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Fabiane Bicca Obetine Baptista

O DISSELENETO DE DIFENILA PROTEGE O MODELO *Caenorhabditis elegans PARA* A DOENÇA DE HUNTINGTON ATRAVÉS DA ATIVAÇÃO ANTIOXIDANTES REDUZINDO A AGREGAÇÃO DE PROTEÍNAS

SANTA MARIA, RS

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Dissertação de mestrado apresentada ao Curso de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção de grau de **Mestre em Ciências Biológicas: Bioquímica Toxicológica.**

Orientador: Professor Dr. Félix Alexandre Antunes Soares

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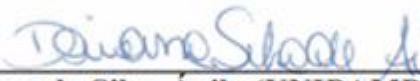
Aprovada em 16 de Junho de 2020

Félix Alexandre Antunes Soares (UFSM)
(Presidente/Orientador)



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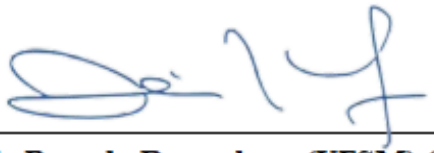

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RESUMO

O DISSELENETO DE DIFENILA PROTEGE O MODELO *Caenorhabditis elegans PARA* A DOENÇA DE HUNTINGTON ATRAVÉS DA ATIVAÇÃO ANTIOXIDANTES REDUZINDO A AGREGAÇÃO DE PROTEÍNAS

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ORIENTADOR: Professor Dr. Félix Alexandre Antunes Soares

A doença de Huntington (DH) é uma doença neurodegenerativa progressiva, autossômica dominante, com um fenótipo distinto, incluindo coreia e distonia, descoordenação, declínio cognitivo e dificuldades comportamentais. Caracterizada pela presença da proteína mutante huntingtina, que resulta de uma repetição da sequência de nucleotídeos (CAG) expandida, conduzindo a uma cadeia de poliglutamina (Poli-Q) de comprimento variável no terminal N. A maioria das doenças neurodegenerativas são caracterizadas pela deposição anormal e consequente agregação de proteínas, que prejudicam a dinâmica das redes proteicas e resultam no desequilíbrio da homeostase celular. Neste trabalho, utilizamos o *Caenorhabditis elegans* devido a sua fácil manipulação e alta homologia de genes e vias de sinalização em relação aos mamíferos. Os vermes foram expostos ao disseleneto de difenila $(PhSe)_2$ nas concentrações de 25, 50 e 100 µM, a partir de adultos jovens e analisados quanto à agregação da poli-Q e viabilidade neuronal e muscular. Baseando-se na teoria de envelhecimento e na agregação de proteínas relacionadas com a DH, e levando em consideração que o $(PhSe)_2$ possui atividade antioxidante, analisamos as possíveis vias envolvidas e relacionada com DH, também analisamos quanto ao seu potencial em aumentar a qualidade e o tempo de vida de *C. elegans.*

PALAVRAS CHAVES: Proteínas, Doenças neurodegenerativas, Sequência de poliglutamina, Selênio.

ABSTRACT

DIPHENYL DISELENIDE PROTECTS THE MODEL *Caenorhabditis elegans* FOR HUNTINGTON'S DISEASE BY ACTIVATING ANTIOXIDANTS REDUCING PROTEIN AGGREGATION

AUTHOR: Fabiane Bicca Obetine Baptista

ADVISOR: Professor Dr. Félix Alexandre Antunes Soares

Huntington's disease (HD) is a progressive, autosomal dominant neurodegenerative disease with a distinct phenotype, including chorea and dystonia, incoordination, cognitive decline and behavioral difficulties. Characterized by the presence of the mutant huntingtin protein, which results from an expanded CAG repeat, leading to a variable length polyglutamine (Poli-Q) chain at the N-terminal. Most neurodegenerative diseases are characterized by abnormal deposition and consequent protein aggregation, which impair the dynamics of protein networks and result in the imbalance of cellular homeostasis. In this work, we used the *Caenorhabditis elegans* experimental model due to its easy manipulation and high homology of genes and signaling pathways in relation to mammals. The worms were exposed to diphenyl diselenide $(PhSe)_2$ at concentrations of 25, 50 and 100 µM, from young adults and analyzed for poly-Q aggregation and neuronal and muscle viability. Based on the theory of aging and the aggregation of proteins related to HD, and taking into account that $(PhSe)_2$ has antioxidant activity, then we will analyze some possible pathways involved related to HD), we will also analyze as to its potential in increasing the quality and life span of *C. elegans.*

Keywords: Neurodegenerative disease, Polyglutamine sequence, Proteinopathies, Selenium.

LISTAS DE FIGURAS

REVISÃO BIBLIOGRÁFICA

ARTIGO

LISTA DE ABREVIATURAS

DA: Doença de Alzheimer

DH: Doença de Huntington

DP: Doença de Parkinson

EROs: Espécies Reativas de Oxigênio

FOXO: Fator de transcrição *Forkhead Box*

GPF: Do inglês proteína verde fluorescente- Green Fluorescente Protein

IGF1 (IIS): Insulin and insulin-like growth factor-1

HSP: Do inglês proteína de choque térmico – Heat Shock Protein

PoliQ: Trato de poliglutamina

SOD- Superóxido dismutase

SUMÁRIO

APRESENTAÇÃO

No item INTRODUÇÃO consta uma revisão sucinta da literatura sobre os temas trabalhados nesta dissertação.

A metodologia realizada e os resultados obtidos que fazem parte desta dissertação estão apresentados no item MANUSCRITO sob a forma de um manuscrito redigido em inglês conforme as normas do periódico **Metallomics** ao qual foi publicado dia 05 de maio de 2020. No mesmo constam as seções: Introdução, Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas.

Os itens CONCLUSÕES e PERSPECTIVAS, encontrados no final desta dissertação, apresentam conclusões gerais sobre os resultados do manuscrito presente neste trabalho e as perspectivas para futuros trabalhos.

As REFERÊNCIAS BIBLIOGRÁFICAS referem-se somente às citações que aparecem no item INTRODUÇÃO desta dissertação.

1. INTRODUÇÃO

A maioria das doenças neurodegenerativas são caracterizadas pela deposição anormal e consequente agregação de proteínas, que prejudicam a dinâmica das redes proteicas e resultam no desequilíbrio da homeostase celular (Douglas e Dillin, 2010). Além dos fatores ambientais e fisiológicos, o envelhecimento adjacente ao estresse cumulativo contribui significativamente para o início tardio das doenças neurodegenerativas, como Doença de Alzheimer (DA), Doença de Parkinson (DP) e Doença de Huntington (DH).

A DH, comumente conhecida como coreia de Huntington, é uma patologia neurodegenerativa que se caracteriza por déficits motores progressivos, distúrbio emocional, demência e morte neuronal (Bates, 2005). A mutação gênica causadora da DH foi localizada no cromossomo 4 (Gusella *et al.*, 1983). Essa mutação resulta numa expansão da sequência de nucleotídeos citosina, adenina e guanina (CAG - que codifica o aminoácido glutamina), resultando em uma proteína mutante com uma sequência de poliglutaminas (poli-Q) no terminal amino da proteína huntingtina. Indivíduos normais apresentam proteína huntingtina com menos de 35 repetições de CAG. Em indivíduos portadores da DH, a proteína huntingtina apresenta mais que 36 repetições da CAG (aproximadamente 38-55) na porção N-terminal da cadeia polipeptídica. Quanto maior essa sequência, mais precoce ocorre o desenvolvimento da doença e mais severa é sua progressão (Vonsattel e Difiglia, 1998).

A DH possui herança autossômica dominante, o alelo normal transmite-se de geração em geração segundo as regras de hereditariedade Mendeliana. O alelo mutante é instável durante a meiose, alterando o seu comprimento na maior parte das transmissões entre 20 gerações, com um aumento de 1-4 unidades ou diminuição de 1-2 unidades do triplete CAG (Gil-Mohapel e Rego, 2011). A DH é uma patologia relacionada ao envelhecimento que normalmente surge após os 40 anos de idade, podendo ocorrer na juventude, sendo mais rara e grave. Uma possível intervenção precoce pode ser útil para desenvolver estratégias terapêuticas iniciais para DH e outras doenças neurodegenerativas mais prevalentes, incluindo DA e DP, que compartilham características comuns como a agregação proteica anormal, neurônio seletivo e início tardio patogenia (Ross e Tabrizi, 2011). A toxicidade da poli-Q parece resultar da

interrupção da rede de proteostase. Portanto, a ação de compostos que restaurem a homeostase seria uma forma de diminuir a toxicidade gerada pela poli-Q.

O Selênio (Se) é um elemento-traço essencial, que auxilia na manutenção do estado redox e defesas antioxidantes, participando da síntese e função de selenoproteínas (Ursini e Bindoli, 1987). Vários efeitos benéficos têm sido relatados a respeito de alguns compostos contendo Se, dentre eles, ação anti-inflamatória, anti-nociceptiva, neuroprotetora, quimiopreventiva, e antioxidante (Commandeur *et al.*, 2001). Os compostos orgânicos de selênio, como disseleneto de difenila $(PhSe)_2$, tem sido amplamente estudados como moléculas protetoras contra o estresse oxidativo, e apresentaram capacidade redutora de peróxidos (Nogueira *et al.*, 2004). Anteriormente, o $(PhSe)_2$ demonstrou atividade benéfica em modelos de DA, utilizando ratos para indução da doença (Ishrat *et al.*, 2009) e também *Caenorhabditis elegans*. No modelo *C. elegans* para a DA, a exposição crônica ao $(PhSe)_2$ reduziu o estresse oxidativo e diminuiu a expressão do peptídeo beta amiloide Aβ1–42 (Zamberlan *et al.*, 2014). Porém não há relatos de que $(PhSe)_2$ tenha diminuído a agregação proteica em doenças neurodegenerativas, como a DH.

Caenorhabditis elegans é um nematódeo pequeno (±1 mm) e estruturalmente simples, no entanto, mostra-se como uma poderosa ferramenta nas áreas de pesquisa em toxicologia, farmacologia e biologia molecular. Seu genoma e suas vias metabólicas e biossintéticas são altamente conservados nos mamíferos com uma semelhança de aproximadamente 60-80%, incluindo vias envolvidas no desenvolvimento celular, na manutenção do sistema nervoso e na apoptose (Nass e Blakely, 2003). Possui diferentes sistemas de neurotransmissão que coordenam seu comportamento, incluindo o sistema dopaminérgico, colinérgico, serotoninérgico, glutamatérgico e também gabaérgico (Vellai *et al.*, 2003). Em geral, *C. elegans* se desenvolvem de ovos a adultos em cerca de 3,5 dias em condições controladas (20 ± 2°C). O desenvolvimento até a idade adulta é, no entanto, dependente da temperatura, variando de < 2,5 dias a 25º C a 6 dias a 15ª C (Figura 1).

Normalmente, após a eclosão do ovo, as larvas passam por quatro estágios larvais (L1, 9 L2, L3 e L4) até adulto jovem e posteriormente adulto capaz de produzir ovos (Riddle *et al.*, 1997). Em seus diferentes estágios larvais, o verme sintetiza uma cutícula que o recobre e que possui várias funções, como suporte, proteção e crescimento, o que pode ser um fator limitante para a utilização do nematódeo em estudos bioquímicos,

devido à dificuldade de rompê-la em condições adequadas (Bhaskaran *et al.*, 2011). Em casos de aglomeração ou falta de alimento, o desenvolvimento larval é interrompido em L2 com formação da 'larva dauer', um estágio de diapausa, cujo crescimento é retomado em ambientes favoráveis (Riddle *et al.*, 1997). Esse nematódeo possui cultivo e manejo simples em laboratório, rápido desenvolvimento, tempo de vida relativamente curta cerca de ±20 dias, genoma completamente sequenciado e toda linhagem celular somática conhecida (Hertweck *et al.*, 2003).

Além disso, como os vermes são transparentes, fusões de 'genes repórter' permitem a visualização direta da morfologia celular e dos padrões de expressão proteica. Ainda, mutantes genéticos e vermes knockouts podem ser facilmente gerados via RNA interferente e estão disponíveis para a pesquisa (Fire *et al.*, 1998). Essas vantagens têm sido exploradas para testar o efeito de diversas substâncias, incluindo antioxidantes naturais e sintéticos, sobre a longevidade de vida do verme (Braeckman *et al.*, 2002).

O primeiro modelo de *C. elegans* para a DH foi gerado pela expressão de um fragmento de huntingtina contendo 150 repetições de Poli-Q em neurônios sensoriais de cabeça (neurônios ASH) e resultou em degeneração nervosa (Faber *et al.*, 1999). A disponibilidade de várias linhagens mutantes semelhantes a doenças em humanos tem sido explorada para testar o efeito de diversas substâncias, incluindo antioxidantes naturais e sintéticos, sobre o tempo de vida do verme (Braeckman *et al.*, 2002).

Desta forma, utilizaremos vermes mutantes que expressam a poli-Q, reproduzindo a agregação da proteína observada em cérebros de portadores da doença, para avaliar o efeito do $(PhSe)_2$ nesta patologia. Como a DH é uma doença relacionada com a idade, genes homólogos em *C. elegans* relacionados com as vias de envelhecimento como a via da sinalização da insulina/DAF-16 (Benedetto *et al.*, 2010) e alguns marcadores relacionados com essa via como a enzima superóxido dismutase 3(SOD-3)(Candas e Li, 2014), um ortólogo de MnSOD em humanos e a as chaperonas envolvidas no controle da homeostase proteica (Douglas e Dillin, 2010) podem ser estudados.

Outro marcador importante relacionada na via DAF-16 são as chaperonas, funcionam como máquinas com ciclos de ligação e liberação de substrato regulados pela ligação e hidrólise do ATP. A HSP16.2 que é da família das proteínas de choque térmico (HSPs), uma molécula de charperona responsivas ao estresse (Henriques, 2010). A eficiência das chaperonas é regulada por co-chaperonas, uma grande classe de proteínas que interagem com as chaperonas para modular o ciclo de nucleotídeos das chaperonas e

para fornecer seletividade ao substrato (Wittung-Stafshede *et al.*, 2003). Essas chaperonas possuem um papel central na proteostase, uma vez que mostram ser essenciais para prevenir o acúmulo de estados proteotóxicos por dobramendos alternados, como ocorre na conformação de proteínas de certas doença neurodegenerativas.

Tendo em vista que a DAF-16 é um dos principais moduladores das vias de envelhecimento e de resposta ao estresse, alguns estudos mostram que ativação da sinalização semelhante à insulina (IGF-1) ativam vias celulares levando à transcrição das redes de chaperonas (Voisine *et al.*, 2010). A integração desses sinais leva à coordenação da resposta adequada, assim garantindo a homeostasia proteica que facilita a resistência ao estresse e a longevidade além da supressão da proteotoxicidade (Tullet *et al.*, 2008).

A localização nuclear de DAF-16 é um pré-requisito para a ativação transcricional de seus genes-alvo, como genes para enzimas antioxidantes como SOD-3 (Murphy *et al.*, 2003), o SOD-3, localizado na cadeia respiratória mitocondrial e está envolvido na remoção de radicais superóxido (Candas e Li, 2014). Portanto, avaliamos o efeito do $(PhSe)_2$ na ativação da via sinalização à insulina (ILS) em *C. elegans,* bem como na modulação da expressão das HSPs e da SOD-3 através de suas propriedades antioxidantes afim de diminuir a agregação de Poli-Q no modelo DH em *C.elegans.*

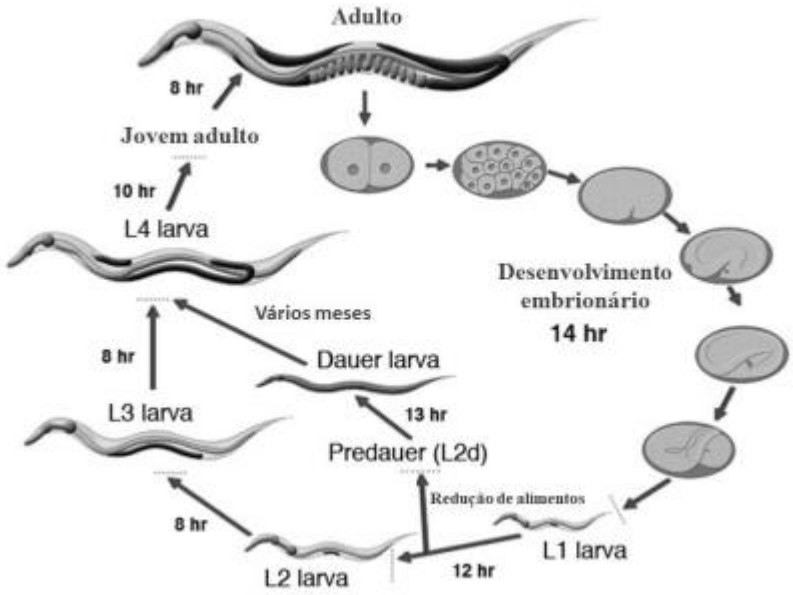

Figura 1- Ciclo de vida do nematoide *Caenorhabditis elegans.*

Fonte: Wormatlas (2017) adapt

2. OBJETIVOS

2.1. Objetivo Geral

O presente estudo tem como objetivo geral, analisar os efeitos do tratamento crônico com o composto disseleneto de difenila $(PhSe)_2$ no modelo de indução de doença de Huntignton, *Caenorhabditis elegans*.

2.2. Objetivos específicos

- Investigar o efeito do $(PhSe)_2$ no tempo e qualidade de vida do nematódeo *C. elgans*;
- Avaliar os possíveis efeitos neuroprotetores do $(PhSe)_2$ em *C. elgans* através de ensaios de sobrevivência neuronal;
- Verificar os níveis de agregação da poli-Q nos músculos *de C. elegans* tradados com o $(PhSe)_2$;
- Avaliar a ativação vias sinalização da insulina / IGF1 (IIS) e a via de sinalização de choque térmico.

3. JUSTIFICATIVA

A DH é atualmente uma doença sem cura, seu tratamento é apenas sintomático e a terapêutica selecionada depende da manifestação clínica. Apesar do aumento da incidência dessa doença e da influência do estresse oxidativo em seu desenvolvimento e progressão, ainda há uma escassez de estudos que explorem a capacidade protetora de compostos antioxidantes no tratamento dessa patologia. O $(PhSe)_2$ vem sendo estudado por possuir várias funções farmacológicas, como antioxidante e neuroprotetora é um composto promissor para estudos relacionados a doenças neurodegenerativas. Desta forma, modelos simples, de baixo custo e fácil manipulação genética como o *C. elegans* permitem explorar o efeito de diferentes compostos e mecanismos de ação que podem ser úteis no tratamento da doença.

4. MATERIAIS E MÉTODOS, RESULTADOS E DISCUSSÃO

Os materiais e métodos, bem como os resultados e discussão encontram-se abaixo no formato de artigo científico publicado em maio de 2020 na revista Metallomics.

4.1 ARTIGO

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Diphenyl diselenide protects a *Caenorhabditis elegans* model for Huntington's disease by activation of the antioxidant pathway and a decrease in protein aggregation

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Huntington's disease (HD) is an autosomal dominant, progressive neurodegenerative disease with a distinct phenotype. It occurs due to a mutation in the huntingtin (or IT19) gene with an abnormal CAG repeat, leading to a variable length N-terminal polyglutamine chain (poly-Q). Like most neurodegenerative diseases, HD is characterized by the abnormal deposition and aggregation of proteins in the cell, which impairs the proteostasis and disrupts cellular homeostasis. In this study, we used *Caenorhabditis elegans* as an animal model due to its easy genetic manipulation and high homology of genes and signaling pathways with mammals. Worms were exposed to diphenyl diselenide $(PhSe)_2$ at 25, 50 and 100 μM, and then we analyzed the polyQ aggregation, neurodegeneration, touch response, reactive oxygen species (ROS) levels, lifespan and health span. In addition, we analyzed the involvement of the transcription factor DAF-16, a FOXO-ortholog, and the downstream heat-shock protein-16.2 (HSP-16.2) and superoxide dismutase-3 (SOD-3). Our data demonstrate that chronic treatment with $(PhSe)_2$ reduced polyQ aggregation in muscle and polyQ mediated neuronal cell death of sensory neurons ASH, as well as maintaining the neuronal function. In addition, $(PhSe)_2$ decreased ROS levels and extended the lifespan and health span of wild type and PolyQ mutant worms. The mechanism proposed is the activation of DAF-16, HSP-16.2 and SOD-3 in whole body tissues to increase the antioxidant capacity and regulation of proteostasis, decreasing PolyQ aggregation and toxicity and reducing ROS levels, leading to an increase in lifespan, and healthspan. Our findings provide new clues for treatment strategies for neurodegenerative diseases and other diseases caused by age-related protein aggregation.

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Significance to metallomics

Selenium (Se) is an essential trace element that plays a role in the maintenance of the redox state and antioxidant defenses of cells, and in the synthesis and function of selenoproteins. Despite many studies revealing neuroprotective effects of the organoselenium compound $(PhSe)_2$, its mechanisms against neurodegenerative diseases are not established. We demonstrated that chronic treatment with $(PhSe)_2$ decreased huntingtin-induced aggregation and neurodegeneration in *C. elegans*. These effects are a consequence of insulin/IGF1 (IIS) signaling activation by $(PhSe)_2$, followed by the overexpression of chaperones and superoxide dismutase.

1. Introduction

Huntington's disease (HD), also known as Huntington's chorea, is an hereditary neurodegenerative disorder characterized by progressive motor deficits, emotional disturbance, dementia, and neuronal death.[1] The HD-causing gene mutation is located on chromosome 4,[2] which results in an expansion of the cytosine, adenine and guanine (CAG) nucleotide sequence encoding the amino acid glutamine, producing a mutant protein with a poly-glutamine sequence (polyQ) at the amino-terminal region of the huntingtin protein. Normal individuals have fewer than 35 CAG repeats, whereas in individuals with HD, the huntingtin protein contains more than 36 CAG replicates in the N-terminal region of

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the polypeptide chain. The longer the CAG sequence is, the earlier the symptoms tend to present and the faster the disease progresses to incapacity.[3] The consequent aberrant folding of huntingtin leads to its deposition as insoluble fibrillary aggregates (inclusions) in the cytoplasm and nucleus of neurons. The pathogenesis of HD has been associated with misfolded proteins, which interfere with various cell functions, including transcriptional regulation and proteasomal degradation.[4] Furthermore, studies have reported that the brains of HD patients contain an abnormally high level of oxidative damage markers.[5] Reactive oxygen species (ROS) including superoxide radicals, hydrogen peroxide and hydroxyl radicals are generated during normal cell metabolism;[6] however, when their levels exceed the neutralizing capacity of an organism's antioxidant defenses, oxidative stress occurs.[7] ROS cause the oxidation of various biomolecules including lipids, proteins and nucleotides, which affects energy homeostasis and causes damage to cells.[8] Protein structures might be modified leading to misfolding and aggregation, which are characteristic of HD and other neurodegenerative diseases.[9]

Thus, compounds that restore protein homeostasis and decrease ROS generation might be possible alternatives to reduce the toxicity generated by polyQ.[4] Current treatments for HD only alleviate the symptoms and do not alter the course or progression of the disease.[10] Therefore, early intervention may help to develop therapeutic strategies for HD and also for other more prevalent diseases, including Alzheimer's (AD) and Parkinson's diseases (PD), which share common characteristics such as abnormal protein aggregation, selective neuron disturbance, and late-onset pathogenesis.[11]

Selenium (Se) is an essential trace element which plays a role in the maintenance of the redox state and antioxidant defenses of cells, and in the synthesis and function of selenoproteins.[12] Organoselenium compounds like 4,4′-dichlorodiphenyldiselenide (p-ClPhSe)$_2$, ebselen ($C_{13}H_9NOSe$) and dyphenil diselenide $(PhSe)_2$ have been extensively studied, mainly due to their beneficial potential.[13–16] However, at high doses these compounds might exert toxicity, related to prooxidant effects.[17] $(PhSe)_2$ is characterized by its antioxidant,[18] anti-inflammatory[19] and neuroprotective[15] activities. Furthermore, this compound decreased the scopolamine-induced memory impairment in mice,[20] and chronic exposure to $(PhSe)_2$ in a *Caenorhabditis elegans* model of Alzheimer's disease reduced oxidative stress and decreased the expression of amyloid beta peptide Aβ1–42.[21] However, the effects of $(PhSe)_2$ on protein homeostasis or in HD models were not investigated.

C. elegans is a small nematode (±1 mm), which is a model organism used in different areas of research including toxicology, pharmacology and molecular biology. Its genome, metabolism and biosynthetic pathways are highly conserved, with a similarity of approximately 60–80% with mammals, including the conservation of pathways involved in cell development, maintenance of the nervous system, aging and apoptosis.[22] Although the worms do not develop HD and other human neurodegenerative diseases, different transgenic strains are available. The HD model of *C. elegans* was obtained by expressing and aggregating the human huntingtin fused to fluorescent proteins in neurons and body wall muscle.[23]

Proteinopathies are devastating neurodegenerative diseases with limited therapeutic options; therefore, $(PhSe)_2$ could be a potential pharmacological strategy. In this study, we investigated the protective activity of $(PhSe)_2$ in a *C. elegans* model of HD, as well as the putative pathways involved, focusing on oxidative stress, protein aggregation and proteostasis.

2. Materials and methods

2.1 Chemicals and reagents

Dimethyl sulfoxide (DMSO), 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA), 5-fluorodeoxyuridine (FUDR) and $(PhSe)_2$ were purchased from Sigma-Aldrich (USA).

2.2 *Caenorhabditis elegans* strains and maintenance

All strains used in this study, including Bristol N2 (wild-type), AM141 (*rmIs133*[(*unc-54p*)*Q40*::*YFP*]), HA759 (*rtIs11*[*osm-10p*::*GFP* + *osm-10p*::*HtnQ150* + *Dpy-20*(+)]), CL2070 (*dvls70* [*hsp-16.2p*::GFP + rol-6(*su1006*)]), TJ356 (*zls356* [*daf-16p::daf-16a/b*::GFP + rol-6(*su1006*)]) and CF1553 (*muIs84* [(*pAd76*)*sod-3p*::GFP + rol-6(*su1006*)]), were obtained from the *C. elegans* Genetics Center of the University of Minnesota (Minneapolis, MN, USA). Age-synchronized worms were obtained by isolating embryos from gravid hermaphrodites using a bleaching solution (1% NaOCl, 0.25 M NaOH). Eggs were allowed to hatch overnight in M9 buffer to obtain animals at the L1 stage.

2.3 Treatment of worms with diphenyl diselenide

Diphenyl diselenide $(PhSe)_2$ powder was diluted in DMSO and applied to the surface of nematode growth medium (NGM) agar plates (DMSO at a 1% final concentration) with *Escherichia coli* OP50 (grown overnight at 36 °C) to obtain final concentrations of 25, 50 and 100 μM $(PhSe)_2$. Synchronized worms at the L1 stage were transferred to treatment or control plates and incubated at 20 °C. For the control plates, DMSO was added at the same volume used in the treatment plates. For analyses performed after the young adult stage, we used FUDR on NGM plates with a final concentration of 12 μM to avoid progeny. Worms were transferred to new plates with FUDR and $(PhSe)_2$ or the vehicle every two days.

2.4 PolyQ toxicity assay

C. elegans strain AM141 was used for the polyQ aggregation assay.[24] Worms were analyzed 48, 72, 96 and 120 h after the L1 larvae stage. Approximately 50 worms per group were transferred to a glass slide in M9 buffer and paralyzed with 5 μL of 50 mM sodium azide. Approximately 10 animals were randomly selected from each treatment and scored for the number of polyQ40::YFP (yellow fluorescent protein) aggregates in muscle cells. The number of aggregates was determined within the total worm, using an Olympus® Fluoview FV10i confocal microscope and ImageJ2X (ImageJ2X software; Rawak Software, Inc., Stuttgart, Germany). Data are expressed as the number of protein aggregates. The fluorescence intensity of each worm

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was analyzed using ImageJ software (ImageJ software; Rawak Software, Inc., Stuttgart, Germany). Data are the average of three independent assays performed each time with 10 worms (n = 30).

C. elegans strain HA759 was used for the neuronal survival assay as described previously.[24] This strain expresses Htn-Q150 (a polyQ tract of 150 residues derived from human huntingtin) together with green fluorescent protein (GFP) strongly in the two ASH sensory neurons (located in the head).[37] Neurodegeneration is considered a loss of fluorescence in one of these neurons. Worms were analyzed 48, 72, 96 and 120 h after the L1 larvae stage. Approximately 50 worms per group were transferred to a glass slide in M9 buffer and paralyzed with 5 μL of 50 mM sodium azide. Approximately 10 nematodes were randomly selected from each treatment and evaluated for neurodegeneration. Data are the average of three independent assays performed each time with 10 worms (n = 30) and are expressed as the percentage of worms with neurodegeneration.

2.5 Touch response

To analyze the response to touch, we used the HA759 and N2 strains. Worms were analyzed 48, 72, 96 and 120 h after the L1 larval stage. The nematode touch response was assessed by gently touching the head region of the animal with a bristle brush. Backward movement was considered a positive response, and non-responding animals were considered to have no adverse reactions when stimulated, with a total of 10 touches performed per worm, with a 10 s rest period between the trials.[25] Three assays were carried out at different times, and 10 worms were analyzed for each experiment. Data are the mean of three independent assays performed each time with 10 worms (n = 30).

2.6 Measurement of reactive oxygen species (ROS)

Intraworm ROS generation was measured in wild-type, AM141, and HA759 strains were analyzed 48, 72, 96 and 120 h after the L1 larvae stage, using the H2DCFDA method as previously described[26] with minor modifications. The worms were collected from the plates and washed three times with fresh M9 buffer, and then 1000 worms were transferred to microtubes containing 990 μL of M9 and 10 μL of 2 mM H2DCFDA (final concentration of 20 mM) and incubated for 2 h. Then, the worms were washed and transferred with M9 to 96-well plates (100 worms per well). The fluorescence intensity was measured with a plate reader (excitation wavelength 488 nm, emission wavelength 510 nm). Data are the average of five independent assays performed each time with 100 worms in triplicate (n = 5).

2.7 Lifespan assay

The lifespan of wild-type, HA759 and AM141 *C. elegans* strains was investigated as previously described.[27] The pre-fertile period of adulthood was used as time zero (t = 0). The worms were maintained on NGM plates containing *E. coli* and $(PhSe)_2$ or the vehicle (control), and were transferred to new plates with FUDR and $(PhSe)_2$ or the vehicle every two days. Nematodes were scored as dead if they did not move after repeated stimulus with a platinum wire. Data are the average of three independent assays performed each time with around 100 worms (n = 300).

2.8 Health span

Behavioral parameters related to the health span were evaluated every 7 days after adulthood in wild-type, HA759 and AM141 strains as described previously.[28]

2.8.1 Pharyngeal pumping. Pharyngeal pumping was assessed directly on the control or treatment plates using a Nikon E200 microscope by observing the number of pharyngeal contractions during a 60 s interval. Data are the average of three independent assays performed each time with 10 worms in triplicate (n = 30).

2.8.2 Thrash frequency. The thrash frequency was selected for analysis of locomotion. Worms from control or $(PhSe)_2$ treatments were individually selected and placed into a drop of M9 and allowed to adapt for 1 min. The number of thrashes over a 20 s period was quantified with a Nikon E200 microscope. A thrash was defined as a change in the direction of bending at the middle of the body. Data are the average of three independent assays performed each time with 10 worms in triplicate (n = 30).

2.9 Subcellular DAF-16 localization

Adult worms of the TJ356 strain were transferred to a glass slide in M9 buffer. Fluorescence images were acquired with an Olympus® Fluoview FV10i Confocal Microscope. Localization of DAF-16::GFP in animals was classified into three categories: (1) only in the cytoplasm, (2) partially in the nucleus (intermediate), or (3) only in the nucleus. Three independent assays were performed, and 10 worms per group were randomly selected for evaluation in each experiment (n = 30). Worms exposed to heat stress at 35 °C for 1 h on NGM plates with *E. coli* were used as positive controls.

2.10 Quantitation of superoxide dismutase-3 (SOD-3) and heat shock protein-16.2 (HSP-16.2)

The expression of SOD-3 and HSP-16.2 was measured in adults of CF1553 and CL2070 strains, respectively, by quantifying the fluorescence of the GFP reporter according to the fluorescence specificity location for each transgenic strain.[29] SOD-3 is the orthologue of human SOD-2. Approximately 50 worms per group were transferred to a glass slide in M9 buffer and paralyzed with 5 μL of 50 mM sodium azide. Worms exposed to heat stress at 35 °C for 1 h on NGM plates with *E. coli* were used as positive controls. Fluorescence images were acquired with an Olympus® Fluoview FV10i Confocal Microscope, and the intensity of fluorescence was quantified using ImageJ2X (ImageJ2X software; Rawak Software, Inc., Stuttgart, Germany). From each group, 10 worms were randomly selected to measure the mean pixel density. The data are expressed as the mean of arbitrary fluorescence units (AFU) per worm after three independent assays performed each time with 10 worms (n = 30).

2.11 Quantitative real-time polymerase chain reaction (qPCR)

The mRNA levels of daf-16, sod-3 and hsp-16.2 were measured in N2 wild-type, AM141 and HA759 strains. *C. elegans* was

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treated with $(PhSe)_2$ from the L1 stage for 72 hours, and the mRNA levels were measured by quantitative real time PCR (qPCR) following a method already described.[30] The animals were washed with M9 buffer into Eppendorf tubes, resuspended in Trizol reagent (Invitrogen) and allowed to settle on ice. The worm pellet was subjected to chloroform extraction and isopropanol precipitation. Total RNA isolation was performed according to the manufacturer's suggested protocol. Total RNA samples were treated with DNase I (Promega) to eliminate DNA contamination. Reverse transcription (RT) of approximately 1 μg total RNA was performed with random primer, dNTPs and M-MLV reverse transcriptase enzyme (Invitrogen), according to the manufacturer's suggested protocol. The gene-specific primers are in Table 1. Every 10 μl PCR mixture containing 5 μl cDNAs (1 : 100), 1× PCR buffer, 0.1 mM dNTPs, 0.2 μM of each primer, 3 mM $MgCl_2$, 0.1× SYBR Green I (Molecular Probes) and 0.5 U platinum *Taq* DNA polymerase (Invitrogen). The qPCR conditions were: 94 °C for 5 min followed by 40 cycles of 15 s at 94 °C, 15 s at 60 °C and 40 s at 72 °C for extension in a Thermocycler StepOne Plus (Applied Biosystems). After amplification, samples were heated from 60 to 95 °C at a 0.3 °C s^{-1} temperature gradient to construct the denaturing curve of the amplified products. All samples were analyzed in triplicate with a non-template control also included. SYBR Green fluorescence (Molecular Probes) was analyzed by StepOne Plus Software version 2.0 (Applied Biosystems) and the Cq value (ΔCq) for each sample was calculated and reported using the ΔΔCq method.[31] Briefly, for each well, a ΔCq value was obtained by the difference in Cq values (ΔCq) between the target gene and the reference gene cdc-42 (cell division cycle 42 protein). The ΔCq mean value obtained from the control group of each gene was used to calculate the ΔΔCq of the respective gene ($2^{-\Delta\Delta Cq}$).

2.11.1 Feeding RNA interference of *daf-16* in neuronal survival assay. RNAi of *daf-16* was conducted using the feeding method already described elsewhere, with empty L4440 as the control.[32,33] Briefly, single colonies of HT115 bacteria containing L4440 plasmids were cultured in Luria Bertani (LB) medium containing 60 μg mL^{-1} ampicillin. On the following day, the culture was used to seed plates containing 100 μg mL^{-1} ampicillin and 1 mM isopropylthiogalactoside (IPTG). The synchronized HA759 L1 larvae worms were then placed on *E. coli* HT115 cells expressing *daf-16* or control RNAi at 20 °C and after 72 hours the neuronal survival assay was performed.

Cel cdc-42_F – CTGCTGGACAGGAAGATTACG
Cel cdc-42_R – CTCGGACATTCTCGAATGAAG

Table 1 Primers used in RT-PCR

Genes	Primers
cdc-42	(Forward) 5′-CTGCTGGACAGGAAGATTACG-3′ (Reverse) 5′-CTCGGACATTCTCGAATGAAG-3′
daf-16	(Forward) 5′-AACTTCAAGCCAATGCCACT-3′ (Reverse) 5′-TGCTGTGCAGCTACAATTCC-3′
sod-3	(Forward) 5′-TCGGTTCCCTGGATAACTTG-3′ (Reverse) 5′-TTCCAAAGGATCCTGGTTTG-3′
hsp-16.2	(Forward) 5′-CTGCAGAATCTCTCCATCTGAGTC-3′ (Reverse) 5′-AGATTCGAAGCAACTGCACC-3′

Cel cdc-42_F – CTGCTGGACAGGAAGATTACG
Cel cdc-42_R – CTCGGACATTCTCGAATGAAG
Cel cdc-42_F – CTGCTGGACAGGAAGATTACG
Cel cdc-42_R – CTCGGACATTCTCGAATGAAG
v Cel cdc-42_F – CTGCTGGACAGGAAGATTACG
Cel cdc-42_R – CTCGGACATTCTCGAATGAAG
Cel cdc-42_F – CTGCTGGACAGGAAGATTACG
Cel cdc-42_R – CTCGGACATTCTCGAATGAAG
Cel cdc-42_F – CTGCTGGACAGGAAGATTACG
Cel cdc-42_R – CTCGGACATTCTCGAATGAAG
Cel cdc-42_F – CTGCTGGACAGGAAGATTACG
Cel cdc-42_R – CTCGGACATTCTCGAATGAAG
Cel cdc-42_F – CTGCTGGACAGGAAGATTACG
Cel cdc-42_R – CTCGGACATTCTCGAATGAAG
vv Cel cdc-42_F – CTGCTGGACAGGAAGATTACG
Cel cdc-42_R – CTCGGACATTCTCGAATGAAG
Cel cdc-42_F – CTGCTGGACAGGAAGATTACG
Cel cdc-42_R – CTCGGACATTCTCGAATGAAG
Cel cdc-42_F – CTGCTGGACAGGAAGATTACG
Cel cdc-42_R – CTCGGACATTCTCGAATGAAG
Cel cdc-42_F – CTGCTGGACAGGAAGATTACG
Cel cdc-42_R – CTCGGACATTCTCGAATGAAG
Cel cdc-42_F – CTGCTGGACAGGAAGATTACG
Cel cdc-42_R – CTCGGACATTCTCGAATGAAG

2.12 Statistical analyses

Statistical analyses were performed using GraphPad Prism Version 6 for Windows (GraphPad Software, USA). Significance was assessed by one- or two-way analysis of variance (ANOVA) followed by Bonferroni's *post hoc* test. The significance for the survival analysis was assessed by the Kaplan–Meier curve followed by the log-rank test. Values of $p < 0.05$ were considered to be statistically significant.

3. Results

3.1 Effect of diphenyl diselenide exposure on polyQ-mediated toxicity

The *C. elegans* AM141 strain has polyQ40-YFP fusion proteins expressed in the muscle cells of the body wall, which aggregate with worm aging.[34,35] We used this transgenic model to investigate whether $(PhSe)_2$ is able to prevent polyQ aggregation at different times past the L1 stage. As shown in Fig. 1a and b, the number of polyQ40::YFP aggregates was significantly decreased after treatment at all times analyzed. The most significant reduction in the number of aggregates was 33.51% at 25 and 50 μM $(PhSe)_2$ and 34.05% at 100 μM $(PhSe)_2$ compared to the control group after 120 h ($p < 0.05$; Fig. 1b).

The *C. elegans* HA759 strain was used to evaluate the protective effect of $(PhSe)_2$ against polyQ-mediated neurotoxicity in ASH sensory neurons. Fig. 2a demonstrates an increase in neuronal survival in treated worms. This effect was more pronounced in the group treated with 100 μM $(PhSe)_2$ for 120 h, which presented 48.67% of worms with

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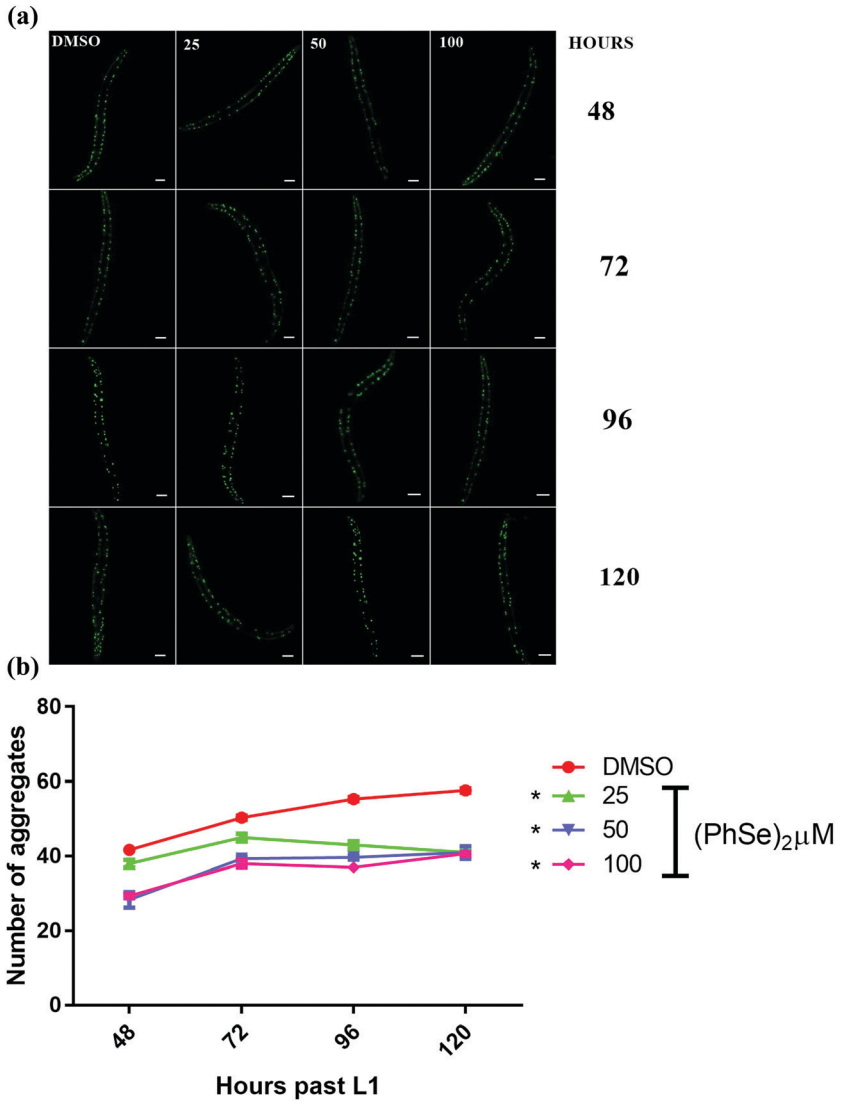

Fig. 1 Diphenyl diselenide decreases the PolyQ aggregation in the body wall muscles. Number of Q40::YFP aggregates in worms of the AM141 strain after treatment with $(PhSe)_2$ starting at L1 for the indicated times. (a) Representative images, and (b) number of Q40::YFP aggregates. Scale bars are 100 μm. Data are the average of three independent assays performed each time with 10 worms (n = 30) and are represented as means ± SEM. * means a significant difference from the control DMSO group by two-way ANOVA followed by Bonferroni's *post hoc* test, $p < 0.05$ for all time points.

neurodegeneration and the control group 93.34% at the same time ($p < 0.05$; Fig. 2b).

3.2 Effect of diphenyl diselenide exposure on touch response behavior

The ASH sensory neurons in *C. elegans* are required for a wide range of avoidance behaviors in response to chemical repellents, highly osmotic solutions and nose touch. The ASH neurons are therefore hypothesized to be polymodal nociceptive neurons.[36] In the HA759 strain, a reduction in the touch response was observed starting at 72 h after the L1 stage in the control worms, while the $(PhSe)_2$-treated groups had a significant increase in the touch response at all concentrations evaluated, being more pronounced at 100 μM $(PhSe)_2$ after 120 hours ($p < 0.05$; Fig. 3a). No differences were observed between untreated and treated worms in the N2 strain ($p < 0.05$; Fig. 3b).

3.3 Effect of diphenyl diselenide on ROS levels

Oxidative stress is correlated with age-dependent diseases, and thus maintaining a balance between ROS generation and anti-oxidant defenses is essential.[37] Therefore, we measured the ROS levels in wild-type (N2), AM141, and HA759 strains. Analyses were performed 48, 72, 96 and 120 h after the L1 stage. There was a significant decrease in ROS levels in the $(PhSe)_2$-treated groups compared to the control at all times and concentrations analyzed for the N2, AM141 and HA759 strains ($p < 0.05$; Fig. 4a–c).

3.4 Effect of diphenyl diselenide exposure on worm lifespan and health span

Aging is known to play an important role in the process of neurodegeneration.[38] Therefore, we investigated whether $(PhSe)_2$ could affect the lifespan and health span of N2, AM141 and

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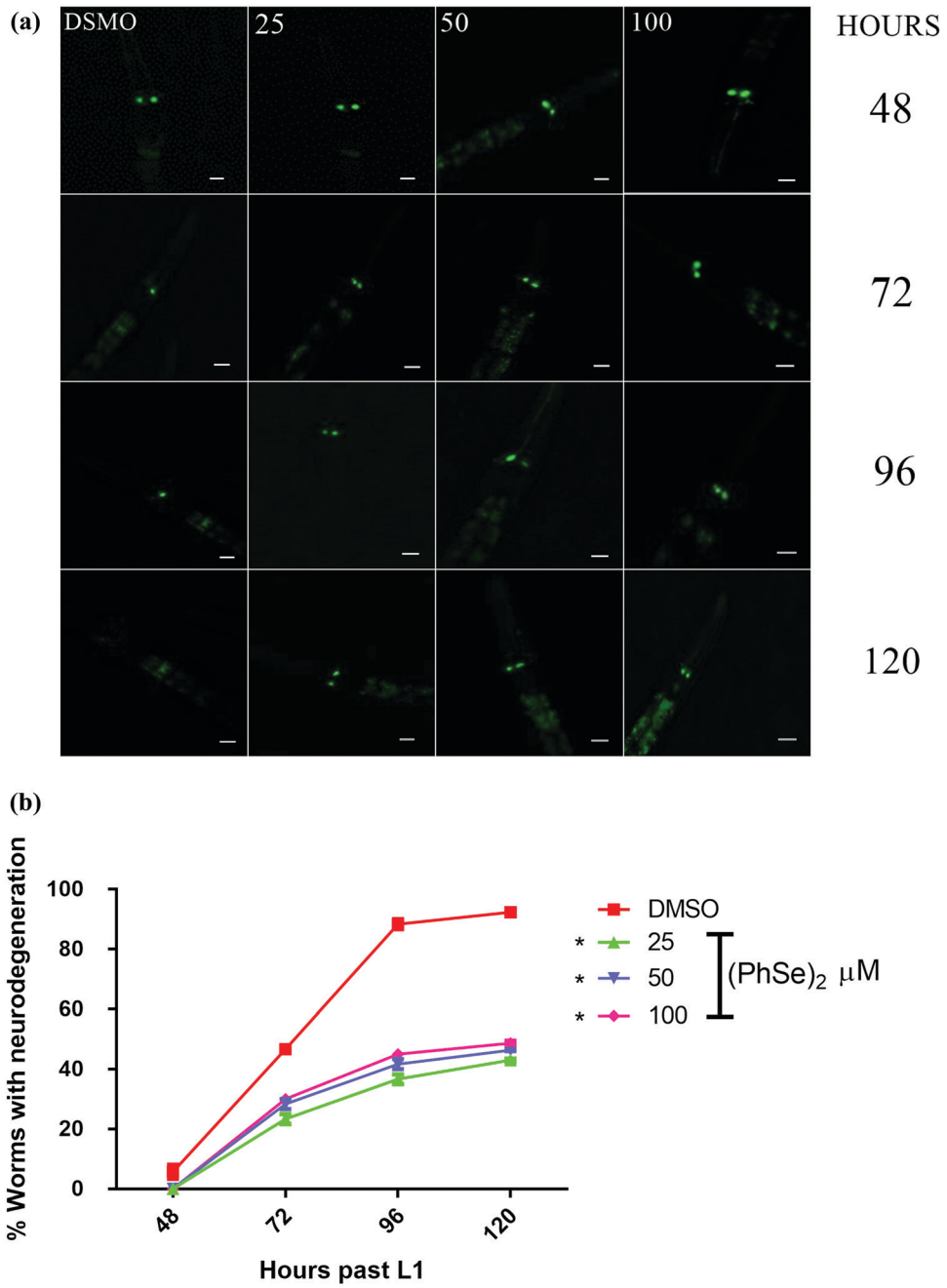

Fig. 2 Diphenyl diselenide protects against polyQ-induced neurodegeneration of ASH neurons. HA759 worms were treated with $(PhSe)_2$ or the vehicle from L1 for the indicated times. Loss of fluorescence of ASH neurons indicates neurodegeneration. (a) Representative images and (b) percentage of worms with neurodegeneration. Scale bars are 100 μm. Data are the average of three independent assays performed each time with 10 worms (n = 30) and are represented as means ± SEM. * means a significant difference from the control DMSO group by two-way ANOVA followed by Bonferroni's *post hoc* test, $p < 0.05$ for all time points.

HA759. The maximum lifespan of wild-type *C. elegans* increased from 28 to 30 days (7.1%) after treatment with $(PhSe)_2$ at all concentrations tested ($p < 0.0001$, Fig. 5a). For the AM141 and HA759 mutant strains, the lifespan also increased from 28 to 30 days (7.1%) after $(PhSe)_2$ treatment at all concentrations tested ($p < 0.05$; Fig. 5b).

It is desirable that an extension in lifespan be accompanied by an extension in the health span, which is the general state of health and vigor until the end of life. Extending the health time and generating resistance to stress is a goal in neurodegenerative diseases such as HD. The health span of the worms was also prolonged after $(PhSe)_2$ treatment. Specifically, treatment with $(PhSe)_2$ delayed the age-related decline in pharyngeal pumping and the number of thrashes starting on day 7 of adulthood in the N2 strain (Fig. 6a and d), as well as in mutant strains AM141 (Fig. 6b and e) and HA759 (Fig. 6c and f).

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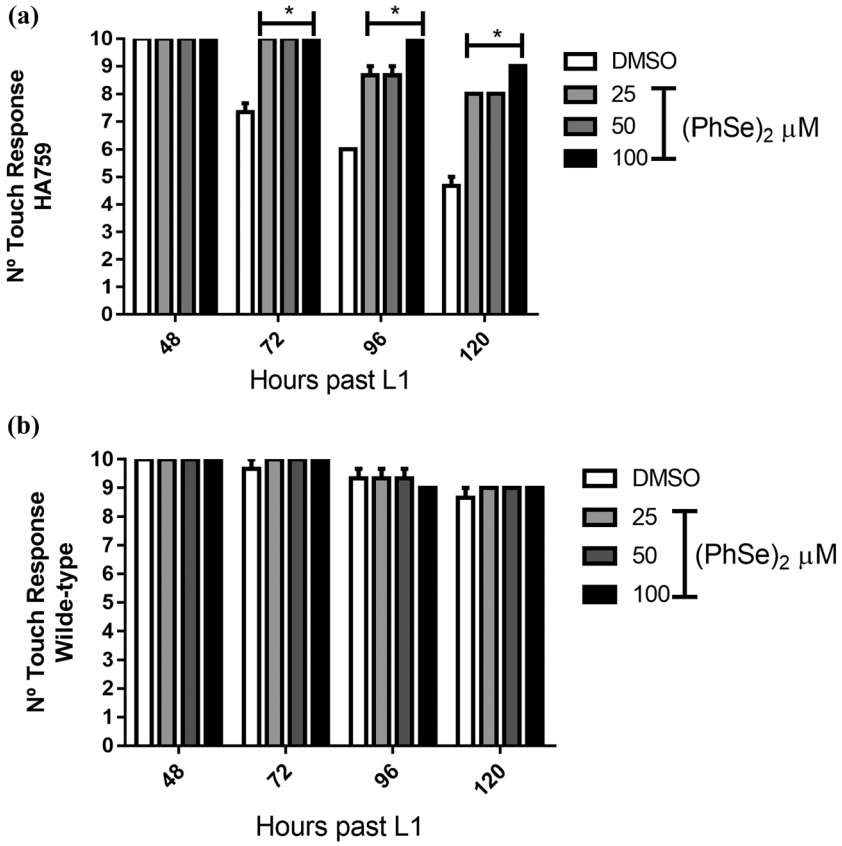

Fig. 3 Effect of diphenyl diselenide on the touch response. Number of touch responses after treatment with $(PhSe)_2$ starting at L1 for the indicated times in (a) HA759 and (b) wild-type (N2) strains. Data are the average of three independent assays performed each time with 10 worms (n = 30) with a total of 10 touches performed per worm. Data are represented as means ± SEM. * means a significant difference from the control DMSO group by two-way ANOVA followed by Bonferroni's *post hoc* test, $p < 0.05$.

3.5 Diphenyl diselenide treatment induced DAF-16/FOXO activation in worms

DAF-16 is the sole ortholog of the FOXO family of transcription factors in the nematode *C. elegans*. When activated, DAF-16/FOXO translocates to the nucleus, upregulating genes involved in longevity, heat shock survival and oxidative stress responses. Representative images with the localization of DAF-16::GFP are shown in Fig. 7a, white arrows indicate nuclear translocation and blue arrows cytoplasmic localization. After treatment with $(PhSe)_2$, we observed a significant migration of DAF-16 to the nucleus at all concentrations analyzed; 76%, 85% and 87% of the animals treated with 25, 50 and 100 μM $(PhSe)_2$ respectively, presented DAF-16 nuclear localization. Heat-shock conditions (positive control) induced a nuclear localization of DAF-16 in 100% of worms (Fig. 7b).

3.6 Treatment with diphenyl diselenide induced SOD-3 and HSP-16.2 overexpression

SOD-3 is a mitochondrial superoxide dismutase enzyme that protects against oxidative stress.[39] SOD-3 expression is under direct control of DAF-16,[40] the major antioxidant pathway in *C. elegans*. Given this, we investigated whether $(PhSe)_2$ would positively regulate the expression of SOD-3. Worms treated with $(PhSe)_2$ had an increase of 98.59%, 91.56% and 95.75% in GFP-labeled SOD-3 fluorescence in relation to the DMSO control at 25, 50 and 100 μM $(PhSe)_2$, respectively ($p < 0.05$, Fig. 8a and b).

Chaperones play a central role in proteostasis, as they have been shown to be essential to prevent proteotoxic accumulation, as occurs in HD. We investigated whether $(PhSe)_2$ would increase HSP-16.2 expression in *C. elegans*. Animals treated with $(PhSe)_2$ showed an increase of 67.80%, 73.27% and 68.81% in GFP-labeled HSP-16.2 fluorescence in relation to the DMSO control at 25, 50 and 100 μM $(PhSe)_2$, respectively ($p < 0.05$, Fig. 8c and d).

3.7 Treatment with diphenyl diselenide increased *daf-16*, *hsp-16.2* and *sod-3* mRNA levels

After 72 h $(PhSe)_2$ treatment, the wild type and mutant worms had an increase in *daf-16*, *hsp-16.2* and *sod-3* mRNA levels compared to the DMSO control.

The *daf-16* mRNA levels had an increase of 15.03%, 59.71% and 90.57% in N2 worms treated with $(PhSe)_2$ compared to the control at 25, 50 μM and 100 μM $(PhSe)_2$, respectively ($p < 0.05$ Fig. 9a). In the AM141 strain the increase was 64.71%, 92.84% and 100% in the treated worms compared to the control at 25, 50 μM and 100 μM $(PhSe)_2$, respectively ($p < 0.05$, Fig. 9b), and in the HA759 strain the increase was

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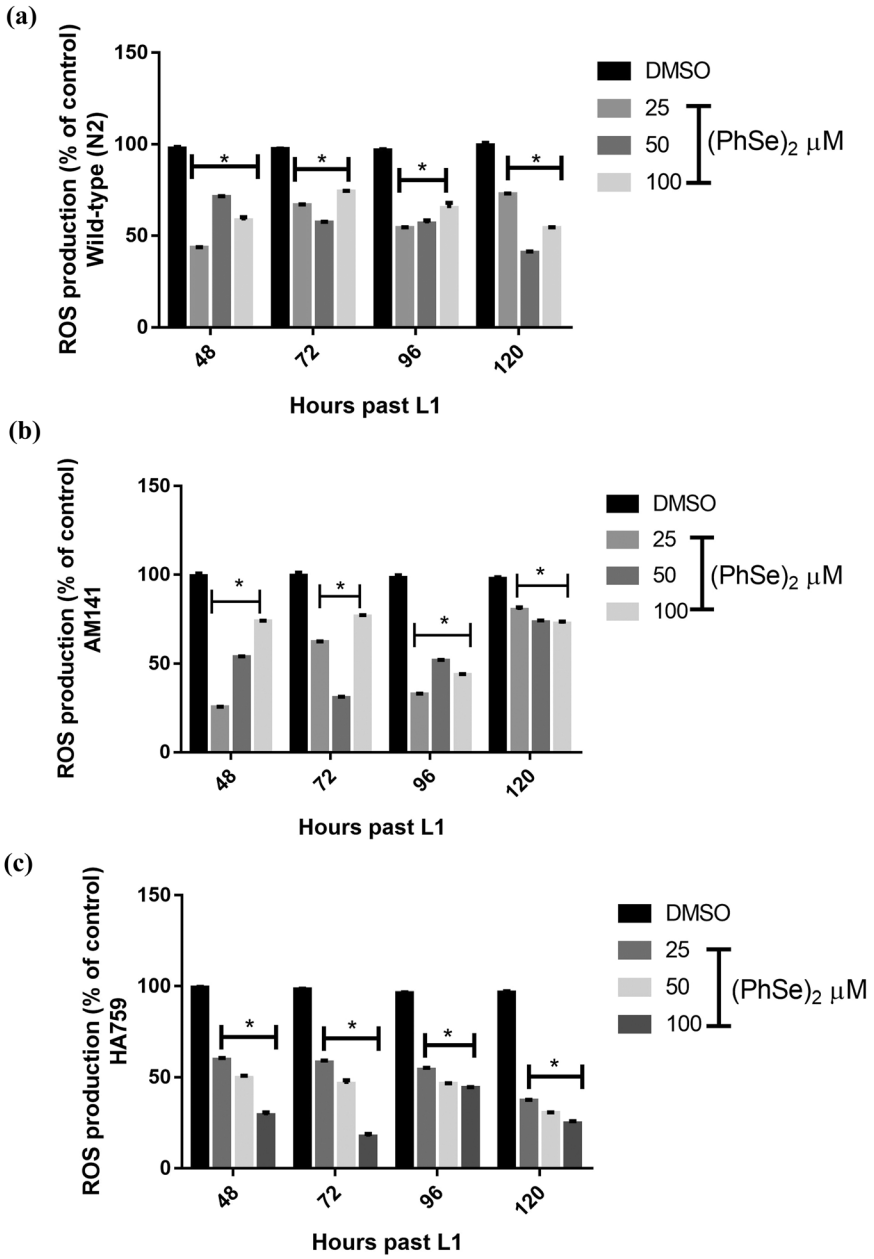

Fig. 4 Diphenyl diselenide decreases reactive oxygen species (ROS) levels in *C. elegans*. ROS levels were measured after treatment with $(PhSe)_2$ starting at L1 for the indicated times in (a) wild-type (N2), (b) AM141 and (c) HA759 strains. Data are the average of five independent assays performed each time with 100 worms in triplicate ($n = 5$) and are represented as means ± SEM. * means a significant difference from the control DMSO group by two-way ANOVA followed by Bonferroni's *post hoc* test, $p < 0.05$.

100.65%, 159.32% and 300.87% in the treated worms compared to the control at 25, 50 μM and 100 μM $(PhSe)_2$, respectively ($p < 0.05$, Fig. 9c).

The *sod-3* mRNA levels had an increase of 91.02%, 192.84% and 203.65% in N2 worms treated with $(PhSe)_2$ compared to the control at 25, 50 μM and 100 μM $(PhSe)_2$, respectively ($p < 0.05$, Fig. 9d). In the AM141 strain the increase was 180.56%, 219.84% and 257.65% in the treated worms compared to the control at 25, 50 μM and 100 μM $(PhSe)_2$, respectively ($p < 0.05$, Fig. 9e), and in the HA759 strain the increase was 70.56%, 94.84% and 135.54% in the treated worms compared to the control at 25, 50 μM and 100 μM $(PhSe)_2$, respectively ($p < 0.05$, Fig. 9f).

The *hsp-16.2* mRNA levels had an increase of 108.02%, 144.28% and 155.59% in N2 worms treated with $(PhSe)_2$ compared to the control at 25, 50 μM and 100 μM $(PhSe)_2$, respectively ($p < 0.05$ Fig. 9g). In the AM141 strain the increase was 372.25%, 467.75% and 661.11% in the treated worms compared to the control at 25, 50 μM and 100 μM $(PhSe)_2$, respectively ($p < 0.05$, Fig. 9h), and in the HA759 strain the increase was 158.56%, 212.66% and 304.49% in the treated worms compared to the control at 25, 50 μM and 100 μM $(PhSe)_2$, respectively ($p < 0.05$, Fig. 9i).

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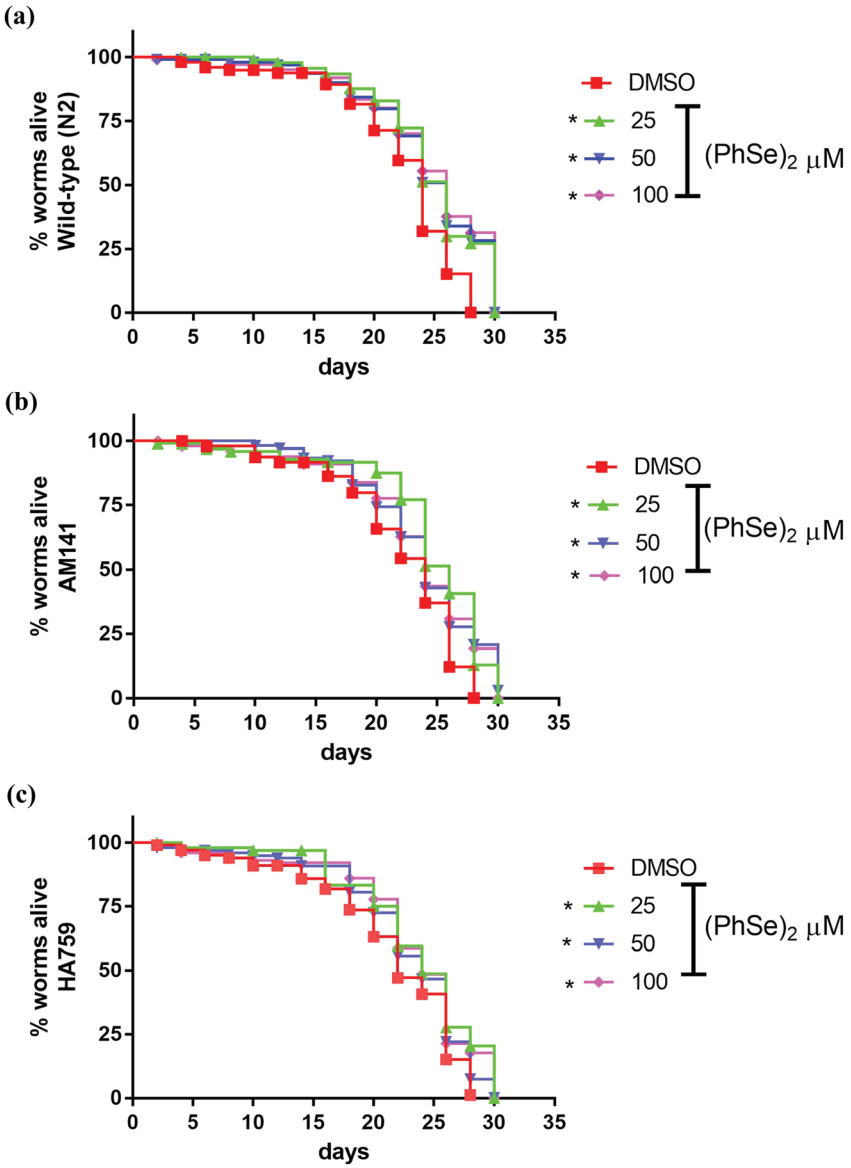

Fig. 5 Diphenyl diselenide increases the lifespan of *Caenorhabditis elegans*. *C. elegans* lifespan of (a) wild-type (N2), (c) AM141 and (c) HA759 strains after treatment with $(PhSe)_2$ starting at L1 for the whole life. The percentage of worms alive was analyzed every 2 days and data are the average of three independent assays with 100 worms in each (n = 300). * represents a significant difference from the control by the log-rank test (Mantel-Cox), $p < 0.0001$ for N2 and $p < 0.05$ for AM141 and HA759 strains.

3.8 Protective effect of diphenyl diselenide against polyQ-mediated neurotoxicity is dependent on DAF-16

To explore the role of DAF-16 in the $(PhSe)_2$ protective effects against polyQ-mediated neurotoxicity, we carried out *daf-16* knockdown through RNAi in the HA759 strain. The results are shown in Fig. 10a and b. It is possible to observe that RNAi of *daf-16* completely inhibited the beneficial effects of $(PhSe)_2$.

4. Discussion

A variety of neurodegenerative diseases, including AD, PD and HD, are known to share some common molecular mechanisms, such as pathogenic protein aggregation and oxidative stress.[41] In the present study, we investigated the protective properties of $(PhSe)_2$ on polyQ-induced toxicity in *C. elegans*. We began by analyzing the number of polyQ aggregates in a *C. elegans* model of HD, and we observed that $(PhSe)_2$ decreased the protein aggregation at all concentrations (25, 50 and 100 μM) and time points (48, 72, 96 and 120 h) tested in worms of the AM141 strain treated from L1 (Fig. 1). We then analyzed the survival of ASH neurons in HA759 worms, which express Http-Q150 (polyQ150 sequence derived from human huntingtin) fused with GFP. Treatment with $(PhSe)_2$ reduced the neurodegeneration caused by Htt-Q150 at all concentrations (Fig. 2), and also maintained the function of ASH sensory neurons, observed in the touch response assay (Fig. 3).

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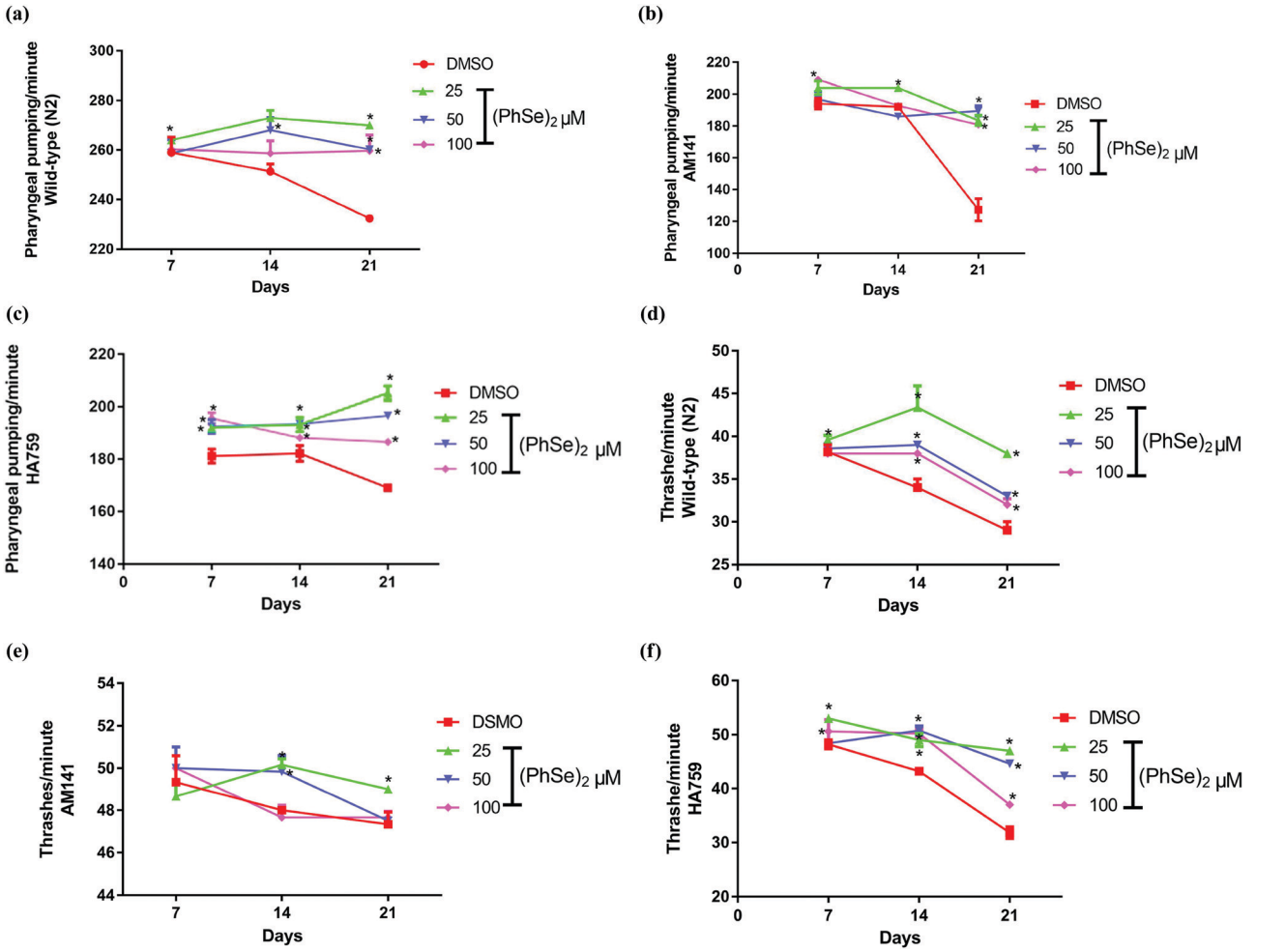

Fig. 6 Diphenyl diselenide prolonged the health span of *Caenorhabditis elegans*. Behavioral parameters related to health span were analyzed after treatment with $(PhSe)_2$ starting at L1 for the indicated times. Pharyngeal pumping rate in (a) wild-type (N2), (b) AM141 and (c) HA759 strains; and thrash frequency in (d) wild-type (N2), (e) AM141 and (f) HA759 strains during aging. Data are the average of three independent assays performed each time with 10 worms in triplicate (n = 30) and are represented as means ± SEM. * means a significant difference between untreated and $(PhSe)_2$ treated worms, by two-way ANOVA followed by Bonferroni's *post hoc* test, $p < 0.05$.

Some studies suggest that ROS play a central role in neuropathologies, specifically in neuronal degeneration and disease progression.[42] Our data demonstrate that treatment with $(PhSe)_2$ decreased the ROS levels of wild type (N2) and PolyQ transgenic strains at all times and concentrations analyzed (Fig. 4a–c). Although oxidative damage to neurons may not be the primary event that initiates neurodegenerative disorders, it seems that oxidative stress participates in the pathogenic cascade of these diseases. The production of ROS is also correlated with aging and age-related diseases, and maintaining a balance in ROS generation is essential for maintaining homeostasis.[43]

The onset of polyQ aggregation and proteotoxicity is age-dependent and might be delayed by extending the lifespan.[44] We analyzed the *C. elegans* lifespan and some parameters related to the health span. It was already shown that mutant strains for HD have the same lifespan as N2.[24] Herein, treatment with $(PhSe)_2$ extended the lifespan of N2, HA759 and AM141 worms at all concentrations tested compared to the vehicle (Fig. 5a–c). Genetic studies with *C. elegans* showed a positive association between aging delay and neuroprotection,[45] as demonstrated in our results with the reduction of PolyQ toxicity during aging.

Moreover, we observed that $(PhSe)_2$ delayed the decline in pharyngeal pumping and the number of thrashes during aging starting on day 7 of adulthood (Fig. 6). It is important that a compound not only increases longevity but also extends the health span, which is the period of general health and vigor into late life.[46] Extending the health span and generating resistance to stress is a goal in neurodegenerative disease treatment.[47] The slower decline of locomotion in $(PhSe)_2$-treated worms during aging compared to the vehicle might be associated with the reduction in the number of polyQ aggregates in body wall muscle cells of AM141 mutants, as well as with the maintenance of neuron integrity in HA759 worms. Decreased locomotion is a conserved feature of aging, as well as of HD. Studies showed that accumulation of structural damage to proteins results in incorrect folding, denaturation

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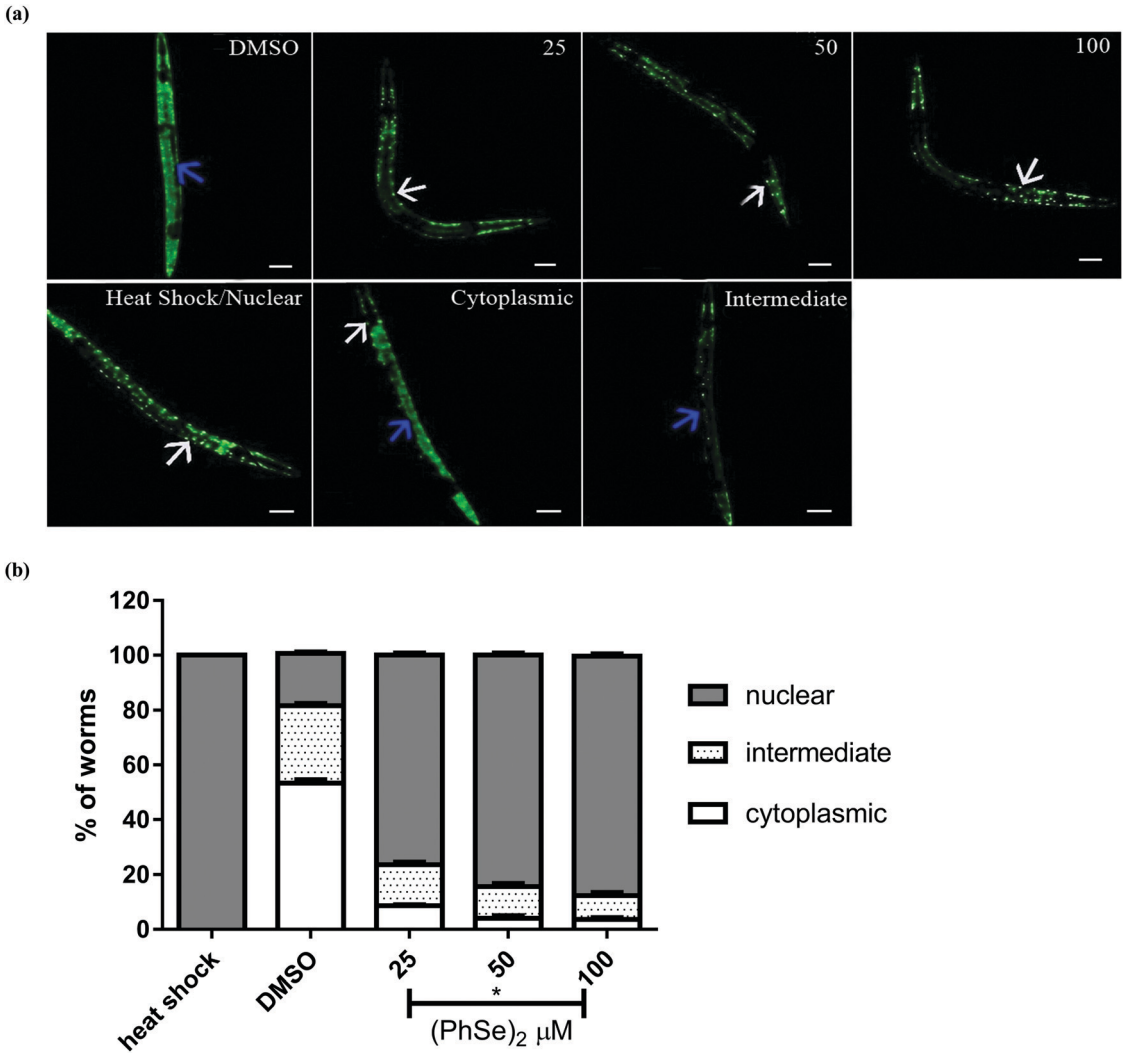

Fig. 7 Diphenyl diselenide induced DAF-16/FOXO activation in worms. Localization of DAF-16::GFP in the TJ356 *C. elegans* strain following $(PhSe)_2$ treatment from L1 until adulthood. Scale bars are 100 μm. (a) Representative images of the groups. White arrows indicate nuclear translocation and blue arrows cytoplasmic localization. (b) Percentage of worms with nuclear, intermediate and cytoplasmic localization of DAF-16::GFP. Worms exposed to heat shock at 35 °C for 1 h were used as a positive control. Data are the average of three independent assays performed each time with 10 worms (n = 30) and are represented as means ± SEM. * means a significant difference between DMSO and $(PhSe)_2$ treated worms, by two-way ANOVA followed by Bonferroni's *post hoc* test, $p < 0.05$.

and aggregation, which leads to locomotor deficits characteristic of neurodegenerative diseases.[48]

The DAF-16 transcription factor, a *C. elegans* homolog of mammalian Forkhead box (FOXO), is thought to be the main target of DAF-2, an insulin/insulin-like growth factor (IGF)-1 receptor homolog. Previous studies reported that DAF-16 plays a pivotal role in the regulation of longevity and is also involved in the formation of less toxic high-molecular-weight protein aggregates,[49] through the activation of antioxidant genes and chaperones, thereby ameliorating polyQ aggregation and toxicity.[50] Our study showed that $(PhSe)_2$ activated DAF-16 transcription factor, since it was translocated to the nucleus (Fig. 7). A previous study demonstrated selenite induced nuclear translocation of the DAF-16/FOXO transcription factor in *C. elegans*.[51] We also demonstrated that $(PhSe)_2$ treatment increased the SOD-3 and HSP-16.2 expression at all concentrations tested (Fig. 8), and caused a large increase in *daf-16*, *sod-3* and *hsp-16.2* mRNA levels in wild type and mutant worms treated with $(PhSe)_2$ (Fig. 9).

When oxidative stress arises as a consequence of a pathological event, there is an energy imbalance associated with changes in the mitochondrial respiratory chain, leading to mitochondrial dysfunction,[52] which has been reported in HD patients. Huntingtin protein aggregates cause changes in the normal flow of mitochondrial traffic, leading to the accumulation of polyQ and making protein aggregates more immobile and functionally inactive.[53] SOD-3 is an ortholog of human MnSOD (SOD-2), involved in the removal of superoxide radicals, and is located in the mitochondrial respiratory chain supercomplex.[54] The role of SOD-3 has been reported to be

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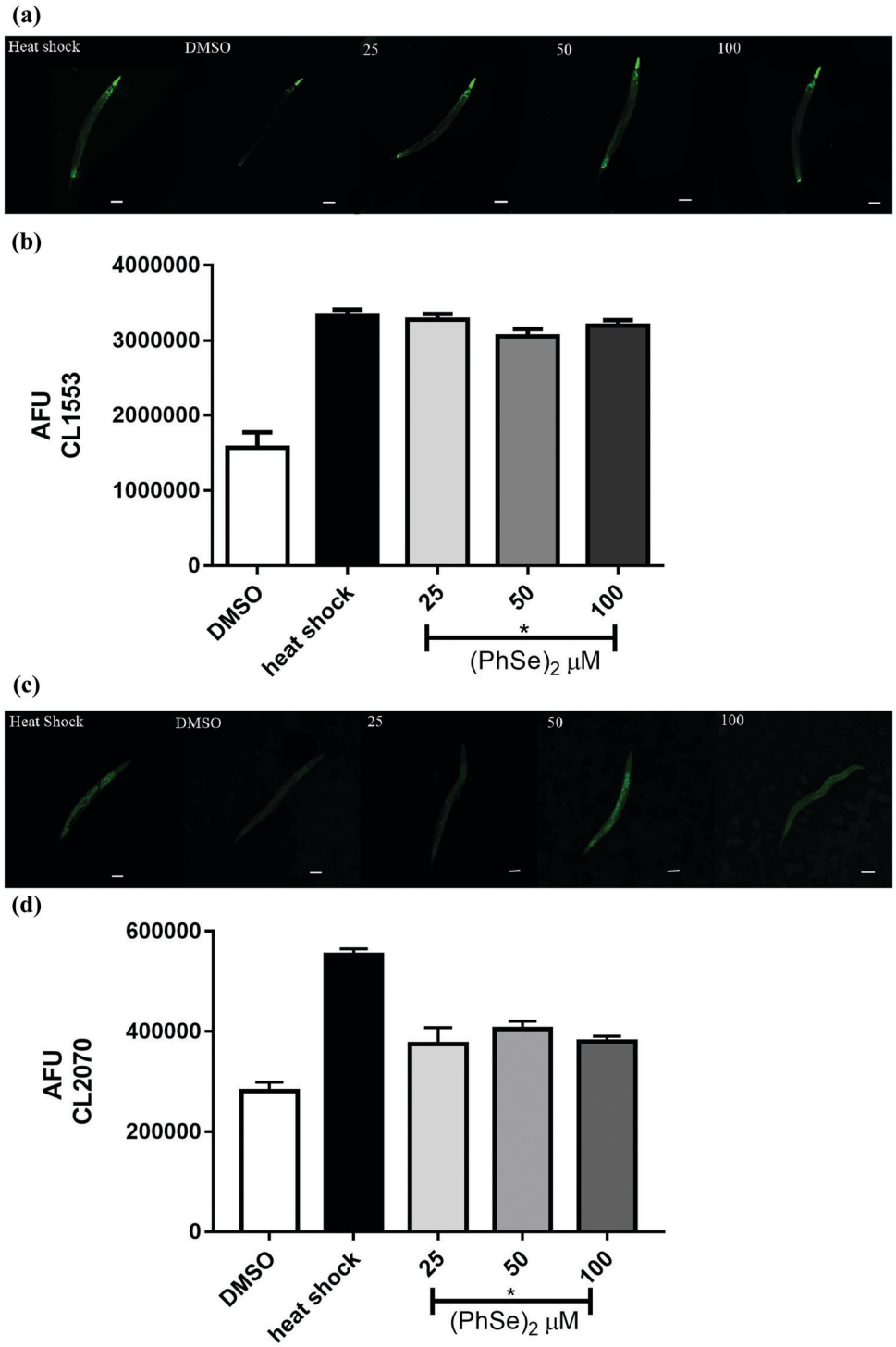

Fig. 8 Diphenyl diselenide induced SOD-3 and HSP-16.2 overexpression. *C. elegans* was treated with $(PhSe)_2$ starting at L1 until adulthood. (a) Representative images of SOD-3::GFP and (b) fluorescence quantification of SOD-3::GFP in the transgenic strain CF1553. (c) Representative images of HSP-16.2::GFP and (d) fluorescence quantification of HSP-16.2::GFP in the transgenic strain CL2070. Worms exposed to heat shock at 35 °C for 1 h were used as positive controls. Scale bars are 100 μm. Data are the average of three independent assays performed each time with 10 worms (n = 30) and are represented as means of arbitrary fluorescence units (AFU) per worm ± SEM. * means a significant difference between DMSO and $(PhSe)_2$ treated worms, by two-way ANOVA followed by Bonferroni's *post hoc* test, $p < 0.05$.

essential in age-related diseases, and MnSOD overexpression efficiently protected mice from 3-nitropropionic acid induced anatomical and metabolic deficits that mimic HD.[55]

Genetic analysis has shown that DAF-16 is one of the essential transcriptional activators for a subset of chaperones, especially HSP-16.2.[56] HSP-16.2 plays a protective role in polyQ

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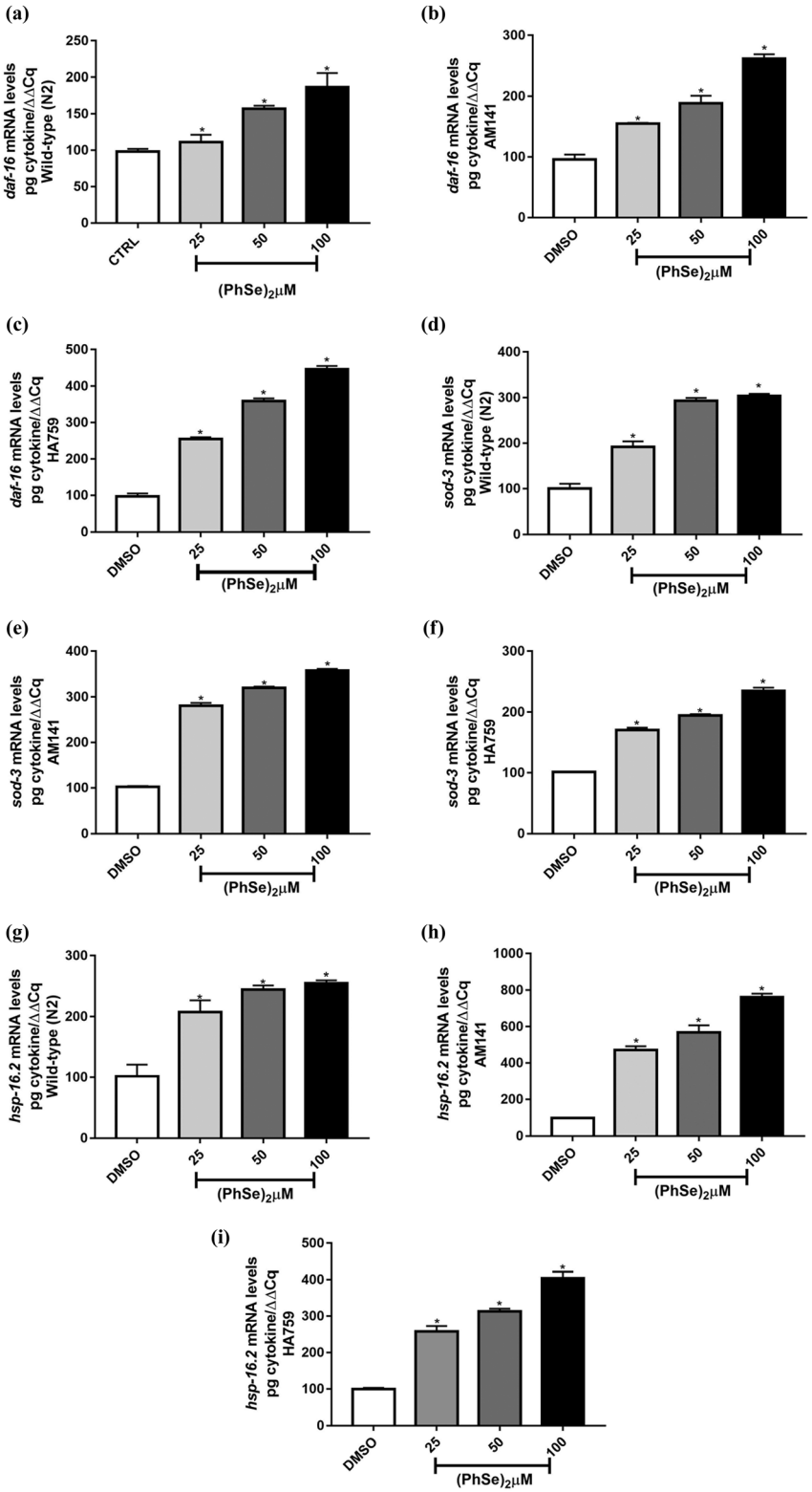

Fig. 9 Treatment with diphenyl diselenide increased the expression of *daf-16*, *sod-3* and *hsp-16.2*. *C. elegans* was treated with $(PhSe)_2$ starting at L1 for 72 hours, and mRNA levels were determined by quantitative real-time PCR using a ΔΔCq method. Expression levels of *daf-16* mRNA in (a) wild-type (N2), (b) AM141 and (c) HA759 strains. Expression levels of *sod-3* mRNA in (d) wild-type (N2), (e) AM141 and (f) HA759 strains. Expression levels of *hsp-16.2* mRNA in (g) wild-type (N2), (h) AM141 and (i) HA759 strains. Data are the percentage of the control derived from three independent assays performed each time with 4000 worms in triplicate ($n = 3$) and are represented as means ± SEM. * means a significant difference between DMSO and $(PhSe)_2$ treated worms, by two-way ANOVA followed by Bonferroni's *post hoc* test, $p < 0.05$.

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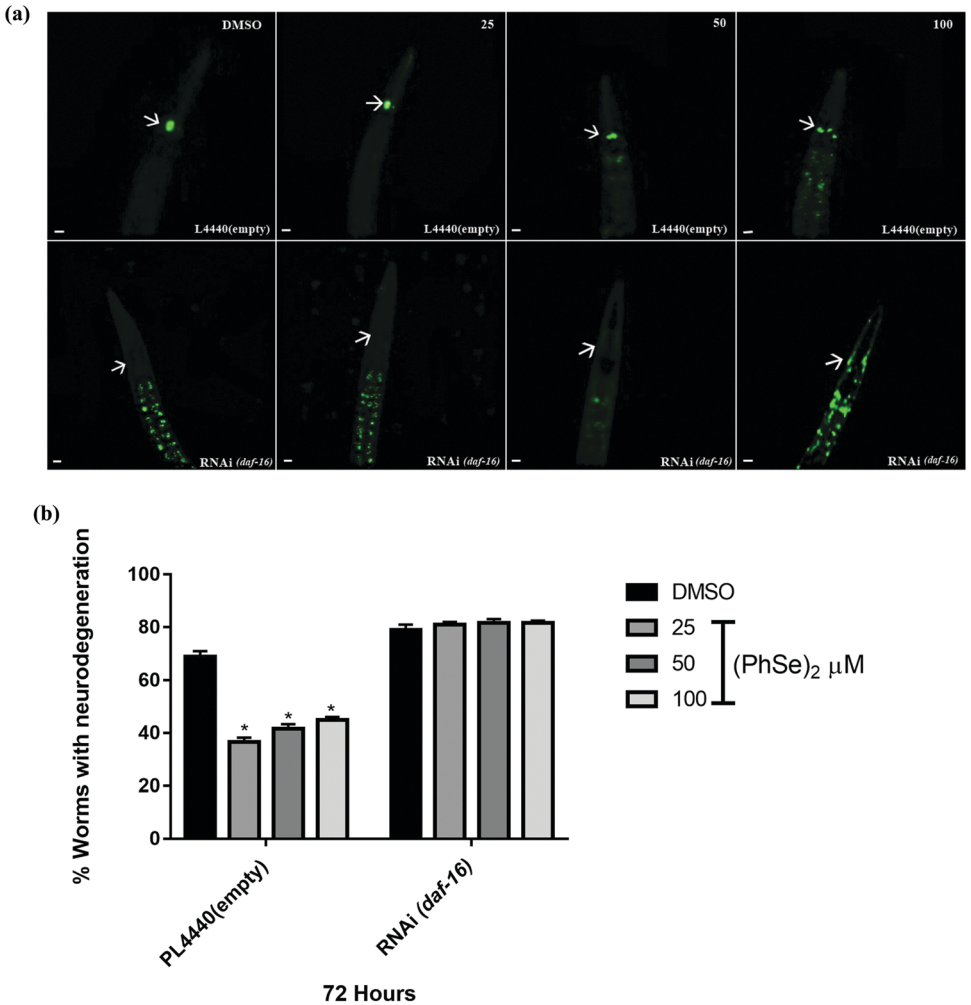

Fig. 10 Protective effect of diphenyl diselenide against polyQ-mediated neurotoxicity depends on DAF-16. The L1 worms from the HA759 strain were fed with PL4440 empty vector or RNAi for *daf-16* and treated with $(PhSe)_2$ or the vehicle for 72 hours. (a) Representative images, arrows indicate live ASH neurons in the PL4440 empty vector and neurodegeneration in RNAi for *daf-16* groups. (b) Percentage of worms with neurodegeneration. Scale bars are 100 μm. Data are the average of three independent assays performed each time with 10 worms (n = 30) and are represented as means ± SEM. * means a significant difference from the DMSO group by two-way ANOVA followed by Bonferroni's *post hoc* test, $p < 0.05$.

diseases, as it promotes folding/refolding of proteins into appropriate conformations, and recovering previously aggregated proteins.[57] Molecular chaperones are also evolutionarily conserved in the cellular response to stress and the regulation of longevity.[58] Some studies show a direct role of these components in the cellular stress response associated with the regulation of lifespan. Overexpression of individual molecular chaperones in *Drosophila melanogaster* and *C. elegans* has been shown to extend the lifespan.[59]

To elucidate the involvement of DAF-16 in $(PhSe)_2$ reduced poly-Q-mediated neurotoxicity, we carried out RNAi for *daf-16* in the HA759 strain. Inactivation of *daf-16* transcription factor blocked the neuroprotective effect of $(PhSe)_2$. Previous studies reported that the knocking down of *daf-2* reduced polyQ toxicity in a DAF-16 dependent manner.[60] Whereas neurons are reported to be resistant to RNAi,[61] we believe the neuroprotective effect induced by $(PhSe)_2$ through DAF-16 activation is not neuron specific, and depends on all worm tissues acting cooperatively or cumulatively (a non-autonomous response).[61]

Therefore, we propose that $(PhSe)_2$ could act through DAF-16 activation to increase the antioxidant capacity and regulation of proteostasis, decreasing PolyQ aggregation and toxicity, and reducing ROS levels, leading to an increase in lifespan, and healthspan.

5. Conclusions

Herein we demonstrated that $(PhSe)_2$ can protect against PolyQ-induced toxicity in *C. elegans* models of HD. The compound reduced neuronal death and maintained the function of ASH sensory neurons in HA759 worms, and decreased polyQ aggregation in AM141 mutants. Furthermore, $(PhSe)_2$ was able to reduce the levels of ROS, and extend the lifespan and health span of wild type and PolyQ mutant worms. These protective

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effects might be attributed to insulin pathway modulation through DAF-16 activation in whole body tissues, and induction of other transcription factors, such as the HSP-16.2 chaperones and the antioxidant enzyme SOD-3. Our findings provide new clues for treatment strategies for neurodegenerative diseases and other diseases caused by age-related protein aggregation.

Author contributions

Fabiane Bicca Obetine Baptista and Larissa Marafiga Cordeiro participated in the research design. Fabiane Bicca Obetine Baptista, Aline Franzen da Silva and Tássia Limana da Silveira conducted the experiments. Fabiane Bicca Obetine Baptista, Leticia Priscilla Arantes and Marina Lopes Machado performed data analysis or contributed to interpretation of results. Fabiane Bicca Obetine Baptista, Leticia Priscilla Arantes, Marina Lopes Machado and Felix Alexandre Antunes Soares wrote or revised the paper.

Conflicts of interest

There are no conflicts to declare.

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5. CONCLUSÃO

Portanto, o presente estudo demostrou que nos modelos transgênicos poliQ em *C. elegans*, o composto $(PhSe)_2$ é capaz de reduzir a morte neuronal bem como manteve a resposta ao toque nos vermes HA759, sugerindo a manutenção dos neurônios sensoriais da ASH e impedindo sua neurodegeneração. No entanto, o efeito neuroprotetor de $(PhSe)_2$ está associado à reduzida agregação de poliQ através da ativação da translocação da DAF-16 mediada pela enzima antioxidante SOD-3 e pela ativação da proteína de choque térmico HSP-16.2. Além disso, mostramos que, devido à sua modulação da DAF-16, o composto pode reduzir os níveis de ROS e, assim, aumentar a longevidade dos vermes e a qualidade de vida. Portanto, nossos dados indicam que $(PhSe)_2$ exerce efeitos protetores contra a toxicidade induzida por PolyQ no modelos *C. elegans* através da via de sinalização de ativação de insulina / IGF1 (IIS).

6. PERSPECTIVAS

Com base nos dados encontrados na presente dissertação, tem-se como perspectivas continuar a investigação dos efeitos de $(PhSe)_2$ no nematoide *C. elegans*, bem como aprofundar seu mecanismo diante a doenças neurodegenerativas, além de testes de moléculas que tenham potencial para proteger frente a doenças neurodegenerativas.

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