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DISSERTAÇÃO DE MESTRADO

Diones Caeran Bueno

Santa Maria, RS, Brasil
2015

TOXICIDADE DE ORGANOCALCOGÊNIOS E SEUS MECANISMOS ATRAVÉS DA EXPRESSÃO GÊNICA EM LEUCÓCITOS HUMANOS

Diones Caeran Bueno

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Orientador: Prof. Dr. João Batista Teixeira da Rocha

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elaborada por

Diones Caeran Bueno

como requisito parcial para a obtenção do grau de
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RESUMO

Dissertação de Mestrado
Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica
Universidade Federal de Santa Maria

TOXICIDADE DE ORGANOCALCOGÊNIOS E SEUS MECANISMOS ATRAVÉS DA EXPRESSÃO GÊNICA EM LEUCÓCITOS HUMANOS

Autor: Diones Caeran Bueno
Orientador: João Batista Teixeira da Rocha
Data e Local da Apresentação: Santa Maria, 6 de fevereiro de 2015.

O selênio (Se) é um micronutriente essencial presente nas selenoproteínas dos organismos vivos, na forma dos aminoácidos selenocisteína e selenometionina. Quimicamente relacionado ao Se, o telúrio (Te) não possui nenhuma função biológica nos mamíferos, porém, compostos orgânicos de Te se mostraram bons agentes antioxidantes. Apesar do ebselen (Ebs), disseleneto de difenila [$(PhSe)_2$] e ditelureto de difenila [$(PhTe)_2$] possuírem atividade mimética a enzima glutationa peroxidase (GPx), exibindo propriedades antioxidantes, estes compostos apresentam efeitos tóxicos em altas concentrações, devido a sua capacidade de oxidar grupos tióis. Porém, os mecanismos de toxicidade destes compostos através da modulação da expressão gênica nunca foram estudados em células humanas. Desta forma, este estudo objetivou avaliar a citotoxicidade e a genotoxidade dos organocalcogênios em leucócitos humanos, e avaliar seus mecanismos através da produção de espécies reativas de oxigênio (EROs) e modulação da expressão de proteínas antioxidantes, além de avaliar a quantidade relativa de organocalcogênio em contato com as células em nosso modelo de exposição *ex vivo*. O teste de exclusão do azul de Trypan e o ensaio cometa foram utilizados para avaliar, respectivamente, a citotoxicidade e a genotoxicidade dos organocalcogênios. A fluorescência da diclorofluoresceína (DCFH) e iodeto de propídeo (IP) foi medida nos leucócitos expostos por citometria de fluxo. A expressão dos genes para as enzimas Catalase, Superóxido Dismutase 1, Glutationa Peroxidase 3, Glutationa Peroxidase 4, Tiorredoxina Redutase 1 e Nrf-2 foram analisados. Uma extração de diclorometano do tampão e do pellet das células foi injetada em um aparelho de GC-MS para avaliar a quantidade de composto em contato com as células. Os organocalcogênios induziram a uma redução da viabilidade celular apenas na concentração de 50 µM, sendo que o efeito maior foi do Ebs, seguido pelo $(PhTe)_2$ e pelo $(PhSe)_2$, enquanto o $(PhTe)_2$ foi o único composto capaz de aumentar a taxa apoptótica dos leucócitos, o que aconteceu em todas as concentrações (10-50 µM). O $(PhTe)_2$ aumentou o índice de dano ao DNA em todas as concentrações testadas (5-50 µM), enquanto o Ebs e o $(PhSe)_2$ o fizeram apenas na concentração de 50 µM. Surpreendentemente, o $(PhSe)_2$ foi o único composto efetivo em aumentar a produção de EROs em todas as concentrações testadas (10-50 µM), o que foi acompanhado por um aumento na expressão da SOD1 e uma diminuição da expressão da CAT. Todos os compostos foram efeitovs em diminuir a expressão da GPX3 e do NFE2L2 (Ebs > $(PhTe)_2$ > $(PhSe)_2$), sendo que nenhum alterou a expressão da GPX4 e da TRXR1. Os organocalcogênios foram encontrados em maior concentração na extração do pellet de leucócitos do que no seu tampão. Concluímos que a toxicidade dos compostos em questão não está diretamente relacionada com a propriedade dos mesmos em produzir EROs.

Palavras-chave: selênio; telúrio; organocalcogênios; toxicidade; ebselen; disseleneto de difenila; ditelureto de difenila; expressão gênica.

ABSTRACT

Master's Dissertation
Graduate Program in Biological Sciences: Toxicological Biochemistry
Federal University of Santa Maria

ORGANOCHALCOGEN TOXICITY AND ITS MECHANISMS THROUGH GENE EXPRESSION IN HUMAN LEUKOCYTES

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Date and Place of Presentation: Santa Maria, February 6th, 2015

Selenium (Se) is an essential micronutrient present in selenoproteins on living beins, in the form of the amino acid selenocysteine and selenomethionine. Chemically related to Se, tellurium (Te) has no biological function in mammals, however, organic Te compounds showed up as good antioxidant agents. Although ebselen (Ebs), diphenyl diselenide [$(PhSe)_2$] and diphenyl ditelluride [$(PhTe)_2$] present antioxidant properties via their glutathione peroxidase (GPx) mimetic activity, these compounds exhibit toxic effects at high concentrations due to its property in oxidizing thiols groups. However, the toxicity mechanism of these compounds through the modulation of gene expression was never studied in human cells. Thus, this study aimed to evaluate the cytotoxicity and genotoxicity of organochalcogens in human leukocytes, and evaluate their mechanisms through the production of reactive oxygen species (ROS) and modulation of antioxidant proteins expression, and to evaluate the relative amount of organochalcogens in contact with the cells in our *ex vivo* exposure model. Trypan's blue exclusion test and comet assay were used to evaluate, respectively, cytotoxicity and genotoxicity induced by the compounds. The fluorescence of dichlorofluorescein (DCFH) and propidium iodide (PI) were measured in leukocytes exposed by flow cytometry. The expression of the genes for the enzymes Catalase, Superoxide Dismutase 1, Glutathione Peroxidase 3, Glutathione Peroxidase 4, Thioredoxin Reductase 1 and Nrf-2 were analyzed. A dichloromethane extraction of the buffer and the pellet of the cells was injected into a GC-MS apparatus to evaluate the amount of compound in contact with cells. The compounds induced a reduction in cell viability only in the concentration of 50 µM, being Ebs the most cytotoxic compound, followed by $(PhTe)_2$ and $(PhSe)_2$, while $(PhTe)_2$ was the only compound able of increasing the apoptotic rate of leukocytes, which happened at all concentrations (10-50 µM). $(PhTe)_2$ increased DNA damage index in all tested concentrations (5-50 µM), while Ebs and $(PhSe)_2$ did it only at 50 µM concentration. Surprisingly, $(PhSe)_2$ was the only compound effective in increasing ROS production in all tested concentrations (10-50 µM), which was accompanied by an increase in the SOD1 expression and a decrease in CAT expression. All compounds were effective in decreasing the expression of GPX3 and NFE2L2 (Ebs > $(PhTe)_2$ > $(PhSe)_2$), and none altered the expression of GPX4 and TRXR1. The compounds were found in higher concentrations in leukocyte pellet extraction than in their buffer. We conclude that the toxicity of the compounds in question is not directly related to their property in inducing production of ROS.

Key-words: selenium; tellurium; organochalcogens; toxicity; ebselen; diphenyl diselenide; diphenyl ditelluride; gene expression.

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LISTA DE SÍMBOLOS E ABREVIAÇÕES

δ-ALAD – δ Aminolevulinato Desidratase
ATP – Adenosina Trifosfato
AVC – Acidente Vascular-Cerebral
CAT – Catalase
CTE – Cadeia Transportadora de Elétrons
DCFH – Diclorofluoresceína
DIO – Iodotironina Deiodinase
DNA – Ácido Desoxirribonucleico
Ebs – Ebselen
EbsSeH – Ebselen Selenol
ERA – Elemento de Resposta Antioxidante
EROs – Espécies Reativas de Oxigênio
Fe^{2+} - Íon ferroso
Fe^{3+} - Íon férrico
GPx – Glutationa Peroxidase
GSH – Glutationa reduzida
H_2O_2 – Peróxido de Hidrogênio
Hg – Mercúrio
ID – Índice de Dano
IP – Iodeto de propídeo
LDH – Lactato Desidrogenase
MeHg – Metilmercúrio
mRNA – Ácido Ribonucleico mensageiro
NADPH – Fosfato de Nicotinamida Adenina Dinucleotídeo Reduzido
Nrf-2 – Fator Nuclear (derivado de Eritroide 2) 2
O_2 – Oxigênio molecular
$O_2^{\bullet -}$ - Ânion superóxido
$OH^{\bullet -}$ - Radical hidroxila
$(PhSe)_2$ – Disseleneto de Difenila
PhSeH – Fenil Selenol
$(PhTe)_2$ – Ditelureto de Difenila
RNA – Ácido Ribonucleico
Se – Selênio
SeCys – Selenocisteína
SISC – Sequência de Inserção da Selenocisteína
SOD – Superóxido Dismutase
T_3 – 3,4,5’-triiodotironina
T_4 – tiroxina
Te – Telúrio
TRx - Tiorredoxina
TRxR – Tiorredoxina Redutase

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1. INTRODUÇÃO

1.1 Selênio e Telúrio

O selênio (Se) é um elemento do grupo dos calcogênios (grupo 16) da tabela periódica. O mesmo foi descoberto em 1817 por Jöns Jacob Berzelius (BERZELIUS, 1818) ao visitar a fábrica de ácido sulfúrico de Gripsholm. Na natureza, o Se é um elemento raro, sendo encontrado em minérios de cobre como um subproduto nos processos de refinação eletrolítica. O mesmo é muito utilizada na indústria na fabricação de células fotoelétricas. Em sua forma elementar, o Se é tóxico para os seres vivos, causando um hálito desagradável de alho em pessoas expostas ao elemento (NOGUEIRA & ROCHA, 2011).

O Se é um elemento essencial para a nutrição animal, incluindo humanos, sendo considerado um micronutriente (COMBS & COMBS, 1984). Ele está presente nos organismos vivos nas selenoproteínas na forma do aminoácido selenocisteína (SeCys) (HOLBEN & SMITH, 1999). Ao contrário da maioria dos aminoácidos incomuns, que são adicionados às proteínas por modificação pós-traducional, a SeCys é considerada o 21º aminoácido por ser adicionada a cadeia polipeptídica durante a síntese proteica, sendo codificada pelo códon UGA, normalmente um códon de parada (LOW & BERRY, 1996). A presença da sequência de inserção da selenocisteína (SISC) na região não traduzida 3' do ácido ribonucleico mensageiro (mRNA) é responsável pela inserção da SeCys no peptídeo nascente (WALCZAK et al., 1996). Atualmente são reconhecidas 25 selenoproteínas no proteoma humano, sendo a maioria delas de função desconhecida (KRYUKOV et al., 2003). As selenoenzimas mais bem descritas são a Glutationa Peroxidase (GPx), a Tiorredoxina Redutase (TRxR) e a Iodotironina Deiodinase (DIO) (ROTRUCK et al., 1973; BERRY et al., 1991; ZHONG et al., 2000). A enzima GPx é responsável pela degradação de peróxido de hidrogênio (H_2O_2) e hidroperóxidos lipídicos com a concomitante oxidação de glutationa (GSH) (ROTRUCK et al., 1973). A TRxR é responsável pela manutenção do ambiente redutor no interior das células através da redução da tiorredoxina (TRx) com elétrons provenientes do fosfato de nicotinamida adenina dinucleotídeo reduzido (NADPH), sendo a TRx então, responsável pela redução de pontes dissulfeto em proteínas (ZHONG et al., 2000). A enzima DIO é responsável por fazer a conversão da tiroxina (T_4) em 3,4,5'-triiodotironina (T_3) na glândula tireoide (BIANCO et al., 2002).

O consumo de Se pelos humanos se dá, principalmente, através de cereais, carnes e peixes, sendo as sementes da castanha-do-pará (*Bertholletia excelsa*) um dos alimentos mais ricos em Se (COMBS, 2001; LEMIRE et al., 2010). Devido as suas funções fisiológicas de defesa contra radicais livres, o consumo de 100 µg/dia de Se na forma de suplemento alimentar é recomendado pela medicina como um antioxidante (RAYMAN, 2000). Com isso, surgiram estratégias terapêuticas usando formas orgânicas e inorgânicas de Se como fármacos antioxidantes com potencial atividade anti-carcinogênica (EL-BAYOUMY, 2001).

Da mesma família periódica do Se, o telúrio (Te) é um calcogênio descoberto em 1782 por Franz-Joseph Müller von Reichenstein e isolado em 1798 por Martin Heinrich Klaproth, tendo abundância e locais de obtenção similares ao Se (KLAPROTH, 1798). É sabido que alguns fungos são capazes de incorporar o Te em teluroproteínas na forma de telurocisteína e telurometionina. Porém, o Te não tem nenhuma função biológica nos mamíferos, sendo altamente tóxico para os mesmos, causando um hálito desagradável de alho e secura na boca em seres humanos expostos ao elemento, além de dores de cabeça, vertigens e sonolência em intoxicações mais pesadas (TAYLOR, 1996; BIENERT et al., 2008). Apesar disso, foi observado que alguns compostos orgânicos de Te podem atuar como moléculas antioxidantes, gerando uma miríade de possibilidades novas dentro da farmacologia dos organocalcogênios (ANDERSSON et al., 1994; NOGUEIRA et al., 2004; BRAGA et al., 2009).

1.2. Farmacologia do Ebs, $(PhSe)_2$ e $(PhTe)_2$

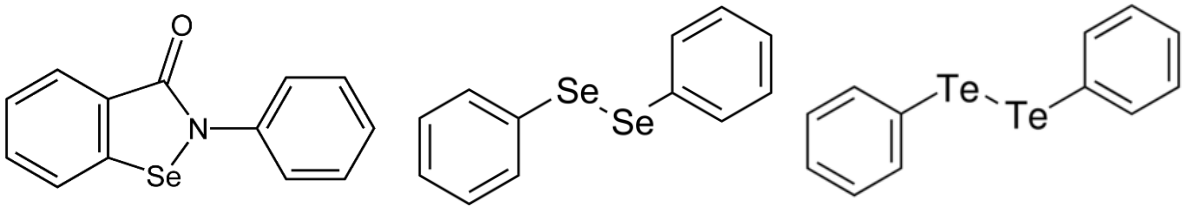
O primeiro composto orgânico de selênio estudado que teve propriedades farmacológicas reconhecidas foi o ebselen (Ebs) (Figura 1) (MÜLLER et al., 1984). Evidências experimentais mostram que este composto pode atuar como antioxidante, anti-inflamatório, neuroprotetor, entre outras propriedades (SCHEWE, 1995; SAITO et al., 1998; BRODSKY et al., 2004; NOGUEIRA et al., 2004). O Ebs já foi muito estudado como um possível fármaco no tratamento do acidente vascular-cerebral (AVC) e atualmente está sob investigação no tratamento do transtorno bipolar (YAMAGUCHI et al., 1998; SINGH et al., 2013).

Recentemente, o $(PhSe)_2$ (Figura 1), um composto cujas evidências têm mostrado propriedades farmacológicas bastante similares ao Ebs, vem sendo investigado como um agende farmacológico baseado em suas propriedades antioxidantes (NOGUEIRA et al., 2004). Vários estudos experimentais mostram que o $(PhSe)_2$ pode atuar como anti-inflamatório, anti-

hiperglicêmico, antioxidante, neuroprotetor, entre outras propriedades (GHISLENI et al., 2003; NOGUEIRA et al., 2003; SANTOS et al., 2005; BARBOSA et al., 2006; BORGES et al., 2008).

Além dos compostos orgânicos de Se, existem vários estudos mostrando os efeitos benéficos de compostos orgânicos de Te (NOGUEIRA et al., 2004; BRAGA et al., 2009). Nesse contexto, o composto $(PhTe)_2$, análogo de Te do $(PhSe)_2$, foi estudado como um antioxidante e antifúngico (PUNTEL et al., 2007; PINTON et al., 2011; ROSSETI et al., 2011).

Os mecanismos pelos quais o Ebs, o $(PhSe)_2$ e o $(PhTe)_2$ exercem seu poder antioxidante possuem certa analogia. Esses compostos são capazes de atuar como miméticos da enzima GPx, ou seja, eles são capazes de degradar H_2O_2 através da oxidação da GSH, sendo este o mecanismo ao qual a maioria dos autores atribui sua atividade antioxidante (SIES, 1993; BRAGA et al., 2009; NOGUEIRA & ROCHA, 2011) (Figura 2). Além disso, foi mostrado que o Ebs e o $(PhSe)_2$ são capazes de atuar como substratos para a enzima TRxR, formando, respectivamente, ebselen selenol (EbsSeH) e fenil selenol (PhSeH), sendo que nesta forma, estes compostos são capazes de atuar como agentes redutores de H_2O_2, de uma forma que não tem relação com sua atividade mimética a GPx (ZHAO & HOLMGREN, 2002; FREITAS et al., 2010) (Figura 3). O selenol nestes compostos também pode atuar como um agente sequestrador de metais, como por exemplo o mercúrio (Hg), existindo muitas evidências na literatura mostrando que estes compostos podem atuar como agentes protetores em casos de intoxicação com o contaminante ambiental metilmercúrio (MeHg) (FARINA et al., 2003; FREITAS et al., 2009).



Ebselen **Disseleneto de difenila** **Ditelureto de difenila**

Figura 1 - Compostos organocalcogênios.

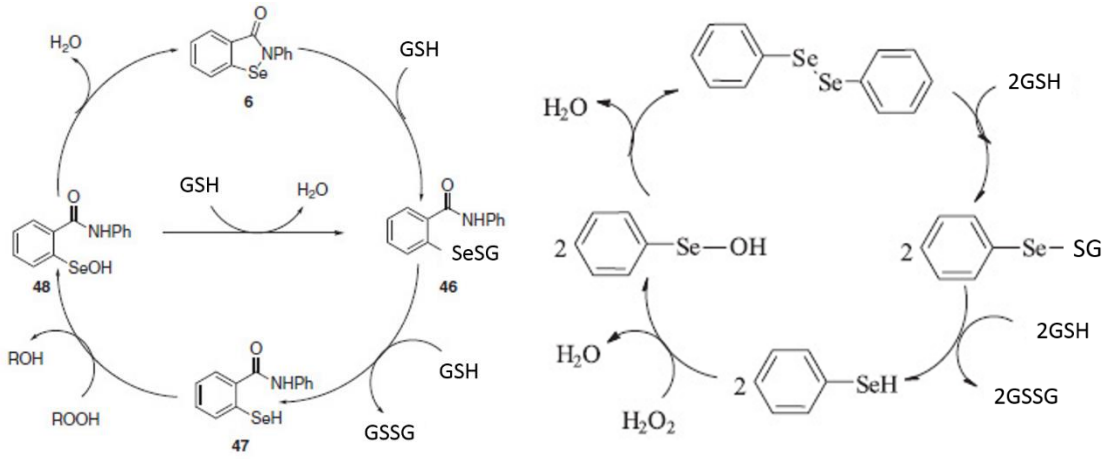


Figura 2 – Esquema mostrando o mecanismo pelo qual os compostos Ebs e $(PhSe)_2$ exercem atividade mimética a enzima GPx (NOGUEIRA et al., 2004, com modificações).

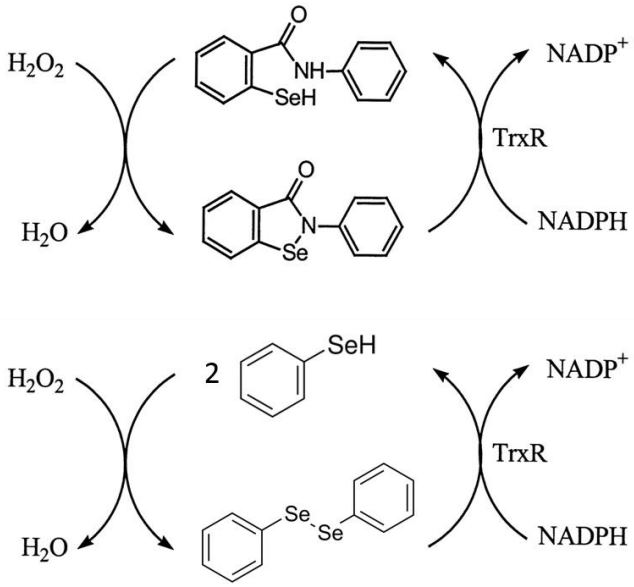


Figura 3 – Esquema mostrando o mecanismo pelo qual os compostos Ebs e $(PhSe)_2$ atuam como substratos para a enzima TRxR (NOGUEIRA et al., 2004, com modificações).

1.3. Toxicologia do Ebs, $(PhSe)_2$ e $(PhTe)_2$

Apesar da ação farmacológica exercida pelos organocalcogênios em questão, estes compostos podem ser altamente tóxicos quando presentes em altas concentrações. A alta reatividade dos grupos selenol e telurol faz com que estes compostos reajam com grupos tiol e/ou selenol de muitas proteínas celulares, gerando espécies reativas de oxigênio (EROs) e,

consequentemente, causando danos oxidativos nas proteínas, peroxidação nos lipídeos de membrana e danos no ácido desoxirribonucleico (DNA) (MACIEL et al., 2003; MEOTTI et al., 2003; NOGUEIRA et al., 2004; ROSA et al., 2007; DEGRANDI et al., 2010; SANTOFIMIA-CASTAÑO et al., 2013). Além de oxidarem tióis não-proteicos, estes compostos são capazes de inibir as enzimas δ-aminolevulinato desidratase (δ-ALAD), Na^+/K^+ ATPase e lactato desidrogenase (LDH) através da oxidação de grupos tióis, deixando o metabolismo celular seriamente comprometido (BARBOSA et al., 1998; NOGUEIRA et al., 2004; BORGES et al., 2005; LUGOKENSKI et al., 2011). Também é observado disfunção mitocondrial devido, principalmente, à inibição dos complexos mitocondriais I e II via oxidação de tióis, o que reduz o potencial de membrana mitocondrial e aumenta a produção de EROs (PUNTEL et al., 2013). O composto $(PhTe)_2$ também se mostrou capaz de inibir a enzima TRxR e de romper a homeostase do citoesqueleto de células nervosas (COMPARSI et al., 2012; PESSOA-PUREUR et al., 2014). Esses compostos também são capazes de induzir neurotoxicidade, afetando o sistema glutamatérgico, onde foi observado inibição da captação de glutamato em microssomas de cérebros de animais tratados (NOGUEIRA et al., 2001; NOGUEIRA et al., 2002).

1.4. Estresse oxidativo e defesas antioxidantes

O estresse oxidativo é uma situação fisiopatológica caracterizada por um desequilíbrio entre a produção de EROs e as defesas antioxidantes. Várias situações patológicas têm como única ou principal causa o estresse oxidativo. Nessas situações, as EROs são produzidas de forma inespecífica como um subproduto da respiração aeróbica, sendo a mitocôndria a principal fonte endógena de EROs (FINKEL & HOLBROOK, 2000).

A mitocôndria é a organela celular de dupla membrana responsável pela produção de adenosina trifosfato (ATP) nas células eucarióticas. Em sua membrana interna, estão incrustadas as enzimas que formam a cadeia transportadora de elétrons (CTE), onde equivalentes reduzidos entregam seus elétrons provenientes de moléculas orgânicas. Os elétrons são transferidos entre os complexos da CTE, sendo o oxigênio molecular (O_2) usado como aceptor final de elétrons. A passagem dos elétrons pela CTE gera o bombeamento de prótons da matriz mitocondrial para o espaço intermembranas, gerando o potencial eletroquímico necessário para a síntese de ATP no processo de fosforilação oxidativa (HATEFI, 1965).

Estimativas a partir de estudos *in vitro* dizem que, em condições normais, cerca de 0,5% dos elétrons que passam pela CTE escapam, principalmente nos complexos I e III. Nesses casos, os elétrons reagem com o O_2, formando a ERO ânion superóxido ($O_2^{\bullet -}$). Sendo um radical livre, i.e., possui um elétron desemparelhado na camada de valência, o $O_2^{\bullet -}$ pode reagir com proteínas e com os lipídeos de membrana, levando a disfunções celulares e danos na membrana plasmática da célula (MURPHY, 2009). A enzima superóxido dismutase (SOD) é responsável por fazer a conversão do $O_2^{\bullet -}$ em peróxido de hidrogênio (H_2O_2), uma forma mais estável e menos reativa que o $O_2^{\bullet -}$ (MCCORD & FRIDOVICH, 1969). O H_2O_2, por sua vez, pode reagir com o íon ferroso (Fe^{2+}), formando o radical hidroxila ($OH^{\bullet -}$) e o íon férrico (Fe^{3+}), processo conhecido como reação de Fenton. O $OH^{\bullet -}$ é a ERO mais danosa as células, pois apresenta uma alta reatividade, causando danos oxidativos em proteínas, peroxidação lipídica, além de quebras de cadeia e modificações oxidativas no DNA, não existindo nenhuma enzima que possa catalisar sua degradação (IMLAY et al., 1988) (Figura 4).

Os organismos vivos dispõem de várias estratégias para amenizar os efeitos danosos das EROs (DAVIES, 2000). A SOD, como mencionada acima, é a enzima responsável por fazer a conversão do $O_2^{\bullet -}$ em H_2O_2, que apesar de ser uma ERO, não é um radical livre, sendo então menos reativo que o $O_2^{\bullet -}$ (MCCORD & FRIDOVICH, 1969). Para evitar que o H_2O_2 reaja com o Fe^{2+}, as enzimas catalase (CAT) e glutationa peroxidase (GPx) fazem a quebra do H_2O_2 em O_2 e água. A CAT é uma enzima contendo heme presente em grandes quantidades nos peroxissomos, enquanto a GPx degrada H_2O_2 com a concomitante oxidação de glutationa (GSH), sendo que algumas isoformas também são capazes de reduzir hidroperóxidos lipídicos (MAY, 1901; ARTHUR, 2000). Além disso, as abundantes peroxirredoxinas são peroxidases dependentes de tiós que também atuam na defesa contra as EROs (POOLE et al., 2011). Ainda, a enzima TRxR atua revertendo as alterações oxidativas causadas em proteínas pelas EROs, em que ela catalisa a redução da TRx, que nessa forma pode reduzir pontes dissulfeto, sulfóxidos em resíduos de cisteína e resíduos de metionina oxidados em proteínas, restaurando suas funções originais (ARNÉR & HOLMGREN, 2000). O controle da atividade dessas enzimas pode estar associado com a expressão da proteína Fator Nuclear (Derivado de Eritroide 2) 2 (Nrf-2), um fator de transcrição responsável pela ativação da expressão gênica de diversas enzimas com ação antioxidante quando há uma grande quantidade de EROs na célula. Essa proteína, na presença de agentes eletrofílicos, se desliga da proteína Keap1 e, no núcleo, se liga aos Elementos de Resposta Antioxidante (ERA), sequência presente na região promotora de genes que codificam proteínas antioxidantes, ativando a transcrição dos mesmos (ISHII et al.,

2000). Além das defesas enzimáticas mencionadas, moléculas orgânicas como vitamina C e bilirrubina, são capazes de sequestrar EROs de forma não-enzimática (BARTOSZ, 2010).

Sabe-se que a produção exacerbada de EROs está associada a ao processo de envelhecimento e também a patofisiologia de inúmeras doenças, como Alzheimer, Parkinson e diabetes (EDREY & SALMON, 2014; GAKI & PAPAVASSILIOU, 2014; ROCHETTE et al., 2014; WANG et al., 2014). Devido a isso, várias estratégias terapêuticas foram e estão sendo desenvolvidas com o objetivo de amenizar o efeito danoso do estresse oxidativo. Essas estratégias envolvem o uso de produto naturais e/ou compostos sintéticos, que atuam tanto no sequestro de EROs bem como na modulação da atividade das enzimas antioxidantes (MAXWELL, 1995).

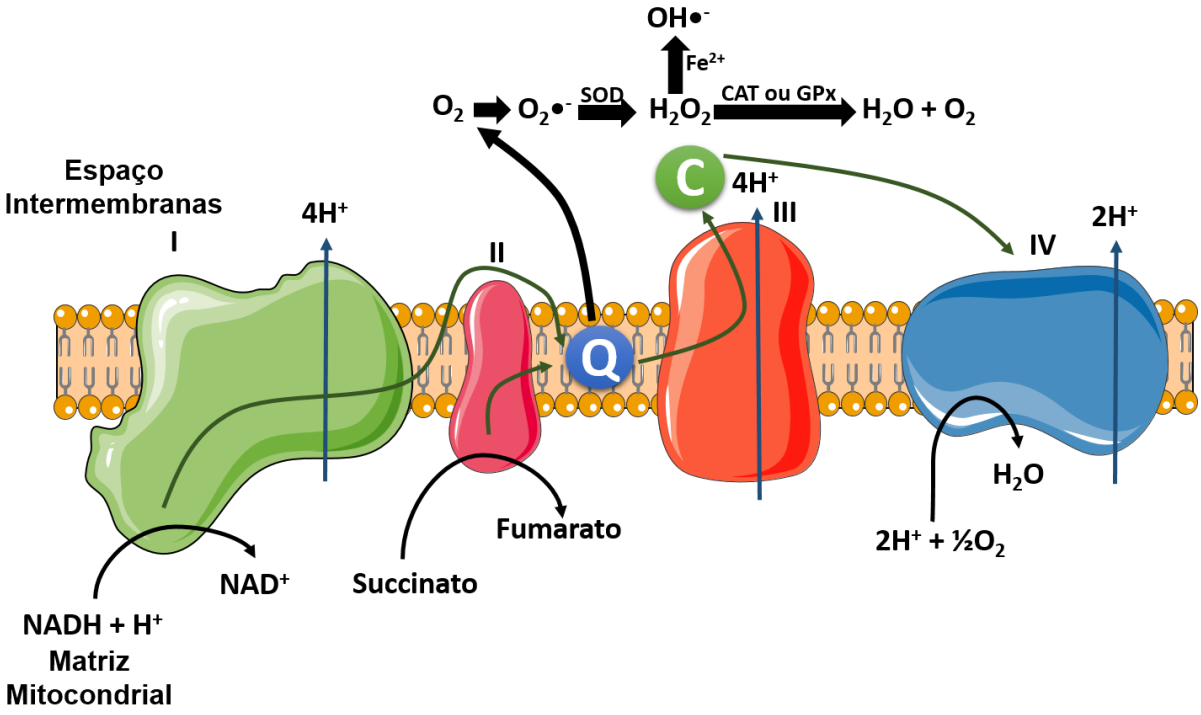


Figura 4 – Esquema mostrando a formação de EROs que acontece quando elétrons vazam da cadeia transportadora de elétrons.

2. JUSTIFICATIVA

Devido ao interesse no uso de compostos orgânicos que contêm Se e Te, a exposição dos humanos aos organocalcogênios pode aumentar no futuro, assim como os riscos relacionados a toxicidade dos mesmos. Apesar dos estudos existentes que foram mencionados anteriormente, os mecanismos de toxicidade dos organocalcogênios, no que diz respeito as alterações causadas na expressão de enzimas antioxidantes, incluindo selenoproteínas, relacionado ao potencial toxicológico destes compostos e geração de EROs, ainda não foram demonstrados em células humanas. Desta forma, este tipo de informação torna-se de grande valia para possíveis testes clínicos com esses compostos. Nesse contexto, o uso de leucócitos isolados de sangue humano é uma alternativa fácil e rápida para testar a toxicidade de organocalcogênios.

Logo, este estudo pretende avaliar e comparar o potencial toxicológico destes compostos usando técnicas simples de citotoxicidade e genotoxicidade em leucócitos isolados de sangue humano e tentar explicar estes resultados através da análise da produção de EROs pelas células e da expressão de enzimas antioxidantes e selenoproteínas, bem como a expressão de fatores de transcrição que atuam em resposta ao estresse oxidativo. E ainda, este estudo pretende quantificar a concentração relativa de composto organocalcogênio encontrada nas células em nosso modelo *ex vivo* de exposição.

3. OBJETIVOS

3.1. Objetivos gerais

Avaliar e comparar a toxicidade a nível celular e genômica dos organocalcogênios Ebs, $(PhSe)_2$ e $(PhTe)_2$ em leucócitos isolados de sangue humano e determinar seus potenciais tóxicológicos.

2.2. Objetivos específicos

- Avaliar a citotoxicidade dos compostos Ebs, $(PhSe)_2$ e $(PhTe)_2$ em leucócitos humanos utilizando o teste de exclusão do azul de Trypan;
- Avaliar a genotoxicidade dos compostos Ebs, $(PhSe)_2$ e $(PhTe)_2$ em leucócitos humanos utilizando o ensaio cometa;
- Analisar a produção de EROs e viabilidade celular dos leucócitos expostos utilizando a citometria de fluxo, usando marcadores fluorescentes diclorofluoresceína diacetato (DCFH-DA) e iodeto de propídeo (IP);
- Analisar a expressão gênica de enzimas antioxidantes como a Catalase (CAT) e Superóxido Dismutase 1 (SOD1), bem como das selenoenzimas Glutationa Peroxidase 3 (GPx3), Glutationa Peroxidase 4 (GPx4) e Tiorredoxina Redutase 1 (TRxR1) em leucócitos humanos tratados com os organocalcogênios;
- Analisar a expressão do Nrf-2 em leucócitos tratados com os organocalcogênios;
- Avaliar a quantidade de composto organocalcogênio nas células tratadas;
- Correlacionar as diferenças nos níveis de mRNA das enzimas em questão com os níveis de citotoxicidade e genotoxicidade, bem como produção de EROs, exibidos pelos leucócitos tratados.

4. METODOLOGIA E RESULTADOS

A metodologia e os resultados obtidos serão apresentados nesta dissertação na forma de um artigo científico publicado no periódico BioMed Research International em 2013 e um manuscrito a ser submetido.

4.1. Artigo publicado no periódico BioMed Research International

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Research Article

Cytotoxicity and Genotoxicity Evaluation of Organochalcogens in Human Leucocytes: A Comparative Study between Ebselen, Diphenyl Diselenide, and Diphenyl Ditelluride

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Organochalcogens, particularly ebselen, have been used in experimental and clinical trials with borderline efficacy. $(PhSe)_2$ and $(PhTe)_2$ are the simplest of the diaryl dichalcogenides and share with ebselen pharmacological properties. In view of the concerns with the use of mammals in studies and the great number of new organochalcogens with potential pharmacological properties that have been synthesized, it becomes important to develop screening protocols to select compounds that are worth to be tested *in vivo*. This study investigated the possible use of isolated human white cells as a preliminary model to test organochalcogen toxicity. Human leucocytes were exposed to 5–50 μM of ebselen, $(PhSe)_2$, or $(PhTe)_2$. All compounds were cytotoxic (Trypan's Blue exclusion) at the highest concentration tested, and Ebselen was the most toxic. Ebselen and $(PhSe)_2$ were genotoxic (Comet Assay) only at 50 μM, and $(PhTe)_2$ at 5–50 μM. Here, the acute cytotoxicity did not correspond with *in vivo* toxicity of the compounds. But the genotoxicity was in the same order of the *in vivo* toxicity to mice. These results indicate that *in vitro* genotoxicity in white blood cells should be considered as an early step in the investigation of potential toxicity of organochalcogens.

1. Introduction

Selenium (Se) is an essential microelement for human and animal nutrition [1]. It is important for selenoprotein synthesis, where it is present as the aminoacid selenocysteine [2]. Several selenoenzymes, such as Glutathione Peroxidase (GPx) and Thioredoxin Reductase (TrxR), are important for the cell defense against oxidative stress [3, 4]. Taking this role of Se in living beings, many therapeutic trials explored the use of inorganic forms of Se as pharmacological agents [5]. However, inorganic forms of Se, such as selenite and selenate, are poorly absorbed and present many toxic effects at high concentrations [6]. Consequently, the interest in organic forms of selenium, that can be less toxic and better absorbed than Se (IV) and Se (VI), has increased.

Tellurium (Te) is chemically related to Se and can be occasionally found in some proteins in bacteria, yeast, and fungi, but no functional telluroproteins have been found in animal cells [7]. In contrast to Se, Te does not have biological function [8]. However, the literature has demonstrated immunomodulatory, antioxidant, and anticancer properties of various organotellurides [9, 10]. Organotellurium compounds can also mimic Glutathione Peroxidase activity [11], and, consequently, these compounds can be potential antioxidants, effective against some cell damaging agents [12–14].

Ebselen and Diphenyl Diselenide ($(PhSe)_2$) are two organoselenium compounds that are recognized as promising pharmacological agents presenting antioxidant, anti-inflammatory, neuroprotective, and other beneficial properties [9]. These compounds can exert their pharmacological effects by

mimicking the native Glutathione Peroxidase enzyme (GPx-like activity) or by being a substrate of TrxR. The selenol intermediate formed after their reduction can reduce the levels of reactive oxygen species (ROS) in the cell and prevent oxidative damage to lipids, proteins, and DNA [15–18]. Diphenyl Ditelluride ($(PhTe)_2$) is an organotellurium compound that also showed antioxidant and other *in vitro* pharmacological properties [9]. Therefore, the experimental use of organoselenium and -tellurium compounds in different models of human diseases has increased [19–23].

On the other hand, ebselen, $(PhSe)_2$, and $(PhTe)_2$ can be toxic when administered at high doses. This toxicity is thought to be associated with inhibition of thiol- and/or selenol-containing enzymes, which can increase ROS formation, lipid peroxidation, and DNA damage [24–27].

However, the quantity of new organoselenium and -tellurium compounds with pharmacological potential that have been synthesized is increasing rapidly. Consequently, information about the toxicity of new organochalcogens is needed. However, we do not have a simple preliminary test to determine the potential toxicity of a great number of new compounds. This point is critical both in view of the time required to perform assays with vertebrates and the need of ethical adherence to the 3R principal in the use of experimental animals. Here we compare the toxicity of ebselen (which has been used in different clinical trials), $(PhSe)_2$ (which is a very simple and pharmacologically active diselenide), and $(PhTe)_2$ (a simple and pharmacologically active ditelluride which is also very toxic *in vivo* to rodents) in human white blood cells to determine whether these cells could be used to do a preliminary screening of potentially toxic new organochalcogens.

In short, the aim of this study was to define the cytotoxic concentrations of ebselen, $(PhSe)_2$, and $(PhTe)_2$ in freshly isolated white human blood cells. Therefore, human leucocytes were exposed to compounds, and their potencial cytotoxic and genotoxic effects were measured using Trypan's Blue Exclusion and Comet Assay Tests.

2. Materials and Methods

2.1. Chemicals. Ebselen, $(PhSe)_2$, $(PhTe)_2$, Trypan's Blue, dextran, and tungstosilicic acid were obtained from Sigma-Aldrich (St. Louis, MO). All the other reagents were obtained from standard chemical suppliers.

2.2. Sample Preparation. Leucocytes were isolated from heparinized venous blood obtained from healthy volunteers. The protocol of study was reviewed and approved by the appropriate institutional review board from Guidelines of the Committee of UFSM (0089.0.243.000-07).

2 mL of dextran 5% (dissolved in Phosphate Buffer Saline 1%) was added to 8 mL of blood. The tube was gently mixed and left to stand at room temperature for 45 min. Afterwards, the supernatant was centrifuged (480 ×g, 10 min) and plasma was discarded. The pellet was washed with erythrocyte lysis solution (NH_4Cl 150 mM; $NaHCO_3$ 10 mM; EDTA 1 mM) and centrifuged (480 ×g, 2 min). The supernatant was discarded and the pellet was washed twice with 1 mL erythrocyte lysis solution. After the second centrifugation, the pellet was suspended in 2 mL Hank's buffer solution (KCl 5.4 mM; Na_2HPO_4 0.3 mM; KH_2PO_4 0.4 mM; $NaHCO_3$ 4.2 mM; $MgCl_2$ 0.5 mM; NaCl 122.6 mM; D-glicose 10 mM, Tris-HCl 10 mM; $CaCl_2$ 1.3 mM; pH 7.4). The concentration of leucocytes was adjusted to 2000 cells/μL.

2.3. Leucocytes Exposure to Organochalcogens. Leucocytes were exposed to ebselen, $(PhSe)_2$, and $(PhTe)_2$ at 5, 10, and 50 μM or an equal volume of DMSO (final concentration of 0.5%) during 3 hours at 37°C. Positive control group was treated with hydrogen peroxide (H_2O_2) 2 mM and sodium azide 1 mM.

2.4. Trypan's Blue Exclusion Test. Trypan's Blue exclusion test was performed according to Mischel and Shiingi [28]. After 3 hours of incubation, 50 μL of Trypan's Blue 0.4% was mixed with 50 μL of leucocytes and left to stand at room temperature for 5 minutes. Cell viability was determined microscopically (400x magnification) and expressed as number of viable cells divided by the total number of cells multiplied by 100.

2.5. Comet Assay. Comet Assay was performed according to Collins [29] with some modifications. After three hours of incubation, 15 μL of the sample was mixed with 90 μL of low-melting point agarose 0.75% and placed in a slide precoated with agarose 1%. A coverslip was added and the samples were left to solidify at 4°C. The coverslips were removed and the slides were placed on a lysis solution (NaCl 2.5 M; EDTA 100 mM; Tris-HCl 8 mM; Triton X-100 1%; pH 10–10.5) during 24 hours at 4°C. Afterwards, the slides were incubated in an electrophoresis solution (NaOH 300 mM; EDTA 1 mM; pH 13.5) for 20 minutes at 4°C and the electrophoresis was performed (25 V; 300 mA; 7 W) for 20 minutes. All the steps were performed in the dark until this moment. After electrophoresis, the slides were washed in a neutralizing solution (Tris-HCl 400 mM; pH 7.5) three times, washed with distilled water, and left to dry. The slides were rehydrated and fixed (Trichloroacetic acid 15%; $ZnSO_4$ 5%; glycerol 5%), washed three with distilled water, and left to dry. Afterwards, the rehydrated slides were stained (Na_2CO_3 5%; NH_4NO_3 0.1%; $AgNO_3$ 0.1%; $H_4[W_{12}SiO_{40}]$ 0.25%; formaldehyde 0.15%). The slides were immersed in acetic acid 1%, washed, and left to dry.

One hundred cells randomly selected were analyzed in each sample according to tail size and intensity in five classes. The damage score for each cell can range between 0 (no damage) and 4 (maximum damage), according to Figure 1. Damage index (DI) was defined as follows: $DI = 1n1 + 2n2 + 3n3 + 4n4$, where $n1$ represents the number of cells with damage level 1, $n2$ represents the number of cells with damage level 2, $n3$ represents the number of cells with damage level 3, and $n4$ represents the number of cells with damage level 4. At least two different individuals analyzed the slides under blind conditions.

2.6. Statistical Analysis. Statistical analyses were performed using analysis of variance (ANOVA) followed by Newman-Keuls multiple test when appropriate. The results are

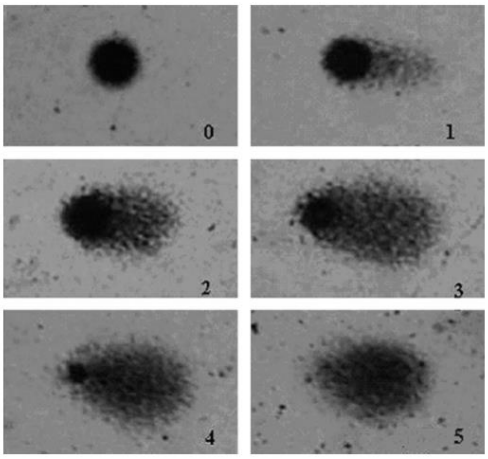

Figure 1: Damage levels considered for analysis in Comet Assay. Level 5 was excluded from our evaluation.

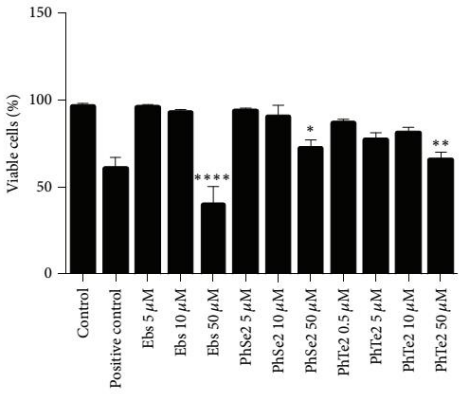

Figure 2: Cellular viability of human leucocytes exposed to organochalcogens for 3 hours. The results are expressed as mean ± SEM from four replicates. One-way ANOVA followed by Newman-Keuls ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{****}P < 0.0001$).

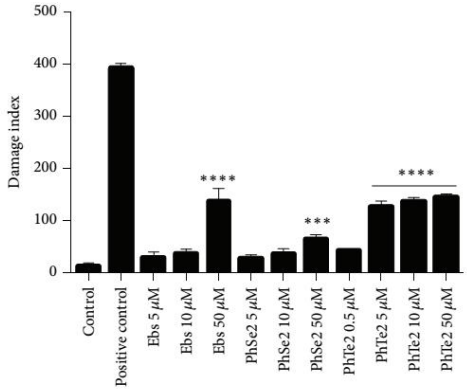

Figure 3: DI of human leucocytes exposed to organochalcogen for 3 hours. Data are expressed as mean ± SEM of four independent experiments done in duplicate. One-way ANOVA followed by Newman-Keuls ($^{***}P < 0.001$ and $^{****}P < 0.0001$).

expressed as mean ± SEM for four independent replicates. The difference was considered significant when $P < 0.05$.

3. Results and Discussion

Organoselenium compounds, such as ebselen and $(PhSe)_2$, are known as pharmacologically active compounds, exhibiting antioxidant, anti-inflammatory, neuroprotective, and antimutagenic properties [9, 20, 22, 30, 31]. At low concentrations, these compounds protect cells against the insults generated by ROS production, depleting H_2O_2 via their GPx-mimic activity [32]. In fact, ebselen was used in clinical trials with borderline efficacy [19]. Therefore, the interest in the use of organochalcogens as therapeutic agents has increased in the last years.

Despite their pharmacological properties, organochalcogens can be hepato-, reno-, and neurotoxic to mammals when administered at high doses [33–36]. Accordingly, $(PhSe)_2$ administration caused genotoxicity and prooxidant effects in mice [37, 38]. These toxic effects of ebselen, $(PhSe)_2$, and $(PhTe)_2$ can be secondary to thiol oxidation of critical target proteins, for instance, lactate dehydrogenase [39], Na^+/K^+ ATPase [9, 40], and δ-aminolevulinic acid dehydratase (δ-ALAD) [24, 41, 42]. Recently, we have demonstrated that $(PhTe)_2$ can also inhibit important antioxidant selenoenzymes [27].

The data available in the literature about organochalcogens toxicity are scarce, mainly in human cells. So, this study examined comparatively the potential cytotoxic and genotoxic effects of ebselen, $(PhSe)_2$, and $(PhTe)_2$ in human leucocytes.

DMSO did not modify cell viability. At 50 μM, ebselen, $(PhSe)_2$, and $(PhTe)_2$ caused a significant decrease in cell viability when compared to the control groups. However, the effect of ebselen (a decrease of about 60%) was higher than that of $(PhSe)_2$ (a decrease of about 20%) and that of $(PhTe)_2$ (a decrease of about 25% in leucocyte viability, Figure 2). At lower concentrations, ebselen, $(PhSe)_2$, and $(PhTe)_2$ did not cause significant decrease in cell viability (Figure 2).

DMSO did not modify damage index (DI) of DNA in human blood leucocytes. Ebselen and $(PhSe)_2$ at 50 μM and $(PhTe)_2$ at 5, 10 and 50 μM caused a significant increase in DI when compared to the control group (Figure 3). Statistical analysis indicated that the effect of 50 μM ebselen and $(PhTe)_2$ on DI was higher than that caused by $(PhSe)_2$ (Figure 3).

At 5 and 10 μM, $(PhTe)_2$ increased DI, whereas ebselen and $(PhSe)_2$ did not cause DNA damage at these concentrations.

Thus, regardless of their structural differences, the toxicity of these compounds can have a common molecular mechanism, that is, oxidation of thiol groups in critical proteins [22, 42, 43]. However, here we observed that ebselen exhibited higher cytotoxicity in human leucocytes than $(PhSe)_2$ and $(PhTe)_2$. The higher toxicity of ebselen may be related to its capacity to induce thiol oxidation on lactate dehydrogenase [39] and mitochondrial complexes I and II [44] more than $(PhSe)_2$ and $(PhTe)_2$, which can cause the impairment of cell respiration and, consequently, cell death. Additionally, we observed that ebselen was more genotoxic than $(PhSe)_2$, and $(PhTe)_2$ was the most genotoxic of the three compounds. A report in the literature shows that $(PhTe)_2$ induces cell death via oncosis [45], which is a different type of cell death than that induced by ebselen [9, 46] and $(PhSe)_2$ [47]. The different genotoxicity potential may be related to differences in the interaction of these compounds with the reparing DNA machinery, in addition to differences in the reactivity with critical thiol-containing proteins.

4. Conclusion

In summary, this study shows that ebselen, $(PhSe)_2$, and $(PhTe)_2$ can cause cytotoxicity and genotoxicity in human leucocytes, that was expressed, respectively, by a decrease in cell viability in Trypan's Blue exclusion test and an increase of DI in Comet Assay, where the cytotoxic effect of ebselen was more pronounced, while $(PhTe)_2$ presented the highest genotoxic effect in freshly isolated human leucocytes. Here, the acute cytotoxicity did not correspond with *in vivo* toxicity of the compounds [9], probably because $(PhTe)_2$ induces cell death by a different way than that induced by ebselen and $(PhSe)_2$, or otherwise they can have some common steps (for instance, oxidation of thiol proteins, but with different potency and perhaps with some different targets). However, the genotoxicity was in the same order of the *in vivo* toxicity to mice (i.e., $(PhTe)_2$ > ebselen > $(PhSe)_2$) [9], confirming that the use of Comet Assay in human leucocytes is a good strategy for a preliminary study of genotoxicity. These results indicate that *in vitro* genotoxicity in white blood cells should be considered as an early step in the investigation of potential toxicity of organochalcogens before performing *in vivo* studies with vertebrates. However, more studies are needed to elucidate the toxic effects of ebselen, $(PhSe)_2$ and $(PhTe)_2$, and their mechanisms of action in different cell types.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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4.2. Manuscrito

Toxicity of organochalcogens in human leukocytes is associated with apoptosis and antioxidant gene expression changes, but not directly related to reactive oxygen species production

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Abstract

Se-containing organic compounds, such as ebselen (Ebs) and diphenyl diselenide [$(PhSe)_2$], have been used as pharmacological agents due to their antioxidant properties. Tellurium (Te) does not have any biological function in mammals, but Te-containing organic compounds, such as diphenyl ditelluride [$(PhTe)_2$], has been used as antioxidant molecules. At high concentrations, these compounds cause toxicity by oxidazing thiol and selenol groups of proteins and ROS production. Here we analyzed whether these compounds could modulate the antioxidant gene expression profile, ROS production and apoptosis in leukocytes isolated from human blood. Since no data is available about their accumulation in isolated leukocytes, we determine their concentration in the cells by CG-MS. Apoptosis (propidium iodide) and ROS production (DCFH-DA) were determined by flow cytometry. The expression of CAT, SOD1, GPX3, GPX4, TRXR1 and NFLE2L2 genes were analyzed by RT-PCR. $(PhTe)_2$ was the only compound able to increase apoptosis rate. $(PhSe)_2$ altered the expression of CAT and SOD1, and this was accompanied by a high ROS production. All compounds decreased the expression of GPX3, but no alteration in GPX4 and TRXR1 expression was detected. All compounds decreased NFE2L2 expression (Ebs > $(PhTe)_2$ > $(PhSe)_2$). We hypothesize that the toxicity induced by these organochalcogens is not directly related to their ability of inducing ROS production.

1. INTRODUCTION

Selenium (Se) is an essential microelement for human and animal nutrition (Combs and Combs, 1984) and it is important for selenoprotein synthesis, where it is present as the amino acid selenocysteine (Holben and Smith, 1999). There are currently 25 known selenoproteins in the human proteome, most of them having unknown functions (Kryukov et al., 2003). Glutathione Peroxidase (GPx) and Thioredoxin Reductase (TrxR) are well-characterized Se-containing enzymes that participate in the cell defense against oxidative stress and in the maintenance of intracellular redox environment (Papp et al., 2007; Zhong et al., 2000).

Tellurium (Te) is chemically related to Se and can be occasionally found in some proteins in bacteria and fungi, but no functional telluroproteins have been found in animal cells. In other words, Te does not have biological functions in mammals (Taylor, 1996; Bienert et al., 2008; Pessoa-Pureur et al., 2014). However, literature has demonstrated pharmacological properties of various organic Te compounds (Nogueira et al., 2004; Jamier et al., 2010; Avila et al., 2012; Pessoa-Pureur et al., 2014). Organotellurium compounds can also mimic GPx activity and, consequently, these compounds can be potential antioxidants, effective against some cell damaging agents, such as Reactive Oxygen Species (ROS) (Andersson et al., 1994; Jacob et al., 2000; Braga et al., 2009).

In the last years, due to the pharmacological effects observed with Se and its chemical similarity with Te, many experimental and therapeutic trials explored the use of inorganic and organic forms of Se and Te as pharmacological agents in pathological situations where oxidative stress is involved (Alfthan et al., 1991; El-Bayoumy, 2001; Nogueira and Rocha, 2011; Pessoa-Pureur et al., 2014).

Ebselen (Ebs) and Diphenyl Diselenide [$(PhSe)_2$] are two organoselenium compounds that are recognized as promising pharmacological agents presenting antioxidant, anti-inflammatory, neuroprotective and other beneficial properties in many experimental models (Nogueira et al., 2004; Chanaday et al., 2008; Nogueira and Rocha, 2011; Rupil et al., 2012). These compounds exert their antioxidant effects by mimicking the native GPx enzyme (GPx-like activity) or by being substrates of TrxR. The selenol intermediate formed after their reduction can reduce the levels of ROS in the cell and prevent oxidative damage to lipids, proteins and DNA (Sies, 1993; Mugesh et al., 2001; Zhao and Holmgren, 2002; De Freitas and Rocha, 2011). Diphenyl Ditelluride [$(PhTe)_2$] is an organotellurium compound that also showed antioxidant and other *in vitro* pharmacological properties (Nogueira et al., 2004). Therefore, the experimental use of organic Se and Te compounds in different models of human diseases

has increased (Yamaguchi et al., 1998; Rossato et al., 2002; Nogueira and Rocha, 2011; Pessoa-Pureur et al., 2014).

On the other hand, Ebs, $(PhSe)_2$, and $(PhTe)_2$ can be toxic when administered at high doses. This toxicity is thought to be associated with inhibition of thiol- and/or selenol containing enzymes, which can lead to increased ROS formation and, consequently, lipid peroxidation and DNA damage (Barbosa et al., 1998; Nogueira et al., 2004; Rosa et al., 2007; Comparsi et al., 2012; Heimfarth et al., 2012a; Heimfarth et al., 2012b; Heimfarth et al., 2013; Pessoa-Pureur et al., 2014)

Due to the interest in organic Se and Te compounds as pharmacological agents, the human exposure to them can increase in the future, as well as the toxicity related to them. Besides the studies mentioned above, there is a lack of information regarding the mechanisms of toxicity of these compounds related to gene expression modulation compared to apoptosis rate and ROS generation in human cells. In these context, freshly isolated human white cells have been used as an alternative model of toxicity, constituting a simple tool for preliminary screening of compounds with potential pharmacological or toxicological properties (Santos et al., 2009a; Santos et al., 2009b; Bueno et al., 2013).

A previous work from our group showed the comparative cytotoxicity and genotoxicity of the organochalcogens Ebs, $(PhSe)_2$, and $(PhTe)_2$ in human leukocytes isolated from human blood cells (Bueno et al., 2013). This study showed that $(PhSe)_2$ was the less toxic compound in both toxicity levels analyzed and that Ebs was the most cytotoxic compound, and $(PhTe)_2$ was the most genotoxic compound. Taking into consideration this background, the aim of this study is to investigate the different toxicological potential of these compounds, hypothesizing that their mechanisms of action are mediated by ROS generation, apoptosis, and modulation of the expression of Catalase (Cat) and Superoxide Dismutase 1 (Sod1), including selenoproteins (Gpx3, Gpx4 and TRxR1), and also the expression of Nuclear Factor (Erythroid-2 like) 2 (Nrf-2), a transcriptional factor that is responsive to electrophile species and to oxidative stress (Nguyen et al., 2003; Kensler et al., 2007; Olalekan Abolaji et al., 2014). Since there is no data about the uptake or absorption of these compound by isolated human leukocytes, we also analyzed the amount of the Ebs, $(PhSe)_2$ and $(PhTe)_2$ found in cells in our experimental model.

2. MATERIALS AND METHODS

2.1. Chemicals

Ebs, $(PhSe)_2$, $(PhTe)_2$, dextran, DCFH-DA, and PI were obtained from Sigma-Aldrich (St. Louis, MO). All the other reagents were obtained from standard chemical suppliers.

The TriZOL®, DNAse I, M-MLVRT (Moloney Murine Leukemia Virus Reverse Transcriptase), and *Taq* DNA Polymerase kits were obtained from Life Technologies (Carlsbad, CA).

2.2. Sample preparation

Leukocytes were isolated from heparinized venous blood obtained from healthy volunteers. The protocol of study was reviewed and approved by the appropriate institutional review board from Guidelines of the Committee of UFSM (0089.0.243.000-07).

Two mL of dextran 5% (dissolved in Phosphate Buffer Saline 1%) was gently mixed with 8 mL of blood and left to stand at room temperature for 45 min. The supernatant was centrifuged (480 x g, 10 min) and plasma was discarded. The pellet was washed with erythrocyte lysis solution (NH_4Cl 150 mM; $NaHCO_3$ 10 mM; EDTA 1 mM) and centrifuged (480 x g, 2 min). The supernatant was discarded and the pellet was washed twice with 1 mL erythrocyte lysis solution. After the second centrifugation, the pellet was suspended in 2 mL Hank's buffer solution (KCl 5.4 mM; Na_2HPO_4 0.3 mM; KH_2PO_4 0.4 mM; $NaHCO_3$ 4.2 mM; $MgCl_2$ 0.5 mM; NaCl 122.6 mM; D-glicose 10 mM, Tris-HCl 10 mM; $CaCl_2$ 1.3 mM; pH 7.4). The concentration of leukocytes was determined in Neubauer chamber and adjusted to 2000 cells/µL.

2.3 Leukocytes exposure to organochalcogens

Leukocytes ($1x10^6$/500 µL) were exposed to Ebs, $(PhSe)_2$ and $(PhTe)_2$ at 10, 25 and 50 µM or an equal volume of dimethyl sulfoxide (DMSO) (final concentration of 0.5%) during 3 hours at 37°C for the tests with flow cytometry. For PCR tests, leukocytes ($2x10^6$/ 500 µL) were exposed to the compounds at 25 µM or an equal volume of DMSO (final concentration of 0.05%) during 3 hours at 37°C.

2.4. Apoptosis rate via PI staining

After incubation, cells were submitted to the hypotonic PI staining method described by Riccardi and Nicoletti (2006) to detect apoptosis. The samples were read in a flow cytometry device BD Accuri™ C6 (BD Life Sciences, USA). At least 150,000 events were recorded. The results are expressed as apoptosis rate compared with the control.

2.5. ROS production

After incubation, the cells were loaded with DCFH-DA 5 µM during 30 minutes at 37°C in the dark. Then, the samples were read in the flow cytometry devide BD Accuri™ C6 (BD Life Sciences, USA). As the device separates the cells according to the size and granularity, we analyzed lymphocytes and granulocytes separately accordingly to Figure 1. At least 500,000 events were recorded. The results are expressed as the percentage of fluorescence emitted by the cells when compared to the control.

2.6. mRNA levels analysis

After incubation time, leukocytes were immediately centrifuged at 480 x g during 5 minutes. The buffer was discarded and the leukocytes were homogenized in 1 mL of TriZOL®.

Total RNA extraction was performed according to manufacturer's instructions. RNA samples were dissolved in 20 µL of water, and the quality/integrity of RNA was confirmed in agarose 1% gel stained with ethidium bromide 0.5 µg/mL and visualized under ultraviolet light. RNA samples were treated with DNase I according to manufacturer's instructions and reverse transcripted in cDNA molecules using M-MLVRT reverse transcriptase enzyme according to manufacturer's instructions.

Gene expression analysis was performed through real-time Polymerase Chain Reaction (PCR). The reaction mixture for PCR reaction contains: 10 µL diluted 1:100 cDNA or water as negative control; 0.2 µM primers; 0.2 mM deoxiribonucleotide triphosphates; 1.5-3 mM $MgCl_2$; 1x PCR buffer; 0.05 U/µL *Taq* DNA Polymerase; and 0.01x SYBR Green. The reaction was submitted to the following cycling conditions: *Taq* DNA Polymerase activation at 95°C during 3 minutes, followed by 40 cycles of 95°C during 15 seconds (denaturation), 58-62°C during 15 seconds (annealing), and 72°C during 15 seconds (extension). Threshold levels were manually determined using StepOne Software v2.0 (Applied Biosystems, NY). SYBR Green

fluorescence and Cq values generation were analyzed using StepOne Software v2.0 (Applied Biosystems, NY).

The analyzed genes were CAT, GPX3, GPX4, NFE2L2, SOD1, and TRXR1. GAPDH and RPL30 were used as reference genes. Primers were designed using Primer3 software v4.0.4 (http://frodo.wi.mit.edu/primer3/) based on published sequences in GenBank (http://www.ncbi.nlm.nih.gov/genbank/) as shown in Table 1.

Results are expressed as relative expression calculated using $2^{-\Delta\Delta Cq}$ formula (Livak and Schmittgen, 2001).

2.7. Gas chromatography-Mass spectrometry (GC-MS) analysis

Leukocytes ($1x10^7$) were exposed to 100 nmol of each compound (final concentration 200 µM) in a volume of 500 µL during 30 minutes at 37°C. Then the samples were centrifuged and the supernatant was transferred to another tube. Both supernatant and pellet were submitted to extraction with 1 mL of dichloromethane and subsequently injected into a GC-MS device. A tube containing 100 nmol of each compound (final concentration 200 µM) and buffer was used as control. The 500 µL solution of the control tube (without addition of leukocytes) was incubated for 30 minutes at 37°C and extracted with 1 ml of dichloromethane.

The mass spectra were obtained in a Shimadzu GC-MS-QP2010 Plus gas chromatograph coupled to a mass detector. The gas chromatographies were run on a 30.0 m x 0.25 mm RTX-5MS column (Thickness: 0.25 µm). The running conditions were: time: 30 minutes; injector temperature: 280°C; initial column oven temperature: 30°C; linear gradient: 20°C/min up to 300°C. Helium (5.4 mL/min; linear velocity: 39.7 cm/s) was employed as carrier gas (Pressure: 68.2 KPa). The result was expressed for molecular ion and its fragments are described as the relations between their atomic mass and corresponding charge (m/z), together with their percent relative abundance (%).

2.8. Statistical Analysis

Statistical analysis were performed using parametric paired t test. The results are expressed as mean ± SEM for six independent experiments performed in triplicates. The difference was considered significant when $P < 0.05$.

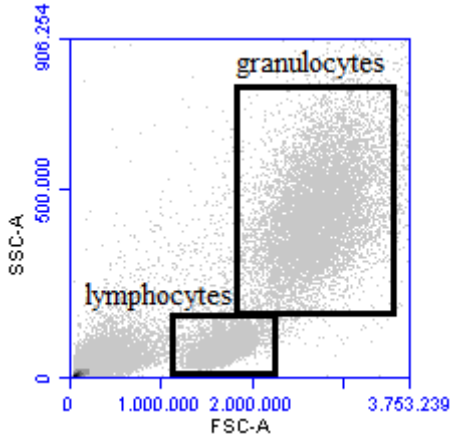

Figure 1 – Leukocytes populations used to measure ROS production via DCFH oxidation in this study.

Table 1

Primers used in gene expression experiment.

Gene	Symbol	Reference sequence	Primer sequence (5'-3')	Amplicon
Catalase	CAT	NM_001752	TGGAAAGAAGACTCCCATCG CCAACGAGATCCCAGTTACC	132 bp
Glyceraldehyde 3-phosphate dehydrogenase	GAPDH	NM_001256799	TTCGTCATGGGTGTGAACC AGTTGTCATGGATGACCTTGG	112 bp
Glutathione peroxidase 3	GPX3	NM_002084	GCAGTATGCTGGCAAATACG AGAATGACCAGACCGAATGG	120 bp
Glutathione peroxidase 4	GPX4	NM_001039847	AGATCCAACCCAAGGGCAAG GACGGTGTCCAAACTTGGTG	72 bp
Nuclear Factor (Erythroid-like 2) 2	NFE2L2	NM_001145412	AACCAGTGGATCTGCCAACT ACGTAGCCGAAGAAACCTCATT	134 bp
Ribosomal Protein L30	RPL30	NM_000989	GATGATCAGACAAGGCAAAGC ACACCAGTTTTAGCCAACATAGC	105 bp
Superoxide dismutase 1	SOD1	NM_000454	GCCAAAGGATGAAGAGAGGCAT ACATCGGCCACACCATCTTT	72 bp
Thioredoxin reductase 1	TRXR1	NM_001093771	GCATCAAGCAGCTTTGTTAGG TCATTCTGTCCCAATCATGC	95 bp

Sequences shown in each cell are, respectively, the forward and the reverse primer.

3. RESULTS

3.1. Apoptosis rate measure

Ebs (Figure 2A) and $(PhSe)_2$ (Figure 2B) did not alter the apoptosis rate in the cells. In contrast, exposure of leukocytes to $(PhTe)_2$ for 3 hours (Figure 2C) increased the apoptosis rate at all concentrations tested ($P < 0.05$).

3.2. ROS production analysis via DCFH oxidation

In lymphocytes, Ebs (Figure 3A) and $(PhSe)_2$ (Figure 3B) induced a significant increase in ROS production. In contrast, $(PhTe)_2$ (Figure 3C) did not alter ROS production in lymphocytes. In granulocytes, Ebs (Figure 4A) did not alter ROS production, but $(PhSe)_2$ (Figure 4B) increased ROS production at all concentrations tested ($P < 0.05$). Similarly, $(PhTe)_2$ induced an increase in ROS production in granulocytes, but this was significant only at 25 µM of $(PhTe)_2$ ($P < 0.05$).

3.3. Catalase (CAT), Superoxide Dismutase 1 (SOD1) and Nrf-2 mRNA expression analysis

$(PhSe)_2$ was the only compound that caused a statistically significant decrease in the expression of CAT mRNA ($P < 0.05$; Figure 5A). There was a tendency to decrease the expression of CAT mRNA in the cells exposed to 25 µM of Ebs or $(PhTe)_2$ (Figure 5A). In contrast to CAT, $(PhSe)_2$ caused a significant increase in the expression of SOD1 ($P < 0.05$; Figure 5B). Ebs caused also a significant decrease in SOD1 mRNA expression ($P < 0.05$); whereas $(PhTe)_2$ did not alter SOD1 expression in human leukocytes (Figure 5B).

All compounds caused a significant decrease in the expression of NFE2L2 gene (Figure 5C). The proportion in which they induced down-regulation was in the order Ebs ($P < 0.001$) > $(PhTe)_2$ and $(PhSe)_2$ ($P < 0.05$).

3.4. Seleno-Enzymes mRNA Expression Analysis

All compounds caused a significant decrease in the expression of GPX3 (Figure 6A), which occurred in the following proportion: Ebs ($P < 0.0001$) > $(PhTe)_2$ ($P < 0.001$) > $(PhSe)_2$ ($P < 0.05$).

In constrast to GPX3, the levels of GPX4 and TRX1 mRNA were not modified by organochalcogens (Figure 6B and C, respectively).

3.5. GC-MS analysis

After the incubation of leukocytes with organochalcogens, the compounds were found almost completely in pellet fraction. These results indicated that the compounds partitioned preferentially to the cells ($P < 0.0001$). This was much more evident for $(PhSe)_2$ and $(PhTe)_2$ ($P < 0.05$ and $P < 0.005$). In effect, only small amounts of the compounds were found in supernatant fractions (Figure 7).

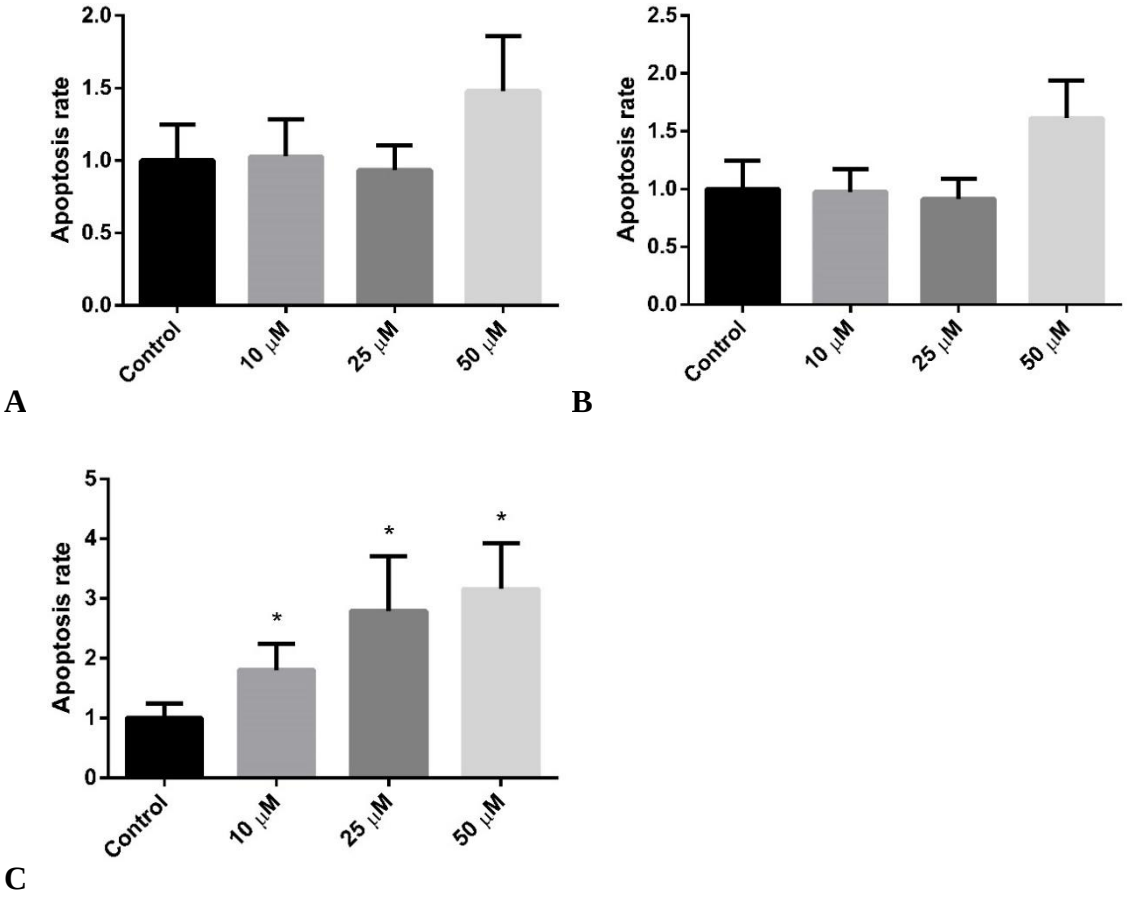

Figure 2 – Apoptosis rate in human leukocytes treated with Ebselen (Ebs; A); Diphenyl Diselenide $(PhSe)_2$, B] and Diphenyl Ditelluride $[(PhTe)_2$, C]. Leukocytes were exposed to 0.5% DMSO or organochalconges for 3 hours at 37°C. Apoptosis was assessed by the PI staining and flow cytometry. The results are shown as Mean ± SEM from 6 independent experiments. Parametric paired t test in comparison with the control group (* $P < 0.05$). The apoptosis rate in control was of 2.69 ± 0.1%.

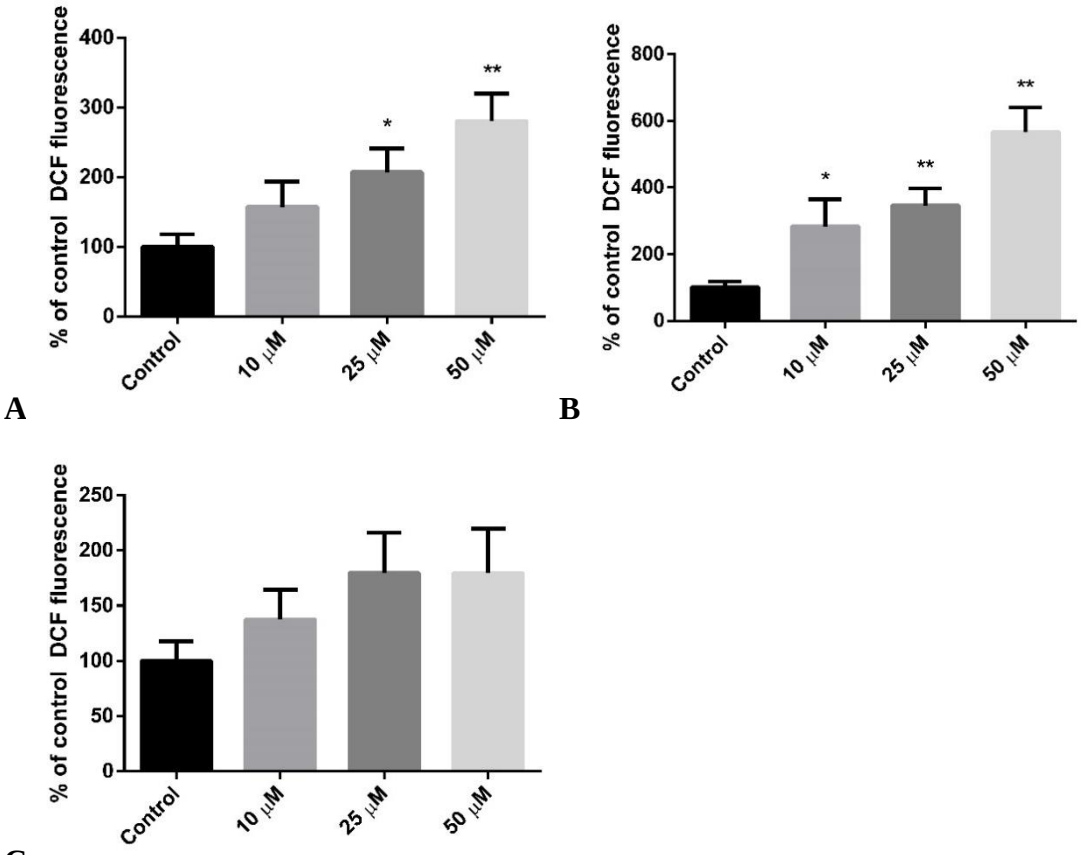

Figure 3 – ROS production in human leukocytes (lymphocytes population) treated with Ebselen (Ebs; A); Diphenyl Diselenide $(PhSe)_2$, B] and Diphenyl Ditelluride $[(PhTe)_2$, C]. Leukocytes were exposed to 0.5% DMSO or organochalconges for 3 hours at 37°C. The ROS production was measured by DCFH fluorescence and flow cytometry. The results are shown as Mean ± SEM from 6 independent experiments. Parametric paired t test in comparison with the control groups (* $P < 0.05$; ** $P < 0.01$). DCFH fluorescence in control was of 383,581.73 ± 409.2.

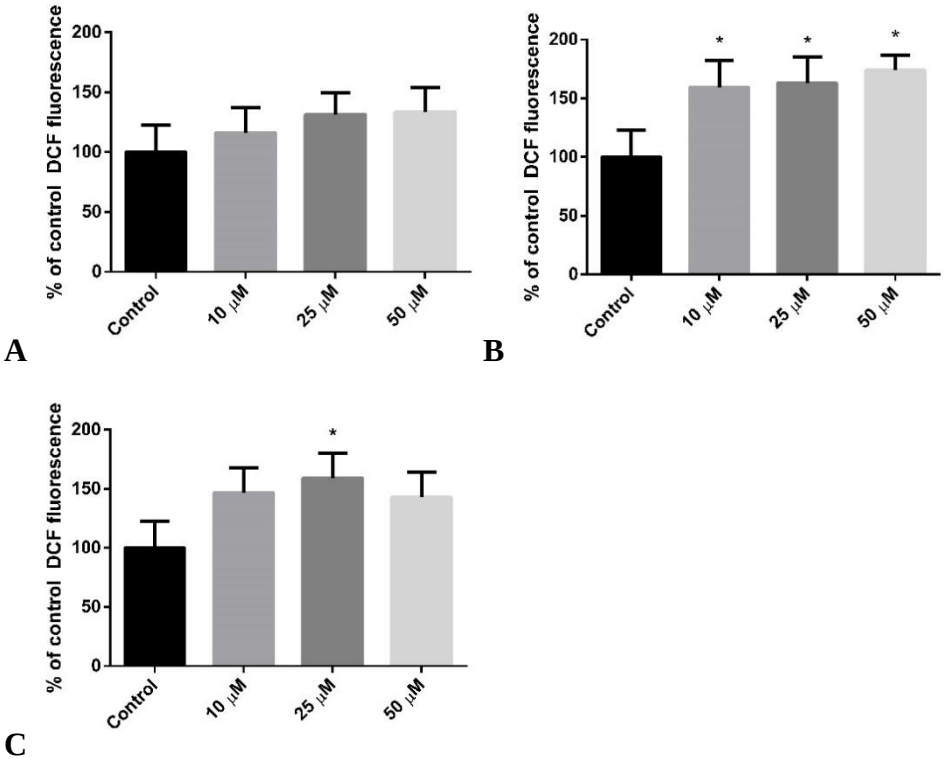

Figure 4 - ROS production in human leukocytes (granulocytes population) treated with Ebselen (Ebs; A); Diphenyl Diselenide $(PhSe)_2$, B] and Diphenyl Ditelluride $[(PhTe)_2$, C]. Leukocytes were exposed to 0.5% DMSO or organochalconges for 3 hours at 37°C. ROS was measured using the DCFH-DA fluorescent probe and flow cytometry. The results are shown as Mean ± SEM from 6 independent experiments. Parametric paired t test in comparison with the control groups (* $P < 0.05$). DCFH fluorescence in control was of 4,685,647.54 ± 1,613.8.

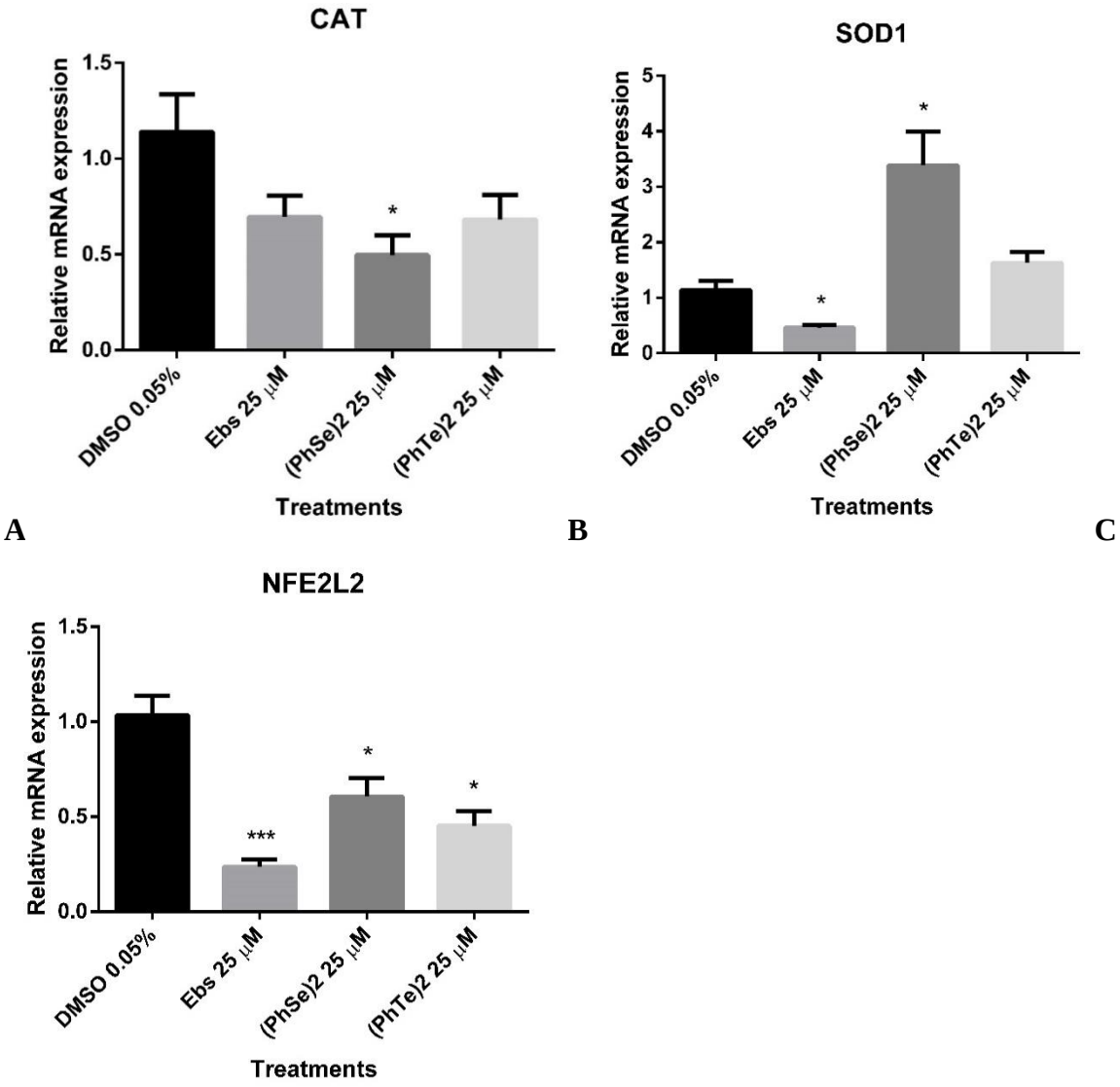

Figure 5 – CAT (A), SOD1 (B), and NFE2L2 (C) mRNA levels in human leukocytes treated with Ebselen (Ebs); Diphenyl Diselenide [$(PhSe)_2$] and Diphenyl Ditelluride [$(PhTe)_2$]. Leukocytes were exposed to 0.05% DMSO or organochalconges for 3 hours at 37°C. The results are shown as Mean ± SEM from 6 independent experiments. Parametric paired t test in comparison with the control groups (* $P < 0.05$; *** $P < 0.001$).

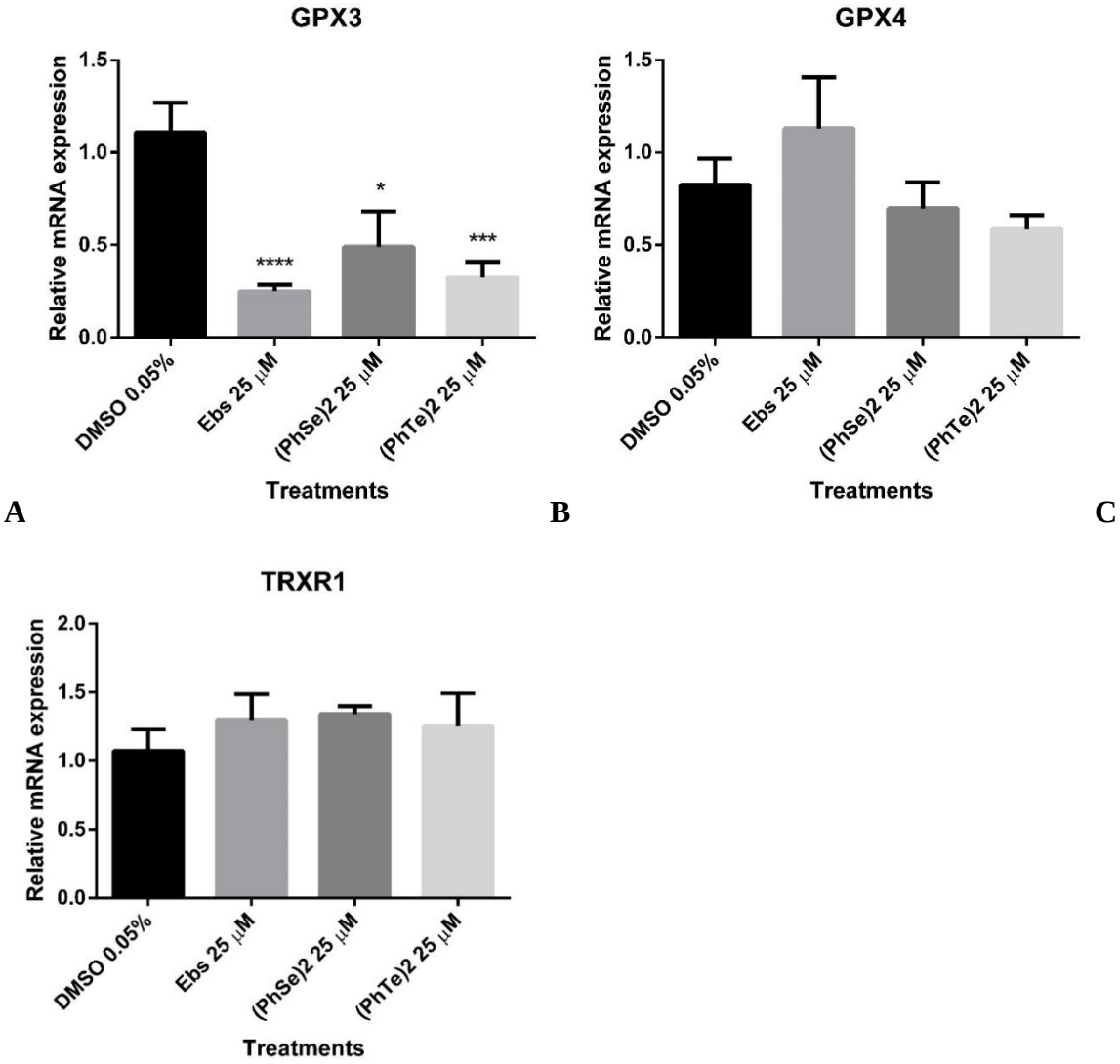

Figure 6 – GPX3 (A), GPX4 (B), and TRXR1 (C) mRNA levels in human leukocytes treated with Ebselen (Ebs); Diphenyl Diselenide [$(PhSe)_2$] and Diphenyl Ditelluride [$(PhTe)_2$]. Leukocytes were exposed to 0.05% DMSO or organochalconges for 3 hours at 37°C. The results are shown as Mean ± SEM from 6 independent experiments. Parametric paired t test in comparison with the control groups (* $P < 0.05$; *** $P < 0.001$; **** $P < 0.0001$).

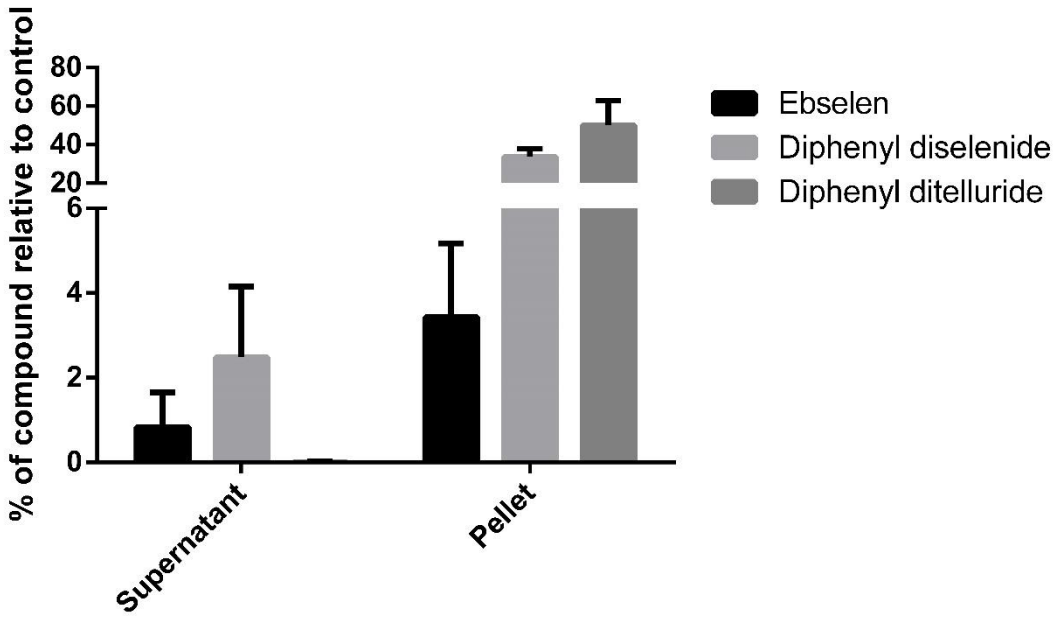

Figure 7 – Relative Levels of Ebselen (Ebs), Diphenyl Diselenide $(PhSe)_2$, and Diphenyl Ditelluride $(PhTe)_2$ in the Supernant and in the Pellet. Leukocytes were exposed to 0.5% DMSO or organochalconges for 3 hours at 37°C. The Leukocytes were centrifuged. Then the supernatants and pellets were extracted with dichloromethane as detailed in material and methods. The control groups for each organochalcogen correspond to the values obtained after extraction of the incubation medium without leukocytes with dichloromethan. The total amount of Ebs, $(PhSe)_2$, and $(PhTe)_2$ recovered from the control supernatants were 22.0 ± 2.8 nmol, 51.7 ± 5.0 nmol, and 35.2 ± 3.4 nmol, respectively (n= 3 for each compound).

4. DISCUSSION

Organochalcogens Ebs, $(PhSe)_2$ and $(PhTe)_2$ can cause thiol oxidation of many cellular components. Consequently, they can decrease the levels of reduced Glutathione (GSH) and inhibit thiol-containing enzymes. For instance, δ-aminolevulinate dehydratase, Na^+/K^+ ATPase, and Lactate Dehydrogenase can be inhibited by organochalcogens (Barbosa et al., 1998; Nogueira et al., 2004; Lugokenski et al., 2011; Rocha et al., 2012). These compounds can, also, interact with mitochondrial complexes I and II, causing mitochondrial dysfunction and enchancing ROS production (Puntel et al., 2013). In a previous study, we analyzed the cytotoxicity and genotoxicity induced by these compounds after 3 hours of incubation in leukocytes. Ebs was the most cytotoxic, whereas $(PhTe)_2$ was the most genotoxic of the three compounds tested (Bueno et al., 2013). Here, we tested the same compounds for the same incubation period and analyzed apoptosis, ROS production, and the expression levels of antioxidant proteins, as well the relative amount of compound found in the cells in our *in vitro* exposure model.

$(PhTe)_2$ is considered more toxic in rodents when compared to Ebs and $(PhSe)_2$ (Nogueira et al., 2004). The results obtained here with PI staining are in agreement with this. Besides Ebs and $(PhSe)_2$ did not induce a significant alteration in apoptosis rate of treated leukocytes, $(PhTe)_2$ increased this parameter at all concentrations tested. In our previous study, Ebs was the compound with the higher toxicity when compared to $(PhSe)_2$ and $(PhTe)_2$ in Trypan's Blue exclusion test (Bueno et al., 2013). The reasons for the disparity between the two tests is still unclear. Roy and Hardej (2011) also observed a discrepancy between Trypan's Blue exclusion test and MTT assay in hippocampal astrocytes exposed to $(PhTe)_2$ and concluded that $(PhTe)_2$ induced astrocytes death via oncosis instead of apoptosis. However, here PI staining indicated that leukocytes underwent apoptosis (Riccardi and Nicoletti, 2006). The discrepancies between the two studies can be related to the type of cell used, time of incubation, and $(PhTe)_2$ concentration used. More studies will be needed to clarify these results. So, the next experiments were performed aiming to explain the organochalcogen-induced cytotoxicity observed here.

Previous study shows that $(PhSe)_2$, when compared to Ebs and $(PhTe)_2$, is less toxic (Nogueira et al., 2004; Lugokenski et al., 2011; Bueno et al., 2013; Puntel et al., 2013; Meinerz et al., 2014). Surprisingly, $(PhSe)_2$ was the only compound able to increase ROS production in both granulocytes and lymphocytes, while Ebs increased ROS production only in lymphocytes. $(PhTe)_2$ increased ROS production only in granulocytes. Consequently, organochalcogen-

induced ROS production in human leukocytes cannot explain their toxicity in isolated cells and, probably, in rodents after *in vivo* administration. Of particular toxicological importance, $(PhTe)_2$ which is the most toxic agent after *in vivo* exposure in mice and rats (Nogueira et al., 2004; Meinerz et al., 2014) did not induce ROS production in isolated human leukocytes.

Then, we performed gene expression tests to explain the different potential toxicity of these compounds. In this study, organochalcogen exposed leukocytes exhibited a complex effect on the expression of CAT and SOD1. $(PhSe)_2$ was the only compound that caused a significant decrease in CAT expression. $(PhSe)_2$ was also capable of increasing SOD1 expression, and Ebs induced a decrease in SOD1 expression. The meaning of this expression regulation in CAT and SOD1 needs to be further investigated.

GPx3 is the extracellular/plasmatic GPx isoform, responsible for H_2O_2 detoxification in plasma and in the extracellular matrix (Yoshimura et al., 1991). In this study, all compounds decreased GPX3 expression, whereas Ebs and $(PhTe)_2$ showed a greater statistical difference from the control than $(PhSe)_2$. The higher toxicity of Ebs and $(PhTe)_2$ when compared to $(PhSe)_2$ observed here can be related, in part, to GPX3 gene expression modulation. Despite the GPx-like activity of these compounds, the decrease in the expression of this selenoenzyme can leave the cell vulnerable to the insults caused by increased generation of ROS in the presence of high concentrations of these organochalcogens.

GPx4 enzyme is the GPx isoform present in cellular membranes, while *TRxR* is a selenoenzyme responsible for reverting the oxidative damage caused in proteins, being responsible for keeping a reducing environment in the living cells (Roveri et al., 1994; Arnér and Holmgren, 2000). It seems that the expression of GPX4 and TRXR1 in the conditions tested here is not directly related to the toxicity exerted by organochalcogens, since any of these compounds significantly altered the expression of these selenoenzymes in human leukocytes. Zhang et al. (2013) showed that $(PhSe)_2$ treatment induced TRXR1 up-regulation in cultured macrophages and monocytes. The difference between these results are probably associated with the differences in time and concentration incubation, as well as the cell types used.

Finally, as we observed that the antioxidant gene expression profile was profoundly altered in the cells exposed to organochalcogens, we aimed to see if the expression of *Nrf-2*, a transcription factor for antioxidant enzymes that is activated under oxidative stress conditions (Ishii et al., 2000), was altered. All the compounds were able to decrease NFE2L2 expression in human leukocytes. So, we can hypothesize that the toxicity induced by Ebs, $(PhSe)_2$, and $(PhTe)_2$ high doses exposure in human leukocytes can be related to a down-regulation in the expression of antioxidant enzyme.

Besides the pharmacological properties of organic Se and Te compounds, they can cause toxic effects when administered at high concentrations (Nogueira et al., 2004). We can observe that these compounds increase ROS production in treated human leukocytes and that, in almost all cases, this is accompanied by a down-regulation of important enzymes that are involved in the cell antioxidant defense. Taking into consideration the data presented here, we can hypothesize that the decrease in cellular viability induced by these compounds is related with the down-regulation of *Nrf-2* and other antioxidant enzymes expression, leaving the cell more vulnerable to ROS damage, and that ROS production is not directly related to their toxicity.

Finally, we also observed that the compounds Ebs, $(PhSe)_2$ and $(PhTe)_2$ accumulates in leukocytes, probably due to their affinity to cellular membranes, and that this affinity increases with the apolarity of the compound. This information show us that organochalcogen were in cells during the incubation period, generating the observed effects. The results presented here indicated that the hydrophobic compounds were taken up by the cells and part of them partionated to the hydrophobic enviroment of biological membranes and that possibly part of them were metabolized to more hydrophilic intermediates (e.g. condensation with GSH or with glucoranidation of the selenol intermediate). It is known that Ebs can be metabolized to two major compounds: 2-glucuronylselenobenzanilide and 4-hydroxy-2-methyl-selenobenzanilide (Terlinden et al., 1988), while $(PhSe)_2$ and $(PhTe)_2$ metabolism knowledge is elusive (Prigol et al., 2012), but it is know that the bond between Se and carbon is broken in $(PhSe)_2$ metabolism (Adams Jr. et al., 1989). However, further studies are needed to clarify if the organochalcogens are undergoing to some chemical transformation in *in vitro* exposure models, such as leukocytes and other cells types, and to identify these metabolites if they exist.

In conclusion, this study shows that the organochalcogens Ebs, $(PhSe)_2$, and $(PhTe)_2$ alter the antioxidant gene expression profile in human leukocytes, that was accompanied by a decrease in *Nrf-2* protein expression, increased ROS production and apoptosis rate, being these results not directly related. We can also conclude that the organochalcogens are efficiently uptake by the human leukocytes in the conditions presented here. However, more studies are needed to better understand the mechanisms of toxicity of these compounds as well as the role of oxidative stress in these effects.

Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgements

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5. DISCUSSÃO

Compostos orgânicos de Se e Te, como Ebs, $(PhSe)_2$ e $(PhTe)_2$, são conhecidos por exibirem atividade farmacológica, mostrando propriedades antioxidantes, anti-inflamatória, neuroprotetora e antimutagênica (ROSSATO et al., 2002; FARINA et al., 2003; NOGUEIRA et al., 2004; FREITAS et al., 2009; NOGUEIRA & ROCHA, 2011). Em baixas concentrações, estes compostos protegem as células contra os danos gerados pela produção de EROs, degradando o H_2O_2 através de sua atividade mimética a GPx (MUGESH & SINGH, 2000). De fato, o Ebs foi usado em testes clínicos com eficácia incerta (YAMAGUCHI et al., 1998). Logo, o interesse no uso de organocalcogênios como agentes terapêuticos aumentou nos últimos anos.

Apesar das propriedades farmacológicas descritas para os compostos orgânicos de Se e Te, estudos prévios mostram que os organocalcogênios Ebs, $(PhSe)_2$ e $(PhTe)_2$ podem causar oxidação de tióis em muitos componentes celulares, diminuindo os níves de GSH, e inibindo várias enzimas que contem tiol, como a δ-ALAD, Na^+/K^+ ATPase e LDH (BARBOSA et al., 1998; MEOTTI et al., 2003; NOGUEIRA et al., 2004; LUGOKENSKI et al., 2011). Estes compostos também podem interagir com os complexos mitocondriais I e II, levando a disfunção mitocondrial e produção aumentada de EROs (PUNTEL et al., 2013). Esses efeitos podem ser atribuídos a alta reatividade dos grupos selenol e telurol, que podem reagir com muitos componentes celulares, causando danos a célula (NOGUEIRA et al., 2004). Assim, o objetivo deste estudo foi determinar o potencial toxicológico do Ebs, $(PhSe)_2$ e $(PhTe)_2$ em leucócitos humanos e, através da análise da expressão de enzimas antioxidantes, determinar os mecanismos moleculares de seus efeitos tóxicos.

Para analisar a citotoxicidade induzida pelos organocalcogênios em leucócitos humanos, utilizamos o teste de exclusão do azul de Trypan e a marcação com iodeto de propídeo (IP). No teste com azul de Trypan, todos os compostos reduziram a viabilidade celular dos leucócitos de forma significativa apenas na concentração de 50 µM, em que o composto Ebs foi o mais citotóxico, seguido pelo $(PhTe)_2$ e pelo $(PhSe)_2$. No teste com IP, em que foi utilizado um protocolo específico para detectar apoptose, o $(PhTe)_2$ foi o único composto capaz de aumentar de forma significativa a taxa apoptótica em todas as concentrações testadas (10, 25 e 50 µM), enquanto os outros compostos não induziram nenhuma alteração na taxa apoptótica dos leucócitos humanos. O $(PhTe)_2$, quando comparado com o Ebs e o $(PhSe)_2$, é o composto mais tóxico *in vivo* (NOGUEIRA et al., 2004). Logo, não está claro para nós o motivo desta

disparidade no resultado da exclusão do azul de Trypan. Roy e Hardej (2011) fizeram a mesma observação com astrócitos de hipocampo de rato tratados com $(PhTe)_2$, comparando os resultados do teste de exclusão do azul de Trypan e do ensaio MTT, e concluiram que o $(PhTe)_2$ induz morte celular por oncose em vez de apoptose. Porém, isto não parece estar acontecendo aqui, já que a marcação com IP é específica para detectar células em processo apoptótico (RICCARDI & NICOLETTI, 2006).

Em seguida, o Ensaio Cometa foi utilizado para avaliar a genotoxicidade induzida pelos organocalcogênios em leucócitos humanos. Enquanto o $(PhTe)_2$ aumentou o índice de dano em todas as concentrações testadas (5, 10 e 50 µM), o Ebs e o $(PhSe)_2$ o fizeram apenas na concentração mais alta. Neste caso, a genotoxicidade dos organocalcogênios está de acordo com a toxicidade *in vivo* desses compostos ($(PhTe)_2$ > Ebs > $(PhSe)_2$) (NOGUEIRA et al., 2004). Logo, o dano no DNA observado nos leucócitos tratados com os organocalcogênios está diretamente relacionado com a toxicidade causada pelos mesmos, sendo que estudos posteriores são necessários para determinar se as quebras na cadeia de DNA são uma consequência direta ou indireta da exposição a esses compostos.

Para determinar a produção de EROs pelas células expostas aos organocalcogênios, utilizamos o marcador fluorescente diclorofluoresceína (DCFH), cuja análise foi realizada por meio da citometria de fluxo. Devido ao fato do citômetro separar os leucócitos em duas populações celulares distintas, nomeadamente linfócitos e granulócitos, a análise foi realizada separadamente para cada tipo celular. Surpreendentemente, o composto $(PhSe)_2$, considerado o menos tóxico entre os três, foi o único capaz de aumentar significativamente a produção de EROs em todas as concentrações testadas (10, 25 e 50 µM) e em ambos os tipos celulares. O Ebs aumentou significativamente a produção de EROs nos linfócitos nas concentrações de 25 e 50 µM, enquanto o $(PhTe)_2$ causou um leve aumento na produção de EROs nos granulócitos na concentração de 25 µM. Como podemos ver, a produção de EROs em leucócitos humanos não está diretamente relacionada com a toxicidade dos mesmos nos leucócitos, e a alta geno- e citotoxicidade do $(PhTe)_2$ parece não estar relacionada com a produção de EROs.

Com o objetivo de explicar os diferentes potenciais toxicológicos dos organocalcogênios em questão, nós analisamos a expressão gênica de enzimas antioxidantes. O $(PhSe)_2$ foi capaz de reduzir de forma significativa a expressão de CAT e de aumentar a expressão da SOD1, enquanto o Ebs reduziu a expressão da SOD1. O $O_2^{\bullet -}$ é uma ERO mais reativa quando comparado com o H_2O_2, sendo a SOD a enzima responsável por catalisar a reação que converte o $O_2^{\bullet -}$ em H_2O_2 (MCCORD & FRIDOVICH, 1969; DRÖGE, 2002). Logo, propomos que o aumento na expressão da SOD1 é um dos mecanismos envolvidos na ação

antioxidante do $(PhSe)_2$, já que um aumento na expressão da SOD1 resultaria numa conversão mais eficiente do instável e altamente reativo $O_2^{\bullet -}$ no menos reativo H_2O_2, estando de acordo com os resultados observados no teste de oxidação da DCFH, já que este marcador fluorescente é oxidado pelo H_2O_2, mas não pelo $O_2^{\bullet -}$ (LEBEL et al., 1992). O Ebs foi capaz de diminuir a expressão da SOD1 e de aumentar a produção de EROs (H_2O_2) nos leucócitos. Logo, o aumento na produção de H_2O_2 nos leucócitos expostos ao Ebs parece não estar relacionado com um aumento na atividade da enzima SOD1.

A CAT é uma das enzimas responsáveis pela degradação do H_2O_2 em água e O_2 (MAY, 1901). O $(PhSe)_2$ pode degradar o H_2O_2 por dois mecanismos: atuando como um mimético da GPx e/ou atuando como um substrato para a enzima TRxR, onde o PhSeH formado pode reduzir o H_2O_2 (WILSON et al., 1989; FREITAS & ROCHA, 2011). Neste estudo, o $(PhSe)_2$ diminuiu significativamente a expressão da CAT em leucócitos humanos, e isso aconteceu concomitantemente com um aumento significativo na produção de EROs. Levando em consideração a toxicidade mais baixa do $(PhSe)_2$ entre os compostos testados aqui, nós assumimos que o $(PhSe)_2$ está aumentando a produção de H_2O_2, o que pode ser explicado pelo aumento na expressão da SOD1, e também que o $(PhSe)_2$ está protegendo as células desse aumento na produção de EROs. Então, nós hipotetizamos que as alterações na expressão da CAT e da SOD1 em uma exposição alta ao $(PhSe)_2$ em sistemas biológicos, juntamente com sua capacidade de induzir oxidação de tióis em muitas proteínas e sua habilidade de aumentar a produção de EROs, pode levar a toxicidade observada na exposição de altas concentrações desse organocalcogênio.

Concluída a análise da expressão das enzimas antioxidantes da fase I, nosso foco se voltou para as enzimas contendo Se. A GPX3 é a isoforma da GPx extracelular/plasmática, responsável pela detoxificação do H_2O_2 no plasma e na matriz extracelular (YOSHIMURA et al., 1991). Neste estudo, todos os compostos diminuíram a expressão da GPX3 em leucócitos humanos, na proporção Ebs > $(PhTe)_2$ > $(PhSe)_2$. A maior toxicidade do Ebs e do $(PhTe)_2$, quando comparado ao $(PhSe)_2$, pode estar relacionada, em parte, a modulação da expressão do gene GPX3. Apesar da atividade mimética a GPx destes compostos, a redução na expressão dessa selenoenzima pode deixar a célula vulnerável aos insultos causados pelo aumento na geração de EROs na presença de altas concentrações desses organocalcogênios.

A enzima GPX4 é a isoforma da GPx presente nas membranas biológicas, enquanto a TRxR é uma selenoenzima responsável por reverter o dano oxidativo causado nas proteínas, sendo assim responsável por manter um ambiente redutor no interior das células vivas (ROVERI et al., 1994; ARNÉR & HOLMGREN, 2000). Nas condições em que os leucócitos

foram expostos aos compostos neste estudo, parece que a expressão dos genes GPX4 e TRXR1 não está diretamente relacionada com a toxicidade exercida por esses organocalcogênios nos leucócitos humanos, sendo que nenhum dos compostos testados foi capaz de alterar de forma significativa a expressão dessas selenoenzimas. Porém, o estudo de Zhang et al. (2013) mostrou que o tratamento com $(PhSe)_2$ aumentou a expressão da TRXR1 em macrófagos e monócitos humanos em cultura. As diferenças entre estes resultados estão provavelmente associadas com as diferenças no tempo de incubação e na concentração, bem como os tipos celulares usados.

Como pode ser observado, o perfil de expressão de genes antioxidantes nos leucócitos foi profundamente alterado pela exposição aos organocalcogênios. Então, nós analisamos a expressão da proteína Nrf-2, um fator de transcrição para enzimas antioxidantes que é ativado em situações de estresse oxidativo (ISHII et al., 2000). Todos os compostos foram capazes de diminuir a expressão do gene NFE2L2 nos leucócitos humanos, onde o efeito do Ebs foi maior que o do $(PhTe)_2$ e $(PhSe)_2$. O $(PhSe)_2$ foi o composto com o menor efeito na expressão do NFE2L2 e com o maior efeito na produção de EROs, logo o $(PhSe)_2$, apesar de reduzir a expressão do NFE2L2 quando comparado ao controle, induziu uma maior produção de EROs quando comparado aos outros compostos e uma menor redução na expressão do NFE2L2. Podemos concluir que a toxicidade induzida pela exposição a altas concentrações de Ebs, $(PhSe)_2$ e $(PhTe)_2$ em leucócitos humanos pode estar relacionada a uma diminuição na expressão de enzimas antioxidantes, deixando a célula vulnerável aos efeitos deletérios das EROs.

Apesar das propriedades farmacológicas dos compostos orgânicos de Se e Te, eles podem causar efeitos tóxicos quando administrados em altas concentrações (NOGUEIRA et al., 2004). Podemos observar que esses compostos aumentam a produção de EROs em leucócitos humanos tratados e que isso está acompanhado, na maioria das vezes, de uma redução na expressão de importantes enzimas envolvidas na defesa antioxidante da célula. Então, levando em consideração os dados apresentados aqui, nós podemos hipotetizar que o decréscimo na viabilidade celular induzida por esses compostos, assim como sua toxicidade *in vivo* demonstrada em outros trabalhos pode estar relacionada com a expressão reduzida do Nrf-2 e, consequentemente, outras enzimas antioxidantes, deixando a célula vulnerável aos efeitos deletérios das EROs. Também podemos concluir que a toxicidade desses compostos não está diretamente relacionada a produção de EROs em leucócitos humanos, sendo que a intensidade da fluorescência da DCFH em células expostas ao Ebs, $(PhSe)_2$ e $(PhTe)_2$ não está de acordo com a citotoxicidade induzida pelos mesmos.

Por fim, nós também observamos que os compostos Ebs, $(PhSe)_2$ e $(PhTe)_2$ acumulam nos leucócitos, provavelmente devido a sua afinidade por membranas celulares, e que essa afinidade aumenta com a apolaridade do composto. Essa informação nos mostra que os compostos organocalcogênios estavam em contato com as células durante o período de incubação, gerando os efeitos observados. Os resultados apresentados aqui indicam que os compostos hidrofóbicos foram absorvidos pelas células e parte deles ficou acumulado no ambiente hidrofóbico das membranas, enquanto outra parte foi possivelmente metabolizada a compostos mais hidrofílicos. É sabido que o Ebs pode ser metabolizado em dois compostos principais: 2-glucoronilselenobenzanilida e 4-hidroxi-2-metil-selenobenzanilida (TERLINDEN et al., 1988), enquanto o conhecimento sobre o metabolismo do $(PhSe)_2$ e do $(PhTe)_2$ é elusivo (PRIGOL et al., 2012), mas sabe-se que a ligação entre o Se e o carbono é quebrada no metabolismo do $(PhSe)_2$ (ADAMS JR. et al., 1989). Assim, mais estudos são necessários para esclarecer se os organocalcogênios sofrem algum tipo de transformação química em modelos de exposição *in vitro*, como leucócitos ou outros tipos celulares, e identificar estes metabólitos se eles existirem.

6. CONCLUSÃO

A partir dos resultados expostos nesta dissertação, podemos concluir:

- Os compostos Ebs, $(PhSe)_2$ e $(PhTe)_2$ são capazes de reduzir a viabilidade celular de leucócitos humanos, sendo o Ebs o mais citotóxico neste caso;
- O $(PhTe)_2$ é o único composto capaz de aumentar a taxa apoptótica em leucócitos humanos;
- Os compostos Ebs, $(PhSe)_2$ e $(PhTe)_2$ são capazes de aumentar o índice de dano ao DNA em leucócitos humanos, sendo o $(PhTe)_2$ o mais genotóxico;
- O $(PhSe)_2$ é o único composto capaz de aumentar a produção de EROs em todas as concentrações testadas e em ambos os tipos celulares, enquanto o Ebs induz apenas nos linfócitos e o $(PhTe)_2$ o faz de forma sutil nos granulócitos. Logo, a toxicidade induzida pelos organocalcogênios nos leucócitos humanos não está diretamente relacionada com suas capacidades em induzir aumento na produção de EROs;
- O $(PhSe)_2$ é capaz de induzir um decréscimo na expressão da CAT, enquanto o Ebs é capaz de diminuir a expressão da SOD1 e o $(PhSe)_2$ é capaz de aumentar;
- Os compostos Ebs, $(PhSe)_2$ e $(PhTe)_2$ são capazes de diminuir a expressão da GPX3 nos leucócitos humanos na seguinte ordem Ebs > $(PhTe)_2$ > $(PhSe)_2$, enquanto nenhum composto é capaz de diminuir a expressão da GPX4 e da TRXR1;
- Os compostos Ebs, $(PhSe)_2$ e $(PhTe)_2$ são capazes de diminuir a expressão do NFE2L2 nos leucócitos humanos, sendo que o efeito do Ebs é maior que o do $(PhTe)_2$ e $(PhSe)_2$;
- Os compostos organocalcogênios se acumulam nas células, já que a maior concentração do composto se apresenta na extração do precipitado de células quando comparada a extração do tampão em que as células estavam incubadas.

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