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O ácido oleico inibe a ferroptose induzida por sobrecarga de ferro em *Caenorhabditis elegans* de maneira dependente de NHR-49

Florianópolis
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Tese submetida ao Programa de Pós-Graduação em Neurociências da Universidade Federal de Santa Catarina como requisito parcial para a obtenção do título de Doutora em Neurociências.

Orientador: Prof. Marcelo Farina, Dr.

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O presente trabalho em nível de Doutorado foi avaliado e aprovado, em 30 de abril de 2023, pela banca examinadora composta pelos seguintes membros:

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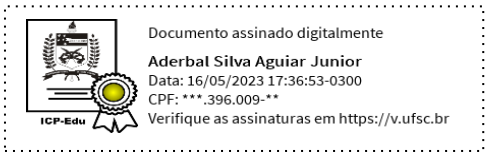
Certificamos que esta é a versão original e final do trabalho de conclusão que foi julgado adequado para obtenção do título de Doutora em Neurociências.



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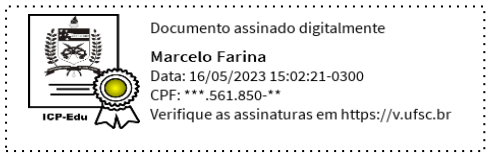

Prof. Marcelo Farina, Dr.
Orientador(a)

Florianópolis, 2023.

Gostaria de dedicar este trabalho especialmente a minha filha Maryane Mann Baptista, meu amor número um e ao seu papai, meu amor número dois. Com todo meu amor e gratidão!

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APRESENTAÇÃO

Este trabalho de tese doutoral está organizado da seguinte forma: (i) Resumo e palavras-chave em língua portuguesa de acordo com a Resolução Normativa Nº 154/2021/CUn, Art. 62, § 2º, e com o Regimento do PPG em Neurociências, Art. 63; (ii) Capítulo 1: breve Introdução em língua portuguesa sobre os tópicos de maior relevância relacionados ao projeto de tese; (iii) Capítulo 2: Artigo científico em língua inglesa contendo os itens Introdução, Materiais e Métodos, Resultados, Discussão, Conclusões e Referências Bibliográficas; (iv) Capítulo 3: Resultados adicionais relacionados aos efeitos do modelo de sobrecarga de ferro e ferroptose sobre neurodegeneração dopaminérgica.

RESUMO EXPANDIDO

Introdução

O ferro é um elemento químico essencial para a manutenção da vida, mas seu excesso causa toxicidade. Este elemento possui papel central na ferroptose, uma forma de morte celular caracterizada pela peroxidação massiva de lipídios. A sobrecarga de ferro (do inglês, *iron overload*) é uma síndrome caracterizada pelo seu excesso no organismo, a qual tem etiologias genéticas e/ou ambientais e causa problemas hepáticos, cardíacos e neuronais. De um ponto de vista clínico e terapêutico, o entendimento acerca dos mecanismos que modulam a toxicidade do ferro em indivíduos com *sobrecarga de ferro* é de extrema importância. Os radicais hidroxila ($^{\bullet}OH$) e lipoperoxila ($LOO^{\bullet}$) representam oxidantes com alta reatividade e possuem papéis centrais na citotoxicidade mediada por ferro. Entretanto, ainda não há evidências de uma relação direta entre os danos orgânicos induzidos pela sobrecarga de ferro e a ferroptose, um tipo de morte celular regulada dependente de ferro e caracterizada pela peroxidação de lipídeos. Evidências experimentais indicam que este tipo de morte celular desempenha um papel importante na progressão de condições patológicas. Interessantemente, agentes quelantes de ferro, que têm sido úteis no tratamento de indivíduos com sobrecarga de ferro, também mostraram efeitos benéficos em modelos de ferroptose. O início e a execução da ferroptose estão intimamente ligados ao metabolismo lipídico. Neste contexto, a enzima acil-coenzima A (CoA) sintetase 4 (ACSL4), que promove a incorporação de ácidos graxos poliinsaturados (PUFA) nas membranas, é necessária para a morte celular ferroptótica em vários contextos. Por outro lado, a enzima ACSL3 promove a incorporação de ácidos graxos monoinsaturados (MUFA) nas membranas, prevenindo a ferroptose. Ainda, a isoforma alfa (α) dos receptores ativados por proliferador de peroxissoma, conhecidos como PPAR (do inglês *peroxisome proliferator-activated receptor* - PPAR-α) representa um elemento-chave mediando a regulação positiva de genes/proteínas envolvidos na beta-oxidação de ácidos graxos, sugerindo que os PPARs podem estar envolvidos na regulação da ferrotose. Esta hipótese é baseada no fato de que o estímulo da lipólise tem sido reportado como um evento que inibe a ferroptose. De forma interessante, o ácido oleico, um MUFA, inibe a ferroptose (ao menos em condições *in vitro* em cultivos celulares) por impedir a incorporação de PUFAs. Estas evidências acima-mencionadas reforçam a ideia de que o metabolismo lipídico tem grande papel na regulação da ferroptose. Há evidências de que o envelhecimento

(mesmo que saudável) desencadeia o acúmulo de ferro em várias regiões cerebrais e tipos de células. Interessantemente, evidências crescentes apontam para o envolvimento da deposição de ferro relacionada a muitas doenças neurodegenerativas; a ferroptose pode ser um novo mecanismo subjacente à neurodegeneração. De particular importância para este projeto, a perda progressiva de neurônios dopaminérgicos parece ser particularmente afetada pelo ferro.

Objetivos

Nosso objetivo geral foi investigar a ocorrência de ferroptose em *C. elegans* submetidos à sobrecarga de ferro, buscando (i) identificar a contribuição deste tipo de morte celular para o dano induzido pela sobrecarga de ferro e (ii) identificar estratégias protetoras para mitigar tal dano com foco em ácidos graxos com propriedades antiferroptóticas, (iii) investigar alterações no sistema dopaminérgico em *C. elegans* expostos a sobrecarga de ferro e os possíveis efeitos neuroprotetores do ácido oleico. Com base nas evidências acima-mencionadas, sugerindo uma possível relação entre a sobrecarga de ferro e o desenvolvimento de ferroptose, a neurodegeneração como uma consequência da ferroptose e o potencial efeito anti-ferroptótico do MUFA ácido oleico, este trabalho de tese teve como objetivos específicos desenvolver um modelo *in vivo* de sobrecarga de ferro baseado no organismo modelo *C. elegans* e caracterizado pela exposição ao ferro exógeno (exposição ao citrato de amônio férrico - FAC) a fim de examinar a possível ocorrência de ferroptose. Ainda se objetivou explorar o impacto do ácido oleico na ferroptose induzida por sobrecarga de ferro *C. elegans* e caracterizar os mecanismos mediadores de tais efeitos. A neurodegeneração tem sido associada ao acúmulo de ferro no encéfalo e, mais recentemente, à ferroptose. Assim, nosso último objetivo foi estudar o sistema dopaminérgico no modelo de sobrecarga de ferro, investigando o possível papel protetor do ácido oleico neste modelo.

Metodologia

Investigou-se se a própria sobrecarga de ferro seria capaz de induzir ferroptose em um modelo *in vivo* usando o nematoide *C. elegans*. Neste estudo, foram examinadas diferentes concentrações de FAC na sobrevivência do nematoide. Isso foi realizado em um curto prazo (4 dias). Após a realização de curvas de sobrevivências e definidas

as condições tempo e concentração, buscou-se avaliar os fenótipos relacionados à ferroptose. Para isso, foi analisado os níveis de GSH e a peroxidação lipídica. Os níveis de GSH totais foram mensurados através do método enzimático baseado na reação de grupos tióis com o reagente de Ellman (DTNB). Para a peroxidação lipídica, os animais foram marcados com o fluoróforo marcador de peroxidação lipídica BODIPY 581/591 C11 e a fluorescência foi analisada através da microscopia confocal. Para confirmar se o modelo estava relacionado com a ferroptose, fez-se o co-tratamento com ferro e o antioxidante lipofílico, ferrostatina-1 (inibidor clássico de ferropose). Para investigar se a suplementação com ácidos graxos modula a ferroptose, avaliou-se o efeito do co-tratamento com ferro e diferentes ácidos graxos (oleico, esteárico e linoleico). Foi demostrado recentemente que NHR-49, o qual é um homólogo funcional do PPAR-α de mamíferos, regula positivamente a expressão de genes envolvidos na β-oxidação de ácidos graxos, bem como na dessaturação e alongamento lipídicos para preservar a homeostase lipídica. Mas, se o NHR-49 está envolvido no efeito antiferroptótico do ácido oleico em *C. elegans* submetidos a sobrecarga de ferro é desconhecido. Em busca de mecanismos moleculares comuns mediando os efeitos do ácido oleico, nosso próximo passo foi testar o impacto da inativação do NHR-49 na sobrevivência dos nematoides, após a sobrecarga de ferro e o co-tratamento com ácido oleico, para isso usamos a cepa VC870 nhr-49(gk405). Para abordar ainda mais o papel da ferroptose na sobrecarga de ferro, avaliamos o efeito do FAC e a exposição ao ácido oleico sobre o sistema dopaminérgico, por meio da cepa BY250 (vtIs7 [P dat-1::GFP]), que expressam a proteína fluorescente verde (GFP) fusionada com o transportador de dopamina (DAT), um marcador de neurônios dopaminérgicos. Tal análise foi realizada em 72h e 96h após a exposição ao FAC, através da técnica de microscopia confocal.

Resultados e Discussão

O ferro pode contribuir para o *pool* de espécies reativas de oxigênio na célula, por exemplo, ele catalisa a decomposição de H_2O_2 para produzir radicais hidroxila. Posteriormente, esses radicais livres podem provocar danos celulares, peroxidação lipídica e eventualmente morte celular. A ferroptose é um tipo de morte celular regulada dependente de ferro que envolve acúmulo de ferro e peroxidação lipídica. Portanto, para investigar se a sobrecarga de ferro causa ferroptose, *C. elegans* foram tratados com FAC, e a ferroptose foi avaliada medindo os níveis de GSH, a

peroxidação lipídica e medidas dos efeitos da ferrostatina-1. Consistente com a ferroptose induzida por sobrecarga de ferro, descobrimos que a exposição aguda ao FAC causou mortalidade nos nematoides, que foi precedida por peroxidação lipídica e depleção de GSH. Esses eventos foram prevenidos por agentes antiferroptóticos (ferrostatina-1 e BHT). Assim, nossos resultados iniciais indicam que a sobrecarga de ferro aumentou a mortalidade de *C. elegans* em um mecanismo dependente de ferroptose. Descobertas recentes demonstraram que os MUFAs podem efetivamente inibir a ferroptose, substituindo os PUFAs nas membranas lipídicas e reduzindo o acúmulo de produtos da peroxidação lipídica. Nossos ensaios revelaram que a adição de ácido oleico, protegeu contra danos mediados pela sobrecarga de ferro/ferroptose. Esses resultados, uma vez extrapolados para condições mais complexas, apontam para a possibilidade do uso de estratégias terapêuticas/nutricionais baseadas no ácido oleico para inibir a ferroptose. Porém, o efeito protetor do ácido oleico contra a mortalidade induzida por sobrecarga de ferro, observado em vermes do tipo selvagem, foi atenuado em vermes sem NHR-49. Esses resultados sugerem que o NHR-49 em *C. elegans* pode estar envolvido nos efeitos protetores do ácido oleico contra a ferroptose. Além disso, investigou-se a relação da ferroptose na neurodegeneração dopaminérgica induzida por sobrecarga de ferro em *C. elegans*. Observou-se que a exposição ao FAC foi capaz de causar neurodegeneração de neurônios dopaminérgicos e o ácido oleico ou a ferrostatina-1 protegeram contra tal efeito. Estes resultados indicam que a ferroptose decorrente da sobrecarga de ferro causa neurodegeneração dopaminérgica em *C. elegans* e o ácido oleico protege devido a ação antiferroptótica.

Palavras-chave: Ferroptose; *Iron overload*; Ácido oleico*;* NHR-49; Neurodegeneração Dopaminérgica.

ABSTRACT

Introduction

Iron is an essential element for life, but its excess causes toxicity. This element plays a central role in ferroptosis, a form of cell death characterized by massive lipid peroxidation. Iron overload is a syndrome characterized by iron excess in the body, which has genetic and/or environmental etiologies and causes liver, heart and neuronal damage. From clinical and therapeutic points of view, understanding the mechanisms that modulate iron toxicity in individuals with iron overload is extremely important. Hydroxyl (•OH) and lipoperoxyl (LOO•) radicals represent highly reactive oxidants for biomolecules and play central roles in iron-mediated cytotoxicity. However, there is still no evidence of a direct relationship between organ damage induced by iron overload and ferroptosis, a type of iron-dependent regulated cell death characterized by lipid peroxidation. Experimental evidence indicates that this type of cell death plays an important role in the progression of pathological conditions. Interestingly, iron chelating agents, which have been useful in treating individuals with iron overload, have also shown beneficial effects in models of ferroptosis. The onset and execution of ferroptosis are closely linked to lipid metabolism. In this regard, the enzyme acyl-coenzyme A (CoA) synthetase 4 (ACSL4), which promotes the incorporation of polyunsaturated fatty acids (PUFA) into membranes, is required for ferroptotic cell death in several contexts. In contrast, the ACSL3 enzyme promotes the incorporation of monounsaturated fatty acids (MUFA) into membranes, preventing ferroptosis. Furthermore, peroxisome proliferator activated receptor alpha (α) isoform, known as PPAR (PPAR-α) represents a key element mediating the upregulation of genes/proteins involved in beta-oxidation of fatty acids, suggesting that PPARs may be involved in the regulation of ferrotosis. This hypothesis is based on the fact that stimulation of lipolysis has been reported as an event that inhibits ferroptosis. Interestingly, oleic acid, a MUFA, inhibits ferroptosis (at least under *in vitro* conditions in cell cultures) by preventing the uptake of PUFAs. These aforementioned evidences reinforce the idea that lipid metabolism plays a major role in the regulation of ferroptosis. There is evidence that aging (even healthy aging) triggers an accumulation of iron in many organisms, including some regions of humans. Interestingly, increasing evidence points to the involvement of iron deposition in the mechanisms underlying many neurodegenerative diseases; ferroptosis may be a new mechanism underlying

neurodegeneration. Of particular importance to this project, the progressive loss of dopaminergic neurons appears to be particularly affected by iron.

Objectives

Our general objective was to investigate the occurrence of ferroptosis in C. elegans subjected to iron overload, seeking (i) to identify the contribution of this type of cell death to the damage induced by iron overload and (ii) to identify protective strategies to mitigate such damage. focusing on fatty acids with antiferroptotic properties, (iii) investigate changes in the dopaminergic system in C. elegans exposed to iron overload and the possible neuroprotective effects of oleic acid. Based on the above-mentioned evidence suggesting a possible relationship between iron overload and the development of ferroptosis, neurodegeneration as a consequence of ferroptosis and the potential anti-ferroptotic effect of the MUFA oleic acid, this thesis work aimed to develop an *in vivo* model of iron overload based on the model organism *Caenorhabditis elegans* (*C. elegans*) and characterized by exposure to exogenous iron (FAC) in order to examine the possible occurrence of ferroptosis. An additional objective was to explore the impact of oleic acid on ferroptosis induced by *C. elegans* iron overload and to characterize the mediating mechanisms of such effects. Finally, we aimed to study dopaminergic neurodegeneration in the iron overload model, investigating the possible protective role of oleic acid in this model.

Methodology

Initially, it was investigated whether iron overload itself (exposure to ferric ammonium citrate - FAC) would be able to induce ferroptosis in an *in vivo* model using the nematode *C. elegans*. In this study, the toxicities of different concentrations of FAC on the nematode were examined. This was performed on a short-term profile (4 days). After carrying out survival curves and defining the time and concentration conditions, we sought to evaluate the phenotypes related to ferroptosis. For this, GSH depletion and lipid peroxidation were analyzed. Total GSH levels were measured using the enzymatic method based on the reaction of thiol groups with Ellman's reagent (DTNB). For lipid peroxidation, the animals were marked with the lipid peroxidation marker fluorophore BODIPY 581/591 C11 and fluorescence was analyzed by confocal microscopy. To confirm whether the model was related to ferroptosis, co-treatment with iron and the lipophilic antioxidant, ferrostatin-1 (classic ferroposis inhibitor) was

performed. To investigate whether supplementation with fatty acids modulates ferroptosis, the effect of co-treatment with iron and different fatty acids (oleic, stearic and linoleic) was evaluated. Nematode survival was evaluated to also test the effects of oleic acid against iron overload mediated damage in knockout worms for NHR-49, which is a functional homolog of mammalian PPAR-α. The effect of iron overload and oleic acid exposure on dopaminergic neurodegeneration was evaluated in BY250 Pdat-1::GFP worms, which express green fluorescent protein (GFP) fused with the dopamine transporter (DAT), a marker of dopaminergic neurons. This analysis was performed at 72h and 96h after exposure to FAC, using the confocal microscopy technique.

Results and discussion

Iron can contribute to the pool of reactive oxygen species in the cell, for example, it catalyses the breakdown of H2O2 to produce hydroxyl radicals. Subsequently, these free radicals can cause cell damage, lipid peroxidation and eventually cell death. Ferroptosis is a type of iron-dependent regulated cell death that involves iron accumulation and lipid peroxidation. Therefore, to investigate whether iron overload causes ferroptosis, C. elegans were treated with FAC, and ferroptosis was assessed by measuring GSH levels, lipid peroxidation, and measures of the effects of ferrostatin-1. Consistent with iron overload-induced ferroptosis, we found that acute exposure to FAC caused nematode mortality, which was preceded by lipid peroxidation and GSH depletion. These events were prevented by antiferroptotic agents (ferrostatin-1 and BHT).Thus, our initial results indicate that iron overload increased *C. elegans* mortality in a ferroptosis-dependent mechanism. We also observed that oleic acid protected against damage mediated by iron overload/ferroptosis. These results, once extrapolated to more complex conditions, point to the possibility of using therapeutic/nutritional strategies based on oleic acid to inhibit ferroptosis. However, the protective effect of oleic acid against iron overload-induced mortality, observed in wild-type worms, was attenuated in worms lacking the NHR-49 nuclear receptor. These results suggest that NHR-49 in *C. elegans* may be involved in the protective effects of oleic acid against ferroptosis. Furthermore, the relationship between ferroptosis and dopaminergic neurodegeneration induced by iron overload in *C. elegans* was investigated. It was observed that exposure to FAC was able to cause neurodegeneration of dopaminergic neurons and oleic acid or ferrostatin-1 protected

against this effect. These results indicate that ferroptosis due to iron overload causes dopaminergic neurodegeneration in *C. elegans* and oleic acid protects due to its antiferroptotic action.

Keywords: Ferroptosis; Iron overload; Oleic acid; NHR-49; Dopaminergic neurodegeneration.

LISTA DE FIGURAS

ABREVIATURAS E SIGLAS

ACSL3 - do inglês A*cyl-CoA synthetase long chain family member 3*

ACSL4 - do inglês acyl-CoA synthetase long chain family member 4

ADE - Neurônios deirídios anteriores

BHT - Hidroxitolueno butilado (do inglês *butylated hydroxytoluene*)

DA - Dopamina

DAT - Transportador de dopamina

DNA - Ácido desoxirribonucléico

DP - Doença de Parkinson

DTNB – Ácido 5, 5-ditio-bis-2-nitrobenzóico

EROs - Espécies reativas de oxigênio

FAC - Citrato de amônio férrico (do inglês ferric ammonium citrate)

Fe^{2}+ - Ferro ferroso

Fe^{3}+ - Ferro férrico

Fer-1 - Ferrostatina -1

FPN - Ferroportina

GFP - Proteína fluorescente verde (do inglês, Green fluorescent protein)

GSH - Glutationa Reduzida

GSSG - Glutationa Oxidada

HFE - Proteína da hemocromatose

HH - Hemocromatose Hereditária

HJV - Hemojuvelina

HNE - 4-hidroxi-2- nonenal

IREs - Elementos reguladores de ferro

IRPs - Proteínas reguladoras de ferro

LIP - Pool de ferro quelatável e redox-ativo, do inglês labile iron pool

MDA – Malondialdeído

MUFAs - Ácidos graxos monoinsaturados

PDE - Neurônios pós-deirídios

PPAR - Receptor ativado por proliferador de peroxissoma

PPAR-alfa - Receptor α ativado por proliferador de peroxissoma

PUFAs Ácidos graxos poliinsaturados

SCD1 - Esteroil CoA dessaturase 1

TfR2 - Receptor de transferrina 2

SUMÁRIO

CAPITULO I

Introdução em língua portuguesa sobre os tópicos de maior relevância relacionados ao projeto de tese

INTRODUÇÃO

1.1 O ferro: essencialidade e toxicidade

O ferro é um metal de transição indispensável para manter a homeostase celular e a saúde humana em geral. Sua capacidade de formar complexos com moléculas orgânicas tem imensas implicações biológicas porque o ferro pode interconverter-se entre suas formas oxidativas mais comuns, ferrosa Fe^{2+} e férrica Fe^{3+} (PAPANIKOLAOU; PANTOPOULOS, 2005). Como componente de várias metaloproteínas, está envolvido em processos bioquímicos essenciais, incluindo proliferação celular (LE; RICHARDSON, 2002), resposta imune (MARTINS et al., 2017; NAIRZ et al., 2018), respiração mitocondrial (VOLANI et al., 2017) e síntese de DNA (LEDERMAN; COHEN; LEE, 1984). A capacidade, do ferro, de aceitar e doar elétrons é imprescindível para esses processos, mas em condições fisiológicas, o Fe^{2+} é altamente reativo e pode catalisar a conversão de peróxido de hidrogênio em espécies como o radical hidroxila (GRENIER; HUOT; MAYRAND, 2000; PAPANIKOLAOU; PANTOPOULOS, 2005). Portanto, a biodisponibilidade do ferro é geralmente limitada pelas células e tecidos, de forma suficiente para suprir as demandas celulares e ao mesmo tempo, evitar excessos desse metal(GALARIS; BARBOUTI; PANTOPOULOS, 2019).

A deficiência de ferro resulta em eritropoiese ineficaz e, consequentemente, anemia (GANZ; NEMETH, 2009). No entanto, o excesso de ferro é tóxico, levando a danos celulares geralmente via formação de radicais livres e consequente oxidação de DNA, proteínas e lipídios (BRITTON; BACON; RECKNAGEL, 1987; WINTERBOURN, 1995). Portanto, a absorção, transporte, uso e armazenamento de ferro são fortemente coordenados por múltiplas proteínas e vias para manter o pool de ferro lábil celular (LIP; um pool de ferro quelatável e redox-ativo) em concentrações não citotóxicas e, ao mesmo tempo, possibilitar a manutenção das funções essenciais (NEMETH; GANZ, 2021).

A quantidade corporal total de ferro, em humanos adultos, é de aproximadamente 3 a 5 g de ferro; a maior parte desse total, cerca de 70%, é utilizada em eritropoiese (do grego '*eritro*' que significa "vermelho" e '*poiesis*' "fazer"- processo que produz glóbulos vermelhos (eritrócitos) (GKOUVATSOS; PAPANIKOLAOU; PANTOPOULOS, 2012), enquanto o restante é armazenado nos hepatócitos (CUMMING, 1999; WANG; PANTOPOULOS, 2011). Sob condições fisiológicas,

estima-se que a absorção de ferro dietético pelos enterócitos duodenais contribui com cerca de 1 a 2 mg/dia (GALARIS; BARBOUTI; PANTOPOULOS, 2019). Estudos recentes mostraram que, em células de mamíferos, o ferro é distribuído para mitocôndrias (aproximadamente 16 μM), citosol (aproximadamente 6 μM), núcleos (aproximadamente 7 μM) e lisossomos (aproximadamente 16 μM) (PETRAT; DE GROOT; RAUEN, 2001; RAUEN et al., 2007). A distribuição de ferro, no tecido, é feita pela transferrina, uma glicoproteína composta por 679 aminoácidos, que liga dois íons de Fe^{3+} mantendo-os em um estado redox-inerte (RAUCH, 2000; WANG; PANTOPOULOS, 2011). Dentro das células, o ferro é armazenado na ferritina, uma proteína citosólica composta por um núcleo de ferro de 6 a 8 nm aproximadamente, que permite o armazenamento de aproximadamente até 4500 íons de ferro (BLISSETT et al., 2018).

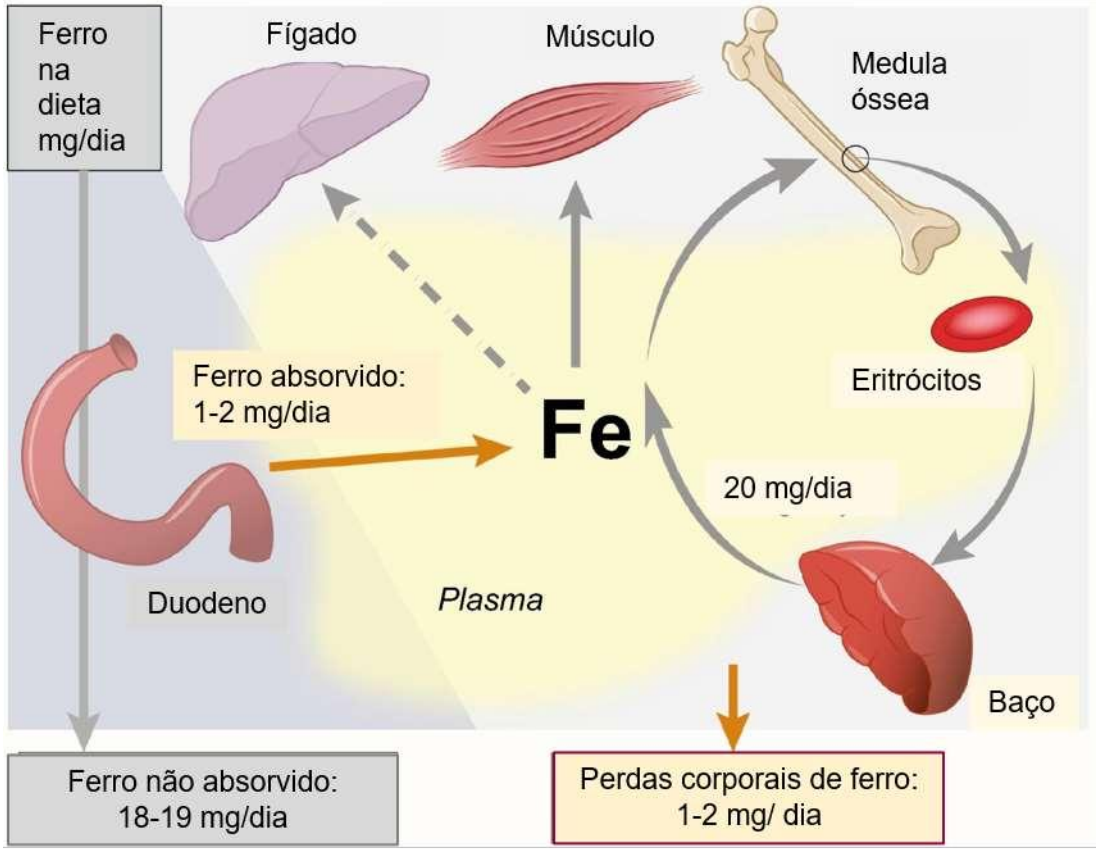

Figura 1.: Homeostase do ferro. O ferro plasmático provém da absorção duodenal e do baço. Adaptado de Brissot & Loréal, (2016).

Apesar dos seres vivos terem desenvolvido um conjunto complexo de estratégias moleculares e fisiológicas para manter o ferro em níveis homeostáticos, evitando deficiência e toxicidade (ANDERSON; LEIBOLD, 2014a; ANDREWS; SCHMIDT, 2007; WALLACE, 2016), os níveis de ferro aumentam com a idade

(JENKINS et al., 2020; KLANG et al., 2014b; WARD et al., 2014a). Essas observações apontam para uma perda da capacidade de manter a homeostase do ferro relacionada à idade, estão de acordo com o fato de que o nível de ferro desregulado desempenha um papel importante em um grande número de condições associadas ao envelhecimento (JAMES et al., 2015; MANGAN, 2021; WAWER; JENNINGS; FAIRWEATHER-TAIT, 2018). De particular importância, o excesso de ferro tem sido associado a várias doenças do sistema nervoso central, como Parkinson (BELAIDI; BUSH, 2016a), Alzheimer (ZHANG; KONG; CHAI, 2018), acidente vascular cerebral (DIETRICH; W G BRADLEY, 1988), Huntington (DONLEY et al., 2021) e esclerose lateral amiotrófica (BUIJS et al., 2017; KWAN et al., 2012).

1.2 Regulação do ferro: peroxidação lipídica impulsionada pelo ferro

A disponibilidade do ferro é regulada nos níveis sistêmico e celular (BRISSOT; LORÉAL, 2016; WILKINSON; PANTOPOULOS, 2014). O sistema hepcidina/ferroportina (FPN) regula a homeostase do ferro no nível sistêmico (SEBASTIANI; WILKINSON; PANTOPOULOS, 2016). A nível celular, a regulação do metabolismo e homeostase intracelular do ferro depende principalmente da interação de proteínas reguladoras de ferro com elementos reguladores de ferro (IRPs/IRES) (ROUAULT; KLAUSNER, 1996).

Quando suas concentrações citoplasmáticas aumentam, o ferro geralmente é armazenado no interior da ferritina, da qual pode ser liberado e suprir possíveis demandas intracelulares (AROSIO; INGRASSIA; CAVADINI, 2009). As ferritinas interagem prontamente com o Fe^{2+}, induzindo sua oxidação e deposição, em uma reação que é catalisada por um centro ferroxidase, determinando assim, o tamanho do LIP (AROSIO; INGRASSIA; CAVADINI, 2009). O LIP está presente no citoplasma, na matriz mitocondrial e nos lisossomos, correspondendo a uma parte muito pequena do ferro celular total, menos de 5% (50-100 μM) (EPSZTEJN et al., 1999; KAKHLON; CABANTCHIK, 2002). Embora corresponda a uma fração pequena do ferro total, em casos extremos de sobrecarga de ferro, o excesso nos níveis de ferro LIP pode resultar em uma formação descontrolada de espécies reativas de oxigênio (ROS), através da reação de Fenton e Haber-Weiss (NAKAMURA; NAGURO; ICHIJO, 2019a). A capacidade de sofrer oxidação e redução cíclica, torna o ferro um elemento chave na reação de Fenton e Haber-Weiss, isso porque, ao assumir estado Fe^{2+} ou Fe^{3+}, ele pode doar ou receber um elétron. Essas reações catalíticas do ferro podem

produzir grandes quantidades de radicais hidroxila ($HO^{\bullet}$), que são altamente reativos com moléculas biológicas (NAKAMURA; NAGURO; ICHIJO, 2019a). O radical hidroxila, reconhecido como o principal produto da reação de Fenton, possui curta duração e é extremamente reativo, podendo oxidar diversos grupos químicos que estejam nas proximidades (GALARIS; BARBOUTI; PANTOPOULOS, 2019).

Os lipídios contendo ligação dupla carbono-carbono (conhecida como insaturação), especialmente ácidos graxos poliinsaturados (PUFAs), são os alvos primários dos $HO^{\bullet}$, dando início à oxidação em cadeia de fosfolipídios poli-insaturados (Figura 2) (YIN; XU; PORTER, 2011). Os PUFAs são particularmente propensos a sofrer os danos oxidativos já que as ligações C-H nas posições bisalílicas são mais fracas nas moléculas e os átomos de hidrogênio, nessas posições, são vulneráveis à abstração por um radical peroxila (YANG et al., 2016). A reação envolve a abstração do hidrogênio de um carbono, com inserção de oxigênio, resultando em radical peroxil lipídicos e hidroperóxidos (AYALA; MUÑOZ; ARGÜELLES, 2014). A peroxidação lipídica produz uma grande variedade de produtos de oxidação, os hidroperóxidos lipídicos são os produtos primários, que podem se tornar aldeídos citotóxicos, como malondialdeído (MDA) ou 4-hidroxi-2- nonenal (HNE), formados como produtos secundários (AYALA; MUÑOZ; ARGÜELLES, 2014; GASCHLER; STOCKWELL, 2017). Esses produtos finais da peroxidação lipídica são danosos para as células: o MDA sendo o produto mais mutagênico, enquanto o 4-HNE, um indutor apoptótico especial, é o mais tóxico (DALLEAU et al., 2013; ESTERBAUER; ECKL; ORTNER, 1990). Além disso, a peroxidação lipídica pode ocorrer enzimaticamente, por enzimas como lipoxigenase (LOX), ciclooxigenase (COX) e citocromo P450s (CYPs), ou pode ocorrer não enzimaticamente, por peroxidação induzida por radicais livres, autoxidação e fotodegradação (MORTENSEN; RUIZ; WATTS, 2023)

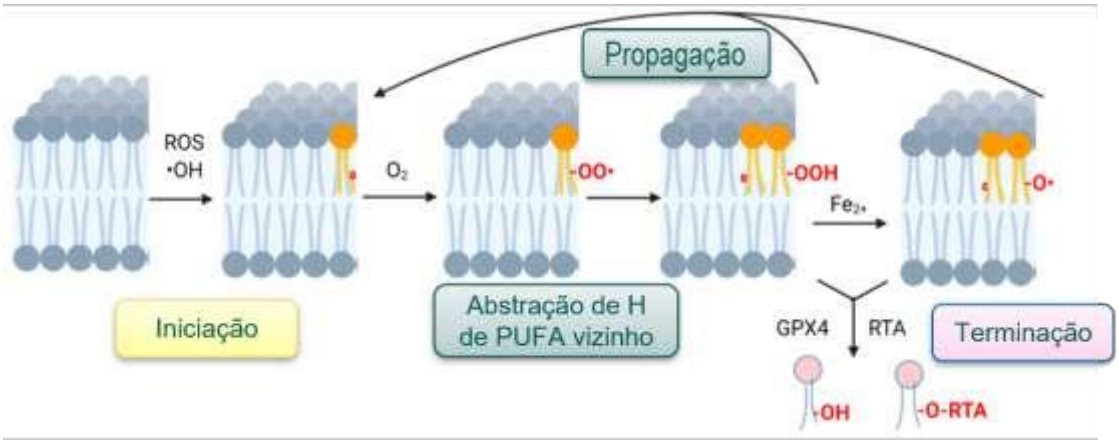

Figura 2.: A peroxidação lipídica é impulsionada pelo ferro: Os ácidos graxos poliinsaturados (PUFAs) são os alvos primários do radical hidroxila (HO·), dando início à oxidação em cadeia de fosfolipídios poli-insaturados. A peroxidação lipídica é terminada por ações de antioxidantes que capturam radicais como a Fer-1 ou por redução catalisada pela atividade da glutationa peroxidase. Adaptado de Mortensen, M. S., Ruiz, J., & Watts, J. L. (2023).

1.3 Sobrecarga de ferro (*iron overload*)

Os distúrbios de sobrecarga de ferro (do inglês, *iron overload*) representam uma variedade de desordens de causa primária (hereditária) ou secundária (congênita ou adquirida), e são reconhecidos como doenças humanas comumente caracterizadas pelo excesso de ferro no organismo (FLEMING; PONKA, 2012; HSU et al., 2022). A sobrecarga de ferro primária representa condições de distúrbios genéticos humanos relacionadas às proteínas envolvidas na homeostase do ferro, como a proteína da hemocromatose (HFE), hemojuvelina (HJV), receptor de transferrina 2 (TfR2), ferroportina-1 e hepcidina e levam ao desenvolvimento de diferentes tipos de hemocromatose (EID; ARAB; GREENWOOD, 2017a; GAO et al., 2009; SWINKELS et al., 2006). A hemocromatose hereditária (HH) se manifesta como a alta absorção inapropriada de ferro da dieta (SIDDIQUE; KOWDLEY, 2012). Os paciente com HH, se não tratados, podem desenvolver cirrose hepática e carcinoma hepatocelular, cardiomiopatia e arritmias, diabetes, artrite e hipogonadismo (AJIOKA; KUSHNER, 2002; KALTWASSER et al., 1998). A sobrecarga de ferro secundária é adquirida como resultado de outra doença, frequentemente associada à terapia transfusional crônica em pacientes com distúrbios hematológicos (GATTERMANN, 2009a). Pacientes com beta-talassemia têm anemia hemolítica crônica e requerem transfusões regulares de sangue, principalmente para a forma grave da doença, a talassemia *major*. Isso resulta em sobrecarga de ferro, pois uma única unidade de concentrado de hemácias,

derivada de 420 mL de sangue total, contém cerca de 200 mg de ferro (BRUZZESE et al., 2023). Em pacientes com talassemia *major*, os esquemas de transfusão comumente empregados liberam uma quantidade total de 100–200 mL/kg de eritrócitos/ano, levando a uma carga anual de ferro de cerca de 116– 232 mg/kg (BRUZZESE et al., 2023). Além disso, nesses pacientes, a regulação negativa da hepcidina também podem estar envolvidos na patogênese da sobrecarga de ferro (SIDDIQUE; KOWDLEY, 2012). Como o excesso de ferro não pode ser excretado ativamente, o uso crônico de transfusões regulares causa uma sobrecarga de ferro, que pode causar danos aos órgãos (GATTERMANN, 2009a; SHANDER; CAPPELLINI; GOODNOUGH, 2009; SIAH et al., 2006).

Outra condição que pode promover *iron overload* é a intoxicação de pessoas saudáveis pela ingestão de uma overdose de suplementos à base de ferro (EID; ARAB; GREENWOOD, 2017b). A toxicidade aguda, decorrente da ingestão acidental de suplementos de ferro foram relatadas principalmente em crianças, no entanto, também foram relatadas a ingestão intencional em adultos em tentativas de suicídio (HALIL et al., 2019; MADIWALE; LIEBELT, 2006). A intoxicação por ferro é grave e pode resultar em morbidades ou na morte por envenenamento (TILNEY; CARPENTER, 2014; YU; GIFFEN, 2021).

As principais alterações decorrentes da *iron overload* no corpo humano estão relatadas em órgãos vitais, como por exemplo, o excesso de depósito de ferro no fígado que pode resultar em doença hepática crônica, cirrose e levar ao carcinoma hepatocelular (KEW, 2009; MCDOWELL; KUDARAVALLI; STICCO, 2021). Também foram relatados danos ao pâncreas, pelo acúmulo de ferro, resultando na elevação dos níveis de glicose no sangue e na diabetes. E ainda, quando o acúmulo de ferro é diagnosticado no músculo cardíaco os danos podem levar à insuficiência cardíaca e a ritmos cardíacos irregulares (DÍEZ-LÓPEZ; COMÍN-COLET; GONZÁLEZ-COSTELLO, 2018). A sobrecarga de ferro em regiões do sistema nervoso pode acelerar o desenvolvimento de doenças neurodegenerativas (CHAUDHARY et al., 2021; JAHANSHAHI; KHALILI; MARGEDARI, 2021; ZECCA et al., 2004).

1.4 Ferroptose e lipídeos/ácidos graxos

A ferroptose, uma forma de morte celular regulada e recentemente descrita, é catalisada por ferro e impulsionada pela peroxidação de fosfolipídios de membrana (Figura 3) (DIXON et al., 2012a). Estudos indicam que a ferroptose apresenta um

papel importante na progressão de condições patológicas renais (ANGELI et al., 2014), hepáticas (QI et al., 2020; TSURUSAKI et al., 2019) e cerebrais (SHEN et al., 2022; SONG et al., 2021), dentre outras. Interessantemente, os agentes quelantes de ferro, que têm sido úteis no tratamento de indivíduos com sobrecarga de ferro (BOLLIG et al., 2017; FISHER et al., 2013), também exibiram efeitos benéficos em modelos experimentais induzidos por estratégias ferroptóticas clássicas, como os compostos erastina [inibidor do trocador cistina-glutamato (ADEDOYIN et al., 2018)] e RSL3 [inibidor da enzima glutationa peroxidase 4 (DÄCHERT et al., 2016)], bem como a depleção de glutationa [importante antioxidante intracelular (SUN et al., 2018)]. No entanto, não está claro se a ferroptose é importante e/ou necessária para a ocorrência do dano celular, tecidual e orgânico observado em pacientes com sobrecarga de ferro. Adicionalmente, não se sabe se estratégias antiferroptóticas são capazes de impedir tal dano.

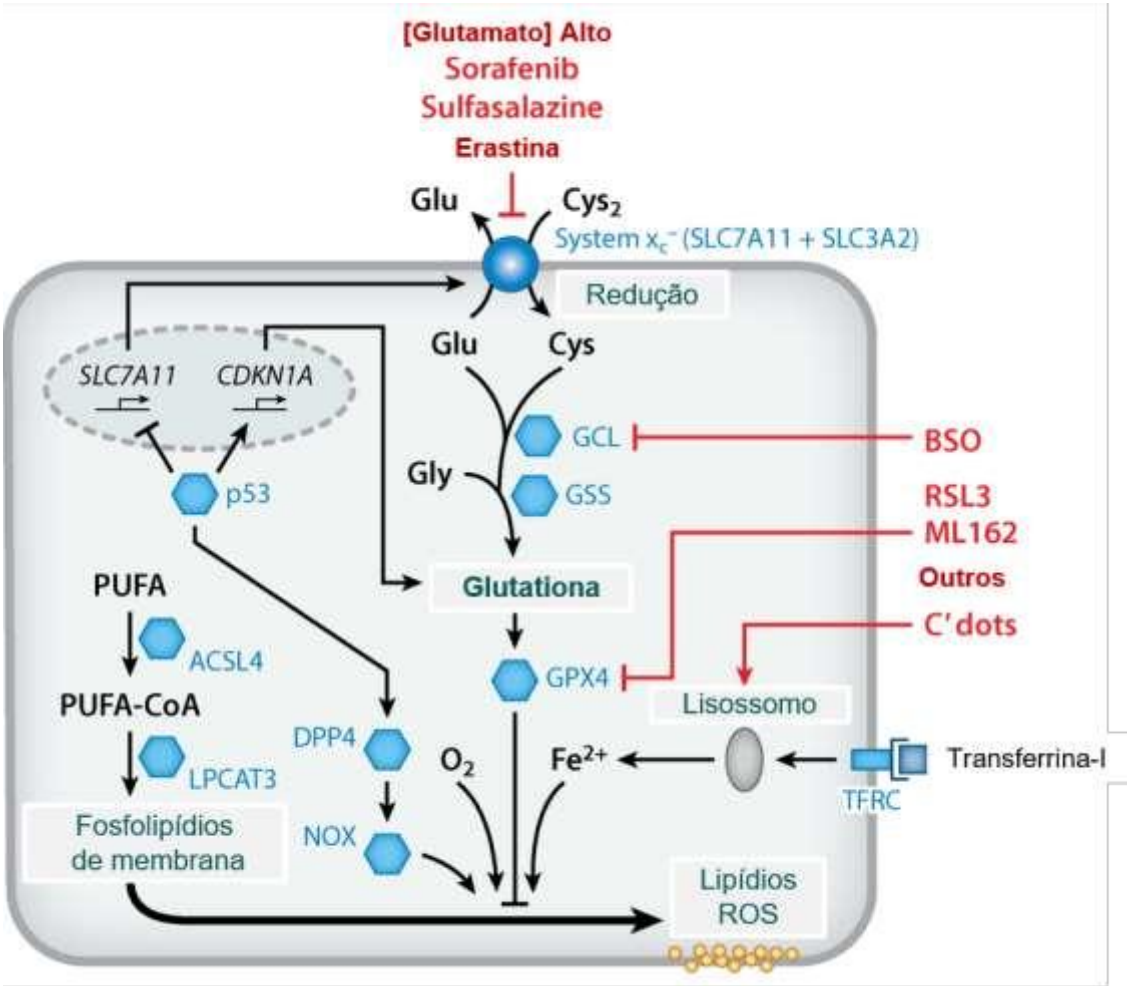

Figura 3.: Principais eventos na via da ferroptose. Os principais reguladores de proteínas da ferroptose são mostrados em azul, enquanto vários indutores de ferroptose são destacados em vermelho. Adaptado de Dixon, S. J., & Stockwell, B. R. (2019).

Os lipídios, que exercem funções vitais na manutenção da homeostase celular devido aos seus papéis como principais reservas de energia metabólica e na composição de membranas, também contribuem para a recepção e transmissão de sinais celulares (SUNSHINE; IRUELA-ARISPE, 2017). Em particular, os lipídios exibem papéis moduladores em formas distintas de morte celular regulada (DONDELINGER et al., 2014; HILDEBRAND et al., 2014; HUANG; FRETER, 2015; MAGTANONG; KO; DIXON, 2016a; YOUNG et al., 2013). Foi demonstrado que a oxidação do lipídio mitocondrial cardiolipina é necessária para a liberação do citocromo c (NOMURA et al., 2000), um evento-chave na morte celular apoptótica (GARRIDO et al., 2006). Além disso, alguns lipídios são capazes de interagir diretamente com a proteína X associada a BCL-2 (Bax), aumentando a permeabilidade da membrana e estimulando o subsequente desmantelamento apoptótico das células (EPAND et al., 2004). No que diz respeito à necroptose, é necessária uma interação direta entre os fosfolipídios da membrana e a proteína MLKL (do inglês, *Mixed Lineage Kinase Domain-Like Protein*) para a execução desse tipo de morte celular regulada (DONDELINGER et al., 2014; HILDEBRAND et al., 2014; MAGTANONG; KO; DIXON, 2016a).

Interessantemente, a ferroptose também é significativamente modulada por lipídios. Por exemplo, a enzima acil-coenzima A (CoA) sintetase 4 (ACSL4), que tem uma preferência para ativar ácidos graxos poliinsaturados (PUFAs) em acil-CoA graxos (um passo essencial antes da incorporação em glicerofosfolipídios), é necessária para a morte celular ferroptótica (DIXON et al., 2015; KAGAN et al., 2016; YUAN et al., 2016). De forma oposta, a enzima acil-coenzima A (CoA) sintetase 3 (ACSL3), que promove a incorporação de ácidos graxos monoinsaturados (MUFA) em membranas, aumenta a resistência da célula ao estresse oxidativo lipídico (UBELLACKER et al., 2020) e à ferroptose (MA et al., 2022). Estes estudos acima-mencionados corroboram o fato de o tratamento com ácido oleico (um MUFA) conferir um fenótipo resistente à ferroptose em cultivos celulares (MAGTANONG et al., 2019b). Sob condições *in vitro*, este estado celular resistente à ferroptose induzido por ácido oleico exógeno é dependente da enzima ACSL3, além de ser caracterizado por níveis reduzidos de fosfolipídios contendo PUFA e sensibilidade reduzida à peroxidação lipídica (MAGTANONG et al., 2019b). No entanto, não está claro se os MUFAs têm efeitos antiferroptóticos em condições *in vivo*. Além disso, os potenciais efeitos moduladores de MUFAs em danos mediados por sobrecarga de ferro nunca

foram explorados. Já a inibição da esteroil CoA dessaturase 1 (SCD1), uma dessaturase lipídica, altera a composição da membrana lipídica celular e modula a ferroptose (TESFAY et al., 2019). Por outro lado, a inibição de β-oxidação pode restaurar a sensibilidade das células tumorais à ferroptose (MIESS et al., 2018). Sabe-se que o receptor nuclear conhecido como receptor α ativado por proliferador de peroxissoma (PPARα) desempenha um papel central no metabolismo de lipídeos e lipoproteínas (GAO; CHEN, 2022; TYAGI et al., 2011). Até o momento, nenhum papel específico foi reconhecido para o PPARα na ferroptose.

1.5 Ferro e neurodegeneração dopaminérgica

No cérebro humano, o tipo de célula mais comum para coloração de ferro em condições normais é o oligodendrócito. Entretanto, os neurônios e micróglias, assim como oligodendrócitos, expressam ferritina, indicando que todos esses tipos de células têm capacidade de armazenar ferro. No envelhecimento saudável, o acúmulo de ferro ocorre de forma “segura” pois o ferro está principalmente ligado à ferritina e à neuromelanina (ZECCA et al., 2001). No entanto, evidências crescentes indicam que o depósito de ferro, maior do que o relatado no envelhecimento saudável, está envolvido nos mecanismos subjacentes a muitas doenças neurodegenerativas (WARD et al., 2014b). Também é relatado que regiões encefálicas ricas em ferro (tais como os núcleos da base) são mais suscetíveis a processos neurodegenerativos, muitas vezes manifestando-se como distúrbios do movimento (BIASIOTTO et al., 2015).

As doenças neurodegenerativas são caracterizadas pela perda progressiva de subconjuntos específicos de neurônios (DUGGER; DICKSON, 2017). Muitas dessas doenças são definidas por acúmulo progressivo de agregados proteicos intracelulares ou extracelulares (por exemplo, β-amilóide e Tau para a doença de Alzheimer, α-sinucleína para a doença de Parkinson e Huntingtina para a doença de Huntington) (DUGGER; DICKSON, 2017).

A doença de Parkinson, descrita pela primeira vez por James Parkinson em 1817, representa a segunda doença degenerativa mais comum do sistema nervoso central e está associada à perda de neurônios dopaminérgicos (TYSNES; STORSTEIN, 2017). Mesmo após significativos avanços na dissecação das bases moleculares da neurodegeneração, como a DP, os mecanismos permanecem pouco compreendidos, dificultando a descoberta de tratamentos eficazes (FORMAN;

TROJANOWSKI; LEE, 2004). A ferroptose pode ser um novo mecanismo subjacente à neurodegeneração. Estudos recentes mostraram que pacientes com DP apresentam no cérebro níveis elevados de ferro e peróxido lipídico em comparação com os grupos controles saudáveis (SARPARAST et al., 2023). Esses atributos são consistentes com a possível ocorrência de ferroptose nestas condições (BUGA et al., 2023). Neste cenário, prevenir a perda progressiva de neurônios dopaminérgicos por interferir na ferroptose é uma nova abordagem de tratamento promissora.

CAPITULO II

Artigo científico em língua inglesa contendo os itens Introdução, Materiais e Métodos, Resultados, Discussão, Conclusões e Referências Bibliográficas

TITLE: OLEIC ACID INHIBITS IRON OVERLOAD-INDUCED FERROPTOSIS IN *CAENORHABDITIS ELEGANS* IN A NHR-49-DEPENDENT MANNER

2. 1 INTRODUCTION

Iron, a transition metal, accomplishes pivotal functions to maintain cellular homeostasis and general human health. The ability to undergo cyclic oxidation and reduction makes iron a key element in several essential biochemical processes, including cell proliferation (LE; RICHARDSON, 2002), immune response (MARTINS et al., 2017; NAIRZ et al., 2018), mitochondrial respiration (VOLANI et al., 2017) and DNA synthesis (LEDERMAN; COHEN; LEE, 1984). However, excess iron is toxic, leading to cell damage generally via free radical formation and consequent oxidation of DNA, proteins and lipids (BRITTON; BACON; RECKNAGEL, 1987; WINTERBOURN, 1995). Therefore, iron uptake, transport, use and storage are tightly coordinated by multiple proteins and pathways to maintain the cellular labile iron pool (LIP; a pool of chelatable and redox-active iron) at non-cytotoxic concentrations (NEMETH; GANZ, 2021).

Despite living organisms have developed a complex set of molecular and physiological strategies to maintain iron at homeostatic levels, avoiding deficiency and toxicity (ANDERSON; LEIBOLD, 2014b; ANDREWS; SCHMIDT, 2007; WALLACE, 2016), iron levels increase with age (JENKINS et al., 2020; KLANG et al., 2014a; WARD et al., 2014a). This evidence, which points to an age-related loss of the capability to maintain iron homeostasis, are in agreement with the fact that disturbed iron plays a major role in a large number of conditions associated with old age (WAWER; JENNINGS; FAIRWEATHER-TAIT, 2018). Of particular importance, excess iron has been associated with several diseases of the central nervous system such as Parkinson's, Alzheimer's, stroke, Huntington's and amyotrophic lateral sclerosis (BELAIDI; BUSH, 2016b; BUIJS et al., 2017; DIETRICH; W G BRADLEY, 1988; DONLEY et al., 2021; KWAN et al., 2012; ZHANG; KONG; CHAI, 2018).

Even though the molecular mechanisms mediating iron-induced toxicity are not fully understood, it is known that its ionic species may compete for binding sites with other essential ions (YE et al., 2017), thus causing structural modifications and disturbances in metal homeostasis (BENNETT; GRALNICK, 2019). In addition, excess labile iron can result in an uncontrolled formation of reactive oxygen species (ROS), via the Fenton and Haber–Weiss reactions (VALKO et al., 2006). Although cells are

equipped with antioxidant defenses to neutralize oxidative damage from ROS (BAYIR et al., 2020; BIRBEN et al., 2012), such systems can be insufficient to properly prevent cellular oxidative damage under excess labile iron. In this scenery, hydroxyl/lipid peroxyl radicals (HO•/LO•) represent oxidants with extremely high reactivity toward biomolecules and major roles in iron-mediated cytotoxicity (LATUNDE-DADA, 2017; NAKAMURA; NAGURO; ICHIJO, 2019b). Despite excess iron displays significant roles in modulating both caspase-dependent apoptosis (LI et al., 2016; YANG et al., 2017) and necroptosis (NAKAMURA; NAGURO; ICHIJO, 2019b; XIE et al., 2005), a pivotal toxic role of iron has been reported in ferroptosis, a recently discovered type of nonapoptotic regulated cell death characterized by increase of redox-active iron, excessive lipid ROS/LOOH and compromised detoxification of lipid peroxides (DIXON et al., 2012b; DIXON; STOCKWELL, 2019). Ferroptosis, which can be inhibited by the specific inhibitor ferrostatin-1 (Fer-1), but not by inhibitors of other forms of regulated cell death (JIANG et al., 2015; SKOUTA et al., 2014), has been recently reported to be involved in either acute or chronic pathological conditions, such as kidney (ANGELI et al., 2014) and liver (CAPELLETTI et al., 2020) ischemia reperfusion injury, hemorrhagic stroke (ALIM et al., 2019), long-term neurodegenerative conditions (MAHONEY-SÁNCHEZ et al., 2021; REICHERT et al., 2020), radiation-mediated cell death (YE et al., 2020), among others.

The initiation and execution of ferroptosis is intimately linked to lipid metabolism (MAGTANONG; KO; DIXON, 2016b). It has been reported that inhibition of steroyl CoA desaturase 1 (SCD1), a lipid desaturase, alters the cellular lipid membrane composition and modulates ferroptosis (TESFAY et al., 2019). Conversely, inhibition of β-oxidation can restore the sensitivity of tumor cells to ferroptosis (MIESS et al., 2018). It is known that the nuclear receptor known as peroxisome proliferator–activated receptor α (PPARα) plays a central role in lipid and lipoprotein metabolism (GAO; CHEN, 2022; TYAGI et al., 2011). Studies in liver from mice deprived of food showed that PPARα transcriptionally activates genes involved in the peroxisomal β-oxidation pathway (KERSTEN et al., 1999; KERSTEN; STIENSTRA, 2017). To date, no specific roles have been recognized for PPARα in ferroptosis.

In addition, ACSL4, which plays a pivotal role in lipogenesis, has been pointed as a driver for the execution of ferroptosis via accumulation of oxidizable PUFA into cellular membrane phospholipids (DOLL et al., 2016). Likewise, other ACSL isoforms promote cell death by ferroptosis, such as ACSL1 (BEATTY et al., 2021). ACSL3,

which also belongs to the acyl-CoAs family, inhibits ferroptosis by decreasing PUFA incorporation into cellular membrane phospholipids (MAGTANONG et al., 2019a).

Exogenous lipids can also regulate ferroptosis. Fatty acids are the key structural components of different lipids, including storage lipids [triacylglycerols (TAGs)], membrane lipids (phospholipids and sphingolipids), and signaling lipids (fatty acyl amides, eicosanoids, and others) (FAHY et al., 2009). Recent *in vitro* data indicate that exogenous monounsaturated fatty acids (MUFAs) inhibit cell death for ferroptosis, an event that requires ACSL3, which preferentially convert MUFAs into fatty acyl-CoA esters for incorporation into membrane phospholipids (MAGTANONG et al., 2019a). Despite these compelling *in vitro* findings, the role of MUFAs in ferroptosis has not been studied *in vivo*.

Iron overload, recognized as a broad syndrome with many different causative etiologies, is commonly characterized by excess of iron in the body (MCDOWELL; KUDARAVALLI; STICCO, 2021). Primary iron overload is due to a genetic predisposition that misregulates key elements involved in iron homeostasis, such as hemochromatosis protein (HFE), hemojuvelin (HJV), transferrin receptor 2, SLC40A1 (Ferroportin-1, Fpn1) and hepcidin (GAO et al., 2009; PAPANIKOLAOU et al., 2003; WAHEED et al., 2002; YUN; VINCELETTE, 2015). In secondary iron overload, hemosiderosis occurs secondary to hematologic disorders associated with ineffective erythropoiesis (GATTERMANN, 2009b). As a result, patients develop secondary hemochromatosis from iron overload upon repeated transfusion. Apart from primary or secondary iron overload, linked particularly to either genetic or hematological causes, the exposure of health individuals to high iron levels (iron overdose) also represent a relevant health concern (EID; ARAB; GREENWOOD, 2017b). Acute iron poisonings, which have been described after accidental ingestion of iron-containing syrups intakes by children and suicidal poisoning (GUMBER et al., 2013; HALIL et al., 2019; TILNEY; CARPENTER, 2014; YU; GIFFEN, 2021), can cause gastro-intestinal, cardiovascular, metabolic, hepatic and central nervous system toxicity (ABHILASH; ARUL; BALA, 2013). Notably, iron has long been reported as a chief responsible of unintentional poisoning death in young children (EID; ARAB; GREENWOOD, 2017b; TENENBEIN, 2005; YUEN; BECKER, 2020).

Although some lines of evidence have indicated that ferroptosis might represent an important event resulting from primary and secondary iron overload (LIU et al., 2018; SUMNEANG et al., 2020; WANG et al., 2017), the occurrence of ferroptosis after

in vivo exposure to iron in health individuals have not yet properly investigated. Moreover, there are no information concerning the putative causal role of ferroptosis in the sequelae resulting from iron exposure in health individuals, although scarce *in vitro* data are available in the literature (HU et al., 2021). Considering that the nematode *Caenorhabditis elegans* (*C. elegans*) shares great orthology with human proteins involved in iron homeostasis, thus representing a convenient tool to evaluate iron metabolism (WANG et al., 2016), here we developed a *C. elegans*-based model of iron overload to investigate the possible occurrence of ferroptosis after exposure to exogenous iron in health individuals, as well as to investigate its causal role in toxicity outcomes. We hypothesized that iron overload in health worms causes ferroptosis and that the blockade of this type of cell death prevents toxicity and mortality. The data presented in this work demonstrate a ferroptosis is a leading event in *in vivo* experimental model of iron overload. Given the clear participation of lipid metabolism and lipids (including the exogenous) in cell death (MAGTANONG; KO; DIXON, 2016b), and also in ferroptosis, (MAGTANONG et al., 2019a), we tested the hypothesis that MUFAs, which have been reported to induce a ferroptosis-resistant phenotype *in vitro* (MAGTANONG et al., 2019a), exhibit beneficial effects against iron overload-mediated damage through their effects on ferroptosis. We found that oleic acid, a MUFA, inhibits iron-overload-mediated damage and mortality *in C. elegans*. Finally, we found that this protection was blunted in worms lacking the nuclear hormone receptor NHR-49, which has homology of function with the mammalian PPARα.

2.2 AIMS

1. Develop a *C. elegans*-based *in vivo* model of iron overload characterized by the exposure to exogenous iron (FAC) in order to examine the possible occurrence of ferroptosis.

2. Explore the impact oleic acid on iron overload-induced ferroptosis *C. elegans*.

3. Characterize mechanisms mediating the potential protective effects of oleic acid against ferroptosis in *C. elegans*.

2.3 MATERIALS AND METHODS

2.3.1 *C. ELEGANS* STRAINS AND CULTURE CONDITIONS

Caenorhabditis elegans strains were maintained for 2–3 generations without starvation at 20°C in Nematode Growth Medium (NGM) plates seeded with Escherichia coli OP50 strain, as previously described (BRENNER, 1974). The N2 strain was a gift from Dr. Felix A.A. Soares (Federal University of Santa Maria, RS, Brazil). *C. elegans strain* (nhr-49(gk405) was purchased from the *Caenorhaditis* Genetics Center (CGC). NGM agar plates were prepared as described in (ADMASU et al., 2018); 100 mm Petri dishes were used. *E. coli* bacteria was cultured overnight in LB at 37° with shaking (160 rpm) for 16 h and, thereafter, 600 µL of liquid culture was seeded on plates to grow overnight at 37°C. Synchronization of nematode cultures was achieved via hypochlorite bleaching of gravid hermaphrodites using standard protocol (STIERNAGLE, [s.d.]).

2.3.2 COMPOUNDS PREPARATION AND TREATMENT

Supplementation with ferric ammonium citrate

Iron-mediated toxicity was induced by treating worms with ferric ammonium citrate (FAC), as described previously (KLANG et al., 2014a). In brief, a stock sterile-filtered solution of FAC was prepared at 4.5 M in M9. The stock solution was used to prepare a concentration curve, from which 100 µL aliquots were spotted onto 3 mL NGM/OP50 *E. coli* plates (final concentrations of 15 to 145 mM FAC prepared in M9). Plates were allowed to dry and used 1 day after plating. Control plates were spotted with 100 µL M9 ammonium citrate. The worms were at the late L4/young adult stage when 20-30 nematodes were transferred to fresh plates containing either M9 ammonium citrate control or FAC. After 48 hours, worms were transferred to fresh-compound treated plates.

Ferrostatin-1 and Butylated hydroxytoluene FAC supplementation

For co-treatment with anti-ferroptotic agents, L4 larval stage worms were placed on NGM agar plates supplemented with FAC (50 mM) and/or Fer-1 (200 µM) or butylated hydroxytoluene - BHT (200 µM) at 20 °C. The 19 mM stock solutions of Fer-1 and BHT were made in DMSO. All stock solutions were stored at -20°C. For each

experiment, each stock solution was diluted to the final concentration in M9 prior to plate pouring. FAC-supplemented NGM agar plates were made ahead of time 24 h and the Fer-1/BHT solution or control DMSO was allowed to absorb into the plates for 30 minutes before adding nematodes.

Fatty Acid Supplementation

For fatty acid supplementation assays, plates were prepared with fatty acids (FAs) according to previously described (DELINE; VRABLIK; WATTS, 2013; PEREZ et al., 2020). In short, Tergitol NP-40 detergent (Sigma-Aldrich) was added to a final concentration of 0.001% in liquid NGM agar medium for both unsupplemented and supplemented plates prior to autoclaving. After autoclaving, FAs [stearic acid (18:0; Cat# 00A1030.06.AG, Labsynth), oleic acid (18:1; Cat# 01A1048.01.BJ, Labsynth), linoleic acid (18:2; Cat# W800075, Sigma-Aldrich)] diluted in ethanol were added to liquid NGM media. Plates (32.8 mm Petri dishes, Cat# K13-0035, Kasvi) containing 3 mL NGM agar supplemented with FAs were dried at room temperature for 24 hours and then seeded with OP50. Final FAs and ethanol concentrations in plates were 0.15 mM and 0.1%, respectively. Two days after seeding OP50, L1 synchronized larvae were transferred to the FAs-containing plates. When L4 stage was reached, FAC treatment was performed as described above.

2.3.3 ACUTE TOXICITY ASSAYS

Worms were scored for mortality at 48-96 hours, the surviving were counted under a stereoscope and lethal dose 50% (LD50) was calculated based on the dose-response survival curve. The worms that did not show movement or reaction to gentle stimulation were scored as dead. All treatments were performed in triplicates and experiments were repeated independently at least three times.

2.3.4 GLUTATHIONE MEASUREMENTS

To measure GSH, the assay was performed according to the previously described method with minor modifications (Rahman et al., 2006; Caito e Aschner, 2015; Jenkins et al., 2020). In brief, synchronized L4 stage animals were transferred to treatment plates and incubated at 20 °C for 48 h. Subsequently, the nematodes were collected in 0.6 mL centrifuge tubes with about 80 nematodes in each sample and then

were all washed three times with 200 µL of M9 buffer solution. The pellet was resuspended in 60 µL of extraction buffer and then vortexed (5 s), frozen with liquid nitrogen, and thawed in a water bath (37 °C); all steps repeated three times. The nematodes were sonicated (40 s on ice) by an ultrasonic probe, centrifuged (10 min, 5200×g, 4 °C) and the supernatant tested for protein estimation and GSH quantification.

Measurement of total glutathione per sample was tested in triplicate in 96-well culture plates using the 5,5′-dithiobis-2-nitrobenzoic acid-GSH disulfide reductase recycling method. The plates were then read in a microplate reader (TECAN) and absorbance was read at 412 nm. The concentration of glutathione was determined using a glutathione standard curve and normalized to total protein level in each sample.

2.3.5 C11 BODIPY 581/591 ANALYSIS

Confocal microscopy was used to visualize the distribution for lipid peroxidation upon oxidation in live worms. For lipid peroxidation, 48 h after exposure on plates supplemented with FAC (50 mM, based on item 3.2), worms were collected, washed three times with buffer M9, and 30 worms were labeled with BODIPY 581/591 C11. After treatment, worms were transferred into 10 µM BODIPY 581/591 C11 solution and stained for 1 h (BEAUDOIN-CHABOT et al., 2019). After labeling, worms were washed three with M9, resuspended in 100 µL M9, and analyzed with confocal. Microscopic slides containing pads of 2% agarose were prepared 30 minutes before mounting worms. Animals were washed with M9 for three times and anesthetized with 10 mM levamisole on agarose pads. Confocal microscopy was carried out on a Leica DMI6000 B microscope coupled with TCS SP5 confocal scanner. Images were captured using a 20x/0.70 objective and oxidized and non-oxidized BODIPY581-591-C11 were excited at 488 and 543 and images were collected from emission at 530(30) and 590(30) nm, respectively.

2.3.6 STATISTICAL ANALYSIS

Results were expressed as mean ± S.D. and graphs and statistical analyzes were generated with GraphPad Prism 9.0 (GraphPad Software Inc., La Jolla, CA, USA). The data presented had normal distribution, according to the Shapiro-Wilk test. Thus, they were evaluated by analysis of variance One-way or Two-way ANOVA,

followed by Dunnett's or Tukey's the post-hoc test. P-values less than 0.05 ($p<0.05$) were considered statistically significant.

2.4 RESULTS

2.4.1 IRON OVERLOAD CAUSES CONCENTRATION- AND TIME-DEPENDENT MORTALITY IN *C. ELEGANS*

Iron overload, which may have either genetic or environmental causes, leads to organ damage or even death in patients. To investigate whether iron overload causes ferroptosis *in vivo*, we performed a protocol based on the exposure of *C. elegans* to FAC. As a first approach to identify concentrations and times required to induce iron-toxicity in *C. elegans*, L4 larval stage worms were placed on agar plates supplemented with FAC (ranging from 15 to 145 mM) during 48 or 96 h. At 48 h exposure to FAC, practically all tested concentrations did not cause significant changes in mortality, with the exception of the highest concentration (145 mM), which caused a significant (although small; 10%) decrease in the survival rate. On the other hand, significant increases in mortality rate were observed at 96 h after FAC treatment (Fig. 1B). Of note, 50 mM FAC, which was unable to change survival at 48 h, significantly decreased the survival rate at 96 h (approximately 40%). Therefore, we selected this concentration (50 mM) in subsequent experiments in an attempt to identify the possible occurrence of ferroptosis-related biochemical changes previously to iron overload-induced mortality in worms (at 48 h or early periods).

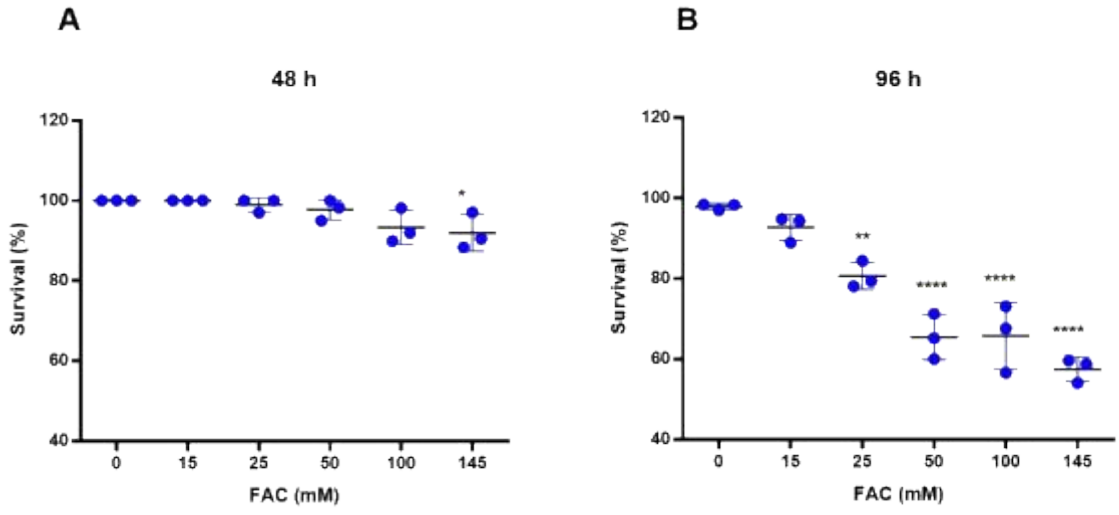

Figure 4. Experimental iron overload causes mortality in C. elegans. Wild type (N2 strain) L4 worms were exposed to different concentrations of ferric ammonium citrate (FAC) during 48 (A) or 96 h (B). Survival rate is presented as % the initial number of alive animals (~25/experiment/group). Data are

represented as mean +/- SD (N = 3). Significant differences were analyzed by one-way ANOVA followed by Dunnett's test. * p < 0.05, ** p < 0.01, **** p < 0.0001 compared to control.

2.4.2 DECREASE OF GLUTATHIONE CONTENT PRECEDES IRON OVERLOAD-INDUCED MORTALITY IN *C. ELEGANS*

Glutathione dyshomeostasis has been reported to display a causal role in ferroptosis (DISTÉFANO et al., 2017). Of note, the depletion of the intracellular pools of both the reduced and oxidized forms of glutathione (GSH and GSSG) represents an event commonly observed in experimental models of ferroptosis, particularly in those based on the inhibition of the cystine-glutamate antiporter known as system Xc^- (STOCKWELL; JIANG, 2020). We observed that 50 mM FAC exposure caused a significant decrease in glutathione levels at 24 h (Fig. 2). On the other hand, no significant changes were observed between control and FAC-exposed worms at 48 h. In addition, within the control group (worms not exposed to FAC), glutathione levels were significantly different between 24 and 48 h, which is in agreement with previous results indicating that total glutathione levels decrease with increased adult age in *C. elegans* (JENKINS et al., 2020). These results indicate that iron overload led to a significant decrease in glutathione levels (a ferroptosis hallmark) in *C. elegans*. The significant decrease of glutathione levels observed at 24 h after treatment with 50 mM FAC (Fig. 2) indicates that this ferroptotic hallmark preceded the increase in worms' mortality, observed at 96 h (Fig. 1B).

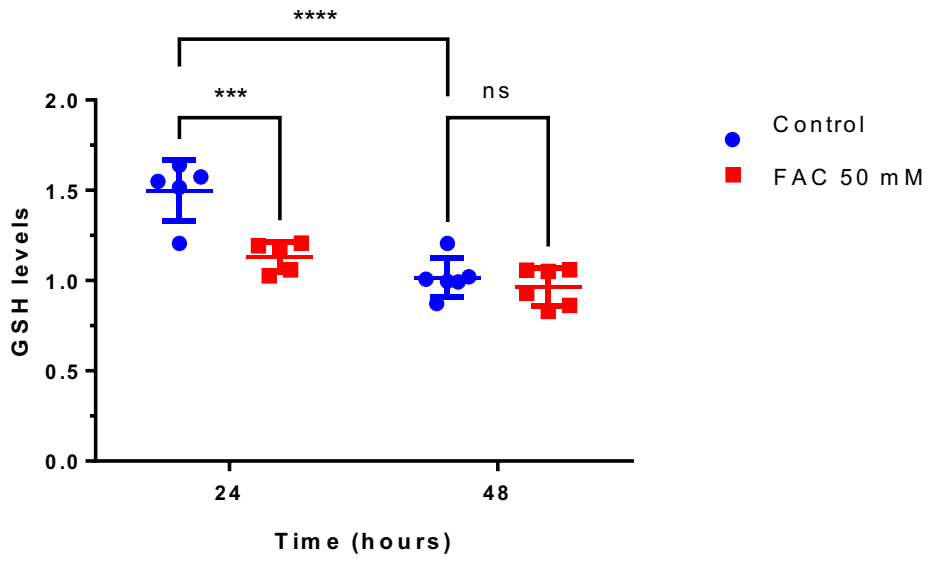

Figure 5. GSH-levels are decrease by iron overload in C elegans. Total glutathione (GSH) decrease following 24 h of FAC (50 mM) exposure. L4 stage animals were transferred to FAC-supplemented plates, incubated for 24 or 48 h - 80 adults per measure were picked. Mean +/- SD (n = 5-6). *** P <

0.0007, and **** P < 0.0001 by two-way ANOVA followed by Tukey's multiple comparisons test. ns = non-significant.

2.4.3 INCREASED LIPID PEROXIDATION PRECEDES IRON OVERLOAD-INDUCED MORTALITY IN *C. ELEGANS*

Lipid peroxidation is a pivotal phenomenon in ferroptosis (YANG; STOCKWELL, 2016). To investigate lipid peroxidation in *C. elegans*, we used BODIPY C11, an oxidation-sensitive fluorescent lipid peroxidation probe (DRUMMEN et al., 2002). Treatment of worms with 50 mM FAC resulted in a significant increase of oxidized lipids compared with control worms (Fig. 3). Of note, such increase in lipid peroxidation was observed at 48 h after 50 mM FAC exposure (Fig. 3), which is characterized by nonsignificant changes in mortality rate (Fig. 1A), indicating that this additional ferroptosis hallmark (lipid peroxidation) also preceded iron overload-induced mortality.

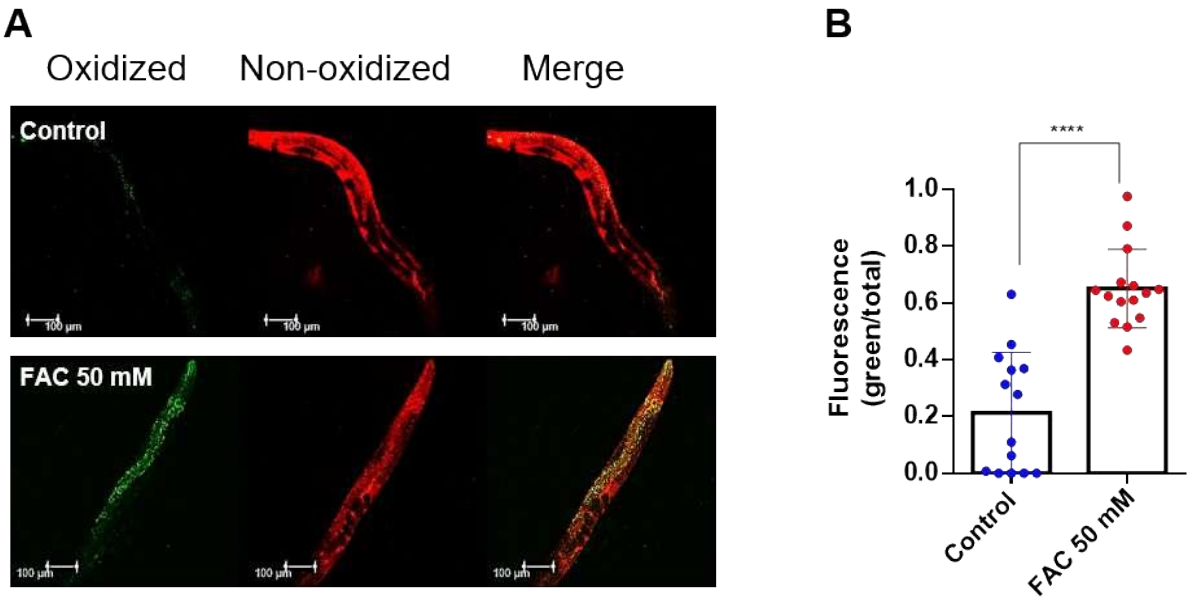

Figure 6. Iron overload induce increment of lipid peroxidation in *C. elegans*. Wild type (N2 strain) L4 worms were exposed to 50 mM FAC during 48 h. (A) Representative confocal microscopy imagens of red and green BODIPY-C11 fluorescence in control and FAC-exposed worms. Green, red and merge fluorescence for each animal is depicted in the left, center and right panels. (B) Lipid peroxidation (BODIPY-C11 staining) is presented as fluorescence [green/(green + red)]. **** p < 0.0001 by Student test. N = 15 worms per group (derived from 3 independent experiments).

2.4.4 ANTI-FERROPTOTIC COMPOUNDS INHIBIT IRON OVERLOAD-INDUCED MORTALITY IN *C. ELEGANS*

Results from Fig. 2 and 3 point to the occurrence of ferroptosis in time-points that precede iron overload-induced mortality in *C. elegans*. To investigate whether ferroptosis has a causal role in iron overload-induced mortality in *C. elegans*, the potential protective effects of two anti-ferroptotic compounds (Fer-1 and BHT; (CHEN et al., 2021; DIXON et al., 2012b; SAPORITO-MAGRIÑÁ et al., 2017)) were evaluated. Fig. 4 shows that both anti-ferroptotic compounds significantly protected against iron overload-induced mortality. Of note, iron overload-induced mortality was fully prevented by 200 µM BHT. This is consistent with ferroptosis being a main cause of mortality in our model; healthy worms exposed to iron overload die via a ferroptosis.

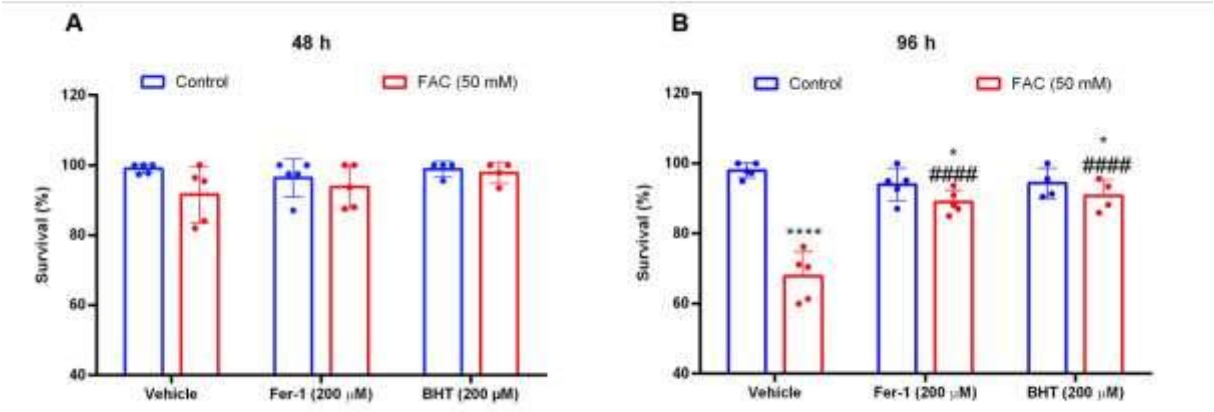

Figure 7. C. elegans models of iron overload develop ferroptosis. Wild type (N2 strain) L4 worms were exposed to 50 mM FAC and/or 200 µM Fer-1/or 200 µM BHT for 48 (A) or 96 h (B). Survival rate is presented as % the initial number of alive animals (~25/experiment/group). Data are represented as mean +/- SD (N = 5). Significant differences were analyzed by two-way ANOVA followed by Tukey's test. * $p < 0.05$, **** $p < 0.0001$ compared to control. #### $p < 0.0001$ compared to worms exposed only to 50 mM FAC.

2.4.5 EXOGENOUS OLEIC ACID PROTECTS AGAINST IRON OVERLOAD-INDUCED LETHALITY IN *C. ELEGANS*

Previously, studies in cultured cells have found that exogenous monounsaturated fatty acids (MUFAs) inhibits ferroptosis induced by RSL3 (YANG et al., 2016), erastin2 and ML 162 (MAGTANONG et al., 2019a). These studies led us to test the hypothesis that dietary exogenous monounsaturated fatty acids (MUFAs) is sufficient to inhibits ferroptosis induced by iron overload. To test this hypothesis, wild type (N2 strain) C. elegans were treated from larval stage 1 (L1) with dietary oleic acid and challenged at stage L4 with 50 or 100 mM FAC, which has known to induce ferroptosis-dependent mortality in C. elegans (Figure 4). Parallel groups were treated with stearic (unsaturated) or linoleic (PUFA) acids for comparison. As expected, experimental iron overload (50 and 100 mM FAC) induced significant mortality in worms at 96 h (Figure 5). Notably, dietary oleic acid (OA, C18:1) significantly inhibited iron overload-induced mortality; this effect was observed at both FAC concentrations (50 and 100 mM). Stearic (SA, C18:0) or linoleic (LA, C18:2) acids exhibited no relevant effects (Figure 5).

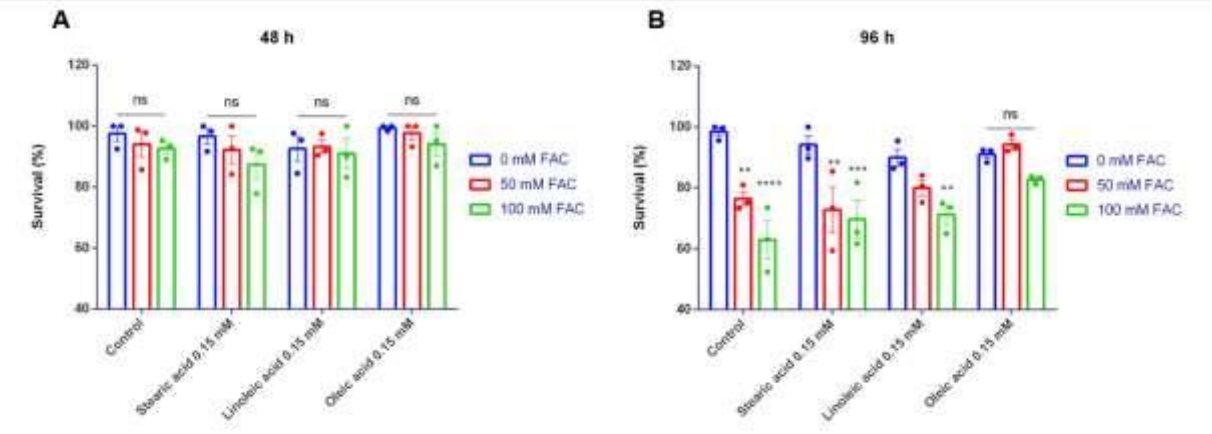

Figure 5. **Oleic acid prevents FAC-induced mortality in *C. elegans***. Wild type (N2 strain) worms were exposed to different 0.15 mM of stearic, linoleic or oleic acid from L1. At the L4 stage, worms were exposed to 50 or 100 mM FAC during 48 (A) or 96 h (B). Survival rate is presented as % the initial number of alive animals (~25/experiment/group). Data are represented as mean +/- SD (N = 3). ** $p < 0.01$, **** $p < 0.0001$ compared to the respective control after two-way ANOVA followed by Tukey's multiple comparisons test.

2.4.6 OLEIC ACID INHIBITS IRON OVERLOAD-INDUCED LIPID PEROXIDATION IN *C. ELEGANS*

Given the anti-ferroptotic effects of oleic acid under *in vitro* conditions (MAGTANONG et al., 2019a; YANG et al., 2016) and the involvement of ferroptosis in our *in vivo* model of iron overload, we hypothesized that the protective effects afforded by oleic acid would be related to the mitigation of ferroptosis. We therefore investigated the effects of dietary fatty acids (oleic, stearic and linoleic) in the accumulation of lipid ROS at 48 h after 50 mM FAC exposure, a condition characterized by lack of changes in the mortality rate (Figure 5). As previously observed (Figure 3), a significant increase in lipid ROS levels was observed in worms exposed to 50 mM FAC for 48 h (Figure 6). Although stearic and linoleic acids did not change FAC-induced increase in lipid ROS, dietary oleic acid significantly decreased iron overload-induced lipid peroxidation in worms (Figure 6), indicating that the protective effects of this MUFA against iron overload-induced mortality is due to its anti-ferroptotic properties. Given the involvement of ferroptosis in our *in vivo* model of iron overload (Figures 4), the observed inhibitory effects of dietary oleic acid toward FAC-induced lipid ROS accumulation and subsequent mortality in *C. elegans* reveal the *in vivo* anti-ferroptotic properties of this MUFA.

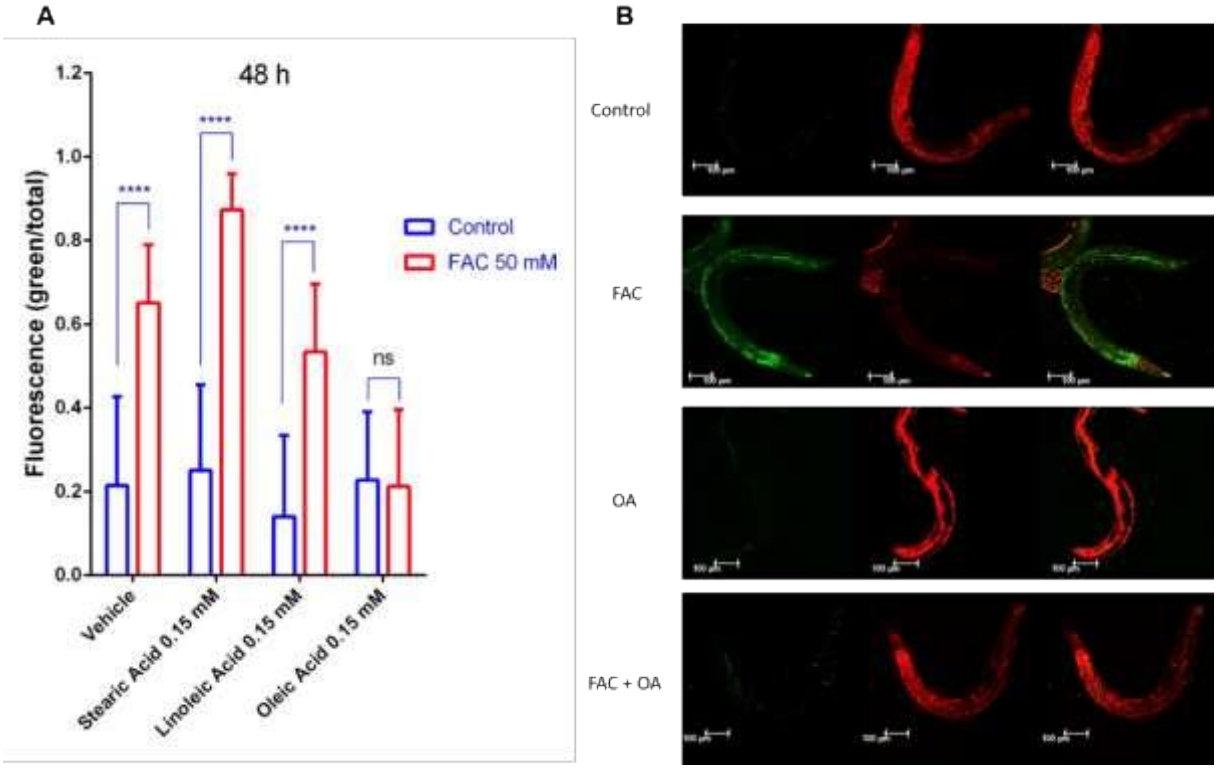

Figure 6. Oleic acid prevents FAC-induced lipid peroxidation in *C. elegans*. (A) Wild type (N2 strain) worms were exposed to vehicle or 0.15 mM of stearic, linoleic or oleic acid from L1. At the L4 stage, worms were exposed to 50 mM FAC during 48 h. Lipid peroxidation (BODIPY-C11 staining) is presented as fluorescence [green/(green + red)]. **** $p < 0.0001$ by one-way ANOVA. N = 15 worms per group (derived from 3 independent experiments). ns = non-significant. (B) Representative confocal microscopy imagens of red and green BODIPY-C11 fluorescence from a (i) control, (ii) FAC (50 mM) and (iii) FAC (50 mM) + oleic acid (0.15 mM)-exposed worms (48 h after FAC exposure).

2.4.7 OLEIC ACID PROTECTS AGAINST IRON OVERLOAD IN A PPAR-α (NHR-49)-DEPENDENT MANNER

MUFAS, including oleic acid, were previously shown to function as ligands for PPARα (JAGANNATHAN et al., 2020; KLIEWER et al., 1997). Unpublished data from our group suggest that the anti-ferroptotic effects of this MUFA is dependent on PPAR-α activation (*in vitro* data). To better understand the protective mechanisms of oleic acid and explore this topic in an *in vivo* scenario, we investigated the protective effects of dietary oleic acid against iron overload-induced mortality in *C. elegans* knocked out for NHR-49, which has homology of function with the mammalian PPAR-α. Remarkably, the protective effect of dietary oleic acid against iron-overload-induced mortality was blunted in worms lacking the nuclear hormone receptor NHR-49 (Figure 7C). These observations suggest that oleic acid protects from iron overload-mediated ferroptosis via both PPAR-α/NHR-49-dependent mechanisms.

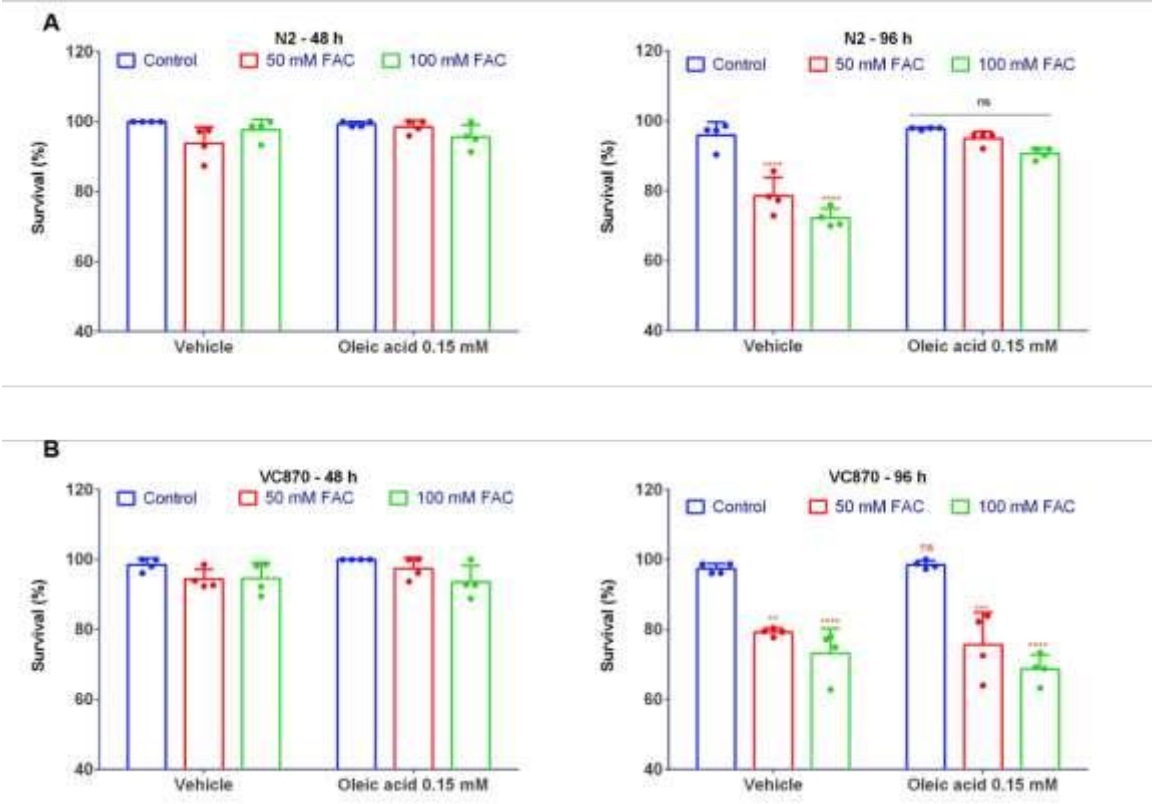

Figure 7. Inhibition of PPAR-α activity decreases the antiferroptotic effects of oleic acid. (A) Wild type (N2 strain, top panels) or (B) NHR-49 mutant (VC870) worms were exposed to different 0.15 mM of oleic acid from L1. At the L4 stage, worms were exposed to 50 or 100 mM FAC during 48 h (left panels) or 96 h (right panels). Survival rate is presented as % the initial number of alive animals (~25/experiment/group). Data are represented as mean +/- SD (N = 6). ** $p < 0.01$, **** $p < 0.0001$ compared to the respective control after two-way ANOVA followed by Tukey's multiple comparisons test.

2.5 DISCUSSION

Iron overload, a broad syndrome characterized by excess iron in the body, may result from genetic predisposition that misregulates key elements involved in iron homeostasis, hematologic disorders associated with ineffective erythropoiesis and acute iron poisonings (EID; ARAB; GREENWOOD, 2017b). Of particular importance, acute iron poisonings have been linked to gastro-intestinal, cardiovascular, metabolic, hepatic and central nervous system toxicity (GAASCH et al., 2007; KIRK et al., 2017; MARSELLA et al., 2011; MURPHY; OUDIT, 2010; NAITO; MASUYAMA; ISHIHARA, 2021; RAJAPURKAR et al., 2012; ROEMHILD et al., 2021) and represent a significant cause of unintentional poisoning death in young children (IANNOTTI et al., 2006; TENENBEIN, 2005). Despite iron overload is characterized by excess iron and ferroptosis is an iron-dependent cell death, the relationship between both events under *in vivo* conditions has not been effectively demonstrated. In this study, we showed that ferroptosis occurs in the context of iron-overload-mediated damage *in vivo* (*C.*

elegans). Moreover, inhibition of ferroptosis with exogenously-added oleic acid protects against such damage by suppressing ferroptosis in a NHR-49-dependent manner.

After showing that iron overload causes mortality in *C. elegans*, we wondered whether ferroptosis occurs in the context of iron-overload-mediated damage in this model. Thus, we standardized an iron exposure model in *C. elegans* and subsequently investigated the occurrence of main characteristics of ferroptosis. Particularly, glutathione represents an important defense against oxidative stress and its depletion is tightly linked to the beginning of ferroptosis (ANGELI et al., 2017; YANG et al., 2014). Moreover, GSH contribute to reduce peroxides through GSH peroxidase-catalyzed reactions (SCHULZ et al., 2000), an event that is critical in ferroptosis. In addition, GSH possesses a buffering role for cytoplasmic ferrous iron (HIDER; KONG, 2011). In the nematode *C. elegans*, GSH synthesis is evolutionarily conserved (FERGUSON; BRIDGE, 2019). So, we evaluate whether acute exposure to FAC could cause GSH depletion. Our results demonstrated that FAC exposure, for up to 24 h, beginning at L4 resulted in a modest, but significant decrease of glutathione (Fig. 2). Remarkably, no differences between groups were observed at 48h after FAC treatment. Our results are consistent with a previous work showing that total GSH levels decrease with increased age in *C. elegans* (JENKINS et al., 2020). Therefore, FAC could not further decrease GSH levels suggesting that can represent a compensatory response (FERGUSON; BRIDGE, 2019; LU, 2009).

A central mechanism of ferroptosis is the accumulation of lipid peroxidation by catalyzing the production of hydroxyl radicals in a Fenton chemistry-dependent manner (DIXON et al., 2012b; STOCKWELL; JIANG, 2020). Lipid peroxidation is widely accepted as an important marker of oxidative stress in cells (GASCHLER; STOCKWELL, 2017) and can be detected using the fluorescent lipid peroxidation sensor C11 BODIPY 581/591 (MAGTANONG et al., 2019a). Previous studies have shown that this peroxidation reaction is dramatically accelerated by iron (GASCHLER; STOCKWELL, 2017; ORTEGA-ARELLANO; JIMENEZ-DEL-RIO; VELEZ-PARDO, 2017; PRATT; TALLMAN; PORTER, 2011). To determine whether FAC treatment results in increased lipid peroxidation, we used the fluorescent dye C11-Bodipy (BAYIR et al., 2020). We found that lipid peroxidation significantly increased at 48 h after 50 mM FAC exposure (Fig. 3), suggesting the occurrence of ferroptosis at a moment that precedes FAC-induced mortality (96 h after FAC exposure). This is consistent with

previous investigations that have demonstrated that FAC treatment significantly increased lipid peroxidation in murine models of hemochromatosis (WANG et al., 2017). Similar results were seen when HT-1080 cells were treated with FAC (FANG et al., 2018). Our results demonstrated FAC could induce lipid peroxidation, suggesting that iron overload is sufficient to elicit ferroptosis in *C elegans*.

Previous studies reported that ferroptosis can be rescued by ferrostatin-1 (Fer-1) (DIXON et al., 2012b). This aromatic amine, as well the antioxidant BHT, was effective decreasing cellular lipid oxidation (CHEN et al., 2021; SAPORITO-MAGRIÑÁ et al., 2017; STOCKWELL et al., 2017). A recent report indicates that the antioxidant BHT was fully effective in preventing phospholipid peroxidation in rat liver mitochondria (SAPORITO-MAGRIÑÁ et al., 2017). Unveiling the causal role of ferroptosis in iron overload-induced toxicity and mortality represents the possibility for discovering new strategies to treat such condition. Although it is known that iron overload may cause toxicity to several organs in humans, such as liver, spleen, heart, bone marrow, pituitary, pancreas, and the central nervous system (GUJJA et al., 2010; YASSIN et al., 2017), as well as mortality (CAPELLETTI et al., 2020), the mechanisms related to such events are not fully understood. The significant protective effects of two anti-ferroptotic compounds (Fer-1 and BHT) in preventing iron overload-induced mortality (Fig. 4) indicate that anti-ferroptosis-based treatments may be useful to mitigate the cellular and organic toxicity elicited by iron overload.

Recently, exogenous MUFAs such as oleic acid (OA) were reported to protect cultured cells from ferroptosis (YANG et al., 2016). Similar to mammalian cells, it has been shown that in *C. elegans* the addition of exogenous MUFAs is sufficient to prevent germ cell ferroptosis (PEREZ et al., 2020). In our study, oleic acid remarkably protected worms from iron overload-induced mortality (Fig. 5). In agreement with previous studies cells (MAGTANONG et al., 2019a), we found that oleic acid prevented lipid peroxidation in *C. elegans* (Fig.6). These results are consistent with the observations that exogenous OA could inhibit ferroptosis (MAGTANONG et al., 2019a; YANG et al., 2016).

Evidence shows that peroxisome proliferator-activated receptors (PPARs) and their downstream signaling are involved in the regulation of lipid metabolism (BERGER; MOLLER, 2002; KOTA; HUANG; ROUFOGALIS, 2005; MUKHERJEE et al., 1997), including in response to oxidative stress (MUZIO; BARRERA; PIZZIMENTI, 2021). Of note, previous studies confirmed that OA may be a natural ligand for PPAR-

α (BERGER; MOLLER, 2002). In fact, oleic acid-derived oleoylethanolamide, which is synthesized from membrane glycerophospholipids, is a high-affinity agonist of the PPAR-α (BOWEN et al., 2017). Moreover, oleoylethanolamide is able to stimulate lipolysis by activating PPAR-**α** (GUZMÁN et al., 2004). Based on these evidences, we reasoned that OA could inhibit ferroptosis via PPAR-**α** activation. In *C. elegans*, NHR-49 is a transcription factor orthologous to mammalian PPAR-**α** (LEE et al., 2016) that have important effects on metabolism, fat storage, and life span (DOERING et al., 2022; VAN GILST et al., 2005). To investigate the role of PPAR-α/NHR-49 in OA-mediated effects on iron-overload-induced ferroptosis, we used the well-characterized mutant (nhr-49(gk405) (HU et al., 2018), which does not express NHR-49. Herein, the protective effect of dietary oleic acid against iron-overload-induced mortality, observed in wild type worms (N2 strain), was blunted in worms lacking the nuclear hormone receptor NHR-49 (Fig. 7), implying that this gene is required for the observed oleic acid protective activity. These results observed in worms indicate that the nuclear receptor-dependent ability of oleic acid in inhibiting ferroptosis, which was also observed in mammalian cultured cells (data not shown), appears to be a conserved event.

2.6 CONCLUSIONS

Based on the results presented above, we conclude that:

- The exposure to FAC could induce ferroptosis in *C. elegans*;
- Iron overload causes ferroptosis-dependent damage and mortality in *C. elegans*;
- The administration of ferroptotic inhibitors exerted protective effects against the death caused by iron-overload;
- Oleic acid inhibits ferroptosis in *C. elegans* in a NHR-49 dependent manner.

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CAPITULO III

Resultados adicionais relacionados aos efeitos do modelo de sobrecarga de ferro e ferroptose sobre neurodegeneração dopaminérgica

CONTEXTUALIZAÇÃO INICIAL

As doenças neurodegenerativas associadas à idade, como a doença de Alzheimer e doença de Parkinson (DP), representam um grande desafio de saúde pública, devido ao aumento demográfico da proporção de indivíduos mais velhos na sociedade. Considerando que a homeostase do ferro desempenha um papel essencial na ferroptose, é importante o desenvolvimento de estratégias para direcionar a ferroptose afim de tratar as doenças associadas à sobrecarga de ferro. Em humanos, a perda da transmissão dopaminérgica no estriado relacionada à perda progressiva de neurônios dopaminérgicos resulta nos sintomas motores da DP (RANGEL-BARAJAS; CORONEL; FLORÁN, 2015). O estudo e a compreensão das modificações neuropatológicas, após a sobrecarga de ferro, pode ter consequências terapêuticas importantes para doenças neurodegenerativas, dado que as evidências epidemiológicas relatadas na literatura indicam a co-exposição a Fe e outros metais tóxicos como um fator de risco para DP (FARINA et al., 2013).

A neurotransmissão dopaminérgica está ligada a vários comportamentos, como locomoção, motivação e atividade de reconhecimento (IVERSEN; IVERSEN, 2007). A eficácia da transmissão dopaminérgica é controlada pelos processos que governam a liberação de dopamina (DA) e também pela regulação das concentrações extracelulares de DA por meio da rápida recaptação pelo transportador de dopamina da membrana plasmática (DAT) (GAINETDINOV, 2008; KRISTENSEN et al., 2011). DAT é um membro da família dos transportadores dependentes de Na + /Cl, contém 12 domínios transmembranares (CHEN et al., 2004; SHIMADA et al., 1991). O transporte de DA mediado por DAT envolve ligação sequencial e cotransporte de dois íons Na + e um íon Cl − com uma molécula de DA (AMARA; SONDERS, 1998; KUHAR et al., 1990). O DAT é expresso exclusivamente nos corpos e terminais celulares dopaminérgicos e pode servir como um marcador seletivo desses neurônios (CILIAX et al., 1995; HOFFMAN et al., 1998; NIRENBERG et al., 1996).

Embora *C. elegans* apresente um sistema nervoso relativamente simples, com 302 neurônios, é considerado um organismo modelo atraente para investigações dos mecanismos moleculares da função neurológica (BARCLAY; MORGAN; BURGOYNE, 2012; BRAUNGART et al., 2004). Estudos relataram semelhanças notáveis nos níveis moleculares e celulares entre neurônios de nematoides e vertebrados. Por exemplo, neurotransmissores clássicos [acetilcolina, glutamato,

ácido γ-aminobutírico (GABA), serotonina e dopamina (DA)], transportadores vesiculares e a maquinaria de liberação de neurotransmissores são semelhantes em estrutura e função entre vertebrados e *C. elegans* (CHEN et al., 2015). O hermafrodita *C. elegans* tem oito neurônios dopaminérgicos: quatro neurônios cefálicos (CEP), dois neurônios deirídios anteriores (ADE), ambos localizados na cabeça e dois neurônios pós-deirídios (PDE) na cauda (ver figura 1) (SULSTON; DEW; BRENNER, 1975). Além disso, o *C. elegans* pode ser geneticamente modificado para expressar proteínas humanas associadas à neurodegeneração ou expressar GFP fusionada a proteínas de interesse. *C. elegans* oferece uma avaliação simples e fácil por transgênicos fluorescentes, como por exemplo, construções combinada a GFP e o transportador DA, DAT-1. As principais cepas incluem BZ555 (egIs1 [dat-1p::GFP]), BY200 (vtIs1 (P dat-1 ::GFP, pRF4(rol-6 (su1006)) e BY250 [vtIs7 ; P dat-1 :: GFP] (CHEN et al., 2015; MARTINS et al., 2022).

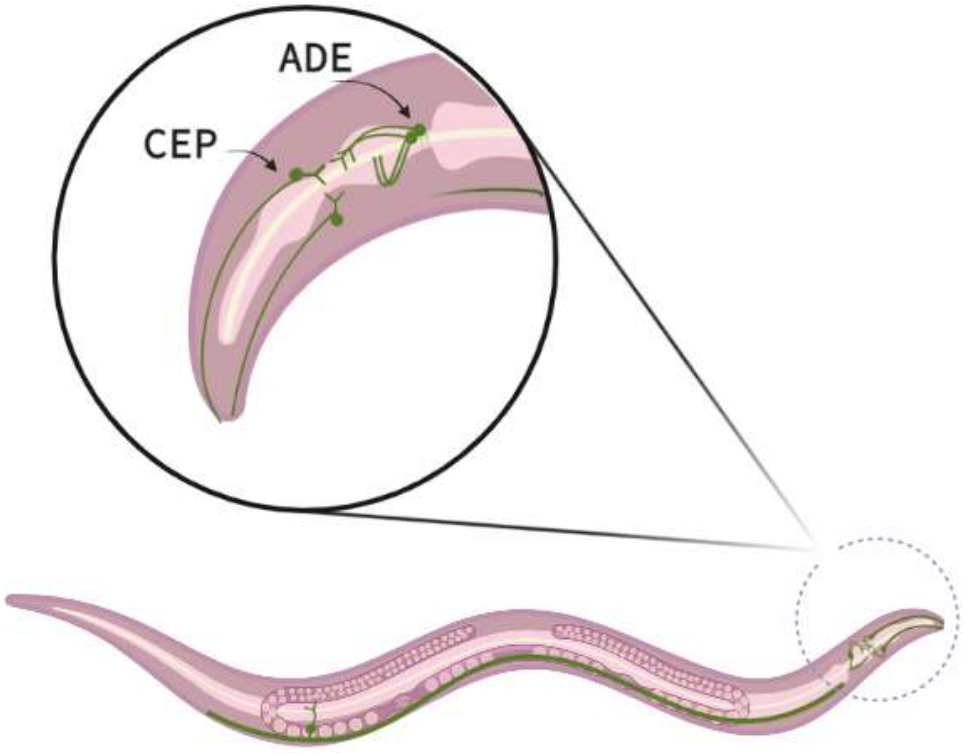


Figura 1: Neurônios dopaminérgicos em hermafroditas adultos de *C. elegans*: Desenho esquemático mostrando a localização dos neurônios dopaminérgicos. No foco em círculo é representado a cabeça e mostra dois pares de neurônios CEP (verde) projetam terminações dendríticas para a ponta da cabeça e um par de neurônios ADE (verde) estende processos ciliados para o bulbo terminal da faringe. Na cauda mostra um par de neurônios PDE (verde) na localização lateral posterior à vulva (Adaptado de Martins et al., 2022).

Nossos dados iniciais, apresentados no capítulo II desta tese, demonstram que nosso modelo experimental de *iron overload* induz significativa toxicidade em *C. elegans* e que a ferroptose representa um evento chave para a ocorrência deste fenômeno. Também observamos que o ácido oleico (um MUFA) protegeu contra o dano mediado por sobrecarga de ferro/ferroptose. Desta forma, iniciamos estudos sobre ferro e neurodegeneração dopaminérgica. Para isso, realizamos um protocolo semelhante (sobrecarga de ferro) em *C. elegans* expressando GFP sob o controle do promotor do transportador de recaptação de dopamina 1 (pdat-1::GFP), utilizando a cepa BY250 Pdat-1::GFP (Figura 2). A cepa BY250 foi usada para avaliar os neurônios dopaminérgicos e explorar os possíveis efeitos neurodegenerativos induzidos por ferroptose. As avaliações morfológicas de neurônios dopaminérgicos foram realizadas de acordo com o protocolo baseado em um roteiro de pontos de 0-6, para quantificar consistentemente as alterações na morfologia dos dendritos de neurônios em *C. elegans* descrito em (BIJWADIA; MORTON; MEYER, 2021).

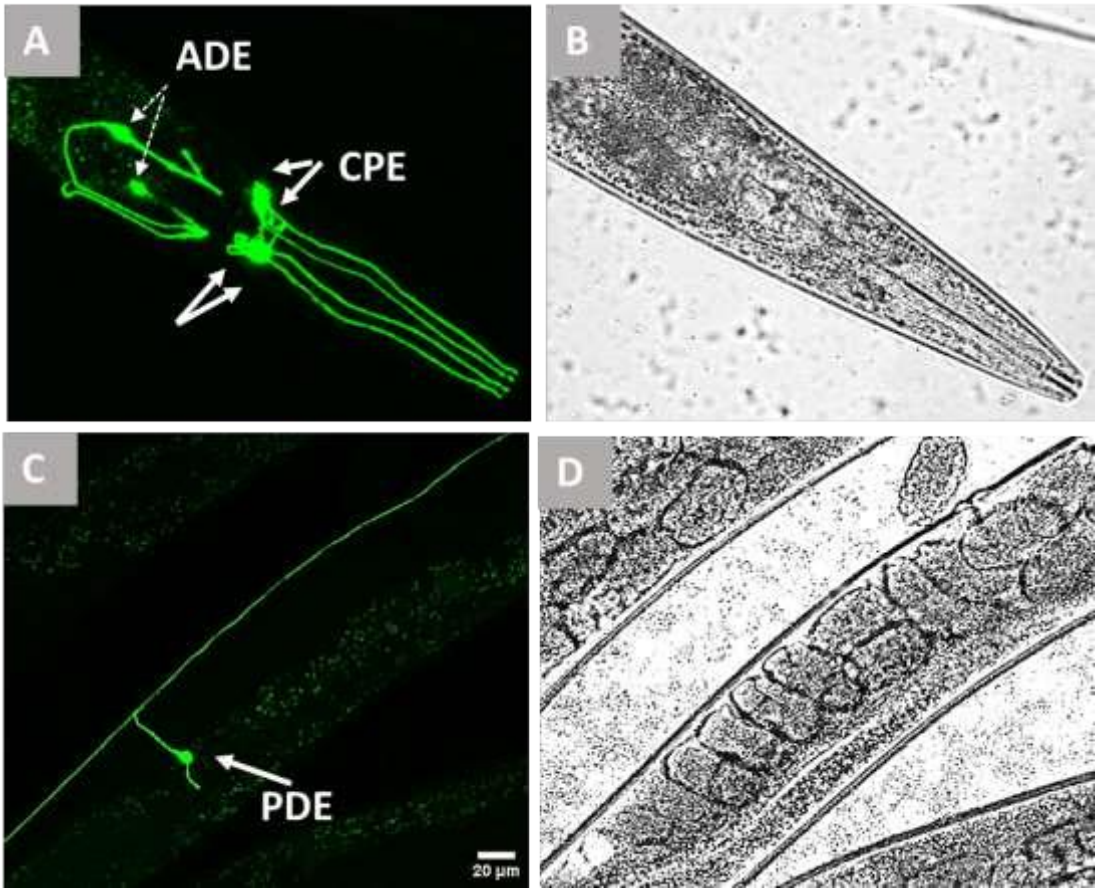


Figura 2: Neurônios dopaminérgicos em adultos hermafroditas de *C. elegans*, vivos, usando fusões de transcrição Pdat-1::GFP. (A) Reconstrução tridimensional de epifluorescência confocal de neurônios dopaminérgicos, localizado na cabeça, em uma linha transgênica Pdat-1 ::GFP (BY250). As setas finas apontam para quatro corpos celulares do CEP. A seta pontilhada indica a localização de dois corpos celulares ADE. (B) Imagem de microscopia de contraste de interferência diferencial do animal em A. (C) Reconstruções tridimensionais de epifluorescência confocal dos neurônios PDE. (D)

Imagem de microscopia de contraste de interferência diferencial do animal em C. (Barras de escala = 20 µm).

RESUMO DOS PRINCIPAIS RESULTADOS

Os principais resultados são resumidos a seguir:

Da mesma forma que os dados anteriores com N2 (citados no Capítulo II), a sobrecarga de ferro aumentou a mortalidade na cepa BY250 Pdat-1::GFP e ácido oleico protegeu contra a mortalidade induzida por sobrecarga de ferro (Figura 3);

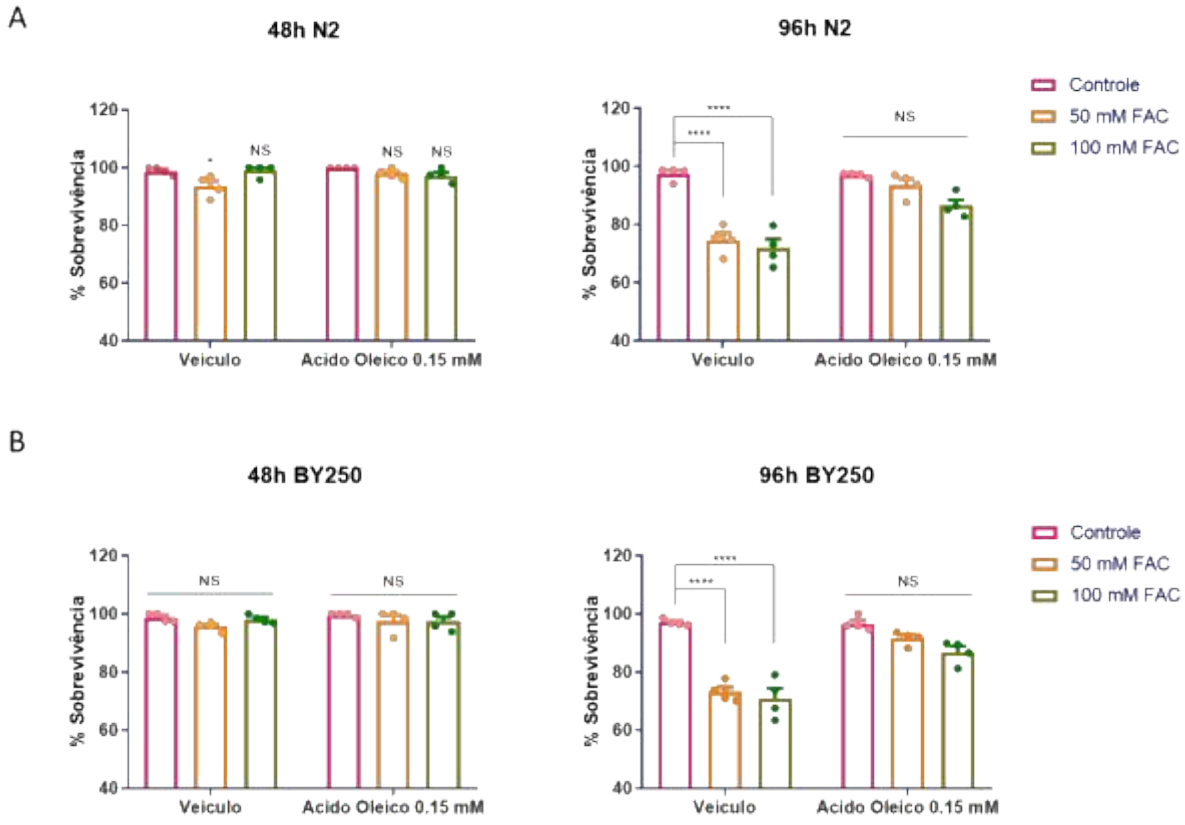
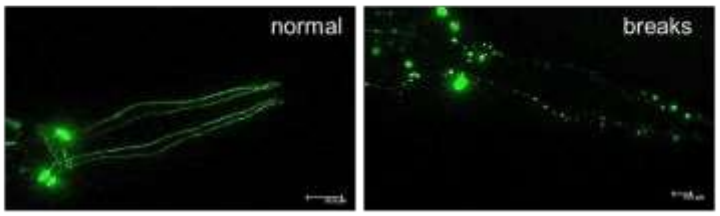


Figura 3: Efeito do FAC na sobrevivência em tipo selvagem (wild-type) (A) e BY250 (B) em *C. elegans* cultivados em meio suplementado com ácido oleico 0,15 mM. Os *C. elegans* foram expostos ao ácido oleico de L1 ao estágio L4, os vermes foram expostos a 50 ou 100 mM de FAC durante 48 ou 96 h. A taxa de sobrevivência é apresentada como % do número inicial de animais vivos (~25/experimento/grupo). Os dados foram analisados por ANOVA two-way seguido pelo teste de comparações múltiplas de Tukey. *P<0,01 e ****P<0,0001. São mostrados resultados representativos de quatro experimentos independentes.

A sobrecarga de ferro foi capaz de causar neurodegeneração de neurônios dopaminérgicos (em pontos de tempo anteriores à mortalidade) e o ácido oleico ou ferrostatina-1 (inibidor específico da ferroptose) protegeu contra a neurodegeneração induzida por sobrecarga de ferro (Figura 4 e 5).

A

Pontuação de Degeneração	
Pontuação	Descrição
0	Nenhum dano observável
1	Irregular (torções e curvas)
2	> 5 bolhas
4	>10 bolhas e/ou quebra removendo <25% do dendrito
5	Quebra, 25-75% de dendrito removido
6	Quebra, >75% de dendrito removido



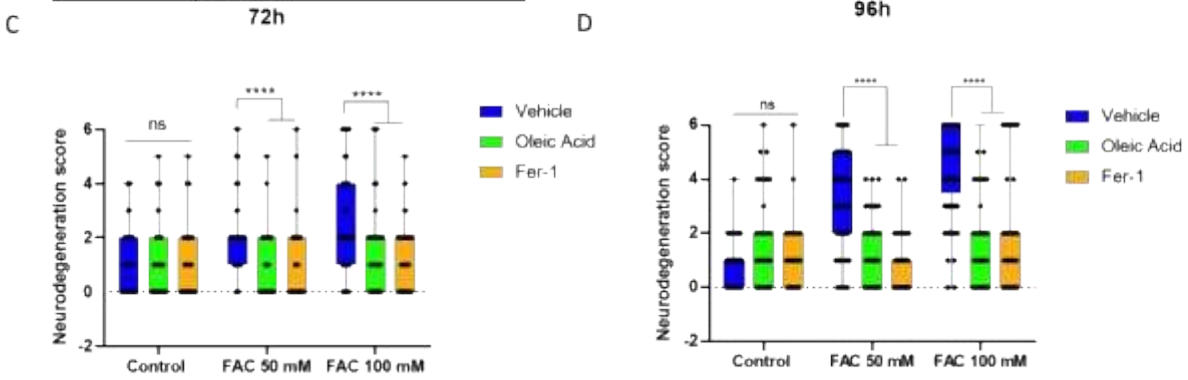
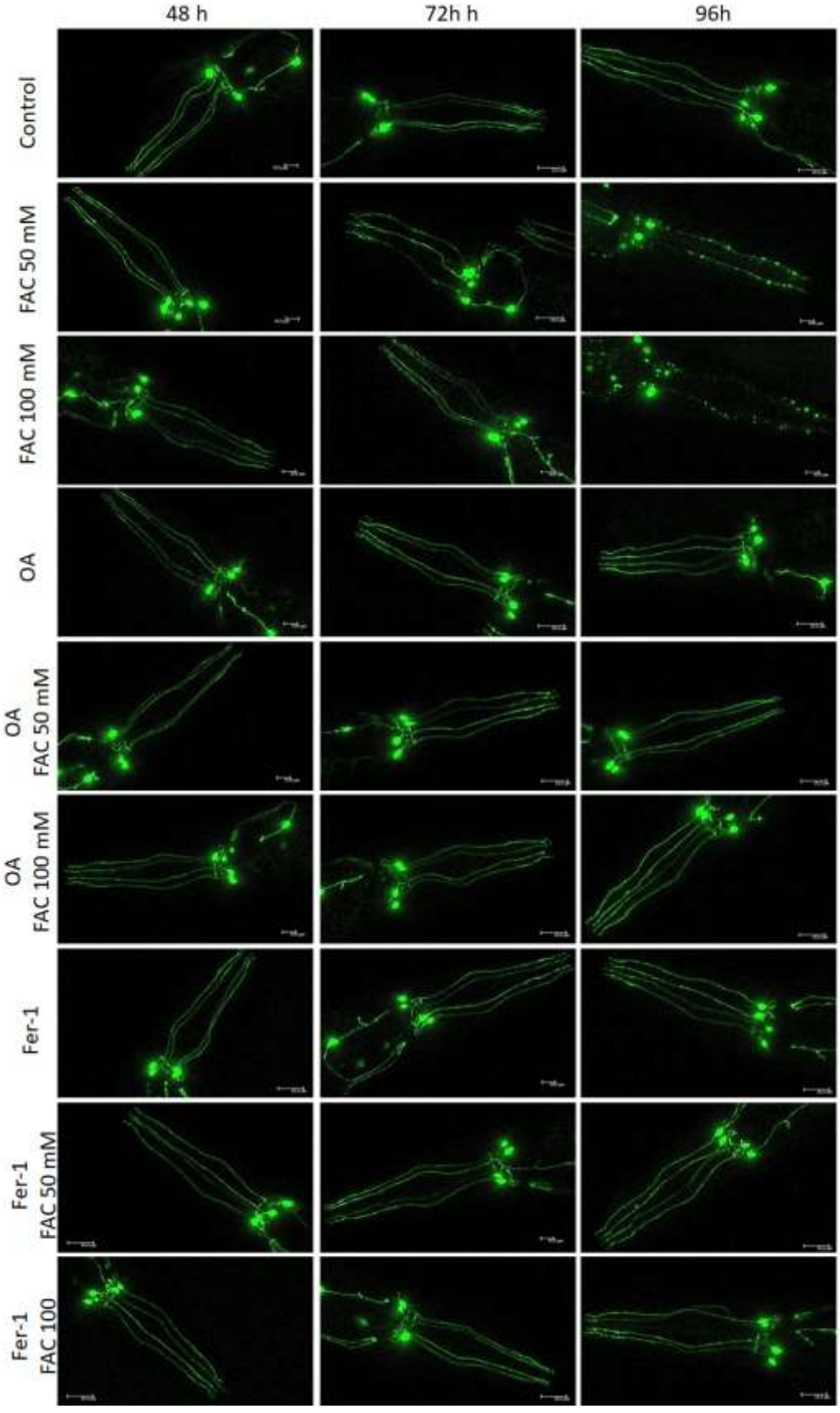


Figura 4: Neurodegeneração dopaminérgica e sua avaliação: *C. elegans* na fase L1 sincronizados, foram expostos a ácido oleico 0,15 mM ou veículo e, no estágio L4, foram tratadas com as doses indicadas de FAC/e 200 µM Fer-1. (A) Descrição da escala de seis pontos utilizada para quantificar os níveis de alteração morfológica do neurônio dopaminérgico e degeneração em C. elegans. pontuação foi realizada conforme descrito em Bijwadia, et al. 2021. (B) Imagens de microscopia confocal representando neurônios dopaminérgicos saudáveis (normais) em comparação com neurônios deformados ou degenerados (quebras). Vermes L4 foram então expostos a FAC por 96 h, e a neurodegeneração foi avaliada em 72 h (C) e 96 h (D) após a exposição. Os dados foram analisados pelo teste de Kruskal-Wallis ANOVA seguido pelo teste de comparações múltiplas de Dunn's. ****P<0,0001.

Figura 5: Imagens representativas de microscopia confocal de alterações em neurônios dopaminérgicos em vermes BY250 Pdat-1::GFP, apresentadas de forma quantitativa na Figura 2.


48 h
72h h
96h
Control
FAC 50 mM
FAC 100 mM
OA
OA
FAC 50 mM
OA
FAC 100 mM
Fer-1
Fer-1
FAC 50 mM
Fer-1
FAC 100

Em resumo, concluímos que a ferroptose induzida pela sobrecarga de ferro causa neurodegeneração dopaminérgica em *C. elegans* e o ácido oleico protege contra esta neurodegeneração, devido a efeitos antiferroptóticos. Na literatura é descrito que a diminuição da taxa locomotora, frente ao alimento, reflete a capacidade dos *C. elegans* de sentir a presença do alimento e ajustar sua atividade locomotora para se alimentar (SAWIN; RANGANATHAN; HORVITZ, 2000). Esse comportamento é mediado exclusivamente por um circuito neural relacionado a dopamina (SULSTON; DEW; BRENNER, 1975). Experimentos futuros terão como foco a padronização de testes comportamentais relacionados aos neurônios dopaminérgicos, para estudar os efeitos funcionais/comportamentais da sobrecarga de ferro e ácido oleico neste protocolo.

Considerando o papel perigoso da sobrecarga de ferro na neurodegeneração dopaminérgica, bem como o papel protetor do ácido oleico, há perguntas adicionais não respondidas:

- As FA dessaturases são capazes de modular (aumentar ou diminuir) a neurodegeneração dopaminérgica? Nossa hipótese é que vermes com níveis aumentados de PUFA (ou diminuídos de MUFA) teriam maior suscetibilidade à neurodegeneração dopaminérgica (sob condições fisiológicas ou após exposições a pequenas concentrações de FAC) e que esse evento é dependente de ferroptose.

- As ferritinas (ftn-1 e ftn-2) são capazes de modular a neurodegeneração dopaminérgica? Nossa hipótese é que vermes sem ftn-1 e/ou ftn-2 teriam maior suscetibilidade à neurodegeneração dopaminérgica (sob condições fisiológicas na idade ou após exposições a pequenas concentrações de FAC) e que esse evento é dependente de ferroptose.

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