Universidade Federal do Rio Grande do Sul

Programa de Pós-Graduação em Biologia Celular e Molecular

AVALIAÇÃO DA CAPTAÇÃO DO ÍON METÁLICO Sn^{2+} E SEUS EFEITOS TÓXICOS E GENOTÓXICOS EM CÉLULAS EUCARIÓTICAS

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"A imaginação é mais importante que o conhecimento. Conhecimento auxilia por fora, mas só o amor socorre por dentro. Conhecimento vem, mas a sabedoria tarda."

(Albert Einstein)

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SUMÁRIO

RESUMO

A resistência ao cloreto estanoso ($SnCl_2$) nas células eucarióticas de levedura *Saccharomyces cerevisiae* é um produto de vários processos metabólicos. O mutante *rad52*Δ, deficiente no mecanismo de reparação recombinacional, incapaz de reparar quebras simples e duplas no DNA, foi o mais sensível ao íon metálico Sn^{2+}. A resistência relativa dos mutantes *rad2Δ* e *rad4Δ*, deficientes na reparação por excisão de nucleotídeos (NER), mostrou-se significativamente elevada, indicando a participação dessa via na reparação dos danos causados pelo $SnCl_2$. Uma série de mutantes defectivos na via de reparação por excisão de bases (BER), combinada com mutantes defectivos em NER, foi utilizada para determinar indiretamente o tipo de lesão causada pelo $SnCl_2$ ao DNA. A DNA N-glicosilase (Ntg2p) mostrou-se, por sua vez, indispensável à reparação das lesões ao DNA induzidas pelo íon metálico Sn^{2+}.

A ausência do fator de transcrição Yap1, responsável pela resposta ao estresse oxidativo em leveduras, ocasionou o aumento da sensibilidade ao íon metálico Sn^{2+}. Além disso, a sensibilidade dos mutantes *sod1*Δ e *sod2*Δ revelou a importância das enzimas antioxidantes Sod1p e Sod2p na resposta aos danos gerados pelo íon metálico Sn^{2+}. A sensibilidade mais elevada ao $SnCl_2$ foi observada quando as células estavam na fase exponencial de crescimento (fase LOG), o que sugere que há dois sistemas independentes (um mediado pela repressão/desrepressão catabólica, e outro, pela ativação da transcrição de genes envolvidos nas vias de defesa contra espécies reativas de oxigênio (EROs)), que atuam em conjunto e contribuem para a resistência aos danos causados pelo íon metálico Sn^{2+}.

Para determinar a concentração do íon metálico Sn^{2+} captada nas células da levedura *S. cerevisiae* e correlacioná-la com a toxicidade desse íon metálico, foi determinado o conteúdo de metal intracelular pelo método de

Emissão de Raios-X Induzida por Partículas Carregadas, ou *Particle Induced X-Ray Emission* (PIXE), desenvolvendo-se um protocolo para as análises. Os resultados obtidos mostraram que a linhagem diplóide XS2316 captou 60% da quantidade total do íon metálico Sn^{2+}, captada pela linhagem haplóide XV184-14c. Isso indica que a linhagem haplóide capta maior quantidade do íon metálico Sn^{2+} do que a linhagem diplóide, o que pode explicar a citotoxicidade diferenciada de ambas as linhagens quando tratadas com $SnCl_2$. Por fim, utilizando-se a mesma metodologia, foi realizada uma análise multielementar para avaliar se havia uma interação entre o íon metálico Sn^{2+} e outros elementos químicos essenciais para a célula. De acordo com as análises por PIXE, após tratamento com $SnCl_2$, a linhagem haplóide XV185-14c apresentou um decréscimo significativo nas quantidades de Mg, Zn, S e Fe, e, por outro lado, um aumento na quantidade de P.

Para verificar como o íon metálico Sn^{2+} é captado, distribuído e destoxificado, foram associadas técnicas moleculares, incluindo ensaios de citotoxicidade e RT-PCR quantitativo (qRT-PCR), com a metodologia do PIXE. Os ensaios de sobrevivência mostraram que Fet4 e Zrt2, ambas proteínas de transporte de baixa afinidade do ferro e zinco, respectivamente, podem internalizar o íon metálico Sn^{2+}. Por sua vez, qRT-PCR mostrou um aumento da expressão gênica de *CCH1* e *MID1*, os quais codificam as proteínas Cch1 e Mid1, envolvidas no transporte de Ca^{2+} na membrana plasmática, sugerindo que essas duas proteínas também podem estar envolvidas na captação do íon metálico Sn^{2+}. Os resultados indicaram que, uma vez dentro da célula, o íon metálico Sn^{2+} pode ser armazenado no vacúolo através da ação da proteína P-type ATPase Pmc1 e quelado no citoplasma pela metalotioneína Crs5. Alternativamente, o íon metálico Sn^{2+} pode gerar EROs, em especial o ânion superóxido ($O_2^{\cdot-}$), o qual pode ser dismutado pela proteína Sod1, formando peróxido de hidrogênio (H_2O_2) e radical hidroxil ($^{\cdot}OH$). Consequentemente, as EROs podem induzir a transcrição de vários genes envolvidos na resposta ao estresse oxidativo, como *SOD1*, *YAP1* e *APN1*.

Para elucidar os mecanismos moleculares envolvidos na genotoxicidade do íon metálico Sn^{2+}, foi avaliada a indução de quebras causadas pelo $SnCl_2$ na presença de formamidopirimidina (FPG) e endonuclease III (ENDO III), bem como a interferência desse íon na reparação das lesões induzidas pelo agente alquilante metilmetanosulfonato (MMS) em células de pulmão de *hamster* chinês (V79) pelo ensaio cometa. Os resultados mostraram que, em concentrações tóxicas, o íon metálico Sn^{2+} induziu somente uma limitada elevação nos sítios sensíveis de FPG e ENDO III, sugerindo que o íon metálico Sn^{2+} preferencialmente não induz lesões oxidativas nas bases nitrogenadas.

Embora a concentração de 50 µM de $SnCl_2$ não tenha produzido um aumento significativo na quantidade de danos ao DNA, ela foi capaz de inibir a reparação dos danos provocados pelo agente alquilante metilmetanosulfonato (MMS) durante o período de pós-tratamento de 24h. Os resultados demonstraram o efeito genotóxico e comutagênico do $SnCl_2$ em células V79. O efeito inibitório do íon metálico Sn^{2+} na reparação dos danos induzidos pelo MMS no DNA sugeriu que esse metal também pode interferir em sistemas de reparação do DNA, que contribuem para o aumento de mutações pela alteração do equilíbrio entre o processo de reparação livre de erro e o sujeito a erro.

ABSTRACT

Resistance to stannous chloride ($SnCl_2$) of the yeast *Saccharomyces cerevisiae* is a product of several metabolic pathways of this unicellular eukaryote. The recombination deficient *rad52*Δ mutant, unable to repair DNA single and double strand breaks was the most sensitive. The relative resistance of mutant strains r*ad2* e *rad4*, deficient in nucleotide excision repair (NER), was rather high, indicating a minor but significant contribution to repair of Sn^{2+} -induced DNA lesions by this repair pathway. A series of mutants defective in different base excision repair (BER) pathway, combined with NER were used to indirectly determine the type of $SnCl_2$-produced DNA lesion. The Ntg2p DNA N-glycosylase seems to be indispensable for repair of Sn^{2+} -induced DNA lesions.

Lack of transcription factor Yap1p, responsible for the oxidative stress response in yeast, led to increase in Sn^{2+} -sensitivity. In addition, sensitivity of the superoxide dismutase mutants *sod1* and *sod2* revealed the importance of these anti-oxidative defence enzymes against Sn^{2+} -imposed DNA damage. The highest $SnCl_2$ -sensitivity, however, was observed in glucose-repressed pre-diauxic shift exponentially growing cells (LOG cells). It has been suggested that two independently acting anti-ROS protective systems (one mediated by glucose repression/de-repression, the other via ROS-inducible transcription activators) are working together, and that both contribute to Sn^{2+} -resistance.

To determine the concentration of Sn^{2+} ions cells of the *S. cerevisiae* and to correlate their quantity with the toxicity, the intracellular metal content of yeast cells was determined by Particle Induced X-ray emission (PIXE). A thick target protocol was developed for PIXE analysis. The PIXE analysis suggested that diploid (XS2316) cells absorbed about 60% of the total amount of Sn^{2+} absorbed by the haploid (XV185-14c) yeast cells. This indicates that Sn^{2+} absorption is better in haploid yeast cells and agrees well with published toxicity results. Finally, we

performed a screening in order to know the putative interactions between Sn^{2+} and other essential elements. According to PIXE analysis, the results for XV185-14c yeast cells indicate a significant loss of intracellular elements such as Mg, Zn, S, Fe and an increase of P levels after 1 h exposure to 25 mM $SnCl_2$ in STAT phase.

In order to verify how Sn^{2+} is uptaken, distributed and detoxified, we associated molecular techniques including survival assay and quantitative real time PCR (qRT-PCR) with PIXE. The survival assay showed that Fet4p and Zrt2p both low-affinity iron and zinc transporters, respectively, may internalize Sn^{2+}. By its turn, qRT-PCR showed an increased in *CCH1* and *MID1* gene expression, which encode for the cytoplasmic transmembrane Ca^{2+} transporters Cch1p and Mid1p, suggesting that both proteins may also take up Sn^{2+}. Our data also indicated that once inside the cell, Sn^{2+} may be taken up from the cytosol by a P-type ATPase to the vacuole (Pmc1p) or may be chelated by Crs5p metallothionein in the cytosol. Alternatively, Sn^{2+} may generate reactive oxygen species (ROS), especially O_2^- which can be dismutate to form H_2O_2 and the highly reactive $^{\bullet}OH$. In consequence, ROS can induce cellular injury and stress-generated activation of many genes, e.g., *SOD1*, *YAP1*, and *APN1*.

In order to clarify the molecular mechanisms of Sn^{2+} genotoxicity, we evaluated the induction of strand breaks, FPG and ENDO III sensitive sites, and the interference with the repair of MMS-caused DNA damage in V79 Chinese *hamster* lung fibroblasts exposed to stannous chloride by comet assay. Our results demonstrated that Sn^{2+} induced only a limited elevation in FPG and ENDO III sensitive sites in toxic concentrations. This suggests that stannous ion does not preferentially induce base modifications that are the substrate of FPG and ENDO III enzymes in V79 cells.

Although 50 μM $SnCl_2$ concentration did not increase significantly the DNA migration by itself in comet assay, it was capable to inhibit the repair of MMS-induced DNA damage during the pos-treatment period of 24h. Our results demonstrate the genotoxic and comutagenic effects of stannous chloride in V79

cells. The inhibitory effect of Sn^{2+} on repair of MMS-induced DNA damage suggests that this metal can also interfere in DNA repair systems thus contributing to increased mutation by shifting the balance from error-free to error-prone repair processes.

INTRODUÇÃO

Quimicamente, os metais são entidades pequenas, bem definidas e, aparentemente, simples (Beyersmann & Hartwig, 2008). No entanto, quando interagem com macromoléculas, tornam-se complexos e de difícil compreensão (Wackett *et al.*, 2004; Beyersmann & Hartwig, 2008). Vários metais são requeridos para o metabolismo, a transdução de sinais e os processos genéticos dos seres vivos, sendo, portanto, elementos químicos essenciais para as células (Eide, 2001; Puig *et al.*, 2007; Riccardi *et al.*, 2008).

Alguns metais, como cálcio, cobalto, cromo, cobre, ferro, potássio, magnésio, sódio, níquel e zinco, são essenciais, servem como micronutrientes, são utilizados em processos redox, estabilizam moléculas através de interações eletrostáticas, são componentes de várias enzimas e regulam a pressão osmótica (Wackett *et al.*, 2004). A transferência de elétrons do ferro e do cobre é fundamental para os processos de respiração e fotossíntese (Rutherford & Bird, 2004; Bird, 2008). O elemento zinco forma centros catalíticos em numerosas enzimas, responsáveis pela ligação ao DNA (Eide, 2006).

O desequilíbrio na concentração intracelular de determinados metais está relacionado a doenças humanas importantes, como hemocromatosis, acrodermatites enteropáticas, anemia severa, doença de Menkes e doença de Wilson (Peña *et al.*, 1999; Wang *et al.*, 2004; Nose *et al.*, 2006; Edison *et al.*, 2008). Além dessas patologias, hereditárias ou adquiridas, tem-se pesquisado cada vez mais a relação entre metais e doenças neurodegenerativas (Waggoner *et al.*, 1999; Salazar *et al.*, 2008).

Na agricultura, os metais desempenham papel crucial na qualidade do solo (Puig *et al.*, 2007; Chen *et al.*, 2008). A deficiência de cálcio, magnésio, ferro, zinco ou cobre pode ser responsável pelo menor desenvolvimento das plantas em solos ácidos (Kim & Guerinot, 2007; Jeong & Guerinot, 2008; Kim *et al.*, 2008). As características físicas e químicas do solo são os principais fatores

que condicionam o desenvolvimento do plantio (Curie & Briat, 2003; Briat *et al.*, 2007). Os solos ácidos e com baixa disponibilidade de nutrientes caracterizam-se pela presença de alguns metais em concentrações tóxicas, constituindo um fator limitante do crescimento vegetal (Grotz & Guerinot, 2006; Chen *et al.*, 2008).

Os metais também são essenciais para o crescimento de microrganismos, estando envolvidos em determinantes de patogenicidade e na capacidade de microrganismos de vida livre competirem com outros organismos por fontes limitadas de ambientes (Posey & Gherardini, 2000; Wackett *et al.*, 2004). Por exemplo, a aderência do protozoário parasita *Trichomonas vaginalis*, causador de uma das mais comuns doenças sexualmente transmissíveis, a tricomoníase, depende dos níveis de ferro presentes no epitélio vaginal (Ong *et al.*, 2007).

Por outro lado, muitos metais que não apresentam funções biológicas conhecidas e não são essenciais podem apresentar um alto potencial tóxico para os microrganismos (Silver & Phung, 2005). A toxicidade desses metais ocorre através do deslocamento de metais essenciais de seus sítios de ligação, ou de interações inapropriadas com formação de complexos, que modificam a atividade biológica de alvos citológicos (Beyersmann & Hartwig, 2008).

Além disso, alguns íons metálicos podem afetar o funcionamento celular, atuando nos ciclos redox e produzindo espécies reativas de oxigênio (EROs), altamente nocivas para as células (Leonard *et al.*, 2004a,b). Portanto, em adição aos elevados níveis, tanto de metais essenciais, quanto de metais não-essenciais, pode haver danos à membrana das células, alteração na especificidade de enzimas, parada de funções celulares e danos à estrutura do DNA (Wiesenberger *et al.*, 2007; Beyersmann & Hartwig, 2008).

Para apresentar efeito fisiológico ou tóxico, a maioria dos íons metálicos necessita entrar na célula. Muitos cátions metálicos divalentes (Mn^{2+}, Mg^{2+}, Ca^{2+}, Fe^{2+} e Zn^{2+}) são estruturalmente muito similares. Para que um sistema de captação de metais consiga diferenciar íons metálicos estruturalmente parecidos, há a necessidade de um refinado sistema de regulação (para revisão,

ver Eide, 1998; Gaither & Eide, 2001; Van Ho *et al.*, 2002;). Dessa forma, a evolução favoreceu a sobrevivência de organismos que desenvolveram mecanismos capazes de garantir a concentração de metais intracelular ótima, através de um balanço entre a entrada de metais do ambiente e a excreção/neutralização dos mesmos na célula (Puig *et al.*, 2008). Esse refinado balanço é chamado de homeostase de metais (Eide *et al.*, 2005).

Os progressos realizados para compreender os mecanismos de ação dos metais, essenciais e não-essenciais, estão sendo alcançados em diversos organismos, especialmente com células de mamíferos. Contudo, a levedura *Saccharomyces cerevisiae* continua sendo um excelente modelo para o estudo de metais (Resnick & Cox, 2000; Vergara & Thiele, 2008; Lauer Júnior *et al.*, 2008; Mielniczki-Pereira *et al.*, 2008), porque muitas das proteínas envolvidas no metabolismo de metais (importação, distribuição, destoxificação e exportação) são conservadas, desde leveduras até humanos (Van Ho *et al.*, 2002; De Freitas *et al.*, 2003).

Uma das principais características que faz com que a levedura seja apropriada para estudos biológicos é que ela teve a sequência completa de seu genoma conhecida em 1996 (Goffeau, 1996). Com aproximadamente 6.200 genes organizados num genoma compacto, seus genes e suas correspondentes funções têm sido anotados e catalogados no banco de dados *Saccharomyces Genome Database*, disponível *on line* através do site http://www.yeastgenome.org/. Mutações recessivas podem ser convenientemente isoladas e manifestadas em linhagens haplóides, estando, esses mutantes individuais e a coleção completa, disponíveis comercialmente para pesquisas na *American Type Culture Collection* (ATTC), *Research Genetics* (Invitrogen) ou *European Saccharomyces cerevisiae Archives for Functional Analysis* (EUROSCARF).

Outras propriedades que fazem da levedura um modelo de estudo eucariótico são: (1) crescimento rápido; (2) organismo unicelular; e (3) organismo não-patogênico. Dessa maneira, técnicas bem definidas e elucidativas têm sido utilizadas nos estudos de regulação gênica de metais até estrutura/função de

proteínas, incluindo sistemas de transporte, distribuição, armazenamento e excreção de metais (Burke *et al.*, 2000; Simm *et al.*, 2007; Bird, 2008).

1 O estanho e seus compostos

O estanho (Sn) é um metal do grupo 14 (grupo IV A) da tabela periódica, com peso atômico de 118,71. O seu ponto de fusão é de 231,9°C, e o seu ponto de ebulição é de 2507°C. Abaixo do seu ponto de fusão, o estanho pode existir em três formas alotrópicas: (1) estanho cinza (estável, abaixo de 13°C); (2) estanho branco (forma metálica maleável e dúctil); e (3) estanho quebradiço ou frágil, que é a forma γ-Sn. Os óxidos de estanho são anfóteros, que, comumente, formam sais estanoso (estado de oxidação 2+) e estânico (estado de oxidação 4+). O estado de oxidação 3+ é extremamente instável (Budavari, 2001).

A crosta terrestre contém aproximadamente 2-3 ppm de estanho, representando um percentual de 0,0006% (Budavari, 2001). A mais importante forma de estanho encontrada na Terra é a forma mineral cassiterita (SnO_2). O estanho, por ser um metal extremamente resistente à maresia, é utilizado para revestir outros metais, protegendo-os da ação da corrosão (El-Makawy *et al.*, 2008). Ele é empregado na composição de vários tipos de ligas com chumbo, cobre, bronze e peltre (liga de estanho utilizada para confeccionar utensílios desse material, como jarros, canecas e potes) (Rüdel, 2003).

Por ser altamente empregado na constituição de ligas metálicas, o estanho está presente nas obturações dentárias e, juntamente com mercúrio, prata, zinco e cobre, forma a amálgama dentária (Von Mayenburg *et al.*, 1991). O estanho também está presente, em quantidades pequenas, em imitações de jóias (Vilaplana *et al.*, 1991; Olivarius *et al.*, 1993).

Na indústria alimentícia, o estanho metálico é utilizado principalmente para revestir latas que acondicionam alimentos e líquidos. As

folhas de flandres (folha de ferro estanhado) são utilizadas no revestimento interno de latas para a conservação dos alimentos (De Groot *et al.*, 1973; Ikem & Egiebor, 2005; Mino, 2006). Essas folhas protegem os alimentos de processos oxidativos, já que é o estanho que sofre oxidação e permite que a cor e o sabor dos alimentos permaneçam inalterados (Blunden & Wallace, 2003). Esse processo garante a qualidade que o consumidor almeja e a durabilidade do produto que os fabricantes esperam.

Em muitos casos, pode haver a transferência de ferro e estanho das folhas de flandres para os alimentos. O grau de transferência dos metais depende de vários fatores, tais como o tipo de produto, o tempo, a temperatura de armazenagem e de o revestimento interno ser plano ou esmaltado (Perring & Basic-Dvorzak, 2002; Mino, 2006). Muitos estudos têm mostrado que, quando os enlatados são abertos, e os alimentos permanecem dentro das latas com posterior refrigeração, aumenta a oxidação do estanho e, consequentemente, a transferência desse metal para os produtos (Perring & Basic-Dvorzak, 2002). O processo de corrosão é aumentado pelo ar, o qual promove o ataque do estanho por ácidos (Boogaard *et al.*, 2003). Portanto, agentes oxidantes, tais como ar, sais férricos, nitratos, sais cúpricos, certos pigmentos e frutas ácidas, promovem a corrosão (Schroeder *et al.*, 1964; Perring & Basic-Dvorzak, 2002; Boogaard *et al.*, 2003).

Os compostos inorgânicos de estanho são empregados na indústria de vidros para fortalecê-los, evitando fissuras. Os compostos inorgânicos servem como bases para as formulações de pigmentos, catalisadores e, na indústria de cosméticos, na fabricação de perfumes e sabões (Rüdel, 2003; Blunden & Wallace, 2003). O óxido de estanho IV (SnO_2) é utilizado nas indústrias de cerâmica e de vidros, sendo agente de polimento e catalisador, empregado na produção de vidros coloridos e esbranquiçados (Blunden & Wallace, 2003). O fluoreto estanoso (SnF_2), por sua vez, está presente nos produtos odontológicos (pastas de dentes) como agente cariostático e também como agente que reduz a dissolução ácida do esmalte dos dentes (Rölla *et al.*, 1983).

Os radiofármacos de tecnécio-99m (^{99m}Tc) tornaram-se, nos últimos trinta anos, importantes ferramentas para o diagnóstico de várias doenças ou disfunções de órgãos e sistemas que compõem o corpo humano (Marques *et al.*, 2001). Os radiofármacos são desenhados através de uma seleção cuidadosa de biomoléculas que se ligam a receptores específicos de órgãos alvo. A fixação do ^{99m}Tc nas macromoléculas de interesse biológico depende da presença de agentes redutores, sendo o cloreto estanoso ($SnCl_2$) o mais frequentemente usado (Bernardo-Filho *et al.*, 1994; Guedes *et al.*, 2006). Tanto o fluoreto estanoso, quanto o cloreto estanoso, são os sais estanhados mais empregados nas pesquisas referentes ao estanho (Yousef, 2005; El-Demerdash *et al.*, 2005; Guedes *et al.*, 2006; Yousef *et al.*, 2007; El-Makawy *et al.*, 2008). A **figura 1** mostra a ligação do radionuclídeo com o fármaco de interesse para formar o radiofármaco:

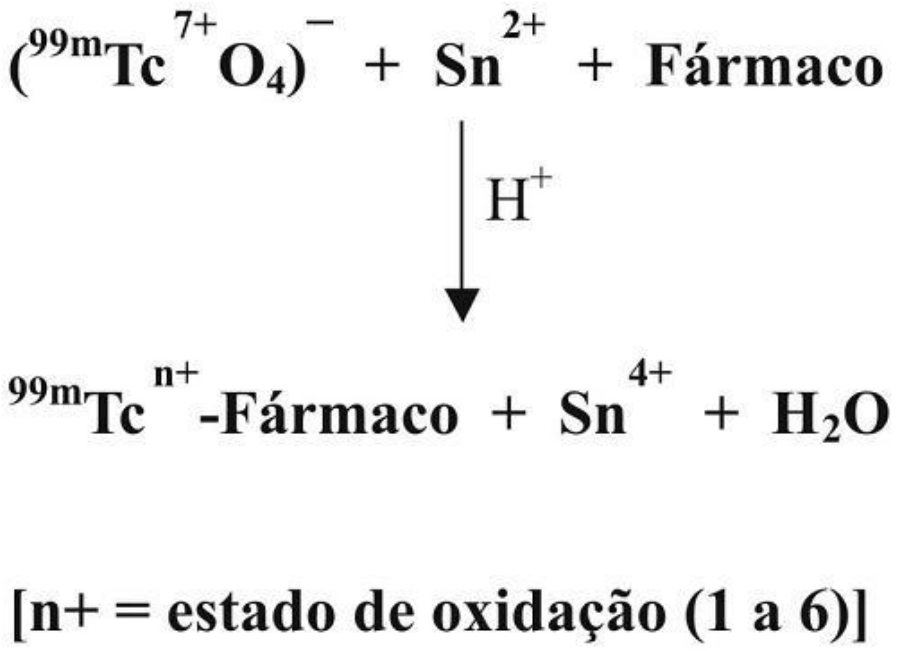

Figura 1: Esquema geral da reação de oxirredução e complexação para a obtenção dos radiofármacos de tecnécio-99m. Figura adaptada de Marques *et al.*, 2001.

O cloreto estânico ($SnCl_4$) é comumente utilizado como material de partida para a produção de compostos orgânicos de estanho (organoestanhados). Exemplos de compostos orgânicos de estanho de importância comercial são: (1) tetraorganoestanhados (R_4Sn); (2) triorganoestanhados (R_3SnX); (3) diorganoestanhados (R_2SnX_2); e (4) mono-organoestanhados ($RSnX_3$). O grupamento R pode ser, dentre outros, metil, butil, octil e fenil. O radical X, por sua vez, pode ser halogênio, óxido, hidróxido, carboxilato, entre outros.

Os compostos tri e diorganoestanhados são os principais compostos orgânicos de estanho empregados comercialmente. Os compostos triorganoestanhados são encontrados em várias preparações biocidas, como fungicidas, inseticidas, herbicidas e molusquicidas, bem como em preservativos da madeira e nas formulações destinadas ao tratamento de cascos de navios e barcos para proteger a parte dos mesmos que fica submersa (Ehman *et al.*, 2007; Moser *et al.*, 2009).

Os compostos diorganoestanhados são utilizados como estabilizadores do cloreto de polivinila (PVC), possibilitando que o mesmo possa ser aquecido e moldado para diferentes fins. Os estabilizadores protegem o PVC do calor e da luz. Esses compostos também estão presentes em reações de esterificação como catalisadores químicos e podem ser espumantes em poliuretano. Os compostos mono-organoestanhados são igualmente empregados como estabilizadores no processo de aquecimento do PVC, assim como na indústria de vidros (Dopp *et al.*, 2007; Moser *et al.*, 2009).

Os compostos orgânicos de estanho também são aplicados como constituintes de desinfetantes, fertilizantes para a agricultura e removedores de limo, tendo, ainda, aplicação em fábricas de cerveja e nas indústrias têxtil, de papel e de processamento do couro (Schroeder *et al.*, 1964; Moser *et al.*, 2009).

2 Exposição ao estanho e legislação vigente

O estanho está presente no ar, na água, no solo, bem como em plantas e animais que vivem na terra ou nas águas. Por ser um elemento químico de ocorrência natural na atmosfera, o estanho pode estar presente nos tecidos humanos (Schroeder *et al.*, 1964; Rüdel, 2003). No entanto, não há evidências científicas de que o estanho seja um elemento essencial para o ser humano, tampouco que haja uma dose diária de consumo recomendada (Boogaard *et al.*, 2003).

A quantidade de estanho a que o homem está exposto depende de vários fatores (para revisão, ver Calloway & McMullen, 1966). As três grandes vias de exposição são a inalatória, a parenteral e a oral, sendo que a oral é a principal via de exposição ao estanho e seus compostos, através do consumo de alimentos enlatados e da ingestão de líquidos enlatados (Boogaard *et al.*, 2003; Ikem & Egiebor, 2005).

Nos alimentos em geral, os níveis de estanho encontrados são inferiores a 4 μg de Sn/g de alimento seco. Em embalagens metálicas, a quantidade de estanho é de 20 a 25 μg de Sn/g de alimento seco (Rader, 1991). Quantidades maiores podem ser encontradas no caso de dietas que incluem uma alta proporção de vegetais e peixes enlatados, nas quais o consumo de estanho pode chegar a cerca de 30 mg de Sn/dia (Rader, 1991).

O limite máximo de ingestão diária para compostos de estanho admitido pela FDA (*Food and Drug Administration*) nos EUA é de 300 μg de Sn/g de alimento seco (Chmielnicka *et al.*, 1981; Burba, 1983). No Reino Unido, esse limite é de 200 μg de Sn/g de alimento seco e 150 μg de Sn/g de líquido seco (Blunden & Wallace, 2003). A Associação Brasileira das Indústrias da Alimentação (ABIA) preconiza um limite de 250 μg de Sn/g de alimento seco, de acordo com a Portaria nº 685, emitida pelo Ministério da Saúde/Secretaria de Vigilância Sanitária, em 27/08/1998 (Publicação no D.O.U. de 28 de agosto de 1998).

Como o estanho é encontrado naturalmente no ambiente, é impossível eliminar totalmente a exposição a esse metal, mas alguns cuidados podem ser empregados para minimizá-la, tais como: (1) diminuir a ingestão de comidas enlatadas, ou, se utilizadas, uma vez abertas, acondicioná-las em embalagens de vidro; (2) reduzir a ingestão de peixes enlatados, tendo em vista que podem advir de águas contaminadas; e (3) evitar o uso de utensílios com revestimento de silicone ao cozinhar, porque contém quantidades consideráveis de compostos organoestanhados.

3 Toxicocinética e toxicidade do estanho

Uma característica de importância biológica desse metal consiste na sua capacidade de formar compostos catiônicos organometálicos de alta solubilidade lipídica, o que o capacita a atravessar membranas biológicas e a exercer seus efeitos tóxicos no interior das células (McLean *et al.*, 1983a,b; Hamasaki *et al.*, 1993).

A absorção, distribuição e retenção de estanho em humanos (Schroeder *et al.*, 1964; Solomons, 1982; Solomons *et al.*, 1983), bem como em animais experimentais de laboratório (Blunden & Wallace, 2003), são dependentes da natureza da exposição. Quando administrado via oral, o estanho é fracamente absorvido no trato gastrointestinal, sendo que mais de 90% do ingerido são eliminados através das fezes e da urina (Blunden & Wallace, 2003).

O estado de valência do estanho pode influenciar a absorção. O estanho divalente (Sn^{2+}) apresenta maior absorção quando comparado com o estanho tetravalente (Sn^{4+}), o que se deve ao fato de o íon Sn^{4+} possuir maior facilidade para formar compostos insolúveis em soluções aquosas do que o íon Sn^{2+} (McLean *et al.*, 1983a).

O estanho apresenta ampla distribuição nos tecidos humanos. Após realizarem a autópsia de vários órgãos humanos, Hamilton *et al.* (1972)

detectaram a presença desse metal nos seguintes locais: cérebro, rins, pulmões, fígado, linfonodos, músculos, testículos, ovários e ossos. Os pesquisadores Fischer & Zimmerman (1969) e Kutzner & Brod (1971) notaram, igualmente, que a distribuição de estanho é bem variada e atinge vários tecidos em animais experimentais de laboratório.

No entanto, estudos mais recentes demonstraram que os órgãos preferenciais são os rins, o fígado (Chiba *et al.*, 1984; Bluden & Wallace, 2003) e os ossos (Yamaguchi *et al.*, 1980; Yamaguchi *et al.*, 1981; Hattori *et al.*, 2001). O estanho é excretado principalmente por meio das fezes e da urina (Bluden & Wallace, 2003), existindo uma pequena fração que pode ser excretada através do leite materno e da bile (Hamilton *et al.*, 1972).

A toxicologia dos compostos de estanho tem sido revisada por muitos pesquisadores. Sabe-se que a ingestão de excessiva quantidade de estanho se deve ao crescente consumo de produtos enlatados. O estanho pode causar irritações gastrointestinais, diarréias, vômitos, náuseas, problemas renais e hepáticos, irritações na pele e olhos (para revisão, ver Blunden & Wallace, 2003; Ikem & Egiebor, 2005).

Estudos têm demonstrado que o estanho pode afetar o metabolismo e os níveis de metais essenciais para a célula. Johnson *et al.* (1982) realizaram estudo clínico com adultos e concluíram que o estanho afeta o metabolismo do zinco. Solomons *et al.* (1983) também realizaram estudo clínico com adultos e notaram que o estanho interfere na excreção fecal do zinco e do ferro. O estudo comprovou que, em alguns voluntários, houve a manifestação de distúrbios gastrointestinas, como náuseas e diarréia. O estudo enfatizou que pessoas em estágio nutricional marginal (gestantes, idosos e crianças) devem ter cuidado com a ingestão de comidas enlatadas.

Yamaguchi *et al.* (1982) estudaram os efeitos do estanho durante um mês em ratos e notaram que há um decréscimo do conteúdo de cálcio na epífise femoral, o que, consequentemente, inibe a formação óssea. Chiba *et al.* (1984), utilizando coelhos adultos, demonstraram que, após administração de estanho

durante uma semana, os níveis de cobre, magnésio, zinco e cálcio decresceram. Vários outros grupos de pesquisa demonstraram que o metabolismo de metais essenciais pode sofrer alterações após exposição ao estanho (Zareba & Chmielnicka, 1989; Hattori *et al.*, 2001).

Além de perturbar o metabolismo de metais essencias, o estanho pode manifestar a sua toxicidade através da formação de EROs (Pungartnik *et al.*, 2005; Yousef, 2005; El-Demerdash *et al.*, 2005; Yousef *et al.*, 2007; El-Makawy *et al.*, 2008). Embora cada metal apresente seu próprio mecanismo de ação (Leonard *et al.*, 2004a), a geração de EROs e, posteriormente, os efeitos na sinalização celular, parecem ser um mecanismo comum a todos os metais (Leonard *et al.*, 2004b).

McLean *et al.* (1983b) sugeriram que o estanho pode catalisar a conversão do oxigênio molecular (O_2) em ânion superóxido ($O_2^{\cdot-}$), ou em peróxido de hidrogênio (H_2O_2). O H_2O_2, também formado durante o metabolismo celular, pode ser convertido no radical hidroxil ($^{\cdot}OH$). Os mesmos autores observaram que tanto o estanho (Sn^{2+}/Sn^{3+}) quanto o ferro (Fe^{2+}/Fe^{3+}) apresentam potenciais de ionização semelhantes (30,498 eV/mol e 30,643 eV/mol, respectivamente), motivo pelo qual o estanho pode exercer a sua toxicidade indiretamente através da formação de $^{\cdot}OH$, lesando macromoléculas importantes para a célula, como a molécula de DNA.

Dantas *et al.* (1996), utilizando um modelo procariótico de estudo (*Escherichia coli*), inferiram que o $SnCl_2$ gera EROs, uma vez que a citotoxicidade dessa linhagem havia sido revertida na presença de diferentes agentes quelantes. El-Demerdash *et al.* (2005), utilizando coelhos como modelo experimental, avaliaram a peroxidação lipídica pela concentração dos produtos de reação com o ácido tiobarbitúrico (TBARS), a atividade da enzima glutationa S-transferase (GST) e os níveis de grupos sulfidril (SH), como parâmetros de estresse oxidativo. Eles concluíram que os coelhos tratados com $SnCl_2$ apresentaram um aumento de

TBARS, diminuição da atividade enzimática da proteína GST e diminuição dos níveis de SH.

Yousef (2005), ao estudar o efeito espermatóxico do $SnCl_2$ em coelhos machos, inferiu que o antioxidante ácido ascórbico (AA) protege parâmetros reprodutivos (motilidade dos espermatozóides, volume de espermatozóides ejaculado, total de espermatozóides normais e mortos, dentre outros) após tratamento concomitante AA + $SnCl_2$.

Yousef *et al.* (2007), em estudo mais amplo, também utilizando coelhos, testaram outras enzimas antioxidantes, como a superóxido dismutase (SOD) e a catalase (CAT), e outros parâmetros bioquímicos, como a aspartato transaminase e a alanina transaminase. Os pesquisadores concluíram que houve diminuição da atividade enzimática de SOD e CAT e aumento dos parâmetros bioquímicos após o grupo teste receber 20 mg de $SnCl_2$ por peso corporal do coelho. Novamente, o estudo empregou AA concomitantemente com $SnCl_2$, notando-se o efeito protetor do AA contra o dano oxidativo gerado pelo $SnCl_2$.

4 Efeitos genotóxicos do estanho

Com relação à genotoxicidade, à mutagenicidade e à carcinogenicidade dos sais de estanho, os resultados descritos na literatura, obtidos a partir de metodologias diferentes, são conflitantes. Nishioka, em 1975, utilizando *Bacillus subtilis* (*Rec assay test*), não constatou efeito mutagênico na concentração de 0,05 M de $SnCl_2$. Mais tarde, Kada *et al.* (1980), empregando o mesmo teste, verificaram que o $SnCl_2$, nas doses de 10 mM e 10 M, não foi mutagênico.

Singh (1983) mostrou que o $SnCl_2$, na concentração de 100 mM, não foi capaz de induzir mutação reversa em células de levedura *S. cerevisiae*. Posteriormente, Tripathy *et al.* (1990) não observaram efeitos genotóxicos do $SnCl_2$ nas concentrações de 50 e 100 mM em células primordiais de *Drosophila*

melanogaster (*Wing spot test*). Os resultados do estudo realizado por Ashby & Tennant (1991), quanto à relação entre a estrutura química e as atividades de mutagenicidade e carcinogenicidade de 301 químicos para o Programa de Toxicologia Nacional dos Estados Unidos (*US-NTP*, *US-National Toxicology Program*), permitiram classificar o $SnCl_2$ em não-mutagênico para o teste de *Salmonella*/Microssoma e carcinogênico para o teste com a glândula tireoide de ratos.

Por outro lado, McLean *et al.* (1983a,b) descreveram que o $SnCl_2$ produz quebras de cadeia simples no DNA, tanto em células ovarianas de *hamster* chinês, quanto em células sanguíneas humanas. O mesmo grupo sugeriu que o íon metálico Sn^{2+} pode formar adutos com o DNA, o que se deve ao fato desse íon, em razão das suas propriedades físico-químicas, formar espécies neutras ou aniônicas, as quais são excelentes doadoras de elétrons. Ademais, esse íon é capaz de coordenar-se com átomos de oxigênio, nitrogênio e fósforo (elementos que compõem o material genético da célula) para formar compostos covalentes.

Os adutos são formados através de ligações covalentes de determinadas classes de substâncias químicas com as bases nitrogenadas do DNA (Schärer, 2005; Räschle *et al.*, 2008). Quimicamente, os compostos ou agentes formadores de adutos podem ser separados em dois grupos: (1) agentes monofuncionais, quando induzem a formação de ligação covalente entre um composto químico e apenas uma base nitrogenada (adutos monofuncionais); e (2) agentes bi ou polifuncionais, quando a substância química possui a habilidade de se ligar covalentemente a duas bases nitrogenadas (adutos bifuncionais), estando elas situadas na mesma fita de DNA (pontes intracadeia) ou em fitas separadas (pontes intercadeias – *interstrand cross-links* ou ICLs) (Sharer, 2005).

Somando-se aos relatos de genotoxicidade dos sais de estanho, Olivier & Marzin (1987) e Bernardo-Filho *et al.* (1994), utilizando o cromoteste SOS, constataram que o $SnCl_2$ e o SnF_2 são capazes de induzir as funções SOS. Dantas *et al.* (1996, 1999) e De Mattos *et al.* (2000) sugeriram que a genotoxicidade induzida *in vivo* pelo $SnCl_2$, em diferentes linhagens de *E. coli*

deficientes em sistemas de reparação de DNA, e as quebras de cadeias de DNA, demonstradas nos estudos *in vitro* de alterações da topologia de DNA plasmidial, ocorrem via formação de EROs.

Cabral *et al.* (1998), analisando o espectro mutacional do $SnCl_2$ em nível molecular, constataram que esse sal gera transversões, transições e deleções em plasmídeo pAC 189. Os autores inferiram que esse efeito mutagênico ocorre, principalmente, via oxidação do DNA e produção de 8-hidroxiguanina (8-oxoG). Do mesmo modo, Caldeira-de-Araújo *et al.* (1996) já tinham evidenciado o aumento da sobrevivência de linhagens de *E. coli* ao $SnCl_2$ na presença de catalase e agentes captadores de metais.

Mais recentemente, Silva *et al.* (2002) observaram que extratos de plantas medicinais (*Cymbopogon citratus*, *Baccharis genistelloides*, *Maytenus ilicifolia*, *Peumus boldus* (*boldine*)) podem reagir com os íons Sn^{2+}, evitando a geração de EROs e, consequentemente, proteger as células de *E. coli* dos efeitos pró-oxidantes do $SnCl_2$. Simões *et al.* (2006) realizaram um estudo similar ao anterior e notaram que extratos de folhas e caule de *Cleome rosea* protegeram o DNA plasmidial pUC 9.1 de ser clivado por 200 µg/mL de $SnCl_2$.

De Mattos *et al.* (2005) fizeram um estudo *in vitro* com DNA de timo de bezerro, nucleotídeos trifosfatados (ATP, TTP, CTP e GTP) e bases nitrogenadas isoladas (adenina, citosina, timina e guanina). Ao observarem o efeito hipercrômico no gráfico de espectroscopia de absorção no ultravioleta, os pesquisadores inferiram que a concentração de 100 µg/mL de $SnCl_2$ foi capaz de desnaturar as fitas duplas de DNA. Em adição, eles propuseram que o íon metálico Sn^{2+} interage diretamente em regiões polianiônicas no DNA, promovendo a abertura da dupla-hélice e expondo as bases nitrogenadas, discutindo, ainda, que pudesse haver geração de EROs nessa região, agravando a ação genotóxica do $SnCl_2$. À exceção da base timina, todas as bases nitrogenadas e todos os nucleotídeos trifosfatados apresentaram alteração no espectro quando tratados com $4,51 \times 10^{-4}$ µg/mL de $SnCl_2$.

Guedes *et al.* (2006) estudaram a ação citotóxica e genotóxica isolada do $SnCl_2$ e conjunta com os *kits* de cintilografia MDP (metilenodifosfonato) e DTPA (ácido dietilenotriaminopentaacético). Linhagens selvagens de *E. coli* foram sensíveis a 1,11 X 10^{-3} M de $SnCl_2$ e resistentes à associação de 1,11 X 10^{-3} M de $SnCl_2$ com 9,49 X 10^{-3} MDP e à associação de 1,11 X 10^{-3} M de $SnCl_2$ com 1,01 X 10^{-3} DTPA. Além dos estudos em procariotos, o grupo estudou as mesmas concentrações mencionadas acima no estudo *in vitro* com DNA plasmidial pUC 9.1. Houve clivagem do DNA plasmidial somente quando tratado isoladamente com $SnCl_2$. Os autores concluíram que há efeito protetor dos kits, o qual mascara a ação isolada do $SnCl_2$. Eles enfatizaram a importância de estudar os efeitos colaterais desse potente agente redutor, empregado em 95% das formulações cintilográficas usadas na medicina nuclear.

Pungartnik *et al.* (2005) estudaram a genotoxicidade do $SnCl_2$ através dos ensaios de *Salmonella*/Microssoma, WP2 Mutoxiteste e da utilização de linhagens haplóides e diplóides de *S. cerevisiae*. No teste de *Salmonella*/Microssoma, o $SnCl_2$ não induziu mutação no quadro de leitura (TA97 e TA98), tampouco substituição de pares de bases (TA100), ao passo que uma resposta positiva foi observada com a linhagem TA102, que detecta mutagênicos oxidativos (Mortelmans e Zeiger, 2000).

No WP2 Mutoxiteste, que emprega linhagem de *Escherichia coli* proficiente no fator de transcrição OxyR (IC188), responsável pelo sistema de resposta adaptativa ao estresse oxidativo, e linhagem deficiente nesse sistema (IC203) (para revisão, ver Martinez *et al.*, 2000), o $SnCl_2$ induziu mutação na linhagem IC203 (*uvrA oxyR*), e não na linhagem IC188 (*uvrA*).

Ao empregar linhagens haplóides e diplóides de *S. cerevisiae*, Pungartnik *et al.* (2005) inferiram que o $SnCl_2$ é um agente mutagênico e convertogênico. Além disso, ao estudar os possíveis tipos de lesões geradas por esse agente químico, o mesmo grupo de pesquisa constatou que o mutante *rad52*Δ, deficiente no mecanismo de reparação recombinacional, incapaz de reparar quebras simples e duplas no DNA, foi o mais sensível.

5 Efeitos citotóxicos em células de *Saccharomyces cerevisiae*, deficientes em sistemas de reparação de DNA

A manutenção da integridade do material genético é fundamental à vida de todos os organismos (Budd *et al.*, 2005). Entre os mecanismos responsáveis por essa manutenção, estão os sistemas de reparação de DNA, os quais apresentam íntima inter-relação com os mecanismos de replicação do DNA (Budd & Campbell, 2000). Esses mecanismos de reparação protegem o DNA de agressões por agentes físicos, como a luz ultravioleta (UVC 254 nm), da ação de agentes químicos, como o metilmetanosulfonato (MMS), dos subprodutos do metabolismo normal da célula, principalmente das EROs, e de alterações ou lesões que podem ocorrer espontaneamente devido à instabilidade das ligações químicas dos nucleotídeos (Friedberg, 2003; Friedberg *et al.*, 2004). A **figura 2** ilustra os principais tipos de lesões ao DNA e a sinalização desencadeada para que haja uma resposta a esses danos:

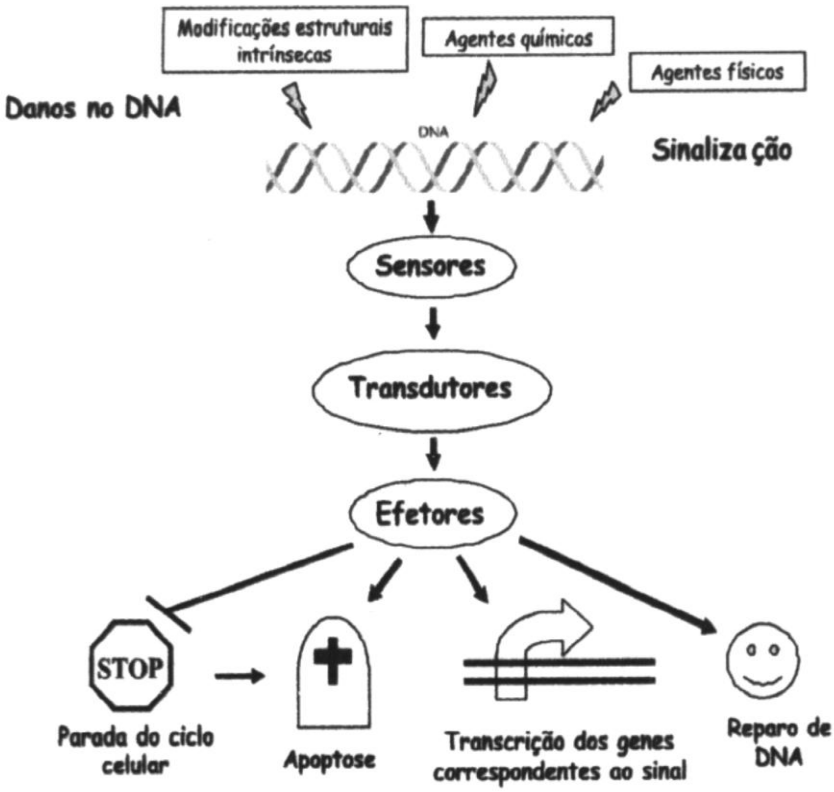

Figura 2: Esquema geral dos mecanismos propostos para sinalização da presença de danos no DNA. As setas representam eventos de ativação, enquanto que a seta cortada representa o evento de inibição. Figura adaptada de Berra & Menck, 2006.

Os sistemas de reparação do DNA, sejam por correção ou tolerância a lesões, foram descritos em todos os seres vivos. A caracterização de vários genes, funcionalmente relacionados à reparação do DNA, revelou alta conservação desses sistemas em espécies filogeneticamente distantes, demonstrando a sua importância ao longo da escala evolutiva (Hoeijmakers, 1993a,b).

A reparação do DNA requer a participação de várias proteínas que, juntas, compõem vias ou mecanismos altamente especializados na reparação de

determinados tipos de lesões (Eisen & Hanawalt, 1999). Sabe-se que algumas vias são usadas para reparar apenas um tipo de dano, enquanto outras são mais abrangentes em suas atuações enzimáticas (Friedberg *et al.*, 2004). Da mesma forma, algumas vias de reparação possuem mecanismos de atuação simples, requerendo apenas algumas enzimas, ao passo que outras são bastante complexas, envolvendo muitos passos enzimáticos e diferentes tipos de complexos proteicos (Eisen & Hanawalt, 1999). Essa diversidade de vias pode ser melhor compreendida quando as mesmas são agrupadas levando-se em conta os mecanismos de ação. Assim, três vias são conhecidas atualmente: a reparação direta, a reparação por excisão e a reparação recombinacional (Eisen & Hanawalt, 1999; Friedberg, 2003).

A reparação direta envolve dois mecanismos principais: (1) a fotorreativação, catalisada por enzimas pertencentes à família das fotoliases/criptocromos (Thompson & Sancar, 2002; Friedberg, 2008); e (2) a reversão de bases alquiladas, catalisadas pelas DNA metiltransferases (Kaina & Christmann, 2002; Christmann *et al.*, 2003). A **figura 3** mostra exemplo de reparação direta, na qual dímeros de pirimidina são reparados pela enzima fotoliase:

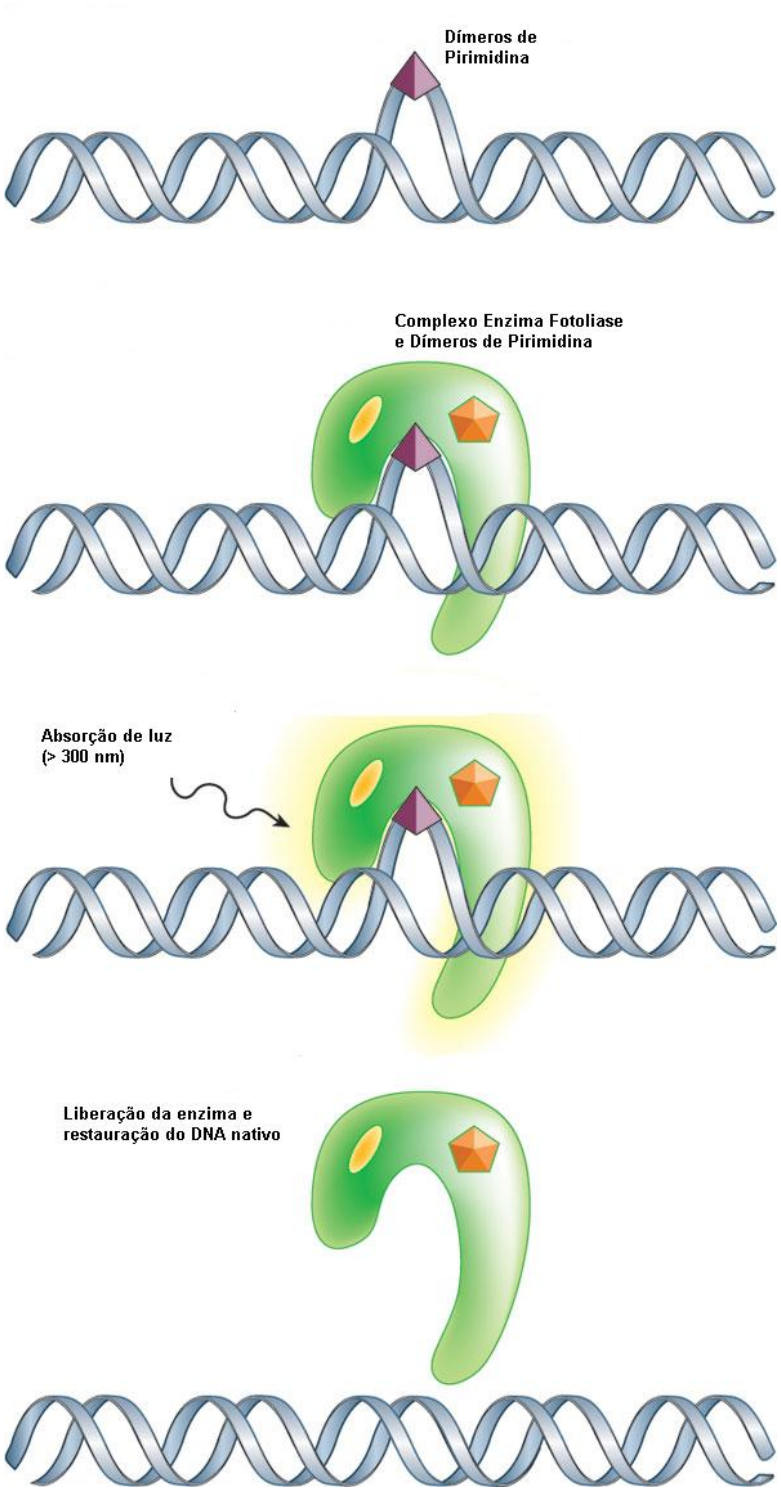

Figura 3: Reversão do dano ao DNA por fotorreativação. O DNA exposto à radiação ultravioleta proporciona a formação de dimerização covalente de pirimidinas adjacentes (dímeros de timina). Essas lesões são reconhecidas pela enzima que monomeriza esses dímeros, restabelecendo a conformação nativa do DNA. Figura adaptada de Friedberg, 2003.

A reparação por excisão-ressíntese é formada por três mecanismos principais: (1) a excisão de bases (*base excision repair* ou BER, para revisão, ver Boiteux & Guillet, 2004); (2) a excisão de nucleotídeos (*nucleotide excision repair* ou NER, para revisão, ver Prakash & Prakash, 2000; Christmann *et al.*, 2003); e (3) a reparação de erros de emparelhamento de bases (*mismatch repair* ou MMR, para revisão, ver Christmann *et al.*, 2003). Tanto as vias de reparação direta, quanto a de excisão, são essenciais para a remoção de bases modificadas e adutos, de forma que respondem por grande parte da atividade de reparação de DNA na célula (Memisoglu & Samson, 2000).

A excisão de bases (BER) é responsável pela reparação de bases nitrogenadas e açúcares modificados dos nucleotídeos trifosfatados (Dizdaroglu, 2005; Dizdaroglu *et al.*, 2008). BER envolve a formação de uma série de complexos enzimáticos e consiste, basicamente, em quatro etapas: (1) reconhecimento da lesão e clivagem da ligação glicosídica por uma DNA glicosilase; (2) clivagem da ligação fosfodiester por uma DNA endonuclease; (3) formação do sítio AP (sítio apurínico ou apirimidínico); e (4) polimerização e posterior ligação do nucleotídeo removido por uma DNA polimerase e DNA ligase, respectivamente. A **figura 4** ilustra o mecanismo BER:

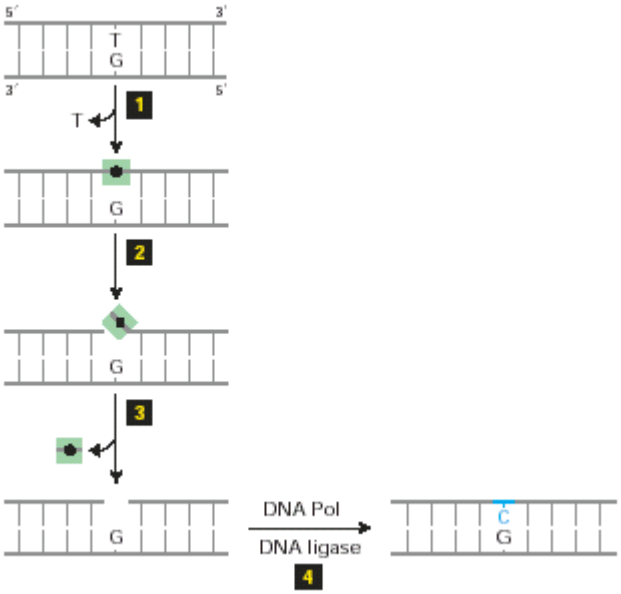

Figura 4: Representação esquemática do mecanismo de excisão de bases (BER) que envolve, basicamente, três etapas: (1) reconhecimento da lesão e clivagem da ligação glicosídica por uma DNA glicosilase; (2) clivagem da ligação fosfodiester por uma DNA endonuclease (3) formação do sítio AP (sítio apurínico ou apirimidínico); e (4) polimerização e posterior ligação do nucleotídeo removido por uma DNA polimerase e DNA ligase, respectivamente. Figura adaptada de Schärer, 2003.

A reparação por NER (Via RAD3) é um mecanismo mais genérico e flexível, que repara lesões que levam a distorções na dupla hélice do DNA. NER envolve a formação de uma série de complexos enzimáticos e consiste em quatro etapas: (1) reconhecimento da lesão; (2) abertura da dupla hélice do DNA; (3) clivagem das extremidades da lesão por endonucleases e liberação do fragmento de DNA; e (4) polimerização e posterior ligação da região removida (Schärer, 2008).

A deficiência em genes envolvidos no mecanismo de NER em humanos causa três síndromes raras recessivas (Xeroderma Pigmentosum, Síndrome de Cockayne e Tricodistrofia) e predisposição ao desenvolvimento de

câncer (De Boer & Hoeijmakers, 2000; Dudásová *et al.*, 2004). A **figura 5** ilustra o mecanismo NER:

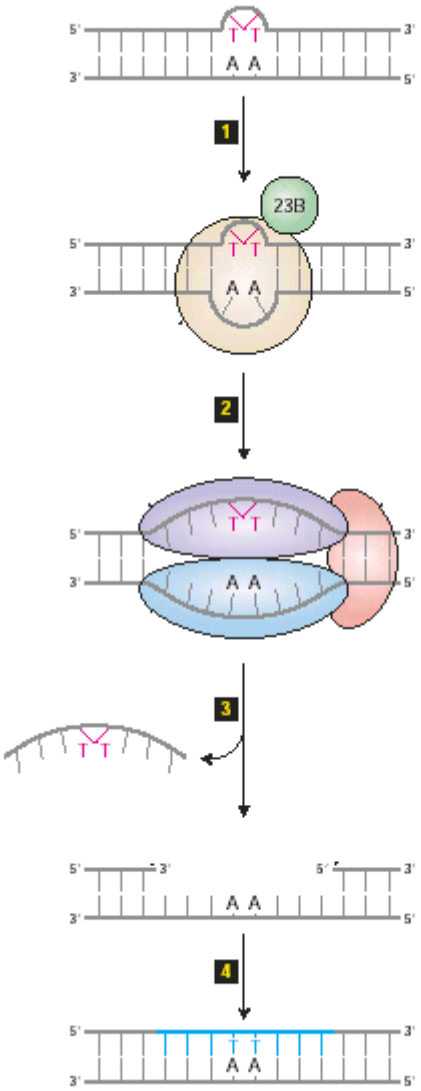

Figura 5: Representação esquemática do mecanismo de excisão de nucleotídeos (NER) que envolve, basicamente, quatro etapas (1): reconhecimento da lesão; (2) abertura da dupla hélice do DNA; (3) clivagem das extremidades da lesão por endonucleases e liberação do fragmento de DNA; e (4) polimerização e posterior ligação da região removida (Schärer, 2008). Figura adaptada de Schärer, 2003 e Gill & Fast, 2007.

A reparação recombinacional (Via RAD52) é recrutada para as lesões do tipo quebra de DNA, geradas por agentes endógenos e físico-químicos, possuindo dois mecanismos principais: (1) a recombinação homóloga (*homologous recombination* ou HR, não-mutagênica); e (2) a recombinação não-homóloga (*non-homologous end joining recombination* ou NHEJ, mutagênica).

A via HR envolve um conjunto de proteínas pertencentes ao grupo de epistasia Rad52 (Symington, 2005). O primeiro evento relacionado à HR é a ressecção nucleolítica da fita 5'-3', promovida por um complexo proteico que contém Rad50, Mre11p e Xrs2p (Complexo MRX; Gill & Fast, 2007). Essa ressecção possui, como objetivo, a geração de extremidades invasivas do tipo 3' fita simples, que resultam no processo recombinacional. Além desse complexo, há a participação de outras proteínas, a exemplo de Rad24, Rad17, Mec3, Ddc1 (para revisão ver, Gill & Fast, 2007; Lehoczký *et al.*, 2007).

A recombinação não-homóloga (NHEJ) representa um dos ramos principais da via recombinacional. Presente em todos os eucariotos, ela é recrutada quando há a necessidade de religar duas extremidades de DNA, resultantes de uma quebra, que não possuem homologia entre si. Além disso, pequenas regiões de homologia (ou micro-homologias, quando abrangem apenas alguns nucleotídeos), existentes entre duas fitas não-homólogas de DNA, podem ser utilizadas pela via NHEJ para a reparação (Shrivastav *et al.*, 2008). Assim, são conhecidas diversas subvias de reparação pertencentes à via NHEJ, cada qual com características próprias, mas cujo resultado final é a reparação de quebras duplas de uma forma sujeita a erros. Embora os mecanismos moleculares da via NHEJ ainda não sejam conhecidos em detalhes, a maquinaria básica da via NHEJ consiste nas proteínas Xrcc4, na DNA ligase IV, no heterodímero Ku70/Ku80, bem como na subunidade catalítica da cinase dependente de DNA (DNA-PKcs) (Dudásová, 2004; Weterings *et al.*, 2009).

Em um primeiro momento, a detecção de quebras duplas no DNA é realizada pelo complexo DNA-PKcs e pelo heterodímero Ku70/Ku80. Esse complexo, em conjunto com as extremidades de DNA, constitui a chamada

sinapse (De Fazio *et al.*, 2002). Uma vez formada a sinapse, diferentes endonucleases são recrutadas para modificar as extremidades de DNA, entre as quais se destacam o complexo MRX e a proteína homóloga de Pso2p/Snm1p, conhecida como Artemis (Mahaney *et al.*, 2009; Weterings *et al.*, 2009). Após a modificação, DNA polimerases e ligases restituem a dupla fita de DNA. A **figura 6** ilustra as vias NHEJ e HR envolvidas na reparação de quebras duplas de DNA:

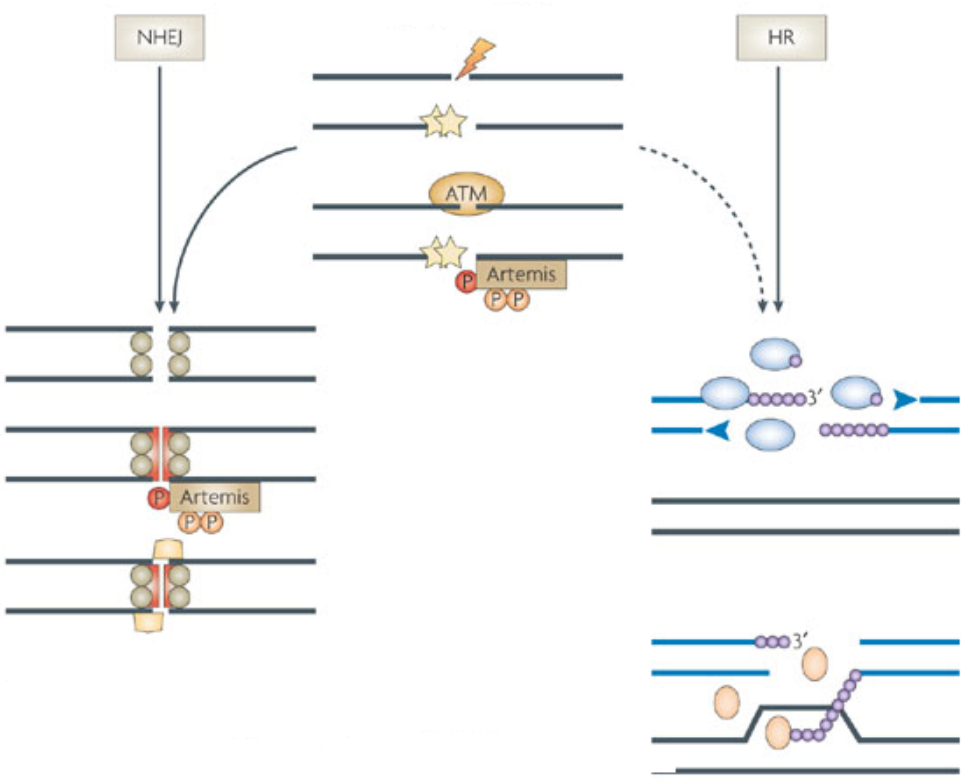

Figura 6: Representação esquemática das duas vias de recombinação não-homóloga (NHEJ) e homóloga (HR). Figura adaptada de Löbrich & Jeggo, 2007.

Há uma outra via de reparação denominada pós-replicativa e/ou mutagênica (via RAD6), também conhecida como sujeita a erros. Mutantes desse grupo são sensíveis a vários agentes químicos e físicos e, em sua maioria, parcial ou totalmente bloqueados na mutagênese induzida. Os alelos mutantes que fazem

parte desse grupo são altamente pleiotrópicos e constituem o sistema de reparação de DNA mais complexo e menos compreendido de reparação de DNA em *S. cerevisiae* (Xiao *et al*., 2000; Andersen *et al*., 2008). O grupo inclui mutantes que são sensíveis à radiação UVC, radiações ionizantes, agentes alquilantes e psoralenos fotoativados, apresentando uma resposta mutagênica diferenciada quando expostos a esses agentes (Game *et al*., 2000; Game & Chernikova, 2009). Xiao *et al*. (2000) sugerem que a via RAD6 é dividida em três subvias representadas pelos genes *RAD5*, *POL30* e *REV3*. As vias RAD5 e POL30 são supostamente livres de erros, enquanto a via REV3 é largamente mutagênica (Brendel *et al*., 2003).

Quando o mecanismo de ação de determinados compostos é desconhecido e não se conhecem os tipos de lesão que eles podem causar no DNA, é possível utilizar, para esse estudo, linhagens da levedura *S. cerevisiae* deficientes em um tipo de reparação. Um método utilizado para a determinação da natureza das lesões induzidas por agentes químicos no DNA consiste em comparar a sensibilidade de mutantes, deficientes em uma via, ou em mais de uma delas, de reparação do DNA, com uma linhagem selvagem isogênica proficiente naquele tipo de reparação. Quando um mutante deficiente em uma via de reparação do DNA apresenta uma sensibilidade maior do que a linhagem selvagem proficiente na reparação, pode-se inferir que a substância estudada induz o tipo de lesão incapaz de ser reparada pela linhagem mutante (Boeira *et al.*, 2002; Pungartnik *et al.*, 2005).

6 Sistemas de transporte de metais em *S. cerevisiae*

Usualmente, os microrganismos utilizam dois sistemas de captação de íons metálicos. Um deles é rápido, inespecífico e comandado por um gradiente quimiosmótico presente na membrana citoplasmática de células procarióticas. Como esse sistema de captação é usado por uma variedade de substratos, ele é

constitutivamente expresso (Nies, 1999). O segundo tipo de sistema de captação apresenta uma alta especificidade ao substrato, é lento e frequentemente utiliza hidrólise de ATP como fonte de energia, sendo produzido pela célula somente quando há necessidade, carência nutricional ou situação metabólica especial (Nies, 2003).

Ainda que os microrganismos possuam sistemas de captação de metais específicos, altas concentrações de metais não-essenciais podem ser transportadas para as células através de sistemas inespecíficos, expressos constitutivamente. Esse "portão aberto" é uma das razões pelas quais os íons metálicos são tóxicos aos microrganismos (Nies, 2003). Como consequência, os microrganismos têm sido forçados a desenvolver fatores homeostáticos de íon metálico, bem como a adquirir resistência metálica (Nies, 1999; Nies, 2003). A **tabela 1** mostra a direção e o tipo de energia utilizados no transporte de íons:

Tabela 1: Principais classes de transportadores e a energia utilizada no transporte de íons.

Direção do transporte	Tipo de energia
Captação ou importação	ATP ou quimiosmótico
Exportação	ATP ou quimiosmótico
Simporte ou antiporte	Quimiosmótico

Dentre os vários mecanismos de resistência a metais (Rosen, 2002), destacam-se os quatro mais empregados pela célula: (1) exclusão do metal através de uma barreira de permeabilidade; (2) ativação de bombas de efluxo; (3) "sequestro" do metal intracelular; e (4) redução enzimática e redução da sensibilidade dos alvos celulares em relação aos íons metálicos. Um, ou mais de um desses mecanismos de resistência a metais, permite a sobrevivência de

microrganismos em ambientes contaminados com metais pesados (Waldron & Robinson, 2009).

Nas células eucarióticas, os íons tóxicos são removidos do citoplasma pela quelação com oligopeptídeos, como glutationa e fitoquelatinas (essa última presente em plantas e em *Schizosaccharomyces pombe,* porém ausente em *S. cerevisiae*), ou por meio de metalotioneínas. Posteriormente, esses complexos são deslocados para o vacúolo através de proteínas transmembrana de transporte (Lin *et al.*, 2006).

6.1 O metabolismo do ferro na levedura *S. cerevisiae*

O elemento ferro é requerido pela maioria dos organismos, sendo que somente algumas espécies de bactérias não o utilizam (Rutherford & Bird, 2004). A habilidade do ferro de ganhar e perder elétrons (metal de transição) faz dele um cofator essencial em reações redox (Philpott & Protchenko, 2008). Sendo muito abundante na crosta terrestre, está disponível na forma férrica Fe^{3+}, a qual é insolúvel para as células. Dessa maneira, dada a sua importância e insolubilidade, procariotos, até mamíferos, tiveram que desenvolver mecanismos que o transformassem da forma insolúvel para a solúvel e que o captassem corretamente (para revisão, ver Eide, 1998; Van Ho *et al.*, 2002; Kosman, 2003; Prá *et al.*, 2008).

Na levedura *S. cerevisiae*, a forma ferrosa (Fe^{2+}) é substrato para as proteínas de transporte, de modo que o Fe^{3+} precisa ser reduzido a Fe^{2+} para ser internalizado. Essa redução ocorre pelas metaloredutases presentes na membrana plasmática das leveduras. Há duas metaloredutases principais, codificadas pelos genes *FRE1* e *FRE2*, mas existem mais cinco metaloredutases (Fre 3, 4, 5, 6 e 7) que podem, igualmente, reduzir o ferro para que o mesmo possa ser internalizado (Van Ho *et al.*, 2002). Os produtos dos genes *FRE* não são específicos para o ferro, podendo reduzir, também, o cobre (Singh, *et al.*, 2007).

Há dois sistemas de captação do ferro: (1) sistema de transporte de alta afinidade; e (2) sistema de baixa afinidade (Singh, *et al.*, 2006). O sistema de alta afinidade é comandado por duas proteínas: Fet3, que é uma ferroxidase que catalisa a oxidação de ferro (proteína oxidase dependente de cobre); e Ftr1, que é uma permease (Singh, *et al.*, 2007). Esse sistema de transporte é específico para o ferro e parece não transportar nenhum outro metal (Stoj *et al.*, 2007).

O sistema de baixa afinidade é expresso quando há um alto conteúdo de ferro extracelular ou anaerobiose, situações em que a proteína Fet4 é responsável pela captação de ferro. Ao contrário do complexo proteico Fet3/Ftr1, Fet4 pode transportar, além do Fe^{2+}, outros metais, tais como: Cu^{+}, Mn^{2+}, Zn^{2+} e Co^{2+} (Van Ho *et al.*, 2002).

As proteínas Fre1, Fre2, Fet3/Ftr1 são reguladas pelos fatores de transcrição *AFT1* e *AFT2 (*Kaplan *et al.*, 2006; Philpott & Protchenko, 2008). Jensen & Culotta (2002) inferiram que a expressão do gene *FET4* pode ser controlada pela proteína Aft1. A **tabela 2** sumariza os principais genes envolvidos no metabolismo do ferro e suas respectivas funções:

Tabela 2: Principais genes envolvidos no metabolismo do ferro em *S. cerevisiae*.

Gene	Função proposta	Localização
AFT1 e *AFT2*	Fator de transcrição responsivo a ferro	Núcleo
FRE1 e *FRE2*	Fe^{2+}/Cu^{2+} redutase	Membrana plasmática
FET3	Multicobre ferroxidase, transporte de alta afinidade	Membrana plasmática
FTR1	Permease, transporte de alta afinidade	Membrana plasmática
FET4	Permease, transporte de baixa afinidade	Membrana plasmática

6.2 O metabolismo do cobre na levedura *S. cerevisiae*

Da mesma forma que o ferro, o cobre é um metal de transição e pode apresentar a forma cuprosa (Cu^{+}) e a forma cúprica (Cu^{2+}). Ele é essencial e potencialmente nocivo, podendo gerar EROs (Thiele & Gitlin, 2008). O cobre é requerido por várias proteínas, dentre elas citocromo oxidase, Fet3 ferroxidase e Cu/Zn superóxido dismutase (Sod1) (Thiele, 2003).

Para ser captado pela célula, o cobre precisa ser reduzido e, da mesma forma do que o ferro, a sua redução se dá pelas ferroredutases Fre1 e Fre2. O sistema de transporte de alta afinidade é comandado pelas proteínas Ctr1 e Ctr3. O sistema de baixa afinidade é governado pelas proteínas Fet4 (sistema de baixa afinidade do ferro) e Smf1 (sistema de alta afinidade do manganês) (Van *et al.*, 2002; Puig & Thiele, 2002, Kim *et al.*, 2008).

Há três chaperonas envolvidas no metabolismo do cobre: (1) proteína Atx1, que entrega cobre para a proteína ATPase transportadora de cobre (Ccc2), presente no complexo de Golgi; (2) proteína Lys7, que entrega cobre para a proteína Cu/Zn superóxido dismutase; e (3) proteína Cox17, que entrega cobre para a mitocôndria (Peña *et al.*, 1999). As chaperonas, ao entregarem cobre para moléculas-alvo, ou para organelas, servem para minimizar a toxicidade desse metal dentro da célula (De Freitas *et al.*, 2003).

Na maioria dos organismos eucarióticos, a principal defesa contra o excesso de metais é a mobilização exercida pelas metalotioneínas (MT), pertencentes à superfamília de proteínas de baixo peso molecular, ricas em resíduos de cisteína e com propriedades de quelar metais (Coyle *et al.*, 2002). Há duas MT na levedura *S. cerevisiae*: Cup1 e Crs5 (Pegani *et al.*, 2007). Ambas proteínas, juntamente com a Cu/Zn superóxido dismutase, exercem papel fundamental quando a célula apresenta níveis nocivos de determinados metais essenciais e não-essenciais.

A regulação transcricional dos genes envolvidos no metabolismo do cobre ocorre, basicamente, por quatro proteínas: (1) Mac1, que regula a transcrição dos genes *CTR1* e *CTR3*, quando há carência de cobre na célula; (2) Ace1, que regula a transcrição dos genes *CUP1*, *CRS5* e *SOD1*, quando há um excesso de cobre na célula; (3) e (4) Aft1 e Aft2, que regulam a expressão dos genes *FRE1*, *FRE2*, *ATX1*, *LYS7* e *COX17* (para revisão, ver Keller *et al.*, 2002; Rutherford & Bird, 2004).

Para contribuir com a homeostase do cobre, a proteína Ctr2 desempenha papel fundamental quando a célula precisa desse metal para exercer as suas funções vitais. A proteína Ctr2 está localizada na membrana do vacúolo, sendo responsável por mobilizar cobre do vacúolo para o citosol (Rees *et al.*, 2004; Rees & Thiele, 2007). Por haver conexão entre o metabolismo do cobre e do ferro (Taylor *et al.*, 2005), a deficiência de ferro pode ativar a transcrição do gene *CTR2* (Rees & Thiele, 2007). A **tabela 3** sumariza os principais genes envolvidos no metabolismo do cobre e suas respectivas funções:

Tabela 3: Principais genes envolvidos no metabolismo do cobre em *S. cerevisiae*.

Gene	Função proposta	Localização
FRE1 e *FRE2*	Fe^{2+}/Cu^{2+} redutase	Membrana plasmática
CTR1 e *CTR3*	Permease, transporte de alta afinidade	Membrana plasmática
FET4 e *SMF1*	Permease, transporte de baixa afinidade	Membrana plasmática
ATX1/CCC2, *LYS7* e *COX17*	Chaperonas	Citoplasma
CUP1 e *CRS5*	Metalotioneínas	Citoplasma
MAC1, *AFT1* e *AFT2*	Fatores de transcrição de genes envolvidos no metabolismo do ferro e do cobre quando há ausência desses metais	Núcleo
ACE1	Fator de transcrição de genes envolvidos no metabolismo do cobre quando há excesso desse metal	Núcleo
CTR2	Permease	Membrana do vacúolo

6.3 O metabolismo do zinco na levedura *S. cerevisiae*

O zinco é elemento químico essencial para todos os organismos. Ele é cofator requerido para a função de mais de trezentas diferentes enzimas (Eide, 2006). Para manter um refinado controle de zinco na célula, há a participação de várias proteínas. O sistema de transporte de alta afinidade é comandado pela proteína Zrt1, pertencente à família ZIP (Zrt-, proteína tipo Irt). A expressão do gene *ZRT1* é aumentada quando a célula apresenta concentrações baixas desse metal (Wu *et al.*, 2008). Três principais proteínas (Zrt2, Fet4 e Pho84) estão envolvidas no sistema de transporte de baixa afinidade, sendo que o zinco não é substrato exclusivo das mesmas (Eide, 2006). O gene *PHO84* codifica uma proteína de alta afinidade envolvida no transporte de fosfato (Jensen *et al.*, 2003).

As proteínas Zrc1 e Cot1, pertencentes à família dos facilitadores de difusão de cátions (CDF – *cation diffusion facilitator*), estão localizadas na membrana do vacúolo, servindo para colocar o excesso de zinco presente no citoplasma para dentro do vacúolo (Eide, 2006). Já a proteína Zrt3 libera zinco do vacúolo para o citosol (Gaither & Eide, 2001; Simm *et al.*, 2007).

O principal fator de transcrição envolvido no metabolismo do zinco é a proteína Zap1. Ela regula vários genes, sendo essa regulação dependente do estado nutricional da célula (Eide, 2001; Herbig *et al.*, 2005). De um modo geral, Zap1 regula a expressão de *ZRT1*, *ZRT2*, *ZRT3*, *ZRC1* e *COT1*. A **tabela 4** sumariza os principais genes envolvidos no metabolismo do zinco e suas respectivas funções:

Tabela 4: Principais genes envolvidos no metabolismo do zinco em *S. cerevisiae*.

Gene	Função proposta	Localização
ZRT1	Permease, transporte de alta afinidade	Membrana plasmática
ZRT2, *FET4* e *PHO84*	Permeases, transporte de baixa afinidade	Membrana plasmática
ZRC1 e *COT1*	Permeases, transporte de zinco do citoplasma para o vacúolo	Membrana do vacúolo
ZRT3	Permease, transporte de zinco do vacúolo para o citoplasma	Membrana do vacúolo
ZAP1	Fator de transcrição de genes envolvidos no metabolismo do zinco	Núcleo

6.4 O metabolismo do cálcio na levedura *S. cerevisiae*

O cálcio está envolvido em vários processos celulares, dentre eles cascatas de sinalização celular e integridade da célula (Zhang & Rao, 2008). O transporte de alta afinidade do cálcio é mediado pelo complexo proteico Cch1/Mid1. O vacúolo serve para armazenar e liberar cálcio, dependendo do estado nutricional da célula. O transporte vacuolar de cálcio resulta da atividade combinada de três proteínas: Yvc1, Pmc1 e Vcx1 (Segarra & Thomas, 2008).

A proteína Yvc1 é responsável pela liberação de cálcio do vacúolo para o citoplasma, enquanto que Pmc1 transporta, com dependência de energia, cálcio para o vacúolo e, por fim, a proteína Vcx1 transporta cálcio para o vacúolo com a ajuda de um gradiente de prótons (Ton & Rao, 2004). A proteína Pmr1

(*plasma membrane related*) foi a primeira proteína secretória a ser identificada, desempenhando papel fundamental na célula. Basicamente, apresenta duas funções: (1) é capaz de exportar cálcio e também manganês quando há um excesso desses elementos na célula, contribuindo para a homeostase do cálcio; e (2) é capaz de suprir a célula no processo de "montagem" de várias proteínas, que requerem cálcio e manganês para desempenhar suas funções (Ton & Rao, 2004; Segarra & Thomas, 2008). Lauer Júnior *et al.* (2008) estudaram o envolvimento dessa proteína secretória na destoxificação do metal pesado cádmio.

Como o cálcio participa de vários eventos de sinalização celular, há um importante sistema que comanda essa cascata de eventos, chamado calmodulina/cálcio (CaM). O complexo CaM liga-se a proteínas-alvo, proporcionando eventos biológicos como apoptose, tráfego intracelular de nutrientes, síntese lipídica, dentre outras (Ton & Rao, 2004). A **tabela 5** sumariza os principais genes envolvidos no metabolismo do cálcio e suas respectivas funções:

Tabela 5: Principais genes envolvidos no metabolismo do cálcio em *S. cerevisiae*.

Gene	Função proposta	Localização
CCH1 e *MID1*	Permease, transporte de alta afinidade	Membrana plasmática
YVC1	Permease, exporta cálcio do vacúolo para o citoplasma	Membrana do vacúolo
PMC1	ATPase, transporta cálcio do citoplasma para o vacúolo	Membrana do vacúolo
VCX1	Bomba de Ca^{2+}/H^{+}, transporta cálcio do citoplasma para o vacúolo	Membrana do vacúolo
PMR1	ATPase, secretora de cálcio e manganês	Membrana do complexo de Golgi

7 Emissão de Raios-X Induzida por Partículas (PIXE)

Recentemente, as técnicas nucleares têm sido utilizadas em pesquisas para a determinação de elementos-traço em materiais biológicos e ambientais (Giulian *et al.*, 2007; Villela *et al.*, 2007; Mielniczki-Pereira *et al.*, 2008). A técnica de Emissão de Raios-X Induzida por Partículas Carregadas, ou *Particle Induced X-Ray Emission (PIXE)*, caracteriza-se por ser um método não-destrutivo e de caráter multielementar (Seven *et al.*, 1995). O material (amostra) a ser analisado é irradiado por partículas carregadas produzidas por um acelerador. A interação dessas partículas com os átomos da amostra faz com que, dentre outros efeitos, elétrons de camadas internas dos átomos da amostra sejam ejetados. Quando as vacâncias resultantes são espontaneamente preenchidas por elétrons de camadas mais externas (processo de desexcitação), são emitidos raios-X com energias características para cada elemento constituinte da amostra (**figura 7**).

Esse método apresenta as seguintes vantagens: (1) exigência de uma pequena quantidade de amostra para a análise; (2) possibilidade de as amostras serem analisadas por outras técnicas, visto que o método não é destrutivo; e (3) possibilidade de análise simultânea, rápida e precisa de vários elementos químicos.

O preparo das amostras depende do tipo de material a ser investigado. Há preparações mais elaboradas, como no caso de proteínas (Follmer *et al.*, 2002), e preparações mais simples, como no caso de sedimentos, solos e sucos (Heuser *et al.*, 2002; Franke *et al.*, 2006). O emprego do PIXE nos estudos de metais essenciais e metais pesados (Viau *et al.*, 2006; Prá *et al.*, 2008; Lauer Júnior *et al.*, 2008) tem contribuído para esclarecer a interação elementar e suas implicações.

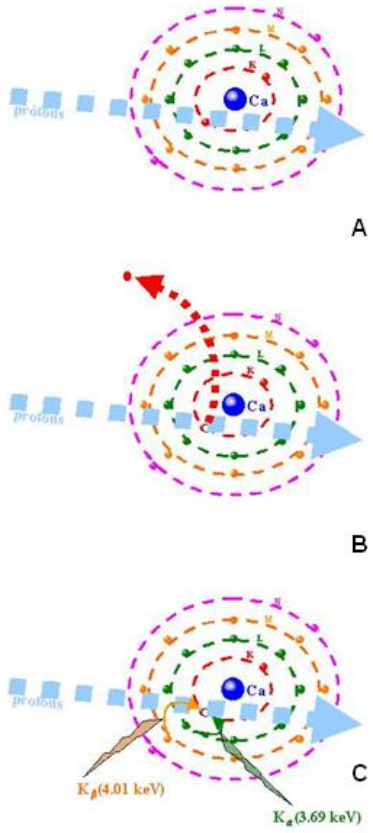

Figura 7: Princípio básico da técnica PIXE. (A) Incidência de um feixe de prótons em um átomo de cálcio. (B) A incidência desse feixe de prótons provoca a emissão de um elétron da camada K de um átomo de cálcio. (C) Podem ocorrer dois modos de preenchimento da vacância deixada nessa camada, com os respectivos raios-X emitidos. Figura extraída de http://www.if.ufrgs.br/pixe/.

OBJETIVOS

1 Objetivo geral

O objetivo geral deste trabalho é avaliar o potencial tóxico e genotóxico do íon metálico Sn^{2+} em células de levedura *Saccharomyces cerevisiae* e em células de pulmão de hamster chinês (V79).

2 Objetivos específicos

- ✓ Testar a citotoxicidade do $SnCl_2$ em mutantes defectivos nas três principais vias de reparação de danos de DNA em *S. cerevisiae*;
- ✓ Testar a citotoxicidade do $SnCl_2$ em mutantes de *S. cerevisiae* defectivos nas enzimas superóxido dismutase mitocondrial e citosólica;
- ✓ Estabelecer um protocolo padrão de análise para detectar o metal estanho nas células de levedura *S. cerevisiae* pela técnica de Emissão de Raios-X Induzida por Partículas (PIXE);
- ✓ Avaliar o potencial mutagênico do $SnCl_2$ em células de pulmão de *hamster* chinês (V79) pelo ensaio cometa alcalino;
- ✓ Avaliar a interferência do $SnCl_2$ na reparação do agente mutagênico metilmetanosulfonato (MMS) em células de pulmão de *hamster* chinês (V79) pelo ensaio cometa alcalino;
- ✓ Analisar o tipo de lesões oxidativas formadas pelo $SnCl_2$ em células de pulmão de *hamster* chinês (V79) pelo ensaio cometa alcalino, com a utilização de enzimas bacterianas endonuclease III (ENDO III) e formamidopirimidina glicosilase (FPG);

- ✓ Analisar se o íon metálico Sn^{2+} interfere no metabolismo de elementos químicos essenciais nas células de levedura *S. cerevisiae* pela técnica de Emissão de Raios-X Induzida por Partículas (PIXE);
- ✓ Testar a citotoxicidade do $SnCl_2$ em mutantes defectivos no metabolismo do ferro, cobre e zinco de *S. cerevisiae*;
- ✓ Analisar a expressão de genes envolvidos no metabolismo do cobre e cálcio em linhagens de *S. cerevisiae* tratadas com $SnCl_2$ por PCR quantitativo (qRT-PCR);
- ✓ Analisar a expressão de genes envolvidos na resposta ao estresse oxidativo em linhagens de *S. cerevisiae* tratadas com $SnCl_2$ por PCR quantitativo (qRT-PCR);
- ✓ Propor um modelo de captação, de geração de danos e de destoxificação em linhagens de *S. cerevisiae* tratadas com $SnCl_2$.

CAPÍTULO I

Sensitivity to Sn^{2+} of the yeast Saccharomyces cerevisiae depends on general energy metabolism, metal transport, anti-oxidative defences, and DNA repair.

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Sensitivity to Sn^{2+} of the yeast *Saccharomyces cerevisiae* depends on general energy metabolism, metal transport, anti-oxidative defences, and DNA repair

C. Viau[1], C. Pungartnik[2], M.C. Schmitt[1], T.S. Basso[2], J.A.P. Henriques[1] & M. Brendel[2,*]
[1]*Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, RS, Brasil;* [2]*Departamento de Biology, Universidade Estadual de Santa Cruz (UESC), Rodovia Ilhéus-Itabuna Km 16, CEP-45662-000, Ilhéus, Bahia, Brasil; *Author for correspondence (E-mail: martinbrendel@yahoo.com.br)*

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Abstract

Resistance to stannous chloride ($SnCl_2$) of the yeast *Saccharomyces cerevisiae* is a product of several metabolic pathways of this unicellular eukaryote. Sensitivity testing of different null mutants of yeast to $SnCl_2$ revealed that DNA repair contributes to resistance, mainly via recombinational (Rad52p) and error-prone (Rev3p) steps. Independently, the membrane transporter Atr1p/Snq1p (facilitated transport) contributed significantly to Sn^{2+}-resistance whereas absence of ABC export permease Snq2p did not enhance sensitivity. Sensitivity of the superoxide dismutase mutants *sod1* and *sod2* revealed the importance of these anti-oxidative defence enzymes against Sn^{2+}-imposed DNA damage while a catalase-deficient mutant (*ctt1*) showed wild type (WT) resistance. Lack of transcription factor Yap1, responsible for the oxidative stress response in yeast, led to 3-fold increase in Sn^{2+}-sensitivity. While loss of mitochondrial DNA did not change the Sn^{2+}-resistance phenotype in any yeast strain, cells with defect cytochrome *c* oxidase (CcO mutants) showed gradually enhanced sensitivities to Sn^{2+} and different spontaneous mutation rates. Highest sensitivity to Sn^{2+} was observed when yeast was in exponential growth phase under glucose repression. During diauxic shift (release from glucose repression) Sn^{2+}-resistance increased several hundred-fold and fully respiring and resting cells were sensitive only at more than 1000-fold exposure dose, i.e. they survived better at 25 mM than exponentially growing cells at 25 μM Sn^{2+}. This phenomenon was observed not only in WT but also in already Sn^{2+}-sensitive *rad52* as well as in *sod1*, *sod2* and CcO mutant strains. The impact of metabolic steps in contribution to Sn^{2+}-resistance had the following ranking: Resting WT cells > membrane transporter Snq1p > superoxide dismutases > transcription factor Yap1p ≥ DNA repair ≫ exponentially growing WT cells.

Introduction

Trace amounts of different metals play a crucial role in cellular metabolism as they constitute ligands of diverse enzymes (Eide & Guerinot 1997). At higher concentrations metal ions, especially some of heavy metals, interfere negatively with cellular metabolism as they may inactivate proteins and damage DNA (McMurray & Tainer 2003). Evolution thus favoured survival of organisms which had developed mechanisms that guaranteed optimal intracellular metal concentration by balancing metal uptake from the environment and metal excretion/neutralization (metal homeostasis (Tomsett & Thurmann 1988)). Modern food preservation relies on sterilization and

packaging of food, and tin plays an important role in this process as it is used for the inner lining of metal containers and for conservation of soft drinks (McLean *et al*. 1983a). Tin thus comes into contact with the packaged food and may form stannous salts. Increased consumption of canned foods, therefore, is held responsible for tin accumulation in humans of wealthy countries (Schroeder *et al*. 1964).

Stannous chloride ($SnCl_2$) is a weak mutagen as defined by its genotoxicity in unicellular prokaryotes (Bernardo-Filho *et al*. 1994; Dantas *et al*. 1996) and eukaryotes (Pungartnik *et al*. 2005) as well as its DNA interactions in mammalian cells (McLean & Kaplan 1979; McLean *et al*. 1983b). Mutagenicity and mitotic gene conversion induced by Sn^{2+} in *Saccharomyces cerevisiae* points to the involvement of error-prone repair mechanisms in the removal of DNA lesions and the involvement in repair of the recombinational Rad52-controlled pathway was also shown (Pungartnik *et al*. 2005).

Metal uptake and metal homeostasis in yeast are controlled by a complex system of metabolic steps, most prominently by membrane transporters (Eide & Guerinot 1997; Van Ho *et al*. 2002) and by intracellular neutralization with thiol-oligopeptides and metallothioneins (Heuchel *et al*. 1994). Thus it is likely that changes in Sn^{2+} uptake or excretion via membrane transporters might also influence sensitivity of yeast cells to this metal. Once in the cell, the genotoxic potential of Sn^{2+} might also be significantly modulated by other, non-DNA repair or membrane transport-related physiological parameters, i.e., the quality and quantity of enzymatic and non-enzymatic scavengers of metal-induced reactive oxygen species (ROS). $SnCl_2$ is known to produce ROS (Dantas *et al*. 1999) most probably via Fenton-like reactions (McLean *et al*. 1983b) and thus the genetic endowment of yeast with anti-oxidative defence systems, e.g., superoxide dismutases, catalase, glutathione, and their oxidative stress-induced expression might contribute to Sn^{2+}-resistance.

Anaerobically growing microorganisms, especially obligate anaerobes, are known to have a higher metal sensitivity than aerobically living microbial species. The facultative anaerobe yeast *S. cerevisiae* can grow both in presence or absence of respiratory metabolism, and thus might be a good model organism to test the influence of general energy metabolism on sensitivity to Sn^{2+}. Mitochondrial activity plays a crucial role in aerobic energy metabolism of eukaryotes and it is thus likely that defects in the respiratory chain located within the inner mitochondrial membrane might directly or indirectly contribute to the generation of ROS (Barros *et al*. 2003) which might be altered by the presence of Sn^{2+}.

All above-mentioned metabolic steps are controlled by proteins encoded in the yeast cell's genome and thus can be influenced by genetic manipulation. Therefore, this unicellular fungus offers itself as an ideal eukaryotic model for the observation of Sn^{2+}-induced effects on its DNA, allowing to determine the relative contribution to Sn^{2+}-resistance of individual protective metabolic pathways.

Materials and methods

Yeast strains and growth conditions

The relevant genotypes of the yeast strains used in this work are given in Table 1. Media, solutions and buffers were prepared according to Burke *et al*. (2000). Complete medium (YPD) was used for routine growth of yeast cells and minimal medium (MM) was supplemented with the appropriate amino acids (synthetic complete medium, SC). To ascertain yeast respiratory competence and for elimination of spontaneously accumulated *petites* all strains were pre-grown on YPglycerol media (glucose replaced by 2% glycerol) before being grown in YPD.

Yeast exposure to $SnCl_2$ and survival

Stationary (STAT) cells were grown in YPD at 30 °C for 72 h. Different times of growth of STAT cells in fresh medium yielded cells in exponentially phase of growth (LOG). LOG cells were microscopically checked for bud appearance and the bud index (% budded cells) was established. Sensitivity of twice saline-washed yeast suspensions to $SnCl_2$ was routinely determined in liquid saline (0.9% NaCl, pH 5.0). Exposure concentration was 25 mM for STAT cells and 25 μM for LOG cells. Exposure time was for 60 min at 30 °C. Thereafter, $SnCl_2$-mediated cell aggregates were de-clumped in phosphate buffer (PB, pH 7.4, 0.067 M) followed by vigorous vortexing before

Table 1. Strains used in this study and their relevant genotypes.

Yeast strains	Genotype	Reference
XS2316 (WT)	*MAT***a** + *leu1 − 1trp5 − 48 + + + his1 − 208* *MAT*α *ade6 leu1-12 + cyh2 met13 lys5-1 his1-208*	Machida & Nakai (1980)
XV185-14c (WT)	*MAT*α *ade2-2 his1-798 lys1-1 trp5-48 hom3-10 arg4-17*	von Borstel *et al.* (1971)
BY10000 (WT)	*MAT*α *his3*Δ1 *lys2*Δ0 *leu2*Δ0 *ura3*Δ0	EUROSCARF
4 BY *rad* mutants	Same genotype as BY10000 but *rad52*Δ, *rad2*Δ, *rad4*Δ, *rad6*Δ	See above
YPH98 (WT)	*MAT***a** *ura3-52 lys2-801 ade2-101 leu2-*Δ*1 trp1-*Δ*1*	Wehner *et al.* (1983)
4NQO sensitive	Same genotype as YPH98 but *snq1*Δ, *snq2*Δ, *snq3*Δ/*yap1*Δ	See above
q1	Same genotype as YPH98 but rho^0	M. Grey, Frankfurt/Main
q2	Same genotype as q1 but *gsh1*Δ	See above
q3	Same genotype as q1 but *gsh1*Δ*lwg1*Δ	See above
q4	Same genotype as q1 but *lwg1*Δ	See above
EG103 (WT)	*MAT*α it leu2-3, 112 his3Δ*1 trp1-289 ura3-52 GAL*$^+$	E.B. Gralla, Los Angeles
EG118 (*sod1*Δ)	*sod1::URA3* all others markers as EG103	See above
EG110 (*sod2*Δ)	*sod2::TRP1* all others markers as EG103	See above
EG133 (*sod1*Δ*sod2*Δ)	*sod1::URA3 sod2*::*TRP1* double mutant, all others markers as EG103	See above
EG223 (*ctt1*Δ)	*ctt1::TRP1* all others markers as EG103	See above
BER/NER (WT)	*MAT*α *ade2-101 his3*Δ*200 ura3*ΔNco lys2ΔBgl	Swanson *et al.* (1999)
Base excision repair mutants	Same genotype as BER/NER WT but *ntg1*Δ, *ntg2*Δ, *ntg1*Δ*ntg2*Δ, *ntg1*Δ*ntg2*Δ*apn1*Δ, *ntg1*Δ*ntg2*Δ*apn1*Δ*rad52*Δ, *ntg1*Δ*ntg2*Δ*apn1*Δ*rad1*Δ, *ntg1*Δ*ntg2*Δ*apn1*Δ*rev3*Δ	See above
W303 (WT)	*MAT***a** *ade2-1 leu2-3, 112 his3-11,15 trp1-1 ura3-1 can1-100*	A. Tzagoloff, New York
Mitochondrial mutants	Same genotype as W303 but *cox14*Δ, *cox15*Δ, *cox16*Δ, *cox17*Δ, *cox18*Δ, *cox20*Δ, *shy1*Δ, *sco1*Δ, *pet100*Δ, *pet117*Δ	See above

dilution in PB and plating (Pungartnik *et al.* 2005). Cells were plated on YPD and survival was determined after 3 d at 30 °C. Presented results are the mean of at least three independent experiments, the standard deviation and statistical analyses were calculated by GraphPad Prism® program.

Spontaneous mutation of mitochondrial mutants

STAT cells were grown in YPD at 30 °C for 72 h, washed twice with saline (0.9% NaCl, pH 5.0) and yeast suspensions plated on media SC (survival) and SC-Trp (spontaneous mutation). Following incubation for 7 d at 30 °C, colonies appearing on SC medium yielded data on cell survival, while those grown on SC-Trp represented the spontaneous mutations. Frequencies of spontaneous genomic mutation in different mitochondrial mutants (deficient of functional CcO) were scored per 10^7 cells. Results are means of three independent experiments, the standard deviation and statistical analyses was performed using the GraphPad Prism® software.

Results and discussion

Repair of Sn^{2+}-induced DNA lesions

The sensitivity of 35 different yeast strains (STAT cells of WT and isogenic mutants) to 60 min exposure at 25 mM of $SnCl_2$ is given in Tables 2 and 3. The range of killing of mutant cells as compared with the WT varied not more than 2 decades (survival between 1 and 90%, Table 2) or not at all (Table 3). Amongst the seven different DNA repair WT strains, survival varied from 25 to 90%, depending on the genetic background of each strain. Six of the WT strains can be roughly allocated to two sensitivity groups (survival either around 25 or 85%), with strain W303 in between (Table 2). These WT sensitivity variations were neutralized when comparing to sensitivities of

Table 2. Sensitivity to $SnCl_2$ (25 mM, 60 min) of STAT cells of different yeast strains.

Strains	$SnCl_2$	*p* value[a]	Relative sensitivity increase[b]
WT (XS2316)	**90.3 ± 3.3**		–
WT (XV185-14c)	**85.0 ± 8.0**		–
WT (W303)	**57.0 ± 5.4**		–
WT (Y10000)	**24.1 ± 1.1**		–
*rad2*Δ	13.5 ± 2.8	<0.05	1.4×
*rad4*Δ	10.3 ± 4.8	<0.01	1.5×
*rad6*Δ	8.1 ± 0.0	<0.0001	1.7×
*rad52*Δ	2.8 ± 0.9	<0.0001	2.5×
WT (YPH98)	**28.2 ± 6.2**		–
*snq1*Δ	14.5 ± 0.5	<0.05	1.5×
*snq2*Δ	23.0 ± 0.4	n.s.	1.1×
*snq3*Δ/*yap1*Δ	1.9 ± 0.2	<0.05	3.0×
WT (EG103)	**26.2 ± 6.2**		–
*sod1*Δ	5.3 ± 0.5	<0.01	2.5×
*sod2*Δ	17.5 ± 0.5	n.s.	1.4×
*sod1sod2*Δ	2.5 ± 0.2	<0.01	3.0×
*ctt1*Δ	49.0 ± 6.1	n.s.	0.6×
*ctt1*Δ*sod1*Δ	26.0 ± 2.3	n.s.	1.0×
WT	**87.2 ± 0.7**		–
*ntg1*Δ	77.4 ± 2.8	<0.01	1.7×
*ntg2*Δ	69.2 ± 2.2	<0.001	2.5×
*ntg1*Δ*ntg2*Δ	70.3 ± 1.6	=0.001	2.5×
*ntg1*Δ*ntg2*Δ*apn1*Δ	70.0 ± 3.4	=0.001	2.5×
*ntg1*Δ*ntg2*Δ*apn1*Δ*rad52*Δ	55.0 ± 1.3	<0.0001	4.0×
*ntg1*Δ*ntg2*Δ*apn1*Δ*rad1*Δ	31.3 ± 0.8	<0.0001	7.0×
*ntg1*Δ*ntg2*Δ*apn1*Δ*rev3*Δ	19.5 ± 0.6	<0.0001	11.0×

[a]Unpaired *t* test (95% interval confidence); statistical analyses comparing each mutant to its isogenic WT.[b]Sensitivity is defined by the inclination of an idealized linear inactivation curve in a semi-log plot. If WT survives 10% and mutant 1%, sensitivity increase of the mutant is by factor 2.n.s. not significantly different from the isogenic WT.

mutant strains, as always a set of WT and WT-derived isogenic mutant strains were compared. Sensitivities (or relative resistance) of mutant strains were calculated by comparison with the WT of semi-log graphs of respective survival curves.

It is known that $SnCl_2$ sensitivity in yeast increases from WT (RAD) < *rad2*Δ < *rad4*Δ < *rad6*Δ < *rad52*Δ in the Y10000 (EUROSCARF) background (Pungartnik *et al*. 2005). The recombination repair-deficient mutant *rad52*Δ had a 2.5-fold higher sensitivity as compared to the WT (Table 2). The relative resistance of mutant strains *rad2* and *rad4*, deficient in nucleotide excision repair (NER) was rather high, indicating a minor but significant contribution to repair of Sn^{2+} -induced DNA lesions by this repair pathway.

A series of mutants defective in different base excision repair (BER) pathways, combined with nucleotide excision repair (BER/NER, constructed elsewhere) were used to indirectly determine the type of $SnCl_2$-produced DNA lesion. Three DNA *N*-glycosylases, encoded by yeast genes *NTG1, NTG2*, and *OGG1* are known to be involved in repair of oxidative DNA damage that results in abasic sites in DNA (You *et al*. 1999, Alseth *et al*. 1999, Boiteux and Guillet 2004). Mutant allele *ntg2*Δ conferred the highest sensitivity (not to be enhanced in the *ntg1*Δ*ntg2*Δ*apn1*Δ triple mutant) demonstrating the necessity of the nucleus-located Ntg2p (Alseth *et al*. 1999) for repair of Sn^{2+} -induced DNA lesions (Table 2), whereas the apurinic site endonuclease Ntg1p, mainly localized in the mitochondria (You *et al*. 1999) and the

Table 3. Mutant alleles not affecting Sn^{2+}-resistance (25 mM, 60 min) of STAT cells.

Strains
q1 WT
q2* *gsh1ΔLWG1*
q3 *gsh1Δlwg1*
q4 *GSH1 lwg1*
*erg3*Δ [EUROSCARF]
*ogg1*Δ [EUROSCARF]
*mag1*Δ [EUROSCARF]
*gsh1*Δ* [EUROSCARF]
*ctt1*Δ [EUROSCARF]

*Grown in SynCo media supplemented with 100 μg GSH/ml.

OGG1-encoded *N*-glycosylase seem dispensable for repair of Sn^{2+}-induced DNA lesions (Tables 2 and 3).

Although all mutants containing *ntg2*Δ in conjunction with mutant alleles of other repair pathways (NER, error-prone or recombinational repair) had statistically significant higher sensitivity than the WT, the *rev3*Δ mutant allele-containing quadruple mutant strain was the most sensitive, thus indicating that the error-prone repair pathway (translesion synthesis (Lawrence 2002)) may make the highest contribution to repair of $SnCl_2$-induced DNA lesions (Table 2); this could explain the observed mutagenicity of $SnCl_2$ (Pungartnik *et al*. 2005). The response to $SnCl_2$-induced oxidative DNA damage thus differs from that introduced by hydrogen peroxide where the contribution of translesion synthesis is smaller than that of recombinational repair (Salmon *et al*. 2004). Introduction of a *rad52*Δ mutant allele, conferring lack of recombinational repair, into the BER triple knockout mutant *ntg1*Δ*ntg2*Δ*apn1*Δ led to a significant increase in sensitivity, demonstrating that the two repair modes contribute (at least in part) independently to removal of $SnCl_2$-induced DNA lesions (Table 2). The same can be said after the introduction of a *rad1*Δ mutant allele that yields an even more sensitive quadruple mutant strain. This indicates that NER, independently from BER or recombinational repair, can remove part of Sn^{2+}-induced DNA damage, most probably abasic sites (Torres-Ramos *et al*. 2000). These overlapping specificities of BER, NER, recombination and error-prone translesion synthesis in repair of damaged bases has already been shown by Swanson *et al*. (1999). Since DNA repair mechanisms were largely conserved during evolution (Eisen & Hanawalt 1999), the repair of Sn^{2+}-induced DNA damage via several different repair pathways in yeast might suggest a similar repair scenario in humans.

Membrane transport proteins influence Sn^{2+} toxicity

Two types of yeast membrane transporters were tested for their putative contribution to Sn^{2+}-uptake/homeostasis. Cells deficient in the facilitated transporter Atr1p/Snq1p (Kanazawa *et al*. 1988, Gömpel-Klein & Brendel 1990) showed significantly increased Sn^{2+}-sensitivity (factor 1.5) while a deletion mutant of the ABC transport protein Snq2p that so far has been shown to mediate resistance to structurally unrelated chemicals like 4-Nitro-quinoline oxide, sulphometuron methyl, triaziquone, and phenanthroline (Servos *et al*. 1993) was practically as resistant as the WT (resistance ranking was WT ≥ *snq2*Δ > *snq1*Δ). Complexity of metal ion homeostasis (Van Ho *et al*. 2002) however, makes it highly likely that other, hitherto unknown transport protein are also involved in Sn^{2+} transport (import/export). Indeed, it has been shown that $SnCl_2$ facilitates the Ca^{2+} entry through the L-type calcium channel under the condition of the membrane depolarization. There is the possibility that Ca^{2+} release from intracellular Ca^{2+} pools is involved in the action of $SnCl_2$ (Hattori *et al*. 2001) and that tin induces considerable changes in the metabolism of endogenous metals such as zinc and copper (Chmielnicka *et al*. 1981).

Lack of adaptive response to oxidative stress leads to Sn^{2+}-sensitivity

Mutants lacking yeast transcription factor Yap1p displayed a 3-fold higher Sn^{2+}-sensitivity than the isogenic WT (Table 2). Under oxidative stress Yap1 is oxidized and rapidly accumulated in the nucleus where it regulates the expression of up to 70 genes encoding proteins involved in oxidative stress response (Wood *et al*. 2004). Thus the yap1 mutant's sensitivity response indicates that anti-ROS defence systems of WT yeast are transcriptionally activated after Sn^{2+}-exposure. A similar response to oxidative stress exists in bacteria where the transcription activator OxyR induces the genes coding for

anti-stress proteins. Bacterial strains lacking a functional OxyR gene are used in the Mutoxitest to detect, via their specific sensitivity phenotype, ROS producing chemicals (Martínez *et al*. 2000). Such mutants also display a significantly higher sensitivity to Sn^{2+} (Pungartnik *et al*. 2005).

Yeast mutants lacking one or two genes encoding anti-oxidative defences (superoxide dismutase mutants *sod1*Δ, *sod2*Δ, and the double mutant *sod1*Δ*sod2*Δ) revealed 2–3-fold higher sensitivity to $SnCl_2$. The yeast strain containing both *sod* mutant alleles exhibited about additive sensitivities of the respective single mutants (Table 2). Judged by the higher sensitivity of *sod1*Δ mutant (Pungartnik *et al*. 2005), cytosolic Sod1p seems more important than mitochondrial Sod2p in protecting against the toxic effects of Sn^{2+} in STAT cells. On the other hand cytoplasmic catalase Ctt1p (Hartig & Ruis 1986) is apparently not involved in detoxification of any Sn^{2+}-induced ROS. In bacteria, H_2O_2 induces a cross-adaptive response to ROS-producing agents, amongst them $SnCl_2$ (Assis *et al*. 2002) suggesting that the OxyR transcription activator, which induces expression of catalase, alkyl hydroperoxide reductase and superoxide dismutase protects against Sn^{2+}-generated oxidative stress. The Yap1 transcription activator in yeast may have the same function (Wu & Mowe-Rowley 1994). However, this protective response to oxidative stress does not render a yeast cell about 1,000-fold more resistant (as calculated in exposure dose necessary for a like-wise killing) to Sn^{2+} as is seen when changing from glucose-repressed LOG to the glucose de-repressed STAT phase (cf. below and Figures 3 and 4). It has been suggested that two independently acting anti-ROS protective systems (one mediated by glucose repression/de-repression, the other via ROS-inducible transcription activators) are working in yeast (Maris *et al*. 2001), and our data imply that both are contributing to Sn^{2+}-resistance.

Defects in respiratory chain lead to Sn^{2+}-sensitivity

Yeast strains containing mutant alleles of genes encoding proteins of mitochondrially located cytochrome c oxidase (CcO-deficient mutants), also showed enhanced and variable sensitivity to

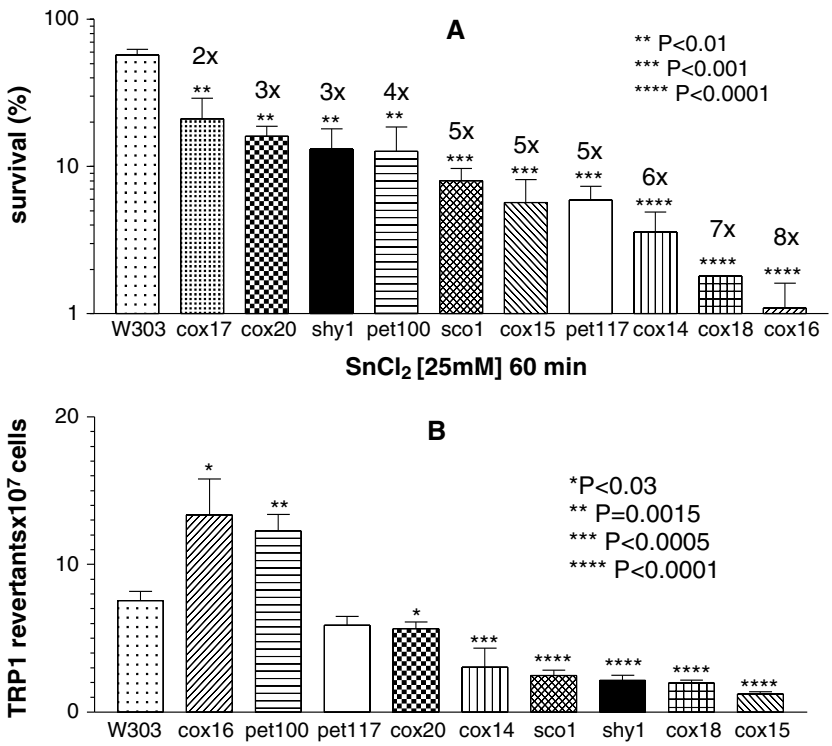

Figure 1. (A) Survival of STAT cells of WT and 9 mitochondrial mutants after 60 min $SnCl_2$ exposure (25 mM). (B) Reversion of tryptophan mutant allele *trp1-1* in mitochondrial mutant strains *(trp1-1)*, per 10^7 survivors. Numbers above the error bar of each column (A) gives the sensitivity increase of the respective mutant. Cells were diluted in PB.

$SnCl_2$ (Figure 1A). Interestingly, this sensitivity could be strongly enhanced in a *cox11/pso7-1* mutant (Pungartnik *et al.* 1999) when introducing the *erg3/pso6* mutant allele (Schmidt *et al.* 1999), as a second mutation (Brendel *et al.* 2003). Alone, the *erg3/pso6* mutant allele confers ergosterol deficiency and renders the mutant not sensitive to Sn^{2+} (Table 3), suggesting a non-protective role of this membrane constituent against the oxidative stress induced by this metal. In combination with *cox11/pso7-1* mutant allele, however, *erg3/pso6* showed a dramatic sensitivity effect. Generally CcO-deficient mutants are thought to produce more H_2O_2 by letting electrons escape from the respiratory chain (Barros *et al.* 2003). This elevated H_2O_2 might act as a mutagen on genomic DNA. We assayed, therefore, for spontaneously induced mutations in the *trp1-1* locus (reversion to trp^+) in mutant alleles of 9 different CcO-encoding genes (Figure 1B). While 2 CcO mutants, *cox16* and *pet100* had indeed higher-than-WT mutability in *trp1-1*, six others, i.e., the majority showed lower-than-WT mutability (Figure 1B), so that a general assumption of higher spontaneous mutation in CcO mutants could not be verified. It is known that Sn^{2+} generates ROS via Fenton-like reactions (McLean *et al.* 1983) and that there is variable content of ROS being produced in different CcO mutants (Barros *et al.* 2003). This might be the reason for, or at least contribute to, the observed variation of Sn^{2+}-sensitivity in the CcO mutants (Figure 1A). It must be emphasized, however, that total lack of respiratory chain activity in rho^0 mutants does not lead to enhanced Sn^{2+}-sensitivity, as isogenic rho^+ and rho^0 strains (YPH98 and q1, respectively) have identical WT-like survival (Tables 2 and 3).

Diauxic shift-induced Sn^{2+}-resistance

The highest $SnCl_2$ -sensitivity, however, was observed in glucose-repressed pre-diauxic shift exponentially growing cells (LOG cells (Figure 2)). On the basis of comparison to the $SnCl_2$ exposure dose required for likewise inactivation of STAT cells we found a more than 1000-fold increase in sensitivity (LOG slightly more sensitive to 25μM Sn^{2+} than STAT at 25 mM (Figure 3)). The significant increase of sensitivity of sod mutants as compared to the WT was the same in LOG cells at 25 μM Sn^{2+} exposure as seen in STAT cells at the

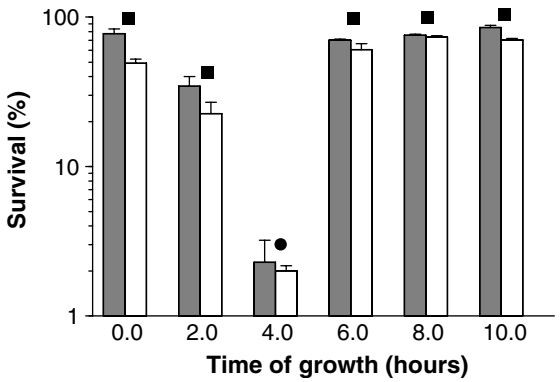

Figure 2. Sensitivity to 20 minutes Sn^{2+}-exposure of haploid WT strain Y10000 (grey column) and *rad52*Δ (white column); (■) STAT cells exposed to 25 mM; (●) LOG cells exposed to 2.5 mM. Cells were diluted in PB.

1000-fold Sn^{2+} exposure dose (Table 2). The high resistance to Sn^{2+} is acquired during and after the diauxic shift, i.e., when the yeast LOG cells are released from glucose repression and many cellular functions are adapted to respiratory metabolism. This resembles the response of yeast cells during diauxic shift-induced resistance against hydroperoxides (Maris *et al.* 2001). This process is independent of one tested repair function (Rad52p) (Figure 2), of the presence of superoxide dismutases (Figure 3), functional cytochrome c oxidase (Figure 4), and the presence of any mitochondrial respiratory metabolism (rho^0 mutants).

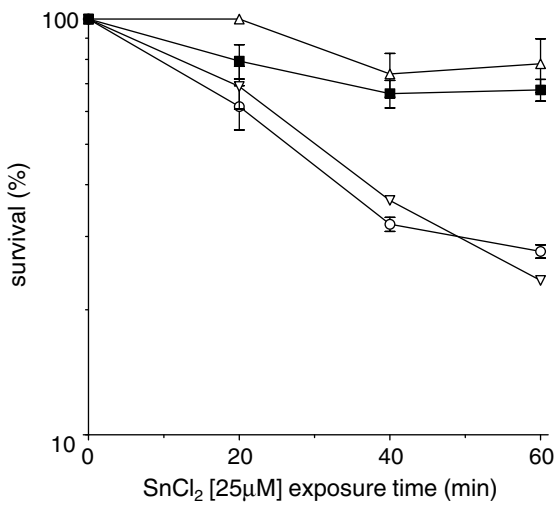

Figure 3. Sensitivity to 25 μM Sn^{2+}-exposure of LOG cells of haploid WT strain EG103 (■); and its isogenic mutants *sod2*Δ (△); *sod1*Δ (▽); and the double mutant *sod1*Δ*sod2*Δ (○).

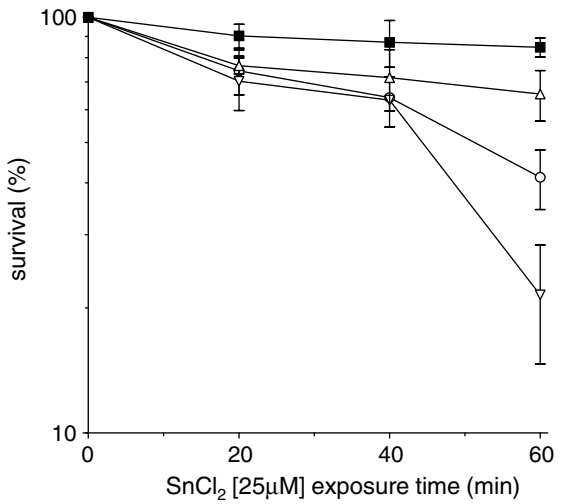

Figure 4. Sensitivity to 25 μM Sn^{2+}-exposure of LOG cells with different defects in cytochrome *c* oxidase WT W303 (■); *cox15*Δ (□); *sco1*Δ (○) and *cox17*Δ (▽).

Glutathione not needed to protect against short-term Sn^{2+}-exposure

Interestingly, mutants with low and extremely low glutathione (GSH) pools showed no enhanced sensitivity to Sn^{2+} (Table 3). Mutant strain q2 (*gsh1*Δ) that lacks the first step of the two-step GSH biosynthesis and relies totally on externally offered (low) GSH showed the same Sn^{2+}-resistance as the q1 WT. The same was true for mutant q3 which is isogenic with mutant strain q2 but, due to a mutational change in the second enzyme of the proline biosynthetic pathway produces a little amount of the dipeptide γ-glutamylcysteine (the product lacking in the *gsh1* mutant (Spector *et al.* 2001)), and hence GSH, and is thus independent of external GSH supplementation. Finally mutant q4 that is WT for GSH biosynthesis and contains only the altered enzyme of the proline pathway, was also WT-like in its Sn^{2+}-resistance phenotype. Thus, GSH is not needed for the protection of yeast against acute, short-term (i.e. 1 h) Sn^{2+}-exposure (Table 3), most probably because catalase provides an overlapping defence system against metal-induced ROS (Grant *et al.* 1998).

The combined action of all above-mentioned protective mechanisms, whose functions were shown in STAT cells, i.e. DNA repair, membrane transport, and defences against ROS, can hardly explain the difference of three orders of magnitude in $SnCl_2$-sensitivity between isogenic LOG and STAT cells. Actually, some protective mechanisms, e.g., Sod1 and Sod2 as well as functionality of cytochrome c oxidase may be totally discounted in this comparison, as the *sod1* and *sod2* mutant alleles (Figure 3) as well as CcO mutants (Figure 4) conferred enhanced $SnCl_2$-sensitivity in STAT and LOG cells alike, regardless of their 1000-fold difference in sensitivity. Thus, this extreme sensitivity of LOG cells (alternatively, the extreme resistance of STAT cells (De Winde *et al.* 1997)) either suggests that cells growing under glucose repression lack at least one, most probably several, unknown mechanism(s) protecting against ROS or other stress (Fuge & Werner-Washburne 1997) or that the protection factors already known to us (c.f. above) have an extremely synergistic interaction (i.e. overlapping functionality) in STAT cells; this would not show us the real protective potential of a single metabolic contribution (as is suggested for the contribution of different repair mechanisms to removal of Sn^{2+}-induced DNA lesions (cf. above and Swanson *et al.* 1999)), but a joint suppression of several of these protective mechanisms (e.g. under glucose repression) would render a LOG cell extremely sensitive. Alternatively or additionally, we might speculate that the high sensitivity of LOG cells could also be, at least partially, due to a more efficient uptake of Sn^{2+} ions as rapidly growing cells might have a more active membrane transport. One step towards clarifying this last question might be the quantitative determination of Sn^{2+}-uptake by molecular dosimetry methods, e.g. via PIXE (particle induced X-ray emission) in isogenic LOG and STAT cells (Viau *et al.* in press). This would also allow us to better assess the genotoxic potential of intracellular Sn^{2+} at different physiological states of the yeast cell. Clearly, there is need for clarifying the types of ROS being directly or indirectly formed by Sn^{2+}, and more information on this may be gained by studying the response of all yeast strains known to have a defect in anti-ROS defence (single or multiple allele mutants) and by complementing this info by *in vitro* biochemical studies.

Despite of the LOG/STAT cells Sn^{2+}-sensitivity/resistance riddle we may summarize our results to partially answer two questions: (1) What type of DNA damage is induced by Sn^{2+}? We know that strand breaks are formed *in vitro* (Dantas *et al.* 1999), and this would best explain the contribution

of recombinational repair; oxidized base damage would explain the necessity of BER repair; some bulky adducts could explain involvement of NER in repair; finally, and most important, translesion synthesis would allow resumption of DNA synthesis at stalled replication forks, at the cost of error-prone repair (mutation). (2) Which species of ROS are generated by intracellular Sn^{2+}? Clearly superoxide anion, as Sod1p, and to a lesser extent, Sod2p are protecting the cells; hydrogen peroxide is most probably generated only in little quantity (or not at all) as cytosolic catalase and GSH are not necessary for protection. Direct base oxidation may occur as indicated by the role of BER in repair, but 8-hydroxyguanine, the specific substrate of Ogg1p, is apparently not formed.

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CAPÍTULO II

Detection and quantitative determination by PIXE of the mutagen Sn^{2+} in yeast cells.

Viau CM, Yoneama ML, Dias JF, Pungartnik C, Brendel M, Henriques JAP.

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Detection and quantitative determination by PIXE of the mutagen Sn^{2+} in yeast cells

C.M. Viau [a], M.-L. Yoneama [b,*], J.F. Dias [b], C. Pungartnik [c], M. Brendel [a,c], J.A.P. Henriques [a,d]

[a] Departamento de Biofísica/Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, UFRGS, Brazil
[b] Instituto de Física, UFRGS, Av. Bento Gonçalves 9500, CEP 91501-970, CP 15051, Porto Alegre, RS, Brazil
[c] Departamento de Ciências Biológicas, Universidade Estadual de Santa Cruz, UESC, Ilhéus, BA, Brazil
[d] Faculdade de Farmácia, Universidade Luterana do Brasil, ULBRA, Porto Alegre, RS, Brazil

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Abstract

The main goal of this work was to determine the concentration of Sn^{2+} ions in cells of the yeast *Saccharomyces cerevisiae* and to correlate their quantity with the genotoxicity of intracellularly accumulated metal ions. The intracellular metal content of yeast cells was determined by PIXE (particle-induced X-ray emission) after cell exposure to $SnCl_2$. To that end, a thick target protocol was developed for PIXE analysis. The samples were irradiated with a 2 MeV proton beam, while the induced X-rays were detected with a high-purity germanium detector. The results of the toxicity of $SnCl_2$ and the PIXE analysis performed with two different yeast strains (haploid and diploid) suggest that the exposure of haploid and diploid yeast to Sn^{2+} induces DNA lesions and that the absorption depends on the genetic background of each strain.

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Keywords: Stannous chloride; Toxicity; *Saccharomyces cerevisiae*; Particle-induced X-ray emission (PIXE)

1. Introduction

Living cells require trace amounts of metal ions for a large variety of biochemical processes [1]. However, over-accumulation of metal ions can be toxic as elevated intracellular levels of many metal ions may negatively interfere with vital processes and thus lead to cell death [2–5]. The regulation of metal ion homeostasis in a cell is a very complicated process that relies on several metal transport proteins and that is highly regulated by transcription factors and protein turnover [6,7].

The mechanisms regulating metal ion resistance in *Saccharomyces cerevisiae* can be roughly classified in four groups: (1) direct binding of metal ions by sulfur-containing amino acids or oligopeptides; (2) transcriptionally activated genes that provide metal ion binding proteins; (3) transmembrane pumps for thiol–metal ion complexes; and (4) proteins involved in proteolytic pathways [8]. Thus, a constellation of proteins, amino acids and oligopeptides with different functions can exert some effect in the metal ion homeostasis.

Usually, the first choice for studies of metal content in cells has been the atomic absorption spectrometry (AAS) technique [9]. Although this technique provides good results for soluble materials, it would demand, in the present case, large amounts of cells and their pre-treatment in order to break the cell walls and free their contents, since the cells themselves are not soluble. These problems can be overcome by choosing the non-destructive and less material-requiring PIXE technique that has already been

* Corresponding author. Tel.: +55 51 33167248; fax: +55 51 33167286.
E-mail address: jfdias@if.ufrgs.br (M.-L. Yoneama).

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used to estimate metal contents in organic and inorganic materials [10–12]. Recently, Kern et al. [13,14] successfully analyzed the cadmium sensitivity phenotypes and the intracellular level of Cd^{2+} in yeast cells through the PIXE technique. In this case, a thin target protocol was applied. Although these targets were suitable for PIXE analysis [13], achieving stringent demands on real thin targets proved to be difficult.

Stannous salts are used in different sectors of human interest, e.g. in food canning and in dentistry (as dentifrices against caries). In nuclear medicine, $SnCl_2$ is employed as reducing agent in labelling radio pharmaceutical agents with technetium-99 (^{99m}TC) [15]. Our work is aimed at analyzing the metal content of Sn^{2+} in yeast cells employing the PIXE technique in the thick target approximation and to correlate its quantity with the toxicity of intracellularly accumulated tin.

2. Experimental

2.1. Target preparation

Media, solutions and buffers were prepared according to [16]. Stationary (STAT) cells were grown in YPD (1% yeast extract, 2% bacto peptone, 2% glucose) at 30 °C for 72 h to a final density of about 2×10^8 cells/mL. Sensitivity of twice saline-washed yeast suspensions to $SnCl_2$ was routinely determined in liquid saline (0.9% NaCl, pH 5.0). Yeast cells of STAT cultures were treated with different $SnCl_2$ concentrations (25 mM for 1 and 2 h, and 50 mM for 1 h). Thereafter, $SnCl_2$-mediated cell aggregates were de-clumped in phosphate buffer (PB, pH 7.4, 0.067 M) followed by vigorous vortexing and washed twice with PB. Some samples were pre-treated with protease (SIGMA, P6911 *Streptomyces griseus*) in a concentration of 10 mg/mL and washed with saline instead of PB. Cells were plated on YPD and survival was determined after 3 days at 30 °C. For PIXE analysis 10 mL of yeast cultures were harvested by centrifugation, freeze-dried, crushed and pressed into pellets. Four independent rounds of sample preparations and PIXE analysis were carried out. The average weight of the pellets was (91.6 ± 0.9) mg.

2.2. Particle-induced X-ray emission (PIXE)

PIXE analysis was carried out at the Ion Implantation Laboratory at the Physics Institute of the Federal University of Rio Grande do Sul. A 3 MV Tandetron accelerator provided a 2 MeV proton beam with an average current of 2 nA for the experiments. Details of the experimental set-up are described in [13,17]. The characteristic X-rays induced by the proton beam were detected by an HPGe detector from EG&G (GLP series, EG&G Ortec, CA, USA), with an energy resolution of 180 eV at 5.9 keV. The detector was positioned at 135° with respect to the beam axis. The electronics consisted of a Telennec 245 amplifier associated with a Genie 2000 multi-channel analyser (Canberra, USA) running in a PC-compatible computer. This detection system was optimised for analysis of X-rays ranging from 6 to 30 keV.

The standardization procedure adopted in this work (the so-called H value method) is described in details elsewhere [18,19]. Since the yeast samples were prepared as pellets, a bovine liver (SRM – NIST 1577b) was used as a standard material for the analysis of all light elements ($18 \leqslant Z \leqslant 28$) present in the yeast samples. In order to minimize matrix effects and therefore improve the quality of the final results [13], the quantification of Sn present in the samples was carried through the analysis of the Sn Kα line of 25.27 keV. Although tin was not present in the NIST 1577b reference material, it was observed that for elements with $Z \geqslant 29$ the H values obtained with this reference material are compatible with those obtained in the thin target approximation using single-element standards from Micromatter. Therefore, the concentration of Sn was evaluated using the standardization obtained with a Sn target of about 50 μg per square centimetre from Micromatter. The limit of detection achieved in this work was of the order of 9 μg per 10^9 cells. In order to determine the matrix composition of the samples, Rutherford backscattering spectrometry (RBS) experiments were carried out using 1.2 MeV He^+ ions. In the framework of surface approximation yield, the C and O contents were evaluated and the final result is consistent with the formula $C_7H_{10}O_3$ presented in [20].

PIXE spectra were analyzed with GUPIX software package developed at the University of Guelph (Canada) and as discussed in [21] and references therein.

3. Results and discussion

The results of the toxicity of stannous chloride and the PIXE analysis performed with two different yeast strains suggest that the cells have indeed absorbed the metal (Table 1). The presence of Sn in untreated yeast samples (blank samples) was not observed by PIXE. Since it is known that Sn is not an essential element for the cells [22,23], it was assumed that the intracellular Sn content, i.e. the fraction of the metal ions that passes through the cytoplasmic membrane and that can cause mutation and toxicity, stems from the cell treatment with $SnCl_2$. As suggested by a recent study with cadmium [13], the Sn ions that remain in the cell walls might be washed out during the last step of the sample preparation.

The results of Table 1 were obtained for PB-treated STAT yeast cells. The diploid (XS2316) cells absorbed about 60% of the total amount of Sn^{2+} absorbed by the haploid (XV185-14c) yeast cells. This indicates that Sn^{2+} absorption is better in haploid yeast cells and agrees well with published genotoxicity results [15]. Our data suggest that the exposure of haploid and diploid yeast to Sn^{2+} induces DNA lesions and that Sn^{2+} absorption apparently depends on the ploidy and the genetic background of each strain [15].

Table 1
PIXE results of Sn content in haploid (XV185-14c) and diploid (XS2316) yeast strains

Yeast strain	$SnCl_2$ treatment	Sn concentration ($mg/10^9$ cells)	LOD[a] ($mg/10^9$ cells)
Haploid	None	<LOD	0.01
Haploid	25 mM, with PB, 1 h	2.4 ± 0.3	0.06
Haploid	25 mM, with protease, 1 h	2.7 ± 0.4	0.06
Diploid	None	<LOD	0.01
Diploid	25 mM, with PB, 1 h	1.5 ± 0.2	0.03
Haploid	25 mM, with PB, 2 h	4.7 ± 0.5	0.05
Haploid	50 mM, with PB, 1 h	4.7 ± 0.5	0.06

These values represent the average of four independent experiments for each strain. The uncertainties quoted were estimated by taking into account the statistical fluctuation, the uncertainty in the mass determination and the fit uncertainty (%) given by the GUPIX code.
[a] Limit of detection.

PIXE experiments of haploid cells treated either with PB or with protease were carried out to verify the effect of these alternative anti-clumping treatments [15] on Sn^{2+} absorption. As shown in Table 1, cellular Sn^{2+} absorption was found to be independent of the treatment. Moreover, the yeast survival rate as a function of $SnCl_2$ exposure time (in minutes) for cells treated with PB and protease were practically identical (Fig. 1). The correct de-clumping by PB of Sn^{2+}-treated cells was controlled by microscopic observation. Therefore, for practical (time of protease digestion) and economical (cost of enzyme) reasons, PB should be used for the de-clumping instead of protease [15].

PIXE analysis of yeast cells treated with 25 mM for 2 h and 50 mM for 1 h revealed that the cells absorbed the same amount of Sn^{2+} in both cases. We could also observe that the amount of Sn^{2+}absorbed by the cells treated with 25 mM for 2 h was approximately twice that absorbed by the cells treated with 25 mM for 1 h. While it is clear that Sn^{2+} is genotoxic and can be defined as a moderate mutagen of low toxicity [15], further investigations on the interference of tin with others metals, i.e. Fe, Zn and Cu are necessary as these metals are essential trace elements required for survival by all pro- and eukaryotes [24].

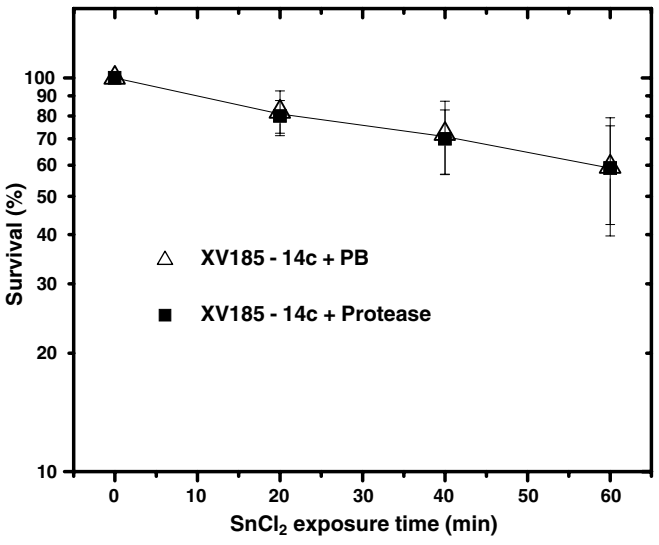

Fig. 1. Toxic effects of 25 mM $SnCl_2$ in stationary haploid XV185-14c yeast cells de-clumped in PB (open triangle) and protease (dark square). The line is only to guide the eye.

In conclusion, we have demonstrated a new application of the PIXE technique for the exact assessment of the real concentration of some cell-bound metals. The method is easy to perform and allows a perfect matching of physical and biological endpoints (genotoxicity or toxicity) after metal exposure. The overall picture of Sn^{2+} metabolism as well as that of other heavy metals in eukaryotic cells is still a challenge to our understanding and demands further studies.

Acknowledgements

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CAPÍTULO III

$SnCl_2$-induced DNA damage and repair inhibition of MMS-caused lesions in V79 chinese hamster fibroblasts.

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GENOTOXICITY AND CARCINOGENICITY

$SnCl_2$-induced DNA damage and repair inhibition of MMS-caused lesions in V79 Chinese hamster fibroblasts

C. M. Viau · Temenouga N. Guecheva · F. G. Sousa · C. Pungartnik · M. Brendel · J. Saffi · João Antonio Pêgas Henriques

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Abstract In order to clarify the molecular mechanisms of Sn^{2+} genotoxicity, we evaluated the induction of strand breaks, formamidopyrimidine DNA glycosylase (Fpg) and endonuclease III (Endo III) sensitive sites, and the interference with the repair of methyl methane sulfonate (MMS)-caused DNA damage in V79 Chinese hamster lung fibroblasts exposed to stannous chloride by comet assay. A concentration-related increase in the DNA damage induced by 2 h $SnCl_2$ treatment at a concentration range of 50–1,000 μM was observed ($r = 0.993$; $P < 0.01$). Significantly elevated DNA migration in relation to the control level was detected at doses 100, 500 and 1,000 μM in normal alkaline and at doses 500 and 1,000 μM in modified (with Fpg and Endo III) comet assay. Although 50 μM $SnCl_2$ concentration did not increase significantly the DNA migration by itself in comet assay, it was capable to inhibit the repair of MMS-induced DNA damage during the post-treatment period of 24 h. Our results demonstrate the genotoxic and comutagenic effects of stannous chloride in V79 cells. The inhibitory effect of Sn^{2+} on repair of MMS-induced DNA damage suggests that this metal can also interfere in DNA repair systems thus contributing to increased mutation by shifting the balance from error-free to error-prone repair processes.

Keywords Stannous chloride · Comet assay · DNA repair · Endonuclease III · Formamidopyrimidine DNA glycosylase

Abbreviations

8-oxoG	8-Oxo-7,8-dihydroguanine
AP sites	Apurinic/apyrimidinic sites
BER	Base excision repair
Endo III	Endonuclease III
Fpg	Formamidopyrimidine DNA glycosylase
MMS	Methyl methane sulfonate
ROS	Reactive oxygen species

Introduction

Stannous (tin II) chloride is used worldwide as preservative in soft drinks, whereas stannous fluoride is used as antimicrobial agent in dentifrices. Stannous chloride is also employed as reducing agent to produce Technetium-99m-labelled radiotracers administered intravenously in nuclear medicine procedures (Harbert et al. 1996). The exposure of the general population to tin is essentially dietary in origin, coming particularly from the consumption of foods stored in tin cans (Blunden and Wallace 2003).

C. M. Viau · T. N. Guecheva · F. G. Sousa · J. Saffi · J. A. P. Henriques (✉)
Departamento de Biofísica/Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, UFRGS, Av. Bento Gonçalves 9500, Porto Alegre 91501-970, Brazil
e-mail: pegas@cbiot.ufrgs.br

T. N. Guecheva
e-mail: tgesheva@gmail.com

C. Pungartnik · M. Brendel
Departamento de Ciências Biológicas, Universidade Estadual de Santa Cruz, UESC, Ilhéus, BA, Brazil

J. Saffi · J. A. P. Henriques
Laboratório de Genética Toxicológica, Universidade Luterana do Brasil, Canoas, RS, Brazil

J. A. P. Henriques
Instituto de Biotecnologia, Universidade de Caxias do Sul, Caxias do Sul, RS, Brazil

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Stannous chloride-induced toxicity and genotoxicity have been tested in various in vivo and in vitro models. Sn^{2+} induces the denaturation of double stranded DNA and leads to alteration of all four types nucleotide triphosphates (de Mattos et al. 2005). In bacteria, negative results were reported for the *Bacillus subtilis* Rec-assay (Kada et al. 1980) and for *Salmonella* assay (reviewed in Ashby and Tennant 1991), whereas genotoxicity of $SnCl_2$ was shown in the SOS chromotest (Olivier and Marzin 1987). Pungartnik et al. (2005) demonstrate that $SnCl_2$ could be defined as a moderate mutagen of low toxicity in *Saccharomyces cerevisiae*. McLean et al. (1983a, b) reported genotoxic effect of $SnCl_2$ in Chinese hamster ovary cells and human white blood cells. $SnCl_2$ induced a dose-dependent increase in the frequency of chromosomal aberrations and teratogenic effects in mice (El-Makawy et al. 2008). In rabbits, decrease in sulfhydryl groups, increase in lipid peroxidation, altered enzyme activities and histopatology were observed after oral administration (El-Demerdash et al. 2005). $SnCl_2$ was carcinogenic in rodent thyroid gland (reviewed in Ashby and Tennant 1991).

Stannous-ion-induced DNA damage was suggested to occur via formation of reactive oxygen species (ROS) (McLean et al. 1983a; Dantas et al. 1996) as found for other metal ions that compromise genetic stability by inducing different types of oxidative DNA damage (reviewed in Beyersmann and Hartwig 2008). Carcinogenic metal compounds are often comutagenic, i.e., they enhance the mutagenicity of other genotoxic agents. Indeed, many carcinogenic metal compounds at low concentrations have been identified as inhibitors of the repair of DNA damage caused either by other xenobiotics or by endogeneous factors (reviewed in Beyersmann and Hartwig 2008). The comet assay [single cell gel electrophoresis (SCGE)] provides a sensitive tool to investigate DNA damage and repair (Tice et al. 2000; Speit and Hartmann 2006). Sensitivity and specificity of the comet assay can be improved by incubation of the cells with lesion-specific enzymes, such as formamidopyrimidine DNA glycosylase (Fpg) and endonuclease III (Endo III) that recognize oxidative damage (Collins et al. 1993). Although increasing number of evidences about Sn^{2+} induced oxidative stress and genotoxicity have been accumulated (McLean et al. 1983a; Dantas et al. 1996; Dantas et al. 1999; Pungartnik et al. 2005; El-Demerdash et al. 2005), there is no data in the literature concerning the possible influence of stannous ions on the repair of DNA damage caused by other genotoxic agents. In order to clarify the molecular mechanisms of Sn^{2+} genotoxicity we evaluated the induction of strand breaks, Fpg and Endo III sensitive sites, and the interference with the repair of methyl methane sulfonate (MMS)-caused DNA damage in V79 Chinese hamster lung fibroblasts exposed to stannous chloride by comet assay.

Materials and methods

Chemical reagents

Stannous chloride (anhydrous), hydrogen peroxide and MMS were purchased from Sigma® (St. Louis, MO, USA). Chemicals for cell culture, low melting point (LMP) and normal-melting-point agarose were purchased from Gibco-BRL® (Grand Island, NY, USA). Fpg, 8,000 U/mL, and Endo III, 10,000 U/mL, were obtained from New England BioLabs® (USA). All other chemicals were of the highest purity grade commercially available.

Cell culture

V79 Chinese hamster lung fibroblast cells (V79 cells) were cultivated in Dulbecco's modifed Eagle's medium (DMEM), supplemented with 10% heat-inactivated foetal bovine serum (FBS), 0.2 mg/mL L-glutamine, 100 IU/mL penicillin, and 100 µg/mL streptomycin. Cells were maintained in a humidified incubator at 37°C with 5% CO_2 and harvested with an aqueous solution containing 0.15% trypsin and 0.08% EDTA.

Cell viability

For cytotoxicity assay, cells were seeded into 96-well micro plates at 2.5×10^4 cells per well in 100 µL DMEM. After 24 h, cultures were treated with 100 µL of $SnCl_2$ at concentrations of 50, 100, 500 and 1,000 µM for 2 h or at 50 µM $SnCl_2$ (concentration used in the DNA repair studies) for 24 h. Cytotoxicity was assessed by means of the thiazolyl blue tetrazolium bromide (MTT) assay (Mosmann 1983). Absorbance was read at a wavelength of 540 nm and semi logarithmically plotted against drug concentrations. Cell growth inhibition was assessed by trypan blue staining. The cells were treated for 2 h at concentrations of 50, 100, 500 and 1,000 µM $SnCl_2$, the medium was removed and cells were washed twice with PBS. Cell number was determined after 24 or 48 h of further incubation in fresh medium by microscopic counting. All values are averages of three independent experiments, each done in triplicate. The viability of the cells was checked concurrently in all experiments at all tested concentrations by trypan blue exclusion assay.

Cell treatment

Stannous chloride was taken from stock prepared daily (25 mM in deionised water) and added to the cell culture medium to give final concentration in the range 50–1,000 µM. The cells were incubated with $SnCl_2$ (50, 100, 500 and 1,000 µM) for 2 h at 37°C. Each experiment

included treatment with hydrogen peroxide at concentration of 10 µM, applied for 5 min at 4°C, as positive control. After the treatment the cells were harvested and used for the comet assay.

For repair kinetics study the V79 cells were treated with MMS at concentration of 8×10^{-5} M for 1 h and post-incubated in DMEM or post-treated in DMEM with 50 µM $SnCl_2$ for 2, 4, or 24 h. Cells not treated with MMS were also incubated in DMEM with or without 50 µM $SnCl_2$ as a control.

Comet assay

The alkaline comet assay was performed as described by Singh et al. (1988). Briefly, 20 µL of cell suspension (~10,000 cells) treated as described above, were mixed with 90 µL LMP agarose, spread on a normal agarose pre-coated microscope slide and placed at 4°C for 5 min to allow for solidification. Cells were lysed in high salt and detergent (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris with 1% Triton X-100 and freshly added 10% DMSO) during 2 h. Slides were removed from lysing solution and washed three times in enzyme buffer (40 mM Hepes, 100 mM KCl, 0.5 mM Na_2EDTA, 0.2 mg/mL BSA, pH 8.0) and further incubated with 70 µL of Fpg (30 min, 37°C) or Endo III (45 min, 37°C) in dilution recommended by the supplier. Subsequently, cells were exposed to alkali (300 mM NaOH/1 mM Na_2EDTA, pH >13, 30 min, 4°C), to allow DNA unwinding and expression of alkali-labile sites. For electrophoresis, an electric field of 78 V/cm was applied for 25 min at 4°C. After electrophoresis, the slides were neutralised and silver stained (Nadin et al. 2001). One hundred cells were scored visually according to the tail length and the amount of DNA present in the tail. Each comet was given an arbitrary value of 0–4 (0, undamaged; 4, maximally damaged), as described by Collins et al. (1995). Damage score was thus assigned to each sample and can range from 0 (completely undamaged: 100 cells × 0) to 400 (with maximum damage: 100 cells × 4). International guidelines and recommendations for the comet assay consider that visual scoring of comets is a well-validated evaluation method as it is highly correlated with computer-based image analysis (Collins et al. 1995; Burlinson et al. 2007).

Statistical analysis

Results are represented as mean ± standard error mean (SEM) of three independent experiments. The statistical analyses were performed by GraphPad Prism® program. Differences in the extent of DNA damage between the control and the treatments in dose-response studies were tested for significance using ANOVA analysis of variance with Dunnett's multiple comparison test and Pearson correlation. For repair studies two-way ANOVA with Bonferroni post-tests were applied. An alpha level of 0.05 was used to determine significance in all statistical analyses.

Results

Cell viability

The viability of the V79 Chinese hamster lung fibroblasts after 2 h stannous chloride treatment at concentration range of 50–1,000 µM was above 98% for all doses tested in MTT assay. The viability of cells incubated with 50 µM $SnCl_2$ for 24 h was 92.7 ± 1.8%. The growth inhibition was observed in dose-dependent manner after 2 h $SnCl_2$ treatment followed by 24 or 48 h of incubation in stannous chloride free medium, reaching 68 and 72%, respectively, for the highest exposure dose (Fig. 1).

DNA damage

The genotoxic effect of Sn^{2+} in V79 cells was estimated by two different parameters—damage score and percent of damaged cells (cells with tail under the test conditions used) (Table 1). Significantly elevated DNA damage was observed at concentration range of 100–1,000 µM $SnCl_2$ for both parameters used. A concentration-related increase in the DNA damage induced by 2 h $SnCl_2$ treatment was observed ($r = 0.993$; $P < 0.01$). The damage score was selected for further evaluation as it is considered a sensitive measure of DNA damage, based on the length of migration and the amount of DNA in the tail (Collins et al. 1995). Furthermore, the visual score (arbitrary units) have shown

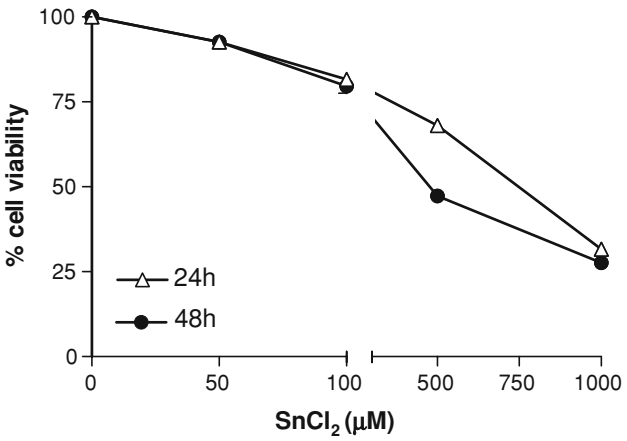

Fig. 1 Effect of $SnCl_2$ on viability of V79 cells. The cells were treated for 2 h at different $SnCl_2$ concentrations, washed, and further incubated in fresh medium for 24 or 48 h. Mean ± SEM of three independent experiments

Table 1 Genotoxic effect of 2 h $SnCl_2$ treatment in V79 cells in comet assay

Treatment	Damage score (0–400)	Damaged cells (%)
Control	59.8 ± 5.4	39.5 ± 4.2
50 μM $SnCl_2$	72.5 ± 2.3	51.5 ± 4.5
100 μM $SnCl_2$	82.0 ± 2.5**	57.5 ± 3.5*
500 μM $SnCl_2$	101.0 ± 2.5**	59.0 ± 3.0*
1,000 μM $SnCl_2$	131.8 ± 4.1**	65.0 ± 4.0**
10 μM H_2O_2	214.3 ± 3.8**	91.8 ± 1.1**

Data are mean ± SEM from three experiments

* $P < 0.05$; ** $P < 0.01$, relative to untreated control

an almost linear relationship with percent tail DNA (reviewed in Møller 2006).

In the modified comet assay (with Fpg and Endo III enzymes), the 2 h Sn^{2+} exposure increase the DNA damage in relation to the basal level at doses 500 and 1,000 μM (Fig. 2), whereas in the comet assay without the use of enzymes the significant increase was detected at 100, 500 and 1,000 μM $SnCl_2$. To verify the ability of Fpg and Endo III to recognize oxidized bases in our test system, control cells were incubated with hydrogen peroxide at concentration of 10 μM for 5 min at 4°C. A significant increase in the damage score in relation to the value in the comet assay without enzyme incubation (214.3 ± 3.8) was observed after incubation with Fpg (290.3 ± 9.0, $P < 0.01$) and Endo III (250.0 ± 3.4, $P < 0.01$).

Repair kinetics studies

In order to study the influence of Sn^{2+} on the persistence of MMS-induced DNA lesions, V79 cells were treated with MMS (1 h at concentration of 8×10^{-5} M), and further incubated in the absence (post-incubation) or presence (post-treatment) of 50 μM $SnCl_2$ for a period of 24 h (Fig. 3). This dose of stannous chloride was chosen for the repair kinetic studies because it did not induce significant elevation of DNA migration in comet assay by itself (Table 1). Non-treated cells were also incubated for 24 h in presence or absence of 50 μM $SnCl_2$ as a control. The MMS-induced DNA damage was reduced significantly after 4 h of post-incubation and reached the almost basal level after 24 h (Fig. 3). In the presence of Sn^{2+} during the post-treatment, enhanced persistence of DNA lesions in relation to the corresponding post-incubation value was detected at both 4 and 24 h ($P < 0.001$) indicating inhibition of the repair of MMS-induced lesions. DNA migration in the non MMS-treated cells incubated with 50 μM $SnCl_2$ slightly increased after 2 h of incubation, and returned to the control level at 4 and 24 h.

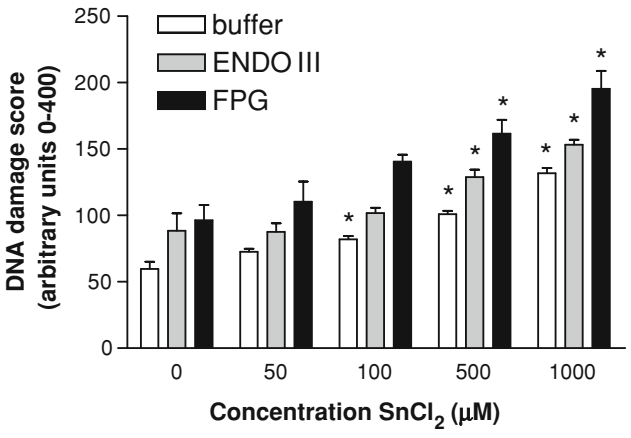

Fig. 2 Induction of DNA strand breaks, Endo III- and Fpg-sensitive sites by 2 h $SnCl_2$ treatment in V79 cells. Mean ± SEM of three independent experiments. Significantly different in relation to the control level, * $P < 0.01$

Discussion

In general, metal genotoxicity is caused by three predominant mechanisms: (1) interference with cellular redox regulation and induction of oxidative stress; (2) inhibition of major DNA repair systems resulting in genomic instability and accumulation of critical mutations; (3) deregulation of cell proliferation (reviewed in Beyersmann and Hartwig 2008). Tin chloride-induced DNA oxidation is suggested to cause mutagenicity in bacteria (Cabral et al. 1998), which is confirmed by the high sensitivity of bacterial tester-strains lacking anti-ROS defences (*Salmonella* TA102 and Mutoxitest strain WP203) (Pungartnik et al. 2005). The importance of anti-ROS defence systems of the yeast cell on

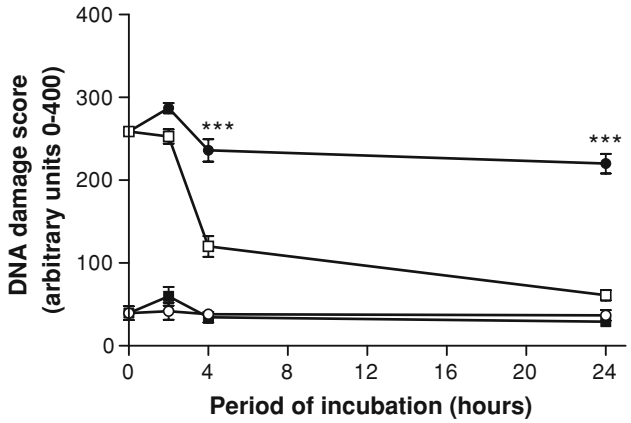

Fig. 3 Repair kinetics of MMS-caused DNA damage in presence of $SnCl_2$ in V79 cells. Cells treated for 1 h with 80 μM MMS and further incubated in DMEM without (*open square*) or with 50 μM $SnCl_2$ (*filled circle*). MMS non-treated cells incubated in DMEM without (*open circle*) or with 50 μM $SnCl_2$ (*filled square*). Mean ± SEM of three experiments. Significantly different from the corresponding post-incubation value, *** $P < 0.001$

$SnCl_2$-induced toxicity was demonstrated by the higher sensitivity of haploid strain containing the *sod1* mutant allele, indicating that the cytosolic superoxide dismutase could be acting to prevent stannous-ion-induced injury (Pungartnik et al. 2005). Moreover, the exponentially growing yeast cells were much more sensitive to Sn^{2+} than stationary cells of the same strain which can be attributed to the maximal expression of anti-ROS defence systems during the stationary phase (Pungartnik et al. 2005).

In our study, genotoxicity of Sn^{2+} in V79 cells in comet assay (Table 1) was observed in the same dose range as in the study of McLean et al. (1983a), where the authors reported extensive DNA strand breaks produced by stannous chloride at concentrations up to 500 μM as detected by alkaline sucrose gradient analysis in Chinese hamster ovary cells. Increased migration in the comet assay was also detected in peripheral blood nuclear cells from patients who had received endovenously injected radiopharmaceuticals containing stannous chloride as reducing agent (Dantas et al. 2002). The increased sensitivity of yeast *rad52Δ* mutant, specific to detect strand breaks-generating mutagens, suggests that $SnCl_2$ directly or indirectly leads to this type of DNA lesion in yeast (Pungartnik et al. 2005). Moreover, the bacterial strain deficient in xthA activity (that accounts for ~90% of the apurinic/apyrimidinic (AP) endonucleolytic activity found in *Escherichia coli*) was extremely sensitive to stannous ion indicating formation of AP sites (Cabral et al. 1998). The alkaline comet assay (pH ≥13) reveals true single-strand breaks and alkali-labile DNA lesions, i.e., AP sites. Thus, the observed increase in DNA migration in comet assay in our study could be explained with either base damage or strand breaks induction by ROS generation or by direct action of Sn^{2+} on DNA in V79 cells.

In order to asses the oxidized base formation after stannous chloride exposure, we incubated the cells with Fpg and Endo III, the enzymes most commonly used to detect oxidative damage in the comet assay. Fpg is specific for oxidized purines, including 8-oxo-7,8-dihydroguanine (8-oxoG) and ring-opened purines while Endo III recognizes oxidized pyrimidines, including thymine glycol and uracil glycol. Moreover, some of the secondary oxidation products of 8-oxoG, the main oxidation product in DNA, are also excised by Fpg (oxaluric acid, oxazolone, hydantoins) and Endo III (oxaluric acid and oxazolone) (reviewed in Kim et al. 2001). The mutant of Ntg 2, the functional homolog of Endo III that was found to repair the oxidation products of 8-oxoG in *Saccharomyces cerevisiae* (Kim et al. 2001), was found to be sensitive to stannous chloride treatment (Viau et al. 2006) indicating that some of these substrates can be formed.

Our results demonstrate that Sn^{2+} induced only a limited elevation in Fpg and Endo III sensitive sites (Fig. 2) in toxic concentrations, differently from Nickel (II), e.g., that induced 3–4-fold elevation in Fpg sensitive sites in human lymphocytes (Woźniak and Błasiak 2002). This can suggest that stannous ion does not induce preferentially base modifications that are substrate of Fpg and Endo III enzymes in V79 cells. The mutation spectrum induced by $SnCl_2$ in *E. coli* reveals high frequency of guanine-involved base substitutions, mainly transversions—G:C → T:A and G:C → C:G (Cabral et al. 1998). High valent metals have been shown to induced secondary oxidation products of 8-oxoG, guanidinohydantoin (Gh) and spiroiminodihydantoin (Sp), that enhanced misincorporation leading to G:C → T:A and G:C → C:G transversions (Hailer et al. 2005). These oxidation products are substrate of the mammalian glycosylases NEIL1 and NEIL2, whereas mammalian homologs of Ogg1 and Nth1 have little or no affinity to Gh and Sp (Hailer et al. 2005). The cyanuric acid, another oxidation product of the unstable 8-oxoG, could be mutagenic DNA lesion yielding G:C–T:A transversions like 8-oxoG (Dherin et al. 2004). Moreover, the authors showed that in mammalian cells the cyanuric acid is substrate of human methylpurine DNA N-glycosylase (Mpg) and not of the oxidized bases-specific enzymes (such as Fpg, Nth, Ntg1, Ntg2, Ogg1, hNth1 and hOgg1) which can be the reason of limited detection of Fpg and Endo III sensitive sites in our experiment.

Since some metal ions were found to inactivate DNA repair (reviewed in Hartwig and Schwerdtle 2002; Hartwig et al. 2002; Beyersmann and Hartwig 2008), possible influence of Sn^{2+} on functionality of DNA repair mechanisms was tested by introducing MMS-induced DNA lesions prior to metal exposure. MMS is monofunctional alkylating agent that alkylates directly the nitrogen and oxygen of DNA bases. The repair of *N*-alkilated bases in living cells occurs mainly via base excision repair (BER) and, to a lesser extent, via nucleotide excision repair (NER) (Sancar et al. 2004), both contributing to comet formation via DNA repair-induced intermediates. BER removes alkilated bases by specific glycosylases, resulting in the formation of AP sites. Subsequently, the AP sites are removed by specific endonucleases, the gaps formed are filled with newly synthesized DNA by polymerases, and the ends are ligated by DNA-ligases. So, the observed increase in DNA strand breaks after MMS treatment reflects the excision of damaged nucleotides, whereas the decline of DNA migration reflects the ligation step.

In our study, the 50 μM $SnCl_2$ concentration did not increase significantly the DNA migration by itself in comet assay (Table 1) but it was capable to inhibit the repair of MMS-induced DNA damage during the post-treatment period of 24 h (Fig. 3). The increased level of DNA strand breaks observed in MMS-treated cells after post-treatment with $SnCl_2$ as compared to MMS-treated and post-incubated cells could indicate that the presence of $SnCl_2$ inhibits

the polymerization and/or ligation steps of MMS-induced DNA damage repair. These steps were shown to be very sensitive towards the action of Cd(II), Pb(II), Co(II), Ni(II) and As(III) (reviewed in Beyersmann and Hartwig 2008). Post-treatment with arsenite increased DNA strand breaks in MMS-treated cells inhibiting in greater extent the ligation step of DNA repair (Lynn et al. 1997). The authors reported that arsenite can inhibit DNA breaks rejoining by interacting with the vicinal dithiol groups of DNA ligase III to inactivate DNA ligation in MMS-treated cells. However, there are no published data for DNA repair enzymes inhibition by Sn^{2+} available.

On the other hand, the increased DNA damage in post-treated cells in our study could reflect accumulation of AP sites (which are converted in strand breaks in comet assay) as a result of endonuclease inhibition. The inhibition of Ape1 (the major mammalian endonuclease for repairing mutagenic and cytotoxic AP sites in DNA) was reported to be caused by lead (McNeill et al. 2008) which, like tin, is post-transition metal from group 14. In addition to AP site incision inhibition lead exposure increased the mutagenicity of MMS (agent known to generate AP sites in DNA) in Chinese hamster ovary AA8 cells (McNeill et al. 2008). Moreover, Ape1 overexpression in AA8 reverses AP sites accumulation and MMS-associated mutagenesis. We found by qRT-PCR significant up-regulation in the expression of *APN1* in stannous chloride treated yeast cells (Viau et al., unpublished results). This transcriptional activation of *APN1* could reflect DNA repair regulation in attempt to overcome the AP accumulation caused by Apn1 inhibition.

Our results demonstrate the genotoxic and comutagenic effect of stannous chloride in V79 cells. The efficient repair of DNA lesions induced by endogenous processes and by environmental factors is an important pre-requisite to maintain DNA integrity. The inhibitory effects of Sn^{2+} on repair of MMS-induced DNA damage suggest that this metal can also interfere in DNA repair systems and thus may contribute to increased mutation by shifting the balance from error-free to error-prone repair processes.

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CAPÍTULO IV

Cellular and molecular mechanisms of uptake and distribution of stannous ions in the yeast Saccharomyces cerevisiae.

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Cellular and molecular mechanisms of uptake and distribution of stannous ions in the yeast *Saccharomyces cerevisiae*

Cassiana Macagnan Viau[1], Jacqueline Moraes Cardone[1], Diego Bonatto[2], Maria-Lúcia Yoneama[3], Jonny Ferraz Dias[3], Cristina Pungartnik[4], Martin Brendel[4], João A. P. Henriques[1,2,5]*

[1] Laboratório de Genotoxicidade-Instituto Royal - Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil

[2] Universidade de Caxias do Sul (UCS), Caxias do Sul, RS, Brazil

[3] Faculdade de Física Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil

[4] Departamento de Ciências Biológicas, Universidade Estadual de Santa Cruz (UESC), Ilhéus, Bahia, Brazil

[5] Faculdade de Farmácia, Universidade Luterana do Brasil (ULBRA), Canoas, RS, Brazil

*Corresponding author: Prof. Dr. João Antonio Pêgas Henriques

Centro de Biotecnologia-UFRGS, Av. Bento Gonçalves 9500, 91501-970 Porto Alegre, Brazil

FAX +55 51 3308-6069

e-mail: pegas@cbiot.ufrgs.br

Abstract

Tin or stannous (Sn^{2+}) compounds are used as catalysts, stabilizers in plastic industries, wood preservatives, agricultural biocides, antifouling paints for ship hulls and in nuclear medicine. Due to the wide application of these compounds, organic and inorganic forms of Sn^{2+} are found in many life forms and have accumulated in the food chain. In order to verify how Sn^{2+} is uptaken, distributed and detoxified, we associated molecular techniques including survival assay and quantitative real time PCR (qRT-PCR) with multielemental procedure namely particle-induced X-ray emission (PIXE). According to PIXE analysis, the results for XV185-14c yeast cells indicate a significant loss of intracellular elements such as Mg, Zn, S, Fe and an increase of P levels after 1 h exposure to 25 mM $SnCl_2$ in stationary (STAT) phase. The survival assay showed that Fet4p and Zrt2p both low-affinity iron and zinc transporters, respectively, may internalize Sn^{2+}. By its turn, qRT-PCR showed an increased in *CCH1* and *MID1* gene expression, which codify for the cytoplasmic transmembrane Ca^{2+} transporters Cch1p and Mid1p, suggesting that both proteins may also take up Sn^{2+}. Our data also indicated that once inside the cell, Sn^{2+} may be taken from the cytosol by a P-type ATPase to the vacuole (Pmc1p) or may be chelated by Crs5p metallothionein in the cytosol. Alternatively, Sn^{2+} may generate reactive oxygen species (ROS), especially O_2^- which can be dismutated to form H_2O_2 and the highly reactive $^{\bullet}OH$. In consequence, ROS can induce direct cellular injury and stress-generated activation of many genes, e.g., *SOD1*, *YAP1*, and *APN1*.

Keywords: stannous ions, metal transport, metallothionein, qRT-PCR, *Saccharomyces cerevisiae*, PIXE

1. Introduction

Life on Earth depends on availability of transition metals [1] as their ability to donate and accept electrons [2] makes them indispensable for metabolic processes. Some metals, such as iron and copper are essential as they serve as micronutrients and are used in redox processes; moreover, they stabilize molecules through electrostatic interactions and serve as cofactors for various enzymes [3-5]. Zinc is not a redox-active metal, but it plays an essential role in protein structure, where hundreds of proteins require it for proper function [6, 7]. Many other metals, e.g., bismuth, boron, mercury, thallium and tin have no biological function [8] and thus are considered nonessential and potentially toxic to microorganisms and higher forms of life [9]. Nonessential metals may be toxic by displacing essential metals from their native binding sites or through ligand interactions [10]. At high concentrations, both essential and nonessential metals can damage cell membranes, change enzyme specificity, and damage the structure of DNA [11].

One of the cellular strategies for metal resistance is the prevention of their entry into the cytoplasm [12]. Uptake of hydrophilic substances is rigidly regulated by specific transmembrane channels or transporters [7, 12]. High-affinity transport systems are active in metal-limited cells, whereas low-affinity metal transporters are predominant in the presence of substrate abundance [1]. A general mechanism for metal detoxification in yeast and in other organisms is the chelation of metal by metallothioneins (MT), a large superfamily of low-molecular weight, cysteine rich metal-chelating peptides [13-15]. Together with MT, the tripeptide glutathione (L-γ-glutamyl-L-cysteinyl-glycine) can also chelate metal ions [16].

The metabolism of endogenous metals can be disturbed by the presence of heavy metals like mercury, bismuth and cadmium [8]. Analogous data for tin are lacking. Tin or stannous ions (Sn^{2+}) can combine with chemicals like chloride, sulfur, or oxygen to form inorganic tin compounds (e.g., stannous chloride, stannous sulfide, stannic oxide) [17]. These

compounds are used in toothpaste, perfumes, soaps, food additives and dyes [18]. In addition, stannous chloride ($SnCl_2$) is widely used in daily human life to conserve soft drinks, in food manufacturing, processing and packaging, and in biocidal preparations. In nuclear medicine, $SnCl_2$ is used as a reducing agent of technetium-99m to manufacture radiopharmaceutical kits [18].

In spite of its widespread use, there are several undesired biological effects of Sn^{2+} ions and derived compounds described in the literature. Using an *in vitro* assay with UV absorption spectra, De Mattos *et al*. [19] demonstrated that Sn^{2+} ions induce the denaturation of double stranded DNA and leads to alteration of all four types of nucleotide triphosphates by an increased and displaced UV absorption peak. In bacteria, negative results were reported for the *Bacillus subtilis* Rec-assay [20] and for *Salmonella* assay [21], whereas genotoxicity of Sn^{2+} ions were shown by the SOS chromotest [22]. McLean *et al*. reported genotoxic effect of Sn^{2+} ions in Chinese hamster ovary cells [23] and human white blood cells [24]. Sn^{2+} ions induced a dose-dependent increase in the frequency of chromosomal aberrations and teratogenic effects in mice [18]. In rabbits, decrease in sulfhydryl groups, lipid peroxidation, altered enzyme activities and histopatology were observed after oral administration of Sn^{2+} [25]. The Sn^{2+} ions were also carcinogenic in rodent thyroid gland [21].

Our recent studies with the yeast *Saccharomyces cerevisiae* have led to a better understanding of Sn^{2+} ions toxicity at the molecular level [26, 27]. Sn^{2+} ions are weakly genotoxic in prokaryotic and eukaryotic microbial test systems [26] and its toxicity and genotoxicity can be correlated with the amount of absorbed metal [28]. Once inside the cell, the indirect effects of Sn^{2+} are related to the generation of reactive oxygen species (ROS) [26, 27] and the interference with DNA repair proteins [29]. Until recently, little was known about Sn^{2+} ions uptake by eukaryotic cells and its intracellular distribution. Also it was not clear if Sn^{2+} ions would influence metabolism of essential elements.

In this work, we use the eukaryotic *S. cerevisiae* to investigate how Sn^{2+} ions are uptaken, distributed and detoxified by this yeast cells. First of all, we performed a screening, using the multielemental technique called particle-induced X-ray emission (PIXE) in order to verify and quantify the levels of essential element perturbed by Sn^{2+} ions. After this analyse we used a cytotoxic assay employing strains deficient in low- and high-affinity transport systems for iron, zinc, and copper as well as for transcription factors that control the expression of genes that code for those transmembrane proteins. Also, we analyzed genes that encoded MT, proteins involved in calcium homeostasis and proteins involved in oxidative stress response.

2. Materials and Methods

2.1 Strains and media. Relevant genotypes of yeast strains used in this work are given in Table 1. Media, solutions and buffers were prepared according to Burke *et al*. [30]. Complete medium (YPD-1% yeast extract, 2% peptone, 2% glucose) was used for routine growth of yeast cells.

2.2 Particle-Induced X-ray Emission (PIXE). For PIXE analysis the yeast cultures were harvested by centrifugation, freeze-dried, crushed and pressed into pellets as described in Viau *et al*. [28]. Exposure concentration was 25 mM for stationary (STAT) cells. The pellets were mounted in a target holder inside a reaction chamber for PIXE experiments which were carried out at the Ion Implantation Laboratory at the Physics Institute of the Federal University of Rio Grande do Sul (Porto Alegre, RS). A 3 MV Tandetron accelerator provided a 2 MeV proton beam with an average current of 2 nA for the experiments. The detection system consisted of two X-rays detectors. One of them is a HPGe detector (GLP series-EG&G Ortec) with a resolution of about 180 eV at 5.9 keV and placed at 135° with respect to the beam direction. This detector was optimized to detect electromagnetic radiation from 6 to 30 keV. Further pulse processing was accomplished with an amplifier model 245 (Tennelec). In addition to this detector, a Si(Li) detector (SLP series-EG&G Ortec), with a resolution of about 155 eV at 5.9 keV and placed at 45° with respect to the beam direction, was employed as well. This detector covered an energy region from 1 to 15.5 keV. In this case, an amplifier model 672 (Ortec) was used. Details of the experimental set-up are described in [28].

2.3 Survival assay. STAT cells were grown in YPD at 30°C for 48 h. Exponential phase of growth (LOG) cells were microscopically checked for bud appearance and the bud index (% budded cells) established. Sensitivity of twice saline-washed yeast suspensions to $SnCl_2$ was routinely determined in liquid saline (0.9% NaCl, pH 5.0). Exposure concentration was 25 μM for LOG cells. Exposure to $SnCl_2$ was for 60 min at 30°C. Thereafter, $SnCl_2$ -mediated

cell aggregates were declumped in phosphate buffer (PB; pH 7.4, 0.067 M) followed by vigorous vortexing before dilution and plating [26]. Cells were plated on YPD and survival was determined after 3 d at 30°C. Presented results are the mean of at least four independent experiments, the standard deviation and statistical analyses were calculated by GraphPad Prism® program.

2.4 RNA extraction and reverse transcription.

The primer sequences are described in Table 2. Two genes involved in metal detoxification, *CUP1* and *CRS5* and its transcriptional activator factor (encoded by the *ACE1* gene) were analyzed. Representative genes involved in calcium metabolism (*CCH1*, *MID1*, *VCX1*, *YVC1*, *PMR1*, *PMC1*, *CMD1*) were previously selected. Genes that codify for important stress proteins were also analyzed (*SOD1*, *SOD2*, *APN1*, *GSH1*, *YAP1*). We evaluated the mRNA gene expression between untreated/treated cells in comparison with a housekeeping gene *ACT1*. Prior to total RNA extraction, *S. cerevisiae* cells were incubated with sorbitol buffer and 200 U of lyticase (Sigma-Aldrich, Steinheim, Germany). Total RNA was extracted from untreated and treated ($SnCl_2$ 25 µM and 1000 µM, 1 h exposure) cells using Qiagen kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions and then treated to eliminate genomic DNA contamination (RQ1 RNase-free DNase, PROMEGA, Madison, USA). Reverse transcription was performed by adding 2 µg total RNA using M-MLV (PROMEGA, Madison, USA) in a reaction mixture containing 10 pmol/µL oligo(dT).

2.5 Quantitative real time PCR (qRT-PCR) analysis.

Quantitative real time PCR (qRT-PCR) was performed using an ABI Prism 7500 Sequence Detection System (SDS) (Applied Biosystems). Amplification was carried out with 20 ng of cDNA in 96-well plates using SYBR green master mix (Applied Biosystems) in 25 µL. Each sample was analyzed in biological triplicate. PCR conditions were: 1 min at 94°C, followed

by 40 cycles at 94°C for 30 s, 60°C for 30 s, 72°C for 30 s, and 60°C for 35 s, followed by a final stage of 15 s at 95°C, 1 min at 60°C, and 15 s at 95°C to determine dissociation curves of the amplified products. The results were analyzed using sequence detection system (SDS) software (Applied Biosystems). Generation of double-stranded DNA was measured in real time by the increase in fluorescence caused by the spontaneous binding of SYBR green. For each measurement, a threshold cycle value (Ct) was determined. This was defined as the number of cycles necessary to reach a point in which the fluorescent signal is first recorded as statistically significant above background. Target gene Ct values were normalized against *ACT1* (housekeeping gene). The results were analyzed using the comparative critical threshold ($2^{-\Delta\Delta Ct}$) method [31, 32] and expressed as fold change compared to untreated cells.

3. Results and discussion

3.1 Disturbance in elemental profile of wild-type yeast cells under Sn^{2+} overload

Initially, we performed a screening in order to know the putative interactions between Sn^{2+} and other essential elements. Despite our growing understanding about the mechanisms that are related to Sn^{2+} homeostasis, the individual genes and gene networks that influence the acquisition, distribution and detoxification of Sn^{2+} remain unknown. To address this question, we have combined molecular methods with spectroscopic methods (PIXE assay). The concentration of seven different metals and non-metals elements was assayed (magnesium, zinc, phosphorus, iron, sulfur, potassium, calcium, and copper; Table 3). According to PIXE analysis, the results for XV185-14c yeast cells indicate a significant loss of intracellular elements such as Mg, Zn, S, Fe and an increase of P levels after 1 h exposure to 25 mM $SnCl_2$ in STAT phase (Table 3).

Unfortunately, the effect of Sn^{2+} on Ca and K metabolism of *S. cerevisiae* could not be observed by PIXE (Table 3). There was a peak shadowing among Ca, K, and Sn. The effect of Sn^{2+} on copper metabolism could not be observed by PIXE technique because

the Cu content was below the detection limit (< limit of detection; Table 3). So, the results of this study provide insights about the putative genes that may be involved in Sn^{2+} metabolism. In this sense, we decided to know what genes could be involved in iron, zinc, and copper metabolism that are related to Sn^{2+} acquisition.

3.2 Yeast lacking iron low-affinity transporter (FET4) and AFT1 transcription factor lead to Sn^{2+}-resistance

After screening a yeast deletion mutants collection for $SnCl_2$ sensitivity/resistance, we observed that yeast cells lacking *FET4* were resistant to 25 µM $SnCl_2$ in LOG phase as compared to the isogenic WT while the *fet3*Δ deletion mutant showed the same sensitivity of the isogenic WT (Fig 1A). Uptake of iron occurs through two distinct systems: a high-affinity uptake system (Km of 0.15 µM) and a low-affinity system (Km 30-40 µM) [33]. *FET4* encodes a transmembrane protein of low-affinity transport [11] and its deletion results in high resistance to Sn^{2+} overload (Fig. 1A), suggesting that Sn^{2+} is a substrate for the Fet4p transporter. Actually, Sn^{2+} would be removed from the medium through the Fet4p because the ionic properties of Sn^{2+} are similar to Fe^{2+}. In this manner Sn^{2+} may enter to cytosol by competing with Fe^{2+} (Table 3).

The high-affinity iron uptake complex in the yeast plasma membrane consists of ferroxidase, Fet3p, and the ferric iron permease, Ftr1p [5, 34]. As opposed to the low-affinity iron transport system, the Fet3p/Ftr1p complex does not participate in Sn^{2+} uptake. The data here provide explicit support for this inference as both WT and *fet3*Δ strains were equally highly sensitive to the presence of Sn^{2+} (Fig. 1A). This agrees with several lines of evidence that confirmed that Fet3p is specific for Fe^{2+} transport [1, 7].

Although iron is abundant in nature, this metal is most commonly found as the virtually insoluble Fe^{3+} hydroxide ($Fe(OH)_3$) [1]. Thus, the iron uptake systems require

strategies to solubilize Fe^{3+}. In *S. cerevisiae*, a reductive mechanism is used. The majority of this activity is provided by two flavocytochromes (Fre1p and Fre2p) that reduce Fe^{3+} to provide Fe^{2+} as a substrate for the high-affinity transport system [1, 11]. The *fre1*Δ, *fre2*Δ, and WT cells were highly sensitive to the presence of Sn^{2+} (Fig. 1A). This data suggest that the products of the *FRE* genes are specific to iron and copper reduction [35] and cannot reduce Sn^{2+}.

To address a possible role of *AFT1* transcription factor on Sn^{2+} metabolism we examined the survival of *aft1*Δ cell. This mutant shows resistance to Sn^{2+} when compared to the WT yeast strain (Fig. 1A). This result strongly suggests that Aft1p, which control the expression of *FET4* gene [36], also alter Sn^{2+} homeostasis.

3.3 Yeast lacking zinc low-affinity transporter (ZRT2) and ZAP1 transcription factors lead to Sn^{2+}-resistance

It was observed by PIXE analysis that Sn^{2+} uptake produced a significant reduction in zinc content (Table 3), leading us to study yeast mutant cells defective in zinc homeostasis. The kinetic studies of Zn^{2+} uptake by cells grown in different concentrations of this ion suggest the presence of at least two uptake systems codified by *ZRT1* and *ZRT2* genes. These two genes encode high-affinity and low-affinity Zn^{2+} transporter systems, respectively [1, 7]. Both *ZRT1* and *ZRT2* genes are regulated at the transcriptional level and are induced in zinc-limited conditions [6]. This regulation is mediated by the Zap1p transcriptional activator [37]. Yeast mutant strain *zrt1*Δ was sensitive to Sn^{2+} ions while yeast *zrt2*Δ and *zap1*Δ strains showed increased resistance to Sn^{2+} when compared to WT (Fig. 1B).

Corroborating our results, expression studies show that Zrt1p is specific for Zn^{2+} and will not transport any other metal [1, 7]. In addition, Zap1p might be involved in Sn^{2+} metabolism (Fig. 1B). If Sn^{2+} causes Zn^{2+} limitation, it may be possible to have a direct

competition between Sn^{2+}/ Zn^{2+} for Zrt2p. The resistance of *zap1*Δ is explained because Zap1p regulates the expression of *ZRT2* [11]; the absence of Zap1p dowregulates *ZRT2* expression, resulting in low Sn^{2+} uptake by the yeast cells (Fig. 1B). It is noteworthy that under zinc-limiting conditions Zap1p increases the expression of three zinc uptake systems encoded by the *ZRT1*, *ZRT2*, and *FET4* genes [11]. Thus, the Fet4p can also be regulated by both *AFT1* and *ZAP1* [36, 11]. Many research groups have demonstrated Sn^{2+} and Zn^{2+} interaction [38-40] and now we are proposing that both metals can be compete for the same substrate.

3.4 Yeast lacking Ctr2 vacuolar copper protein and Mac1 transcription factors lead to Sn^{2+}-resistance

The connection between iron and copper metabolisms led us to investigate the possible influence of Sn^{2+} on copper homeostasis. Mutants *ctr1*Δ and *ctr3*Δ, lacking the high-affinity copper transport-mediating Ctr1p and Ctr3p, had the same sensitivity to Sn^{2+} like WT (Fig. 1C). This suggests that both proteins are not involved in Sn^{2+} transport. Our data are in accordance with the literature, which states that these high-affinity plasma membrane permeases are specific for reduced copper (Cu^{1+}) and cannot transport any other metal [3].

Interestingly, absence of copper-responsive transcription activator, encoded by yeast gene *MAC1*, resulted in increased resistance to Sn^{2+} (Fig. 1C). It was intriguing to us because we expected a strong sensitivity in this yeast strain. The lack of *mac1*Δ sensitivity to Sn^{2+} may arise from one of two scenarios. First, Mac1p may regulate additional proteins present at the plasma membrane that may transport Sn^{2+} into the cell; second, Sn^{2+} is internalized into yeast cells by Fet4p and Zrt2p (Fig. 1A, B) and goes to the vacuole by Pmc1p (see below-Fig. 2A) and may not exit to cytosol via Ctr2p (Fig. 1C). The *S. cerevisiae* Ctr2p serves to mobilize copper stores to cytosolic copper chaperones [4] and *ctr2*Δ strain

grew in the presence of Sn^{2+} ions (Fig. 1C), supporting our idea that Sn^{2+} is directed to vacuole and may not exit via Ctr2p.

It is important to note that Cu^{1+} could compete with Fe^{2+} for Fet4p-mediated transport [1, 7], so, a secondary consequence of Sn^{2+} overload is copper deficiency and mobilization of cooper from vacuole to cytosol.

3.5 Disturbance in calcium metabolism by Sn^{2+} overload

Disturbance in calcium metabolism by Sn^{2+} overload was tested by assaying expression of some genes involved in calcium metabolism by qRT-PCR (Fig. 2A). The plasma membrane, high-affinity Ca^{2+} influx is mediated by a protein complex consisting of Cch1p and Mid1p [41]. Both proteins might be involved in uptake of Sn^{2+} as transcript levels of *CCH1* increased following exposure to 1000 µM Sn^{2+} ($p < 0.001$) and expression of *MID1* increased at both Sn^{2+} exposures ($p < 0.05$ and $p < 0.001$, respectively; Fig.2A). The results presented here provide strong evidence that Sn^{2+} could be captured and internalized through Ca^{2+} channel via Cch1p and Mid1p; because Sn^{2+} has similar coordination chemistry as Ca^{2+} and thus might bind to protein ligands in a similar fashion. It is expected that both divalent ions compete for the Ca^{2+} transports. If Sn^{2+} is in excess, it is expected that it enters more than Ca^{2+}, resulting in an increase in the levels of Cch1p and Mid1p. Once inside the cell, both Ca^{2+} and Sn^{2+} activate the calmodulin/calcineurin complex which promotes the expression of genes related to stress tolerance and Ca^{2+} homeostasis by means of the transcription factor Tcn1/Crz1p [42]. The altered expression profile of *PMC1* that encodes the vacuolar plasma membrane Ca^{2+}-ATPase (PMCA), supports this idea. Expression of *PMC1* was up-regulated in presence of excess Sn^{2+} (Fig. 2A). So, excess of Sn^{2+} could be sequestered from cytoplasm to vacuole via Pmc1p and may not exit by Ctr2p (Fig. 1C).

By contrast the level of *VCX1*, a gene coding for another vacuolar Ca^{2+}-sequestration protein (Vcx1p), a Ca^{2+}/H^{+} exchanger driven by the proton electrochemical gradient set up by the vacuolar H^{+}-ATPase [43], was down regulated in presence of excess Sn^{2+} (Fig. 3B). To avoid decrease in cytoplasmic Ca^{2+} level, there is a diminution of Vcx1p in an attempt not to bring Ca^{2+} to the vacuole. In addition, transcript levels of *YVC1* were decreased following exposure to excess Sn^{2+} (Fig. 2A). This gene encodes Yvc1p, a homolog of the mammalian transient receptor potential (TRP)-like channel, that releases Ca^{2+} from the vacuole into the cytosol. We considered that if Sn^{2+} enters with Ca^{2+} via Mid1p and Cch1p, there is low content of Ca^{2+}, so Vcx1p does not brings Ca^{2+} into the vacuole and, because the activation of *CCH1* and *MID1*, it is not necessary release Ca^{2+} into the cytoplasm via Yvc1p.

Transcription levels of *PMR1* appeared to be not affected in the presence of Sn^{2+} ions (Fig. 2A). This gene codes for Pmr1p (plasma membrane ATPase related protein) that mediates high-affinity Ca^{2+} transport under normal physiological conditions. Our data have shown that Pmr1p do not capture the excess of intracellular Sn^{2+} by means of secretory vesicles. Reduced expression of *CMD1* was observed after Sn^{2+} treatment (Fig. 2A). The *CMD1* gene encodes a calmodulin that, in association with Ca^{2+}, participates in diverse cellular processes [44]. Keeping the idea that Sn^{2+} disrupted the Ca^{2+} homeostasis and also influenced Ca^{2+} signalling in the yeast *S. cerevisiae*, calmodulin participates in calcium-dependent stress response pathways through activation of the yeast CaM kinases Cmk1 and Cmk2. Dephosphorylation by calcineurin results in nuclear translocation of Tcn1/Crz1p, analogous to mammalian NFATc, and transcriptional activation of more than 160 target genes involved in cell wall and lipid synthesis, ion and small molecule transport, vesicle trafficking, and other signaling proteins [44].

It is important to note that cellular Ca^{2+} and Mg^{2+} levels appear linked in many circumstances, such that high Mg^{2+} results in low Ca^{2+} and vice versa [41]. Thus, in our

situation, Sn^{2+} overload, there was a significant loss of intracellular Mg^{2+} content (Table 3) and this depletion may be linked to induction of calcineurin/Crz1p. Wiesenberger *et al.* [41] provide evidence that calcineurin/Crz1p signalling is crucial for yeast cells to cope with Mg^{2+} depletion stress.

3.6 Crs5 metallothionein detoxifies Sn^{2+}

In most eukaryotic organisms, the main defense against metal is the mobilization exerted by MT [46]. A general mechanism for metal detoxification in yeast and in other organisms is the chelation of metal by MT, a large superfamily of low-molecular weight, cysteine rich metal-chelating peptides [45, 46]. To verify the involvement of Cup1p and Crs5p MT, we decided to study their expression by qRT-PCR when yeast cells were faced with Sn^{2+} overload. In addition, we analyzed their copper-sensing transcription factor encoded by *ACE*.

Levels of *ACE1* mRNA increased in response to Sn^{2+} overload (Fig. 2B). High Sn^{2+} levels activate Ace1p, which in turn increases expression of *CRS5* and *SOD1*, a metallothionein-like protein and Cu,Zn-SOD, respectively [45]. The Crs5 metallothionein, non-homologous to the Cu-thionein Cup1p can chelate excess of Sn^{2+} whereas Cu,Zn-SOD controls the level of ROS (Fig. 2B, C). We suggest that Sn-Crs5 complexes are obtained in conditions of Sn^{2+} overload. Our data are in accordance with the literature that attests Crs5p an efficient good divalent metal-binding ability, thus yielding homometallic, highly chiral and stable Zn and Cd complexes when expressed in media rich in these metal ions [45]. *CUP1* mRNA levels did not change in response to Sn^{2+} overload (Fig. 2B). These results corroborate data from the literature, which show that Cup1p detoxifies copper and only protects against the divalent metal cadmium when it is over-expressed [46].

3.7 Sn^{2+} overload and oxidative stress

The ability of Sn^{2+} to transfer and gain electrons from molecular oxygen and form ROS, makes it potentially toxic [23, 47]. So, besides disturbing essential elements, 'free' Sn^{2+} can rapidly catalyze superoxide radical (O^{2-}) production, which is a precursor of hydrogen peroxide (H_2O_2). This H_2O_2 can then react with Sn^{2+} by Fenton reaction to generate hydroxyl radical ($^{\bullet}OH$). Thus we decided to investigate some genes involved in stress response (Fig. 2C).

Expression of *SOD1* -encoded superoxide dismutase was up-regulated at both Sn^{2+} exposure concentrations ($p < 0.01$ and $p < 0.001$, respectively) while the *SOD2* transcript levels remained practically unchanged under these conditions (Fig. 2C). Considering the higher sensitivity of *sod1*Δ mutant [26], cytosolic Sod1p seems more important than mitochondrial Sod2p in protecting against the toxic effects of ROS in STAT cells [27].

The *YAP1* transcription factor was also analysed by qRT-PCR (Fig. 2C). Significant up-regulation in the expression of *YAP1* ($p < 0.001$ at both treatments) was observed (Fig. 2C). Under oxidative stress Yap1p is oxidized and accumulates rapidly in the nucleus where it regulates the expression of up to 70 genes encoding proteins involved in oxidative stress response [48]. Previous results show a low resistance to Sn^{2+} of Yap1 lacking cells, suggesting that the toxic effect of this metal is associated with an indirect oxidative stress produced by lack of mobilization of components of the antioxidant defence system [27]. A similar response to oxidative stress exists in bacteria where the transcription activator OxyR induces the genes coding for anti-stress proteins. Bacterial strains lacking a functional OxyR gene are therefore used in the Mutoxitest to detect ROS-producing chemicals, via enhanced bacterial sensitivity phenotype [49]. Such OxyR lacking mutants also display a significantly higher sensitivity to Sn^{2+} [26]. In the present work we observed increased levels of *YAP1* mRNA.

Unexpectedly *GSH1* mRNA levels remained unchanged in presence of Sn^{2+} ions (Fig. 2C), as Yap1p controls the synthesis of *GSH*. [50]. We assume, therefore, that no Sn^{2+} -glutathione complexes are formed or only formed to a lesser extent. Indeed mutants with low and extremely low GSH pools showed no enhanced sensitivity to Sn^{2+} [27]. Thus, GSH seems not needed in protection of yeast against acute, short-term (e.g., 1 h) Sn^{2+} -exposure (Fig. 2C), most probably because catalase and MTs provide an overlapping and potent defence against Sn^{2+} -induced ROS.

Looking a little further into protective mechanisms we have verified that base excision repair (BER) plays an important role in removal of Sn^{2+} generated oxidative base damage in DNA (Fig. 2C). By using qRT-PCR, we observed the increased expression of *APN1* after both Sn^{2+} exposure conditions ($p < 0.01$ and $p < 0.001$, respectively). The Apn1p DNA repair enzyme of *S. cerevisiae* acts on abasic sites and oxygen radical damaged bases [51]. Apurinic/apyrimidinic endonucleases cleave the DNA backbone at abasic sites [52].

3.8 General scheme of biological consequence of Sn^{2+} intoxication in yeast cells

Based on our results, we propose that Sn^{2+} ions might reduce the biochemical and cellular functions of essential elements indicating that it can probably attach to similar binding sites in the enzymes. This implies that Sn^{2+} shows ionic properties similar to divalent ions like Zn^{2+}, Ca^{2+}, and Fe^{2+}. Our data allowed us to propose the following model for the control of intracellular Sn^{2+} levels (Fig. 3). Apparently some essential metal transporters (Fet4p, Zrt2p, Cch1p, and Mid1p) cannot discriminate Sn^{2+} and import it via the cytoplasma membrane. In this manner, cells take up Sn^{2+}, Fe^{2+}, Zn^{2+}, and Ca^{2+}. It is expected that these divalent ions should compete for its transporters. Once inside the cell, Sn^{2+} may be chelated by Crs5p or, alternatively, can generate ROS, especially O_2^- which can dismutate to form H_2O_2 and the highly reactive $^{\bullet}OH$. In consequence, ROS can induce direct cellular injury and stress-

generated activation of many genes, e.g., *SOD1*, *YAP1* and *APN1*. Sn^{2+} is cleared from the cytosol by P-type ATPase to the vacuole (Pmc1p) and not by P-type ATPase to the Golgi (Pmr1p). As a second consequence, Sn^{2+} activates the calmodulin/calcineurin complex and the participation of transcription factors (Mac1p, Aft1p, Zap1p, Ace1p) that sense the imbalance promoted by Sn^{2+}.

In summary, we have identified here a novel physiological role Sn^{2+} overload and how *S. cerevisiae* faces with this condition. These data further demonstrate the complex integration of different signaling pathways *in vivo* that allows yeast to respond appropriately to its changing environment. Additional biochemical experiments are now being performed in order to clarify how Sn^{2+} interacts with Mg and S elements and which genes may be involved in this process. Because of the increase in P levels (Table 3) we are further investigating the potential participation of Pho84p, a high affinity inorganic phosphate transporter that plays a role in metal homeostasis [56] and its possible role in the transport of Sn^{2+}.

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Figure legends

Figure 1 Sensitivity to 25 μM Sn^{2+}-exposure of LOG cells of mutants with different defects in metal transport and regulation of: **A)** iron WT (■); *Δfet4* (□); *Δfet3* (*); *Δaft1* (○); *Δfre1* (Δ); *Δfre2* (+); **B)** zinc WT (■); *Δzrt1*(○); *Δzrt2* (*); *Δzap1* (Δ); and **C)** copper WT (■); *Δctr1* (□); *Δctr2* (○); *Δctr3* (Δ); *Δmac1* (*). In some points, error bars (standard deviation) do not exceed the symbol's size.

Figure 2 Relative expression levels of: **A)** *CRS5*, *CUP1*, and *ACE1* genes; **B)** *CCH1*, *MID1*, *PMC1*, *VCX1*, *YVC1*, *PMR1*, and *CMD1* genes; **C**) *SOD1*, *SOD2*, *YAP1*, *APN1*, and *GSH1* genes in the *S. cerevisiae* strain XV185-14c. Gene expression is quantified using qRT-PCR and comparative critical threshold ($2^{-\Delta\Delta Ct}$) method. The *ACT1* gene was used as internal control and the ratio of the fold-change without treatment was standardized to 1.0. White bars (without treatment), hatched bars (25 μM $SnCl_2$) and black bars (1000 μM $SnCl_2$). These values represent the average of four independent experiments; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. All treatment compared to control.

Figure 3 Sn^{2+} signaling and transport pathways in the yeast *S. cerevisiae*. Sn^{2+} enters the cytosol via the plasma membrane channel complex Cch1/Mid1p, Fet4p and Zrt2p. Once inside the cell, Sn^{2+} can generate ROS that activate of *SOD1*, *YAP1* and *APN1* genes. Stannous itself can be detoxified by Crs5p a *S. cerevisiae* metallothionein (MT) or can be sequestered to the vacuole by P-type ATPase Pmc1p. Sn^{2+} can cause imbalance in intracellular zinc, copper, calcium, magnesium, and iron levels and these disturbances vary with activity of the yeast genes *AFT1*, *MAC1*, and *ZAP1*.

Figure 1

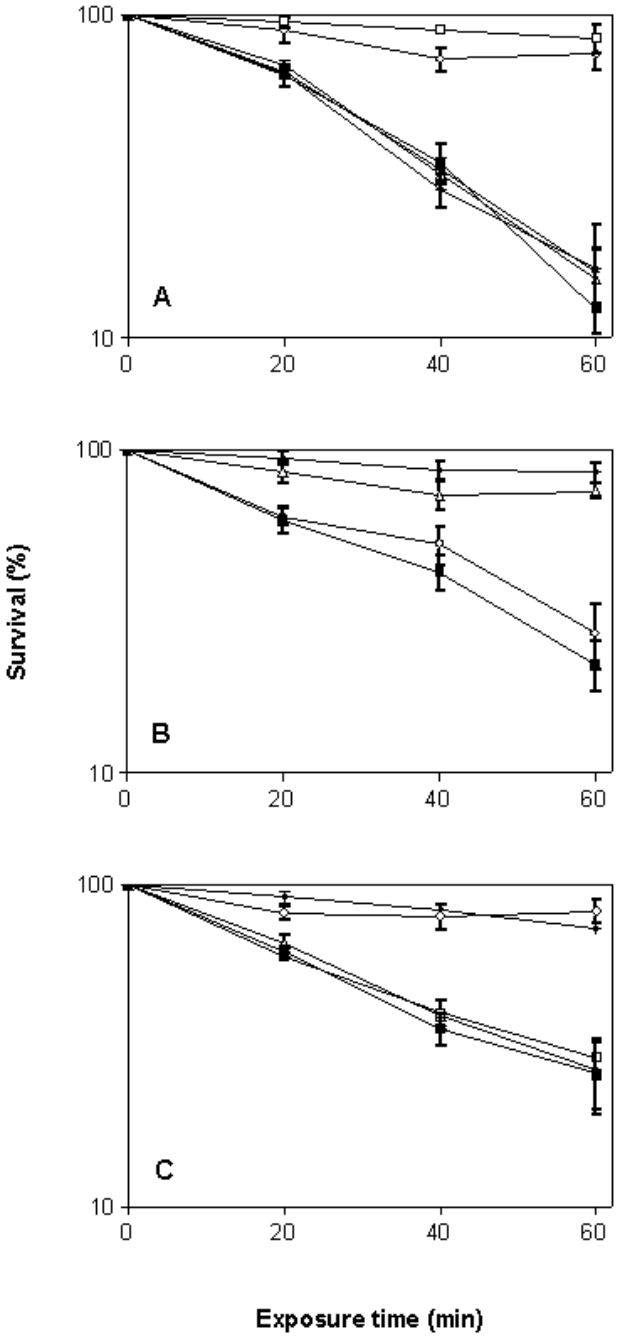
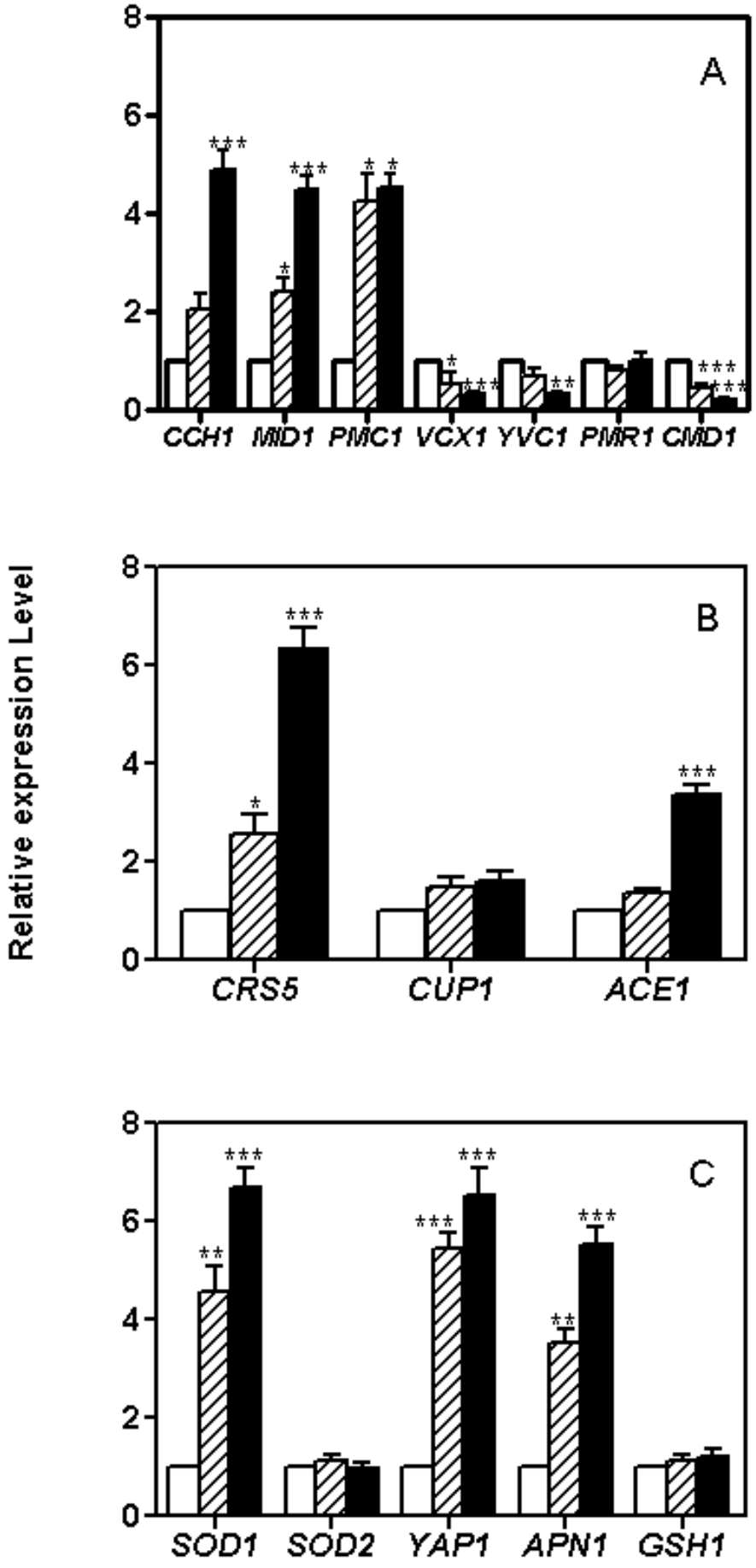


Figure 2


Relative expression Level
A
CCH1
MID1
PMC1
VCX1
YVC1
PMR1
CMD1
B
CRS5
CUP1
ACE1
C
SOD1
SOD2
YAP1
APN1
GSH1

[28]

Figure 3

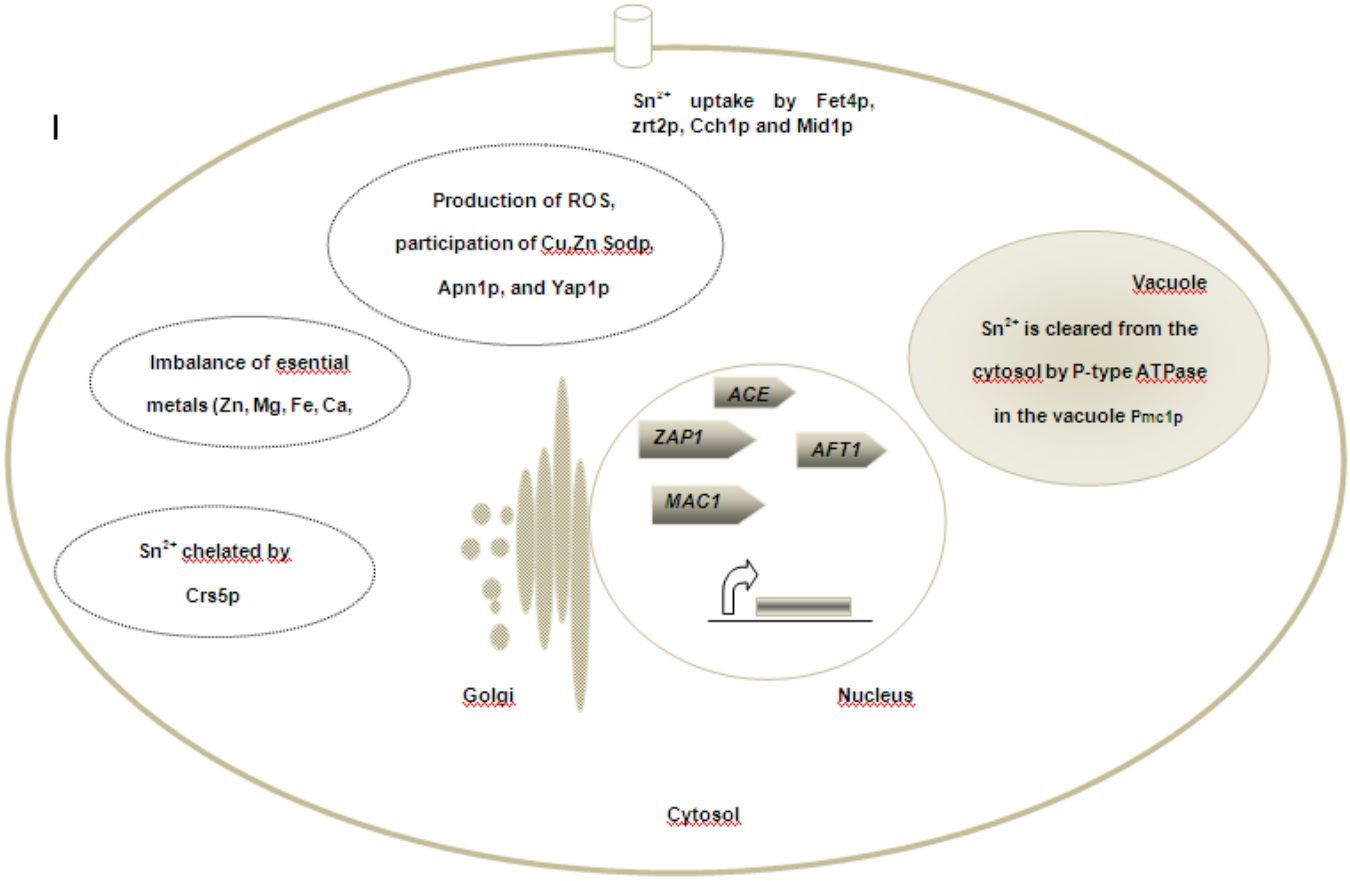

Sn2+ uptake by Fet4p, zrt2p, Cch1p and Mid1p
Production of ROS, participation of Cu,Zn Sodp, Apn1p, and Yap1p
Imbalance of esential metals (Zn, Mg, Fe, Ca,
Sn2+ chelated by Crs5p
Vacuole
Sn2+ is cleared from the cytosol by P-type ATPase in the vacuole Pmc1p
ACE
ZAP1
AFT1
MAC1
Golgi
Nucleus
Cytosol

Table 1

Strains used in this study and their relevant genotypes

Yeast Strains	Genotype	Reference
XV185-14c	*MATα: ade2-2 his1-798 lys1-1 trp5-48 hom3-10 arg4-17*	Von Borstel et al., 1971
DY1457 (WT)	MAT*a: ade6 can1-100oc his3 leu2 trp1 ura3*	From David J Eide
DEY1394	MAT*a: fet3: :HIS3 ade6 can1-100oc his3 leu2 trp1ura3*	From David J Eide
DDY33	MAT*a: fet4: :LEU2 ade6 can1-100oc his3 leu2 trp1ura3*	From David J Eide
ZHY1	MAT*a: zrt1: :LEU2 ade6 can1-100oc his3 leu2 trp1ura3*	From David J Eide
ZHY2	MAT*a: zrt2: :HIS3 ade6 can1-100oc his3 leu2 trp1ura3*	From David J Eide
ZHY6	MAT*a: zap1: :TRP1 ade6 can1-100oc his3 leu2 trp1ura3*	From David J Eide
DEY1524	MAT*a: aft1: :TRP1 ade6 can1-100oc his3 leu2 trp1ura3*	From David J Eide
SEY6210 (WT)	MAT*α: ura3-52 leu2-3,-112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9*	From Dennis J Thiele
DTY189	*MATα: ura3-52 leu2-3,-112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ctr1 : : URA3*	From Dennis J Thiele
SKY6	*MATα: ura3-52 leu2-3,-112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ctr3 : : LEU2*	From Dennis J Thiele
ctr2	*MATα: ura3-52 leu2-3,-112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ctr2 : : HIS3*	From Dennis J Thiele
SKY33	*MATα: ura3-52 leu2-3,-112 his3-Δ200 mac1 : : HIS3 trp1-Δ901 lys2-801 suc2-Δ9*	From Dennis J Thiele
BY4741	*MATa: hi3sΔ1 leu2Δ0 met15Δ0 ura3Δ0*	EUROSCARF
ΔFre1	*MATa: hi3sΔ1 leu2Δ0 met15Δ0 ura3Δ0fre1 : : HIS3*	From Dennis J Thiele
ΔFre2	*MATa: hi3sΔ1 leu2Δ0 met15Δ0 ura3Δ0fre2 : :* KanMX6	From Dennis J Thiele

Table 2

***S. cerevisiae* primers used in real time PCR**

Target gene	Sequence
ACT1	FW 5'-ATGGAAGATGGAGCCAAAGC-3'
	RV 5'-TCTGCCGGTATTGACCAAAC-3'
CUP1	FW 5'-TCATTTCCCAGAGCAGCATG-3'
	RV 5'-TGCCAATGCCAATGTGGTAG-3'
CRS5	FW 5'-AAGGCGAATGTTGTAAGGACTC-3'
	RV 5'-TCACAAGTGCACGTGGTTTC-3'
ACE1	FW 5'-AGCTGTTGGCAGCAAGGAAG-3'
	RV 5'-GAGACCATTGACATGCGCTG-3'
CCH1	FW 5'-GCTAAATCTACTCATTGCAGTGTTG-3'
	RV 5'-TGGGAATTTTGTCTGATTCG-3'
MID1	FW 5'-TAAGCGACGTCACTGCAGAAG-3'
	RV 5'-GACAGGACTGAATGCCTCAAC-3'
PMR1	FW 5'-ACTGTACACTTATTGTCGACAGGG-3'
	RV 5'-GCCATATTTCTGGGAAATCC-3'
VCX1	FW 5'-TGCCTTTAGAGCCACCCTAC-3'
	RV 5'-TGTTGCTCAAACAAGGCGTG-3'
PMC1	FW 5'-ACAGTCGCCGCTGTTGTTTC-3'
	RV 5'-CTGGTAATCGTTAGCGGCAC-3'
YVC1	FW 5'-GCCGGAAAACAATGGATCTC-3'
	RV 5'-GCCAGCTTCACAACTTTTGG-3'
CMD1	FW 5'-GCGGAGATTAAACCATCACC-3'
	RV 5'-GCCCAGTGAAGCAGAAGTAAA -3'
SOD1	FW 5'-CGTGGATAACGACGCTTCTG-3'
	RV 5'-ACATGGTGCTCCAACTGACG-3'
SOD2	FW 5'-TTGAACAACGTCCAGCTTGC-3'
	RV 5'-CTTTGGCAAAGGCAATCGAC-3'
APN1	FW 5'-ACCCAAATAACCTTGACCCAAG-3'
	RV 5'-CATACATTTGCAGCAGGCTAC-3'
GSH1	FW 5'-AAAGAATTTTGCTGTTGTTGGC-3'
	RV 5'-TCAATTTGTTACGCCAATCC-3'
YAP1	FW 5'-TCCGCTTGATAACGACAACG-3'
	RV 5'-AAGGGAATCCAAAGTAGCGC-3'

Table 3

PIXE results of metal content in the haploid XV185-14c yeast strain after 25 mM $SnCl_2$

Element	Untreated cells (ppm)[a]	Treated cells (ppm)[a]
Mg	1407 ± 119.1	1042 ± 119.21*
Zn	335 ± 24.2	242 ± 38.31*
P	11999 ± 305	20209 ± 1230.64**
Fe	33.62 ± 5.87	24.55 ± 8.1***
S	2566 ± 123.2	2172 ± 221.74 *
K	14286	?
Ca	143	?
Cu	< LOD[b]	< LOD[b]

[a] These values represent the average of four independent experiments; [b] Limit of detection; * p < 0.05; ** p < 0.01; *** p < 0.001, [?] not determined.

DISCUSSÃO

Os processos vitais seriam impossíveis na ausência de determinados íons metálicos (Rutherford & Bird, 2004; Puig *et al.*, 2008; Turski & Thiele, 2009). No entanto, nas sociedades industrializadas, os metais pesados mostram-se potencialmente prejudiciais à saúde humana (Ikem & Egiebor, 2005; Mielniczki-Pereira *et al.*, 2008). Particularmente, o estanho e seus compostos têm despertado o interesse da comunidade científica por estarem presentes em vários segmentos da vida cotidiana (Dopp *et al.*, 2007; Yousef *et al.*, 2007; El-Makawy *et al.*, 2008).

A família 14 da classificação periódica é de extrema importância: três de seus membros, o carbono, o estanho e o chumbo, são conhecidos da humanidade desde a antiguidade mais remota em sua forma elementar. O estanho, em particular, teve papel fundamental, juntamente com o cobre, na transição do neolítico à idade do bronze e no desenvolvimento civilizatório que se seguiu desde então. O estanho também esteve presente sob a forma de compostos inorgânicos nas tecnologias mais antigas. O óxido de estanho (IV) (SnO_2), por exemplo, tem sido usado há milênios para opacificar cerâmicas e esmaltes vítreos (Blunden & Wallace, 2003).

A exposição da população ao estanho ocorre, basicamente, pela dieta, por meio do consumo de alimentos e líquidos enlatados. Há vários agravantes que ocasionam um contato ainda maior com esse metal: determinados tipos de alimentos, como o tomate enlatado, apresentam uma quantidade maior de estanho por já terem sido expostos a esse metal e seus compostos (utilização de formulações de agrotóxicos com organoestanhados); peixes enlatados também podem conter maior quantidade de estanho em virtude da contaminação das águas com produtos utilizados nos cascos dos navios, barcos e similares a fim de evitar a ação da corrosão (utilização de formulações que contêm compostos de estanho) (Yousef *et al.*, 2007; Nakanishi, 2008). No presente trabalho, foi utilizada

a levedura *Saccharomyces cerevisiae* como modelo eucariótico de estudo, com o objetivo de investigar os tipos de danos que o íon metálico Sn^{2+} gera nos componentes celulares, principalmente em nível do material genético da célula, bem como verificar a entrada desse íon na célula, a sua distribuição e a destoxificação.

Para avaliar o tipo de lesão causada pelo íon metálico Sn^{2+}no DNA, analisou-se a sensibilidade dos mutantes de *S. cerevisiae* defectivos nas três principais vias de reparação de danos ao DNA: (1) reparação por excisão de nucleotídeos (NER), representada pelos genes *RAD2* e *RAD4*; (2) reparação sujeita a erro, representada pelo gene *RAD6*; (3) reparação recombinacional, representada pelo gene *RAD52*. Observou-se que os mutantes *rad2*Δ, *rad4*Δ e *rad6*Δ apresentaram uma sensibilidade intermediária ao íon metálico Sn^{2+} quando comparada com a da linhagem selvagem isogênica (BY10000) (Capítulo I, tabela 2). Dessa forma, infere-se que a reparação por excisão de nucleotídeos e a reparação sujeita a erro podem estar envolvidas na reparação das lesões geradas pelo íon metálico Sn^{2+}. O envolvimento da via de reparação sujeita a erro corrobora a mutagenicidade observada na levedura *S. cerevisiae* (Pungartnik *et al.*, 2005).

No entanto, a acentuada sensibilidade do mutante *rad52*Δ, deficiente na reparação de quebras simples e duplas na estrutura do material genético da célula (Aylon & Kupiec, 2004), ao íon metálico Sn^{2+}, quando comparada com a da linhagem selvagem isogênica (BY10000) (Capítulo I, tabela 2), sugere que as lesões provocadas pelo íon metálico Sn^{2+} são do tipo quebra simples ou dupla, que mobilizam as células a acionar o mecanismo de reparação recombinacional. Dantas *et al.* (1999) e De Mattos *et al.* (2000) já haviam demonstrado, *in vitro,* que o íon metálico Sn^{2+} gera quebras no DNA plasmidial.

As quebras simples ou duplas geradas pelo íon metálico Sn^{2+} no DNA são devidas a: (1) uma ação direta do íon metálico Sn^{2+} nas fitas de nucleotídeos, clivando-as e/ou (2) uma ação indireta do íon metálico Sn^{2+} por meio da formação de EROs, as quais, sujeitas à reparação, formariam, igualmente,

quebras simples e duplas no DNA. Corroborando o fato do íon metálico Sn^{2+} apresentar uma ação genotóxica direta no DNA, o estudo realizado em células de fibroblastos de *hamster* chinês (células V79) e ensaio cometa, nas doses de 100, 500 e 1000 µM de $SnCl_2$, mostrou que houve geração de quebras no DNA, evidenciadas pelo aumento do número de danos e porcentagem de células lesadas (capítulo III, tabela 1).

McLean *et al.* (1983a) também reportaram quebras no DNA produzidas pelo íon metálico Sn^{2+} em concentrações acima de 500 µM, detectadas por gradiente de sacarose alcalino em células de ovário de *hamster* chinês. Os autores discutiram que o íon metálico Sn^{2+} pode coordenar com vários átomos (oxigênio, nitrogênio, halogênio), formando compostos covalentes, e apresentar o potencial de formar pontes intercadeias (*interstrand cross-links* ou ICLs) (DNA-DNA, DNA-proteína). Dessa forma, infere-se que o íon metálico Sn^{2+} age diretamente sobre o DNA, provocando quebras simples e duplas.

Mais recentemente, Dantas *et al.* (2002a) realizaram um estudo com voluntários adultos, que haviam recebido formulações intravenosas (*kits* cintilográficos, contendo ^{99m}Tc e o íon metálico Sn^{2+}) para realização de exames de cintilografia. Os pesquisadores, utilizando o ensaio cometa, constataram um aumento do número de danos ao DNA, em linfócitos, depois de 2 horas da aplicação da formulação e um decréscimo do mesmo após 24 horas. O grupo de pesquisa sugeriu que a diminuição do número de danos deveu-se ao reparo das lesões ou eliminação por apoptose. Dantas *et al.* (2002b) analisaram o efeito genotóxico do $SnCl_2$ em células K562 (provenientes de pacientes com leucemia mielogênica crônica e que superexpressam enzimas envolvidas na resposta a estresse oxidativo) pelo ensaio cometa e sugeriram que o efeito genotóxico ocorreu devido à formação de EROs e não somente à ação direta do íon metálico Sn^{2+}.

Como a grande maioria dos metais (Beyersmann & Hartwig, 2008), a genotoxicidade do íon metálico Sn^{2+} deve-se também à formação de EROs e, consequentemente, à indução de estresse oxidativo. McLean *et al.* (1983a,b)

sugeriram, pela primeira vez, que a ação indireta do íon metálico Sn^{2+} poderia explicar a genotoxicidade observada. Sendo assim, o íon metálico Sn^{2+} pode reagir com o oxigênio (O_2) e formar uma das EROs mais comuns, o ânion superóxido ($O_2^{\cdot\cdot}$), segundo a reação $Sn^{2+} + O_2 \Rightarrow Sn^{4+} + O_2^{\cdot\cdot}$. O íon metálico Sn^{2+} também pode reagir com o peróxido de hidrogênio (H_2O_2), produzido em condições normais da célula durante o metabolismo da mesma, e formar a espécie mais reativa e nociva à célula, o radical hidroxil ($^{\cdot}OH$), na reação química Tipo Fenton: $Sn^{2+} + H_2O_2 \Rightarrow Sn^{4+} + {}^{\cdot}OH + OH^{-} + Sn^{4+}$.

Com o intuito de esclarecer se há uma maior contribuição do mecanismo indireto do $SnCl_2$, com formação de bases oxidadas que possam ser reconhecidas por enzimas específicas, na reparação por BER, realizou-se o ensaio cometa modificado. Após serem lisadas, as células V79 foram incubadas com glicosilases e endonucleases, que reconhecem bases oxidadas, permitindo que o ensaio cometa apresentasse maior especificidade e sensibilidade (Blasiak *et al.*, 2004) (Capítulo III, figura 2). Utilizaram-se a enzima formamidopirimidina DNA glicosilase (FPG), que é específica para bases púricas oxidadas, e a enzima endonuclease III (ENDO III), que é específica para bases pirimídicas oxidadas (para revisão, ver Kim *et al.*, 2001; Dizdaroglu, 2005; Dizdaroglu *et al.*, 2008).

O estudo demonstrou que houve a formação de poucos sítios sensíveis às duas enzimas FPG e ENDOIII (Capítulo III, figura 2), porque não houve um aumento acentuado do índice de dano ao DNA pelo íon metálico Sn^{2+}. Dessa forma, as lesões provocadas pelo íon metálico Sn^{2+} são um fraco substrato para essas enzimas testadas, ou o íon metálico Sn^{2+} pode estar gerando a lesão 8-oxoguanina (8-oxoG) e essa, por ser muito instável (Hailer *et al.*, 2005), pode estar formando subprodutos que são fracos substratos para as enzimas empregadas no estudo. No capítulo I, tabela 3, mostrou-se que o mutante defectivo na proteína Ogg1 N-glicosilase de *S. cerevisiae* é resistente ao íon metálico Sn^{2+}, reforçando a idéia de que há pouca formação de 8-oxoG e/ou formação de produtos secundários provenientes da mesma.

Outra hipótese é que há uma maior contribuição da ação direta do íon metálico Sn^{2+} na estrutura do DNA com formação de quebras simples e duplas, o que estaria mobilizando a reparação recombinacional (Capítulo I, tabela 2; Capítulo III, tabela 1). Com isso, há a formação de pouca base oxidada, o que justificaria a participação de BER, que, por sua vez, levaria à excisão de nucleotídeos, que também poderia ser responsável pela participação da reparação recombinacional.

A DNA N-glicosilase Ntg2 de *S. cerevisiae*, homóloga funcional da ENDO III bacteriana, parece estar envolvida na reparação das lesões causadas pelo íon metálico Sn^{2+} (Capítulo I, tabela 2). Novamente, percebe-se uma ação conjunta das vias de reparação do DNA para reparar as lesões formadas pelo íon metálico Sn^{2+}. Portanto, além das vias de reparação recombinacional, NER e reparação sujeita a erro, há a participação de BER. Ao analisar os quádruplos mutantes (Capítulo I, tabela 2), constatou-se que a reparação sujeita a erro (síntese translesão – Rev3) contribuiu, majoritariamente, para reparar as lesões provocadas pelo íon metálico Sn^{2+}, uma vez que o quádruplo mutante *ntg1*Δ*ntg2*Δ*apn1*Δ*rev3*Δ foi mais sensível do que a linhagem selvagem (Capítulo I, tabela 2).

A significativa sensibilidade do mutante *sod1*Δ (Capítulo I, tabela 2, figura 3) sugeriu que há a formação do $O_2^{\cdot-}$, corroborando os achados de McLean *et al.* (1983). Além disso, a maior sensibilidade do duplo mutante (*sod1*Δ*sod2*Δ) em relação à do simples mutante *sod1*Δ (Capítulo I, tabela 2) demonstrou que a proteína superóxido dismutase mitocondrial (Sod2) também tem uma participação mínima na proteção contra os danos oxidativos gerados pelo íon metálico Sn^{2+}.

No capítulo IV, figura 2c, analisaram-se as expressões dos genes *SOD1*, *SOD2*, *YAP1*, *APN1* e *GSH1* pela técnica de PCR quantitativo (qRT-PCR). A expressão do gene *SOD1* aumentou nas duas condições de tratamento (25 e 1000 µM de $SnCl_2$), fato que, juntamente com a sensibilidade do mutante *sod1*Δ (Capítulo I, tabela 2), reforça a idéia de que o íon metálico Sn^{2+} gera $O_2^{\cdot-}$.

Novamente, a participação da Sod2 parece ser mínima e/ou o seu nível basal é suficiente para dismutar o $O_2^{\cdot-}$ gerado, uma vez que não houve alteração da expressão do gene *SOD2* nas duas condições do estudo (Capítulo IV, figura 2c).

Sabe-se que, em condições de estresse oxidativo, a proteína Yap1 é oxidada e se acumula rapidamente no núcleo, onde regula a transcrição de mais de setenta genes que codificam proteínas envolvidas nas respostas antiestresse oxidativo (Wood *et al.*, 2004; Ma *et al.*, 2007). Em ambas as concentrações de estudo, 25 e 1000 µM de $SnCl_2$, houve um aumento acentuado da expressão do gene *YAP1* (Capítulo IV, figura 2c). A sensibilidade acentuada do mutante *yap1*Δ corrobora a idéia de participação desse fator de transcrição, numa tentativa de restabelecer o equilíbrio (pró-oxidante X antioxidante), alterado pelo íon metálico Sn^{2+} (Capítulo I, tabela 2). Pungartnik *et al.* (2005) mostraram que o $SnCl_2$ foi mutagênico, em células procarióticas, para a linhagem IC203, que não apresenta o fator de transcrição OxyR, responsável pela regulação da transcrição de vários genes envolvidos na resposta a agentes oxidativos (Blanco *et al.*, 1998; Martinez *et al.*, 2000), bem como mutagênico para a linhagem TA102 de *S. typhimurium*, que detecta mutágenos oxidativos e agentes alquilantes (Martinez *et al.*, 2000).

A sensibilidade mais acentuada ao íon metálico Sn^{2+} foi observada quando as células estavam em metabolismo fermentativo (fase logarítmica ou exponencial) (Capítulo I, figuras 2, 3 e 4). Sabe-se que as células adquirem resistência a compostos que geram danos oxidativos durante e logo após a fase diáuxica, quando as células são desreprimidas (desrepressão catabólica), e as funções celulares são adaptadas ao metabolismo respiratório (Maris *et al.*, 2000). Independentemente dos mecanismos de reparação e das enzimas antioxidativas envolvidos na reparação das lesões causadas pelo íon metálico Sn^{2+}, há uma maior contribuição do estado fisiológico (metabólico) da célula para a resistência a esse íon.

Nota-se que alguns metais podem inativar determinados mecanismos de reparação, por se ligarem a determinadas enzimas que participam do processo (para revisão, ver Hartwig *et al.*, 2002; Hartwig & Schwerdtle, 2002;

Beyersmann & Hartwig, 2008). No capítulo III, figura 3, 50 µM de $SnCl_2$, concentração escolhida por não aumentar significativamente o índice de dano ao DNA, foi capaz de retardar a reparação dos danos ocasionados pelo agente mutagênico clássico MMS. Dessa forma, infere-se que o íon metálico Sn^{2+} inibe a etapa de polimerização e/ou ligação das extremidades que foram clivadas para remoção das lesões provocadas pelo MMS. Já foram relatados outros metais e semimetais capazes de inibir as DNA polimerases e as DNA ligases, tais como: Cd^{2+}, Co^{2+}, Ni^{2+}, As^{2+} e, inclusive, o Pb^{2+}, pertencente à família do Sn^{2+} (Beyersmann & Hartwig, 2008).

Além das etapas de polimerização e ligação, a etapa de reconhecimento da lesão pode ser afetada por metais. Hartmann & Hartwig (1998) demonstraram que 50 µM de Ni^{2+} e 0,5 µM de Cd^{2+} foram capazes de inibir a interação de um oligonucleotídeo sintético lesado por radiação UV com a proteína XPA do complexo NER, que reconhece a lesão. No caso do íon metálico Sn^{2+}, além de o mesmo gerar dano oxidativo no DNA, a sua genotoxicidade se manifesta por agir com enzimas importantes que garantem a integridade do material genético. Em concentrações baixas e não tóxicas, o íon metálico Sn^{2+} pode ser classificado como um agente comutagênico (Capítulo III, figura 3).

Por outro lado, o aumento do índice de dano ao DNA, após as células serem pós-tratadas com o íon metálico Sn^{2+}, pode ser reflexo da acumulação de sítios apurínicos/pirimídicos (sítios AP), os quais podem ser convertidos em quebras no ensaio cometa, como resultado da inibição de endonucleases. McNeill *et al.* (2007) mostraram que a principal endonuclease mamária, Ape1, é inibida pelo Pb^{2+}. Além disso, esses pesquisadores demonstraram que a superexpressão de Ape1 pode reverter a acumulação de sítios AP e a mutagenicidade associada ao MMS. No capítulo IV, figura 2c, percebeu-se um aumento da expressão do gene *APN1*, que poderia ser explicado pela tentativa de a célula suprir a enzima Apn1, inibida pelo íon metálico Sn^{2+}. Esse aumento também poderia ser explicado pela participação de Apn1 na

reparação, por BER, das lesões indiretas causadas pelo íon metálico Sn^{2+} via formação de EROs, mencionada anteriormente.

Sabe-se que, ao ligarem-se às proteínas da membrana, os metais pesados podem modificar os canais proteicos ou os receptores de membranas, assim como alterar a permeabilidade e, consequentemente, o fluxo de compostos das células (Györi *et al.*, 2000). O estanho pode entrar na célula e, ao acumular-se, competir com íons divalentes endógenos e alterar processos metabólicos intracelulares. A técnica de Emissão de Raios X Induzida por Partícula (PIXE) foi empregada para realizar uma análise multielementar e propor com quais elementos químicos essenciais o íon metálico Sn^{2+} poderia estar interagindo (Capítulo IV, tabela 3). Houve um decréscimo significativo da concentração dos elementos químicos Mg, Zn, Fe e S, enquanto que a concentração do elemento P aumentou após o tratamento com $SnCl_2$. Infelizmente, não puderam ser estudados os elementos Ca, K e Cu por essa técnica, porque, com relação aos dois primeiros elementos, houve uma sobreposição dos picos com o elemento Sn, e o Cu ficou abaixo do limite de detecção (Capítulo IV, tabela 3).

A metodologia PIXE também foi usada para tentar elucidar o porquê das diferenças de sensibilidade entre as linhagem selvagens ao $SnCl_2$, evidenciadas no Capítulo I, tabelas 2 e 3. Notou-se que a linhagem selvagem haplóide XV185-14c é capaz de captar maior quantidade do íon metálico Sn^{2+} do que a linhagem diplóide XS2316 (Capítulo II, tabela 1), o que explicaria a diferença de sensibilidade ao íon metálico Sn^{2+}. A XS2316 capta menor quantidade de metal sendo, consequentemente, mais resistente a ele (Capítulo I, tabela 2). Portanto, a capacidade de captar maior ou menor quantidade de metal pode definir a sensibilidade das linhagens frente a um agente tóxico.

A partir da análise multielementar realizada pela metodologia PIXE, optou-se por estudar mutantes defectivos em sistemas de transporte dos metais Fe, Zn e Cu e seus respectivos fatores de transcrição (Capítulo IV, figura 1 a,b,c). Se há interação do íon metálico Sn^{2+} com os elementos químicos Fe^{2+} e Zn^{2+}, pode haver competição pelas enzimas que carreiam esses metais. E, realmente, o

íon metálico Sn^{2+} pode ser substrato para as enzimas Fet4 e Zrt2 (transportadores de baixa afinidade do ferro e zinco, respectivamente). Tanto o mutante *fet4*Δ quanto o mutante *zrt2*Δ de *S. cerevisiae* foram resistentes a 25 µM de $SnCl_2$, quando comparados com a linhagem selvagem (Capítulo IV, figura 1 a,b). A proteína Fet4 não é específica para Fe^{2+}, porque outros metais de transição (Cu^{2+}, Mn^{2+}, Zn^{2+} e Co^{2+}) podem competir com o Fe^{2+} para serem transportados (Hassett *et al.*, 2002; Philpott, 2006). A proteína Zrt2 não é específica para Zn^{2+}, podendo transportar também Fe^{2+}, Cu^{2+}, Mn^{2+}, Mg^{2+}, Ni^{2+} e Co^{2+} (Eide, 2006; Simm *et al.*, 2007). Dessa forma, como o íon metálico Sn^{2+} está em excesso na célula, pode haver uma competição entre ele e o Fe^{2+} e/ou Zn^{2+} pelos sistemas de transporte de baixa afinidade na levedura *S. cerevisiae* (Capítulo IV, figura 1 a, b), explicando, também, a menor captação desses íons após o tratamento das células com $SnCl_2$ (Capítulo IV, tabela 3).

A proteína Smf1 de *S. cerevisiae* é responsável pelo sistema de captação do Mn^{2+} de alta afinidade e, ao contrário dos outros sistemas de alta afinidade, pode, igualmente, transportar outros cátions divalentes como Fe^{2+}, Cd^{2+}, Mg^{2+} e Zn^{2+} (Culotta *et al.*, 2005). Essa proteína poderia, de alguma forma, estar contribuindo para a captação do íon metálico Sn^{2+} e ser, portanto, um alvo de futuros estudos. O gene *PHO84* de *S. cerevisiae* seria também um excelente candidato para investigações futuras, já que codifica um transportador de alta afinidade para o fosfato inorgânico (Mouillon & Persson, 2006; Wykoff *et al.*, 2007). Como o conteúdo de fosfato aumentou após tratamento com $SnCl_2$, cogita-se que o íon metálico Sn^{2+} possa estar sendo carreado por esse transportador juntamente com o fosfato. Jensen *et al.* (2003), ao analisarem o comportamento do mutante *pho84*Δ de *S. cerevisiae* após suplementação com o íon Mn^{2+}, observaram que o mutante apresentava resistência a elevadas concentrações do metal. Logo, os pesquisadores inferiram que o sistema de alta afinidade Pho84 poderia estar envolvido na captação de metais divalentes como o manganês. Interessante notar que o aumento dos níveis de fosfato na célula, após tratamento com o íon metálico Sn^{2+}, implica uma diminuição dos níveis de magnésio, uma vez que,

quando há acumulação de fosfato, há uma menor captação do magnésio (Capítulo IV tabela 3) (Beeler *et al.*, 1997; Simm *et al.*, 2007).

Sabe-se que há uma intrínseca relação entre o metabolismo do cobre e do ferro (Stoj *et al.*, 2007; Sedlák *et al.*, 2008). Por essa razão, apesar de não ter sido possível verificar a influência do íon metálico Sn^{2+} pela metodologia PIXE, evidenciou-se uma diminuição dos níveis de ferro (Capítulo IV, tabela 3). A partir disso, decidiu-se estudar mutantes defectivos no transporte de cobre e seus fatores de transcrição. O sistema de alta afinidade para o cobre, codificado pelos genes *CTR1* e *CTR3*, parece não estar envolvido na captação do íon metálico Sn^{2+}, porque os mutantes defectivos nesse transporte apresentaram a mesma sensibilidade da linhagem selvagem isogênica (Capítulo IV, figura 1c). No entanto, a proteína Ctr2 parece estar envolvida no metabolismo do íon metálico Sn^{2+}. A proteína Ctr2 se localiza na membrana do vacúolo e, quando há baixos níveis de cobre no citoplasma, ela é responsável por enviar cobre do vacúolo para o citoplasma (Turski & Thiele, 2009).

No capítulo IV, figura 1c, notou-se uma resistência do mutante *ctr2*Δ a 25 µM de $SnCl_2$. Dessa forma, sugere-se que: (1) o íon metálico Sn^{2+} entra na célula através dos transportadores de baixa afinidade e vai para o vacúolo, não causando toxicidade; e/ou (2) se há a carência de ferro após tratamento com o íon metálico Sn^{2+}, a célula entende que deva ativar os sistemas de alta afinidade para captar maior quantidade de ferro (ferroredutases e transportadores de alta afinidade, que, por sua vez, não carreiam Sn^{2+}, entrando mínima quantidade do metal). Nesse contexto, Rees & Thiele (2007) demonstraram que os níveis de expressão *CTR2*, *ATX1* e *CCC2* aumentam quando há carência de ferro. A proteína Atx1 é uma chaperona, que entrega cobre para a proteína Ccc2, presente no complexo de Golgi, que, por sua vez, entrega cobre para a ferroxidase apoFet3, responsável pelo sistema de alta afinidade do ferro (Kim *et al.*, 2008). Avaliar a expressão gênica, por qRT-PCR, de *CTR2*, *ATX1* e *CCC2*, poderia fundamentar melhor a hipótese mencionada no item 2.

Os fatores de transcrição Aft1, Zap1 e Mac1 podem estar envolvidos no metabolismo do íon metálico Sn^{2+} (Capítulo IV, figura 1 a, b, c). No mutante *aft1*Δ, não há a regulação da transcrição do gene *FET4* e, portanto, a proteína Fet4 não estaria transportando o íon metálico Sn^{2+}, o que explicaria a resistência do mutante (Capítulo IV, figura 1a). No mutante *zap1*Δ, não está sendo regulada a expressão do *ZRT2*, porque a proteína Zrt2 não estaria transportando o íon metálico Sn^{2+}, fato que justificaria a resistência do mutante (Capítulo IV, figura 1b). Interessantemente, no mutante *mac1*Δ, podem estar ocorrendo duas possibilidades: (1) a ausência da proteína Mac1 está deixando de regular outra proteína que poderia estar envolvida na captação do íon metálico Sn^{2+}; ou (2) a ausência da proteína Mac1 está deixando de regular a proteína Ctr2, que impede que o íon metálico Sn^{2+} saia do vacúolo.

O fator de transcrição Ace1 parece estar envolvido no metabolismo do íon metálico Sn^{2+}. A expressão do gene *ACE1* foi aumentada na condição de tratamento 1000 µM de $SnCl_2$ (Capítulo IV, figura 2b). Sabe-se que, no excesso de cobre, Ace1 ativa a transcrição de *CRS5* e *CUP1* (metalotioneínas) e de *SOD1* (Pegani *et al.*, 2007). O íon metálico Sn^{2+} pode estar sendo destoxificado pela proteína Crs5, enquanto que a proteína Cup1 parece não estar envolvida nesse processo (Capítulo IV, figura 2b). No entanto, a proteína glutationa parece não participar na destoxificação do íon metálico Sn^{2+}, provavelmente porque há várias proteínas com funções similares que, de alguma forma, garantem o "sequestro" do íon no citoplasma (Capítulo IV, figura 2c). Corroborando esses achados, mutantes defectivos em várias etapas da biossíntese da glutationa foram resistentes ao tratamento de 25 mM de $SnCl_2$ (Capítulo I, tabela 3), o que reforça a idéia de que Sod1 e Crs5 são capazes de defender as células contra os danos provocados pelo íon metálico Sn^{2+}.

Ao analisar a interferência do íon metálico Sn^{2+} no metabolismo do cálcio, sugere-se que as proteínas de membrana plasmática, Mid1 e Cch1, que transportam Ca^{2+}, podem transportar o íon metálico Sn^{2+}. A expressão dos genes *MID1* e *CCH1* aumentou após tratamento com $SnCl_2$ (Capítulo IV, figura 2a). Duas

hipóteses que justificariam esse aumento de expressão podem ser aventadas: (1) devido à semelhança química dos elementos, o íon metálico Sn^{2+} poderia ser substrato para as proteínas Mid1 e Cch1 e capaz de ser internalizado juntamente com o Ca^{2+}; com maior facilidade por estar em excesso; e (2) a depleção do Mg^{2+} (Capítulo IV, tabela 3) seria capaz de induzir genes envolvidos na captação de Ca^{2+}, via ativação do sistema fosfatase calcineurina/Crz1, uma vez que a diminuição dos teores de Mg^{2+} em *S. cerevisiae* promove a ativação de vários genes regulados por esse sistema (Wiesenberger *et al.*, 2007).

A defosforilação da fosfatase calcineurina pelo complexo calmodulina/Ca faz com que haja translocação de dois fatores de transcrição (Crz1/Tcn1) do citoplasma para o núcleo (Kafadar & Cyert, 2004). Esses fatores de transcrição reconhecem regiões específicas de genes-alvo, chamadas de elemento responsivo dependente de calcineurina (CDRE – *calcineurin-dependent response element*), de forma que, consequentemente, ocorre a transcrição de mais de cento e sessenta genes envolvidos em diversos processos metabólicos, por exemplo da homeostase de íons, do transporte de vesículas no citoplasma, da síntese de lipídios, do transporte de pequenas moléculas e da manutenção da parede celular (Cyert, 2003; Ton & Rao, 2004).

Pungartnik *et al.* (2005) evidenciaram uma forte interação do íon metálico Sn^{2+} com a parede celular da levedura *S. cerevisiae*, não observada, no entanto, com relação a outros metais (Co^{2+}, Cd^{2+}, Ni^{2+}, Fe^{2+}, Cu^{2+} e Ca^{2+}). Dessa maneira, essa forte interação poderia prejudicar a integridade da parede celular da levedura. Attramadal & Svatun (1980) mostraram a rápida ligação do íon metálico Sn^{2+} com polímeros polianiônicos presentes na parede celular e na cápsula do agente etiológico da cárie dental (*Streptococcus mutans*), sugerindo perturbações de estruturas celulares causadas por esse metal. Portanto, a perturbação da integridade da parede celular provocada pelo íon metálico Sn^{2+} levaria à ativação do sistema fosfatase calcineurina/Crz1, numa tentativa de restabelecer os danos provocados por esse metal tóxico. Faz-se necessário investigar os principais componentes da parece celular de levedura (manoproteínas, glicanos, quitina, por

exemplo) após tratamento com $SnCl_2$, bem como a expressão de genes presentes na parede celular da levedura (FIT1, FIT2 e FIT3, que codificam proteínas facilitadoras transportadoras de ferro para o espaço periplasmático) (Protchenko *et al.*, 2001; Philpott & Protchenko, 2007).

O transporte vacuolar de Ca^{2+} ocorre devido à ação de três proteínas: (1) Pmc1, transportadora de Ca^{2+} dependente de ATP do citoplasma para o vacúolo; (2) Vcx1, transportadora de Ca^{2+} para dentro do vacúolo, que funciona dependente de energia proveniente de um gradiente de prótons (antiporter); e (3) Yvc1, que atua como canal facilitador de liberação de Ca^{2+} do vacúolo para o citoplasma (Ton & Rao, 2004). Ao analisar os genes que codificam essas proteínas, nota-se que, após tratamento com 25 e 1000 µM de $SnCl_2$, houve um aumento da expressão de *PMC1* e diminuição de *VCX1* e *YVC1* (Capítulo IV, figura 2a). Sugere-se que, num primeiro momento, o íon metálico Sn^{2+} possa estar sendo levado para o vacúolo, numa tentativa de minimizar os seus efeitos tóxicos, motivo pelo qual há um aumento da expressão do gene *PMC1*. Secundariamente, como houve aumento de genes que respondem à calcineurina/Crz1, por depleção de Mg^{2+} e por aumento de P (Capítulo IV, tabela 3), há uma tentativa da célula de evitar uma hipercalcemia, aumentando a expressão do gene *PMC1*. Entretanto, Vcx1 e Yvc1 operam inversamente à Pmc1 com o intuito de não deixar a célula desprovida de Ca^{2+}, porque, uma vez em excesso, há entrada de maior quantidade do íon metálico Sn^{2+} do que do Ca^{2+}.

Ao analisar o gene *PMR1*, que codifica a principal Ca^{2+} ATPase presente no complexo de Golgi (Ton & Rao, 2004), nota-se que não há variação da expressão gênica por qRT-PCR (Capítulo IV, figura 2a). Isso implica que Pmr1 não exporta o íon metálico Sn^{2+}, diferentemente do que ocorre com o Cd^{2+} (Lauer Júnior *et al.*, 2008).

Portanto, com o estudo realizado com o agente $SnCl_2$ e células de *S. cerevisiae* e V79, pode-se concluir que a toxicidade e genotoxicidade desse agente deriva de dois mecanismos principais: (1) a ação direta do íon metálico Sn^{2+}, que parece predominar sobre a sua ação indireta, e que se caracteriza por

gerar quebras simples e duplas na fita de nucleotídeos, as quais podem ser reparadas via RAD52 (livre de erros) e via RAD6 – tipo Rev3 – (sujeita a erro), corroborando achados anteriores que apontam o $SnCl_2$ como um agente mutagênico moderado. A ação direta igualmente explicaria a toxicidade do íon metálico Sn^{2+}, que seria captado pelos mesmos transportadores, ao promover um desequilíbrio metálico por apresentar similaridades químicas com outros metais essenciais. Não menos importante, o íon metálico Sn^{2+} pode interferir diretamente em enzimas de reparação, tornando-as incapazes de reparar lesões geradas por outros agentes mutagênicos clássicos, como o MMS; e (2) a ação indireta do íon metálico Sn^{2+}, com formação de EROs, que poderia justificar a participação de BER, que, por sua vez, levaria a quebras e poderia, também, envolver a via recombinacional.

CONCLUSÕES

1 Conclusão geral

Com este estudo, conclui-se que o $SnCl_2$ é um agente genotóxico de baixa toxicidade. Os danos que o íon metálico Sn^{2+} gera ao DNA são quebras do tipo simples e duplas, as quais são, predominantemente, reparadas via RAD52. Há, também, a formação de espécies reativas de oxigênio, das quais resultam quebras na estrutura do material genético, o que canalizaria para a via de reparação recombinacional (via RAD52).

2 Conclusões específicas

- ✓ As células de levedura *Saccharomyces cerevisiae* em metabolismo fermentativo são extremamente sensíveis ao íon metálico Sn^{2+} quando comparadas em metabolismo respiratório;
- ✓ O mutante *rad52*Δ foi o mais sensível ao íon metálico Sn^{2+}, quando comparado com a linhagem selvagem isogênica e com outros mutantes de outras vias de reparação, o que permite inferir que o íon metálico Sn^{2+} causa quebras simples e duplas no DNA;
- ✓ A moderada sensibilidade dos mutantes *rad2*Δ e *rad4*Δ, bem como a do mutante *rad6*Δ, sugere a participação da via de reparação por excisão de nucleotídeos e da via sujeita a erros na remoção dos danos provocados pelo íon metálico Sn^{2+};
- ✓ Os testes realizados com o íon metálico Sn^{2+} demonstram que ele pode interferir na homeostase de outros elementos químicos essenciais, tais como magnésio, zinco, fósforo, ferro e enxofre;

- ✓ As proteínas Fet4, Zrt2, Cch1, Mid1 podem estar envolvidas na captação do íon metálico Sn^{2+};
- ✓ Os fatores de transcrição Mac1, Zap1, Aft1, Ace1 e Yap1 podem estar envolvidos no metabolismo do íon metálico Sn^{2+};
- ✓ As proteínas Sod1 e Apn1, mobilizadas em condições de estresse oxidativo, podem estar envolvidas no metabolismo do íon metálico Sn^{2+};
- ✓ A metalotioneína Crs5 de *Saccharomyces cerevisiae* parece destoxificar o íon metálico Sn^{2+};
- ✓ O íon metálico Sn^{2+} parece não ser exportado pela célula através da proteína cálcio ATPase, Pmr1, presente no complexo de Golgi;
- ✓ O íon metálico Sn^{2+} é um agente comutagênico e parece interferir na atividade de enzimas de reparação do DNA.

PERSPECTIVAS

Para melhor compreender como o íon metálico Sn^{2+} é internalizado, distribuído e destoxificado na célula da levedura *Saccharomyces cerevisiae*, faz-se necessário aprofundar os estudos sobre a interação desse íon com outros elementos químicos essenciais, realizando:

- ✓ A análise da expressão dos genes *COT1*, *ZRC1* e *CCC1*, presentes na membrana do vacúolo, responsáveis pela retirada de zinco, cobalto e ferro do citoplasma por qRT-PCR;
- ✓ O isolamento de organelas, tais como o vacúolo e a mitocôndria, para analisar a distribuição do íon metálico Sn^{2+} por espectrometria de massas com fonte de plasma (ICP-MS);
- ✓ A análise dos elementos Ca, K e Cu, que não puderam ser estudados pela metodologia PIXE, por espectrometria de massas com fonte de plasma (ICP-MS), quando as células de *Saccharomyces cerevisiae* são tratadas com o íon metálico Sn^{2+};
- ✓ A medição da quantidade de Ca^{2+} nos mutantes *cch1*Δ, *mid1*Δ e *pmc1*Δ, após tratamento com o íon metálico Sn^{2+};
- ✓ A análise da expressão de *SMF1* e *PHO84*, cujos produtos, possivelmente, estão envolvidos no transporte do íon metálico Sn^{2+} na membrana plasmática da célula de *Saccharomyces cerevisiae*.

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ANEXO I

Genotoxicity of stannous chloride in yeast and bacteria.

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Genotoxicity of stannous chloride in yeast and bacteria

C. Pungartnik [a,c], C. Viau [a], J. Picada [a], A. Caldeira-de-Araújo [a,b], J.A.P. Henriques [a], M. Brendel [a,c,*]

[a] *Dept. de Biofísica, Centro de Biotecnologia, UFRGS, Av. Bento Gonçalves 9500, 91507-970 Porto Alegre, RS, Brazil*
[b] *Dept. de Biofísica e Biometria, Instituto de Biologia Roberto Alcantara Gomes, UERJ, Av. 28 de Setembro, 87 fundos, CEP20551-130 Rio de Janeiro, RJ, Brazil*
[c] *Departamento de Ciências Biológicas, Universidade Estadual de Santa Cruz, Rodovia Ilhéus-Itabuna, Km 16, Ilhéus, BA CEP45650-000, Brazil*

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Abstract

Stannous chloride was found genotoxic in microbial test systems of the yeast *Saccharomyces cerevisiae*, in one strain of *Salmonella typhimurium* and in the Mutoxitest of *Escherichia coli*. Five isogenic haploid yeast strains differing only in a particular repair-deficiency had the following ranking in Sn^{2+}-sensitivity: *rad52Δ* > *rad6Δ* > *rad2Δ* > *rad4Δ* > *RAD*, indicating a higher relevance of recombinogenic repair mechanisms than nucleotide excision in repair of Sn^{2+}-induced DNA damage. Sn^{2+}-treated cells formed aggregates that lead to gross overestimation of toxicity when not undone before diluting and plating. Reliable inactivation assays at exposure doses of 25–75 mM $SnCl_2$ were achieved by de-clumping with either EDTA- or phosphate buffer. Sn^{2+}-induced reversion of the yeast *his1-798*, *his1-208* and *lys1-1* mutant alleles, in diploid and haploid cells, respectively, and putative frameshift mutagenesis (reversion of the *hom3-10* allele) was observed. In diploid yeast, $SnCl_2$ induced intra-genic mitotic recombination while inter-genic (reciprocal) recombination was very weak and not significant. Yeast cells of exponentially growing cultures were killed to about the same extend at 0.1% of $SnCl_2$ than respective cells in stationary phase, suggesting a major involvement of physiological parameters of post-diauxic shift oxidative stress resistance in enhanced Sn^{2+}-tolerance. Superoxide dismutases, but not catalase, protected against $SnCl_2$-induced reactive oxygen species as *sod1Δ* had a three-fold higher sensitivity than the WT while the *sod2Δ* mutant was only slightly more sensitive but conferred significant sensitivity increase in a *sod1Δ sod2Δ* double mutant. In the *Salmonella* reversion assay, $SnCl_2$ did not induce mutations in strains TA97, TA98 or TA100, while a positive response was seen in strain TA102. $SnCl_2$ induced a two-fold increase in mutation in the Mutoxitest strain IC203 (*uvrA oxyR*), but was less mutagenic in strain IC188 (*uvrA*). We propose that the mutagenicity of $SnCl_2$ in yeast and bacteria occurs via error-prone repair of DNA damage that is produced by reactive oxygen species.

Keywords: Stannous chloride; *Saccharomyces cerevisiae*; Cell clumping; Genotoxicity; Oxidative stress; Superoxide dismutase

* Corresponding author. Tel.: +55 73 3680 5328; fax: +55 73 3680 5328.
E-mail address: martinbrendel@yahoo.com.br (M. Brendel).

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1. Introduction

Stannous ions (Sn^{2+}), as fluoride or chloride salts, are present in our daily life. $SnCl_2$, also known as tin chloride, is chemically classified as low risk agent [1]. It has been used in food industry to conserve soft drinks [2], in sensitising of glass and plastics before metallizing [3] and in manufacture of tin chemicals, colour pigments, pharmaceuticals and sensitised paper; Sn^{2+} in the form of SnF_2 has been mainly used in dentistry (as dentifrices) as a highly effective anti-microbial agent against caries [4]. $SnCl_2$ is also employed as reducing agent in production of Technetium-99m-labelled radio-pharmaceuticals [5].

Early reports showed toxicity of tin chloride in rats, i.e. pathological changes in liver, kidney and brain edema [6], pancreatic atrophy [7], increased incidence of changes in fatty acids and vacuoles in the proximal convoluted tubules [8]. Rabbits fed with one or more grams of $SnCl_2$ every 6–10 days died after 1–2 months with gastritis, degeneration of liver and kidney and paralysis of the hind legs [9].

In an attempt to determine Sn^{2+}-induced toxicity/genotoxicity and/or carcinogenicity/teratogenicity, various in vivo and in vitro models have been tested during the past 25 years. Negative results were reported for the *Bacillus subtilis* Rec-assay [10] and for yeast [11] where $SnCl_2$ could induce neither mutation nor mitotic gene conversion. Also, no genotoxic effects were found in the *Drosophila melanogaster* wing spot test [12]. However, since McLean and Kaplan [13] showed rapid and prolonged suppression of DNA synthesis in $SnCl_2$-treated Human Raji cells, many reports indicated an interaction between genomic material and $SnCl_2$, thus suggesting its putative genotoxicity. McLean et al. [2,14] described that $SnCl_2$ produced single-strand breaks in DNA of Chinese hamster ovary cells and caused DNA damage in human white blood cells; genotoxicity of $SnCl_2$ was shown in the SOS chromotest [15]. Ashby and Tennant [16] found carcinogenic activity in rat thyroid cells but reported negative results for the *Salmonella* assay. $SnCl_2$-induced cytotoxicity/genotoxicity in different DNA repair mutants of *Escherichia coli* [17,18] and DNA damage was suggested to occur via formation of oxygen radicals [19,20]. In vitro, $SnCl_2$ induced strand breaks in plasmid DNA and it was suggested that this type of DNA lesion could also be introduced by $SnCl_2$ in vivo [21].

In order to further evaluate the genotoxic potential of $SnCl_2$ and to confirm suggested mechanisms of its action on DNA and on repair of induced lesions, we chose the standard bacterial genotoxicity test systems *Salmonella* and Mutoxitest as well as the yeast *Saccharomyces cerevisiae* as a well-defined eukaryotic model system, this unicellular organism offers large numbers of isogenic DNA repair mutants and molecularly well-defined alleles for mutagenicity testing and, in the diploid phase, also allows the study of mutagen-induced recombinational processes.

2. Materials and methods

2.1. Strains and media

The relevant genotypes of the bacteria and yeast used in this study are given in Table 1. Media, solutions and buffers were prepared according to Burke et al. [22]. Complete medium (YPD) was used for routine growth of yeast cells and minimal medium (MM) was supplemented with the appropriate amino acids (synthetic complete medium, SC). To ascertain yeast respiratory competence and for the elimination of spontaneously accumulated *petites*, all strains were pre-grown on YPG media (glucose replaced by 3% glycerol) before being propagated in YPD. *E. coli* strains WP2 *uvrA*/pKM101 (IC188) and WP2 *uvrA oxyR*/pKM101 (IC203) were obtained from M. Blanco, Instituto de Investigaciones Citológicas, Spain.

2.2. $SnCl_2$ solubilization

Two types of stannous chloride were used: $SnCl_2{\cdot}2H_2O$ and anhydrous $SnCl_2$ (Sigma, St. Louis, MO). As $SnCl_2{\cdot}2H_2O$ is poorly soluble in water (less than its own molecular weight of water; The Merck Index, 2001), it was routinely dissolved in the following manner: 0.1 g was dissolved in 1 mL of cold absolute ethanol (EtOH), and immediately and carefully heated not to reach the boiling point. Upon agitation, the initially turbid solution becomes clear. After cooling, 1 mL of water (1:1) was carefully and slowly added in order to avoid precipitation. This stock solution (250 mM in 50% EtOH) had to be freshly prepared for

Table 1
Strains used in this study and their relevant genotypes

	Genotype	Reference
Yeast strains		
BY10000	*MATα his3Δ1, lys2Δ0, leu2Δ0, ura3Δ0*	EUROSCARF
4 BY *rad* mutants	Same genotype as BY10000 but *rad52Δ*, *rad2Δ*, *rad4Δ*, *rad6Δ*	See above
XS2316	$\frac{MATa}{MAT\alpha}\ \frac{+leu1-1\ trp5-48+++}{ade6\ leu1-12+cyh2\ met13\ lys5-1}\ \frac{his1-208}{his1-208}$	[32]
XV185-14c	*MATα ade2-2, his1-798, lys1-1, trp5-48, hom3-10, arg4-17*	[29]
EG103 (WT)	*MATα leu2-3,112 his3Δ1 trp1-289 ura3-52 GAL*$^+$	E.B.Gralla
EG118 (*sod1Δ*)	*sod1::URA3* all others markers as EG103	See above
EG110 (*sod2Δ*)	*sod2::TRP1* all others markers as EG103	See above
EG133 (*sod1Δ sod2Δ*)	*sod1::URA3 sod2::TRP1* double mutant, all others markers as EG103	See above
EG223 (*ctt1Δ*)	*ctt1::TRP1* all others markers as EG103	See above
Bacterial strains		
IC203	*trpE65, uvrA,oxyR*, pKM101	[42]
IC188	*trpE65, uvrA*, pKM101	See above
TA102	*hisG428, rfa*, pKM101, pAQ1	[23]

each experiment. Final concentration of $SnCl_2{\cdot}2H_2O$ was either 2.5 or 25 mM (concentration of EtOH never exceeded 5% and did not affect cell survival). When concentrations >25 mM of $SnCl_2$ were used, the anhydrous salt was directly added to the cell suspension. Since equimolar concentrations of anhydrous $SnCl_2$ and $SnCl_2{\cdot}2H_2O$ had the same genotoxicity we preferably used the readily water-soluble anhydrous stannous salt, thus avoiding use of ethanol and of heating.

2.3. Salmonella/microsome mutagenicity assay

Mutagenicity was assayed by the pre-incubation procedure proposed by Mortelmans and Zeiger [23]. $SnCl_2$ was dissolved in water immediately prior to use. Hundred microliter of tester-strain cultures ($(1–2) \times 10^9$ cells/mL) was incubated at 37 °C with different amounts of $SnCl_2$ (125–5000 μM) for 20 min, without shaking. The reaction was stopped and the cells were dis-aggregated by adding 800 μL of 0.067 M sodium phosphate buffer, pH 7.4 (PB). Then, 2 mL of molten top agar (0.6% agar, 0.5% NaCl, 50 μM histidine, 50 μM biotin, pH 7.4, 45 °C) was added and the mixture poured immediately onto a plate of minimal agar (1.5% agar, Vogel–Bonner E medium, containing 2% glucose). The positive controls were 4-nitroquinoline-oxide (0.5 μg/plate) for TA98, TA97 and TA102, and sodium azide (5 μg/plate) for TA100. The plates were incubated in the dark at 37 °C for 48 h before counting.

2.4. E. coli mutagenicity assay—WP2 Mutoxitest

The assays were performed according to Blanco et al. [24], with a modified pre-incubation procedure [25]. Hundred microliter of a fresh overnight culture was incubated without shaking at 37 °C with different amounts of $SnCl_2$ (125–5000 μM) for 20 min. The reaction was stopped and the cells were dis-aggregated by adding 800 μL of PB. Two milliliter of molten top agar at 45 °C (supplemented with 0.05 mM tryptophan) was added and poured into plates containing minimal ET4 agar (15 g Difco agar and 4 g glucose/L of Vogel–Bonner E medium). The plates were incubated for 48 h at 37 °C. The *tert*-butylhydroperoxide (*t*-BOOH, 50 μg/plate) was used as positive control. All mutagenicity assays were carried out in triplicate, on at least two occasions.

2.5. Bacterial data analysis

Mutagenicity data were analyzed with *Salmonel* software [26]. A compound was considered positive for mutagenicity only when (a) the number of revertants was at least double the spontaneous yield (MI ≥ 2; MI = mutagenic index: number of colonies in the treated sample/number of colonies in the negative control); (b) a significant analysis of variance ($p \leq 0.05$) was found for the treated and negative control data; and (c) a reproducible positive dose–response ($p \leq 0.01$) was present, as evaluated by the *Salmonel* software [27].

2.6. Yeast Exposure to $SnCl_2$ and survival

Stationary (STAT) cells were grown in YPD at 30 °C for 72 h and exponentially growing (LOG) cells were obtained after 4.5-h incubation of YPD freshly inoculated with 2×10^6 STAT cells/mL. LOG cells were only used when having a minimum of 20–30% buds at $(1–2) \times 10^7$ cells/mL. Sensitivity of twice saline-washed yeast suspensions to $SnCl_2$ was routinely determined in liquid saline (0.9% NaCl, pH 5.0). Exposure concentrations ranged from 25 to 75 mM for STAT and from 25 to 250 μM for LOG cells. Exposure time was up to 90 min at 30 °C. Thereafter, $SnCl_2$-mediated cell aggregates were de-clumped in phosphate buffer (PB, pH 7.4, 0.067 M) or in EDTA solution (0.25 M) before dilution and plating. Routinely, $SnCl_2$-exposed cell suspensions were diluted into PB, followed by vigorous vortexing, except when stated otherwise. Cells were plated on YPD and survival was determined after 3 days at 30 °C. Presented results are the mean of at least three independent experiments and the error bars represent standard deviation as calculated by GraphPad Prism® program.

2.7. Point and frameshift mutation in haploid yeast

Two alleles, *his1-798* and *lys1-1* were used for point mutagenesis. The suppressible *ochre* nonsense mutant allele *lys1-1* can be reverted either by locus-specific sequence alteration (true reversion) or by a forward mutation in a suppressor gene [28,29]. Distinction between true reversions and forward (suppressor) mutations at the *lys1-1* locus was according to [30], where the reduced adenine content of the SC-Lys medium shows true reversions as red and suppressor mutations as white colonies. The *hom3-10* mutant allele of haploid strain XV185-14c was used for assaying putative frameshift mutagenesis. It is believed that *hom3-10* contains a frameshift mutation due to its response to a range of diagnostic mutagens [29]. Survival was determined on SC (3–5 day, 30 °C) and mutation induction (HIS, LYS or HOM revertants) on media lacking the appropriate amino acid (7–10 day, 30 °C). Forward mutation was measured with the Canavanine Resistance (CAN-) Assay (*CAN1* → *can1*^{r}). Concentration of canavanine in complete synthetic media fully supplementing all auxotrophies of the yeast cells was 20–60 mg/L (SC – ARG + CAN) [31]. Stop of $SnCl_2$ reaction and de-clumping was as described above. Mutant frequencies were scored per surviving cells. Results are means of three independent experiments. Statistical analyses were performed using the GraphPad Prism® software.

2.8. Induced mutation and recombination in diploid yeast

Mitotic recombination (inter- and intra-genic), as well as *his1-208* reversion was determined in diploid strain XS2316 [32]. Mutant and recombinant frequencies were scored per surviving cell. Washed yeast suspensions ($(2–4) \times 10^8$/mL) were treated with 25–75 mM $SnCl_2$ as follows: 5 mL of STAT saline-washed cells were incubated with 25, 50 or 75 mM $SnCl_2$ for up to 60 min. At fixed times, 1.0 mL aliquots were withdrawn from the cell suspension and added to 1 mL or 9.0 mL of PB (the latter dilution for 50 and 75 mM of $SnCl_2$) to stop the reaction and to de-clump the cell aggregates. The diluted cell suspension was vortexed vigorously, diluted in 1:10 steps with PB, and plated on three media (SC, SC–Leu and SC + Cyh). Following incubation for 4–7 day at 30 °C, colonies appearing on SC medium yielded data on cell survival, while those grown on SC–Leu and SC + Cyh gave the incidence of intra-genic recombination (mitotic gene conversion) and inter-genic recombination (crossing-over), respectively. For assaying, the exact frequency of reciprocal crossing-over it was necessary to eliminate possible revertants to cycloheximide-resistance resulting from mutation at the *CYH2* locus, as well as from monosomy of chromosome VII. Therefore, cycloheximide-resistant colonies were replica-plated on SC–Lys, SC–Met and SC–Ade media to confirm the recombination origin of *cyh2* homozygocity. Results are means of three independent experiments. Mitotic recombination statistical analyses were performed using the GraphPad Prism® software.

3. Results

3.1. Genotoxicity in bacteria

In *Salmonella typhimurium* tester-strains, 12.5–500 μM $SnCl_2$ was not mutagenic in frameshift-sensitive strains TA98 and TA97 and in TA100 (base pair substitution mutation). The only positive

Table 2
Mutagenicity of $SnCl_2$ in *S. typhimurium* TA102 and in *E.coli* IC188 and IC203 strains

Substance	Dose (mM)	TA102		IC188		IC203	
		Rev./plate[a]	MI[b]	Rev./plate[a]	MI[b]	Rev./plate[a]	MI[b]
NC[c]		367 ± 14		133 ± 21		146 ± 26	
$SnCl_2$	0.125	ND		150 ± 30	1.1	179 ± 55	1.2
	0.25	ND		157 ± 33	1.2	174 ± 66	1.2
	0.50	ND		179 ± 8	1.3	201 ± 66	1.4
	1.00	442 ± 50	1.2	202 ± 35	1.5	231 ± 27*	1.6
	1.25	463 ± 36*	1.3	185 ± 5*	1.4	298 ± 41*	2.0
	2.50	482 ± 18**	1.3	ND		ND	
	3.75	515 ± 16**	1.4	ND		ND	
	5.00	733 ± 41**	2.0	ND		ND	
PC[d]		1923 ± 210	5.2	1250 ± 172	9.1	2137 ± 288	14.6

ND, not determined.
[a] Number of revertants/plate: mean of three plates ± S.D.
[b] MI, mutagenic index: number of colonies induced in the sample/number of spontaneous colonies (negative control).
[c] NC, negative control: distilled water.
[d] PC, positive control: 4-nitroquinoline 1-oxide (0.5 μg/plate) for TA102; *tert*-butylhydroperoxide (50 μg/plate) for IC188 and IC203.
* $p < 0.05$.
** $p < 0.01$ (ANOVA).

mutagenic response with an MI of 2.0 was observed in strain TA102, which detects oxidative and alkylating mutagens and ROS [25] at a $SnCl_2$ dose of 500 μM (Table 2). In this result, also a significant response for analysis of variance ($p \leq 0.05$) and a positive dose–response ($p \leq 0.01$) was observed. When using the oxidant-sensitive *E. coli* strain IC188 (WP2 *vrA*/pKM101) and its derivative, IC203 (WP2 *uvrA oxyR*/pKM101) with enhanced sensitivity to ROS, we found a dose-dependent mutation induction by $SnCl_2$ (Table 2). However, an MI of 2.0 was reached only in strain IC203 and only at 125 μM of $SnCl_2$, the highest dose tested.

3.2. Genotoxicity in yeast

Survival of $SnCl_2$-treated STAT cells of haploid yeast is influenced by cell aggregation and by DNA repair (Fig. 1). Sn^{2+}-induced cell aggregation, when not corrected for (undone by diluting in PB or EDTA buffer) exaggerated the toxic effect. The extent of correction of survival by proper de-clumping of the Sn^{2+}-treated yeast cells for the WT and the *rad4* mutant that is deficient in nucleotide excision repair (NER) is shown in Fig. 1 (inset). Clumping efficiency is the same for both strains as the relative increase in sensitivity of Sn^{2+}-treated *rad4Δ* to the isogenic *RAD* WT re-

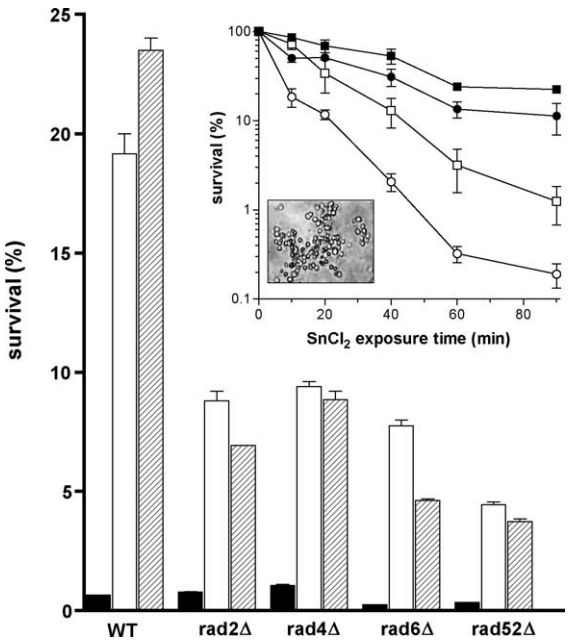

Fig. 1. Survival of STAT cells of WT and 4 DNA repair mutants after 90 min $SnCl_2$ exposure (25 mM) after dilution in saline (black columns), EDTA buffered saline (open columns), and phosphate buffer (hatched columns). *Inset*: Survival of Sn^{2+} -treated WT (squares) and *rad4Δ* (circles), diluted in either phosphate buffer (filled symbols) or in saline (open symbols). *Photo*: Clumping haploid WT yeast after exposure to 25 mM $SnCl_2$.

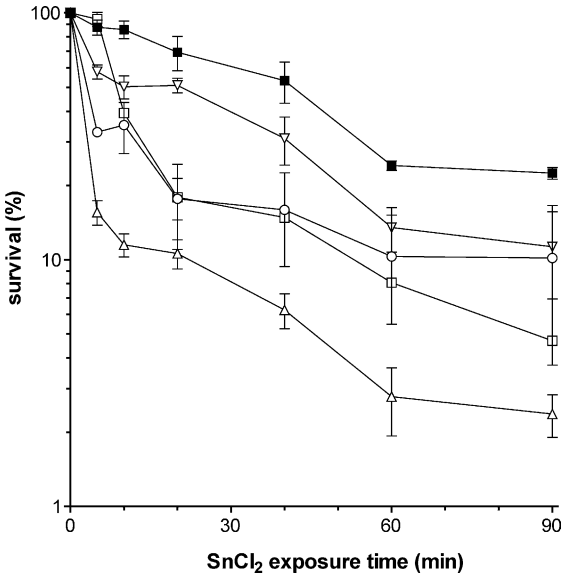

Fig. 2. Sensitivity to 25 mM $SnCl_2$ of five isogenic yeast strains. *RAD* (WT (■)); *rad4Δ* (▽); *rad2Δ* (◯); *rad6Δ* (□); *rad52Δ* (Δ). In some points, error bars (standard deviation) do not exceed the size of the symbol. Cells were diluted in PB.

mained constant with a dose-modifying factor of about 1.6 (Fig. 1; inset).

The three main modes of DNA repair of *S. cerevisiae* each contribute to the observed resistance in the WT (*RAD*) strain. Fig. 2 shows sensitivity to Sn^{2+} of five isogenic yeast strains, four of which carry each one specific deletion of a DNA repair gene of *S. cerevisiae*. All four repair-deficient mutants were significantly more Sn^{2+}-sensitive than the isogenic WT, with *rad52Δ* exhibiting the highest sensitivity amongst the isogenic repair mutants.

While Sn^{2+} was clearly mutagenic in two histidine reversion assays, it was more so for the *his1-798* mutant allele (Fig. 3). Survival of both strains was in the order of 70–80%, with the diploid being more resistant (Fig. 3). Clearly, mutation induction was exposure dose-dependent (Fig. 4). Sn^{2+} also reverted the putative frameshift allele *hom3-10* and the ochre *lys1-1* mutant allele in haploid strain XV185-14c at the highest exposure concentration (75 mM) at still low levels of toxicity (Fig. 5), in the absence of significant induction of ochre suppressor mutations (data not shown).

Under the same conditions we could not observe Sn^{2+}-induced forward mutagenesis in the CAN-assay.

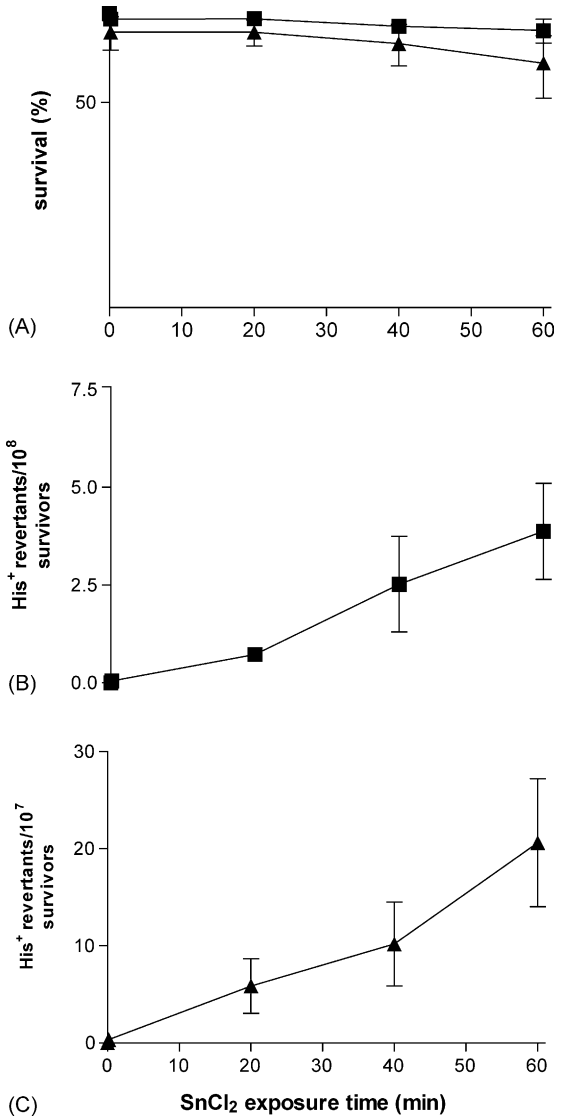

Fig. 3. Genotoxic effects of 25 mM $SnCl_2$ in STAT yeast cells of haploid XV185-14c (▲) and diploid XS2316 (■). (A) Survival; (B and C) reversion of different *his1* mutant alleles, depending on exposure time; (B and C) have the same abscissa. Cells were diluted in PB.

In fact, we found negative mutation induction, i.e. the frequency/survivors of *can1*^{R} mutations declined with increasing exposure to Sn^{2+} (Table 3). This was also observed when plating on lower CAN concentrations (from 60 μg/mL down to 20 μg/mL) for all five EUROSCARF strains (data not shown). The correct declumping by PB of Sn^{2+}-treated cells was controlled

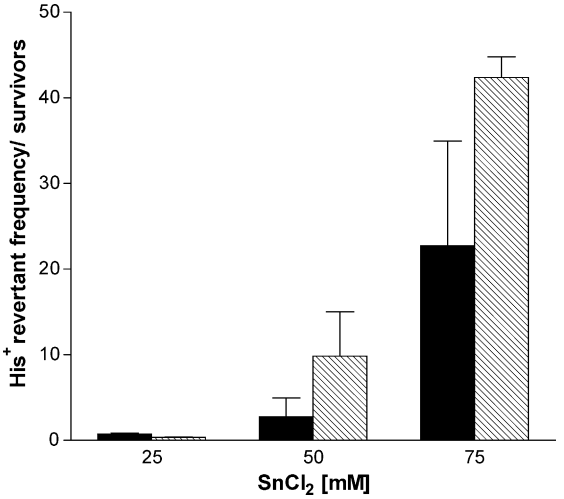

Fig. 4. Reversion of two different histidine mutant alleles depending on exposure concentration of Sn^{2+} in STAT cells (20 min) in haploid XV185-14c (*his1-7* [*his1-798*], per 10^7 survivors), hatched and diploid XS2316 (*his1-1* [*his1-208*], per 10^8 survivors), solid columns, respectively. Cells were diluted in PB.

by microscopic observation. The same yeast suspensions showed forward mutation to CAN-resistance (*CAN1* → *can1*^{R}) when treated with UVC (Table 3).

Possible recombinogenic effects of Sn^{2+} were investigated by using the diploid yeast strain XS2316 [32], which detects two forms of mitotic recombination (crossing-over and gene conversion), in addition to reversion by mutation. There was a slight increase in mitotic crossing-over at 50 and 75 mM Sn^{2+}-exposure

Table 3
Differential response of the *rad52Δ* strain in the CAN forward mutation assay after $SnCl_2$ and UVC exposure

	Survival (%)	*can1*^{R}/*10*^{7} *survivors*	*can1*^{R} mutants*
$SnCl_2$ (25 mM)**			
Zero (control)	100	82	329
20 min	22	45	358
40 min	15	27	188
60 min	9.1	22	175
UVC (J/m^2)**			
Zero (control)	100	82	1615
10	80	175	2800

* Mutant numbers scored on SC+CAN agar plates [60 μg/mL CAN].
** Cell suspensions were diluted with PB.

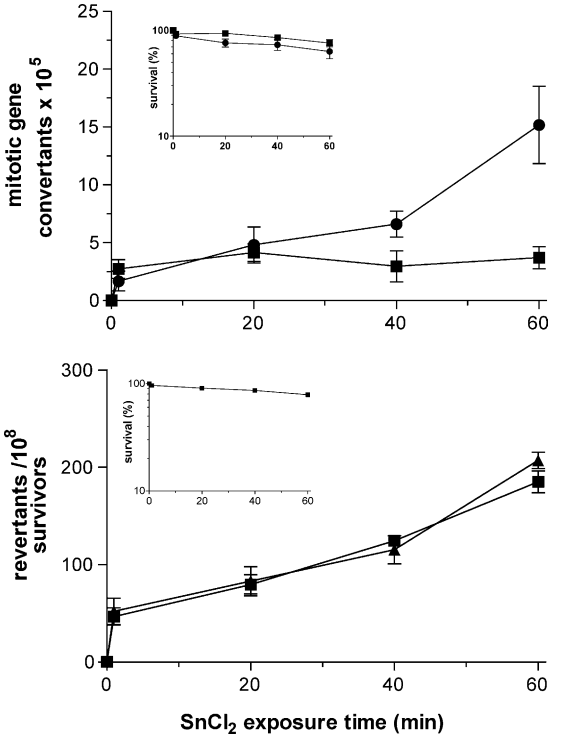

Fig. 5. $SnCl_2$-induced mitotic gene conversion in diploid strain XS2316 (upper panel): (●) 50 mM $SnCl_2$; (■) 25 mM $SnCl_2$. Lower panel: $SnCl_2$ (75 mM)-induced reversion to prototrophy of (■) *hom3-10* and (▲) *lys1-1* (locus-specific revertants) in haploid strain XV185-14c. All experiments include a 2-min exposure dose-sample to assess for eventual early effects of $SnCl_2$. Cells were diluted in PB.

concentrations while UVC-induced mitotic recombination was highly significant (Table 4). $SnCl_2$ at 50 mM resulted in significant mitotic gene conversion (Fig. 5; upper panel). Increasing the concentration of Sn^{2+} from 25 to 50 and to 75 mM, while maintaining the same exposure time, resulted in significantly higher reversion frequencies for both *his1* mutant alleles (Fig. 4).

Compared to STAT cells of the haploid DNA repair-proficient WT strain XV185-14c, pre diauxic-shift LOG cells were found slightly more sensitive at an exposure concentration of 25 μM, i.e. at 0.1% of the $SnCl_2$ concentration (Fig. 6). Thus, the difference in sensitivity to $SnCl_2$ was much greater between STAT and LOG cells of the same WT strain than amongst isogenic STAT phase cells of strains with different capacity of DNA repair, where the most sensitive *rad52Δ* mutant showed an about three-fold higher

Table 4
Induction of mitotic recombination by $SnCl_2$ in diploid *S.cerevisiae* strain XS2316

Agent (dose)	Treatment	Survival (%)	Crossing-over/10^5 survivors
UVC (J/m^2)			
	0	100.0	42.8 ± 1.8 (231)
	40	75.5	1944 ± 189 (1008)***
	60	67.6	2534 ± 276 (1112)***
$SnCl_2$ (mM)	Time (min)		
25	0	100.0	16.6 ± 5.0 (352)
	1	92.3	24.6 ± 5.0 (472)
	20	93.7	16.7 ± 1.0 (468)
	40	85.4	22.2 ± 2.6 (488)
	60	75.9	21.9 ± 5.7 (446)
50*	0	100.0	35.7 ± 8.3 (475)
	1	89.0	24.4 ± 6.0 (392)
	20	76.3	29.3 ± 9.3 (650)
	40	73.1	29.8 ± 0.7 (642)
	60	63.2	22.9 ± 4.5 (645)
75**	0	100.0	28.9 ± 1.2 (493)
	1	98.7	29.3 ± 2.2 (518)
	20	78.7	39.0 ± 2.8 (534)
	40	80.6	39.1 ± 18.9 (609)
	60	64.1	33.4 ± 16.0 (435)

UVC data is average of three experiments; data for 25, 50 and 75 mM $SnCl_2$ treatments are from an average of two independent experiments. Numbers in parenthesis represent the absolute number of mitotic recombinants in all plates per dose.
* $p < 0.05$.
** $p < 0.01$ unpaired *t*-test.
*** $p < 0.001$ one-way ANOVA.

Sn^{2+}-sensitivity compared to that of the *RAD* WT (Fig. 2).

The importance of anti-ROS defence systems of the yeast cell on $SnCl_2$-induced toxicity could be demonstrated by the higher sensitivity of haploid strains containing the *sod1* and *sod2* mutant alleles. Lack of cytosolic superoxide dismutase (*sod1Δ*) and, to a marginal extent, of mitochondrial superoxide dismutase (*sod2Δ*) sensitised the STAT cells to Sn^{2+} (Fig. 7). The *sod2Δ* single mutant may confer slight but insignificant sensitivity, but when in conjunction with the *sod1* mutant allele it increases the sensitivity of the double mutant significantly (Fig. 7), suggesting that Sod1p can apparently handle some of the Sod2p substrate, thus masking the potential contribution of Sod2p in coping with Sn^{2+}-induced oxi stress. On the other hand, lack of catalase did not impose any Sn^{2+}-sensitivity, as the respective *ctt1Δ* mutant had WT-sensitivity (Fig. 7).

4. Discussion

$SnCl_2$ has been shown to be genotoxic when applied to single cells, be it of mammalian [13,14] or bacterial origin [17,18]. However, up to now mutation induction by $SnCl_2$ in the genetically well-established test system *S. cerevisiae* has not been described and data on putative DNA-repair of $SnCl_2$-induced DNA damage has been lacking. Our results clearly show that Sn^{2+} is both toxic and mutagenic in yeast, and that DNA lesions induced by it are repaired by different repair mechanisms.

In order to obtain realistic experimental conditions we had to overcome a Sn^{2+}-specific complication that stemmed from the high reactivity of Sn^{2+} ions with the cell's surface, causing cell aggregates of up to 50 cells (Fig. 1; inset, photo). These clumps, however, could be undone by addition of chelating agents, e.g. EDTA or PB plus vortexing, and this procedure lead to a realistic

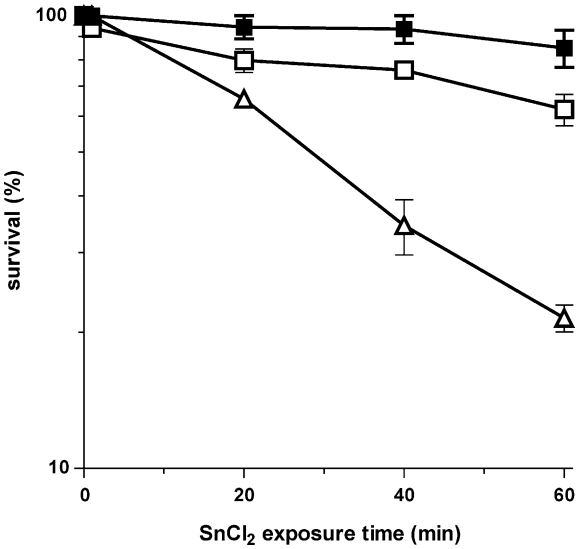

Fig. 6. Growth phase-related sensitivity to Sn^{2+} exposure of haploid strain XV185-14c (*RAD*). (■) STAT cells exposed to 25 mM; (□) LOG cells exposed to 25 μM; (Δ) LOG cells exposed to 250 μM $SnCl_2$. Cells were diluted in PB.

representation of surviving single cells (Fig. 1). Rapid binding of Sn^{2+}, apparently to poly-anionic polymers of the cell wall and cell capsule of *Streptococcus mutans*, most of it removable by washing with EDTA, has already been reported [33]. Similar processes, involving mannoproteins, might occur in Sn^{2+}-induced yeast cell clumping (Viau, unpublished data). This is suggested by the finding that post-treatment protease digestion could undo the clumps as efficiently as EDTA or PB (data not shown). Induction of cell aggregation seems to be a typical characteristic of Sn^{2+} (SnF_2 does likewise), whereas Co^{2+}, Cd^{2+}, Ni^{2+}, Fe^{2+}, Ca^{2+}, Al^{3+} and Cu^{2+} did not induce this (data not shown). These aggregation processes may have contributed to some negative results presented in literature (i.e. *B. subtilis* and *Salmonella* [34,16]) and the only report that $SnCl_2$ is not genotoxic in yeast [11] might have resulted from an experimental shortcoming, in that $SnCl_2$ was applied on agar media where several putative chelating agents in the media would neutralize most, if not all, Sn^{2+}-activity. This might also have happened in the *B. subtilis* Rec-assay [10,34] that uses filter disks on agar medium and, therefore these negative results may be questionable.

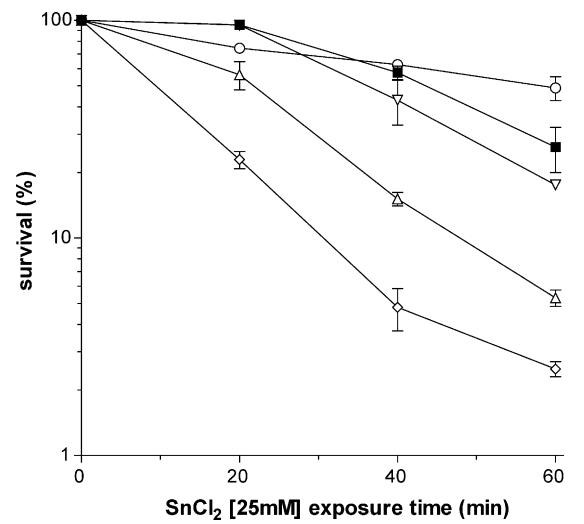

Fig. 7. Sensitivity to 25 mM $SnCl_2$ of haploid STAT cells of yeast. WT (■); *sod1Δ* (Δ); *sod2Δ* (▽); *sod1Δ sod2Δ* (◊); *ctt1Δ* (◯). Cells were diluted in PB.

After correcting for clumping we found that $SnCl_2$ has significant genotoxic potential as all isogenic DNA repair mutants of *S. cerevisiae* were more sensitive than the WT (Fig. 2). The recombination-deficient *rad52Δ* mutant, unable to repair DNA single- and double-strand breaks [35], was the most sensitive while *rad6Δ*, representing a blocked error-prone repair [36], displayed intermediate Sn^{2+}-sensitivity; mutants *rad2Δ* and *rad4Δ*, both deficient in NER [37], showed only a weak (i.e. less than two-fold) and nearly identical sensitivity response as compared to the isogenic WT (Fig. 1; inset and Fig. 2). The *rad6* mutant showed some variability in its $SnCl_2$-sensitivity, perhaps owing to its slow-growth characteristics and tendency to accumulate suppressors [38], and responded to EDTA-treatment with significantly improved survival (Fig. 1) (at the same declumping efficiency as PB). We also want to point out that different WT strains, i.e. EUROSCARF versus XY185-14C differed significantly in $SnCl_2$-sensitivity (Figs. 1 and 3). Of seven WT strains tested by us, six fell into two sensitivity groups: three strains, amongst them the EUROSCARF WT, showing around 80% survival (25 mM $SnCl_2$, 60 min), while three others, amongst them XV185-14c, had around 35% survival at the same treatment. One, W303, had intermediate sensitivity with a survival at about 55% (data not shown).

The sensitivity ranking of the five isogenic EUROSCARF strains indicates a major repair contribu-

tion of recombinational processes (RAD52 pathway) whereas $SnCl_2$-induced DNA lesions seem to be a poor substrate for NER. Error-prone repair is clearly involved in lesion removal (Fig. 2) and the observed mutation induction (Figs. 3–5). Gene conversion requires DNA synthesis and during repair of damaged DNA this apparently includes error-prone repair enzymes [39]. Thus, processes associated with translesion synthesis, including the activity of polzeta might be involved, and a homoallelic diploid *rev3* mutant might be a good candidate for testing the putative involvement of polzeta in Sn^{2+}-induced mitotioc gene conversion.

The yeast *rad52Δ* mutant's specific sensitivity to strand breaks-generating mutagens suggests that $SnCl_2$ directly or indirectly leads to this type of DNA lesion in yeast as it does in vitro in plasmid DNA [20,21]. Tin chloride-induced DNA oxidation is suggested to cause mutagenicity in bacteria [18,19]; this is confirmed by the high sensitivity of bacterial tester-strains lacking anti-ROS defences (*Salmonella* TA102 and Mutoxitest strain WP203; Table 2). Sn^{2+}-induced toxicity can be prevented by ROS scavengers (i.e. thiourea, sodium benzoate and dipyridyl [40]) as well as by chelating agents (i.e. glucoheptonic acid [41] and EDTA [21]). Our finding that $SnCl_2$ was mutagenic for strain IC203 and non-mutagenic for IC188 may again indicate oxidative mutagenesis, since both strains have the same genetic background and only differ in that the transcription regulator of many genes involved in the response to oxidants, OxyR is missing in strain IC203 [42]. The fact that H_2O_2 is able to induce a cross-adaptive response against $SnCl_2$-promoted lethality [43] suggests that OxyR participates, via regulating catalase, alkyl hydroperoxide reductase and superoxide dismutase, in the defense against Sn^{2+}-generated ROS. Finally, the about extremely high resistance to Sn^{2+} of STAT cells as compared to LOG cells of the same strain (Fig. 6) may also be due, at least in part, to the maximal expression of anti-ROS defence systems in the former [44].

From Figs. 3 and 4 we can deduce that the *his1-798* missense mutant allele can be reverted by Sn^{2+} much more readily than the *his1-208* chain termination mutant allele of the homozygous diploid, which is also true for spontaneously arising His^+ revertants in haploids containing these mutant alleles [45]. Mutation induction by Sn^{2+} in these two His reversion systems clearly depends on exposure dose, be it variable exposure time at constant dose (Fig. 3) or variable concentration at constant time (Fig. 4). Treatment with $SnCl_2$ also induced reversion of a putative frameshift allele (*hom3-10*) and locus-specific reversion of the ochre allele *lys1-1* (Fig. 5) while induction of ochre suppressor mutations was negligible (data not shown). The moderate mutagenicity of Sn^{2+} was achieved at low cytotoxicity so that selection of spontaneous mutants can be ruled out.

The significant mutagenicity of Sn^{2+} in yeast certainly justifies further research as to the types of DNA lesions induced by it and the cellular repair responses elicited. The strong induction of Sn^{2+}-resistance during diauxic shift and growth to STAT phase, that leads to a better survival at a 1000-fold higher Sn^{2+} exposure dose (Fig. 6), suggests that cellular defences against ROS, i.e. expression and activity of enzymes not involved in DNA repair, play a much more prominent role in Sn^{2+}-resistance than does the DNA repair machinery. This high resistance to oxi-stress is a typical hallmark of resting yeast cells [46]. However, the ratio of metal uptake as well as the metal binding to the outer cell wall (mannoproteins) may vary between LOG and STAT cells and thus might greatly influence sensitivity as well. This question must be addressed and may be answered by molecular dosimetry; with this approach we might be able to determine not only the amount of totally cell-bound Sn^{2+} but also the fraction of the metal ions that passes the cytoplasmic membrane, i.e. that is actually taken up by the cells (LOG versus STAT).

In summary, our results clearly show that Sn^{2+} ions are genotoxic in our pro- and eukaryotic microbial test systems. Tin chloride can thus be defined as a moderate mutagen of low toxicity. Most probably, DNA damage is caused by ROS and is preferentially repaired via recombinational and error-prone processes in haploid and diploid yeast.

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ANEXO II

Genotoxicity of aminohydroxynaphthoquinones in bacteria, yeast, and Chinese hamster lung fibroblast cells.

da Costa Medina LF, Viau CM, Moura DJ, Saffi J, Stefani V, Brandelli A, Henriques JA.

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Genotoxicity of aminohydroxynaphthoquinones in bacteria, yeast, and Chinese hamster lung fibroblast cells

Luis Fernando da Costa Medina [a,1], Cassiana Macagnan Viau [a,1], Dinara Jaqueline Moura [a], Jenifer Saffi [a,b], Valter Stefani [c], Adriano Brandelli [d], João Antonio Pêgas Henriques [a,b,*]

[a] *Laboratório de Genotoxicidade-Instituto Royal-Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil*
[b] *Laboratório de Genética Toxicológica, Universidade Luterana do Brasil, Canoas, RS, Brazil*
[c] *Laboratório de Novos Materiais Orgânicos, Departamento de Química Orgânica, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil*
[d] *Laboratório de Bioquímica e Microbiologia Aplicada, Departamento de Ciência de Alimentos, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil*

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Abstract

There are few studies on the biological activity of aminohydroxy derivates of 1,4-naphthoquinone (1,4-NQ) on prokaryotic and eukaryotic cells. We determined the mutagenic activity of 5-amino-8-hydroxy-1,4-naphthoquinone (ANQ) and 5-amino-2,8-dihydroxy-1,4-naphthoquinone (ANQ-OH) as compared to the unsubstituted 1,4-NQ in *Salmonella*/microsome assay. Potential mutagenic and recombinogenic effects and cytotoxicity were analyzed in haploid and diploid cultures of the yeast *Saccharomyces cerevisiae*. In *Salmonella*/microsome assay, 1,4-NQ was not mutagenic, whereas aminohydroxynaphthoquinones were weakly mutagenic in TA98 and TA102 strains. In haploid yeast in stationary growth phase (STAT), mutagenic response was only observed for the *hom3* locus at the highest dose. In diploid yeast, aminohydroxynaphthoquinones did not induce any recombinogenic events, but 1,4-NQ was shown to be a recombinogenic agent. These results suggest that aminohydroxynaphthoquinones are weak mutagenic agents only in prokaryotic cells. The cytotoxicity of 1,4-NQ in yeast stationary cells was more significant in diploid cells as compared to that observed in haploid cells. However, ANQ and ANQOH were slightly cytotoxic in all treatments. Genotoxicity of these naphthoquinone compounds was also determined in V79 Chinese hamster lung fibroblast cells using standard Comet, as well as modified Comet assay with the bacterial enzymes formamidopyrimidine DNA-glycosylase (FPG) and endonuclease III (ENDOIII). Both 1,4-NQ and ANQ induced pronounced DNA damage in the standard Comet assay. The genotoxic effect of ANQ-OH was observed only at the highest dose. In presence of metabolic activation all substances showed genotoxic effects on V79 cells. Post-treatment of V79 cells with ENDOIII and FPG proteins did not have a significant effect on ANQ-OH-induced oxidative DNA damage as compared to standard alkaline Comet assay. However, all naphthoquinones were genotoxic in V79 cells in the presence of metabolic activation and post-treatment with enzymes, indicating that all compounds induced oxidative DNA damage in V79 cells. Our data suggest that aminohydroxynaphthoquinone pro-oxidant activity, together with their capability of DNA intercalation, have an important role in mutagenic and genotoxic activities.

Keywords: *Salmonella* microsome/Ames; *Saccharomyces cerevisiae*; Mutagenesis; Comet assay; Quinones

1. Introduction

Naphthoquinones (NQs) and their derivates play an important role in several biological processes, such as photosynthesis, respiratory chain-dependent energy production, allelopathy, and defense [1–3]. During the last decades, a large number of natural and synthetic NQs and its derivates have been extensively studied as to their antitumoral [4], antiparasitic [5], and antimicrobial [6–9] activities.

The classic mechanism of NQs toxicity is to produce reactive oxygen species (ROS). NQs can be reduced to semiquinones by NADPH–cytochrome P-450 reductase or to hydroquinones by DT-diaphorase NAD(P)H: quinoneoxireductase (EC 1.6.99.2).

* Corresponding author at: Centro de Biotecnologia – UFRGS, Avenue Bento Gonçalves 9500, 91501-970 Porto Alegre, Brazil. Fax: +55 51 3308 6069.
E-mail address: pegas@cbiot.ufrgs.br (J.A.P. Henriques).
[1] These authors contributed equally to this work.

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In the presence of oxygen, reduced NQs auto-oxidize, producing superoxide anion radical. This redox cycling of quinones promotes oxidative stress conditions, resulting in destabilization of lysosomes, release of cathepsin D, and mitochondrial membrane potential decrease [10,11]. Furthermore, the capacity to produce free radicals is influenced by the nature and the position of substituents in the quinone molecule [12].

Cell membranes are often permeable to NQs, which, once present in the cell, may form adducts with DNA, leading to abasic sites and single or double strand breaks [12–14]. NQs also react with proteins and lipids, destroying their functionality, thereby causing cell death [15].

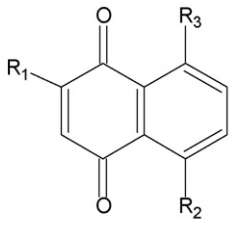
Tikkanen et al. [16] investigated the influence of substituents, such as hydroxy, methyl or both moieties in different positions of NQs molecules. Upon metabolic activation, naphthazarin (5,8-dihydroxy-1,4-naphthoquinone) (NTZ) and plumbagin (2-methyl-5-hydroxy-1,4-naphthoquinone) induced frameshift mutations in *Salmonella typhimurium* strain TA98. Hakura et al. [17] demonstrated that 4-amino-1,2-naphthoquinone was mutagenic in TA97, TA100, and TA102 strains. However, few studies were conducted to determine the mutagenic activity of amino-hydroxy substituents in this class of compounds.

Considering the use of some quinones as antineoplastic agents [4] and their ability to form adducts with DNA [12,13], we conducted a study on the toxic and genotoxic potential of two aminohydroxynaphthoquinones – 5-amino-8-hydroxy-1,4-naphthoquinone (ANQ) and 5-amino-2,8-dihydroxy-1,4-naphthoquinone (ANQ-OH) – by employing the three test organisms: *Salmonella*/microsome assay, *Saccharomyces cerevisiae*, and permanent lung fibroblast cell line derived from Chinese hamsters (V79 cells). The genotoxicity of these compounds was determined using standard Comet assay (single-cell gel—SCG). Standard Comet assay sensitivity and specificity can be improved by incubating lysed cells with lesion-specific endonucleases, which recognize particular damage bases and create breaks [18]. The most commonly used endonucleases in the modified Comet assay are formamidopyrimidine DNA-glycosylase (FPG, also known as MutM), and endonuclease III (ENDOIII, also known as NTH). FPG is specific for oxidized purines, specially for 8-oxo-7,8-dihydroguanine (8-oxoGua), and ENDOIII recognizes oxidized pyrimidines, including thymine glycol and uracil glycol [18,19]. Using this technique, we were able to determine the specific oxidative lesion induced by these naphthoquinones in mammalian culture cells, and thereby obtain insight as to their toxicity mechanisms.

2. Materials and methods

2.1. Chemicals

The compounds 5-amino-8-hydroxy-1,4-naphthoquinone, CAS [68217-36-7] (ANQ), and 5-amino-2,8-dihydroxy-1,4-naphthoquinone, CAS [500733-87-9] (ANQ-OH) (Fig. 1) were synthesized as described. A mixture of sulfur (7.5 g) and fuming sulfuric acid (60% SO_3, 85 mL) is added dropwise, with stirring to an ice-cooled slurry of 1,5-dinitropnaphthalene (20.0 g) in concentrated sulfuric acid (45 mL), as well as crushed ice. The solution is continuously stirred for 1 h, and then it is warmed at 50 °C for 10 min. The mixture is allowed to stand at room temperature for 18 h for cooling, and finally poured on crushed ice. The solution is filtered and extracted with chloroform in a liquid/liquid extractor. Crude quinine is purified by short-column chromatography in silica gel (chloroform) [20]. 1,4-Naphthoquinone (1,4-NQ), CAS [130-15-4], was purchased from ACROS (Geel, Belgium). D-Biotin, aflatoxin B1, amino acids (L-histidine, L-threonine, L-methionine, L-tryptophan, L-leucine and L-lysine), nitrogen bases (adenine and uracil), cycloheximide, methyl methane sulfonate (MMS), hydrogen peroxide (H_2O_2), 4-nitroquinoline-oxide (4-NQO), cyclophosphamide (CP), and sodium azide were purchased from Sigma (St. Louis, MO, USA). FPG and ENDOIII were obtained from New England BioLabs (USA). Nutrient broth no. 2 was obtained from Oxoid (MD, USA), and Yeast extract, Bacto-peptone and Bacto-agar were obtained from Difco Laboratories (Detroit, USA). Dulbecco's modified Eagle Medium (DMEM), fetal bovine serum (FBS), trypsin–EDTA, L-glutamine, antibiotics, and trypan blue (TB) were purchased from Gibco BRL (Grand Island, NY, USA). The S9 fraction, prepared from livers of Sprague–Dawley rats, pre-treated with the polychlorinated biphenyl mixture Araclor 1254, was purchased from Moltox (Annapolis, MD, USA). For all treatments, 5 mg/mL dimethylsulfoxide (DMSO, Merck, Darmstadt) stock solutions of NQ were prepared immediately prior to use. All others reagents were of an analytical grade.



	1,4-NQ	ANQ	ANQ-OH
R_1	H	H	OH
R_2	H	NH_2	NH_2
R_3	H	OH	OH

Fig. 1. Chemical structures of 1,4-naphthoquinones tested. (1,4-NQ) 1,4-naphthoquinone, (ANQ) 5-amino-8-hydroxy-1,4-naphthoquinone and (ANQ-OH) 2,8-dihydroxy-5-amino-1,4-naphthoquinone.

2.2. Strains

S. typhimurium TA97, TA98, TA100, and TA102, described in [21], were kindly provided by Ames and Maron (University of California, Berkeley, CA, USA). *S. cerevisiae* strain XV185-14c (MATα *ade2-2 arg4-17 his1-7 lys1-1 trp5-48 hom3-10*) [22] was used in mutagenicity assay. Induced mitotic recombination was measured in diploid yeast strain XS2316 (MATa/α *his1-1/his1-1 leu1-1/leu1-12 +/cyh2 trp5-48 +/met13*) [23]. Chinese hamster lung fibroblasts (V79 cells) were cultivated under standard conditions in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum and 2 mM L-glutamine [24].

2.3. Yeast culture media

Media, solutions, and buffers were prepared as previously described [25]. Complete YPD medium, containing 0.5% yeast extract, 2% bacto-peptone, and 2% glucose was used for routine growth of yeast cells. For plates, the medium was solidified with 2% bacto-agar. Minimal medium (MM), containing 0.67% yeast nitrogen base without amino acids, 2% glucose, and 2% bacto-agar, was supplemented with the appropriate amino acids. Synthetic complete medium (SC) consisted of MM supplemented with 2 mg adenine, 2 mg arginine, 5 mg lysine, 1 mg histidine, 2 mg leucine, 2 mg methionine, 2 mg uracil, 2 mg tryptophan, and 24 mg threonine per 100 mL MM. For mutagenesis of the strain

XV-185-14c, omission media, lacking lysine (SC-lys), histidine (SC-his), or homoserine (SC-hom), were used. For recombinogenesis, leucine was omitted from the synthetic complete medium (SC-leu) or supplemented with 0.2% (w/v) cycloheximide (SC + Cyh). Cells were harvested and washed with phosphate buffer saline solution pH 7.4. Cell titer and percentage of budding cells were determined by microscopic counting/observation, using a Neubauer chamber.

2.4. Growth and survival assay in S. cerevisiae

Stationary phase (STAT) cultures were obtained by inoculation of liquid YPD bearing a single colony. After 48 h incubation at 30 °C with aeration by shaking, cultures contained 1–2 × 10^8 cells/mL. Cells were harvested by centrifugation, and washed with PBS. Cytotoxicity of the compounds was assayed by suspending 2 × 10^8 STAT cells/mL in PBS containing different compound concentrations, and incubation with aeration by rotary shaking at 30 °C for 4 h. Cell number was determined by microscopic count and colony formation on solid SC. Plates were incubated at 30 °C for 3–5 days before counting.

2.5. Salmonella/microsome mutagenic assay

Mutagenicity was assayed by the pre-incubation procedure [26]. S9 metabolic activation mixture (S9 mix) was prepared according to Maron and Ames [21]. The appropriate concentrations of compounds were obtained by dilution in DMSO stock solution. One hundred microliters of test bacterial cultures (1–2 × 10^9 cells/mL) were incubated in the dark at 37 °C with different amounts of naphthoquinones (0.01–20 μg per plate) in the presence or absence of the S9 mix for 20 min, without shaking. Then, 2 mL of soft agar (0.6% agar, 0.5% NaCl, 50 μM histidine, 50 μM biotin, pH 7.4, 42 °C) were added, and the contents of the test tube were immediately poured onto a plate of minimal agar (1.5% agar, Vogel-Bonner E medium, containing 2% glucose). Aflatoxin B1 (0.5 μg/plate) was used as positive control for all strains in the metabolic assay with S9 mix. In the absence of the S9 mix, the positive control was 4-NQO (0.5 μg/plate), except in TA 100, where sodium azide (5 μg/plate) was used as positive control. Plates were incubated in the dark at 37 °C for 48 h before revertant colony counting.

2.6. Detection of aminohydroxynaphthoquinone induced reverse mutation in S. cerevisiae

Mutagenesis was measured in haploid cells of STAT strain XV185-14c, as described in Section 2.4. A suspension of 2 × 10^8 STAT cells/mL was incubated at 30 °C for 4 h with different concentrations of naphthoquinones. Cell survival was determined in SC (3–5 days, 30 °C), and mutation induction (*LYS*, *HIS* or *HOM* revertants) in appropriate omission media (5–7 days, 30 °C). Whereas *his 1–7* is a non-suppressible missense allele and reversions result from mutations at the locus itself [27], *lys1-1* is a suppressible ochre nonsense mutant allele [28], which can be reverted either by locus-specific or by forward mutation in a suppressor gene [22,29]. True reversions and forward (suppressor) mutations at *lys1-1* locus were differentiated according to Schuller and von Borstel [30]. It is believed that *hom3–10* contains a frameshift mutation due to its response to a range of diagnostic mutagens [22]. Assays were repeated at last three times, and plating was performed in triplicate for each dose.

2.7. Detection of induced mitotic recombination in S. cerevisiae

Suspension of STAT diploid XS2316 (2 × 10^7 cells/mL) cells was incubated at 30 °C for 4 h in PBS, which contained different NQ concentrations. Treated cells were diluted in PBS, plated on three different media (SC, SC-leu and SC + cyh), and incubated at 30 °C for 5–7 days. Colonies grown on SC medium indicated cell survival and colonies grown on SC-leu and SC + cyh were scored for intragenic mitotic recombination (mitotic gene conversion) and intergenic recombination (crossing-over), respectively. The exact frequency of reciprocal crossing-over was determined by subtracting possible revertants to cycloheximide-resistance, resulting from reversion at the *CYH2* locus, as well as from monosomy of chromosome VII. Therefore, cycloheximide-resistant colonies were replica-plated on a series of plates with SC-lys, SC-met, and SC-ade media to confirm the recombination origin of *cyh2* homozygocity. Irradiation at UV 254 nm (30 J/m^2 and 60 J/m^2) was used as positive control. Assays were repeated three times, and plates were performed in triplicate for each dose.

2.8. Treatment of V79 cells and trypan blue exclusion

V79 cells were maintained in tissue-culture flasks at 37 °C in a humidified atmosphere containing 5% CO_2, and were harvested by treatment with 0.15% trypsin and 0.08% of EDTA PBS. Cells (2 × 10^5 cells) were seeded into each flask and grown/incubated 1 day prior to treatment. The assays performed in the presence of metabolic activation used 3% final S9-mix concentration.

The concentrations used for each sample were based on trypan blue exclusion, which was determined as described by Robichova and Slamenova [31]. Treated and washed, as well as control V79 cells were trypsinized, stained with trypan blue (0.4%), and the number of viable (uncolored) and dead (colored) cells was counted. The ratio of number of viable cells/all cells results in the percentage of viable cells. Dose was considered cytotoxic when cell survival was <70%.

2.9. Comet assay

Alkaline Comet assay was performed as described by Singh et al. [32], with minor modifications [33]. At the end of the treatment, cells were washed with ice-cold PBS, and trypsinized with 100 μL trypsin (0.15%). After 30 s, 100 μL complete medium was added and the cells were gently re-suspended. Immediately thereafter, cells (10^6 cells/mL) were suspended in 0.75% low melting agarose (LMA), and spread on agarose-precoated microscope slides. Cells were lysed (2.5 M NaCl, 100 mM EDTA and 10 mM Tris, pH 10.0, with freshly added 1% Triton X-100 and 10% DMSO) at 4 °C for 24 h. In the modified Comet assay, slides were removed from the lysing solution, and washed three times in enzyme buffer (40 mM hepes, 100 mM KCl, 0.5 mM Na_2EDTA, 0.2 mg/mL BSA, pH 8.0), drained, and incubated with 70 μL FPG (30 min 37 °C) or ENDOIII (45 min 37 °C). Slides were placed in a horizontal electrophoresis chamber, containing freshly made alkaline buffer (300 mM NaOH and 1 mM EDTA, pH 13.0) at 4 °C for 20 min. An electric current of 300 mA and 25 V (0.90 V/cm) was applied for 20 min to allow DNA migration. Slides were neutralized (0.4 M Tris, pH 7.5), and stained with silver [34]. Images of 100 randomly selected cells (50 cells from each of two replicate slides) were analyzed per group. Cells were also visually scored into five classes, according to tail size: (1) class 0: undamaged, without a tail; (2) class 1: tail shorter than the diameter of the head (nucleus); (3) class 2: tail length 1 − 2*x* the diameter of the head; (4) class 3: tail longer than 2*x* the diameter of the head; (5) class 4: comets with no heads. Damage frequency (%) was calculated based on number of cells with tails as a percentage of total number of scored cells.

International guidelines and recommendations for the Comet assay consider that visual scoring of comets is a well-validated evaluation method as it is highly correlated with computer-based image analysis [35,36]. Damage index is based on migration length and on the amount of DNA in the tail, and it is considered a sensitive DNA measure. Therefore, a damage index (DI) was assigned to each comet according to its class, and ranged from 0 (completely undamaged: 100 cells × 0) to 400 (with maximum damage: 100 cells × 4) [37]. Results are expressed as mean and standard deviation of three independent experiments. DMSO was used as negative control, whereas MMS and CP were used as positive controls, without or with metabolic activation, respectively.

2.10. Statistical analysis

Mutagenicity data were analyzed using Salmonel software [38]. A compound was considered positive for mutagenicity only when: (a) the number of revertants was at least double the spontaneous yield (MI ≥ 2; mutagenic index (MI): number of induced colonies in the sample/number of spontaneous in negative control), (b) analysis of variance yielded significant response ($P \leq 0.05$) and (c) a reproducible positive dose response ($P \leq 0.01$) was present,

Table 1
Induction of his$^+$ revertants in *Salmonella typhimurium* TA100 and TA97 by naphthoquinones, with and without metabolic activation

Substance	Dose (μg/plate)	TA100		TA100 + S9		TA97		TA97 + S9	
		Rev/plate[a]	MI[b]	Rev/plate	MI	Rev/plate	MI	Rev/plate	MI
NC[c]		208 ± 6		175 ± 10		287 ± 31		203 ± 68	
PC[d]	0.5	592 ± 62**	3.38	362 ± 21**	2.12	751 ± 33**	2.62	509 ± 24**	2.51
1,4-NQ	0.1	240 ± 28	1.15	199 ± 11	1.06	362 ± 27	1.26	301 ± 51	1.48
	0.5	232 ± 16	1.12	193 ± 10	1.11	360 ± 30	1.27	358 ± 59	1.76
	1	124 ± 19	0.58	187 ± 11	1.07	361 ± 10	1.26	340 ± 41	1.68
	2	97 ± 41	0.47	204 ± 8	1.17	267 ± 16	0.93	345 ± 27	1.70
ANQ	1	216 ± 2	1.04	185 ± 12	1.06	324 ± 10	1.13	315 ± 63	1.55
	5	154 ± 44	0.74	175 ± 9	1.00	388 ± 12	1.35	320 ± 43	1.58
	10	155 ± 34	0.75	213 ± 23	1.22	347 ± 26	1.21	314 ± 53	1.54
	20	61 ± 28	0.30	271 ± 29	1.55	324 ± 30	1.09	366 ± 104	1.80
ANQOH	1	263 ± 2	1.27	161 ± 14	0.92	361 ± 33	1.26	280 ± 43	1.38
	5	328 ± 44	1.58	218 ± 25	1.25	375 ± 24	1.31	284 ± 51	1.40
	10	323 ± 35	1.55	222 ± 22	1.27	335 ± 55	1.17	275 ± 53	1.35
	20	367 ± 28	1.77	330 ± 27	1.89	338 ± 63	1.18	284 ± 38	1.40

Data significant in relation to negative control (solvent) at $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.
[a] Number of revertants/plate: mean of three independent experiments ± S.D.
[b] MI mutagenic index: number of *his*$^+$ induced in the sample/number of spontaneous *his*$^+$ in the negative control.
[c] Negative control: dimethylsulfoxide.
[d] Positive control (4NQO—S9mix to TA98 and TA102 and aflatoxin B1 + S9).

as evaluated by *Salmonel* software. Cytotoxic effect was considered when MI ≤ 0.6.

Descriptive statistical analysis for mutagenesis assays in *S. cerevisiae* and Comet assay was expressed as means and standard deviation from three independent experiment data submitted to one-way ANOVA and Tukey's multiple comparison tests, using GraphPad Prism version 4.00, GraphPad Software (San Diego, USA).

3. Results

3.1. Salmonella/microsome assay

When the NQs dose range was evaluated with the TA 100 strain, with and without metabolization, cytotoxicity was observed in concentrations higher than 3 μg/mL for 1,4-NQ and 30 μg/mL for ANQ and ANQOH (data not shown). No mutagenicity of NQ compounds was observed in TA97a

Table 2
Induction of his$^+$ revertants in *S. typhimurium* TA102 and TA98 by naphthoquinones, with and without metabolic activation

Substance	Dose (μg/plate)	TA102		TA102 + S9		TA98		TA98 + S9	
		Rev/plate[a]	MI[b]	Rev/plate	MI	Rev/plate	MI	Rev/plate	MI
NC[c]		310 ± 31		380 ± 5		32 ± 1		46 ± 4	
PC[d]	0.5	2910 ± 300***	9.38.38	1173 ± 15***	3.08	301 ± 35***	9.40	562 ± 31***	12.2
1,4-NQ	0.1	400 ± 3	1.29	719 ± 70	1.89	27 ± 2	0.85	49 ± 6	1.06
	0.5	421 ± 5	1.35	681 ± 21	1.79	29 ± 3	0.90	42 ± 1	0.91
	1	462 ± 10	1.49	516 ± 42	1.36	32 ± 8	1.00	44 ± 5	0.95
	2	429 ± 5	1.38	509 ± 53	1.33	25 ± 6	0.78	49 ± 7	1.07
ANQ	1	287 ± 30	0.93	838 ± 72*	2.21	42 ± 1	1.31	47 ± 2	1.02
	5	255 ± 20	0.82	781 ± 54*	2.06	60 ± 7	1.88	53 ± 4	1.15
	10	290 ± 4	0.94	1106 ± 64**	2.91	59 ± 4	1.84	93 ± 14*	2.02
	20	268 ± 15	0.86	1155 ± 140**	3.04	119 ± 20**	3.71	144 ± 6**	3.11
ANQOH	1	309 ± 27	0.99	891 ± 128**	2.35	34 ± 3	1.06	47 ± 9	1.02
	5	302 ± 26	0.97	790 ± 151**	2.08	48 ± 5	1.50	39 ± 3	0.84
	10	281 ± 21	0.91	844 ± 88**	2.22	76 ± 3**	2.37	48 ± 8	1.04
	20	308 ± 39	0.99	886 ± 122**	2.33	159 ± 4**	4.96	60 ± 7	1.30

Data significant in relation to negative control (solvent) at $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.
[a] Number of revertants/plate: mean of three independent experiments ± S.D.
[b] MI mutagenic index: number of *his*$^+$ induced in the sample/number of spontaneous *his*$^+$ in the negative control.
[c] Negative control: dimethylsulfoxide.
[d] Positive control (4NQO—S9mix to TA98 and TA102 and aflatoxin B1 + S9).

Table 3
Naphthoquinone-induced reversion of point mutation for *his1–7*, frameshift mutation (*hom3–10*) and ochre allele (*lys1–1*) in haploid strain XV185-14c of *Saccharomyces cerevisiae* in stationary phase (STAT)

Agent	Treatment (μg/mL)	Survival (%)	His1/10^7 survivors[a]	Hom3/10^7 survivors[a]	Lys1/10^7 survivors[b]
STAT					
DMSO[b]	0	100	7.73 ± 0.87	1.19 ± 0.06	4.36 ± 0.62
4NQO[c]	1	86.8	275.5 ± 1.36***	4.91 ± 2.60**	51.42 ± 2.61**
1,4-NQ	1	85.7	7.51 ± 1.76	1.05 ± 0.67	5.11 ± 1.05
	10	80.2	6.23 ± 2.74	3.05 ± 0.95	4.82 ± 0.86
	15	86.5	5.85 ± 2.51	1.44 ± 1.01	3.45 ± 0.45
	30	4.5	8.86 ± 2.04	8.86 ± 2.00**	7.74 ± 0.84
ANQ	1	103.0	4.14 ± 0.92	1.63 ± 0.55	5.03 ± 2.02
	10	92.7	6.82 ± 2.29	1.45 ± 0.27	4.23 ± 1.55
	30	97.9	7.03 ± 1.53	2.01 ± 1.17	5.12 ± 0.48
	60	97.0	8.31 ± 1.93	1.70 ± 0.79	4.12 ± 0.41
ANQ-OH	1	96.4	5.01 ± 0.85	1.45 ± 0.29	5.18 ± 0.61
	10	101.0	4.38 ± 0.83	1.48 ± 0.65	6.30 ± 0.41
	30	103.1	4.38 ± 1.35	0.71 ± 0.02	3.66 ± 1.16
	60	99.1	5.66 ± 2.21	1.16 ± 2.60	5.85 ± 0.54

Data significant in relation to negative control group (solvent) at $*P<0.05$; $**P<0.01$; $***P<0.001$/one-way ANOVA with Tukey's post-test.
[a] Locus-specific revertants.
[b] Locus non-specific revertants (suppression by forward mutation).
[c] Mean and S.D. per three independent experiments.

(detects frameshift mutations in C–C–C–C–C–C; +1 cytosine), or in TA100 (base-pair substitution mutation results from the substitution of a leucine [GAG] by a proline [GGG]), in absence or presence of metabolic activation. However, at their highest concentration, 1,4-NQ (1–2 μg/plate) and ANQ (20 μg/plate) were toxic to TA100, without metabolic activation (Table 1). Table 2 shows the absence of mutagenicity effects of all NQS compounds on TA102, which detects oxidative and alkylating mutagens [21], in the absence of metabolic activation. ANQ and ANQ-OH were able to induce mutagenicity in TA102, in the presence of metabolic activation. However, ANQ induced mutagenic response in TA98 (detects frameshift in DNA target –C–G–C–G–C–G–C–G), in the presence and absence of metabolic activation, whereas for ANQ-OH this effect was only detected in absence of S9-mix.

3.2. Cytotoxic and mutagenic effects in S. cerevisiae

The results of the cytotoxicity and mutagenicity tests in the yeast *S. cerevisiae* are shown in Tables 3 and 4. Cytotoxicity of naphthoquinones in yeast STAT cells was more significant in diploid cells as compared to that observed in haploid cells. This cytotoxic effect was critical in diploid cells, when 1,4-NQ concentration was higher than 2 μg/mL. However, ANQ and ANQ-OH showed mild cytotoxicity in all treatments (Tables 3 and 4). None of the naphthoquinones was mutagenic in strain XV185-14c during treatment in PBS (STAT)

Table 4
Induction of crossing-over (+/*cyh2*) and gene conversion (*leu-1/leu1–12*) by naphthoquinones in diploid yeast XS2316 in stationary phase

Substance	Treatment	Survival (%)	Crossing-over/10^5 survivors[a]	Gene conversion/10^5 survivors[a]
NC		100	56 ± 1.00	22 ± 1.07[b]
UVC (J/m^2)[c]	30 J	77.9	708 ± 44.0**	691 ± 85.3**
UVC (J/m^2)[c]	60 J	56.3	1150 ± 32.0***	1210 ± 87.1***
1,4-NQ	0.5 μg/mL	79.1	20.2 ± 13.0	20.8 ± 8.3
	1 μg/mL	71.0	42.0 ± 3.43	17.1 ± 1.53
	2 μg/mL	2.03	1180 ± 21***	50.0 ± 10.0
ANQ	1 μg/mL	79.7	30.2 ± 12.5	33.1 ± 1.59
	5 μg/mL	85.1	35.9 ± 10.7	14.4 ± 7.00
	10 μg/mL	81.2	42.8 ± 18.2	10.0 ± 1.00
	30 μg/mL	81.0	71.5 ± 18.4	24.4 ± 12.0
ANQ-OH	1 μg/mL	84.3	48.4 ± 0.45	26.8 ± 6.10
	10 μg/mL	72.0	33.6 ± 0.19	27.6 ± 5.40
	30 μg/mL	68.0	47.9 ± 1.29	23.9 ± 2.60

Data significant in relation to negative control group (solvent) at $*P<0.05$; $**P<0.01$; $***P<0.001$/one-way ANOVA with Tukey's post-test.
[a] Mean and S.D. per three independent experiments.
[b] Negative control.
[c] Positive control.

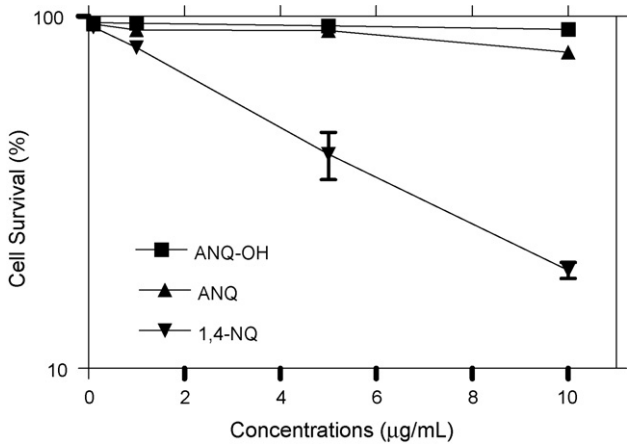


Fig. 2. Cell survival of V79 after exposure to naphthoquinones for 3 h in the absence of metabolization.

(Table 3), except for locus *hom3*$^+$ at the highest NQ concentration ($P < 0.01$).

3.3. Recombinogenic effects in S. cerevisiae

The recombinogenic effect of naphthoquinones was investigated in diploid STAT cells. Table 4 shows that 1,4-NQ induced crossing-over at highest dose ($P < 0.001$); however, 1,4-NQ cytotoxicity was high (2% survival). Aminohydroxynaphthoquinone did not induce any recombinogenic events in STAT yeast cells.

3.4. Trypan blue exclusion (TB)

Cytotoxicity of naphthoquinones for the 3-h treatment without the S9 mix in V79 cells is shown in Fig. 2. Significant cytotoxicity was induced in cells treated with 1,4-NQ at concentration of 5 μg/mL or higher. ANQ and ANQ-OH did not show any cytotoxic effect in any concentration.

3.5. NQ induced DNA damage in V79 cells

Table 5 shows DNA damage induced by compounds tested in V79 cells, without metabolic activation. Both 1,4-NQ and ANQ caused pronounced DNA damage in the standard Comet assay. A concentration-dependent increase in damage index and damage frequency was observed for both compounds. As compared to the negative control, concentrations >0.1 μg/mL of 1,4-NQ, and 5 μg/mL of ANQ induced significant DNA damage. V79 cells treated with 0.1–5 μg/mL ANQ-OH did not show any significant DNA damage. Increased DNA damage was observed only at the highest dose (Table 5). In presence of metabolic activation, all substances caused genotoxic effects in V79 cells (Table 6). The extent of DNA damage in cells exposed to these compounds was concentration dependent.

3.6. DNA repair enzymes recognize oxidative DNA damage

Fig. 3A–C shows mean DNA damage caused by NQs, expressed as DNA damage index after treatment with DNA-repair enzymes ENDOIII and FPG, without metabolic activation. It can be seen in Fig. 3 that the post-incubation results with the enzymes clearly shows increased DNA migration of the positive control H_2O_2. It shows that the enzyme activity was appropriated. The results indicate that the extent of oxidative DNA damage caused by NQs, as recognized by ENDOIII and FPG in V79 cells, was significantly higher (Fig. 3A). Moreover, a high level of DNA damage was recognized by FPG was observed at intermediate ANQ doses (Fig. 3B). However, both

Table 5
Effect of naphthoquinones in V79 cells exposed for 3 h and evaluated by standard Comet assay without metabolic activation

Substance	Treatment	Damage index[a]	Damage frequency (%)[a]
NC[b]		66.67 ± 3.51[a]	51.33 ± 7.37[a]
MMS[c]	4.0×10^{-5} M	318.33 ± 92.1***	100.00 ± 0.0***
1,4-NQ	0.01 μg/mL	63.66 ± 26.57	48.33 ± 14.01
	0.1 μg/mL	147.33 ± 40.20**	76.66 ± 5.03
	1 μg/mL	316.66 ± 75.08***	97.33 ± 4.61***
	5 μg/mL	341.00 ± 70.15***	99.33 ± 1.15***
ANQ	0.1 μg/mL	27.00 ± 4.24	21.00 ± 1.41
	1 μg/mL	34.00 ± 19.79	28.00 ± 16.97
	5 μg/mL	145.50 ± 44.54**	75.50 ± 23.30
	10 μg/mL	325.50 ± 4.95***	100.00 ± 0.0***
ANQ-OH	0.1 μg/mL	41.66 ± 25.85	32.66 ± 18.58
	1 μg/mL	66.67 ± 15.00	48.00 ± 7.55
	5 μg/mL	80.00 ± 40.28	49.33 ± 18.14
	10 μg/mL	166.00 ± 36.37**	81.00 ± 9.6

Data significant in relation to negative control (DMSO: solvent) group at *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$/one-way Tukey's multiple comparison test.
[a] Mean values and S.D. obtained from average of 100 cells per experimental—total of three experiments for each substance.
[b] Negative control.
[c] Positive control.

Table 6
Effect of naphthoquinones in V79 cells exposed for 3 h and evaluated by standard Comet assay with metabolic activation

Substance	Treatment	Damage index[c]	Damage frequency (%)[a]
NC[b]		33.3 ± 8.28	23.0 ± 5.79
CP[c]	4.5×10^{-5} M	272.5 ± 9.19***	87.0 ± 1.10***
1,4-NQ	0.01 μg/mL	63.3 ± 7.45	50.6 ± 1.41
	0.1 μg/mL	142.6 ± 4.24**	75.5 ± 2.12**
	1 μg/mL	244.3 ± 12.16***	88.3 ± 0.70***
	5 μg/mL	282.0 ± 1.41***	93.7 ± 2.0***
ANQ	0.1 μg/mL	45.0 ± 0.08	36.3 ± 3.24
	1 μg/mL	93.5 ± 12.02*	62.0 ± 4.55*
	5 μg/mL	223.0 ± 12.72***	92.3 ± 4.95***
	10 μg/mL	268.5 ± 0.70***	90.6 ± 4.24***
ANQ-OH	0.1 μg/mL	27.8 ± 5.65	21.3 ± 2.12
	1 μg/mL	150.6 ± 3.53**	77.5 ± 0.70**
	5 μg/mL	189.6 ± 2.82***	84.3 ± 1.41***
	10 μg/mL	212.0 ± 15.86***	88.5 ± 0.7***

Data significant in relation to negative control (DMSO: solvent) group at *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$/one-way Tukey's multiple comparison test.
[a] Mean values and S.D. obtained from average of 100 cells per experimental—total of three experiments for each substance.
[b] Negative control.
[c] Positive control.

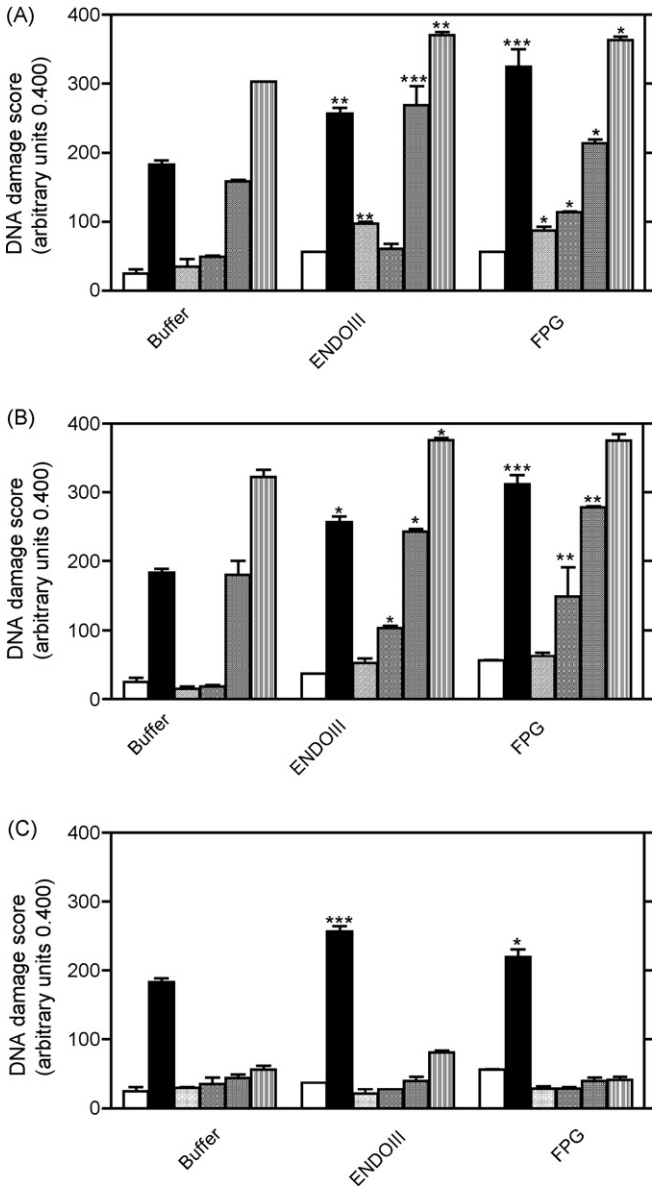


Fig. 3. (A) Effect of ENDOIII and FPG post-treatment on 1,4-naphthoquinone (1,4-NQ), with metabolic activation induced DNA migration in modified Comet assay. Columns from left to right: solvent; positive control (H_2O_2 10 μM); NQ: 0.01 μg/mL, 0.1 μg/mL, 1 μg/mL and 5 μg/mL. (B) Effect of ENDOIII and FPG post-treatment on 5-amino-8-hydroxy-1,4-naphthoquinone (ANQ) with metabolic activation induced DNA migration in modified Comet assay. Columns from left to right: solvent; positive control (H_2O_2 10 μM); ANQ: 0.1 μg/mL, 1 μg/mL, 5 μg/mL and 10 μg/mL. (C) Effect of ENDOIII and FPG post-treatment on 5-amino-2,8-dihydroxy-1,4-naphthoquinone (ANQ-OH) with metabolic activation induced DNA migration in modified Comet assay. Columns from left to right: solvent; positive control (H_2O_2 10 μM); ANQ-OH: 0.1 μg/mL, 1 μg/mL, 5 μg/mL and 10 μg/mL. Mean ± S.D. of three independent experiments. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$/one-way ANOVA Tukey's multiple comparison test. All asterisks indicate significant data after enzyme treatment when compared the same concentration in buffer conditions.

enzymes were not able to detect ANQ-OH-induced oxidative DNA damage in the absence of metabolic activation (Fig. 3C).

Fig. 4A–C shows mean DNA damage caused by NQS expressed as DNA damage index after treatment with DNA-repair enzymes ENDOIII and FPG, with metabolic activation. We observed that all NQS at higher concentrations cause oxidative DNA damage, as recognized by FPG enzyme (Fig. 4A–C). A significant level of DNA damage, recognized by ENDOIII, was only observed for ANQ and ANQ-OH at highest concentrations (Fig. 4B and C). 1,4-NQ-incubated cells treated with ENDOIII, in the presence of S9-mix, did not show significant increase in strand breaks at all tested concentrations (Fig. 4B). It is important to note that H_2O_2 was only used as a control for enzyme activity, and therefore was not included in figure. This positive control demonstrated significant increase in ENDOIII and FPG sites as compared to incubation in reaction buffer (data not shown). Cyclophosphamide (CP) is an alkylating agent widely used as positive control in tests using metabolic activation [39,40]. The damage generated by CP is expected to be recognized only by FPG, as few authors reported that FPG is able to recognize and to remove alkylating damage as well [18]. We found a significant difference between buffer level and incubation with FPG after CP treatment (Fig. 4A–C).

4. Discussion

Quinone cellular toxicity has been extensively studied, and it is generally accepted to be a function of (a) the capacity of quinones to produce ROS, and (b) the electrophilicity of quinones, which enables them to form adducts with cellular macromolecules [41]. However, there are few studies on amino-hydroxynaphthoquinone mutagenesis [17]. In the present study, we investigated the influence of hydroxy and amino substituents on 1,4-naphthoquinone genotoxicity in prokaryotic and eukaryotic cell systems.

In *Salmonella*/microsome assay, ANQ and ANQ-OH induced mutagenesis, in the presence of the S9-mix, in the strain TA102, which is sensitive to oxidative mutagens [21]. The reduction of these compounds by cytochrome P-450 produces a semiquinone radical, and, in the presence of oxygen, most semiquinone rapidly auto-oxidize, producing a hydroxyl and a superoxide anion radical [42]. Both ANQ and ANQ-OH were mutagenic in TA98 (sensitive to frameshift mutagens), without metabolic activation, whereas in the presence of S9-mix, only ANQ exhibited mutagenicity. This confirms results of Tikkanen et al. [16], who showed that naphthazarin and plumbagin induced frameshift mutations in strain TA98, with metabolic activation. The only tested naphthoquinone with an amino substituent – 4-amino-1,2-naphthoquinone – was mutagenic in strains TA97, TA100, and TA104 [17].

Chemical compounds bearing planar topologies and electrophilicity are often capable of intercalating between DNA bases [43]. As aminohydroxynaphthoquinone is an electrophilic molecule with an aromatic planar structure, inducing frameshift mutation only in *S. typhimurium* (where access to DNA is facilitated), we suggest that ANQ and ANQ-OH are weak intercalator mutagens.

1,4-NQ did not induce mutagenesis in any of the tested *Salmonella* strains, in the presence or absence of metabolic activation, confirming the results of Sakai et al. [44]. However, Hakura et al. [17], using a pre-incubation variant of the Ames-test, observed 1,4-NQ mutagenic activity in TA100, both

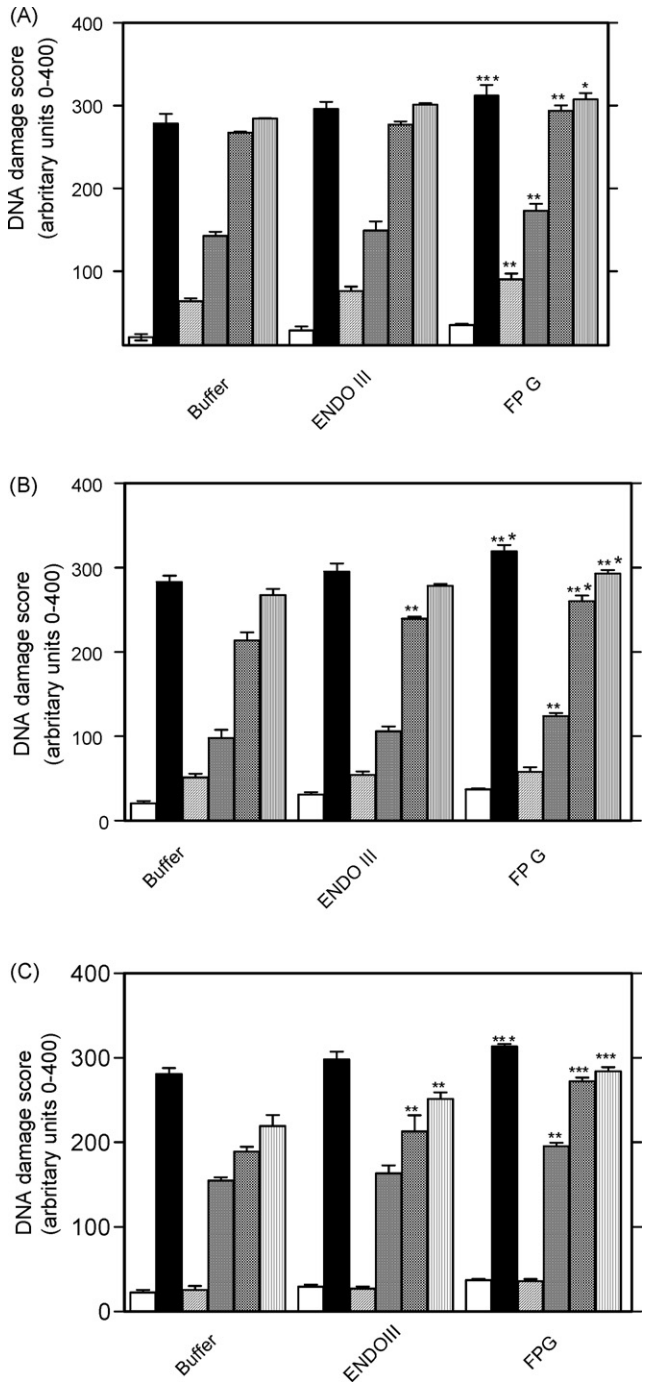


Fig. 4. (A) Effect of ENDOIII and FPG post-treatment on 1,4-naphthoquinone (1,4-NQ), with metabolic activation induced DNA migration in modified Comet assay. Columns from left to right: solvent; positive control (cyclophosphamide 6.0×10^{-5} M); NQ: 0.01 μg/mL, 0.1 μg/mL, 1 μg/mL and 5 μg/mL. (B) Effect of ENDOIII and FPG post-treatment on 5-amino-8-hydroxy-1,4-naphthoquinone (ANQ), with metabolic activation induced DNA migration in modified Comet assay. Columns from left to right: solvent; positive control (cyclophosphamide 6.0×10^{-5} M); ANQ: 0.1 μg/mL, 1 μg/mL, 5 μg/mL and 10 μg/mL. (C) Effect of ENDOIII and FPG post-treatment on 5-amino-2,8-dihydroxy-1,4-naphthoquinone (ANQ-OH) with metabolic activation induced DNA migration in modified Comet assay. Columns from left to right: solvent; positive control (cyclophosphamide 6.0×10^{-5} M); ANQ-OH: 0.1 μg/mL, 1 μg/mL, 5 μg/mL and 10 μg/mL. Mean ± S.D. of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$/one-way ANOVA Tukey's multiple comparison test. All asterisks indicate significant data after enzyme treatment when compared the same concentration in buffer conditions.

in the presence and absence of metabolic activation, and in TA97 and TA102 with metabolic activation. These controversial results may be explained by the fact that 1,4-NQ mutagenic activity is relatively weak, as compared to its cytotoxicity. In the yeast *S. cerevisiae*, 1,4-NQ induced mutagenesis and recombinogenesis (crossing-over) were also coupled with higher cytotoxicity (4.5–2% survival). This higher 1,4-NQ cytotoxicity, observed mainly in yeast diploid cells, could be associated to a more efficient one-electron reduction to semiquinone by NADPH–cytochrome P-450 reductase, leading to a better capacity to produce free radicals in the presence of oxygen (10–12). In addition, it is known that the diploid cells of the yeast *S. cerevisiae* have a higher level of cytochrome P-450 than to the haploid cells [45].

All *S. cerevisiae* strains were less sensitive to aminohydroxynaphthoquinones than to 1,4-NQ, indicating that the introduction of amino and hydroxy groups decreases toxicity in bacteria, yeasts, and mammalian cell lines. Lower 1,4-NQ toxicity after mono or dimethyl substitution (at positions 2 or 2 and 3, respectively) has been reported [12], and this was attributed to a lower rate of redox cycling caused by the lower redox potential imposed by methyl substitution, or to a lower rate of electrophilic addition. Consistent with this last assertion, Rodriguez et al. [46] suggested that 1,4-NQ toxicity depends not only on the rate of superoxide production, but also on the electrophilicity of the quinone molecule.

The alkaline SCG assay is a sensitive procedure to quantify DNA damage in cells, which includes alkali-labile sites (ALS) and DNA single- and double-strand breaks [35]. The standard Comet assay showed a significant increase in DNA damage after 1,4-NQ and ANQ treatment, without metabolization (Table 5). By employing ENDOIII and FPG proteins, usually applied in the modified Comet assay, we found that 1,4-NQ and ANQ promoted an important additional increase in strand breaks, without S9-mix, suggesting that these breaks are caused by ROS (Fig. 3A and B). This observation, along with the fact that this increase was not observed in all tested doses, suggests that, in addition to naphthoquinone-induced oxidative DNA damage, other types of lesions are involved in the genotoxic effects of these compounds. Indeed, it was shown that NQs are able to produce adducts with DNA, which may lead to a basic sites and single or double strand breaks [13,14]. 1,4-NQ-generated oxygen-free radicals can attack DNA at both pyrimidine and purine bases, and lead to strand breaks (Fig. 3A). At intermediate concentrations, ANQ predominantly induced damage in purines (Fig. 3B). Based on these results, we suggest that amino and hydroxy groups (at positions C8 and C5, respectively) facilitate DNA oxidative damage by free radicals, such as superoxide radical ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2). H_2O_2 is not toxic by itself, but its high *in vivo* reactivity, through the Fenton reaction, where it reacts with partially reduced metal ions, generates hydroxyl radical ($^{\bullet}OH$), the most important radical in ROS-related DNA damage [47]. These radicals cause a variety of base lesions in DNA. In the case of ANQ, the lesion produced is probably 8-oxo-Gua, the preferred substrate for FPG [48]. Interestingly, ANQ-OH showed a relevant genotoxic effect in the standard Comet assay at the highest dose, but it had no significant genotoxic effects

in the modified Comet assay, without S9 metabolic activation (Table 5 and Fig. 3C), indicating that the introduction of one additional hydroxy group at the C2 position does not produce a genotoxic molecule.

However, all NQs with metabolic activation presented important genotoxic potential in V79 cells (Table 6), and, as indicated by the presence of FPG sensitive sites, all tested compounds at higher doses can predominantly damage purines, which is suggestive of their ability to produce ROS (Fig. 4A–C).

Therefore, naphthoquinone capacity to produce free radicals is strongly influenced by the substituents present in the quinoid molecule and by its reduction. The compound 1,4-NQ is able to generate free radicals, reduced or not to semiquinone. Nevertheless, ANQ, and particularly ANQ-OH, must be reduced to semiquinone intermediates and enter in a process called redox cycling, generating superoxide anion, hydrogen peroxide, and hydroxyl radical, in order to cause oxidative stress [12]. We suggest that the presence of the hydroxy group in position C2 can stabilize the quinoid form, and modify the one-electron reduction of ANQ-OH, which can be associated a lesser efficient one-electron reduction. A similar effect was described for 2-hydroxy-1,4-naphthoquinone [12]. Furthermore, Roberg et al. [49] showed that NTZ induced oxidative stress in human foreskin fibroblasts, which caused lysosomal membrane destabilization, releasing proteases and causing loss of membrane potential in mitochondria, resulting in cell death. Considering their structural similarities with NTZ, we suggest similar effects in 1,4-NQ and ANQ-treated V79 cells.

Our results show that the presence of an amino group at C5 and a hydroxy group at different positions of 1,4-naphthoquinone decreases the toxicity of this new molecule in all test systems. Aminohydroxynaphthoquinones are weak mutagens in prokaryotic cells, whereas in mammalian cells, all NQs are genotoxic in the presence of metabolic activation. Aminohydroxynaphthoquinone pro-oxidant activity, together with their capability of DNA intercalation, has an important role in mutagenic and genotoxic effects.

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ANEXO III

Curriculum Vitae

Cassiana Macagnan Viau

possui graduação em Farmácia Industrial pela Pontifícia Universidade Católica do Rio Grande do Sul (2002) e mestrado em Biologia Celular e Molecular pela Universidade Federal do Rio Grande do Sul (2005). Atualmente realiza o seu doutorado em Biologia Celular e Molecular pela Universidade Federal do Rio Grande do Sul. Tem experiência na área de Genética, com ênfase em Genética Molecular e de Microorganismos, atuando principalmente nos seguintes temas: saccharomyces cerevisiae, biologia molecular, mutagenicidade, toxicidade de metais e microbiologia.

(Texto informado pelo autor)

Última atualização em 05/04/2009

Endereço para acessar este CV:
http://lattes.cnpq.br/7688401789334705

Dados Pessoais

Nome	Cassiana Macagnan Viau
Nome em citações bibliográficas	VIAU, C. M.
Sexo	feminino
Filiação	Luiz Volnei Mattos Viau e Joselina Apolônia Macagnan Viau
Nascimento	07/01/1978 - Porto Alegre/RS - Brasil
Carteira de Identidade	1050658457 SJTC - RS - 17/10/1995
CPF	66977037091
Endereço residencial	Rua Henrique Dias 201, ap 602 Bom Fim - Porto Alegre 90035-121, RS - Brasil Telefone: 51 30135335
Endereço profissional	- - Brasil
Endereço eletrônico	e-mail para contato : cassianaviau@yahoo.com.br

Formação Acadêmica/Titulação

2005 Doutorado em Biologia Celular e Molecular.
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil
Título: Efeitos Tóxicos e Genotóxicos do Cloreto de Estanho em células de levedura e mamíferos, Ano de obtenção: 2009
Orientador: João Antonio Pêgas Henriques
Bolsista do(a): Conselho Nacional de Desenvolvimento Científico e Tecnológico
Palavras-chave: qRT-PCR, EXPRESSÃO GÊNICA
Áreas do conhecimento : Biologia Geral,Genética,Toxicidade de Metais

2004 - 2005 Mestrado em Biologia Celular e Molecular.
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil
Título: Efeitos Tóxicos e Genotóxicos do Cloreto de Estanho (SnCl2) em bactéria e levedura, Ano de obtenção: 2005
Orientador: João Antonio Pêgas Henriques
Bolsista do(a): Conselho Nacional de Desenvolvimento Científico e Tecnológico
Palavras-chave: Saccharomyces cerevisiae, Stannous chloride, Mutagenic
Áreas do conhecimento : Microbiologia

1998 - 2002 Graduação em Farmácia Industrial.
Pontifícia Universidade Católica do Rio Grande do Sul, PUC RS, Porto Alegre, Brasil
Título: Identificação de Flavonóides em Extratos de Própolis do Rio Grande do Sul
Orientador: Ivone Sartor

Atuação profissional

1. **Escola Técnica FACTUM - FACTUM**

Vínculo institucional

2009 - Atual Vínculo: Celetista formal , Enquadramento funcional: Professor , Carga horária: 20, Regime: Parcial
Outras informações:
Leciona a disciplina de Produtos Farmacêuticos I.

2. Escola Profissional UNIPACS - UNIPACS

Vínculo institucional

2006 - 2008 Vínculo: Assessor pedagógico , Enquadramento funcional: autônomo , Carga horária: 4, Regime: Parcial
Outras informações:
Elaboração do Projeto Político Pedagógico do Curso de Farmácia para a Faculdade VIPACS de propriedade de Antônio de Medeiros Nazário.

3. Pontifícia Universidade Católica do Rio Grande do Sul - PUC RS

Vínculo institucional

2001 - 2002 Vínculo: Bolsista de Iniciação , Enquadramento funcional: Estudante de iniciação científica do CNPq , Carga horária: 20, Regime: Parcial

2001 - 2001 Vínculo: Bolsista voluntário , Enquadramento funcional: Estudante de iniciação científica voluntário , Carga horária: 20, Regime: Parcial

2000 - 2001 Vínculo: Bolsista , Enquadramento funcional: Estudante de iniciação científica da FAPERGS , Carga horária: 20, Regime: Parcial

Atividades

08/2001 - 07/2002 Projetos de pesquisa, Pontifícia Universidade Católica do Rio Grande do Sul

Participação em projetos:
Investigação de Quercetina em Extratos de Própolis do Rio grande do Sul Bolsista do CNPq

01/2001 - 07/2001 Estágio, Pontifícia Universidade Católica do Rio Grande do Sul

Estágio:
Monitoria da disciplina de Biotecnologia para Farmácia

01/2001 - 07/2001 Projetos de pesquisa, Pontifícia Universidade Católica do Rio Grande do Sul

Participação em projetos:
Isolamento e Determinação de Compostos Biologicamente Ativos no Polygonum punctatum

05/2000 - 02/2001 Projetos de pesquisa, Pontifícia Universidade Católica do Rio Grande do Sul

Participação em projetos:
Obtenção do Polifenol trans-Resveratrol Bolsista da FAPERGS

08/1999 - 12/1999 Estágio, Pontifícia Universidade Católica do Rio Grande do Sul

Estágio:
Monitoria Voluntária da disciplina de Química Orgânica II

Projetos

2001 - 2002 Investigação de Quercetina em Extratos de Própolis do Rio grande do Sul Bolsista do CNPq

Descrição: Análise de extratos de própolis de abelhas criadas na Reserva do Pró-Mata em São Francisco de Paula-RS. Análise por FTIR, HPLC, TLC.
Situação: Concluído Natureza: Pesquisa
Alunos envolvidos: Graduação (6);
Integrantes: Cassiana Macagnan ViauMarina Scopel; André Arigony Souto (Responsável)
Financiador(es): Conselho Nacional de Desenvolvimento Científico e Tecnológico-CNPq

2001 - 2001 Isolamento e Determinação de Compostos Biologicamente Ativos no Polygonum punctatum

Descrição: Isolamento e caracterização de compostos isolados do Polygonum punctatum
Situação: Concluído Natureza: Pesquisa
Alunos envolvidos: Graduação (5);
Integrantes: Cassiana Macagnan ViauMarina Scopel; André Arigony Souto (Responsável)
Financiador(es): Pontifícia Universiadade Católica do Rio Grande do Sul-PUCRS

2000 - 2001 Obtenção do Polifenol trans-Resveratrol Bolsista da FAPERGS

Descrição: Síntese do trans-resveretrol e seus derivados.
Situação: Concluído Natureza: Pesquisa
Alunos envolvidos: Graduação (3);
Integrantes: Cassiana Macagnan ViauMarina Scopel; André Arigony Souto (Responsável); Leandro Taso
Financiador(es): Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul-FAPERGS

Áreas de atuação

1. Genética Molecular e de Microorganismos
2. Expressão Gênica
3. Farmácia
4. Microbiologia
5. Química Orgânica

Idiomas

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

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

Produção em C, T& A

Produção bibliográfica

Artigos completos publicados em periódicos

1. Medina LFC, VIAU, C. M., Moura DJ, Saffi J, Stefani V, Brandelli A, Henriques, JAP
Genotoxicity of aminohydroxynaphthoquinones in bacteria, yeast, and Chinese hamster lung fibroblast cells.. Mutation Research. Genetic Toxicology and Environmental Mutagenesis. , v.650, p.140 - 149, 2008.
Palavras-chave: Salmonella microsome, Saccharomyces cerevisiae, Mutagenesis, comet assay, Quinones

2. VIAU, C. M., M-L Yoneama, Dias, JF, PUNGARTNIK, C., Brendel, M, Henriques, JAP
Detection and quantitative determination by PIXE of the mutagen Sn2+ in yeast cells. Nuclear Instruments & Methods in Physics Research. Section B. Beam Interactions with Materials and Atoms. , v.249, p.706 - 709, 2006.
Palavras-chave: Stannous chloride, Saccharomyces cerevisiae, Toxicidade de Metais
Áreas do conhecimento : Microbiologia,Biologia Geral,Física Atômica e Molecular

3. VIAU, C. M., PUNGARTNIK, C., SCHMITT, M. C., Basso, TS, Henriques, JAP, Brendel, M
Sensitivity of Sn2+ of the yeast Saccharomyces cerevisiae depends on geral energy metabolism, metal tranposrt, anti-oxidative defences, and DNA repair. BioMetals (Oxford). , v.19, p.705 - 714, 2006.
Palavras-chave: Toxicidade de Metais, Saccharomyces cerevisiae, SnCl2
Áreas do conhecimento : Microbiologia,Biologia Geral,Genética

4. PUNGARTNIK, C., VIAU, C. M., Picada J, Caldeira-de-Araújo, A, Henriques, JAP, Brendel, M
Genotoxicity of stannous chloride in the yeast and bacteria. Mutation Research. Genetic Toxicology and Environmental Mutagenesis. , v.583, p.146 - 157, 2005.
Palavras-chave: Stannous chloride, Saccharomyces cerevisiae, Cell clumping, Genotoxicity, Oxidative stress, Superoxide dismutase
Áreas do conhecimento : Biologia Geral,Microbiologia,Genética

Artigos aceitos para publicação

1. VIAU, C. M., Guecheva TN, Sousa FG, PUNGARTNIK, C., Brendel, M, Saffi J, Henriques, JAP
SnCl2-induced DNA damage and repair inhibition of MMS-caused lesions.. Archives of Toxicology. , 2009.
Palavras-chave: Stannous chloride, comet assay, DNA repair, Endonuclease III, Formamidopyrimidine DNA glycosylase

Trabalhos publicados em anais de eventos (resumo)

1. VIAU, C. M., Guecheva TN, PUNGARTNIK, C., Brendel, M, Henriques, JAP
SnCl2 INDUCES OXIDATIVE DAMAGE PREDOMINANTLY TO PURINES AND INTERFERES WITH DNA REPAIR SYSTEMS IN V79 CELLS In: VIII Congresso Brasileiro de Mutagênese Carcinogênese e Teratogênese Ambiental, 2007, Mangaratiba RJ.
SnCl2 INDUCES OXIDATIVE DAMAGE PREDOMINANTLY TO PURINES AND INTERFERES WITH DNA REPAIR SYSTEMS IN V79 CELLS. , 2007.
Palavras-chave: Chinese hamster lung fibroblast cells, Oxidative stress, Enzima ENDOIII, Enzima FPG
Áreas do conhecimento : Toxicidade de Metais,Genotoxicidade
Referências adicionais : Brasil/Português. Meio de divulgação: Impresso

2. VIAU, C. M., CARDONE, J. M., PUNGARTNIK, C., Brendel, M, Henriques, JAP
THE YEAST Saccharomyces cerevisiae, A MODEL ORGANISM FOR STANNOUS (Sn) METABOLISM STUDIES In: VIII Congresso Brasileiro de Mutagênese Carcinogênese e Teratogênese Ambiental, 2007, Mangaratiba RJ.
THE YEAST Saccharomyces cerevisiae, A MODEL ORGANISM FOR STANNOUS (Sn) METABOLISM STUDIES. , 2007.
Palavras-chave: Real time PCR, Saccharomyces cerevisiae, SnCl2, metallothioneins (MTs), Crs5 protein, Cup1 protein

Áreas do conhecimento : Toxicidade de Metais,Microbiologia Genética
Referências adicionais : Brasil/Português. Meio de divulgação: Impresso

3. VIAU, C. M., Guecheva TN, PUNGARTNIK, C., Brendel, M, Henriques, JAP
Genotoxic Effects of Stannous Chloride (SnCl2) in V79 Cells in Modified Comet Assay Employing Endo III and Fpg Enzymes In: XXXV Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular, 2006, Águas de Lindóia.
XXXV Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular. , 2006.
Palavras-chave: Ensaio Cometa Modificado, Enzima FPG, Enzima ENDOIII
Áreas do conhecimento : Genotoxicidade
Referências adicionais : Brasil/Português. Meio de divulgação: Impresso
Apresentação de pôster intitulado: "Genotoxic Effects of Stannous Chloride (SnCl2) in V79 Cells in Modified Comet Assay Employing Endo III and Fpg Enzymes".

4. VIAU, C. M., M-L Yoneama, Dias, JF, PUNGARTNIK, C., Brendel, M, Henriques, JAP
Interplay betwen tin (Sn) and others essential metals in the yeast Saccharomyces cerevisiae In: 25 Reunião de Genética de Microrganismos, 2006, São Pedro.
25 Reunião de Genética de Microrganismos. , 2006.
Palavras-chave: Toxicidade de Metais, Saccharomyces cerevisiae, Stannous chloride, PIXE
Áreas do conhecimento : Toxicidade de Metais,Genética Molecular e de Microorganismos,Física Atômica e Molecular
Referências adicionais : Brasil/Português. Meio de divulgação: Impresso
Apresentação de pôster intitulado: "Interplay betwen tin (Sn) and others essential metals in the yeast Saccharomyces cerevisiae".

5. PUNGARTNIK, C., VIAU, C. M., SOUTO, A. A., Dias, JF, Brendel, M, Henriques, JAP
Analysis of Stannous Chloride Effects in the Yeast Saccharomyces cerevisiae In: VII Congresso Brasileiro de Mutagênese, Carcinogênese e teratogênese Ambiental, 2005, Natal.
Congresso Brasileiro de Mutagênese, Carcinogênese e teratogênese Ambiental. , 2005.
Palavras-chave: Toxicidade de Metais, SnCl2, Saccharomyces cerevisiae
Áreas do conhecimento : Microbiologia,Genética,Ciência e Tecnologia de Alimentos Enlatados
Referências adicionais : Brasil/Português. Meio de divulgação: Impresso
Apresentação de pôster intitulado: "Analysis of Stannous Chloride Effects in the Yeast Saccharomyces cerevisiae",

6. VIAU, C. M., PUNGARTNIK, C., Picada J, Brendel, M, Henriques, JAP
Evaluation of Potential Genotoxicity of Stannous Chloride (SnCl2) in the yeast and Bacteria In: VII Congresso Brasileiro de Mutagênese, Carcinogênese e Teratogênese Ambiental, 2005, Natal.
VII Congresso Brasileiro de Mutagênese, Carcinogênese e Teratogênese Ambiental. , 2005.
Palavras-chave: Toxicidade de Metais, Saccharomyces cerevisiae, Genotoxicidade
Áreas do conhecimento : Microbiologia,Genética
Referências adicionais : Brasil/Português. Meio de divulgação: Impresso
Apresentação de pôster intitulado: "Evaluation of Potential Genotoxicity of Stannous Chloride (SnCl2) in the yeast and Bacteria".

7. SCHMITT, M. C., VIAU, C. M., PUNGARTNIK, C., Henriques, JAP, Brendel, M
Cellular Activation and Deactivation of Genotoxicity of Tin Chloride in Saccharomyces cerevisiae In: XXIV Reunião de Genética de Microorganismos, 2004, Gramado.
XXIV Reunião de Genética de Microorganismos. , 2004.
Palavras-chave: Saccharomyces cerevisiae, Genotoxicidade, Mutagenicidade
Áreas do conhecimento : Genética,Microbiologia,Física Atômica e Molecular
Referências adicionais : Brasil/Português.
Apresentação de pôster intitulado: "Cellular Activation and Deactivation of Genotoxicity of Tin Chloride in Saccharomyces cerevisiae".

8. VIAU, C. M., PUNGARTNIK, C., SOUTO, A. A., Brendel, M, Henriques, JAP
FTIR as a Tool for the Analysis of Effects of Stanoous Chloride in the Yeast Saccharomyces cerevisiae In: International Workshop on Vibrational Spectroscopy Applied to Microbiology and Biomedical Research, 2004, La Plata.
International Workshop on Vibrational Spectroscopy Applied to Microbiology and Biomedical Research. , 2004.
Palavras-chave: FTIR, Saccharomyces cerevisiae, SnCl2
Áreas do conhecimento : Química Inorgânica
Referências adicionais : Argentina/Inglês. Meio de divulgação: Impresso
Apresentação de pôster intitulado: "FTIR as a Tool for the Analysis of Effects of Stanoous Chloride in the Yeast Saccharomyces cerevisiae".

9. PUNGARTNIK, C., VIAU, C. M., SOUTO, A. A., Henriques, JAP, Brendel, M
Interaction of genotoxic SnCl2 with cell wall and membranes of the yeast Saccharomyces cerevisiae In: 11° International Conrgess on Yeast, 2004, Rio de Janeiro.
11° International Conrgess on Yeast. , 2004.
Palavras-chave: FTIR, Saccharomyces cerevisiae, SnCl2
Áreas do conhecimento : Genética,Microbiologia,Biologia Geral
Referências adicionais : Brasil/Inglês. Meio de divulgação: Impresso

10. VIAU, C. M., PUNGARTNIK, C., SOUTO, A. A., Henriques, JAP, Brendel, M
Monitoring Macromolecular Changes in Saccharomyces cerevisiae by FTIR Spectroscopy In: XXIV Reunião de Genética de Microorganismos, 2004, Gramado.
XXIV Reunião de Genética de Microorganismos. , 2004.
Palavras-chave: FTIR, Genotoxicidade, Saccharomyces cerevisiae
Áreas do conhecimento : Microbiologia Genética,Química Espectral
Referências adicionais : Brasil/Português.
Apresentação de pôster intitulado: "Monitoring Macromolecular Changes in Saccharomyces cerevisiae by FTIR Spectroscopy"

11. PUNGARTNIK, C., VIAU, C. M., SCHMITT, M. C., Henriques, JAP, Brendel, M
Sensitivityto Sn2+ ions of the yeast Saccharomyces cerevisiae depend on general energy metabolism, metal transport, anti-oxidative defences, and DNA capacity In: 11 International Congress on Yeast, 2004, Rio de Janeiro.
11 International Congress on Yeast. , 2004.
Palavras-chave: DNA repair, Metal transport, general energy metabolism
Áreas do conhecimento : Biologia Geral,Microbiologia,Genética
Referências adicionais : Brasil/Inglês. Meio de divulgação: Impresso

12. VIAU, C. M., PUNGARTNIK, C., SOUTO, A. A., Brendel, M, Henriques, JAP
Treatment of the yeast cells with stannous chloride leads to physicochemical changes of membranes, proteins, and nucleic acids and results in toxicity and genotoxicity In: 50 Congresso Brasileiro de Genética, 2004, Costão do Santinho.
50 Congresso Brasileiro de Genética. , 2004.
Palavras-chave: Saccharomyces cerevisiae, Stannous chloride, FTIR, Toxicidade de Metais
Áreas do conhecimento : Biologia Geral,Química
Referências adicionais : Brasil/Português. Meio de divulgação: Impresso
Apresentação de pôster intitulado: "Treatment of the yeast cells with stannous chloride leads to physicochemical changes of membranes, proteins, and nucleic acids and results in toxicity and genotoxicity".

13. VIAU, C. M., SCHMITT, M. C., PUNGARTNIK, C., Henriques, JAP, Brendel, M
DNA and Membrane Damage Induced by Stannous Chloride In: V Congresso Ibero-Americano de Biofísica, 2003, Rio de Janeiro.
V Congresso Ibero-Americano de Biofísica. , 2003.
Palavras-chave: Stannous chloride, Saccharomyces cerevisiae, Mutagenic
Áreas do conhecimento : Microbiologia
Referências adicionais : Brasil/Português.

14. SCHMITT, M. C., VIAU, C. M., PUNGARTNIK, C., Henriques, JAP, Brendel, M
Stannous Chloride Induces Oxidative Stress in Cox Deficient Yeast Strains In: V Congresso Ibero-Americano de Biofísica, 2003, Rio de Janeiro.
V Congresso Ibero-Americano de Biofísica. , 2003.
Palavras-chave: Saccharomyces cerevisiae, Stannous chloride, Mutagenic
Áreas do conhecimento : Microbiologia
Referências adicionais : Brasil/Português.

15. SCOPEL, M., VIAU, C. M., SOUTO, A. A., Blochtein, B
Determinação do Teor de Flavonóides Totais em Extratos de Própolis da Reserva do Pró-Mata (Rio Grande do Sul) In: 6 Congresso de Produtos Farmacêuticos e Cosméticos do Rio Grande do Sul, 2002, Porto Alegre.
6 Congresso de Produtos Farmacêuticos e Cosméticos do Rio Grande do Sul. , 2002.
Palavras-chave: Própolis, Cromatografia emn camada delgada, FTIR, HPLC
Áreas do conhecimento : Química Orgânica,Biologia Geral
Referências adicionais : Brasil/Português. Meio de divulgação: Impresso
Apresentação de pôster intitulado: "Determinação do Teor de Flavonóides Totais em Extratos de Própolis da Reserva do Pró-Mata (Rio Grande do Sul)".

16. SCOPEL, M., VIAU, C. M.
Isolamento e Determinação de Compostos Bioativos no Polygonum punctatum In: XIII Salão de Iniciação Científica - UFRGS, 2002, Porto Alegre.
XIII Salão de Iniciação Científica - UFRGS. , 2002.
Palavras-chave: Polygonun punctatun, Farmacognosia, Cromatografia emn camada delgada
Áreas do conhecimento : Química Orgânica
Referências adicionais : Brasil/Português.
Apresentação de pôster intitulado: "Isolamento e Determinação de Compostos Bioativos no Polygonum punctatum".

17. VIAU, C. M., SOUTO, A. A.
The bee species (Apini and Meliponini) determine the propolis profile In: XIV Salão de IniciaÇão Científica UFRGS, 2002, Porto Alegre.
XIV Salão de IniciaÇão Científica UFRGS. , 2002.
Palavras-chave: Própolis, Cromatografia emn camada delgada, Farmacognosia, FTIR
Áreas do conhecimento : Botânica,Farmacognosia,Análise Toxicológica
Referências adicionais : Brasil/Inglês. Meio de divulgação: Impresso
Apresentação do pôster intitulado: "The bee species (Apini and Meliponini) determine the propolis profile".

18. SCOPEL, M., VIAU, C. M.
Determinação de Flavonóides Totais e Quercetínicos em Extratos de Própolis Comerciais e Nativos In: II Salão de Iniciação Científica - PUCRS, 2001, Porto Alegre.
II Salão de Iniciação Científica - PUCRS. , 2001.
Palavras-chave: Flavonóides, Quercetina, Própolis
Áreas do conhecimento : Química Orgânica
Referências adicionais : Brasil/Português. Meio de divulgação: Impresso
Apresentação do pôster intitulado: "Determinação de Flavonóides Totais e Quercetínicos em Extratos de Própolis Comerciais e Nativos".

19. VIAU, C. M., SCOPEL, M., SOUTO, A. A.
Determinação de Quercetina em Extratos de Própolis do Rio Grande do Sul In: I Semana da Química, 2001, Porto Alegre.
I Semana da Química. , 2001.
Palavras-chave: Própolis, Farmacognosia
Áreas do conhecimento : Química Orgânica,Biologia Geral
Referências adicionais : Brasil/Português. Meio de divulgação: Impresso
Apresentação de pôster intitulado: "Determinação de Quercetina em Extratos de Própolis do Rio Grande do Sul".

20. VIAU, C. M., SCOPEL, M.
Isolamento e Determinação de Compostos Biologicamente Ativos no P. punctatun In: II Salão de Iniciação Científica - PUCRS, 2001, Porto Alegre.
II Salão de Iniciação Científica - PUCRS. , 2001.
Palavras-chave: Polygonun punctatun, Farmacognosia, Cromatografia em camada delgada (TLC)
Áreas do conhecimento : Química Orgânica
Referências adicionais : Brasil/Português. Meio de divulgação: Impresso
Apresentação do pôster intitulado: "Isolamento e Determinação de Compostos Biologicamente Ativos no P. punctatun".

21. VIAU, C. M., SCOPEL, M.
Síntese dos Derivados (Hidroxi-fenil)-propionaldeídos In: XII Salão de Iniciação Científica - UFRGS, 2000, Porto Alegre.
XII Salão de Iniciação Científica - UFRGS. , 2000.
Palavras-chave: Síntese Orgânica, Aldeído insaturado, Reação de Redução
Áreas do conhecimento : Química Orgânica
Referências adicionais : Brasil/Português. Meio de divulgação: Impresso
Apresentação do pôster intitulado: "Síntese dos Derivados (Hidroxi-fenil)-propionaldeídos".

Produção Técnica

Demais produções técnicas

1. VIAU, C. M.
Análise dos Mecanismos envolvidos na Tolerância ao Estresse Químico Gerado no Processo de Produção de Bioetanol por Leveduras etanologênicas, 2007. (Relatório de pesquisa)
Palavras-chave: Leveduras etanologênicas, Bioetanol, Furfural, 5-hidroximetilfurfural (HMF), 2,5-bis-hidroximetilfurano (FDM)
Referências adicionais : Brasil/Português. Meio de divulgação: Impresso
Elaboração do Projeto de Pesquisa para o Programa de Pós-Graduação em Biologia Celular e Molecular referente ao Exame de Qualificação, como requisito parcial para obtenção do título de doutor.

Eventos

Participação em eventos

1. **PCR em tempo real: métodos analíticos e quantitativos**, 2008. (Encontro)

2. **Curso de Biossegurança e Segurança Laboratorial**, 2005. (Encontro)

3. Apresentação de Poster / Painel no(a) **6 Congresso de Produtos Farmacêuticos e Cosméticos do Rio Grande do Sul**, 2002. (Congresso)
Determinação do Teor de Flavonóides Totais rm Extratos de Própolis da Reserva do Pró-Mata (Rio Grande do Sul)..

4. **Curso de Boas Práticas de Fabricação**, 2002. (Encontro)

5. Apresentação de Poster / Painel no(a) **I Semana da Química**, 2001. (Encontro)
Determinação de Quercetina em Extratos de Própolis do Rio Grande do Sul.
Palavras-chave: Química Orgânica, Meio Ambiente
Áreas do conhecimento : Análise e Controle de Medicamentos
Setores de atividade : Fabricação de produtos químicos inorgânicos

6. **V Semana da Faculdade de Farmácia**, 2000. (Encontro)
Palavras-chave: Quimioterapia, Pesquisa, Administração Farmacêutica
Áreas do conhecimento : Análise e Controle de Medicamentos
Setores de atividade : Outro

7. **IV Semana da Faculdade de Farmácia**, 1999. (Encontro)
Palavras-chave: Medicamentos, Âmbito Profissional
Áreas do conhecimento : Análise e Controle de Medicamentos
Setores de atividade : Desenvolvimento de produtos tecnológicos voltados para a saúde humana

8. **III Semana da Faculdade de Farmácia da PUCRS**, 1998. (Encontro)
Palavras-chave: Medicamentos, Legislação, Correlatos
Áreas do conhecimento : Análise e Controle de Medicamentos
Setores de atividade : Outro

Organização de evento

1. VIAU, C. M.
Avaliação da Captação do Sn2+ e sua interação com outros elementos químicos essenciais na levedura Saccharomyces cerevisiae, 2007. (Exposição, Organização de evento)
Palavras-chave: Chinese hamster lung fibroblast cells, DNA repair, SnCl2
Áreas do conhecimento : Genotoxicidade,Toxicidade de Metais
Referências adicionais : Brasil/Português. Meio de divulgação: Meio digital

2. VIAU, C. M.
Teste Cometa para Avaliação de Dano em DNA - Teoria e Prática, 2007. (Exposição, Organização de evento)
Referências adicionais : Brasil/Português. Meio de divulgação: Impresso
Participação como organizadora e palestrante do curso "Teste Cometa para Avaliação de Dano em DNA - Teoria e Prática" no período de 19 a 30 de novembro de 2007, com carga horária total de 40 horas.

3. VIAU, C. M.
Efeitos Tóxicos e Genotóxicos do Cloreto de Estanho na Levedura Saccharomyces cerevisiae, 2005. (Exposição, Organização de evento)
Palavras-chave: Toxicidade de Metais, Genotoxicidade, Stannous chloride, Saccharomyces cerevisiae
Áreas do conhecimento : Microbiologia,Genética
Referências adicionais : Brasil/Português. Meio de divulgação: Outro
Apresentação do Seminário intitulado: "Efeitos Tóxicos e Genotóxicos do Cloreto de Estanhona Levedura Saccharomyces cerevisiae".

Totais de produção

Produção bibliográfica

Artigos completos publicado em periódico	4
Artigos aceitos para publicação	1
Trabalhos publicados em anais de eventos	21

Produção Técnica

Relatório de pesquisa	1

Eventos

Participações em eventos (congresso)	1
Participações em eventos (encontro)	7
Organização de evento (exposição)	3

Outras informações relevantes

1 Experiência Profissional (11/2002 - 08/2003) Drogaria Régis Gazineu ME FARMACÊUTICA TITULAR Experiência Profissional Drogaria ALARCON LTDA FARMACÊUTICA TITULAR

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