Universidade Federal do Rio Grande do Sul

Programa de Pós-Graduação em Biologia Celular e Molecular

Avaliação do efeito do micronutriente Ferro (Fe) na viabilidade celular e estabilidade genômica de culturas celulares de fibroblasto pulmonar (MRC5) e hepatorcarcinoma (HepG2) humanos

TESE DE DOUTORADO

Ana Lúcia Vargas Arigony

Porto Alegre, março de 2013.

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Orientador: João Antonio Pêgas Henriques

Porto Alegre, março de 2013.

É exatamente disso que a vida é feita: de momentos! Momentos os quais temos que passar, sendo bons ou não, para o nosso próprio aprendizado, por algum motivo. Nunca esquecendo do mais importante:
Nada na vida é por acaso...
Chico Xavier

Se as coisas são inatingíveis... ora!
Não é motivo para não querê-las...
Que tristes os caminhos, se não fora
A presença distante das estrelas!
Mario Quintana

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ESTRUTURA DA TESE

O presente trabalho está dividido na seguinte forma: Introdução Geral, Objetivos (gerais e específicos), dois capítulos escritos na forma de artigos científicos contendo uma página de apresentação sobre o assunto, Discussão Geral, Conclusões (gerais e específicas), Perspectivas, Referências Bibliográficas e Anexo.

A Introdução Geral discorre a respeito do micronutriente de interesse nessa tese, o ferro e seu papel no organismo como um todo, abrangendo desde as fontes de ferro provenientes da alimentação, passando por todos os processos de metabolismo, incluindo seu efeito redox e papel na estabilidade genômica.

O Capítulo I refere-se ao artigo de revisão sobre a influência de micronutrientes na integridade genômica e no desenvolvimento de culturas celulares, traçando paralelos entre os níveis de micronutrientes comumente encontrados em culturas celulares e os seus níveis fisiológicos em humanos.

No Capítulo II estão ilustrados os resultados obtidos avaliando-se a influência do ferro na viabilidade celular e estabilidade genômica de duas linhagens celulares humanas, um fibroblasto pulmonar não tumoral (MRC5) e um hepatocarcinoma (HepG2).

A Discussão Geral contempla os comentários sobre os resultados apresentados nos dois Capítulos e a sua importância para a contribuição científica deste estudo. Após, estão descritas as Conclusões e as Perspectivas geradas por este trabalho, as Referências Bibliográficas utilizadas na elaboração desta tese e o Anexo (*Curriculum Vitae*).

RESUMO

Micronutrientes, vitaminas e minerais, são indispensáveis para as vias de metabolismo do DNA e, além disso, são tão importantes para a manutenção da vida quanto os macronutrientes. Na ausência dos nutrientes adequados, a instabilidade genômica compromete a homeostase, ocasionando doenças crônicas e certos tipos de câncer. Meios de cultura celular tem por finalidade mimetizar o ambiente *in vivo*, proporcionando aos modelos *in vitro* condições adequadas para que se avalie a resposta celular aos diferentes estímulos. O artigo de revisão sumariza e discute os micronutrientes usados na suplementação das culturas celulares e sua influência na a viabilidade celular e a estabilidade genômica, focando nos estudos *in vitro* previamente realizados. Nestes estudos, os meios de cultura celular incluem certas vitaminas e minerais em concentrações distintas das fisiológicas *in vivo.* Em muitos meios de cultura comumente usados, a única fonte de micronutrientes é o Soro Fetal Bovino (SFB), o qual contribui com 5-10% da composição final do meio. Atenção insuficiente tem sido direcionada à composição de SFB, micronutrientes e culturas celulares como um todo, ou à influência de micronutrientes na viabilidade e genética de culturas celulares. Estudos adicionais avaliando melhor o papel de micronutrientes no nível molecular e a sua influência na estabilidade genômica de células ainda se fazem necessários. O micronutriente foco dessa tese é o Ferro (Fe), que por sua vez é um micronutriente essencial, sendo requerido para o crescimento, desenvolvimento e condições normais de funcionamento das células. Tanto seu excesso quanto a sua deficiência podem causar estresse oxidativo e dano ao DNA. Uma vez que os meios de cultura usualmente utilizados para culturas celulares têm níveis de Fe abaixo das concentrações encontradas no soro fisiológico humano, os objetivos deste estudo foram a avaliação do papel da suplementação com Fe na viabilidade celular, na produção de espécies reativas de oxigênio (ERO), na atividade da catalase, na integridade genômica, na expressão de proteínas de reparo de DNA que contém *clusters* Fe/S em sua estrutura (TFIIH e MutyH) e na expressão de receptores de absorção de Fe (CD71 e Nramp2). Duas linhagens celulares – MRC5

(fibroblasto pulmanar humano) e HepG2 (hepatocarcinoma) - e dois tipos de suplementação com Fe foram utilizados, holo-Transferrina (h-Tf) e $FeSO_4$. Ambas suplementações foram capazes de aumentar os níveis intracelulares de Fe e a viabilidade genômica. A suplementação com Fe também aumentou a formação de ERO, sem alterar a atividade da catalase. No entanto, este aumento de ERO não foi acompanhado por genotoxicidade. No que se refere à expressão de proteínas de reparo ao DNA, os resultados sugerem que o pré-tratamento com h-Tf ou $FeSO_4$ não exercem influência direta na expressão de TFIIH ou MutyH. Entretanto, na expressão de receptores de Fe, os resultados preliminares indicam que CD71 é uma via prioritária de absorção de Fe, estando relacionada com a homeostase de Fe, enquanto Nramp2 parece ter um papel secundário. Devido à importância fisiológica da h-Tf na homeostase do Fe e o acúmulo de ERO menos pronunciado, sugere-se que h-Tf seja uma melhor forma para a suplementação de Fe nas culturas *in vitro*. Estudos adicionais se fazem necessários para a melhor elucidação do papel do Fe na viabilidade celular e estabilidade genômica.

Palavras-chave: micronutrientes, ferro, viabilidade celular, estabilidade genômica, MRC5, HepG2, h-Transferrina, $FeSO_4$.

ABSTRACT

Micronutrients, including minerals and vitamins, are indispensable to DNA metabolic pathways and thus are as important for life as macronutrients. Without the proper nutrients, genomic instability compromises homeostasis, leading to chronic diseases and certain types of cancer. Cell-culture media try to mimic the i*n vivo* environment, providing *in vitro* models used to infer cells' responses to different stimuli. The review summarizes and discusses studies of cell-culture supplementation with micronutrients that can increase cell viability and genomic stability, with a particular focus on previous *in vitro* experiments. In these studies, the cell-culture media include certain vitamins and minerals at concentrations not equal to the physiological levels. In many common culture media, the sole source of micronutrients is fetal bovine serum (FBS), which contributes to only 5-10% of the media composition. Minimal attention has been dedicated to FBS composition, micronutrients in cell cultures as a whole, or the influence of micronutrients on the viability and genetics of cultured cells. Further studies better evaluating micronutrients' roles at a molecular level and its influence on the genomic stability of cells is still required. The micronutrient focus on this thesis is Iron (Fe), which is an essential micronutrient and is required for growth, development, and normal cellular functioning. Either excess or deficiency of iron can cause oxidative stress and DNA damage. Since the cell media commonly used for cell culture has a lower iron concentration than the human serum, this study aimed to evaluate the role of iron supplementation on viability, reactive oxygen species (ROS) production, catalase activity, genome integrity and the expression of iron-bearing DNA repair proteins (TFIIH and MutyH) and proteins associated with iron absorption (CD71 and Nramp2). Two human cell lines – MRC5 (normal lung fibroblast) and HepG2 (hepatocellular carcinoma) and 2 sources of iron - holo-Transferrin (h-Tf) or $FeSO_4$ were used. Both iron supplements were able to increase intracellular iron levels and cell viability. Iron supplementation increased the formation of ROS, but did not alter catalase activity. However, this increase was not accompanied by genotoxicity. Regarding the DNA repair protein expressions, the results suggest that 24h

pre-treatment with h-Tf or $FeSO_4$ has no role in the TFIIH or MutyH expressions. Although, in iron receptor proteins expression, the preliminary data could indicate that CD71 is priority related with Fe homeostasis while Nramp2 seems to have a secondary role. Due to h-Tf physiological role in the iron homeostasis and the less pronounced ROS accumulation, h-Tf could be a better iron supplier *in vitro*. Additional studies are still required to better elucidate the role of Fe in cell viability and genomic stability.

Key words: micronutrients, Iron, cell viability, genomic stability, MRC5, HepG2, h-Transferrin, $FeSO_4$

LISTA DE ABREVIATURAS E SIGLAS

8-OHdG	8-hydroxy-2′-deoxyguanosine (8-hidroxideoxiguanosina)
8-oxodG	8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-Gua: 8-oxo-guanosina)
AD	Alzheimer's disease (Doença de Alzheimer)
AlkB	Alpha-ketoglutarate-dependent dioxygenase
APE1	Apurinic/apyrimidinic endonuclease 1
apo-Tf	apo-Transferrina
B12	Cianocobalamina
B7	Biotina
B9	Ácido fólico/folato
BACH1	Basic leucine zipper transcription factor 1
BCRP	Breast cancer resistance protein
BER	Base excision repair (Reparação por excisão de bases)
C	Control (Controle)
CAT	Catalase
CBMN	Cytokinesis-Blocked Micronucleus
CD71	Cluster of differentiation 71
Cu	Cobre
DCYTB	Duodenal cytochrome b
DF	Damage frequency (Frequência de dano)
DI	Damage index (Índice de dano)
DMEM	Dulbecco's Modified Eagle Medium
DMEM/HAM F-12	Dulbecco's Modified Eagle Medium/Ham's Nutrient Mixture F-12
DMT1	Divalent metal transporter 1 (Trasportador de metais divalentes 1)
DNA	Deoxyribonucleic acid (Ácido desoxiribonucléico)
ENDO III	Endonuclease III
ERCC2	Excison repair cross complementing 2
FancJ	Fanconi anemia group J protein
Fe	Ferro
Fe/S	Ferro/Enxofre
Fe2+	Ferro ferroso/forma reduzida
Fe3+	Ferro férrico/forma oxidada
$FeSO_4$	Sulfato ferroso
FLVCR	Feline leukemia virus subgroup C receptor
FPG	Formamidopyrimidine DNA glycosylase
FPN	Ferroportina
GPAT	Glycerol-3-phosphate acyltransferase, mitochondrial
GSH	Glutationa
H_2O_2	Peróxido de Hidrogênio
HAM F-10	Ham's Nutrient Mixture F-10
HAM F-12	Ham's Nutrient Mixture F-12
HCP1	Haem carrier protein 1
HepG2	Human hepatocellular liver carcinoma cell line

HO-	Hidroxila
h-Tf	holo-Transferrina
IRE	Iron Responsives Elements (Elementos responsivos a Ferro)
IRP	Iron Responsive Proteins (Proteínas responsivas a Ferro)
LIP	Labile Iron Pool
MEM	Minimum Essential Medium
MMR	Mismatch Repair
MMS	Metil metano sulfonado
MRC5	Human fetal lung fibroblast cell line
mRNA	RNA mensageiro
mtDNA	DNA mitocondrial
MutyH	mutY homolog
NER	Nucleotide excision repair (Reparação por excisão de nucleotídeos)
Nramp2	natural resistance-associated macro-phage protein
NTHL1	Nth endonuclease III-like
OH•	Radical hidroxila
PDT	Population doubling time
PRIM2	Primase, DNA, polypeptide 2
RLI1	Fe-S ribosome biogenesis protein Rli1
RNA	Ribonucleic acid (Ácido Ribonucléico)
ROS/ERO	Reactive oxygen species (Espécies reativas de oxigênio)
RPMI	Roswell Park Memorial Institute medium
RRM1	Ribonucleotide reductase M1
RRM2	Ribonucleotide reductase M2
RRM2B	Ribonucleotide reductase M2 B (TP53 inducible)
S	Enxofre
SB	Single- and double strand breaks
SDH	Sucinato desidrogrenase
SFB/FBS	Soro Bovino Fetal/Fetal Bovine Serum
SOD	Superóxido dismutase
Tf	Transferrina
TFIIH	Multi-protein complex transcription initiation factor
TfR1	Transferrin receptor 1
TP53	Tumor protein 53
TR/RT	Transferrin receptor (Receptor de Transferrina)
TYW1	tRNA-yW synthesizing protein 1 homolog
Vitamin A	Ácido retinóico/retinol/alfa e beta caroteno
Vitamin C	Ácido ascórbico
Vitamin E	Tocoferol/tocotrienol
WRN	Werner syndrome
XPD	Xeroderma pigmentosum complementary group D

SUMÁRIO

1. INTRODUÇÃO GERAL

1.1. O Micronutriente Ferro

O Ferro (Fe) é um micronutriente de importância crucial para os organismos vivos, uma vez que é cofator de numerosas proteínas ou enzimas envolvidas na respiração, na síntese de DNA e muitos outros processos críticos do metabolismo celular (PRA *et al.*, 2009; PRA *et al.*, 2012). Em decorrência de sua natureza de metal divalente, pode atuar como um componente redox de proteínas e é intimamente envolvido em processos metabólicos que requerem transferência de elétrons (MACKENZIE *et al.*, 2008), incluindo o transporte de oxigênio, a fosforilação oxidativa e o metabolismo xenobiótico (HENTZE *et al.*, 2004).

O Fe é constituinte de uma gama de proteínas importantes como a hemoglobina, citocromos, oxigenases, flavoproteínas, redoxinas e enzimas de reparação do DNA (PRA *et al.*, 2009; PRA *et al.*, 2012). Como metal de transição, participa da transferência de elétrons via reações de oxidação-redução que resultam na flutuação do Fe entre seu estado férrico (Fe^{3+}) e sua forma ferrosa (Fe^{2+}) (PAPANIKOLAOU & PANTOPOULOS, 2005). Esta característica é amplamente responsável pela significância biológica do Fe, permitindo que o mesmo participe da produção de energia intracelular. Contudo, é igualmente responsável pela toxicidade observada em condições em que o Fe está em excesso.

A maior parte do Fe citoplasmático encontra-se em sua forma reduzida (Fe^{2+}), fornecendo ao organismo um excelente substrato para a oxidação (PAPANIKOLAOU & PANTOPOULOS, 2005). A doação de elétrons, por sua vez, leva a formação de espécies reativas de oxigênio (ERO), onde Fe^{2+} pode interagir com peróxido de hidrogênio (H_2O_2) e produzir Fe^{3+}, ânion hidroxila (^{-}OH) e radical hidroxila (OH•), caracterizando tanto a reação de Fenton quanto a de Haber-Weiss, onde o Fe atua como um catalisador (Fig. 1) (FOY & LABHASETWAR, 2011).

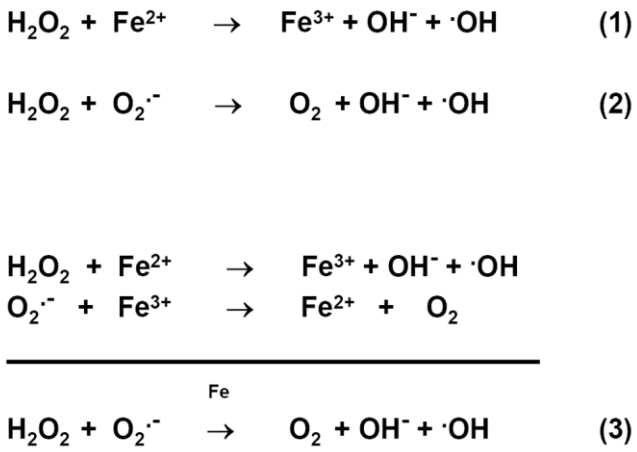

Figura 1. Espécies reativas de oxigênio e ciclagem redox do Fe. A reação de Fenton está representada pela equação (1), a reação de Haber-Weiss, pela equação (2), e o Fe como catalizador da reação de Haber-Weiss pela equação (3). A reação de Fenton descreve a decomposição do peróxido de hidrogênio ao radical extremamente reativo hidroxila, na presença de ferro ferroso.

A reação de Fenton pode ser mediada ainda por outros metais, como o cobre, sendo neste caso referida como *Fenton*-like. O acúmulo dos produtos de tal reação pode levar ao dano oxidativo do DNA, prejudicando a síntese de proteínas, lipídeos de membrana e carboidratos (HALLIWELL & GUTERRIDGE, 2007).

Devido a este potencial reativo, tem sido atribuído ao Fe, quando em excesso, participação no desenvolvimento da carcinogênese, aterosclerose e desordens neurodegenerativas, como mal de Parkinson e Alzheimer [para revisão, ver FLEMING & PONKA (2012); PRA *et al.* (2012)]. É importante observar que tanto o acúmulo de Fe no organismo quanto a sua deficiência podem ser deletérios (PRA *et al.*, 2012). Uma vez que o Fe está intimamente envolvido com a produção de energia e o transporte de oxigênio, sua deficiência é um problema sério capaz de gerar dano celular, redução do crescimento e proliferação das células, hipóxia e até mesmo, morte celular (MACKENZIE *et al.*, 2008; PRA *et al.*, 2011). A dualidade do Fe, sendo tanto essencial quanto tóxico, levou a elaboração de sistemas fisiológicos e celulares complexos a fim de garantir níveis adequados de Fe ao organismo e, simultaneamente, prevenindo a sua sobrecarga (HENTZE *et al.*, 2004).

1.1.1. Fontes de Ferro na Dieta

O Fe é obtido pela alimentação tanto em sua forma heme, de origem animal, cuja principal fonte são as carnes vermelhas, entre elas, o fígado, como na sua forma não-heme, inorgânica - sulfatos, óxidos e íons livres. Estima-se que aproximadamente 85-90% do Fe na dieta seja do tipo não-heme (CHENG, 2009), sendo, neste caso, proveniente de feijões, espinafre, frutas secas e cereais. (WINGARD *et al.*, 1995). O Fe férrico (Fe^{3+}) é mais facilmente encontrado na forma heme, enquanto o Fe ferroso (Fe^{2+}), em vegetais (CHENG, 2009). O Fe^{2+} é melhor absorvido na camada mucosa do intestino do que o Fe^{3+} (CHENG, 2009).

É importante observar que, na atualidade, a fim de combater a anemia proveniente da carência de Fe, muitos alimentos industrializados são enriquecidos com Fe e em paralelo, muitas pessoas consomem suplementos alimentares contendo Fe (PRA, 2008).

1.1.2. Homeostase do Ferro - Absorção, Transporte e Armazenamento

A manutenção do equilíbrio homeostático do Fe requer somente que 1-3 mg sejam absorvidos diariamente para compensar as perdas das células escamosas. Uma vez que não existe mecanismo regulado de excreção de Fe, sua absorção é altamente controlada (FLEMING & PONKA, 2012).

O Fe^{3+} da dieta é primeiramente absorvido pelos enterócitos duodenais (Fig. 2), sendo reduzido na membrana apical pela enzima ferroreductase - DCYTB (*Duodenal cytochrome b*), que altera o estado de oxidação do Fe. Então, o Fe^{2+} é carregado para dentro da célula pelo transportador de metal divalente 1 - DMT1 (*Divalent metal transporter 1*) (FLEMING & PONKA, 2012).

A absorção intestinal do Fe heme é mediada pelo HCP1 (*Haem carrier protein 1*). Contudo os mecanismos ainda não foram plenamente elucidados (FLEMING & PONKA, 2012). É possível que uma porção heme intacta seja liberada do enterócito através de dois exportadores de heme -

BCRP (*Breast cancer resistance protein*) e FLVCR (*Feline leukemia virus subgroup C receptor*) (CAIRO *et al.*, 2006).

Dentro do enterócito, a maior parte do Fe^{2+} proveniente de qualquer fonte é armazenada sob a forma de ferritina, o mesmo pode vir a ser perdido no desprendimento dos enterócitos senescentes ou ser transportado através da superfície basolateral do enterócito para alcançar o plasma, através da ferroportina (FPN). Neste caso, deve ser subsequentemente convertido a Fe^{3+} pela hepaestina, uma ferroxidase associada à membrana para então, ser transportado aos demais tecidos pela transferrina (Tf) (DE DOMENICO *et al*., 2008).

A regulação de cada uma dessas etapas (redução, absorção, armazenamento e transporte) é mediada por sinais que refletem a tensão de oxigênio nos enterócitos, os níveis de Fe intracelulares e as demandas de Fe sistêmicas (FLEMING & PONKA, 2012).

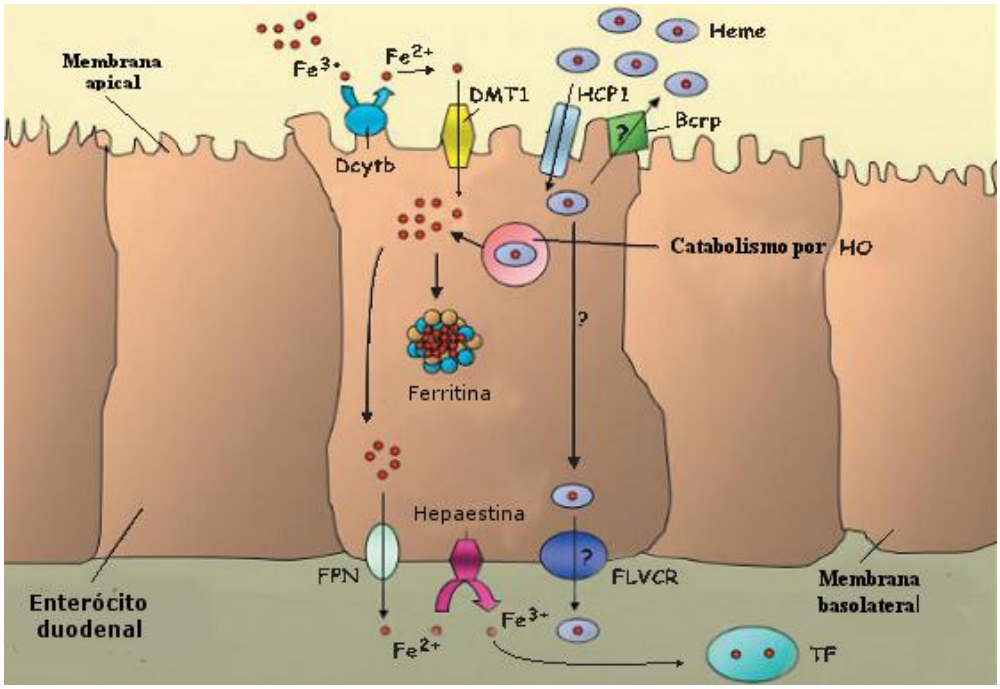

Figura 2. Absorção intestinal do Fe nos enterócitos e as respectivas enzimas envolvidas nas etapas de redução, absorção, armazenamento e transporte. Adaptada de CAIRO *et al.* (2006).

Normalmente, a quantidade de Fe absorvida diariamente, em adultos normais, equivale à quantidade excretada e o Fe do organismo é

continuamente reciclado através de um eficiente sistema de reutilização deste metal das fontes internas, principalmente do Fe proveniente da hemoglobina das hemácias após hemólise intra e extravascular (CANÇADO & CHIATTONE, 2002).

A maior parte do Fe plasmático destina-se à medula óssea, sendo que 80% do Fe liga-se ao heme e passa a fazer parte da hemoglobina como Fe funcional, e os 20% restantes permanecem ligados à transferrina como Fe de transporte (ANDREWS, 1999; WANG & PANTOPOULOS, 2011). Aproximadamente 25% do Fe do organismo de um adulto normal encontra-se armazenado, principalmente no fígado e no baço (CANÇADO & CHIATTONE, 2002). Quando necessário, esse Fe retorna ao plasma e dirige-se à medula óssea para a formação de novas hemácias. Essa liberação do Fe armazenado ocorre sob duas formas: forma lenta, proveniente do Fe de depósito e forma rápida (“pool” lábil), que é o mecanismo que o organismo utiliza em situações de estresse (DUNN *et al.*, 2007)

Fundamentalmente, duas proteínas são as mais importantes para evitar a sobrecarga e garantir o metabolismo adequado do Fe - a Tf e a ferritina. Enquanto a função da Tf é transportar o Fe livre entre os locais de absorção e utilização e/ou armazenamento, a ferritina tem a função específica de armazenar o Fe não utilizado (ARREDONDO & NUNEZ, 2005).

A ferritina existe principalmente no citosol, mas também pode ocorrer no núcleo celular e no exterior das células. Em todos esses locais, ela armazena o Fe, impedindo que o mesmo participe de reações de estresse oxidativo (PAPANIKOLAOU & PANTOPOULOS, 2005). Ainda assim, em torno de 3-5% do conteúdo celular de Fe existe na forma lábil - LIP (*Labile Iron Pool*), ou seja, ligado fracamente a substâncias como fosfatos, nucleotídeos, hidroxilas, aminas e grupos sulfidrila (ARREDONDO & NUNEZ, 2005). Quando a capacidade de estocagem de Fe pela ferritina é eventualmente saturada, forma-se um complexo de Fe com fosfatos e formas hidróxidas, conhecido por hemosiderina (KOHGO *et al.*, 2008).

A Tf plasmática (apo-Tf) tem, pelo menos, dois importantes papéis na

fisiologia do Fe. Num primeiro momento, ao ligar-se imediatamente ao Fe disponibilizado no plasma (holo-Tf ou h-Tf), limita a capacidade do Fe de gerar radicais tóxicos quando livremente disponível. Além disso, é capaz de transportar e direcionar o Fe para as células que estão expressando o receptor de Tf (RT ou Transferrin receptor - TR) da membrana plasmática (DE DOMENICO *et al.*, 2008). É importante observar que RT é uma glicoproteína de membrana cuja única função claramente estabelecida é mediar a absorção do Fe ligado à Tf, permitindo um controle rigoroso na homeostase do Fe intracelular (MENEGHINI, 1997).

A absorção do Fe ligado a Tf requer a ligação da Tf ao RT, internalizando a Tf por endocitose, e a liberação do Fe da proteína ocorre em função da diminuição do pH no endossomo (Fig. 3). Com exceção de células altamente diferenciadas, RT são expressos em provavelmente todas as células, embora uma grande variação possa ocorrer entre os diferentes tipos celulares (PONKA & LOK, 1999; FLEMING & PONKA, 2012). RT são altamente expressos em eritrócitos imaturos, tecidos da placenta e células em divisão, tanto normais quanto malignas. Em células proliferativas não-eritrocitárias, a expressão de RT é regulada pós-transcricionalmente pela presença de Fe intracelular (FLEMING & PONKA, 2012).

Após a endocitose do macrocomplexo Fe^{3+} – Tf – RT, o mesmo é encapsulado em endossoma, no qual o Fe^{3+} é então liberado e novamente reduzido a sua forma mais solúvel Fe^{2+} (WATKINS *et al.*, 1992; DANCIS *et al.*, 1994; WANG & PANTOPOULOS, 2011). A Tf sem o Fe retoma sua forma "apo" e é reciclada de volta ao plasma, via fusão entre o endosoma e a membrana plasmática (WANG & PANTOPOULOS, 2011).

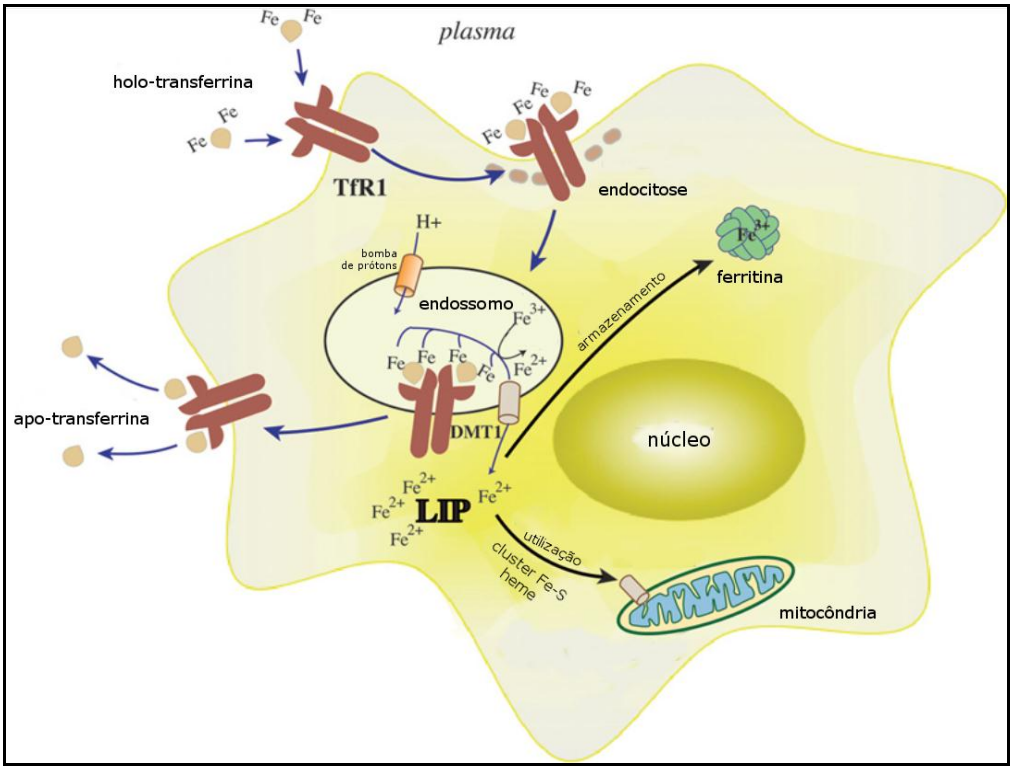

Figura 3: Absorção do ferro intracelular via ciclo da transferrina. Adaptada de WANG & PANTOPOULOS (2011).

Em síntese, uma vez no citosol, o Fe pode ter quatro destinos: a) ser utilizado para a síntese de proteínas com grupamentos heme, grupamentos Fe/S ou Fe em outros arranjos moleculares; b) ser estocado na ferritina; c) se associar ao LIP, estando preso a ligantes com fraca afinidade por ele; ou ainda, d) ser novamente exportado para a corrente sanguínea, em direção a outros locais de uso ou de armazenamento como o fígado (PRA, 2008; WANG & PANTOPOULOS, 2011).

É interessante observar que, em humanos, a homeostase do Fe é regulada no organismo como um todo e especificamente, no nível intracelular (JACOLOT *et al.*, 2008).

A manutenção da homeostase de Fe intracelular pode ser considerada, possivelmente, o melhor mecanismo conhecido de controle pós-transcricional em vertebrados, no qual os níveis de Fe ativam proteínas sem a necessidade de sintetizá-las, ocorrendo uma ativação apenas pela mudança estrutural da proteína (MENEGHINI, 1997). Quando há elevação da

concentração de Fe no citosol, este interage com proteínas responsivas ao Fe - IRP (*Iron Responsive Proteins*) que se deslocam de regiões específicas - elementos responsivos ao Fe - IRE (*Iron Responsives Elements*) da porção 3' do mRNA que codifica o receptor de transferrina, levando à redução da sua estabilidade e da sua taxa de tradução (WANG & PANTOPOULOS, 2011). O resultado é uma diminuição da síntese do receptor de transferrina, o que evita que mais Fe seja transferido para o interior das células, prevenindo-se assim a produção de OH•, que são extremamente reativos e exercem uma variedade de efeitos tóxicos nas células (CALTAGIRONE *et al.*, 2001). Nessa mesma condição, ocorre o contrário com o mRNA da ferritina, cuja estabilidade é aumentada como resultado da formação de um complexo, entre Fe e proteínas citosólicas, o qual se liga à porção 5' do transcrito, situação em que a sua taxa de tradução aumenta, elevando-se assim o conteúdo intracelular de ferritina (MACKENZIE *et al.*, 2008).

O controle pós-transcricional da síntese de RT e ferritina permite que o Fe seja mantido numa faixa de concentração de compatibilidade fisiológica, evitando sua carência e igualmente, seu excesso [para revisão, ver WANG & PANTOPOULOS (2011)].

1.2. Ferro e Estabilidade Genômica

1.2.1. Carência e Excesso de Ferro

Do ponto de vista nutricional, tanto a carência quanto o excesso de Fe tem consequências dramáticas e são de grande significância epidemiológica (CAIRO *et al.*, 2006). Enquanto a deficiência de Fe é a principal desordem nutricional no mundo, a hemocromatose hereditária, por sua vez, que leva ao acúmulo excessivo de Fe no organismo, é a doença genética mais comum entre os homens (PRA *et al.*, 2011).

Claramente, o balanço adequado de Fe no organismo é crítico para a produção normal de hemácias e a manutenção da saúde em geral (CAIRO *et al.*, 2006). A deficiência de Fe pode resultar na cessação da síntese de hemoglobina nas células eritrocitárias e a indução de apoptose em células eritrocitárias e não-eritrocitárias (LE & RICHARDSON, 2002).

A deficiência de Fe raramente é devida a defeitos inerentes ao metabolismo de Fe (ANDREWS, 2000), mas comumente é causada por ingestão insuficiente de Fe através da dieta (ou absorção defectiva) ou perda sanguínea crônica, originando a anemia (CARVALHO *et al.*, 2006; PRA *et al.*, 2009).

As quantidades médias de Fe que devem ser absorvidas diariamente pelo organismo, para os homens adultos e para as mulheres em idade fértil, são cerca de 1,0 mg e 1,5 mg, respectivamente, sendo que, gestantes e crianças em fase de crescimento, tem demandas, proporcionalmente, aumentadas (PRA *et al.*, 2012). Os níveis considerados saudáveis de Fe disponível no soro humano podem variar numa faixa de 8-30 µmol/L e valores abaixo de 5 µmol/L caracterizam anemia (CUNZHI *et al.*, 2003; WU *et al.*, 2004).

A anemia por deficiência de Fe, a qual é prevalente no mundo afetando quase um bilhão de pessoas, em especial crianças e jovens mulheres, está incluída entre aquelas deficiências de micronutrientes reconhecidas como problemas nutricionais prioritários (CAIRO *et al.*, 2006; PRA *et al.*, 2012). Particularmente, a deficiência de Fe é a causa primária de anemia nutricional e a deficiência nutricional mais comum no mundo.

Contudo, a deficiência de Fe tem sido igualmente associada com o desbalanço de funções fisiológicas, incluindo a síntese e reparação de DNA, possivelmente aumentando o risco de alguns tipos de cânceres [para revisão, ver PRA *et al.*(2009)], comprometimento do desenvolvimento cerebral e imunidade. Além disso, a anemia também tem demonstrado aumentar o dano ao DNA em adultos, como evidenciado pelo aumento de dano ao DNA em pacientes com anemia (PRA *et al.*, 2011).

No que diz respeito ao sistema imune, a relação entre o Fe e a imunidade ainda é controversa. Enquanto alguns autores afirmam que a deficiência de Fe predispõe à infecções, outros sugerem que o excesso de Fe pode aumentar o risco de infecções e também sua gravidade, pois os microrganismos, assim como os animais, necessitam de Fe para o

desenvolvimento das funções vitais como síntese de DNA e transporte de elétrons (POWER *et al.*, 1991; OPPENHEIMER, 2001; PRA, 2008).

Existem ainda evidências de que a deficiência de Fe pode prejudicar o desenvolvimento comportamental e cognitivo de crianças (AKMAN *et al.*, 2004), possivelmente causando defeitos na mielinização que podem resultar em prejuízo cognitivo a longo prazo (ORTIZ *et al.*, 2004). A deficiência de Fe e a anemia também podem aumentar o estresse oxidativo e o dano ao DNA, possivelmente aumentando o risco de carcinogênese, em especial, de câncer no trato gastro-intestinal (PRA *et al.*, 2009).

Por outro lado, o excesso de Fe está igualmente associado a diversas patologias, incluindo doenças hepáticas e cardíacas, câncer, doenças neurodegenerativas, diabetes, anormalidades hormonais e do sistema imune (VALKO *et al.*, 2005). Em geral, a sobrecarga de Fe, medida tanto pela ferritina plasmática quanto pela saturação da Tf, está fortemente associada ao aumento da mortalidade e de forma ainda controversa, ao aumento de câncer (MAINOUS *et al.*, 2005).

Além disso, evidências acumuladas demonstram que uma desregulação no metabolismo cerebral do Fe é uma das causas iniciais para a morte neuronal em algumas doenças neurodegenerativas (KE & QIAN, 2007). Os erros encontrados no metabolismo cerebral do Fe nessas doenças tem uma patogenia multifatorial, incluindo fatores genéticos e não-genéticos. Os distúrbios no metabolismo do Fe podem ocorrer em níveis múltiplos, incluindo a absorção e a liberação, armazenamento, metabolismo intracelular e regulação. O aumento da concentração do Fe em determinadas regiões do cérebro, por sua vez, está associado a uma cascata de eventos deletérios, levando a morte neuronal observada em doenças como Parkinson e Alzheimer (KE & QIAN, 2007; SCHRODER *et al.*, 2012).

O excesso de Fe no ambiente intracelular é ainda mais danoso, pois o acúmulo de ERO acima da capacidade antioxidante da célula pode desencadear uma série de eventos moleculares, incluindo, dano ao DNA (ver item 1.2.3 desta tese) (FLEMING & PONKA, 2012).

1.2.2. *Clusters* de Ferro e Enxofre (Fe/S)

Reconhecidamente, todo organismo vivo desenvolveu maquinários complexos e especializados para orquestrar a biogênese de *clusters* Fe/S de forma segura, eficiente e específica, a fim de garantir a sua entrega para as proteínas que são funcionalmente dependentes destes grupos prostéticos (SHEFTEL *et al.*, 2010). Os *clusters* de Fe/S são importantes grupos prostéticos para proteínas que estão envolvidas em inúmeros processos celulares, incluindo o transporte de elétrons, catálise enzimática e regulação proteica (ROUAULT & TONG, 2005). Além disso, são indispensáveis em três processos centrais para a vida na terra - respiração, fotossíntese e fixação de nitrogênio. Em eucariotos, proteínas Fe/S estão presentes na mitocôndria, no citosol, no retículo endoplasmático e no núcleo, exercendo funções específicas e relevantes em cada um desses locais (Fig. 4), como por exemplo, a síntese de RNA e DNA, bem como a reparação do DNA.

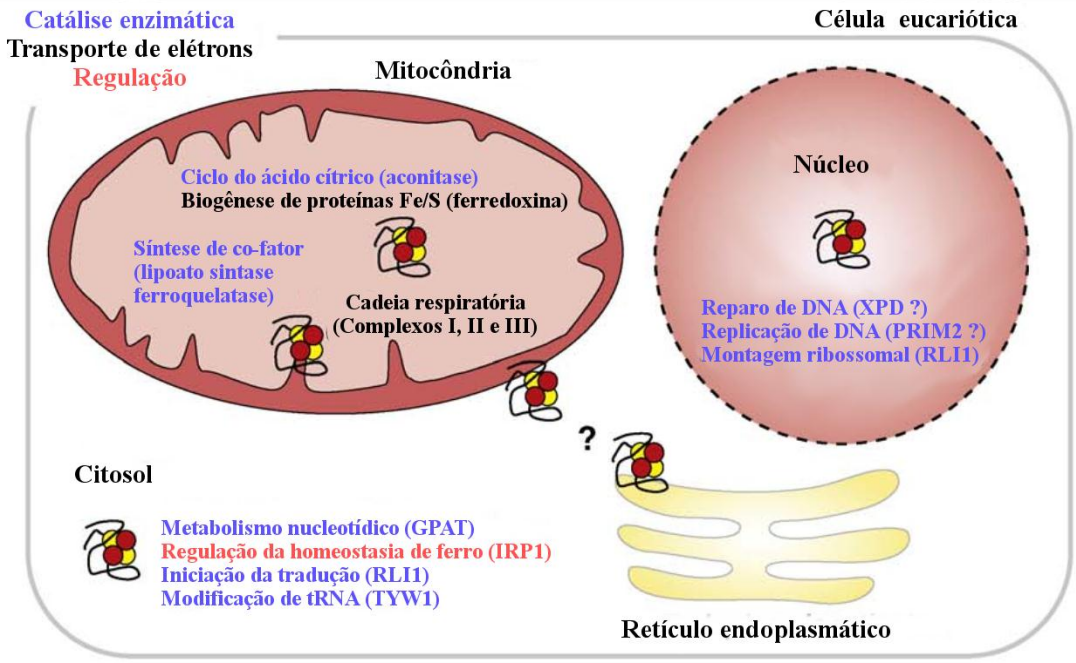

Figura 4. Localização e função de proteínas Fe/S em células de mamíferos. Proteínas Fe/S estão localizadas na mitocôndria, citosol, núcleo e no retículo endoplasmático. Elas executam as diversas funções indicadas na catalase enzimática (azul), transferência de elétrons (preto) e na regulação da expressão de genes (vermelho). As funções de duas proteínas Fe/S localizadas fora da mitocôndria e do retículo endoplasmático ainda são desconhecidas. Adaptada de SHEFTEL *et al.* (2010).

No que diz respeito aos processos de reparação do DNA, existem proteínas que possuem *cluster* Fe/S. Entre elas, duas helicases - ERCC2 (*excision repair cross-complementing rodent repair deficiency, complementation group 2*, também conhecida como XPD - *xeroderma pigmentosum complementary group D*) e BACH1 (*Basic leucine zipper transcription factor 1* também conhecida com FancJ - *Fanconi anemia group J protein*), que desempenham importantes papéis no sistema de reparação por excisão de nucleotídeos (NER) (RUDOLF *et al.*, 2006). Enquanto, mutY homologo (MutyH) e Nth endonuclease tipo III (NTHL1), além de também possuírem *cluster* Fe/S, são DNA glicosilases (ASPINWALL *et al.*, 1997) requeridas para o reconhecimento e reparação de lesões no DNA, ambas atuando no sistema de reparação por excisão de bases (BER). MutyH também tem importante papel no reparo de DNA causado por desemparelhamento de bases (mismatch repair - MMR) (CHEADLE & SAMPSON, 2003; BRZOSKA *et al.*, 2006; WHITE, 2009).

Cada uma destas proteínas envolvidas na reparação contém *cluster* de 4 átomos de Fe ligados a 4 átomos de enxofre [4Fe-4S]. Este *cluster* é importante para o funcionamento da proteína, embora seu papel ainda não seja plenamente compreendido. No caso das proteínas de reparação do DNA, nas quais o *cluster* Fe/S está espacialmente localizado a uma distância considerável do sítio ativo da enzima para desempenhar uma ação direta na catálise (WOLSKI *et al.*, 2008), tem sido proposto que o *cluster* Fe/S exerce uma ação estrutural, alinhando os domínios ativos do DNA ligado à enzimas com o substrato (LUKIANOVA & DAVID, 2005).

É interessante observar, também, que a integridade do DNA tem sido diretamente relacionada à biossíntese de Fe/S mitocondrial (VEATCH *et al.*, 2009). Além disso, uma correlação entre as proteínas Fe/S e câncer existe em pacientes com mutações nas subunidades da succinato desidrogenase (SDH), por exemplo (POLLARD *et al.*, 2005). Em linhas gerais, existem evidências suficientes demonstrando a relação entre a biogênese de proteínas Fe/S e diferentes doenças. Contudo, pesquisas complementares se fazem necessárias para elucidar melhor o papel do *cluster* e proteínas Fe/S

na manutenção do DNA, na patogenia do câncer e de outras doenças (SHEFTEL *et al.*, 2010).

1.2.3. Estresse Oxidativo, Dano e Reparação de DNA

A toxicidade do Fe já está bem estabelecida e devidamente descrita na literatura (para revisão, ver (MENEGHINI, 1997; PAPANIKOLAOU & PANTOPOULOS, 2005; VALKO *et al.*, 2005; FLEMING & PONKA, 2012; PRA *et al.*, 2012)). Como mencionado, a ciclagem entre Fe^{+2} (doador de elétrons) e Fe^{+3} (aceptor de elétrons), por um lado faz do Fe um participante ideal para diversas reações biológicas, mas por outro lado o caracteriza como uma substância altamente tóxica em condições oxidativas. De fato, tanto o seu excesso quanto a sua deficiência podem causar estresse oxidativo e subsequente dano ao DNA (HALLIWELL & GUTERRIDGE, 2007; PRA *et al.*, 2009; PRA *et al.*, 2012).

Íons metálicos podem induzir danos ao DNA por dois mecanismos - gerando danos ao DNA diretamente ou induzindo a formação de ERO, levando ao dano indireto ao DNA, possivelmente através de reações de Fenton-*like.* As ERO causam peroxidação lipídica, oxidação de amino ácidos com consequente *cross-links* de proteínas, além de fragmentação de proteínas e dano ao DNA (FLEMING & PONKA, 2012). Muitas das modificações no DNA induzidas por ERO tem demonstrado potencial mutagênico. Considerando-se que o processo de mutagênese é largamente reconhecido como um dos primeiros fatores no desenvolvimento de câncer, a correlação entre ERO, Fe, dano ao DNA e câncer tem recebido crescente atenção nas últimas décadas (PARK & PARK, 2011).

O Fe especificamente é capaz de produzir quebras simples e duplas no DNA, aumentar a frequência de micronúcleos, além de ser o metal de transição que mais induz a formação de 8-hidroxideoxiguanosina (8-OHdG) e que igualmente, resulta em maior número de quebras duplas do que simples (para revisão, ver PRA *et al.* (2012)). A sobrecarga de Fe parece ainda induzir dano cumulativo ao DNA mitocondrial (mtDNA), prejudicando a síntese de subunidades da cadeia respiratória e levando a disfunção da

respiração mitocondrial (GAO *et al.*, 2009; GAO *et al.*, 2010). Além disso, concentrações insuficientes de Fe também causam significante mal funcionamento mitocondrial (WALTER *et al.*, 2002).

Contudo, apesar do Fe gerar ERO, que por sua vez estão diretamente relacionados à formação de lesões no DNA, ele também pode ser um cofator para peroxidases, como a catalase, uma importante enzima antioxidante (PRA *et al.*, 2012). Adicionalmente, o Fe ainda está relacionado diretamente à modulação da atividade de proteínas de síntese e de reparação do DNA (PRA *et al.*, 2009; PRA *et al.*, 2012), como 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).

Existem ainda proteínas envolvidas na reparação do DNA que contém Fe, tanto em bactérias, como a AlkB (*Alpha-ketoglutarate-dependent dioxygenase*), quanto a sua homologa em humanos (SUNDHEIM *et al.*, 2008). Evidências demonstraram que a ligação de AlkB com seu substrato, DNA metilado, é diminuída se o Fe^{2+} no sítio ativo desta proteína é substituído por Cu^{2+} (BLEIJLEVENS *et al.*, 2007). Em outro estudo, empregando ensaios bioquímicos, foi demonstrado que a atividade endonucleásica de APE1 (*Apurinic/apyrimidinic endonuclease 1)* purificada pode ser inibida pela presença de Fe^{2+} (MCNEILL *et al.*, 2004).

A privação de Fe reduziu a atividade da ribonucleotídeo redutase, bem como a síntese de DNA e a proliferação celular (STRAND *et al.*, 2004). Além disso, em algumas enzimas de reparação ao DNA (XPD, FANCJ, MutyH, NTHL1), *clusters* Fe/S parecem estar envolvidos no reconhecimento do dano ou desempenham alguma outra função (HIOM, 2010). No caso da XPD, pelo menos, o *cluster* Fe/S não é requerido para a estabilidade da enzima, nem para a ligação com o substrato de quebras simples de DNA, mas parece ser essencial para a sua atividade de helicase (HIOM, 2010).

De acordo com experimentos realizados por PRA (2008), a expressão dos genes de reparo WRN (*Werner syndrome*) e MutyH apontam

associação notória entre a dose de Fe e o nível de mRNA transcrito. O gene WRN, por sua vez, codifica uma DNA helicase e possui também *cluster* Fe/S. Esta DNA helicase atua no reparo de quebras duplas, recrutando a maquinaria de reparo para essas lesões, bem como, interage diretamente com a proteína TP53 (*Tumor protein 53*). PRA (2008) observou uma curva tipo “U” descendente e ascendente para o nível de expressão do gene MutyH, com nível mínimo na concentração de 5,4 µmol/L de Fe proveniente de suplementação com h-Tf e aumento significativo na concentração máxima avaliada (23 µmol/L).

Nesse sentido, é interessante observar que mutações em enzimas de reparo que requerem Fe estão relacionadas ao aumento no risco de câncer, como no xeroderma pigmentoso (XPD), na anemia de Fanconi (FANCJ) e no grupo de complementação das poliposes adenomatosas de cólon (MutyH) (HIOM, 2010). De tal forma, em linha com a hipótese de que a deficiência de Fe está relacionada com o aumento no risco de câncer, existem evidências preliminares que relacionam anemia em mães com o aumento de leucemia em crianças (WEN *et al.*, 2002) e, também, que pessoas idosas com baixa ingestão de Fe, tem maiores riscos de desenvolver câncer gastrointestinal (PRA *et al.*, 2009). Na verdade, muitas outras vias metabólicas que podem modular a estabilidade genômica e o risco de câncer parecem ser influenciadas pela deficiência de Fe (PRA *et al.*, 2012).

O Fe está envolvido em diferentes mecanismos genotóxicos e antigenotóxicos (Fig. 5), demonstrando claramente a importância de níveis adequados desse micronutriente para a manutenção da estabilidade genômica como um todo.

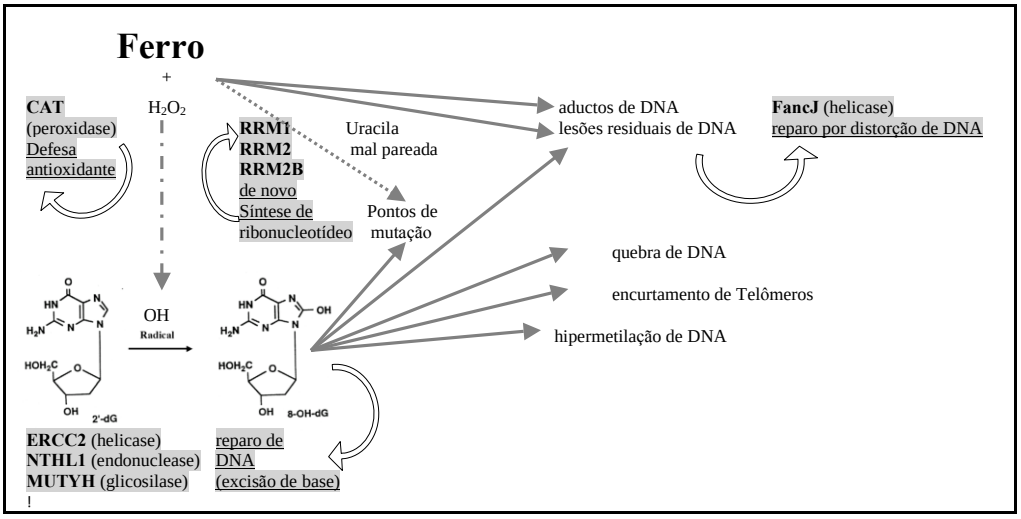

Figura 5: Mecanismos genotóxicos e anti-genotóxicos do ferro. As linhas contínuas indicam danos ao DNA induzidos por Fe. Linhas pontilhadas indicam possíveis danos ao DNA induzidos por deficiência de Fe. Linhas mistas indicam que tanto a sobrecarga de Fe quanto a carência podem estar aumentando a formação de 8-hidroxideoxiguanosina (8-OHdG) e associadas a lesões de bases oxidadas. Ferro pode induzir quase todo tipo de dano ao DNA tanto diretamente ou pela formação do radical hidroxila. Linhas curvas e texto com fundo cinza indicam mecanismos antioxidante e de reparação de DNA nos quais o Fe desempenha algum papel (genes em negrito, enzimas entre parênteses e vias sublinhadas). Adaptada de PRA *et al.* (2012).

1.3. O Papel do Ferro nas Culturas Celulares

O Fe é um importante co-fator requerido para inúmeras funções celulares essenciais, além de ser um micronutriente essencial a vida da maioria dos organismos (CAIRO *et al.*, 2006; OGLESBY-SHERROUSE & MURPHY, 2013). No entanto, como já foi descrito anteriormente nessa tese, o Fe pode ser também danoso uma vez que catalisa reações formando ERO. Portanto, a homeostase intracelular do Fe e o equilíbrio da disponibilidade de Fe sistêmica são mecanismos bastante regulados pelo organismo (CAIRO *et al.*, 2006; OGLESBY-SHERROUSE & MURPHY, 2013).

Nas culturas celulares, o Fe é igualmente requerido. Contudo, a manutenção de níveis adequados para o desenvolvimento celular, garantido a disponibilidade do micronutriente em nível benéfico para a célula - evitando o excesso ou a privação - é frequentemente negligenciado na formulação dos meios de cultura e ainda pouco estudado na literatura. Na grande maioria das

culturas celulares, a “entrega” de Fe para as células acaba sendo exclusivamente através da suplementação com soro fetal bovino (SFB), no qual existem níveis nem sempre conhecidos de Tf ligada ao Fe.

Basicamente, o papel do Fe nas culturas celulares refere-se a respiração e ao metabolismo celular, incluindo transporte de oxigênio, síntese de DNA e transporte de elétrons (LIEU *et al.*, 2001). Tanto a carência de Fe quanto a sua sobrecarga no ambiente intracelular induz, por exemplo, o dano ao DNA mitocondrial, causando uma deterioração funcional da mitocôndria e comprometendo seriamente a respiração celular (PRA *et al.*, 2011; PRA *et al.*, 2012). Na completa ausência de Fe, as células param de proliferar e eventualmente, morrem. Na presença de níveis elevados, ocorre o acúmulo de ERO, gerando danos ao DNA e mutagenicidade (HUANG, 2003; PRA *et al.*, 2009).

Em recentes estudos com células neuronais foi demonstrado o papel da Tf como suplemento no meio de cultura para melhorar a proliferação assim como o desenvolvimento celular (SILVESTROFF *et al.*, 2012; SILVESTROFF *et al.*, 2013). A presença de Tf demonstrou ser capaz de controlar tanto a proliferação celular quanto o comprometimento da linhagem ou a diferenciação celular, dependendo do momento em que o tratamento fosse conduzido (SILVESTROFF *et al.*, 2012). Adicionalmente, foi observado que o aumento da proliferação das células estava relacionado a um incremento, também, no tamanho das mesmas, e este efeito, por sua vez, era mediado pela incorporação da Tf nas células por um dos receptores de Tf celulares (TfR1) (SILVESTROFF *et al.*, 2012).

Contudo, conforme descrito no Capítulo I, concentrações elevadas do micronutriente Fe (normalmente acima de 10 µmol/L), podem ser genotóxicas e comprometer o desenvolvimento de diferentes tipos de células em cultura. Estudo com outros micronutrientes, como o Zinco, por exemplo, nos meios de cultura, corroboram para a necessidade de determinação de níveis ótimos de micronutrientes para as células (SHARIF *et al.*, 2011; SHARIF *et al.*, 2012; SHARIF *et al.*, 2012)

Tanto no caso do Fe como para outros micronutrientes, estabelecer e manter os níveis fisiológicos são mandatórios para a manutenção da saúde (FENECH, 2002; AMES, 2003; FENECH, 2005). Adicionalmente, é valioso aprofundar o entendimento sobre o papel dos micronutrientes em nível celular.

O conhecimento acerca da influência dos micronutrientes nas culturas pode ser útil ao se desenvolver um ambiente de cultivo ideal para as células *in vitro*. Igualmente, é importante elucidar os possíveis efeitos nas células quando ocorrem alterações nas concentrações dos micronutrientes nos meios de cultura. O Capítulo I dessa tese faz uma revisão sobre a influência de micronutrientes, viabilidade celular e estabilidade genômica, sendo que o Fe está entre os micronutrientes descritos. Além disso, paralelos são traçados considerando-se os níveis fisiológicos de micronutrientes e as concentrações praticadas no cultivo de células.

Apesar do Fe ser claramente um micronutriente essencial para todas as células (CAIRO *et al.*, 2006; PRA *et al.*, 2012), é evidenciado no Capítulo I que os níveis do mesmo, comumente usados nas culturas celulares, se encontram em concentrações inferiores as do soro sanguíneo humano; gerando alguns questionamentos que merecem ser devidamente endereçados: i) o ambiente de cultivo celular apresenta-se inadequado quanto à concentração de Fe? ii) esse perfil pode ser denominado de "anêmico" para o desenvolvimento celular? O Capítulo II, por sua vez, visa responder estas e outras questões pertinentes ao avaliar o efeito do micronutriente Fe na viabilidade celular e na estabilidade genômica de duas linhagens celulares.

2. OBJETIVOS

2.1. Objetivo Geral

Avaliar a influência da suplementação do meio de cultura com o micronutriente Fe na viabilidade celular e na estabilidade genômica de culturas celulares.

2.2. Objetivos Específicos

1. Elaborar revisão bibliográfica a respeito da influência de micronutrientes na viabilidade celular e estabilidade genômica de culturas celulares.
2. Avaliar a influência de diferentes fontes do micronutriente Fe, ligado a h-Tf (orgânico) *versus* $FeSO_4$ (inorgânico), bem como diferentes concentrações deste micronutriente, na viabilidade celular de culturas de fibroblastos pulmonares (MRC5) e hepatocarcinoma (HepG2) humanos, a fim de encontrar uma faixa adequada para o cultivo celular.
3. Quantificar o Fe intracelular, após a exposição a diferentes suplementações de Fe, com o auxílio da técnica de ultravioleta com absorção atômica.
4. Avaliar a influência na viabilidade e proliferação celular pelos ensaios: redução do sal tetrazolato*3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide* (MTT) e clonogênico e, igualmente, observar o tempo de duplicação populacional (do inglês: *population doubling time* - PDT).
5. Verificar se as diferentes suplementações de Fe, no meio de cultura, influenciam na geração de espécies reativas de oxigênio pelo o ensaio de oxidação do 2',7'-Diclorofluoresceína (DCFH).
6. Avaliar se a suplementação com Fe altera a resposta antioxidante celular pela análise da atividade da enzima catalase.
7. Investigar a influência das diferentes suplementações de Fe na

estabilidade genômica utilizando os ensaios cometa alcalino e citoma de micronúcleos em células binucleadas.

8. Verificar se as diferentes suplementações de Fe alteram a expressão de proteínas que possuem *cluster* de ferro/enxofre (Fe/S) em sua estrutura e que tenham relação com as vias de reparação do DNA, como a TFIIH e MutyH, aplicando-se a técnica de *Western Blotting*.
9. Verificar a influência da suplementação com Fe na expressão de proteínas/receptores celulares para Fe, como CD71 e Nramp2, pela técnica de *Western Blotting*.
10. Investigar o potencial antigenotóxico do pré-tratamento de células MRC5 com diferentes suplementações de Fe e posterior exposição a agentes genotóxicos, como o metil metano sulfonado (MMS) ou o Peróxido de Hidrogênio (H_2O_2), pelo ensaio cometa alcalino modificado.

CAPÍTULO I – "**The Influence of Micronutrients in Cell Culture: A Reflection on Viability and Genomic Stability"**

Ana Lúcia Vargas Arigony, Iuri Marques de Oliveira, Miriana Machado, Diana Lilian Bordin, Lothar Bergter, Daniel Prá, João Antonio Pêgas Henriques

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Existem muitos estudos avaliando a influência de micronutrientes sobre a estabilidade genômica *in vivo,* geralmente pela avaliação da relação entre os níveis da dieta e da suplementação sobre a estabilidade genômica. Contudo, há carência de conhecimento sobre os efeitos de diferentes concentrações plasmáticas de micronutrientes sobre as células, condição que pode ser avaliada em modelos *in vitro*. As culturas celulares são usadas para se tentar predizer os possíveis efeitos de dadas substâncias ou condições no organismo vivo e devem refletir ao máximo as condições fisiológicas do organismo humano. No entanto, muitos dos meios de culturas usualmente utilizados para o cultivo celular apresentam algum nível de desbalanço na concentração de micronutrientes, possivelmente, impactando no desenvolvimento celular e gerando inclusive, instabilidade genômica.

Sendo assim, foi elaborada uma revisão da literatura, priorizando os estudos realizados *in vitro* com micronutrientes, a fim de demonstrar que os mesmos podem aumentar a viabilidade celular e a estabilidade genômica, dependendo das concentrações usadas. O artigo que segue avaliou ainda os níveis atualmente utilizados nas culturas celulares e os possíveis impactos de sua carência ou excesso no desenvolvimento das células usadas como modelos experimentais.

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Review Article

The Influence of Micronutrients in Cell Culture: A Reflection on Viability and Genomic Stability

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Micronutrients, including minerals and vitamins, are indispensable to DNA metabolic pathways and thus are as important for life as macronutrients. Without the proper nutrients, genomic instability compromises homeostasis, leading to chronic diseases and certain types of cancer. Cell-culture media try to mimic the *in vivo* environment, providing *in vitro* models used to infer cells' responses to different stimuli. This review summarizes and discusses studies of cell-culture supplementation with micronutrients that can increase cell viability and genomic stability, with a particular focus on previous *in vitro* experiments. In these studies, the cell-culture media include certain vitamins and minerals at concentrations not equal to the physiological levels. In many common culture media, the sole source of micronutrients is fetal bovine serum (FBS), which contributes to only 5–10% of the media composition. Minimal attention has been dedicated to FBS composition, micronutrients in cell cultures as a whole, or the influence of micronutrients on the viability and genetics of cultured cells. Further studies better evaluating micronutrients' roles at a molecular level and influence on the genomic stability of cells are still needed.

The Influence of Micronutrients in Cell Culture: A Reflection on Viability and Genomic Stability

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Contents

Abstract

Micronutrients, including minerals and vitamins, are indispensable to DNA metabolic pathways and thus are as important for life as macronutrients. Without the proper nutrients, genomic instability compromises homeostasis, leading to chronic diseases and certain types of cancer. Cell-culture media try to mimic the i*n vivo* environment, providing *in vitro* models used to infer cells' responses to different stimuli. This review summarizes and discusses studies of cell-culture supplementation with micronutrients that can increase cell viability and genomic stability, with a particular focus on previous *in vitro* experiments. In these studies, the cell-culture media include certain vitamins and minerals at concentrations not equal to the physiological levels. In many common culture media, the sole source of micronutrients is fetal bovine serum (FBS), which contributes to only 5-10% of the media composition. Minimal attention has been dedicated to FBS composition, micronutrients in cell cultures as a whole, or the influence of micronutrients on the viability and genetics of cultured cells. Further studies better evaluating micronutrients' roles at a molecular level and influence on the genomic stability of cells are still needed.

Keywords: vitamins, minerals, DNA damage, cell viability, genomic stability.

1. Introduction

Micronutrients, essential nutrients that are needed in small amounts, are as important for life as macronutrients. Micronutrients comprise all of the vitamins, such as A, D, and E, as well as the minerals, such as calcium, zinc and iron. The *in vivo* role of micronutrients is well established, and several studies have examined the effects of micronutrients on genomic stability [1-21]. Approximately 40 micronutrients are required in the human diet, and for each micronutrient, proper metabolism demands an optimal level of intake. A micronutrient deficiency distorts the metabolism in numerous and complicated ways, many of which may lead to DNA damage**.**

Micronutrients are required for optimal macronutrient metabolism because of micronutrients' critical role in intermediate metabolism. Invariably, metabolism requires the concomitant involvement of one or more vitamins and minerals. Chronic degenerative disease etiology and the rate of pathogenesis are thus intimately associated with micronutrient imbalances. Nutrition research has recently highlighted the role of several nutrients in regulating the genomic machinery [22]. More specifically, a number of vitamins and micronutrients are substrates and/or cofactors in the metabolic pathways regulating DNA synthesis and/or repair and gene expression [23]. A deficiency in such nutrients may result in the disruption of genomic integrity and alteration of DNA methylation, thus linking nutrition with the modulation of gene expression. In many cases, the response to a nutrient deficiency also seems to be genotype-specific. Gene-nutrient interactions are thus a fascinating example of physiological responses to the environment/diet at the molecular level [22].

Minerals and vitamins are indispensable to DNA metabolic pathways [24, 25]. Although there is still no clear evidence for a diet that optimally protects against DNA damage, in terms of either proportions or combinations of specific micronutrients, many studies conducted *in vitro* and in animal models have demonstrated the roles of micronutrients in maintaining genomic stability. For example, vitamin C and E deficiencies are known to cause DNA oxidation and chromosomal damage [26, 27]. Vitamin D exhibits antioxidant activity, stabilizes chromosomal structure and prevents DNA double-strand breaks [28]. Similarly, magnesium is an essential cofactor in DNA metabolism that plays a role in maintaining the high fidelity of DNA transcription [29]. Whereas either an excess of or a deficiency in iron may cause DNA breaks [30], a

carotenoid-rich diet reduces DNA damage [31], but excess retinol may be carcinogenic in certain individuals [32]. In a final example, vitamin B-12 deficiency is associated with the formation of micronuclei [5, 24], and reduced transcobalamin II in the serum is associated with chromosomal abnormalities [33].

Given the importance of micronutrients *in vitro*, the optimization of cell viability and genomic stability warrants further studies. Cell-culture media mimicking the *in vivo* environment may help to generate *in vitro* models of a cell's response to different stimuli. The composition of these media includes certain vitamins and minerals, but unfortunately, in many common culture media, the only source of micronutrients is fetal bovine serum (FBS), which contributes to only 5-10% of the media composition. Moreover, the appropriate proportion of micronutrients is not always provided because the precise composition of each batch of FBS is in fact extremely variable [34].

Certain micronutrients, such as calcium, folate, magnesium and iron, have been reported as key elements in cellular processes, including the proliferation, survival and even differentiation of cell cultures [35-38]. However, the particular concentration of micronutrients in a culture as well as the cell type may trigger different responses. Further studies of micronutrients' roles at a molecular level and influence on genomic stability are still required.

2. Aims and Scope

This review summarizes and discusses studies showing the influence of some micronutrients on cell viability and genomic stability, with a particular focus on *in vitro* models. *In vivo* evidences are presented to illustrate the relevance of the nutrients to genomic stability. Articles were retrieved from PubMed using the following search terms: micronutrients, vitamins, minerals, cell culture, proliferation, viability and genomic stability. Additional publications were collected by cross-referencing the primary articles retrieved. The review does not aim to include all nutrients that could influence genomic stability, then only the following nutrients were included - vitamins A, B7, B9, B12, C and E and minerals Cu, Fe, Mg, Se and Zn. According to Friso *et al.* [39], an imbalance of such dietary nutrients as folate, zinc, vitamin C and selenium can alter genomic and/or gene-specific DNA methylation, resulting in many different molecular effects on gene expression and integrity, in turn affecting cell growth, tissue differentiation, cancer incidence and aging. To better

address the selected micronutrients' effects in cell viability and genomic stability, we considered the information available regarding either their deficiency or excess.

3. Micronutrients and their influence on genomic stability

DNA damage is one of the most important factors that can compromise homeostasis, resulting in chronic (e.g., atherosclerosis) and even degenerative diseases, including Alzheimer's disease (AD) and certain types of cancer [40]. A deficiency in or imbalance of certain micronutrients has been described as mimicking radiation or chemicals, causing single- and double-strand breaks (SB) or lesions in DNA, or even both [20].

In Table 1, micronutrients whose imbalances cause DNA damage are listed, as well as the nutrients' food sources and possible health effects. In general, micronutrients can either act directly on the genome to prevent mutations or protect the genome indirectly by serving as enzyme cofactors in the cellular processes that modulate transformation [41, 42]. Therefore, any imbalance may result in a degree of DNA damage.

The role of diet in determining genomic stability is more important than previously imagined. It has been found that diet affects all pathways relevant to genomic stability, including exposure to dietary carcinogens, activation and detoxification of carcinogens, DNA repair, DNA synthesis and cell apoptosis [23, 43]. All of these critical pathways are dependent not only on enzymes but also on substrates and cofactors, a few of which are only available at the right concentration when the dietary intake of key minerals and vitamins is adequate [44]. As a result, a dietary deficiency in certain micronutrients required for DNA maintenance may exert effects similar to inherited genetic disorders that impair the activity of enzymes required for genomic stability [23, 45-47]. Additionally, such a deficiency may damage DNA to a similar extent as significant exposure to known carcinogens, such as ionizing radiation [43].

Table 1: Micronutrients linked to genomic stability: dietary requirements, and effects of deficiency and excess.

Micronutrient	EAR for adults (not pregnant or lactating)	General health effects of deficiency	Effects of deficiency related to genome instability	UL for Adults	Effects of excess related to genome instability	References
Vitamin						
Vitamin A	500-625 RAE	Blindness, impaired immunity, dermal alterations	Increased sensitivity to DNA-damaging agents	3000 RAE	Congenital malformations while in pregnancy. Cancer risk increase for smokers	[48-50]
Vitamin B7 (biotin)	30 µg*	Dermal alterations, immune dysfunction, neurological symptoms, and congenital malformations during pregnancy	Chromatin structural alterations	NA (safe up to 20,000 µg)	Congenital malformations. Increase in DNA damage	[51-54]
Vitamin B9	320 DFE	Anemia and other hematological alterations, pregnancy complication (e.g. neural tube defect)	Uracyl misincorporation in DNA; DNA strand breaks	1000 DFE	Increased cancer risk (promotion effect)	[43, 51, 55, 56]
Vitamin B12	2 µg	From lack of energy to irreversible severe damage to nervous system	DNA strand breaks	1000 µg	Unknown	[43, 51]
Vitamin C	60-75 mg (95-110 if smoker)	Dermatological alterations associated to collagen synthesis and immune impairment	DNA strand breaks	2000 mg	DNA damage related to oxidative stress	[43, 49, 55]
Vitamin E	12 mg	Increase in chronic disease risk	DNA strand breaks	1000 mg	DNA damage related to oxidative stress	[43, 49, 57, 58]

*Adequate Intake not EAR.

EAR: Estimated Adequate Requirement; DFE: Dietary Folate Equivalents; RAE: Retinol Activity Equivalents; UL: Upper Level; NA: not available.

Table 1: Micronutrients linked to genomic stability: dietary requirements, and effects of deficiency and excess (Cont.).

Micronutrient	EAR for adults (not pregnant or lactating)	General health effects of deficiency	Effects of deficiency related to genome instability	UL for Adults	Effects of excess related to genome instability	References
Mineral						
Copper	700 µg	Anemia and other blood dysfunctions, impaired growth and neurological alterations	Oxidative DNA damage increase	10000 µg (under review)	DNA damage associated to oxidative stress, particularly to liver	[59, 60]
Iron	6-8.1mg	Anemia and other blood dysfunctions, impaired growth and neurological alterations	DNA damage increase	45 mg	DNA damage associated to oxidative stress, particularly to liver	[21, 43, 60]
Magnesium	255-350 mg	Rare because Mg deficiency is unusual	DNA repair deficiency	NA	Unknown	[61, 62]
Selenium	45 µg	Decreased activity of gluthathione peroxidase leading to increased risk of degenerative diseases and impairment in immunity	DNA strand breaks	400 µg	Tumor incidence seems to be reduced in high doses supplementation	[49, 63]
Zinc	6.8-9.4 mg	Dermal alterations, growth retardation, immune dysfunction, neurological symptoms, and night blindness, and adverse outcomes during pregnancy	DNA strand breaks	40 mg	DNA damage increase	[43, 60, 64]

EAR: Estimated Adequate Requirement; NA: not available.

Vitamin A

Vitamin A is also referred to as retinoic acid, retinol, retinal, α- and β-carotene, lycopene, lutein, zeaxanthin, β-cryptoxanthin, or astaxanthin. The role of vitamin A and provitamin A (carotenoids) in DNA damage has recently been reviewed by Collins and Azqueta [65]. The well-established antioxidant properties of vitamin A have facilitated studies measuring oxidative damage both *in vivo*, in animal studies and human clinical trials, and *in vitro*. Whereas high concentrations of provitamin A carotenoids can cause DNA damage, perhaps by acting as prooxidants, non-vitamin A carotenoids can significantly reduce such damage [66].

The functions of vitamin A are related to night, day, and color vision; epithelial-cell integrity against infections; the immune response; hemopoiesis; skeletal growth; male and female fertility; and embryogenesis. Paradoxically, either an excess of or a deficiency in retinoic acid results in similar malformations in certain organs, including the mammalian kidney [67]. Many eye pathologies are due to vitamin A deficiency, including night blindness, conjunctival xerosis and corneal injuries. Similarly, hypervitaminosis A, resulting from the storage of excess vitamin A in the body, can damage various systems. Very large doses of vitamin A, especially in young children, can increase the intracranial pressure, leading to headache, nausea and vomiting [68]. It has also been established that adequate vitamin A intake is required for normal organogenesis, immune function, tissue differentiation, and vision. Given these requirements, vitamin A deficiency, which is widespread in the developing world, is responsible for at least one million instances of unnecessary death and blindness each year [69].

Vitamin B7

Vitamin B7, also known as biotin, act as a cofactor for the biotin-dependent enzymes pyruvate carboxylase, propionyl-CoA carboxylase, crotonyl-CoA carboxylase and two isozymes of acetyl-CoA carboxylase [70]. These enzymes catalyze key steps in important metabolic pathways, including fatty-acid biosynthesis, gluconeogenesis and amino-acid metabolism [71]. Vitamin B7 deficiency due to inadequate dietary intake or congenital defects in biotin absorption or metabolism results in the inactivation of all five biotin-dependent enzymes. This condition is known as multiple carboxylase deficiency (MCD) [72, 73], whose symptoms include ketoacidosis, lactic acidosis, feeding difficulties,

skin rashes and neurological abnormalities, such as subependymal cysts, hypotonia, seizures and ataxia. In severe cases, or if MCD is left untreated, the condition can lead to coma or death [74].

It has been demonstrated that biotin plays a role in DNA-strand breaks and the cellular response to strand breaks (SB). More specifically, biotin supplementation increased DNA breaks in cell cultures, although it is unknown whether this finding is relevant to whole organisms [75]. In contrast, *in vivo*, a high biotin intake in combination with a low intake of multiple other nutrients has been associated with increased genomic stability [76]. Biotin deficiency rarely occurs spontaneously in animals, including humans [77], but can be induced by consuming large amounts of raw egg white, which contains avidin, known to inhibit biotin absorption from the intestinal tract, or by taking anticonvulsants [78].

Vitamin B9

A deficiency in vitamin B9, also known as folic acid or folate, is common in people who consume few fruits and vegetables. Vitamin B9, as well as other vitamins from the B complex, plays an important role in genomic stability, and a deficiency can cause chromosomal breaks in human genes [79]. Vitamin B9 deficiency can also lead to (a) an elevated rate of DNA damage and altered DNA methylation, both of which are risk factors for cancer [79-81], possibly including colon cancer [82], or (b) an increased homocysteine concentration, an important risk factor for cardiovascular disease [83]. These defects may also play a significant role in developmental and neurological abnormalities [79, 80]. However, in animals with existing pre-neoplasic or neoplasic lesions, folic-acid supplementation increases the tumor burden [84]. In contrast, the adequate intake of vitamin B9 can increase genomic stability and possibly reduce cancer risk [85-88] because vitamin B9 is a key carbon donor during nucleotide biosynthesis [89].

Vitamin B12

Vitamin B12, or cyanocobalamin, deficiency is associated with pernicious anemia and neurological pathologies varying from a minor decrease in cognitive function to neurodegenerative disorders, although the role of vitamin B12 in these conditions requires further investigation [90, 91]. The lack of understanding of the underlying molecular

mechanisms may be due to the experimental limitations of the available classical cell-culture models [90]. Nevertheless, vitamin B12 is known to play an important role in genomic stability, and a deficiency in vitamin B12 can lead to DNA damage [82]. Vitamin B12 is also required for the synthesis of methionine and *S*-adenosyl methionine, the common methyl donor required for the maintenance of the DNA methylation patterns that determine gene expression and DNA conformation [92].

Despite controversies in the literature regarding the prevalence of vitamin B12 deficiency, this deficiency seems to be more common among people aged 65–76 years [93]. However, the symptoms of vitamin B12 deficiency caused by poor diet, digestive problems, and/or inadequate absorption in elderly people can be nonspecific, rendering a diagnosis more difficult. Furthermore, neurological symptoms may appear before anemia; in fact, only approximately 60% of elderly people with vitamin B12 deficiency are anemic [93, 94]. In cell-culture models, sufficient vitamin B12 can be provided to the cells by the FBS [90].

Vitamin C

Vitamin C, also known as ascorbate or ascorbic acid, is a micronutrient required for innumerable biological functions, specifically serving as a cofactor for certain important enzymes [95]. One type enzyme is the prolyl hydroxylases, which play a role in collagen biosynthesis and the downregulation of hypoxia-inducible factor (HIF)-1, a transcription factor that regulates many genes responsible for tumor growth, energy metabolism, and neutrophil function and apoptosis. Vitamin C-dependent inhibition of the HIF pathway may provide alternative or additional approaches to controlling tumor progression, infection, and inflammation [95].

As vitamin C exhibits antioxidant properties that provide protection against oxidative stress-induced cell damage by scavenging reactive oxygen species (ROS), the effects of this vitamin on cancer chemoprevention [96, 97] and cancer treatment [98] as well as sepsis [99] and neurodegenerative diseases (e.g., Alzheimer's disease) [100] have been studied. In fact, ingesting inadequate levels of vitamin C can mimic radiation exposure. In the literature, numerous human supplementation studies have used biomarkers of oxidative damage to DNA, lipids (lipid oxidation releases mutagenic aldehydes), and protein. Although these studies have yielded both positive and negative

results, if the fact that blood-cell saturation occurs at approximately 100 mg/day is taken into consideration, the evidence suggests that this level of vitamin C intake minimizes DNA damage [20]. Unfortunately, vitamin C deficiency is common in poor communities, so measures to improve the consumption of vitamin C-rich foods should be considered [101].

Vitamin E

Vitamin E, which comprises compounds from the tocopherol and tocotrienol families, is required to prevent peripheral neuropathy and hemolytic anemia in humans, which arise due to vitamin E deficiency. Vitamin E functions as a vital lipid-soluble antioxidant, scavenging hydroperoxyl radicals in the lipid milieu. The human symptoms of vitamin E deficiency suggest that this vitamin's antioxidant properties play a major role in protecting erythrocyte membranes and nervous tissues [95]. Additionally, these antioxidant properties play a role in genomic stability, particularly because vitamin E is a potent peroxyl-radical scavenger. Vitamin E is also a chain-breaking antioxidant that prevents the propagation of free radicals in membranes and plasma lipoproteins [102].

Recently, Ni and Eng [103] demonstrated that α-tocopherol can selectively protect SDH(var+) cells from oxidative damage and apoptosis and rebalance the redox metabolites nicotinamide adenine dinucleotide (NAD^+ and NADH). Another interesting recent study [104] evaluated the amount of the oxidation product 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) formed from the DNA nucleoside deoxyguanosine (dG) after vitamin exposure. In the case of vitamin E, no DNA damage was induced in cultured cells. Taken together, these results reinforce the role of this vitamin in maintaining DNA integrity and stability. Although the direct comparison of the study outcomes is complicated by varying definitions of vitamin E deficiency, the available data suggest that children and the elderly are most vulnerable to this deficiency and that men may be at higher risk than women [105].

Copper

Copper is an essential trace element, serving as a cofactor for many enzymes in different biological processes. In contrast to iron, the copper concentration not only in the blood but also in individual organs is maintained at constant levels beginning in early childhood, indicating the presence of robust homeostatic mechanisms [106]. Adequate copper intake permits the normal utilization of dietary iron, as intestinal iron absorption, iron release from stores (e.g., in the macrophages of the liver and spleen), and iron incorporation into hemoglobin are copper-dependent processes. In addition to preventing anemia, copper assists in blood coagulation and blood-pressure control; the crosslinking of connective tissue in the arteries, bones, and heart; defense against oxidative damage; energy transformation; the myelination of the brain and spinal cord; reproduction; and hormone synthesis. In contrast, inadequate copper intake has adverse effects on the metabolism of cholesterol and glucose, blood-pressure control and heart function, bone mineralization, and immunity [107].

The excessive accumulation of copper in the body can contribute to the development of cancer due to copper's role in causing DNA damage [108]. Curiously, in addition to the robust mechanisms maintaining copper homeostasis and copper's rapid excretion, mammals express copper-dependent enzymes that are central players in antioxidant defense. Thus, whereas copper can induce ROS formation when involved in Fenton-like or Haber-Weiss reactions, copper-dependent processes can also help to clear ROS [106]. For further information on the relationship between copper and DNA damage, please refer to the recent review published by Linder [106].

Iron

Iron is a crucial nutritional element for all life forms that plays a critical role in the cell, including electron transport and cellular respiration, proliferation and differentiation, and the regulation of gene expression [3]. Iron can undergo univalent redox reactions, resulting in oxidized and reduced forms known as ferric (Fe^{3+}) and ferrous (Fe^{2+}) iron, respectively. Due to iron's oxi-reduction, which can contribute to ROS generation, as well as iron's role in Fenton and Haber-Weiss reactions, this nutrient is also potentially deleterious. These reactions occur when an inorganic nutrient, such as Fe^{2+} or Cu^{+}, is in excess and donates an electron to H_2O_2, leading to OH production. The ROS generated

by Fenton chemistry can contribute to major pathologies, such as cancer, atherosclerosis, and neurodegenerative diseases [38].

Free radicals can cause serious damage to the genome. Depending on the dose and type, inorganic nutrients can protect against or contribute to oxidative stress [109]. Peroxidases and especially catalase, which use heme-iron as a cofactor, decompose H_2O_2. If the resultant reactive species are not efficiently removed, these species can induce the formation of the more active OH or peroxynitrite, which may result in DNA oxidation. Therefore, deficiencies in such nutrient-dependent antioxidant enzymes can increase oxidative stress and favor the genomic instability [110].

In addition, iron is a co-factor of many important enzymes related to DNA repair mainly as clusters iron-sulphur. For example, the glycosilases MutyH and NTHL1 involved in base excision repair (BER) and mismatch repair (MMR) and the helicases ERCC2 and BACH1 acting in the nucleotide excision repair (NER) possess iron-sulphur clusters in their structure [111, 112]. The increased DNA damage sensitivity in cells with impaired Fe/S protein biogenesis may include the loss of nucleotide excision repair because maturation of XPD is defective. Since the Fe/S cluster of XPD is required for its DNA helicase activity in vitro [111].

Although excess iron can cause oxidative DNA damage in rats and has been associated with an increased risk of cancer and heart disease in humans [20], iron deficiency also appears to lead to oxidative DNA damage and is associated with cognitive dysfunction in children. The importance of iron in normal neurological function has been well established, as neurons require iron for many physiological processes, including electron transport and axonal myelination, and as a cofactor for many enzymes involved in neurotransmitter synthesis [113, 114]. In contrast, inadequate iron intake results in anemia, immune dysfunction, and adverse pregnancy outcomes, such as premature birth. Maintaining physiological iron levels via dietary intake is thus mandatory for health. However, iron deficiency is still very common in the human population, particularly among children and pregnant women [115].

Magnesium

Magnesium is indispensable to life, as this micronutrient is involved in many important biological processes. Magnesium has multiple functions in all cellular

processes, including DNA replication and protein synthesis, and also serves as a cofactor for DNA-repair proteins and in the maintenance of a cell's redox status, cell-cycle regulation and apoptosis [29]. Magnesium deficiency or the displacement of Mg^{2+} by other toxic, divalent metal ions leads to increased genomic instability, which has been implicated in many diseases [116] and may result in inhibited DNA repair, oxidative stress, accelerated aging, and increased cancer risk [29, 117]. Studies have indicated that higher magnesium consumption may protect against certain inflammatory disorders, such as insulin resistance [118], hypertension [119], diabetes mellitus [120] and cardiovascular disease [119].

Magnesium is not genotoxic at physiologically relevant concentrations and in fact maintains low mutation frequencies by facilitating high-fidelity replication and by supporting all DNA-repair processes and chromosomal segregation during mitosis [29]. In fact, it is an essential cofactor in NER, BER, MMR processes, where magnesium is required for the removal of DNA damage [121]. All downstream activities of major base excision repair proteins, such as apurinic/apyrimidinic endonuclease, DNA polymerase beta, and ligases, require magnesium. Thus, this element may act as a regulator for the base excision repair pathway for efficient and balanced repair of damaged bases, which are often less toxic and/or mutagenic than their subsequent repair product intermediates [122]. Magnesium is also important for the fidelity of DNA replication, impacting cell cycle and apoptosis [61]

Animal and human epidemiological studies have demonstrated inverse correlations between magnesium levels and cardiovascular disease [29] or the incidence of certain types of cancer, including colorectal cancer [123, 124]. Additionally, magnesium deficiency is one risk factor for premature aging [29]. The relationship between magnesium levels and tumorigenesis is more complex, with magnesium deficiency increasing tumor incidence in animals and humans, whereas magnesium promotes the growth of preexisting tumors due to profound changes in magnesium homeostasis in tumor cells. Thus, the protective effects of magnesium are restricted to the early stages of tumor development [29]. According to Ford and Mokdad [125], despite the role of magnesium in maintaining good health, historically, much of the population of the United States has not consumed adequate amounts of this nutrient. Additionally, there are significant racial and ethnic disparities in magnesium intake.

Selenium

The trace element selenium is another well-established micronutrient essential for mammalian health [126]. Selenium is a constituent of the small group of selenocysteine-containing selenoproteins [127], including glutathione peroxidase, thioredoxin reductase, selenoprotein P and selenoprotein R, which are primarily involved in antioxidant activity and the maintenance of a cell's redox state [128-131]. Due to selenium's key role in redox regulation and antioxidant function, this nutrient is critical for membrane integrity, energy metabolism and protection against DNA damage [127]. However, in certain cases, selenium can also lead to oxidative DNA damage [20], increased infection risk and altered mood [132]. Whether selenium exerts positive or negative effects *in vivo* or *in vitro* is related to dose. Interest in organoselenide chemistry and biochemistry has increased over the last two decades, mainly because a variety of organoselenium compounds can be used as antioxidants, enzyme inhibitors, neuroprotective, antitumoral, or anti-infectious agents, as well as cytokine inducers and immunomodulators [126, 133-136]. In fact, an interaction with the zinc finger structures of DNA repair proteins may occur by essential trace elements such as certain selenium compounds, which appear to exert anticarcinogenic properties at low concentrations but may compromise genetic stability at higher concentrations [137].

Selenium deficiency alone is not common in developed countries, but an inadequate intake of this mineral has been associated with the development of cancer, asthma and coronary disease, among other chronic conditions [138]. When required, dietary supplementation must be performed carefully, given the intrinsic toxicity of high selenium levels [139].

Zinc

Zinc is one of the most important micronutrients due to the prevalence of zinc-dependent enzymes in metabolic processes; zinc's vital role in several bodily functions, such as vision, taste perception, cognition, cell reproduction, growth, and immunity; and the beneficial effect of zinc supplementation on many disease states [140]. In fact, zinc is a component of over 300 proteins, including over 100 DNA-binding proteins with zinc fingers, Cu/Zn superoxide dismutase, the estrogen receptor, and the synaptic transmission protein [20]. Zinc also has a crucial role in the biology of p53, in that p53

binds to DNA through a structurally complex domain stabilized by zinc atom, possibly increasing the response to anticancer drugs [141].

Zinc deficiency is a health problem in many communities, especially among adolescents, due to the pubertal growth spurt [140]. At the molecular level, there is evidence of a relationship between zinc deficiency and increased chromosomal breaks, possibly due to increased oxidative damage stemming from a loss in the activity of Cu/Zn superoxide dismutase or the zinc-containing DNA-repair enzyme Fapy glycosylase, which repairs oxidized guanine [20]. Unfortunately, nearly half of the world's population is at risk of inadequate zinc intake, so public-health programs are urgently needed to reduce zinc deficiency [140].

Summary of the effects of the selected micronutrients on genomic stability

Taking the preceding discussion and other evidence from the literature into account, the adequate intake of micronutrients seems to have an important role in genomic stability. In contrast, an imbalance of the same micronutrients may also negatively impact the DNA, possibly via oxidative stress, consequently causing or contributing to different human diseases. It is thus highly relevant to elucidate the mechanism underlying the response to and repair of oxidative stress and this mechanism's relationship to the DNA damage response pathways, all of the inorganic nutrients (vitamins and minerals) and disease, including carcinogenesis. An understanding of the possible influences on genomic stability, even in cell culture, is also in current demand.

4. Cell-culture medium and micronutrients that increase genomic stability: is the concentration relevant?

According to Ferguson and Fenech [142], the last decade of studies on micronutrients and genomic stability have improved dietary recommendations based on the prevention of DNA damage or the maintenance of genomic integrity. In light of this, the development of *in vivo* and especially *in vitro* models to more robustly evaluate DNA damage is necessary.

Table 2 presents interesting data regarding the micronutrients that may interfere with genomic stability and the micronutrient concentration values found in typical cell-

culture media, FBS and human serum. Unfortunately, data are not available for all of the micronutrients in the media, and even the proportions of micronutrients in FBS, as an organic product, are not all well characterized. Additionally, as demonstrated by Bryan *et al.* [34], the concentration of many micronutrients in FBS can vary significantly between batches.

Although cell-culture media attempt to provide an environment similar to the *in vivo* milieu of cell development, there is an evident imbalance of micronutrients between the media and human serum. Certain micronutrients are present in these media at concentrations higher than those found in human serum (e.g., vitamins B7 and B12), whereas other nutrients are present at significantly lower concentrations than in human serum (e.g., iron and zinc). A recent study [104] called attention to the composition of multivitamin supplements, which may trigger unwanted health outcomes due to the synergistic oxidative effects of the component vitamins and metals. In this research, the vitamins' chemical oxidation potencies were studied by measuring the amount of the oxidation product 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) formed from the DNA nucleoside deoxyguanosine (dG) after vitamin exposure. The micronutrients evaluated by the authors were the vitamins A, B1, B2, B3, B6, B12 and C; β-carotene; folic acid; and α-, δ- and γ-tocopherol. The minerals copper, iron and zinc were also examined. All of these micronutrients were tested in cell culture, alone or in combination, taking the human serum levels of each micronutrient into account. The main conclusion reported was that certain vitamins, alone or in combination with metals (e.g., vitamin C and copper), can induce DNA damage. However, cells in culture and *in vivo* have distinct needs for nutrients and growth factors, as the cells' activity in each environment may differ due to interactions with other cells or parts of the larger organism. Thus, examining physiological concentrations of micronutrients *in vitro* may not be the most appropriate approach.

As mentioned above, each cell type may have a distinct requirement for micronutrients. Depending of the origin of the cell and its role *in vivo*, the cell may specifically have a higher affinity for one micronutrient over another. In the case of iron, for example, which is stored in specific tissues, including the spleen, liver and bone marrow [143], the primary cells or immortal cell lines derived from these tissues may have a greater need for this specific micronutrient. In the case of certain neuronal cells, which require iron for cell development [144], the demand for iron may also be higher than in other cell types. Although the evaluation of micronutrients' influence on DNA damage and

integrity as well as on cell development, including the related enzymes and proteins, should be continued, the micronutrient concentrations relevant not only to human but also to cell-culture genomic stability must be considered.

Table 2. Concentrations (in µmol/L) of micronutrients that can increase genomic stability in traditional cell-culture media and FBS *versus* human serum.

Micronutrients	Cell Culture Medium*								10% FBS**	Mean Human Serum Concentration***	Status Cell Culture Medium *versus* Human Serum
	MEM	DMEM	L-15	M-199	HAM F-10	HAM F-12	RPMI-1640	DMEM/ HAM F12			
Vitamins											
Vitamin A	NA	NA	NA	3.1×10^{-1}	NA	NA	NA	NA	3.0×10^{-2}	2.0	Lower
Vitamin B7 (Biotin)	NA	NA	NA	4.1×10^{-2}	1.0×10^{-1}	3.0×10^{-2}	8.2×10^{-1}	1.0×10^{-2}	Trace	4.0×10^{-4}	Higher
Vitamin B9	2.3	9.1	2.3	2.3×10^{-2}	3.0	3.0	2.3	6.0	Trace	5.0×10^{-3}	Higher
Vitamin B12	NA	NA	NA	2.8×10^{-1}	1.0	1.0	4.0×10^{-3}	5.0×10^{-1}	Trace	3.0×10^{-4}	Higher
Vitamin C	NA	NA	NA	1.4×10^{-2}	NA	NA	NA	NA	Trace	50.0	Lower
Vitamin E	NA	NA	NA	NA	NA	NA	NA	NA	0.0003	30.0	Unknown
Minerals											
Copper	NA	NA	NA	NA	1.0×10^{-2}	1.0×10^{-2}	NA	5.0×10^{-3}	Trace	14.0	Lower
Iron	NA	2.5×10^{-1}	NA	1.7	3.0	3.0	NA	1.6	3.0	23.0	Lower
Magnesium	8.0×10^{2}	8.0×10^{2}	1.8×10^{2}	NA	6.2×10^{2}	6.1×10^{2}	4.1×10^{2}	1.1×10^{3}	Trace	8.0×10^{2}	Lower/Similar
Selenium	NA	NA	NA	NA	NA	NA	NA	$3 .0 \times 10^{-2}$	3.0×10^{-2}	11.0	Lower
Zinc	NA	NA	NA	NA	1.0×10^{-1}	3.0	NA	1.5	Trace	17.0	Lower

NA: not available.

* MEM: Minimum Essential Medium. DMEM: Dulbecco's Modified Eagle Medium. L-15: Leibovitz Medium 15. M-199: Medium-199. HAM F-10 and F-12: Ham's Nutrient Mixture F-10 and F-12. RPMI-1640: Roswell Park Memorial Institute medium. DMEM/HAM F-12: Dulbecco's Modified Eagle Medium/Ham's Nutrient Mixture F-12. The vitamin and mineral concentrations described were obtained from the webpages of the key suppliers.

** The values for the vitamin A, vitamin E and selenium concentration in FBS were found in [145], and the iron concentration in FBS was determined analytically.

*** The references citing the micronutrient concentrations in human serum are as follows: vitamins A [146], B7 [147], B9 [148], B12 [149], C and E [150]; Mg [151]; and Cu, Fe, Se and Zn [152]. The concentration of the vitamins and minerals in the media were obtained from the manufacturers.

5. Could changes in a culture's micronutrient composition influence the viability and genetics of the cultured cells?

Cells are typically maintained at an appropriate temperature and CO_2 concentration (usually 37°C and 5% CO_2 for mammalian cells) in an incubator. Beyond these parameters, the most commonly varied factor in culture systems is the growth medium. The recipes for growth medium can vary in pH, glucose concentration, growth factors, and the presence of other nutrients and micronutrients. The development of synthetic basal formulations for mammalian cell-culture applications has been facilitated by the contributions of many investigators. In particular, the definition of the minimally required nutrients by Harry Eagle in the 1950s spawned an iterative process of continuous modification and refinement of the exogenous environment to cultivate new cell types and support the emerging applications of cultured mammalian cells. This process led to the development of highly potent, basal nutrient formulations capable of sustaining serum-free cell proliferation and biological production [153]. However, the growth factors most often used to supplement cell-culture media are still derived from animal blood, such as FBS. FBS has become the supplement of choice for cell culture-based research, containing an array of proteins, growth factors and ions necessary for cell viability and proliferation *in vitro*, including certain vitamins and minerals [154]. Currently, the use of these ingredients is minimized or eliminated wherever possible in favor of chemically defined media, but this substitution is not always possible.

Bryan *et al.* [34] stated that one of the major obstacles to obtaining human cells of a defined and reproducible standard, and thus suitable for use in medical therapies, is the routine necessity of supplementing cell-culture media with FBS. In this study, FBS variants were evaluated, in terms of both elemental (micronutrient) composition and the variants' effects on the expression of a group of proteins associated with the antigenicity of primary human umbilical vein endothelial cells (HUVECs). A combination of inductively coupled plasma mass spectrometry (ICPMS) and flow cytometry was used to achieve these experimental objectives. Statistically significant differences in antigenic expression during cell culture were demonstrated for a set of trace elements in FBS (e.g., lithium, boron, magnesium, phosphorus, sulfur, potassium, titanium, vanadium, chromium, manganese, iron, copper, zinc, gallium and selenium). The lack of reproducibility and the variation in protein expression in the primary human cells was attributed to the FBS supplementation.

Culture conditions for cell lines are known to affect gene expression [155-157], while stem cells grown in different types of serum exhibit variable differentiation and proliferation characteristics [158, 159] the same cell line, if cultivated in different conditions, can present different phenotypes either. Nevertheless, the cellular requirement for a specific micronutrient is directly correlated with the cell type, the rate of cell grow and the stage of cell differentiation. In light of this, it is important to observe that minimal attention has been dedicated to the composition of FBS and the micronutrient supplementation of media in cell cultures or the fact that micronutrients can influence the viability and genomic stability of cultured cells.

In **Tables 3** and **4,** a few examples of the effects of vitamins and minerals in cell culture and on genomic stability, drawn from the literature, are highlighted.

Table 3: Examples from the literature of vitamins' effects in cell culture and on genomic stability.

Micronutrient	Main effects on cell viability and genomic stability	Cell type	Additional information regarding the form and concentration of the micronutrient evaluated	Status in relation to physiological concentration	References
Vitamin A	Enhanced the levels of 8-oxo-dG DNA damage but significantly inhibited M1dG formation especially after induction of M1dG by H_2O_2 or B[a]P; increased production of reactive oxygen species and formation of promutagenic DNA lesions.	Lung epithelial cells	beta-carotene (5 µmol/L)	Similar	[160, 161]
	Caused oxidation of dG and cytotoxicity, giving rise to an almost complete cell death.	Leukemia cells (HL-60)	retinol (2 µmol/L) and ascorbic acid (50 µmol/L)	Similar	[162]
	Induced apoptosis by increasing apoptotic protein p53 and decreasing anti-apoptotic Bcl-2 as well as nuclear ATM; also induced DNA fragmentation.	Gastric cancer cells (AGS)	beta-carotene (100 µmol/L)	Higher	[163]
	DNA damage on HepG2 which were also concordant to increased apoptosis and necrosis of cells	Hepatocarcinoma cells (HepG2)	beta-carotene (4 µmol/L)and 8 µmol/L)	Similar	[164, 165]
	Reduced levels of total DNA adducts and increased apoptosis levels in cells co-exposed to benzo(a)pyrene and retinoic acid		retinoic acid (1 µmol/L)	Lower	
Vitamin B7 (biotin)	Increased strand breaks and cellular response to strand breaks	T-lymphocyte cell line (Jurkat)	25 $x10^{-6}$ µmol/L and 0.01 µmol/L	Lower and Higher	[75]
	Affects biotinylation of proteins, gene expression and metabolism of interleukin-2; Rates of proliferation and apoptosis were not affected by biotin status		25 $x10^{-6}$ µmol/L, 25 $x10^{-5}$ µmol/L and 0.01 µmol/L	Lower and Higher	[166]
Vitamin B9	Increased levels of excision repair and apoptosis	Lymphocytes	folate (<2.3 x 10^{-3} µmol/L)	Lower	[167, 168]
	Decreased apoptosis and increased cell proliferation	Neural stem cells (NSCs)	folic acid (8.4 x 10^{3} µmol/L)	Higher	[169, 170]
	High concentration accelerated growth; increased metabolic activity, proliferation and apoptosis; and decreased differentiation	Human colon cancer cells (HT29)	folic acid (0.021 µmol/L and 0.21 µmol/L) with other micronutrients involved in folate-methionine cycle	Similar and Higher	[56]

Table 3: Examples from the literature of vitamins' effects in cell culture and on genomic stability (Cont.).

Micronutrient	Main effects on cell viability and genomic stability	Cell type	Additional information regarding the form and concentration of the micronutrient evaluated	Status in relation to physiological concentration	References
Vitamin B12	Reduced cell proliferation and increased differentiation	Neuroblastoma cells (NIE115)	vitamin B12 (total absence)	Lower	[90]
	Chronic exposure inhibited neurotoxicity	Retina cells (primary cultures from fetal rats)	methylcobalamin (1 µmol/L)	Higher	[171]
	Absence is likely to result both in reduced cell proliferation and cell death, as inhibition of DNA synthesis generally results in apoptosis	Human erythroleukemic (K562) and murine lymphoma (BW5147) cell lines	cobalamin (total absence and 3.7×10^{-3} µmol/L)	Lower and Higher	[172]
Vitamin C	Physiological concentrations of AA were not toxic while high concentrations of AA induced DNA strand breakage in a dose-dependent manner, whereas AA2P were not genotoxic	Human dermal fibroblasts (HDFs)	ascorbic acid (AA) and ascorbic acid 2-phosphate (AA2P) (total absence or 20, 100, 500 µmol/L)	Lower, Similar and Higher	[173]
	Enhanced DNA-protein crosslinks and cytotoxicity	Chinese hamster cells (V79)	ascorbic acid (1000 µmol/L)	Higher	[174]
	Decreased number of 8-hydroxydeoxyguanosine adducts	Mouse keratinocyte cell line	ascorbic acid (2,27 µmol/L and 4,54 µmol/L)	Lower	[175]
	Protective effect against DNA damage induced by X-ray treatment	Human lymphoblastoid cells (Raji)	ascorbic acid (60 µmol/L)	Similar	[176]
Vitamin E	Protective effect against DNA damage induced by H_2O_2 treatment	Raji cells	α-Tocopherol (30 µmol/L)	Similar	[176]
	Reduced DNA fragmentation and apoptotic body formation, possibly favoring DNA repair	African green monkey kidney (Vero), human colon carcinoma (Caco-2) and dysplastic oral keratinocyte (DOK) cells	vitamin E (25 µmol/L)	Similar	[177]
	Reduced apoptosis and autophagy	Cultured trophoblasts and villous explants obtained from human placentas at term	vitamin E (50 µmol/L) with vitamin C (50 µmol/L)	Higher	[178]

Vitamin A

For vitamin A, but possibly applicable to many other micronutrients, the studies presented in **Table 3**, conducted at low concentrations, tend to show protective effects, whereas higher concentrations are associated with increased DNA damage [65]. This finding is consistent with the known ability of β-carotene to act as a prooxidant, rather than as an antioxidant, at high concentrations and under high oxygen tension [179]. The physiological concentrations of micronutrients should always be evaluated and, if possible, at least used as a maximum in studies evaluating the viability and genomic stability of cell cultures. However, as can be verified in **Table 2**, there is a lack of data regarding the presence of vitamin A in cell-culture media.

Vitamin B7 (Biotin)

Biotin plays an important role in regulating gene expression, thus mediating certain aspects of cell biology and fetal development [180]. The effects of biotin deficiency are detailed in **Table 3** and are related to decreased rates of cell proliferation, impaired immune function, and abnormal fetal development. An excess of biotin is also mentioned and can exert reproductive and teratogenic effects. However, as can be verified in **Table 2**, cell-culture media containing higher levels of biotin than human serum are common. More studies evaluating the effects of the high biotin levels in cell cultures are necessary.

Vitamin B9

Folate depletion appears to enhance carcinogenesis, whereas folate supplementation above what is presently considered to be the basal requirement confers a protective effect [181]. A few examples of folate deficiency and supplementation are described in **Table 3**, and the relationship between this vitamin and cell proliferation and apoptosis has been demonstrated. Furthermore, as can be verified in **Table 2**, the folate levels in the cell-culture media evaluated are typically higher than those levels found in human serum. It is well established that folate deficiency can influence the genomic stability of cultured cells [82, 182], yet there is still a lack of data evaluating whether folate levels above the physiological range can impair cell growth. Elevated levels of folic acid should be examined, as in tumor-prone animals, both folate deficiency and supplementation promote the progression of established neoplasms [84, 183]. As a folate

overload is more common than a deficiency in *in vitro* studies, the former should be most thoroughly evaluated.

Vitamin B12

Vitamin B12 deficiency has been described as similar to chemicals that damage DNA by causing single- and double-strand breaks [20]. As demonstrated in **Table 3**, in a cellular model designed to better understand vitamin B12 deficiency in the brain, the growth and differentiation of neuronal cells were affected [90]. Additionally, supplementation with certain cobalamin compounds protected the cells from neurotoxicity and increased cell growth [171, 172]. Unfortunately, *in vitro* research demonstrating a directly link between vitamin B12 deficiency or overload and genomic stability in human cells has not yet been published. Based on **Table 2**, however, high concentrations of vitamin B12 are more common in cell-culture media than in human serum.

Vitamin C

In **Table 3**, a few examples of the influence of vitamin C in cell cultures are provided. Different concentrations of this vitamin result in distinct responses, ranging from DNA damage (at higher concentrations) to the protection of DNA (at lower concentrations). Importantly, the concentration of vitamin C in current cell cultures is not available in **Table 2**, as possibly only trace levels are present in media. As the cellular response to vitamin C may be dose-dependent, a similar concentration of this vitamin in culture media to that in human serum should be evaluated.

Vitamin E

In vivo vitamin E supplementation is still being discussed [184], and more *in vitro* studies will be required to better understand the protective effects of vitamin E on cell viability and genomic stability. Nevertheless, certain results (**Table 3**) have been consistent with the concept that α-tocopherol, combined with ascorbic acid or alone, can protect against oxidative DNA damage [176] and reduce apoptosis and autophagy [178] under certain conditions. Unfortunately, the current *in vitro* concentration of vitamin E is also not available in **Table 2**, as possibly only trace levels are present in media. Given this observation, it is interesting to observe that the *in vitro* studies of vitamin E described in

Table 3 adopted concentration values similar to that of human serum (approximately 30 µmol/L) and that the results were positive for the cell cultures.

Table 4: Examples from the literature of minerals' effects in cell culture and on genomic stability.

Micronutrient	Main effects on cell viability and genomic stability	Cell type	Additional information regarding the form and concentration of the micronutrient evaluated	Status in relation to physiological concentration	References
Copper	Increased cytotoxicity and ROS formation	HepG2	50,100,150 and 200 µmol/L	Higher	[185]
	Reduced mitochondrial activity and cell viability and increased DNA damage	Chinese hamster ovary cells (CHO-K1)	24.55, 35.40, 48.31, 89.23, 116.77, 170.75, 339.45 and 450.35 µmol/L	Higher	[186]
	Increased the DNA damage in a dose-dependent manner and also reduced rates of DNA synthesis and histone acetylation	Leukemia cells (HL-60)	total absence, 10, 20, 50, 100 and 200 µmol/L	Lower, Similar and Higher	[187]
Iron	Inhibited DNA synthesis in proliferative cells	Human lymphocytes	iron suphate (22.38, 44.76 and 89.52 µmol/L)	Similar and Higher	[188]
	Possibly accelerated aging process and death at concentrations > 10 µmol/L, whereas 5 µmol/L increased protein content	Cerebellar granule cells	ferric nitrilotriacetate (5, 10, 15, 20 and 40 µmol/L are shown	Lower, Similar and Higher	[189]
	Genotoxic effects	Primary nontransformed colon cells and preneoplasic colon adenoma cell line (LT97)	ferric nitrilotriacetate (10, 100, 250, 500 and 1000 µmol/L)	Lower and Higher	[190]
Magnesium	Inhibited cell proliferation and promoted endothelial dysfunction by generating proinflammatory, prothrombotic and proatherogenic environment	Human endothelial cells	magnesium sulphate (100, 500 and 1000 µmol/L)	Lower and Higher	[191]
	Inhibited growth more drastically in normal than in transformed cells and altered cell-cycle progression	Normal (HC11) and transformed (MCF-7) breast epithelial cell lines	total absence, 10, 30, 50, 100, 300 and 500 µmol/L	Lower	[192]
	Inadequate concentration accelerated cell senescence	Normal human fibroblasts (IMR-90)	100, 400 and 800 µmol/L	Lower and Similar	[193]
	Incision repair completely inhibited in absence of Mg^{2+} as well as at very high concentrations, whereas optimal concentrations essential in all steps of NER	Human lymphoblastoid (AHH1) and clonal human epithelial adenocarcinoma (HeLa S3) cell lines	400 and 800 µmol/L	Lower and Similar	[194]

Table 4: Examples from the literature of minerals' effects in cell culture and on genomic stability (Cont.).

Micronutrient	Main effects on cell viability and genomic stability	Cell type	Additional information regarding the form and concentration of the micronutrient evaluated	Status in relation to physiological concentration	References
Selenium	Methylseleninic acid, L-selenocystine, selenodiglutathione or selenite induced cell death in micromolar concentrations, whereas selenomethionine or ebselen was not toxic within the concentration range tested	HepG2, human hepatoma cell line (Huh-7), mouse hepatoma (Hepa 1-6)	sodium selenite, L- or DL-selenocystine, selenodiglutathione, selenomethyl-selenocysteine, sodium selenate, L- or DL-selenomethionine, methylseleninic acid, ebselen, selenomethionine, selenodiglutathione (0.1 x10^{-3} to 1000 µmol/L)	Lower, Similar and Higher	[195]
	Induces G1-cell cycle arrest and apoptosis via multiple signaling pathways, which may play a key role in methylselenol-induced inhibition of cancer cell proliferation and tumor cell invasion	Human sarcoma cell line (HT1080)	seleno-L-methionine (SeMet) (total absence, 1.25, 2.5 and 5 µmol/L)	Lower	[196]
	Decrease in cell damage and protection against oxidative stress	HepG2 cells	Selenium methylselenocysteine (0,01, 0,1, 1 and 10 µmol/L)	Lower and Similar	[197]
			Selenium methylselenocysteine (1 µmol/L)	Lower	[198]
Zinc	Increased oxidative DNA damage; disrupted p53, NFκB, and AP1 DNA binding; decreased DNA repair	Rat glioma cell line (C-6)	Zn sulfate and Zn carnosine (4.0 µmol/L)	Lower	[199]
	Decreased cell growth and viability, increased DNA SB an cytotoxicity in Zn-depleted cultures as well as at concentrations of 32 and 100 µM; Reduced genomic damage in cultures supplemented with 4 or 16 µM	Human lymphoblastoid cell line (WIL2-NS)	Zn sulfate and Zn carnosine (total absence, 0.4, 4.0, 16.0, 32.0 and 100.0 µmol/L)	Lower, Similar and Higher	[200]
	Decreased cell viability in Zn-depleted cultures (0 µM) as well as at concentrations of 32 and 100 µM for both Zn compounds and increased DNA SB, apoptotic and necrotic cells in Zn-depleted cultures	Primary human oral keratinocyte cell line (HOK)			[201]

Copper

As can be verified in **Table 2**, there is a marked lack of copper in common cell-culture media, even when supplemented with FBS. Thus, cells in culture are typically exposed to an environment deficient in a micronutrient critical for the formation of detoxifying enzymes, which may impact cell development and possibly genomic stability and survival rates. It is important to note that the copper concentrations evaluated in cell culture (**Table 4**) are generally above the human physiological range, so toxic effects in cultures should be expected. Thus, the optimization of the copper concentration in cell cultures is necessary to maintain cell viability and genomic stability and to avoid the deleterious effects of this metal.

Iron

In **Table 4**, it is important to note that the results of Lima *et al.* [188] may be expected in a cell culture in which the requirements for micronutrients are quite different from those *in vivo*. In this study, the concentrations evaluated were generally higher than the values measured in human serum (**Table 2**), and even the lowest concentration of iron applied for the authors (22.38 µmol/L) would be considered high for cells in culture. For HL-60 leukemia cells, as demonstrated in [202], the iron concentration range for optimal cell proliferation is very narrow (2-3 µmol/L). In contrast, in the studies in which the iron levels were between 5 and 10 µmol/L, these levels generally benefitted the cultures analyzed, or at least no damage was observed [188-190].

Magnesium

As presented in **Table 4**, several studies on the effects of magnesium deficiency on cultured cells have demonstrated reduced oxidative stress, cell-cycle progression, cell growth, and cell viability [191, 192, 203-208]. Killilea and Ames [193] specifically investigated the consequences of long-term and moderate magnesium deficiency in normal human cells in comparison with more typical magnesium levels, using a concentration observed in normal human serum (0.8 mmol/L). No alterations were observed in the cells cultured in the medium containing normal magnesium levels. Additionally, based on studies conducted either in bacteria or in mammalian cells in culture, there is no evidence for the genotoxic effects of magnesium salts at

physiologically relevant doses [29], indicating that adequate micronutrient levels in cell-culture media may improve cell viability and genomic stability. As shown in **Table 2**, the levels of magnesium currently found in cell-culture media are very similar to those levels in human serum, which is very unusual for micronutrients in general.

Selenium

The differential toxicities elicited by selenocompounds need to be taken into account in *in vivo* and *in* vitro supplementation studies [195]. The references in **Table 4** evaluated different forms of selenium and certain salts that may be more toxic to the cellular environment than others. Due to the importance of selenium as well as many other micronutrients discussed in this review, the micronutrient concentration in the media, as well as the FBS, intended for cell culture should be controlled and adjusted to the physiological range, if applicable. By comparing the human serum concentration of selenium in **Table 2** with those concentrations described in the experiments cited in **Table 4**, it is apparent that the concentrations below the physiological range benefitted the cell culture, although high concentrations of selenium compounds potentially negatively affected tumor cells.

Zinc

The role of zinc in genomic stability was recently reviewed by Sharif *et al.* [209]. Additionally, a few brief examples of zinc's influence on cell viability and genomic stability are provided in **Table 4**. A possible conclusion from the *in vitro* assays is that when the zinc concentration used is below the human serum value (**Table 2**), the results tend to be beneficial for the cultured cells. In contrast, zinc concentrations above the physiological level can damage cultured cells. Again, it is interesting observe that certain cell-culture media (e.g., HAM F-10 and F-12), even when supplemented with FBS, cannot provide enough of this micronutrient for appropriate cell development and genomic stability once the concentration falls below the physiological range.

6. What must be done: limitations of the available evidence and conclusions

Micronutrients are clearly important for cell development and genomic stability, and many of the micronutrients mentioned are necessary for the DNA synthesis and repair mechanisms. **Table 5** provides an overview of the current data regarding the effects of deficiencies or excesses of the micronutrients addressed in this review on genomic stability. The micronutrient levels found in the discussed cell-culture media and the status of research on each micronutrient are also highlighted. Evidently, much research has been performed, but more specific studies focusing on cell cultures are still required.

Table 5: Overview of the data addressed in this review.

Micronutrient	Evidence of genomic instability induction		Concentration in common cell-culture media versus physiological concentration	Optimal concentration proposed for cell culture
	Deficiency	Excess		
Vitamin A	+	+	Lower	Studied
Vitamin B7	+	+	Higher	Requires more studies
Vitamin B9	+	+	Higher	Studied
Vitamin B12	+	NA	Higher	Studied
Vitamin C	+	+	Unknown	Studied
Vitamin E	-	+	Unknown	Studied
Copper	+	+	Lower	Studied
Iron	+	+	Lower	Studied
Magnesium	NA	+	Similar	Studied
Selenium	+	-	Lower	Studied
Zinc	+	+	Lower	Studied

NA: Not available.

(-) Negative: the available data indicate no effect.

(+) Positive: the available data indicate an effect.

Even though there are some highly enriched media available as basal media for serum-free cell culture, like Medium 199 or Ham F-12 nutrient mixture, the most common source of micronutrients currently used in cell cultures is still FBS. The limitations of FBS in providing adequate micronutrient concentrations have been analyzed and described in the literature [34]. Given that cell- and tissue-culture models are generally important in scientific research, the development of standards *in vitro* methods is mandatory. These

new standards will decrease dependence on animal serum, a supplement with an undefined, variable composition that can considerably influence experimental results [210]. Furthermore, according to van der Valk *et al*. [210], an improved exchange of information regarding newly developed serum-free media may be beneficial. It has also become clear that nearly every cell type has distinct requirements for media supplementation, and especially, as discussed in this review, for micronutrient supplementation. A universal cell- and tissue-culture medium may not be feasible, as different cell types have different receptors involved in cell survival, growth and differentiation and release different factors into the surrounding environment.

Besides this, it is important to highlight that although the formulations of the classical cell culture media are unchanged for a long time, since their development, the quality and purity of single components used as supplements, are likely to have increased considerably. However, some losses of important substances could have occurred, including trace elements, vitamins, growth factors, and lipids and this should be better addressed before define a serum-free media. In fact, the threshold for developing and using a new, well-defined medium, given that the current FBS-supplemented culture media work well, is high [210]. At the very least, an evaluation of FBS composition, in terms of micronutrients and possibly other factors, should be strongly considered in the laboratories that focus on *in vitro* studies. Knowledge of the micronutrient composition of FBS may help to minimize the bias in experimental results. However, maintaining both successful and consistent cell cultures can be difficult, as FBS is a complex natural product and may vary between batches, even if obtained from a single manufacturer. More specifically, the quality and concentration of both bulk and specific proteins in cell cultures can affect cell growth [211]. Adjusting the *in vitro* micronutrient levels to physiological values will guarantee a better environment for cell development, mimicking the *in vivo* milieu.

Further studies on the effects of micronutrients on cell viability, proliferation and stability, as well as gene expression and integrity are still required, but the information already available is a sufficient call to action. As mentioned by Ferguson and Fenech [142], most investigations have been limited to studying the effects of single micronutrients and have not considered genetic consequences. Thus, there is an important need for studies that also examine nutrient-nutrient and nutrient-gene interactions. Determining the physiological range of such significant micronutrients as iron

and then adjusting the concentrations currently found in cell-culture media may be beneficial for *in vitro* assays. More specifically, the viability and genomic stability of cell lines and primary cultures may be improved. Depending on the cell type (primary, immortalized, tumor or normal) and origin (lung, hepatic, neural or other), the requirement for a micronutrient may vary widely, so this subject should be carefully evaluated. Finally, the form of the micronutrient used in supplementation media may also influence experimental results. For example, according to Jacobs *et al.* [212], whether iron has toxic effects is directly related to the presence of a chelating agent, which reduces the concentration of free ferric ion and promotes the formation of ferritin.

Once the relationship between an *in vivo* imbalance of micronutrients and genomic stability, which may cause many diseases, including cancer, is established, it will be mandatory to better understand *in vitro* micronutrient supplementation. In fact, certain simple questions, such as *'is the concentration of this micronutrient sufficient for the development of this cell?'* or '*are the levels of this micronutrient similar to the levels observed in human serum?',* may aid the proper design of *in vitro* studies.

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CAPÍTULO II – "**The Effect of Iron on Viability and Genomic Stability of Human Pulmonary Fibroblasts (MRC5) and Hepatocellular Carcinoma (HepG2) Cell Cultures"**

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O Fe é o micronutriente cerne desta tese de doutorado, em torno do qual estudos prévios já haviam sido realizados pelo grupo e muitos questionamentos ainda exigiam ser devidamente endereçados.

Considerando-se a dualidade desse micronutriente, o qual pode ser tanto essencial quanto tóxico para a manutenção da vida, e observando-se sua homeostase altamente controlada, fica claro que sensíveis variações em suas concentrações podem desencadear processos de oxi-redução no organismo. De tal forma, o acúmulo de ERO e a instabilidade genômica são efeitos deletérios associados tanto à deficiência de Fe quanto a sobrecarga do mesmo, principalmente no organismo vivo. Contudo, análises mais aprofundadas a respeito de seu papel no cultivo de células ainda se faziam necessárias.

Sendo assim, o artigo que se segue, consolida todos os dados experimentais obtidos ao longo desse trabalho, a fim de avaliar a influência do Fe tanto na viabilidade quanto na estabilidade genômica celular. Para tanto, duas linhagens celulares foram selecionadas - um fibroblasto pulmonar (MRC5) e um hepatocarcinoma (HepG2), e diferentes suplementações de Fe em duas formas distintas (uma orgânica e outra inorgânica) foram analisadas.

The Effect of Iron on Viability and Genomic Stability of Human Pulmonary Fibroblasts (MRC5) and Hepatocellular Carcinoma (HepG2) Cell Cultures

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Abstract

Iron is an essential micronutrient and is required for growth, development, and normal cellular functioning. Either excess or deficiency of iron can cause oxidative stress and DNA damage. Since the cell media commonly used for cell culture has a lower iron concentration than the human serum, this study aimed to evaluate the role of iron supplementation on viability, reactive oxygen species (ROS) production, catalase activity, genome integrity and the expression of iron-bearing DNA repair proteins (TFIIH and MutyH) and proteins associated with iron absorption (CD71 and Nramp2). Two human cell lines – MRC5 (normal lung fibroblast) and HepG2 (hepatocellular carcinoma) and 2 sources or iron holo-Transferrin (h-Tf) or $FeSO_4$ were used. Both iron supplements were able to increase intracellular iron levels and cell viability. Iron supplementation increased the formation of ROS, but did not alter catalase activity. However, this increase was not accompanied by genotoxicity. Regarding the DNA repair protein expressions, the results suggest that 24h pre-treatment with h-Tf or $FeSO_4$ has no role in the TFIIH or MutyH expressions. Although, in iron receptor proteins expression, the preliminary data could indicate that CD71 is priority related with Fe homeostasis while Nramp2 seems to have a secondary role. Due to h-Tf physiological role in the iron homeostasis and the less pronounced ROS accumulation, h-Tf could be a better iron supplier *in vitro*. Additional studies are still required to better elucidate the role of Fe in cell viability and genomic stability.

1. Introduction

Iron (Fe) is an essential micronutrient required for growth, development, and normal cellular functioning, including genome stability. Excess or deficiency of Fe can cause oxidative stress and DNA damage [1]. Furthermore, low Fe intake also results in anemia, immune dysfunction, and adverse pregnancy outcomes, such as prematurity. Regarding reactive oxygen species (ROS) generation, Fe has a controversial role since while its excess seems to lead to oxidative DNA damage in rats and an increase risk of human cancer and heart disease [2], Fe deficiency may also lead to oxidative DNA damage [3]. In fact, Fe can enhance the ROS accumulation and this can be related to DNA damage, however, iron is also a co-factor of some DNA-repair enzymes and could be at the same moment, favoring DNA repair pathways [1]. Some enzymes have a iron-sulphur cluster in their structure [1], such as ERCC2/XPD which is a subunit of the transcription factor TFIIH and act as a helicase in nucleotide excision repair (NER) [4, 5] and MutyH, that acts as a DNA glycosylase in mismatch repair and also in base excision repair (BER) [6-8].

In the case of iron, as well as other micronutrients, it is well established that the maintenance of the physiological levels is mandatory for health [7, 8] but also, is clear that understanding the role of micronutrients in cells could help to find an ideal environment for cells *in vitro*, as well as to understand the cellular effects evoked by alterations in micronutrients concentration. Despite of this, the levels of iron commonly used in culture media are under physiological levels of human blood serum, which could vary between 8 µmol/L until 30 µmol/L, while values below 5 µmol/L characterize anemia [9, 10].

The main source of iron to cells in culture is serum supplementation, which can show very different iron levels. Cell culture conditions are known to affect gene expression, and the same cell line, if cultivated in different conditions, can present different phenotypes [11-13]. In the same way, stem cells grown in different types of serum exhibit variable differentiation and proliferation characteristics [14, 15]. In spite of these aspects, minimal attention is given to the selection of Fetal Bovine Serum (FBS) or other supplements for growing *in vitro* cultured cell lines. Researchers are usually concerned that their cells are growing at optimal rates and without phenotypic alterations. However, minimal alterations in gene expression and, as a consequence, in subtle cellular process, caused by slight environmental differences of cell culture may explain why some laboratories obtain different data using the same cell lines grown under superficially similar conditions [16]. In light of this, it is important to notice that cell culture media containing 5-10% serum can provide iron in a concentration ranging, in average, 2 - 6 µmol/L, suggesting that the culture conditions could have an "*anemic profile"* for cell growth. The few data [9-11] investigating

and optimizing the concentrations of iron for genomic stability *in vitro* led to the hypothesis that supplementation with iron could increase the proliferation and genomic stability in cell cultures.

Different sources of iron supplementation have been evaluated in the cell medium [9-12] and the data from media suppliers website, as www.sigmaaldrich.com and www.invitrogen.com, provides us with some inorganic salts used, as ferric nitrate ($FeNo_3$) and ferrous sulfate ($FeSO_4$). Other possible source of iron is Transferrin (Tf), which is the principal iron transporter in serum. Each Tf molecule can bind two molecules of iron and when bounded to iron, it is designated as h-Tf [3]. Once h-Tf is the main transporter of iron *in vivo*, it was one of the forms evaluated in this study. Besides this, it was important to evaluate an inorganic iron common used not only for media culture supplementation, but also, for anemia treatment *in vivo.* In light of this, $FeSO_4.7H_2O$, which is the form of iron listed by the European Parliament and Council (2002) as a compound allowed in commercial supplements and recently used also by other researchers [13] in their study, was the chosen one. Additionally, the expression of iron absorption proteins was evaluated - Transferrin receptor protein1 (TfR1) also known as cluster of differentiation 71 (CD71) [14] and natural resistance-associated macro-phage protein (Nramp2, also known as DMT1 for divalent metal transporter) [15]. The transferrin receptor (CD71) is an integral membrane protein that mediates the uptake of transferrin-iron complexes. Two transferrin receptors have been cloned (TfR1 and TfR2); however, TfR1 is considered the major protein responsible for iron uptake owing to its higher affinity and expression pattern [14].

Based in these considerations, in this study was investigated the effect of supplementation with iron, from two specific sources: bounded to Transferrin (h-Tf) and an inorganic iron - iron sulphate ($FeSO_4$), on viability, oxidative species production, catalase activity, genome integrity and the expression of DNA repair proteins (TFIIH and MutyH) and iron absorption proteins (CD71 and Nramp2) of two human cell lines – MRC5 (normal lung fibroblast) and HepG2 (hepatocellular carcinoma). It is important to point out that either catalase or the DNA repair proteins selected to be evaluate in this study have a heme moiety or a Fe/S cluster, respectively, in their structures.

2. Materials and Methods

2.1. Cell Culture

Regarding the cell lines selected for the study, HepG2 were chosen because cells derived from liver, as well as those from the gastrointestinal tract and renal tubules, are actively involved in uptake, transport, detoxification and secretion of metal compounds. Moreover, these cells are particularly susceptible to metal toxicity representing a suitable model for studying the effects of

these compounds [16, 17] and also because liver is the major site of iron storage *in vivo* [18]. Parallel experiments in normal human diploid fibroblast-like MRC5 cells were conducted to evaluate the role of iron supplementation also in a non-tumoral cell line.

MRC5 and HepG2 cell were cultured in high glucose Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactived FBS, 1% penicillin/streptomycin and 0.1% fungizone at 37^{o}C and 5% CO_2 in a humidified incubator. Cells were grown to semi-confluence before being detached by Trypsin/EDTA treatment. Cells suspensions were seeded in different concentrations according to the well plate used, the test proposal and the time of treatments.

2.2. Cell Culture Media Supplementation with Iron

DMEM with 10% of FBS was used as control (C), which iron concentration (~4.0 µmol/L) was previously determined by Flame Atomic Absorption Spectrometry. Iron supplementation was set around 7 µmol/L once this value is near the physiological and also, above the anemia level in human serum [19, 20]. Besides, the value evaluated is below 10 µmol/L, which has already been described to induce DNA damage to cells in culture [9]. Then, around 3.0 µmol/L of iron from either h-Tf or $FeSO_4$ were added to the media to reach a final concentration of ~7.0 µmol/L.

The iron concentration values are approximated because, although they have been analytical determined, the natural viscosity of the medium could interfere in the iron solubility and availability. The cells were exposed to the iron treatments for periods between 24h, 96h, 6 and 10 days, depending on the purpose of the assay. All the tests were performed in triplicates. The results for all the experiments are expressed in mean ± standard deviation.

2.3. Intracellular Iron Quantification

For MRC5 cells, the intracellular iron was determined based on Jacolot *et al.* [21] with some adaptations. Briefly, after exposure to iron for 24h and 96h, cells were washed twice with 2 mL phosphate-buffered saline (PBS), trypsinized and recovered by centrifugation, then washed again twice with PBS. Mineralization was obtained after addition of 1.5 mL of concentrated HNO_3 followed by ultrasound with heating (80°C) for 40 minutes. After the digestion, the samples were analyzed by Flame Atomic Absorption Spectrometry (248.3 nm). The control was the non treated cells.

2.4. Clonogenic Assay

Clonogenic assay or colony formation assay was used to test the proliferative capacity of single cells after treatment [22]. MRC5 cells were harvested from a stock culture and plated into 6-well plate in an initial concentration of 250 cells/mL. After attachment (24h) of the cells to the dishes, they had the media replaced - one received the h-Tf media, another $FeSO_4$ media and a third group of cells just had the media replaced by the same media (non extra iron supplemented), as C. After treatment, the dishes were incubated at 37ºC, 5% CO_2 for 10 days, a time equivalent to at least six potential cell divisions. The media were replaced every 3 days. Finally, the media were removed, the cells were rinsed carefully with PBS that was removed and the colonies were fixed with methanol and finally, stained with crystal violet 0.1%. The plates with colonies were leaved to dry in normal air at room temperature (20ºC), the colonies containing more than 50 cells were counted and their survival calculated as a percentage relative to control treatment.

2.5. MTT viability assay

Cell viability was evaluated with the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay which is based on the formation of a visible dark blue formazan product generated by the action of cellular mitochondrial dehydrogenase as a marker for living cells [23]. For 24h of treatments, 1 x 10^4 cells/mL (MRC5 and HepG2) were seeded in 96-well; for 96h of treatments, the concentration plated for MRC5 was 5 x 10^3 cells/mL in 24-well plate while the concentration used for HepG2 was 1 x 10^4 cell/mL in 24-well plate. After cell attachment, culture medium was replaced by C, h-Tf and $FeSO_4$ treatments. After incubation, the media were removed, the cells were washed once with PBS, the MTT solution (5 mg/ml) was added and the culture was incubated for further 3h. Culture medium was discarded and was replaced with 200 µmol/L of dimethyl sulphoxide (DMSO). Absorbance at 540 nm was measured by ELISA Reader.

2.6. Population Doubling Time (PDT)

The population doubling time (PDT) refers to the total number of times the cells in the population have doubled *in vitro* [24]. This is usually a very crude estimate rounded off to the nearest whole number, being based on the following formula: n = 3.32 (log UCY - log I) + X, where n = the final PDT number at end of a given subculture, UCY = the cell yield at that point, I = the cell number used as inoculum to begin that subculture, and X = the doubling level of the inoculum used to initiate the subculture being quantified. Firstly, 1 x 10^4 cells/mL (MRC5 and HepG2) were plated in 6-well culture plates and after 24h, the iron treatments (h-Tf, $FeSO_4$) and the C were added and

the cells were leaved in culture during 6 days. The cells were trypsinized and counted using a Neubauer chamber and the PDT was calculated using the log formula.

2.7. Determination of ROS levels by flow cytometry analysis

ROS accumulation in cells was assessed using the probe 2,7-dichlorofluorescein (DCF). The membrane-permeable diacetate form of the dye (reduced DCF (DCFH-diacetate)) was added to the perfusate at a final concentration of 10 µmol/L. Within the cell, esterases cleave the acetate groups on DCFH-diacetate, thus trapping the reduced probe (DCFH) intracellularly [25]. ROS in the cells oxidize DCFH, yielding the fluorescent product DCF [26]. Briefly, for 24h of treatments, 1 x 10^4 cells/mL (MRC5 and HepG2) were plated in 24-well culture plates; for 96h of treatments, the concentration plated for MRC5 was 5 x 10^3 cells/mL in 24-well plate while the concentration used for HepG2 was 1 x 10^4 cell/mL in 24-well plate. After, growing overnight, the cells were treated with Iron supplemented media (h-Tf and $FeSO_4$) and C and leave in culture for the acute or chronic period of test. Then, the cells were washed twice with PBS and incubated with 10 µmol DCFH-DA in PBS at 37°C for 30 min, protected from light. After incubation, cells were washed with PBS, harvested with trypsin/EDTA and evaluated by flow cytometry using a GUAVA flow cytometer and GUAVA Cytosoft (Millipore, Billerica, MA). Ten thousand cells were measured for each experimental condition. Relative ROS production was expressed as the change in fluorescence of experimental groups compared with that of the appropriate control (100%).

2.8. Catalase-activity (Cat activity)

For MRC5 cells, the iron treatments (h-Tf and $FeSO_4$) and C were tested for Cat activity where the H_2O_2 degradation ability is determined. The assay was performed according to the method described by Aebi [27]. Its principle is based on the determination of H_2O_2 decomposition rate at 240nm. This reaction was conducted at constant temperature (30^{o}C) for 1 min. One unit of catalase decomposed 1 µmol of H_2O_2 per mg of protein in 1 min at pH 7.0.

2.9. Alkaline Comet Assay

MRC5 and HepG2 cells were evaluated by the exposure to the iron treatments (h-Tf and $FESO_4$) and C. Briefly, for 24h of treatments, 1 x 10^4 cells/mL (MRC5 and HepG2) were plated in 24-well culture plates; for 96h of treatments, the concentration plated for MRC5 was 5 x 10^3 cells/mL in 24-well plate while the concentration used for HepG2 was 1 x 10^4 cell/mL in 24-well plate. The alkaline comet assay used in this study followed the general guidelines proposed by Singh [28] with some modifications. Every step was carried out under indirect light and the slides

were coded and analyzed without knowledge of the sample identity. Volumes of 20 µL of treated and non-treated cells were added to 80 µL of 1% low melting point agarose, at 37°C and the mixtures were layered onto slides pre-coated with 1.5% normal agarose, covered with a coverslip, and left for 15 min at 4 °C to solidify. The coverslips were then removed carefully and the slides were immersed in a lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris [pH 10], 1% N-lauroylsarcosine sodium, 1% Triton X-100 and 10% DMSO) for 1 h. The alkaline comet assay was performed at pH 13. The positive control used was hydrogen peroxide (H_2O_2) (200 µmol/L), 1 hour.

Additionally, to verify if iron 24h pre-treatment with h-Tf, $FeSO_4$ and C followed by mutagens exposure H_2O_2 in a concentration of 150 µmol/L for 1h or to Methyl Methanesulfonate (MMS), in the concentration of 80 µmol/L for 1h) induced oxidative DNA lesions in the MRC5 cells, the modified comet assay was performed with lesion-specific repair enzymes. Formamido pyrimidine glycosylase (FPG) was used for detection of oxidized purines while Endo III, for detection of oxidized pyrimidine. These enzymes recognize and introduce breaks at sites of oxidative damage and therefore can detect oxidative lesions in the DNA [29]. The test was done in the same manner as described in alkaline comet and another step was added: after lysis the slides were washed three times in enzyme buffer (40 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 100 mM KCl, 0.5 mM EDTA, 0.2 mg/ml bovine serum albumin, pH 8.0), drained and incubated at 37°C in this buffer with 60 µl of FPG (1µg/ml solution) for 30 min and EndoIII (1 µg/ml solution) for 45 min and then electrophoresis was performed, as described above. The same staining and scoring procedures were done.

Cells were visually scored according to tail length into five classes: (1) class 0: undamaged, without a tail; (2) class 1: with a tail shorter than the diameter of the head (nucleus); (3) class 2: with a tail length 1–2x the diameter of the head; (4) class 3: with a tail longer than 2x the diameter of the head and (5) class 4: comets with no heads. A value (damage index) was assigned to each comet according to its class. Damage index ranged from 0 (completely undamaged: 100 cells x 0) to 400 (with maximum damage: 100 cells x 4). The damage frequency (%) was calculated based on the number of tailed versus tailless cells [30].

2.10. Western Blotting Analysis

MRC5 cell line where tested, in the concentration of 1 x 10^4 cells/mL in 24-well plate, in order to verify if iron 24h pre-treatment with h-Tf, $FeSO_4$ and C followed by mutagens exposure (H_2O_2 in a concentration of 150 µmol/L for 1h or MMS, in the concentration of 80 µmol/L for 1h) induced the expression of DNA repair proteins of interest or iron receptor proteins selected for this study. Cells were washed with PBS and collected from plates with RIPA buffer (Radio Immuno

Precipitation Assay buffer) containing 0.1% SDS (Sodium dodecyl sulphate), 150mM Sodium chloride, 1% Triton X-100, 0.5% Sodium deoxycholate, 50mMTris, pH 8.0. Proteins were quantified using the BCA assay (Pierce, Rockford, IL). Equal amounts of total protein (40 µg) were resolved in a SDS–PAGE and transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ). After the electrophoretic transfer, the membrane was blocked in 5% skim milk powder with 0.1% Tween 20. The blots were then probed at 4°C overnight with the primary antibody for: TFIIH (1:100); MutyH (1:200); CD71 (1:200); Nramp2 (1:100), all purchased from Santa Cruz Biotechnology, INC. Secondary antibodies were goat anti-mouse IgG-HRP (1:5000, Santa Cruz, CA). Optical density of the bands was obtained using Bio-Rad software (Quantity One; Hercules, CA). The data is presented as ratio of samples to ß-actin.

2.11. Cytokinesis-Blocked Micronucleus (CBMN) Assay

The CBMN Assay was performed according to Thomas and Fenech [31], with minor modifications. MRC5 cells (3 x 10^5 cells/mL) in logarithmic growth phase were seeded in 6-well plates. In this assay, only h-Tf treatment was compared to the C for 24h and 96h. Cultures were then washed twice with medium, and Cytochalasin-B (Cyt-B) was added to a final concentration of 3 µg/mL. Cultures were harvested 21h after Cyt-B addition. Cells were separated from the bottle by trypsinization, and the cell suspension was centrifuged at 1000 x g for 5 min. Cells were resuspended in 75 mmolKCl, and maintained at 4 °C for 3 min (mild hypotonic treatment). They were then centrifuged, and a methanol/acetic acid (3:1) solution was slowly added. This fixation step was repeated twice, and cells were finally resuspended in a small volume of methanol/acetic acid, dropped on clean slides, and stained with 3% Giemsa (pH 6.8) for 5 min. Slides were mounted, and codified prior to microscopic analysis.

For h-Tf treatment and C, a total of 1000 cells were scored and classified to determine the ratios of mononucleate, binucleate (BN), multinucleate, apoptotic and necrotic cells. These ratios were used to determine the replication index (RI), a biomarker of cytostatic effect, and cytotoxicity events were assessed by the frequency of necrotic and apoptotic cells. A total of 1000 BN cells were scored for genome-damage indices - Micronucleus (MNi), Nucleoplasmatic Bridges (NPBs) and Nuclear Buds (NBuds). Scoring criteria for the CBMN assay were followed as previously described [32].

2.11. Statistical analysis

Statistical analysis was done, when applicable, using the one-way analysis of variance followed by Dunnett's t test. T test was used for comparison between two groups. Results with *P* <0.05 were considered significant. All statistical analysis was performed using Graphpad Prism 5 (GraphPad Inc, San Diego, CA). The parameters were expressed as mean ± standard deviation (sd).

3. Results

3.1. Intracellular iron levels quantification

The intracellular iron levels after exposure to the treatments were evaluated in order to verify if the cells were really uptaken the extra iron added to the medium. As an initial screening, this assay was performed only in MRC5 cells. In **Figure 1** is demonstrated that both treatments were able to significantly increase the intracellular iron levels in relation to the C, in 24h and 96h. The cells exposed to the iron treatments by chronic period (**Fig. 1B**) obtained more iron from the media when compared to acute treatment cells (**Fig. 1A**). No significant difference between hTf and $FeSO_4$ treatment was observed. The text was not performed with HepG2 because it was an initial screening.

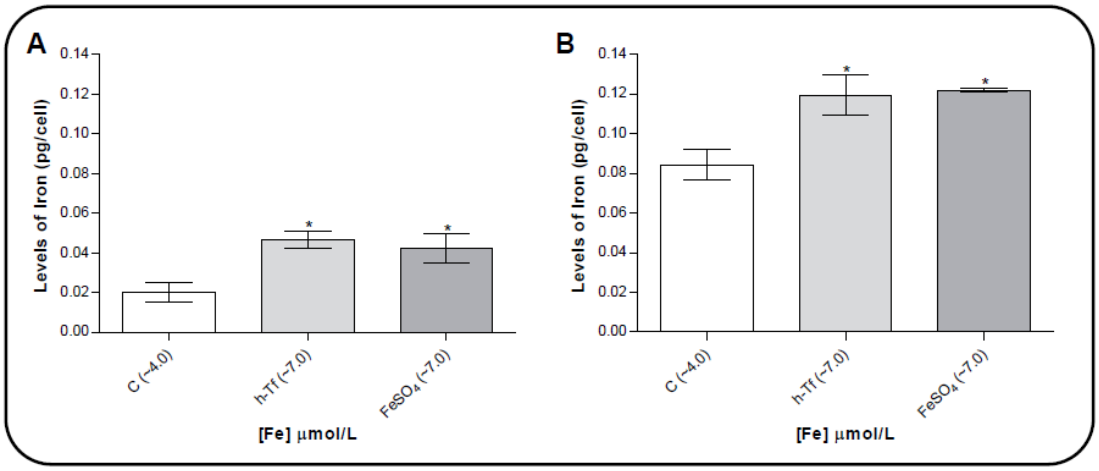

Figure 1. Levels of intracellular iron in MRC5 cells after the treatment with h-Tf or $FeSO_4$. In (A) 24h of treatment; in (B) for 96h of treatment; *Statistical significant difference in comparison to control, Student *t* test, $P < 0.05$.

3.2. Effect of Iron supplementation on cell proliferation and viability

The results of the clonogenic assay with MRC5 cell are presented in **Figure 2**. We observed that the viability was improved for both supplementation proposed – h-Tf or$FeSO_4$ - for acute treatment (24h) and for the chronic period of time evaluated, in this case, 10 days ($P < 0.05$). The assay was not performed with HepG2 due to its growing pattern. No significant difference between hTf and $FeSO_4$ treatment was observed.

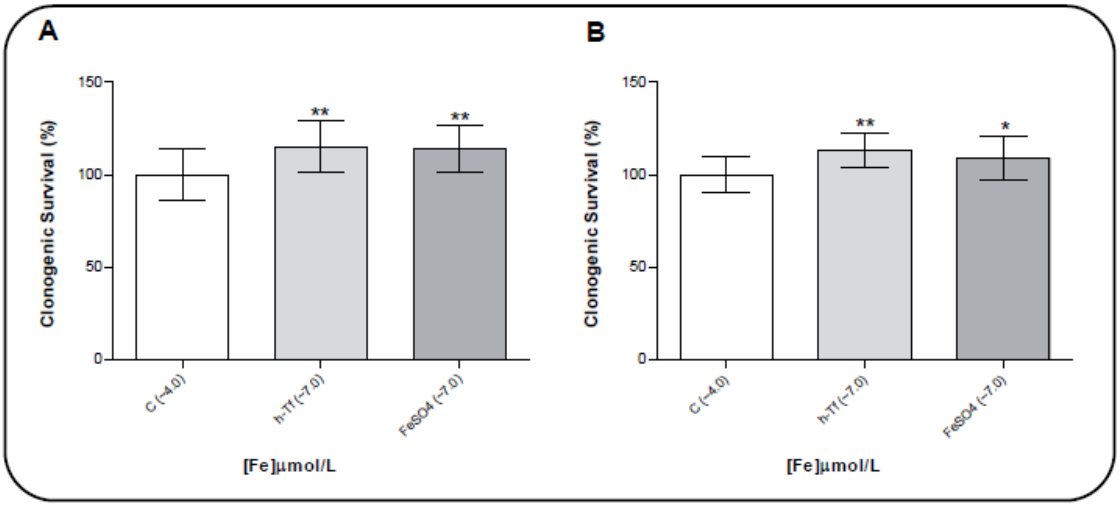

Figure 2. Clonogenic assay for MRC5 cell line exposed to different iron treatments (h-Tf and $FeSO_4$) in (A) the acute treatment (24h) and in (B) the chronic treatment (10 days); *Statistical significant difference in comparison to control, Student *t* test, $P < 0.05$; **$P < 0.01$.

To verify the proliferation and viability of both cell lines, the MTT assay was applied (**Fig. 3** and **4**). While after 24h of treatment we did not observe alterations in cell viability, both supplementations, h-Tf or $FeSO_4$, increased the cell viability after96h of exposure. Besides this, to confirm that the cells were growing and proliferating normally in the media supplemented with iron, the PDT assay was verified. The **Figure 5** showed that proliferative rate of both MRC5 and HepG2 were not altered by iron treatments and that the doubling times remain similar to C - around 27h for MRC5 and 48h for HepG2. No significant difference between hTf and $FeSO_4$ treatment was observed.

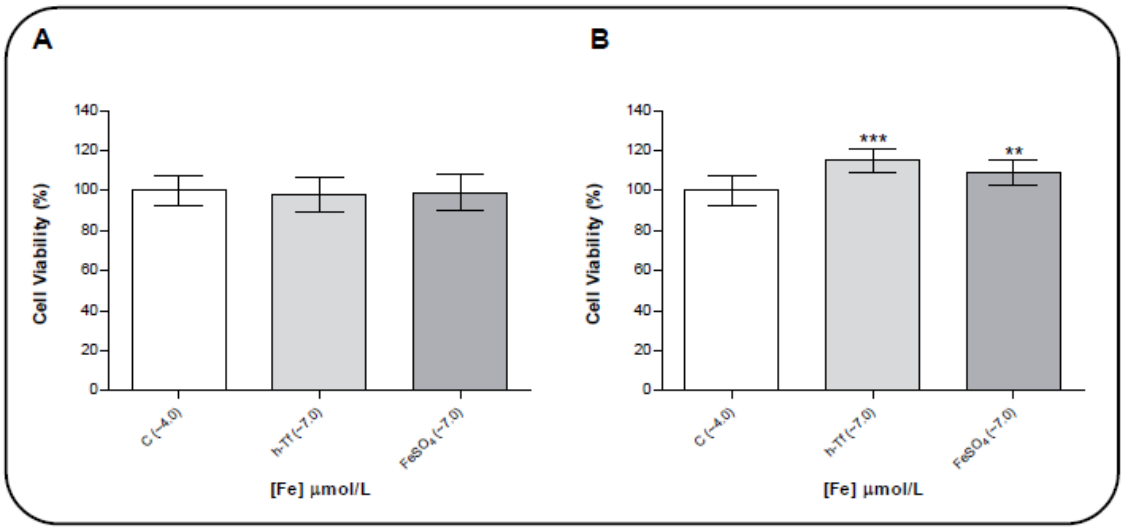

Figure 3. Cell viability evaluation by MTT assay for MRC5. In (A) the acute treatment (24h) and in (B) the chronic treatment (96h); **Statistical significant difference in comparison to control, Student *t* test, $P < 0.01$; ***$P < 0.001$.

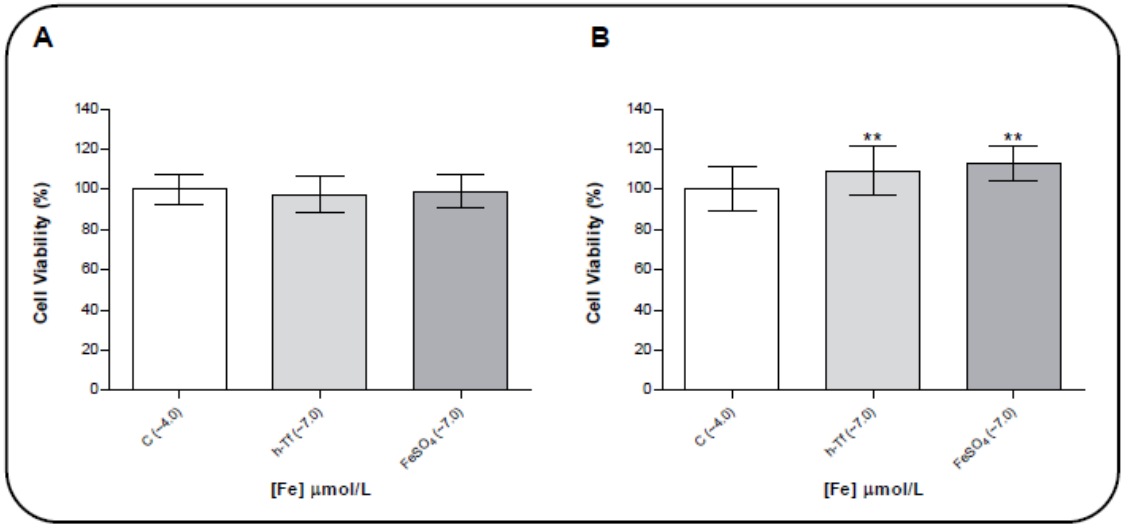

Figure 4. Cell viability evaluation by MTT assay for HepG2. In (A) the acute treatment (24h) and in (B) the chronic treatment (96h); **Statistical significant difference in comparison to control, Student *t* test, $P < 0.01$.

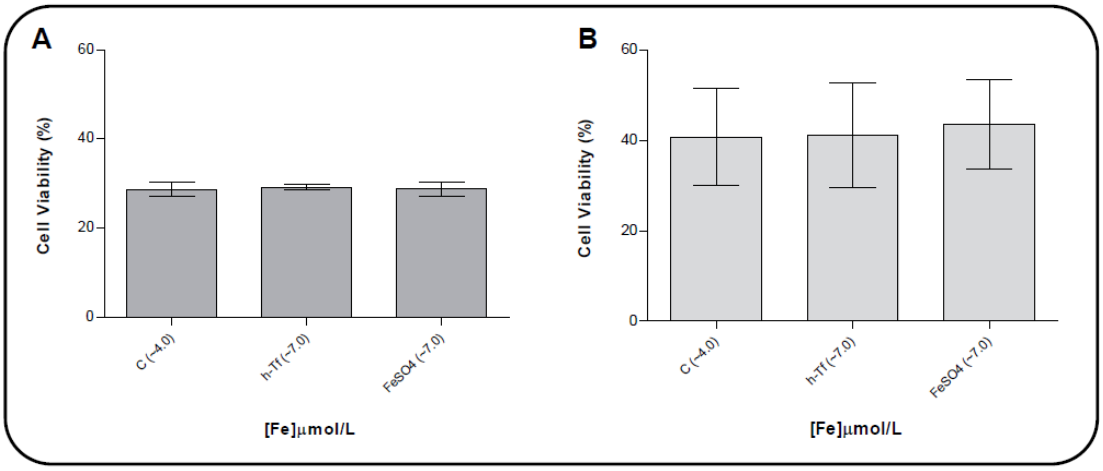

Figure 5. Cell viability evaluation by PDT assay (6 days of culture); in (A) MRC5 and in (B) HepG2.

3.3. Effect of Iron on ROS accumulation and Catalase Activity

The ROS accumulation assay showed that, exposure to iron treatments evaluated, in 24h or 96h, induced an increase in the free radicals formation in MRC5 and HepG2 cells (**Fig. 6** and **7)**. For MRC5, even though h-Tf presented a clear tendency of increase in the ROS accumulation, only $FeSO_4$ treatment showed statistic differences when compared to the C, for MRC5 cell line ($P < 0.01$) (**Fig. 6**). Interestingly, from 24h to 96h, it is possible to observe that the ROS levels were decreased, most accentuated for $FeSO_4$ treatment, in both cell lines profiles.

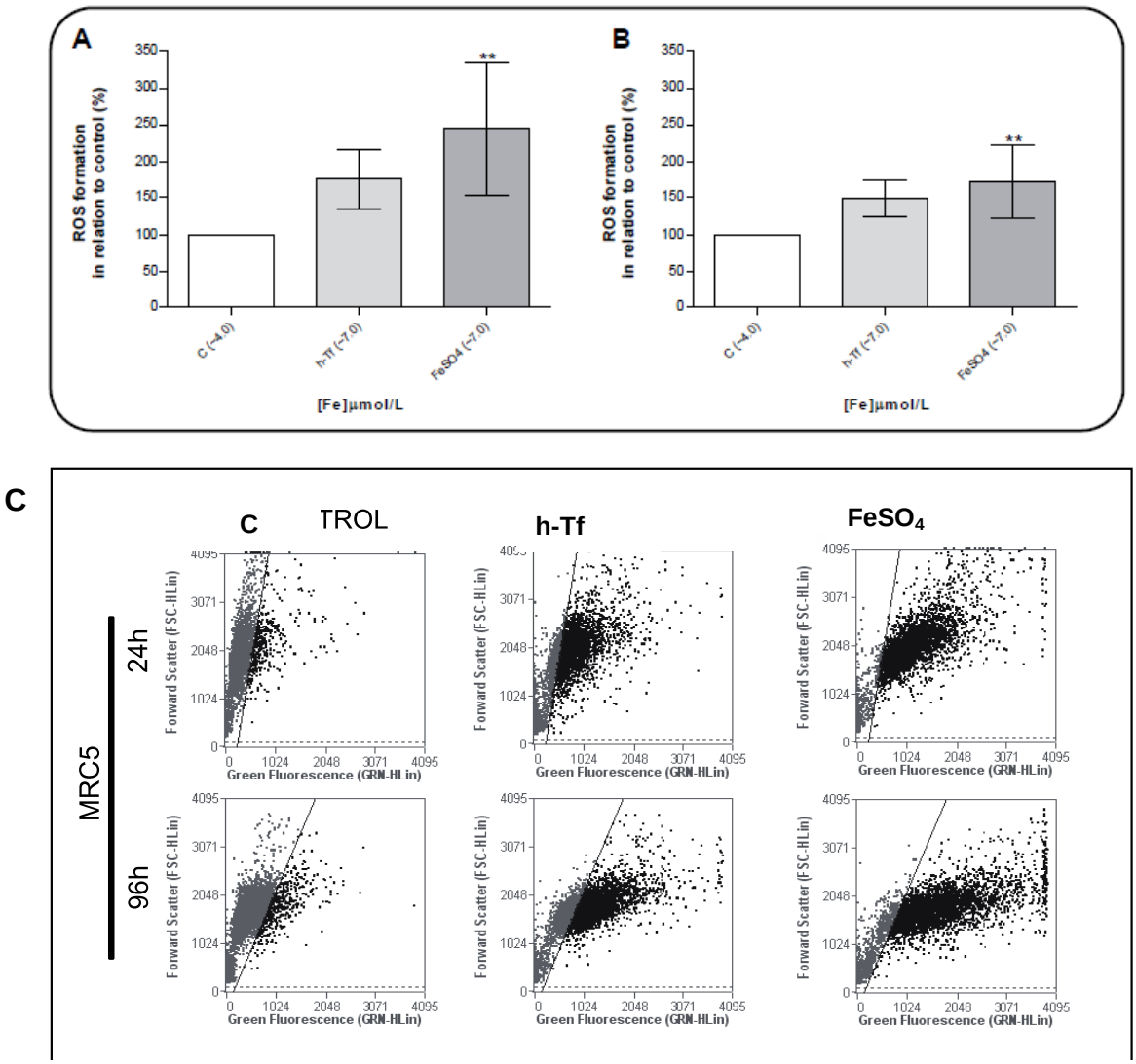

Figure 6. Evaluation of ROS formation in MRC5 cell line treated with h-Tf and $FeSo_4$. In **(A)** 24h experiments; In **(B)** 96h experiments; In **(C)** are shown representative plots from flow cytometry, as indicated; **Statistical significant difference in comparison to control, Student *t* test, $P < 0.01$.

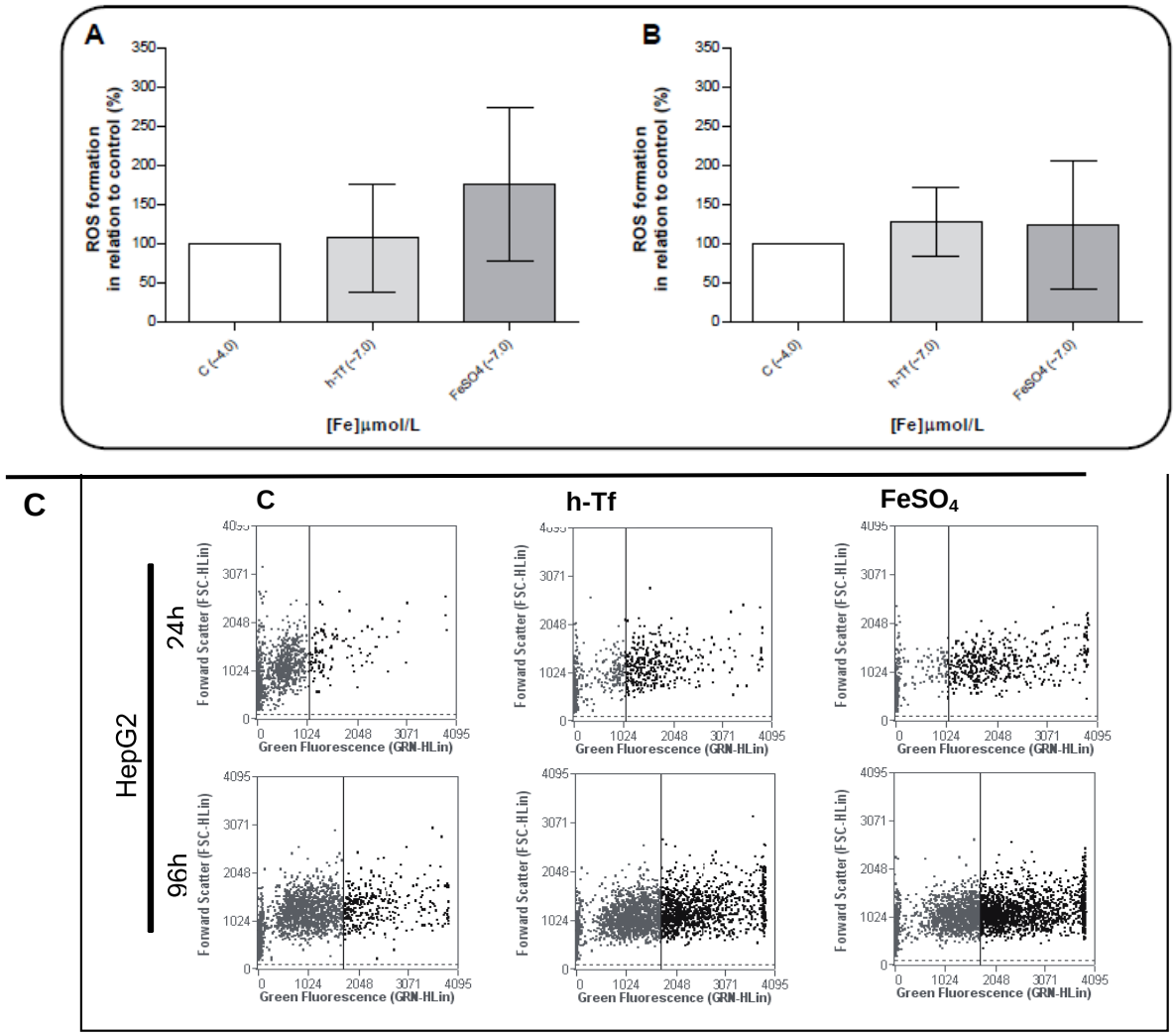

Figure 7. Evaluation of ROS formation in HepG2 cell line treated with h-Tf and $FeSO_4$. In **(A)** 24h experiments; In **(B)** 96h experiments; In **(C)** are shown representative plots from flow cytometry, as indicated.

Then, the catalase activity was verified in order to evaluate if the ROS formation in MRC5 cells, after Fe exposure, might influence the cell antioxidant profile. In addition, Fe is known to be a co-factor of this enzyme [1]. In the **Figure 8** cells treated with h-Tf or $FeSO_4$ by 24h showed no difference in the catalase activity when compared to the C. Once detoxificaying enzymes are required almost immediately after the increasing of ROS, the catalase activity in MRC5 was measured only after 24h of treatments.

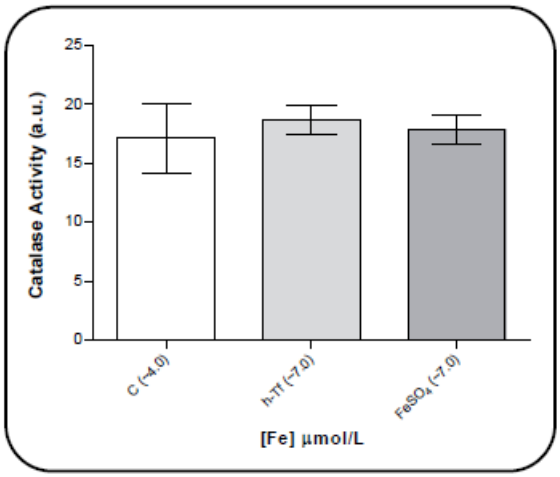

Figure 8. Catalase activity in MRC5 cell line, for 24h treatment with h-Tf or $FeSO_4$.

3.4. Effect of iron on genomic stability measured by Comet Assay

To evaluate the DNA damage potential of Fe treatments, the alkaline comet assay was performed in MRC5 and HepG2 cell lines. As showed in **Figures 9** and **10**, there are no significant differences in the damage index (DI) and frequency index (FI) between any of the iron treatments (h-Tf or $FeSO_4$) and C for 24h and 96h. On the other hand, when cells were pre-treated with Fe and after exposed to genotoxic agents (H_2O_2 and MMS), no protective effects on DNA damage index was demonstrated (**Fig. 11A**). In the presence of H_2O_2, h-Tf pre-treatment seems to increase the damage caused by the H_2O_2, when compared to the C, while $FeSO_4$ did not increase the damage index already observed for H_2O_2 alone. However, neither h-Tf nor $FeSo_4$ pre-treatments increased the DNA damage induced by the alkylating agent (MMS).

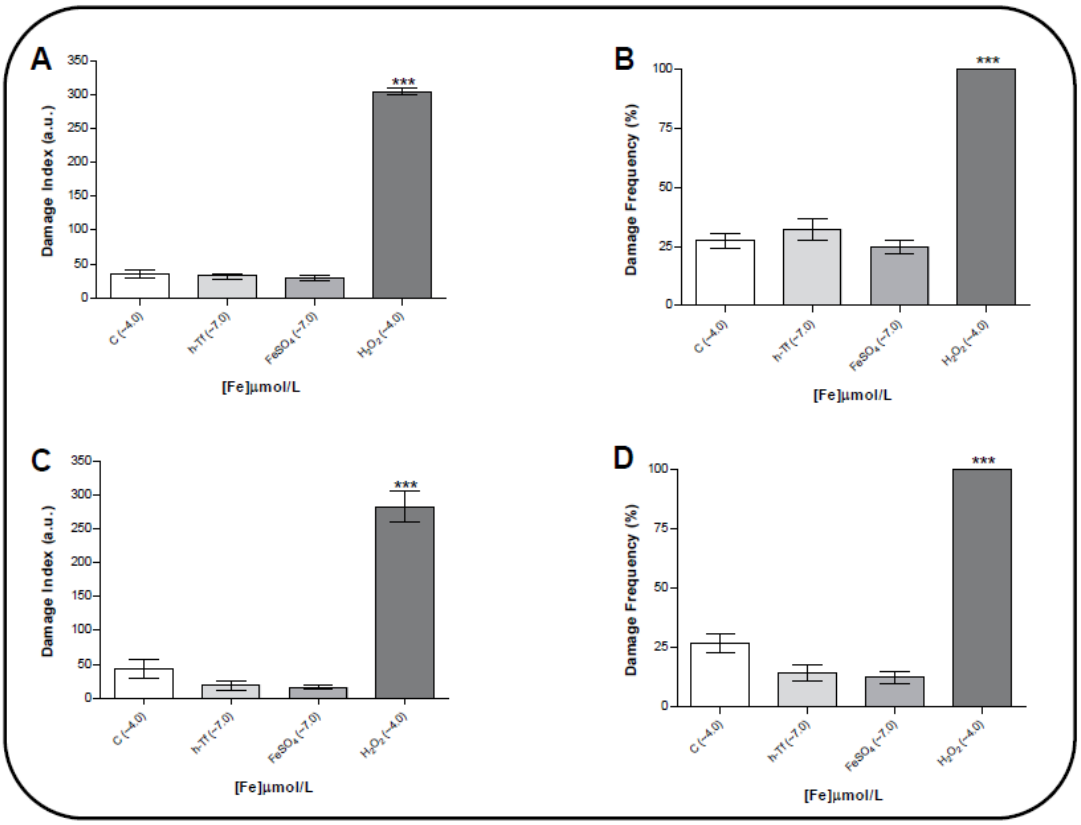

Figure 9. DNA damage evaluation by Comet assay in MRC5 cell line treated with h-Tf and $FeSO_4$. (A) Damage Index and (B) Damage Frequency for 24h; (C) Damage Index and (D) Damage Frequency for 96h; ***Statistical significant difference in comparison to positive control (H_2O_2), ANOVA Dunnett Multiple Comparison Test, $P < 0.001$.

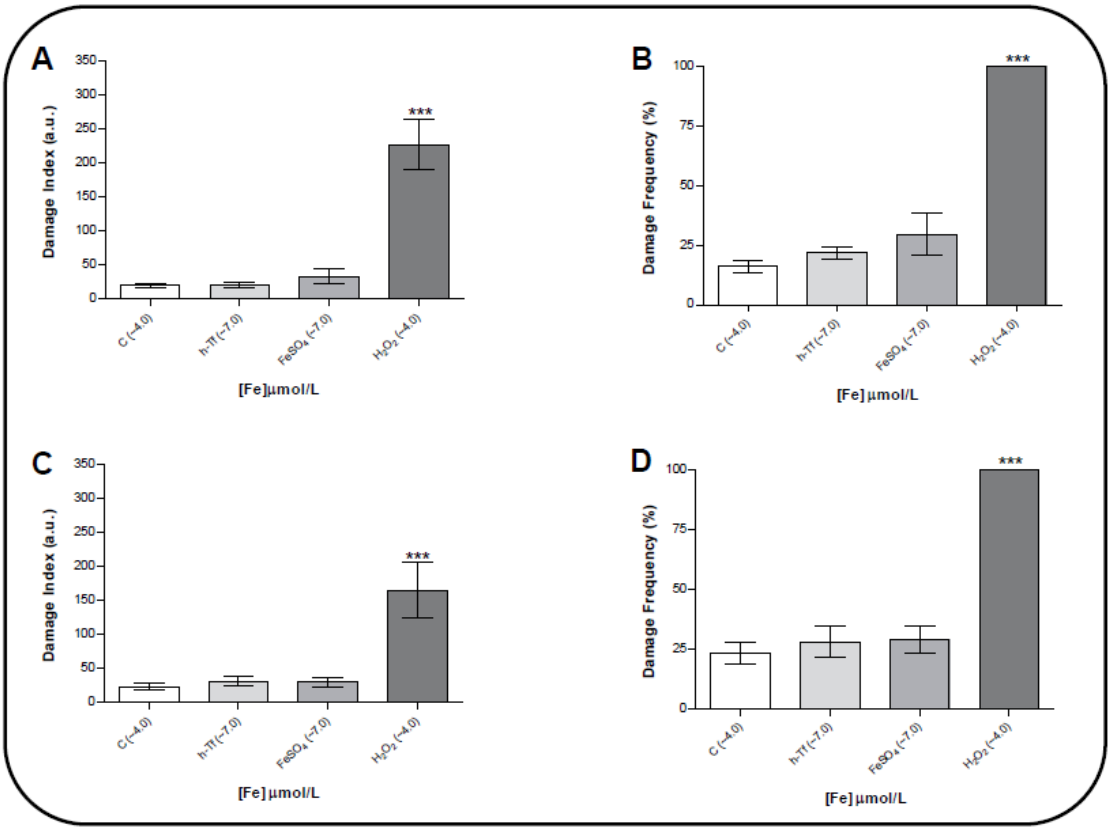

Figure 10. DNA damage evaluation by Comet assay in HepG2 cell line treated with h-Tf and $FeSO_4$. (A) Damage Index and (B) Damage Frequency for 24h; (C) Damage Index and (D) Damage Frequency for 96h. ***Statistical significant difference in comparison to positive control (H_2O_2), ANOVA Dunnett Multiple Comparison Test, $P < 0.001$.

In addition to the alkaline comet assay, the modified enzyme comet assay was performed in order to evaluate if iron supplementation cause oxidative DNA damage. Then, slides of the same groups used in the alkaline comet assay were incubated with lesion-specific enzymes and the difference of DNA breaks formation was evaluated. As illustrated in **Figure 11B**, grey bars, all groups showed an increase in the DI when ENDO III, that detects oxidized pyrimidine, was used, but only H_2O_2 demonstrated a significant effect in comparison to the C (**P< 0.01). When FPG was used (**Fig. 11C**, grey bars), both positive controls H_2O_2 and MMS showed and increase in DI. These results can be related to FPG properties of oxidized purines and also alkylated bases. Besides this, Fe pre-treatments seem to decrease the DNA damage induced by H_2O_2 when ENDO III or FPG enzymes were used.

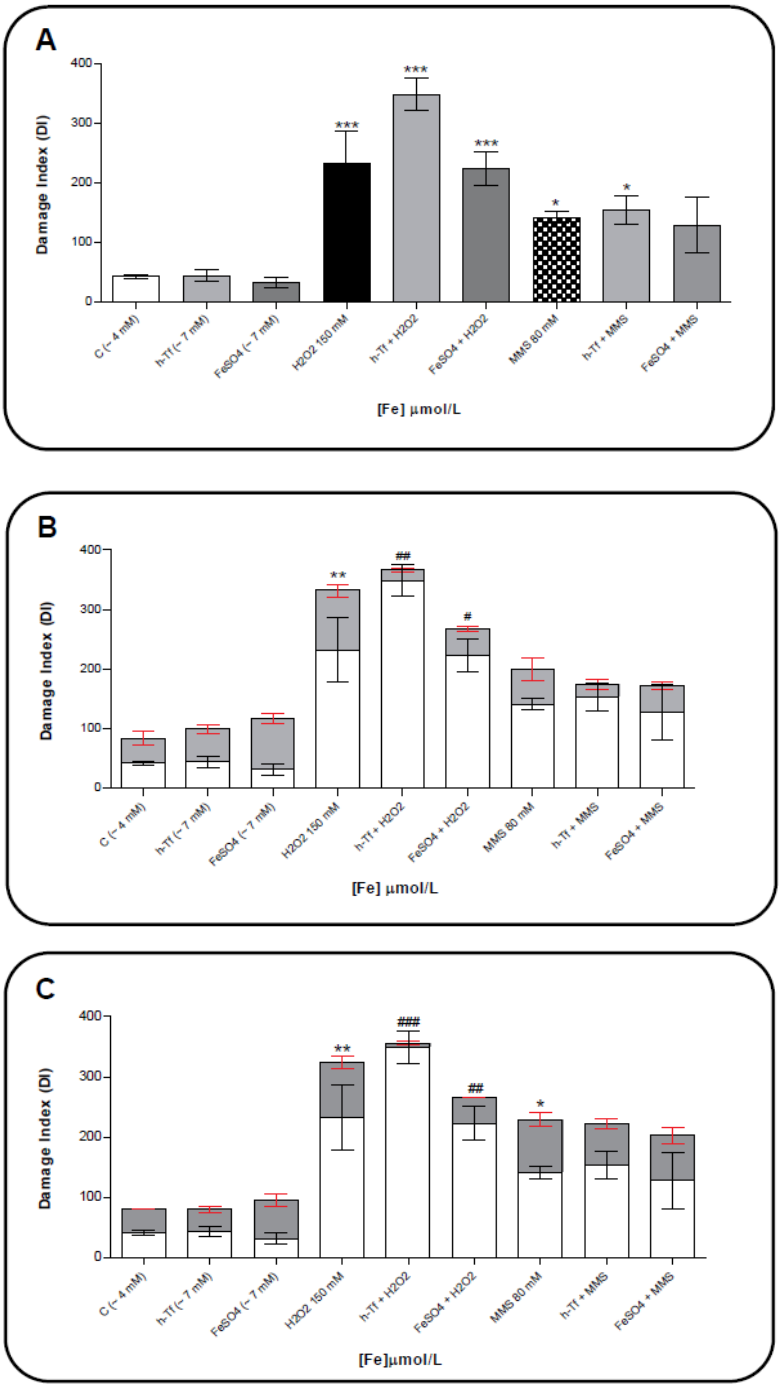

Figure 11. DNA damage evaluation by alkaline comet assay in MRC5 treated with h-Tf and $FeSO_4$. In (A), Damage index without enzyme. In (B), with Endo III enzyme and in (C), with FPG enzyme. Grey bars illustrate the additional DNA damage after enzyme exposure. *Statistical significant difference in comparison to control (C), ANOVA Dunnett Multiple Comparison Test (*P*< 0.05, ***P*< 0.01, ****P*< 0.001). $^{\#}$Statistical significant difference in comparison to positive control (H_2O_2), ANOVA Dunnett Multiple Comparison Test (*P*< 0.05, $^{\#\#}$*P*< 0.01, $^{\#\#\#}$*P*< 0.001).

3.5. Effect of iron on DNA damage repair proteins and iron receptors protein expression by Western Blotting assay

Western blotting was performed in MRC5 cells to evaluate if h-Tf and $FeSO_4$ 24h pre-treatments could influence the expression of TFIIH and MutyH, DNA repair proteins which present a Fe/S cluster in their structure, and CD71 and Nramp2, cellular absorption proteins for iron.

As illustrated in **Figure 12** (upper panel), all treatment groups showed similar TFIIH expression levels with exception for h-Tf pre-treatment followed by H_2O_2 or MMS exposure. For MutyH, all groups presented an increase in the expression of the protein (**Fig. 12**, lower panel). The quantification results presented for DNA repair proteins were the mean ± sd from two replicates (the figures are representative results).

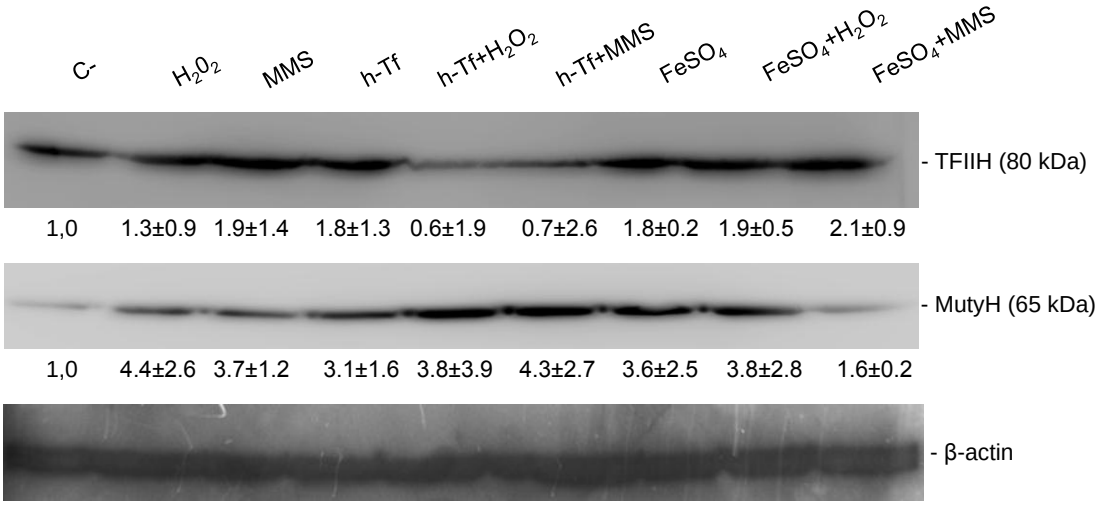

Figure 12. Expression of DNA repair proteins in MRC5 cells pre-treated with h-Tf and $FeSO_4$, for 24h. TFIIH (upper panel) and MutyH (second panel) were assessed by immunoblotting with actin (last panel) serving as internal control for equal loading. Optical density of the bands was obtained using Bio-Rad software.

The expression levels of CD71, h-Tf receptor, were demonstrated in **Figure 13** (upper panel), where all groups showed increased expression levels, being more pronounced in the presence of H_2O_2. For Nramp2, the divalent metal receptor, all groups presented a less pronounced increase in the expression of the protein (**Fig. 13**, lower panel). The results for iron proteins receptors are preliminary and require more replicates.

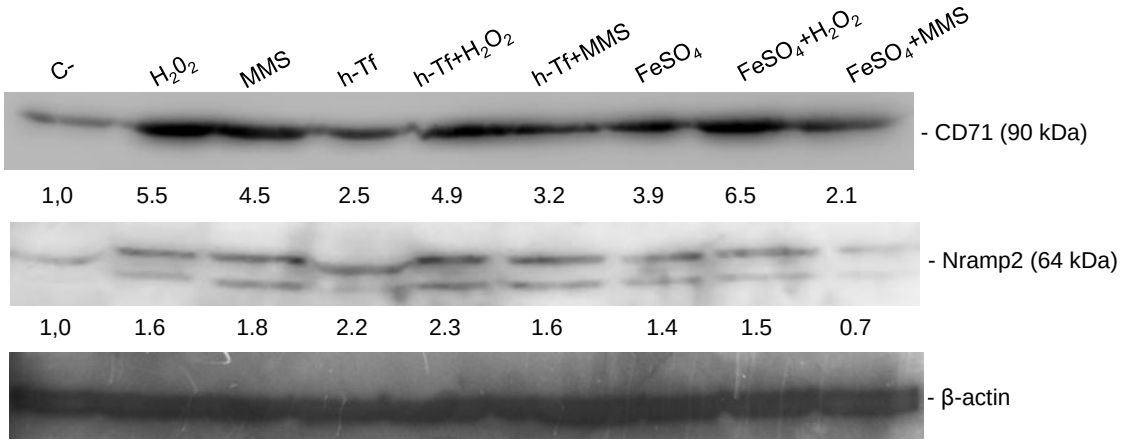

Figure 13. Expression of DNA iron receptor proteins in MRC5 cells pre-treated with h-Tf and $FeSO_4$, for 24h. CD71 (upper pannel) and Nramp2 (second pannel) were assessed by immunoblotting with actin (last pannel) serving as internal control for equal loading. Optical density of the bands was obtained using Bio-Rad software.

3.6. Effect of iron from h-Tf on genomic stability measured by CBMN assay

In order to further verify the effect of iron on baseline levels of cytotoxicity and chromosome damage, the CBMN assay was applied in the MRC5 cell line treated with h-Tf for 24h and 96h. As demonstrated in **Table 1**, the replication index is similar between the h-Tf treatment and C, demonstrating that the iron released by h-Tf did not cause cytostatic effect in MRC5 in both periods of exposure. Furthermore, no differences were observed regarding h-Tf treatment and C in the percentages of necrotic and apoptotic cells after 24h or 96h of treatment. MNi, NPBs and NBuds in binucleated cells were scored to measure chromosome damage and the results showed also no differences between h-Tf and control (**Tab. 1**).

Table 1. DNA damage evaluation by Micronucleus Assay and Cytome evaluation for MRC5 cells exposed to h-Tf for 24h and 96h.

	24h			**96h**		
	C	h-Tf	*p* value	C	h-Tf	*p* value
Replication index (‰)	100 ± 6.1	98.8 ± 5.0	0.4429	100 ± 14.8	94 ± 15.1	0.2209
Nuclear bridges (‰)	5.8 ± 3.7	5.2 ± 4.2	0.5000	1.4 ± 1.5	1.9 ± 2.2	0.3103
Nuclear buds (‰)	2.2 ± 1.7	1.4 ± 2.8	0.1714	0.9 ± 0.78	1.0 ± 0.45	0.3249
Micronucleus (‰)	0.7 ± 1.5	0.7 ± 0.8	0.1714	0.7 ± 0.81	0.4 ± 0.70	0.2008
Necrotic cells (%)	0.2 ± 0.0	0.0 ± 0.0	0.0908	0.3 ± 0.26	0.6 ± 0.57	0.2898
Apoptotic cells (%)	0.2 ± 0.28	0.1 ± 0.14	0.5000	0.2 ± 0.16	0.3 ± 0.38	0.3400

4. Discussion

Micronutrients, including iron, have been reported as key elements in cellular processes like proliferation, survival and even differentiation of cell cultures [33-36]. Besides this, it is well recognized that minerals and vitamins are indispensable to DNA metabolic pathways [37, 38], and in the case of Fe, either an excess of or a deficiency may cause DNA damage [39]. Here, we reported the influence of different sources of Fe supplementation on cell viability, oxidative stress, genomic stability, expression of DNA repair protein, which contains Fe/S cluster in their structure, and finally, iron receptor proteins expression.

In this study, we observed that the iron supplements evaluated, h-Tf and $FeSO_4$, were being uptaken by the MRC5 cell line almost in the same rate, as demonstrated in the intracellular iron quantification. Besides, both iron treatments were able to improve cell viability and proliferation, in MRC5 and HepG2 cell lines, in around 10-20%, without interfering with the doubling time. These PDT results are in agreement with literature [36, 37].]. In line, there is evidence that iron deprivation reduces cell proliferation and DNA synthesis by inhibiting rebonucleotide reductase, an iron-containing enzyme [40].

Previous findings suggested that Fe has a role in ROS formation due to its participation in Fenton reaction [41], and evidences indicate that oxidative stress resulting from an increased Fe levels, possibly in association with defects in antioxidant defense mechanisms, could cause cell death in some degenerative diseases and could be related to some types of cancer [42]. Interestingly, both Fe supplementations, h-Tf (Fe^{3+}) and $FeSO_4$ (Fe^{2+}), increased the ROS levels in the cells evaluated (**Fig. 6** and **Figure 7**), however, without compromising the cell proliferation, and viability, as already demonstrated. Besides both Fe supplementations had increased the ROS accumulation, only the inorganic form significantly increased it in MRC5 cells.

In order to understand if there was any adaptive mechanism taken place when cells are exposed to basal levels of oxidants, catalase activity was evaluated in MRC5 cells. Fe is present as a co-factor in several enzymes and proteins, including catalase, which has a heme moiety [43, 44]. Besides the subtle decrease in the ROS accumulation in MRC5 cells, from 24h to 96h of exposure, no difference in the catalase activity was observed (**Fig. 8**).

In addition, the damage index (DI) and damage frequency (DF) of the h-Tf and $FeSO_4$ supplementation were evaluated by the alkaline comet assay. The results (**Fig. 9** and **10**) suggested no genotoxicity of Fe at the treated forms and concentration evaluated. However, when MRC5 cell were pre-treated with h-Tf and then followed by H_2O_2, the DI was significantly increased

(**Fig. 11A**). For $FeSO_4$, the DI was similar to H_2O_2 (**Fig. 11A**). A possible explanation for this result could be related to the LIP [45, 46] as well as the kinetics of iron release from transferrin bound to the TfR at endosome [47]. The main form of Fe in LIP is, on average Fe^{+2} state because of the reductive environment of the cell, although transient Fe^{+3} is expected as a result of cellular oxidations [48].

Moreover, iron bounded to h-Tf (Fe^{3+}) has to be reduced to Fe^{2+} before being used for the cell. This process of iron reduction could initially delay the release of Fe^{2+} in the cell, but eventually, could maintaining in a basal order the availability of an instable form of iron (Fe^{2+}) inside the cell. In this case, the DNA damage increase observed when cells were pre-treated with h-Tf and further exposed to H_2O_2 might be related to the reaction of H_2O_2 in the presence of a continuous release of Fe^{2+}, possibly leaving Fe^{3+}/ Fe^{2+} available to enter in the nucleus [46]. When H_2O_2 enters the nucleus, it is likely to oxidize Fe^{2+} in the DNA vicinity, producing OH•. Besides this, large quantities of OH• radical could be formed in the nucleus during certain phases of the cell cycle [49, 50]. Then, the DNA damage induced by this radical, as single- and double strand breaks and 8-hydroxydeoguanosine (8-OHdG) [51, 52], could trigger DNA repair. Furthermore, Fe^{2+} seems to be more easily acquired by the mitochondria from the cytosol [45] and also, could be required by a large number of enzymes present in the cytosol which bind Fe^{2+} [46]. This fact suggests that, while high-affinity uptake system for Fe^{2+} predominantly supplies iron for heme and Fe/S cluster synthesis, the uptake system for Fe^{3+} is considerably more restricted and have a compensatory role [45]. Finally, Fe^{2+} from inorganic salt could also, act as a pro-oxidant activating the antioxidants defenses in the first 24h and then, after the iron pre-treatment, when cells are finally exposed to the H_2O_2, the damage is less pronounced.

In the presence of enzymes - ENDO III and FPG (**Fig. 11B** and **11C**, respectively), both iron pre-treatments demonstrated a decrease in the oxidative damage when compared to H_2O_2. These results indicate that, the oxidative damage was not the main cause of the increase rate in damage observed in the h-Tf pre-treatment followed by H_2O_2. In fact, this could be attributed mainly to strand-breaks caused by OH• radical. Moreover, BER DNA-repair enzymes could be activated as a result of ROS accumulation [8] that is likely to have occurred with cells pre-treated with h-Tf and $FeSO_4$, once the DI after the exposure to enzymes (gray bar in **Fig. 11B** and **11C**) was lower than H_2O_2. The most common DNA base modification formed by oxidation is 8-OHdG and if unrepaired, post-replicative mispairing could signalize for BER pathway [53]. FPG protein has been used to assess oxidative DNA base damage because it detects 8-OHdG and other oxidatively damaged purines. Besides, FPG also detects alkylation damage with high sensitivity in the comet assay [53]. In the case of MMS, in the FPG modified comet assay (**Fig. 11C**), the DI profile remains similar to MMS alone while for ENDO III (**Fig. 11B**), a small decrease in the

oxidized pyrimidine was demonstrated. It is well recognized that MMS is an alkylating agent, but a weak oxidative stress inducer [54].

Additionally, we evaluated the expression of the DNA repair proteins that bear Fe/S cluster in their structure [1]. The less pronounced expression of TFIIH expression protein (**Fig. 12**, upper panel), which belongs to NER pathway [4, 5], for the h-Tf pre-treatment followed by H_2O_2 or MMS, could suggest that other repair pathway is upregulated, as BER, or even that, the efficiency of TFIIH is increased in the iron presence (once Fe is a co-factor). Moreover, the increased levels of MutyH expression (**Fig. 12**, second panel) were expected because this enzyme has an important role in mismatch repair and mainly, as a glycosylase in BER, also repairing alkylating damage [6-8]. As demonstrated, the pre-treatment with h-Tf or $FeSO_4$ were not improving the expression of this protein, but could be discussed that, in some way, they were improving the efficiency of the pathway as a whole. Indeed, less oxidative DNA damage was observed in the comet assay with enzymes, for oxidized pyrimidine (**Fig. 11B**) and especially for oxidized purines (**Fig. 11C**).

Analyzing the preliminary data from the iron receptors expression, CD71 (**Fig. 13**, upper panel) and Nramp2 (**Fig. 13**, second panel), it is possible to observe that the expression of both proteins was more pronounced in the presence h-Tf pre-treatment than in the presence of $FeSO_4$. The increase of the CD71 for h-Tf pre-treatment was expected, once this protein is the main iron-bind to Tf receptor (TfR1) in the cell membrane. In addition, the expression increased for Nramp2 in the presence of h-Tf could be explained by the fact that Nramp2 is required to release the Fe^{2+} from the endosome, where the h-Tf-TfR complex is present [55]. Furthermore, it is possible that the protein levels were triggered to the endosome rather than to the plasmatic membrane. In the case of $FeSO_4$, in the presence of H_2O_2, a higher increase in the CD71 expression was observed (**Fig. 13**, upper panel) as well as for H_2O_2 itself. This result could indicate that in cell cultures, H_2O_2 activates iron regulatory protein-1 (IRP1) [56], a central cytoplasmic regulator of cellular iron metabolism, to bind to mRNA iron-responsive elements (IRE) [57] signaling for iron receptor expression improve (TRf1/CD71) [55]. Nramp2, which is a divalent metal receptor [15], showed no increase in expression in the presence of $FeSO_4$, even alone or followed by the mutagenic agents (**Fig. 13**, second panel), however this receptor is also related to iron homeostasis [58] and it was expected to be more pronounced, especially in the presence of $FeSo_4$. Indeed, iron receptor proteins expression preliminary results could suggest that CD71 is priority related with Fe homeostasis while Nramp2 seems to have a secondary role. In agreement, Nramp2 is not strictly controlled at post-transcriptional level by iron concentration, as CD71 is through iron responsive elements [43].

Based in the collection of data presented at this moment, we judge h-Tf to be the best form of iron to be used as a supplementation of extra iron for cells in culture. To further validate this, the CBMN assay was applied to verify the h-Tf non-genotoxic and non-cytotoxicity profile, as the comet assay was previously pointing. In its current basic form the CBMN assay can provide, using simple morphological criteria, the following measures of genotoxicity and cytotoxicity: chromosome breakage, chromosome loss, chromosome rearrangement (nucleoplasmic bridges), gene amplification (nuclear buds), cell division inhibition, necrosis and apoptosis [59]. The micronucleus and cytome results (**Tab. 1**) showed no differences between h-Tf and control in terms of mis-repaired of DNA strand-breaks or any other parameters evaluated, corroborating to our proposal of h-Tf as an ideal form of iron supplementation for cell cultures.

In summary, both iron supplementations were shown to improve cell viability without increasing the genomic instability. Due to its physiological role in iron homeostasis, h-Tf could be the better iron supplier *in vitro*. The pattern of iron absorption, release, use and storage is possibly the key to understand the better form of iron to be used in the cell cultures for improve viability and genomic stability. Most, if not all organisms have developed a tightly regulated system to maintain iron within a physiological range. The cytosolic Fe^{2+} levels must be sufficient to enable cellular function, but low enough to prevent damage caused by highly reactive $OH\bullet$ [46]. Results of this study also suggest that ROS accumulation in h-Tf supplementations was less expressive and iron intracellular absorption was mildly higher, when in comparison to $FeSO_4$. Further studies are still required to improve the current knowledge regarding the metabolism of iron *in vitro* and its role for cells viability and genomic stability. The h-Tf supplementation suggested should also be evaluated in cell cultures that require a continuous iron supply for proliferation and differentiation, such as mesenchymal stem cells.

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3. DISCUSSÃO GERAL

Existem evidências suficientes demonstrando a relação entre o estado nutricional, a concentração de micronutrientes e o desenvolvimento de diversas patologias (FENECH, 2002; FERRAZ, 2010) Determinadas vitaminas e minerais atuam na manutenção da estabilidade genômica, portanto, com potencial ação na prevenção de doenças, incluindo o câncer (FERRAZ, 2010). Contudo, vitaminas e minerais, quando administrados em excesso ou fora de matrizes alimentares, podem favorecer o acúmulo de ERO e influenciar processos de dano ao DNA (FRANKE, 2006).

Especificamente a respeito do micronutriente Fe, é sabido que o mesmo tanto é essencial quanto potencialmente tóxico para os organismos, exigindo que sua homeostase seja mantida de forma bastante orquestrada. Para administrar esse paradoxo, os níveis de aquisição, utilização e estocagem do Fe devem ser finamente regulados de acordo com a sua disponibilidade e necessidade (OGLESBY-SHERROUSE & MURPHY, 2013).

Visto que o Fe e outros micronutrientes influenciam a estabilidade genômica (PAPANIKOLAOU & PANTOPOULOS, 2005), redigiu-se um artigo com o objetivo de revisar o papel de vitaminas e minerais chaves para a estabilidade genômica, dando-se um enfoque mais voltado para o seu papel no desenvolvimento celular. Os dados apresentados no Capítulo I demonstram claramente que tanto a deficiência de micronutrientes como sua sobrecarga podem ter impactos na viabilidade celular e na estabilidade genômica de células em cultura (Tab. 3 e 4). Isto reforça a hipótese de que o desequilíbrio observado nos níveis de micronutrientes usualmente encontrados em meios de cultivo celular (Tab. 2, Capítulo I) frente aos valores fisiológicos humanos pode, de alguma forma, estar gerando vieses nas análises feitas em estudos *in vitro.*

No caso do micronutriente de interesse desta tese, o Fe, além dos dados relatados no Capítulo II, que discorrem sobre os efeitos na viabilidade celular e estabilidade genômica das culturas avaliadas, é interessante destacar o papel ainda controverso a respeito do mesmo no que se refere à proliferação bacteriana. Ao delinearmos os estudos experimentais, muitos ensaios preliminares foram conduzidos, inclusive para se determinar a concentração de Fe a ser usada na suplementação dos meios de cultura. Justamente, ao se preparar os meios de cultura de tratamento com Fe,

verificou-se, em dadas situações, um maior índice de contaminação por bactérias nesses meios, em especial, naqueles que recebiam o sal inorgânico na forma de sulfato ferroso ($FeSO_4$).

Na verdade, o Fe é um micronutriente virtualmente essencial para todo organismo vivo, inclusive os microrganismos. Embora o corpo humano contenha Fe em abundância, a maior parte está ligada a hemoglobina, mioglobina e citocromos e, além disso, não está disponível em uma forma que possa contribuir para o crescimento de microrganismos (JURADO, 1997; ZHANG & ENNS, 2009). A disponibilidade do ferro férrico (Fe^{3+}) é ainda limitada pela Tf, que pela sua alta capacidade de ligação ao Fe plasmático, contribui para a imunidade inata contra diversos microrganismos patogênicos no ambiente hospedeiro (GILES & CZUPRYNSKI, 2004). Interessante observar que, durante o curso de uma infecção, as concentrações de Tf plasmática são aumentadas e a expressão de RT, diminuída, resultando numa rápida redução nas concentrações de Fe^{3+} no plasma (para valores menores que 10^{-15} mol/L) (JURADO, 1997) sendo que este valor está abaixo do limite de Fe^{3+} requerido para suportar a maioria dos crescimentos microbianos (10^{-6} mol/L) (JURADO, 1997). Esse fenômeno tem uma repercussão fisiológica importante, visto que em situações de infecção (proteína C-reativa aumentada) os valores de referência para diagnóstico de anemia, medida pela ferritina sérica, são alterados (HOEN, 1999; SHANTHI *et al.*, 2013).

Nesse sentido, as contaminações indesejadas de algumas de nossas culturas podem, possivelmente, ser explicadas pela afinidade que microrganismos demonstram por ambas formas do micronutriente Fe, mas preferencialmente por Fe na forma ferrosa (Fe^{2+}) (VELAYUDHAN *et al.*, 2000), tanto quanto as células que estavam sendo avaliadas. Corroborando com este fato, verificou-se que as culturas que recebiam a suplementação proveniente de Fe inorgânico, $FeSO_4$, não ligado à Tf, claramente contaminavam com maior facilidade. A saber, o Fe inorgânico não está “preso” e, portanto, é de fácil utilização pelos possíveis microrganismos contaminantes, contrariamente ao Fe ligado a h-Tf, que parece ser a via mais rapidamente absorvida pela célula.

Observando-se os valores de Fe intracelular para as células MRC5, tratadas com h-Tf (Fig. 1, Capítulo II), conjugados a expressão aumentada de CD71 (receptor de h-Tf - glicoproteína de membrana envolvida na absorção celular de Fe via endocitose) verificada

no *western blotting* (Fig. 13, Capítulo II), pode-se inferir que a absorção do Fe^{3+} ligado a Tf parece ser realmente primordial para as células. Contudo, é importante observar que a Tf liga-se preferencialmente a Fe^{3+}, mas também pode ligar-se a Fe^{2+} (KELTER *et al.*, 2007) e, portanto, menos Fe^{2+}/Fe^{3+} permanece disponível no meio. Com base nesse dado, pode-se inferir adicionalmente que o consumo do micronutriente por algum microrganismo oportunista estaria potencialmente minimizado frente a suplementação com h-Tf. Além da expressão de CD71 estar aumentada frente ao pré-tratamento com h-Tf, observou-se um incremento também na expressão de Nramp2 (receptor de íons dimetálicos - proteína de membrana com função transportadora de cátions divalentes, como o Fe^{2+}) (Fig. 13, Capítulo II). Neste sentido, pode-se inferir que não se trata da expressão de Nramp2 presente na membrana celular e sim, possivelmente, da proteína presente no endossomo (ARREDONDO & NUNEZ, 2005), no qual o complexo h-Tf-RTf é encapsulado (Fig. 3, Introdução). Após a redução de Fe^{3+}, para que ocorra a liberação de Fe^{2+} para o citosol é necessário que o mesmo seja transportado através de DMT1 (Nramp2). Sugere-se assim que os mecanismos envolvidos na absorção e utilização de Fe pela célula via Tf sejam mais elegantemente regulados.

Uma vez que o Fe^{2+} proveniente do Fe ligado a h-Tf ou não ligado a Tf (NTBI, non-transferrin binding iron) chega ao citosol, ele permanece temporariamente ligado fracamente a proteínas ou quelantes de baixo peso molecular, antes de ser devidamente estocado na ferritina, consumido em processos metabólicos mitocondriais (SHVARTSMAN & IOAV CABANTCHIK, 2012) ou usado na síntese de hemoproteínas ou outras enzimas que usam Fe como cofator (PRA *et al.*, 2012). Mesmo com um refinado sistema de transporte e estocagem, em torno de 3-5% do conteúdo celular de Fe existe na forma lábil (LIP), sendo citotóxico em função de sua habilidade em catalisar a formação de radicais hidroxila a partir de H_2O_2 pela reação de Fenton (ARREDONDO & NUNEZ, 2005; PRA, 2008). As células mantêm o LIP sob rigoroso controle pela regulação da expressão de ferritina e do RTf (MACKENZIE *et al.*, 2008). Sendo assim, uma expressão aumentada em CD71, frente a uma maior disponibilidade de Fe^{3+} ligado a Tf no meio de cultura sugere que, possivelmente, para a homeostasia celular adequada, uma demanda maior por Fe estava sendo sinalizada intracelularmente visando uma maior absorção do micronutriente.

O tratamento com $FeSO_4$ também demonstrou níveis intracelulares de Fe aumentados quando comparado ao controle (Fig. 1, Capítulo II), mas a expressão do receptor de metais divalentes (Nramp2) curiosamente não foi tão notória para os tratamentos com $FeSO_4$ nestes ensaios preliminares (Fig 13, Capítulo II). É importante destacar que a principal forma de Fe presente no LIP é, em geral, Fe^{+2} devido ao ambiente redutor celular, embora Fe^{+3} transiente seja esperado como um resultado de oxidação celular (ARREDONDO & NUNEZ, 2005). De certa forma, este resultado poderia ser um indicativo de que a célula não aumentou a expressão do receptor de Fe divalente a fim de evitar um aumento substancial no LIP e com isso, evitar toxicidade proveniente de ERO intracelular.

Ainda no que se refere à absorção de Fe intracelular, resultados preliminares (não apresentados) sugerem que semeaduras celulares iniciais distintas, para um mesmo tempo de exposição aos tratamentos de Fe, resultam em valores de Fe intracelular maiores para um menor número de células semeadas. Uma vez que o suprimento de Fe disponível no meio é limitado, um maior número de células semeadas gera, possivelmente, uma "competição" por Fe. Por outro lado, quanto mais tempo as células ficam expostas ao meio, maior é a taxa de Fe intracelular observada (Fig. 1A e 1B, Capítulo II). Acredita-se no entanto que as taxas de Fe intracelular atinjam um platô, uma vez que esse recurso no meio celular é finito.

Adicionalmente, ao se analisar as informações disponíveis na literatura e descritas no Capítulo I, que discorre sobre a influência de micronutrientes na viabilidade celular, geração de ERO e danos oxidativos, conjuntamente a alguns dos resultados demonstrados no Capítulo II, verifica-se que, apesar do Fe absorvido gerar acúmulo de ERO (Fig. 6 e 7, Capítulo II), a presença desses ERO, até um determinado nível, estaria apenas corroborando com a proliferação celular (TRACHOOTHAM *et al.*, 2008), pois não se verificou dano às células no cometa alcalino (Fig. 9 e 10). A formação de ERO é um processo intrínseco à vida e os ERO possuem atividades biológicas fundamentais, por exemplo, relacionadas à defesa contra patógenos e à sinalização celular (EVANS *et al.*, 2004). Adicionalmente, um aumento da taxa metabólica, que correlaciona-se com a proliferação celular, deve aumentar a síntese de ERO (EVANS *et al.*, 2004).

Por outro lado, evidências indicam, já de longa data, que o estresse oxidativo em geral, mas que também pode ser resultante de um aumento nos níveis de Fe, quando em

associação com defeitos nos mecanismos de defesa antioxidante, pode estar relacionado com morte celular em algumas doenças degenerativas (JOMOVA & VALKO, 2011) e a alguns tipos de câncer (PRA *et al.*, 2009; JOMOVA & VALKO, 2011). Entre o período de tratamento de 24h e 96h, verificou-se um decaimento no acúmulo de ERO de ambas as culturas, tanto na presença de h-Tf quanto de $FeSO_4$ ERO (Fig. 6A e 6B; Fig. 7A e 7B, Capítulo II), apesar de a atividade da enzima catalase não estar aumentada (Fig. 8, Capítulo II). Esses resultados poderiam ser justificados possivelmente pela ação de outros mecanismos de defesa compensatórios que estivessem ativados. A catalase foi avaliada uma vez que possui um grupamento heme, ou seja, o micronutriente Fe é um de seus co-fatores e, além disso, há evidências de que anêmicos possuem redução na atividade da enzima (GOTH & BIGLER, 2007). Contudo, de acordo com a literatura, ERO produzidas por Fe também podem ser removidas por SODs, peroxidases, além de catalases, sendo que SOD parece ter um importante papel de proteção (HENLE & LINN, 1997; HIDER & KONG, 2013).

Em relação à suplementação com h-Tf, observou-se um menor acúmulo de ERO tanto na cultura de MRC5 quanto em HepG2 em relação ao tratamento com $FeSO_4$. Nesse contexto, é importante destacar que um dos principais papeis da Tf é justamente se ligar ao Fe^{3+}, evitando assim que o mesmo encontre-se disponível para reagir e gerar ERO, sendo esta uma importante estratégia antioxidante do organismo (FRANKE *et al.*, 2006).

Os níveis de Fe intracelular verificados para h-Tf foram levemente superiores aqueles encontrados para $FeSO_4$ (Fig.1, Capítulo II), entretanto, os níveis de ERO se mostraram inversamente proporcionais. Dessa forma, já seria possível sugerir que, aparentemente, a suplementação com h-Tf seria a via primordial e mais facilmente reconhecida pela célula para absorção de Fe, mantendo os níveis de ERO dentro de uma faixa que não compromete a viabilidade celular nem a estabilidade genômica. Adicionalmente, poderia inferir-se que a via de absorção mediada por h-Tf resultasse em uma absorção mais orquestrada de Fe, gerando em um LIP menor. De fato, a absorção parece ser favorecida. Contudo, os resultados demonstrados no cometa alcalino, onde houve pré-tratamento com h-Tf e $FeSO_4$ seguido de exposição aos mutágenos H_2O_2 e MMS (Fig. 11A, Capítulo II), sugerem uma cinética de liberação do Fe^{3+}. O Fe^{3+} ligado a Tf, principalmente frente ao H_2O_2, cuja lesão predominante é a quebra de fita, mas

algumas bases também são oxidadas (IMLAY *et al.*, 1988; HENLE & LINN, 1997; NAKAMURA *et al.*, 2003), gerou um aumento bastante significativo no índice de dano, possivelmente, devido a um aumento nas quebras em fitas de DNA. Por outro lado, $FeSO_4$ apresentou padrão de dano semelhante ao tratamento com H_2O_2 (Fig. 11A, Capítulo II).

É importante salientar que o ensaio cometa não é capaz de distinguir lesões que são induzidas *per se* pelo tratamento daquelas que são induzidas pela atividade de enzimas de reparo do DNA, pois quebras simples são formadas como etapa intermediária nos reparos por excisão (COLLINS *et al.*, 2008). O Fe, por sua vez, reage com peróxidos produzindo radicais livres que são altamente reativos, como HO• , a espécie reativa de oxigênio mais reativa de todas. Por sua vez, radicais HO• podem gerar lesões no DNA, causando quebras no DNA, depurinação/depirimidinação e modificações químicas nas bases ou nos açúcares, além de peroxidação lipídica e modificações proteicas (WELCH *et al.*, 2002; JOMOVA & VALKO, 2011). Adicionalmente, o Fe está sempre apto a reagir com moléculas que contenham oxigênio, mesmo que o Fe e/ou o oxigênio estejam livres ou complexados com ligantes fracos ou até mesmo dentro de grandes biomoléculas, como lipídeos, proteínas, açúcares ou DNA e RNA (HALLIWELL & GUTERRIDGE, 2007).

Uma teoria possível para se tentar elucidar o porquê de h-Tf na presença de H_2O_2 ter demonstrado efeito aditivo de danos ao DNA, talvez esteja relacionada à forma do Fe ligado a h-Tf *versus* $FeSO_4$. Pode-se inferir que a cinética de liberação de Fe no meio intracelular, bem como a formação de LIP, estejam envolvidas nesse evento. Esta hipótese é devidamente discutida no Capítulo II. No caso de $FeSO_4$, o Fe disponibilizado para a célula é Fe^{2+} enquanto a h-Tf transporta Fe^{3+}. A h-Tf, para liberar o Fe no citosol, precisa que o mesmo seja reduzido no endossomo. Sendo assim, pode-se inferir que no caso de $FeSO_4$, tão logo o Fe seja absorvido pela célula, seja instantaneamente utilizado pela célula e/ou, armazenado na ferritina (Fig. 2, Introdução). Após as 24h do pré-tratamento, quando finalmente o mutágeno vai entrar em contato com a célula, possivelmente não reste tanto Fe^{2+} disponível livremente no interior celular para reagir com o H_2O_2 e, portanto, não se observa um aumento tão expressivo nos danos ao DNA. Por outro lado, considerando esse "atraso" na liberação do Fe ligado a Tf, pode-se inferir que, após o mesmo período de pré-tratamento, quando finalmente ocorre a exposição ao mutágeno, ainda exista Fe disponível livremente no meio intracelular ou até mesmo, no

núcleo (HIDER & KONG, 2013). Uma absorção levemente maior de Fe, conforme demonstrado pelo nível intracelular quantificado (Fig. 1, Capítulo II), pode ser igualmente uma explicação alternativa ao maior nível de dano nas células pré-tratadas com h-Tf em relação àquelas pré-tratadas com $FeSO_4$.

Para os pré-tratamentos com MMS, mutágeno reconhecidamente usado para verificação de danos alquilantes ao DNA, e em menor escala, também usado para avaliação de danos oxidativos (FRANKE *et al.*, 2005), ambos pré-tratamentos tiveram perfil semelhante ao do MMS (Fig. 11A, Capítulo II). Sendo assim, sugere-se que a presença de Fe no meio de cultura, previamente à exposição ao MMS, não demonstrou influência sobre danos alquilantes ao DNA. A hipótese de que a suplementação com Fe pudesse modular alquilransferases, que contêm Fe como co-fator, reduzindo o dano induzido pelo MMS, não foi comprovada no tempo de reparo avaliado.

O perfil de danos frente à ENDO III e FPG foi bastante semelhante para todos os tratamentos de Fe (Fig. 11B e 11C, Capítulo II), não sendo verificadas diferenças entre danos em pirimidinas *versus* purinas, e denotando-se apenas um decréscimo nos danos oxidativos quando comparados ao H_2O_2. Nos tratamentos com MMS quando expostos à FPG, que por sua vez reconhece danos oxidativos, mas também bases alquiladas (SPEIT *et al.*, 2004), foi possível observar uma diferença maior quando se subtraíram os danos com enzimas dos resultados sem enzimas, sugerindo que MMS cause danos alquilantes mais frequentes em purinas, mas para os quais nem h-Tf nem $FeSO_4$ demonstraram efeito protetor.

Uma vez que o Fe é co-fator de inúmeras enzimas e proteínas (PRA *et al.*, 2012), como já mencionado, entre elas enzimas de reparação ao DNA, também verificou-se a expressão de duas proteínas que apresentam *clusters* Fe/S em sua estrutura e cujos resultados estão igualmente descritos no Capítulo II. Basicamente, o objetivo foi avaliar se a presença de Fe de alguma forma poderia estar favorecendo o reparo por alguma das vias em que as proteínas eleitas estivessem envolvidas.

Com base nas informações descritas na literatura (PRA *et al.*, 2012), foram selecionadas uma helicase - ERCC2 (também conhecida como XPD) - que desempenha importante papel no reparo de DNA por excisão de nucleotídeos (NER), sendo uma das subunidades do fator de transcrição TFIIH (ASSFALG *et al.*, 2012; COMPE & EGLY,

2012), e uma glicosilase – MutyH -, que além de ser uma enzima relacionada ao reparo por desemparelhamento de bases (MMR), é também uma DNA glicosilase requerida para o reconhecimento e reparo de DNA que atua no BER removendo adeninas quando mal-pareadas com 8-oxo-Gua (CHEADLE & SAMPSON, 2003; BRZOSKA *et al.*, 2006; WHITE, 2009). No momento em que a adenina é substituída pela citosina, a 8-oxo-Gua já é excisada pela proteína FPG, que atua em desemparelhamentos do tipo 8-oxo-Gua:A (HENLE & LINN, 1997; SPEIT *et al.*, 2004).

O NER é capaz de reconhecer e reparar um grande número de lesões não necessariamente relacionadas estruturalmente. Neste caso, as lesões são reconhecidas não por sua natureza química, mas pelo grau de distorção promovido na hélice de DNA. Essas lesões incluem dímeros de pirimidina e 6-4 fotoprodutos (citosina-timina), timina-glicóis, adutos gerados por psoralenos fotoativos, purinas-cisplatinas, agentes alquilantes, e adutos formados por carcinógenos policíclicos, além de pontes intercalantes (HENRIQUES & SAFFI, 2003; ROCCA *et al.*, 2010). O NER ocorre quando a remoção da base defeituosa é feita pela incisão endonucleolítica nos dois lados da lesão, com liberação dos nucleotídeos, seguida pelo preenchimento da região por ação da DNA-polimerase. As principais etapas deste processo de reparação compreendem: reconhecimento da lesão, incisão do dano (3’ e 5’), excisão do dano (em torno de 24 a 32 nucleotídeos), síntese de reparação (ressíntese de DNA) e ligação (HENRIQUES & SAFFI, 2003; ROCCA *et al.*, 2010).

O BER é um dos mecanismos de reparo mais utilizados para o reparo de danos oxidativos e alquilação, sendo considerado a via primoridal para esse tipo de lesão e atuando igualmente na remoção de 8-OHdG (OCK *et al.*, 2012). Caracteriza-se pela excisão única e exclusivamente da base lesada. O BER, a exemplo de outros sistemas de reparo, envolve a clivagem e a ressíntese da porção de DNA danificada. Uma série de enzimas denominadas DNA-glicosilases reconhecem a lesão e promovem a hidrólise da ligação N-glicosil, que liga a base ao esqueleto de fosfato-açúcar do DNA. Como consequência, temos um sítio abásico que é reconhecido por uma AP-endonuclease, capaz de produzir quebras na ligação fosfodiester a 5’ ou 3’ do sítio básico (SATOH & LINDAHL, 2004). Desse modo, a estrutura do DNA fica rompida até que a DNA polimerase ressintetize a cadeia e esta seja religada. O ensaio cometa detecta principalmente quebras no DNA, que podem ter resultado de genotoxicidade ou da

atividade de reparo. Portanto, um aumento de genotoxicidade detectado pelo ensaio cometa eventualmente também pode indicar um aumento na atividade de reparo, uma vez que as quebras detectadas podem ser provenientes de excisão das cadeias de DNA pelas enzimas de reparo (TICE *et al.*, 2000; COLLINS *et al.*, 2008).

Os resultados de *Western Blotting* para a expressão das proteínas de reparo eleitas, após tratamentos com h-Tf e $FeSO_4$, por períodos de 24h e 96h (resultados preliminares não mostrados), não indicaram nenhuma alteração na expressão das mesmas com base simplesmente na maior disponibilidade de um co-fator, no caso, o Fe. Sendo assim, decidiu-se fazer um pré-tratamento com h-Tf e $FeSO_4$ (24h), seguido de exposição a mutágenos (procedimento idêntico ao usado no ensaio cometa), para verificar-se a expressão das proteínas envolvidas com a reparação de DNA. Analisando-se os resultados obtidos (Fig. 12, Capítulo II), é possível verificar que a via de reparação mais favorecida frente aos pré-tratamentos e exposição aos mutágenos, H_2O_2 e MMS, foi BER, uma vez que a expressão de MutyH está visivelmente aumentada, em especial, na presença de H_2O_2. Contudo, a presença do pré-tratamento de Fe, tanto com h-Tf quanto com $FeSO_4$, não demonstrou ter nenhuma influência em particular sobre a expressão de MutyH. No caso da proteína relacionada ao NER, não houve aumento em sua expressão. Curiosamente, o pré-tratamento com h-Tf seguido de exposição aos mutágenos, H_2O_2 e MMS, demonstrou um decaimento na expressão de TFIIH (Fig. 12, Capítulo II). Nesse sentido, pode-se sugerir que na presença de Fe, em sendo este micronutriente um cofator para TFIIH, a eficiência desta enzima possa estar aumenta, apesar de sua expressão ter diminuído. Ou ainda, que outra via está sendo sinalizada, diferentemente de NER, como é o caso de BER.

No Capítulo I, é demonstrado que micronutrientes são capazes de influenciar a viabilidade celular e a estabilidade genômica *in vitro* de maneira dependente da concentração e da forma de micronutriente estudada (Tab. 3 e 4, Capítulo I). Contudo, muitos micronutrientes ainda requerem mais estudos *in vitro* (Tab. 5, Capítulo I), apesar de *in vivo* já se ter um conhecimento mais robusto, conforme demonstrado na Tabela 1 (Capítulo I).

No caso do Fe, os resultados do Capítulo II vêm a somar nesse sentido, uma vez que usando modelos celulares distintos, MRC5 e HepG2, e duas formas de suplementação de Fe, h-Tf e $FeSO_4$, demonstrou-se um aumento de 10-20% na

proliferação e viabilidade celular. Além de ter-se investigado outras interações relevantes (como formação de ERO e expressão de proteínas) a fim de se elucidar melhor o papel do micronutriente Fe na estabilidade genômica de culturas celulares, demonstrando que, não somente concentrações distintas (resultados preliminares não descritos), mas também, diferentes formas de Fe usado na suplementação de meios de cultura, podem interferir nos resultados finais de qualquer estudo que considere um modelo celular *in vitro*.

Na verdade, pode-se afirmar que a forma de suplementação usada para fornecer qualquer micronutriente para o meio celular pode ser uma importante variável nos resultados de uma pesquisa com células. Há décadas está estabelecido que a diferença no efeito tóxico do Fe está diretamente relacionada com a presença de agentes quelantes, os quais servem para reduzir a concentração de Fe^{3+} e promover a formação de ferritina (JACOBS *et al.*, 1978; MAY & QU, 2010). Agentes quelantes não somente removem o Fe do organismo, mas também sequestram e ligam-se fortemente ao Fe lábil, prevenindo a geração de ERO. A suplementação dos meios de cultura com quelato de Fe pode ser considerada, mas normalmente essa forma de Fe apresenta preços mais elevados junto aos fornecedores, não favorecendo o seu uso em larga escala como suplemento de culturas celulares. Importante observar que, *in vivo,* existe um complexo sistema de proteínas responsáveis pelo transporte e armazenamento de Fe e de tal forma, o mesmo raramente encontra-se livre no organismo, uma vez que é danoso (MENEGHINI, 1997; PRA *et al.*, 2012). Sendo assim, cuidado semelhante deve existir no momento de se formular meios de cultura celular que contenham concentrações mais elevadas de Fe do que as atualmente existentes.

No campo da biotecnologia industrial, existe um grande interesse em encontrar componentes alternativos que possam substituir suplementos derivados de animal (ex.: SFB) nos meios de cultura, sendo que a h-Tf é uma alternativa bastante estudada. A h-Tf humana tem demonstrado uma grande capacidade para promover o crescimento celular, quando comparada ao análogo bovino (h-Tf proveniente do SFB) nas culturas celulares humanas (BRANDSMA *et al.*, 2011). Recentemente, um estudo demonstrou que a suplementação com Tf influencia positivamente o cultivo de células neuronais, sugerindo que a mesma deveria ser fortemente considerada como um fator de amplificação de células neuronais *in vitro* (SILVESTROFF *et al.*, 2013).

Em resumo, neste trabalho foram encontrados resultados interessantes no que se refere à suplementação de meio de cultura celular com o micronutriente Fe, tanto na sua forma h-Tf quanto como $FeSO_4$. A proliferação e viabilidade celular foram aumentadas e, apesar do acúmulo de ERO observado, não houve comprometimento na estabilidade genômica das células. Além disso, considerando-se que os níveis de Fe intracelular verificados para a h-Tf foram levemente superiores aos do $FeSO_4$, mas por outro lado, os níveis de ERO acumulados nas células tratadas com h-Tf foram inferiores aquelas que foram expostas a $FeSO_4$, pode-se inferir que a h-Tf seja a forma primordial para a suplementação dos meios de cultura. Adicionalmente, h-Tf tem papel importante na homeostase do Fe. Corroborando com essa inferência, pode-se ainda mencionar o fato de que o Fe ligado a Tf dificilmente é usado por microrganismos oportunistas presentes nas culturas. A concentração e a forma de Fe sugeridos neste estudo deveriam ser futuramente usadas em outras culturas celulares, em especial, aquelas que requerem um suprimento constante desse micronutriente para a sua diferenciação, como precursores neuronais (SILVESTROFF *et al.*, 2013) e células tronco mesenquimais (CALLENS *et al.*, 2010).

4. CONCLUSÕES

4.1. Conclusão geral

O micronutriente Fe desempenhou importante papel na viabilidade celular e na estabilidade genômica, sendo que a suplementação com h-Tf numa concentração próxima a fisiológica (7 µmol/L) demonstrou ser benéfica para as linhagens MRC5 e HepG2.

4.2. Conclusões específicas

- Ambas formas de suplementação de Fe, h-Tf e $FeSO_4$, foram captadas por MRC5.

- Igualmente, h-Tf e $FeSO_4$ aumentaram a proliferação e viabilidade de ambas linhagens (MRC5 e HepG2).

- h-Tf gerou menos ERO, possivelmente, por estar ligada a uma forma de Fe mais estável (Fe^{3+}).

- Pré-tratamento de h-Tf demonstrou ter uma cinética de liberação de Fe no meio intracelular mais lenta do que $FeSO_4$, apesar de transportar uma forma mais estável de Fe, sendo que na presença de H_2O_2 aumentou dano ao DNA.

- Pré-tratamentos de h-Tf e $FeSO_4$ não parecem ter influência na expressão das proteínas de reparo TFIIH e MutyH, quando seguidos de exposição as mutágenos, na concentração e períodos de tratamentos avaliados.

- h-Tf aumentou a expressão de CD71 e Nramp, sugerindo que a absorção de Fe ligado a Tf seja uma via preferencial pela célula para a obtenção do micronutriente.

- H_2O_2 extracelular é capaz de ativar o mecanismo pós-transcricional de regulação da homeostase de Fe, relacionado a IRP-IRE.

- O tratamento com h-Tf, em concentração próxima a fisiológica, foi capaz de aumentar a proliferação e viabilidade celular, não sendo nem citotóxico nem genotóxico para as linhagens avaliadas, MRC5 e HepG2.

5. PERSPECTIVAS

Adicionalmente aos resultados obtidos e às conclusões geradas, algumas questões surgiram ao longo do trabalho, instigando a necessidade de estudos complementares.

- Testar outras formas de Fe na suplementação de culturas celulares, como quelatos de Fe.

- Avaliar a influência da forma e concentração propostas de Fe em culturas de células primárias ou ainda, de células não diferenciadas, como células-tronco mesenquimais.

- Verificar a cinética de reparo das células no ensaio comenta, frente aos pré-tratamentos seguidos de exposição à mutágenos.

- Avaliar a atividade de outras defesas antioxidantes, como a GSH, SOD e GPx.

- Complementar os dados de expressão de proteínas receptoras de Fe celular e identificar o local em que as mesmas são expressas nas células.

- Mensurar as espécies de Fe intracelular - Fe^{2+} e Fe^{3+}.

- Verificar a formação de micronúcleos frente aos pré-tratamentos com Fe seguidos de exposição aos mutágenos, a fim de entender se o dano causado pelos mutágenos é reparável ou não e se o pré-tratamento com Fe favorece de alguma forma esse processo.

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ANEXO – *Curriculum Vitae* – Ana Lúcia Vargas Arigony

Ana Lúcia Vargas Arigony

Curriculum Vitae

Março/2013

Ana Lúcia Vargas Arigony
Curriculum Vitae

Dados pessoais

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Formação acadêmica/titulação

2008 Doutorado em Biologia Celular e Molecular.
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil
Título: Avaliação do Efeito do Micronutriente Ferro (Fe) na Viabilidade Celular e Estabilidade Genômica de Culturas Celulares de Fibroblasto Pulmonar (MRC5) e Hepatocarcinoma (HepG2), Ano de obtenção: 2013
Orientador: Prof. Dr. João Antônio Pêgas Henriques

2003 - 2005 Mestrado em Ciências Farmacêuticas.
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil
Título: Determinação Química e Biológica de Bauhinia forficata Link sub. pruinosa, Ano de obtenção: 2005
Orientador: Prof. Dr. José Ângelo da Silveira Zuanazzi
Bolsista do(a): Conselho Nacional de Desenvolvimento Científico e Tecnológico

1998 - 2002 Graduação .
Pontifícia Uiniveridade Católica do Rio Grande do Sul, PUC-RS, Brasil
Título: Homeopatia: uma alternativa de tratamento para animais de produção
Orientador: MSc. Denise Milão

Atuação profissional

1. Souza Cruz S.A. - BAT

Vínculo institucional

2006 - Atual Vínculo: Empregado , Enquadramento funcional: Especialista

em Assuntos Regulatórios , Carga horária: 44, Regime: Integral

2. Universidade do Vale do Rio dos Sinos - UNISINOS

Vínculo institucional

2010 - Atual Vínculo: Professor Convidado , Enquadramento funcional: Professor Convidado, Regime: Parcial

3. Emporio Body Store - BODY STORE

Vínculo institucional

2003 - 2004 Vínculo: Consultor , Enquadramento funcional: Consultoria para P&D de cosméticos , Carga horária: 8, Regime: Parcial

4. Riopasa Importadora de Medicamentos LTDA - RIOPASA

Vínculo institucional

2005 - 2006 Vínculo: Empregado , Enquadramento funcional: Responsável Técnico , Carga horária: 44, Regime: Integral

5. Medicor Produtos Hospitalares - MEDICOR

Vínculo institucional

2005 - 2006 Vínculo: Empregado , Enquadramento funcional: Responsável Técnica , Carga horária: 8, Regime: Parcial

Áreas de atuação

1. Biologia Celular e Molecular
2. Genética
3. Homeopatia
4. Farmacognosia
5. Controle de Qualidade
6. Farmacologia

Idiomas

Inglês Compreende Bem , Fala Bem , Escreve Bem , Lê Bem

Espanhol Compreende Razoavelmente , Fala Pouco , Escreve Pouco , Lê Razoavelmente

Francês Compreende Razoavelmente , Fala Razoavelmente , Escreve Razoavelmente , Lê Razoavelmente

Prêmios e títulos

2009 Prêmio Golden Leaf: A Planta Piloto Industrial sob o enfoque de um Grupo Multifuncional, Souza Cruz S.A. uma empresa do grupo British American Tobacco

Producão

Produção bibliográfica

Artigos completos publicados em periódicos

1. MONTANHA, J., RATES, S., PROVENSI, G., DRESCH, A. P., MENDEZ, A., RECH, S., VARGAS, C., GERMANI, J., STEPPE, M., HEINECK, I., CHAVES, C., LIMBERGER, Renata, **ARIGONY, A. L. V.**, PETERSEN, R., VIERA, S., SPALDING, S., OPPE, T., COSTA, T. D.
A Iniciação Científica na Faculdade de Farmácia da Universidade Federal do Rio Grande do Sul. Caderno de Farmácia. , v.21, p.89 - 93, 2005.

2. **ARIGONY, A. L. V.**, MILAO, D.
Homeopatia: uma alternativa de tratamento para animais de produção. Revista Afargs. , v.18, p.4 - 7, 2004.

Trabalhos publicados em anais de eventos (resumo)

1. **ARIGONY, A. L. V.**, Joao Antonio Pegas Henriques, MILANO, L.
Avaliação do Efeito do Micronutriente Ferro (Fe) na Viabilidade Celular e Estabilidade Genômica de Culturas Celulares de Fibroblasto Pulmonar (MRC5) e Hepatocarcinoma (HepG2) In: XIV Semana do PPGBCM, 2012, Porto Alegre.
Caderno de Resumos da XIV Semana do PPGBCM. , 2012.

2. **ARIGONY, A. L. V.**, LIMA, M., SCHULER, G., JUCHEM, A., TRINDADE, C., Diana Bordin, MACHADO, M., Daniel Prá, Joao Antonio Pegas Henriques
Avaliação da Influência de Meio de Cultura Suplementado com Ferro na Viabilidade Celular e na Estabilidade Genômica das Linhagens Mrc5 & HepG2 In: XIII Reunião Anual do Programa de Pós-Graduação em Biologia Celular e Molecular da UFRGS, 2011, Porto Alegre.
Livro de Resumos da XIII Reunião Anual do PPGBCM. , 2011.

3. LIMA, M., MACHADO, M., Diana Bordin, Daniel Prá, **ARIGONY, A. L. V.**, Joao Antonio Pegas Henriques
Avaliação da Viabilidade e Proliferação Celular nas Linhagens HepG2 e MRC5 em Cultura Suplementada com Ferro In: XIII Reunião Anual do Programa de Pós-Graduação em Biologia Celular e Molecular da UFRGS, 2011, Porto Alegre.
Livro de Resumos da XIII Reunião do PPGBCM. , 2011.

4. **ARIGONY, A. L. V.**, JAEGER, C., Diana Bordin, Daniel Prá, Joao Antonio Pegas Henriques
Influência de Ferro Inorgânico e holo-Transferrina na Viabilidade e Estabilidade Genômica de Culturas Celulares In: XII Reunião Anual do Programa de Pós-graduação em Biologia Celular e Molecular do Centro de Biotecnologia da UFRGS, 2010, Porto Alegre.
Livro de Resumos PPGBCM. , 2010.

5. **ARIGONY, A. L. V.**, Daniel Prá, Diana Bordin, Manoela Peletti-Figueiró, Daniel Marinovic, Luiza Moreira, Ana Paula Franco Lambert, Diego Bonatto, Denise Cantarelli Machado, Joao Antonio Pegas Henriques
Influência de ferro inorgânico na proliferação e na estabilidade genômica de células de medula óssea humana in vitro In: XI Reunião Anual do Programa de Pós-graduação em Biologia Celular e Molecular do Centro de Biotecnologia da UFRGS, 2009, Porto Alegre.
Livro de Resumos PPGBCM. , 2009.

6. **ARIGONY, A. L. V.**, ZUANAZZI, J. A., MENEZES, F., ALMEIDA, A. C.
Atividade Antioxidante de Extratos Etanólico e Butanólico de Bauhinia forficata Link In: VIII Simposio Argentino y XI Simposio Latino Americano de Farmacobotânica, 2004, Buenos Aires.
VIII Simposio Argentino y XI Simposio Latino Americano de Farmacobotânica. , 2004. p.70 - 70

7. **ARIGONY, A. L. V.**, HENRIQUES, A., CHAVES, C., SUYENAGA, E., ZUANAZZI, J. A., APEL, M., MAHMUD, N., KERBER, V.
Flavonóides e Terpenóides de Heterothalamus psiadioides Less In: Congresso Ítalo-Latino-Americano de Etnomedicina Nuno Alvares Pereira, 2003, Rio de Janeiro.
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Apresentação de trabalho e palestra

1. **ARIGONY, A. L. V.**, Joao Antonio Pegas Henriques, MILANO, L., CHIELA, E., LENZ, G.
Avaliação do Efeito do Micronutriente Ferro (Fe) na Viabilidade Celular e Estabilidade Genômica de Culturas Celulares de Fibroblasto Pulmonar (MRC5) e Hepatocarcinoma (HepG2), 2012. (Congresso,Apresentação de Trabalho)

2. **ARIGONY, A. L. V.**, LIMA, M., SCHULER, G., Diana Bordin, MACHADO, M., Daniel Prá, Joao Antonio Pegas Henriques
Efeitos da Suplementação com Ferro na Viabilidade Celular e Genotoxicidade em Linhagens - HepG2 e MRC5, 2011. (Congresso,Apresentação de Trabalho)

3. **ARIGONY, A. L. V.**, Ana Paula Franco Lambert, Moura, D., Mariana Lemos, Silva, J.A.S., Lambert, B.F., Lubianca, J., Diego Bonatto, Denise Cantarelli Machado, Joao Antonio Pegas Henriques
Genotoxicity of Neuronal Induction in Adipose Derived Stem Cells in vitro, 2009. (Congresso,Apresentação de Trabalho)

4. **ARIGONY, A. L. V.**, Daniel Prá, Ana Paula Franco Lambert, Diana Bordin, Daniel Marinovic, Manoela Peletti-Figueiró, Luiza Moreira, Diego Bonatto, Denise Cantarelli Machado, Joao Antonio Pegas Henriques
Influência de ferro inorgânico na proliferação e na estabilidade genômica de células de medula óssea humana in vitro, 2009. (Congresso,Apresentação de Trabalho)

5. **ARIGONY, A. L. V.**, MILAO, D.
Homeopatia: uma alternativa de tratamento para animais de produção, 2003. (Outra,Apresentação de Trabalho)

6. **ARIGONY, A. L. V.**, CARNEIRO, M. C.
O pH em gráficos. Equiligraphs: uso de planilhas de cálculo no estudo do equilíbrio iônico em solução, 2000. (Outra,Apresentação de Trabalho)

Educação e Popularização de C&T

Trabalhos publicados em anais de eventos (resumo)

1. **ARIGONY, A. L. V.**, Joao Antonio Pegas Henriques, MILANO, L.
Avaliação do Efeito do Micronutriente Ferro (Fe) na Viabilidade Celular e Estabilidade Genômica de Culturas Celulares de Fibroblasto Pulmonar (MRC5) e Hepatocarcinoma (HepG2) In: XIV Semana do PPGBCM, 2012, Porto Alegre.
Caderno de Resumos da XIV Semana do PPGBCM. , 2012.

Apresentação de trabalho e palestra

1. **ARIGONY, A. L. V.**, Joao Antonio Pegas Henriques, MILANO, L., CHIELA, E., LENZ, G. **Avaliação do Efeito do Micronutriente Ferro (Fe) na Viabilidade Celular e Estabilidade Genômica de Culturas Celulares de Fibroblasto Pulmonar (MRC5) e Hepatocarcinoma (HepG2)**, 2012. (Congresso,Apresentação de Trabalho)

Participação em eventos, congressos, exposições, feiras e olimpíadas

1. **Cell Culture as Alternative Model for Animal Experimentation**, 2012. (Outra)
.

2. **X Cell Stress Society International Workshop on the Molecular Biology of Stress Responses,**, 2012. (Simpósio)

Orientações e supervisões

Orientações e supervisões concluídas

Trabalhos de conclusão de curso de graduação

1. Paulo Eduardo Colling. **Revisão Bibliográfica de Echinodorus macrophyllus**. 2003. Curso (Farmácia) - Universidade Federal do Rio Grande do Sul

Iniciação científica

1. Camila Jaeger. **Influência de Ferro Inorgânico e Transferrina na Viabilidade de Culturas Celulares**. 2010. Iniciação científica (Biologia) - Pontifícia Universidade Católica do Rio Grande do Sul

Eventos

Participação em eventos

1. **Programa de Gestão Empresarial - Fundação Dom Cabral/Souza Cruz**, 2012. (Outra)
.

2. **X Cell Stress Society International Workshop on the Molecular Biology of Stress Responses,**, 2012. (Simpósio)
.

3. **Cell Culture as Alternative Model for Animal Experimentation**, 2012. (Outra)
.

4. Apresentação de Poster / Painel no(a) **XIII Reunião Anual do Programa de Pós-**

Graduação em Biologia Celular e Molecular da UFRGS, 2011. (Outra)
Avaliação da Influência de Meio de Cultura Suplementado com Ferro na Viabilidade Celular e na Estabilidade Genômica das Linhagens Mrc5 & HepG2.

5. Apresentação Oral no(a) **II Congresso Sul de Toxicologia Clínico-Laboratorial**, 2011. (Congresso)
Efeitos da Suplementação com Ferro na Viabilidade Celular e Genotoxicidade em Linhagens - HepG2 e MRC5.

6. **Curso Sinalização Celular no Câncer ministrado pelo LabSinal UFRGS**, 2011. (Outra)
.

7. **57 Congresso Brasileiro de Genética**, 2011. (Congresso)
.

8. **II Reunião Brasileira de Citogenética**, 2011. (Outra)
.

9. **Curso Aprendendo Citogenética Clássica: Rotina dos Procedimentos in vitro**, 2011. (Outra)
.

10. **Curso Mutagênese Ambiental: biomonitores e biomarcadores**, 2011. (Outra)
.

11. **Curso Técnicas para estudar instabilidade genética em células**, 2011. (Outra)
.

12. Apresentação de Poster / Painel no(a) **XII Reunião Anual do Programa de Pós-graduação em Biologia Celular e Molecular do Centro de Biotecnologia da UFRGS**, 2010. (Outra)
Influência de Ferro Inorgânico e holo-Transferrina na Viabilidade e Estabilidade Genômica de Culturas Celulares.

13. **Curso Condução Eficaz de Auditorias**, 2010. (Outra)
.

14. **International Conference on Nutrigenomics and 10th International Conference on Mechanisms of Antimutagenesis and Anticarcinogenesis**, 2010. (Congresso)
.

15. Apresentação de Poster / Painel no(a) **IX Congresso Brasileiro da SBMCTA**, 2009. (Congresso)
Influência de Ferro Inorgânico na Proliferação e na Estabilidade Genômica de Células de Medula Óssea Humana in vitro.

16. Apresentação de Poster / Painel no(a) **XI Reunião Anual do Programa de Pós-graduação em Biologia Celular e Molecular do Centro de Biotecnologia da UFRGS**, 2009. (Outra)
Influência de ferro na proliferação e na estabilidade genômica de células de medula óssea humana in vitro.

17. **Curso de Biossegurança - IPB/PUCRS**, 2009. (Outra)
.

18. **Curso de Epigenética e Mutagênese ministrado no IX Congresso Brasileiro da SBMCTA**, 2009. (Outra)
.

19. **Mini-Curso de Citogenética aplicada à mutagênese e análise de genotoxicidade**,

2009. (Outra)
.

20. **XVIII Congresso Mundial de Epidemiologia/ VII Congresso Brasileiro de Epidemiologia**, 2008. (Congresso)
.

21. **Course on Epidemiologic Methods and New Directions**, 2008. (Seminário)
.

22. Conferencista no(a) **O Farmacêutico como pesquisador na área fumageira**, 2007. (Outra)
O Farmacêutico como pesquisador na área fumageira.

23. **Jornada Farmacêutica**, 2007. (Encontro)
.

24. **NBR ISO/IEC 17025:2005**, 2007. (Outra)
.

25. **NBR ISO 9001, NBR ISO 14001:2004, OHSAS 18001:1999 e Legislação Ambiental**, 2007. (Outra)
.

26. **IV Workshop de Analistas de Resíduos de Agrotóxicos do Estado do RS**, 2007. (Simpósio)
.

27. Conferencista no(a) **Entidades Farmacêuticas**, 2006. (Outra)
Apresentando a Associação dos Farmacêuticos do Rio Grande do Sul (AFARGS).

28. **Curso Mercado de produtos cosméticos e diretrizes para criação de empresas**, 2006. (Outra)
.

29. Apresentação de Poster / Painel no(a) **VIII Simposio Argentino y XI Simposio Latino Americano de Farmacobotánica**, 2004. (Simpósio)
Atividade Antioxidante dos Extratos Etanólico e Butanólico de Bauhinia forficata Link.

30. Moderador no(a) **XXX Semana Acadêmica de Estudos Farmacêuticos**, 2004. (Simpósio)
Mesa Redonda - Panorama da Pesquisa na Faculdade de Farmácia.

31. **VIII Simposio Argentino y XI Simposio Latino Americano de Farmacobotánica**, 2004. (Simpósio)
.

32. **XVII Fórum da Liberdade**, 2004. (Encontro)
.

33. **Strategy in the search of bio-active plants contituents**, 2004. (Outra)
.

34. Apresentação de Poster / Painel no(a) **XII Congresso Ítalo-Latino-Americano de Etnomedicina "Nuno Álvares Pereira"**, 2003. (Congresso)
Flavonóides e Terpenóides de Heterothalamus psiadioides Less..

35. Apresentação Oral no(a) **VII Semana Acadêmica da Faculdade de Farmácia PUCRS**, 2003. (Outra)
Homeopatia: uma alternativa de tratamento em animais de produção.

36. **Fórum Social Mundial**, 2003. (Encontro)
.

37. **VII Semana Acadêmica da Faculdade de Farmácia da PUCRS**, 2003. (Outra)
.

38. **Curso de Introdução à Homeopatia na Clínica de Pequenos e Grandes Animais**, 2003. (Outra)
.

39. **Curso Pré-congresso: Metodologia de Estudo em Etnobotânica e Etnofarmacologia**, 2003. (Congresso)
.

40. **Palestra Marketing em Farmácia de Manipulação**, 2002. (Outra)
.

41. **Palestra Controle de Qualidade em Farmácia de Manipulação**, 2002. (Outra)
.

42. Moderador no(a) **VI Semana Acadêmica da Faculdade de Farmácia da PUCRS**, 2001. (Outra)
Mesa Redonda - A Participação do Farmacêutico no Hospital.

43. **VI Semana Acadêmica da Faculdade de Farmácia**, 2001. (Outra)
.

44. **Workshop de equinos "O futuro do cavalo de esporte"**, 2001. (Outra)
.

45. Apresentação Oral no(a) **XII Salão de Iniciação Científica da UFRGS**, 2000. (Outra)
Equiligraphs: Uso de planilhas de cálculo no estudo do equilíbrio iônico em solução.

46. Apresentação de Poster / Painel no(a) **I Salão de Iniciação Científica da PUCRS**, 2000. (Outra)
O Ph em gráficos. Equiligraphs: Uso de planilhas de cálculo no estudo do equilíbrio iônico em solução..

47. **Curso Formulações Dermatológicas: da matéria prima ao produto final**, 2000. (Outra)
.

48. **IX Encontro Estadual de Farmacêuticos e Bioquímicos, VII Congresso Catarinense de Farmacêuticos e Bioquímicos e I Encontro de Farmacêuticos e Bioquímicos do Mercosul**, 2000. (Congresso)
.

49. **I Simpósio de Manipulação Veterinária**, 2000. (Simpósio)
.

50. **Curso "O Papel do Farmacêutico na Promoção do Uso Racional de Medicamentos"**, 1999. (Outra)
.

51. **VIII Encontro Estadual de Farmacêuticos e Bioquímicos, VI Congresso Catarinense de Farmacêuticos e bioquímicos**, 1999. (Congresso)
.

52. **III Semana da Faculdade de Farmácia da PUCRS**, 1998. (Encontro)

Bancas

Participação em banca de trabalhos de conclusão

Curso de aperfeiçoamento/especialização

1. **ARIGONY, A. L. V.**
Participação em banca de Carolina Ubal e Renata Puntel. **"Emprego dos inibidores da bomba de prótons no tratamento de refluxo gastroessofágico"**, 2006
(Farmácia) Pontifícia Universidade Católica do Rio Grande do Sul

2. **ARIGONY, A. L. V.**
Participação em banca de Ana Claudia Saling e Ariane Rodrigues Sallaberry. **"Fluconazol e Itraconazol na Candidíase Vulvovaginal"**, 2006
(Farmácia) Pontifícia Universidade Católica do Rio Grande do Sul

3. **ARIGONY, A. L. V.**
Participação em banca de José Gilberto e Rafael da Fonseca. **"Omeoprazol nas Patologias Gástricas"**, 2006
(Farmácia) Pontifícia Universidade Católica do Rio Grande do Sul

4. **ARIGONY, A. L. V.**
Participação em banca de Katiane Cella e Tatiana Paveglio. **"Osteoartrose: uma revisão sobre patologia e tratamento"**, 2006
(Farmácia) Pontifícia Universidade Católica do Rio Grande do Sul

5. **ARIGONY, A. L. V.**
Participação em banca de Aline Cecília e Débora Brandão. **"Tratamento de úlceral venosa com formulações transdérmicas manipuladas"**, 2006
(Farmácia) Pontifícia Universidade Católica do Rio Grande do Sul

Graduação

1. **ARIGONY, A. L. V.**
Participação em banca de Tatiana Costa Copat. **"Fontes de cálcio como suplemento dietético**, 2007
(Farmácia) Pontifícia Universidade Católica do Rio Grande do Sul

2. **ARIGONY, A. L. V.**
Participação em banca de Rodrigo Seibt. **Determinação do Teor de Metilxantinas em Amostras de Guaraná, erva-mate, chá-preto e chá-verde**, 2003
(Farmácia) Universidade Federal do Rio Grande do Sul

Outras informações relevantes

1 Experiência Prática em Consultoria, Assessoria, Pesquisa e Desenvolvimento em Indústria de Cosméticos; Experiência em Importadora e Distribuidora de produtos para a saúde e medicamentos, Autorização de Funcionamento, Registros junto ao Ministério da Saúde, Visas municipais e estaduais, Regulamentação junto ao Conselho Regional de Farmácia; Segundo lugar em Concurso Público para vaga de Professor Substituto da disciplina de Farmacotécnica Homeopática na UFRGS. Atuação na área de Scientific Affairs de Indústria Fumageira.