UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE CIÊNCIAS NATURAIS E EXATAS
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA TOXICOLÓGICA

EFEITOS DO DIFENIL DISSELENETO SOBRE A DISFUNÇÃO MITOCONDRIAL NA INSUFICIÊNCIA HEPÁTICA AGUDA INDUZIDA POR PARACETAMOL EM CAMUNDONGOS

TESE DE DOUTORADO

Nélson Rodrigues de Carvalho

Santa Maria, RS, Brasil

2015

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Nélson Rodrigues de Carvalho

Tese apresentada ao Curso de Doutorado Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção de grau de **Doutor em Bioquímica Toxicológica**

Orientador: Prof. Félix Alexandre Antunes Soares

Santa Maria, RS, Brasil

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A Comissão Examinadora, abaixo assinada, aprova a Tese de doutorado

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elaborada por
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como requisito parcial para obtenção do grau de
Doutor em Bioquímica Toxicológica

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RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica
Universidade Federal de Santa Maria, RS, Brasil

EFEITOS DO DIFENIL DISSELENETO SOBRE A DISFUNÇÃO MITOCONDRIAL NA INSUFICIÊNCIA HEPÁTICA AGUDA INDUZIDA POR PARACETAMOL EM CAMUNDONGOS

AUTOR: NÉLSON RODRIGUES DE CARVALHO
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Local e Data da Defesa: Santa Maria, 26 de fevereiro de 2015.

A insuficiência hepática aguda (IHA) induzida por paracetamol (APAP) é um processo complexo que envolve depleção de glutationa (GSH), mudanças no metabolismo energético e disfunção mitocondrial, o que resulta na incapacidade de manter o funcionamento adequado do órgão. Neste contexto, a utilização de compostos orgânicos de selênio como o difenil disseleneto $(PhSe)_2$ tem se destacado nos últimos anos, devido as propriedades antioxidantes e efeitos hepatoprotetores, no entanto, o mecanismo pelo qual $(PhSe)_2$ age não está totalmente esclarecido. Assim, este estudo busca aprofundar nossos conhecimentos sobre as ações do $(PhSe)_2$ na disfunção mitocondrial assim como a sinalização intracelular durante a IHA induzida por APAP. Para tanto, estabelecemos primeiramente um parâmetro comparativo entre o composto orgânico de selênio e o antídoto clássico (N-acetil cisteina, NAC), em homogenato. O $(PhSe)_2$ foi tão efetivo quanto NAC reduzindo os marcadores de dano oxidativo, auxiliado na manutenção dos níveis de GSH e aumentando o tempo de sobrevivência após a intoxicação por APAP. O tratamento com $(PhSe)_2$ reduziu alterações morfológica, minimizou o dano quando analisamos histologicamente o tecido hepático e determinou uma redução nos níveis plasmáticos dos indicadores de dano hepatocelular (AST e ALT). Além disso, o $(PhSe)_2$ foi eficaz na redução significativa do dano oxidativo ao limitar a peroxidação lipídica, formação de espécies reativas de oxigênio e nitrogênio, carbonilação de proteínas mitocondriais e viabilidade mitocondrial após a IHA induzida por APAP. Neste contexto, os níveis de antioxidantes não enzimáticos, tais como GSH, e enzimáticos, tais como as enzimas catalase, manganês superoxido dismutase, glutationa peroxidase e glutationa redutase, também foram mantidos semelhantes ao grupo controle. Em geral os resultados observados neste estudo indicam que um importante mecanismo pelo qual o $(PhSe)_2$ exerce os seus efeitos terapêuticos está relacionado a manutenção da atividade do sistema de defesa antioxidante e inibição da transição de permeabilidade mitocondrial (MPT) indicados pela redução do inchaço mitocondrial, preservação da atividade dos complexos respiratórios I, II e ATPase, e manutenção do gradiente de H^+ com a formação do potencial de membrana mitocondrial ($\Delta\psi m$). Também observamos que o $(PhSe)_2$ limita a perda do funcionamento bioenergético mitocondrial com a manutenção dos níveis adequados de fosforilação oxidativa (OXPHOS) e ativa a via das proteínas do choque térmico aumentando a expressão de HSP70, a qual apresenta um efeito modulador importante sobre a MPT preservando a viabilidade mitocondrial. O tratamento com $(PhSe)_2$ foi efetivo em preservar níveis apropriados de citocinas envolvidas na recuperação do tecido hepático, tais como fator de necrose tumoral alfa (TNF- α), interleucina 6 (IL-6) e fator nuclear kappa B (NF-κB). Além disso, a manutenção bioenergética celular poderia estar associada com os elevados níveis transcricionais do receptor gama ativado por proliferador de peroxissoma (PGC-1α) que auxilia a restaurar os níveis de fator nuclear respiratório 1 (NRF1) os quais estão envolvidos no processo de biogênese mitocondrial. Por fim, o $(PhSe)_2$ poderia ser uma importante alternativa terapêutica a qual auxiliaria na recuperação do fígado, controle de qualidade mitocondrial e manutenção da homeostase e saúde celular.

Palavras Chaves: Paracetamol; biogênese mitocondrial; HSP70; estresse oxidativo; difenil disseleneto.

ABSTRACT

Thesis of Doctor's Degree
Graduation Program in Biological Sciences: Toxicological Biochemistry
Federal University of Santa Maria, RS, Brazil

EFFECTS OF DIPHENYL DISELENIDE ON MITOCHONDRIAL DYSFUNCTION IN THE ACUTE LIVER FAILURE INDUCED BY ACETAMINOPHEN IN MICE

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Date and Place of the Defense: Santa Maria, 26th February 2015.

Acute liver failure (ALF) induced by acetaminophen (APAP) is a complex process associated with glutathione (GSH) depletion, energetics metabolism changes and mitochondrial dysfunction, resulting in the impairment of maintenance of tissue normal function. On this matter, organoselenium compounds, such as diphenyl diselenide $(PhSe)_2$, have been highlighted in the last years due to the antioxidant properties and the hepatoprotective effects, however, the $(PhSe)_2$ hepatoprotection mechanism remains unclear. So, this work was aimed to deepen into understanding of the effects of $(PhSe)_2$ on the mitochondrial dysfunction as well as the signaling pathway during the ALF induced by APAP. Firstly, it was performed a comparative study between the organoselenium compound and the classical antidote (N-acetylcysteine, NAC) in the liver homogenate. $(PhSe)_2$ presented similar results to the NAC reducing the oxidative damage markers, maintaining the GSH levels and enhancing the survival after the APAP overdose. The treatment with $(PhSe)_2$ reduced plasmatic levels of transaminases (aspartate and alanine aminotransferase) and the morphological/histological changes. In addition, $(PhSe)_2$ was able to reduce significantly the oxidative damage such as lipid peroxidation, reactive oxygen and nitrogen species generation, mitochondrial protein carbonylation and mitochondrial viability after ALF induced by APAP. In this context, the levels of non enzymatic antioxidants, such as GSH, and enzymatic antioxidants, such as catalase, Mn superoxide dismutase, glutathione peroxidase and glutathione reductase remained to the control levels. In general, the results noticed in this work the probably $(PhSe)_2$ mechanism is closely related with the maintenance of antioxidant defense system and inhibition of mitochondrial transition permeability (MPT) indicated by reduction of mitochondrial swelling, activity preservation of respiratory complexes I, II and ATPase, and maintenance of H^+ gradient with the mitochondrial membrane potential ($\Delta\psi m$) generation. It was observed that $(PhSe)_2$ was able to limit the impairment of mitochondrial bioenergetics function with the normalization of oxidative phosphorilation (OXPHOS) and activation of heat shock protein pathway through the enhance of HSP70 levels, which in turn, modulates the MPT protecting the mitochondrial viability. $(PhSe)_2$ treatment was able to maintain the appropriated levels of cytokines associated with the liver recovery, such as tumoral necrosis factor alfa (TNF-α), interleukin 6 (IL-6) and nuclear factor kappa B (NF-κB). Moreover, the integrity of cellular bioenergetic function could be associated with the increase of peroxisome proliferator-activated receptor-γ coactivator (PGC-1α), helping to restore the nuclear respiratory factor 1 (NRF1) levels associated with the mitochondrial biogenesis. Finally, $(PhSe)_2$ could be a useful therapeutic alternative that would contribute to the liver recovery, controlling the quality of mitochondrial function and maintaining homeostasis and cellular health.

Keywords: Acetaminophen; mitochondrial biogenesis; HSP70; oxidative stress; diphenyl diselenide.

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ARTIGO

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LISTA DE APREVIATURAS

$^{\cdot}OH$ - Radical hidroxila
$(PhSe)_2$ – Difenil disseleneto
ADP – adenosina difosfato
AINEs - Antiinflamatórios não esteroidais
ALT – Alanina aminotransferase
APAP - Paracetamol, N-acetil-p-acetaminofeno
ARE - Elementos de resposta antioxidantes
AST – Aspartato aminotransferase
ATP - Adenosina trifosfato
ATP – adenosina trifosfato
Ca^{2+} - Cálcio
CAT - Catalase
DCF-oxid - Diclorofluoresceína oxidada
ERNs - Espécies reativas de nitrogênio
EROs - Espécies reativas de oxigênio
ERs - Espécies reativas
GCS – Gama glutamil cisteína sintase
GPx - Glutationa Peroxidase
GR - Glutationa Redutase
GSH - Glutationa reduzida
GST - Glutationa S-transferase
H_2O - Água
H_2O_2 - Peróxido de hidrogênio
HO-1 – Hemeoxigenase 1
HOCl - Ácido hipocloroso
HSF-1 – do inglês "*Heat shock factor 1*", Fator 1 do choque térmico
HSP70 – do inglês "*heat shock protein*", proteinas do choque térmico
IHA – Insuficiência hepática aguda
IL6 -Interleucina 6
LPO – Lipoperoxidação
MnSOD - Manganês superóxido dismutase
MPO - Mieloperoxidase
MPT – do inglês "*mitochondrial permeability transition*"Transição de Permeabilidade Mitocondrial
NAC – N-acetil cisteina
NAPQI – N acetil p benzoquinoneimina
NF-κB - Fator nuclear kappa B
NOS - Óxido nítrico sintase
NQO1 – NAD(P)H:quinona oxidorredutase 1
NRF1 - Fator nuclear respiratório 1
Nrf2 - Fator de transcrição nuclear 2
O_2 - Oxigênio
$O_2^{\cdot-}$ - Ânion superóxido
OCl^- - Hipoclorito
$ONOO^-$ - Peróxinitrito
OXPHOS – do inglês "*oxidative phosphorilation*", fosforilação oxidativa
PGC-1α - Receptor gama ativado por proliferador de peroxissoma
SeH – selenol
SINITOX – Sistema nacional de informações toxicológicas
SOD - Superóxido dismutase
TBARS - Substâncias reativas ao ácido tiobarbitúrico
TNF-α – Fatore de necrose tumoral alfa
TrxR – Tiorredoxina redutase
ΔΨm - Potencial de membrana mitocondrial

E SIGLAS

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APRESENTAÇÃO

No item INTRODUÇÃO, está descrita apresentação sucinta deste trabalho, e no item REVISÃO BIBLIOGRÁFICAS será apresentado os temas trabalhados nesta tese.

Os resultados que fazem parte desta tese estão apresentados sob a forma de artigo e manuscrito científico, os quais se encontram alocados no item ARTIGO E MANUSCRITO CIENTÍFICO. As seções Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas, encontram-se nos respectivos artigo e manuscrito científico e representam a íntegra deste estudo.

Os itens, DISCUSSÃO E CONCLUSÕES, no final desta tese, apresentam interpretações e comentários gerais sobre os resultados contidos neste trabalho.

As REFERÊNCIAS BIBLIOGRÁFICAS se referem somente às citações que aparecem nos itens INTRODUÇÃO, REFERÊNCIAS BIBLIOGRÁFICAS e DISCUSSÃO desta tese.

1. INTRODUÇÃO

Paracetamol (N-acetil-p-acetaminofeno; APAP) é uma fármaco amplamente empregada devido às propriedades analgésica e antipirética, porém, sem apresentar atividade antiinflamatória significativa clinicamente (Larson et al., 2005). A fácil aquisição e relativamente baixo custo tornam o APAP um dos fármacos mais consumidos segundo dados da ANVISA (Agência Nacional de Vigilância Sanitária) (Schuh, 2007). O consumo tanto acidental quanto intencional de altas doses de APAP podem comprometer o fígado, uma vez que este órgão apresenta uma demanda energética e metabólica elevada causando o comprometimento de sua função, levando a insuficiência hepática aguda (IHA) (de Achaval and Suarez-Almazor, 2011; Nourjah et al., 2006).

Em condições normais, a metabolização hepática do APAP ocorre primeiramente pelo processo de glicuronidação e sulfatação, os quais aumentam a solubilidade do fármaco, eliminado do fígado e da corrente sanguínea principalmente via urina e bile (Larson et al., 2005). Durante a overdose, o APAP é principalmente metabolizado no fígado via Citocromo P4502E1, resultando em um intermediário altamente reativo, o N-acetil-p-benzoquinona imina (NAPQI). O NAPQI, por sua vez, reage diretamente com glutationa reduzida (GSH) depletando seus níveis hepáticos. O não reestabelecimento de GSH faz com que o NAPQI comece a acumular e formar ligações covalentes com proteínas celulares, modificando a estrutura e função das mesmas (Gardner et al., 2012; Moyer et al., 2011). Desta forma, a IHA conduz ao desequilíbrio redox hepático causando a disfunção bioenergética mitocondrial (Cheng and Ristow, 2013; Jaeschke, 1990). Estudos demonstram que a toxicidade do APAP é um processo multifatorial envolvendo a pronunciada formação de espécies reativas de oxigênio e nitrogênio (ERO/ERN), estresse oxidativo e a transição de permeabilidade mitocondrial (MPT) (da Rosa et al., 2012; Jaeschke et al., 2012).

A toxicidade do APAP consiste de duas fases cruciais: primeiramente há uma depleção de GSH e ligação covalente do NAPQI a proteínas alvos, e por último um aumento da MPT e nitração de proteínas. Neste contexto, a intoxicação hepática induzida pro APAP pode causar uma redução na capacidade fosforilativa e fluxo de elétrons pela cadeia respiratória. O aumento da MPT está associado com o colapso do potencial de membrana mitocondrial ($\Delta\psi m$) e depleção de ATP, o qual, por sua vez, contribui para aumentar o estresse oxidativo mitocondrial e rompe a homeostase do Ca^{2+} resultando na liberação de fatores pro-apoptóticos (Bajt et al., 2011). É importante destacar que a MPT é mediada pelo estresse oxidante e pode aumentar o mesmo. Portanto, durante a MPT ocorre com a liberação de superóxido ($O_2^{\bullet-}$), o qual pode induzir a produção de peroxinitrito ($ONOO^-$) e nitração de proteínas, eventos letais

para a célula (Jaeschke et al., 2012). Assim, a MPT exacerba o desperdício energético e compromete a capacidade de reestabelecimento da produção de energia celular (Kim et al., 2003). Evidentemente, a limitação da adaptação metabólica após a IHA induzida por APAP causa a ruptura de processos que requerem alta demanda metabólica como a proliferação celular. A adaptação metabólica tem sido demonstrada como um processo complexo e bem orquestrado aceitando como principais fatores reguladores os níveis de GSH, formação de especies reativas e a viabilidade mitocondrial (Han et al., 2013).

Classicamente, a N-acetilcisteina (NAC) é o tratamento convencional escolhido para intoxicação aguda com APAP. Contudo, o NAC apresenta sua eficácia limitada a uma janela de tempo de tratamento e situações (Woodhead et al., 2012). Considerando este fato, o uso de compostos orgânicos de selênio poderia emergir como uma medida terapêutica alternativa. Estudos têm demonstrado as propriedades antioxidantes e antiinflamatórias dos compostos orgânicos de selênio, tais como Difenil disseleneto $(PhSe)_2$ e Ebselen (Ebs) (Borges et al., 2006; Brandão et al., 2009; Meotti et al., 2004). Em particular, $(PhSe)_2$, um diaril disseleneto lipofílico, com baixa toxicidade e demonstra-se eficiente em neutralizar peróxido de hidrogênio (H_2O_2) e outros hidroperóxidos orgânicos (Brandão et al., 2009; Meotti et al., 2004). Evidências demonstram um efeito promissor do $(PhSe)_2$ no tratamento da IHA induzida por APAP (da Rosa et al., 2012; da Silva et al., 2012). Contudo, os efeitos terapêuticos do $(PhSe)_2$ sobre o comprometimento bioenergético mitocondrial e a consequente sinalização intracelular durante a resposta adaptativa à IHA induzida por APAP permanece incerta. Ao mesmo tempo que tem sido demonstrado um efeito modulador do $(PhSe)_2$ sobre a abertura do poro de MPT (Puntel et al., 2010), o seu envolvimento na resposta adaptativa relacionada ao Fator de transcrição nuclear 2 (Nrf2) e elementos de resposta antioxidantes (ARE) tem sido explorado (de Bem et al., 2013). Contudo, a resposta celular adaptativa não se resume somente à translocação nuclear do Nrf2, mas há também um importante envolvimento de proteínas do choque térmico (HSP) (Dobrachinski et al., 2014), e uma interação envolvendo moléculas pleiotrópicas como citocinas e fatores de crescimento, tais como Fator de necrose tumoral (TNF)-α, Interleucinas (IL) 1 e 6 e a translocação do Fator nuclear, Kappa-B (NF-κB) (Michalopoulos, 2007; Polimeno et al., 2000). Além disso, há o envolvimento de fatores reguladores da biogênese mitocondrial, tais como, fator nuclear respiratório 1 (NRF1) e 2 (NRF2) assim como receptor-γ ativado por proliferador de peroxissoma (PGC-1α) (Glaser et al., 2010). Assim, permanece desconhecido se o envolvimento destas vias de sinalização estariam associados com o mecanismo pelo qual o $(PhSe)_2$ restaura o funcionamento bioenergético mitocondrial.

2. JUSTIFICATIVA

As estratégias terapêuticas contra intoxicação por APAP se restringem apenas a utilização de NAC, a qual visa o reestabelecimento dos níveis de glutationa para contra-atacar o efeito tóxico do metabólito intermediário reativo. No entanto, a gênese da hepatotoxicidade demonstra-se complexa e multifatorial induzindo estresse oxidativo, disfunção mitocondrial e comprometimento energético celular. Assim compostos orgânicos de selênio surgem como alternativas eficazes, mas o mecanismo permanece incerto. Por sua vez, a elucidação dos mecanismos pelos quais $(PhSe)_2$ exerce os seus efeitos terapêuticos é de suma importância clínica. Neste contexto, a manutenção do sistema de defesa antioxidante, redução da comprometimento bioenergético mitocondrial e um estimulo adaptativo via HSP70 e genes marcadores de biogênese mitocondrial poderiam ser cruciais para o efeito hepatoprotetor do $(PhSe)_2$.

3. REVISÃO BIBLIOGRÁFICA

3.1. Sobre o paracetamol e intoxicações

Medidas terapêuticas para controlar a dor, inflamação e outras condições patológicas têm sido utilizada a milênios pelo homem principalmente através da utilização de produtos naturais, contudo o rápido avanço dos conhecimentos científicos levaram a descoberta e desenvolvimento dos compostos analgésicos e antiinflamatórios, como por exemplo o paracetamol (APAP). Sintetizado pela Johns Hopkins University, em 1877, o APAP, apresenta propriedades analgésica e antitérmica, cujo emprego na medicina teve início com Von Mering, no ano de 1893. Mesmo com seu advento, foi apenas após 1949 que se tornou popular. No Brasil, a utilização do APAP e seu conhecimento pela população em geral teve início nos anos 80, passando a ser amplamente utilizado na década de 90 (Andrade Filho et al., 2013; Brunton et al., 2003; Raffa, 1996). Seu potencial analgésico e antitérmico assemelha-se ao dos aintiinflamatórios não esteroidais (AINES), como a aspirina. No entanto, difere destes por apresentar fraca ação antiinflamatória, antiagregante plaquetária e gastrotóxica (Brunton et al., 2003; Kis et al., 2005). Naturalmente o APAP, ganhou aceitação popular como uma terapia alternativa de analgesia para pacientes sensíveis à aspirina. Atualmente, encontra-se disponível no mercado uma ampla gama de fármacos que apresentam em sua composição APAP, principalmente compostos antigripais, sendo utilizado principalmente na pediatria (Sebben et al., 2010).

Devido a sua popularidade e venda livre, intoxicações por APAP tornaram-se um problema de saúde crescente em todo o mundo, sendo uma das principais causas de insuficiência hepática aguda (IHA) no mundo ocidental (Ayonrinde et al., 2005; Schmidt, 2005). Dados da literatura demonstram que os maiores índices de intoxicação intencional ocorrem com jovens, do sexo feminino, costumando ser a sua primeira tentativa de suicídio (Ayonrinde et al., 2005; Schmidt, 2005; Townsend et al., 2001). Além disso, a falta de conhecimento dos efeitos adversos e administração incorreta são fatores que colaboram para acentuar os efeitos nocivos do fármaco no organismo. Conforme pesquisas do Sistema Nacional de Informações Toxicológicas (SINITOX), o abuso no consumo de medicamentos foram responsáveis por pelo menos 4251 óbitos no período que compreende de 2002 à 2011. Neste mesmo período podemos observar um aumento nos casos de intoxicação na região Sudeste, esta região registrou uma média anual de 45,1% dos relatos de intoxicação humana, seguida pela região Sul com uma participação percentual de 26%, região Nordeste com 18%, região Centro-Oeste com valores de 9,1% e região Norte com 1,7% (Figura 1). Análises

estatísticas deste período também revelaram uma taxa anual média de óbito de 481 indivíduos associado com intoxicação.

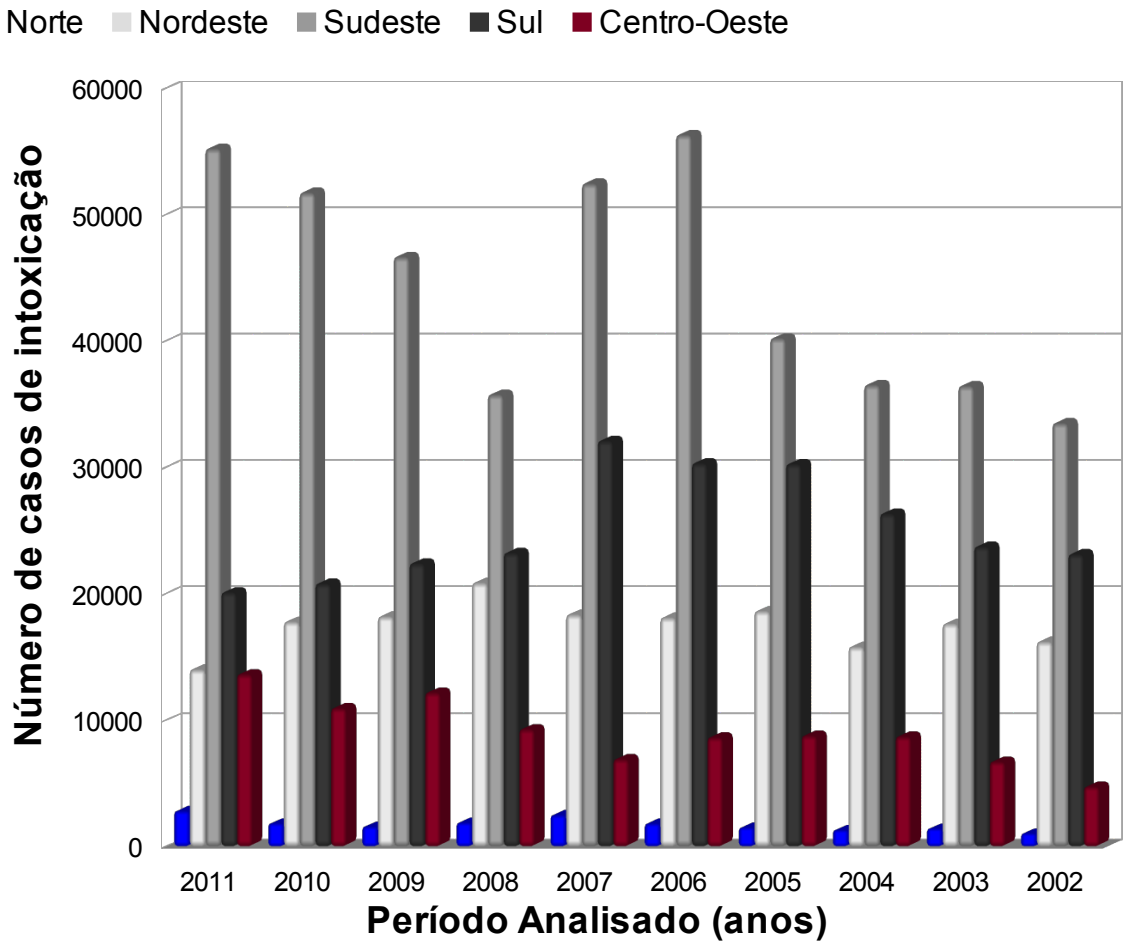

Figura 1 - Casos registrados de intoxicação humana no Brasil no **período de 2002 a 2011.** Fonte: Ministério da Saúde/ Fundação Oswaldo Cruz/ SINITOX (Figura **adaptada).**

Quando analisamos os dados provenientes da pesquisa realizada pela SINITOX ainda neste período o meio de intoxicação na qual os indivíduos foram expostos, é possível observar que os índices de intoxicação medicamentosa aumentam gradativamente alcançando uma porcentagem de 28,57% em 2011 e uma média anual neste período de 10 anos de 27.23% (Tabela 1) em uma lista que apresenta agentes intoxicantes como por exemplo agrotóxicos de uso agrícola e doméstico, drogas de abuso, metais, plantas e produtos químicos industriais. Na mesma tabela podemos observar os dados correlatos no mesmo período, mas relacionados ao estado do Rio Grande do Sul. Desta forma observamos que aproximadamente 31.3% dos casos de intoxicação humana no período que compreende de 2002 à 2011 foram relacionados com intoxicação medicamentosa (Tabela 1).

Tabela 1 - Casos registrados de intoxicação humana no Brasil e Rio Grande do Sul no período de 2002 a 2011.

Ano	Brasil			Rio Grande do Sul		
	Total das intoxicações	Total de intoxicações por medicamento	%	Total das intoxicações	Total de intoxicações por medicamento	%
2011	105875	30249	28.57	20137	6526	32.41
2010	103184	27710	26.68	19372	5964	30.79
2009	106928	27845	26.04	18786	5936	31.60
2008	105488	29620	28.04	19199	6111	31.83
2007	128158	37703	29.42	20341	6065	29.82
2006	132081	39176	29.66	19644	6031	30.70
2005	115548	39499	26.4	18883	5814	30.79
2004	109322	28899	26.43	15616	5258	33.67
2003	105071	27677	26.34	14466	4598	31.78
2002	94471	23397	24.77	14634	4398	30.05
Total	**1106126**	**311775**	**27.23**	**181078**	**56701**	**31.30**

Fonte: Ministério da Saúde/ Fundação Oswaldo Cruz/ SINITOX (tabela adaptada).

Além disso, nos últimos 20 anos, países como Estados Unidos, Dinamarca e Reino Unido apresentam dados crescentes de relatos de intoxicação medicamentosa causada por APAP levando a insuficiência hepática e aumentando o índice de hospitalizações e mortes (Larson et al., 2005), e estudos recentes sugerem um dramático aumento de transplante de fígado e considerável morbidade e mortalidade associados com a overdose de APAP nos Estados Unidos e muitos outros países (de Achaval and Suarez-Almazor, 2011; Nourjah et al., 2006).

Tabela 2 - Distribuição dos casos registrados no CIT/RS envolvendo intoxicações e exposições por paracetamol segundo o ano de ocorrência.

Ano	Intoxicações		Exposições		Total	
	N° de casos	%*	N° de casos	%*	N° de casos	%*
2001	30	53.57	26	46.43	56	100
2002	41	67.21	20	32.79	61	100
2003	53	69.74	23	30.26	76	100
2004	53	69.74	23	30.26	76	100
2005	99	84.62	18	15.38	117	100
2006	122	91.04	12	8.96	134	100
Total	398	76.54	122	23.46	520	100

*Percentuais referentes ao ano, exceto em Total, neste valor é referente à soma dos anos.

Fonte: Adaptado de SCHUH, C. D. Intoxicações e Exposições por Paracetamol: Análise de Seis Anos de Registros do Centro de Informações Toxicológicas do Rio Grande do Sul – CIT/RS. 2007.36 f.

No estado do Rio Grande do Sul, as intoxicações por APAP, no ano de 2008, somaram 440 casos, segundo dados do Centro de Informações Toxicológicas do Rio Grande do Sul

(CIT/RS), de um total de 6.111 casos registrados de intoxicações por medicamentos. Uma análise dos registros de intoxicações e exposições por APAP no período de janeiro de 2001 a dezembro de 2006 realizado por SCHUH (2007) demonstrou um acentuado crescimento anual de hospitalizações causadas por intoxicação ocasionada por esse fármaco, passando de 56 registros no primeiro ano de estudo para 134 casos no último ano, além de uma prevalência no sexo feminino (SCHUH 2007). Os resultados podem ser analisados em relação ao ano de ocorrência (Tabela 2).

3.2. Da metabolização até a hepatotoxicidade

A utilização do APAP em doses terapêutica é relativamente segura com raros casos de efeitos adversos. Assim, APAP apresenta sua absorção rápida e quase que completamente pelo trato gastrointestinal (Brunton et al., 2003). Contudo, evidencias da literatura demonstram que doses tóxicas podem variar entre 4-10 g em adultos, e a recomendação é que não se ultrapasse 4 g/dia para adultos e 100 mg/kg para crianças (Fontana, 2008). Desta forma, aproximadamente 95 % do APAP ingerido, em condições normais é metabolizado via glicuronidação e sulfatação, e 5-10 % do remanescente pode ser oxidado via sistema citocromo P450 para formar o metabolito reativo NAPQI, o qual pode ser eficientemente neutralizado pela conjugação com GSH e excretado do organismo via bile e urina (Budnitz et al., 2011; Larson et al., 2005). Por outro lado, em doses hepatotóxicas ocorre a saturação da vias de metabolização (glicuronidação e sulfatação), passando a um aumento exponencial da formação de NAPQI (Gardner et al., 2012; Moyer et al., 2011). Evidências da literatura demonstram que 90 % do GSH celular encontra-se na forma reduzida, representando uma concentração limitante para os processos bioquímicos (Bounous et al., 1989), o aumento do conjugação do metabolito reativo com a GSH associado com a incapacidade de repor níveis adequados devido a alta demanda, podem conduzir ao desequilíbrio redox intracelular, sendo que a hepatotoxicidade emerge quando os níveis de GSH caem abaixo de 70 % do normal (Jaeschke, 1990; Walubo et al., 2004). Consequentemente o o aumento exacerbado de NAPQI consiste em um dos eventos primários da IHA induzida por APAP, podendo promover necrose centrolobular (Anundi et al., 1993; da Rosa et al., 2012). Este processo, no entanto, não exclui a participação significativa de outro mecanismos para causar a hepatotoxicidade, tais como a acumulação de neutrófilos e células de Kuppfer que contribuem para o processo inflamatório associado com a hepatotoxicidade e aumentando a formação de especies reativas de oxigênio e nitrogênio (EROs/ERNs) (Brown et al., 2010; Jaeschke, 1990), conduzindo ao comprometimento funcional do fígado após a overdose e refletindo em condições

fisiopatológicas em outros órgãos como rins e cérebro (da Silva et al., 2012; Ghosh et al., 2010; Panatto et al., 2011). Além disso, o NAPQI pode reagir com componentes macromoleculares da célula (proteínas, lipídios e DNA), comprometendo a maquinaria metabólica necessária para o processo de adaptação e acentuado o desperdício energético no tecido hepático.

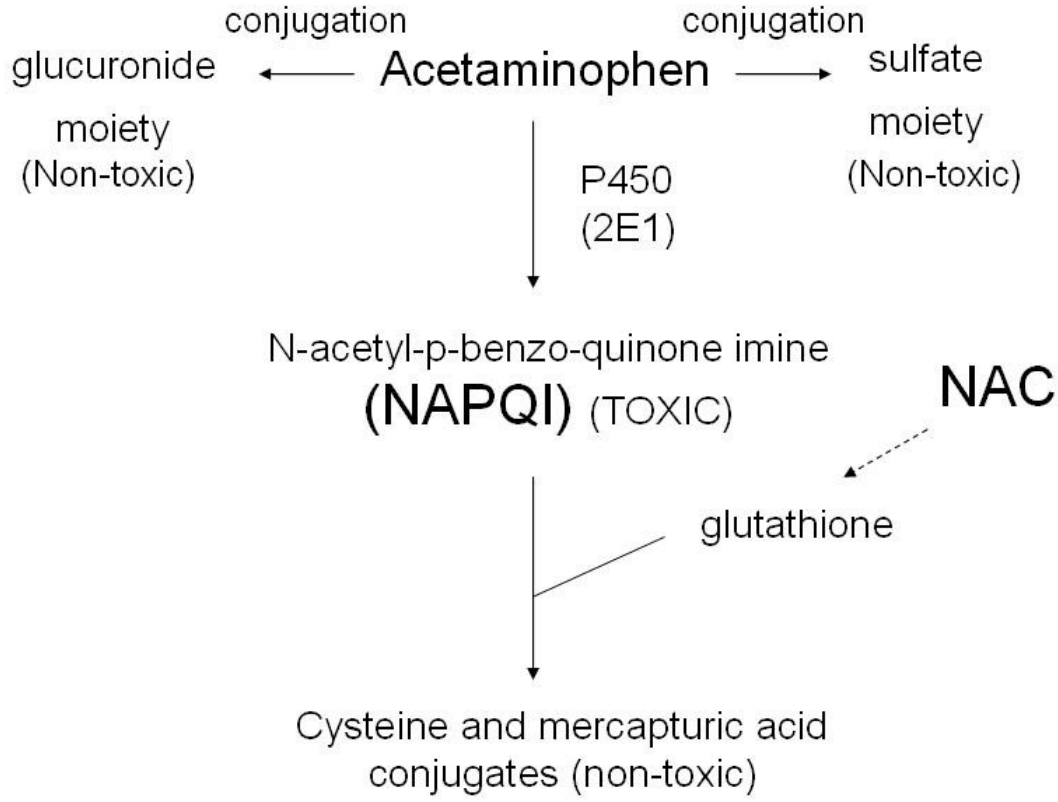

Figura 2 - Metabolismo do APAP.
Fonte: Adaptado de Katzung BC, Masters SB, Trevor AJ: *Basic & Clinical Pharmacology, 11 th Edition*: http: www.accessmedicine.com

3.3. Estresse oxidativo e seu envolvimento com a hepatotoxicidade

É amplamente aceito que a formação do metabólito reativo do APAP seja o estímulo inicial para hepatotoxicidade (Knight et al., 2003). No entanto, trabalhos têm demonstrado a importância do estresse oxidativo na gêneses de condições patológicas (Carvalho et al., 2010; Puntel et al., 2011). O dano oxidativo está intimamente associado com a IHA induzida por APAP, uma vez que durante a overdose ocorre um desequilíbrio redox na célula, causando uma diminuição da capacidade neutralizadora e também um aumento pronunciado na geração de espécies reativas (Jaeschke, 2003). Durante intoxicação induzida por APAP ocorre o comprometimento da função de enzimas do sistema de defesa antioxidante citosólico e mitocondrial agravando o dano celular (Michael Brown et al., 2012). A perda de função de

determinadas proteínas pode ser decorrente da conjugação com NAPQI, mas há também um declínio na atividade enzimática que pode ser correlacionado com a redução da disponibilidade de substrato, como é o caso das enzimas dependentes de GSH (Olaleye and Rocha, 2008). Por outro lado, o dano oxidativo pode prejudicar a funcionalidade dos componentes das membrana celulares e comprometer a permeabilidade seletiva através da peroxidação lipídica (LPO), como é o caso da inativação da função enzimática da Na^+/K^+-ATPase, acarretando sérios danos a cérebro de camundongos após a intoxicação com APAP. Classicamente, a atividade de enzimas oxidases como a citocromo P450 envolvida na metabolização do APAP apresentam como subprodutos EROs que podem causar a LPO (Kuthan et al., 1978). A geração de ERNs são fatores importantes durante a IHA induzida por APAP, em especial $ONOO^-$ (Abdelmegeed et al., 2013; Jaeschke, 2003). A geração exacerbada de $ONOO^-$ está associada com a inibição da enzima Mn-Superóxido Dismutase (MnSOD), localizada na matriz mitocondrial, e consequentemente afeta a manutenção da homeostase redox (MacMillan-Crow et al., 1998; Ramachandran et al., 2011). Neste contextos, salientamos que ERNs desempenham um papel fisiológico importante na modulação da atividade dos componentes da cadeia respiratória mitocondrial, por exemplo o óxido nítrico (NO) que é capaz de modular a atividade da citocromo oxidase de forma especifica. No entanto, durante a IHA induzida por APAP ocorre uma acentuada produção de $ONOO^-$ o que acarreta em ataques oxidativos e nitração de forma inespecífica sobre todos os complexos da cadeia respiratória, incluindo a ATP sintase, o que pode consequentemente causar uma perda de potencial de membrana ($\Delta\psi m$), escape de elétrons e redução na produção de ATP mitocondrial (Brown and Borutaite, 2002).

3.4. Disfunção mitocondrial como um mecanismo para a hepatotoxicidade

A hepatotoxicidade induzida por APAP demonstra-se como um processo complexo causando danos direta e indiretamente a célula. O desequilíbrio redox do tecido hepático é frequentemente acompanhado pela LPO, a qual acarreta em serias consequências para a viabilidade da membrana celular e mitocondrial, comprometendo ainda mais a homeostase bioenergética do tecido hepático (Abdelmegeed et al., 2013; Arakawa et al., 2012; Ramachandran et al., 2011) Durante esta condição fisiopatológica o dano hepatocelular pode ser instalado através do prejuízo funcional da fisiologia mitocondrial, devido a limitada capacidade de tamponamento redox e parcial dependência da capacidade antioxidante citosólica (Vendemiale et al., 1996).

A manutenção do $\Delta\psi m$ está associada com a capacidade dos complexos da cadeia

respiratória bombear os prótons para o espaço intermembrana, este processo ocorre através da liberação de energia proveniente da oxidação de coenzimas reduzidas (NADH e $FADH_2$) as quais são constantemente produzidas principalmente pela metabolização de substratos energéticos via Ciclo do Ácido Cítrico. Neste contexto, a intoxicação por APAP tem demonstrado inibir enzimas importantes envolvidas na produção das coenzima reduzidas (Balaji Raghavendran et al., 2005). Também foi demonstrado inibir a enzima aconitase, a qual demonstra-se sensível a variações acentuadas no estado redox mitocondrial (Brown and Borutaite, 2002). Levando em consideração isso, observamos que a reduzida proteção mitocondrial é seguida por uma redução significativa da capacidade de produção energética celular através do rompimento do $\Delta\psi m$ e inibição da fosforilação oxidativa (OXPHOS), prejudicando o tecido hepático que é altamente dependente do metabolismo aeróbico (Dumas et al., 2011). A intoxicação por APAP causa uma reduzida disponibilidade das coenzimas reduzidas e tem sido demonstrada reduzir a atividade dos complexos da cadeia respiratória mitocondrial, o que proporciona uma redução da capacidade bombeadora de prótons para o espaço intermembrana, prejudicando a produção ATP e $\Delta\psi m$ (Panatto et al., 2011).

Em condições normais EROs são produzidas em níveis relativamente baixos e resultam da auto-oxidação dos componentes da cadeia respiratória durante o fluxo de elétrons com produção de superóxido ($O_2^{\bullet-}$) e o peróxido de hidrogênio (H_2O_2), que na presença de ferro pode produzir o radical hidroxil ($HO^{\bullet}$), altamente reativo (Gutteridge and Halliwell, 1994; Halliwell and Gutteridge, 2006; Puntel et al., 2009). Todavia, quando em condições patológicas como durante a disfunção mitocondrial induzida por APAP, a decomposição destas espécies reativas é superada pela produção, tornando a cadeia respiratória mitocondrial uma das principais fontes de EROs celular durante IHA (Al-Belooshi et al., 2010). É importante destacar que o acentuado escape de elétrons com a consequente formação de $O_2^{\bullet-}$ na presença de NO pode tornar favorável a formação de $ONOO^-$, o qual é altamente reativo (Halliwell, 2006). Assim, a hepatotoxicidade induz mudanças simultâneas na taxa de respiração, síntese de ATP, fluidez e permeabilidade da membrana decorrente de danos oxidativos.

Frequentemente quando analisamos estes processos patológicos a nível mitocondrial, eles estão associados com a abertura do poro de transição de permeabilidade mitocondrial (MPT). O poro de transição de permeabilidade consiste em um canal complexo, apresentando em sua composição o canal de ânion voltagem dependente na membrana mitocondrial externa, translocador de nucleotídeo de adenina na membrana mitocondrial interna e ciclofilina D na matriz (Kowaltowski et al., 2009). A MPT é um processo que está

intimamente envolvido com IHA induzida por APAP, evidências da literatura demonstram um significativo efeito hepatoprotetor com a utilização do bloqueador da abertura do mega canal (Masubuchi et al., 2005). Desta forma, fatores como uma redução da razão GSH/GSSG, NAD(P)H/NAD(P)$^+$ e a dissipação do gradiente de prótons e a perda do $\Delta\psi m$ além de modificações de grupos tiois das proteínas da membrana interna são fatores que podem proporcionar um ciclo vicioso e desencadear MPT (Puntel et al., 2010). Assim, a modulação da MPT é um elemento chave no controle da viabilidade celular independentemente do fenótipo de morte celular.

3.5. Alterações no funcionamento bioenergético mitocondrial hepático

O metabolismo aeróbico é essencial para a manutenção do funcionamento correto de diversos órgão. Alterações neste metabolismo podem conduzir a falência do órgão. A ruptura deste processo tem sido associada a inúmeras condições patológicas tais como isquemia e reperfusão, lesão muscular e lesão cerebral por trauma (Dobrachinski et al., 2014; Mazzeo et al., 2009; Puntel et al., 2013, 2011). Modelos experimentais *in vitro* demonstram que AINES, mesmo em baixas concentração são capazes de alterar a fisiologia mitocondrial, reduzindo o fluxo de elétrons, induzindo desacoplamento do cadeia respiratória e aumentando a produção de EROs (Berson et al., 2006; Masubuchi et al., 2002). Durante a intoxicação por APAP a produção energética via oxidação aeróbica encontra-se comprometida, e a administração substratos glicolíticos demonstrou-se benéfica (Kon et al., 2004), indicando que a depressão energética pode ser compensada com o desvio do metabolismo aeróbico para a via glicolítica. Diversos mecanismos podem estar envolvidos no processo de depressão energética associada com intoxicação por APAP, dentre eles a inibição direta do metabolismo mitocondrial, danos aos componentes da membrana e a MPT que produz o desacoplamento da cadeia respiratória resultando na redução da capacidade da OXPHOS.

Localizada em todas células eucarióticas, as mitocôndria não são importantes somente na manutenção energética da célula, ela desempenha papel fundamental na sobrevivência à diversas condições patológicas (Galluzzi et al., 2012; Lemasters et al., 1999; López-Armada et al., 2013). Assim, a adaptação da fisiologia da organela pode ocorrer através do processo de biogênese que envolve a ação coordenada de ambos genomas nuclear e mitocondrial. O coativador alfa do receptor gama ativado por proliferador de peroxissoma (PGC-1α) atua como um regulador principal, o qual pode interagir com o fator respiratório nuclear 1 (NRF1), estimulando a transcrição de muitos genes mitocondriais, bem como o fator de transcrição mitocondrial A (Tfam), regulador direto de replicação e transcrição do DNA mitocondrial.

Neste contexto, a biogênese abrange também o aumento da organela e síntese de enzimas relacionadas à sua função (Scarpulla, 2008). Desta forma, diversas condições fisiológicas podem promover a ativação de PGC-1α, dentre estas o desequilíbrio redox é um processo central para a biogênese mitocondrial (Lin et al., 2005). Este processo compreenderia uma complexa rede que visa uma adaptação à condição patologica e poderia fornecer a célula uma maior resistência e maior capacidade de manutenção bioenergética, envolvendo um aumento da capacidade de sintetizar ATP, a qual poderia modular o processo de morte celular evidenciado durante a IHA induzida por APAP. O PGC-1α como um fator regulador de transcrição está envolvido com a ativação de NRF1 o qual é importante para promoção da biogênese mitocondrial e formação de subunidades dos complexos respiratórios (Finck and Kelly, 2006; Kelly and Scarpulla, 2004). Estudos recentes têm salientado a importância do PGC-1α como um modulador do processo inflamatório e supressor da geração de EROs (Baldelli et al., 2013; Handschin et al., 2007a, 2007b).

3.6. Adaptação celular contra hepatotoxicidade induzida por APAP

Diversos mecanismos contribuem para proteção contra a disfunção bioenergética mitocondrial durante a hepatotoxicidade induzida por APAP. Como o tecido hepático é altamente versátil e seu funcionamento é dinâmico, o fígado passa constantemente por diversas situações de estresse fisiológicos e decorrente do metabolismo de xenobióticos, assim, as respostas adaptativas celulares emergem como uma forma para o tecido superar o estresse e retornar ao metabolismo normal. Para adaptar e sobreviver, a célula apresenta uma complexa rede de sinalização envolvendo fatores reguladores que permitem a expressão de genes que codificam enzimas importantes para o sistema de defesa antioxidante, e o aumento na concentração citoplasmática dessas proteínas fazem parte de um complexo processo de recuperação do tecido em condições fisiopatológicas, assim, os fatores mais importantes para sobrevivência são as proteínas de choque térmico (HSP) e Fator de transcrição nuclear 2 (Nrf2) (Chan et al., 2001; Tolson et al., 2006).

Na hepatotoxicidade induzida por APAP tem sido atribuído ao Nrf2 um papel fundamental como mediador da hepatoproteção, esse por sua vez é um fator transcrição essencial para a indução coordenada de genes que codificam enzimas de resposta ao estresse ou citoprotetoras como a NAD(P)H:quinona oxidorredutase-1 (NQO1), superóxido dismutase (SOD), glutationa S-transferase (GST), glutationa peroxidase (GPx), heme oxigenase-1 (HO-1), γ glutamil cisteína sintase (GCS), catalase e tioredoxina (Baird et al., 2014; Barbour and Turner, 2014). Animais geneticamente deficientes dessa proteína apresentam uma maior

sensibilidade a agentes intoxicantes (Chan et al., 2001).

Em condições fisiológicas normais o Nrf2 encontra-se no citoplasma associado a sua proteína inibitória o Keap-1. Sob estresse oxidante, o Nrf2 é translocado para o núcleo desencadeando a expressão dos elementos de resposta antioxidante (ARE) (Baird et al., 2014; Chan et al., 2001). Assim, a quebra da associação entre Nrf2-Keap1 pode ocorrer quando houver um desequilíbrio da homeostase redox intracelular, o que torna o Nrf2 um fator de transcrição redox sensível. A utilização de compostos naturais e sintéticos promove a translocação do Nrf2 para o núcleo através de modificações redox (Baird et al., 2014; Greco et al., 2011; Yang et al., 2009). Compostos orgânicos de selênio como ebselen, um análogo do $(PhSe)_2$, tem demonstrado ativar a via do Nrf2-ARE resultando em uma proteção complementar ao dano oxidativo induzido por xenobióticos (Kim et al., 2009). Levando em consideração que o $(PhSe)_2$ compartilha propriedades com ebselen, o seu efeito hepatoprotetor poderia ser similar com a ativação da via Nrf2-ARE, porém, há limitadas evidências na literatura. O provável mecanismo de ativação envolveria a atividade mimética da GPx, a qual por meio da oxidação de grupos tióis críticos para a retenção citosólica do fator de transcrição são oxidados (de Bem et al., 2013).

Assim, o Nrf2 representaria um importante alvo terapêutico contra a hepatotoxicidade induzida por APAP, contudo ele não é a única via que possui um efeito hepatoprotetor representativo (Tolson et al., 2006; Zhang et al., 2011), e alguns estudos tem demonstrado compostos com efeitos benéficos para a funcionalidade hepática os quais possuem um efeito hepatoprotetor independente de Nrf2 (Li et al., 2012, 2014).

As proteínas do choque térmico (HSP) em especial a HSP70 parecem também ter um papel importante durante a hepatotoxicidade induzida por APAP (Tolson et al., 2006). A indução desta via conduz a uma adaptação com expressão de proteínas citoprotetoras que detectam, previnem e contra-atacam as consequências do estresse térmico, oxidativo e eletrofílico, promovendo a sobrevivência. Assim, as HSP apresentam sua expressão controladas pelo fator 1 de choque térmico (HSF-1), o qual encontra-se inativo quando localizado no citosol, após a sinalização indutora o fator de transcrição é fosforilado no núcleo e promove a expressão da HSP70 (Joly et al., 2010; Yaglom et al., 2007). A função chaperona da HSP70 auxilia no enovelamento correto e reenovelamento de muitas proteínas que foram afetadas pela condição estressora. As HSP apresentam-se naturalmente expressas nas células em condições fisiológicas (aproximadamente 1-2%), podendo compreender durante o estresse oxidativo de 4-6% das proteínas na célula. Elas são encontrados na maioria, se não em todos os compartimentos celulares de eucariotos incluindo o núcleo, mitocôndria, retículo

endoplasmático e citosol (Kim and Kim, 2011).

A HSP70 é também uma importante proteína anti-apoptótica que age na modulação da morte celular, desempenhando um efeito inibitório sobre apoptose mediada pela TNF-α e translocação dar Bax (Joly et al., 2010; Yaglom et al., 2007), assim, evidencias sugerem que HSP70 pode prevenir a MPT e modular a expressão de citocinas.

A ativação da HSP70 parece possuir um papel vital na proteção contra a hepatotoxicidade induzida por APAP, uma vez que animais com deleção do gene codificante desta proteína são mais suscetíveis a intoxicação por APAP (Tolson et al., 2006). Além disso, o pré-tratamento com estresse térmico (43 ºC) aumenta a expressão de HSP70 e diminui a toxicidade induzida por agentes hepatotoxicantes (Salminen et al., 1996), outros estudos demonstraram que HSP70 participa da resposta imune modulando o estresse oxidativo envolvido no processo inflamatório (da Rosa et al., 2012; Gelain et al., 2011).

O acentuada resposta inflamatória no tecido hepático após a intoxicação por APAP é um processo mediado principalmente por neutrófilos seguido por monócitos/macrófagos e outas células do sistema imune (Williams et al., 2014). Como um mecanismo para acentuar o dano celular o APAP não inibe a ativação de neutrófilos, como fazem outros anti-inflamatórios (Brunton et al., 2003). Para o funcionamento hepático correto estas células do sistema imune são fundamentais para a defesa celular bem como para o processo de recuperação do tecido lesado, contudo com uma resposta inflamatória acentuada, no caso da intoxicação, ocorre um desequilíbrio entre a lesão e o reparo do tecido, o que contribui para a extensão do dano tecidual (Williams et al., 2010). Desta forma, a hepatotoxicidade é promovida pela resposta inflamatória resultando no aumento da ativação da cascata de sinalização intracelular e consequentemente liberação de citocinas (García-Mediavilla et al., 2007).

Ambos APAP e o metabólito NAPQI podem ativar um resposta imune no fígado. Classicamente, a citotoxicidade mediada por APAP é desencadeada por uma morte celular necrótica com a liberação de fatores pró-inflamatórios e uma significativa depleção dos níveis de ATP (Hoque et al., 2012). Desta forma, a perda da integridade mitocondrial é um evento critico para o processo de morte celular (Brookes et al., 2004), e protegendo a mitocôndria poderíamos obter uma prevenção do dano tecidual durante a inflamação. Neste contexto, a disfunção mitocondrial e exacerbada formação de EROs pode promover uma ativação de macrófagos no tecido afetado (Kim et al., 2014). Além disso, quando ocorre um aumento na produção de $O_2^{\bullet-}$ mitocondrial, este pode atuar como um modulador do estado pró-inflamatório provocando mudanças do sistema redox intracelular (Brookes et al., 2004;

López-Armada et al., 2013). Outro fator é a excessiva acumulação mitocondrial de Ca^{2+} que também pode induzir a disfunção mitocondrial e aumentar a produção de EROs e ativando o NF-kB causando a inflamação no tecido (Maass et al., 2005). No entanto, a manutenção de níveis apropriados de NF-kB parece ser fundamental para regulação do metabolismo energético celular (Mauro et al., 2011). Quando inativo NF-kB encontra-se sequestrado no citosol, porém ao ser translocado para o núcleo ele ativa a transcrição de genes de mediadores inflamatórios e inibidores da apoptose (Moretti et al., 2012). Como consequência dos múltiplos genes alvos induzidos por NF-kB, sua desregulação tem sido implicada em diversas condições fisiopatológica.

Devido a sua versatilidade, as citocinas são consideradas moléculas pleiotrópicas envolvidas em uma ampla gama de atividades biológicas, incluindo inflamação, crescimento e diferenciação e apoptose. Por exemplo, TNF-α e IL-6 são moléculas chaves na rede de citocinas, os quais estão envolvidos nos processos que estimulam a proliferação das células endoteliais hepática e a regulam a divisão celular (Tarlá et al., 2006). Alterações nos níveis de TNF-α estão associados com uma deficiência na produção de ATP (Valerio et al., 2006), enquanto que, os animais modificados geneticamente para IL-6 são mais sensíveis a IHA induzida por APAP (James et al., 2003). Assim, do ponto de vista metabólico, a participação de citocinas é importante para que ocorra a adaptação e a consequente sobrevivência celular.

3.7. Difenil disseleneto

Selênio é um micronutriente de fundamental importância para a saúde, cuja principal fonte é a dieta, sendo localizado no grupo 16 da tabela periódica e podendo ser encontrado sob diferentes estados de oxidação (Papp et al., 2007). Selênio é componente essencial de enzimas antioxidantes como Glutationa peroxidase (Gpx) e Tiorredoxina Redutase (TrxR) (Nogueira and Rocha, 2011). Assim, compostos orgânicos de selênio, a partir dos anos 80, passaram a receber mais interesse devido as suas propriedades bioquímicas, farmacológicas e toxicológicas (Nogueira et al., 2004). Em especial os compostos Ebselen e Difenil disseleneto [$(PhSe)_2$] (Figura 3), os quais apresentaram promissoras propriedades antioxidantes miméticas das seleno-enzimas GPx e TrxR em diversos modelos experimentais (Nogueira and Rocha, 2011). No entanto, efeitos tóxicos também são observados quando compostos orgânicos de selênio são administrados em altas doses, estes efeitos estão comumente relacionados ao consumo excessivo dos grupos tiois, GSH e NAD(P)H endógenos, podendo disparar processos como a morte celular por apoptose, com a abertura do poro de MPT (Nogueira and Rocha, 2010).

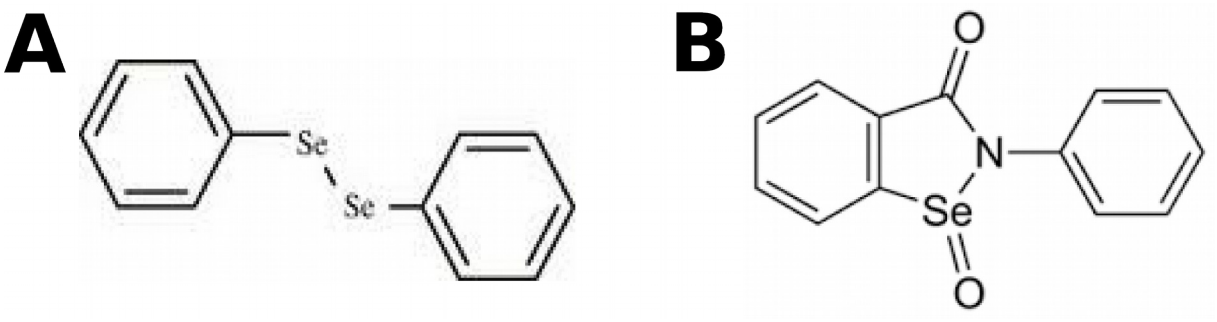

Figura 3 – Estruturas do Difenil disseleneto (A) e Ebselen (B).
Fonte: Nogueira e Rocha. 2010.

Durante o ciclo de reação do $(PhSe)_2$ ocorre a formação do intermediário selenol com alto poder redutor, e este por sua vez pode interagir com moléculas pró-oxidantes (Nogueira et al., 2004). O mecanismo catalítico do $(PhSe)_2$ envolve a reação com grupo tiol (RSH) ou moléculas de GSH, formando selenilsufeto que pode reagir com um segundo grupo tiol, gerando o selenol (SeH), o qual é capaz de reagir eficientemente com peróxido de hidrogênio (H_2O_2) e hidroperóxidos lipídeos (Figura 4). Outra característica importante desse composto é que $(PhSe)_2$ mostrou-se menos tóxico que o ebselen e podem ser melhores nucleófilos (e, portanto antioxidantes) do que os antioxidantes clássicos (Nogueira and Rocha, 2010).

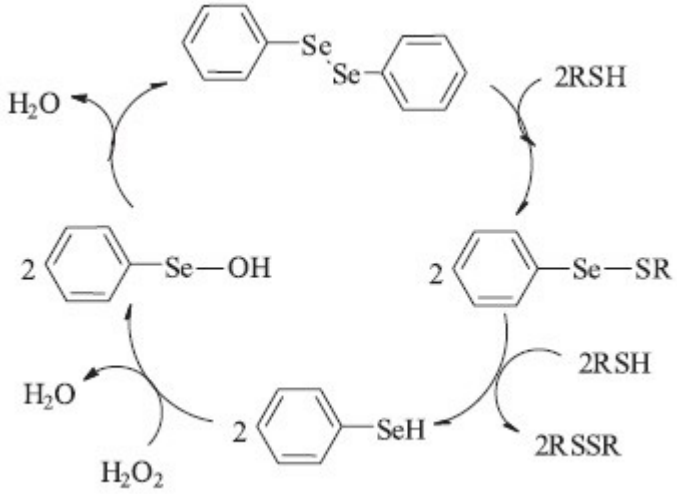

Figura 3 – Atividade mimética da GPx do Difenil disseleneto.
Fonte: Nogueira e Rocha. 2010.

Trabalhos anteriores trazem evidências sugerindo que o $(PhSe)_2$ assim como ebselen apresentam suas propriedades antioxidantes intimamente associadas com a atividade mimética TrxR, formando o intermediário selenol/selenoato através da oxidação de NADPH, que pode novamente ser reoxidado a difenil disseleneto pela atividade da enzima TrxR (Nogueira and Rocha, 2011, 2010; Zhao and Holmgren, 2002). Além disso, estudos sugerem que a formação do intermediário selenol via atividade mimética da TrxR poderia decompor H_2O_2 de forma mais eficiente do que a atividade mimética da GPx (de Freitas and Rocha, 2011; Sausen de

Freitas et al., 2010), a atividade TrxR do $(PhSe)_2$ poderia explicar efeito hepatoprotetor observado após a intoxicação por APAP sem a redução nos níveis de GSH citosólicos (da Rosa et al., 2012; Rosa et al., 2007). Neste contexto, a atividade mimética da GPx para o $(PhSe)_2$ está diretamente associada a sua concentração, podendo em alguns casos causar efeitos danosos para o tecido (Rosa et al., 2007).

Atualmente o antídoto mais eficaz para auxiliar no tratamento da intoxicação hepática induzida por APAP é a N-acetilcisteina (NAC). Contudo, NAC apresenta sua eficácia limitada e o uso do medicamento por via sistêmica pode ser seguido ocasionalmente por reações de hipersensibilidade como náusea, vômito, diarreia, irritação gastrintestinal e, raramente, reações como urticária e broncoespasmo (Woodhead et al., 2012). Por este motivo, se faz necessário buscar alternativas terapêutica para controlar o dano hepático causado por APAP. A utilização de fitoterápicos têm sido considerada, no entanto, os protocolos experimentais consistem em pré-tratamentos com doses muito elevadas seguido pela intoxicação, o que torna pouco viável clinicamente (Bulku et al., 2012; Gamal el-din, 2003; Küpeli et al., 2006). Considerando estes fatos, o uso de compostos orgânicos de selênio emergem como uma medida terapêutica alternativa, principalmente porque muitos destes compostos apresentarem propriedades antioxidantes e antiinflamatórias (Borges et al., 2006; Brandão et al., 2009; Meotti et al., 2004). Em especial o $(PhSe)_2$ que possui uma absorção rápida (Prigol et al., 2012), uma característica importante para o tratamento da overdose, uma vez que os períodos iniciais da intoxicação são fundamentais para o sucesso do tratamento. O $(PhSe)_2$ tem sido demonstrado um tratamento efetivo para intoxicação por APAP em camundongos, mesmo quando administrado após ingestão da dose tóxica, atuando como um antioxidante com alta capacidade neutralizadora de EROs (da Rosa et al., 2012; da Silva et al., 2012). Outros trabalhos demonstraram que a administração de $(PhSe)_2$ também pode proporciona um efeito protetor principalmente pela modulação do dano oxidativo, aumentando a sobrevivência de animais tratados com uma dose hepatotóxica de APAP (Rocha et al., 2005; Wilhelm et al., 2009).

Devido ao papel importante da disfunção mitocondrial, a qual não é somente uma causa, mas também uma consequência da IHA induzida por APAP, a modulação deste processo é fundamental para que ocorra a adaptação celular. No entanto, os efeitos dos compostos orgânicos de selênio sobre a funcionalidade mitocondrial ainda permanece incerto. Estudos sugerem um potencial efeito tóxico com a indução da MPT (Kim et al., 2003; Puntel et al., 2010), os quais podem estar diretamente relacionados com a concentração do composto (Morin et al., 2003; Puntel et al., 2010). Por outro lado, compostos orgânicos de selênio

modulam a expressão de diferentes selenoproteinas (Zhang et al., 2013), assim como podem ser capaz de preservar a função mitocondrial reduzindo a lesão cerebral após um evento de isquemia/reperfusão (Mehta et al., 2012). O $(PhSe)_2$ em cultura de células induz uma adaptação significativa contra o estresse oxidativo com a modulação de Nrf2-ARE (de Bem et al., 2013), por sua vez o tratamento com $(PhSe)_2$ foi efetivo em reduzir a disfunção mitocondrial induzida por APAP em cérebro (da Silva et al., 2012). No entanto, ainda não foi demonstrado se o $(PhSe)_2$ seria uma alternativa terapêutica eficaz na proteção contra disfunção mitocondrial induzida por APAP no fígado.

4. OBJETIVOS

4.1. Objetivo Geral

Investigar por quais mecanismos o Difenil disseleneto modula o comprometimento bioenergético e a disfunção mitocondrial em na insuficiência hepática aguda induzida por paracetamol.

4.2. Objetivos Específicos

Analisar os efeitos do difenil disseleneto como estratégia terapêutica na reversão da insuficiência hepática aguda induzida por paracetamol através do desenvolvimento dos parâmetros bioquímicos destacados nos objetivos específicos seguintes.

- Avaliar a eficiência terapêutica do $(PhSe)_2$ em comparação com o antídoto clássico em homogenato de fígado frente o estresse oxidativo;
- Avaliar a influência do $(PhSe)_2$ na redução da disfunção mitocondrial induzida por APAP;
- Determinar a influência do $(PhSe)_2$ sobre a função bioenergética mitocondrial após a indução da insuficiência hepática aguda;
- Determinar o possível mecanismo de resposta adaptativa envolvido na hepatoproteção induzida por (PhSe)2 na intoxicação hepática induzida por APAP.

5. RESULTADOS

Os resultados que fazem parte desta tese serão apresentados sob a forma de um artigo científico e um manuscrito, os quais se encontram aqui organizado. Os itens Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas, encontram-se no artigo científico e no manuscrito.

5.1. Artigo: Nova Abordagem Terapêutica: Difenil Disseleneto Reduz A Disfunção Mitocondrial Na Insuficiência Hepática Aguda Induzida Por Paracetamol

Artigo científico publicado na revista PLOs ONE

New Therapeutic Approach: Diphenyl Diselenide Reduces Mitochondrial Dysfunction in Acetaminophen-Induced Acute Liver Failure

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New Therapeutic Approach: Diphenyl Diselenide Reduces Mitochondrial Dysfunction in Acetaminophen-Induced Acute Liver Failure

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Abstract

The acute liver failure (ALF) induced by acetaminophen (APAP) is closely related to oxidative damage and depletion of hepatic glutathione, consequently changes in cell energy metabolism and mitochondrial dysfunction have been observed after APAP overdose. Diphenyl diselenide [$(PhSe)_2$], a simple organoselenium compound with antioxidant properties, previously demonstrated to confer hepatoprotection. However, little is known about the protective mechanism on mitochondria. The main objective of this study was to investigate the effects $(PhSe)_2$ to reduce mitochondrial dysfunction and, secondly, compare in the liver homogenate the hepatoprotective effects of the $(PhSe)_2$ to the *N*-acetylcysteine (NAC) during APAP-induced ALF to validate our model. Mice were injected intraperitoneal with APAP (600 mg/kg), $(PhSe)_2$ (15.6 mg/kg), NAC (1200 mg/kg), APAP+$(PhSe)_2$ or APAP+NAC, where the $(PhSe)_2$ or NAC treatment were given 1 h following APAP. The liver was collected 4 h after overdose. The plasma alanine and aspartate aminotransferase activities increased after APAP administration. APAP caused a remarkable increase of oxidative stress markers (lipid peroxidation, reactive species and protein carbonylation) and decrease of the antioxidant defense in the liver homogenate and mitochondria. APAP caused a marked loss in the mitochondrial membrane potential, the mitochondrial ATPase activity, and the rate of mitochondrial oxygen consumption and increased the mitochondrial swelling. All these effects were significantly prevented by $(PhSe)_2$. The effectiveness of $(PhSe)_2$ was similar at a lower dose than NAC. In summary, $(PhSe)_2$ provided a significant improvement to the mitochondrial redox homeostasis and the mitochondrial bioenergetics dysfunction caused by membrane permeability transition in the hepatotoxicity APAP-induced.

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Introduction

Acetaminophen (*N*-acetyl-p-aminophenol; APAP) is a drug widely employed as an analgesic and antipyretic that can induce acute liver failure (ALF) when high doses are ingested [1]. Recent data suggest a dramatic increase in ALF, liver transplants and considerable morbidity and mortality associated with APAP overdoses in the United States and many other countries [2,3]. During overdoses, APAP is mainly metabolized in the liver by cytochrome P450, resulting in a highly reactive intermediate, N-acetyl-p-benzoquinone imine (NAPQI). NAPQI reacts directly with glutathione (GSH), causing a depletion of GSH in the liver. This redox imbalance in the liver has been shown to play a major role in ALF associated with APAP [3]. If glutathione is not replenished, NAPQI begins to form covalent bonds with cellular proteins, modifying their structure and function [4,5]. In addition, the accumulation of neutrophils and Kuppfer cells contribute to the inflammatory process and reactive species (RS) generation in the hepatocytes [5,6,7]. The hepatic injury is associated with damage to subcellular organelles including mitochondria, because mitochondria are responsible for cellular energy metabolism and represent a remarkable source of intracellular RS generation in mammalian cells, effects on this organelle are critical with regard to APAP-mediated liver injuries [8].

The compound N-acetylcysteine (NAC) is the treatment of choice for acute poisoning with APAP [2]. NAC administration is beneficial for preventing or reducing ALF by increasing GSH and thiols levels, reduces the histological changes caused by oxidative stress induced by APAP overdose [1,9,10]. The efficacy of NAC and the prognosis are dependent on three factors, the type of APAP ingestion (acute vs. chronic), the dose of APAP ingestion and the elapsed time from APAP ingestion to the initiation of NAC treatment [9]. In clinical situations, NAC is administered after the occurrence of an APAP overdose, making the study of alternative therapies attractive.

Considering the fact that NAC efficacy is limited to a narrow window of time and situations [10,11]. The use of organoselenium compounds could emerge as an alternative therapy. Several studies have demonstrated both the antioxidant and the anti-inflammatory properties of organoselenium compounds such as diphenyl diselenide [$(PhSe)_2$] and ebselen (Ebs) [12,13,14]. In particular, $(PhSe)_2$, the simplest of diaryl diselenides and a lipophilic organic compound of selenium, has demonstrated low toxicity in different experimental models. For example, the calculated LD_{50} in mice is 655 mg/kg when administered intraperitoneally [15]. The hepatoprotective is associated with the biochemical and pharmacological properties of the organoselenium compounds to scavenge hydrogen peroxide and other organic hydroperoxides originate from the powerful nucleophile intermediates that involve the selenol-selenolate group, which play critical roles in their glutathione peroxidase– and thioredoxin reductase–like activities [13,15]. Earlier work from our laboratory has shown that $(PhSe)_2$ is effective for the treatment of cellular damage caused by APAP [16,17]. However our study uses for the first time the $(PhSe)_2$ as a possible target to the mitochondrial dysfunction in hepatic failure caused by APAP in a new therapeutic approach.

Previously, the APAP toxicity was shown to consist of two crucial phases: the initial GSH depletion and covalent binding of NAPQI to target proteins and the subsequent increase in the mitochondrial permeability transition (MPT) and nitration of proteins [7]. In this condition, the impairment of GSH antioxidant system has been noticed to enhance the susceptibility to mitochondrial dysfunction from oxidant stress and resulting in the collapse of mitochondrial membrane potential ($\Delta\psi_m$) and ATP depletion [18]. It should be noted that MPT is mediated by oxidant stress [19]. Therefore, MPT occurs with the release of superoxide, which in turn can lead to peroxynitrite ($ONOO^-$) production and tyrosine nitration, a lethal event for the cell [8]. Moreover, both oxidative damage and NAPQI have been reported to produce MPT through the oxidation of critical thiols to disulfides, which appears to be a prerequisite for membrane permeabilization [20]. Currently, it has been suggested that organoselenium present modulatory effects in relation to mitochondrial oxidative stress; however, these studies were conducted using *in vitro* models [21,22]. Thus, there is no evidence in the literature demonstrating the effects of $(PhSe)_2$ on liver mitochondrial dysfunction caused by APAP intoxication.

Thus, considering that relatively few studies have focused on the mechanisms by which these organoselenium compounds exert their pharmacological effects on APAP-induced ALF [16,17], this study was designed to evaluate the benefits of the $(PhSe)_2$ treatment under the mitochondrial dysfunction, and subsequently, compare in liver homogenate the hepatoprotective effects with *N*-acetylcysteine (NAC) during APAP-induced ALF to validate our model. This work may contribute to a better understanding of the $(PhSe)_2$ mechanism of action and open new perspectives for its application.

Materials and Methods

Materials

$(PhSe)_2$ (98%), thiobarbituric acid (TBA), 2′-7′-dichlorofluorescein (DCFH), trichloroacetic acid (TCA) and nucleotides were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of analytical grade and obtained from standard commercial suppliers.

Animals

Seven-week-old male adult Swiss albino mice (30–40 g) from our own breeding colony were used. The animals were kept on a separate animal room, on at 12 h light/dark cycle, at temperature of 22±2°C, with free access to food and water. Mice were acclimated for 7 days before initiation of any procedures. This study was approved by the Ethics and Animal Welfare Committee of Federal University of Santa Maria, Brazil.

Experimental procedure

Briefly, the mice were randomly divided into the following groups: Control (vehicle); diphenyl diselenide [$(PhSe)_2$]; N-acetylcysteine (NAC); acute liver failure (induced by APAP); acute liver failure treated with diphenyl diselenide [APAP+$(PhSe)_2$] and acute liver failure treated with N-acetylcysteine (APAP+NAC). All the solutions were administered by the intraperitoneal (i.p.) route. Injections were administered at 9:00 a.m. in order to remove any confounding factors of circadian rhythm. The APAP, $(PhSe)_2$ and NAC doses were described earlier [9,16,17]. Each group contained 7 different mice/group. Mice in the control, $(PhSe)_2$ and NAC groups were injected intraperitoneally (i.p.) with saline 0.9% (20 ml/Kg), and mice in the APAP, APAP+$(PhSe)_2$ and APAP+NAC groups were injected i.p. with 600 mg/Kg APAP (20 ml/Kg in saline 0.9%). One hour after saline and APAP treatment, mice were injected i.p. with 15.6 mg/Kg $(PhSe)_2$ (2.5 mL/Kg in canola oil) in the $(PhSe)_2$ and APAP+$(PhSe)_2$. In addition, studies were done with a higher dose of NAC to validate our model. One hour after saline and APAP treatment, mice were injected i.p. with 1200 mg/kg NAC (20 ml/kg in saline 0.9%) in the NAC and APAP+NAC. The feed was available ad libitum and animals were not fasted prior to dosing. The biochemical analyses were carried out 4 h as previous studies had shown toxicity was apparent at this time [16,17]. The animals were killed by cervical dislocation and blood was collected by cardiac puncture using heparin-rinsed 1-mL syringes (20-gauge needles) and centrifuged. The plasma was used for determination of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities using a commercial kit (Labtest®, Diagnostica S.A., Minas Gerais, Brazil).

Survival

For survival studies, mice were injected with 600 mg/kg and after 1 h treated with $(PhSe)_2$ or NAC. Then, thirty minutes later the mice were returned to their cages and fed with food and water *ad libitum*. To determine the effect of $(PhSe)_2$ and NAC on mortality of APAP-administrated mice, the survival rate after APAP administration was evaluated for 48 h.

Liver homogenates preparation

At the end of the treatment period the liver was removed and quickly dissected, placed on ice, and immediately homogenized in cold 10 mM Tris–HCl pH 7.4. Homogenates were centrifuged at 2,000×g for 10 min to yield the low-speed supernatant fractions that were used for different biochemical assays in all trials. Besides, aliquots of liver preparations were frozen (−20°C) for posterior analysis.

Isolation of liver mitochondria

Mice liver mitochondria were isolated at 4°C by differential centrifugation [23]. The animals were sacrificed by cervical dislocation. The livers were rapidly removed (within 1 min) and immersed in ice-cold "Ionic Medium" containing 100 mM Sucrose, 10 mM EDTA, 46 mM KCl and 100 mM Tris-HCl, pH 7.4. The tissue was minced using surgical scissors and then

extensively washed. The tissue was then homogenized in a power-driven, tight-fitting Potter Elvehjem homogenizer with Teflon pestle. The resulting suspension was centrifuged for 7 min at 2,000×g in a Hitachi CR 21E centrifuge. After centrifugation, the supernatant was recentrifuged for 10 min at 12,000×g. The pellet was resuspended in "Ionic Medium+BSA" containing 100 mM sucrose, 10 mM EDTA, 46 mM KCl, 0.1% bovine serum albumin free fatty acid and 100 mM Tris-HCl, pH 7.4, and recentrifuged at 12,000×g for 10 min. The supernatant was decanted, and the final pellet was gently washed and resuspended in "Suspension Medium" containing 230 mM mannitol, 70 mM sucrose, and 20 mM Tris-HCl, pH 7.4. The pellet was washed three times with ice suspension medium buffer to get intact mitochondria, which it has been suggested that after three washings, influence of contaminating microsomes on NAD(P)H oxidation, reactive oxygen species (ROS) production and membrane permeabilization becomes negligible [24]. An aliquot of the resulting mitochondrial suspension were separated and rapidly frozen at −80°C for later biochemical analysis of GSH content, TBARS, protein carbonyls and mitochondrial enzymes.

Measurement of lipid peroxidation (LPO)

Liver homogenate and mitochondrial membrane LPO were quantified measuring the malondiadehyde (MDA). In summary, liver homogenate and mitochondria protein were incubated in 300 µl of a medium consisting of 175 mM KCl and 10 mM Tris-HCl, pH 7.4, and then, were added to color reaction. Thiobarbituric acid reactive substances (TBARS) levels were measured at 532 nm using a standard curve of MDA [25].

Measurement of ROS production

ROS generation was determined spectrofluorimetrically in liver homogenate and mitochondria, using H_2DCF-DA levels as an index of the peroxide production by cellular components (1 µM) [26]. Briefly, the liver homogenate and mitochondria were added to standard medium and the fluorescence was determined at 488 nm for excitation and 525 nm for emission, with slit widths of 3 nm.

Measurement of reduced glutathione (GSH)

GSH levels were determined in liver homogenate and mitochondria with fluorescence detection after reaction of the supernatants from deproteinized containing H_3PO_4/NaH_2PO_4–EDTA, with *O*-Phthalaldehyde (OPT) [27]. In brief, 250 mg of liver were homogenized in 3.75 mL phosphate–EDTA buffer (100 mM NaH_2PO_4, 5 mM EDTA, pH 8.0) plus 1 mL H_3PO_4 (25%), and isolated liver mitochondria (0.5 mg protein) resuspended in 1.5 mL phosphate-EDTA buffer and 500 µl H_3PO_4 (4.5%) were rapidly centrifuged at 100,000×g (Hitachi, TL-100 ultracentrifuge) for 30 min. For GSH determination, 100 µl of supernatant was added to 1.8 ml phosphate buffer and 100 µl OPT. After thorough mixing and incubation at room temperature for 15 min, the solution was transferred to a quartz cuvette and the fluorescence was measured at 420 and 350 nm emission and excitation wavelengths, respectively. GSH contents were determined from comparisons with a linear GSH standard curve.

Measurement of antioxidant enzyme activities

The activities of antioxidant enzymes, total superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), glutathione reductase (GR), and glutathione peroxidase (GPx) have been measured in liver homogenates.

Liver homogenate total SOD activity was measured by the capacity of inhibiting auto-oxidation of adrenaline to adrenochrome at 480 nm [28]. The liver supernatant (5 µg protein) was added to a medium containing 2 mM EDTA, 50 mM $NaHCO_3$/Na_2CO_3 buffer (pH 10.3) and adrenaline (4 mM).

The CAT enzyme activity was determined in liver homogenate in according to the method previously proposed [29]. Liver homogenate (5 µg protein) was added to a medium containing potassium phosphate buffer (50 mM KH_2PO_4, 50 mM K_2HPO_4; pH 7.4) and H_2O_2 (1 mM). The kinetic analysis of CAT was started after H_2O_2 addition. The CAT activity was determined using the molar extinction coefficient 36 $M^{-1}cm^{-1}$ and the reaction was measured at 240 nm.

Glutathione-S-transferase (GST) activity was determined spectrophotometrically [30]. GST activity was quantified in liver homogenates (5 µg protein) in a reaction mixture containing 1 mM 1-chloro-2,4-dinitrobenzene (CDNB), and 1 mM glutathione as substrates in 0.1 M sodium phosphate buffer, pH 6.5, at 37°C. Enzyme activity was calculated by the change in the absorbance value from the slope of the initial linear portion of the absorbance time curve at 340 nm for 5 min. Enzyme activity was determined using the molar extinction coefficient 9,6 $mM^{-1}cm^{-1}$ and expressed as nmol CDNB/min/mg Prot.

Glutathione peroxidase (GPx) activity was determined spectrophotometrically at 340 nm by NADPH consumption for 2 min at 30°C [31]. The liver homogenate supernatant (5 µg protein) was added to medium containing 0.1 M phosphate buffer (0.1 M KH_2PO_4, 0.1 M K_2HPO_4 and 5 mM EDTA, pH 7.0), 1 mM GSH, 0.15 mM NADPH, 0.1 U/mL GR and 1 mM sodium azide. So, the reaction was initiated by adding the H_2O_2 to a final concentration of 0.4 mM. The GPx activity was determined using the molar extinction coefficient 6220 $M^{-1}cm^{-1}$ and expressed as nmol/min/mg protein.

For the measurement activity of glutathione reductase (GR) activity, the liver homogenate supernatant (5 µg protein) was added to medium containing 0.15 M phosphate buffer (0.15 M K_2HPO_4 and 1.5 mM EDTA, pH 7.0) and 0.15 mM NADPH. The measurements were made at 340 nm and initiated with addition of 20 mM GSSG, at 30°C for 2 min [32]. GR activity was determined using the molar extinction coefficient 6220 M^{-1} cm^{-1} and expressed as nmol/min/mg protein.

Measurement of mitochondrial protein carbonyls

The oxidative damage to proteins was measured by the quantification of carbonyl groups based on the reaction with dinitrophenylhidrazine (DNPH) assay [33]. The mitochondria were divided into two portions containing 1 mg of protein in each. To one portion, 1 ml of 2 N HCl was added and incubated at room temperature shaking intermittently for 1 h. The other portion was treated with 1 ml of 10 mM DNPH in 2 N HCl and incubated by shaking intermittently for 1 h at room temperature. After incubation the mixture was precipitated with 10% TCA and centrifuged. The precipitate was washed thrice with 1 ml of ethanol:ethyl acetate (1:1). The final protein precipitate was dissolved in denaturation buffer (3% SDS and 150 mM NaH_2PO_4; pH 6.8) and the absorption at 370 nm (DNPH-treated sample minus sample blank) was determined. Carbonyl content was calculated using the molar extinction coefficient of 22,000 M^{-1} cm^{-1} and expressed as nmol carbonyls/mg mitochondrial protein.

Mitochondrial transmembrane electrical potential ($\Delta\psi_m$)

Mitochondrial $\Delta\psi_m$ was estimated by fluorescence changes in Safranine – *O* (10 µM) recorded by RF-5301 Shimadzu

spectrofluorometer (Kyoto, Japan) operating at excitation and emission wavelengths of 495 and 586, with slit widths of 5 nm [34]. The mitochondria (0.5 mg protein) were added and 30 second latter mitochondrial respiration was induced by the addition of succinate and glutamate. Mitochondrial preparation, which was held on ice, was well maintained and did not change over the course of 5–6 hours, as determined by their ability to maintain a stable transmembrane potential in the presence of oxidizable substrates.

Mitochondrial swelling

Measurement of mitochondrial swelling was performed in RF-5301 Shimadzu spectrofluorometer at 600 nm and slit 1.5 nm for excitation and emission. The mitochondria (0.1 mg protein) were incubated in the presence of 100 μM Ca^{2+} [19].

Oxygen consumption of liver mitochondria

The oxygen consumption of liver mitochondria was measured using an oxymeter (Hansatech model with a Clark-type electrode) at 30°C. The cuvete containing aerated medium consisting of 225 mM mannitol, 75 mM sucrose, 10 mM KCl, 10 mM Tris-HCl, 10 mM K_2HPO_4, 5 mM $MgCl_2$, 0.1 mM EDTA (pH 7.4) was added 0.1 mg mitochondrial protein. Pyruvate (5 mM), glutamate (5 mM) and succinate (5 mM) were placed in the medium to increase the respiratory state.

Assessment of mitochondrial activity (MTT reduction assay)

This assay is based on the ability of mitochondrial enzymes to metabolize MTT into formazan, a reaction that takes place only in functionally intact mitochondria. The mitochondrial samples (0.1 mg protein) were incubated with 20 mM succinate at 30°C for 1 hour. After that, color was quenched with DMSO, and readings were reported as the difference in absorbance between 570 and 630 nm, and then, expressed in percent of the control [35].

Measurement of mitochondrial antioxidant enzyme activities

The activities of antioxidant enzymes in liver mitochondria were measured by the same methods described above. The enzyme activities in isolated mitochondria were measured after disruption of mitochondria by freeze-thawing (3x), following centrifugation at 2,000xg for 1 minute at 4°C, and the mitochondrial supernatant (0.1 mg protein/mL) was add to reaction medium.

Mitochondrial MnSOD activity was measured as described previously [28]. The isolated mitochondria were assayed after incubation with 1 mM KCN. At this concentration cyanide inhibits the CuZnSOD isoform of the enzyme, but does not affect the MnSOD isoform [36].

Mitochondrial GPx activity was measured as described previously [31].

For the GR activity measurement, the mitochondria supernatant was added to reaction medium as described previously [32].

Mitochondrial complex I and complex II assays

The samples were frozen and thawed three times, and mitochondrial electron transfer chain activity detection was performed as described below.

The activity of complex I (NADH dehydrogenase) was measured by following the oxidation of NADH [37,38]. Approximately 0.1 mg protein of mitochondria was added to a solution containing 35 mM potassium phosphate buffer (pH 7.4) and 1.3 mM 2,6 dichloroindophenol (DCIP) in a final volume of 1 mL. The reaction was initiated with the addition of 0.15 mM NADH. Absorbance at 600 nm was monitored for 2 min to follow the rate of oxidation of NADH, and the activity was determined using an extinction coefficient of 6.22 mM^{-1} cm^{-1}. After thawing, the mitochondria were found to be completely permeable to NADH.

The activity of complex II (succinate dehydrogenase) was determined by following the reduction of DCIP by succinate [39]. The reaction mixture consisted of 50 mM potassium phosphate buffer pH 7.0, 1 mM KCN, 0.05 mM DCIP, 16 mM succinate and 0.1–0.5 mg protein of mitochondrial. Absorbance changes were followed at 600 nm, using an extinction coefficient of 19.1 mM^{-1} cm^{-1} for dichloroindophenol.

Mitochondrial ATPase activity

The mitochondrial ATPase activity was measured as the hydrolysis rate of ATP to ADP + Pi [40]. Mitochondria were incubated in buffer consisting of 50 mM Tris-HCl, pH 7.4, 75 mM KCl and 0.4 mM EDTA; 6.0 mM $MgCl_2$. After pre-incubating 0.2–0.25 mg protein of mitochondrial in the reaction mixture for 2 min at 37°C, the reaction was started by adding 6.0 mM ATP and carried out for 10 min. At the end of the incubation period, the reaction was terminated by adding 0.1 ml of 5% (w/v) sodium dodecyl sulphate [41]. A control was performed in same conditions in order to obtain the non-enzymatic hydrolysis of ATP. Inorganic phosphate (Pi) production was measured using the method based on the determination of the Pi released to the reaction medium by the hydrolysis of the ATP [42]. The activity was measured spectrophotometrically at 405 nm. The values were calculated in relation to a standard curve constructed with Pi at known concentration sand also corrected by the protein content.

Protein Determination

Protein content was determined using bovine serum albumin (BSA) as standard [43].

Statistical analysis

Statistical analysis was performed using GraphPad (version 5.0 for Macintosh OSX, GraphPad Software, San Diego, CA). Significance was assessed by one-way analysis of variance (ANOVA), followed by Newman–Keuls's Test for post-hoc comparison. Values of $p<0.05$ were considered statistically significant.

Results

Effects of $(PhSe)_2$ and NAC on Survival after APAP overdose

Mice were monitored for 48 h to determine the effects of (PhSe)2 and NAC on the survival of mice following an APAP overdose. The mice received 600 mg/kg APAP intraperitoneally in a single dose. The APAP group mortality was pronounced when compared to the control group, which was 100% in approximately 8 h (Fig. 1). Treatment with (PhSe)2 dramatically extended the percent survival after the lethal APAP dose. All (PhSe)2 mice receiving APAP survived up to 37.5 h after treatment. A similar protection was reported following administration of NAC 1 h after the acute APAP overdose (Fig. 1). The NAC mortality was 78% compared to the control group. It is important to note that neither the (PhSe)2 and NAC controls altered the mice survival during the experimental period (data not shown).

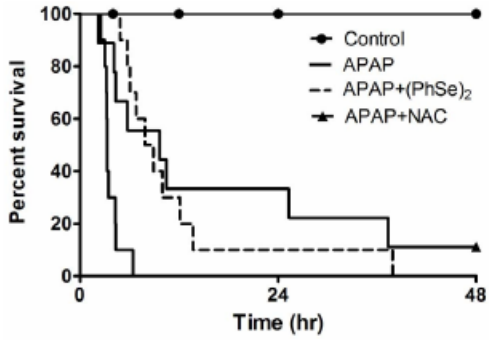

Figure 1. Effects of treatment with $(PhSe)_2$ and NAC on the survival following lethal doses of acetaminophen. Mice were given acetaminophen (600 mg/kg, i.p.) and 1 h after were treated with or without $(PhSe)_2$ (15.6 mg/kg, i.p.) and NAC (1200 mg/kg, i.p.). Survival was followed for 48 h, n = 10 per group.
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Effects of $(PhSe)_2$ and NAC on Liver Injury Induced by APAP after 4 hours

In the present study, animals developed hepatotoxicity 4 h after a single intraperitoneal dose of 600 mg/kg APAP, as judged from the increase in plasma AST and ALT activities. In the APAP mice treated with $(PhSe)_2$, the AST and ALT activity values did not significantly differ from the controls, confirming that the $(PhSe)_2$ prevented the liver from APAP-induced injury (Table 1). Further studies evaluated whether the protection afforded by the $(PhSe)_2$ for APAP hepatotoxicity was comparable to the NAC. The increase in plasma AST and ALT values induced by APAP was also prevented in the APAP+NAC group (Table 1). The levels of hepatotoxicity markers indicated that the $(PhSe)_2$ and NAC were able to reduce the liver injury when administered following an APAP overdose (Table 1). In the present report the group treated with $(PhSe)_2$ or NAC did not show hepatotoxic effects (Table 1).

Table 1. Effects of $(PhSe)_2$ and NAC on the plasmatic transaminases levels after 4 hours.

	AST (IU/L)	ALT (IU/L)
Control	82.5±38.4	12.6±0.8
$(PhSe)_2$	122.8±75.6	13.2±1.9
NAC	93.7±22.2	16.1±2.3
APAP	673.9±134.3*	256.2±57.1*
APAP+$(PhSe)_2$	149.8±60.5#	12.0±1.1#
APAP+NAC	80.7±12.8#	19.8±6.1#

Mice were given acetaminophen (600 mg/kg, i.p.) and 1 h after treated with $(PhSe)_2$ (15.6 mg/kg, i.p.); or NAC (1200 mg/kg, i.p.), and were killed at 4 h after the APAP treatment. Data are expressed as means ± SEM, (n = 7). Significance was assessed by one-way analysis of variance (ANOVA), followed by Newman-Keuls's Test for post hoc comparison. Significant differences are indicated by *p≤0.05 when compared with control group. Significant difference is indicated by #p≤0.05 when compared with APAP group.
doi:10.1371/journal.pone.0081961.t001

Effects of $(PhSe)_2$ and NAC on Markers of the Oxidative Damage and Glutathione Redox System in Liver Homogenate following APAP Overdose

Lipid peroxidation (TBARS) caused by APAP is commonly associated with ROS generation in the liver [17]. Our results demonstrated that APAP induces a considerable increase in values of TBARS and ROS after 4 h (Table 2). Treatment with $(PhSe)_2$ or NAC 1 h after the APAP dose diminished the TBARS and ROS generation to a level comparable to the control levels (Table 2). In addition, the APAP administration induced a pronounced increase in the CAT activity levels compared to the control, $(PhSe)_2$ and NAC mice (Table 2), while the total SOD activity levels declined 4 h after APAP treatment (Table 2). However, the $(PhSe)_2$ and NAC mice following APAP administration were able to normalize the activity levels of CAT and total SOD in the liver homogenate after 4 h (Table 2). These results suggested that $(PhSe)_2$ protects against APAP toxicity by maintaining the markers of the oxidative damage at control levels, and similar levels were observed between the APAP+$(PhSe)_2$ and APAP+NAC groups (Table 2).

The glutathione redox system is a major cellular antioxidant system that combats ROS and xenobiotics in the cell. APAP depleted the liver homogenate GSH levels when compared to the control (Table 3). $(PhSe)_2$ and NAC administration 1 h after the APAP dose prevented the depletion of GSH (Table 3). APAP also decreased the GPx, GR and GST activity levels, suggesting impairment in the liver homogenate redox homeostasis (Table 3). Treatment with $(PhSe)_2$ following the APAP dose produced activity levels of GPx and GR that were similar to the control levels. Treatment with NAC resulted in a similar prevention of decline of GPx and GR activity (Table 3). However, the GST activity levels the APAP+$(PhSe)_2$ and APAP+NAC groups remained similar to the APAP group in the liver homogenate (Table 3). Therefore, these results suggested that administration of $(PhSe)_2$ 1 h after APAP treatment was sufficient to reduce the extent of the biochemical changes mediated by APAP.

Effects of $(PhSe)_2$ on Liver Mitochondrial Oxidative Damage and Changes in Antioxidant Enzyme Activities Induced by APAP

Oxidative stress and mitochondrial dysfunction are critical events during APAP-mediated liver injury. To investigate the effects $(PhSe)_2$ on the redox balance, we measured the markers of oxidative stress and the activity of antioxidant enzymes in the liver mitochondria. The administration of APAP to the mice resulted in significantly increased levels of TBARS, protein carbonylation and an accumulation of ROS in the liver mitochondria, indicating that APAP induced oxidative stress in the liver mitochondria. The $(PhSe)_2$ treatment significantly abolished all the effects in the mice exposed to APAP (Fig. 2A, 2B and 2C). The APAP overdose depleted the mitochondrial GSH levels at 4 h when compared to control levels, but administration of $(PhSe)_2$ prevented the collapse of the mitochondrial glutathione redox balance caused by the APAP hepatotoxicity (Fig. 3).

Because antioxidant enzymes contribute to the maintenance of redox equilibrium, we next measured the activity of various enzymes involved in the scavenging of ROS (GPx, GR and MnSOD) and observed that APAP administration significantly reduced the activity of the antioxidant enzymes that were analyzed in the mitochondria. The activities of the enzymes reached values that significantly differed from those in the control group. Treatment with $(PhSe)_2$ prevented this outcome, and enzyme activity values in the APAP+$(PhSe)_2$ group did not significantly

Table 2. Effects of $(PhSe)_2$ and NAC on oxidative damage markers in liver homogenate after 4 hours.

	TBARS (nmol MDA/mg Prot)	ROS (μmol DCF/mg Prot)	CAT (μmol H_2O_2/min/mg Prot)	SOD (U/mg Prot)
Control	0.3±0.10	4.1±0.7	170.3±9.9	141.2±11.4
$(PhSe)_2$	0.2±0.03	4.9±1.1	186.9±21.6	171.1±15.8
NAC	0.3±0.03	3.5±0.7	149.8±24.1	144.9±9.7
APAP	1.4±0.2*	8.8±0.3*	254.4±13.9*	70.6±3.3*
APAP+$(PhSe)_2$	0.8±0.1#	4.7±0.8#	190.3±21.4#	113.5±7.3#
APAP+NAC	0.5±0.1#	4.3±0.9#	170.1±29.4#	158.8±17.3#

Mice were given acetaminophen (600 mg/kg, i.p.) and 1 h after treated with $(PhSe)_2$ (15.6 mg/kg, i.p.); or NAC (1200 mg/kg, i.p.), and were killed at 4 h after the APAP treatment. Data are expressed as means ± SEM, (n = 7). Significance was assessed by one-way analysis of variance (ANOVA), followed by Newman-Keuls's Test for post hoc comparison. Significant differences are indicated by *p≤0.05 when compared with control group. Significant difference is indicated by #p≤0.05 when compared with APAP group.

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differ from the control values (Fig. 4A, 4B and 4C). Therefore, effects of $(PhSe)_2$ on the antioxidant enzyme activities contributed to the maintenance of the redox equilibrium in the liver mitochondria.

Effects of $(PhSe)_2$ on APAP-Induced Liver Mitochondrial Dysfunction

Next, we analyzed the effects of $(PhSe)_2$ on APAP-induced liver mitochondria dysfunction. Because mitochondrial respiration and ATP production depend on the transmembrane electrical potential and mitochondrial membrane integrity, the $\Delta\psi_m$ and mitochondrial swelling were analyzed. A marked decrease of $\Delta\psi_m$ and considerable swelling were observed in the liver mitochondria of the mice exposed to APAP compared to the control group. Treatment with $(PhSe)_2$ after APAP exposure prevented the loss of $\Delta\psi_m$ and prevented the mitochondrial swelling (Fig. 5 and Fig. 6, respectively). To determine whether APAP overdoses cause changes in the mitochondrial bioenergetics function, the NAD(P)H redox and mitochondrial activity were measured. APAP administration caused a significant decrease of mitochondrial NAD(P)H redox status and mitochondrial activity, but $(PhSe)_2$ treatment following the APAP exposure prevented those effects (Fig. 7).

To further elucidate the mechanism by which APAP impairs the mitochondrial bioenergetics function, the impact of the hepatotoxicity was assessed with regard to the electron transport chain (complex I and II) and the mitochondrial ATPase activity. The activities of complex I (NADH dehydrogenase), complex II (succinate dehydrogenase) and mitochondrial ATPase were significantly reduced in the mice with APAP-induced liver injuries, while the activity values of these enzymes did not significantly differ from the control in the APAP+$(PhSe)_2$ group (Fig. 8).

Because of the observed changes in the mitochondrial electron transport chain, the mitochondrial aerobic capacity could also be affected upon exposure to APAP. Therefore, the rates of glutamate/pyruvate and succinate-supported O_2 consumption in liver mitochondria preparations were monitored (Table 4). APAP administration caused a significant depletion of the rate of mitochondrial oxygen consumption induced for the substrates of complex I (glutamate and pyruvate) and the substrate of complex II (succinate). The treatment with $(PhSe)_2$ was able to restore the oxygen consumption to values that did not significantly differ from those in the control group (Table 4). Overall, these experiments suggested that mitochondrial dysfunction plays a crucial role in mitochondrial swelling, which is consistent with the changes that were observed in the membrane potential, NADH redox state, and mitochondrial activity and O_2 consumption, indicating that mitochondria undergo permeabilization following APAP-induced hepatotoxicity. However, the treatment with $(PhSe)_2$ even after 1 h was able to reduce the mitochondrial dysfunction.

Discussion

$(PhSe)_2$ delivers a hepatoprotective effect against APAP toxicity, but the mechanism remains unclear [17]. The aim of the present

Table 3. Effects of $(PhSe)_2$ and NAC on the Glutathione redox system in liver homogenate after 4 hours.

	GSH (nmol GSH/mg Prot)	GPx (nmol NADPH/min/mg Prot)	GR (nmol NADPH/min/mg Prot)	GST (nmol CDNB/min/mg Prot)
Control	36.1±0.4	381.4±34.0	28.1±3.1	566.1±93.4
$(PhSe)_2$	32.3±1.9	400.3±24.7	29.8±3.1	588.2±96.1
NAC	27.7±2.9	372.7±47.2	32.1±5.2	658.5±30.7
APAP	10.7±1.5*	147.7±19.1*	15.3±2.1*	298.8±20.7*
APAP+$(PhSe)_2$	30.1±1.8#	365.0±37.5#	30.6±3.3#	372.7±26.3*
APAP+NAC	33.5±2.8#	335.1±32.5#	32.1±3.8#	385.2±34.6*

Mice were given acetaminophen (600 mg/kg, i.p.) and 1 h after treated with $(PhSe)_2$ (15.6 mg/kg, i.p.); or NAC (1200 mg/kg, i.p.), and were killed at 4 h after the APAP treatment. Data are expressed as means ± SEM, (n = 7). Significance was assessed by one-way analysis of variance (ANOVA), followed by Newman-Keuls's Test for post hoc comparison. Significant differences are indicated by *p≤0.05 when compared with control group. Significant difference is indicated by #p≤0.05 when compared with APAP group.

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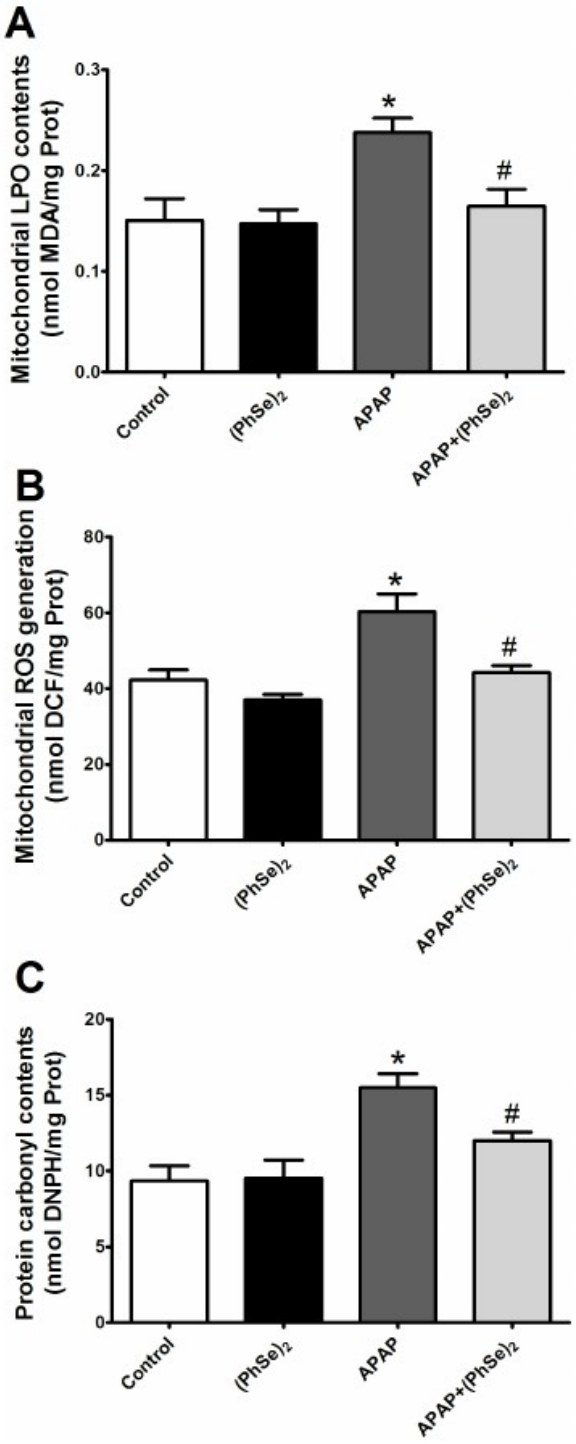

Figure 2. Effects of treatment with APAP and $(PhSe)_2$ on oxidative damage markers in liver mitochondria of mice. (A) TBARS. (B) Oxidized H_2DCF-DA. (C) Protein carbonyls. Mice were given acetaminophen (600 mg/kg, i.p.) and 1 h after were treated with or without $(PhSe)_2$ (15.6 mg/kg, i.p.), and were killed at 4 h after the APAP treatment. Data are expressed as means ± SEM, (n = 5). Significance was assessed by one–way analysis of variance (ANOVA), followed by Newman-Keuls's test for post hoc comparison. Significant differences are indicated by $^{*}p<0.05$ when compared with control group. Significant difference is indicated by $^{\#}p<0.05$ when compared with APAP group.

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study was to evaluate the ability of $(PhSe)_2$ to reduce the mitochondrial dysfunction and compare at the liver homogenate level the hepatoprotective effects of $(PhSe)_2$ to the clinically used antidote NAC during APAP-induced ALF to validate our model.

The effects of APAP are dose dependent, with the LD_{50} estimated to be 400 mg/kg, so doses above this threshold are considered lethal [9]. After exposure to low doses of APAP, the APAP absorption is usually rapid, approximately 40–60 min, while APAP overdoses often result in slightly longer absorption times, typically within 2 h [1]. Thus, 4 h after the APAP overdose, the liver damage induced leakage of AST and ALT into the plasma, confirming that the hepatic tissue was functionally impaired when compared to those of the control, APAP+$(PhSe)_2$ and APAP+NAC groups. As glucuronidation and sulfation routes become overwhelmed, the formation of NAPQI increases exponentially, with the peak levels at 4 h following the overdose [8]. Consequently, this process is followed by the perturbation of the cytosolic and mitochondrial GSH redox systems, i.e., the impairment of GSH levels and the activity of GSH-dependent enzymes (e.g., GR, GPx and GST). Additionally, the reduced activity of the total SOD and the enhanced CAT in the liver homogenate 4 h after the APAP overdose lead to a severe redox imbalance and an accumulation of RS that can exacerbate a complex cascade of reactions, culminating with lipid peroxidation and hepatocellular damage [8].

Organoselenium compounds have emerged as an alternative therapy to APAP overdoses; therefore, it is critical to establish a

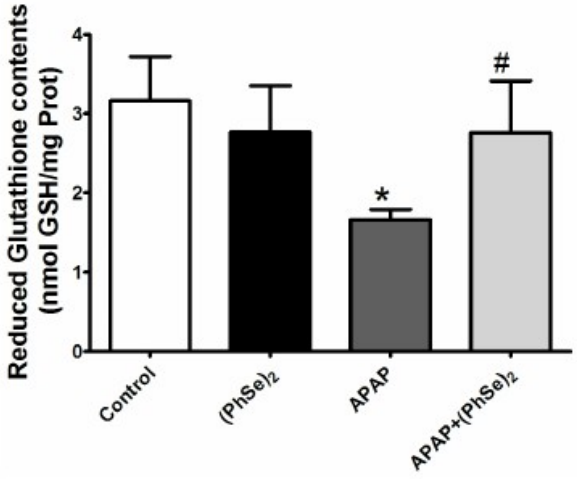

Figure 3. Effects of treatment with APAP and $(PhSe)_2$ on reduced glutathione (GSH) levels in liver mitochondria of mice. Mice were given acetaminophen (600 mg/kg, i.p.) and 1 h after were treated with or without $(PhSe)_2$ (15.6 mg/kg, i.p.), and were killed at 4 h after the APAP treatment. Dates are expressed as means ± SEM, (n = 5). Significance was assessed by one-way analysis of variance (ANOVA), followed by Newman-Keuls's test for post hoc comparison. Significant differences are indicated by $^{*}p<0.05$ when compared with control group. Significant difference is indicated by $^{\#}p<0.05$ when compared with APAP group.

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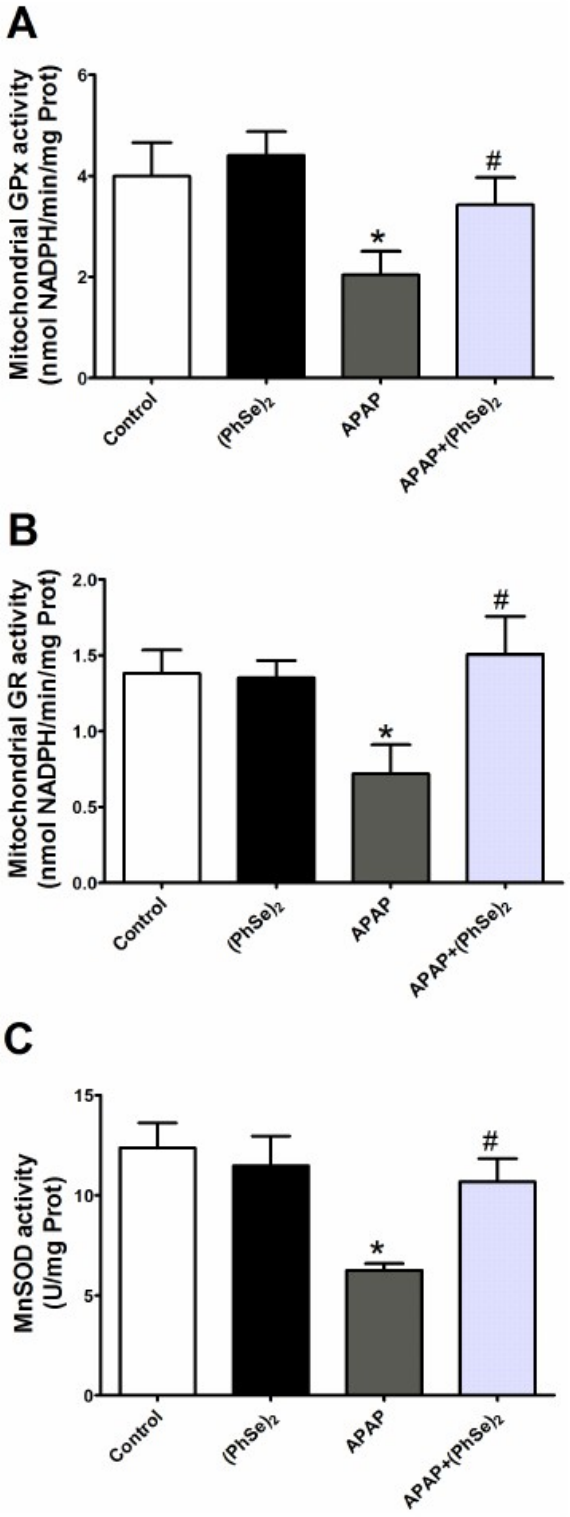

Figure 4. Effects of treatment with APAP and $(PhSe)_2$ on antioxidant enzyme activities in liver mitochondria of mice. (A) Glutathione peroxidase (GPx) activity. (B) Glutathione reductase (GR) activity. (C) Mn Superoxide dismutase activity. Mice were given acetaminophen (600 mg/kg, i.p.) and 1 h after were treated with or without $(PhSe)_2$ (15.6 mg/kg, i.p.), and were killed at 4 h after the APAP treatment. Dates are expressed as means ± SEM, (n = 5). Significance was assessed by one-way analysis of variance (ANOVA), followed by Newman-Keuls's test for post hoc comparison. Significant differences are indicated by $^{*}p<0.05$ when compared with control group. Significant difference is indicated by $^{\#}p<0.05$ when compared with APAP group.

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comparative parameter of $(PhSe)_2$ and NAC, the standard clinical antidote for APAP. There are few agents similar to NAC that are able to reduce ALF when administered following an APAP overdose [5,44]. The effectiveness of $(PhSe)_2$ was similar to the classic antidote, and we observed a significant improvement in the oxidative damage markers and antioxidant enzyme activity levels in the liver homogenate. These results corroborate with the remarkable capacity of the $(PhSe)_2$ to minimize all the parameters linked to oxidative stress [45]. The present study is the first to show that $(PhSe)_2$ was effective at a lower dose than NAC when administered 1 h after APAP. It has been demonstrated that the selenol-selenolate intermediate group from organoselenium compounds is biochemical and physiologically more nucleophilic than the thiol-thiolate groups from cysteine residues, including from NAC [15]. Our results clearly demonstrated a depression of GST activity 4 h after the APAP overdose, and neither the $(PhSe)_2$ nor the NAC treatment showed a protective effect in relation to the GST activity. The fact that the GST activity was not returned to the control level might be beneficial, as GST-null mice were previously reported to show resistance to APAP-induced hepatotoxicity [46]. Moreover, the increased activity of GR after 4 h in the APAP+$(PhSe)_2$ group would serve to increase the cellular levels of GSH, which is consistent with prior results from our lab [17].

Thus, according with our results, we believed that the $(PhSe)_2$ presents the therapeutic effects closely related to the three important points: maintenance of mitochondrial GSH, reduction of oxidative stress and inhibition of mitochondrial transition permeability. Firstly, the maintenance of mitochondrial GSH contributes to improve the redox homeostasis in liver, since the mitochondrial GSH pool is limited [47]. Previous reports asserted that the selective mitochondrial GSH depletion induces a significant increase of susceptibility in APAP overdose [48]. In addition, the GSH depletion precedes APAP toxicity [7]. Therefore, the concentration of intracellular GSH is a key determinant of the extent of APAP-induced hepatic injury [49]. Secondly, the abolishment of increase in oxidative markers (i.e., ROS, LPO and carbonyl proteins) is a consequence of the maintenance of antioxidant enzyme system (i.e., MnSOD, GPx and GR), which contributes to the reduction of the susceptibility to mitochondrial membrane permeability from oxidant stress [18,50]. Decreased levels of MnSOD have been shown to significantly increase APAP toxicity, which is consistent with the generation of superoxide occurring primarily in the mitochondria with APAP toxicity [51]. Indeed, this condition could induce the mitochondrial dysfunction and mitochondrial structural degeneration [6,7]. Finally, the inhibition of the MTP due to $(PhSe)_2$ antioxidant properties that prevent a vicious cycle, which leads to a dissipation of the H^+ gradient, impairing the oxidative phosphorylation system which is related to the bioenergetics control.

In this context, due to the $(PhSe)_2$ ability to undergo oxidation–reduction cycles with concomitant scavenging of the hydroperoxides the reduction of mitochondrial oxidative damage would rescue the functionality of tricarboxilic acid cycle enzymes and the

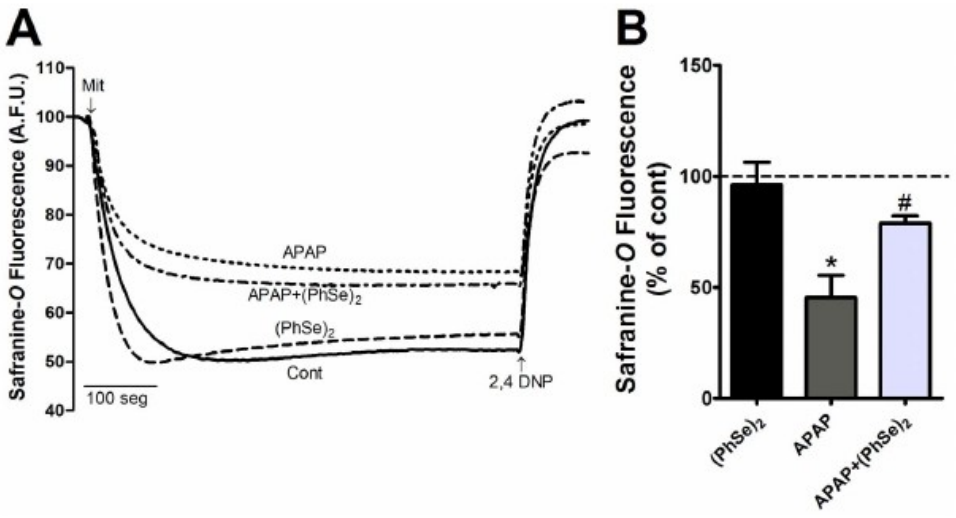

Figure 5. Effects of treatment with APAP and $(PhSe)_2$ on the mitochondrial membrane potential in liver mitochondria of mice. (A) The traces are representative of five independent experiments. (B) Means of the five experiments mitochondrial transmembrane electrical potential ($\Delta\psi_m$). Mice were given acetaminophen (600 mg/kg, i.p.) and 1 h after were treated with or without $(PhSe)_2$ (15.6 mg/kg, i.p.), and were killed at 4 h after the APAP treatment. Mitochondria (0.5 mg/ml) were incubated in the reaction medium containing 230 mM Mannitol, 70 mM Sucrose, 0.02 mM EDTA, 1 mM K_2HPO_4, 20 mM Tris-HCl, pH 7.4 and was energized by 5 mM Glutamate and 5 mM Succinate. The mitochondria and 2,4 DNP (100 μM) were added where indicated by arrows. Dates are expressed as means ± SEM, (n = 5). Significance was assessed by one-way analysis of variance (ANOVA), followed by Newman-Keuls's test for post hoc comparison. Significant differences are indicated by $^{*}p<0.05$ when compared with control group. Significant difference is indicated by $^{\#}p<0.05$ when compared with APAP group.
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intramitochondrial redox status [52], besides, $(PhSe)_2$ improved the mitochondrial antioxidant defense system and, so that can reduce the limited ability of both the H^+ pump and bioenergetics function. Results from the present study, such as improvement of the mitochondrial bioenergetics function ($\Delta\psi_m$, mitochondrial activity and NAD(P)H redox status) and normalization of oxygen consumption at sites 1 (glutamate/pyruvate) and 2 (succinate) supports the idea of an improved energy coupling of the

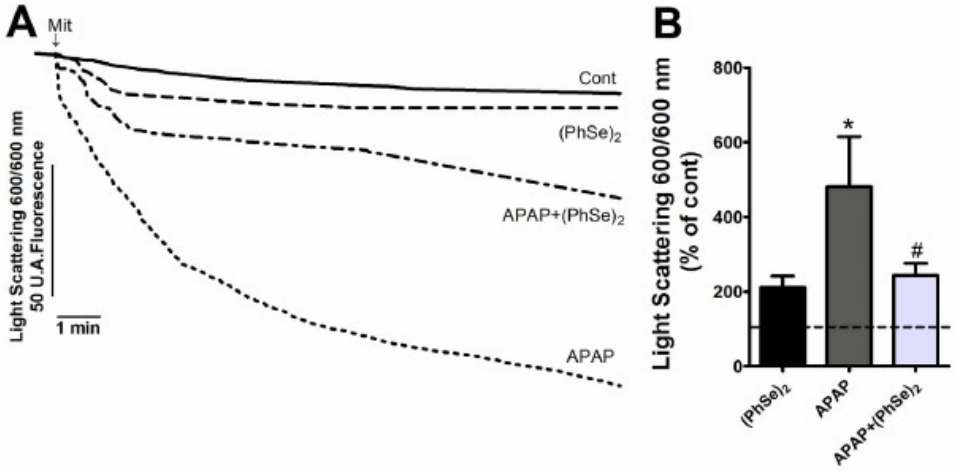

Figure 6. Effects of treatment with APAP and $(PhSe)_2$ on PTP opening in liver mitochondria based on swelling measurements. (A) The traces are representative of five independent experiments. (B) Means of the five experiments swelling. Mice were given acetaminophen (600 mg/kg, i.p.) and 1 h after were treated with or without $(PhSe)_2$ (15.6 mg/kg, i.p.), and were killed at 4 h after the APAP treatment. Mitochondria (0.1 mg/ml) were incubated in the reaction medium containing 230 mM Mannitol, 70 mM Sucrose, 1 mM K_2HPO_4, 20 mM Tris-HCl, pH 7.4 and was energized by 5 mM Glutamate and 5 mM Succinate. The light scattering was monitored after adding $CaCl_2$ (100 μM). Dates are expressed as means ± SEM, (n = 5). Significance was assessed by one-way analysis of variance (ANOVA), followed by Newman-Keuls's test for post hoc comparison. Significant differences are indicated by $^{*}p<0.05$ when compared with control group. Significant difference is indicated by $^{\#}p<0.05$ when compared with APAP group.
doi:10.1371/journal.pone.0081961.g006

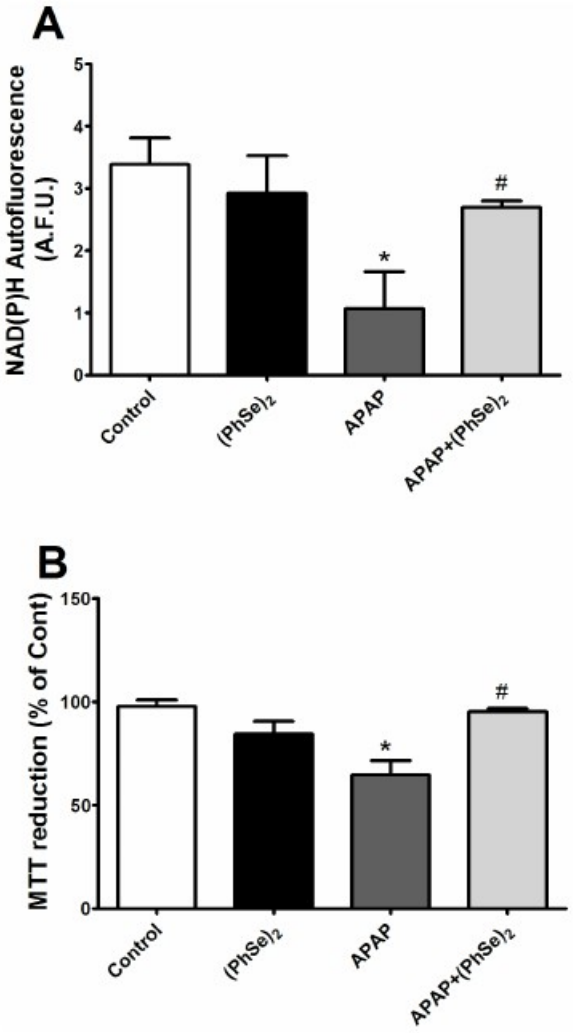

Figure 7. Effects of treatment with APAP and $(PhSe)_2$ on mitochondria function markers in liver mitochondria of mice. (A) Pyridine nucleotide autofluorescence (NAD(P)H redox). (B) Mitochondrial activity (MTT reduction). Mice were given acetaminophen (600 mg/kg, i.p.) and 1 h after were treated with or without $(PhSe)_2$ (15.6 mg/kg, i.p.), and were killed at 4 h after the APAP treatment. Dates are expressed as means ± SEM, (n = 5). Significance was assessed by one-way analysis of variance (ANOVA), followed by Newman-Keuls's test for post hoc comparison. Significant differences are indicated by $^{*}p<0.05$ when compared with control group. Significant difference is indicated by $^{\#}p<0.05$ when compared with APAP group.
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respiratory chain, reducing the electron escape, which reflected an improvement at the level of the oxidative phosphorylation system, substantiated by the maintenance of the mitochondrial ATPase activity. The deleterious effects on the outer and inner membrane affect the mitochondrial energy metabolism, disrupting the integrity of the respiratory chain, and induce a remarkable degree of mitochondrial swelling in the APAP group, which is consistent with the occurrence of mitochondrial membrane depolarization. One of the hallmarks of permeability transition is the exacerbated ROS generation that results in a decrease of the protein-SH and

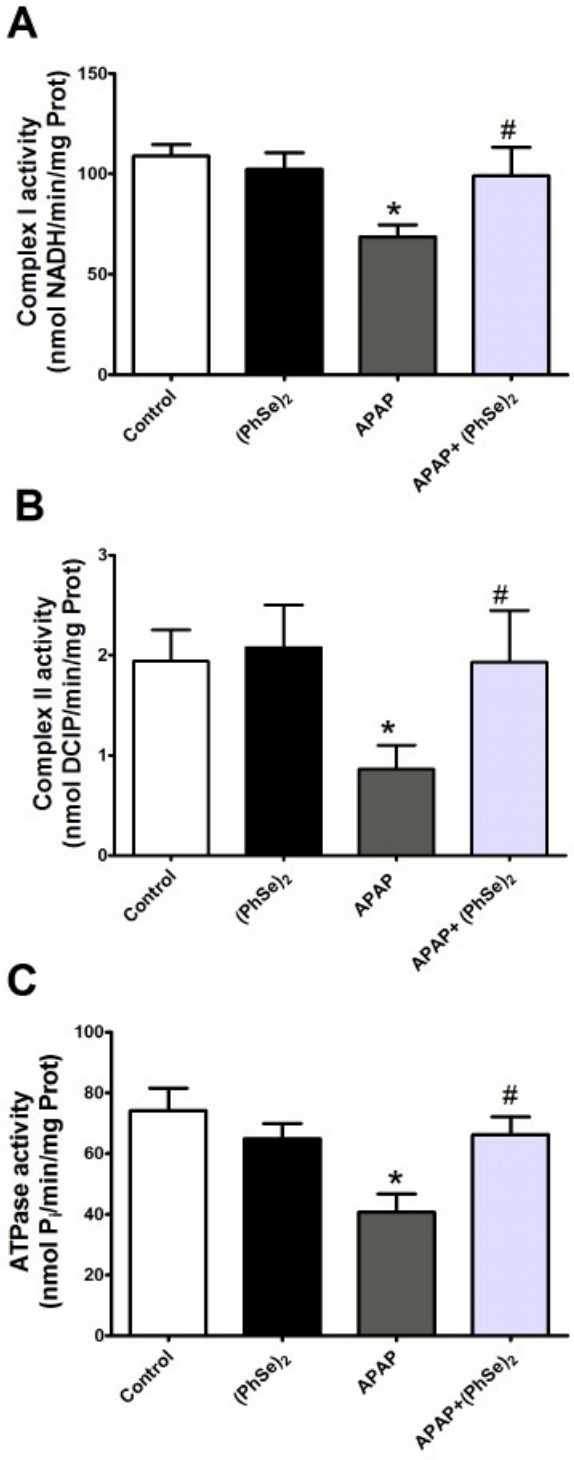

Figure 8. Effects of treatment with APAP and $(PhSe)_2$ on the activity of respiratory chain enzymes in liver mitochondria of mice. (A) Complex I (NADH dehydrogenase) activity. (B) Complex II (succinate dehydrogenase) activity. (C) Mitochondrial ATPase activity. Mice were given acetaminophen (600 mg/kg, i.p.) and 1 h after were

treated with or without $(PhSe)_2$ (15.6 mg/kg, i.p.), and were killed at 4 h after the APAP treatment. Dates are expressed as means ± SEM, (n = 5). Significance was assessed by one-way analysis of variance (ANOVA), followed by Newman-Keuls's test for post hoc comparison. Significant differences are indicated by $^{*}p<0.05$ when compared with control group. Significant difference is indicated by $^{\#}p<0.05$ when compared with APAP group.
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NAD(P)H redox [53].Thus, $(PhSe)_2$ could reduce MPT, associated with the changes in the intramitochondrial oxidized redox state [53,54].

Indeed, organoselenium compounds have demonstrated the ability to reduce LPO, ROS generation in the respiratory chain and the release of Fe^{2+}/citrate-induced cytochrome c [21,22]. Thus, $(PhSe)_2$ exerts its effects by preserving the mitochondrial membrane integrity. Organoselenium compounds can reduce phospholipid hydroperoxides, thus protecting biomembranes from peroxidative degradation [15], consequently, causing a decrease in the collapse of $\Delta\psi_m$ and ROS production in the mitochondrial respiratory chain, which act as negative modulators of the MPT. The mechanism of action involved in the hepatoprotective effect of $(PhSe)_2$ is related to its thiol peroxidase-like activity, i.e., its ability to react with peroxide after its transformation to the selenol-selenolate intermediate via either a direct interaction with GSH or another reducing thiol or by its reduction via NADPH-catalyzed thioredoxin reductase activity [55,56]. The mitochondrial dysfunction is a consequence during the ALF induced by APAP and there is an interrelationship between the oxidative stress and MPT pore opening caused by intoxicant agents, which together can deplete NAD(P)H and affect the GSH redox status and cause a loss of $\Delta\psi_m$ [18,57]. Additionally, the $(PhSe)_2$ treatment displayed a remarkable maintenance of redox balance as well as antioxidant enzyme function, since the redox imbalance is related to the control of cell death [51], and posing a threat for both the mitochondria and the cell, with severe consequences for the proper function of organs and consequently the organism.

Notably, the treatment with $(PhSe)_2$ enhances survival, extending the therapeutic window for chemical intervention. Our results demonstrate a remarkable effect extending the survival after APAP administration from 8 to 37.5 h. In line with our results, the $(PhSe)_2$ administration prevents the secondary toxic effects of APAP metabolism, delaying the onset of toxic phase. Previous studies have shown that the organoselenium compounds cause a partial inhibition of cytochrome P450 [58,59]. $(PhSe)_2$ inhibited *in vitro* cytochrome P450 metabolism in rat microsomes and the IC_{50} was reported as 78 μM for microsomal activity inhibition [59]. However, another elegant study demonstrated that the ebselen presented protective effect when co-treated with APAP in hepatocytes, and this condition was probably not caused by direct reaction with APAP or inhibition of cytochrome P450 but by reduction of NAPQI by selenol intermediate [60]. Since $(PhSe)_2$ shares with ebselen some chemical properties and has about twofold greater glutathione peroxidase–like activity and is also less toxic to rodents than ebselen, so, it is reasonable to suggest the formation of powerful nucleophile selenol-selenolate intermediate following by fast reduction of NAPQI to APAP, the $(PhSe)_2$ could be interfering with NAPQI formation, which reduces the toxicity, and then, increasing the urinary excretion of the APAP-glucuronide metabolite. In according to Li *et al.*, selenol-selenolate intermediate was much more a reductant than a nucleophile towards NAPQI when compared with GSH [60]. It has been demonstrated that sodium selenite protected via enhanced glucuronidation of APAP thereby diverting the amount of APAP converted to NAPQI [61].

In summary, our study is the first to compare $(PhSe)_2$ with NAC with regard to effectiveness as an antidote for APAP toxicity. $(PhSe)_2$ was effective at a lower dose than NAC when administered 1 h after APAP. Data from the present research indicate that $(PhSe)_2$ administration delayed the onset of the toxic phase, reducing APAP-induced mitochondrial dysfunction in mice and suggesting that the beneficial effects of the organoselenium treatment resulted from its antioxidant properties. The $(PhSe)_2$ significantly improved the cellular and mitochondrial redox homeostasis and reduced the mitochondrial bioenergetics dysfunction caused by membrane permeability transition associated with APAP overdose. These results may help to better understand the role of mitochondrial dysfunction in APAP hepatotoxicity and support the possibility that organoselenium $(PhSe)_2$ could be used as an adjuvant therapy to protect the liver from APAP-induced injuries.

Author Contributions

Conceived and designed the experiments: NRC FAS JGG CLDC. Performed the experiments: NRC EFdR MHdS CCT. Analyzed the data: NRC CLDC SCP JLM JGG FAS. Contributed reagents/materials/analysis tools: FAS. Wrote the paper: NRC CLDC JGG FAS.

Table 4. Effects of treatment with $(PhSe)_2$ and APAP on the respiratory rates of liver mitochondrial after 4 hours.

	Rate 1	Rate 2
	Respiration with Glut/Pyr (nmol O_2/min/mL)	Respiration with Succ (nmol O_2/min/mL)
Control	3.9±0.2	10.9±1.4
$(PhSe)_2$	3.3±0.4	8.8±1.8
APAP	2.5±0.2*	5.6±1.1*
APAP+$(PhSe)_2$	3.6±0.5#	8.1±1.2#

Mice were given acetaminophen (600 mg/kg, i.p.) and 1 h after were treated with or without $(PhSe)_2$ (15.6 mg/kg, i.p.), and were killed at 4 h after the APAP treatment. Data are expressed as means ± SEM, (n = 7). Significance was assessed by one-way analysis of variance (ANOVA), followed by Newman-Keuls's Test for post hoc comparison. Significant differences are indicated by *p≤0.05 when compared with control group. Significant difference is indicated by #p≤0.05 when compared with APAP group.
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References

1. Larson AM, Polson J, Fontana RJ, Davern TJ, Lalani E, et al. (2005) Acetaminophen-induced acute liver failure: results of a United States multicenter, prospective study. Hepatology 42: 1364–1372.
2. Nourjah P, Ahmad SR, Karwoski C, Willy M (2006) Estimates of acetaminophen (paracetamol)-associated overdoses in the United States. Pharmacoepidemiology and Drug Safety 15: 398–405.
3. de Achaval S, Suarez-Almazor M (2011) Acetaminophen overdose: a little recognized public health threat. Pharmacoepidemiology and Drug Safety 20: 827–829.
4. Moyer AM, Fridley BL, Jenkins GD, Batzler AJ, Pelleymounter LL, et al. (2011) Acetaminophen-NAPQI hepatotoxicity: a cell line model system genome-wide association study. Toxicological Sciences 120: 33–41.
5. Brown JM, Ball JG, Hogsett A, Williams T, Valentovic M (2010) Temporal study of acetaminophen (APAP) and S-adenosyl-L-methionine (SAMe) effects on subcellular hepatic SAMe levels and methionine adenosyltransferase (MAT) expression and activity. Toxicology and Applied Pharmacology 247: 1–9.
6. Jaeschke H (1990) Glutathione disulfide formation and oxidant stress during acetaminophen-induced hepatotoxicity in mice in vivo: the protective effect of allopurinol. Journal of Pharmacology and Experimental Therapeutics 255: 935–941.
7. Jaeschke H, Bajt ML (2006) Intracellular signaling mechanisms of acetaminophen-induced liver cell death. Toxicological Sciences 89: 31–41.
8. Jaeschke H, McGill MR, Ramachandran A (2012) Oxidant stress, mitochondria, and cell death mechanisms in drug-induced liver injury: lessons learned from acetaminophen hepatotoxicity. Drug Metabolism Reviews 44: 88–106.
9. Chan KM, Han XD, Kan YW (2001) An important function of Nrf2 in combating oxidative stress: Detoxification of acetaminophen. Proceedings of the National Academy of Sciences of the United States of America 98: 4611–4616.
10. San-Miguel B, Alvarez M, Culebras JM, Gonzalez-Gallego J, Tunon MJ (2006) N-acetyl-cysteine protects liver from apoptotic death in an animal model of fulminant hepatic failure. Apoptosis 11: 1945–1957.
11. Woodhead JL, Howell BA, Yang Y, Harrill AH, Clewell HJ 3rd, et al. (2012) An analysis of N-acetylcysteine treatment for acetaminophen overdose using a systems model of drug-induced liver injury. The Journal of Pharmacology and Experimental Therapeutics 342: 529–540.
12. Meotti FC, Stangherlin EC, Zeni G, Nogueira CW, Rocha JBT (2004) Protective role of aryl and alkyl diselenides on lipid peroxidation. Environmental Research 94: 276–282.
13. Brandao R, Santos FW, Oliveira R, Roman SS, Nogueira CW (2009) Involvement of non-enzymatic antioxidant defenses in the protective effect of diphenyl diselenide on testicular damage induced by cadmium in mice. Journal of Trace Elements in Medicine and Biology 23: 324–333.
14. Borges LP, Nogueira CW, Panatieri RB, Rocha JBT, Zeni G (2006) Acute liver damage induced by 2-nitropropane in rats: Effect of diphenyl diselenide on antioxidant defenses. Chemico-Biological Interactions 160: 99–107.
15. Nogueira CW, Zeni G, Rocha JBT (2004) Organoselenium and organotellurium compounds: Toxicology and pharmacology. Chemical Reviews 104: 6255–6285.
16. Da Silva MH, Da Rosa EJ, De Carvalho NR, Dobrachinski F, Da Rocha JB, et al. (2011) Acute Brain Damage Induced by Acetaminophen in Mice: Effect of Diphenyl Diselenide on Oxidative Stress and Mitochondrial Dysfunction. Neurotoxicity research 21: 334–344.
17. da Rosa EJ, da Silva MH, Carvalho NR, Bridi JC, da Rocha JB, et al. (2012) Reduction of acute hepatic damage induced by acetaminophen after treatment with diphenyl diselenide in mice. Toxicologic Pathology 40: 605–613.
18. Bajt ML, Ramachandran A, Yan HM, Lebofsky M, Farhood A, et al. (2011) Apoptosis-inducing factor modulates mitochondrial oxidant stress in acetaminophen hepatotoxicity. Toxicological Sciences 122: 598–605.
19. Votyakova TV, Reynolds IJ (2005) Ca2+-induced permeabilization promotes free radical release from rat brain mitochondria with partially inhibited complex I. Journal of Neurochemistry 93: 526–537.
20. Kim JY, Park JH (2003) ROS-dependent caspase-9 activation in hypoxic cell death. Febs Letters 549: 94–98.
21. Boireau A, Dubedat P, Bordier F, Coimbra M, Meunier M, et al. (1999) Effects of ebselen, a glutathione peroxidase mimic, in several models of mitochondrial dysfunction. Annals of the New York Academy of Sciences 893: 254–257.
22. Boireau A, Marechal PM, Meunier M, Dubedat P, Moussaoui S (2000) The anti-oxidant ebselen antagonizes the release of the apoptogenic factor cytochrome c induced by Fe2+/citrate in rat liver mitochondria. Neuroscience letters 289: 95–98.
23. Bhattacharya SK, Thakar JH, Johnson PL, Shanklin DR (1991) Isolation of Skeletal-Muscle Mitochondria from Hamsters Using an Ionic Medium Containing Ethylenediaminetetraacetic Acid and Nagarse. Analytical Biochemistry 192: 344–349.
24. Kruglov AG, Teplova VV, Saris NE (2007) The effect of the lipophilic cation lucigenin on mitochondria depends on the site of its reduction. Biochemical Pharmacology 74: 545–556.
25. Ohkawa H, Ohishi N, Yagi K (1979) Assay for Lipid Peroxides in Animal-Tissues by Thiobarbituric Acid Reaction. Analytical Biochemistry 95: 351–358.
26. Dionisio N, Garcia-Mediavilla MV, Sanchez-Campos S, Majano PL, Benedicto I, et al. (2009) Hepatitis C virus NS5A and core proteins induce oxidative stress-mediated calcium signalling alterations in hepatocytes. Journal of Hepatology 50: 872–882.
27. Hissin PJ, Hilf R (1976) Fluorometric Method for Determination of Oxidized and Reduced Glutathione in Tissues. Analytical Biochemistry 74: 214–226.
28. Misra HP, Fridovich I (1972) The generation of superoxide radical during the autoxidation of hemoglobin. Journal of Biological Chemistry 247: 6960–6962.
29. Aebi H (1984) Catalase in vitro. Methods in enzymology 105: 121–126.
30. Habig WH, Pabst MJ, Jakoby WB (1974) Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. The Journal of Biological Chemistry 249: 7130–7139.
31. Flohe L, Gunzler WA (1984) Assays of glutathione peroxidase. Methods in Enzymology 105: 114–121.
32. Carlberg I, Mannervik B (1985) Glutathione reductase. Methods in Enzymology 113: 484–490.
33. Levine RL, Garland D, Oliver CN, Amici A, Climent I, et al. (1990) Determination of Carbonyl Content in Oxidatively Modified Proteins. Methods in Enzymology 186: 464–478.
34. Akerman KEO, Wikstrom MKF (1976) Safranine as a Probe of Mitochondrial-Membrane Potential. Febs Letters 68: 191–197.
35. Bernas T, Dobrucki J (2002) Mitochondrial and nonmitochondrial reduction of MTT: interaction of MTT with TMRE, JC-1, and NAO mitochondrial fluorescent probes. Cytometry 47: 236–242.
36. Geller BL, Winge DR (1984) Subcellular distribution of superoxide dismutases in rat liver. Methods in Enzymology 105: 105–114.
37. Bottje W, Iqbal M, Tang ZX, Cawthon D, Okimoto R, et al. (2002) Association of mitochondrial function with feed efficiency within a single genetic line of male broilers. Poultry science 81: 546–555.
38. Galante YM, Hatefi Y (1978) Resolution of complex I and isolation of NADH dehydrogenase and an iron—sulfur protein. Methods in Enzymology 53: 15–21.
39. Fischer JC, Ruitenbeek W, Berden JA, Trijbels JM, Veerkamp JH, et al. (1985) Differential investigation of the capacity of succinate oxidation in human skeletal muscle. Clinica Chimica Acta 153: 23–36.
40. Morin C, Zini R, Simon N, Charbonnier P, Tillement JP, et al. (2000) Low glucocorticoid concentrations decrease oxidative phosphorylation of isolated rat brain mitochondria: an additional effect of dexamethasone. Fundamental & Clinical Pharmacology 14: 493–500.
41. Katyare SS, Satav JG (1989) Impaired mitochondrial oxidative energy metabolism following paracetamol-induced hepatotoxicity in the rat. British Journal of Pharmacology 96: 51–58.
42. Atkinson A, Gatenby AD, Lowe AG (1973) The determination of inorganic orthophosphate in biological systems. Biochimica Et Biophysica Acta 320: 195–204.
43. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72: 248–254.
44. McGill MR, Williams CD, Xie Y, Ramachandran A, Jaeschke H (2012) Acetaminophen-induced liver injury in rats and mice: comparison of protein adducts, mitochondrial dysfunction, and oxidative stress in the mechanism of toxicity. Toxicology and Applied Pharmacology 264: 387–394.
45. Nogueira CW, Rocha JB (2011) Toxicology and pharmacology of selenium: emphasis on synthetic organoselenium compounds. Archives of Toxicology 85: 1313–1359.
46. Arakawa S, Maejima T, Fujimoto K, Yamaguchi T, Yagi M, et al. (2012) Resistance to acetaminophen-induced hepatotoxicity in glutathione S-transferase Mu 1-null mice. Journal of Toxicological Sciences 37: 595–605.
47. Fernandez-Checa JC, Kaplowitz N (2005) Hepatic mitochondrial glutathione: transport and role in disease and toxicity. Toxicology and Applied Pharmacology 204: 263–273.
48. Zhao P, Kalhorn TF, Slattery JT (2002) Selective mitochondrial glutathione depletion by ethanol enhances acetaminophen toxicity in rat liver. Hepatology 36: 326–335.
49. Vendemiale G, Grattagliano I, Altomare E, Turturro N, Guerrieri F (1996) Effect of acetaminophen administration on hepatic glutathione compartmentation and mitochondrial energy metabolism in the rat. Biochemical Pharmacology 52: 1147–1154.
50. Hong SW, Lee HS, Jung KH, Lee H, Hong SS (2012) Protective Effect of Fucoidan against Acetaminophen-Induced Liver Injury. Archives of Pharmacal Research 35: 1099–1105.
51. Ramachandran A, Lebofsky M, Weinman SA, Jaeschke H (2011) The impact of partial manganese superoxide dismutase (SOD2)-deficiency on mitochondrial oxidant stress, DNA fragmentation and liver injury during acetaminophen hepatotoxicity. Toxicology and Applied Pharmacology 251: 226–233.
52. Raghavendran HRB, Sathivel A, Devaki T (2005) Antioxidant effect of Sargassum polycystum (Phaeophyceae) against acetaminophen induced changes in hepatic mitochondrial enzymes during toxic hepatitis. Chemosphere 61: 276–281.
53. Puntel RL, Roos DH, Folmer V, Nogueira CW, Galina A, et al. (2010) Mitochondrial dysfunction induced by different organochalchogens is mediated by thiol oxidation and is not dependent of the classical mitochondrial permeability transition pore opening. Toxicological Sciences 117: 133–143.

54. Morin D, Zini R, Ligeret H, Neckameyer W, Labidalle S, et al. (2003) Dual effect of ebselen on mitochondrial permeability transition. Biochemical Pharmacology 65: 1643–1651.
55. de Freitas AS, Rocha JBT (2011) Diphenyl diselenide and analogs are substrates of cerebral rat thioredoxin reductase: A pathway for their neuroprotective effects. Neuroscience Letters 503: 1–5.
56. de Freitas AS, Prestes AD, Wagner C, Sudati JH, Alves D, et al. (2010) Reduction of Diphenyl Diselenide and Analogs by Mammalian Thioredoxin Reductase Is Independent of Their Gluthathione Peroxidase-Like Activity: A Possible Novel Pathway for Their Antioxidant Activity. Molecules 15: 7699–7714.
57. Ueda S, Masutani H, Nakamura H, Tanaka T, Ueno M, et al. (2002) Redox control of cell death. Antioxidants & Redox Signaling 4: 405–414.
58. Kuhn-Velten N, Sies H (1989) Optical spectral studies of ebselen interaction with cytochrome P-450 of rat liver microsomes. Biochemical Pharmacology 38: 619–625.
59. Prigol M, Nogueira CW, Zeni G, Bronze MR, Constantino L (2012) In vitro metabolism of diphenyl diselenide in rat liver fractions. Conjugation with GSH and binding to thiol groups. Chemico-Biological Interactions 200: 65–72.
60. Li QJ, Bessems JG, Commandeur JN, Adams B, Vermeulen NP (1994) Mechanism of protection of ebselen against paracetamol-induced toxicity in rat hepatocytes. Biochemical Pharmacology 48: 1631–1640.
61. Schnell RC, Park KS, Davies MH, Merrick BA, Weir SW (1988) Protective effects of selenium on acetaminophen-induced hepatotoxicity in the rat. Toxicol Appl Pharmacol 95: 1–11.

5.2. Manuscrito: Difenil Disseleneto Através Da Hsp70 E Associado Com Genes Promotores Da Biogênese Mitocondrial Previnem A Disfunção Mitocondrial Após A Insuficiência Hepática Induzida Por Paracetamol

Manuscrito científico submetido para publicação

Diphenyl Diselenide Through The Hsp70 And Associated With Promoter Genes Of Mitochondrial Biogenesis Prevents The Mitochondrial Dysfunction After Liver Failure Induced By Acetaminophen

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Diphenyl Diselenide Prevents Mitochondrial Dysfunction After Acetaminophen-Induced Liver Failure through Hsp70 And In Association With The Promoters of Genes Involved In Mitochondrial Biogenesis

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Abstract

Physiopathological conditions such as acute liver failure (ALF) induced by acetaminophen (APAP) can often impair the bioenergetics of mitochondrial function. Studies have demonstrated that Diphenyl diselenide [$(PhSe)_2$], an organoselenium compound, protects against APAP-induced ALF. The current study was aimed to elucidate the beneficial effects of $(PhSe)_2$ and identify a signaling pathway involved in the prevention of ALF-induced by APAP overdose. Mice were injected intraperitoneally with APAP (600 mg/kg), $(PhSe)_2$ (15.6 mg/kg) or APAP+$(PhSe)_2$. Samples were collected at 2 and 4 h after APAP administration. $(PhSe)_2$ treatment reduced reactive oxygen and nitrogen species levels enhanced by APAP-overdose. $(PhSe)_2$ was also able to abolish the decline of mitochondrial oxidative phosphorylation (OXPHOS). Surprisingly, the hepatoprotective effects of $(PhSe)_2$ may not be associated with the Nrf2-ARE pathway, which suggests that the generation of a selenol intermediate could significantly contribute to liver recovery via another pathway such as that involving HSP70, which is critical for the maintenance of cellular homeostasis and inhibits the mitochondrial permeability transition. $(PhSe)_2$ also maintained appropriate levels of cytokines, such as tumoral necrosis factor alpha (TNF-α), interleukin 6 (IL-6) and nuclear factor kappa B (NF-κB), that are involved in liver recovery. In addition, $(PhSe)_2$ elevated the levels of peroxisome proliferator-activated receptor-γ coactivator (PGC-1α), helping to restore the levels of nuclear respiratory factor 1 (NRF1) associated with mitochondrial biogenesis. In summary, the $(PhSe)_2$ treatment maintained the bioenergetics of mitochondrial function, most likely through the decomposition of reactive species, increasing the expression of HSP70, and modulating of key cytokines and the promoter of genes related to mitochondrial dynamic.

Keywords: Diphenyl Diselenide; HSP70; mitochondrial biogenesis; Nrf2; oxidative phosphorylation.

Introduction

Mitochondrial bioenergetic dysfunction is a physiopathological condition that occurs in many diseases, and results in the inability of cells to maintain energy production (Cheng and Ristow, 2013). Because the liver, a highly metabolic and energy-demanding organ, is affected by acetaminophen (APAP) overdose, the mitochondrial dysfunction that manifests during acute liver failure (ALF) may contribute to alterations in energy metabolism and the presence of hepatocellular necrosis (Jaeschke et al., 2012). APAP hepatotoxicity is closely related to the N-acetyl-p-benzoquinone imine (NAPQI), a highly reactive metabolite (Larson et al., 2005). Different studies have demonstrated that APAP toxicity is a multifactorial process, involving the development of oxidative stress (da Silva et al., 2012; Jaeschke et al., 2012; Larson et al., 2005), that consequently triggers injury progression. The impairment of mitochondrial viability is associated with the mitochondrial permeability transition (MPT) (Kon et al., 2004).

The emergence of MPT increases energy wasting and limits the ability of cells to reestablish energy metabolism (Jaeschke et al., 2012). Evidently, the disruption of maintenance the bioenergetics metabolism after APAP-induced ALF results in the inhibition of processes with high energy demands, such as cell proliferation. Previous studies have demonstrated that cellular adaptation is a complex and well-orchestrated process in which the major limiting factors are glutathione (GSH) levels, the generation of reactive oxygen and nitrogen species (ROS/RNS) and mitochondrial viability (Han et al., 2013). Organoselenium compounds, in particular diphenyl diselenide [$(PhSe)_2$], may be useful agents for controlling the initiation of cell death through modulation of the MPT opening (Puntel et al., 2010). $(PhSe)_2$ is known to antagonize the effects of APAP poisoning (Carvalho et al., 2013). Thiol-disulfite modifications (arising from the glutathione peroxidase-like and thioredoxin reductase-like activities) of $(PhSe)_2$ appear to be the most direct mechanism of this compound, these activities are manifested through the generation of selenol/selenolate intermediate species. The reactivity of these intermediates makes them more powerful nucleophiles compared to known thiol/thiolate groups (Nogueira and Rocha, 2011).

APAP overdose represents a profound bioenergetic challenge to the cell that induces oxidative phosphorylation (OXPHOS) deficiency and disrupts the signaling pathways that permit survival during ALF (Hanawa et al., 2008; Jaeschke et al., 2012). Nonetheless, the adaptive liver response that promotes cell survival involves a complex interaction of transcription factors, such as heat shock proteins (HSP), nuclear factor erythroid 2-related

factor 2 (Nrf2) and antioxidant response elements (ARE) (de Bem et al., 2013) with cytokines and growth factors that are pleiotropic molecules. These include tumoral necrosis factor (TNF)-α, interleukins (IL) 1 and 6 and involve the translocation of the nuclear factor, Kappa-B (NF-κB) (Michalopoulos, 2007; Polimeno et al., 2000).

The functional role of $(PhSe)_2$ in improving mitochondrial dysfunction has already been reported (Carvalho et al., 2013; da Silva et al., 2012). $(PhSe)_2$ is able to freely cross cell membranes and enter into cells, where it preserves the cellular redox balance by maintaining mitochondrial homeostasis (Carvalho et al., 2013; Tirosh et al., 2007). However, whether $(PhSe)_2$ regulates the mitochondrial metabolic adaptation required for cell survival still is unknown, $(PhSe)_2$ has been shown to be an enhancer of mitochondrial biogenesis, promoting the activation of mitochondrial transcription factors including, nuclear respiratory factor-1 (NRF1) and -2 (NRF2), the mitochondrial transcription factor A and the peroxisome proliferator-activated receptor-γ coactivator (PGC-1α) (Glaser et al., 2014). Additionally, treatment with selenium induces an up-regulation of HSP70 levels (Kumar et al., 2014; Yousuf et al., 2007). The maintenance of HSP70 levels are closely related to the inhibition of apoptosis (Martindale and Holbrook, 2002), and the involvement of cytokines, such as IL-6,t has been highlighted in the modulation of HSP70 activation (Masubuchi et al., 2003). Thus, it is important to understand the mechanism by which $(PhSe)_2$ exerts a hepatoprotective effect against the impairment of mitochondrial bioenergetics and influences the intracellular signaling pathways involved in the adaptive response, as the changes in these signaling pathways could induce altered metabolic circuitries and antagonize the liver regenerative process.

Given the demonstrated ability of $(PhSe)_2$ to protect against APAP toxicity, the current study was aimed to elucidate the mechanism by which $(PhSe)_2$ protects mitochondrial bioenergetics and the signaling pathway involved in this process.

Materials and methods

Materials

$(PhSe)_2$ (98%), thiobarbituric acid (TBA), 2′-7′-dichlorofluorescein (DCFH), trichloroacetic acid (TCA) and nucleotides were purchased from Sigma Chemical Co. (St. Louis, MO). All antibodies utilized in this study were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other chemicals were of analytical grade and

obtained from standard commercial suppliers.

Animals

Seven-week-old male adult Swiss albino mice (30-40 g) from our own breeding colony were used. The animals were kept on a separate animal room, on at 12 h light/dark cycle, at temperature of 22 ± 2 ºC, with free access to food and water. Mice were acclimated for 7 days before initiation of any procedures. This study was approved by the Ethics and Animal Welfare Committee of Federal University of Santa Maria, Brazil.

Experimental protocol

Mice were randomly separated into four groups: control (vehicle); diphenyl diselenide [$(PhSe)_2$]; acute liver failure induced by APAP (APAP) and acute liver failure treated with diphenyl diselenide [APAP+$(PhSe)_2$]. All the solutions were administered by the intraperitoneal (i.p.) route. Injections were administered at 9:00 a.m. in order to remove any confounding factors of circadian rhythm. Mice in the control and $(PhSe)_2$ groups received saline 0.9% (20 ml/kg), and mice in the APAP and APAP+$(PhSe)_2$ groups received 600 mg/kg APAP (20 ml/kg in saline 0.9%). One hour after saline and APAP treatment, mice received 15.6 mg/kg $(PhSe)_2$ (2.5 ml/kg in canola oil) in the $(PhSe)_2$ and APAP+$(PhSe)_2$ groups. APAP and $(PhSe)_2$ doses have been used in previous studies (Carvalho et al., 2013; Chan et al., 2001; da Rosa et al., 2012).Food was available *ad libitum* and animals were not fasted prior to dosing.

At 2 and 4 h following APAP administration, animals were killed by cervical dislocation and blood was collected by cardiac puncture using heparin-rinsed 1-ml syringes (20-gauge needles) and centrifuged. The plasma was used for determination of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities using a commercial kit (Labtest®, Diagnostica S.A., Minas Gerais, Brazil). Excised liver of mice was washed in buffered normal saline and weighed to obtain the absolute liver weights. Relative weights were calculated with the formula: Relative organ weight = (Absolute organ weight/Body weight at sacrifice)X100%.

Histopathology

The caudal portion of the left lobe from liver tissues of mice (n = 5 per each group) was fixed in 10% formalin. For light microscopy examination, tissues were embedded in paraffin, sectioned at 6 μm section and stained with hematoxylin and eosin (H&E) to analysis qualitative of alterations histopathological were taken and analyzed by a pathologist (LoGuidice and Boelsterli, 2011).

Isolation of liver mitochondria

Mice liver mitochondria were isolated at 4ºC as previously described (Carafoli and Gazzotti, 1970), with few modifications. The livers of animals sacrificed was removed and immersed in medium containing 320 mM Succrose, 1 mM EDTA, 1 EGTA and 10 mM Tris-HCl, pH 7.4. The tissue was minced using surgical scissors, extensively washed and homogenized in a power-driven, tight-fitting Potter Elvehjem homogenizer with Teflon pestle. The resulting suspension was centrifuged for 10 min at 2,500 rpm in a Hitachi CR21E centrifuge. After centrifugation, the supernatant was recentrifuged for 10 min at 10,000 rpm. The pellet was resuspended in a medium containing 350 mM sucrose, 1 mM EGTA, 1 mM EDTA, 1 mM ADP, 1 mg/ml bovine serum albumin free fatty acid (BSA) and Tris-HCl, pH 7.4, and recentrifuged at 10,000 rpm for 10 min. The supernatant was decanted, and the final pellet was gently washed and resuspended in 250 mM succrose and 10 mM Tris-HCl, pH 7.4.

Mitochondria respiration rates and phosphorylation efficiency

Oxygen consumption was monitored polarographically with a Clark-type electrode (Hansatech, UK), in a closed glass chamber equipped with magnetic stirring, thermostated at 30°C. Liver mitochondria were incubated in 1 ml of medium containing 10 mM Tris-HCl (pH 7.4), 320 mM mannitol, 8 mM K_2HPO_4, and 4 mM $MgCl_2$, 0.08 mM EDTA, 1 mM EGTA, 0.2 mg/ml BSA (da-Silva et al., 2004). Respiration of mitochondria (1 mg of protein/ml) was initiated with a cocktail of NAD^+-linked substrates at 5mM (malate and glutamate) and phosphorylating (state III) respiration was initiated by addition of 200 nmol ADP. Next, the oligomycin-sensitive respiration (state IV) was initiated by addition of 1 μg/ml oligomycin. Respiration rates are given in nmol oxygen/min/mg protein. O_2 consumption recordings allowed the calculation of the rates of state III respiration, state IV respiration, and respiratory control ratio (RCR = state III/state IV).

Measurement of ROS production

ROS generation was determined spectrofluorimetrically in liver mitochondria, using H_2DCF-DA levels as an index of the peroxide production by cellular components (1µM) (Dionisio et al., 2009). Briefly, mitochondria were added to standard medium containing 10 mM Tris-HCl (pH 7.4), 10 mM KCl, 200 mM sucrose and the fluorescence was determined at 488 nm for excitation and 525 nm for emission, with slit widths of 3 nm.

Determination of mitochondrial Glutathione

The mitochondrial suspension was treated with 0.5ml of 13% trichloroacetic acid and centrifuged at 13000 rpm for 10 min at 4ºC . Aliquots (100 µl) of the supernatant were mixed with 2 ml of 100 mM NaH_2PO_4 buffer, pH 8.0, containing 5mM EDTA. One hundred microliters of *O*-phthalaldehyde (1 mg/ml) was added and fluorescence was measured 15 min later using the 350/420nm excitation/emission wavelength pair, with slit widths of 3 nm (Hissin and Hilf, 1976).

Measurement of liver nitrite and nitrate levels (NOx)

The liver samples were rapidly dissected on ice and homogenized in 500 µl of 200 mM $ZnSO_4$ plus 500 µl of acetonitrile (Guerra et al., 2006). The samples were centrifuged at 16,000 x g at 4°C for 30 min and the supernatants were used for the assays. The resulting pellet was suspended in 3M NaOH for protein determination with bovine serum albumin as the standard (Bradford, 1976). NOx content in the supernatant was estimated in a medium containing 400 µl of 2% VCl_3 (in 5% HCl), 200 µl of 0.1% N-(1-naphthyl) ethylene-diamine dihydrochloride, 200 µl of 2% sulfanilamide (in 5% HCl). After incubating at 37°C for 60 min, nitrite levels were determined spectrophotometrically at 540 nm, based on the reduction of nitrate to nitrite by VCl_3 (Guerra et al., 2006). Tissue nitrite and nitrate levels were expressed as nmol NOx/mg of protein.

Western blot analysis

Western blotting was performed with minor modifications (Franco et al., 2010; Zemolin et al., 2012). The liver samples were homogenized at 4°C in 300 µl of buffer (pH

7.0) containing 50 mM Tris, 1 mM EDTA, 0.1 mM phenylmethyl sulfonyl fluoride, 20 mM Na_3VO_4, 100 mM sodium fluoride and protease inhibitor cocktail (Sigma, MO). The homogenates were centrifuged at 1000 x g for 10 min at 4°C and the supernatants (S1) collected. After total protein determination using bovine serum albumin as standard) (Bradford, 1976), β-mercaptoethanol was added to samples to a final concentration of 8%. Then samples were frozen at -80°C for further analysis. The proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Then, membranes were incubated with specific primary antibodies for the determination of HSP70, NQO1, Nrf2 and β-actin protein expression. The blots were developed using secondary antibody linked to peroxidase and luminescence was captured in a Carestream Image Station 4000MM PRO molecular imaging system (New Haven, CT).

RNA Isolation

Total RNA was isolated from liver using Trizol® reagent (Invitrogen®), Carlsbad, CA) immediately after euthanasia. Isolation of total RNA was performed accordingly to the manufacturer's suggestion protocol.

Analysis of mRNA expression by q-PCR (Quantitative Real-Time PCR)

Gene specific primer sequences were based on published sequences in GenBank Overview (http://www.ncbi.nlm.nih.gov/genbank/) designed with Primer3 program version 0.4.0 (http://frodo.wi.mit.edu/primer3/) and custom made by Invitrogen® (Table 1). β-actin served as reference gene. Total RNA samples were treated with DNase I (Invitrogen®) to remove genomic DNA contamination in the presence of RNase inhibitor. Reverse transcription (RT) of approximately 2 μg total RNA was performed using random primer, RNase inhibitor, dNTPs, and M-MLV reverse transcriptase enzyme (Invitrogen®), accordingly to the manufacturer's suggested protocol. RT products (cDNAs) were maintained at -20°C. Quantitative real- time PCR were performed in 20 μl PCR mixture containing 1 μl RT product (cDNAs) as template, 1 x PCR Buffer, 25 μM dNTPs, 0.2 μM of each primer, 1.5–2.5 mM $MgCl_2$ (supporting information, Table 1), 0.1 x SYBR Green I (Molecular Probes®), and 1U Taq DNA Polymerase (Invitrogen®). PCR mixtures were subjected to PCR at 95°C for 5 min followed of 40 cycles of 15 s at 95°C, 15 s at annealing temperature appropriated to each primer sequence, and 25 s at 72°C for extension in a Thermocycler

StepOne Plus (Applied Biosystems, Foster City, CA). All samples were analyzed as technical duplicate with a no- template control also included. SYBR Green fluorescence was analyzed by StepOne Plus Software version 2.0 (Applied Biosystems, Foster City, CA), and Cq value (ΔCq) for each sample was calculated and reported using ΔΔCq method (Livak and Schmittgen, 2001). Briefly, for each well, a ΔCq value was obtained by the difference in Cq values (ΔCq) between the target gene and the reference gene. The ΔCq mean value obtained from the control group of each gene was used to calculate the ΔΔCq of the respective gene ($2^{-\Delta\Delta Cq}$).

Protein determination

Protein content was determined using bovine serum albumin (BSA) as standard (Bradford, 1976).

Statistical analysis

Statistical analysis was performed using GraphPad (version 5.0 for Macintosh OSX, GraphPad Software, San Diego, CA). Significance was assessed by one-way analysis of variance (ANOVA), followed by Newman–Keuls's Test for post-hoc comparison. Values of $p < 0.05$ were considered statistically significant.

Results

Effects of $(PhSe)_2$ treatment on APAP-induced acute liver injury

A single administration of APAP resulted in acute liver injury manifested by the substantial increase of plasma AST and ALT levels at the 2 and 4 h time periods (Figure 1A and B). The relative liver weight was similar for all the treatment groups at 2 h. However, APAP significantly increased the relative liver weights at 4 h when compared to control group (Figure 2). $(PhSe)_2$ prevented the increased levels of AST and ALT and maintained the relative liver weights at control levels compared to the APAP group.

Effects of $(PhSe)_2$ treatment on liver histopathology

Histopathological assessments were performed on the livers of all groups. The livers of mice in the control (Figure 3A and E; Table 2) and $(PhSe)_2$ (Figure 3B and F; Table 2) groups showed normal architecture, with an absence of hepatocellular injury at the 2 and 4 h time periods. Beginning at 2 h, the histopathological analysis of the livers from mice in the APAP group (Figure 3C; Table 2) revealed the presence of zonal necrosis, indicated by presence of eosinophilic hepatocytes and nuclear pyknosis around central vein. Treatment with $(PhSe)_2$ resulted in a reduction of the APAP-induced changes, with the preservation of the hepatic laminae and reduction in sinusoidal congestion, vacuolar degeneration, and nuclear pyknosis (Figure 3D, Table 2). By 4 h (Figure 3G; Table 2), the APAP group presented marked lesions, and the area of necrosis was more defined and greater in extent. In contrast, the area of necrosis was contained to the cells surrounding the central vein in the mice that received $(PhSe)_2$ 1 h after APAP administration (Figure 3H; Table 2), indicating that $(PhSe)_2$ prevented the further progression of necrosis.

Effects of APAP and $(PhSe)_2$ treatment on the mitochondrial GSH content

$(PhSe)_2$ treatment did not alter the mitochondrial GSH content at either the 2 or 4 h time periods when compared to the control (Figure 4). APAP treatment significantly depressed the mitochondrial GSH contents in comparison to the control levels at the 2 and 4 h time periods. APAP+$(PhSe)_2$ administration induced a less marked decrease in GSH levels compared to the APAP group at 2 h, and the mitochondrial GSH contents returned to levels comparable to those of the control at 4h.

Effects of APAP and $(PhSe)_2$ treatment on mitochondrial bioenergetics

APAP treatment caused a significant decline in state III respiration (using complex I substrate, glutamate/malate and ADP) in comparison to the control levels at 2 and 4 h following APAP administration. At 2 h, the state III respiration in the APAP+$(PhSe)_2$ treatment group was partially protected from APAP toxicity, as indicated by a less marked reduction in the state III levels compared to the APAP group. At 4 h, state III in the APAP+ $(PhSe)_2$ group returned to the control levels (Figure 5A). At both 2 and 4 h, state IV (oligomycin-sensitive) respiration was significantly increased in the APAP group; whereas, the APAP+$(PhSe)_2$ animals presented similar levels of state IV respiration to the control group (Figure 5B). APAP overdose induced a significant depletion of RCR (state III/state IV) at the

2 and 4 h time periods. When $(PhSe)_2$ was administered following APAP, the RCR returned to control levels (Figure 5C).

Effects of APAP and $(PhSe)_2$ treatment on mitochondrial ROS generation

The production of reactive species in the liver mitochondria was similar for all groups at 2 h, but at 4 h APAP induced a pronounced increase in the generation of ROS when compared to the control group (Figure 6A). Treatment with $(PhSe)_2$ following APAP treatment maintained the levels of mitochondrial ROS generation at levels that were comparable to the control group at 4 h.

Effects of APAP and $(PhSe)_2$ treatment on liver NOx generation

The hepatic NOx (nitrite + nitrate) levels were measured after APAP poisoning (Figure 6B). At both 2 h and 4 h the APAP injury induced a significant increase in the NOx levels when compared to the control group. The treatment with $(PhSe)_2$ following APAP was able to reduce the levels of liver NOx.

Effects of APAP and $(PhSe)_2$ treatment on liver HSP70, Nrf2 and NQO-1 protein levels

Figures 7A and B show representative blots of the immunoreactive bands for HSP70, NQO-1, Nrf2 and ß-actin (loading control) in liver at 2 h and 4 h. The western blot analyses revealed that the protein levels of NQO-1 and Nrf2 in the $(PhSe)_2$ group were similar to those of the control at both time periods, while the $(PhSe)_2$ group showed a increased amounts of HSP70 in the livers at 2 h. The APAP-induced ALF only produced increased in levels of HSP70, while the NQO-1 and Nf2 remained unchanged at 2 h (Figure 7C, D and E, respectively). Additionally, after 4 h, the HSP70 levels were even higher in APAP group. The treatment with $(PhSe)_2$ in the APAP+$(PhSe)_2$ group presented a significant increase of the HSP70 levels at 4 h, but were lower than the APAP group. The APAP-overdose caused a significant increase of NQO-1 and Nrf2 levels in liver at 4 h. Nevertheless, the APAP+$(PhSe)_2$ group showed NQO-1 and Nrf2 levels that were similar to the control group and significantly lower than the APAP group (Figures 7D and E).

Effects of APAP and $(PhSe)_2$ treatment on the expressions of IL-6, TNF-α and NF-κB mRNA in the livers

To investigate the tissue recovery after the APAP-induced ALF, the molecular biomarkers involved in this process were studied. On one hand, APAP overdose caused a meaningful reduction in the mRNA levels of IL-6, TNF-α and NF-κB. Treatment with $(PhSe)_2$ induced an increase of the IL-6 mRNA levels, partially prevented the decrease of TNF-α expression and kept NF-κB at the control levels (Figures 8A, B and C, respectively).

Effects of APAP and $(PhSe)_2$ treatment on markers of mitochondrial biogenesis

Because tissue recovery is an energy-dependent process, we evaluated the expression of mitochondrial biogenesis markers. Though APAP administration did not change the expression of PGC-1α, a significant reduction of NRF1 was noticed in the livers after APAP overdose. By contrast, the expression of PGC-1α increased in the APAP+$(PhSe)_2$ animals, whereas the NRF1 levels returned to the control levels in this group (Figure 8D and E, respectively).

Discussion

The liver is a central organ involved in xenobiotic detoxification and it is important that efficient bioenergetics as well as the cellular signaling pathways that underlie the metabolic adaptation of the liver are maintained (Hanawa et al., 2008). In the current study we investigated the beneficial effects of $(PhSe)_2$ on mitochondrial bioenergetics and the signaling pathways involved in the protection against APAP-induced ALF. Our results demonstrated that $(PhSe)_2$ treatment effectively reestablished the GSH redox status in a faster manner. Moreover, $(PhSe)_2$ inhibited NOx production in the liver and consequently prevented energy wasting during APAP overdose. Surprisingly, the hepatoprotection was not critically dependent on the Nrf2 pathway. Instead, our results suggest that the cell survival and maintenance of function after treatment of APAP-induced ALF with $(PhSe)_2$ was associated with HSP70. We demonstrated for the first time that $(PhSe)_2$ treatment after APAP overdose modulated the adaptive response of the liver, stimulated the expression of PGC-1α and restored the NRF1 levels, thereby contributing to the recovery of mitochondrial bioenergetics.

Different studies have suggested that $(PhSe)_2$ exerts a protective against APAP-

overdose (Carvalho et al., 2013; da Rosa et al., 2012; da Silva et al., 2012). Although there is general agreement that the generation of NAPQI is important during the early phase of toxicity, the subsequent decline of bioenergetics metabolism cannot be completely attributed to the direct effects of NAPQI, as the combination of factors (such as increased levels of ROS/RNS) may contribute to the initiation or promotion APAP-induced ALF. Evidence suggests that the effects of $(PhSe)_2$ are mediated by generation of a the selenol intermediate through the glutathione peroxidase- and thioredoxin reductase-like activities (Dobrachinski et al., 2014; Nogueira and Rocha, 2010), though $(PhSe)_2$ could use the thioredoxin system far more efficiently than GSH (Dobrachinski et al., 2014; Zhao and Holmgren, 2002; Zhao et al., 2002). The selenol intermediate reacts with peroxynitrite at a second order rate constant of 2.7 x $10^5 M^{-1}s^{-1}$, at pH 7.4 and 37°C (de Bem et al., 2013). $(PhSe)_2$ acts as a scavenger of RNS through the thioredoxin reductase-like activity, which could be an important strategy by which GSH levels are maintained. Thus, the hepatic GSH network is continuously remodeled and plays a significant role in satisfying the requirements of mitochondrial dynamics, protecting the OXPHOS and allowing for the exchange of molecule across the membrane (Ribas et al., 2014; Zhang et al., 2012).

The activation of Nrf2-ARE is an important strategy for preventing the imbalance of mitochondrial dynamics during APAP-induced hepatotoxicity (Chan et al., 2001; Gum and Cho, 2013). Surprisingly, the adaptive response induced by $(PhSe)_2$ was not critically dependent on Nrf2-ARE. Previous works have demonstrated Nrf2-independent mechanisms of cellular protection (Li et al., 2014; Reisman et al., 2009). Additionally, $(PhSe)_2$ may prevent mitochondrial energy wasting by activating a complementary redox-sensitive pathway, such as that involving HSP70. HSP70 is activated by thermal stress, environmental redox changes and H_2O_2, and its induction is Nrf2-independent (Stuart et al., 1994). Because the enhanced susceptibility of HSP70-null mice to APAP has been previously reported (Tolson et al., 2006). HSP70 could protect the mitochondrial dynamics, by inhibiting protein oxidation and promoting improved metabolic adaptation by the liver.

APAP-induced ALF results in significant changes to the mRNA levels of NF-κB and TNF-α which may impair the energy metabolism network involving the OXPHOS and thereby lead to an energetic crisis and cell death (Boulares et al., 2000; Mauro et al., 2011; Palomer et al., 2009). Our results suggest that $(PhSe)_2$ exerts a protective effect that is mediated by HSP70 activation. Previously, HSP70 have demonstrated a potent effect on the activity of cytokines (Asea et al., 2002), and may restore NF-κB levels, up-regulate IL-6 levels, and modulate TNF-α levels. It has been recognized that the effects of HSP70 are

related to the cellular amount of this protein (Guzhova et al., 1997). Increased levels of HSP70 act as a useful tool in the cellular adaptation that occurs through negative feedback associated with NF-κB and TNF-α modulation (Senf et al., 2008; Van Molle et al., 2002). Thereby, the increased levels of IL-6 mRNA levels could antagonize cell death and, tissue damage while accelerating liver regeneration, due to the effect of IL-6 on HSP70 responsiveness (Masubuchi et al., 2003). An association between higher levels of HSP70 and the inhibition of MPT that occurs through the suppression of caspase activation and DNA fragmentation has been observed (Hong et al., 2002; Masubuchi et al., 2003; Tolson et al., 2006). This, could be an important mechanisms involved in the modulation of metabolic reorganization, cell viability and proliferative activity that occur under $(PhSe)_2$ treatment. Early studies on this topic have shown that organoselenium compounds have a direct effect in mediating the activation of NF-κB and the increase in levels of IL-10 which in turn inhibits TNF-α (Rupil et al., 2012; Tiegs et al., 1998). The lower levels of TNF-α expression, observed in the $(PhSe)_2$-treated animals, could be related to an increase in PGC-1α transcription, since TNF-α has been shown to act as a negative modulator of PGC-1α expression (Palomer et al., 2009). The activation of PGC-1α could lead the reestablishment of the NRF1 levels. Current studies have highlighted the ability of $(PhSe)_2$ to induce an increase in the number of mitochondria by stimulating mitochondrial biogenesis (Glaser et al., 2014; Kumari et al., 2012; Mendelev et al., 2012). Thus, it is possible that the $(PhSe)_2$ treatment could simultaneously co-activate PGC-1α and NRF1 to normalize state III and state IV respiration, thereby preventing the intensification of non-phosphorylating energy wasting that contributes to the deficiency of OXPHOS and allowing for all oxygen consumed to remain dedicated to ATP synthesis-related processes (Panatto et al., 2011). In this context, the maintenance of mitochondrial integrity inhibits the energy wasting, which is inversely correlated with the efficiency of ATP synthesis but positively correlated with ROS generation under conditions of oxidative stress (Hernández-Muñoz et al., 2003; Tait and Green, 2012).

On the basis of these findings it is possible to suggest that the hepatoprotection mediated by $(PhSe)_2$ treatment is connected to both HSP70 activation and mitochondrial biogenesis. Pretreatments with selenium induces the overexpression of HSP70 (Chen et al., 2014; Giffard and Yenari, 2004; Kumar et al., 2014), and the protection of OXPHOS by $(PhSe)_2$ is essential for the activity of HSP70, an ATP-dependent chaperones. Moreover, the translocation of HSP70 across the outer and inner mitochondria membranes is dependent of $\Delta\psi m$ and ATP levels (Stuart et al., 1994). Due to the cellular bioenergetic crisis observed in the APAP-induced ALF, activation of HSP70 as well as the Nrf2-ARE do not translate into a

significant reduction of redox impairment. In this way, $(PhSe)_2$ induces an adaptive response through the upregulation of PGC-1α and the reestablishment of NRF1, two important factors that contribute to liver recovery. Thereby, $(PhSe)_2$ controls the quality of mitochondrial function and maintain homeostasis and cellular health.

In conclusion, $(PhSe)_2$ treatment after APAP-overdose plays a hepatoprotective role through the preservation of bioenergetics efficiency. For the first time, our results elucidate the mechanism by which $(PhSe)_2$ changes the expression of HSP70 and manipulates the activity of transcription factors associated with mitochondrial biogenesis that are not critically dependent on the Nrf2-ARE pathway.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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References

Asea, A., Rehli, M., Kabingu, E., Boch, J.A., Baré, O., Auron, P.E., Stevenson, M.A., Calderwood, S.K., 2002. Novel Signal Transduction Pathway Utilized by Extracellular HSP70. J. Biol. Chem. 277, 15023–15034. doi:10.1074/jbc.M200497200

Boulares, A.H., Giardina, C., Inan, M.S., Khairallah, E.A., Cohen, S.D., 2000. Acetaminophen Inhibits NF- B Activation by Interfering with the Oxidant Signal in Murine Hepa 1-6 Cells. Toxicol. Sci. 375, 370–375.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Carafoli, E., Gazzotti, P., 1970. Loss and maintenance of energy-linked functions in aged mitochondria. Biochem. Biophys. Res. Commun. 39, 842–6.

Carvalho, N.R., da Rosa, E.F., da Silva, M.H., Tassi, C.C., Dalla Corte, C.L., Carbajo-Pescador, S., Mauriz, J.L., González-Gallego, J., Soares, F.A., 2013. New therapeutic approach: diphenyl diselenide reduces mitochondrial dysfunction in acetaminophen-induced acute liver failure. PLoS One 8, e81961. doi:10.1371/journal.pone.0081961

Chan, K., Han, X.D., Kan, Y.W., 2001. An important function of Nrf2 in combating oxidative stress: detoxification of acetaminophen. Proc. Natl. Acad. Sci. U. S. A. 98, 4611–4616.

Chen, X., Yao, H., Yao, L., Zhao, J., Luan, Y., Zhang, Z., Xu, S., 2014. Selenium deficiency influences the gene expressions of heat shock proteins and nitric oxide levels in neutrophils of broilers. Biol. Trace Elem. Res. 161, 334–40. doi:10.1007/s12011-014-0150-1

Cheng, Z., Ristow, M., 2013. Mitochondria and metabolic homeostasis. Antioxid. Redox Signal. 19, 240–2. doi:10.1089/ars.2013.5255

Da Rosa, E.J.F., da Silva, M.H., Carvalho, N.R., Bridi, J.C., da Rocha, J.B., Carbajo-Pescador, S., Mauriz, J.L., González-Gallego, J., Soares, F.A.A., 2012. Reduction of acute hepatic damage induced by acetaminophen after treatment with diphenyl diselenide in mice. Toxicol. Pathol. 40, 605–13. doi:10.1177/0192623311436179

Da Silva, M.H., da Rosa, E.J.F., de Carvalho, N.R., Dobrachinski, F., da Rocha, J.B.T., Mauriz, J.L., González-Gallego, J., Soares, F.A.A., 2012. Acute brain damage induced by acetaminophen in mice: effect of diphenyl diselenide on oxidative stress and mitochondrial dysfunction. Neurotox. Res. 21, 334–44. doi:10.1007/s12640-011-9288-1

da-Silva, W.S., Gómez-Puyou, A., de Gómez-Puyou, M.T., Moreno-Sanchez, R., De Felice, F.G., de Meis, L., Oliveira, M.F., Galina, A., 2004. Mitochondrial bound hexokinase activity as a preventive antioxidant defense: steady-state ADP formation as a regulatory mechanism of membrane potential and reactive oxygen species generation in mitochondria. J. Biol. Chem. 279, 39846–55. doi:10.1074/jbc.M403835200

De Bem, A.F., Fiuza, B., Calcerrada, P., Brito, P.M., Peluffo, G., Dinis, T.C.P., Trujillo, M., Rocha, J.B.T., Radi, R., Almeida, L.M., 2013. Protective effect of diphenyl diselenide against peroxynitrite-mediated endothelial cell death: a comparison with ebselen. Nitric Oxide 31, 20–30. doi:10.1016/j.niox.2013.03.003

Dionisio, N., Garcia-Mediavilla, M. V, Sanchez-Campos, S., Majano, P.L., Benedicto, I., Rosado, J.A., Salido, G.M., Gonzalez-Gallego, J., 2009. Hepatitis C virus NS5A and core proteins induce oxidative stress-mediated calcium signalling alterations in hepatocytes. J. Hepatol. 50, 872–82. doi:10.1016/j.jhep.2008.12.026

Dobrachinski, F., da Silva, M.H., Tassi, C.L.C., de Carvalho, N.R., Dias, G.R.M., Golombieski, R.M., da Silva Loreto, E.L., da Rocha, J.B.T., Fighera, M.R., Soares, F.A.A., 2014. Neuroprotective Effect of Diphenyl Diselenide in a Experimental Stroke Model: Maintenance of Redox System in Mitochondria of Brain Regions. Neurotox. Res. doi:10.1007/s12640-014-9463-2

Franco, J.L., Posser, T., Missau, F., Pizzolatti, M.G., Santos, A.R.S., Souza, D.O., Aschner, M., Rocha, J.B.T., Dafre, A.L., Farina, M., 2010. Structure–activity relationship of flavonoids derived from medicinal plants in preventing methylmercury-induced mitochondrial dysfunction. Environ. Toxicol. Pharmacol. 30, 272–278.

Giffard, R.G., Yenari, M.A., 2004. Many mechanisms for hsp70 protection from cerebral ischemia. J. Neurosurg. Anesthesiol. 16, 53–61.

Glaser, V., Martins, R. de P., Vieira, A.J.H., Oliveira, E. de M., Straliotto, M.R., Mukdsi, J.H., Torres, A.I., de Bem, A.F., Farina, M., da Rocha, J.B.T., De Paul, A.L., Latini, A., 2014. Diphenyl diselenide administration enhances cortical mitochondrial number and activity by increasing hemeoxygenase type 1 content in a methylmercury-induced neurotoxicity mouse model. Mol. Cell. Biochem. 390, 1–8. doi:10.1007/s11010-013-1870-9

Guerra, G.P., Mello, C.F., Sauzem, P.D., Berlese, D.B., Furian, A.F., Tabarelli, Z., Rubin, M.A., 2006. Nitric oxide is involved in the memory facilitation induced by spermidine in rats. Psychopharmacology (Berl). 186, 150–8. doi:10.1007/s00213-006-0376-5

Gum, S. Il, Cho, M.K., 2013. Recent updates on acetaminophen hepatotoxicity: the role of nrf2 in hepatoprotection. Toxicol. Res. 29, 165–72. doi:10.5487/TR.2013.29.3.165

Guzhova, I. V, Darieva, Z.A., Melo, A.R., Margulis, B.A., 1997. Major stress protein Hsp70 interacts with NF-kB regulatory complex in human T-lymphoma cells. Cell Stress Chaperones 2, 132–139.

Han, D., Dara, L., Win, S., Than, T.A., Yuan, L., Abbasi, S.Q., Liu, Z.-X., Kaplowitz, N., 2013. Regulation of drug-induced liver injury by signal transduction pathways: critical role of mitochondria. Trends Pharmacol. Sci. 34, 243–53. doi:10.1016/j.tips.2013.01.009

Hanawa, N., Shinohara, M., Saberi, B., Gaarde, W. a, Han, D., Kaplowitz, N., 2008. Role of JNK translocation to mitochondria leading to inhibition of mitochondria bioenergetics in acetaminophen-induced liver injury. J. Biol. Chem. 283, 13565–77. doi:10.1074/jbc.M708916200

Hernández-Muñoz, R., Sánchez-Sevilla, L., Martínez-Gómez, A., Dent, M.A.R., 2003. Changes in mitochondrial adenine nucleotides and in permeability transition in two models of rat liver regeneration. Hepatology 37, 842–51. doi:10.1053/jhep.2003.50145

Hissin, P.J., Hilf, R., 1976. A fluorometric method for determination of oxidized and reduced glutathione in tissues. Anal. Biochem. 74, 214–26.

Hong, F., Kim, W.-H., Tian, Z., Jaruga, B., Ishac, E., Shen, X., Gao, B., 2002. Elevated interleukin-6 during ethanol consumption acts as a potential endogenous protective cytokine against ethanol-induced apoptosis in the liver: involvement of induction of Bcl-2 and Bcl-x(L) proteins. Oncogene 21, 32–43. doi:10.1038/sj.onc.1205016

Jaeschke, H., McGill, M.R., Ramachandran, A., 2012. Oxidant stress, mitochondria, and cell death mechanisms in drug-induced liver injury: lessons learned from acetaminophen hepatotoxicity. Drug Metab. Rev. 44, 88–106. doi:10.3109/03602532.2011.602688

Kon, K., Kim, J.-S., Jaeschke, H., Lemasters, J.J., 2004. Mitochondrial permeability transition in acetaminophen-induced necrosis and apoptosis of cultured mouse hepatocytes. Hepatology 40, 1170–9. doi:10.1002/hep.20437

Kumar, G.S., Kulkarni, A., Khurana, A., Kaur, J., Tikoo, K., 2014. Selenium nanoparticles involve HSP-70 and SIRT1 in preventing the progression of type 1 diabetic nephropathy. Chem. Biol. Interact. 223C, 125–133. doi:10.1016/j.cbi.2014.09.017

Kumari, S., Mehta, S.L., Li, P.A., 2012. Glutamate induces mitochondrial dynamic imbalance and autophagy activation: preventive effects of selenium. PLoS One 7, e39382. doi:10.1371/journal.pone.0039382

Larson, A., Polson, J., Fontana, R., 2005. Acetaminophen-induced acute liver failure: results of a United States multicenter, prospective study. Hepatology 42, 1364–72. doi:10.1002/hep.20948

Li, S., Li, J., Shen, C., Zhang, X., Sun, S., Cho, M., Sun, C., Song, Z., 2014. tert-Butylhydroquinone (tBHQ) protects hepatocytes against lipotoxicity via inducing autophagy independently of Nrf2 activation. Biochim. Biophys. Acta 1841, 22–33. doi:10.1016/j.bbalip.2013.09.004

Livak, K., Schmittgen, T., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402–408. doi:10.1006/meth.2001.1262

LoGuidice, A., Boelsterli, U. a, 2011. Acetaminophen overdose-induced liver injury in mice is mediated by peroxynitrite independently of the cyclophilin D-regulated permeability transition. Hepatology 54, 969–78. doi:10.1002/hep.24464

Martindale, J.L., Holbrook, N.J., 2002. Cellular response to oxidative stress: Signaling for suicide and survival. J. Cell. Physiol. 192, 1–15.

Masubuchi, Y., Bourdi, M., Reilly, T.P., Graf, M.L.M., George, J.W., Pohl, L.R., 2003. Role of interleukin-6 in hepatic heat shock protein expression and protection against acetaminophen-induced liver disease. Biochem. Biophys. Res. Commun. 304, 207–212. doi:10.1016/S0006-291X(03)00572-2

Mauro, C., Leow, S.C., Anso, E., Rocha, S., Thotakura, A.K., Tornatore, L., Moretti, M., De Smaele, E., Beg, A.A., Tergaonkar, V., Chandel, N.S., Franzoso, G., 2011. NF-κB controls energy homeostasis and metabolic adaptation by upregulating mitochondrial respiration. Nat. Cell Biol. 13, 1272–9. doi:10.1038/ncb2324

Mendelev, N., Mehta, S.L., Idris, H., Kumari, S., Li, P.A., 2012. Selenite stimulates mitochondrial biogenesis signaling and enhances mitochondrial functional performance in murine hippocampal neuronal cells. PLoS One 7, e47910. doi:10.1371/journal.pone.0047910

Michalopoulos, G.K., 2007. Liver regeneration. J. Cell. Physiol. 213, 286–300. doi:10.1002/jcp.21172

Molle, W. Van, Wielockx, B., Mahieu, T., Takada, M., Taniguchi, T., Sekikawa, K., Libert, C., 2002. HSP70 Protects against TNF-Induced Lethal Inflammatory Shock. Immunity 16, 685–695.

Nogueira, C.W., Rocha, J.B.T., 2010. Diphenyl diselenide a janus-faced molecule. J. Braz. Chem. Soc. 21, 2055–2071. doi:10.1590/S0103-50532010001100006

Nogueira, C.W., Rocha, J.B.T., 2011. Toxicology and pharmacology of selenium: emphasis on synthetic organoselenium compounds. Arch. Toxicol. 85, 1313–59. doi:10.1007/s00204-011-0720-3

Palomer, X., Alverez-Guaria, D., Rodríguez-Calvo, R., Coll, T., Laguna, J.C., Davidson, M.M., Chan, T.O., Feldman, A.M., Vázquez-Carrera, M., 2009. TNF- a reduces PGC-1 a expression through NF- k B and p38 MAPK leading to increased glucose oxidation in a human cardiac cell model. Cardiovasc. Res. 81, 703–712. doi:10.1093/cvr/cvn327

Panatto, J.P., Jeremias, I.C., Ferreira, G.K., Ramos, A.C., Rochi, N., Gonçalves, C.L., Daufenbach, J.F., Jeremias, G.C., Carvalho-Silva, M., Rezin, G.T., Scaini, G., Streck, E.L., 2011. Inhibition of mitochondrial respiratory chain in the brain of rats after hepatic failure induced by acetaminophen. Mol. Cell. Biochem. 350, 149–54. doi:10.1007/s11010-010-0689-x

Polimeno, L., Capuano, F., Marangi, L.C., Margiotta, M., Lisowsky, T., Ierardi, E., Francavilla, R., Francavilla, A., 2000. The augmenter of liver regeneration induces mitochondrial gene expression in rat liver and enhances oxidative phosphorylation capacity of liver mitochondria. Dig. Liver Dis. 32, 510–517. doi:10.1016/S1590-8658(00)80009-2

Puntel, R.L., Roos, D.H., Folmer, V., Nogueira, C.W., Galina, A., Aschner, M., Rocha, J.B.T., 2010. Mitochondrial dysfunction induced by different organochalchogens is mediated by thiol oxidation and is not dependent of the classical mitochondrial permeability transition pore opening. Toxicol. Sci. 117, 133–43. doi:10.1093/toxsci/kfq185

Reisman, S.A., Aleksunes, L.M., Klaassen, C.D., 2009. Oleanolic acid activates Nrf2 and protects from acetaminophen hepatotoxicity via Nrf2-dependent and Nrf2-independent processes. Biochem. Pharmacol. 77, 1273–1282. doi:10.1016/j.bcp.2008.12.028

Ribas, V., García-Ruiz, C., Fernández-Checa, J.C., 2014. Glutathione and mitochondria. Front. Pharmacol. 5, 151. doi:10.3389/fphar.2014.00151

Rupil, L.L., Bem, A.F. De, Roth, G.A., 2012. Diphenyl diselenide-modulation of macrophage activation: Down-regulation of classical and alternative activation markers. Innate Immun. 18, 627–637. doi:10.1177/1753425911431285

Senf, S.M., Dodd, S.L., McClung, J.M., Judge, A.R., 2008. Hsp70 overexpression inhibits NF-kB and Foxo3a transcriptional activities and prevents skeletal muscle atrophy. FASEB J. 22, 3836–3845. doi:10.1096/fj.08-110163

Stuart, R.A., Cyr, D.M., Neupert, W., 1994. Hsp70 in mitochondrial biogenesis: From chaperoning nascent polypeptide chains to facilitation of protein degradation. Experientia 50, 1002–1011. doi:10.1007/BF01923454

Tait, S.W.G., Green, D.R., 2012. Mitochondria and cell signalling. J. Cell Sci. 125, 807–15. doi:10.1242/jcs.099234

Tiegs, G., Küsters, S., Künstle, G., Hentze, H., Kiemer, A.K., Wendel, A., 1998. Ebselen Protects Mice Against T Cell-Dependent, TNF-Mediated Apoptotic Liver Injury. J. Pharmacol. Exp. Ther. 287, 1098–1104.

Tirosh, O., Levy, E., Reifen, R., 2007. High selenium diet protects against TNBS-induced acute inflammation, mitochondrial dysfunction, and secondary necrosis in rat colon. Nutrition 23, 878–86. doi:10.1016/j.nut.2007.08.019

Tolson, J.K., Dix, D.J., Voellmy, R.W., Roberts, S.M., 2006. Increased hepatotoxicity of acetaminophen in Hsp70i knockout mice. Toxicol. Appl. Pharmacol. 210, 157–62. doi:10.1016/j.taap.2005.10.001

Yousuf, S., Atif, F., Ahmad, M., Nasrul Hoda, M., Badruzzaman Khan, M., Ishrat, T., Islam, F., 2007. Selenium plays a modulatory role against cerebral ischemia-induced neuronal damage in rat hippcampus. Brain Res. 1147, 218–225.

Zemolin, A.P.P., Meinerz, D.F., de Paula, M.T., Mariano, D.O.C., Rocha, J.B.T., Pereira, A.B., Posser, T., Franco, J.L., 2012. Evidences for a role of glutathione peroxidase 4 (GPx4) in methylmercury induced neurotoxicity in vivo. Toxicology 302, 60–67.

Zhang, H., Limphong, P., Pieper, J., Liu, Q., Rodesch, C.K., Christians, E., Benjamin, I.J., 2012. Glutathione-dependent reductive stress triggers mitochondrial oxidation and cytotoxicity. FASEB J. 26, 1442–51. doi:10.1096/fj.11-199869

Zhao, R., Holmgren, A., 2002. A novel antioxidant mechanism of ebselen involving ebselen diselenide, a substrate of mammalian thioredoxin and thioredoxin reductase. J. Biol. Chem. 277, 39456–62. doi:10.1074/jbc.M206452200

Zhao, R., Masayasu, H., Holmgren, A., 2002. Ebselen: a substrate for human thioredoxin reductase strongly stimulating its hydroperoxide reductase activity and a superfast thioredoxin oxidant. Proc. Natl. Acad. Sci. U. S. A. 99, 8579–84. doi:10.1073/pnas.122061399

Figure legends and tables

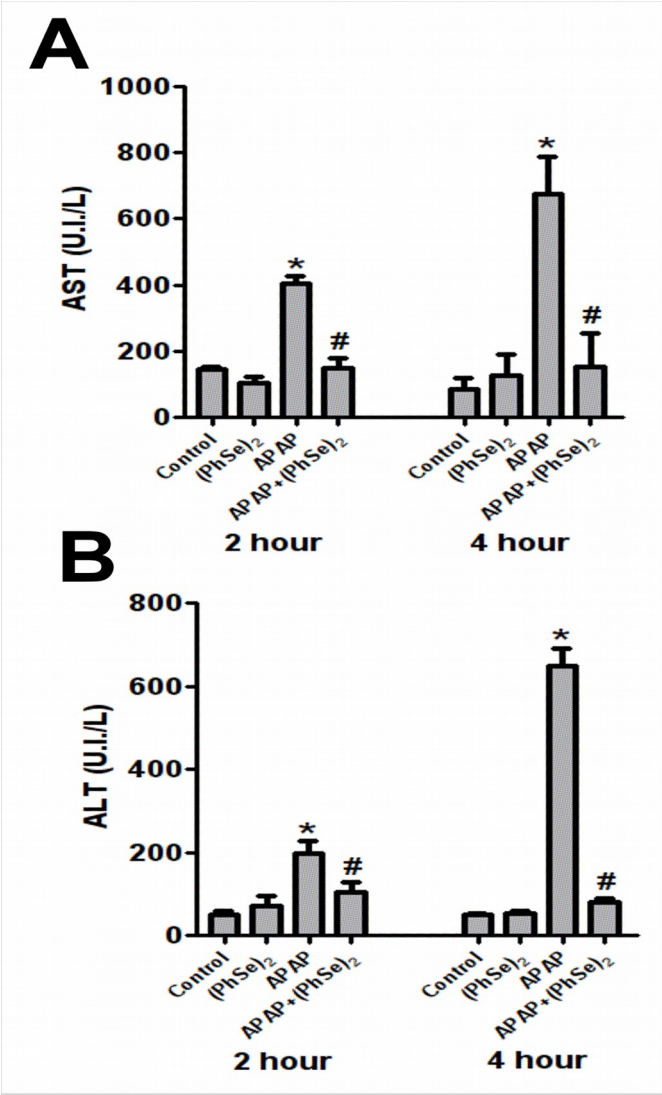

FIGURE 1: Effects of treatment with APAP and $(PhSe)_2$ on transaminase levels. A) Aspartate aminotransferase (AST), B) Alanine aminotransferase (ALT). APAP was given alone (600 mg/kg, i.p.) or in combination with $(PhSe)_2$ (15.6 mg/kg, i.p., 1 hr after APAP). Mice were killed at 2 h and 4 h following APAP administration. Data are expressed as means ± SEM, (n=5). Significance was assessed by one–way analysis of variance (ANOVA), followed by Newman-Keuls's test for post hoc comparison. Significant differences are indicated by $^{*}p < 0.05$ when compared with control group. Significant differences are indicated by $^{\#}p < 0.05$ when compared with APAP group.

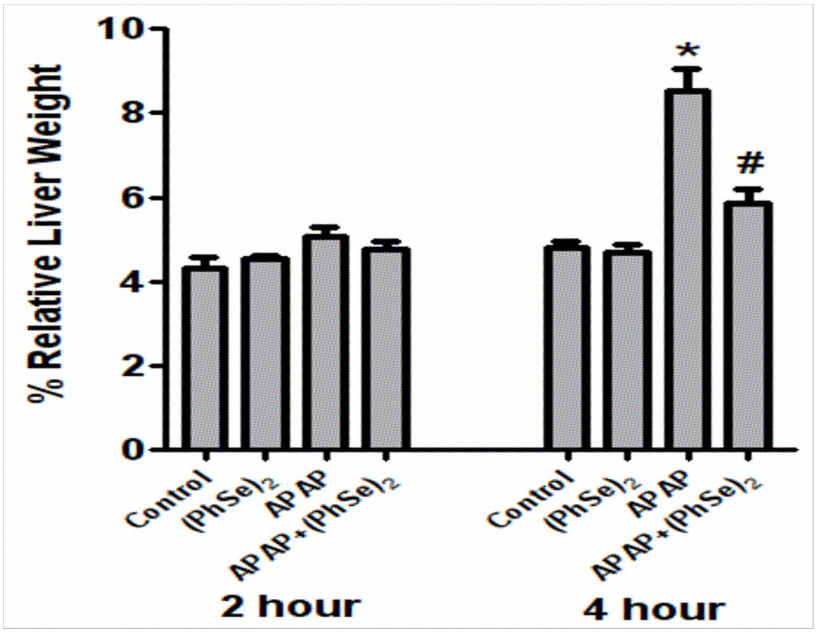

FIGURE 2: Macroscopic effects of treatment with APAP and $(PhSe)_2$ on relative liver weight. APAP was given alone (600 mg/kg, i.p.) or in combination with $(PhSe)_2$ (15.6 mg/kg, i.p., 1 hr after APAP). Mice were killed at 2 h and 4 h following APAP administration. Data are expressed as means ± SEM, (n=5). Significance was assessed by one–way analysis of variance (ANOVA), followed by Newman-Keuls's test for post hoc comparison. Significant differences are indicated by $^{*}p < 0.05$ when compared with control group. Significant differences are indicated by $^{\#}p < 0.05$ when compared with APAP group.

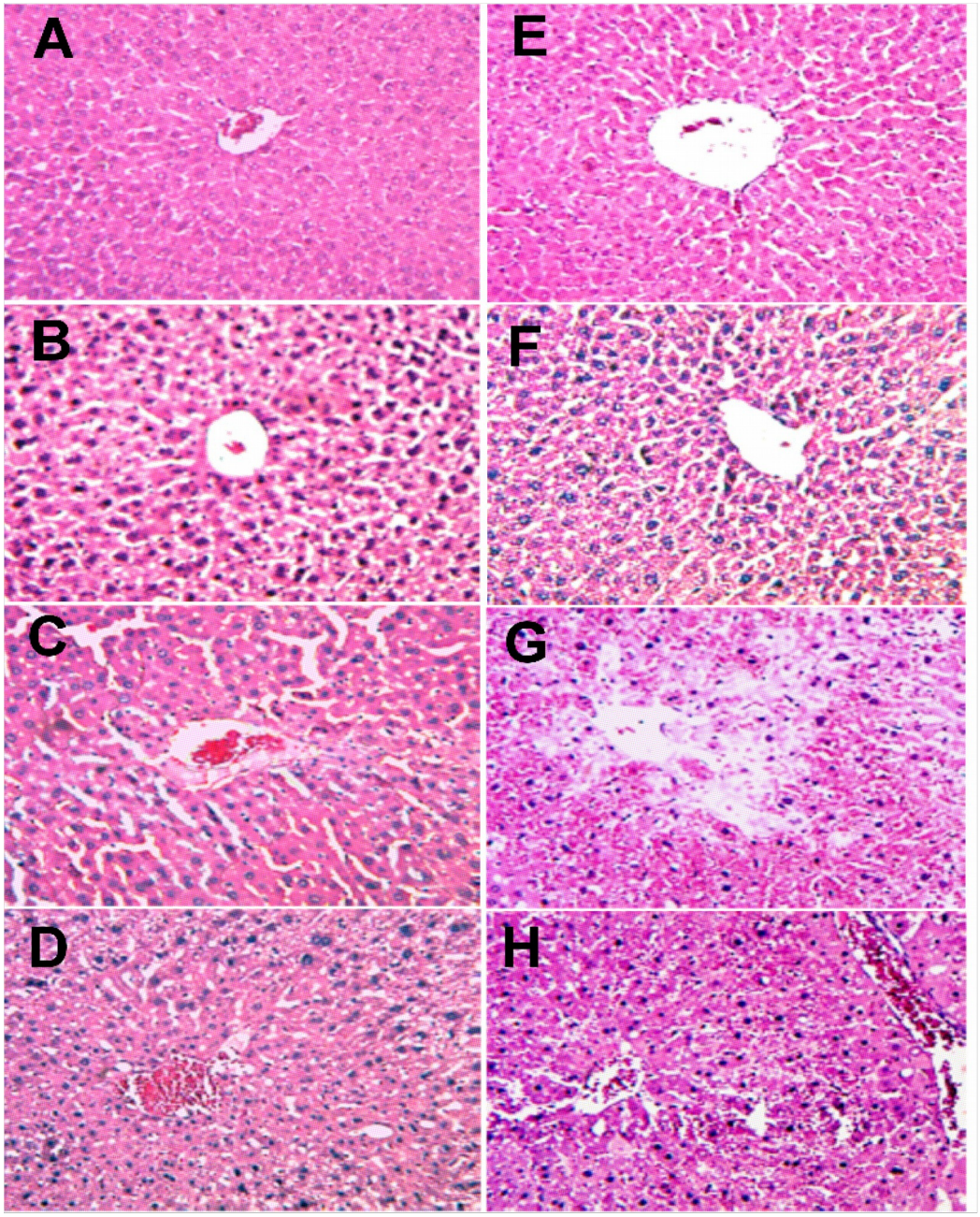

FIGURE 3: Effects of APAP and $(PhSe)_2$ on liver histology. All images correspond to 10 μm H&E-stained slices at 200 x magnification, collected 2 h (figure A-D) and 4 h (figure E -H) after APAP administration. APAP was given alone (600 mg/kg, i.p.) or in combination with $(PhSe)_2$ (15.6 mg/kg, i.p., 1 hr after APAP). The slides are representative of all animals in the specified group. Normal morphology was observed in Control (A and E), $(PhSe)_2$-treated (B and F), APAP (C and G) and APAP+$(PhSe)_2$ group (D and H).

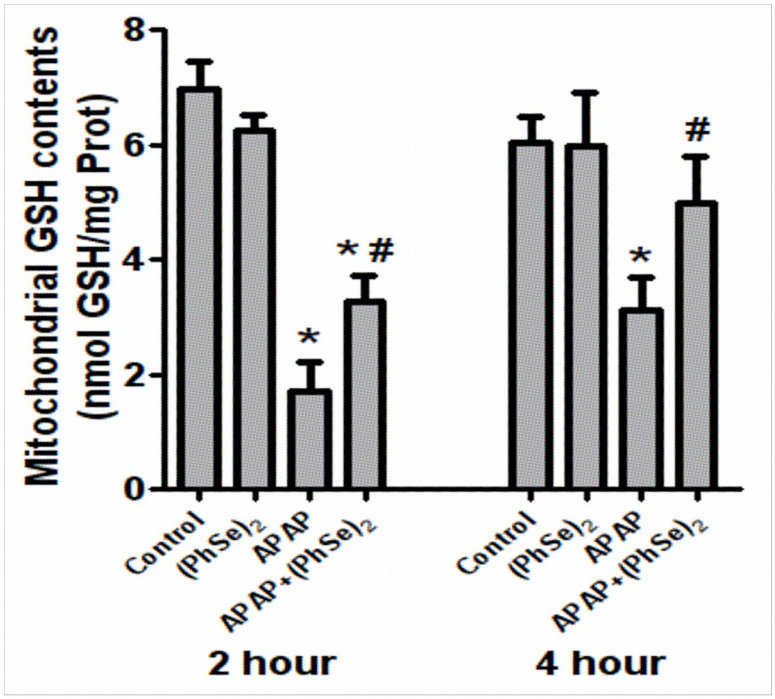

FIGURE 4: Effects of treatment with APAP and $(PhSe)_2$ on mitochondrial GSH concentration. (APAP was given alone (600 mg/kg, i.p.) or in combination with $(PhSe)_2$ (15.6 mg/kg, i.p., 1 hr after APAP). Mice were killed at 2 h and 4 h following APAP administration. Data are expressed as means ± SEM, (n=5). Significance was assessed by one–way analysis of variance (ANOVA), followed by Newman-Keuls's test for post hoc comparison. Significant differences are indicated by $^{*}p < 0.05$ when compared with control group. Significant differences are indicated by $^{\#}p < 0.05$ when compared with APAP group.

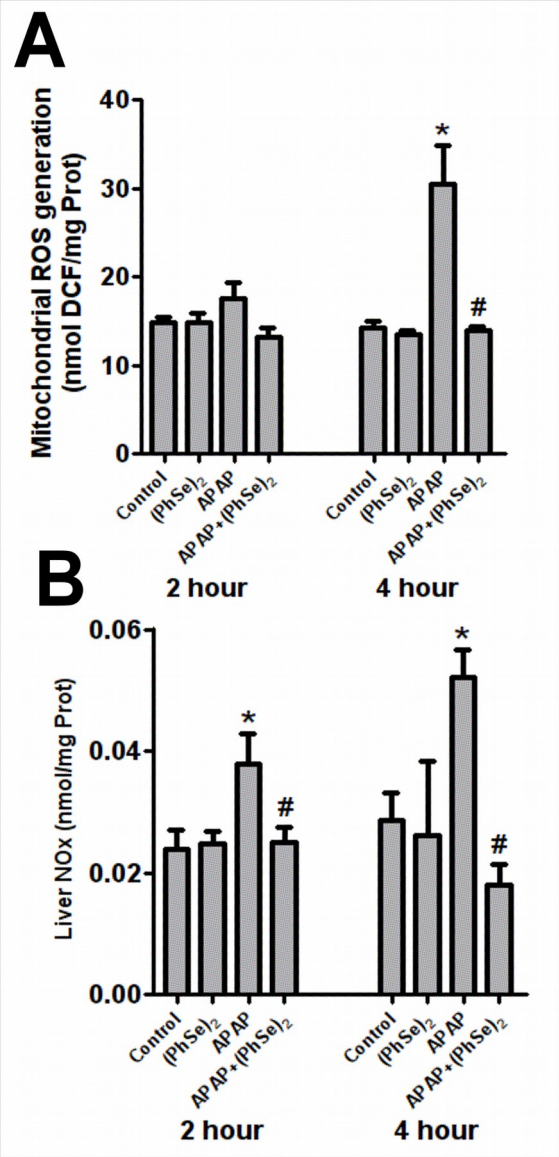

FIGURE 5: Effects of treatment with APAP and $(PhSe)_2$ on mitochondrial reactive oxygen and nitrogen species generation. A) Mitochondrial ROS generation and B) Liver NOx generation. APAP was given alone (600 mg/kg, i.p.) or in combination with $(PhSe)_2$ (15.6 mg/kg, i.p., 1 hr after APAP). Mice were killed at 2 h and 4 h following APAP administration. Data are expressed as means ± SEM, (n=5). Significance was assessed by one–way analysis of variance (ANOVA), followed by Newman-Keuls's test for post hoc comparison. Significant differences are indicated by $^{*}p < 0.05$ when compared with control group. Significant differences are indicated by $^{\#}p < 0.05$ when compared with APAP group.

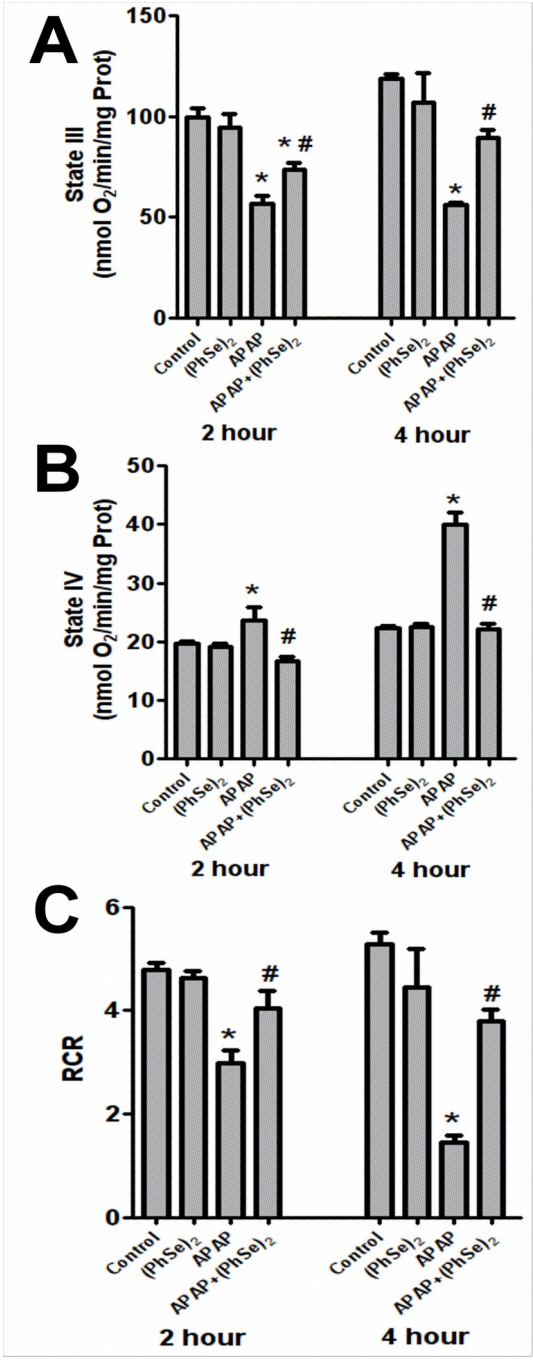

FIGURE 6: Effects of treatment with APAP and $(PhSe)_2$ on mitochondrial bioenergetics function. A) State III, B) State IV and C) RCR (State III/State IV). APAP was given alone (600 mg/kg, i.p.) or in combination with $(PhSe)_2$ (15.6 mg/kg, i.p., 1 hr after APAP). Mice were killed at 2 h and 4 h following APAP administration. Data are expressed as means ± SEM, (n=5). Significance was assessed by one–way analysis of variance (ANOVA), followed by Newman-Keuls's test for post hoc comparison. Significant differences are indicated by $^{*}p < 0.05$ when compared with control group. Significant differences are indicated by $^{\#}p < 0.05$ when compared with APAP group.

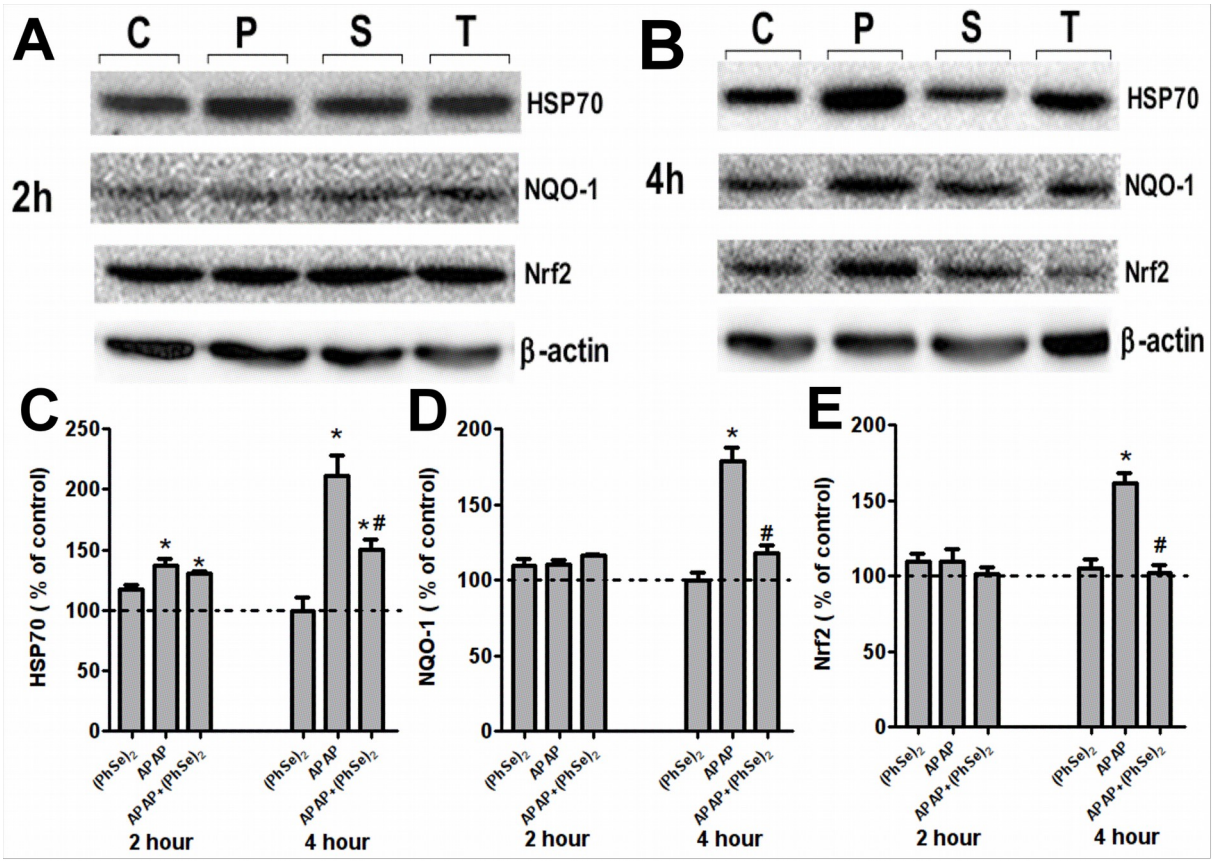

FIGURE 7: Effects of treatment with APAP and $(PhSe)_2$ on HSP70, NQO1 and Nrf2 protein concentration. APAP was given alone (600 mg/kg, i.p.) or in combination with $(PhSe)_2$ (15.6 mg/kg, i.p., 1 hr after APAP). Mice were killed at 2 h and 4 h following APAP administration. Representative immunoblots showing the expression levels of HSP70, NQO1 and Nrf2 are presented in panels A and B (C, P, S and T represent groups control, APAP, $(PhSe)_2$ and APAP+$(PhSe)_2$, respectively) at 2 hr and 4 hr. (C) Densitometric analysis of HSP70 immunoreactive bands. (D) Densitometric analysis of NQO1 immunoreactive bands. (E) Densitometric analysis of Nrf2 immunoreactive bands. Specific protein levels were normalized by β-actin immunocontent and expressed as a percentage of control. Data are expressed as means ± SEM, (n=5). Significance was assessed by one–way analysis of variance (ANOVA), followed by Newman-Keuls's test for post hoc comparison. Significant differences are indicated by *$p < 0.05$ when compared with control group. Significant differences are indicated by #$p < 0.05$ when compared with APAP group.

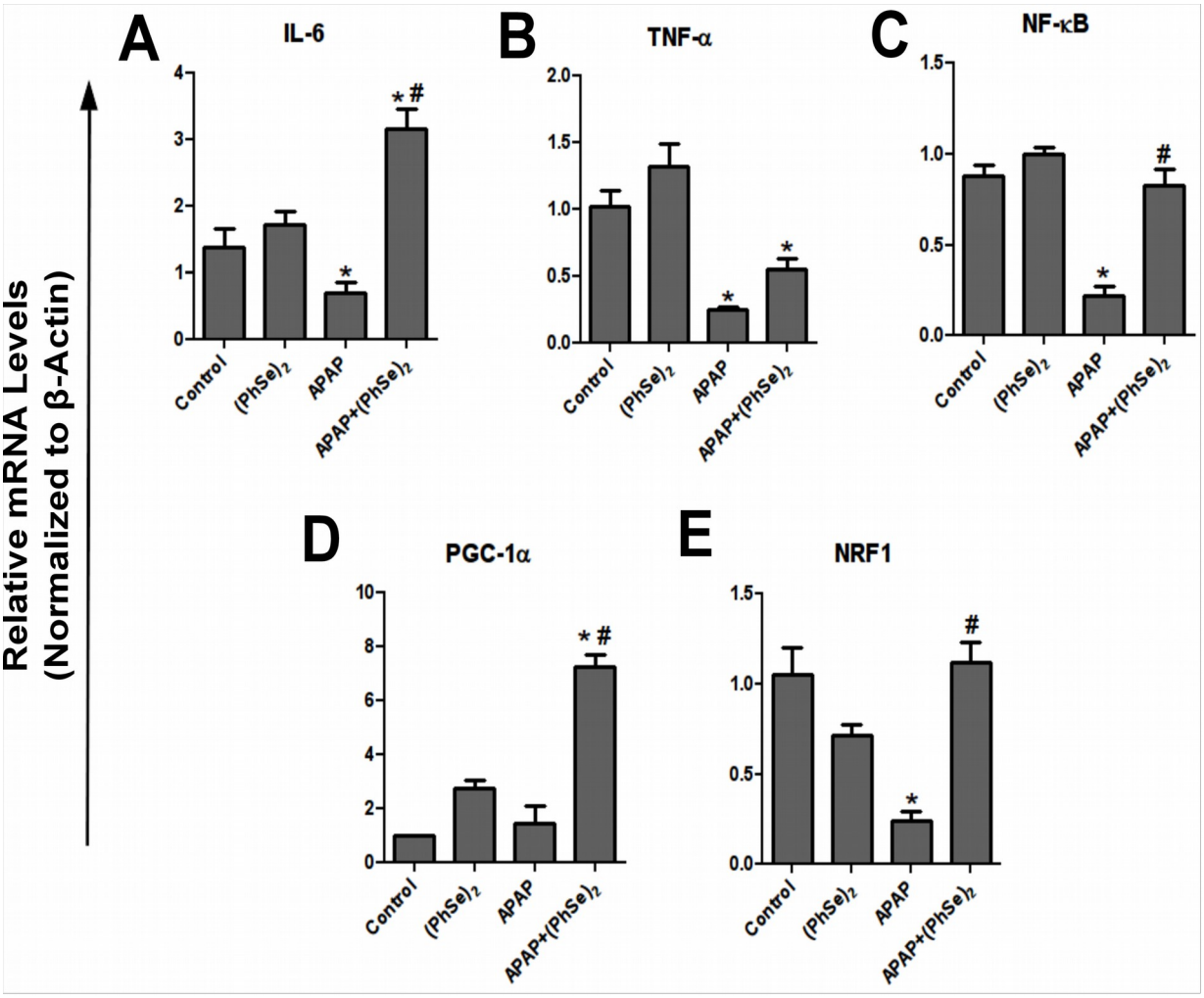

FIGURE 8: Effects of treatment with APAP and $(PhSe)_2$ on IL-6, TNF-α and NF-κB mRNA levels. APAP was given alone (600 mg/kg, i.p.) or in combination with $(PhSe)_2$ (15.6 mg/kg, i.p., 1 hr after APAP). Mice were killed at 2 h and 4 h following APAP administration. A) IL-6, B) TNF-α, C) NF-κB, D) PGC-1α and E) NRF1. Relative mRNA expression levels were normalized to β-actin. Data are expressed as means ± SEM, (n=4). Significance was assessed by one–way analysis of variance (ANOVA), followed by Newman-Keuls's test for post hoc comparison. Significant differences are indicated by *$p < 0.05$ when compared with control group. Significant differences are indicated by #$p < 0.05$ when compared with APAP group.

TABLE 1: Primer sequences for real-time PCR.

Genes	Forward primer (5'–3')	Reverse primer (5'–3')
β-Actin	GCTACAGCTTCACCACCACA	AAGGAAGGCTGGAAAAGAGC
Interleukin 6	AGTTGCCTTCTTGGGACTGA	CAGAATTGCCATTGCACAAC
NF-κB	TTTTCGACTACGCAGTGACG	CCAAGTGCAGAGGTGTCTGA
TNF-α	CGTCAGCCGATTTGCTATCT	CGGACTCCGCAAAGTCTAAG
NRF1	CAACAGGGAAGAAACGGAAA	GCACCACATTCTCCAAAGGT
PGC-1α	ATGTGTCGCCTTCTTGCTCT	ATCTACTGCCTGGGGACCTT

TABLE 2: Hepatotoxicity score of mice treated with APAP alone (600 mg/kg, i.p.) or in combination with $(PhSe)_2$ (15.6 mg/kg, i.p., 1 hr after APAP).

Treatment	Histopathology Score (post APAP overdose)					
	0	1+	2+	3+	4+	5+
2 hr						
Control (n=5)	5	0	0	0	0	0
$(PhSe)_2$ (n=5)	5	0	0	0	0	0
APAP (n=5)	0	0	2	2	1	0
APAP+$(PhSe)_2$ (n=5)	1	2	2	0	0	0
4 hr						
Control (n=5)	5	0	0	0	0	0
$(PhSe)_2$ (n=5)	5	0	0	0	0	0
APAP (n=5)	0	0	0	1	3	1
APAP+$(PhSe)_2$ (n=5)	0	1	2	2	0	0

Note: The criteria for scoring the liver injury were: 0, no injury; 1+,minimal injury (only few hepatocytes affected); 2+, mild injury (centrilobular necrosis in some lobules, 1-2 rings of necrotic cells); 3+ moderate injury (centrilobular necrosis in most lobules, 2-3 rings of necrotic cells); 4+ marked injury (centrilobular necrosis in all lobules, 3-4 rings of necrotic cells); 5+ severe injury (panlobular confluent necrosis, > 5 rings of necrotic cells and hemorrhage).Values are the total number of animals with the indicated score.

6. DISCUSSÃO

Compostos orgânicos de selênio têm demonstrado efeitos benéficos sobre o tratamento contra hepatotoxicantes, mas o mecanismo permanece incerto (Brandão et al., 2009; da Rosa et al., 2012). No atual trabalho nos investigamos os efeitos do $(PhSe)_2$ sobre a disfunção mitocondrial, funcionamento bioenergético e a via de sinalização envolvida na proteção contra a IHA induzida por APAP.

Atualmente a única modalidade terapêutica aceita para o tratamento da hepatotoxicidade induzida por APAP é a NAC, contudo, este apresenta uma janela terapêutica limitada, e a reversão dos níveis de GSH nem sempre são suficientes para impedir a progressão da IHA induzida por APAP (Gum and Cho, 2013). A partir do presente estudo, estabelecemos um parâmetro comparativo entre o $(PhSe)_2$ e o antídoto clássico para hepatotoxicidade induzida por APAP, uma vez que existem poucos agentes eficazes no tratamento contra a intoxicação (Brown et al., 2010; Terneus et al., 2008). A eficiência do $(PhSe)_2$ foi similar ao antídoto clássico (Figura 1-**Artigo**) é possível observar que o tratamento manteve os níveis normais de transaminases no plasma durante todo o protocolo experimental (Tabela 1, **Artigo**), evitando o aumento acentuado que ocorre com a progressão da intoxicação e proporcionou a manutenção da morfologia hepática (redução do edema hepático) e reduziu o dano a arquitetura microscópica conforme demonstrado na analise histológica evidenciando um menor escore de dano celular (Figura 1-3, **Manuscrito**). Estudos anteriores indicam que estes efeitos podem estar relacionados a redução da atividade da enzima mieloperoxidase envolvida no processo inflamatório decorrente da intoxicação por APAP (da Rosa et al., 2012). Observamos também em nosso estudo uma significante melhora nos marcadores de dano oxidativo (LPO e EROs) e níveis de atividades de enzimas antioxidantes no homogenato de fígado (CAT, SOD, GPx, GR) demonstrando que o $(PhSe)_2$ foi efetivo em doses mais baixa que a NAC quando administrado 1 h após APAP (Tabela 2 e 3, **Artigo**). Evidências da literatura demonstram que o intermediário selenol-selenoato formado a partir do ciclo redox dos compostos orgânicos de selênio são mais nucleofílicos do que os grupos tiol-tiolato proveniente de resíduos de cisteína como a NAC (Nogueira et al., 2004). Assim, em acordo com nossos resultados é plausível sugerir que o $(PhSe)_2$ desempenha seu efeito terapêutico relacionado primeiramente a três importantes pontos: manutenção dos níveis de GSH, redução do estresse oxidativo e inibição da MPT.

A manutenção dos níveis citosólicos e o rápido reestabelecimento mitocondrial dos níveis de GSH contribuem para uma melhora da homeostase redox do fígado (Tabela 3,

Artigo; Figura 4, **Manuscrito**), uma vez que as reservas de GSH mitocondriais são limitado estando intimamente relacionado com as reservas citosólicas, sendo que a depleção de GSH precede a toxicidade de APAP (Jaeschke and Bajt, 2006; Vendemiale et al., 1996). Neste contexto, embora a formação de NAPQI seja importante durante a hepatotoxicidade devido a depleção dos níveis de GSH hepático, a subsequente disfunção mitocondrial e queda do metabolismo bioenergético não podem ser completamente atribuídos aos efeitos diretos do NAPQI e a combinação de fatores como o aumento de EROs/ERNs contribuem significativamente para a iniciação e/ou promoção da IHA induzida por APAP (Figura 5A e B, **Manuscrito**). Várias linhas de evidências sugerem que os efeitos hepatoprotetores do $(PhSe)_2$ sejam mediados pela geração do intermediário selenol através da atividades miméticas da GPx e TrxR (Dobrachinski et al., 2014; Nogueira and Rocha, 2010), mas poderia usar de forma mais eficientemente o sistema da tiorredoxina do que a GSH (Dobrachinski et al., 2014; Zhao and Holmgren, 2002; Zhao et al., 2002). O intermediário selenol através da atividade mimética da TrxR poderia eficientemente neutralizar as EROs/ERNs poupando a utilização de GSH, prevenindo a LPO e carbonilação de proteínas mitocondriais (Figura 2 e 3, **Artigo**). Assim, estes efeitos poderiam ser observados através de um aumento na sobrevivências de 8 para 37.5 h dos animais tratados com $(PhSe)_2$, refletindo em uma maior janela terapêutica para intervenção terapêutica similares ao grupo tratado com NAC (Figura 1, **Artigo**).

A MPT limita a capacidade de reestabelecimento de bioenergético celular (Jaeschke et al., 2012). O rompimento da manutenção da OXPHOS após a IHA induzida por APAP causa inibição de processos que requerem uma maior demanda energética como proliferação celular e transporte de moléculas transmembrana (Fernandez-Checa and Kaplowitz, 2005; Galluzzi et al., 2012; Ribas et al., 2014). Estudos demonstram que a adaptação metabólica é um processo complexo e bem orquestrado tendo como fatores limitantes os níveis de GSH, geração de EROs/ERNs e viabilidade mitocondrial (Han et al., 2013). Assim, as propriedades antioxidantes do $(PhSe)_2$ somando-se à manutenção do sistema de defesa enzimático mitocondrial (MnSOD, GPx e GR) previnem a dissipação do $\Delta\psi m$ e a perda de permeabilidade seletiva da membrana mitocondrial (Figura 3, 5-6, **Artigo**). Além disso, a limitada produção de EROs/ERNs observada no tratamento com $(PhSe)_2$ poderia evitar o comprometimento funcional de enzimas do ciclo de Krebs as quais podem contribuir para a manutenção do NAD(P)H redox mitocondrial (Figura 7A, **Artigo**) e ciclo redox da GSH (Balaji Raghavendran et al., 2005).

A manutenção bioenergética assim como a sinalização envolvida na adaptação

metabólica é importante para um órgão como o fígado, uma vez que ele desempenha um papel importante na destoxificação de xenobióticos e apresenta uma alta demanda energética aeróbica (Hanawa et al., 2008). A ativação de vias como Nrf2-ARE e HSP70 são importantes estratégias para prevenção do desequilíbrio da dinâmica fisiológica mitocondrial após a hepatotoxicidade induzida por APAP (Chan et al., 2001; Gum and Cho, 2013). Surpreendentemente, a resposta adaptativa induzida pelo $(PhSe)_2$ não foi criticamente dependente de Nrf2-ARE (Figura 7A, B, D e E, **Manuscrito**), a qual tem sido apontada como um dos principais mecanismos de proteção mediado por compostos orgânicos de selênio (de Bem et al., 2013)(Kim et al., 2009). No entanto, estudos anteriores têm demonstrado uma proteção celular independente de Nrf2 (Li et al., 2014; Reisman et al., 2009). Neste contexto, $(PhSe)_2$ poderia evitar a disfunção bioenergética mitocondrial pela ativação de uma via redox-sensível complementar como a HSP70 (Figura 7C, **Manuscrito**), a qual é ativada por estresse térmico, mudanças redox e H_2O_2 tendo sua indução independe de Nrf2 (Stuart et al., 1994). Assim, HSP70 poderia auxiliar na proteção da dinâmica mitocondrial, reduzindo a oxidação de proteínas e promovendo uma melhor adaptação metabólica ao fígado, isso é evidenciado em animais com deleção do gene da HSP70 os quais são mais sensíveis a intoxicação (Tolson et al., 2006).

Assim, a IHA induzida por APAP pode causar significantes alterações nos níveis de mRNA de NF-κB e TNF-α os quais podem promover o comprometimento do metabolismo energético causando uma depressão da OXPHOS levando a uma crise energética e morte celular (Boulares et al., 2000; Mauro et al., 2011; Palomer et al., 2009). Nossos resultados sugerem que um dos possíveis mecanismos de hepatoproteção do $(PhSe)_2$ poderiam ser mediados pela ativação de HSP70. Dados da literatura demonstram que HSP70 possui uma atividade de citocina potente (Asea et al., 2002), a qual poderia restaurar os níveis de NF-κB, aumentar a expressão de IL-6 e modular os níveis de TNF-α (Figura 8A, B e C, **Manuscrito**). Desta forma, os efeitos de HSP70 sobre a sinalização de citocinas está intimamente relacionado com sua concentração (Guzhova et al., 1997). Níveis aumentados de HSP70 podem modular os níveis de NF-κB e TNF-α por um mecanismo de regulação negativa (Senf et al., 2008; Van Molle et al., 2002). Além disso, o aumento nos níveis de IL-6 poderiam antagonizar a morte celular, dano tecidual e acelerar a regeneração do fígado devido a sensibilização do gene regulador da expressão de HSP70 (Masubuchi et al., 2003). Desta forma, os altos níveis de HSP70 poderiam prevenir a MPT pela inibição da ativação de caspases e fragmentação do DNA (Hong et al., 2002; Masubuchi et al., 2003; Tolson et al., 2006), o que poderia modular a reorganização metabólica, viabilidade celular e capacidade de

proliferativa.

Estudos anteriores têm demonstrado um efeito direto de compostos orgânicos de selênio mediado pela ativação de NF-κB e um aumento dos níveis de IL-10 os quais inibem TNF-α (Rupil et al., 2012; Tiegs et al., 1998). Além disso, os baixos níveis de TNF-α, observados nos animais tratados com $(PhSe)_2$ poderia estar relacionado com o aumento da transcrição de PGC-1α (Figura 8D, **Manuscrito**), uma vez que TNF-α tem demonstrado agir como um modulador negativo da expressão de PGC-1α (Palomer et al., 2009). Desta forma, PGC-1α é um sinal que influencia a ativação de fatores de transcrição e o reestabelecimento dos níveis de NRF1 (Figura 8E, **Manuscrito**), o que afeta a taxa de transcrição do material genético, influencia a ativação de fatores de estabilidade do mRNA, altera a eficiência da tradução e a cinética mitocondrial de importação de proteínas e, ainda pode ter um efeito mais direto sobre a mitocôndria, iniciando a replicação ou transcrição do DNA mitocondrial ou ter efeito direto sobre a tradução do mRNA mitocondrial e na montagem de enzimas componentes da cadeia respiratória o que contribuí para a recuperação do tecido hepático e controle de qualidade da função mitocondrial (Baldelli et al., 2013; Finck and Kelly, 2006; Mootha et al., 2003).

Em acordo com nossos resultados o aumento na expressão de PGC-1α e a normalização de NRF1 observados no tratamento com $(PhSe)_2$ poderiam restaurar o estado III e estado IV resultando em valores maiores de RCR (Figura 6, **Manuscrito**), evitando a intensificação do consumo de oxigênio em condições não fosforilativas proporcionando um melhor acoplamento do fluxo de elétrons na cadeia respiratória e permitindo que a maior parte do oxigênio consumido permanece dedicado a síntese de ATP reduzindo o escape de elétrons com a consequente formação de EROs (Panatto et al., 2011). A manutenção da integridade mitocondrial é refletida pela restauração da atividade dos complexos I, II e ATPase inibindo o desperdício energético o qual é inversamente proporcional a síntese de ATP (Figura 7B e 8, **Artigo**), mas positivamente correlacionado com a geração de especies reativas durante o estresse oxidante (Hernández-Muñoz et al., 2003; Tait and Green, 2012). Desta forma, nossos resultados sugerem que o efeito hepatoprotetor do $(PhSe)_2$ possa ser via ativação da HSP70 que apresenta sua atividade dependente de ATP, assim, a preservação da OXPHOS, que favorece a síntese de ATP, essencial para manutenção da atividade da HSP70. Além disso, tem sido demonstrado que HSP70 deve ser translocado através das membranas mitocondriais externa e interna, sendo esta etapa um passo dependente do $\Delta\psi m$, integridade da membrana e níveis de ATP (Stuart et al., 1994). Por outro lado, embora a intoxicação por APAP aumente a expressão de HSP70 e Nrf2-ARE, isto não se traduz em uma redução do comprometimento

redox devido a crise energética que se estabelece no tecido hepático. Levando em consideração estes dados, trabalhos anteriores demonstram que o pré-tratamento com selênio induz um aumento na expressão de HSP70 (Chen et al., 2014; Giffard and Yenari, 2004; Kumar et al., 2014) e o tratamento com $(PhSe)_2$ foi capaz de induzir uma aumento no número de mitocôndrias e estimular a biogênese (Glaser et al., 2014; Kumari et al., 2012; Mendelev et al., 2012), aumentando o número de evidências de que o $(PhSe)_2$ em conjunto com a HSP70 poderiam agir como um moduladores da MPT, e desta forma, induzir uma resposta adaptativa pelo aumento dos níveis de PGC-1α e reestabelecimento dos níveis de NRF1, fatores importantes para a recuperação do fígado, controle de qualidade mitocondrial e manutenção da homeostase e saúde celular.

7. CONCLUSÃO GERAL

De acordo com os resultados apresentados nesta tese podemos concluir que:

- A utilização do $(PhSe)_2$ demonstrou-se tão eficaz quanto o antídoto clássico contra intoxicação aguda induzida por APAP, restaurando a funcionalidade do sistema redox mitocondrial e citosólico após a intoxicação;
- A disfunção mitocondrial associada a transição de permeabilidade mitocondrial e ao comprometimento do sistema redox mitocondrial foram revertido pelo tratamento com $(PhSe)_2$;
- O prejuízo funcional dos componentes da cadeia respiratória e o consequente desequilíbrio bioenergético foi eficientemente revertido com o $(PhSe)_2$;
- Demonstramos pela primeira vez um efeito modulador da ativação da HSP70 e genes promotores da biogênese mitocondrial resultando em efeitos fundamentais para o reestabelecimento energético celular envolvidos na manutenção da homeostase do tecido hepático a qual foi profundamente afetada durante a insuficiência hepática aguda induzida por APAP.

8. PERSPECTIVAS

Tendo em vista os resultados obtidos neste estudo, as perspectivas para estudos futuros são:

- Investigar a interação de APAP e álcool em camundongos;
- Avaliar os efeitos sobre o estresse oxidativo induzido por APAP sobre retículo endoplásmatico e a interação com a mitocôndria de fígado;
- Investigar a interação de APAP com a cafeína, uma vez esta combinação é utilizada em diversos medicamentos e não está totalmente esclarecido se a combinação APAP e cafeína pode aumentar o dano hepático;
- Investigar a interação de APAP e cafeína sobre os parâmetros de funcionalidade mitocondrial.

9. REFERÊNCIAS BIBLIOGRÁFICAS

Abdelmegeed, M. a, Jang, S., Banerjee, A., Hardwick, J.P., Song, B.-J., 2013. Robust protein nitration contributes to acetaminophen-induced mitochondrial dysfunction and acute liver injury. Free Radic. Biol. Med. 60, 211–22. doi:10.1016/j.freeradbiomed.2013.02.018

Al-Belooshi, T., John, A., Tariq, S., 2010. Increased mitochondrial stress and modulation of mitochondrial respiratory enzyme activities in acetaminophen-induced toxicity in mouse macrophage cells. Food Chem. … 48, 2624–32. doi:10.1016/j.fct.2010.06.031

Andrade Filho, A., Campolina, D., Dias, M.B., 2013. Toxicologia Na Prática Clínica, 2nd ed. Folium, Belo Horizonte.

Anundi, I., Lähteenmäki, T., Rundgren, M., Moldeus, P., Lindros, K.O., 1993. Zonation of acetaminophen metabolism and cytochrome P450 2E1-mediated toxicity studied in isolated periportal and perivenous hepatocytes. Biochem. Pharmacol. 45, 1251–1259. doi:10.1016/0006-2952(93)90277-4

Arakawa, S., Maejima, T., Fujimoto, K., Yamaguchi, T., Yagi, M., Sugiura, T., Atsumi, R., Yamazoe, Y., 2012. Resistance to acetaminophen-induced hepatotoxicity in glutathione S-transferase Mu 1-null mice. J. Toxicol. Sci. 37, 595–605.

Asea, A., Rehli, M., Kabingu, E., Boch, J.A., Baré, O., Auron, P.E., Stevenson, M.A., Calderwood, S.K., 2002. Novel signal transduction pathway utilized by extracellular HSP70. Role of toll-like receptor (TLR) 2 and TLR4. J. Biol. Chem. 277, 15028–15034. doi:10.1074/jbc.M200497200

Ayonrinde, O.T., Phelps, G.J., Hurley, J.C., Ayonrinde, O.A., 2005. Paracetamol overdose and hepatotoxicity at a regional Australian hospital: a 4-year experience. Intern. Med. J. 35, 655–60. doi:10.1111/j.1445-5994.2005.00947.x

Baird, L., Swift, S., Llères, D., Dinkova-Kostova, A.T., 2014. Monitoring Keap1-Nrf2 interactions in single live cells. Biotechnol. Adv. 32, 1133–1144. doi:10.1016/j.biotechadv.2014.03.004

Bajt, M.L., Ramachandran, A., Yan, H.-M., Lebofsky, M., Farhood, A., Lemasters, J.J., Jaeschke, H., 2011. Apoptosis-inducing factor modulates mitochondrial oxidant stress in acetaminophen hepatotoxicity. Toxicol. Sci. 122, 598–605. doi:10.1093/toxsci/kfr116

Balaji Raghavendran, H.R., Sathivel, A., Devaki, T., 2005. Antioxidant effect of Sargassum polycystum (Phaeophyceae) against acetaminophen induced changes in hepatic mitochondrial enzymes during toxic hepatitis. Chemosphere 61, 276–81. doi:10.1016/j.chemosphere.2005.01.049

Baldelli, S., Aquilano, K., Ciriolo, M.R., 2013. Punctum on two different transcription factors regulated by PGC-1α: nuclear factor erythroid-derived 2-like 2 and nuclear respiratory factor 2. Biochim. Biophys. Acta 1830, 4137–46. doi:10.1016/j.bbagen.2013.04.006

Barbour, J.A., Turner, N., 2014. Mitochondrial Stress Signaling Promotes Cellular Adaptations. Int. J. Cell Biol. 2014, 156020. doi:10.1155/2014/156020

Berson, A., Cazanave, S., Tinel, M., Grodet, A., Wolf, C., Pessayre, D., 2006. The Anti-Inflammatory Drug, Nimesulide (4-Nitro-2- phenoxymethane-sulfoanilide), Uncouples Mitochondria and Induces Mitochondrial Permeability Transition in Human Hepatoma Cells: Protection by Albumin 318, 444–454. doi:10.1124/jpet.106.104125.1999

Borges, L.P., Nogueira, C.W., Panatieri, R.B., Rocha, J.B.T., Zeni, G., 2006. Acute liver damage induced by 2-nitropropane in rats: effect of diphenyl diselenide on antioxidant defenses. Chem. Biol. Interact. 160, 99–107. doi:10.1016/j.cbi.2005.12.010

Boulares, A.H., Giardina, C., Inan, M.S., Khairallah, E.A., Cohen, S.D., 2000. Acetaminophen Inhibits NF- B Activation by Interfering with the Oxidant Signal in Murine Hepa 1-6 Cells. Toxicol. Sci. 375, 370–375.

Bounous, G., Batist, G., Gold, P., 1989. Immunoenhancing property of dietary whey protein in mice: role of glutathione. Clin. Invest. Med. 12, 154–61.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Brandão, R., Santos, F.W., Oliveira, R., Roman, S.S., Nogueira, C.W., 2009. Involvement of non-enzymatic antioxidant defenses in the protective effect of diphenyl diselenide on testicular damage induced by cadmium in mice. J. Trace Elem. Med. Biol. 23, 324–33. doi:10.1016/j.jtemb.2009.06.006

Brookes, P.S., Yoon, Y., Robotham, J.L., Anders, M.W., Sheu, S.-S., 2004. Calcium, ATP, and ROS: a mitochondrial love-hate triangle. Am. J. Physiol. Cell Physiol. 287, C817–33. doi:10.1152/ajpcell.00139.2004

Brown, G.C., Borutaite, V., 2002. Serial Review: Nitric Oxide in Mitochondria. Free Radic. Biol. Med. 33, 1440–1450.

Brown, J.M., Ball, J.G., Hogsett, A., Williams, T., Valentovic, M., 2010. Temporal study of acetaminophen (APAP) and S-adenosyl-L-methionine (SAMe) effects on subcellular hepatic SAMe levels and methionine adenosyltransferase (MAT) expression and activity. Toxicol. Appl. Pharmacol. 247, 1–9. doi:10.1016/j.taap.2010.04.018

Brunton, L., Chabner, B., Knollman, B., 2003. Goodman & Gilman: Manual de Farmacologia e Terapêutica, 1st ed. Artmed, Rio de Janeiro.

Budnitz, D.S., Lovegrove, M.C., Crosby, A.E., 2011. Emergency department visits for overdoses of acetaminophen-containing products. Am. J. Prev. Med. 40, 585–92. doi:10.1016/j.amepre.2011.02.026

Bulku, E., Stohs, S.J., Cicero, L., Brooks, T., Halley, H., Ray, S.D., 2012. Curcumin exposure modulates multiple pro-apoptotic and anti-apoptotic signaling pathways to antagonize acetaminophen-induced toxicity. Curr. Neurovasc. Res. 9, 58–71.

Carafoli, E., Gazzotti, P., 1970. Loss and maintenance of energy-linked functions in aged mitochondria. Biochem. Biophys. Res. Commun. 39, 842–6.

Carvalho, N., Puntel, G., Correa, P., Gubert, P., Amaral, G., Morais, J., Royes, L., da Rocha, J., Soares, F., 2010. Protective effects of therapeutic cold and heat against the oxidative damage induced by a muscle strain injury in rats. J. Sports Sci. 28, 923–35. doi:10.1080/02640414.2010.481722

Carvalho, N.R., da Rosa, E.F., da Silva, M.H., Tassi, C.C., Dalla Corte, C.L., Carbajo-Pescador, S., Mauriz, J.L., González-Gallego, J., Soares, F.A., 2013. New therapeutic approach: diphenyl diselenide reduces mitochondrial dysfunction in acetaminophen-induced acute liver failure. PLoS One 8, e81961. doi:10.1371/journal.pone.0081961

Chan, K., Han, X.D., Kan, Y.W., 2001. An important function of Nrf2 in combating oxidative stress: detoxification of acetaminophen. Proc. Natl. Acad. Sci. U. S. A. 98, 4611–4616.

Chen, X., Yao, H., Yao, L., Zhao, J., Luan, Y., Zhang, Z., Xu, S., 2014. Selenium deficiency influences the gene expressions of heat shock proteins and nitric oxide levels in neutrophils of broilers. Biol. Trace Elem. Res. 161, 334–40. doi:10.1007/s12011-014-0150-1

Cheng, Z., Ristow, M., 2013. Mitochondria and metabolic homeostasis. Antioxid. Redox Signal. 19, 240–2. doi:10.1089/ars.2013.5255

Da Rosa, E.J.F., da Silva, M.H., Carvalho, N.R., Bridi, J.C., da Rocha, J.B., Carbajo-Pescador, S., Mauriz, J.L., González-Gallego, J., Soares, F.A.A., 2012. Reduction of acute hepatic damage induced by acetaminophen after treatment with diphenyl diselenide in mice. Toxicol. Pathol. 40, 605–13. doi:10.1177/0192623311436179

Da Silva, M.H., da Rosa, E.J.F., de Carvalho, N.R., Dobrachinski, F., da Rocha, J.B.T., Mauriz, J.L., González-Gallego, J., Soares, F.A.A., 2012. Acute brain damage induced by acetaminophen in mice: effect of diphenyl diselenide on oxidative stress and mitochondrial dysfunction. Neurotox. Res. 21, 334–44. doi:10.1007/s12640-011-9288-1

da-Silva, W.S., Gómez-Puyou, A., de Gómez-Puyou, M.T., Moreno-Sanchez, R., De Felice, F.G., de Meis, L., Oliveira, M.F., Galina, A., 2004. Mitochondrial bound hexokinase activity as a preventive antioxidant defense: steady-state ADP formation as a regulatory mechanism of membrane potential and reactive oxygen species generation in mitochondria. J. Biol. Chem. 279, 39846–55. doi:10.1074/jbc.M403835200

De Achaval, S., Suarez-Almazor, M., 2011. Acetaminophen overdose: a little recognized public health threat. Pharmacoepidemiol. Drug Saf. 20, 827–9. doi:10.1002/pds.2162

De Bem, A.F., Fiuza, B., Calcerrada, P., Brito, P.M., Peluffo, G., Dinis, T.C.P., Trujillo, M., Rocha, J.B.T., Radi, R., Almeida, L.M., 2013. Protective effect of diphenyl diselenide against peroxynitrite-mediated endothelial cell death: a comparison with ebselen. Nitric Oxide 31, 20–30. doi:10.1016/j.niox.2013.03.003

De Freitas, A.S., Rocha, J.B.T., 2011. Diphenyl diselenide and analogs are substrates of cerebral rat thioredoxin reductase: a pathway for their neuroprotective effects. Neurosci. Lett. 503, 1–5. doi:10.1016/j.neulet.2011.07.050

Dionisio, N., Garcia-Mediavilla, M. V, Sanchez-Campos, S., Majano, P.L., Benedicto, I., Rosado, J.A., Salido, G.M., Gonzalez-Gallego, J., 2009. Hepatitis C virus NS5A and core proteins induce oxidative stress-mediated calcium signalling alterations in hepatocytes. J. Hepatol. 50, 872–82. doi:10.1016/j.jhep.2008.12.026

Dobrachinski, F., da Silva, M.H., Tassi, C.L.C., de Carvalho, N.R., Dias, G.R.M., Golombieski, R.M., da Silva Loreto, E.L., da Rocha, J.B.T., Fighera, M.R., Soares, F.A.A., 2014. Neuroprotective Effect of Diphenyl Diselenide in a Experimental Stroke Model: Maintenance of Redox System in Mitochondria of Brain Regions. Neurotox. Res. doi:10.1007/s12640-014-9463-2

Dumas, J.-F., Goupille, C., Julienne, C.M., Pinault, M., Chevalier, S., Bougnoux, P., Servais, S., Couet, C., 2011. Efficiency of oxidative phosphorylation in liver mitochondria is decreased in a rat model of peritoneal carcinosis. J. Hepatol. 54, 320–7. doi:10.1016/j.jhep.2010.08.012

Fernandez-Checa, J.C., Kaplowitz, N., 2005. Hepatic mitochondrial glutathione: transport and role in disease and toxicity. Toxicol. Appl. Pharmacol. 204, 263–73. doi:10.1016/j.taap.2004.10.001

Finck, B.N., Kelly, D.P., 2006. PGC-1 coactivators: inducible regulators of energy metabolism in health and disease. J. Clin. Invest. 116, 615–22. doi:10.1172/JCI27794

Fontana, R.J., 2008. Acute liver failure including acetaminophen overdose. Med. Clin. North Am. 92, 761–94, viii. doi:10.1016/j.mcna.2008.03.005

Franco, J.L., Posser, T., Missau, F., Pizzolatti, M.G., Santos, A.R.S., Souza, D.O., Aschner, M., Rocha, J.B.T., Dafre, A.L., Farina, M., 2010. Structure–activity relationship of flavonoids derived from medicinal plants in preventing methylmercury-induced mitochondrial dysfunction. Environ. Toxicol. Pharmacol. 30, 272–278.

Galluzzi, L., Kepp, O., Trojel-Hansen, C., Kroemer, G., 2012. Mitochondrial control of cellular life, stress, and death. Circ. Res. 111, 1198–207. doi:10.1161/CIRCRESAHA.112.268946

Gamal el-din, a, 2003. Protective effect of arabic gum against acetaminophen-induced hepatotoxicity in mice. Pharmacol. Res. 48, 631–635. doi:10.1016/S1043-6618(03)00226-3

García-Mediavilla, V., Crespo, I., Collado, P.S., Esteller, A., Sánchez-Campos, S., Tuñón, M.J., González-Gallego, J., 2007. The anti-inflammatory flavones quercetin and kaempferol cause inhibition of inducible nitric oxide synthase, cyclooxygenase-2 and reactive C-protein, and down-regulation of the nuclear factor kappaB pathway in Chang Liver cells. Eur. J. Pharmacol. 557, 221–9. doi:10.1016/j.ejphar.2006.11.014

Gardner, C.R., Mishin, V., Laskin, J.D., Laskin, D.L., 2012. Exacerbation of acetaminophen hepatotoxicity by the anthelmentic drug fenbendazole. Toxicol. Sci. 125, 607–12. doi:10.1093/toxsci/kfr301

Gelain, D.P., de Bittencourt Pasquali, M.A., M Comim, C., Grunwald, M.S., Ritter, C., Tomasi, C.D., Alves, S.C., Quevedo, J., Dal-Pizzol, F., Moreira, J.C.F., 2011. Serum heat shock protein 70 levels, oxidant status, and mortality in sepsis. Shock 35, 466–70. doi:10.1097/SHK.0b013e31820fe704

Ghosh, J., Das, J., Manna, P., Sil, P.C., 2010. Acetaminophen induced renal injury via oxidative stress and TNF-alpha production: therapeutic potential of arjunolic acid. Toxicology 268, 8–18. doi:10.1016/j.tox.2009.11.011

Giffard, R.G., Yenari, M.A., 2004. Many mechanisms for hsp70 protection from cerebral ischemia. J. Neurosurg. Anesthesiol. 16, 53–61.

Glaser, V., Martins, R. de P., Vieira, A.J.H., Oliveira, E. de M., Straliotto, M.R., Mukdsi, J.H., Torres, A.I., de Bem, A.F., Farina, M., da Rocha, J.B.T., De Paul, A.L., Latini, A., 2014. Diphenyl diselenide administration enhances cortical mitochondrial number and activity by increasing hemeoxygenase type 1 content in a methylmercury-induced neurotoxicity mouse model. Mol. Cell. Biochem. 390, 1–8. doi:10.1007/s11010-013-1870-9

Glaser, V., Nazari, E.M., Müller, Y.M.R., Feksa, L., Wannmacher, C.M.D., Rocha, J.B.T., de Bem, A.F., Farina, M., Latini, A., 2010. Effects of inorganic selenium administration in methylmercury-induced neurotoxicity in mouse cerebral cortex. Int. J. Dev. Neurosci. 28, 631–7. doi:10.1016/j.ijdevneu.2010.07.225

Greco, T., Shafer, J., Fiskum, G., 2011. Sulforaphane inhibits mitochondrial permeability transition and oxidative stress. Free Radic. Biol. Med. 51, 2164–71. doi:10.1016/j.freeradbiomed.2011.09.017

Guerra, G.P., Mello, C.F., Sauzem, P.D., Berlese, D.B., Furian, A.F., Tabarelli, Z., Rubin, M.A., 2006. Nitric oxide is involved in the memory facilitation induced by spermidine in rats. Psychopharmacology (Berl). 186, 150–8. doi:10.1007/s00213-006-0376-5

Gum, S. Il, Cho, M.K., 2013. Recent updates on acetaminophen hepatotoxicity: the role of nrf2 in hepatoprotection. Toxicol. Res. 29, 165–72. doi:10.5487/TR.2013.29.3.165

Gutteridge, J.M.C., Halliwell, B., 1994. Antioxidants in Nutrition, Health and Disease. New York.

Guzhova, I. V, Darieva, Z.A., Melo, A.R., Margulis, B.A., 1997. Major stress protein Hsp70 interacts with NF-kB regulatory complex in human T-lymphoma cells. Cell Stress Chaperones 2, 132–139. doi:10.1379/1466-1268(1997)002<0132:MSPHIW>2.3.CO;2

Halliwell, B., 2006. Oxidative Stress and neurodegeneration: were are we now? J. Neurochem. 97, 1634–1548.

Halliwell, B., Gutteridge, J.M.C., 2006. Free Radicals in Biology and Medicine, 4th ed.

Han, D., Dara, L., Win, S., Than, T.A., Yuan, L., Abbasi, S.Q., Liu, Z.-X., Kaplowitz, N., 2013. Regulation of drug-induced liver injury by signal transduction pathways: critical role of mitochondria. Trends Pharmacol. Sci. 34, 243–53. doi:10.1016/j.tips.2013.01.009

Hanawa, N., Shinohara, M., Saberi, B., Gaarde, W. a, Han, D., Kaplowitz, N., 2008. Role of JNK translocation to mitochondria leading to inhibition of mitochondria bioenergetics in acetaminophen-induced liver injury. J. Biol. Chem. 283, 13565–77. doi:10.1074/jbc.M708916200

Handschin, C., Chin, S., Li, P., Liu, F., Maratos-Flier, E., Lebrasseur, N.K., Yan, Z., Spiegelman, B.M., 2007a. Skeletal muscle fiber-type switching, exercise intolerance, and myopathy in PGC-1alpha muscle-specific knock-out animals. J. Biol. Chem. 282, 30014–30021.

Handschin, C., Choi, C.S., Chin, S., Kim, S., Kawamori, D., Kurpad, A.J., Neubauer, B.M., Hu, J., Mootha, V.K., Kim, Y.B., Kulkarni, R.N., Shulman, G.I., Spiegelman, B.M., 2007b. Abnormal glucose homeostasis in skeletal muscle-specificPGC-1alpha knockout mice reveals skeletal muscle-pancreatic beta cell crosstalk. J. Clin. Invest. 117, 3463–3474.

Hernández-Muñoz, R., Sánchez-Sevilla, L., Martínez-Gómez, A., Dent, M.A.R., 2003. Changes in mitochondrial adenine nucleotides and in permeability transition in two models of rat liver regeneration. Hepatology 37, 842–51. doi:10.1053/jhep.2003.50145

Hissin, P.J., Hilf, R., 1976. A fluorometric method for determination of oxidized and reduced glutathione in tissues. Anal. Biochem. 74, 214–26.

Hong, F., Kim, W.-H., Tian, Z., Jaruga, B., Ishac, E., Shen, X., Gao, B., 2002. Elevated interleukin-6 during ethanol consumption acts as a potential endogenous protective cytokine against ethanol-induced apoptosis in the liver: involvement of induction of Bcl-2 and Bcl-x(L) proteins. Oncogene 21, 32–43. doi:10.1038/sj.onc.1205016

Hoque, R., Sohail, M.A., Salhanick, S., Malik, A.F., Ghani, A., Robson, S.C., Mehal, W.Z., 2012. P2X7 receptor-mediated purinergic signaling promotes liver injury in acetaminophen hepatotoxicity in mice. Am. J. Physiol. Gastrointest. Liver Physiol. 302, G1171–9. doi:10.1152/ajpgi.00352.2011

Jaeschke, H., 1990. Glutathione disulfide formation and oxidant stress during acetaminophen-induced hepatotoxicity in mice in vivo: the protective effect of allopurinol. J. Pharmacol. Exp. Ther. 255, 935–41.

Jaeschke, H., 2003. The role of oxidant stress and reactive nitrogen species in acetaminophen hepatotoxicity. Toxicol. Lett. 144, 279–288. doi:10.1016/S0378-4274(03)00239-X

Jaeschke, H., Bajt, M.L., 2006. Intracellular signaling mechanisms of acetaminophen-induced liver cell death. Toxicol. Sci. 89, 31–41. doi:10.1093/toxsci/kfi336

Jaeschke, H., McGill, M.R., Ramachandran, A., 2012. Oxidant stress, mitochondria, and cell death mechanisms in drug-induced liver injury: lessons learned from acetaminophen hepatotoxicity. Drug Metab. Rev. 44, 88–106. doi:10.3109/03602532.2011.602688

James, L.P., Lamps, L.W., McCullough, S., Hinson, J.A., 2003. Interleukin 6 and hepatocyte regeneration in acetaminophen toxicity in the mouse. Biochem. Biophys. Res. Commun. 309, 857–863. doi:10.1016/j.bbrc.2003.08.085

Joly, A.-L., Wettstein, G., Mignot, G., Ghiringhelli, F., Garrido, C., 2010. Dual role of heat shock proteins as regulators of apoptosis and innate immunity. J. Innate Immun. 2, 238–47. doi:10.1159/000296508

Kelly, D.P., Scarpulla, R.C., 2004. Transcriptional regulatory circuits controlling mitochondrial biogenesis and function. Genes Dev. 18, 357–68. doi:10.1101/gad.1177604

Kim, L.S., Kim, J.H., 2011. Heat shock protein as molecular targets for breast cancer therapeutics. J. Breast Cancer 14, 167–74. doi:10.4048/jbc.2011.14.3.167

Kim, S.-J., Park, C., Han, A.L., Youn, M.-J., Lee, J.-H., Kim, Y., Kim, E.-S., Kim, H.-J., Kim, J.-K., Lee, H.-K., Chung, S.-Y., So, H., Park, R., 2009. Ebselen attenuates cisplatin-induced ROS generation through Nrf2 activation in auditory cells. Hear. Res. 251, 70–82. doi:10.1016/j.heares.2009.03.003

Kim, S.R., Kim, D.I., Kim, S.H., Lee, H., Lee, K.S., Cho, S.H., Lee, Y.C., 2014. NLRP3 inflammasome activation by mitochondrial ROS in bronchial epithelial cells is required for allergic inflammation. Cell Death Dis. 5, e1498. doi:10.1038/cddis.2014.460

Kim, T.-S., Yun, B.Y., Kim, I.Y., 2003. Induction of the mitochondrial permeability transition by selenium compounds mediated by oxidation of the protein thiol groups and generation of the superoxide. Biochem. Pharmacol. 66, 2301–2311. doi:10.1016/j.bcp.2003.08.021

Kis, B., Snipes, J.A., Busija, D.W., 2005. Acetaminophen and the cyclooxygenase-3 puzzle: sorting out facts, fictions, and uncertainties. J. Pharmacol. Exp. Ther. 315, 1–7. doi:10.1124/jpet.105.085431

Knight, T.R., Fariss, M.W., Farhood, A., Jaeschke, H., 2003. Role of lipid peroxidation as a mechanism of liver injury after acetaminophen overdose in mice. Toxicol. Sci. 76, 229–36. doi:10.1093/toxsci/kfg220

Kon, K., Kim, J.-S., Jaeschke, H., Lemasters, J.J., 2004. Mitochondrial permeability transition in acetaminophen-induced necrosis and apoptosis of cultured mouse hepatocytes. Hepatology 40, 1170–9. doi:10.1002/hep.20437

Kowaltowski, A.J., de Souza-Pinto, N.C., Castilho, R.F., Vercesi, A.E., 2009. Mitochondria and reactive oxygen species. Free Radic. Biol. Med. 47, 333–43. doi:10.1016/j.freeradbiomed.2009.05.004

Kumar, G.S., Kulkarni, A., Khurana, A., Kaur, J., Tikoo, K., 2014. Selenium nanoparticles involve HSP-70 and SIRT1 in preventing the progression of type 1 diabetic nephropathy. Chem. Biol. Interact. 223C, 125–133. doi:10.1016/j.cbi.2014.09.017

Kumari, S., Mehta, S.L., Li, P.A., 2012. Glutamate induces mitochondrial dynamic imbalance and autophagy activation: preventive effects of selenium. PLoS One 7, e39382. doi:10.1371/journal.pone.0039382

Küpeli, E., Orhan, D.D., Yesilada, E., 2006. Effect of Cistus laurifolius L. leaf extracts and flavonoids on acetaminophen-induced hepatotoxicity in mice. J. Ethnopharmacol. 103, 455–60. doi:10.1016/j.jep.2005.08.038

Kuthan, H., Tsuji, H., Graf, H., Ullrich, V., 1978. Generation of superoxide anion as a source of hydrogen peroxide in a reconstituted monooxygenase system. FEBS Lett. 91, 343–5.

Larson, A., Polson, J., Fontana, R., 2005. Acetaminophen-induced acute liver failure: results of a United States multicenter, prospective study. Hepatology 42, 1364–72. doi:10.1002/hep.20948

Lemasters, J.J., Qian, T., Bradham, C. a, Brenner, D. a, Cascio, W.E., Trost, L.C., Nishimura, Y., Nieminen, a L., Herman, B., 1999. Mitochondrial dysfunction in the pathogenesis of necrotic and apoptotic cell death. J. Bioenerg. Biomembr. 31, 305–319.

Li, G., Lee, M.-J., Liu, A.B., Yang, Z., Lin, Y., Shih, W.J., Yang, C.S., 2012. The antioxidant and anti-inflammatory activities of tocopherols are independent of Nrf2 in mice. Free Radic. Biol. Med. 52, 1151–8. doi:10.1016/j.freeradbiomed.2011.12.005

Li, S., Li, J., Shen, C., Zhang, X., Sun, S., Cho, M., Sun, C., Song, Z., 2014. tert-Butylhydroquinone (tBHQ) protects hepatocytes against lipotoxicity via inducing autophagy independently of Nrf2 activation. Biochim. Biophys. Acta 1841, 22–33. doi:10.1016/j.bbalip.2013.09.004

Lin, J., Handschin, C., Spiegelman, B.M., 2005. Metabolic control through the PGC-1 family of transcription coactivators. Cell Metab. 1, 361–70. doi:10.1016/j.cmet.2005.05.004

Livak, K., Schmittgen, T., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402–408. doi:10.1006/meth.2001.1262

LoGuidice, A., Boelsterli, U. a, 2011. Acetaminophen overdose-induced liver injury in mice is mediated by peroxynitrite independently of the cyclophilin D-regulated permeability transition. Hepatology 54, 969–78. doi:10.1002/hep.24464

López-Armada, M.J., Riveiro-Naveira, R.R., Vaamonde-García, C., Valcárcel-Ares, M.N., 2013. Mitochondrial dysfunction and the inflammatory response. Mitochondrion 13, 106–18. doi:10.1016/j.mito.2013.01.003

Maass, D.L., White, J., Sanders, B., Horton, J.W., 2005. Role of cytosolic vs. mitochondrial Ca2+ accumulation in burn injury-related myocardial inflammation and function. Am. J. Physiol. Heart Circ. Physiol. 288, H744–51. doi:10.1152/ajpheart.00367.2004

MacMillan-Crow, L. a, Crow, J.P., Thompson, J. a, 1998. Peroxynitrite-mediated inactivation of manganese superoxide dismutase involves nitration and oxidation of critical tyrosine residues. Biochemistry 37, 1613–1622.

Martindale, J.L., Holbrook, N.J., 2002. Cellular response to oxidative stress: Signaling for suicide and survival. J. Cell. Physiol. 192, 1–15.

Masubuchi, Y., Bourdi, M., Reilly, T.P., Graf, M.L.M., George, J.W., Pohl, L.R., 2003. Role of interleukin-6 in hepatic heat shock protein expression and protection against acetaminophen-induced liver disease. Biochem. Biophys. Res. Commun. 304, 207–212. doi:10.1016/S0006-291X(03)00572-2

Masubuchi, Y., Nakayama, S., Horie, T., 2002. Role of mitochondrial permeability transition in diclofenac-induced hepatocyte injury in rats. Hepatology 35, 544–51. doi:10.1053/jhep.2002.31871

Masubuchi, Y., Suda, C., Horie, T., 2005. Involvement of mitochondrial permeability transition in acetaminophen-induced liver injury in mice. J. Hepatol. 42, 110–6. doi:10.1016/j.jhep.2004.09.015

Mauro, C., Leow, S.C., Anso, E., Rocha, S., Thotakura, A.K., Tornatore, L., Moretti, M., De Smaele, E., Beg, A.A., Tergaonkar, V., Chandel, N.S., Franzoso, G., 2011. NF-κB controls energy homeostasis and metabolic adaptation by upregulating mitochondrial respiration. Nat. Cell Biol. 13, 1272–9. doi:10.1038/ncb2324

Mazzeo, A.T., Beat, A., Singh, A., Bullock, M.R., 2009. The role of mitochondrial transition pore, and its modulation, in traumatic brain injury and delayed neurodegeneration after TBI. Exp. Neurol. 218, 363–70. doi:10.1016/j.expneurol.2009.05.026

Mehta, S.L., Kumari, S., Mendelev, N., Li, P.A., 2012. Selenium preserves mitochondrial function, stimulates mitochondrial biogenesis, and reduces infarct volume after focal cerebral ischemia. BMC Neurosci. 13, 79. doi:10.1186/1471-2202-13-79

Mendelev, N., Mehta, S.L., Idris, H., Kumari, S., Li, P.A., 2012. Selenite stimulates mitochondrial biogenesis signaling and enhances mitochondrial functional performance in murine hippocampal neuronal cells. PLoS One 7, e47910. doi:10.1371/journal.pone.0047910

Meotti, F.C., Stangherlin, E.C., Zeni, G., Nogueira, C.W., Rocha, J.B.T., 2004. Protective role of aryl and alkyl diselenides on lipid peroxidation. Environ. Res. 94, 276–82. doi:10.1016/S0013-9351(03)00114-2

Michael Brown, J., Ball, J.G., Wright, M.S., Van Meter, S., Valentovic, M. a, 2012. Novel protective mechanisms for S-adenosyl-L-methionine against acetaminophen hepatotoxicity: improvement of key antioxidant enzymatic function. Toxicol. Lett. 212, 320–8. doi:10.1016/j.toxlet.2012.05.018

Michalopoulos, G.K., 2007. Liver regeneration. J. Cell. Physiol. 213, 286–300. doi:10.1002/jcp.21172

Mootha, V.K., Lindgren, C.M., Eriksson, K.-F., Subramanian, A., Sihag, S., Lehar, J., Puigserver, P., Carlsson, E., Ridderstråle, M., Laurila, E., Houstis, N., Daly, M.J., Patterson, N., Mesirov, J.P., Golub, T.R., Tamayo, P., Spiegelman, B., Lander, E.S., Hirschhorn, J.N., Altshuler, D., Groop, L.C., 2003. PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. Nat. Genet. 34, 267–73. doi:10.1038/ng1180

Moretti, M., Bennett, J., Tornatore, L., Thotakura, A.K., Franzoso, G., 2012. Cancer: NF-κB regulates energy metabolism. Int. J. Biochem. Cell Biol. 44, 2238–43. doi:10.1016/j.biocel.2012.08.002

Morin, D., Zini, R., Ligeret, H., Neckameyer, W., Labidalle, S., Tillement, J.-P., 2003. Dual effect of ebselen on mitochondrial permeability transition. Biochem. Pharmacol. 65, 1643–1651. doi:10.1016/S0006-2952(03)00114-X

Moyer, A.M., Fridley, B.L., Jenkins, G.D., Batzler, A.J., Pelleymounter, L.L., Kalari, K.R., Ji, Y., Chai, Y., Nordgren, K.K.S., Weinshilboum, R.M., 2011. Acetaminophen-NAPQI hepatotoxicity: a cell line model system genome-wide association study. Toxicol. Sci. 120, 33–41. doi:10.1093/toxsci/kfq375

Nogueira, C., Zeni, G., Rocha, J., 2004. Organoselenium and organotellurium compounds: toxicology and pharmacology. Chem. Rev.

Nogueira, C.W., Rocha, J.B.T., 2010. Diphenyl diselenide a janus-faced molecule. J. Braz. Chem. Soc. 21, 2055–2071. doi:10.1590/S0103-50532010001100006

Nogueira, C.W., Rocha, J.B.T., 2011. Toxicology and pharmacology of selenium: emphasis on synthetic organoselenium compounds. Arch. Toxicol. 85, 1313–59. doi:10.1007/s00204-011-0720-3

Nourjah, P., Ahmad, S.R., Karwoski, C., Willy, M., 2006. Estimates of acetaminophen (Paracetomal)-associated overdoses in the United States. Pharmacoepidemiol. Drug Saf. 15, 398–405.

Olaleye, M., Rocha, B., 2008. Acetaminophen-induced liver damage in mice: effects of some medicinal plants on the oxidative defense system. Exp. Toxicol. Pathol. 59, 319–27. doi:10.1016/j.etp.2007.10.003

Palomer, X., Alverez-Guaria, D., Rodríguez-Calvo, R., Coll, T., Laguna, J.C., Davidson, M.M., Chan, T.O., Feldman, A.M., Vázquez-Carrera, M., 2009. TNF- a reduces PGC-1 a expression through NF- k B and p38 MAPK leading to increased glucose oxidation in a human cardiac cell model. Cardiovasc. Res. 81, 703–712. doi:10.1093/cvr/cvn327

Panatto, J.P., Jeremias, I.C., Ferreira, G.K., Ramos, A.C., Rochi, N., Gonçalves, C.L., Daufenbach, J.F., Jeremias, G.C., Carvalho-Silva, M., Rezin, G.T., Scaini, G., Streck, E.L., 2011. Inhibition of mitochondrial respiratory chain in the brain of rats after hepatic failure induced by acetaminophen. Mol. Cell. Biochem. 350, 149–54. doi:10.1007/s11010-010-0689-x

Papp, L.V., Lu, J., Holmgren, A., Khanna, K.K., 2007. From selenium to selenoproteins: synthesis, identity, and their role in human health. Antioxid. Redox Signal. 9, 775–806. doi:10.1089/ars.2007.1528

Polimeno, L., Capuano, F., Marangi, L.C., Margiotta, M., Lisowsky, T., Ierardi, E., Francavilla, R., Francavilla, A., 2000. The augmenter of liver regeneration induces mitochondrial gene expression in rat liver and enhances oxidative phosphorylation capacity of liver mitochondria. Dig. Liver Dis. 32, 510–517. doi:10.1016/S1590-8658(00)80009-2

Prigol, M., Brüning, C.A., Martini, F., Nogueira, C.W., 2012. Comparative excretion and tissue distribution of selenium in mice and rats following treatment with diphenyl diselenide. Biol. Trace Elem. Res. 150, 272–7. doi:10.1007/s12011-012-9464-z

Puntel, G.O., Carvalho, N.R., Amaral, G.P., Lobato, L.D., Silveira, S.O., Daubermann, M.F., Barbosa, N. V, Rocha, J.B.T., Soares, F. a a, 2011. Therapeutic cold: An effective kind to modulate the oxidative damage resulting of a skeletal muscle contusion. Free Radic. Res. 45, 125–38. doi:10.3109/10715762.2010.517252

Puntel, G.O., Carvalho, N.R., Dobrachinski, F., Salgueiro, A.C.F., Puntel, R.L., Folmer, V., Barbosa, N.B. V, Royes, L.F.F., Rocha, J.B.T., Soares, F. a a, 2013. Cryotherapy reduces skeletal muscle damage after ischemia/reperfusion in rats. J. Anat. 222, 223–30. doi:10.1111/joa.12009

Puntel, G.O., de Carvalho, N.R., Gubert, P., Palma, A.S., Dalla Corte, C.L., Avila, D.S., Pereira, M.E., Carratu, V.S., Bresolin, L., da Rocha, J.B.T., Soares, F. a A., 2009. Butane-2,3-dionethiosemicarbazone: an oxime with antioxidant properties. Chem. Biol. Interact. 177, 153–60. doi:10.1016/j.cbi.2008.09.028

Puntel, R.L., Roos, D.H., Folmer, V., Nogueira, C.W., Galina, A., Aschner, M., Rocha, J.B.T., 2010. Mitochondrial dysfunction induced by different organochalchogens is mediated by thiol oxidation and is not dependent of the classical mitochondrial permeability transition pore opening. Toxicol. Sci. 117, 133–43. doi:10.1093/toxsci/kfq185

Raffa, R.B., 1996. A novel approach to the pharmacology of analgesics. Am. J. Med. 101, 40S–46S.

Ramachandran, A., Lebofsky, M., Weinman, S. a, Jaeschke, H., 2011. The impact of partial manganese superoxide dismutase (SOD2)-deficiency on mitochondrial oxidant stress, DNA fragmentation and liver injury during acetaminophen hepatotoxicity. Toxicol. Appl. Pharmacol. 251, 226–33. doi:10.1016/j.taap.2011.01.004

Reisman, S.A., Aleksunes, L.M., Klaassen, C.D., 2009. Oleanolic acid activates Nrf2 and protects from acetaminophen hepatotoxicity via Nrf2-dependent and Nrf2-independent processes. Biochem. Pharmacol. 77, 1273–1282. doi:10.1016/j.bcp.2008.12.028

Ribas, V., García-Ruiz, C., Fernández-Checa, J.C., 2014. Glutathione and mitochondria. Front. Pharmacol. 5, 151. doi:10.3389/fphar.2014.00151

Rocha, J.B.T., Gabriel, D., Zeni, G., Posser, T., Siqueira, L., Nogueira, C.W., Folmer, V., 2005. Ebselen and diphenyl diselenide change biochemical hepatic responses to overdosage with paracetamol. Environ. Toxicol. Pharmacol. 19, 255–61. doi:10.1016/j.etap.2004.07.006

Rosa, R.M., Hoch, N.C., Furtado, G.V., Saffi, J., Henriques, J.A.P., 2007. DNA damage in tissues and organs of mice treated with diphenyl diselenide. Mutat. Res. 633, 35–45. doi:10.1016/j.mrgentox.2007.05.006

Rupil, L.L., de Bem, A.F., Roth, G.A., 2012. Diphenyl diselenide-modulation of macrophage activation: Down-regulation of classical and alternative activation markers. Innate Immun. doi:10.1177/1753425911431285

Salminen, W., Voellmy, R., Roberts, S., 1996. Induction of hsp 70 in HepG2 Cells in Response to Hepatotoxicants. Toxicol. Appl. Pharmacol. 141, 117–123. doi:10.1006/taap.1996.0267

Sausen de Freitas, A., de Souza Prestes, A., Wagner, C., Haigert Sudati, J., Alves, D., Oliveira Porciúncula, L., Kade, I.J., Teixeira Rocha, J.B., 2010. Reduction of Diphenyl Diselenide and Analogs by Mammalian Thioredoxin Reductase Is Independent of Their Gluthathione Peroxidase-Like Activity: A Possible Novel Pathway for Their Antioxidant Activity. Molecules 15, 7699–7714. doi:10.3390/molecules15117699

Scarpulla, R.C., 2008. Transcriptional paradigms in mammalian mitochondrial biogenesis and function. Physiol. Rev. 88, 611–38. doi:10.1152/physrev.00025.2007

Schmidt, L.E., 2005. Age and paracetamol self-poisoning. Gut 54, 686–90. doi:10.1136/gut.2004.054619

Schuh, D.C., 2007. Intoxicações e exposições por paracetamol: Análise de seis anos de registros no centro de informações toxicológicas de Santa Catarina - CIT/SC.

Sebben, V.C., Lugoch, R. de W., Schlinker, C.S., Arbo, M.D., Vianna, R.L., 2010. Validação de metodologia analítica e estudo de estabilidade para quantificação sérica de paracetamol. J. Bras. Patol. e Med. Lab. 46, 143–148. doi:10.1590/S1676-24442010000200012

Senf, S.M., Dodd, S.L., McClung, J.M., Judge, A.R., 2008. Hsp70 overexpression inhibits NF-kappaB and Foxo3a transcriptional activities and prevents skeletal muscle atrophy. FASEB J. 22, 3836–3845. doi:10.1096/fj.08-110163

Stuart, R.A., Cyr, D.M., Neupert, W., 1994. Hsp70 in mitochondrial biogenesis: From chaperoning nascent polypeptide chains to facilitation of protein degradation. Experientia 50, 1002–1011. doi:10.1007/BF01923454

Tait, S.W.G., Green, D.R., 2012. Mitochondria and cell signalling. J. Cell Sci. 125, 807–15. doi:10.1242/jcs.099234

Tarlá, M.R., Ramalho, F.S., Ramalho, L.N.Z., Silva, T.C., Brandão, D.F., Ferreira, J., Silva, O.C., Zucoloto, S., 2006. A molecular view of liver regeneration 1 Uma visão molecular da regeneração hepática. Acta Cirúrgica Bras. 21, 58–62.

Terneus, M. V, Brown, J.M., Carpenter, a B., Valentovic, M. a, 2008. Comparison of S-adenosyl-l-methionine (SAMe) and N-acetylcysteine (NAC) protective effects on hepatic damage when administered after acetaminophen overdose. Toxicology 244, 25–34. doi:10.1016/j.tox.2007.10.027

Tiegs, G., Küsters, S., Künstle, G., Hentze, H., Kiemer, A.K., Wendel, A., 1998. Ebselen protects mice against T cell-dependent, TNF-mediated apoptotic liver injury. J. Pharmacol. Exp. Ther. 287, 1098–1104.

Tirosh, O., Levy, E., Reifen, R., 2007. High selenium diet protects against TNBS-induced acute inflammation, mitochondrial dysfunction, and secondary necrosis in rat colon. Nutrition 23, 878–86. doi:10.1016/j.nut.2007.08.019

Tolson, J.K., Dix, D.J., Voellmy, R.W., Roberts, S.M., 2006. Increased hepatotoxicity of acetaminophen in Hsp70i knockout mice. Toxicol. Appl. Pharmacol. 210, 157–62. doi:10.1016/j.taap.2005.10.001

Townsend, E., Hawton, K., Harriss, L., Bale, E., Bond, A., 2001. Substances used in deliberate self-poisoning 1985-1997: trends and associations with age, gender, repetition and suicide intent. Soc. Psychiatry Psychiatr. Epidemiol. 36, 228–34.

Valerio, A., Cardile, A., Cozzi, V., Bracale, R., Tedesco, L., Pisconti, A., Palomba, L., Cantoni, O., Clementi, E., Moncada, S., Carruba, M.O., Nisoli, E., 2006. TNF-?? downregulates eNOS expression and mitochondrial biogenesis in fat and muscle of obese rodents. J. Clin. Invest. 116, 2791–2798. doi:10.1172/JCI28570

Van Molle, W., Wielockx, B., Mahieu, T., Takada, M., Taniguchi, T., Sekikawa, K., Libert, C., 2002. HSP70 protects against TNF-induced lethal inflammatory shock. Immunity 16, 685–695. doi:10.1016/S1074-7613(02)00310-2

Vendemiale, G., Grattagliano, I., Altmare, E., Turturro, N., Guerrieri, F., 1996. Effect of Acetaminophen Administration on Hepatic Glutathione compartmentation and mitochondrial energy metabolism in the rat. Biochem. Pharmacol. 52, 1147–1154.

Walubo, a, Barr, S., Abraham, a M., Coetsee, C., 2004. The role of cytochrome-P450 inhibitors in the prevention of hepatotoxicity after paracetamol overdose in rats. Hum. Exp. Toxicol. 23, 49–54.

Wilhelm, E. a, Jesse, C.R., Leite, M.R., Nogueira, C.W., 2009. Studies on preventive effects of diphenyl diselenide on acetaminophen-induced hepatotoxicity in rats. Pathophysiology 16, 31–7. doi:10.1016/j.pathophys.2008.12.002

Williams, C.D., Bajt, M.L., Sharpe, M.R., McGill, M.R., Farhood, A., Jaeschke, H., 2014. Neutrophil activation during acetaminophen hepatotoxicity and repair in mice and humans. Toxicol. Appl. Pharmacol. 275, 122–33. doi:10.1016/j.taap.2014.01.004

Williams, C.D., Farhood, A., Jaeschke, H., 2010. Role of caspase-1 and interleukin-1beta in acetaminophen-induced hepatic inflammation and liver injury. Toxicol. Appl. Pharmacol. 247, 169–78. doi:10.1016/j.taap.2010.07.004

Woodhead, J.L., Howell, B.A., Yang, Y., Harrill, A.H., Clewell, H.J., Andersen, M.E., Siler, S.Q., Watkins, P.B., 2012. An analysis of N-acetylcysteine treatment for acetaminophen overdose using a systems model of drug-induced liver injury. J. Pharmacol. Exp. Ther. 342, 529–40. doi:10.1124/jpet.112.192930

Yaglom, J.A., Gabai, V.L., Sherman, M.Y., 2007. High levels of heat shock protein Hsp72 in cancer cells suppress default senescence pathways. Cancer Res. 67, 2373–81. doi:10.1158/0008-5472.CAN-06-3796

Yang, C., Zhang, X., Fan, H., Liu, Y., 2009. Curcumin upregulates transcription factor Nrf2, HO-1 expression and protects rat brains against focal ischemia. Brain Res. 1282, 133–41. doi:10.1016/j.brainres.2009.05.009

Yousuf, S., Atif, F., Ahmad, M., Nasrul Hoda, M., Badruzzaman Khan, M., Ishrat, T., Islam, F., 2007. Selenium plays a modulatory role against cerebral ischemia-induced neuronal damage in rat hippcampus. Brain Res. 1147, 218–225.

Zemolin, A.P.P., Meinerz, D.F., de Paula, M.T., Mariano, D.O.C., Rocha, J.B.T., Pereira, A.B., Posser, T., Franco, J.L., 2012. Evidences for a role of glutathione peroxidase 4 (GPx4) in methylmercury induced neurotoxicity in vivo. Toxicology 302, 60–67.

Zhang, H., Limphong, P., Pieper, J., Liu, Q., Rodesch, C.K., Christians, E., Benjamin, I.J., 2012. Glutathione-dependent reductive stress triggers mitochondrial oxidation and cytotoxicity. FASEB J. 26, 1442–51. doi:10.1096/fj.11-199869

Zhang, Q., Chen, L., Guo, K., Zheng, L., Liu, B., Yu, W., Guo, C., Liu, Z., Chen, Y., Tang, Z., 2013. Effects of different selenium levels on gene expression of a subset of selenoproteins and antioxidative capacity in mice. Biol. Trace Elem. Res. 154, 255–61. doi:10.1007/s12011-013-9710-z

Zhang, Y., Ahn, Y.-H., Benjamin, I.J., Honda, T., Hicks, R.J., Calabrese, V., Cole, P.A., Dinkova-Kostova, A.T., 2011. HSF1-dependent upregulation of Hsp70 by sulfhydryl-reactive inducers of the KEAP1/NRF2/ARE pathway. Chem. Biol. 18, 1355–61. doi:10.1016/j.chembiol.2011.09.008

Zhao, R., Holmgren, A., 2002. A novel antioxidant mechanism of ebselen involving ebselen diselenide, a substrate of mammalian thioredoxin and thioredoxin reductase. J. Biol. Chem. 277, 39456–62. doi:10.1074/jbc.M206452200

Zhao, R., Masayasu, H., Holmgren, A., 2002. Ebselen: a substrate for human thioredoxin reductase strongly stimulating its hydroperoxide reductase activity and a superfast thioredoxin oxidant. Proc. Natl. Acad. Sci. U. S. A. 99, 8579–84. doi:10.1073/pnas.122061399