT TGG GTT TGG GTT) and reverse (GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT TAC CCT); for *36B4*: forward (CAG CAA GTG GGA AGG TGT AAT CC) and reverse (CCC ATT CTA TCA TCA ACG GGT ACA A); and for *cytochrome b*: forward (TAT CCG CCA TCC CAT ACA TT) and reverse (GGT GAT TCC TAG GGG GTT GT). Cycling conditions were: 10 min at 95°C, followed by 40 cycles of 95°C for 15 sec, 60°C for all amplicons.

2.4.3. Gene expression

cDNA was synthesized from 1.3 µg total RNA using a High-Capacity cDNA Archive Kit® (ABI, Foster City, CA), according to the manufacturer's instructions. mRNA expression was measured by quantitative real-time PCR (qRT-PCR) using TaqMan® assays (ABI, Foster City , CA). WRN (Hs00172155_m1), MUTYH (xxx), TFRC (Hs00174609_m1) and FTL (Hs00830226_gH) target gene assays (VIC-labelled) were used; *18S RNA* (H_0001) was used as an endogenous control (FAM-labelled) for all reactions. 2 µL cDNA was aliquoted into final reactions volumes of 20 µL per well, together with TaqMan® universal PCR master mix. qRT-PCR was performed using a ABI 7300 Sequence Detection System equipped with the software SDS Ver. 1.9 (ABI, Foster City, CA), following the standard thermal cycling profile: 50◦C for 2 min and 95 ◦C for 10 min for enzyme activation, followed by denaturing at 95 ◦C for 15 s, then annealing and elongation at 60 ◦C for 1 min, with a total of 40 cycles.

2.4.3. Gene expression

Telomere length, mtDNA copy number or relative RNA content (gene expression) were calculated using the comparative $\Delta\Delta C_T$ method. For this method, the endogenous control was used as the comparator (i.e. ΔC_T values of controls were averaged and set as 1 for comparative expression determination). Briefly, a ΔC_T value was calculated for each individual tested by C_T*target* - C_T*36B4* (telomere length or mtDNA copy number) or C_T*target* - C_T*18SRNA* (mRNA level). -$\Delta\Delta C_T$ was calculated by the difference of ΔC_T between experimental and comparator. The relative expression value is shown as $2^{-\Delta\Delta CT}$.

2.5. In silico prediction of secondary structures of selected mRNAs

DNA primary sequences corresponding to 5' untranslated region (5'-UTR) of human transferrin receptor (GenBank acession number gi|4507456|), ferritin light chain (gi|56682960|), MUTYH (gi|115298646|), and Werner Syndrome helicase (WRN; gi|110735438|) were compared using different algorithms implemented in T-COFFEE [local alignment, 27], and CLUSTALW version 2 [global alignment, 28]. Possible RNA secondary structures present in the 5'-UTR of genes described above were predicted using the CONTRAfold program [29].

2.6. Statistical analyses

One-Way ANOVA followed by Dunnet post-tests or Kruskal-Wallis test followed by Dunn post-tests were used to compare gene expression and DNA damage end-points. Linear or polynomial curve fitting equations were used to evaluate dose-response correlations. The software Graph Prism 4.0 was used for statistical analyses and to plot graphs. The level of significance was of $P<0.05$. Values are expressed as average ± standard error of the mean. All values were normalized according to the average value in control to express relative differences. All data were normalized regarding control values (0 hTf). mRNA levels are expressed as folds to control. Primary DNA damage and MN frequency are expressed as % of control.

3. Results and discussion

There was significant increase in TFRC mRNA level in concentrations 0.7-10.5 µM hTf (3.4-23 µM Fe), and no effect in FTL mRNA level, excepting an increase for the highest

concentration (Fig. 1 A and B). Regarding WRN mRNA, there was a progressive decrease in mRNA level to a maximum at 4 μM hTf (10 μM Fe), with a tendency to increase at the maximal concentration. MUTYH mRNA level showed a descendent and ascendant typical "U"-shaped curve, with minimal mRNA level at 1.7 μM hTf (5.4 μM Fe) and a significant increase in the maximal concentration (Fig. 1 C and D).

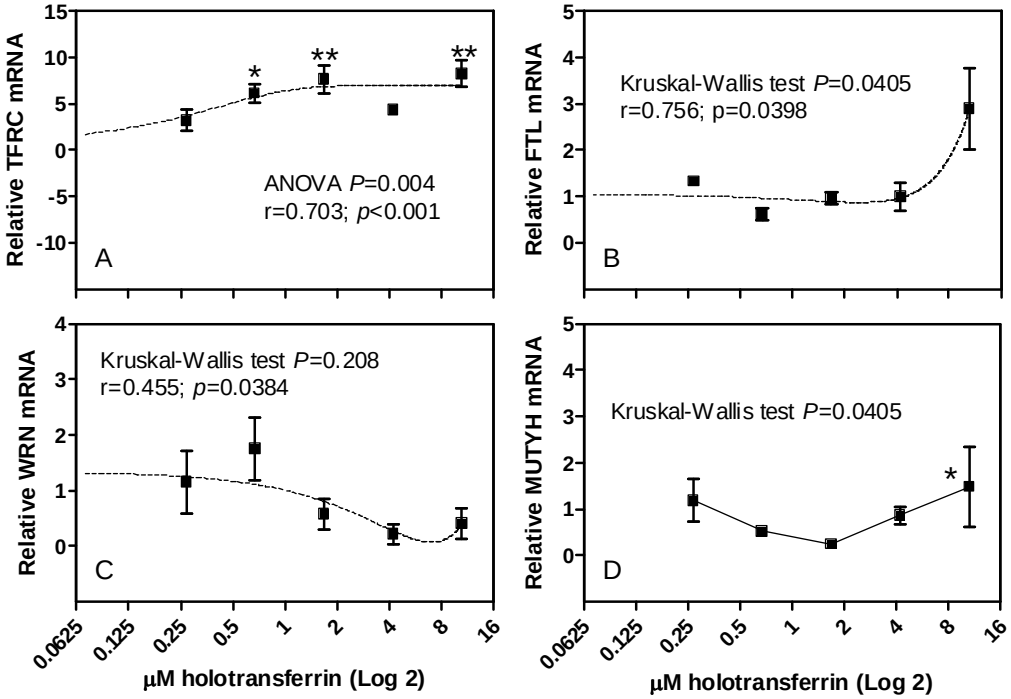

Figure 1. Relative mRNA level in WIL2-NS cells exposed for 14 days to various concentrations of iron as holotransferrin. A) transferrin receptor, B) ferritin light chain, C) WRN, D) MUTYH. mRNA levels are expressed as folds to control cells.

WRN mRNA level correlated positively to primary DNA damage level and MN frequency (Fig. 2. A and B). MUTYH mRNA level, on the contrary, tended to be lower in situations of increased primary DNA damage or increased MN frequency (Fig 2. C and D). Interestingly, there was a strong inverse association between telomere length and MUTYH mRNA level (Fig 2. E).

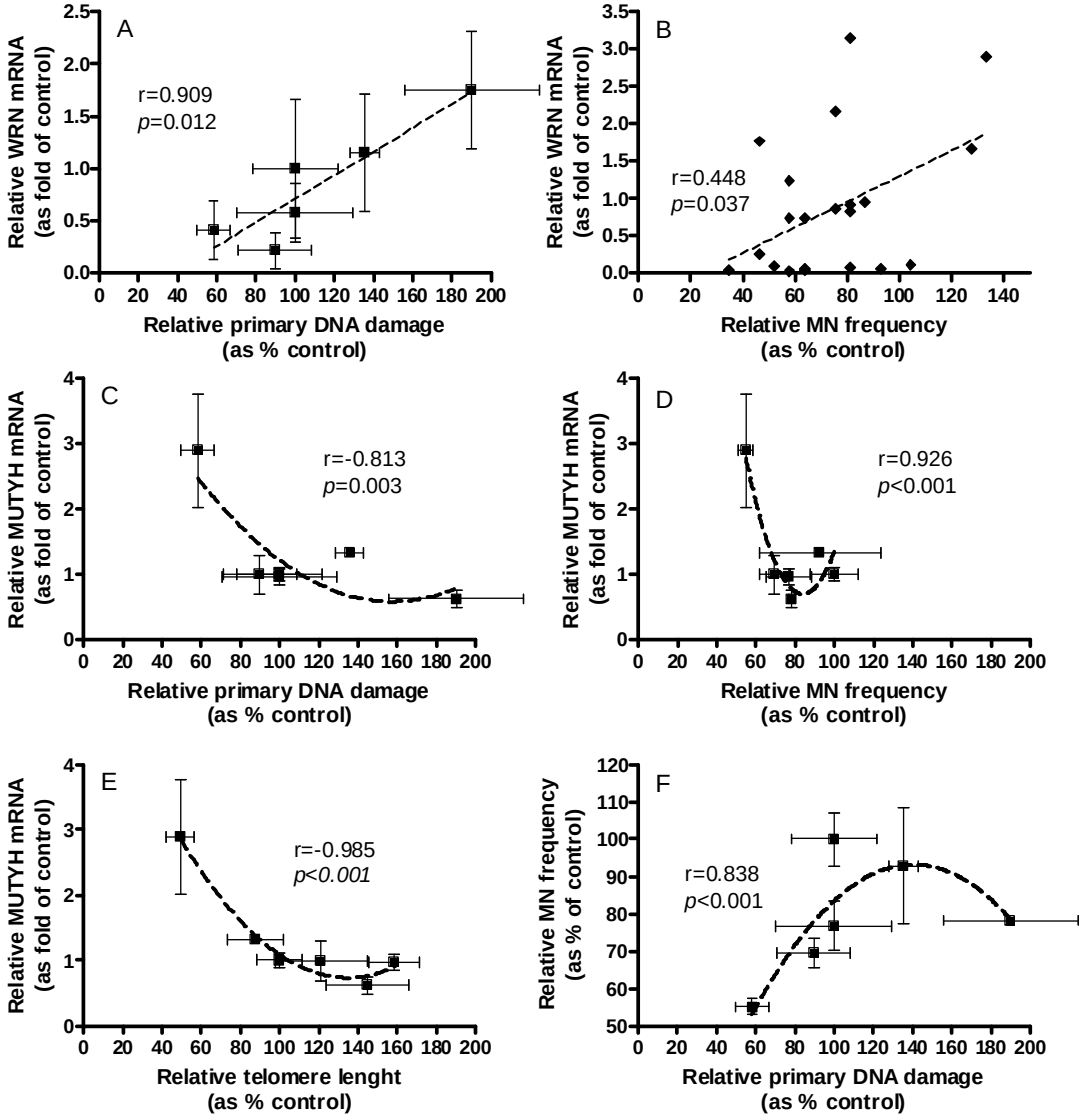

Figure 2. Correlations between the mRNA levels and genomic stability end-points in WIL2-NS cells exposed for 14 days to various concentrations of iron as holotransferrin. A) primary DNA damage (comet assay) versus WRN mRNA level; B) MN frequency versus WRN mRNA level, C) primary DNA damage versus MUTYH mRNA level, D) MN frequency versus MUTYH mRNA level, E) telomere length versus MUTYH mRNA level, F) primary DNA damage versus MN frequency. mRNA levels are expressed as folds to control cells and genomic stability end-points are expressed as percentage of control cells.

DNA alignment of 5'- and 3'-untranslated region (5'-UTR and 3'-UTR, respectively) of TRFL, FTL, MUTYH, and WRN genes using two different alignment algorithms do not indicate any similarity in the primary sequences analyzed. However, it was possible to observe in the 5'-UTR of WRN and in the 3'-UTR MUTYH genes the presence of secondary RNA structures similar to those found in iron-responsive elements (IREs) of TRFL and FTL (Fig. 3), indicating that both WRN and MUTYH could also be regulated by iron metabolism by a homologous post-transcription mechanism. To date, in spite of the characterization of roles of iron in DNA repair genes, there was no evidence of iron regulation over DNA repair enzymes.

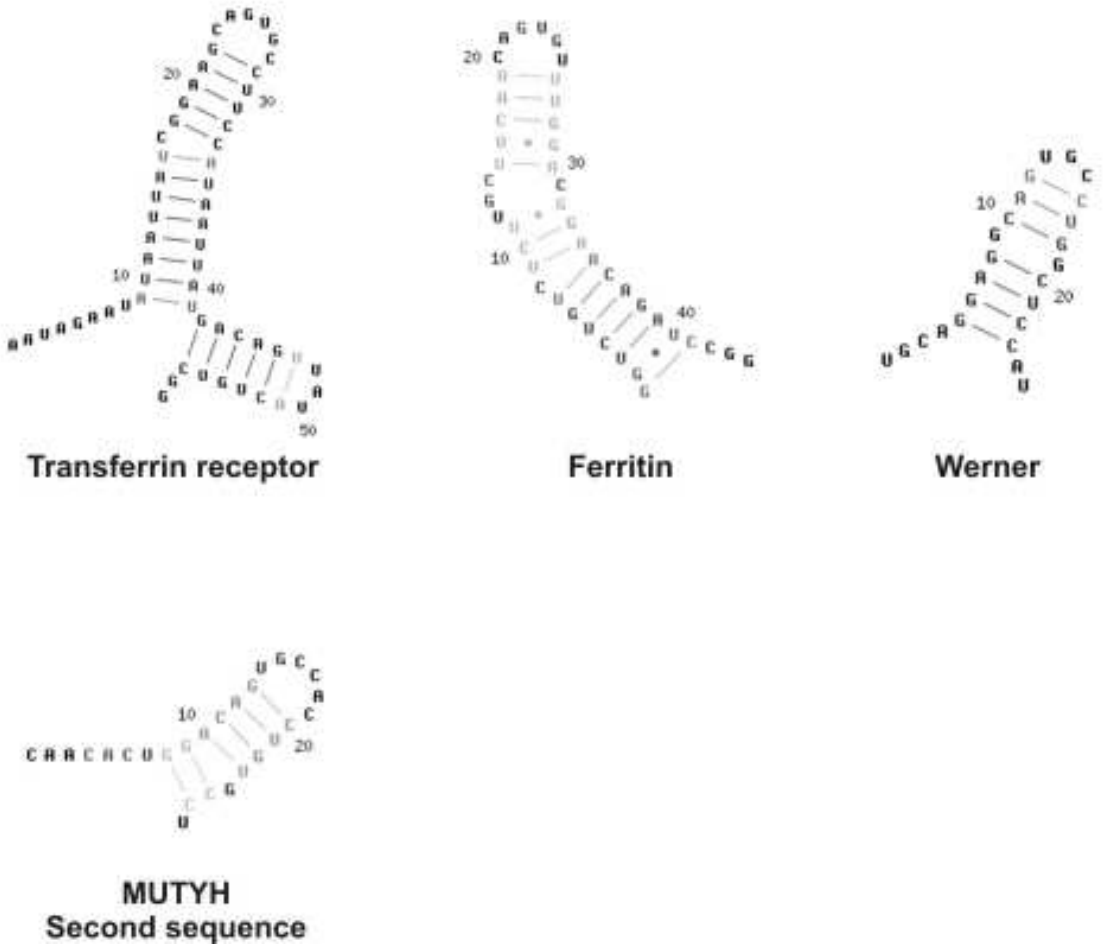

Figure 3. Predicted mRNA secondary structure of the evaluated genes, indicating iron responsive elements motifs.

Two cytoplasmic RNA-binding proteins, iron-regulatory protein-1 (IRP-1) and IRP-2, respond to changes in cellular iron availability and coordinate the expression of mRNAs that harbor IRP-binding sites, iron-responsive elements (IREs). IRPs operate at post-transcriptional level under the influence of iron, nitric oxide, and oxidative stress Under low iron conditions, IRP-IRE interaction can have two opposing effects. When IREs reside in 5' untranslated regions (UTR), translation is inhibited by a feedback regulation such as for ferritin heavy or light chain. In this case, a low chelatable "free" cytoplasmic iron pool blocks the formation of its own deposit site (ferritin). On the other hand, when IREs are sited in the 3' UTR, low iron leads to the inhibition of mRNA degradation, as in the case of TfRC [30].

Similarly to FTL, WRN gene has its IRE located in 5' UTR, which would account for a translation inhibition under low iron situations. On the contrary, MUTYH has its IRE in 3' UTR as TFRC, which could account for an inhibition of mRNA degradation at low iron situations, therefore, increasing the mRNA level. It has been shown for a long time that anemic individual have increased serum transferrin receptor [31]. We have shown for the first time that TFRC mRNA level can be increased with hTf supplementation at physiological level. Moreover, we hypothesize MUTYH mRNA degradation could be associated to a reduction in oxidative stress level and, the related telomere erosion (Fig. 2E). Either iron deficiency or iron overload have been associated to increased oxidative stress and mtDNA damage [32,33]. Moreover, chronic oxidative stress can generate BER intermediates [34] and

telomere primary sequence are highly sensitive to oxidation and Fe_{2+}/H_2O_2-mediated oxidations generate 7-fold more lesions in telomeric sequences $(TTAGGG)_x$ than in control sequences [35]. In spite of the fact, there are no studies linking MUTYH to telomere maintenance, MUTYH codes for a DNA glycosylase involved in a exclusive postreplication repair pathway base excision repair system (BER) targeted for removal of the adenine base missincorporated DNA to 8-oxo-7,8-dihydroguanine (8-oxoG) in the newly synthesized daughter strand of DNA [36]. 8-oxoG level is strongly correlated with the level of cellular libile iron in human lymphocytes [37]. MUTYH interacts with several DNA repair enzymes, including RPA, MMR heterodimer MSH2/MSH6, APE1, PCNA, and bears an iron-sulphur [12,36]. Moreover, it has been hypothesized the [4Fe-4S] cluster in MutY glycosylase, the bacterial orthologue of MUTYH, can function in DNA mediated electron transfer, whose inhibition serves as the driver force for the action of the enzyme. Upon its binding to intact DNA, the enzyme becomes oxidized and releases an electron in a DNA-mediated reaction. DNA breaks, base damages or mismatches inhibit DNA-mediated electron transfer resulting in local procession of the DNA glycosylase towards the damage site [2].

The analysis of the transcription pattern of colonic cells upregulated by iron deprivation in rats by Collins & Hu [38] showed that only 2 out of more than 200 genes are regulated by IRE and, conversely, most are the regulation of iron metabolism induced had a strong statistical enrichment for Sp1 and Sp1-like binding sites in experimental promoters as compared to background sequences; many of them with multiple highly conserved SP1 and FOX binding sites [38].

Sp1 is an ubiquitous zinc-finger transcription factor that binds to the GC-box motif (5′-GGGGCGGGG-3′), which is especially critical for the transcriptional regulation of housekeeping genes and the genes that generally do not contain TATA—or CAAT—boxes within their proximal promoter. A number of genes that code for the enzymes involved in glucose metabolism and DNA synthesis fall into this category. Interestingly as other zinc finger domains, sp1 binding is dependent of GC composition of DNA binding sequence and is reduced in tissue of old rats, possibly by the oxidation of their cysteine residues. At increasing levels of intracellular oxidants with age, the ability of the cysteine residues to maintain the reduced thiol status begins to be compromised. The resultant change in the finger conformation impairs the DNA binding ability of these transcription factors, thereby altering their gene regulatory function [39].

WRN upstream region (UR) contains a number of cis-regulatory elements, including 7 sp1, 9 retinoblastoma control elements (RCE) and 14 AP2 motifs. Two sp1 motifs locate close to the transcription initiation sites are indispensable for WRN promoter activity and bind specifically to sp1 proteins. In addition to the modulation by Sp1 and Rb, p53 also controls WRN expression [9]. One interesting finding in this sense is that WRN function is abolished by cpG-island promoter hypermetilation, increasing chemotheraphy efficiency on cancer cells [40]. The downregulation of mRNA level in response to increased iron concentration could be explained by Sp1 regulation.

It has been seen an inverse correlation between the expression levels of WRN and the resistance to genotoxic insults both in vitro and in vivo [40]. WRN interacts with DNA-PKcs and Ku 70/80, two key enzymes for repairing double strand breaks (DSB), trought non-homologous end-joining. WRN suffers post-transcriptional regulation and interacts with several other enzymes such as DNA polymarese delta, PCNA, RPA and TRF2 [40]. Moreover, serially passaged fibroblasts from WS patients show average increased heterogeneity and decreased size regarding telomere length, possibly due to the fact WRN plays a helicase role in unwinding telomeres' terminal loops [40], possibly through the interaction with TRF2 [41]. However, we did not see a clear relationship between WRN mRNA level and telomere length.

Two interesting features also support a role of iron in regulating the expression of DNA repair genes: i) many genes contain, including iron or oxidative stress-regulated ones, contain RTGR sites in promoters [42]. RTGR sequences exist in the promoter of AP endonucleases [43], that interact with MUTYH in BER [6] and are essential to hematopoietic maturation [44]. Moreover, RTGR motif is directly involved in the regulation of certain chemoprotective responses mediated by the antioxidant-responsive element (ARE)(Henle et al, 1999)[45]; ii) there are evidences of a circuitry between oxidative metabolism and DNA repair. For instance, aconitase, a key iron regulator also know as IRP1, plays a dual role in yeast cells acting as an iron metabolism regulator and as a component of mtDNA that protects this genome against excessive accumulation of point mutations and ssDNA and suppresses reductive recombination [46].

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