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

AVALIAÇÃO DOS MECANISMOS DE TOXICIDADE DO DISSELENETO DE DIFENILA $(PhSe)_2$ E CLORETO DE FENILSELÊNIO ZINCO (PhSeZnCl) EM *Saccharomyces cerevisiae*

DISSERTAÇÃO DE MESTRADO

Letícia Selinger Galant

Santa Maria, RS, Brasil

2015

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Letícia Selinger Galant

Dissertação apresentada ao Curso de Mestrado do Programa de Pós-Graduação em Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM) como requisito parcial para a obtenção do grau de **Mestre em Bioquímica Toxicológica.**

Orientador: Prof. Dr. João Batista Teixeira da Rocha

Santa Maria, RS, Brasil

2015

Universidade Federal de Santa Maria
Centro de Ciências Naturais e Exatas
Programa de Pós-Graduação em Bioquímica Toxicológica

A Comissão Examinadora, abaixo assinada, aprova a Dissertação de Mestrado

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elaborada por
Letícia Selinger Galant

como requisito parcial para a obtenção do grau de **Mestre em Bioquímica Toxicológica**

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Santa Maria, 21 de agosto de 2015.

AGRADECIMENTOS

Aos meus pais Vânio e Dalva, por todo ensinamento de valores e comprometimento com os objetivos, além de todo amor, compreensão e carinho, vocês são a minha inspiração.

Aos meus irmãos Anderson e Kênia pelo apoio e parceria nas horas mais difíceis de nossas vidas, sempre estivemos juntos.

Ao meu namorado Gustavo, pelo amor e paciência.

A minha família “adotiva” de Santa Maria que me acolheu com todo carinho, meus colegas de laboratório: João Matheus, Aline, Aninha, Andréia, Marcos, Diego, Diones, Emili, Lilian, Pablo, Letícia, Claudia, Claudinha, Bruna e Jean Paul, muito obrigada pelos ensinamentos, paciência e momentos de descontração.

Ao meu orientador Prof. João Batista Teixeira da Rocha, pelos ensinamentos profissionais e oportunidade da realização deste trabalho em seu laboratório.

Aos professores do Programa de Pós-Graduação em Bioquímica Toxicológica, que contribuíram para a minha formação.

Agradeço a Universidade Federal de Santa Maria e ao Programa de Pós-Graduação em Bioquímica Toxicológica pela possibilidade de realização deste mestrado.

RESUMO

Dissertação de Mestrado
Programa de Pós-Graduação em Bioquímica Toxicológica
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AVALIAÇÃO DOS MECANISMOS DE TOXICIDADE DO DISSELENETO DE DIFENILA $(PhSe)_2$ E CLORETO DE FENILSELÊNIO ZINCO (PhSeZnCl) EM *Saccharomyces cerevisiae*

Autor: Letícia Selinger Galant
Orientador: Prof. Dr. João Batista Teixeira da Rocha
Data e Local da Apresentação: Santa Maria, 21 de agosto de 2015.

O selênio (Se) é um microelemento encontrado em diversos tecidos animais na forma de selenoproteínas, como por exemplo, a glutationa peroxidase (GPx). Compostos orgânicos de selênio, como o disseleneto de difenila $(PhSe)_2$ e o cloreto de fenilselênio zinco (PhSeZnCl) tem apresentado atividade mimética a GPx na degradação de peróxidos de hidrogênio. Estes efeitos protetores são dependentes da dose estudada, sendo que altas concentrações destes compostos podem proporcionar a oxidação de grupos tiol de proteínas e consequentemente aumentar a produção de espécies reativas de oxigênio (EROs). Porém, os estudos dos mecanismos celulares na toxicidade destes compostos não foram completamente compreendidos. Desta forma, este estudo objetivou investigar os mecanismos de toxicidade do $(PhSe)_2$ e PhSeZnCl, a partir da produção de EROs e alterações morfológicas na levedura *Saccharomyces cerevisiae*. As amostras foram incubadas por 1, 2, 3, 4, 6 e 16 horas com $(PhSe)_2$, nas concentrações de 2, 4, 6 e 10 µM. O PhSeZnCl apenas foi incubado por 16 horas nas concentrações de 4, 8, 12 e 20 µM. O crescimento celular foi analisado por espectrofotometria. Através da citometria de fluxo foi analisado a produção de EROs pela fluorescência da diclorofluoresceína diacetato (DCFH-DA), a permeabilidade da membrana por iodeto de propídeo (PI), o tamanho e a granulosidade celular. O conteúdo total de tióis foi analisado pela reação colorimétrica com DTNB. O $(PhSe)_2$ foi capaz de inibir o crescimento celular a partir de 2 h de incubação em 10 µM seguido por um aumento na permeabilidade da membrana. O aumento do tamanho e granulosidade celular foram observados em 3 h de incubação. Porém a produção de EROs foi observada apenas em 16 h de incubação na concentração de 10 µM. O conteúdo total de tióis aumentou apenas em 6 µM de $(PhSe)_2$ em 16 h de incubação. Quando as leveduras foram tratadas com PhSeZnCl, este composto apenas apresentou toxicidade na concentração de 20 µM em todos os parâmetros testados. Concluímos que a toxicidade do $(PhSe)_2$ não esta diretamente relacionada a produção de EROs. O PhSeZnCl aparentemente é menos tóxico que o $(PhSe)_2$.

Palavras-chave: Selênio, disseleneto de difenila, cloreto de fenilselênio zinco, toxicidade, *Saccharomyces cerevisiae*.

ABSTRACT

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

EVALUATION OF TOXICITY MECHANISMS DIPHENYL DISELENIDE $(PhSe)_2$ AND CHLORIDE FENILSELENIUM ZINC (PhSeZnCl) IN *Saccharomyces cerevisiae*

Author: Letícia Selinger Galant
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Date and Place of Presentation: Santa Maria, August 21, 2015.

Selenium (Se) is a microelement present in different animal tissues in the form of selenoproteins, for example, the glutathione peroxidase (GPx). Organic selenium compounds, such as diphenyl diselenide $(PhSe)_2$ and fenilselenium zinc chloride (PhSeZnCl) have presented GPx mimetic activity in the degradation of hydrogen peroxide. However the protective effects are dependent on the dose evaluated. High concentrations of these compounds can provide oxidation in protein thiol groups and leading to increase of reactive oxygen species (ROS) production. However, studies of cellular mechanisms in the toxicity of these compounds were not completely understood. Thus, this study aimed to investigate the mechanisms of toxicity $(PhSe)_2$ and PhSeZnCl, through the ROS production and morphological alterations in *Saccharomyces cerevisiae*. The samples were incubated for 1, 2, 3, 4, 6 and 16 hours with $(PhSe)_2$ in concentrations of 2, 4, 6 and 10 µM. PhSeZnCl was only incubated at 16 hours in concentrations of 4, 8, 12 and 20 µM. Cell growth was analyzed by spectrophotometry. Through flow cytometry was analyzed ROS production by fluorescence of dichlorofluorescein diacetate (DCFH-DA), cell membrane permeability by propidium iodide (PI), cells size and granularity. Total thiol groups were analyzed by the colorimetric reaction with DTNB. $(PhSe)_2$ was able to inhibit cell growth after 2 h incubation in 10 µM followed by an increase in cell membrane permeability. The increase in cell size and granularity was observed after 3 h of incubation. However the ROS production was observed only at 16 h of incubation in 10 µM. The total of thiol groups increased in 6 µM of $(PhSe)_2$ after 16 h of incubation. When yeast cells were treated with PhSeZnCl, this compound showed toxicity only at concentrations of 20 µM in all parameters tested. We concluded that the toxicity of $(PhSe)_2$ is not directly related to production of ROS. PhSeZnCl apparently is less toxic than the $(PhSe)_2$.

Key-words: Selenium, diphenyl diselenide, fenilselênium zinc chloride, toxicity, *Saccharomyces cerevisiae*.

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INTRODUÇÃO

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APRESENTAÇÃO

No item **INTRODUÇÃO** está descrito uma revisão sucinta sobre os temas trabalhados nesta dissertação. No final deste item estão apresentados a justificativa do trabalho e os objetivos geral e específicos.

Os **RESULTADOS e DISCUSSÃO** estão dispostos na forma de manuscrito submetido à publicação. As seções Introdução, Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas encontram-se no manuscrito e representam a integra deste estudo.

No item **CONCLUSÕES** são apresentadas as conclusões gerais do presente trabalho.

As **REFERÊNCIAS BIBLIOGRÁFICAS** apresentadas no final da tese referem-se somente as citações que aparecem ao item **INTRODUÇÃO**.

1. INTRODUÇÃO

1.1 Toxicologia

1.1.1 Histórico

A história da toxicologia é acompanhada da história da própria civilização, desde a pré-história o homem já conhecia os efeitos tóxicos dos venenos de determinados animais e plantas. Logo o homem começou a utilizar estas substâncias venenosas como armas de caça. Com o progresso da civilização e consequentemente as descobertas toxicológicas, deu-se inicio a busca por antídotos. Na antiguidade, por exemplo, os experimentos com substâncias tóxicas e seus prováveis antídotos eram realizados em escravos.

A toxicologia foi evoluindo de forma muito lenta e até mesmo no século XVIII seus conhecimentos eram evidenciados empiricamente (OGA, 2003). Ao longo dos anos, inúmeros cientistas contribuíram com métodos científicos e sistemáticos para o entendimento da toxicologia mecanística. O século XX é marcado pelo avanço tecnológico em síntese química de fármacos, conservantes, corantes, praguicidas e herbicidas. Consequentemente, o homem tornou-se mais exposto a agentes químicos, ocasionando um aumento nos casos de intoxicação. Atualmente a toxicologia tem ganhado ênfase em estudos de carcinogenicidade, genotoxicidade, teratogenicidade, bem como estudos que buscam compreender o comportamento de determinada substância química no organismo (FAVERO, et al., 2005; SAVEGNAGO, et al., 2006; LIPPMAN, et al., 2009; BUENO, et al., 2013;).

Atualmente, um grande número de pesquisadores tem direcionado suas pesquisas aos compostos orgânicos de selênio, a fim de compreender os efeitos destes compostos no organismo (NOGUEIRA, et al., 2004; ROSA, et al., 2005; NOGUEIRA; ROCHA, 2010; BUENO, et al., 2013; BARTOLINI, et al., 2015a).

1.2 Selênio

1.2.1 Histórico

O elemento químico selênio foi descoberto pelo químico sueco Jons Jacob Berzelius em 1817. Enquanto preparava ácido sulfúrico, Berzelius notou a precipitação de um novo resíduo o qual ele denominou *selenè* (palavra grega para a lua) (BOYD, 2011). Desde então ocorreu um grande interesse pelas propriedades químicas do selênio (Se), descobrindo-se que este elemento compartilha muitas propriedades químicas e físicas com o elemento enxofre. O selênio encontra-se localizado no grupo 16 da tabela periódica, podendo apresentar quatro formas de oxidação o Se^{0}, o Se^{+6}, o Se^{+4} e o Se^{-2}.

Em 1847 foi descrito a síntese do primeiro composto contendo selênio, o etilselenol (STADTMAN, 1974). Desde então, inúmeros compostos contendo selênio foram sintetizados. Na década de 30 foram identificadas propriedades tóxicas do selênio em animais que alimentavam-se de plantas que continham grande quantidade de Se acumulada (OLDFIELD,1987).

Mais de cem anos se passaram entre a descoberta do selênio e o seu reconhecimento como essencial para função celular na maioria dos mamíferos. Em 1957 o alemão Klaus Schawarz identificou o selênio como um elemento essencial para a dieta de ratos, protegendo a degeneração necrótica do fígado (SCHWARTZ; FOLTZ, 1957).

A primeira constatação de que o selênio encontra-se em proteínas, como o aminoácido selenocisteína, foi em 1978 por Frostrom e colaboradores. Neste estudo, a glutationa peroxidase (GPx) hepática foi isolada, mostrando a natureza química do selênio. Posteriormente, foi estabelecido que todas as selenoproteínas de células animais contém pelo menos um resíduo de selenocisteína na sua estrutura (LU; HOLMGREN, 2009; NOGUEIRA; ROCHA, 2010).

1.2.2 Disponibilidade de selênio no ambiente

Embora na natureza o selênio seja considerado um elemento raro, na crosta terrestre a maior parte deste elemento está associado a sulfetos. A presença ou ausência de selênio no solo é dependente da decomposição de material orgânico, lixiviação e processos associados à formação do solo (SHAMBERGER, 1981).

O selênio é emitido na atmosfera na forma volátil de dimetilseleneto, dióxido de selênio ou selênio elementar durante a queima de combustíveis fósseis (ANDREN, et al.,1975). Em amostras de águas pluviais, o selenito e o selenato são

as formas de selênio mais encontradas (CUTTER; CHURCH, 1986). Na indústria, o selênio pode ser isolado a partir da lama que forma no ânodo durante a eletrolítica refinação do cobre. No entanto o maior uso do selênio reside na fabricação de vidro, em que ele é usado como dopante para produzir tons de vermelho. Ainda, o Se é utilizado na indústria elétrica e de borracha (BOYD, 2011).

1.2.3 Selênio nos organismos vivos

Com o avanço do interesse da pesquisa sobre o selênio observou-se os seus efeitos benéficos, sendo este um elemento essencial para o funcionamento adequado do organismo.

A Junta de Alimentação e Nutrição da Academia de Ciências dos Estados Unidos propôs a ingestão diária de 50 a 200 µg de selênio para adultos, sendo este valor livre de efeitos tóxicos (FOOD AND NUTRITION BOARD INSTITUTE OF MEDICINE, 2000). Em muitos países, carnes, frutos do mar, arroz, macarrão e pão são comuns fontes de selênio na dieta, no Brasil a semente de *Bertholletia excelsa* H.B.K., popularmente conhecida como Castanha do Pará, é uma das fontes mais ricas em selênio (BOYD, 2011).

Nos tecidos animais o selênio está presente em duas formas, selenocisteína (SeCys) e selenometionina (SeM). A SeM é obtida através do consumo de vegetais. Por sua vez, a SeCys, um análogo da cisteína, pode ser sintetizada no organismo ou ingerida através do consumo de produtos de origem animal. A SeCys é a forma biologicamente ativa do elemento (HATFIELD et al., 2014; PILLAI et al., 2014).

O resíduo de SeCys compõe selenoproteínas com atividades biológicas importantes como a glutationa peroxidase (GPx), a tioredoxina redutase e a iodotironina deionidase. Atualmente, sabe-se que os humanos possuem pelo menos 25 selenoproteínas (URSINI et al., 1982; MAY et al., 1998; LU; HOLMGREN, 2009; HATFIELD et al., 2014; LABUNSKYY et al., 2014; PILLAI et al., 2014; GLADYSHEV, 2011).

Porém, mesmo o Se sendo essencial para o bom funcionamento do organismo, o risco de contaminação ocupacional com este elemento motiva os estudos toxicológicos.

1.2.4 Toxicidade de Selênio

As dietas contendo selênio devem ser monitoradas, pois uma alta ou baixa ingestão de formas de selênio pode gerar graves patologias no organismo.

A deficiência de selênio pode causar doenças cardíacas como, por exemplo, a doença de Keshan, doenças imunes, diabetes, e câncer em diversos tecidos (SALONEN et al., 1982; NAVARRO-ALARCÓN et al., 1998; NAVARRO-ALARCÓN; LÓPEZ-MARTÍNEZ, 2000). Por outro lado, o excesso de selênio pode levar a *selenose*. Essa doença foi registrada pela primeira vez em 1961 em partes da população de Enshi County, província de Hubei, China. Os sintomas mais comuns foram perda de unhas e cabelos, perda da sensação de dor na pele, dormência dos membros, tontura e vertigem (ZHENG et al., 1999; FINKELMAN et al., 2007).

Porém a concentração de selênio a ser consumida é um fator crítico na atividade biológica do elemento, sendo que a concentração benéfica é muito próxima da concentração que é considerada tóxica (NOGUEIRA; ROCHA, 2010). Alguns estudos demostraram que a toxicidade de compostos que contém selênio esta na sua capacidade de catalisar a oxidação de grupos tióis em proteínas, gerando espécies reativas de oxigênio e diminuindo as defesas antioxidantes (BARBOSA et al., 1998; NOGUEIRA et al., 2003; NOGUEIRA et al., 2004; ROSA et al., 2005; PUNTEL et al., 2013).

1.2.5 Compostos orgânicos de Selênio:

1.2.5.1 Disseleneto de Difenila $(PhSe)_2$

Compostos orgânicos de selênio, chamados organoselênios, ganharam grande interesse a partir dos anos 80, quando estudos revelaram que estes poderiam possuir atividade antioxidante semelhante com a selenoenzima GPx (MULLER et al., 1984).

O disseleneto de difenila $(PhSe)_2$ é um organocalcogênio que contém Se ligado a um anel aromático (Figura 1). Muitos estudos mostram efeitos benéficos do $(PhSe)_2$, em mamíferos, apresentando atividade neuroprotetora (FREITAS; ROCHA, 2011), antiúlcera (SAVEGNAGO et al., 2006), e hepatoprotetora (BORGES et al., 2005), atuando também na decomposição de peróxidos na presença de tióis e redução da peroxidação lipídica (FARINA et al., 2002; SANTOS et al., 2005).

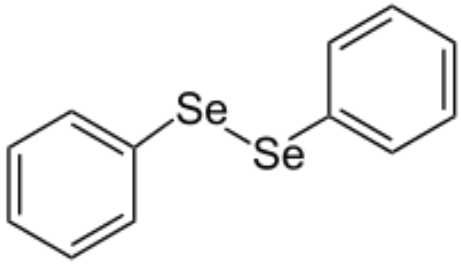


Figura 1: Estrutura química do disseleneto de difenila $(PhSe)_2$.

Estes efeitos protetores do $(PhSe)_2$ estão associados a ação antioxidante e mimética da GPx, ou seja a capacidade de degradar o H_2O_2 (NOGUEIRA et al., 2004). A interação de grupos tiol como, por exemplo, a glutationa (GSH) com disselenetos pode gerar grupos selenol-selenolato intermediários. Estes grupos podem decompor H_2O_2 e peróxidos orgânicos (Figura 2). Porém, os efeitos protetores do $(PhSe)_2$ são dependentes da dose estudada, sendo que altas concentrações podem causar toxicidade através da indução do estresse oxidativo (NOGUEIRA; ROCHA, 2010).

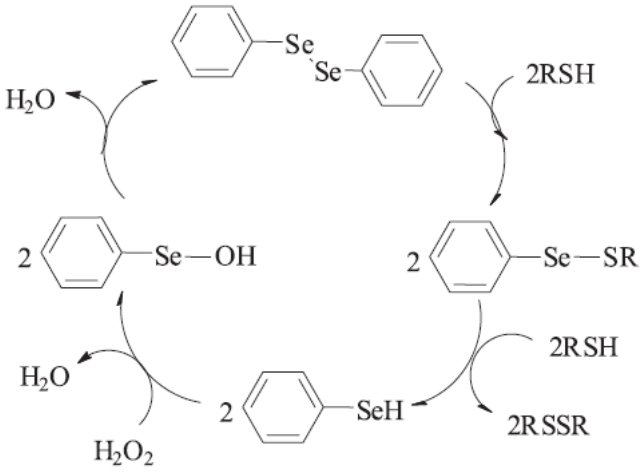
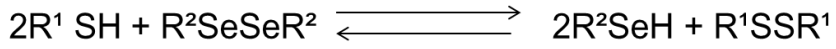


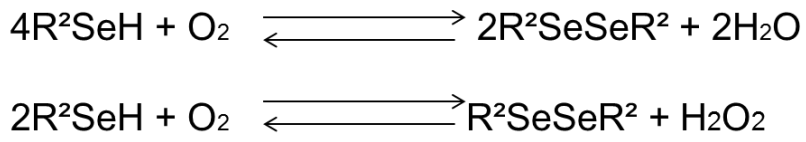
Figura 2: Ciclo catalítico do $(PhSe)_2$ na degradação de H_2O_2, ação mimética a GPx (NOGUEIRA; ROCHA, 2010).

Dados da literatura, relatam que o $(PhSe)_2$ pode agir como um pro-oxidante, diminuindo a GSH livre, sensibilizando mutantes de *S. cerevisiae* com defesas antioxidantes como a superóxido dismutase (SOD) e a GPx além de provocar genotoxicidade em doses altas (ROSA et al., 2004; ROSA et. al., 2005; BUENO et al., 2013).

O efeito tóxico do $(PhSe)_2$ pode estar associado ao fato deste composto formar selenol e este ser altamente reativo com grupamentos dissulfeto de proteínas celulares, podendo causar alterações conformacionais nestas, e consequentemente gerar espécies reativas de oxigênio, as quais podem ser danosas para proteínas, lipídeos e ácidos nucleicos. (NOGUEIRA et al., 2004; NOGUEIRA; ROCHA, 2010). O $(PhSe)_2$ ainda interage com grupamentos tióis mesmo na ausência de peróxidos. Nogueira e Rocha (2010), demostraram a estequiometria desta reação, como representado no Esquema 1. Contudo, a formação de quatro moléculas de selenol pode reduzir oxigênio à água. Porém dois equivalentes de selenol podem formar a molécula de H_2O_2 (Esquema 2).



Esquema 1



Esquema 2

1.2.5.2 Cloreto de Fenilselênio Zinco (PhSeZnCl)

O composto nomeado recentemente por cloreto de fenilselênio zinco (PhSeZnCl) (Figura 4), tem despertado curiosidade em estudos bioquímicos (SANTI et al., 2008) pelo fato de conter selênio em sua estrutura e apresentar ação semelhante a selenoproteína GPx na degradação de hidroperóxidos e peróxidos orgânicos.

Este composto possui uma molécula de zinco que pode desempenhar um papel ativo no ciclo catalítico do H_2O_2 (Figura 5) (TIDEI et al., 2012). A reação de redução de peróxidos forma o intermediário ácido selênico (PhSeOH), que reage com a molécula de GSH formando o sulfeto selenil (PhSeSG). Uma segunda molécula de GSH reage com a ligação Se-S regenerando um selenol ativo e a produção da glutationa oxidada (GSSG).

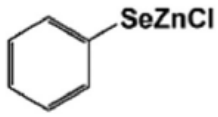


Figura 3: Estrutura química do PhSeZnCl

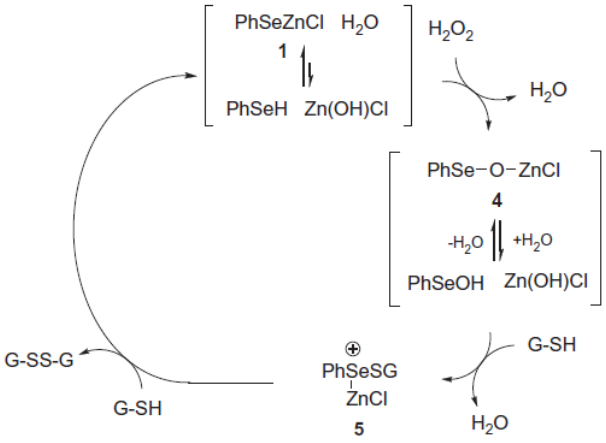


Figura 4: Ciclo catalítico de PhSeZnCl proposto por TIDEI et al., 2012.

Estudos recentes tem mostrado que este composto possui alta eficiência catalítica tendo como substratos a GSH e o H_2O_2. Porém também obteve uma grande reatividade com grupos tiol, podendo apresentar citotoxicidade em culturas de células cancerígenas, via aumento da produção de espécies reativas de oxigênio, depleção de grupamentos tiol e indução de apoptose (BARTOLINI et al., 2015).

1.3 Espécies Reativas de Oxigênio (EROs) e Estresse Oxidativo

Substâncias como o peróxido de hidrogênio (H_2O_2) e radicais livres como o ânion superóxido (O_2^-), o radical hidroxila (OH^-), oxido nítrico (NO) e peroxinitrito ($ONOO^-$) são exemplos de espécies reativas de oxigênio, pois estas substâncias são altamente reativas com outras biomoléculas (DROGE, 2002).

As EROs podem ser produzidas nos organismos, por fontes exógenas como a radiação, os agrotóxicos, os medicamentos, a poluição ambiental, ou por fontes endógenas como a degradação de ácidos graxos e a oxidação de moléculas entre outros (HALLIWELL; GUTTERIDGE, 1999). Ao longo da cadeia transportadora de elétrons, as EROs podem ser geradas por erros no processo de redução univalente

do oxigênio, dentre eles o O_2^-, OH^- e o H_2O_2, as quais são altamente prejudiciais às células (AMES et al., 1993).

O H_2O_2 é considerado um oxidante fraco, porém ele pode oxidar alguns grupos tiol e aminoácidos. Além de ser reativo com íons de Fe^{+2}, podendo gerar Fe^{+3} e o OH^- o qual é um dos radicais mais danosos para as células, esta reação é conhecida como reação de Fenton ($Fe^{+2} + H_2O_2 \rightarrow Fe^{+3} + OH + OH^-$).

As EROs estão balanceadas com defesas antioxidantes encontradas no organismo. Essas defesas podem ser enzimas como a superóxido dismutase (SOD), a catalase (CAT) e a GPx, ou não enzimáticas, por exemplo, a GSH, vitamica C, vitamina E, entre outras. Quando estas enzimas estão em menor quantidade nos organismos algumas biomoléculas podem sofrer danos irreparáveis (VALKO et al., 2006).

As membranas celulares contém grande quantidade de ácidos graxos insaturados que podem ser alvos de EROs, formando hidro ou lipoperóxidos que são altamente reativos e consequentemente levam a uma cascata oxidativa nas células, podendo ocasionar a perda de algumas características, criando fendas iônicas nas membranas, alterando a sua permeabilidade e consequentemente levando ao rompimento e extravasamento do conteúdo celular (HALLIWELL; GUTTERIDGE, 1999).

Um distúrbio no balanço entre pró-oxidantes e antioxidantes em favor do primeiro gera o estresse oxidativo, o que pode resultar em adaptação ou lesão celular (HALLIWELL; GUTTERIDGE, 2007).

O estresse oxidativo tem sido implicado em diversas patologias, como o câncer, diabetes mellitus, envelhecimento, enfisema pulmonar, doenças neurodegenerativas, (HALLIWELL; GUTTERIDGE, 1999; DROGE, 2002; VALKO, 2006).

Desta forma, o estresse oxidativo é uma condição celular onde as defesas antioxidantes encontradas nas células, não conseguem remover o excesso de EROs, causando danos às estruturas celulares como lipídios, proteínas e até mesmo o DNA, podendo causar morte celular (HALLIWELL; GUTTERIDGE, 2007).

1.4 Defesas Antioxidantes: Sistema Glutationa Peroxidase (GPx)

Com os efeitos danosos das EROs as células possuem enzimas específicas que atacam diretamente as EROs, formando produtos menos agressivos, as principais enzimas antioxidantes são: superóxido dismutase (SOD), catalase (CAT) e o sistema da glutationa peroxidase (GPx). A SOD converte o O_2^- em H_2O_2 e oxigênio molecular, enquanto CAT atua juntamente com a GPx não permitindo a produção OH^- a partir do peróxido de hidrogênio (MICHIELS et al., 1994).

A GPx é uma selenoenzima, o aminoácido selenocisteina esta localizado no seu sitio ativo, sendo responsável por degradar H_2O_2 evitando assim que este reaja com íons de Fe^{+2}, gerando radicais livres pela reação de Fenton podendo levar a danos nas membranas, proteínas e até mesmo ao núcleo celular (FLOHE et al., 1973; URSINI et al., 1982; HALLIWELL; GUTTERIDGE, 2007).

O sistema de degradação de peróxidos pela GPx é um sistema complexo que depende também do fluxo de glutationa (GSH) livre na célula. A reação de detoxificação de peróxidos se inicia quando o sitio ativo da GPx forma o intermediário selenol (GPx-Se^-), este reage com peróxidos reduzindo-o a álcool, formando o ácido selênico (GPx-Se-OH). O ácido selênico reage com uma molécula de GSH formando o selenosulfeto (GPx-Se-SG) e água, sendo este atacado por outra molécula de GSH regenerando a forma ativa da enzima e liberando uma molécula de glutationa oxidada (GSSG) (Figura 1) (NOGUEIRA; ROCHA, 2010).

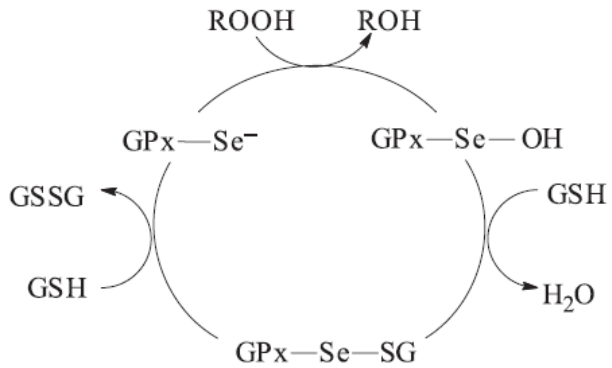


Figura 5: Ciclo catalítico da GPx (NOGUEIRA; ROCHA, 2010).

1.5 Levedura *Saccharomyces cerevisiae* como organismo modelo

Atualmente a introdução de organismos modelos como o *Danio rerio* (peixe zebra), *Caenorhabditis elegans* (verme), *Drosophila melanogaster* (mosca) e

Saccharomyces cerevisiae (levedura) tem ganhado grande interesse em estudos bioquímicos e toxicológicos (ROSA, et al., 2005; ZAMBERLAN, et al., 2014; JIANG, et al., 2015; ALARABY, et al., 2015).

A levedura *S. cerevisiae* é um eucarioto unicelular que possui sistema aeróbico facultativo, que respiram na presença de oxigênio, porém podem fazer fermentação em situações de anaerobiose. O uso de *S. cerevisiae* na pesquisa tem vantagens que a tornam um excelente organismo modelo. Dentre estas, destaca-se: o crescimento rápido, tratamentos ocorrem de forma estéril, genoma totalmente sequenciado, facilitando a modificação genética (GOFFEAU et al., 1996). Desta forma, os genes de interesse podem ser alterados, retirados ou inseridos no genoma (SHERMAN, 1991).

S. cerevisiae tem sido um organismo modelo muito utilizado para o entendimento dos mecanismos celulares, como por exemplo, a regulação do ciclo celular, possuindo grande importância na compreensão de doenças complexas como o câncer (HARTWELL, 2002; MATTIAZZI et al., 2012).

Embora o genoma de *S. cerevisiae* é aproximadamente 200 vezes menor que o genoma humano, alguns estudos demostraram que esta espécie de levedura possui similaridade com a sequência de aminoácidos de proteínas de células de humanos (GOFFEAU et al., 1996; HARTWELL, 2002). Sendo assim é importante o uso deste organismo modelo para o entendimento de diversos mecanismos moleculares que ocorrem em células humanas.

Estudos utilizando leveduras tem contribuído para elucidar o papel das EROs na toxicologia de compostos organoselênios (ROSA et al., 2004; ROSA et al., 2005; AZAD et al., 2014; ROSSETI et al., 2015).

Rosa e colaboradores (2004; 2005) mostraram que o $(PhSe)_2$ pode induzir mutação em cepas de leveduras *S. cerevisiae*, sensibilizar leveduras mutantes de GSH, bem como causar depleção de GSH intracelular possivelmente induzindo o estresse oxidativo.

Desta forma a levedura *S. cerevisiae* é um organismo modelo econômico no qual podemos explorar o mecanismo celular envolvido na toxicidade de determinados compostos.

2. JUSTIFICATIVA

Devido ao grande interesse pelos efeitos farmacológicos de compostos que contem selênio, se faz necessário o entendimento dos mecanismos envolvidos na toxicidade dos mesmos. Os mecanismos celulares como alteração da integridade da membrana, morfologia e produção de EROs envolvidos na toxicidade destes compostos ainda não foram totalmente elucidados. Desta forma, estas informações podem ajudar na formulação de compostos de selênio com ação menos tóxicas na oxidação de biomoléculas.

A utilização de organismos modelos como a levedura *S. cerevisiae* pode facilitar este entendimento, visto que é um organismo de fácil manipulação no laboratório e seu genoma já está totalmente sequenciado sendo possível futuramente estudar os genes que estão ligados à toxicologia de compostos de selênio.

3. OBJETIVOS

3.1 Objetivo geral

Investigar a toxicidade do $(PhSe)_2$ e PhSeZnCl, bem como o envolvimento na produção de espécies reativas de oxigênio e sua relação temporal com alterações bioquímicas e morfológicas em *Saccharomyces cerevisiae*.

3.2 Objetivos específicos

- Avaliar o efeito do $(PhSe)_2$ e PhSeZnCl sobre o crescimento de *Saccharomyces cerevisae*, por espectrofotômetria.

- Avaliar a produção de espécies reativas de oxigênio em *Saccharomyces cerevisiae* expostas ao $(PhSe)_2$ e PhSeZnCl, utilizando diclorofluoresceína diacetato (DCFH-DA) como marcador de fluorescência em citometria de fluxo e microscopia de fluorescência.

- Avaliar a permeabilidade da membrana celular de *Saccharomyces cerevisiae* expostas ao $(PhSe)_2$ e PhSeZnCl, utilizando o iodeto de propídeo (PI) como marcador de fluorescência em citometria de fluxo.

- Avaliar o tamanho e granulosidade celular de *Saccharomyces cerevisiae* expostas ao $(PhSe)_2$ e PhSeZnCl, por citometria de fluxo.

- Avaliar o conteúdo total de tiol em *Saccharomyces cerevisiae* expostas ao $(PhSe)_2$ e PhSeZnCl.

4. ARTIGO CIENTÍFICO

4.1 Manuscrito

DIPHENYL DISELENIDE $(PhSe)_2$ INDUCING REACTIVE OXYGEN SPECIES IS PRECEDED BY CHANGES IN CELL MORPHOLOGY AND PERMEABILITY IN *Saccharomyces cerevisiae*

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Keywords

Diphenyl diselenide; phenylselenium zinc chloride; toxicity; ROS production; *Saccharomyces cerevisiae*.

Abstract

Organic selenium compounds such as diphenyl diselenide $(PhSe)_2$ and phenylselenium zinc chloride (PhSeZnCl) have antioxidant activity. They can imitate the catalytic cycle of glutathione peroxidase (GPx). However, at high concentrations organoselenium can cause toxicity by oxidizing thiol groups of proteins and increase the production of reactive oxygen species (ROS). Here, we analyzed the toxicity of $(PhSe)_2$ and PhSeZnCl in yeast *Saccharomyces cerevisiae*. Cell growth of *S. cerevisiae* after 1, 2, 3, 4, 6 and 16 hours of treatment with 2, 4, 6, and 10µM of $(PhSe)_2$. For comparative purpose, PhSeZnCl was analyzed only at 16 hours of incubation at equivalent concentrations of selenium (i.e., 4, 8, 12, and 20 µM). ROS production (DCFH-DA), size, granularity and cell membrane permeability (propidium iodide) were determined by flow cytometry. Total thiol was analysed by Ellman's reaction. $(PhSe)_2$ inhibited cell growth at 2 h (10 µM) of incubation, followed by increase in cell membrane permeability. The increase of cell size (10 µM) and granularity (6 and 10 µM) was observed after 3 h of incubation. However ROS production occurs only at 16 hours of incubation (10 µM) with $(PhSe)_2$ indicating that ROS overproduction is more likely a consequence of $(PhSe)_2$ toxicity and not its determinant. All these parameters tested showed that only concentration of 20 µM induced toxicity in samples incubated with PhSeZnCl. In summary, the results suggests that $(PhSe)_2$ toxicity in *S. cerevisiae* is time and concentration dependent, presenting more toxicity when compared with PhSeZnCl.

1. INTRODUCTION

The interest in the biochemistry and pharmacology of Selenium (Se) has increased considerably in the last decades (Mugesh et al. 2001; Nogueira et al. 2004; Nogueira and Rocha, 2011). Selenium is an essential dietary element and it is found as component of about 25 selenoproteins (Hatfield et al. 2014; Labunskyy, et al., 2014; Pillai, et al., 2014; Gladyshev, 2011). The majority of selenoproteins have only one selenocysteine residue as part of their biologically active site, which normally participates in redox reactions (Hatfield et al. 2014). In contrast to mammals, fungi do not have selenoproteins in their genome (Kryukov, et al., 2003; Araie and Shiraiwa, 2009; Hatfield, et al., 2014).

In view of the prominent antioxidant activity, organoseleno compounds have gained great interest in biochemical studies, which can be in part due to their versatility to perform “catalytic cycles” similar to glutathione peroxidase (GPx) (Sies, et. al., 1993; Muller, et. al. 1984; Nogueira, et al., 2004; Bartolini, et. al., 2015a; Nogueira and Rocha, 2010; Rosa, et. al., 2007a, 2007b). For instance, diphenyl diselenide $(PhSe)_2$ is an organoselenium with a very simple structure. After being reduced to benzenoselenophenol by different mechanisms (Nogueira and Rocha, 2010; 2011), it can efficiently react with hydrogen peroxide, mimicking the catalytic cycle of GPx enzyme (Nogueira, et al., 2004; Nogueira and Rocha, 2010; 2011; Santi et al., 2013). Different laboratories have shown its beneficial effects in mammalian and non-mammalian models of diseases (Nogueira and Rocha, 2010; Nogueira and Rocha, 2011; Stefanello, et. al., 2015; Fiuza, et al. 2015; Zamberlan, et. al., 2014; Mancini, et al., 2014; Dias et al., 2014; Hort, et al.,2014; Dobrachinski, et al., 2014; Silvestre, et. al., 2014; Godoi, et al., 2013; Rupil, et al., 2012; Chanaday, et al., 2011).

The protective effects of $(PhSe)_2$ are concentration- or dose-dependent, wherein exposure to high doses can cause toxicity (Nogueira and Rocha; 2010;2011). High levels of $(PhSe)_2$ can be pro-oxidant, decreasing the level of non-protein -SH groups in rodents (Maciel et al., 2000). Similarly, in *S. cerevisiae,* a few number of studies have indicated that $(PhSe)_2$ depleted GSH levels and increased the sensitivity to oxidative stress of yeasts (Rosa et al., 2004; 2005). Moreover, genotoxic action of $(PhSe)_2$ have also been reported in yeast, bacteria and white human blood cells (Rosa, et al., 2004; 2005; Bueno, et al., 2013). In *Candida*

albicans, it has been shown that $(PhSe)_2$ decreased cell growth and enhanced reactive oxygen species (ROS) production, which was associated with an increase in plasma membrane permeability (Rosseti, et al., 2015). However, data about whether cell toxicity, induced by $(PhSe)_2$, is the cause or consequence of oxidative stress are still elusive in the literature. Thus, additional studies are required to understand the mechanism(s) of toxicity of selenocompounds.

Particularly, the evaluation of role played by oxidative stress in cellular toxicity of $(PhSe)_2$ and its analog, phenylselenium zinc chloride **(**PhSeZnCl), is critical to guide the future of synthesis of new compounds with GPx-like activity and with low toxicity (Nogueira and Rocha, 2010). The investigation of PhSeZnCl toxicity is of particular importance because it can be an alternative formulation of diselenide with less pro-oxidant action against thiol groups of biomolecules (Tidei, et al., 2012; Bartolini, et al., 2015a). This compound has been reported to exhibit GPx mimetic activity with higher catalytic efficiency than its diselenide analog (Tidei et al. 2012). However, PhSeZnCl can also oxidize thiol groups and trigger the formation of ROS in human cell cultures (Bartolini, et al., 2015a; Tidei, et al., 2012; Santi et al., 2008).

Here we evaluated the mechanism(s) of toxicity of two related compounds of selenium (i.e., $(PhSe)_2$ and PhSeZnCl) in *Saccharomyces cerevisiae*. Specifically, we determined whether oxidative stress preceded or followed cellular toxicity induced by $(PhSe)_2$.

2. MATERIALS AND METHODS

2.1 Chemicals

The $(PhSe)_2$, 2′,7′-Dichlorofluorescin diacetate, Diamidino-2-phenylindole, propidium iodide, 5,5'- dithio-bis (2-nitrobenzoic acid) were obtained from Sigma - Aldrich Brazil. The PhSeZnCl compound was synthesized as described early (Santi, et al., 2008; Santi, et al., 2012). Yeast extract bacto-peptone, and bacto-agar were obtained from Difco Laboratories (Detroit, MI). All other reagents were of analytical grade.

2.2 Yeast Growth

Stationary yeast cultures were obtained by inoculation of isolated haploid colony (By4147) in liquid complete medium (YPD) containing 1% yeast extract, 2% peptone and 2% glucose. The yeast were kept overnight at 30°C with shaking at 250 rpm until the stationary growth phase was reached.

2.3 Treatment with $(PhSe)_2$ or PhSeZnCl

An aliquot was inoculated in fresh YPD to a final cell density of approximately ~$1x10^5$ cells/mL. The cells were then treated with 0.05% of dimethyl sulfoxide (DMSO), 2, 4, 6 or 10 µM of $(PhSe)_2$ (dissolved in DMSO) or with 4, 8, 12 e 20 µM of PhSeZnCl (dissolved in distilled water) (Santi et al., 2008). The treatments were performed for 1, 2, 3, 4, 6 or 16 h. Then, cells were harvested and washed twice with PBS solution. The samples were shaken at 30^{o}C during the treatment periods and cell density was determined by measuring the absorbance at 600 ηm (SHIMADZU UV-1650 PC spectrophotometer).

2.4 Determination of ROS by flow cytometer and microscope of fluorescence

The ROS generation in yeast was assessed by incubating yeast (~$0.7x10^7$ cells/mL or 0.5 of optical density) with 20 µM of dichlorofluorescein diacetate (DCFH-DA) in the dark. After 2 h of incubation, the fluorescence of the cells was determined

in Accuri flow cytometer (Becton-Dickinson, San Jose, USA). The signal from DCFH was detected with FL1 (530/30) band pass filter, and the events (100,000) were determined using Cell Quest software (Becton-Dickinsin, San Jose, CA). The size and granularity cell were expressed in arbitrary units. For cell localization of ROS, an aliquot of samples was incubated with 10 µg/ml of the nuclear marker, 4'-6-Diamidino-2-phenylindole (DAPI), for 20 min at room temperature. The microscope imaging was performed using an IX81 inverted microscope coupled with a charge-coupled device camera (Olympus, Tokyo, Japan). The DAPI staining was observed under 340 ηm excitation and 488 ηm emission, and DCFH-DA staining was viewed under 480 ηm excitation and 510 ηm emission at 60 x magnification. The pictures were captured with Olympus cell Sens (®) software.

2.5 Determination of cell membrane permeability by flow cytometer

The cell membrane permeability was determined by staining with propidium iodide (PI) 30 µmol/L, for 4^{o}C at room temperature. Using flow cytometer, the events (100,000) were recorded, and the stained cell were detected with FL2 (585/40) band pass filter. Since the yeasts in the control group presented a basal PI staining, positive PI cells were considered above 99% of fluorescence of the control group in order to compare $(PhSe)_2$- and PhSeZnCl-treated cells.

2.5 Determination of total thiols

The analysis of total thiol concentration or sulfhydryl groups (SH) was based on method described by Elmman (Elmman, 1959). Briefly, thiols interact with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), forming a highly colored anion with maximum peak at 412 ηm. Briefly, the cells were treated with $(PhSe)_2$ and PhSeZnCl concentration, and after 16 h, the samples were washed twice with 100 mM Tris-buffered saline (TBS, pH = 8,0). The cell density was adjusted to 10^7 cells in all treatments groups. Cells were lysed with glass beads (0.5 mm), and centrifuged at 3,400 rpm at 4°C for 6 min. The supernatant (100µL) was transferred to 96 well microplate, and mixed with 100 µL of TBS plus 10 µL of DTNB (10 mM dissolved in ethanol 100%). Then, the absorbance from thiols interacting with DTNB was read at 412 ηm. The concentration of thiol groups was calculated using a curve of cysteine

and the results were expressed in ηmol/L. Finally, the total content of thiols were normalized by number of estimated cells in according to table of Burke (Burke et al., 2005).

2.6 Statistical analysis

Statistical results were determined by STATISTICA/w 5.0 software, and expressed as means ± standard error of the mean (SEM). The tests with PhSeZnCl compound were analyzed by one-way ANOVA with Sidak's multi comparison. The tests with $(PhSe)_2$ were evaluated by two-way ANOVA (compound concentration and time as factors) following Tukey's *post hoc* test. Statistical significance was set at $p < 0.05$ level.

3. RESULTS

3.1 Cell growth

After 2 h exposure to 2, 4, 6 and 10 µM diphenyl diselenide $(PhSe)_2$, only 10 µM of $(PhSe)_2$ caused inhibition of cell growth (**Fig. 1**). At 3, 4 and 6h of incubation with 4, 6 and 10 µM of $(PhSe)_2$ occurred a significant inhibitory effect on yeasts. Exposure to 6 µM and 10 µM of $(PhSe)_2$ for 16 h caused also significant inhibition of cell growth. In contrast, the exposure to PhSeZnCl caused cell growth inhibition of *S. cerevisiae* only at 20 µM (**Fig. 2**) in 16 h of incubation. The results demonstrate that low concentration of $(PhSe)_2$ inhibited yeast growth in a time-dependent manner. After 16 h of exposure, the inhibition of cell growth was distinct between $(PhSe)_2$ and PhSeZnCl, indicating that $(PhSe)_2$ is more toxic to *S. cerevisiae* than PhSeZnCl.

3.2 Production of ROS in S. cerevisiae

A significant increase in ROS production was observed after 16h of incubation with the highest concentration of $(PhSe)_2$ (10 µM) (**Fig. 3**, **Fig. 4)**, as can be seen in the representative histogram of **Figure 5**. Moreover, the exposure of *S. cerevisiae* to 20 µM of PhSeZnCl for 16 h increased the production of ROS (**Fig. 6**). All these results indicate that the cell growth inhibition induced by both compounds was causally associated to oxidative stress, but these effects were more strongly promoted by $(PhSe)_2$.

3.3 Effect of $(PhSe)_2$ and PhSeZnCl on morphology of S. cerevisiae determined by Flow Cytometry.

The exposure of yeasts to $(PhSe)_2$ caused an increase in size of cells, which was both time and concentration dependent. After 3 h of incubation, 10 µM $(PhSe)_2$ produced a significant increase in cell size. At 6 h of exposure to the chalcogenide, the increase in cell size was significant at 6 µM and 10 µM. However, after 16 h of incubation with $(PhSe)_2$, only 10 µM caused a significant increase in cell size (**Fig. 7A** and **Fig. 8).**

The cellular granularity of samples incubated with 6 µM and 10 µM $(PhSe)_2$ increased from 3 to 6 h of incubation. The exposure to $(PhSe)_2$ for 16 h caused a significant increase in cell granularity only in 10 µM (**Fig. 7B** and **Fig. 9**). For the case of PhSeZnCl, it was observed an elevation in the cell size and cell granularity after incubation with 20 µM of PhSeZnCl (**Fig 10A** and **10B**).

3.4 Effect of $(PhSe)_2$ and PhSeZnCl in cell membrane permeability of S. cerevisiae

The ability of selenium compounds to increase the cell membrane permeability was analyzed by nuclear staining with propidium iodide (PI) by flow cytometry.
The **Figure 11** and **12** depicts that 10 µM of $(PhSe)_2$ increased the permeability of cell membrane from 2 to 16 hours of incubation. Similarly, exposure to 6 µM $(PhSe)_2$ increased the cell permeability to PI from 2 to 6 hours of exposure. In contrast, the treatment of yeasts with PhSeZnCl did increase the permeability of cell membrane to PI only after exposure to2 0 µM for 16 h of incubation (**Fig. 13**).

3.5 Determination of total thiol

To analyze the possible oxidation of total thiol groups from yeast cells by selenium compounds, the content of –SH was evaluate in samples incubated for 16 h with different concentrations of $(PhSe)_2$ and PhSeZnCl. The results were expressed correcting the total thiols per estimated number of cells (**Fig. 14** and **15**). The **Figure 14** shows that the $(PhSe)_2$ was able to increase the total thiol concentration after exposure to 6 µM. The results of samples incubated with PhSeZnCl indicated an increase in total thiols, but this effect was not significant (**Fig. 15**). The increase in the total thiols could be a defense mechanism of samples in order to increase their antioxidant capacity with enzymes that have thiol groups, leading to scavenger of excessive production of ROS promoted by selenium compounds. However, in the highest concentrations of $(PhSe)_2$ (10 µM) and of PhSeZnCl (20 µM) this cellular antioxidant capacity is probably exhausted.

4. DISCUSSION

The biological effects of organic selenium compounds have been extensively studied in recent decades due to their antioxidant properties (Chanaday, et al., 2011; Ibrahim,et. al., 2015; Rosa, et al., 2007; Fiuza, et., al., 2015; Machado, et. al., 2009). However, the cytotoxicity of selenium compounds has been also reported in different models (Nogueira and Rocha, 2011; Rosa, et al., 2004; 2005; 2007a; Santos, et al., 2009). $(PhSe)_2$ is a very simple molecule that has gained great interest in toxicological studies because this selenium compound can exhibit pro-oxidant properties. In fact, organoselenium compounds, including diselenides, can trigger the production of superoxide radicals and hydrogen peroxide. At least in part, the production of these reactive species can result from the oxidation of selenol intermediates formed after the reduction of parent compounds by thiols (Yan and Spallholz, 1993; Spallholz, 1994; Nogueira, et al., 2004; Wallenberg, et al., 2010). In this regard, mammalian thioredoxin reductase and glutaredoxin reductase have been demonstrated to metabolize diselenide compounds to selenol intermediates (Zhao and Holmgren, 2002; Freitas, et al., 2010; Wallenberg, et al., 2010). In H-157 cells, Wallenberg and collaborators (2010) have demonstrated that selenite and GSSeSG were cytotoxic tentatively by the selenide (HSe^-)- or selenol/selenolate (-SeH/Se^-)-mediate induction of superoxide formation.

Overproduction of ROS can promote damage to DNA, proteins and cell membranes, which can culminate in cell death (Imlay, 2003). Here we have observed that the cytotoxic effects of $(PhSe)_2$ in *S. cerevisiae* depended on the time incubation and the concentrations tested. Our results indicated that $(PhSe)_2$ inhibited cell growth of *S. cerevisiae,* starting at 6 µM after 16h of incubation. Indeed, *S. cerevisiae* cells exposed to $(PhSe)_2$ exhibited an increase in size and granularity, which was associated with an elevation in cell membrane permeability. These morphological and molecular changes caused by $(PhSe)_2$ occurred at early times of exposures. Although ROS production tended to increase after 3 and 4 hours of exposure to $(PhSe)_2$, the increase was modest not statistically significantly. Taken together, these results suggest that changes in cell membrane permeability and morphology preceded ROS overproduction. Recently, Rosseti and collaborators (2015) have assessed the effects of $(PhSe)_2$ in the pathogenic fungus *Candida albicans.* The

authors suggested that ROS promoted permanent damage to cell membranes, which was associated with cell death.

At 16h, cell growth was inhibited by 6 and 10 µM of $(PhSe)_2$; however, the increase in cell size, granularity, cell membrane permeability and ROS levels, were observed only at 10 µM of $(PhSe)_2$. It is interesting to note that cells exposed to 6 µM of $(PhSe)_2$ had a significant increase in total content of thiol groups. Similarly, Barbosa and collaborators (2006) demonstrated an increase in non-protein–SH groups (NP-SH) in liver, kidney and blood of mice treated with low doses of $(PhSe)_2$, suggesting that the cells developed antioxidant adaptations to counteract ROS production. This may indicate that surviving cells increased the expression of antioxidant defenses, such as thiol-containing proteins. For instance, pro-oxidant condition or exposure to electrophiles have been associated with activation of transcription factors such as nuclear transcription related factor 2 (Nrf2) (Ehren and Maher, 2013; De Bem, et al., 2013; Habib, et al., 2015; Bartolini, et al., 2015b), which may also promote the synthesis of reduced glutathione in mammals cells. Here it is important to emphasize that $(PhSe)_2$ can target thiol-containing proteins, particularly those containing vicinal thiol groups (Rocha et al., 2012) and keap-1, which binds Nrf2 and impedes its translocation to the nucleus, contains vicinal thiol groups.

However, at high concentrations of $(PhSe)_2$ (10 µM), these antioxidant defenses can be exhausted. Accordingly, exposure to high levels of toxic electrophiles have been reported to modify the expression of Nrf2-related genes and decrease the levels of thiol groups in different animal models (Huang et al., 2012; Jin, et al., 2013; Abolaji, et al., 2014; 2015).

PhSeZnCl has been reported to exhibit higher efficiency in decomposing peroxide than $(PhSe)_2$ (Bartolini, et al., 2015a). However, the PhSeZnCl toxicity has not yet been studied in detail. Here, we observed that PhSeZnCl inhibited cell growth and increased ROS production, membrane permeability, cell size and granularity only when tested at high concentration (20 µM). At 20 µM of PhSeZnCl, which in terms of selenium corresponds to 10 µM of $(PhSe)_2$, the compound was toxic to *S. cerevisiae* cells after 16 h incubation. In contrast, at a low concentration (12 µM), PhSeZnCl did not produce overt signs of toxicity in *S. cerevisiae*, whereas equivalent concentrations of $(PhSe)_2$ (6 µM) were toxic. Accordingly, Bartolini and collaborators (2015a) have reported that 20 µM of PhSeZnCl inhibited cell viability and increased ROS production in K562 human erytholeukemia cells and nontumor MEFs cells

culture. Taken together, these results may indicate that PhSeZnCl can be a safer source of thiol-peroxidase-like intermediates than the related compound $(PhSe)_2$. However, more studies are needed to support this proposition.

As mentioned above, $(PhSe)_2$ is a pro-oxidant compound (a relatively weak electrophile) and it can induce oxidative stress by reacting with thiol groups. Here, observed that $(PhSe)_2$ increased oxidative stress (as determined by DCFH oxidation), but this effect was secondary to changes in cell morphology and membrane permeability. Together these results suggest that ROS formation can contribute for $(PhSe)_2$-induced cell death in *S. cerevisiae*, but ROS participation seems to follow biochemical and morphological modifications caused by oxidation of thiol groups of target proteins. Here, PhSeZnCl was less toxic than $(PhSe)_2$ and these results indicate that PhSeZnCl should be evaluated with more detail in animal models of toxicity in invertebrates and vertebrates.

Acknowledgements

This paper is under the financial support of CNPq, CAPES, and FAPERGS.

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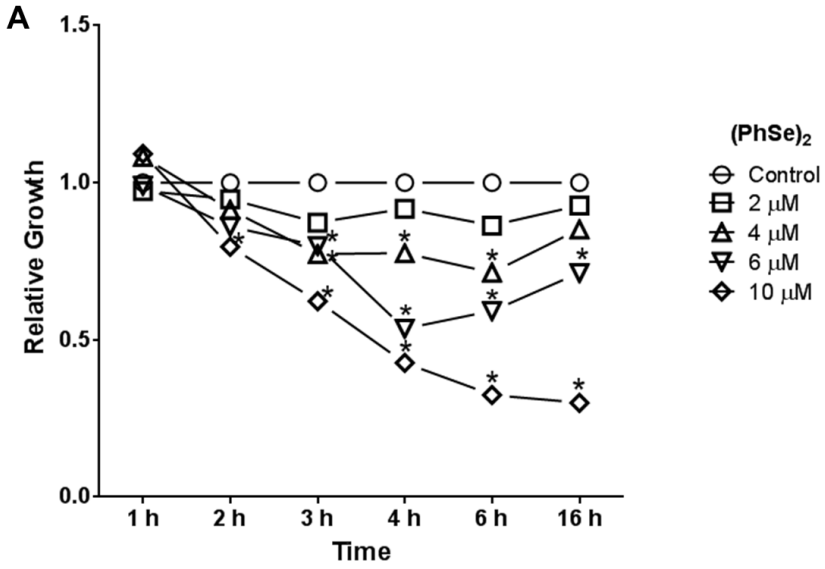
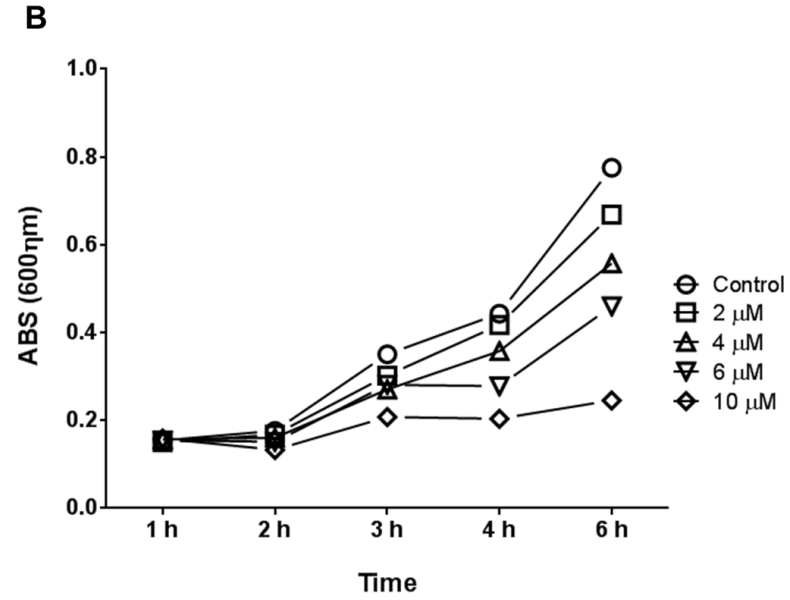

A
Relative Growth
1.5
1.0
0.5
0.0
1 h
2 h
3 h
4 h
6 h
16 h
Time
(PhSe)2
Control
2 μM
4 μM
6 μM
10 μM


B
ABS (600ηm)
1.0
0.8
0.6
0.4
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1 h
2 h
3 h
4 h
6 h
Time
Control
2 μM
4 μM
6 μM
10 μM

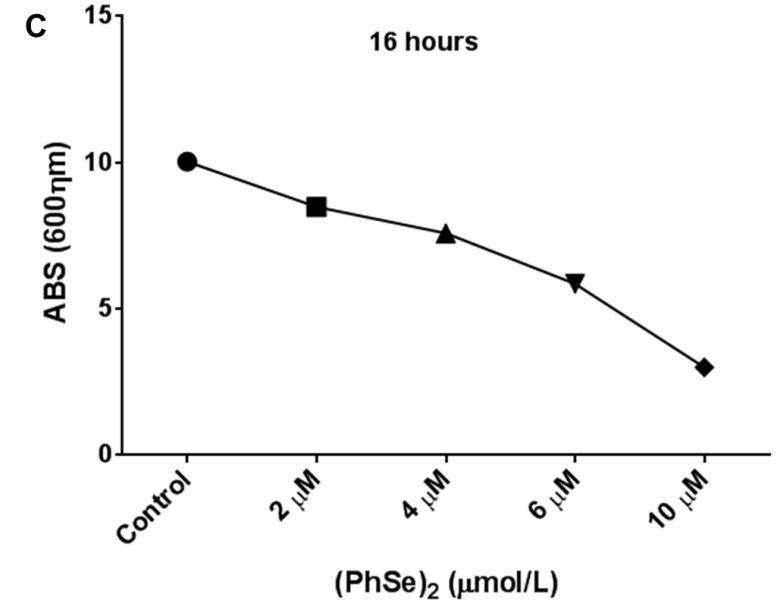
C
16 hours
ABS (600ηm)
15
10
5
0
Control
2 μM
4 μM
6 μM
10 μM
(PhSe)2 (μmol/L)

Fig.1. Inhibitory effect of diphenyl diselenide $(PhSe)_2$ on cell growth after 1, 2, 3, 4, 6 and 16 h of exposure to $(PhSe)_2$. Cell growth was analyzed by measuring the cells density in relation to the control (relative growth 1) as represented in (A). (B) Average of optical density values from 1 to 6 h incubation with $(PhSe)_2$. (C) Average of optical density values for 16 h incubation with $(PhSe)_2$. Data are expressed as mean ± SEM of ten independent experiments (n=10). (SEM was less than 10% of the respective means and it was omitted for the sake of clarity). Data were analyzed by two-way ANOVA followed by Tukey's *post hoc* test. * $p < 0.01$.

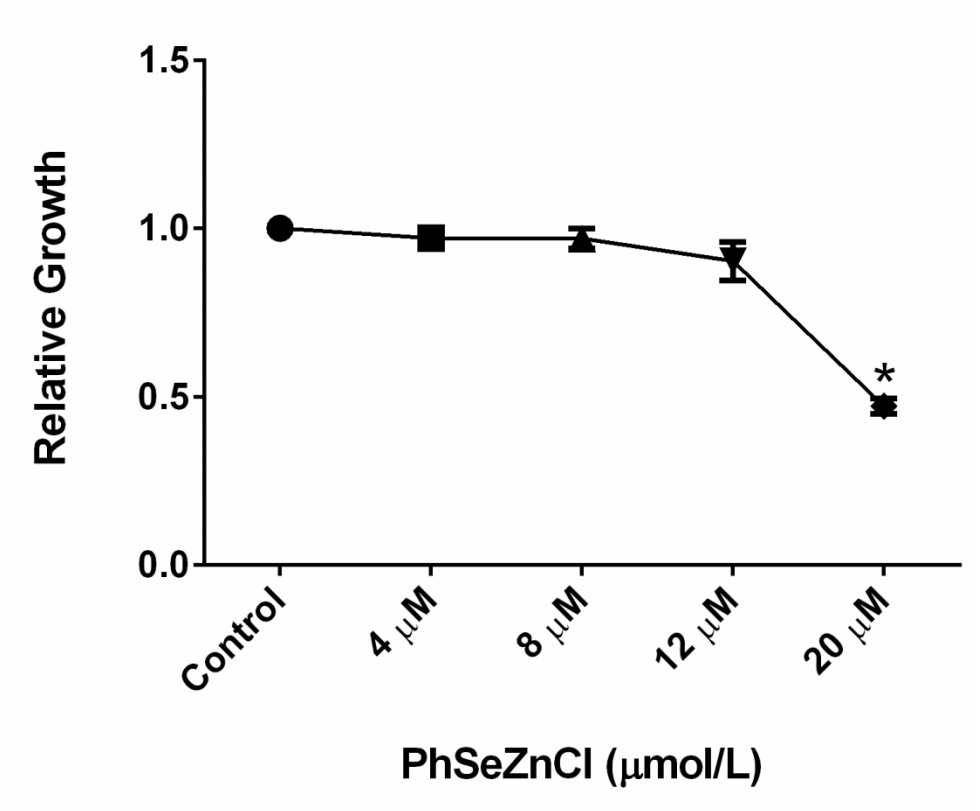

Fig.2. Inhibitory effect of PhSeZnCl on cell growth of *S. cerevisiae* after 16 hours of exposure. Cell growth was analyzed by measuring the cells density in relation to the control (relative growth 1). Values are expressed as mean ± SEM of ten independent experiments (n=10). Data were analyzed by one-way ANOVA followed by Sidak's *post hoc* test. * $p < 0.01$.

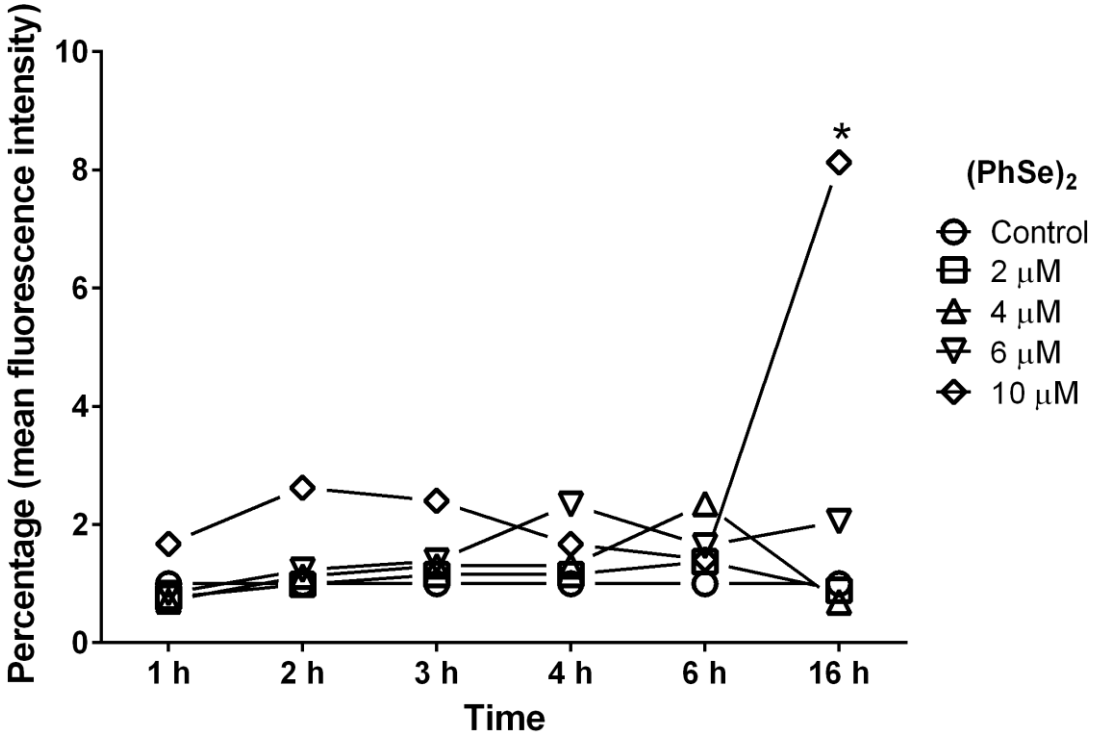

Fig.3. Effect of exposure to $(PhSe)_2$ in the production ROS in *S. cerevisiae.* After exposure to $(PhSe)_2$, cells were incubated with DCFH-DA for 2h and examined by flow cytometry. Values are expressed as percentage of mean fluorescence intensity (FL1) of eight independent experiments (n=8) (SEM was less than 10% of the respective means and it was omitted for the sake of clarity). Data were analyzed by two-way ANOVA followed by Tukey's *post hoc* test. *$p < 0.01$.

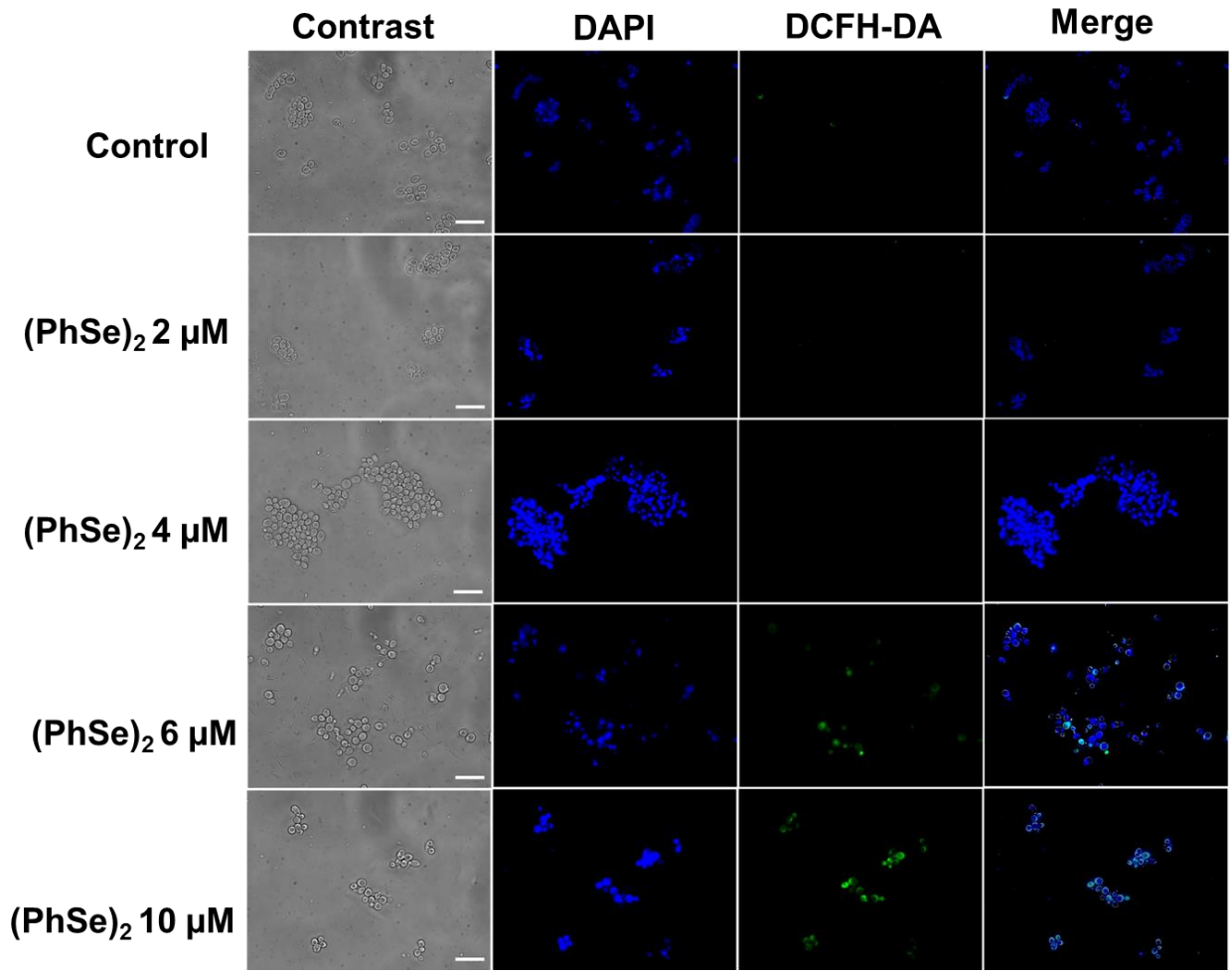

Fig.4. Production of ROS in *S. cerevisiae* exposed to different concentrations of $(PhSe)_2$ for 16h. The cell nuclei were stained by DAPI (blue color) and ROS were stained by DCFH-DA (green color). The images from left to right represent the cells observed in phase contrast, blue fluorescence (DAPI), green fluorescence (DCFH-DA) and merge images of blue and green fluorescence. The white bars represent 10 µm.

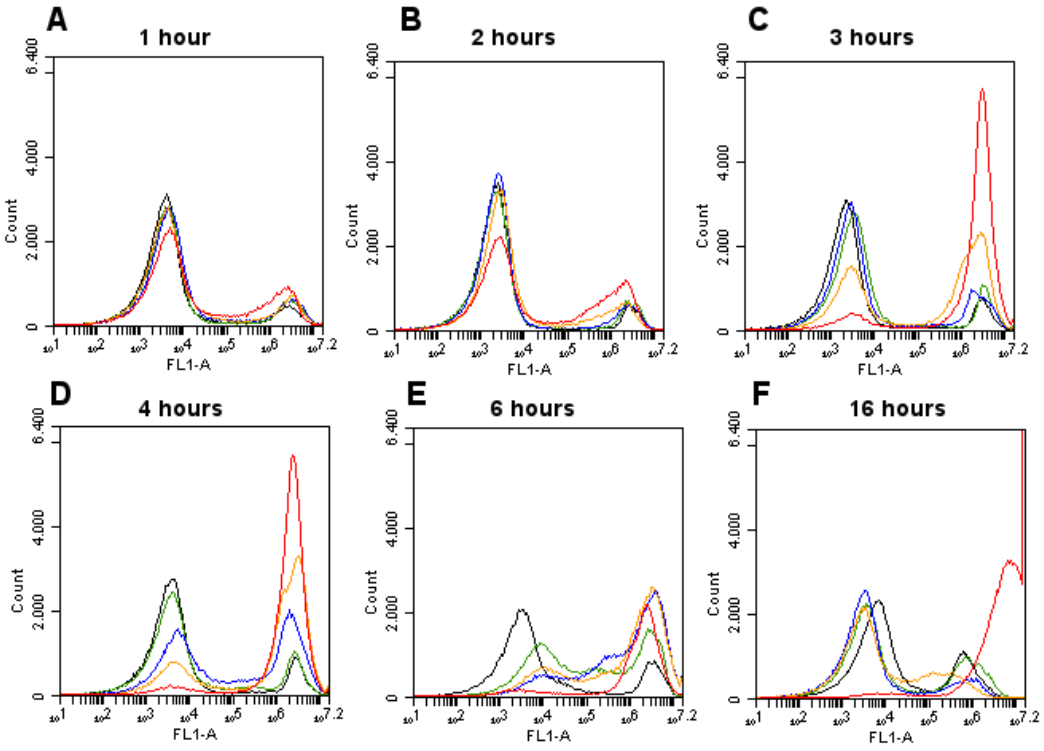

Fig.5. ROS Production *S. cerevisiae* exposed to diphenyl diselenide $(PhSe)_2$ for different periods. Data are the representative histograms of DCFH fluorescence intensity (X axis) versus cell number (Y axis) of one typical experiment from 8 (n=8). The black line corresponds to control, green to 2 µM, blue to 4 µM, orange to 6 µM and red to 10 µM of $(PhSe)_2$. The panels represent the results after time exposure to $(PhSe)_2$ in 1 (A), 2 (B), 3 (C), 4 (D), 6 (E) and 16 h (F).

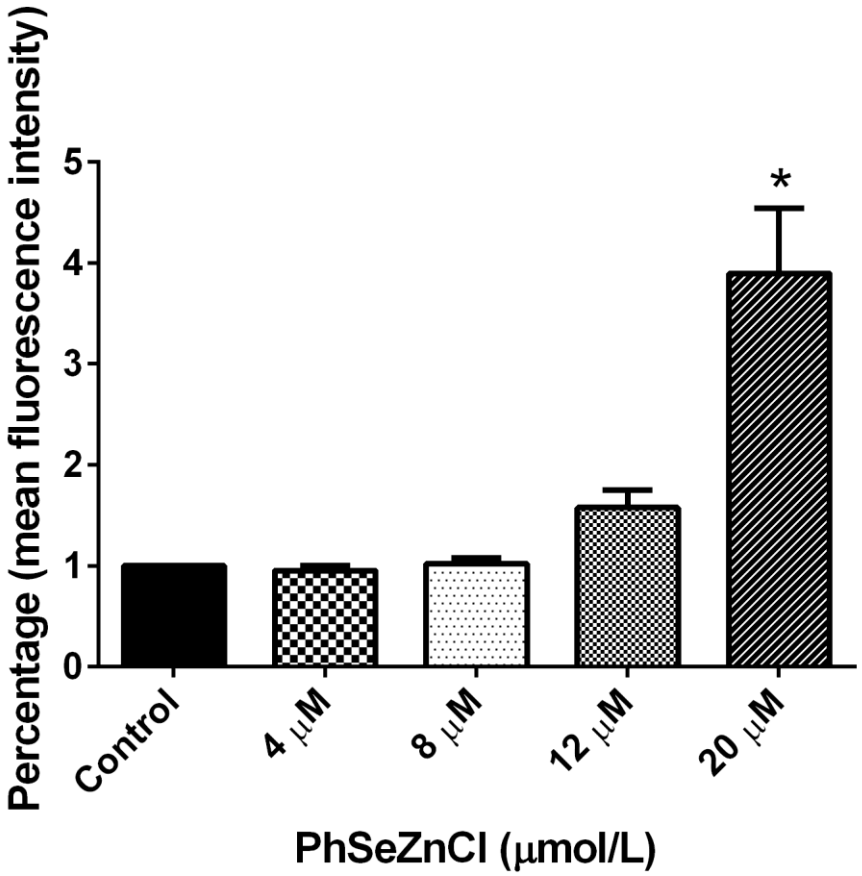

Fig.6. Effect of PhSeZnCl in the production ROS in *S. cerevisiae.* After exposure to PhSeZnCl, cells were incubated with DCFH-DA for 2h and examined by flow cytometry. Values are expressed as mean ± SEM of ten independent experiments (n=10). Data were analyzed by one-way ANOVA followed by Sidak's *post hoc* test. * $p < 0.01$.

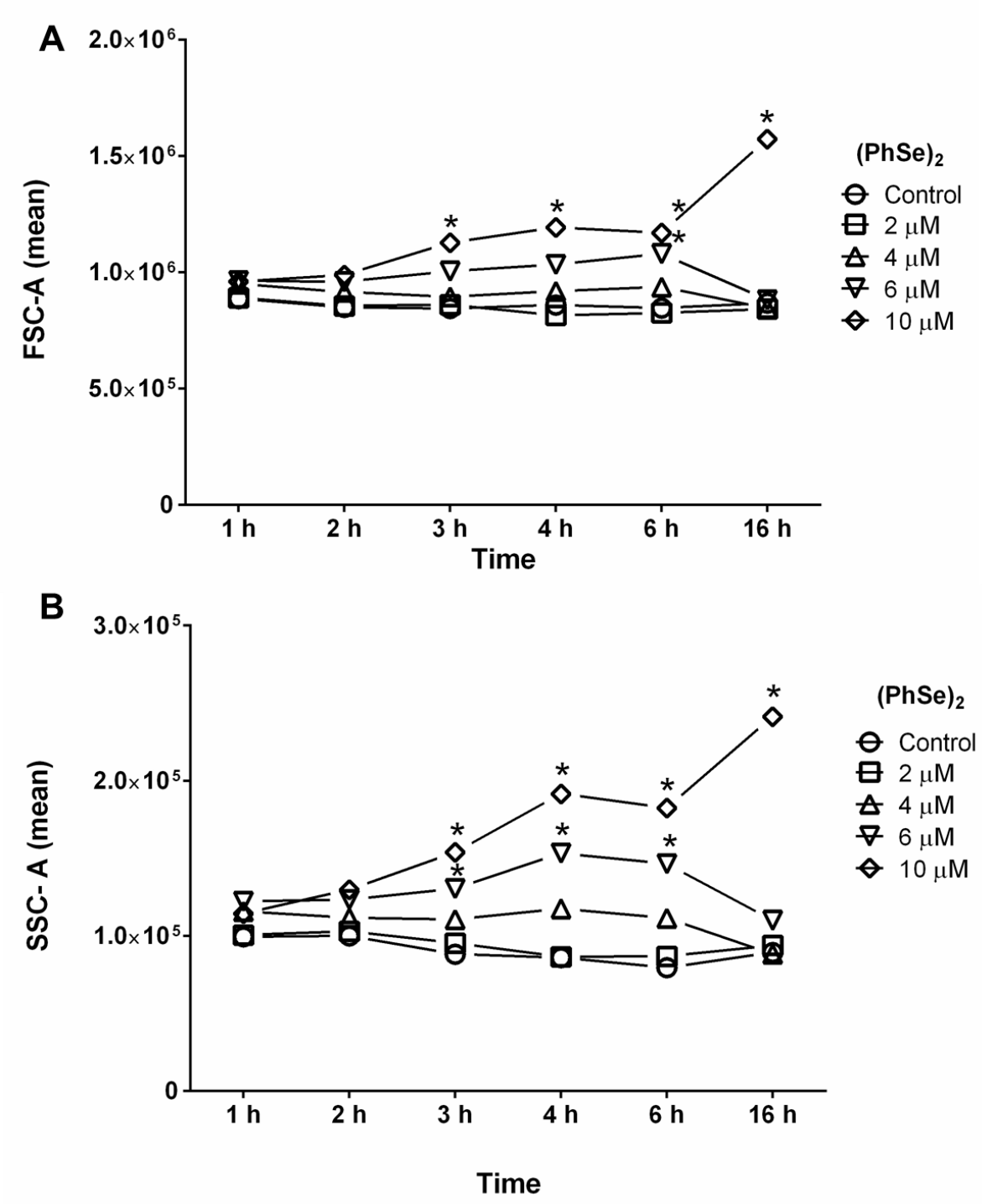

Fig.7. Effect on morphology of cells incubated for different times with the concentrations of $(PhSe)_2$. After exposure to $(PhSe)_2$, cells were examined by flow cytometry. The cell size is represented in (A) and cell granularity is demonstrated in (B). Values are expressed only as mean of ten independent experiments (n=10) (SEM was less than 10% of the respective means and it was omitted for the sake of clarity). Data were analyzed by two-way ANOVA followed by Tukey's *post hoc* test.* p <0.01.

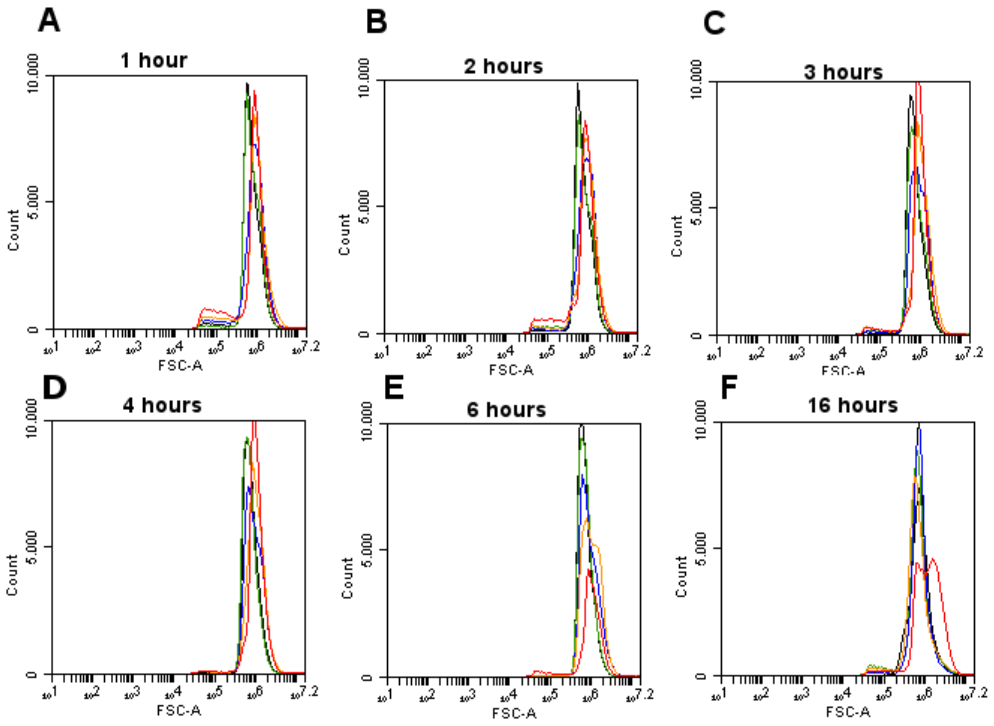

Fig.8. Cell size of *S. cerevisiae* exposed to diphenyl diselenide $(PhSe)_2$ for different periods. Data are the representative histograms of size (Forward Scatter, FSC) (X axis) versus cell number (Y axis) of one typical experiment from 10 (n=10). The black line corresponds to control, green to 2 µM, blue to 4 µM, orange to 6 µM and red to 10 µM of $(PhSe)_2$. The panels represent the results after time exposure to $(PhSe)_2$ in 1 (A), 2 (B), 3 (C), 4 (D), 6 (E) and 16 h (F).

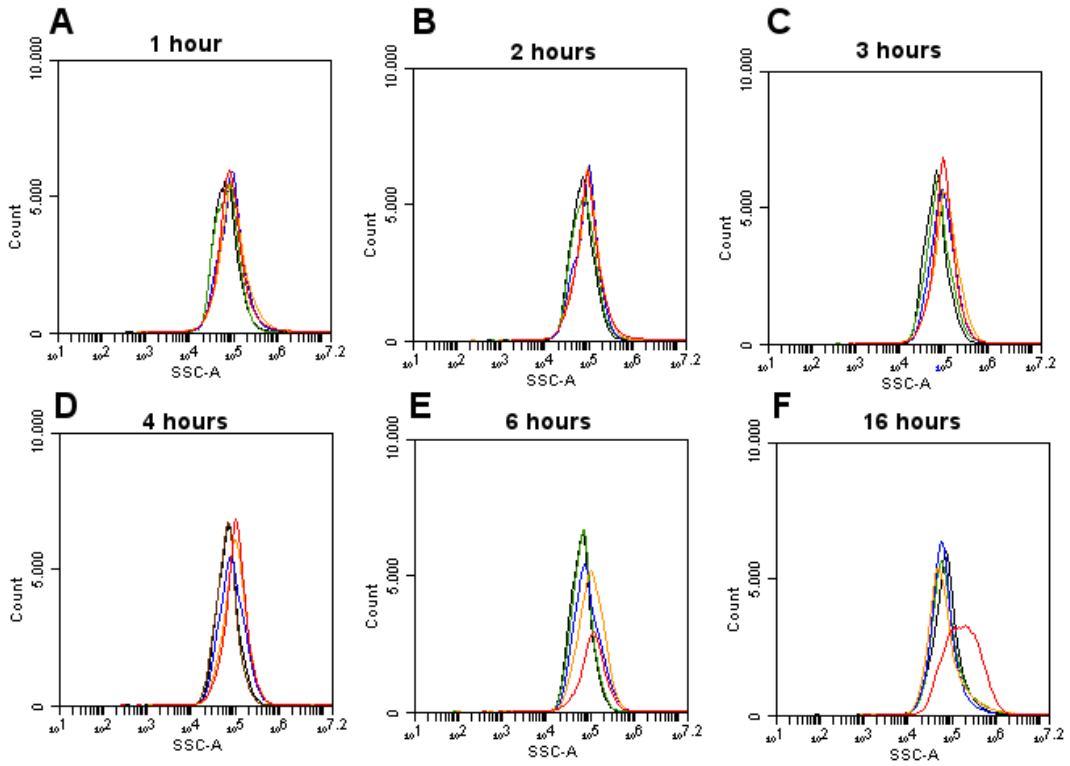

Fig.9. Cell granularity of *S. cerevisiae* exposed to diphenyl diselenide $(PhSe)_2$ for different periods. Data are the representative histograms of size (Side Scatte, SSC) (X axis) versus cell number (Y axis) of one typical experiment from 10 (n=10). The black line corresponds to control, green to 2 µM, blue to 4 µM, orange to 6 µM and red to 10 µM of $(PhSe)_2$. The panels represent the results after time exposure to $(PhSe)_2$ in 1 (A), 2 (B), 3 (C), 4 (D), 6 (E) and 16 h (F).

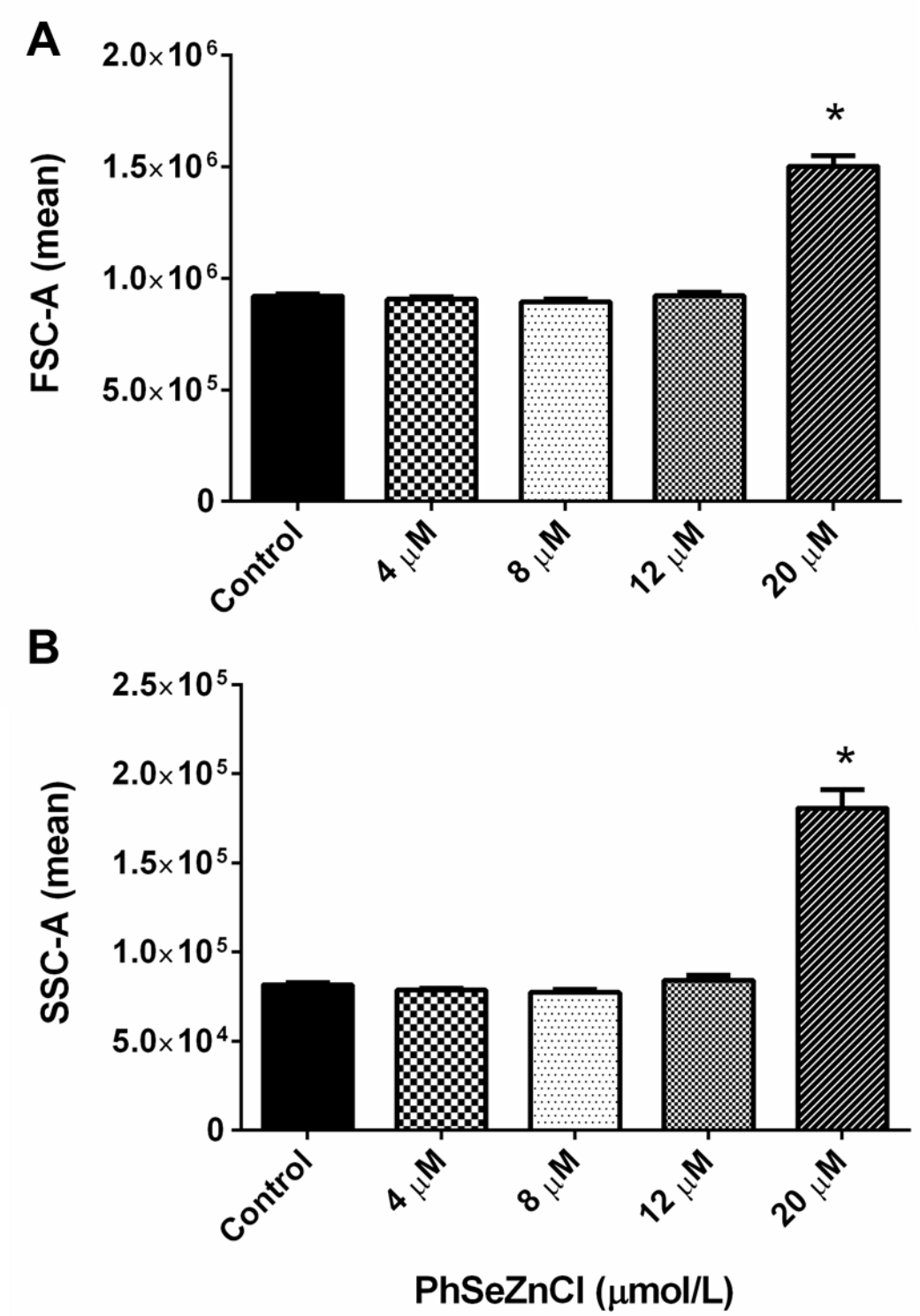

Fig.10. Effects on cell morphology of *S. cerevisiae* after 16h of exposure. Cells were treated with PhSeZnCl and examined by flow cytometry. The cell size is represented in (A) and cell granularity is demonstrated in (B). Values are expressed only as mean ± SEM of ten independent experiments (n=10). Data were analyzed by one-way ANOVA followed by Sidak's *post hoc* test. * $p < 0.01$.

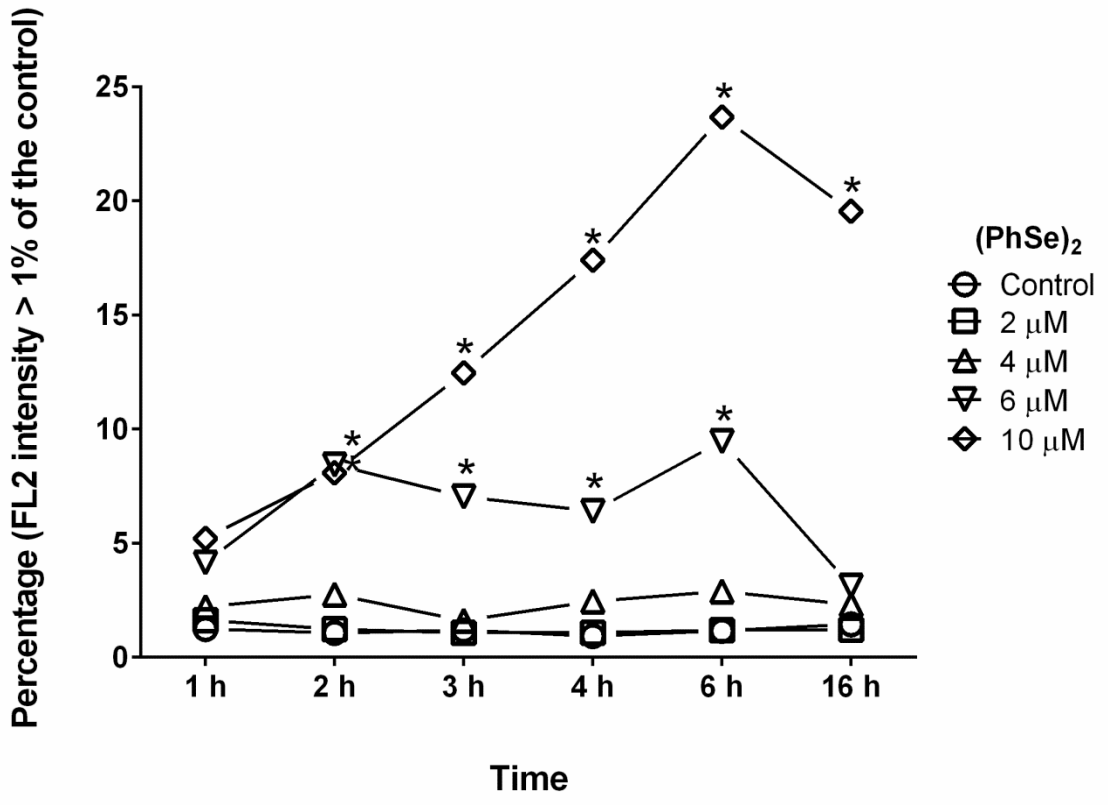

Fig.11. Effect of $(PhSe)_2$ on cell membrane permeability in *S. cerevisiae*. After exposure to different concentrations of $(PhSe)_2$, cells were incubated with PI for 1 h and examined by flow cytometry. Values are expressed only as mean of ten independent experiments (n=10) (SEM was less than 10% of the respective means and it was omitted for the sake of clarity). Data were analyzed by two-way ANOVA followed by Tukey's *post hoc* test.* $p < 0.01$.

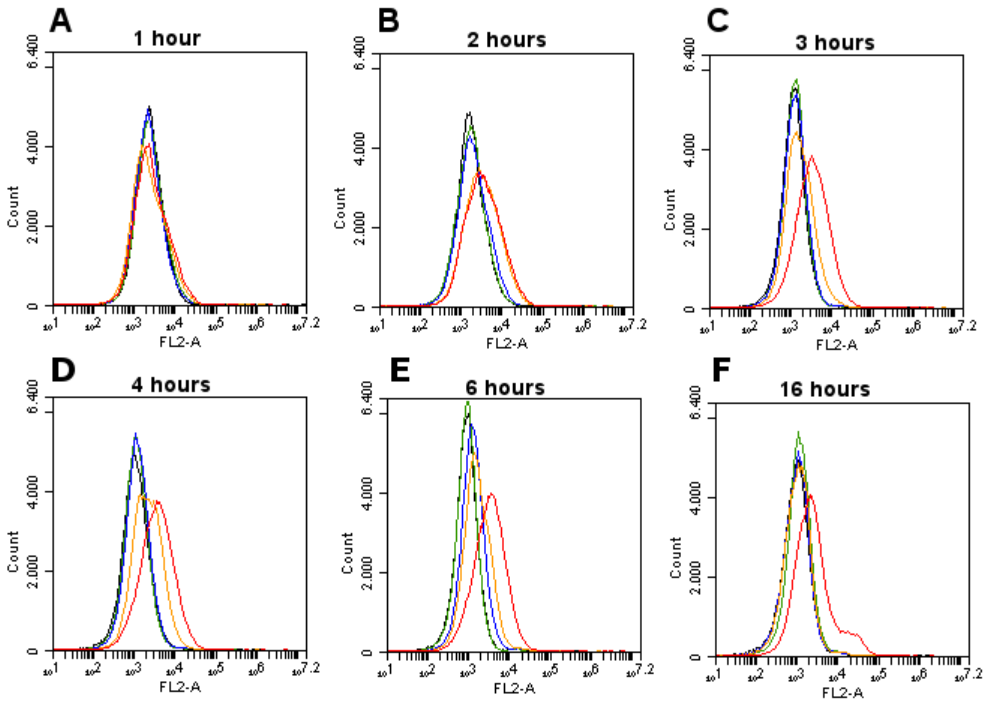

Fig.12. Cell membrane permeability of *S. cerevisiae* exposed to diphenyl diselenide $(PhSe)_2$ for different periods. Data are the representative histograms of PI fluorescence intensity (X axis) versus cell number (Y axis) of one typical experiment from 10 (n=10). After exposure to $(PhSe)_2$, cells were incubated with PI for 1 h. The black line corresponds to control, green to 2 µM, blue to 4 µM, orange to 6 µM and red to 10 µM of $(PhSe)_2$. The panels represent the results after time exposure to $(PhSe)_2$ in 1 (A), 2 (B), 3 (C), 4 (D), 6 (E) and 16 h (F).

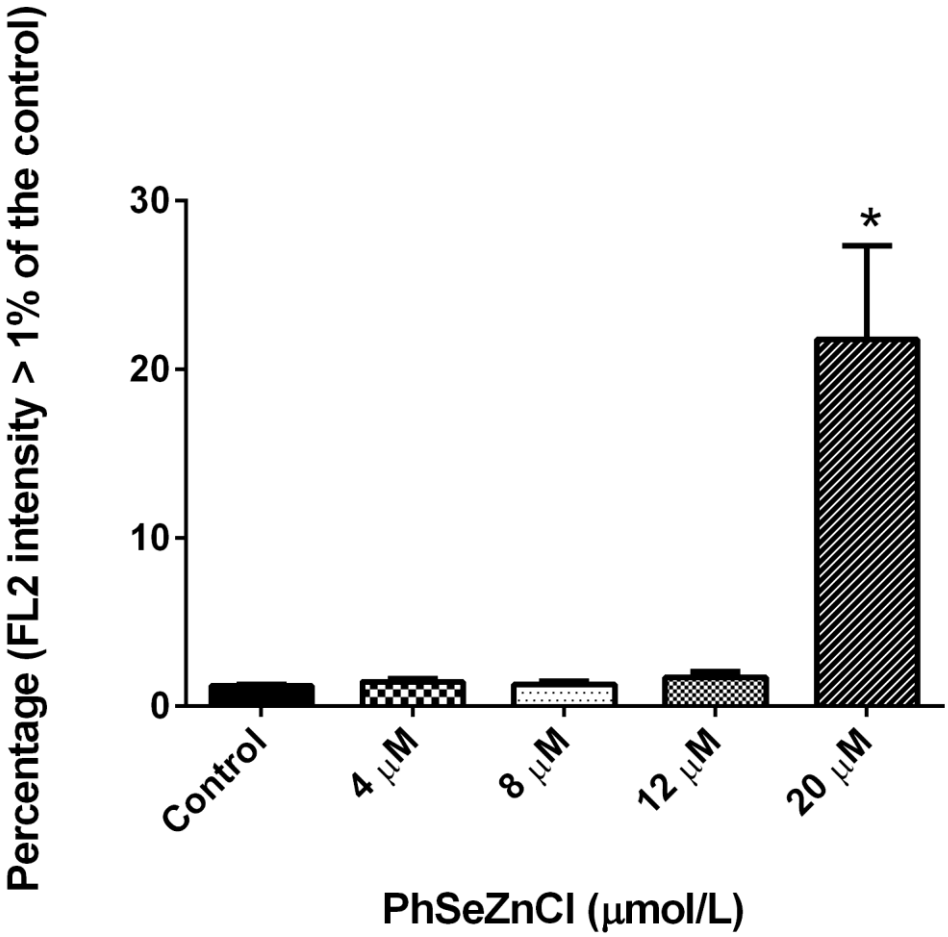

Fig.13. Effect on cell membrane permeability of *S. cerevisiae* incubated for 16 h with different concentrations of PhSeZnCl. After exposure to PhSeZnCl, cells were incubated with PI for 1 h. Values are expressed only as mean ± SEM of ten independent experiments (n=10). Data were analyzed by one-way ANOVA followed by Sidak's *post hoc* test. * $p < 0.01$.

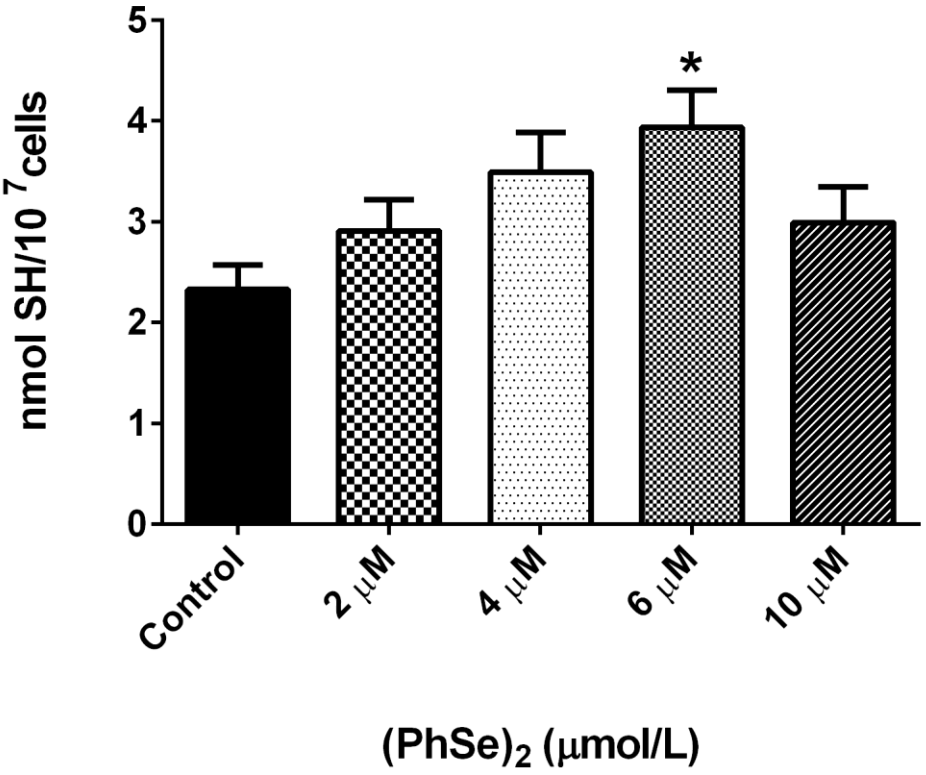

Fig.14. Determination of total thiols in samples after exposure to different concentrations of $(PhSe)_2$ for 16 h. Values are expressed as mean ± SEM of ten independent experiments (n=10). Data were analyzed by one-way ANOVA followed by Sidak's *post hoc* test. * $p < 0.01$.

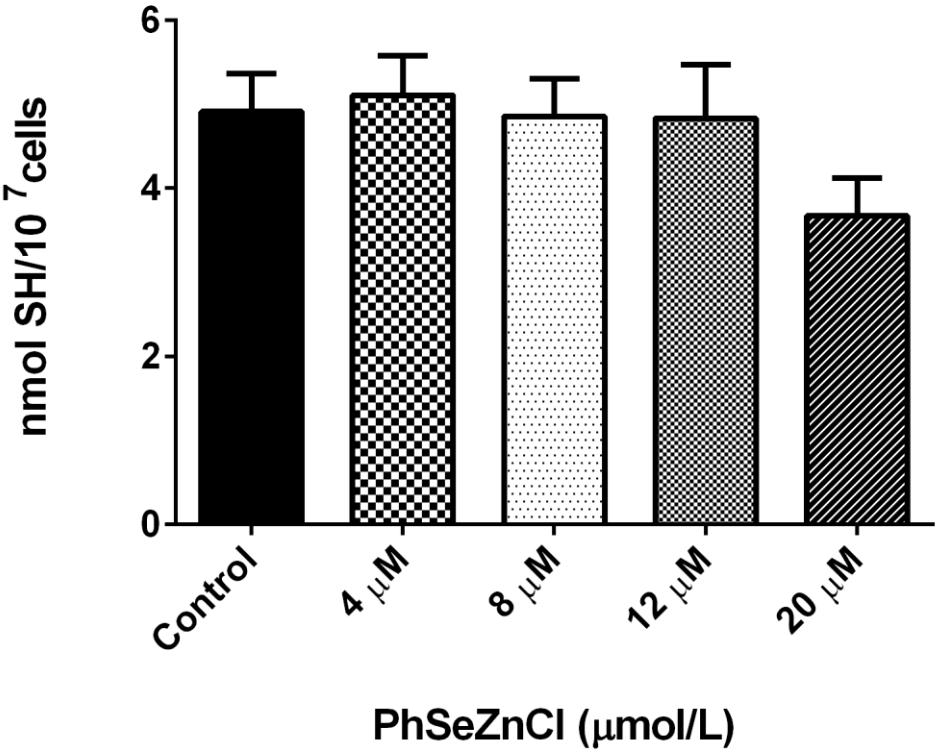

Fig.15. Determination of total thiols in samples after exposure to different concentrations of PhSeZnCl for 16 h. Values are expressed as mean ± SEM of ten independent experiments (n=10). Data were analyzed by one-way ANOVA followed by Sidak's *post hoc* test. * $p < 0.01$.

5. CONCLUSÃO

A partir dos resultados expostos nesta dissertação, podemos concluir:

- O $(PhSe)_2$ inibe o crescimento celular de leveduras *S. cerevisiae* depois de 2 horas de incubação;
- A inibição do crescimento celular é acompanhada pelo aumento da permeabilidade da membrana, tamanho e granulosidade celular;
- O $(PhSe)_2$ apenas aumenta a produção de EROs em 16 horas de incubação na concentração mais alta (10 µM);
- O $(PhSe)_2$ é capaz de aumentar o conteúdo total de tióis na concentração de 6 µM em 16 horas de incubação;
- O PhSeZnCl apresentou toxicidade inibindo o crescimento celular, aumentando a produção de EROs, permeabilidade da membrana, tamanho e granulosidade celular na concentração de 20 µM, em amostras incubadas por 16 horas.

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