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Larissa Marafiga Cordeiro

AÇÃO DA RUTINA EM UM MODELO DE EXPOSIÇÃO AOS METAIS COBRE E ZINCO EM *Caenorhabditis elegans* COM SUPERPRODUÇÃO DE HUNTINGTINA

Santa Maria, RS, Brasil

2023

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

Orientador: Prof. Dr. Félix Alexandre Antunes Soares

Santa Maria, RS

2023

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_____
Félix Alexandre Antunes Soares, Dr. (UFSM)
(Presidente/Orientador)

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Denis Broock Rosemberg, Dr. (UFSM)

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Paula Rossini Augusti, Dra. (UFRGS)

Santa Maria, RS
2023

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Daqui a milhões de anos, quando os humanos inevitavelmente também forem extintos, que sejamos lembrados como uma espécie gloriosa e não como uma cicatriz profunda na história do planeta.

(Tito Aureliano em Realidade Oculta)

APRESENTAÇÃO

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

O DESENVOLVIMENTO da tese está apresentado sob a forma de dois artigos, os quais se encontram alocados no item ARTIGOS CIENTÍFICOS. As seções Materiais e Métodos, Resultados, Discussão, Conclusão e Referências Bibliográficas, encontram-se nos próprios artigos e representam a íntegra deste estudo.

O item DISCUSSÃO apresenta interpretações e comentários gerais sobre os trabalhos científicos aqui incluídos.

Os itens CONCLUSÕES e PERSPECTIVAS, encontrados no final desta tese, apresentam interpretações e comentários gerais sobre a investigação desenvolvida.

As REFERÊNCIAS BIBLIOGRÁFICAS referem-se somente às citações que aparecem no item INTRODUÇÃO e DISCUSSÃO, uma vez que o artigo científico contém as suas próprias referências.

RESUMO

AÇÃO DA RUTINA EM UM MODELO DE EXPOSIÇÃO AOS METAIS COBRE E ZINCO EM *Caenorhabditis elegans* COM SUPERPRODUÇÃO DE HUNTINGTINA

AUTORA: Larissa Marafiga Cordeiro
ORIENTADOR: Professor Dr. Félix Alexandre Antunes Soares

A doença de Huntington (DH) é uma doença neurodegenerativa progressiva e hereditária causada devido a uma mutação no gene da huntingtina que contém uma expansão anormal das repetições da citosina-adenina-guanina (CAG), levando a uma cadeia de poliglutamina (poliQ) de comprimento variável. A mutação confere funções tóxicas à proteína huntingtina mutante, causando neurodegeneração. A patologia de DH resulta em perda de células neuronais, alterações motoras, demência e atualmente é uma doença sem cura. É sabido que o acúmulo de metais é encontrado nas regiões patologicamente afetadas de muitas doenças neurodegenerativas. Os metais essenciais Cobre e Zinco são necessários em pequenas concentrações para funções metabólicas, podendo, entretanto, produzir efeitos tóxicos em concentrações elevadas. Além disso existe uma grande preocupação quanto a poluição ambiental por metais, sendo que os ambientes urbanos são locais sujeitos a significativos níveis de contaminação e, consequentemente, são as áreas de maior exposição de metais a população, seja pela inalação de poeira ou pela ingestão de solo contaminado através de alimentos. A rutina é um flavonóide encontrado em várias plantas, como o trigo sarraceno, alguns chás e frutas, sendo assim, de fácil acesso e baixo custo a toda população. Portanto, esse trabalho investigou o efeito da rutina sobre efeitos neurotóxicos causados por Cobre e Zinco em um modelo de DH em *Caenorhabditis elegans*. A avaliação da mistura de metais de forma crônica e concentrações permitidas pela legislação brasileira referentes a solos residencias foi investigada pela primeira vez. No geral, a exposição aos metais e sua mistura levou a alterações locomotoras, corporais, além de um atraso no desenvolvimento dos vermes, afetou comportamentos relacionados aos neurônios ASH e neurônios receptores de toque. Ademais, causou um aumento nos agregados proteicos musculares e neuronais, levando a neurodegeneração. Verificou-se o efeito neuroprotetor da rutina, o flavoinoide foi capaz de diminuir os agregados proteicos e a neurodegeneração. Propôs-se que a rutina atue através de mecanismos que envolvam propriedades antioxidantes, através do aumento da expressão de enzimas antioxidantes e chaperonas, além de atuar contra os metais. Em conjunto, nossos dados fornecem novas indicações sobre os valores orientadores da qualidade do solo, analisados de forma crônica e em mistura de metais, além de novas estratégias para futuros tratamentos de doenças neurodegenerativas causadas pela agregação de proteínas relacionadas a metais.

Palavras-chave: Neuroproteção. Neurodegeneração. Flavonoides. *C. elegans*. Mistura de metais. Solo Brasileiro.

ABSTRACT

ACTION OF RUTIN IN A MODEL OF EXPOSURE TO COPPER AND ZINC METALS IN *Caenorhabditis elegans* WITH HUNTINGTIN OVERPRODUCTION

AUTHOR: Larissa Marafiga Cordeiro

ADVISOR: Professor Dr. Félix Alexandre Antunes Soares

Huntington's disease (HD) is a progressive, hereditary neurodegenerative disease caused due to a mutation in the huntingtin gene that contains an abnormal expansion of cytosine-adenine-guanine (CAG) repeats, leading to a polyglutamine (polyQ) chain of variable length. The mutation confers toxic functions on the mutant huntingtin protein, causing neurodegeneration. HD pathology results in loss of neuronal cells, motor changes, dementia and is currently a disease with no cure. It is known that metal accumulation is found in the pathologically affected regions of many neurodegenerative diseases. The essential metals Copper and Zinc are necessary in small concentrations for metabolic functions, but can, however, produce toxic effects in high concentrations. Furthermore, there is great concern regarding environmental pollution by metals, as urban environments are places subject to significant levels of contamination and, consequently, are the areas with the greatest exposure of metals to the population, whether through inhalation of dust or ingestion of contaminated soil through food. Rutin is a flavonoid found in several plants, such as buckwheat, some teas and fruits, making it easily accessible and low cost to the entire population. Therefore, this work investigated the effect of rutin on neurotoxic effects caused by Copper and Zinc in a HD model in *Caenorhabditis elegans*. The evaluation of the mixture of metals in chronic form and concentrations permitted by Brazilian legislation regarding residential soils was investigated for the first time. In general, exposure to metals and their mixture led to locomotor and body changes, in addition to a delay in the development of the worms, and affected behaviors related to ASH neurons and touch receptor neurons. Furthermore, it caused an increase in muscle and neuronal protein aggregates, leading to neurodegeneration. The neuroprotective effect of rutin was verified, the flavonoid was able to reduce protein aggregates and neurodegeneration. It has been proposed that rutin acts through mechanisms involving antioxidant properties, through increased expression of antioxidant enzymes and chaperones, in addition to acting against metals. Taken together, our data provide new insights into the guiding values of soil quality, analyzed chronically and in mixed metals, as well as new strategies for future treatments of neurodegenerative diseases caused by the aggregation of metal-related proteins.

Keywords: Neuroprotection. Neurodegeneration. Flavonoids. *C. elegans*. Mixture of metals. Brazilian soil.

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS E SIGLAS

AMPc - Adenosina monofosfato cíclico

CAG - Citosina, adenina e guanina

C. elegans - *Caenorhabditis elegans*

CAT - Catalase

CONAMA - Conselho Nacional do Meio Ambiente

DA - Doença de Alzheimer

DH - Doença de Huntington

DMT-1 - Transportador de metal bivalente 1

DP - Doença de Parkinson

DW – Doença de Wilson

ELA – Esclerose lateral amiotrófica

EROs – Espécies reativas de oxigênio

GFP - Proteína verde fluorescente, do inglês *green fluorescent protein*

GSH – Glutationa reduzida

GSSG – Glutationa oxidada

GPx – Glutationa peroxidade

INOS- Óxido nítrico sintase induzível

MDA - Malondialdeído

Htt - Proteína huntingtina

HTT – gene, do inglês *huntingtin gene*

mHtt – Proteína huntingtina mutante

PoliQ - Poliglutamina

SOD – Superóxido dismutase

SNC - Sistema nervoso central

PET - Tomografia por emissão de pósitrons, do inglês *Early Positron emission tomography*

PTEs - Elementos potencialmente tóxicos, do inglês *potentially toxic elements*

TBZ – Tetrabenazina

YFP – Proteína amarelo fluorescente, do inglês *yellow fluorescent protein*

SUMÁRIO

1. INTRODUÇÃO

A doença de Huntington (DH) é uma patologia neurodegenerativa que se caracteriza por alterações motoras progressivas, movimentos involuntários anormais, demência e morte neuronal. Conhecida comumente como coréia de Huntington (‘khoreia’ é a palavra grega para dança), foi primeiramente descrita com características clínicas da doença e o padrão de transmissão familiar (BATES, 2005).

No entanto, foi apenas em 1983 que a mutação gênica causadora da DH foi localizada no cromosssomo 4 (GUSELLA et al., 1983) e, subsequentemente, isolada em 1993 pelo Huntington’s Disease Collaborative Research Group. Este grupo identificou uma mutação na porção 5’ do gene IT15 ou “Interesting Transcript 15” no braço curto do cromossomo 4, que codifica a proteína Huntingtina (Htt). 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 (poliQ) no terminal amínico da proteína Htt. Os indivíduos não portadores da doença apresentam proteína huntingtina com menos de 35 repetições. Já nos afetados pela desordem, a proteína apresenta mais que 36 repetições (aproximadamente 38-55) de resíduos de glutamina na porção N-terminal da cadeia polipeptídica. Quanto maior essa sequência, mais cedo ocorre o desenvolvimento da doença e mais severa é sua progressão (VONSATTEL; DILIGLIA, 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; REGO, 2011).

A DH é uma patologia idade-dependente que normalmente surge após os 40 anos, porém pode ocorrer na juventude sendo mais rara e grave. O envelhecimento é um processo controlado por fatores genéticos, e influenciado por fatores ambientais. Teoricamente, este processo deriva-se do acúmulo gradual de falhas e danos nas células, influenciado tanto pelo estresse oxidativo e metabólico aos quais as células são expostas, de maneira cumulativa, quanto pelo declínio dos mecanismos celulares de defesa contra esses (MATTSON; CHAN; DUAN, 2002). A maioria das doenças neurodegenerativas como a DH, Doença de Alzheimer (DA), Doença de Parkinson (DP), por exemplo, são caracterizadas pelo acúmulo e agregação

de proteínas, que interrompem a dinâmica das redes de proteínas e resultam na desestabilização da homeostase celular (DOUGLAS; DILLIN, 2010). A proteína Htt mutante (mHtt), considerada o principal fenótipo característico da DH, forma agregados tanto no núcleo quanto no citoplasma, que devido à sua natureza insolúvel se acumulam e se enredam para formar inclusões (DIFIGLIA et al., 1997). Outros mecanismos, como dano oxidativo, também contribuem para a disfunção neuronal e, eventualmente, a morte (TASSET; SÁNCHEZ; TÚNEZ, 2009). Além disso, o acúmulo de metais é frequentemente encontrado nas regiões patologicamente afetadas de muitas doenças neurodegenerativas (XIAO et al., 2013). Embora esteja estabelecido que a homeostase de metais seja alterada em doenças neurodegenerativas, o perfil de deposição de metais difere para cada distúrbio. Na DH foram encontradas anormalidades no tecido ou deposição de Cobre (Cu) e Zinco (Zn), que podem ser o resultado de processos da doença ou a causa da doença (WHITE et al., 2017).

Os metais essenciais Cu e Zn atuam como um cofator de enzimas e estão envolvidos em uma série de processos fisiológicos, como transporte de elétrons, transporte de oxigênio e síntese de neurotransmissores (BANCI, 2013). Embora os metais sejam importantes para animais e plantas, geralmente são necessários em pequenas quantidades (WRIGHT; BACCARELLI, 2007). Além disso, existe uma grande preocupação quanto à poluição ambiental por metais pesados sendo um problema ambiental global crítico, como a contaminação ambiental influencia diretamente a saúde humana, esta tem sido uma questão de grande importância (GOULDING; BLAKE, 1998). Há uma estreita relação entre solos urbanos e a saúde da população (POGGIO et al., 2008). Ambientes urbanos são locais sujeitos a significativos níveis de contaminação e, consequentemente, são as áreas de maior exposição de metais a população, seja pela inalação de poeira ou pela ingestão de alimentos provenientes de solo contaminado (RASMUSSEN; SUBRAMANIAN; JESSIMAN, 2001).

Cobre é um oligoelemento essencial e atua como cofator de várias enzimas (como citocromo c oxidase e superóxido dismutase (SODs)) (DESAI; KALER, 2008). O cérebro concentra metais pesados, incluindo cobre, para uso metabólico (BUSH, 2000). Cu é de grande importância para o desenvolvimento e função normal do cérebro. No entanto, níveis elevados de Cu podem resultar na geração de espécies reativas de oxigênio (EROs), danos ao DNA e disfunção mitocondrial. O cobre excessivo tem sido associado a doenças como DA, esclerose lateral amiotrófica (ELA), DH, DP e doença de Wilson (DW) em humanos (DESAI; KALER, 2008). O Cu pode aumentar a auto-agregação de proteínas precursoras de amiloide e peptídeo β-amilóide (HA; RUY; BEUM, 2007). Além disso, os níveis de Cu também são mais elevados em pacientes com DH em comparação com os controles (FOX et al., 2007). O

cobre pode promover alteração da conformação, agregação e/ou atividade redox da huntingtina mutante (HUANG et al., 1999).

Zinco é o oligoelemento mais abundante no cérebro, onde desempenha várias funções, interagindo com várias proteínas conferindo-lhes propriedades catalíticas ou estruturais (DANSCHER et al., 1997). Além disso, 300 enzimas são dependentes desse metal, muitas delas expressas no sistema nervoso central (SNC) (HAGMEYER; HADERSPECK; GRABRUCKER, 2015). Porém, a homeostase alterada do zinco é sugerida como um fator de risco para DA, envelhecimento e outros distúrbios neurodegenerativos. Sob a fisiologia normal do SNC, controles homeostáticos são colocados em prática para evitar o acúmulo de zinco em excesso ou sua deficiência (SZEWCZYK, 2013). Além disso, foi visto um aumento na concentração de Zn no sangue de pacientes com DH (SQUADRONE et al., 2020). As concentrações de Zn também foram maiores em análises de cérebro post-mortem de pacientes (ROSAS et al., 2012).

A contaminação ambiental por metais vem sendo associada a uma maior prevalência de doenças no mundo (ZAR; FERKOL, 2014). Da mesma forma, a toxicidade dos metais pesados tem consequências severas e de longo prazo no cérebro, resultando em comprometimento cognitivo (ORTEGA et al., 2020). Uma vez que a exposição a metais pode ocorrer através do consumo de alimentos cultivados em solos contaminados, no Brasil existe a Resolução número 420 do Conselho Nacional do Meio Ambiente (CONAMA), que dispõe sobre critérios e valores orientadores de qualidade do solo quanto à presença de substâncias químicas e estabelece diretrizes para o gerenciamento ambiental de áreas contaminadas por essas substâncias em decorrência de atividades antrópicas (Resolução n. 420, CONAMA, 2009). Os principais poluentes que prejudicam o solo e expõem as pessoas a doenças são os agrotóxicos (20%), derivados do petróleo (16%), resíduos industriais (12%) e metais (12%) (Associação Brasileira de águas subterrâneas, 2010).

Atualmente, há uma busca crescente por alternativas contra esses toxicantes. Os flavonoides são substâncias pertencentes à classe dos compostos fenólicos e estão presentes em vegetais e frutas, que são as principais fontes dessas substâncias (BARREIROS; DAVID, 2006). Flavonoides também podem atuar como quelantes de íons metálicos de transição (MIRA et al., 2002). A rutina (3, 3′,4 ′, 5, 7- pentahydroxyflavone-3-rhamnoglucoside) é um flavonol que se encontra em muitas plantas típicas, como trigo mourisco, *Passiflora incarnata* (também conhecida como flor da paixão), maçã e chás. Foi relatado que a rutina tem várias propriedades farmacológicas, incluindo atividades antioxidantes, citoprotetoras, anti-inflamatórias, imunomoduladoras e neuroprotetora (KATSUBE et al., 2006;

TRUMBECKAITE et al., 2006; BISHNOI et al., 2007). A rutina atenuou sintomas semelhantes aos da DH induzidos por ácido 3- nitropropiônico em ratos (SUGANYA; SUMATHI, 2017), além de exercer efeitos protetores contra a toxicidade induzida por poliQ em *Caenorhabditis elegans (C. elegans)* (CORDEIRO et al., 2020). Tais resultados sugerem que a rutina pode ser um composto promissor para prevenir ou tratar doenças neurodegenerativas. No entanto, pouco se sabe sobre seus mecanismos subjacentes e seus efeitos na homeostase de proteínas.

No momento atual, o tratamento para DH é apenas sintomático e a terapêutica selecionada depende da manifestação clínica dominante. Os agentes depletores de dopamina têm sido o grupo farmacológico mais utilizado para o controle dos movimentos coreicos. Antidepressivos, antagonistas do glutamato, antiepilépticos e outros fármacos são utilizados na DH para tratamento sintomático (COPPEN; ROOS, 2017). As estratégias terapêuticas destinadas a prevenir ou atrasar a degeneração neuronal representam uma escolha razoável para o tratamento de doenças neurodegenerativas. Por conseguinte, existe um interesse crescente no uso de antioxidantes naturais, incluindo compostos fenólicos encontrados em vegetais que podem prevenir a morte celular (SANDHIR; MEHROTRA, 2013). Devido ao grande número de espécies vegetais e a presença de diferentes compostos antioxidantes em seus extratos, aumentam-se as chances de identificação de substâncias com atividades neuroprotetoras. Assim, muitas patologias que hoje permanecem sem um tratamento adequado, poderão vir a ser tratadas de forma mais eficientes a partir de novos fármacos derivados de substâncias naturais (OLIVEIRA et al., 2007).

C. elegans é um nematódeo pequeno (±1 mm quando adulto), de fácil manipulação, curto ciclo de vida, fácil de cultivo e rápido tempo de geração. É estruturalmente simples, no entanto mostra-se como uma poderosa ferramenta nas áreas de pesquisa em toxicologia, farmacologia e biologia molecular (NASS; BLAKELY, 2003). É um modelo *in vivo* para estudar o papel dos processos de envelhecimento no desenvolvimento de proteinopatias neurodegenerativas (VAN PELT et al., 2020). O primeiro modelo de *C. elegans* para a DH foi gerado pela expressão de um fragmento de huntingtina contendo 150 repetições de poliQ em neurônios sensoriais de cabeça (neurônios ASH) e resultou em neurodegeneração (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 (BRACKMAN et al., 2002).

2. OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar os efeitos neuroproterores da rutina no modelo de Doença de Huntington em *Caenorhabditis elegans* após exposição crônica a metais pesados.

2.2 OBJETIVOS ESPECÍFICOS

- Avaliar o papel neuroprotetor da rutina em neurônios ASH e seus mecanismos de ação envolvidos;
- Avaliar os possíveis efeitos neurotóxicos da exposição a cobre e zinco de forma isolada ou em uma mistura;
- Investigar o papel da exposição aos metais na progressão da DH em modelo de *C. elegans*;
- Avaliar os efeitos neuroprotetores da rutina na neurodegeneração e agregação de proteínas em modelo de DH em *C. elegans* exercidas pela exposição a cobre e zinco;

3. REVISÃO DA LITERATURA

3.1 DOENÇAS NEURODEGENERATIVAS

As doenças neurodegenerativas são caracterizadas pela perda progressiva e irreversível de certos neurônios, o que leva a um déficit progressivo de funções do SNC. Dentre elas, as mais comuns são a DH, DA, DP e ELA. As diferentes doenças neurodegenerativas afetam regiões distintas do cérebro, por exemplo, a DA afeta o córtex cerebral, a DP e DH afetam os gânglios da base, e a ELA afeta o sistema motor (PIEVANI et al., 2014; ODDONE; IMBRIANI, 2015). Embora ainda não bem elucidados, os mecanismos envolvidos na morte neuronal envolvem eventos em comuns nas doenças neurodegenerativas, incluindo agregação de proteínas mal formadas (por exemplo, a huntingtina na DH, o peptideo βA na DA e a α-sinucleína na DP), estresse oxidativo e inflamação (ROSS; POIRIER, 2004). Porém, os sintomas além da classificação da doença variam dependendo da área afetada.

Devido ao aumento da expectativa de vida da população, somado a fatores ambientais e genéticos tem aumentado a incidência do desenvolvimento das doenças neurogenerativas (CHECKOWAY; LUNDIN; KELADA, 2011). À medida que a população mundial envelhece o número de indivíduos em risco para demência também aumenta. Estima-se que 35,6 milhões de pessoas viviam com demência em 2010 e esses números quase dobram a cada 20 anos, podendo chegar a 65,7 milhões de pessoas em 2030 (PRINCE et al., 2013).

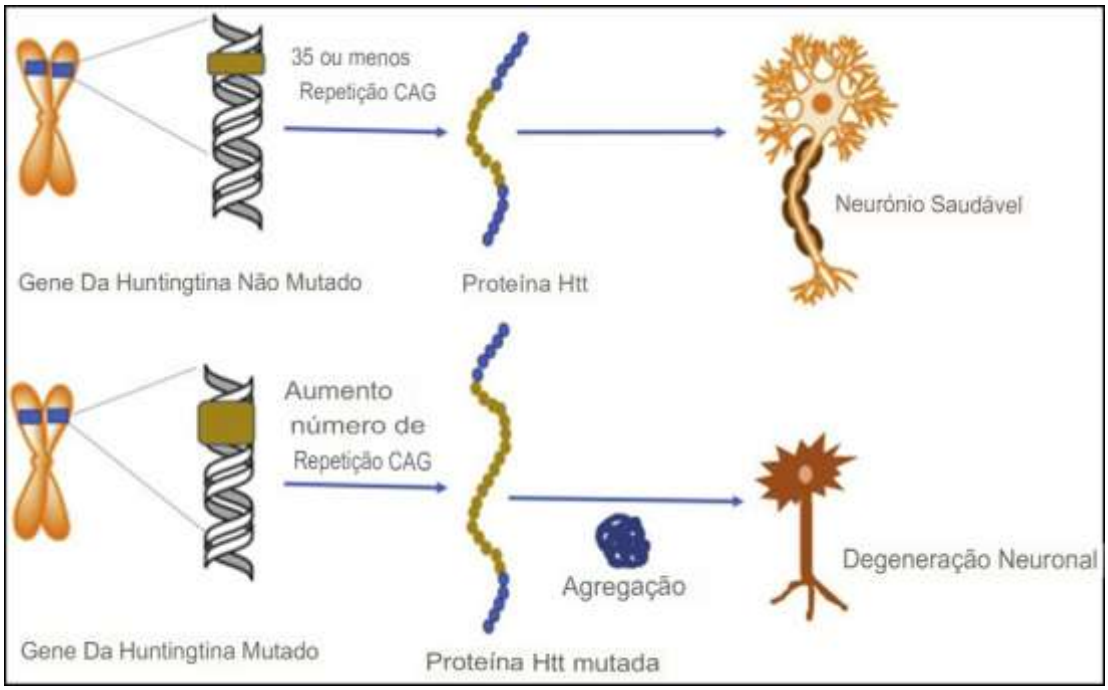
A maioria das pessoas com demência vive em países de baixa e média renda, como é o caso do Brasil, o maior e mais populoso país da América Latina. Na América Latina estima-se um aumento de quatro vezes no número de indivíduos com demência até 2050 (PARRA et al., 2018). Na DH, por exemplo, a prevalência mínima no estado do Rio Grande do Sul (RS) foi estimada em 1,85:100.000 habitantes (CASTILHOS et al., 2019).

3.1.1 Doença de Huntington

A DH é uma doença neurodegenerativa genética, herdada de forma autossômica dominante, ou seja, para ser afetado basta herdar uma cópia do gene mutante de um dos pais afetados pela doença. É uma patologia causada por uma expansão de repetições do trinucleotídeo CAG no gene da huntingtina (HTT) no cromossomo 4. Isso resulta na produção de uma proteína huntingtina mutante (mHtt) com uma repetição de poliglutamina (poliQ) anormalmente longa, conforme a Figura 1 (MACDONALD et al., 1993). Indivíduos

com mais de 36 repetições (aproximadamente 38-55) desenvolverão a doença, enquanto não portadores da doença apresentam proteína huntingtina com até 35 repetições de resíduos de glutamina (TELENIUS et al., 1994). O comprimento da repetição CAG é o que determina se um indivíduo desenvolverá a doença, sendo também o principal determinante da taxa de patogênese que leva aos sinais motores característicos que fundamentam o diagnóstico clínico (ANDREW et al., 1993).

Figura 1: Aumento do número de repetições CAG levando à Doença de Huntington



Fonte: Adaptado de Kohli *et al*. (2021)

A proteína Htt é expressa em todos os tipos de células do corpo, tanto no nível tecidual quanto subcelular, em todos os estágios de desenvolvimento. Suas funções ainda não são bem elucidadas, porém, parece interagir com diferentes proteínas que estão envolvidas em processos como transporte intracelular e sinalização celular (CATTANEO; ZUCCATO; TARTATI, 2005; HARJES; WANKER, 2003). Além disso, foi descrita por estar relacionada no desenvolvimento do cérebro, com um papel crucial na formação de sinapses excitatórias corticais e estriatais (GATTO et al., 2020). Sabe-se que a mHtt tem um ganho tóxico de função desencadeando o processo neurodegenerativo, além disso, essa proteína é mais resistente à degradação, levando a formação de agregados intracelulares que causam

degeneração e morte neuronal (CISBANI; CICCHETTI, 2012). Embora o papel fisiológico da huntingtina ainda não tenha sido definido totalmente, Bano e colaboradores (2011) relataram que ela está associada ao desenvolvimento de mamíferos, camundongos que tinham a supressão completa da expressão do gene vieram a óbito quando embriões (BANO et al., 2011).

Dentro das células cerebrais, a mHtt é mal dobrada e forma agregados com propriedades tóxicas (HATTERS, 2008). O dobramento incorreto sobrecarrega o sistema de degradação ubiquitina-proteassômica, sendo necessário para a homeostase celular da reciclagem de proteínas (BENCE; SAMPAT; KOPITO, 2001). A mHtt também se agrega com outras proteínas, incluindo a proteína de ligação responsiva ao AMPc (CREB), podendo esgotar várias proteínas diferentes disponíveis para a célula (STEFFAN et al., 2000; NUCIFORA et al., 2001).

A neuropatologia mais proeminente na DH ocorre na parte estrial dos gânglios da base e a atrofia é acompanhada por extensa perda neuronal, a qual se torna mais grave à medida que a doença progride, levando a atrofia a um grande alargamento dos ventrículos laterais (THOMAS et al., 1995; VIS et al., 2005). Durante os estágios mais avançados da doença, se estende a uma variedade de regiões cerebrais, incluindo o hipotálamo e o hipocampo (VONSATTEL et al., 1985). A DH é caracterizada por distúrbios na movimentação, sintomas cognitivos e distúrbios psiquiátricos. Os defeitos motores incluem coreia e perda de coordenação, e os pacientes também demonstram dificuldade de fala e deglutição (YANAGISAWA, 1992). Os sintomas cognitivos podem ser detectados anos antes do diagnóstico e a capacidade cognitiva diminui à medida que a doença progride (BONNER et al., 2013). Sintomas psiquiátricos, como depressão, psicose e transtorno obsessivo-compulsivo também são comuns na DH (HARRINGTON et al., 2013; EPPING et al., 2013).

O comprometimento do eixo metabólico no cérebro é uma importante manifestação da patogênese da DH. Distúrbios metabólicos no cérebro de pacientes foram relatados antes mesmo que a causa genética da doença fosse descoberta. Os primeiros estudos de tomografia por emissão de pósitrons (do inglês *Early Positron Emission Tomography - PET*) mostraram um metabolismo de glicose reduzido no corpo estriado e no córtex cerebral de pacientes com DH sem sintomas clínicos evidentes relacionados à doença (KUHL et al., 1985; KUWERT et al., 1990; GRAFTON et al., 1992; ANTONINI et al., 1996). Além disso, a presença de produtos de oxidação de lípidos, ácidos nucleicos e proteínas (lipofuscina, malondialdeído, carbonilos de proteínas, entre outros) demonstra uma relação com estresse

oxidativo em cérebros de pacientes, o que contribui ainda mais para déficits na função cerebral (HERSCH et al., 2006; BROWNE; BEAL, 2006; CHEN et al., 2007).

Por ser uma doença complexa, a patogênese a jusante do gene mutante da huntingtina é problemática, envolvendo múltiplas vias, incluindo fragmentação anormal de proteínas e neuroinflamação (PAN et al., 2021). Até o momento, nenhum ensaio clínico foi bem sucedido na identificação de tratamentos modificadores da doença (KUMAR et al., 2020; STAHL et al., 2020), sendo assim o tratamento atual da DH baseia-se principalmente no manejo sintomático. A Tetrabenazina (TBZ), um inibidor do transportador vesicular de monoamina, foi aprovada pela Food and Drug Administration (FDA) dos Estados Unidos da América (EUA) para o tratamento da coreia na DH em 2008 e é o medicamento aprovado para esta utilização até à data (POTKIN et al., 2018). Por outro lado, vale ressaltar que efeitos colaterais foram observados com TBZ, incluindo depressão e parkinsonismo, provavelmente ser devido à redução concomitante de outras monoaminas, como serotonina e norepinefrina (WYANT et al., 2017). Esses efeitos colaterais não devem ser menosprezados, pois os pacientes com DH já correm maior risco de depressão, ansiedade e suicídio em comparação com a população em geral (GALTS et al., 2019).

3.1.2 Doença de Huntington e metais

Os metais geralmente podem ser divididos em dois grupos: metais essenciais e não essenciais. Os essenciais incluem cobre e zinco, sendo importantes para processos biológicos como transporte de oxigênio e elétrons, além de atuar como cofatores de enzimas. Alguns metais ainda, como o zinco, tem um papel na ativação de células de defesa e na regulação da resposta inflamatória (CHEN; MIAH; ASCHNER, 2016). Embora sejam metais importantes nos processos biológicos e no metabolismo, eles geralmente são necessários em pequenas quantidades. Quando em excesso, os metais podem se acumular em vários órgãos, incluindo o cérebro onde podem induzir uma série de eventos intracelulares deletérios, incluindo estresse oxidativo, disfunção mitocondrial, fragmentação do DNA, dobramento incorreto de proteínas, desregulação da autofagia e ativação da apoptose (ANGELI et al., 2014; ZHANG et al., 2013; SEO et al., 2013; GUNTEL et al., 2010). Esses efeitos alteram a neurotransmissão e podem levar a neurodegeneração e a neurotoxicidade induzida por metais tem sido associada a diversas doenças neurológicas, como, DH, DA, ELA, DP, DW, entre outras (SHAW et al., 2013; AUTHIER et al., 2001; STRAUSAK et al., 2001; OKUDA et al., 1997; CHEN et al., 2015).

O excesso de cobre tem sido associado a diferentes doenças neurodegenerativas (VALENSIN et al., 2016) evidências demonstraram que Cu pode aumentar a auto-agregação de proteínas precursoras amiloides e peptídeo β amiloide (HA; RYU; PARK, 2007) além disso, níveis aumentados do metal foram encontrados no líquido cefalorraquidiano de pacientes com DA (ROOS; VESTERBERG; NORDBERG, 2006). Do mesmo modo, o Cu interage com a α-sinucleína e promove agregação, podendo levar a DP. Notavelmente, em pacientes com DH os níveis do metal também são maiores em comparação com os controles (FOX et al., 2007), sendo que o cobre poderia promover alterações na conformação, agregação e/ou atividade redox da mHtt, assim como a interação da β amiloide com o metal, induzindo a oligomerização da β amiloide (HUANG et al., 1999a; HUANG et al., 1999b).

O papel do Cu e das proteínas de ligação ao Cu em pacientes com DH ainda não é totalmente esclarecido. Experimentos *in vitro* demonstram que o Cu interage com a Htt selvagem, diminuindo a solubilidade dos fragmentos da proteína (FOX et al., 2007) e aumentando a agregação. Anteriormente, acreditava-se que os agregados eram inertes, porém foi demonstrado que o Cu também pode se ligar a Htt após a agregação, agravando ainda mais sua insolubilidade (MITOMI et al., 2012). Além disso, existem dois resíduos na proteína Htt que podem se ligar ao Cu (His82 e Met8) e, quando esses sítios sofrem mutação em modelo de DH em *Drosophila melanogaster*, os efeitos tóxicos da mHtt são evitados (XIAO et al., 2013) evidenciando que nem o Cu nem a extensão poliQ sozinhos causaram sintomas da doença, mas a combinação da mutação e exposição ao metal foi tóxica, sugerindo que a terapia relacionada ao Cu poderia ser benéfica para os pacientes.

De mesma relevância, o Zn atua como cofator para mais de 300 enzimas e metaloproteínas, regulando a resposta antioxidante e transcrição gênica. No entanto, níveis aumentados de Zn promovem a produção de EROs, além de interromper atividades de enzimas metabólicas e ativar processos apoptóticos (MARREIRO et al., 2017). O desbalanço da homeostase do Zn vem sendo associada a DA, isquemia cerebral, trauma cerebral, demência do tipo vascular, epilepsia (WRIGHT; BACCARELLI, 2007; MIZUNO; KAWAHARA, 2013), DP e ELA (SIKORA et al., 2020; KANEKO et al., 2015). Além disso, as doenças neurodegenerativas citadas tem em comum características e mecanismos semelhantes a DH que fazem com que as proteínas mal dobradas causem a morte neuronal e neurodegeneração. Níveis aumentados de Zn foram detectados no sangue de pacientes com DH, indicando que a mHtt pode prejudicar a homeostase do Zn (SQUADRONE et al., 2020)

ademais sugere-se que a interrupção da homeostase do Zn vesicular pode contribuir para a disfunção sináptica e neurodegeneração na DH (NIU et al., 2020).

3.2 CONTAMINAÇÃO AMBIENTAL POR METAIS

A contaminação ambiental por metais aumentou desde 1900 (NRIAGU, 1979) devido à rápida urbanização e industrialização, o que acarretou que mais metais fossem liberados no meio ambiente. As concentrações de metais pesados em solos em todo o mundo frequentemente excedem seus valores normativos e, embora nem todos os metais pesados sejam derivados de fontes antropogênicas, as concentrações variam largamente (SILVA et al., 2020). Além disso, os valores padronizados variam de país para país. No Brasil, o CONAMA é o órgão que estabelece padrões de controle da poluição ambiental, sendo que a resolução número 420 de 28 de dezembro de 2009 preconiza valores orientadores de qualidade para solos agrícolas, residenciais e industriais e águas subterrâneas (Conselho Nacional do Meio Ambiente, 2009).

A contaminação do meio ambiente por metais ocorre principalmente através da queima de combustíveis fósseis, lixo municipal, pesticidas, fundição de mineração, esgoto, entre outros (NAILA et al., 2019). A aplicação de pesticidas e fertilizantes é uma maneira que promove o acúmulo de Cu no solo, sendo que a presença de Cu em fungicidas existe principalmente na forma de Cu triclorofenol, misturas de Cu e Zn e sulfato de Cu (QIN et al., 2021). A transferência de metais do solo através da cadeia alimentar ou deposição atmosférica pode causar efeitos crônicos, dentre eles relacionados à mutagenicidade e carcinogenicidade (DAMEK-POPRAWA; SAWICKA-KAPUSTA, 2013).

Embora certos elementos sejam essenciais para a saúde humana, a elevação da concentração pode atingir níveis de risco potencial à saúde (WITKOWSKA; SLOWIK; CHILICKA, 2021). Ao contrário dos poluentes orgânicos, os elementos potencialmente tóxicos (do inglês *potentially toxic elements – PTEs*) são persistentes no ambiente e suas concentrações se acumulam ao longo do tempo (LEE et al., 2006) e a presença prolongada desses elementos representa um desafio para a saúde das populações urbanas (WOSZCZYK; SPYCHALSKI; BOLUSPAEVA, 2018). A inalação direta, ingestão, contato com a pele, cadeia alimentar e ingestão de água contaminada são as principais vias de exposição humana aos elementos potencialmente tóxicos em ambientes urbanos (ABRAHAMS, 2002; MCLAUGHIN et al., 2000; POGGIO et al., 2009).

Os estudos de solos urbanos e residenciais em várias cidades ao redor do mundo demonstram concentrações significativas de cobre, cádmio, zinco, chumbo, mercúrio e outros metais (AJMONE-MARSAN; BIASIOLI, 2010; AWADH et al., 2013). Em áreas urbanas, os metais também podem entrar no solo por meio de emissões industriais, transportes, queima de combustíveis fósseis e lixo municipal. O chumbo e o cobre provêm principalmente do tráfego e da combustão. Degradação de materiais (cabos e tubos) e incineradores são outra fonte de contaminação urbanas dos solos (WONG; LI; THORNTON, 2006).

Um dos grandes problemas dos valores orientadores da qualidade do solo no mundo todo se refere à concentração de um elemento isolado. Desta maneira, a avaliação de um único metal não deve ser tomada como critério 100% confiável para a avaliação de riscos potenciais à saúde, principalmente devido à exposição a diversos metais ao mesmo tempo. Avaliações que consideram os efeitos combinados de poluentes refletem melhor os efeitos existentes de exposições ambientais do que avaliações que determinam a toxicidade de produtos individuais (SCHNUG; LEINAAS; JENSEN, 2014).

Organismos no ambiente são frequentemente expostos a misturas de metais, essas exposições podem ser prejudiciais embora as concentrações desses metais possam estar abaixo da concentração sem efeito observado (KORTENKAMP, 2008). Esse conceito é chamado de toxicidade de misturas (BEYER et al., 2014), entretanto a maioria dos testes de toxicidade é realizada usando metais individuais no ambiente, ignorando os efeitos potenciais das misturas especialmente em concentrações muito baixas. Sendo assim, os efeitos de misturas especialmente em concentrações baixas são subestimados. A toxicidade de metais é uma grande preocupação, pois eles não se degradam prontamente no ambiente e bioacumulam, além de causar efeitos deletérios como carcinogênese, mutagênese e problemas neurológicos (JUDAH et al., 2014; NOTARACHILLE et al., 2014; TYLER; ALLAN, 2014). Um parâmetro considerado chave ao avaliar a toxicidade de misturas metálicas é o modo de ação do metal (BALISTRIERI; MEBANE, 2014; CHARLES et al., 2014). O modo de ação são os processos vitais iniciados pela interação do metal com o receptor e o processo por meio de alterações anatômicas no organismo, resultando em efeitos letais e subletais. Basicamente, é a resposta produzida em um organismo exposto ao metal ou as características do mecanismo necessário para a produção de uma resposta biológica (BORGERT et al., 2004). Este modo de ação é usado na avaliação de risco para prever a toxicidade de misturas tóxicas.

A combinação de metais pode produzir efeitos aditivos, sinérgicos ou antagônicos, manifestados em uma toxicidade geral, distinta da toxicidade dos componentes individuais da mistura. O efeito aditivo é quando a toxicidade da mistura é igual à soma da toxicidade dos metais individualmente, já efeito sinérgico ou antagônico é quando a toxicidade da mistura é maior ou menor do que a soma da toxicidade dos metais individualmente, respectivamente (WALKER et al., 2016).

3.3 COMPOSTOS FENÓLICOS

Estudos demonstram que as doenças neurodegenerativas são multifatoriais e que o estresse oxidativo está interligado com os mecanismos dessas doenças (BARNHAM; MASTERS; BUSH, 2004). Por esse motivo, compostos antioxidantes de fontes naturais, como carotenoides, compostos fenólicos e vitaminas tem sido amplamente estudados e já demonstram efeitos benéficos principalmente associados ao envelhecimento (ABBAS; WINK, 2009; POWOLNY et al., 2011).

Devido à diversidade de espécies vegetais e a presença de diferentes compostos bioativos em seus extratos, aumenta o número de substâncias potencialmente bioativas. Dentre os principais compostos com atividades biológicas presentes nos extratos, podemos citar os compostos fenólicos, que possuem atividades antioxidantes, cardioprotetoras, anti-inflamatórias e desempenham relação com as doenças neurodegenerativas (SCALBERT; JOHNSON; SALTMARSH, 2005).

Dentre os compostos fenólicos, os flavonoides apresentam efeito neuroprotetor, antioxidante, anti-inflamatório, além de desempenhar um papel significativo na prevenção de doenças cardiovasculares, isso pode ser devido principalmente aos seus efeitos antiaterogênicos, antitrombóticos e antioxidantes (KHAN et al., 2021; CORDEIRO et al., 2020).

As propriedades antioxidantes dos flavonoides estão relacionadas à sua capacidade de eliminação de radicais, atividade redutora de metais e quelação de metais, que estão envolvidos na geração de radicais hidroxila reativos. Estas propriedades surgem da estrutura química polifenólica constituída pelo sistema de anéis C6-C3-C6. Em particular, a atividade antioxidante depende do número e da posição dos grupos hidroxila na estrutura. Por exemplo, alguns radicais são reduzidos via transferência de prótons através da clivagem homolítica do grupo catecol (3´-OH e 4´-OH) presente em algumas estruturas flavonoides (SOUZA et al., 2004). No entanto, embora a porção catecol seja um requisito importante

para a atividade antioxidante, alguns flavonoides desprovidos de grupos catecol também apresentam desempenho notável de eliminação de radicais devido à capacidade de transferir átomos de hidrogênio, ou à transferência simultânea de hidrogênio/elétron ou transferência sequencial de elétrons com perda de prótons (SAMSONOWICZ et al., 2017).

3.3.1 Rutina

Rutina (3,3',4',5,7-pentahidroxiflavona-3-rutinosídeo) é um composto pertencente a classe dos flavonoides, encontrada principalmente em fontes naturais, por exemplo, laranja, limões, uvas, frutas vermelhas e pêssegos (KREFT; KNAPP; KREFT, 1999). É um componente nutricional vital das plantas (HARBORNE, 1986) e recebeu o nome da planta *Ruta graveolens,* que também contém rutina. Quimicamente, é um glicosídeo composto por flavonol quercetina aglicona junto com o dissacarídeo rutinose (GANESHPURKAR; SALUJA, 2017). Além disso, possui diversos efeitos biológicos como, atividades antioxidantes, antimicrobianas, anticarcinogênicas, antitrombóticas, cardioprotetoras e neuroprotetoras (NEGAHDARI et al., 2021).

Ao longo dos anos, mecanismos foram sugeridos como responsáveis por suas atividades antioxidantes em modelos *in vivo* e *in vitro*. Primeiramente, foi relatado que sua estrutura química pode eliminar EROs diretamente (HANASAKI; OGAWA; FUKUI, 1994). Em segundo lugar, a rutina aumenta a produção de glutationa (GSH) e menciona-se que os sistemas celulares de defesa oxidativa aumentem devido ao aumento da expressão de enzimas antioxidantes, como a catalase (CAT) e superóxido dismutase (SOD) (AL-ENAZI, 2014). Finalizando, a rutina inibe a xantina oxidase, que está envolvida na geração de EROs (KOSTIC et al., 2015).

Devido a sua atividade neuroprotetora, a rutina exerce efeitos benéficos em diferentes modelos de doenças neurodegenerativas, incluindo a DA e a DH. Em modelo de DA, a rutina inibiu a agregação da β amiloide, além de prevenir danos mitocondriais e reduzir a produção de EROs, malonaldeído (MDA), óxido nítrico sintase induzível (iNOS), glutationa dissulfeto (GSSG) e citocinas pró inflamatórias. Além disso, a rutina aumentou os níves de CAT, SOD, glutationa peroxidase (GPx) e GSH (WANG et al., 2012). Em um modelo *in vivo,* Xu e colaboradores, (2014) demonstraram que após a administração oral de rutina na dose diária de 100 mg/kg por 6 semanas, houve redução no déficit de memória de camundongos transgênicos, além de redução nos níveis de oligoméricos β amiloide (XU et al., 2014). Em um modelo de DA utilizando peixe-zebra, Richetti e colaboradores, (2011)

demonstraram que a rutina não afetou a locomoção geral do animal e preveniu a amnésia induzida por escopolamina (RICHETTI et al., 2011).

A administração oral de rutina (25 mg/kg e 50 mg/kg) reduziu a oxidação de proteínas e melhorou o sistema antioxidante, bem como atenuou as mudanças comportamentais restaurou a atividade das enzimas do complexo mitocondrial em animais expostos ao 3-nitropropiônico (3-NP), (SUGANYA; SUMATHI, 2017; SUGANYA; SUMATHI, 2014). Recentemente, os mesmos pesquisadores demonstraram que a rutina atenuou as alterações induzidas por 3-NP no peso corporal, movimento, níveis de antioxidantes e memória. Além disso, demonstraram que a rutina aliviou o dano estriatal, reduzindo os peróxidos lipídicos, nitrito, proteína ácida fibrilar glial (GFAP) e a atividade da acetilcolinesterase (SUGANYA; SUMATHI, 2017).

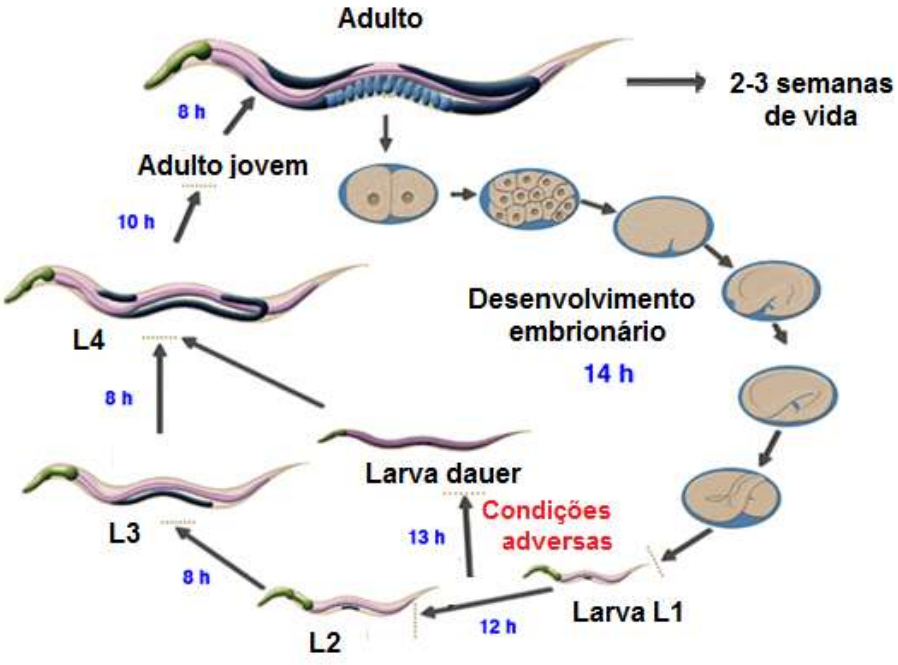
Um efeito relevante da rutina e atualmente ainda pouco estudado é a sua propriedade como quelante de metais. Estudos demonstram que os flavonoides podem atuar como antioxidantes devido ás suas propriedades quelantes. Kostyuk e colaboradores (2001) descobriram que os complexos de rutina e epicatequina com ferro (II), ferro (III), cobre (II) e zinco (II) são mais eficazes do que flavonoides livres na eliminação de radicais livres. Esses complexos protegem os glóbulos vermelhos de forma mais eficaz contra o amianto, que causa danos oxidativos *in vitro* (KOSTYUK et al., 2021). Além disso, Prakash e colaboradores (2020) demonstraram que a formação de quelatos é consequência da interação entre rutina e íons metálicos. A quelação de metais pode ser crucial para prevenir a formação de radicais livres que danificam biomoléculas, uma vez que a interação entre flavonoides e metais de transição forma complexos que impedem que os íons metálicos participem dos processos de produção de radicais livres, exibindo assim um comportamento antioxidante (PRAKASH et al., 2020).

3.4 O *Caenorhabditis elegans*

O *Caenorhabditis elegans* foi introduzido em 1963 por Sydney Brenner como um modelo para o estudo do desenvolvimento e neurobiologia (BRENNER, 1974). Atualmente, é um organismo usado para análise genética, toxicologia, neurotoxicidade e para estudos relacionados à homeostase de metais (LEUNG et al., 2008). *C. elegans* é um pequeno nematoide (±1 mm) de vida livre que vive em solo úmido e usa bactérias como fonte de alimento. A uma temperatura controlada (±20 ºC), *C. elegans* desenvolve-se de ovos a adultos em cerca de 2,5 dias. As larvas normalmente, após a eclosão do ovo, passam por

quatro estágios larvais (L1, L2, L3 e L4) para o estágio de adulto jovem e depois para o estágio de produção de ovos (RIDDLE et al., 1997), conforme a Figura 2. Em condições adversas, como temperaturas extremas ou escassez de alimentos o desenvolvimento larval para no estágio L2, produzindo então a larva dauer que é um estágio de diapausa, retomando o crescimento quando está novamente em um ambiente favorável (RIDDLE et al., 1997).

Figura 2 – Ciclo de vida de *C. elegans*



Fonte: Adaptado de Worm Atlas. Disponível em: <https://www.wormatlas.org/hermaphrodite/introduction/IMAGES/introfig6leg.htm>. Acesso em: 2 agost. 2023.

O hermafrodita adulto é anatomicamente simples com 959 células somáticas que formam diferentes tecidos (SULSTON et al., 1983), seu genoma é composto por aproximadamente 20.000 genes (MITANI, 2017) e suas vias metabólicas e biossintéticas são altamente conservadas em comparação com os mamíferos (RIDDLE et al., 1997; NASS; BLAKELY, 2003). Aproximadamente 60-80% dos genes humanos tem ortólogos no genoma de *C. elegans* (KALETTA; HENGARTNER, 2006). Através da utilização de construções transgênicas, também é possível estudar os mecanismos envolvidos na neurotoxicidade de diferentes doenças humanas que não possuem um gene ortólogo em *C. elegans.*

C. elegans possui um sistema nervoso simples com 302 neurônios (WHITE et al., 1986) além de um sistema de neurotransmissores conservado, como o colinérgico, dopaminérgico, glutamatérgico, gabaérgico e serotoninérgico (RIDDLE, 1997). Além disso, animais *knockouts* e mutantes genéticos podem ser gerados e estão disponíveis para a pesquisa (FIRE et al., 1998), como estirpes expressando diferentes comprimentos de poliQ ligada a YFP (do inglês *yellow fluorescent protein)* ou GFP (do inglês *green fluorescent protein*) expressas nos músculos dos vermes (MORLEY et al., 2002) ou neurônios (BRIGNULL et al., 2006). Além disso, através da estirpe HA759 podemos observar a fluorescência GFP como um marcador de sobrevivência de neurônios ASH (CHEN et al., 2015). *C elegans* é, portanto um modelo altamente sensível para realizar estudos relacionados à neurodegeneração.

Através da translucidez do corpo de *C. elegans* é possível a visualização do nível celular por meio de técnicas não invasivas, o que torna possível a visualização das estruturas celulares, anatomia e de transcritos marcados com proteínas fluorescentes em tempo real. *C. elegans* é um organismo de fácil manipulação genética, através da construção de estirpes transgênicas com deleção ou superexpressão de genes de interesse (PRAITIS et al., 2001; KAYMAK et al., 2016).

3.4.1 *Caenorhabditis elegans* como modelo para Doença de Huntington

C. elegans é um importante modelo para a compreensão dos mecanismos moleculares que regulam as respostas ao estresse, doenças neurogenerativas e envelhecimento. Várias proteínas humanas propensas à agregação associadas a doenças neurodegenerativas são expressas em diversos tecidos de *C. elegans* para entender sobre a agregação e o dobramento incorreto de proteínas (DIMITRIADI; HART, 2010; LI; LE, 2013; LUBLIN; LINK, 2012). *C. elegans* possui fatores de transcrição altamente conservados que regulam a resposta à longevidade, estresse e homeostase proteica, elucidando seu papel na proteotoxicidade e neurodegeneração (DIMITRIADI; HART, 2010; LI; LE, 2013).

Dentre os modelos de neurodegeneração existe um bem descrito em *C. elegans*, como da DH. Através da estirpe AM141, a qual contém o transgene unc-54p::Q40::YFP, o modelo expressa 40 repetições da poliQ nas células musculares da parede do corpo do verme e mostra um fenótipo agregado fluorescente ao atingir a idade adulta (NOLLEN et al., 2004). A estirpe AM101, contém o transgene F25B3.3p::Q40::YFP, o modelo superexpressa 40

repetições da poliQ pan-neuronalmente (GIDALEVITZ et al., 2006). Além disso, a estirpe HA759, a qual expressa Htt-Q150 (um trato de poliQ com 150 repetições derivado da huntingtina humana) fortemente expressa em neurônios ASH e fracamente em outros neurônios, levando à morte neuronal de ASH. A fluorescência GFP é utilizada como um indicador para a sobrevivência desses neurônios (VOISINE et al., 2007).

Os neurônios sensoriais ASH são considerados como neurônios nociceptivos polimodais. A detecção de estímulos sensoriais aversivos no ambiente é uma característica fundamental do sistema nervoso animais, permitindo-lhes evitar substâncias químicas nocivas e condições perigosas. Os animais usam neurônios especializados e estruturas sensoriais chamadas nociceptores para detectar uma variedade de estímulos aversivos e dolorosos, incluindo produtos químicos tóxicos. Uma característica dos neurônios nociceptores é que eles são frequentemente polimodais e podem responder a muitos tipos diferentes de estímulos sensoriais (HILLIARD et al., 2004). *C. elegans* possui um par desses neurônios na cabeça, onde se faz necessário para evitação de repelentes químicos e voláteis (octanol), choque osmótico e estimulação mecânica na ponta do nariz do verme (BARGMANN et al, 1990 ; KAPLAN; HORVITZ, 1993; TROEMEL et al, 1997; HART et al, 1999; SAMBONGI et al, 1999; HILLIARD et al, 2002).

Muitas vias conservadas entre mamíferos e *C. elegans* estão envolvidas na patogenia da DH, entre elas podemos citar o fator de transcrição DAF-16 (um homólogo de *C. elegans* do Forkhead box (FOXO) de mamíferos), o fator de transcrição de choque térmico HSF-1 e as proteínas de choque térmico (HSPs). Nollen e colaboradores (2004) demonstraram que o knockdown de HSF-1 aumentou a agregação da proteína poliQ em modelos de DH em *C. elegans.* Da mesma forma, a redução de agregados proteicos pelo silenciamento de *age-1* de maneira dependente de DAF-16, demonstrou a correlação entre as vias associadas ao envelhecimento e a agregação da poliQ. Também, o silenciamento de HSP-70 aumentou a agregação de proteínas em modelos poliQ (NOLLEN et al., 2004). A indução das HSPs é regulada pelos fatores de transcrição HSF-1 e DAF-16 (MORLEY; MORIMOTO, 2004; SEO et al., 2013).

É sabido sobre o envolvimento das HSPs na homeostase proteica de *C. elegans* que expressam poliQ (BOASQUIVIS et al., 2018). Foi demonstrado que HSP-16.2 em particular desempenha um papel protetor contra poliQ (TAKEUCHI et al., 2017). Nosso grupo de pesquisa já demonstrou o envolvimento do extrato hidroalcoólico de *Ilex paraguariensis* na expressão dessa chaperona em modelo de *C. elegans* (Machado et al., 2019). Além disso, estudos anteriores demonstraram que DAF-16 está envolvido na formação de agregados

proteicos menos tóxicos (KARAGOZ; RUDIGER, 2015), por meio da ativação de genes antioxidantes e proteínas de choque térmico, diminuindo assim a agregação e toxicidade da poliQ (ZECIC; BRAECKMAN, 2020). Sob estresse, a proteína DAF-16 é fosforilada e ativada e se acumula no núcleo, ativando assim sua própria função para regular genes-alvo a jusante, como a SOD-3 que desempenha um papel importante no estresse e envelhecimento (CHIRUMBOLO, 2010). Análises genéticas demonstraram que DAF-16 é um importante ativador transcricional de um subcojunto de chaperonas, como a HSP 16.2. A HSP 16.2 é uma chaperona protetora contra modelos de poliQ, pois pode promover o dobramento/redobramento de proteínas na conformação adequada e pode restaurar as proteínas previamente agregadas (KIM; KIM; LEE, 2016).

3.4.2 *Caenorhabditis elegans* e metais

O desbalanço da homeostase de metais ocorre quando os níveis de metais aumentam ou diminuem além dos limites normais. Devido a sua importância, os metais traços desempenham papeis vitais em processos bioquímicos e neurais. Quando em homeostase, os metais facilitam a função cerebral normal, protegendo contra EROs, regulando a expressão gênica e ativando enzimas. O desequilíbrio na homeostase leva a danos celulares induzidos pela formação de EROs e danos oxidativos (GROCHOWSKI et al., 2019).

Uma enorme vantagem de utilizar o *C. elegans* como modelo de exposições a metais e neurotoxicidade é a simplicidade de seu sistema nervoso, que possui 302 neurônios e cerca de 5.000 sinapses (WHITE et al., 1986). Apesar da simplicidade, *C. elegans* possui um sistema nervoso completo, com 4 classes funcionais de neurônios com base em seus circuitos: 1: neurônios motores, transmitem sinais sinápticos as células musculares; 2: neurônios sensoriais, convertem sinais ambientais em estímulos internos; 3: interneurônios, recebem e transmitem sinais entre os neurônios; 4: neurônios polimodais, possuem duas ou mais das funções citadas acima (CHEN et al., 2013).

O papel de *C. elegans* como um biossensor para avaliação de riscos ambientais causados por metais vem sendo explorado por biomarcadores, como GSH, metalotioneína (MT), HSPs e transportadores envolvidos na desintoxicação de metais. Desta maneira, o *C. elegans* demonstra ser um modelo importante para pesquisas toxicológicas (BEYERSMANN; HARTWING, 2008).

C. elegans possui proteínas ortólogas altamente conservadas envolvidas no metabolismo de metais, como o transportador de metal bivalente 1 (DMT-1), que possui 2 genes de metalotioneína (*mtl-1*e *mtl-2*), onde demonstram um papel essencial na proteção contra a toxicidade de metais. Os dois genes tem funções diferentes, onde *mtl-1* é expresso constitutivamente no bulbo faríngeo na ausência de exposições a metais, portanto, pode atuar como sensor de metais. A expressão de *mtl-1* e *mtl-2* é aumentada na região do intestino após a exposição ao metal. Ambas as isoformas tem preferência por ligação de metais, o *mtl-1* para Zn (II) e *mtl-2* para Cd (II) (ZEITOUN et al, 2010). Em relação ao metabolismo do ferro, *C. elegans* possui ferritina (FTN-1 e FTN-2) e transportador de ferro (FPN-1.1, FPN-1.2 e FPN-1.3) (ANDERSON; LEIBOLD, 2014).

4. DESENVOLVIMENTO

O desenvolvimento que faz parte desta tese está apresentado sob a forma de dois artigos científicos. Os itens Introdução, Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas, encontram-se nos próprios artigos.

O artigo 1 foi publicado na revista Nutritional Neuroscience e encontra-se no formato da mesma.

O artigo 2 foi publicado na revista NeuroToxicology e encontra-se no formato da mesma.

4.1 ARTIGO CIENTÍFICO 1

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Neuroprotective effects of rutin on ASH neurons in *Caenorhabditis elegans* model of Huntington's disease

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Neuroprotective effects of rutin on ASH neurons in *Caenorhabditis elegans* model of Huntington's disease

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ABSTRACT

Huntington's disease (HD) is an autosomal dominant, progressive neurodegenerative disease. It occurs due to a mutated huntingtin gene that contains an abnormal expansion of cytosine-adenine-guanine repeats, leading to a variable-length N-terminal polyglutamine (polyQ) chain. The mutation confers toxic functions to mutant huntingtin protein, causing neurodegeneration. Rutin is a flavonoid found in various plants, such as buckwheat, some teas, and apples. Our previous studies have indicated that rutin has protective effects in HD models, but more studies are needed to unravel its effects on protein homeostasis, and to discern the underlying mechanisms. In the present study, we investigated the effects of rutin in a *Caenorhabditis elegans* model of HD, focusing on ASH neurons and antioxidant defense. We tested behavioral changes (touch response, movement, and octanol response), measured neuronal polyQ aggregates, and assessed degeneration using a dye-filling assay. In addition, we analyzed expression levels of heat-shock protein-16.2 and superoxide dismutase-3. Overall, our data demonstrate that chronic rutin treatment maintains the function of ASH neurons, and decreases the degeneration of their sensory terminations. We propose that rutin does so in a mechanism that involves antioxidant activity by controlling the expression of antioxidant enzymes and other chaperones regulating proteostasis. Our findings provide new evidence of rutin's potential neuroprotective role in the *C. elegans* model and should inform treatment strategies for neurodegenerative diseases and other diseases caused by age-related protein aggregation.

KEYWORDS

C. elegans; neurodegenerative diseases; polyQ; flavonoids; natural compounds; neurodegeration; proteinopathies; aggregation of proteins

1. Introduction

Huntington's disease (HD) is an autosomal dominant neurodegenerative disease caused by the expansion of a cytosine-adenine-guanine (CAG) trinucleotide repeat. CAG encodes the amino acid glutamine, and mutant versions of the huntingtin protein (Htt) possess a polyQ sequence in the amino-terminal region [1]. Most neurodegenerative diseases (ND) are characterized by the pathogenic accumulation and aggregation of proteins, which disrupt the dynamics of protein networks, and result in destabilization of cellular homoeostasis [2]. The mutant Htt (mHtt), considered to be the major characteristic phenotype of HD, forms aggregates in both the nucleus and the cytoplasm, due to its insoluble nature, and these aggregates accumulate and entangle together to form inclusions [3]. Other mechanisms, such as oxidative damage, have also been found to contribute to neuronal dysfunction and, eventually, death [4].

Currently, there are no disease-modifying treatments available, other than some approaches to address certain specific symptoms of HD. There is an ongoing search, therefore, for new, low-toxicity products capable of acting on the most diverse biochemical targets. Dietary consumption of flavonoids and/or foods rich in flavonoids has been shown to improve cognitive abilities, and to inhibit or delay senescence and neurodegenerative disorders [5].

Rutin (3,3′,4′,5,7-pentahydroxyflavone-3-rhamnoglucoside) is a flavonol found in many plants, such as buckwheat, apple, and tea. It has several reported pharmacological properties, including antioxidant, cytoprotective, anti-inflammatory, immunomodulatory, and neuroprotective activities [6]. Our group recently showed that rutin exerted antioxidant properties, and activated protein degradation (autophagy), in a *Caenorhabditis elegans* model of HD [7], and that it also attenuated 3-nitropropionic acid-induced HD-like symptoms in rats [8], suggesting that it may be a promising compound for

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preventing or treating neurodegenerative diseases. However, more studies are needed to unravel its effects on protein homeostasis and the underlying mechanisms.

Reactive oxygen species (ROS) are formed as a natural by-product of metabolism, and have significant roles in cell signaling and homeostasis. Breaking the balance can be deleterious, and is termed oxidative stress. Under these conditions, excessive free radicals can damage the cell membrane via lipid peroxidation, modifying signal and structural proteins, leading to misfolding and aggregation, which are characteristics of ND [9]. Antioxidants could, therefore, be useful for the cell. Furthermore, heat-shock proteins are involved in controlling protein folding and play an important role in ensuring proteostasis [10].

C. elegans has highly conserved transcription factors regulating stress resistance responses, longevity, and protein homeostasis, allowing for the elucidation of their role in protein toxicity and neurodegeneration [11]. It is a reliable *in vivo* model system to study the development of HD related to aging [7, 12], and the availability of several disease-like mutant strains allows us to test the effects of various compounds, including natural and synthetic antioxidants. Furthermore, transgenic *C. elegans* models that express an N-terminal human Htt fragment with different numbers of CAG repeats have been used to model HD. The models generally express the repeats in specific neurons, such as the ASH sensory neurons, which are multi-modal sensory neurons that mediate avoidance to chemo- and mechanosensory stimuli [13]. Transgenic *C. elegans* lines expressing different lengths of polyQ-YFP (yellow fluorescent protein) or GFP (green fluorescent protein) from integrated arrays in muscle (polyQm) [14] or neuronal (polyQn) [15] cells have been utilized in genetic screens to address the mechanisms underlying the impact of aggregation-prone proteins on cellular function. *C. elegans* is therefore a highly sensitive system to conduct pharmacological screens for suppression of polyQ-induced physiological dysfunctions.

The extent of the global burden of neurodegenerative diseases underscores the urgent need for strategies to identify disease-modifying factors, and in this context, the pharmacological potential of rutin warrants further research. In the present study, we investigated neurodegeneration in ASH neurons, and the putative pathways involved (focusing on oxidative stress), via behavioral assays and vital dyes.

2. Materials and methods

2.1. Chemicals and reagents

Ethanol, 5-fluorodeoxyuridine (FUDR), 1-*octanol*, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate (DiD), and rutin (synthetic font) were purchased from Sigma-Aldrich (USA).

2.2. C. elegans strains and maintenance

We used the following *C. elegans* strains: Bristol N2 (wild-type), AM141 (rmIs133[unc-54p::Q40::YFP]), AM101 (rmIs110[F25B3.3p::Q40::YFP]), HA759 (rtIs11[osm-10p::GFP + osm-10p::HtnQ150+Dpy-20 (+)]), CL2070 (dvIs70[hsp-16.2p::GFP + rol-6 (su1006)]), and CF1553 (muIs84[(pAd76)sod-3p::GFP + rol6(su1006)]). All were obtained from the *Caenorhabditis* Genetics Center (University of Minnesota, Minneapolis, MN, USA). Age-synchronized worms were obtained by isolating embryos from gravid hermaphrodites, using bleaching solution (1% NaOCl, 0.25 M NaOH) [16]. Eggs were allowed to hatch overnight in M9 buffer to obtain worms in the first larval stage (L1).

2.3. Treatment of worms with rutin

Treatment of worms was conducted as previously reported [7]. Rutin powder was diluted in absolute ethanol and added to the surface of nematode growth medium (NGM) agar plates with *E.coli* OP50 (already grown overnight at 37°C) to obtain final concentrations of 15, 30, 60, and 120 µM (9.16–73.26 mg rutin/mL agar), and 1% ethanol. Synchronized L1 worms were transferred to either treatment or control plates, and incubated at 20°C until adulthood. In control plates, ethanol was added in the same volume used in the treatment plates. For analysis after the young adult stage, we used FUDR in plates with a final concentration of 12 µM to avoid progeny, and worms were transferred to new plates with FUDR and rutin or vehicle every two days.

2.4. Behavioral tests

Ten worms per treatment were analyzed for the following behavior parameters: touch response, number of thrashes [17–19], and octanol response [20, 21]. A set of four experiments were performed individually.

2.4.1. Touch response

To analyze the response to touch we used the N2, HA759, and AM101 strains. The worms' touch response was assessed by gently touching the head region of the animal with a bristle brush. Indicates how many times worms responded to ten touches with a 10-second rest period between trials [19]. Four assays were carried out at different times, and ten worms were analyzed for each experiment.

2.4.2. Thrash frequency

A test of thrash frequency was applied to N2, AM141, AM101, and HA769 strains. Worms from control or rutin treatments were individually placed into a drop of M9, and allowed to adapt for 1 min. A thrash was defined as a change in the direction of bending at the middle of the body [18], and the number of thrashes over a 20-second period was counted with the aid of a Nikon E200 microscope. Four assays were carried out at different times, and 10 worms were analyzed for each experiment.

2.4.3. Octanol response

Response to 1-octanol was assessed as previously described, with minor modifications [20, 21]. Well-fed worms were transferred to food-free intermediate NGM agar plates to remove any remaining *E. coli* OP50. After 1 min, the worms were transferred to new food-free NGM agar plates and allowed to adapt for 5 min. The assay consisted of submerging a bristle brush in 30% 1-octanol (dissolved in 100% ethanol, v/v), and placing it in front of a forward-moving worm. The latency time until the backward movement was counted. At least ten worms were analyzed in triplicate, and the means of each triplicate were considered. The experiment was repeated on four different days, thus at least 40 worms were observed in this assay. The third day of the adult stage was chosen, in order to align with previously observed patterns of neurodegeneration of ASH neurons [7].

2.5. Measurement of polyQ aggregates

The number of neuronal polyQ aggregates in the nerve-ring neurons of individual worms of strain AM101 was counted in young adult worms, and on day 4 of adulthood [22]. The worms were mounted onto a glass slide, and paralyzed with 5 µL of 50 mM sodium azide. Approximately 10 worms were randomly selected in each treatment, and scored for the number of polyQ40::YFP aggregates in the nerve-ring neurons, with the aid of an Olympus® Fluoview FV10i confocal microscope, and ImageJ. Three independent experiments were performed individually.

2.6. Dye filling

To visualize degeneration in Htn-Q150 worms (strain HA759), we performed the DiD dye-filling assay [12] on the third day of adulthood. DiD is a lipophilic vital dye, which is taken up by the sensory endings of the ASH neurons unless they are absent or have defective sensory endings. By counting the percentage of GFP-expressing ASH neurons that fail to take up the dye, it is possible to quantify the number of live neurons that have degenerated, based on sensory process retraction [23].

Worms were incubated for 1 h in 2 mg/ml DiD, then washed three times with M9 to de-staining. The worms were mounted onto a glass slide and paralyzed with 5 µL of 50 mM sodium azide. Approximately 10 ASH neurons were examined in three different experiments, using an Olympus® Fluoview FV10i confocal microscope. The percentage of dye-filling defective ASH neurons was used as a measure of the average of the dye-filling defect for the corresponding groups.

2.7. Quantification of superoxide dismutase-3 and heat-shock protein-16.2 expression

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 (GFP expression in the head, tail and around the vulva, and whole body, respectively) for each transgenic strain [24]. 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 mL 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, five worms were randomly selected to measure the mean pixel density. The data are expressed as the mean of the arbitrary fluorescence units (AFU).

2.8. 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 a Dunnet *post hoc* test. Values of $p < .05$ were considered to be statistically significant.

3. Results

3.1. Effect of rutin on touch response

As shown in Figure 1(A), no differences were observed in N2, but in HA759 (HtnQ150 over-expressed specifically in the ASH neurons) the touch response was

significantly increased by rutin treatment at 30 µM ($p < .05$; Figure 1(B)). In the AM101 strain (Q40 over-expressed pan-neuronally) there was no significant difference in response among young adult worms ($p < .05$; Figure 1(C)), but among worms at day 4 of adulthood there was a significant increase induced by all concentrations of rutin ($p < .05$; Figure 1(D)).

3.2. Effect of rutin on thrash frequency

As shown in Figure 2(A), no differences were observed in N2, but in AM141 (Q40 over-expressed in the body wall muscle), the thrash frequency significantly increased after rutin treatment at 30 and 60 µM concentrations ($p < .05$; Figure 2(B)). The control group rate was 115 thrashes per minute, while in the group with 30 and 60 µM rutin, it was 162 and 146, respectively. Rutin also increased the thrashing movement of AM101 (Q40 over-expressed pan-neuronally) at 15 and 120 µM in the young adult stage (Figure 2(C)), while in worms at day 4 of adulthood the number of thrashes per minute was significantly increased to 159, 177, and 149, at 30, 60 and 120 µM respectively, compared to control worms with 122 thrashes per minute ($p < .05$; Figure 2(D)). In the HA759 (HtnQ150 over-expressed specifically in the ASH neurons) the thrash frequency significantly increased by all concentrations of rutin ($p < .05$; Figure 2(E)).

3.3. Effect of rutin on octanol response

We performed the octanol response behavioral assay in N2, and HA759 (HtnQ150 over-expressed specifically in the ASH neurons) strains at day 3 of adulthood. As shown in Figure 3(A), there was a significant decrease at 15 and 30 µM in the N2 strain ($p < .05$; Figure 3 (A)). While in mutant HA759 there was a significant decrease in latency response (in seconds) by the treatment with rutin in all concentrations ($p < .05$; Figure 3 (B)).

3.4. Effect of rutin on polyQ-mediated neurotoxicity

Figure 4(A) shows representative images of neuronal polyQ aggregation in young adult worms and worms at day 4 of adulthood. Rutin significantly decreased polyQ aggregation in AM101 (Q40 over-expressed pan-neuronally) young adult worms at 15, 30, and 60 µM (Figure 4(B), $p < .05$), with an average of 1.3, 1.2, and 1.3 aggregates respectively, compared to 1.8 aggregates in control worms. Worms at day 4 of adulthood were also evaluated. Figure 4(C) shows that all rutin concentrations decreased the number of aggregates relative to the control. The average number of aggregates observed was 6.5, 4.8, 4.9, and 5.7 in worms treated with 15, 30, 60, and 120 µM of rutin, respectively, compared to an average of 11.2 aggregates in control worms.

3.5. Effect of rutin on dye-filling defects in ASH neurons

Figure 5(A) shows representative images of a viable GFP-labeled ASH neuron with DiD dye uptake, and a degenerated GFP-labeled ASH neuron with a DiD dye-filling defect. Worms treated with rutin had a decrease of 19%, 7.3%, and 16% in neurodegeneration in ASH neurons at 15, 30, and 60 µM rutin, respectively, compared to 52.6% of the control (Figure 5(B), $p < .05$).

3.6. Effect of rutin on SOD-3 and HSP-16.2 expression

Figure 6(A) shows representative images of SOD-3::GFP. Worms treated with rutin at concentrations of 15, 30, and 60 µM had an increase of 34%, 48% and 32% GFP-labeled SOD-3 fluorescence in relation to the control at 15, 30 and 60 µM rutin, respectively (Figure 6(B), $p < .05$). Figure 7(A) shows representative images of HSP-16.2::GFP. Worms treated with rutin showed an increase of 40%, 26%, 39% and 32% in GFP-labeled HSP-16.2 fluorescence in relation to the control at 15, 30, 60 and 120 µM rutin, respectively (Figure 7(B), $p < .05$).

4. Discussion

Several neurodegenerative diseases are known to share some common molecular mechanisms, such as pathogenic protein aggregation and oxidative stress [25]. In our study, we investigated the protective properties of rutin in polyQ-induced neurodegeneration in *C. elegans*. We focused on sensory ASH neurons, and assessed the impact of rutin on the function and survival of ASH neurons via behavioral assays and vital dyes. The sensory system is necessary for the maintenance of the worm's life, due to the foraging behavior, and ASH sensory neurons are largely responsible for controlling the response to sensory stimuli [26]. Besides being involved in mechanosensory behaviors, such as touch response and response to odorants (e.g. 1-octanol), ASH neurons also modulate mechanoreceptor behaviors such as movement. The ability of ASH to respond to both chemical and mechanical cues defines them as polymodal nociceptors, analogous to the

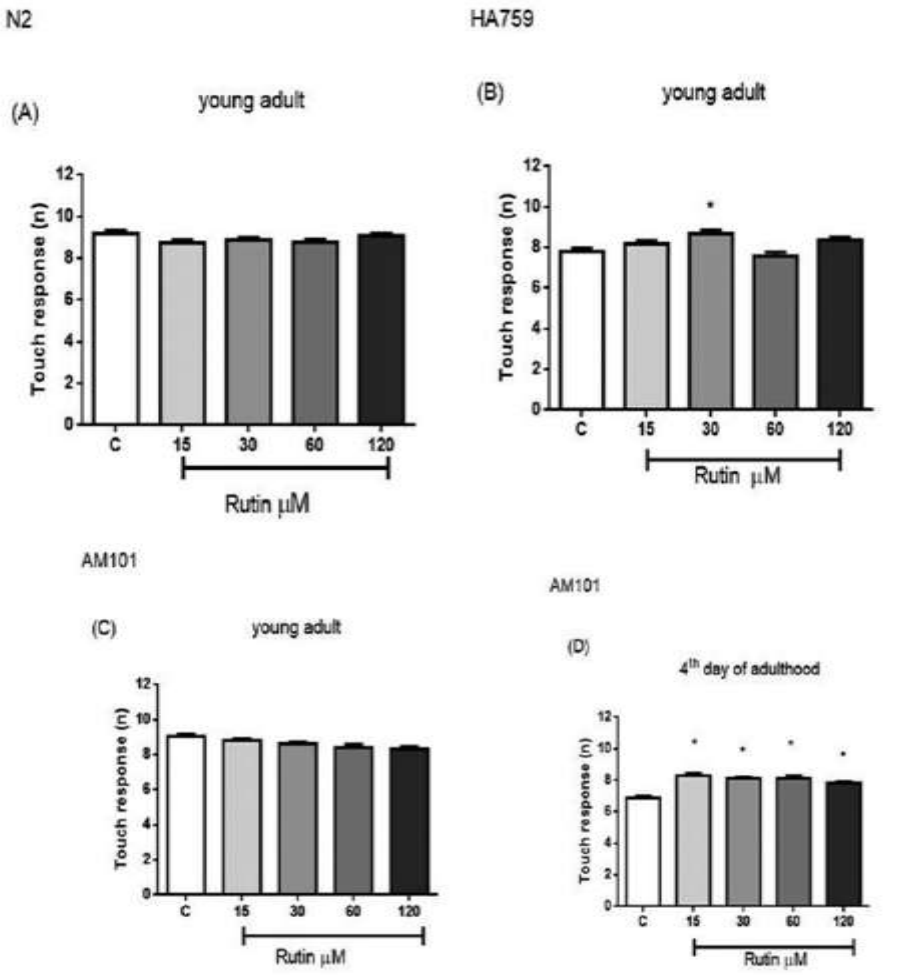

Figure 1. Effect of rutin on touch response behavior in (A) Wild type (N2), (B) HA759, and (C) AM101 young adult worms, and in (D) AM101 4-day-adults. Data are derived from four independent assays with 10 worms per group in each experiment. Results are represented as means ± S.E.M. *$p < .05$ compared to control (one-way ANOVA followed by Dunnett's *post hoc* test).

pain-sensing polymodal nociceptive neurons in vertebrates. We demonstrated that rutin can maintain the function of ASH neurons, observed in behaviors as the touch response assay. Our data demonstrate that rutin increased touch response in HA759 (HtnQ150 over-expressed specifically in the ASH neurons) and AM101 (Q40 over-expressed pan-neuronally) worms in day 4 of adulthood (Figure 1). Our data demonstrate that rutin increased movement in AM141 (Q40 over-expressed in the body wall muscle), AM101 (Q40 over-expressed pan-neuronally) worms in young adult stage and day 4 of adulthood, and in HA759 strain (HtnQ150 over-expressed specifically in the ASH neurons) (Figure 2), in addition, decreased the latency time in response to 1-Octanol in HA759 worms at day 3 of adulthood (Figure 3). The discovery of compounds that may preserve the function of neurons in intact organisms opens up a new direction in drug-screening methodology.

An important event in ND involves the formation of free radicals, oxidative stress, and the misfolding, aggregation, and accumulation of proteins [27]. Previous studies have already demonstrated that rutin could act as a neuroprotector compound in Alzheimer's disease and HD animal models [7, 28], and that it can protect the striatum from oxidative/nitrosative insults caused by 3-nitropropionic acid in a HD model in rats, in addition to improving motor activity, memory, and

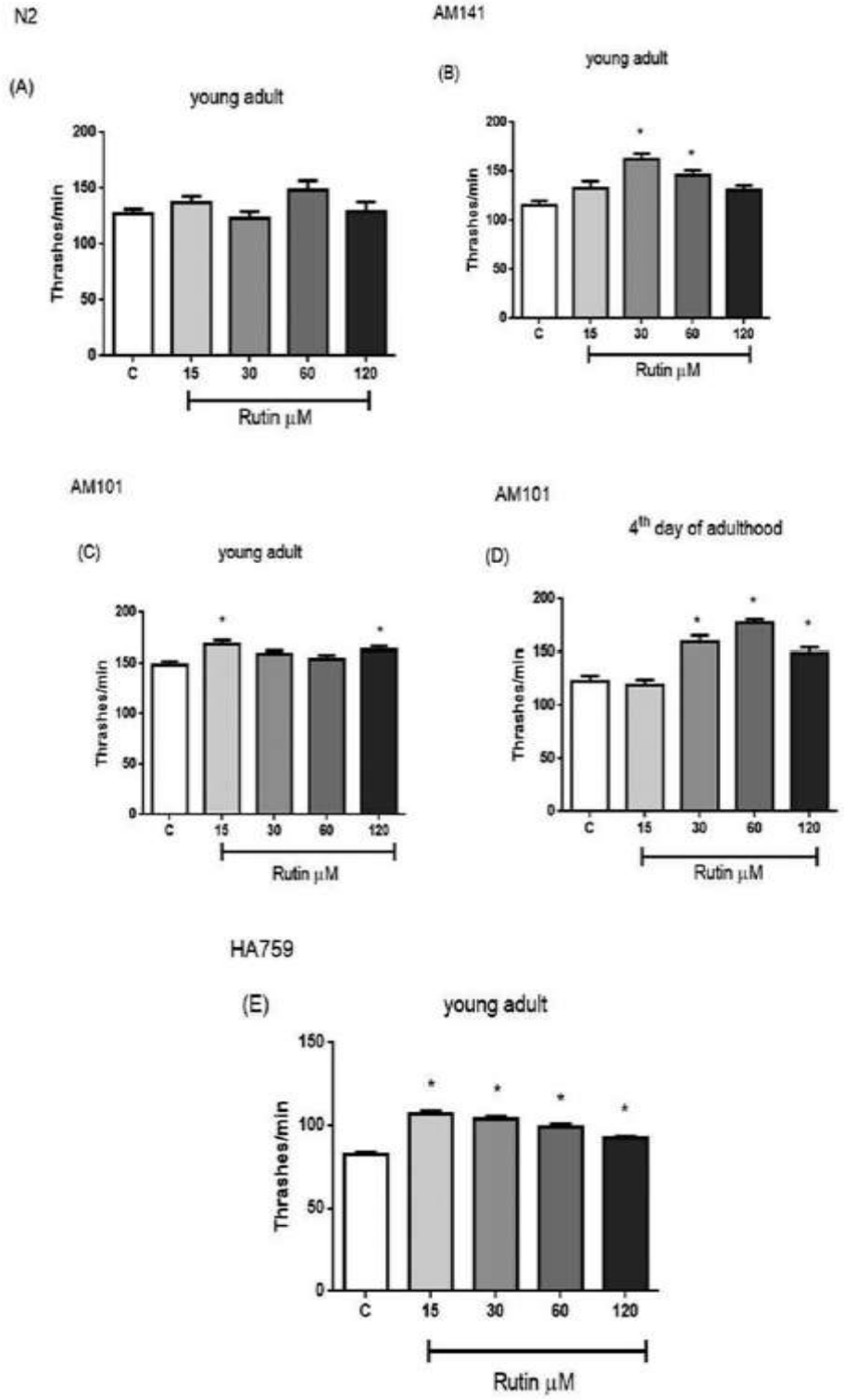

Figure 2. Effect of rutin on movement in (A) Wild type (N2), (B) AM141, (C) AM101 young adult worms, (D) AM101 4-day-adults and in (E) HA759. Data are derived from four independent assays with 10 worms per group in each experiment. Results are represented as means ± S.E.M. *$p < .05$ compared to control (one-way ANOVA followed by Dunnett's *post hoc* test).

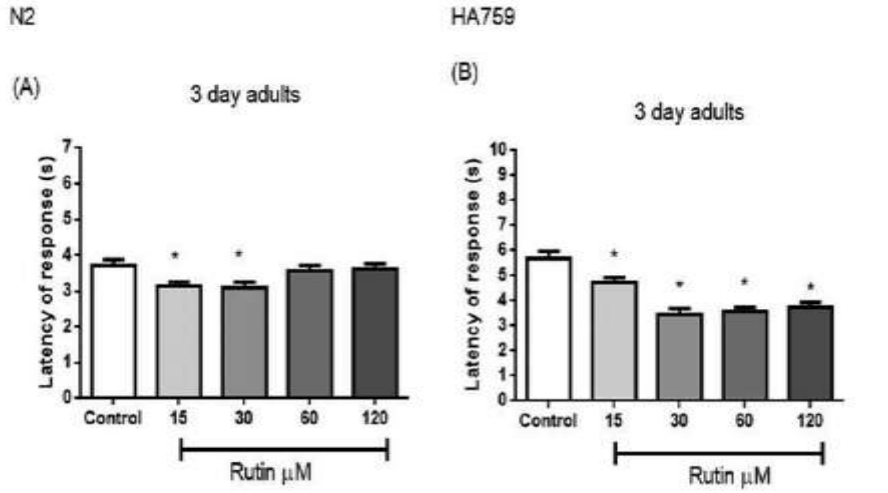

Figure 3. Effect of rutin on the latency of response (in seconds) to octanol in (A) Wild type (N2) and (B) HA759 3-day-adult worms. Data are derived from four independent experiments with 10 worms in each experiment. Results are represented as means ± S.E.M. *$p < .05$ compared to the control group (one-way ANOVA followed by Dunnett's *post hoc* test).

learning [8]. Rutin (and/or its metabolites) may also be able to cross the blood–brain barrier, and thereby modify the cognitive and behavioral symptoms of ND [29].

Landon et al [30] showed that pan-neuronal 40Q-neuron worms performed poorly in learning tasks, suggesting that polyQ disrupts long-term potentiation in *C. elegans*, as it does in mouse models of HD [30]. Collectively, these results indicate that this worm may be a good model for vertebrate neurodegenerative diseases. Furthermore, the generation of a pan-neuronal model in *C. elegans* has made it possible to compare tissue-specific responses to polyQ proteins. When polyQ proteins are expressed in muscle cells, both aggregation and toxicity are length-dependent, with the same pathogenic threshold of 35–40 repeats in young adult worms [14]. Therefore, that polyQ pathogenesis in neurons occurs at the same threshold as seen in muscle cells. Our data demonstrate clearly that in addition to the improvement in behavioral tasks (Figures 1–3), rutin decreased the number of polyQ aggregates in neurons (Figure 4). Viewed together with our previous results [7] and corroborating the work of Brignull et al. [15] – this work suggests that the threshold for aggregation and toxicity in *C. elegans* is not a unique feature of tissue type, but is instead a feature of polyQ proteins.

The idea that some polyphenolic compounds may interfere with protein aggregation is not new. Flavonoids are considered to be the major category of dietary polyphenols; for example, rutin is a citrus flavonoid glycoside, which is a low-molecular-weight polyphenolic compound. Wanker et al. [31] found that epigallocatechingallate (EGCG), a green tea polyphenol, modulates misfolding and oligomerization of the expanded polyQ proteins, resulting in efficient suppression of polyQ aggregation *in vitro* [31]. EGCG suppressed aggregation formation of the polyQ proteins, and polyQ-induced cytotoxicity, in HD models of yeast and *Drosophila* [31], and in the SCA3 model of *C. elegans* [32]. Curcumin inhibited tau [33], α-synuclein [34], and Htt protein [35], and acted as an activator of molecular chaperones in polyQ diseases [33, 35]. Finally, our own studies have shown that chronic rutin treatment reduced polyQ protein aggregation in muscle, and reduced polyQ-mediated neuronal death in ASH neurons [7]. No specific drugs are available to counter the pathology of polyQ aggregates, and current treatments have multiple side effects; there is an urgent need, therefore, to find natural modulators with neuroprotective effects against polyQ diseases.

The sensory endings of the ASH and several other *C. elegans* neurons are exposed to the environment, allowing the neurons to take up lipophilic vital dyes, including DiD [12]. These fluorescent compounds accumulate in cellular membranes, facilitating rapid visualization of these cells; neurons that fail to take up the dye are therefore either absent or have defective sensory endings [36, 37]. We examined the effect of rutin on the dye-filling of ASH neurons in 3-day-old worms containing HtnQ150 (the Htt fragment containing a

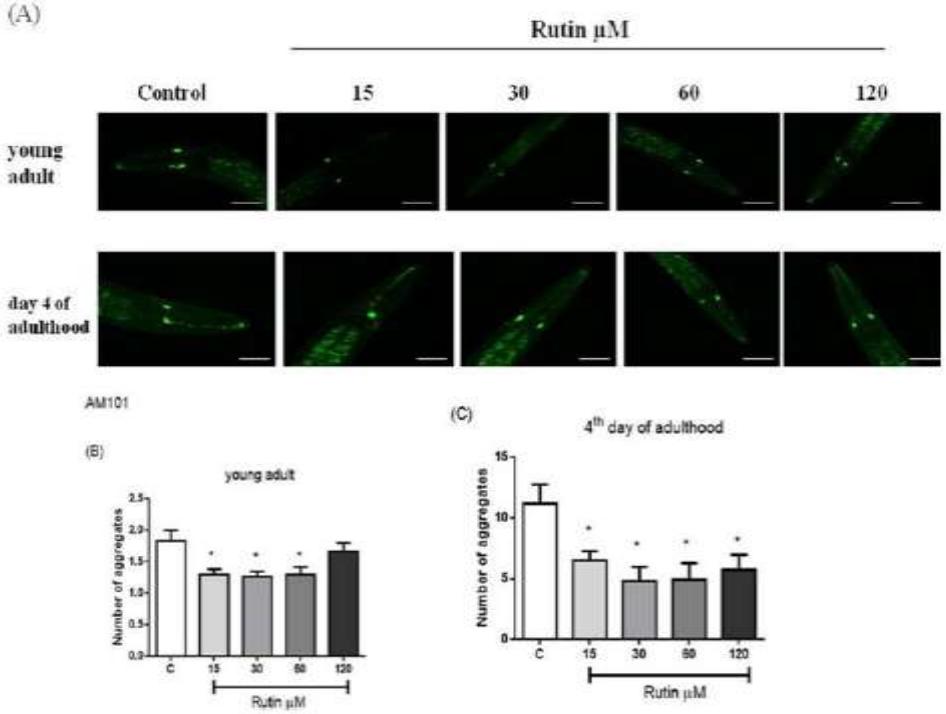

Figure 4. Effect of rutin on neuronal PolyQ aggregation in AM101 strain. (A) representative images and quantification of aggregates in (B) young adults and (C) 4-day-adult worms. Data are derived from three independent experiments with 10 worms each experiment. Results are shown as means ± S.E.M. *$p < .05$ compared to the control group (one-way ANOVA followed by Dunnett's *post hoc* test).

polyQ tract of 150 residues), and found a significant decrease in the dye-filling defect (Figure 5). We conclude that rutin plays an important role in attenuating the degeneration of sensory terminations in these neurons.

Evidence for accumulative oxidative damage related to the development of HD has been demonstrated [38]. When oxidative stress arises from a pathological event, there is an energy imbalance associated with changes in the mitochondrial respiratory chain, leading to mitochondrial dysfunction [39], and this has been reported in HD patients. Htt 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 [40]. Here, we demonstrated that rutin treatment increased the expression of SOD-3 (Figure 6). SOD-3 is an ortholog of human MnSOD (SOD-2), involved in the removal of superoxide radicals, and is found in the mitochondrial respiratory chain super-complex [41]. SOD-3 is thought to play an essential role in age-related diseases, and MnSOD overexpression efficiently protected mice from 3-NP-induced anatomical and metabolic deficits that mimic HD [42]. Increased SOD-3 expression has also been shown to be related to a decrease in protein aggregation [43]. Nevertheless, rutin treatment in SOD-3 knockout worms would be useful to strengthen the conclusion.

To prevent aberrant accumulation of misfolded proteins that would otherwise be toxic, cells have a highly conserved and integrated protective system that maintains cellular protein homeostasis (proteostasis). Among these mechanisms are molecular chaperones, which play a central role in maintaining the cellular proteostasis that assists refolding of misfolded proteins, and which also mediate degradation through autophagy and proteasome machinery [44]. Since polyQ diseases are caused by misfolding and aggregation of the disease-associated proteins, activation of cellular protective mechanisms that maintain proteostasis, including molecular chaperones, is expected to be one a key therapeutic approach [45]. It has been demonstrated that HSP-16.2 in particular plays a protective role against polyQ [46], and in the present study rutin treatment was able to increase the expression of this chaperone

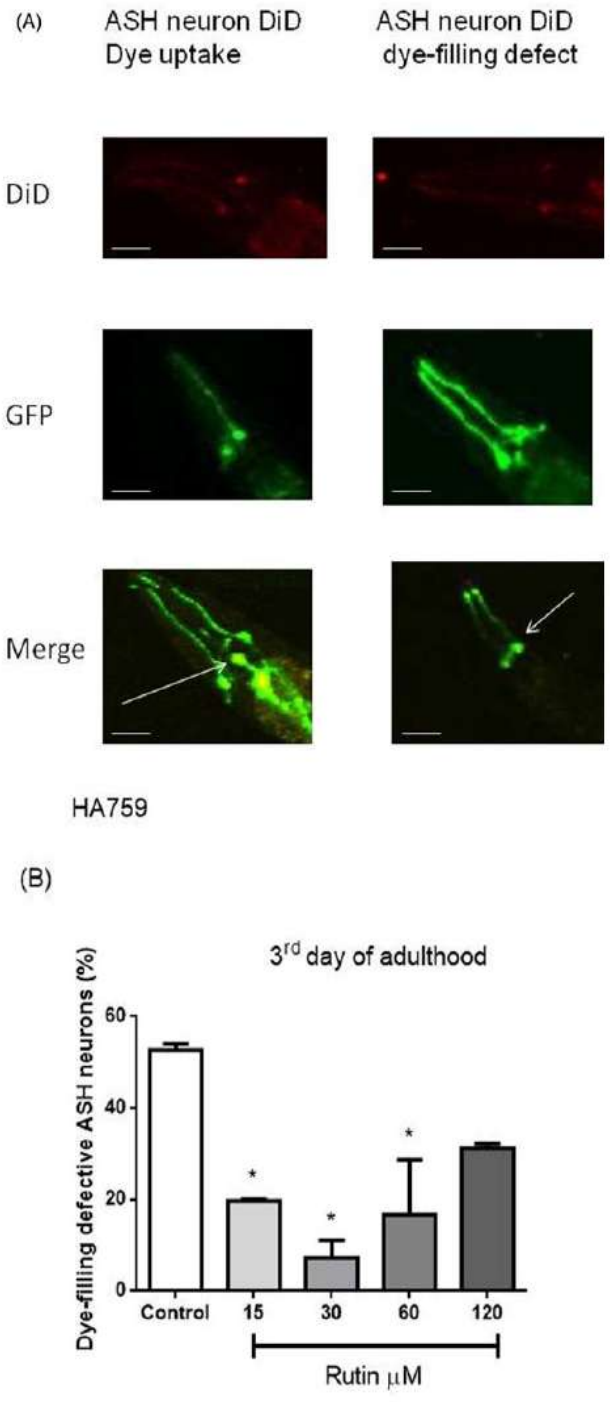

Figure 5. Effect of rutin on dye-filling defect in ASH neurons. (A) Representative images of viable GFP-labeled ASH neuron with DiD dye uptake (left panel, see arrow on merged image) and degenerated GFP-labeled ASH neuron with DiD dye-filling defect (right panel, see arrow on merged image) and (B) percentage dye-filling defective ASH neurons. Data are derived from three independent experiments with 10 worms each experiment. Results are shown as means ± S.E.M. $^{*}p < .05$ compared to the control group (One-way ANOVA followed by Dunnett's *post hoc* test).

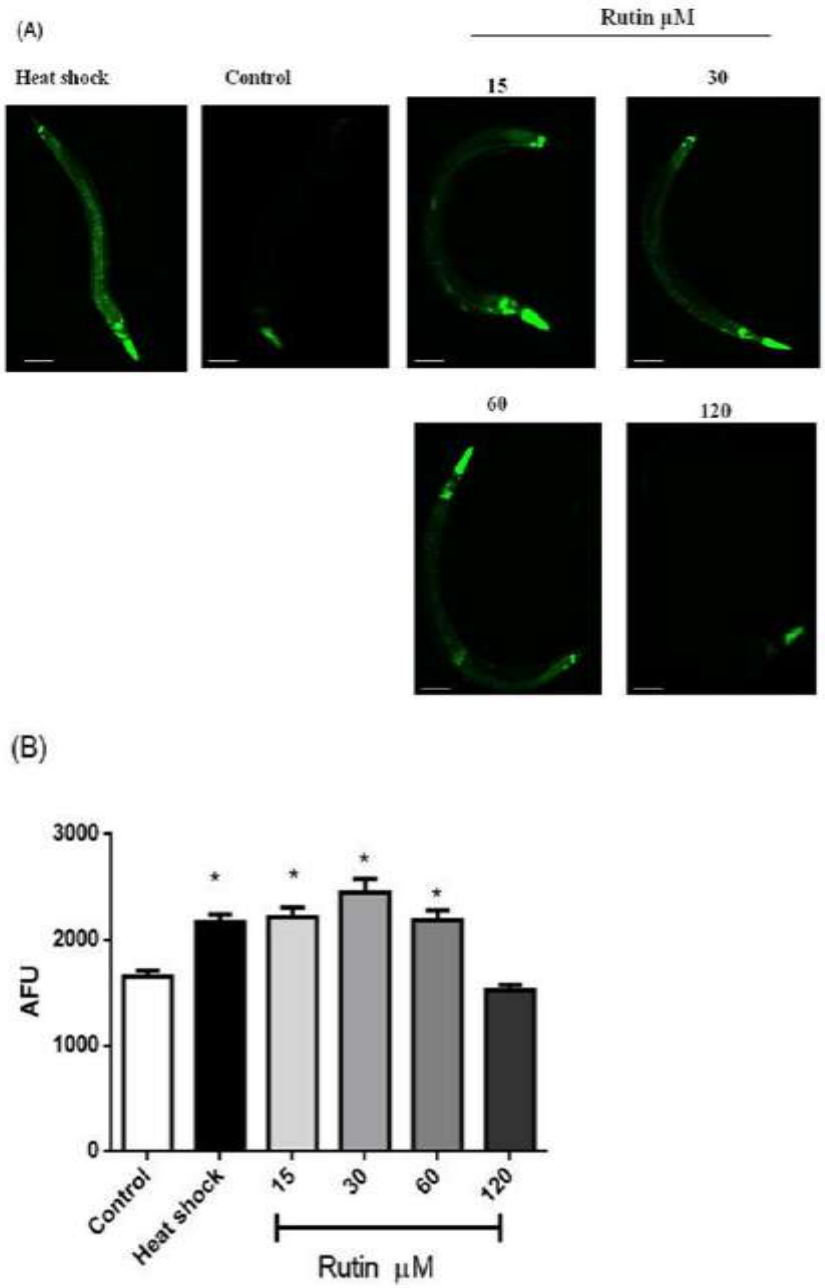

Figure 6. Effect of rutin on the expression of SOD-3 antioxidant enzyme. Worms exposed to heat shock at 35°C for 1 h were used as a positive control. (A) representative images and (B) quantification of SOD-3 expression. Data are expressed as means of arbitrary fluorescence units (AFU) ± S.E.M. derived from four independent assays with five worms per group in each experiment. *$p < .05$ compared to the control group (one-way ANOVA followed by Dunnett's *post hoc* test).

in *C. elegans* at all concentrations tested (Figure 7). Despite this, RT–PCR reactions would help to strengthen the conclusion.

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

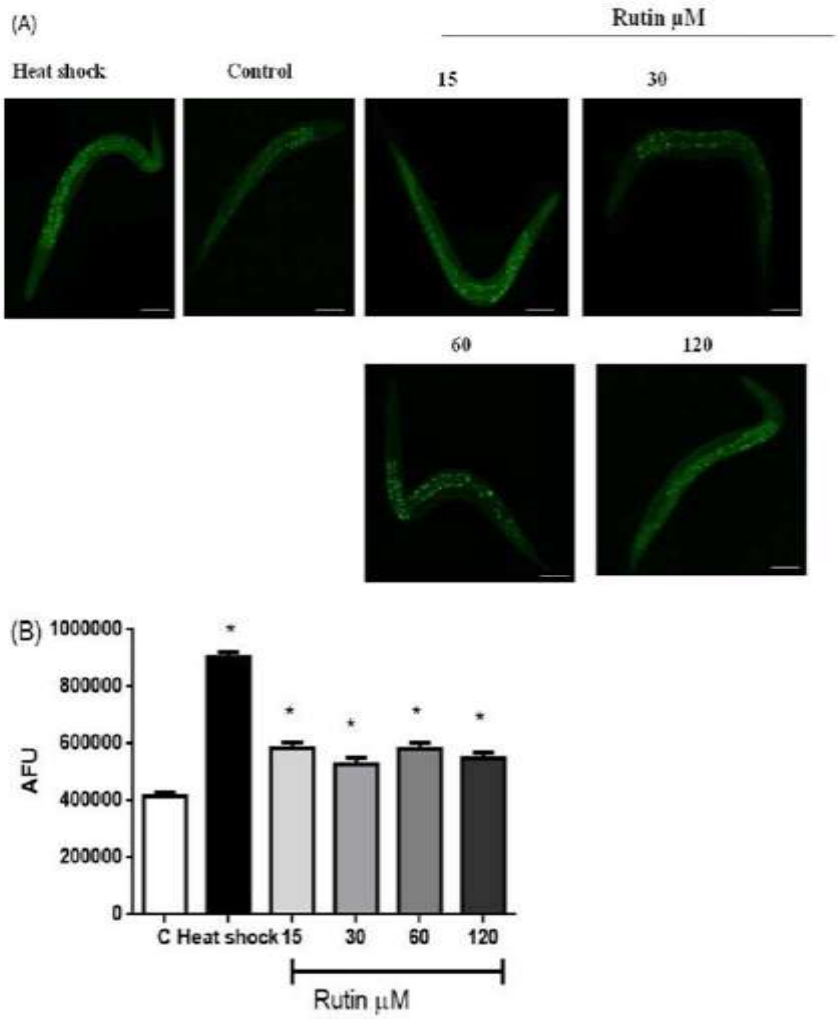

Figure 7. Effect of rutin on the expression of heat shock protein (HSP-16.2). Worms exposed to heat shock at 35°C for 1 h were used as a positive control. (A) representative images and (B) quantification of HSP-16.2 expression. Data are expressed as means of arbitrary fluorescence units (AFU) ± S.E.M. derived from three independent assays with 10 worms per group in each experiment. *$p < .05$ compared to the control group (one-way ANOVA followed by Dunnett's *post hoc* test).

growth factor (IGF)-1 receptor homolog [47]. 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 [48], via the activation of antioxidant genes and chaperones, thereby ameliorating polyQ aggregation and toxicity [49]. Our previous study demonstrated rutin-induced nuclear translocation of the DAF-16/FOXO transcription factor in *C. elegans* [7], which is essential for activating downstream genes. Under stress, DAF-16 proteins are phosphorylated and activated, and accumulate in the nucleus, thereby activating own functions to regulate downstream target genes, such as *sod-3*, which play an important role in metabolism, oxidative stress, and aging [50]. Genetic analysis has shown that DAF-16 is one of the essential transcriptional activators for a subset of chaperones, especially HSP-16.2. HSP-16.2 plays a protective role in polyQ diseases, as it promotes folding/refolding of proteins into appropriate conformations, and recovers previously aggregated proteins [51]. Molecular chaperones are also evolutionarily conserved in the cellular response to stress, and in the

regulation of longevity, and some studies show a direct role of these components in the cellular stress response associated with the regulation of lifespan [52].

We demonstrated previously that rutin exerts its protective effects against Htn toxicity through the overexpression of antioxidant enzymes and chaperones, as well as via the decrease of ROS [7]. Suganya and Sumathi [48] reported that oral administration of rutin significantly decreased protein oxidation and improved the endogenous antioxidant defense system in a rat model of HD induced by 3-NP. Other studies have also shown that rutin protects neuronal cells from amylin-induced neurotoxicity and oxidative stress, indicating a beneficial potential of this compound in other proteinopathies [53]. We propose, therefore, that rutin may increase the cell's antioxidant capacity through the overexpression of antioxidant enzymes and chaperones regulating proteostasis, consequently decreasing polyQ aggregation and toxicity, in addition to maintaining the function of ASH neurons.

5. Conclusions

Our results demonstrate the neuroprotective effects of rutin in *C. elegans* transgenic models of HD. The compound was able to maintain the function of the ASH neurons while decreasing the neurodegeneration or disturbance of their sensory terminations. The mechanisms proposed here are the modulation of SOD-3 and HSP-16.2 expression, through the increase of nuclear localization of DAF-16. Our findings provide new avenues for treatment strategies for neurodegenerative diseases and other diseases caused by age-related protein aggregation.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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4.2 ARTIGO CIENTÍFICO 2

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Toxicity of copper and zinc alone and in combination in *Caenorhabditis elegans* model of Huntington's disease and protective effects of rutin

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Metal mixture

ABSTRACT

Copper (Cu) and Zinc (Zn) are required in small concentrations for metabolic functions, but are also toxic. There is a great concern about soil pollution by heavy metals, which may exposure the population to these toxicants, either by inhalation of dust or exposure to toxicants through ingestion of food derived from contaminated soils. In addition, the toxicity of metals in combination is questionable, as soil quality guidelines only assess them separately. It is well known that metal accumulation is often found in the pathologically affected regions of many neurodegenerative diseases, including Huntington's disease (HD). HD is caused by an autosomal dominantly inherited CAG trinucleotide repeat expansion in the huntingtin (HTT) gene. This results in the formation of a mutant huntingtin (mHTT) protein with an abnormally long polyglutamine (polyQ) repeat. The pathology of HD results in loss of neuronal cells, motor changes, and dementia. Rutin is a flavonoid found in various food sources, and previous studies indicate it has protective effects in HD models and acts as a metal chelator. However, further studies are needed to unravel its effects on metal dyshomeostasis and to discern the underlying mechanisms. In the present study, we investigated the toxic effects of long-term exposure to copper, zinc, and their mixture, and the relationship with the progression of neurotoxicity and neurodegeneration in a *C. elegans*-based HD model. Furthermore, we investigated the effects of rutin post metal exposure. Overall, we demonstrate that chronic exposure to the metals and their mixture altered body parameters, locomotion, and developmental delay, in addition to increasing polyQ protein aggregates in muscles and neurons causing neurodegeneration. We also propose that rutin has protective effects acting through mechanisms involving antioxidant and chelating properties. Altogether, our data provides new indications about the higher toxicity of metals in combination, the chelating potential of rutin in the *C. elegans* model of HD and possible strategies for future treatments of neurodegenerative diseases caused by the aggregation of proteins related to metals.

1. Introduction

Some metals are essential in multiple physiological processes, such as electron and oxygen transport, enzyme activity, and neurotransmitter synthesis (Banci, 2013). However, they are needed in small concentrations (Wright and Baccaueli, 2007). Among the major environmental risk factors of neurodegenerative diseases, metals are gaining increasing attention because a larger percentage of the population is being exposed to them either by inhalation of dust or by ingestion of contaminated soil through food (Ramussen et al., 2001). Chronic metal poisoning can be attributed to long-term exposure to low levels of metals, this type of poisoning is asymptomatic and is associated with several disorders

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(Vonsattel and DiFiglia, 1998). Patients with neurodegenerative diseases have been observed to accumulate metals in their nervous system (Douglas and Dillin, 2010) suggesting a role for metals in these diseases.

In Huntington's disease (HD), tissue abnormalities and deposition of Copper (Cu) and Zinc (Zn) in brain regions have been found, which can be a result of disease initiation and progression (White et al., 2017; Huang et al., 1999; Squedrone et al., 2020). HD is caused by an expanded repeat of the cytosine-adenine-guanine trinucleotide (CAG) encoding a mutant protein with a polyglutamine sequence (polyQ). Mutant huntingtin contains an abnormally long sequence of polyQ, which is associated to its toxic properties, leading to dysfunction and death of neurons (Vonsattel and DiFiglia, 1998). HD and other neurodegenerative diseases (ND), such as Parkinson's and Alzheimer's diseases, are known for the accumulation and aggregation of proteins that disrupt the process of protein networks and destabilize homeostasis (Douglas and Dillin, 2010).

Elevated levels of metals may promote changes in the conformation, aggregation, and/or redox activity of proteins like Htt (Wright and Baccarelli, 2007; Huang et al., 1999; Squedrone et al., 2020). However, the exact mechanisms by which metals induce neurotoxicity are not fully understood, despite being reported as consequences of toxic exposures and dyshomeostasis in essential metal metabolism (Farina et al., 2013a). An additional aspect is that the effects of metals are usually evaluated on an individual metal basis, while people are, in fact, exposed to an environment of mixed toxicants, a situation more complicated to study. It is important to highlight that changes in the level of a single metal can have significant effects on the homeostasis of other metals, since various metals (such Cu and Zn) share the same membrane transporters or are controlled by overlapping signaling pathways (Sanders et al., 2015).

Regarding HD therapy, there are no treatments available, only approaches for the symptomatic phase of the disease without altering its progression. Therefore, there is a great interest in the search for new products capable of acting on different targets, with new mechanisms of action, with low cost and low toxicity. Researchers have directed attention to the dietary consumption of flavonoids with potential to modulate neuronal functions and delay neurodegenerative diseases (Ayas et al., 2019).

Rutin (3,3',4',5,7-pentahydroxyflavone-3-rhamnoglucoside) is one of the most common flavonoids available in the diet, generally found in plants, like buckwheat, fruits such as apples, and some teas. It has several pharmacological activities, such as reduction of oxidative stress, prevention of neuroinflammation (Javed et al., 2012), decrease of neurodegeneration (Xin Xu,. et al., 2014), and neuroprotection (Cordeiro et al., 2020, 2021). Recently, our group showed that rutin has antioxidant effects in addition to activating autophagy (protein degradation) in a *Caenorhabditis elegans* (*C. elegans*) model of HD (Cordeiro et al., 2020). Furthermore, we demonstrated protective effects on ASH sensory neurons (Cordeiro et al., 2021). Suganya and Sumathi, (2017) also demonstrated in a rat model that rutin attenuated HD symptoms induced by 3-nitropropionic acid (Suganya and Sumathi, 2017), suggesting the compound is advantageous for preventing or delaying neurodegenerative diseases. Additionally, it was shown an important property of flavonoids, the ability to form complexes with transition metals increasing their antioxidant activity (Arce-Rodriguez and Saldiaz, 2021). However, more studies are needed to unravel its effects on protein homeostasis, metals homeostasis, and the underlying mechanisms.

C. elegans has homologs to 60–80 % mammalian genes (Harris et al., 2004) and conserved transition factors regulating stress resistance responses, longevity, protein and metal homeostasis (Li and Le, 2013). It contains many of the transporters and stress response genes critical for xenobiotic and metal detoxification, including metallothioneins (MTs), transporters involved in metal homeostasis. Furthermore, the several disease-like mutant strains available, such as transgenic strains to study HD by human huntingtin expression and aggregation, enable investigation of the effects of various compounds on the disease course.

There are *C. elegans* strains expressing different lengths of polyQ linked to YFP (yellow fluorescent protein) or GFP (green fluorescent protein) from matrices integrated into muscle (polyQm) (Morley et al., 2002) or neurons (polyQn) (Brignull et al., 2006). *C. elegans* models for HD demonstrate that polyQ interrupts the function and changes the morphology of ASH neurons. Through the HA759 strain, we can observe GFP fluorescence as a marker of the survival of these neurons (Chen et al., 2015a). *C. elegans* is, therefore, a highly sensitive system to conduct studies related to metal-induced neurodegeneration and pharmacological interventions for delaying or attenuating dysfunctions induced by polyQ.

Herein, we investigated the toxic effects of long-term exposure of *C. elegans* to copper and zinc separately and in combination, their influence on neurodegeneration in models of HD, and the possible neuroprotective effect of rutin.

2. Materials and methods

2.1. Chemicals and reagents

Rutin (synthetic font), 5-fluorodeoxyuridine (FUDR), ethanol, 1-octanol, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate (DiD), Copper chloride ($CuCl_2$) and Zinc chloride ($ZnCl_2$) were purchased from Sigma-Aldrich (USA).

2.2. C. elegans strains and maintenance

We used the following *C. elegans* strains in this study: Bristol N2 (wild-type), HA759 (pqe-1(rt13) III; rtIs11 V[osm-10p::GFP+osm-10p::HtnQ150 +Dpy-20(+)]), AM141 (rmIs133[unc-54p::Q40::YFP]), AM101 (rmIs110[F25B3.3p::Q40::YFP]), and SK4005 (zdIs5[mec-4::GFP + lin-15(+)]. All of them were obtained from the *Caenorhabditis* Genetics Center (University of Minnesota, Minneapolis, MN, USA). Age-synchronized worms were collected by isolating embryos from gravid hermaphrodites using bleaching solution (1 % NaOCl, 0.25 M NaOH) (Zamberlan et al., 2016). Eggs were allowed to hatch overnight in M9 buffer to obtain the first larval stage (L1).

2.3. Exposure to metals

Copper and Zinc were diluted in distilled water and combined to warm nematode growth medium (NGM) before pouring into petri dishes. After agar solidification, *E.coli* OP50 was added as a food source and grown overnight at room temperature. The highest concentration tested for each metal followed the limits stipulated by the Brazilian National Environment Council (CONAMA) for residential soils. In detail, the maximum concentration allowable in soil for Copper is 400 mg/kg (6 mM), and for Zinc is 1000 mg/kg (15 mM). The dose-response curves were prepared with a 1:2 dilution ratio, i.e., 6, 3, and 1 mM of Cu, and 15, 7, and 3 mM of Zn. The chronic exposure to these metals was carried out separately or in combination, from the first larval stage of the animals (L1) to the young adult stage (after 48 h at 20 ± 2 °C).

2.4. Treatment of worms with rutin

Treatment of worms was conducted as previously reported (Cordeiro et al., 2020). Rutin powder was diluted in absolute ethanol and added to the surface of NGM agar plates with *E.coli* OP50 (already grown overnight at room temperature) to obtain final concentrations of 60 μM rutin and 1 % ethanol. This concentration was chosen because it demonstrated a neuroprotective and antioxidant effect, as described in (Cordeiro et al., 2020). Control plates were prepared with the same volume of ethanol. After exposure to metals, young adult worms were washed with M9, transferred to the agar plates, and incubated at 20ºC. For analysis after the young adult stage, we used FUDR in the agar with a

final concentration of 12 μM to avoid progeny, and worms were transferred to new plates with FUDR every two days.

2.5. Survival analysis

Wild-type (N2) L1-stage animals were placed on exposure (copper, zinc or metal mixture) or control plates with *E. coli* OP50 and incubated at 20 °C until the young adulthood for further analysis. Approximately 100 worms were observed for survival rate. The worms were gently touched with a platinum wire, and the absence of response was classified as death (Park et al., 2017). Three independent experiments were carried out.

2.6. Behavioral assessment in Wormlab® software

Protocol was conducted as previously reported (Silveira et al., 2021). Well-fed worms were transferred to a NGM plate without food and allowed to move freely. After 30 s of adaptation, the animals were recorded in a Motic Images Plus 3.0 Megapixel camera with a resolution of 2048 × 1536, containing 1.5 frames/s, for 1 min. The videos were analyzed in Wormlab® software (Version 2.0.1, MBF Bioscience, Williston, VT) by skeletonized capture, where more than one point on the animal is assessed. Wormlab® is a software that enables tracking and image analysis to collect animal data from videos and images, enabling the automation of data search. Evaluation focused on morphological (length and average width), and locomotion parameters (track length and speed). Approximately 30 worms were examined individually in three independently performed experiments.

2.7. Development

The larval stages were evaluated in animals from N2, AM141, AM101 and HA759 strains observing the development of the vulva with an optical microscope. The result was presented as a percentage of the animals in each larval stage. Three independent experiments were carried out, with approximately 100 worms per group.

2.8. Octanol response

Response to 1-octanol was assessed as previously described, with minor modifications (Chao et al., 2004; Kumsta et al., 2017). Well-fed worms were transferred to food-free intermediate NGM agar plates to remove any remaining *E. coli* OP50. After 1 min, the worms were transferred to new food-free NGM agar plates and allowed to adapt for 5 min. The assay consisted of submerging a bristle brush in 30 % 1-octanol (dissolved in 100 % ethanol, v/v), and placing it in front of a forward-moving worm. The latency time until the backward movement was counted. At least ten worms were analyzed in triplicate, and the means of each triplicate were considered. The experiment was repeated in 3 different days, thus at least 30 worms were observed in this assay. The third day of adulthood was chosen in order to align with previously observed patterns of neurodegeneration of ASH neurons (Cordeiro et al., 2020).

2.9. Touch response

To analyze the touch response, we used N2, HA759, and SK4005 strains. The behavior was assessed by gently touching the head region of the animal with a bristle brush and counting how many times worms responded to ten touches with a 10-second rest period between trials (Bratu et al., 2014). Three assays were carried out at different times, and ten worms were analyzed in each experiment.

2.10. Measurement of polyQ aggregates

The number of neuronal polyQ aggregates in the nerve-ring neurons of individual worms of AM101 strain was counted in young adults, and on day 4 of adulthood (Kumsta et al., 2017). Additionally, animals of AM141 strain were used to count the number of polyQ aggregates in the muscles (Zhang et al., 2012) at the young adult stage and at the 3rd day of adulthood. The worms were mounted onto a glass slide, and paralyzed with 5 μL of 50 mM sodium azide. Approximately ten were randomly selected in each treatment and scored for the number of polyQ40::YFP aggregates with an Olympus® Fluoview FV10i confocal microscope and ImageJ software. Three independent experiments were performed individually.

2.11. Dye filling

To visualize neurodegeneration in Htn-Q150 worms (strain HA759), we performed the DiD dye-filling assay (Faber et al., 1999) on the third day of adulthood. DiD is a lipophilic vital dye, which is taken up by the sensory endings of the ASH neurons unless they are absent or have defective sensory endings. By counting the percentage of GFP-expressing ASH neurons that fail to take up the dye, it is possible to quantify the number of live neurons that have degenerated, based on sensory process retraction (Jia et al., 2007).

Worms were incubated for 1 h in 2 mg/mL DiD, then washed three times with M9 buffer to de-staining. They were mounted onto a glass slide and paralyzed with 5 μL of 50 mM sodium azide. Approximately ten ASH neurons were examined in three different experiments, using an Olympus® Fluoview FV10i confocal microscope. The percentage of dye-filling defective ASH neurons was used as a measure of the average of the dye-filling defect for the corresponding groups.

2.12. Metal quantification by inductively coupled plasma mass spectrometry (ICPMS)

2.12.1. Standards

For the calibration curve, a multielement solution (SCP33MS, PlasmaCAL, Quebec, Canada) was used in concentrations from 0.1 μg/L to 50 μg/L. To verify the accuracy of the instrument's response, a standard reference solution (NIST 1643 f, National Institute of Standards and Technology, Gaithersburg, USA) diluted by a factor of 10 was used. In addition, sample dilutions in factors of 10 and 100 times were performed, and the concentrated sample was introduced to verify whether the sample matrix exerted any effect during determinations by ICP-MS.

2.12.2. Sample preparation procedure

After 48 h-exposure to metals, approximately 3000 young adult animals of N2, HA759 and AM101 strains were washed 3 times with M9 and once with ultrapure water, previously distilled, deionized in an ion exchange column and subsequently purified in a Milli-Q system (Millipore, United States). The worms were pelleted and dried in an oven at 37 °C. For the extraction in acid medium, polypropylene flasks with a maximum capacity of 2 mL (CRALPLAST®) were used. After collecting the samples, 260 μL of P.A. grade nitric acid (65 %, 14.4 mol/L, Dinâmica, Indaiatuba, São Paulo) was added and purified in a distillation system below the boiling point. Subsequently, the samples were allowed to rest for 12 h and then completed to 2 mL with ultrapure water, previously distilled, deionized in an ion exchange column and subsequently purified in a Milli-Q system (Milipore, USA), with resistivity of 18.2 MΩ cm.

2.12.3. Instrumentation

For the quantification of the elements Cr, Fe, Mn, Cu, Cd, Pb, Zn and Ni, an inductively coupled plasma mass spectrometer (model Elan DRC II, Perkin Elmer Sciex, Canada) equipped with a concentric nebulizer (Meinhard Associates, USA) and baffled-type cyclonic nebulization chamber (Glass Expansion Inc., Australia), quartz torch with 2.0 mm internal diameter injector tube and Argon (99.999 %, White Martins-Praxair, Brazil), was used for plasma generation. Table 1 describes the

Table 1
Instrumental conditions used for elemental determination by ICP-MS.

Parameters	ICP-MS
RF power, W	1300
Main argon flow, L/min	12.0
Auxiliary argon flow, L/min	1.30
Nebulizer gas argon flow, L/min	1.05
Fogging chamber	Cyclonic
Nebulizer	Concentric
Sampling cone and skimmer	Pt
Dwell time, ms	20
Replicate scans	5
Readings per replicate	5
Replicated	3

instrumental conditions used in determinations with ICP-MS.

2.13. Statistical analyses

Statistical analyses were performed using GraphPad Prism Version 8 for Windows (GraphPad Software, USA). Significance was assessed by one- or two-way analysis of variance (ANOVA), followed by a Dunnett's or Tukey's post-hoc test. Values of $p < 0.05$ were considered to be statistically significant.

3. Results

3.1. Effect of metals on survival

As shown in Fig. 1A and B, no differences in survival were observed in N2 strain after exposure to different concentrations of copper (1–6 mM) and zinc (3–15 mM). However, groups exposed to a combination of both metals had a significant decrease in the percentage of worms alive ($p < 0.05$; Fig. 1C). Considering these results, the concentrations chosen for the subsequent assays were 1 mM Copper, 3 mM Zinc and Mix group (3 mM Zn+1 mM Cu).

3.2. Effect of metals exposure on body parameters of worms

We demonstrated in Figs. S1 and S2 that exposure to metals altered

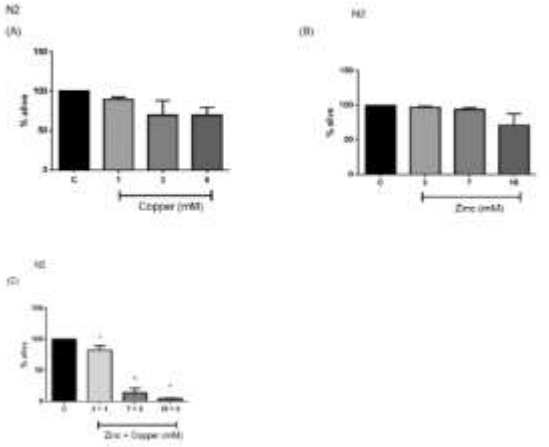

Fig. 1. Survival of worms of the N2 strain exposed in agar plates to different concentrations of (A) Copper, (B) Zinc, and (C) mix of Copper and Zinc starting in L1 stage. Analysis was assessed 48 h later. Data are representative of three independent experiments performed in triplicate. Results are represented as means ± S.E.M. *$p < 0.05$ compared to control (One-way ANOVA followed by Dunnett's post-hoc test).

body parameters of the worms. As shown in Fig. S1A, all groups (Cu, Zn, and Mix) had a significant decrease in mean worm length of the N2 strain ($p < 0.05$). While in the mutant strains, AM141 (Q40 over-expressed in the body wall muscle), AM101 (Q40 overexpressed pan-neuronally) and HA759 (HtnQ150 over-expressed specifically in the ASH neurons) (Fig S1B-D), there was a significant decrease in mean worm length in the Cu and Mix groups ($p < 0.05$).

In addition, we can observe in Fig. S2A that there was a significant decrease in mean width in all groups (Cu, Zn, and Mix) of N2 strain ($p < 0.05$). While in the mutants, mean width was decreased in AM141 (Q40 over-expressed in the body wall muscle) worms exposed to the Mix ($p < 0.05$; Fig S2B), AM101 (Q40 overexpressed pan-neuronally) exposed to Cu and Mix ($p < 0.05$; Fig S2C), and HA759 (HtnQ150 over-expressed specifically in the ASH neurons) exposed to Cu, Zn, and Mix ($p < 0.05$; Fig S2D).

3.3. Effect of metals exposure on locomotion parameters of worms

We demonstrated in Figs. S3 and S4 that exposure to metals altered locomotion parameters of the worms. As shown in Fig. S3A, there was a significant decrease in track length in the Cu and Mix groups of the N2 strain ($p < 0.05$). While in the mutants, in AM141 (Q40 over-expressed in the body wall muscle) strain there was no significant difference (Fig S3B), in AM101 (Q40 overexpressed pan-neuronally) we demonstrated a significant decrease in the Cu group ($p < 0.05$; Fig S3C) and in HA759 (HtnQ150 over-expressed specifically in the ASH neurons) there was no significant difference (Fig S3D).

Furthermore, we can observe in Fig. S4 A a significant decrease in worm speed in the Mix group in N2 strain ($p < 0.05$). While in the mutants, in AM141 (Q40 over-expressed in the body wall muscle) and AM101 (Q40 overexpressed pan-neuronally) strains, we can observe a decrease only in the Cu group (Fig S4B and C, $p < 0.05$), while in the HA759 (HtnQ150 over-expressed specifically in the ASH neurons) strain there was a significant increase in the Zn group ($p < 0.05$; Fig S4D).

3.4. Effect of metals on development

As shown in Fig. 2A-D, there was a significant developmental delay in all the strains exposed to the metal mix ($p < 0.05$).

3.5. Effect of metals on octanol response

We performed the octanol avoidance response assay in N2 and HA759 (HtnQ150 over-expressed specifically in the ASH neurons) strains in the young adult stage and day 3 of adulthood. In N2, as shown in Fig. 3A and B, there was no significant difference in any of the larval stages ($p < 0.05$). While in the young adult stage of HA759 strain, there was a significant increase in latency response (in seconds) in the mix group ($p < 0.05$, Fig. 3C), indicating dysfunction in the neurons. On the 3rd day of adulthood there was no significant difference ($p < 0.05$; Fig. 3D).

3.6. Effect of metals on touch response

As shown in Fig. 4A, in N2 strain we observed a significant decrease in touch response in the mix group ($p < 0.05$), while in HA759 (HtnQ150 over-expressed specifically in the ASH neurons) strain we observed a decrease in the Cu and mix groups ($p < 0.05$, Fig. 4B). Likewise, in the SK4005 (mec-4::GFP expressed in touch neurons) strain, we observed a significant decrease in all groups compared to the control ($p < 0.05$, Fig. 4C).

3.7. Effect of metals on polyQ-mediated neurotoxicity and effect of rutin

Fig. 5A shows representative images of muscle polyQ aggregation in young adult worms and worms at day 3 of adulthood. Mix group

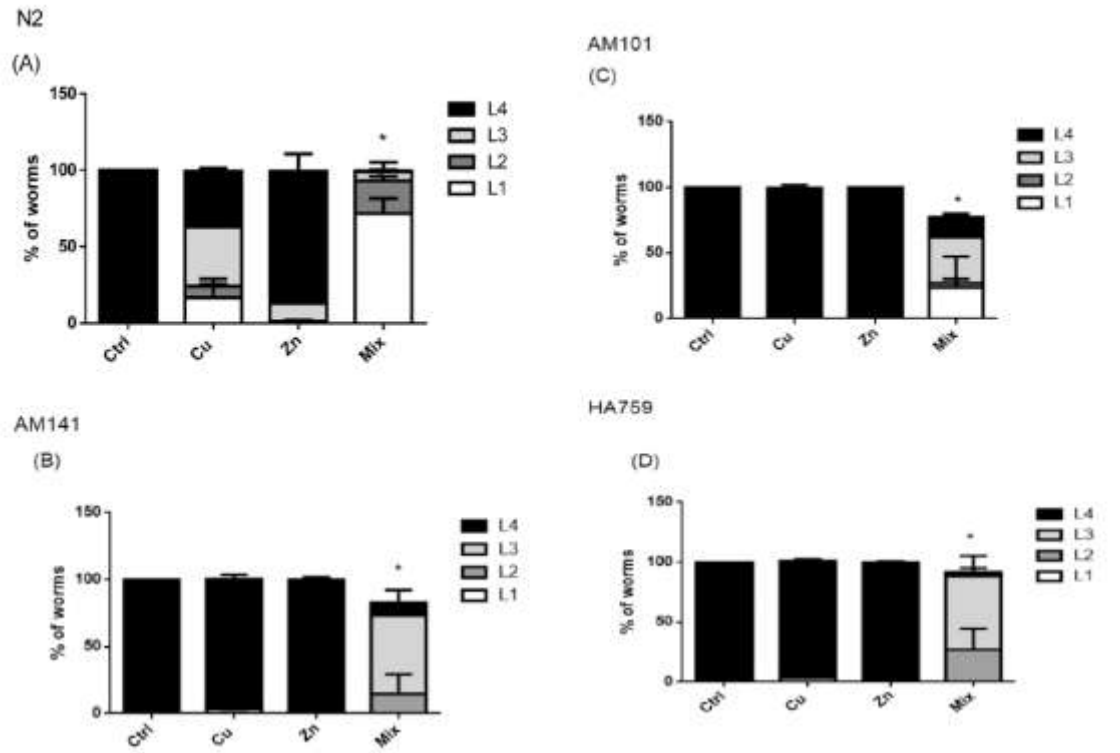

Fig. 2. Effect of metals exposure on development of (A) N2, (B) AM141, (C) AM101, and (D) HA759 strains exposed to copper, zinc, and mix (copper + zinc) for 48 h from L1. The worms were analyzed using a microscope and scored through the larval stages observing vulva development. (*) represents a significantly difference between control and metal-exposed in L4 stage by two-way ANOVA followed by Dunnett's Multiple Comparison Test ($p < 0.05$). Data are expressed as means ± SEM.

increased significantly polyQ aggregation in AM141 (Q40 over-expressed in the body wall muscle) young adult worms (Fig. 5B, $p < 0.05$), with an average of 3.3 aggregates compared to 2.1 aggregates in control worms. Worms at day 3 of adulthood were also evaluated, and Fig. 5C shows that there was no significant difference in relation to control.

In addition, we analyzed the number of polyQ neuronal aggregates in young adult worms and worms at day 4 of adulthood, as demonstrated in representative images (Fig. 6A). Fig. 6B shows a significant increase in the zinc group of young adult animals ($p < 0.05$) with an average of 2.8 aggregates compared to 1.7 aggregates in control group. However, in day 4 of adulthood, we observed a significant increase in the copper group (Fig. 6C, $p < 0.05$) with an average of 12.6 aggregates compared to 11.2 aggregates in control group. The effect of rutin after exposure to metals was also analyzed in 4-days adults. We demonstrated, in Fig. 6C, a decrease in the number of polyQ neuronal aggregates in all groups after treatment with 60 μM of rutin ($p < 0.05$), with an average of 4.3 aggregates (copper group), 6.2 aggregates (zinc group) and 7 aggregates (mix group) compared to 11.2 aggregates in control group.

3.8. Effect of metals on dye-filling defects in ASH neurons

Fig. 7A shows representative images of a viable GFP-labeled ASH neuron with DiD dye uptake, and a degenerated GFP-labeled ASH neuron with a DiD dye-filling defect. Worms exposed to zinc and mix had a significant increase of 44 % and 46 % in degeneration in ASH neurons, respectively, compared to 32 % of the control in young adult stage (Fig. 7B, $p < 0.05$). Likewise, 3 day adult worms of zinc and mix groups had a significant increase of 72 % and 64 % in ASH neurodegeneration, respectively, compared to 54 % in the control (Fig. 7C, $p < 0.05$). After exposure to metals, we analyzed the effect of 60 μM rutin on neurodegeneration in 3-day adult worms, and Fig. 7C demonstrates that there was a decrease on dye-filling defects in ASH neurons in all groups ($p < 0.05$), with a decrease of 37 % (Cu), 40 % (Zn) and 47 % (mix) compared to 54 % of the control.

3.9. Quantification by ICPMS

As shown in Fig. 8A, in the N2 strain, we observed a significant increase in Fe and Zn in worms exposed to the mixture of 3 mM Zn + 1 mM Cu ($p < 0.05$). In strain HA759 (HtnQ150 over-expressed specifically in the ASH neurons) we observed a significant increase of Fe in the group exposed to Zn ($p < 0.05$, Fig. 8B). In contrast, in the AM101 (Q40 overexpressed pan-neuronally) strain, we did not observe significant differences (Fig. 8C).

4. Discussion

The neurotoxicity of metals and also their occupational and ecological roles have been demonstrated by several in vitro and in vivo studies (Cicero et al., 2007). However, the investigations were mainly focused on an isolated contaminant, while in real environments, organisms are exposed to mixtures of many metals. Although some of them are essential for vital functions, in excessive amounts they can be harmful or even lethal, with a very small margin between essentiality and toxicity (Angima, 2010).

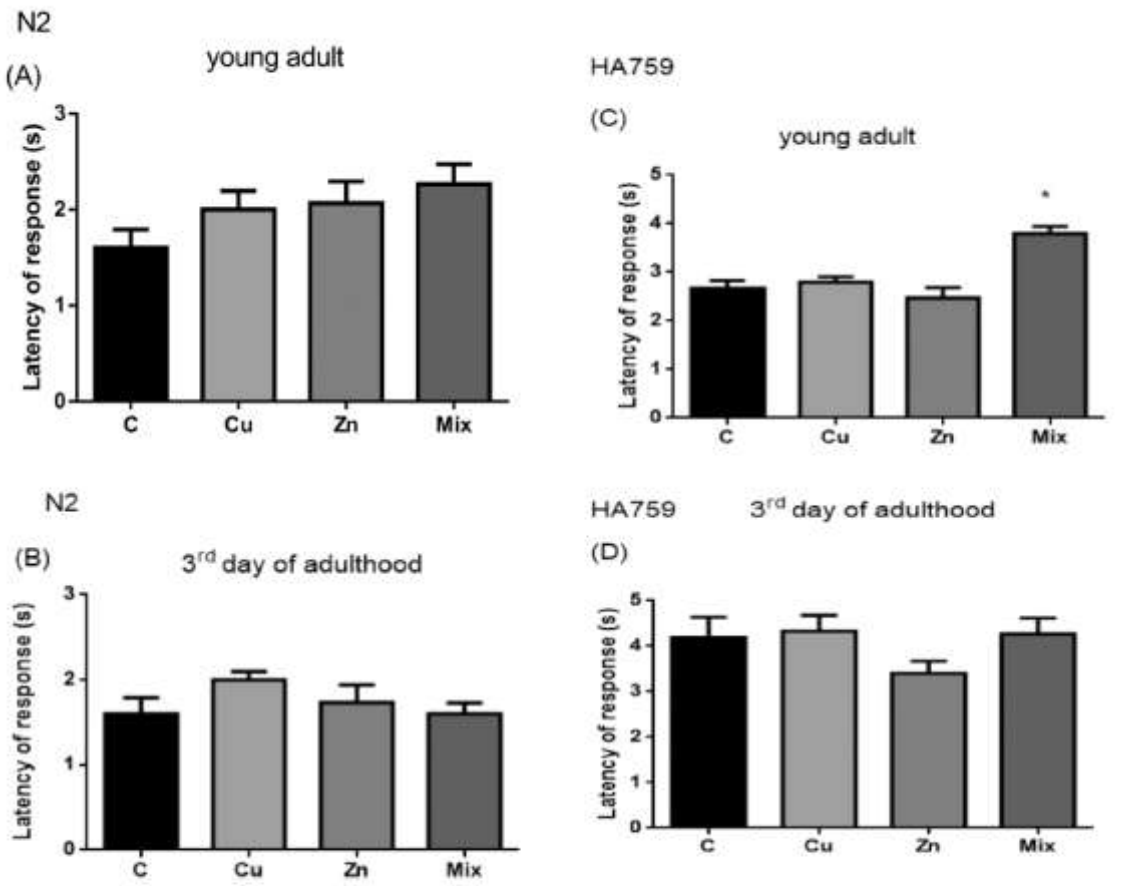

Fig. 3. Effects of metals exposure on the latency of response (in seconds) to octanol in N2 young adults (A) and 3-day-adult worms (B), and in HA759 young adults (C) and 3-day-adult worms (D). Data are representative of three independent experiments with 5 worms per experiment. Results are represented as means ± S.E.M. *$p < 0.05$ compared to control group (One-way ANOVA followed by Dunnett's post-hoc test).

Our data demonstrate that copper and zinc individually did not cause mortality in *C. elegans* after long-term exposure (from L1 to adult) to the concentrations considered safe by the Brazilian legislation for residential soils (6 mM Cu and 15 mM Zn) (Fig. 1A and B). However, the combination of these metals was toxic to worms even at lower concentrations (3 mM Zn + 1 mM Cu) (Fig. 1C). The mixture affected survival and body parameters (mean worm length and width) in wild-type (N2) and HD mutants AM141 (Q40 over-expressed in the body wall muscle), AM101 (Q40 over-expressed pan-neuronally), and HA759 (HtnQ150 over-expressed specifically in the ASH sensory neurons) (Figs. S1 and S3), locomotor parameters (track length and speed) in N2 strain (Figs. S3 and S4), suggesting that exposure is capable of causing generalized toxicity. Furthermore, delayed the development of wild-type and mutant worms (Fig. 2).

The current guidelines for metal exposure worldwide just consider levels of toxicants separately, and not their possible interactive effects. Despite the toxic potential of mixtures is gaining attention (Baas et al., 2010), studies in this issue are still relatively uncommon. Furthermore, many data of metal mixtures were based on acute (Norwood et al., 2003; Vijver et al., 2011) rather than chronic exposure that is more relevant for risk assessment (Nys et al., 2016). Since each metal may affect a variety of metabolic pathways, interactive effects of metals in mixtures can be additive, antagonistic, or synergistic, all resulting in different toxicity responses (Chu and Chow, 2002). Herein, we showed that low levels of combined Cu and Zn are more toxic compared to isolated higher concentrations. In addition, the morphological and behavioral parameters of worms were more sensible than the survival endpoint for toxicity.

C. elegans responds to stimuli like odors (1-octanol) and a variety of toxic compounds, such as metals (Tobin and Bargmann, 2004) via several sensory neurons in its head (Ward et al., 1975). Besides being involved in mechanosensory behaviors such as octanol response, ASH neurons also modulate behaviors such as movement, called mechanoreceptor behaviors. ASH neurons are defined as polymodal nociceptors, since they can respond to chemical and mechanical signals, analogous to polymodal pain-sensitive nociceptive neurons in vertebrates. Our data demonstrate that the mixture of metals increased the latency time in response to 1-octanol in HA759 young adult worms (HtnQ150 over-expressed specifically in the ASH neurons) (Fig. 3). However, we do not observe the same effect in animals at day 3 of adulthood. Hilliard et al. (2005) described that the property of many primary sensory neurons is sensory adaptation, leading to a reduction in response sensitivity after persistent stimulation (repeated or prolonged), corroborating with our data.

Gentle touch is sensed in the nematode *C. elegans* by six touch receptor neurons (TRNs; these cells are the 2 ALM, 2 PLM, 1 AVM, and 1 PVM neurons) (Chalfie and Sulston, 1981). Touch is transuded in the

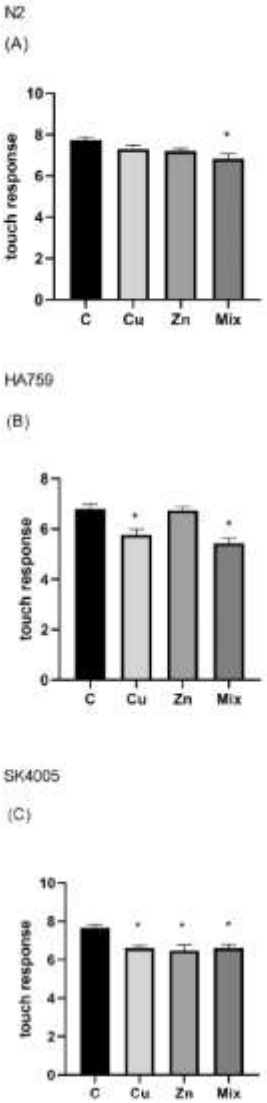

Fig. 4. Effects of metals on touch response in (A) Wild type N2, (B) HA759, and (C) SK4005 young adult worms. Data are derived from three independent assays with 10 worms per group in each experiment. Results are represented as means + S.E.M. *$p < 0.05$ compared to control (one-way ANOVA followed by Dunnett's post hoc test).

TRNs by the activation of a trimeric channel formed by two degenerin/epithelial sodium channel (DEG/ENaC) proteins, MEC-4 and MEC-10 (O'Hagan et al., 2005; Arnadóttir et al., 2011; Chen et al., 2015b). The touch receptor neurons respond to stimuli delivered anywhere on the length of their processes. Corroborating the idea, MEC-4 (tagged with YFP) is present in puncta distributed along touch receptor neuron processes (Chelur et al., 2002). Our data show that combined Cu and Zn decreased touch response in HA759 young adult worms (HtnQ150 over-expressed specifically in the ASH neurons) and in SK4005 (express mec-4 with GFP in the touch neurons ALM, PLM, AVM, and PVM) (Fig. 4). This suggests that in addition to affecting ASH neurons, the mixture of metals also affects touch receptor neurons.

Cu and Zn homeostasis is strictly regulated and their accumulation or deficiency leads to neurodegenerative diseases (ND) (Fraga, 2005; Wegrzynowicz et al., 2012). Many proteins require metal ions for proper folding and/or for catalytic activity, and protein misfolding and aggregation might occur when the homeostasis of essential metal ions is disturbed (Greenough et al., 2013; Waldron et al., 2009). Increase in copper level was observed in human brains with HD and rodent models of HD (Dexter et al., 1991; Fox et al., 2007; Pérez et al., 1996), and Xiao et al (Xiao et al., 2013). found a direct interaction between increased intracellular Cu and increased mutant huntingtin aggregation (Xiao et al., 2013). It remains unclear whether zinc homeostasis affects mHtt in the brain, but it was demonstrated high levels of this metal may bind to amyloid- β and increase the formation of fibrillar aggregation (Cuajungco and Faget, 2003; Jomova et al., 2010). AD and HD have in common features and mechanisms that the misfolded proteins cause neuronal death.

Our data showed that the mixture of low levels of Cu and Zn increased polyQ protein aggregation in muscle in young adult worms (Fig. 5). The presence of insoluble proteins in the younger animals, even at relatively low amounts, suggests that protein aggregation is not limited to mid-life or old animals but already occurs in early stages. It is considered that these insoluble proteins have a function in young adults, but these results are consistent with reports showing that a decline in proteostasis begins in young *C. elegans* animals (Ben-Zvi et al., 2009). However, we did not observe a significant effect of metals on the 3-day adult animals when the animals were no longer in contact with the metals.

Previous studies have demonstrated that poor performance on learning tasks was characteristic of worms with pan-neuronal 40Q, suggesting that polyQ disrupts long-term potentiation in *C. elegans*, as in mouse models of HD (Landon et al., 2019). Overall, the protein aggregation effects suggest this worm is a good model for neurodegenerative diseases in vertebrates. Furthermore, by generating *C. elegans* models in a pan-neuronal fashion, tissue-specific responses to polyQ proteins can be compared. In addition to studying protein aggregates in muscle, we also studied polyQ aggregates in neurons. Our data demonstrate that zinc caused an increase in the number of neuronal aggregates in young adult animals and copper in 4-day adult animals (Fig. 6), suggesting a residual effect of Cu. Pedersen et al. (2011) demonstrated that Aβ begins to aggregate instantly with trace concentrations of Cu compared to the characteristic lag period observed in aqueous environments without metal ions present (Pedersen et al., 2011). Similarly, Sarell and colleagues (Sarell et al., 2010) observed that substoichiometric concentrations of Cu^{2+} induce acceleration in both the nucleation and elongation phases of amyloid fibril formation (Sarell et al., 2010). Pfalzer et al., (2022) demonstrated that the interactions among essential metals may modulate HD progression. *C. elegans* transgenic strains that simulate neurodegeneration-associated aggregation have proven invaluable in assessing mechanisms of age-dependent proteostasis failure (Ayyadevara et al., 2015; Kikis et al., 2010; Guthrie et al., 2011).

In ASH and other *C. elegans* neurons, the sensory endings are exposed to the environment, allowing the neurons to take up vital lipophilic dyes, including DiD (Faber et al., 1999). In cell membranes these fluorescent compounds accumulate, facilitating the rapid visualization of these cells, and when there is a failure to absorb the dye by neurons, it is considered absent or defective sensory endings (Starich et al., 1995). We examined the effect of metals on the dye-filling in ASH neurons in young adult and 3-day-old adult worms containing HtnQ150 (the Htt fragment containing a polyQ tract of 150 residues), and found a significant increase in the dye-filling defect in the zinc and mixture metals groups (Fig. 7), characterizing neurodegeneration.

Due to the essential metal interdependency, dyshomeostasis of a single metal will result in aberration dyshomeostasis of others (Pfalzer et al., 2022). For instance, increased Cu can replace Zn on Zinc-dependent enzymes, which alter the functional status of those

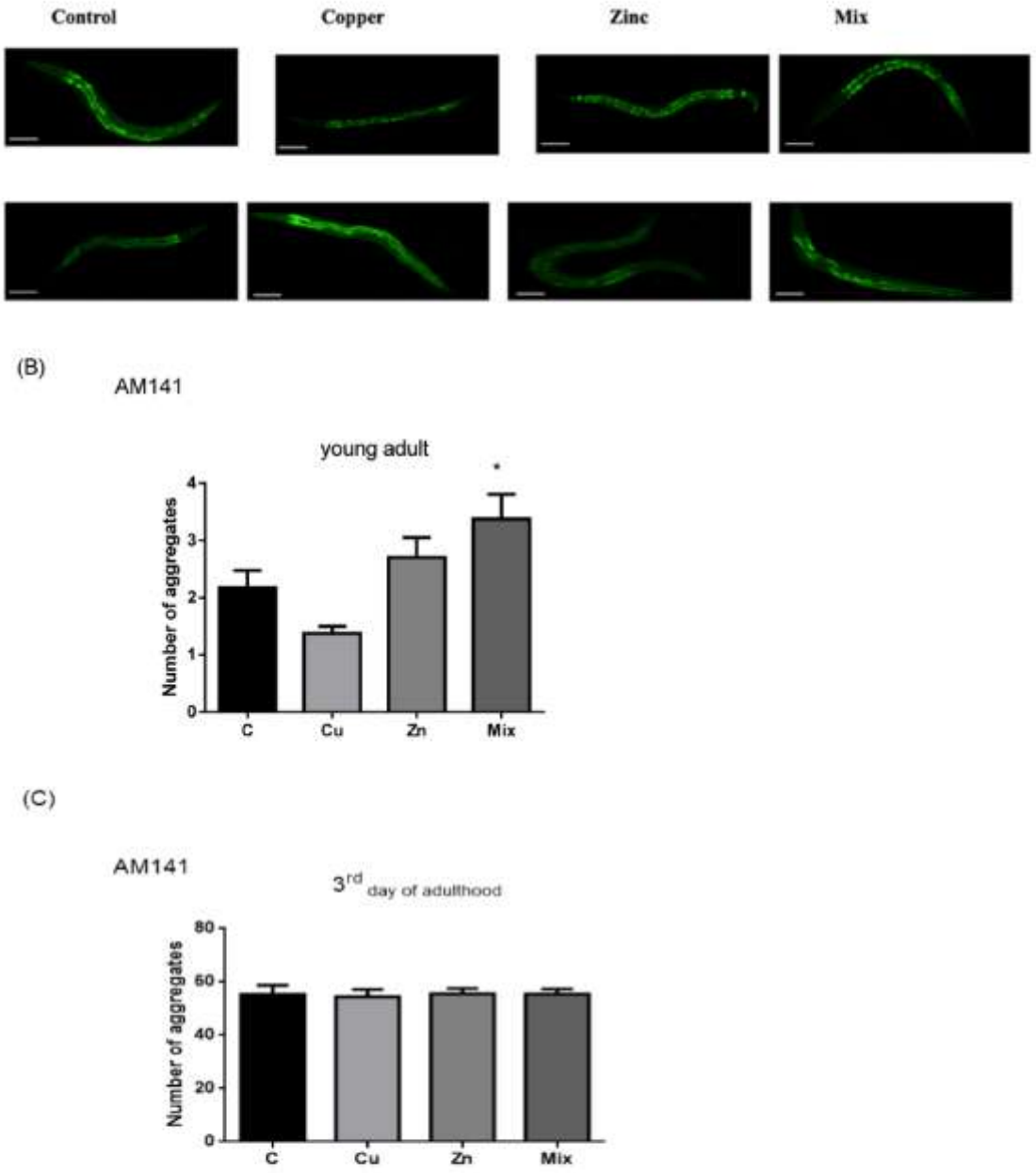

Fig. 5. Effects of metals exposure on polyQ aggregation in muscle. (A) Fluorescence micrographs of transgenic nematodes expressing Q40:YFP in body wall muscle cells, and number of aggregates in (B) young adults and (C) 3-day-adult worms. Data are derived from three independent experiments with 10 worms in each experiment. Results are shown as means ± S.E.M. *$p < 0.05$ compared to control group (One-way ANOVA followed by post-hoc Dunnett's).

proteins (Chung et al., 2010). In this way, global essential transition metal (ETM) homeostasis is a vital and delicate process, because the balance of each ETM depends on the balance of other ETMs as a consequence of their complex interaction and shared biological activities. In fact, aberrant ETM homeostasis is a central feature in ND, resulting in unbalanced distributions of some of these metals in the brain, as significant amounts accumulate in specific areas and lead to metal deficiency in others (Lopes de Andrade et al., 2021). Currently, it is commonly accepted that oxidative stress (OS) represents a mutual hallmark of various ND (Pimentel et al., 2012), and that redox-active ETM dyshomeostasis is associated with their etiology and pathogenesis (Braidy et al., 2017). The disruption of ETM homeostasis may cause

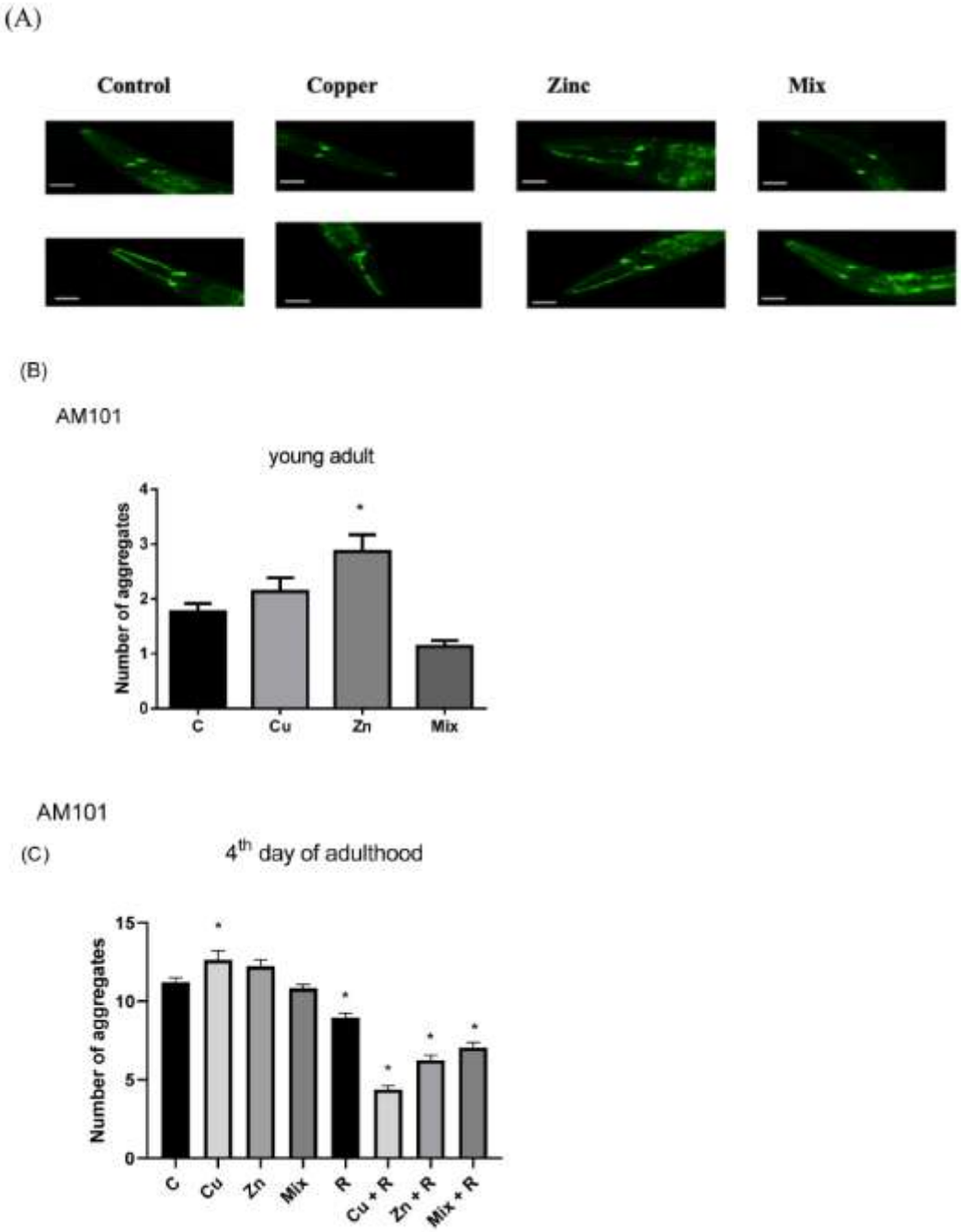

Fig. 6. Effects of metals exposure on neuronal polyQ aggregates. (A) representative images and quantification of aggregates in (B) young adults and in (C) 4-day-adults exposed only to metals and exposed to metals (L1 to young adult) followed by post treatment (young adult to fourth day of adulthood) with rutin (60 μM). Data are derived from three independent experiments with 10 worms each experiment. Results are shown as means ± S.E.M. *$p < 0.05$ compared to control group (One-way ANOVA followed by post-hoc Dunnett's). R – rutin.

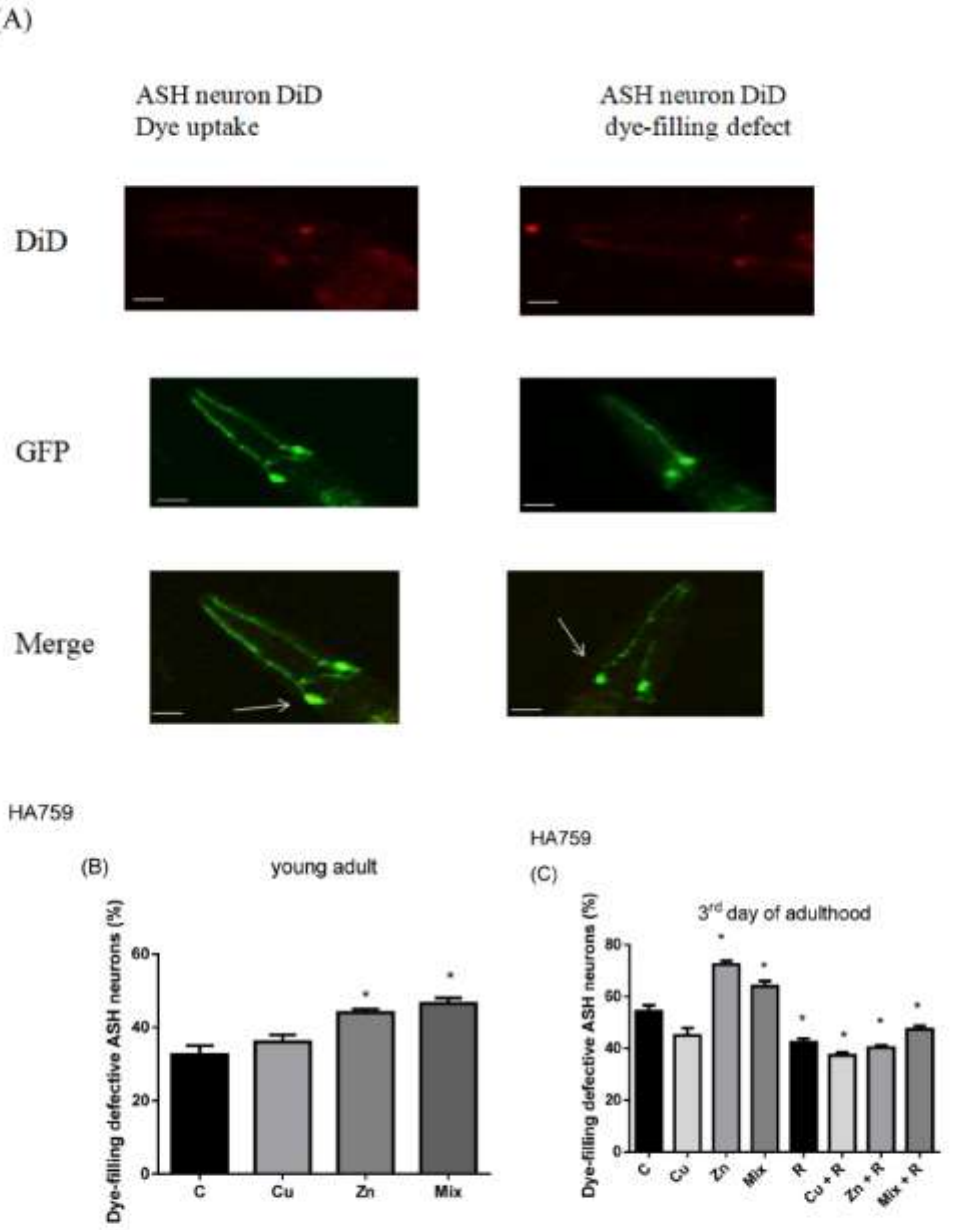

Fig. 7. Effects of metals exposure on dye-filling defect in ASH neurons. (A) Representative images of viable GFP-labeled ASH neuron with DiD dye uptake (left panel, see arrow on merged image) and degenerated GFP-labeled ASH neuron with DiD dye-filling defect (right panel, see arrow on merged image), (B) percentage of dye-filling defective ASH neurons in young adults, and (C) 3-day adults exposed only to metals and exposed to metals (L1 to young adult) followed by post treatment (young adult to third day of adulthood) with rutin (60 μM). Data are derived from three independent experiments with 10 worms each experiment. Results are shown as means ± S.E.M. *$p < 0.05$ compared to the control group (One-way ANOVA followed by Dunnett's post hoc test). R= rutin.

two major features associated with ND: dysfunction of metalloproteins and aberrant metal-protein interactions, which can lead to protein aggregation and uncontrolled ROS production (Bowman et al., 2011; Garza-Lombó et al., 2018; Pokusa and Trančíková, 2017). Mainly and less explored, the effect of an increase or decrease of an individual ETM, due to dyshomeostasis, is not restricted only to that metal alone. In addition, it causes an overall homeostatic imbalance of several metals presumably due to the loss of their regulation across cell membranes (Mitra et al., 2014). Further, the simultaneous accumulation of ETM in the same areas of the brain and the successive increase in ROS production is greater than could be induced by a single metal, which is relevant to the progression of ND (Lopes de Andrade et al., 2021).

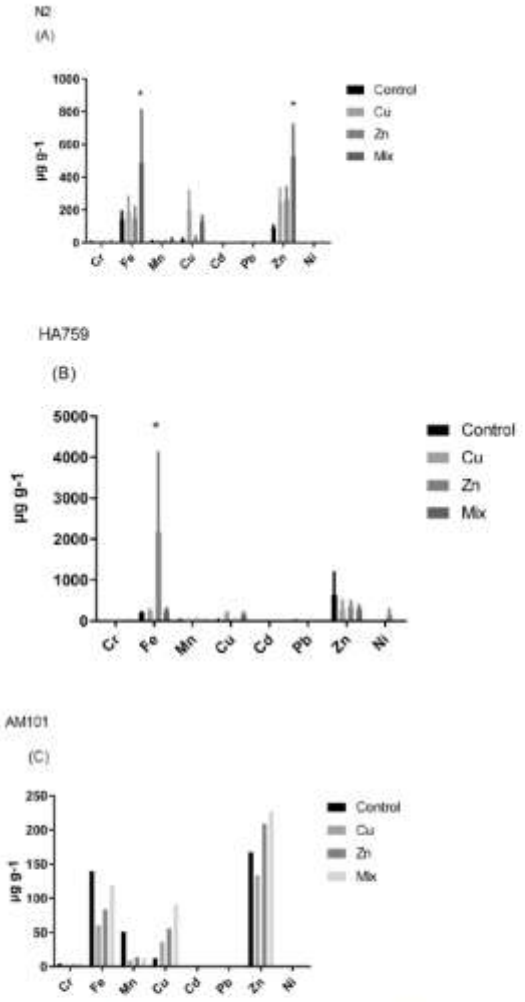

Fig. 8. Level of metals (in µg/g) in (A) N2, (B) HA759, and (C) AM101 48 h after exposure to zinc, copper, and mix. Results are represented as means ± S.E. M. *$p < 0.05$ compared to control (Two-Way ANOVA test followed by Tukey's post hoc test).

In addition, little is known about the molecular mechanisms of trace element interactions, and to what extent their profiles change during aging. Our study investigated the accumulation of metals in wild-type worms and mutant strains for HD model, in order to assess the level of metals and disease progression. In wild-type worms, exposure to the copper and zinc mixture resulted in increased Fe and Zn levels. While in the HA759 mutant strain (HttQ150 over-expressed specifically in the ASH neurons) exposure to Zn caused an increase in the Fe level (Fig. 8). There is uncertainty about the potential competition between iron, zinc, and copper when all three elements are used together. Current knowledge about the interaction between Zn to Fe and Cu to Fe at the cellular level and in the whole organism is incomplete (Espinoza et al., 2012). The interaction between iron, copper and zinc absorption may be explained by competitive binding to the transporter protein DMT1, which participates in divalent metal transport (Fe, Cu, Zn, Mn, Pb) (Gunshin et al., 1997). Zn, Cu, and Fe are essential elements that exhibit important interactions and possible competitive inhibition of transport and bioavailability (Reinstein et al., 1984; Brewer et al., 1985). Other studies have provided information about uptake of Fe, Zn, and Cu at the cellular level. Using Caco-2 cells (a human cell line of intestinal epithelium), it was shown that Zn promotes the transepithelial flux of Fe by increasing DMT1 gene expression and concentration (Yamaji et al., 2001). Cu uptake by Caco-2 cells was reduced by ~50 % when DMT1 expression was blocked with an antisense oligonucleotide (Arredondo et al., 2003). In addition, there is evidence that Fe and Cu compete for DMT1 transport at the enterocyte (Arredondo et al., 2006). Zn could affect tissue utilization of Fe because competition for DMT1 may occur not only in intestinal cells, but in other tissues and organs (Espinoza et al., 2012). Thus, the potential competition in transport of Fe, Cu and Zn as well as homeostasis-related proteins needs to be considered, especially in the context of co-exposure.

Furthermore, neurodegenerative disorders involve a multifactorial etiology due to a complex interaction between genetic and environmental factors, and among the possible environmental factors, metals have been extensively studied (Farina et al., 2013b). Metals are gaining increasing attention because a larger percentage of population is exposed to industrial and chemical pollution through food, air and water (Kwok, 2010). Of particular importance, oxidative stress and neurodegeneration have been reported as consequences of toxic exposures to essential metals, along with dyshomeostasis in essential metal metabolism (Farina et al., 2013b). Hence, it is of considerable importance the metal chelation.

Rutin is an antioxidant flavonoid with well-known metal chelating potential in vivo. The chelation of metals can be crucial in the prevention of radical generation, which damages target biomolecules. Moreover, natural chelators may be better than the synthetic ones due their lower toxicity (Prakash et al., 2020). Our data showed that rutin was able to decrease neuronal polyQ aggregates in worms exposed to copper, zinc, and the mixture (Fig. 6), in addition to decreasing neurodegeneration (Fig. 7). The interaction of certain flavonoids with transition metals create flavonoid-metal complexes, which may act as more potent free radical scavengers than the flavonoid alone (Prakash et al., 2020). For example, it was demonstrated that the antioxidant capacity is higher in the case of rutin-Zn complex, rutin-Cu(II) complex and quercetin-Cu(II) complex (Bratu et al., 2014). Also, complexes with flavonoids lead to a reduction of toxic metals bioavailability. Some studies have confirmed that flavonoids can act as antioxidants because of their chelating properties, such as rutin. Kostyuk et al., (2001) found that complexes of rutin and epicatechin with iron(II), iron(III), copper (II) and zinc(II) are more effective in free radicals scavenging than the free flavonoids. These complexes show increased protection of red blood cells against oxidative damage induced by asbestos in vitro (Kostyuk et al., 2001).

Our group has already demonstrated that rutin exerts its protective effects against Htn toxicity through overexpression of the antioxidant enzyme SOD-3 and the chaperone HSP 16.2 (Cordeiro et al., 2021), and reduction in ROS levels (Cordeiro et al., 2020). Previous studies reported that rutin significantly decreased protein oxidation after oral administration and improved the endogenous antioxidant system in a rat model of HD induced by 3-NP (Suganya and Sumathi, 2017). We therefore propose, that rutin is a flavonoid capable of forming complexes with transition metals increasing its antioxidant activity. This effect is an important property of some natural compounds, consequently decreasing polyQ aggregation and toxicity, in addition to neurodegeneration.

5. Conclusions

Our results demonstrated for the first time the neurotoxic effects of Cu and Zn in *C. elegans* even at concentrations below those considered safe by the Brazilian National Environment Council, and the

involvement of these essential metals in the progression of HD in a *C. elegans* model. Additionally, rutin, known as a natural and easily accessible antioxidant, exerted protection against metals toxicity. Thus, our findings reinforce the need to investigate the effects of essential metals in long-term exposures, focusing on their mixtures, and suggest the revision of guidelines about safe concentrations of metals. Furthermore, we provided new avenues for treatment strategies against neurodegenerative diseases through flavonoids with chelating properties.

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CRediT authorship contribution statement

Larissa Marafiga Cordeiro: Conceptualization, Methodology, Validation, Investigation, Formal analysis, Writing – original draft, Visualization, Writing – review & editing. **Marcell Valandro Soares:** Conceptualization. **Aline Franzen da Silva:** Conceptualization, Investigation. **Luiza Venturini dos Santos:** Investigation. **Larissa Ilha de Souza:** Investigation. **Tássia Limana da Silveira:** Investigation. **Fabiane Bieca Obetine Baptista:** Investigation. **Gabriela Vitória de Oliveira:** Investigation. **Cristiane Pappis:** Investigation. **Valderi Luiz Dressler:** Resources. **Leticia Priscilla Arantes:** Writing – review & editing, Supervision. **Fuli Zheng:** Writing – review & editing, Conceptualization. **Felix Alexandre Antunes Soares:** Writing – review & editing, Conceptualization, Methodology, Validation, Resources, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.neuro.2023.06.005.

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4.2.1 Supplementary material

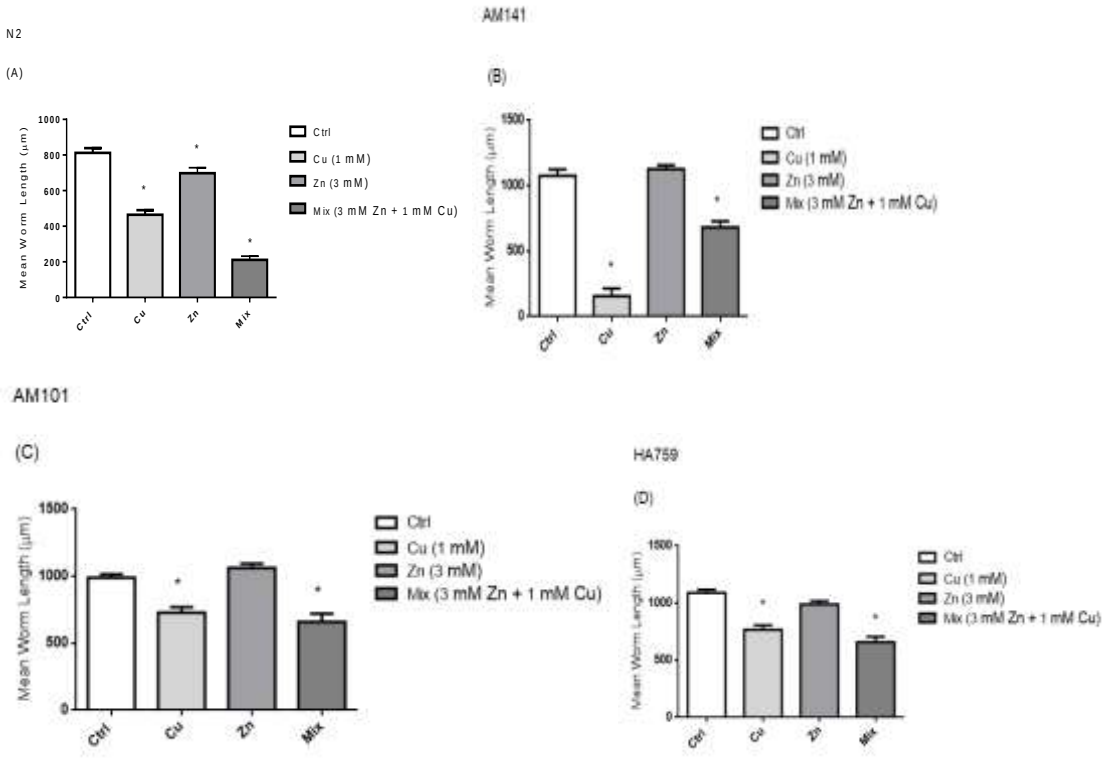


Figure S1. Effect of metals in mean length of (A) N2, (B) AM141, (C) AM101, and (D) HA759 worms exposed in agar plates from L1 stage until young adult. Analysis was conducted on young adult animals. Data are representative of three independent experiments performed in triplicate. Results are represented as means ± S.E.M *$p<0.05$ compared to control (One-way ANOVA followed by Dunnett's post-hoc test).

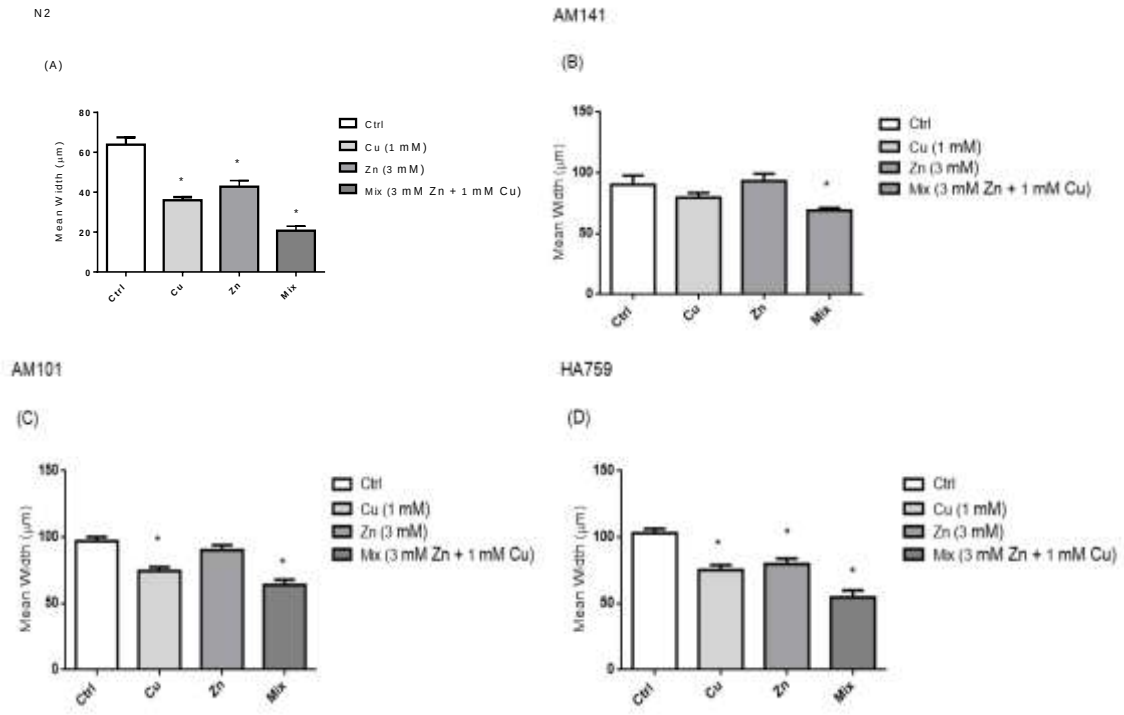


Figure S2. Effect of metals in mean width of (A) N2, (B) AM141, (C) AM101, and (D) HA759 worms exposed in agar plates from L1 stage until young adult. Analysis was conducted on young adult animals. Data are representative of three independent experiments performed in triplicate. Results are represented as means ± S.E.M *p<0.05 compared to control (One-way ANOVA followed by Dunnett's post-hoc test).

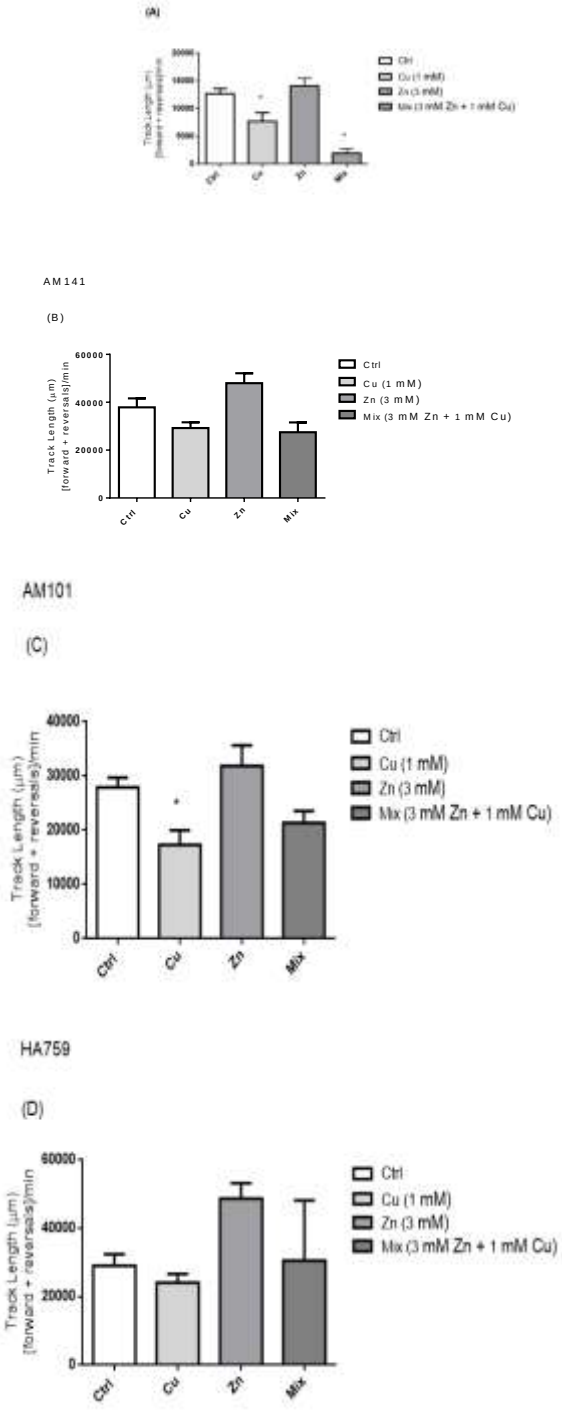
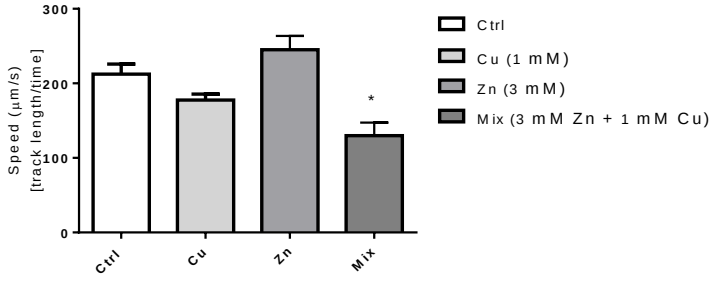


Figure S3. Effect of metals exposure in track length of worms exposed in agar plates from the L1 stage until young adult. Analysis was performed on young adult animals. (A) N2, (B) AM141, (C) AM101 and (D) HA759 strains. Data are representative of three independent experiments performed in triplicate. Results are represented as means ± S.E.M *$p<0.05$ compared to control (One-way ANOVA followed by Dunnett's post-hoc test).

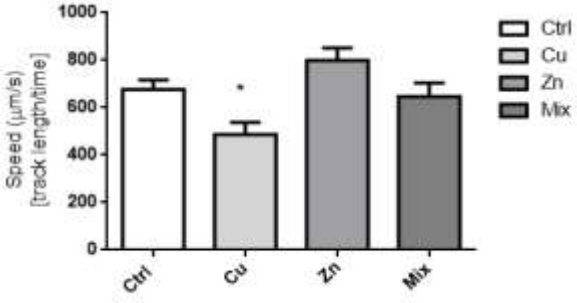
N2

(A)



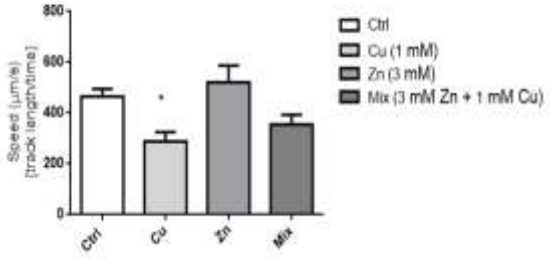
AM141

(B)



AM101

(C)



HA759

(D)

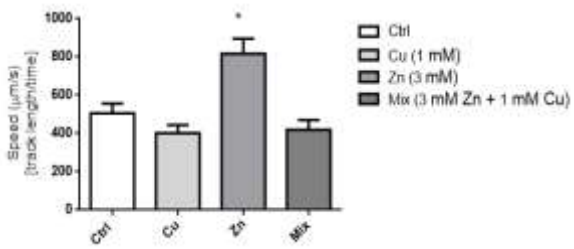


Figure S4. Effect of metals exposure in speed of worms exposed in agar plates from the L1 stage until young adult. Analysis was performed on young adult animals. (A) N2, (B) AM141, (C) AM101 and (D) HA759 strains. Data are representative of three independent experiments performed in triplicate. Results are represented as means ± S.E.M *p<0.05 compared to control (One-way ANOVA followed by Dunnett's post-hoc test).

5. DISCUSSÃO

Os resultados desta tese trazem elucidações sobre a relação entre a toxicidade dos metais cobre e zinco em combinação e a progressão da DH, além do papel neuroprotetor da rutina e seu potencial papel como quelante de metais. Sabendo que existem mecanismos em comum entre a patogenia da DH e a neurotoxicidade causada por metais, como o estresse oxidativo (BONO-YAGUE et al., 2020), e também baseado nas evidências de que a rutina confere atividade antioxidante (ENOGIERU et al., 2018) e efeitos neuroprotetores (CORDEIRO et al., 2020), buscamos avaliar os efeitos neuroprotetores da rutina no modelo de DH em *C. elegans* após a exposição crônica dos metais cobre e zinco. Nesta sessão serão discutidos de maneira geral os resultados de maior relevância a cada trabalho, estabelecendo as possíveis relações entre eles.

No primeiro estudo da tese, investigamos o efeito da rutina em um modelo de DH em *C. elegans* com foco nos neurônios ASH e na defesa antioxidante. Foram avaliados ensaios comportamentais relacionados aos neurônios ASH, agregados poliQ neuronais, avaliação da neurodegeneração, além de níveis de expressão da HSP 16.2 e a enzima antioxidande SOD-3.

Os neurônios ASH são responsáveis pelo controle da resposta aos estímulos sensoriais, importante para a manutenção da vida do animal, devido ao comportamento de forrageamento. A detecção dos estímulos aversivos no ambiente é uma característica fundamental do sistema nervoso animal, permitindo-lhe evitar substâncias químicas nocivas e condições adversas. Os animais usam neurônios especializados e estruturas sensoriais chamadas nociceptores para detectar uma ampla variedade de estímulos aversivos, incluindo produtos tóxicos. Uma característica dos neurônios nociceptores é que eles são polimodais e podem responder a diferentes tipos de estímulos sensoriais, análogos aos neurônios nociceptivos polimodais sensíveis a dor em vertebrados. Além de estarem envolvidos em comportamentos mecanorreceptores, como o movimento, os neurônios ASH também modulam comportamentos mecanossensoriais, como resposta ao toque e resposta a odores (como o 1-octanol) (HILLIARD et al., 2005).

O tratamento de animais com rutina nas estirpes mutantes demonstrou que a rutina foi capaz de manter o funcionamento dos neurônios ASH, observada em comportamentos como o ensaio de resposta ao toque e a latência da resposta ao odorante octanol. Semelhante ao envelhecimento neuronal humano, o envelhecimento do sistema nervoso de *C. elegans* sofre mudanças significativas na integridade sináptica e na citoarquitetura neuronal com o avanço da idade (BÉNARD et al., 2012). Pesquisadores demonstraram que a expressão da Htt humana com 150 repetições da poliQ em *C. elegans* faz com que os neurônios sensoriais ASH envelhecidos mudem sua morfologia e aumentem de duas a três vezes seu tamanho normal, em alguns casos, conteúdos intracelulares são perdidos. Além disso, esses animais também exibem déficits severos na resposta ao toque (MIYASAKA et al., 2005). Ademais, estudos sugerem que a capacidade dos neurônios de funcionar adequadamente se correlaciona com o envelhecimento neuronal saudável (CHEW et al., 2013). Corroborando com nossos dados, a rutina também demonstrou efeito positivo ao proteger a morfologia neuronal dos oligômeros tóxicos da tau em um modelo de camundongo com DA (SUN et al., 2021).

Como mencionado anteriormente, os neurônios ASH também são conhecidos por modular comportamentos de mecanorreceptores, como o movimento (EZAK; FERKEY, 2010). A estirpe mutante AM141 tem sido usada para modelar aspectos da toxicidade da poliQ, uma vez que comprimentos variados de poliQ são expressos em células musculares do verme (MORLEY et al., 2002). A maioria das proteínas relacionadas a doenças são propensas à agregação e tendem a se unir em espécies agregadas que podem ser facilmente visualizadas no animal. Quando a proteína é expressa nas células musculares, a toxicidade dessas proteínas geralmente resulta em dano tecidual e subsequentemente paralisia ou movimento descoordenado (CHEN et al., 2015). Além disso, Gatrell e colaboradores (2020) demonstraram que a presença da poliQ nas células musculares reduz o movimento em *C. elegans* (GATRELL et al., 2020). Em nosso estudo, a rutina claramente demonstrou melhorar tarefas comportamentais.

O modelo de expressão da poliQ de forma pan-neuronal em *C. elegans* permite a investigação simultânea da patogênese da poliQ no nível de neurônios individuais, além do efeito no comportamento de um animal vivo. Brignull e colaboradores (2020) demonstraram que em neurônios específicos, a proteína (Q40) forma agregados e causa diversos defeitos comportamentais (BRIGNULL et al., 2020). O tratamento com rutina nas concentrações testadas foi capaz de diminuir os agregados poliQ neuronais. A literatura mostra que outros compostos polifenólicos também foram capazes de atenuar a agregação proteica. A epigalocatequina (EGCG) um polifenol presente no chá verde demonstrou modular o

dobramento incorreto das proteínas poliQ expandidas *in vitro* (EHRNHOEFER et al., 2006), além de suprimir a formação de agregados poliQ no modelo SCA3 (ataxia espinocerebelar do tipo III) em *C. elegans* (BONANOMI et al., 2014). A curcumina demonstrou inibir a α-sinucleína (DAVID et al., 2005) e a proteina Htt (ONO et al., 2004), além de ativar chaperonas moleculares em modelos de doenças de agregação da poliQ (FRAUTSCHY; COLE, 2010; ONO et al., 2004).

As terminações sensoriais dos neurônios ASH são expostas ao ambiente, também chamados de neurônios sensoriais ambientalmente expostos, o que permite a utilização de corantes lipofílicos, como o 1,1′-Dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanin-perchlorat (DiD) (CHALFIE; SULSTON, 1981). Esse composto fluorescente é captado pelas terminações sensoriais, sendo que os neurônios que falham em absorver o corante estão ausentes ou tem defeitos nas terminações sensoriais. O corante DiD cora os neurônios que expressam OSM-10 (envolvido na via de sinalização osmossensorial), e de forma mais fraca outras classes de neurônios (FABER et al., 1999). Evidenciamos que a rutina foi capaz de atenuar a degeneração dessas terminações sensoriais. Nossos estudos anteriores já demonstraram o efeito da rutina como neuroprotetor reduzindo a morte neuronal mediada por poliQ em neurônios ASH (CORDEIRO et al., 2020). Além disso, estudos demonstraram que a suplementação com rutina atenuou a neurotoxicidade induzida por colistina em modelo de mamíferos, considerando que os prováveis mecanismos subjacentes estão associados aos efeitos antioxidantes, anti-inflamatórios e anti-apoptóticos da rutina nos tecidos cerebrais (ÇELIK et al., 2020).

O estresse oxidativo tem um papel importante na patogênese das doenças neurodegenerativas, pois o excesso de EROs pode causar peroxidação lipídica da membrana, desnaturação proteica, danos aos ácidos nucleicos e pode alterar a função neuronal e prejudicar o sistema nervoso (BENZER et al., 2018; DAI et al., 2015; JIANG; LI, 2014). Estudos apoiam a relação entre o estresse oxidativo e a DH (ROTBLAT et al., 2014; JOHRI; BEAL, 2012; CHEN et al., 2007), propondo que o estresse oxidativo elevado seja um mecanismo nos estágios finais da patogênese da DH (JOHRI; BEAL, 2012). Assim, compostos naturais com efeito antioxidante são levados em consideração para atenuar eventos decorrentes da patologia. Foi relatado que a estrutura química da rutina pode eliminar diretamente EROs (HANASAKI; FUKUI, 1994), além isso, as enzimas antioxidantes como a SOD são a primeira linha de defesa contra agentes tóxicos, metabolizando-os em subprodutos inócuos (RODRIGUEZ et al., 2004).

A resposta ao estresse oxidativo em *C. elegans* é regulada por diferentes vias, como a tipo insulina/IGF-1 (IIS). A IIS controla processos biológicos, incluindo metabolismo, desenvolvimento, reprodução e resistência ao estresse. Peptídeos semelhantes à insulina se ligam ao DAF-2, ortólogo do receptor de insulina/IGF-1 em *C. elegans* (KIMURA et al., 1997), ativando sua atividade de tirosina quinase. Essa ativação desencadeia uma cascata de eventos de fosforilação através de diferentes quinases (AGE-1, PDK-1 e AKT-1/2) que promovem o sequestro citoplasmático dependentes de fosforilação dos fatores DAF-16/FOXO, SKN-1 e HSF-1, impedindo sua atividade transcricional (OGG et al., 1997). De outra forma, a perda de sinalização resulta em fenótipos citoprotetores resistentes ao estresse oxidativo (MOHRI-SHIOMI; GARSIN, 2008). A ativação de DAF-16 aumenta a expressão de diferentes genes envolvidos na resposta antioxidante, incluindo a SOD-3 e proteínas de choque térmico como a HSP 16.2 (HASSAN et al., 2009). Nossos resultados demonstram que ambas as expressões foram significativamente reguladas pela rutina, confirmando seu papel antioxidante. Corroborando com nossos achados, outros flavonoides também modulam as expressões como o EGCG (ZHANG et al., 2009), miricetina, kaempferol e quercetina (GRUNZ et al., 2012).

Tomados em conjunto, os resultados do primeiro artigo indicam que a rutina possui efeito neuroprotetor mantendo as funções dos neurônios ASH, além de diminuir a degeneração das terminações sensoriais. É proposto então que a rutina aja em um mecanismo que envolve sua função antioxidante relacionada à expressão de enzimas antioxidantes e chaperonas que regulam a proteostase. Tendo em vista esses efeitos, no segundo artigo da tese procuramos investigar o papel da exposição aos metais cobre e zinco na progressão da DH, além de avaliar os efeitos neuroprotetores da rutina na neurodegeneração induzida por esses toxicantes. É sabido que níveis excessivos de metais se acumulam no cérebro podendo causar uma variedade de eventos intracelulares deletérios, incluindo dobramento incorreto de proteínas e estresse oxidativo (WRIGHT; BACCARELLI, 2007). Esses efeitos podem alterar a neurotransmissão e causar neurodegeneração que pode se manifestar de diferentes maneiras, com problemas cognitivos, disfunção de aprendizado e memória e distúrbios do movimento. Atualmente, a neurotoxicidade induzida por metais vem sendo associada a diferentes doenças neurológicas, incluindo a DH (DESAI; KALER, 2008).

A toxicidade dos metais vem sendo estudada, porém, as pesquisas se concentram em um contaminante de forma isolada enquanto no ambiente estamos expostos a misturas. Em nosso estudo, demonstramos que mesmo em concentrações permitidas pelo CONAMA para

solos residenciais, a mistura de metais foi tóxica aos *C. elegans*, afetando parâmetros corporais e locomotores, além de atrasar o desenvolvimento larval. As legislações atuais em todo o mundo para exposições a metais consideram níveis de substâncias tóxicas de forma isolada, ao invés de seus possíveis efeitos interativos, tendo em vista que cada metal pode afetar diferentes vias metabólicas, os efeitos interativos dos metais em misturas podem ser aditivos, sinérgicos ou antagônicos, levando a uma resposta tóxica diferente. Um parâmetro que deveria ser sempre considerado na estimativa da toxicidade de misturas de metais é o modo de ação do metal (BALISTRIERI; MEBANE, 2014). O modo de ação basicamente é a resposta produzida no organismo exposto a um tóxico ou as características do mecanismo necessário para a produção da resposta biológica (BORGERT et al., 2004). O modo de ação nem sempre é aplicado em previsões da toxicidade de misturas tóxicas na avaliação do risco. Além disso, muitas análises são consideradas apenas para exposição aguda.

Devido à toxicidade apresentada pela mistura de metais, analisamos comportamentos específicos para avaliar o efeito dos metais em neurônios individuais. Nossos dados demonstraram que a mistura de metais aumentou o tempo de latência em resposta ao 1-octanol em animais HtnQ150 com superexpressão especificamente nos neurônios ASH. Ademais, demonstramos que a mistura de metais além de afetar neurônios ASH também afeta neurônios receptores de toque. Seis neurônios receptores de toque são sensíveis ao toque suave (CHALFIE; SULSTON, 1981), dois pares estão localizados anteriormente á vulva (ALML e ALMR) e na cauda (PLML e PLMR), um terceiro par (AVM e PVM) encontra-se no meio anterior e posterior do verme (SULSTON; HORVITZ, 1997). O complexo de canais mecanorreceptores contêm proteínas necessárias para transduzir o estímulo do toque, as principais subunidades são codificadas pelos genes *mec-4* e *mec-10*. Os neurônios receptores de toque respondem aos estímulos em qualquer lugar ao longo de seus processos. Além disso, *mec-4* está presente ao longo dos processos de neurônios receptores de toque (CHERLUR et al., 2002). Dessa maneira, sugerimos que a mistura de metais afeta a mecanossensação de toque em *C. elegans*.

Já é conhecido o fato de que as proteínas são os principais alvos dos metais. Os metais podem interferir na atividade biológica das proteínas nativas dobradas através de diferentes modos de interação, como: ligar-se a tióis livres ou outros grupos funcionais em proteínas; deslocar íons metálicos essenciais em metaloproteínas ou ainda catalisar a oxidação das cadeias laterais de aminoácidos (WYSOCKI; TAMÁS, 2010; SHARMA; GOLOUBINOFF; CHRISTEN, 2011; JACOBSON et al., 2012). Pesquisas demonstraram que metais pesados inibem o redobramento de proteínas quimicamente desnaturadas *in*

vitro, interferindo no enovelamento de proteínas *in vivo* e causando a agregação de proteínas nascentes em células vivas (HOLLAND et al., 2007). Ao interferir no dobramento de proteínas nascentes ou não nativas, os metais afetam a homeostase de proteínas. Além disso, os metais podem aumentar a propensão de agregação de proteínas associadas a doenças e possivelmente promover a progressão de patologias neurodegenerativas (BREYDO; UVERSKY, 2011). Em nosso trabalho, demonstramos que a mistura de metais causou um aumento nos agregados proteicos musculares, além disso, Zn e Cu causaram um aumento nos agregados neuronais, reforçando a literatura atual onde está cada vez mais claro que os metais afetam a homeostase de proteínas, esse mecanismo de toxicidade resulta na formação e acumulação de agregados proteicos tóxicos (TAMÁS et al., 2014). Da mesma forma, demonstramos que o tratamento com a rutina foi capaz de diminuir os agregados neuronais, confirmando seu papel neuroprotetor.

O acúmulo de Cu em cérebros de pacientes com DH pode levar à interação com locais de ligação de baixa afinidade em várias biomoléculas. Interações aberrantes de Cu-proteína foram relacionadas na patogênese da DA e PD (HUANG et al., 1999; VALENTINE; HART, 2003). O Cu pode alterar a conformação, atividade redox e/ou agregação da mHtt. Isso é semelhante à interação da β-amilóide com o Cu, que induz a oligomerização da β-amilóide onde se acredita contribuir para a patogênese da DA (HUANG et al., 1999). A recaptação de Zn nos neurônios ainda não é bem compreendida, mas sabe-se que a recaptação desbalanceada pode levar, por exemplo, ao acúmulo extracelular que interfere no comportamento do receptor NMDA e pode gerar agregação da β-amilóide (BARNHAM; BUSH, 2014).

Como mencionado anteriormente, neurônios anfídeos (neurônios gustativos e olfativos localizados na cabeça de *C. elegans*) podem absorver corantes lipofílicos (HEDGECOK et al., 1985), esses corantes podem marcar todas as partes do neurônio. Sabe-se que a neurodegeneração se correlaciona com o comprimento da poliQ e é detectada pela falha do neurônio em absorver o corante (Faber et al., 1999). Em nossos resultados, demonstramos defeito no preenchimento do corante após a exposição à mistura de metais, caracterizado neurodegeneração. A interação Cu/Zn é uma associação conhecida, pode promover a agregação de β-amilóide e a formação de placas, além de estar envolvido no estresse oxidativo, outro mecanismo que leva à neurodegeneração e à demência (NÚNEZ et al., 2012). Além disso, corroborando com nossos dados anteriores, onde demonstramos o papel neuroprotetor da rutina, aqui ela foi capaz de diminuir o processo neurodegenerativo em todas as concentrações testadas após a exposição aos metais.

Compreende-se que a homeostase de metais seja um processo vital, visto que o desbalanço de um único metal resultará no desequilíbrio de outros metais (PFALZER et al., 2022). Além disso, o desequilíbrio nas concentrações de metais é uma característica das doenças neurodegenerativas, ocasionando distribuições desequilibradas de alguns metais no cérebro, por outro lado, quantidades significativas se acumulam em áreas específicas e levam à deficiência de metais em outras (LOPES DE ANDRADE; SANTOS; ASCHNER, 2021). Em nosso estudo, a exposição à mistura de cobre e zinco resultou no aumento dos níveis de ferro (Fe) e Zn, enquanto na estirpe mutante (HtnQ150 superexpressa nos neurônios ASH) a exposição ao Zn levou ao aumento nos níveis de Fe. A literatura atual sobre as interações entre Zn e Cu com Fe ainda não é bem descrita (ESPINOZA et al., 2012). A interação entre a absorção de Fe, Zn e Cu é sugerida através da ligação competitiva com o transportador DMT1 (GUNSHIN et al., 1997). Cu, Zn e Fe são metais essenciais que demonstram interações e possíveis inibições competitivas de biodisponibilidade e transporte (REINSTEIN et al., 1984; BREWER et al., 19985). Arredondo e colaboradores (2006) demonstraram que a incubação de células Caco-2 (linhagem celular do epitélio intestinal) com Cu, Fe ou Zn afeta o metabolismo do Fe. Fe, Cu e Zn afetaram a absorção um do outro no modelo de epitélio intestinal. Interações inibitórias entre esses metais podem surgir quando são administradas doses altas de um único metal ou quando o fornecimento é uma mistura de metais (ARREDONDO et al., 2006).

Avaliando os resultados obtidos em ambos os trabalhos, inferimos que a rutina exibe efeitos neuroprotetores ao mitigar a neurodegeneração e o número de agregados proteicos neuronais, além de manter a funcionabilidade dos neurônios ASH. Propomos que ela atua de maneira antioxidante ao aumentar a expressão de enzimas antioxidantes e chaperonas que regulam a proteostase e também como quelante de metais. Pesquisadores confirmaram que flavonoides podem se comportar como antioxidantes devido as suas propriedades quelantes. Kostyuk e colaboradores (2001) demonstraram que os complexos de rutina e epicatequina com ferro (II), ferro (III), cobre (II) e zinco (II) são mais eficazes na eliminação de radicais livres do que os flavonoides livres (KOSTYUK et al., 2001). Outro estudo, Prakash e colaboradores (2020) demonstraram que a formação de quelato é o resultado da interação da rutina com íons metálicos. A quelação de metais pode ser fundamental na prevenção da geração de radicais livres, sendo observada que as interações entre os flavonoides e íons de metais de transição formam complexos que impedem a

participação de íons metálicos em processos de geração de radicais, demonstrando assim um comportamento antioxidante (PRAKASH et al., 2020).

Até o momento, estudos sobre o efeito das misturas de metais em baixas concentrações em longo prazo ainda são escassos. Pela primeira vez, demonstramos que mesmo em concentrações abaixo do que é permitido pelo Conselho Nacional do Meio Ambiente há efeitos neurotóxicos em *C. elegans* provocados pela mistura de cobre e zinco após exposição crônica. Apesar da melhor compreensão das exposições a metais, a legislação ainda se baseia principalmente na avaliação de metais individuais, excluindo possíveis interações. Sendo assim, através desse trabalho foi possível delinear pontos importantes sobre a toxicidade da mistura de cobre e zinco de forma crônica (i) envolvimento dos metais na progressão da DH, através do aumento de agregados proteicos e neurodegeneração, (ii) sugerir a revisão das diretrizes sobre as concentrações ditas seguras de metais em solos residenciais.

6. CONCLUSÃO

Podemos concluir que este trabalho endossa evidências do papel neuroprotetor da rutina contra a toxicidade da huntingtina mutante. Em linhas gerais, nos modelos de DH em *C. elegans* propostos, a molécula atua por meio de seu potencial antioxidante, indução de enzimas antioxidantes e de chaperonas, além do seu papel importante na atenuação da degeneração das terminações sensoriais nos neurônios ASH. Ademais, demonstramos a relação entre a exposição crônica a cobre e zinco em baixas concentrações e a progressão da toxicidade da poliglutamina, e o efeito neuroprotetor da rutina. Pela primeira vez, é demonstrado que a exposição a uma mistura desses metais mesmo em concentrações abaixo das permitidas pelo Conselho Nacional do Meio Ambiente alteram parâmetros toxicológicos importantes. Isso reforça a necessidade do estudo dos efeitos neurotóxicos dos metais pesados de maneira crônica e em concentrações subletais e a revisão de legislações internacionais.

7. PERSPECTIVAS

A partir dos resultados obtidos nesta tese, as perspectivas para trabalhos futuros são:

- Quantificar a rutina e quercetina em *C. elegans* por cromatografia líquida de alta eficiência (HPLC);
- Elucidar as interações entre os metais ferro, zinco e cobre;
- Delinear os mecanismos específicos pelos quais os metais alteram a homeostase, a fim de compreender os processos patológicos da Doença de Huntington;

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