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AVALIAÇÃO DOS EFEITOS DA RUTINA EM MODELO DE DOENÇA DE HUNTINGTON EM *Caenorhabditis elegans*

Santa Maria, RS, Brasil

2019

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

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“Ao fim do dia podemos aguentar muito mais do que pensamos que podemos.”

(Frida Kahlo)

APRESENTAÇÃO

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

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

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

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

RESUMO

Dissertação de Mestrado

Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica

Universidade Federal de Santa Maria

AVALIAÇÃO DOS EFEITOS DA RUTINA EM MODELO DE DOENÇA DE HUNTINGTON EM *Caenorhabditis elegans*

AUTOR: Larissa Marafiga Cordeiro
ORIENTADOR: Prof. Dr. Félix Alexandre Antunes Soares
Local e Data da Defesa: Santa Maria, 19 de julho, 2019

A doença de Huntington (DH) é um transtorno neurodegenerativo progressivo e hereditário predominantemente causado pela expansão de uma repetição CAG, que codifica a poliglutamina (poliQ). Essa patologia resulta em perda de células neuronais, alterações motoras, demência e atualmente é uma doença sem cura. A rutina é um flavonoide encontrado em plantas, como *Passiflora incarnata*, trigo sarraceno, alguns chás e também em maçãs. Ela é conhecida por possuir atividades antioxidantes, citoprotetoras, anticancerígena e neuroprotetoras. Assim, nós investigamos o efeito da exposição crônica de rutina *in vivo* no modelo de DH utilizando linhagens transgênicas do nematoide *Caenorhabditis elegans*. O *C elegans* é um nematódeo pequeno (±1 mm), de fácil manipulação, curto ciclo de vida, fácil de cultivo e rápido tempo de geração. Os animais foram tratados a partir do estágio L1 com 15, 30, 60 e 120 μM de rutina até a idade adulta. Avaliamos ensaios comportamentais, a agregação poliQ, o dano oxidativo, o nível de neurodegeneração, o tempo de vida e investigamos o possível papel das vias de sinalização da autofagia e da insulina / IGF1 (IIS) nos efeitos benéficos induzidos pela rutina. Em geral, nossos dados demonstram que o tratamento com rutina reduziu a agregação da proteína poliglutamina (poliQ) no músculo, reduziu a morte neuronal mediada por poliQ em neurônios sensoriais de ASH e prolongou a vida útil em *C. elegans*. Os possíveis mecanismos envolvidos nas ações descritas são: atividade antioxidante *per se* do extrato, ativação da degradação proteica (autofagia), e ainda as vias de sinalização da insulina / IGF1 (IIS). Esses achados demonstram que a exposição crônica a rutina pode desempenhar um papel útil na prevenção da DH e também fornecer possíveis caminhos a serem explorados para a busca de novas terapias contra as proteinopatias relacionadas ao envelhecimento.

ABSTRACT

EVALUATION OF RUTIN EFFECTS IN A HUNTINGTON DISEASE MODEL IN *Caenorhabditis elegans*

AUTHOR: Larissa Marafiga Cordeiro
ADVISOR: Prof. Dr. Félix Alexandre Antunes Soares
Place and Date of the Defense: Santa Maria, July 19th, 2019

Huntington's disease (DH) is a progressive and hereditary neurodegenerative disorder predominantly caused by the expansion of a CAG repeat, which encodes polyglutamine (polyQ). This pathology results in loss of neuronal cells, motor alterations, dementia and is currently a disease without cure. The rutin is a flavonoid found in plants such as *Passiflora incarnata*, buckwheat, some teas and also in apples. It is known to have antioxidant, cytoprotective, anticancer and neuroprotective activities. Thus, we investigated the effect of *in vivo* rutin chronic exposure on the DH model using transgenic lines of the nematode *Caenorhabditis elegans*. The *C elegans* is a small nematode (± 1 mm), easy to handle, short life cycle, easy to grow and fast generation time. The animals were treated from the L1 stage with 15, 30, 60 and 120 μM rutin until adulthood. We evaluated behavioral assays, polyQ aggregation, oxidative damage, neurodegeneration level, lifespan, and investigated the possible role of autophagy and insulin / IGF1 (IIS) signaling pathways in the beneficial effects induced by the rutin. In general, our data demonstrate that routine treatment reduced polyglutamine (polyQ) protein aggregation in muscle, reduced polyQ mediated neuronal death in ASH sensory neurons, and extended lifespan in *C. elegans*. The possible mechanisms involved in the actions described are: antioxidant activity per se of the extract, activation of protein degradation (autophagy), and insulin / IGF1 (IIS) signaling pathways. These findings demonstrate that chronic exposure to rutin may play a useful role in the prevention of DH and also provide possible avenues to be explored for the search for new therapies against aging-related proteinopathies.

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Introdução

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LISTA DE ABREVIATURAS

DA: Doença de Alzheimer

DH: Doença de Huntington

DP: Doença de Parkinson

EROs: Espécies Reativas de Oxigênio

FOXO: Fator de transcrição *Forkhead Box*

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

N2: cepa selvagem de *C. elegans*

PoliQ: Trato de poliglutamina

ROS: Reactive Oxygen Species

SUMÁRIO

1. INTRODUÇÃO

A doença de Huntington (DH) é uma patologia neurodegenerativa que se caracteriza por alterações motoras progressivas, distúrbio emocional, 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 (TANZI et al., 1983) e, subsequentemente, isolada em 1993 pelo “Huntington’s Disease Collaborative Research Group”. Esse 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. Essa mutação resulta numa expansão da sequência de nucleotídeos citosina, adenina e guanina (CAG - que codifica o aminoácido glutamina), resultando em uma proteína mutante com uma sequência de poliglutaminas (poli-Q) no terminal amínico da proteína huntingtina. Os indivíduos normais (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; DIFIGLIA, 1998). A DH possui herança autossômica dominante, o alelo normal transmite-se de geração em geração segundo as regras de hereditariedade Mendeliana. O alelo mutante é instável durante a meiose, alterando o seu comprimento na maior parte das transmissões entre 20 gerações, com um aumento de 1-4 unidades ou diminuição de 1-2 unidades do triplete CAG (GIL-MOHAPEL; REGO, 2011).

A DH é uma patologia relacionada ao envelhecimento que normalmente surge após os 40 anos de idade, podendo ocorrer na juventude, sendo mais rara e grave. Uma possível intervenção precoce pode ser útil para desenvolver estratégias terapêuticas iniciais para DH e outras doenças neurodegenerativas mais prevalentes, incluindo Doença de Alzheimer e Doença de Parkinson, que compartilham características comuns como a agregação proteica anormal, morte neuronal e início tardio (ROSS; TABRIZI, 2011). 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 et al., 2002).

A associação de eventos que levam a patogênese da doença de Huntington ainda não está clara, porém relatos anteriores sugerem que eventos excitotóxicos, mudanças no metabolismo energético e disfunção mitocondrial podem estar envolvidos na fisiopatologia da DH (KUMAR, 2009). Mutações no DNAmt e o estresse oxidativo contribuem para o envelhecimento e aumentam o fator de risco para a doença (LIN; BEAL, 2006).

Espécies reativas de oxigênio (EROs) são gerados durante o metabolismo celular normal (KONTOS, 1989) seus níveis fisiológicos podem ser eliminados por enzimas antioxidantes, no entanto, quando os níveis de EROs superam a capacidade de neutralização das defesas antioxidantes de um organismo, ocorre o estresse oxidativo (WINYARD et al., 2005). Estas espécies reativas, quando em excesso, causam a oxidação de biomoléculas como, lipídeos, proteínas e nucleotídeos, o que afeta a homeostase energética e causa danos celulares (STADTMAN, 2001). Aumentos anormais nos níveis de marcadores para danos oxidativos têm sido relatados no cérebro de portadores de DH (TELLEZ-NAGEL et al., 1974; FERRANTE et al., 1997).

Além disso, a disfunção da autofagia é uma característica consistente nos modelos animais em HD (CORTES; SPADA, 2014). A disfunção de carregamento de autofagossomos tem sido observada em modelos celulares e animais de HD, causando uma degradação proteica autofágica prejudicada (VICENTE et al., 2010). A ativação da autofagia pode levar ao aumento da depuração de proteínas tóxicas. A ativação indireta da autofagia por certas substâncias químicas tem mostrado reduzir a toxicidade do poliQ (RAVIKUMAR et al., 2014). Park et al, demonstraram que a rutina induz a autofagia em várias linhagens celulares de câncer, como leucemia (THP-1), oral (CA9-22) e pulmonar (A549), linhagens de células de câncer (PARK et al., 2016). A autofagia é essencial para a remoção de organelas danificadas e proteínas tóxicas ou agregadas, entregando-as ao lisossomo para degradação. Consequentemente, a autofagia tornou-se um alvo para o tratamento de doenças neurodegenerativas que envolvem a agregação de proteínas (NAH et al., 2015).

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. O tratamento é puramente sintomático e a terapêutica selecionada em cada momento dependente da manifestação clínica dominante (COPPEN; ROOS, 2017). As estratégias terapêuticas destinadas a prevenir ou atrasar a degeneração neuronal representam uma escolha razoável

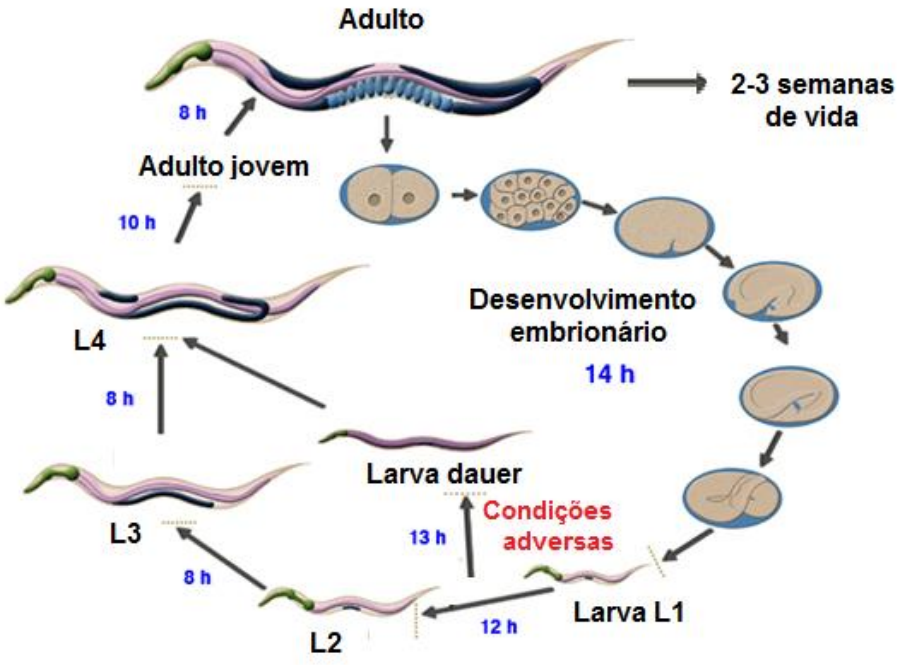
para o tratamento de doenças neurodegenerativas. Por conseguinte, existe um interesse crescente no uso de antioxidantes naturais, incluindo polifenóis encontrados em plantas medicinais e alimentos que podem prevenir a morte celular (SANDHIR et al., 2013). Devido ao grande número de espécies vegetais 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 de origem vegetal (SIMÕES et al., 2004).

Flavonoides são substâncias pertencentes a uma classe de produtos naturais que atualmente podem ser consideradas micronutrientes. Estão presentes na dieta humana rica em vegetais e frutas, que são as principais fontes dessas substâncias (BARREIROS et al., 2006). 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, 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; ALEKSANDROV et al., 1986; CRUZ et al., 1998; CHEN et al., 2000; BISHNOI et al., 2007). A literatura já descreve que a rutina reprimiu atividade de citocinas pró-inflamatórias pela diminuição da produção de TNF-α e IL-1β em micróglia de ratos. Tal efeito parece ser útil no tratamento da doença de Alzheimer como é evidente pela prevenção da citotoxicidade oligomérica β-amilóide (WANG et al., 2012). A rutina também causou a atenuação da inflamação induzida por estreptozotocina, diminuindo a atividade da proteína ácida fibrílica glial, interleucina-8, ciclooxigenase-2, óxido nítrico sintase induzível e fator nuclear-kB e, assim, evitou mudanças anatômicas no hipocampo de ratos. Tal efeito pode ser útil para evitar déficits cognitivos e revela-se benéfico no tratamento da "demência esporádica do tipo Alzheimer" (JAVED et al., 2012). Porém, ainda não foram descritos estudos utilizando o tratamento crônico com rutina no modelo de doença de Huntignton em *Caenorhabditis elegans.*

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. Seu genoma e suas vias metabólicas e biossintéticas são altamente conservados nos mamíferos com uma semelhança de aproximadamente 60-80%,

incluindo vias envolvidas no desenvolvimento celular, na manutenção do sistema nervoso e na apoptose (NASS; BLAKELY, 2003). Possui diferentes sistemas de neurotransmissão que coordenam seu comportamento, incluindo o sistema dopaminérgico, colinérgico, serotoninérgico, glutamatérgico e também gabaérgico (RIDDLE, 1997). *C. elegans* é um organismo simples tanto anatomicamente como geneticamente e essa simplicidade, aliada à sua translucidez, permitem a compreensão do desenvolvimento do organismo. A geração facilitada de cepas com mutações e a existência de diversas cepas transgênicas expressando a proteína verde fluorescente (do inglês GFP) fundida a genes promotores que codificam proteínas de interesse, por exemplo, a cepa TJ356 (DAF-16, fator de transcrição que atua na via de sinalização mediada por insulina / IGF-1 (IIS) e regula a formação de dauer, longevidade, metabolismo de gordura, resposta ao estresse), tornam o organismo ainda mais atrativo (The *C. elegans* Sequencing Consortium, 1998).

Figura 1 - Ciclo de vida de *C. elegans*



Adaptado de http://www.sfu.ca/biology/faculty/hutter/hutterlab/research/Celegans.html

Os embriões de *C. elegans* se desenvolvem rapidamente após 14 horas. O primeiro estágio larval é completado após mais 12 horas e os animais passam por quatro ciclos antes de se tornar em adultos. Sob condições adversas ou na ausência de alimentos, pode adaptar-se a uma via de desenvolvimento alternativa que leve à larva dauer, que não se alimenta, mas pode

sobreviver a condições adversas por vários meses (até quatro meses). Na presença de alimento o desenvolvimento normal é retomado, os animais saem do estágio larval dauer e se tornam o quarto estágio larval normal antes de se tornarem adultos. Animais adultos são hermafroditas e produzem seus ovos. Ao longo de 3-4 dias, cerca de 300 ovos são colocados. A vida útil geral de *C. elegans* é 2-3 semanas (Figura 1).

C. elegans é um modelo *in vivo* para estudar o papel dos processos de envelhecimento no desenvolvimento de proteinopatias neurodegenerativas (KRAEMER et al., 2013). 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 degeneração nervosa (FABER et al., 1999). A disponibilidade de várias linhagens mutantes semelhantes a doenças em humanos tem sido explorada para testar o efeito de diversas substâncias, incluindo antioxidantes naturais e sintéticos (BRAECKMAN et al., 2002). Desta forma, utilizamos vermes mutantes que expressam a poliQ, reproduzindo a agregação da proteína observada em cérebros de portadores da DH para avaliar o efeito da rutina nesta patologia e os mecanismos envolvidos.

2. OBJETIVOS

2.1 Objetivo Geral

Estudar os efeitos da rutina no modelo de doença de Huntington em *Caenorhabditis elegans.*

2.2 Objetivos específicos

- Avaliar os possíveis efeitos neuroprotetores da rutina em *C. elegans* através de ensaios de sobrevivência neuronal.
- Verificar os níveis de agregação da poliQ nos músculos de *C. elegans* tratados com a rutina e elucidar os possíveis mecanismos de ação da rutina.

3. MANUSCRITO

Rutin protects *Caenorhabditis elegans* model for Huntington's disease through the insulin/IGF1 (IIS) signaling pathway and autophagy activity

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Abstract

Huntington's disease (HD) is inherited neurodegenerative disease, and it is characterized by excessive motor movements and cognitive and emotional deficits. HD is caused by an abnormally long polyglutamine (polyQ) expansion in the huntingtin (Htt) protein, which confers toxic functions to mutant Htt leading to neurodegeneration. Rutin is a flavonoid found in plants, buckwheat, some teas and also in apples. Although previous studies have already indicated that rutin has some protective effects in HD's models, the underlying mechanisms are still unknown. In our study, we investigated the effects of rutin in *Caenorhabditis elegans* model of HD. We assessed polyQ aggregation, oxidative damage, neurodegeneration level and lifespan, and investigated the possible role of autophagy and insulin / IGF1 (IIS) signaling pathways in the beneficial effects induced by rutin. Overall, our data demonstrate that chronic rutin treatment reduced polyglutamine (polyQ) protein aggregation in muscle, reduced polyQ-mediated neuronal death in ASH sensory neurons, and extended lifespan. The possible mechanisms involved are antioxidant activity, activation of protein degradation (autophagy) and insulin/IGF1 (IIS) signaling pathways. These findings indicate that rutin consumption might be helpful in preventing HD and also provide possible pathways to be explored to search for new therapies against proteinopathies related to aging.

Keywords: *C. elegans*; Flavonoids; Neurodegeration; Proteinopathies.

Introduction

Huntington's disease (HD) is an autosomal dominant progressive neurodegenerative disorder, characterized by motor dysfunction, emotional disturbances, abnormal involuntary movements, dementia, and weight loss [1, 2]. The genetic mutation responsible for HD is the redundant CAG repeats (≥36), which encode elongated polyglutamine (polyQ) tracts within the mutant huntingtin protein [3]. Most neurodegenerative diseases such as HD, Alzheimer's disease (AD), Parkinson's disease (PD), for instance, are characterized by the pathogenic accumulation and aggregation of proteins, which disrupt the dynamics of protein networks and result in destabilization of cellular homoeostasis [4]. Once HD is monogenic, fully penetrant and more amenable to early intervention, it can serve as a model to develop early therapeutic strategies for other more prevalent neurodegenerative diseases, including AD and PD, which share such features as abnormal protein aggregation, selective neuronal vulnerability and delayed onset [5].

Reactive Oxygen Species (ROS) are balanced with antioxidant systems to keep their level under control in living organisms. The antioxidant systems are both enzymatic and non-enzymatic. Breaking the balance by over production of ROS and/or reduction of antioxidants can be deleterious, and is termed oxidative stress. Under these conditions, excessive free radicals could freely pass through the plasma membrane, damaging the cell membrane via lipid peroxidation, modifying signal and structural proteins to lead to misfolding and aggregation, a characteristic of neurodegenerative diseases [6]. The main intracellular pathways for the degradation and recycling of proteins are the ubiquitin/proteasome system (UPS) and the autophagy-lysosomal pathway [7]. Thus, antioxidants and compounds acting in the pathway of autophagy could be useful for the cell.

Currently, the available treatments for HD only address the symptoms and do not alter the course or progression of the disease. In this context, the search for new products capable of acting on several biochemical targets, with new mechanisms of action and low toxicity are important. Recently, there has been intense interest in the potential of flavonoids to modulate neuronal function and prevent against age-related neurodegenerative diseases [8]. The use of flavonoid-rich plant or food extracts in humans and animal dietary supplementation studies have shown improvements in cognition function possibly by protecting vulnerable neurons, enhancing existing neuronal function or by stimulating neuronal regeneration [9].

Rutin (3, 3′,4 ′, 5, 7- pentahydroxyflavone-3-rhamnoglucoside) is a flavonol found in many typical plants such as buckwheat, apple, and tea. It has several reported

pharmacological properties, including antioxidant, cytoprotective, anti-inflammatory, immunomodulatory and neuroprotective activities [10]. It was previously shown that rutin exerted antioxidant and anticholinesterase activities in *Caenorhabditis elegans* [11] and attenuated 3-Nitropropionic acid-induced HD-like symptons in rats [12], suggesting that rutin may be a promising compound for preventing or treating neurodegenerative diseases. However, little is known regarding its underlying mechanisms and its effects on protein homeostasis.

Caenorhabditis 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 [13,14]. Although *C. elegans* is a tiny nematode (~1 mm in length), it contains about two-thirds of the potential counterparts of human disease genes and nearly one-third of its somatic cells are neurons. It is a reliable *in vivo* model system to study the development of HD related to aging [15,16]. The availability of several disease-like mutant strains has been explored to test the effect of various compounds, including natural and synthetic antioxidants [17].

Considering that neurodegenerative diseases remains with limited therapeutic options and that rutin possesses a promising pharmacological potential, herein we investigated the protective activity of this compound in *C. elegans* model of HD and the putative pathways involved, focusing on oxidative stress and autophagy.

Materials and methods

Chemicals and reagents

Ethanol, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA), 5-fluorodeoxyuridine (FUDR) and rutin were purchased from Sigma-Aldrich (USA).

C. elegans strains and maintenance

The strains used in this study: Bristol N2 (wild-type), AM141 {rmIs133[unc-54p::Q40::YFP]}, HA759 {rtIs11[osm-10p::GFP+osm-10p::HtnQ150+Dpy-20(+)]}, TJ356 [daf-16p::daf-16a/b:: GFP + rol-6], DA2123 [lgg-1p::GFP::lgg-1 + rol 6(su1006)] were obtained from the *C. elegans* Genetics Center (CGC, 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) [18]. Eggs were allowed to hatch overnight in M9 buffer to obtain animals in L1 stage.

Treatment of worms with rutin

Rutin powder was obtained commercially from Sigma Aldrich, diluted in absolute ethanol and added to the surface of nematode growth medium (NGM) agar plates with *E.coli* OP50 (already grown overnight at 36 °C) to obtain final concentrations of 15, 30, 60 and 120 μM (9.16 to 73.26 mg rutin/mL agar) and 1% ethanol. Synchronized worms in L1 stage were transferred to treatment or control plates and incubated at 20ºC until adulthood. Rutin was added to the surface of plates every 24 hours at the same concentrations. In control plates, ethanol was added in the same volume used in the treatment plates. For analysis after young adult stage we use FUDR in the plates in the final concentration of 12 μM to avoid progeny and worms were transferred to new plates with FUDR and rutin or vehicle every two days.

Behavioral tests

Behavioral tests were performed in order to select non-toxic concentrations to further assays. Ten nematodes per treatment were analyzed for the following behavior parameters: defecation cycle length, pharyngeal pumping and number of thrashes [19,20]. A set of three experiments were performed individually.

Neuronal Survival Assay

C. elegans strain HA759 was used for neuronal survival assay as described [21]. This strain expresses both GFP and Htn-Q150 (a polyQ tract of 150 residues derived from human huntingtin) strongly in ASH neurons, leading to neurodegeneration and cell death [59]. The GFP expression is monitored to assess ASH neuronal viability. The presence or absence of GFP fluorescence indicates live or dead neurons.

Worms were analyzed 24, 48, 72 and 120 hours after L1 larvae. Nematodes were collected, mounted onto a glass slide, paralyzed with 5 μL of 50 mM sodium azide and the presence or absence of GFP (green fluorescent protein) fluorescence in bilateral ASH neurons was examined using Olympus® Fluoview FV10i confocal microscope. Approximately 10 nematodes were randomly selected from each treatment and scored for live/dead ASH neurons. A set of three experiments were performed individually.

PolyQ Aggregation Assay

C. elegans strain AM141 was used for the polyQ aggregation assay [21]. Worms were analyzed 24, 48, 72 and 120 hours after L1 larvae. The nematodes were collected, mounted onto a glass slide and paralysed with 5 μL of 50 mM sodium azide. Approximately 10 animals were randomly selected in each treatment and scored for the number of polyQ40::YFP aggregates in muscle cells using Olympus® Fluoview FV10i confocal microscope and ImageJ. Three independent experiments were performed individually.

Lifespan assay

The lifespan of *C. elegans* strains HA759, AM141 and N2 was investigated as previously described [22]. The pre-fertile period of adulthood was used as time zero (t=0). The worms were kept on NGM plates containing rutin or vehicle (control) and *E. coli* and transferred to new plates with FUDR and rutin or vehicle every two days. Nematodes were scored as dead if they did not move after repeated stimulus with a platinum wire. Experiments were performed at least in triplicate with 100 nematodes each.

Measurement of reactive oxygen species

Reactive oxygen species (ROS) levels were measured in AM141, HA759 and N2 strains at 24, 48, 72 and 120 hours after L1 larvae. With 2′,7′-dichlorofluorescein diacetate (DCFDA), following a previously described method [23] with minor modifications. Worms were collected from plates, transferred to micro tubes and washed three times with M9. The samples were sonicated on ice (15 minutes, 10 seconds interval in amplitude 30%) and after centrifugation (4ºC, 15.000g and 30 minutes) the supernatants (lysates) were collected. Aliquots (5 μL) were transferred to black 96-well plates containing M9 and incubated for 1 hour with 20 μM DCFDA (final concentration) at 20°C. ROS-associated fluorescence levels were measured in a microplate reader at 485 nm excitation and 520 nm emission wavelengths at room temperature [23]. Data were normalized to protein content determined by the Bradford method [24]. Analyses were carried in duplicate and the experiment was independently repeated three times.

DAF-16/FOXO localization

C. elegans strain TJ356 was used for DAF-16 localization. Once DAF-16/FOXO is translocated from the cytoplasm to the nucleus, it would activate some downstream targets that may be involved in stress resistance and lifespan, for example [60].

Young adult worms were transferred to a glass slide and paralyzed with 5 μL of 50 mM sodium azide. Fluorescence images were acquired with an Olympus® Fluoview FV10i confocal microscope housed in air-conditioned room (20°C). Ten worms per group were randomly selected for evaluation and scored according the localization of DAF-16::GFP: totally in the cytoplasm, partially in the nucleus (intermediate) or totally in the nucleus. The percentage of worms with nuclear localization of DAF-16 was plotted. Worms exposed to heat stress at 35 °C for 30 minutes on NGM plates with OP50 were used as positive control. The assay was performed in triplicate.

Autophagy

Autophagy was evaluated by quantifying the number of GFP-positive foci of the *gfp::lgg-1* transgene in seam cells. The gene *lgg-1* encodes a protein necessary for the degradation of cellular components by autophagy. Under normal conditions, DA2123 worms exhibit diffuse fluorescence in the cytoplasm of various tissues. The formation of pre-autophagosomic and autophagosomic structures can be observed and counted with the appearance of focal points of fluorescence (puncta) [25]. Young adult worms were transferred to a glass slide and paralyzed with 5 μL of 50 mM sodium azide. Ten worms per group were randomly selected for evaluation and assays were performed in triplicate. Fluorescence images were acquired with an Olympus® Fluoview FV10i confocal microscope housed in air-conditioned rooms (20°C) and the number of autophagosomes were quantified by ImageJ program.

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 Tukey test. Significance for survival analysis was assessed by the Kaplan-Meier curve followed by the log rank test for trend. Values of $p < 0.05$ were considered to be statistically significant.

Results

Effect of rutin in behavioral assessment

No differences in defecation cycle length, pharyngeal pumping and number of thrashes were observed in worms treated with 15, 30, 60 and 120 μM of rutin compared to untreated worms (data not shown).

Effect of rutin on polyQ-mediated neurotoxicity

We evaluated the protective effect of rutin against polyQ-mediated neurotoxicity. As shown in Figure 1, the percentage of animals with neurodegeneration in HA759 (HtnQ150) strain was significantly decreased by rutin treatment at 120 hours at all concentrations ($p<0.05$). In control group, 85% of animals showed degeneration in ASH neurons, while the percentage of animals with neurodegeneration significantly decreased to 57.3%, 41.6%, 43.3% and 23% at 15, 30, 60 and 120 μM of rutin, respectively.

Rutin also significantly decreased polyQ aggregation in AM141 (Q40) transgenic strain at 120 hours at all concentrations (Fig 2, $p<0.05$). The average number of aggregates observed was 57%, 42.3, 37 and 37 for worms treated with 15, 30, 60 and 120 μM of rutin respectively, compared to an average of 62.6 aggregates in control worms.

Effect of rutin on *C. elegans* lifespan

Maximum lifespan was extended from 28 (control) to 30 (treated) days by treatment with 15, 30 and 120 **μ**M in the wild-type strain (Figure 3A). Similarly, an increase in lifespan also was observed in AM141 (Figure 3B) and HA759 (Figure 3C) mutants. Both were extended from 28 (control) to 30 (treated).

Effect of rutin on ROS production

There was a significant decrease in ROS levels after treatment with rutin in N2 (WT) strain at 30 μM and 120 μM (24 hours), 30 μM (48 hours) and 15 and 30 μM (120 hours after L1 larvae) (Figure 4A, $p<0.05$). ROS production was also quantified in HA759 (HtnQ150) and AM141 (Q40) strains. Figure 4B shows a significant decrease in ROS levels in HA759 mutant worms treated with rutin at concentrations of 30 μM (24 hours), all concentrations (48 hours) and 15 μM (72 hours after L1) ($p<0.05$). Measurement of ROS production in AM141 mutants demonstrated a significant decrease in ROS levels within the first 24 hours, followed by an increase in 48 and 120 hours after L1 (Figure 4C).

Effect of rutin on DAF-16 localization

After treatment with rutin, we observed a significant DAF-16 activation and migration to the nucleus ($p<0.05$, Figure 5). The percentage of animals with nuclear localization of DAF-16::GFP was 31.8%, 16.2%, 21.4% and 50.6% for worms treated with 15, 30, 60 and 120 μM of rutin respectively, compared to 4.4% for control worms.

Effect of rutin on Autophagy

The number of GFP-positive foci in the seam cells of the GFP::LGG-1 transgene was significantly increased in 11.2, 8.8 and 11.3 in worms treated with 30, 60 and 120 μM of rutin respectively, compared with control group.

Discussion

In this study, we investigated the protective effects of rutin against the onset and progression of huntingtin (Htn) toxicity. Despite the simplicity of *C. elegans* model does not replicate all the aspects of HD, like the motor symptoms, this nematode is a powerful model to study target pathways and compounds that affect Htn pathology [63]. Our data depict that all the concentrations of rutin tested (15-120μM) did not exert any toxic effects in *C.elegans* behavioral assays performed, thus these concentrations were used in further analysis. Herein, rutin was able to reduce polyQ-mediated neuronal death in ASH neurons (Fig 1) and the polyQ40 aggregation (Fig 2) in *C. elegans* transgenic strains. Previous studies already demonstrated that rutin could act as a neuroprotector compound in AD models [26] and protected the striatum from oxidative/nitrosative insults caused by 3-NP in the HD model in rats, in addition to, improved the motor, memory and learning [12]. Due to the ability of rutin and/or its metabolites to cross the blood brain barrier, it has also been shown to modify the cognitive and various behavioral symptoms of neurodegenerative diseases [26]. Also, neuroprotective potential of rutin has been demonstrated against both oxidative stress and neuronal injury induced by neurotoxins [32].

Aging is considered a risk factor for developing many diseases, and has been associated with a decline in a variety of pathways relevant to neurodegenerative disorders [33,34], especially those that are critical for handling misfolded proteins [35]. The deficits in homeostatic mechanisms caused by aging could overlap or operate independently from those induced by disease-associated forms of Htn, but in either case, aged cells would be predicted to be more susceptible than younger ones [67]. Thus, compounds that retard aging may also slow the progression of HD, and the effects of rutin on *C. elegans* lifespan were also

investigated (Fig 3). Our results showed that rutin increased lifespan in wild-type, and AM141 (Q40) and HA759 (HtnQ150) mutants. Li et al, previously demonstrated the beneficial effect of rutin against age-related metabolic dysfunctions [58]. Since rutin is an important constituent in many kinds of human food materials, it might be a potent therapy to delay aging and consequently the onset and development of HD and other age-related diseases.

Evidence for accumulative oxidative damage related to the development of HD have been demonstrated [33]. Mitochondria are the major intracellular source of free radicals, and are also particularly susceptible to oxidative stress. Abnormal increases in marker levels for oxidative damage have been reported in the brains of HD carriers, and mitochondrial dysfunction is one important facet of the pathogenesis [38]. Reactive oxygen species (ROS) are generated during normal cell metabolism [37]. However, when levels of ROS exceed the neutralizing capacity of an organism's antioxidant defenses, biomolecules might be oxidized, affecting energetic homeostasis and causing cell damage [41]. Despite that oxidative damage to neurons may not be the primary event initiating neurodegenerative disorders, it seems that oxidative stress participates in the pathogenetic cascade of these diseases [39].

Thus, antioxidant compounds might be helpful in preventing or treating HD. Here, we demonstrate that rutin may be exerting its protective effects against Htn toxicity through an antioxidant activity by decreasing ROS (Fig 4). It is according with the study of Suganya and Sumathi [64], which reported that oral administration of rutin significantly decreased protein oxidation and improved endogenous antioxidant defence system in a rat model of HD induced by 3-nitropropionic. 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 proteinophaties [31].

Furthermore, in our study, rutin increased the nuclear localization of transition DAF-16 (Fig 5), indicating its activation. It has been demonstrated that DAF-16 plays pivotal roles in activating antioxidant genes, regulating longevity, and in ameliorating polyQ aggregation and toxicity [40]. DAF-16, the homologue of mammalian FOXO transcription factor, is thought to be the main target of the DAF-2 pathway, an insulin/insulin-like growth factor (IGF)-1 receptor homolog, which signals through a conserved phosphatidylinositol 3-kinase (PI 3-kinase)/Akt pathway. The insulin IGF-1/FOXO pathway (IIS) is initiated by changes in IGF-1 levels, which induces IGF-1 receptors to start a phosphorylation cascade that deactivates the FOXO transcription factor. When the IIS signaling is suppressed,

FOXO/DAF-16 is not phosphorylated and migrates to the nucleus activating many transcription factors, like ROS-scavenging enzymes [41, 42].

DAF-16 is also involved in the formation of less toxic high-molecular weight protein aggregates [43]. Because the transcription factor DAF-16 also controls molecular chaperone expression, pharmacological activation of DAF-16 directly or indirectly via the suppression of the IIS pathway may be a viable method to increase the buffering capacity of the proteostasis network and simultaneously to increase lifespan and reduce the aggregation and toxicity of disease-associated proteins [44]. Therefore, the effects of rutin on DAF-16 activation might be contributing to decrease the PolyQ aggregation and toxicity through induction of antioxidant transcription factors and chaperones, responsible for maintaining the proper conformation of proteins in the cell.

The aggregates in the HD brains apparently result from the accumulation of misfolded proteins with age and are therefore an indicator of the impaired intracellular capacity to clear or remove misfolded proteins [65]. The presence of toxic and insoluble protein aggregates is a common feature of more than 20 neurodegenerative conditions [27,28,29]. In HD, the proteolytic cleavage of Htn results in the formation of polyQ oligomers, aggregates and inclusions [30]. The effects of progressive deterioration of protein homeostasis are thought to play a role in age-related neurodegenerative diseases. Additionally to inducting chaperones to regulate protein dynamics, we demonstrated for the first time that rutin increases autophagy *in vivo* (Fig 6).

As well as, Park et al demonstrated that rutin induces autophagy in several cancer cell lines such as leukemia (THP-1), oral (CA9-22), and lung (A549) cancer cell lines (61). Other flavonoids have also been studied for promoting induction in autophagy. For example, quercetin induces protective autophagy in gastric cancer cells by modulation of Akt-mTOR signaling and hypoxia-induced factor 1α (HIF-1α) signaling [62]. Autophagy may lead to the clearance and degradation of potentially harmful protein aggregates [45] therefore being important in the protective maintenance of neural cells [46]. It is an important process in a variety of human diseases caused by toxic, aggregate-prone, intracytosolic proteins, which become inaccessible to the proteasome when they oligomerise. There is increasing evidence that the lysosomal degradation pathway of autophagy may also be important in the degradation of polyQ aggregates [47,48,49], and thus, autophagy up regulation may be a therapeutic strategy for HD and related conditions, where the mutant aggregate prone proteins are autophagy substrates [55].

Interestingly, Essick et al [46] reported that cellular oxidative stress and increased generation of ROS are important stimuli of autophagy during periods of nutrient deprivation, ischemia/reperfusion, hypoxia, and in response to cell stress [50]. During response to cell stress, there is an increased generation of mitochondrial derived hydrogen peroxide (H_2O_2) through a PI3K/beclin1 dependent pathway. This leads to oxidation and consequent inhibition of ATG4, ultimately promoting ATG8-PE (Autophagy-related protein 8) conjugation and enhancing autophagy [46]. We believe that the variations in ROS levels showed in Figure 5 might be associated with an increase in autophagy activity. Depending on ROS concentration, molecular species and subcellular localization, cell components and signaling pathways might be affected positively or negatively [66].

Combined, our data suggest that rutin, a flavonoid present in food and of low cost, exerts protective effects against PolyQ-induced toxicity, and may help prevent or treat HD. Rutin can act through its antioxidant potential, induction of antioxidant defenses and chaperones by the insulin/IGF1 (IIS)/ DAF-16/FoxO signaling pathway and increase in autophagy activity, in addition to delay aging and possibly the onset of age-related diseases.

Conclusion

This study demonstrated the protective effects of rutin in *C. elegans* transgenic models of HD. The mechanisms are associated with the flavonoid antioxidant potential, modulation of the insulin/IGF1 (IIS)/ DAF-16/FOXO signaling with consequent activation of antioxidant defenses and chaperones, and increase in autophagy activity. Rutin also retarded *C. elegans* aging, which may delay the onset of age-related diseases.

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Conflict of Interest statement

The authors declare that they have no conflict of interest.

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Figure Legends

Figure 1. Effect of rutin on the survival of ASH neurons. (A) Micrographs of HA759 nematodes expressing Htt-Q150 in ASH neurons. Death of ASH neurons is assessed by loss of bilateral GFP fluorescence. (B) Nematodes of HA759 strain were treated with rutin (15-120 µM) from L1 and observed in different hours post larvae L1. Data are representative of three independent experiments with 10 worms each and are presented as mean ± SD. # and * represents significant difference from ethanol in 72 h and 120 h, respectively, by two-way ANOVA followed by Tukey's post-hoc test, $p<0.05$).

Figure 2. Effect of rutin on polyQ aggregation in muscle. (A) Fluorescence micrographs of transgenic nematodes expressing Q40::YFP in body wall muscle cells with or without rutin treatment for the indicated times. (B) Nematodes of AM141 strain were treated with rutin (15-120 µM) from L1 and observed in different hours post larvae L1. Data are representative of three independent experiments with 10 worms each and are presented as mean ± SD. # and * represents significant difference from ethanol in 72 h and 120 h, respectively, by two-way ANOVA followed by Tukey's post-hoc test, $p<0.05$.

Figure 3. Effect of rutin on *C. elegans* lifespan in (A) wild-type, (B) AM141 and (C) HA759 strains. Worms were treated with rutin (15-120 µM) from L1. Data are representative of three independent experiments with 100 worms each. * represents significant difference from ethanol (vehicle) by log-rank (Mantel-Cox) test, $p<0.001$.

Figure 4. Effect of rutin on reactive oxygen species (ROS) production in (A) wild-type (N2), (B) HA759 and (C) AM141 strains. Worms were treated with rutin (15-120 µM) from L1 and analyzed in different hours post adult stage. Aliquots of worm's lysates were incubated for 1 hour with 20 µM DCFDA (final concentration) at 20°C and fluorescence levels were

measured in duplicate in a microplate reader at 485 nm excitation and 520 nm emission wavelengths at room temperature. Data were normalized to protein content and are reported in arbitrary fluorescence units (AFU) as mean ±SD from 3 independent assays * represents significant difference from ethanol by two-way ANOVA followed by Tukey's post-hoc test, $p<0.05$).

Figure 5. Effect of rutin on DAF-16 localization. (A) Representative images of the transgenic strain TJ356. (B) Nematodes of TJ356 strain were treated with rutin (15-120 µM) from L1 and observed at young adult stage. Control worms exposed to heat shock at 37°C for 30 minutes were used as positive control. Data are representative of three independent experiments with 10 worms each and are presented as percentage of worms with nuclear localization of DAF-16 (mean ± SD). * represents significant difference from ethanol by one-way ANOVA followed by Tukey's post-hoc test, $p<0.05$).

Figure 6. Effect of rutin on autophagy. (A) Representative images of autophagosomes (GFP::LGG-1 puncta). (B) Nematodes of DA2123 strain were treated with rutin (15-120 µM) from L1 and analyzed at the young adult stage. Number of GFP::LGG-1 puncta were counted in seam cell. Data are representative of three independent experiments and are presented as mean ± SD. * represents significant difference from ethanol by one-way ANOVA followed by Tukey's post-hoc test, * $p < 0.05$.

Fig 1A

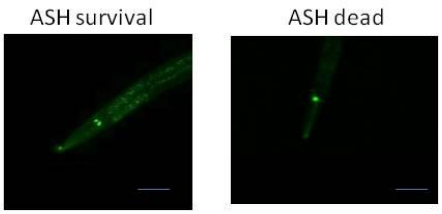

ASH survival
ASH dead

Fig 1B

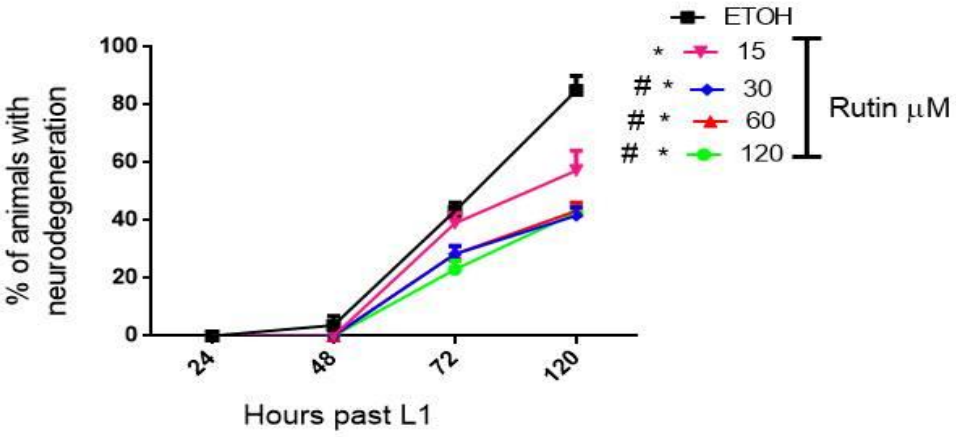

% of animals with neurodegeneration
100
80
60
40
20
0
24
48
72
120
Hours past L1
ETOH
* 15
# * 30
# * 60
# * 120
Rutin μM

Fig 2A

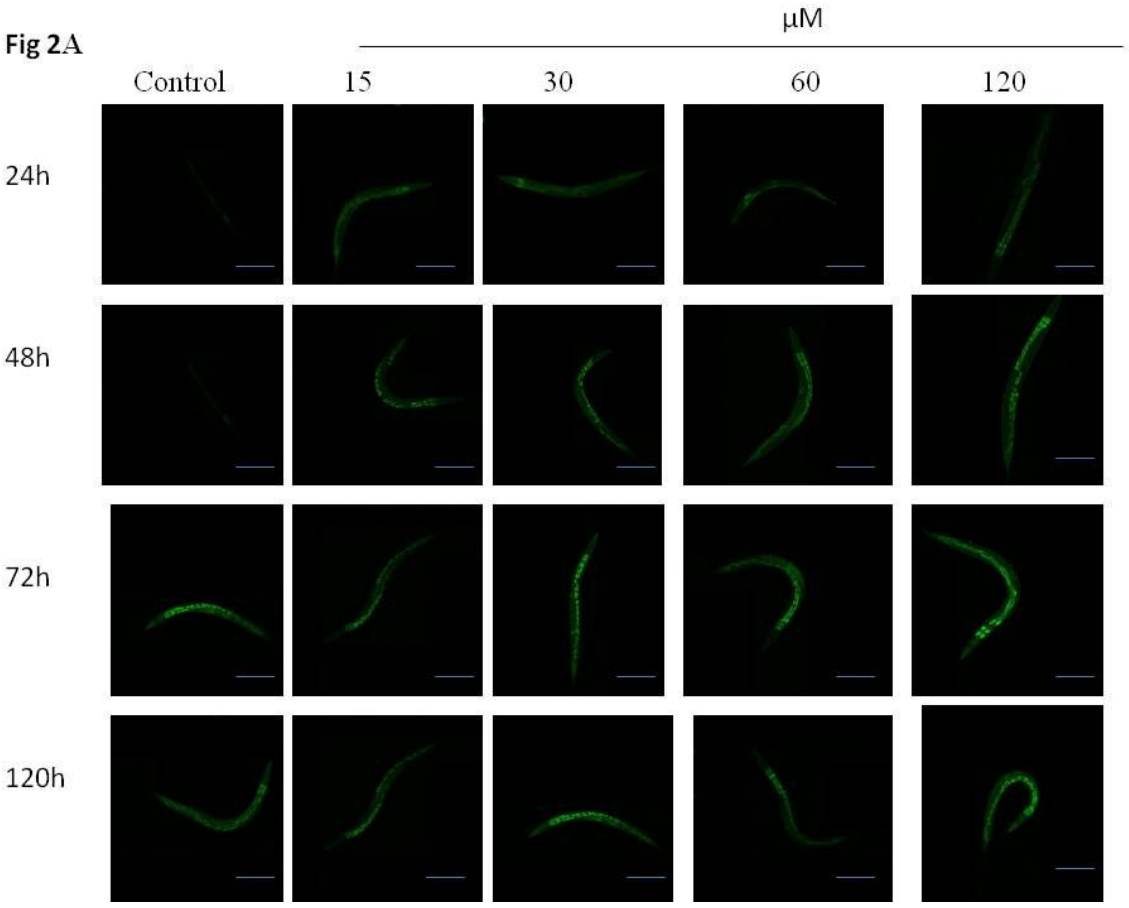

µM
Control
15
30
60
120
24h
48h
72h
120h

Fig 2B

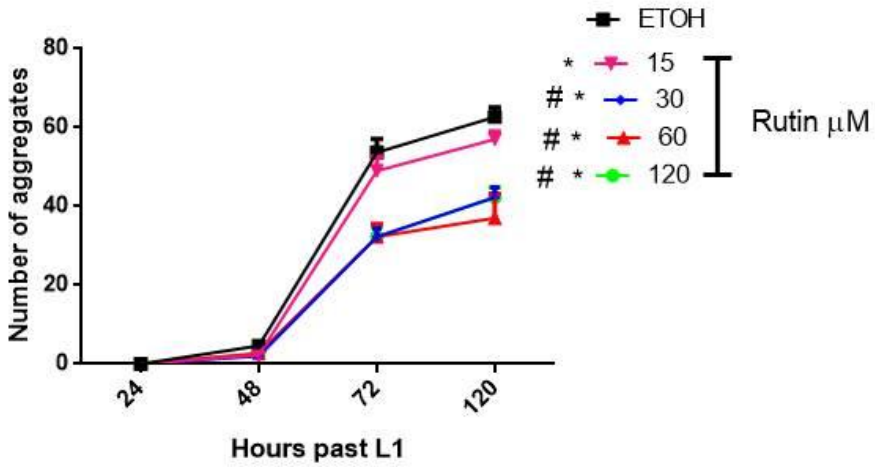

ETOH
15
30
60
120
Rutin µM
Number of aggregates
0
20
40
60
80
24
48
72
120
Hours past L1

Fig 3

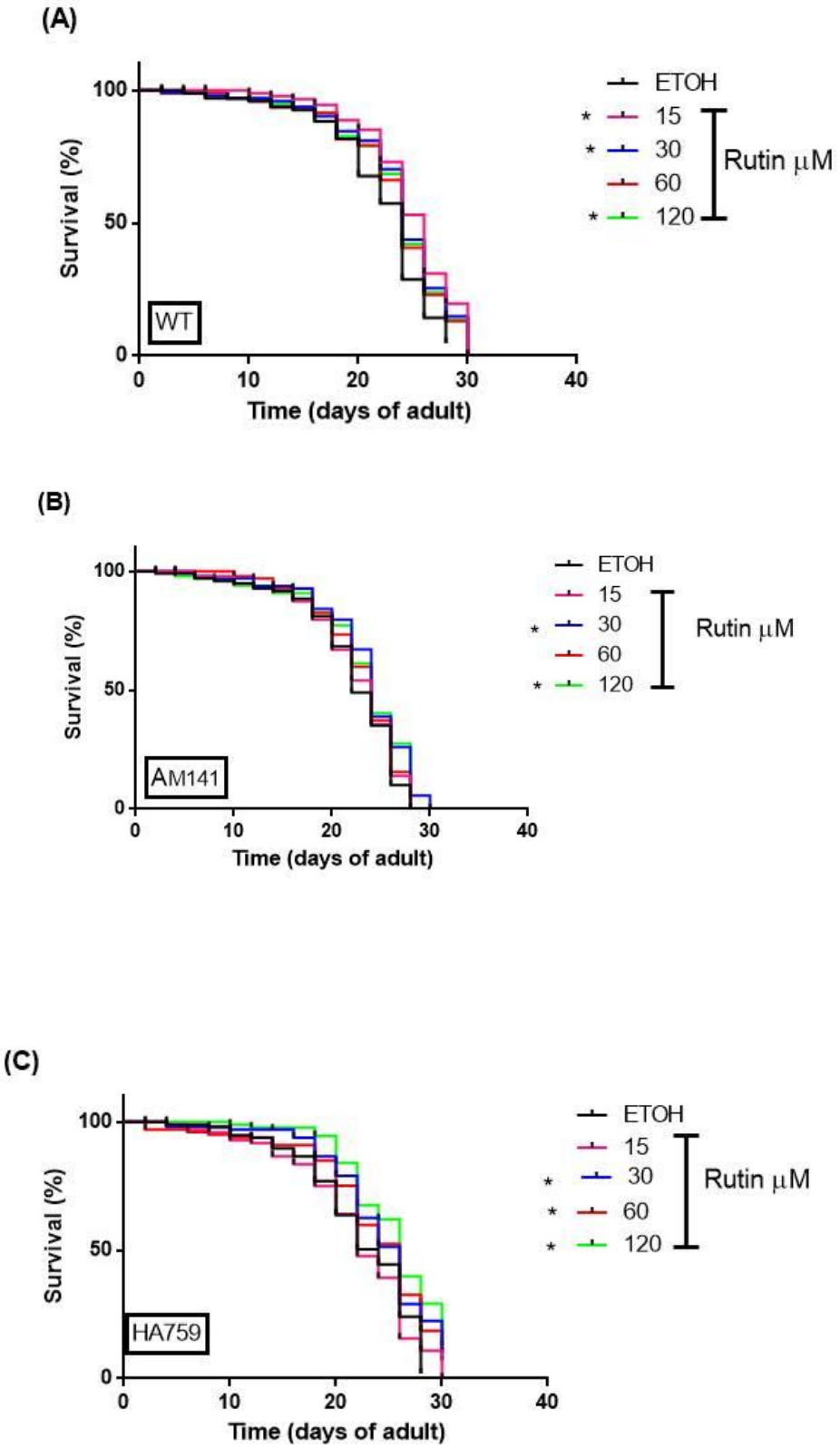

(A)
Survival (%)
100
50
0
0
10
20
30
40
Time (days of adult)
WT
ETOH
* 15
* 30
60
* 120
Rutin μM
(B)
Survival (%)
100
50
0
0
10
20
30
40
Time (days of adult)
AM141
ETOH
15
* 30
60
* 120
Rutin μM
(C)
Survival (%)
100
50
0
0
10
20
30
40
Time (days of adult)
HA759
ETOH
15
* 30
* 60
* 120
Rutin μM

Fig 4

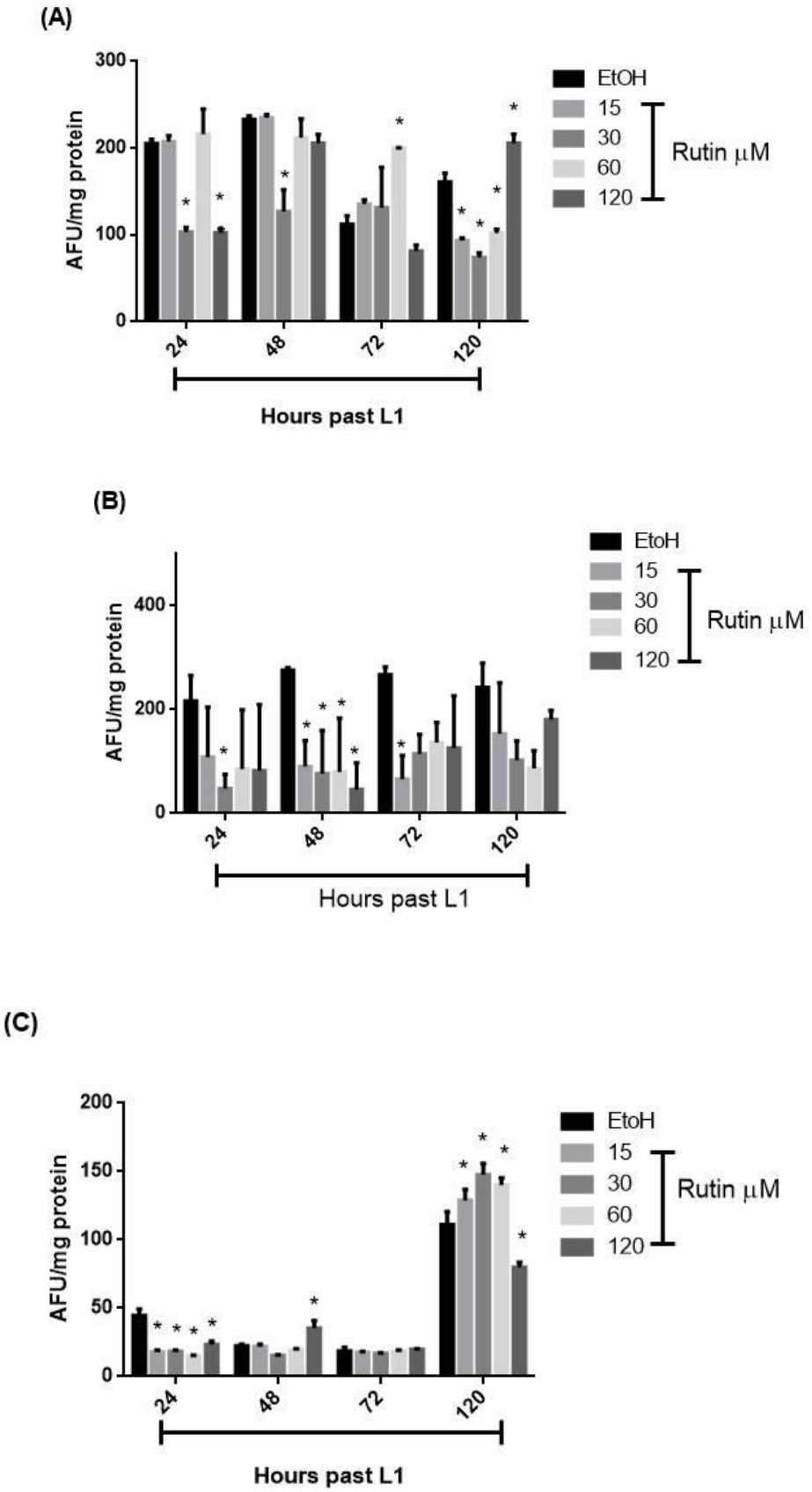

(A)
AFU/mg protein
300
200
100
0
24
48
72
120
Hours past L1
EtOH
15
30
60
120
Rutin μM
(B)
AFU/mg protein
400
200
0
24
48
72
120
Hours past L1
EtoH
15
30
60
120
Rutin μM
(C)
AFU/mg protein
200
150
100
50
0
24
48
72
120
Hours past L1
EtoH
15
30
60
120
Rutin μM

Fig 5A

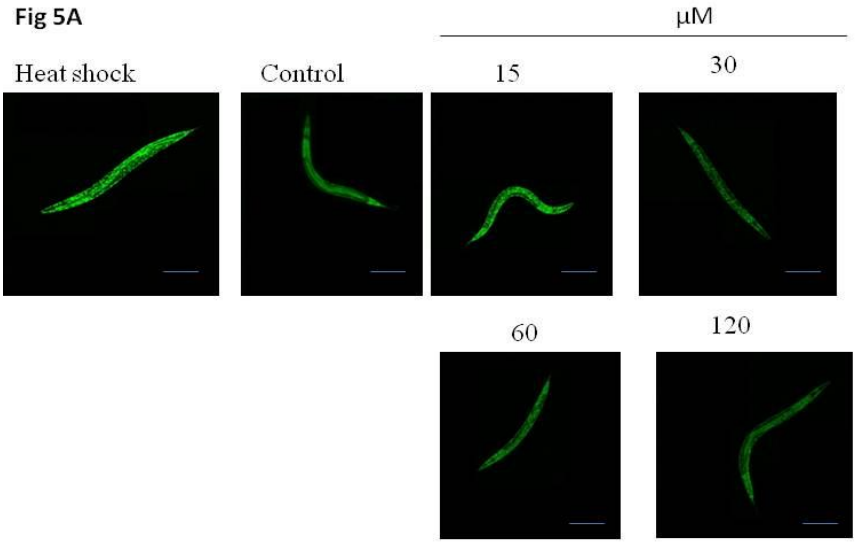

µM
Heat shock
Control
15
30
60
120

Fig 5B

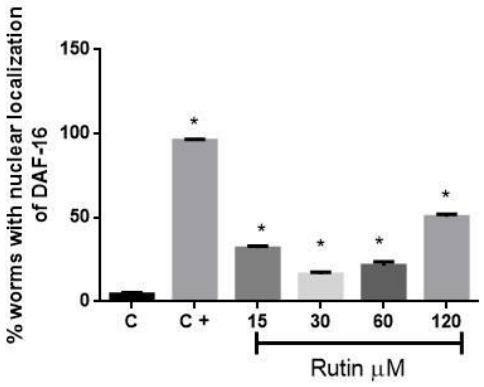

% worms with nuclear localization of DAF-16
150
100
50
0
C
C +
15
30
60
120
Rutin µM

Fig 6A

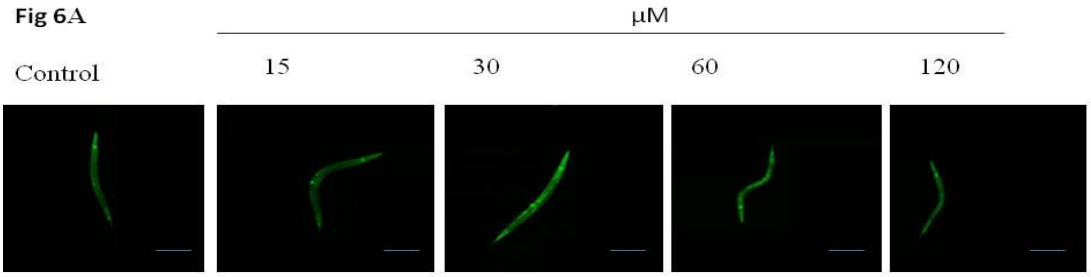
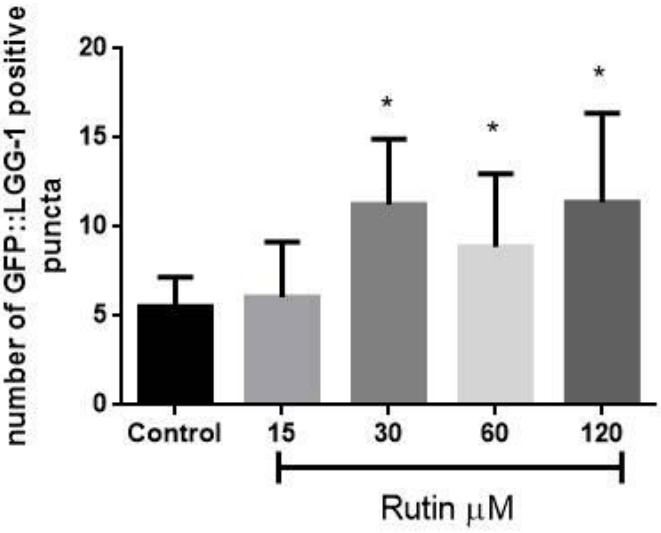

μM
Control
15
30
60
120

Fig 6B


number of GFP::LGG-1 positive puncta
20
15
10
5
0
*
*
*
Control
15
30
60
120
Rutin μM

4. CONCLUSÃO

Os resultados apresentados neste trabalho indicam o possível potencial terapêutico da rutina na prevenção da Doença de Huntington. Foi mostrado, pela primeira vez que o tratamento crônico com a rutina foi eficaz em diminuir a agregação da poliQ e a neurodegeneração no nematódeo *Caenorhabditis elegans*. Esse efeito se deve, provavelmente, ao potencial antioxidante da rutina, modulando a sinalização da insulina / IGF1 (IIS) / DAF-16/FOXO e aumentando a atividade da autofagia. Além de a rutina retardar o envelhecimento de *C elegans*, o que pode retardar o aparecimento de doenças relacionadas à idade. Mais estudos são necessários a fim de comprovar sua eficácia, bem como elucidar o mecanismo exato de atuação da rutina.

5. PERSPECTIVAS

Tendo em vista os resultados obtidos neste trabalho, as perspectivas para trabalhos posteriores em modelos de DH em *C. elegans* são:

- Elucidar outros mecanismos de ação da rutina;
- Investigar o aumento das espécies reativas do oxigênio (EROs) induzida pela rutina;
- Investigar os efeitos da rutina em modelos de DH em mamíferos.

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