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**AVALIAÇÃO DOS EFEITOS NEUROTÓXICOS DO FERRO *IN VIVO* EM
*Caenorhabditis elegans***

DISSERTAÇÃO DE MESTRADO

Daiandra de Almeida Fagundez

**Urugaiana, RS, Brasil
2014**

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Dissertação apresentada ao programa de Pós-graduação *Stricto Sensu* em Bioquímica da Universidade Federal do Pampa, como requisito parcial para obtenção do Título de Mestre em Bioquímica

Orientadora: Profa. Dra. Daiana Silva de Ávila

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Área de concentração: Bioprospecção Molecular

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Banca examinadora

Dedico este trabalho

A minha família, aos meus amigos e a todos que de alguma forma se sentem parte deste trabalho.

“Mas com certeza, para nós, que compreendemos o significado da vida, os números não têm tanta importância.”

Antoine de Saint-Exupéry em *O pequeno príncipe*

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RESUMO

Dissertação de Mestrado
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AVALIAÇÃO DOS EFEITOS NEUROTÓXICOS DO FERRO *IN VIVO* EM *Caenorhabditis elegans*

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Local e Data da Defesa: Uruguaiana, 27 de junho, 2014

Ferro (Fe) é um metal importante para a homeostase do organismo e existe em abundância no ambiente. Níveis moderados de Fe obtidos a partir de alimentos são necessários para a fisiologia celular normal; no entanto, os níveis elevados de Fe(II)/Fe(III) podem causar efeitos citotóxicos através de redução de H₂O₂ a radical hidroxil (OH •), fenômeno conhecido como Reação de Fenton. Mesmo sendo amplamente utilizado os efeitos tóxicos do ferro não são bem compreendidos. Buscando modelos experimentais alternativos que possam substituir e oferecer novas possibilidades de ensaios de toxicidade de xenobióticos, o nematoide *Caenorhabditis elegans* tem sido utilizado como um organismo bioindicador valioso. Assim, este estudo avaliou os efeitos tóxicos de Fe usando *C. elegans* analisando diferentes parâmetros, a fim de contribuir para a investigação da toxicidade induzida por Fe e validar este modelo visando, em última instância, a busca de alvos terapêuticos mais eficazes do que aqueles usado atualmente. Nosso estudo descreveu que a DL50 Fe em exposição aguda (30min) foi de 1.2 mM. Doses subletais de Fe diminuíram significativamente o tempo de vida dos vermes e sua capacidade reprodutiva em comparação com vermes não-expostos. Também foi observado que os animais expostos ao Fe diminuem a atividade locomotora e a sensibilidade mecânica, o que sugere uma possível disfunção do sistema

nervoso. Alterações na expressão de importantes enzimas antioxidantes e o aumento da peroxidação lipídica sugerem que o estresse oxidativo leva a danos neuronais, que podem ser a causa do comportamento alterado e dos demais efeitos encontrados.

Palavras-chave: toxicidade de ferro, estresse oxidativo, locomoção, exposição aguda, *Caenorhabditis elegans*

ABSTRACT

Dissertation of Master's Degree
Post-Graduation Program in Biochemistry
Federal University of Pampa

AVALIAÇÃO DOS EFEITOS NEUROTÓXICOS DO FERRO *IN VIVO* EM *Caenorhabditis elegans*

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Place and Date of Defense: Uruguaiiana, June 27th, 2014

Iron (Fe) is an important metal to the organism homeostasis and exists abundantly in the environment. Moderate levels of Fe obtained from food are necessary for normal cell physiology; however, abnormally high levels of Fe(II)/Fe(III) can be cytotoxic effects via reducing H₂O₂ to the highly cytotoxic hydroxyl radical (OH•) (Fenton catalysis). Consequently leading to oxidative stress. Fe is ubiquitous toxicant in the environment and also widely used in food products, however its effects to the nervous system is not well understood. Seeking for alternative experimental models that may substitute and offer new possibilities to assay xenobiotics toxicity, the nematode *Caenorhabditis elegans* has been found favorable as a valuable bioindicator organism. Hence, this study evaluated the toxic effects of Fe using *C. elegans* and investigating different parameters in order to contribute to the investigation of Fe-induced toxicity and to validate this model aiming, ultimately, the search for therapeutic targets that are more effective than those currently used. Our study depicted that the Fe LD₅₀ in acute exposure (30min) was 1.2 mM, as we verified that worms can uptake this metal. Furthermore, sublethal Fe concentrations decreased significantly the worms lifespan and brood size compared to non-exposed worms. We also observed that animals

exposed to Fe showed decreased locomotor activity and decreased mechanic sensitivity, which suggests possible dysfunction of the nervous system. In agreement, we found cholinergic and dopaminergic alterations in the worms. In summary, we suggest that iron exposure leads to damage of certain neurons, which could be the cause of altered behavior and of the defects of reproduction.

Keywords: iron toxicity, oxidative stress, locomotion behavior, acute exposure, *Caenorhabditis elegans*

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SUMÁRIO

INTRODUÇÃO

O ferro (Fe) é um metal importante para a fisiologia da célula aeróbica e existe em abundância no ambiente. Como micronutriente, o Fe desempenha um papel fundamental como componente dos complexos da cadeia respiratória mitocondrial (FARINA et al., 2013) e também é componente estrutural da catalase e de proteínas transportadoras ou de estoque de oxigênio (hemoglobina e mioglobina).

Níveis elevados de Fe pode causar efeitos tóxicos em muitos organismos (HU et al., 2008), como por exemplo a intoxicação aguda em crianças ou exposição crônica, especialmente a contaminação do ambiente. Positivamente, todo o Fe absorvido é armazenado ou transportado por proteínas e os níveis de Fe livre intra e extracelular são muito baixos. Porém, mesmo baixos níveis de Fe livre podem causar efeitos tóxicos em diferentes tipos de células (HU et al., 2008).

Sabe-se que o acúmulo de Fe provoca danos através da produção dos radicais livres pela reação de Fenton (AISEN; ENNS; WESSLING-RESNICK, 2001; FRAGA; OTEIZA, 2002), onde Fe^{2+} reduz o peróxido de hidrogênio para a forma altamente citotóxica, o radical hidroxila (OH •). De acordo, uma grande quantidade de dados da literatura o Fe é um importante fator etiológico associado à indução de estresse oxidativo e morte celular em situações patológicas (JOHNSTONE; MILWARD, 2010; JOMOVA; VALKO, 2011a; MESQUITA et al., 2012). Além disso o Fe parece estar envolvido na patogênese de algumas doenças neurodegenerativas, como Parkinson e Manganismo (CORDOVA et al., 2013; FARINA et al., 2013), pois a deposição de Fe tem sido observada em regiões do cérebro afetadas nestas doenças degenerativas crônicas (BARTZOKIS et al., 1997; PFEFFERBAUM et al., 2009; SCHIPPER, 2012).

Vários estudos mostram o *Caenorhabditis elegans*, um nematoide de vida livre do solo, como um modelo potencial para testes de toxicidade e ainda como método alternativo para substituir modelos animais (COLBERT; BARGMANN, 1997; WANG, D. Y.; WANG, 2008; GUO; YANG; WANG, 2009; LI et al., 2009). O *C. elegans* é um animal bem caracterizado em nível genético, fisiológico, molecular e de desenvolvimento (RIDDLE, D. L. et al., 1997). Foi o primeiro organismo multicelular a ter o seu genoma completamente sequenciado, e ainda possui um elevado nível de

conservação do seu genoma em relação aos mamíferos (BRENNER, 1974; BETTINGER et al., 2004)

As vantagens da sua utilização em relação aos modelos de invertebrados incluem ciclo curto de vida, tamanho pequeno, fácil cultivo e avaliação comportamental. O sistema nervoso de *C. elegans* é composto de 302 neurônios (WHITE et al., 1986) que podem ser especificamente marcados com proteína verde fluorescente (GFP) e observados por microscopia de fluorescência. Neste estudo, foram avaliados os efeitos tóxicos de Fe usando *C. elegans* através de diferentes parâmetros para melhor compreender a toxicidade induzida por Fe, bem como para validar este modelo para auxiliar a pesquisa de toxicologia ambiental e para o desenvolvimento de novos alvos terapêuticos que possam ser mais eficazes do que os atualmente usados, como deferroxamine, que possui toxicidade já constadada em muitos estudos (WHITTEN et al., 1965; WESTLIN, 1966; SUMMERS et al., 1979; OLIVIERI et al., 1986). Nossos resultados mostraram que o Fe é capaz de causar alterações na postura de ovos, longevidade e locomoção possivelmente como reflexo de danos no sistema nervoso, principalmente nos neurônios dopaminérgicos, mesmo em um curto período de tempo de exposição a concentrações de FeSO₄.

2. REVISÃO BIBLIOGRÁFICA

2.1. Ferro

O Ferro (Fe), pertence ao grupo VIII da tabela periódica e é um dos elementos mais abundantes na crosta terrestre (WEBER; ACHENBACH; COATES, 2006) e o mais abundante dos metais de transição da tabela periódica (WACHTERSCHAUER, 2007). Desempenha um papel indispensável no desenvolvimento de plantas e animais (FRAGA; OTEIZA, 2002), sendo requerido principalmente para o transporte de elétrons e de oxigênio (AISEN; ENNS; WESSLING-RESNICK, 2001).

O Fe é um mineral essencial para a constituição de diversas proteínas e enzimas, especialmente no grupamento heme da hemoglobina e dos citocromos. A deficiência

deste metal pode causar, por exemplo, uma séria disfunção no transporte de oxigênio pelas hemácias (anemia ferropriva), levando à fraqueza muscular, diminuição da capacidade respiratória e tontura (OLIVEIRA; OSORIO, 2005) por outro lado, o excesso de ferro pode causar danos ao coração, fígado, sistema endócrino e sistema nervoso (KOHGO et al., 2008; KALDARA-PAPATHEODOROU; TERROVITIS; NANAS, 2010). Esse acúmulo de Fe pode ser causado por excesso de ingestão do metal, exposição ocupacional ou por doença hereditária, mais conhecida por hemocromatose (RAMM; RUDELL, 2010).

Metais de transição, como Fe ou cobre (Cu), podem doar ou aceitar elétrons livres em reações intracelulares, catalisando a formação de radicais livres, como na reação de Fenton (Figura I). Visto que a maior parte do Fe intracelular está na forma férrica (Fe^{3+}), o metal é reduzido para a forma ferrosa (Fe^{2+}) para participar da reação de Fenton (Kumar, 2012). Quando a concentração intracelular de superóxido é elevada, a reação favorece a redução de Fe^{3+} a Fe^{2+} perpetuando a reação de Fenton e formando mais radicais hidroxila (CASTELLANI et al., 2007), o que no sistema nervoso central, por exemplo, pode levar ao estresse oxidativo e conseqüentemente a neurodegeneração (NUNEZ et al., 2012), uma vez que este metal se deposita em áreas específicas do cérebro, principalmente no *globus pallidus* (SCHIPPER, 2012).

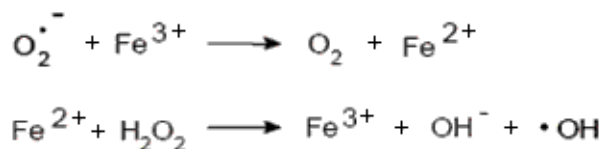


Figura I: Reação de Fenton. Fonte: Krishnamoorthy et al., 2009

Além do estresse oxidativo desencadeado pela reação de Fenton, uma quantidade elevada de Fe pode ser liberada em regiões específicas do cérebro após hemorragia local causada por trauma cerebral ou acidente vascular cerebral (HALLIWELL, 1992; WAGNER et al., 2003; CARBONELL; RAMA, 2007). Após o episódio hemorrágico, eritrócitos são liberados no interior do parênquima cerebral ocorrendo em seguida a hemólise, hemoglobina, heme e Fe são então libertados no espaço extracelular do cérebro, causando uma sobrecarga local de Fe (HALLIWELL, 1992).

Como detalhado anteriormente, o Fe é altamente abundante no ambiente e sua exigência para o bom funcionamento do corpo humano é normalmente excedida após a ingestão. A fim de evitar a sobrecarga de Fe, a absorção de Fe é estreitamente regulada por uma complexa e ainda não totalmente compreendida interação entre carga corporal de Fe e mecanismos de absorção gastrointestinal (DE DOMENICO; MCVEY WARD; KAPLAN, 2008; NUNEZ et al., 2012). Esta regulação é extremamente importante, pois não há processos celulares para excreção do metal (DE DOMENICO; MCVEY WARD; KAPLAN, 2008; FINBERG, 2011; FLEMING; PONKA, 2012; MESQUITA et al., 2012). Muitos genes ortólogos aos humanos já foram identificados em *C. elegans* (SHAYE; GREENWALD, 2011) e muitos dos principais genes e vias que regulam o metabolismo do ferro em mamíferos são conservados neste animal. Dada a natureza fundamental do metabolismo do ferro, não é de estranhar que muitas proteínas envolvidas na absorção intestinal de ferro, armazenamento e exportação sejam altamente conservadas entre *C. elegans* e mamíferos (ANDERSON, C. P.; LEIBOLD, 2014)(Figura II).

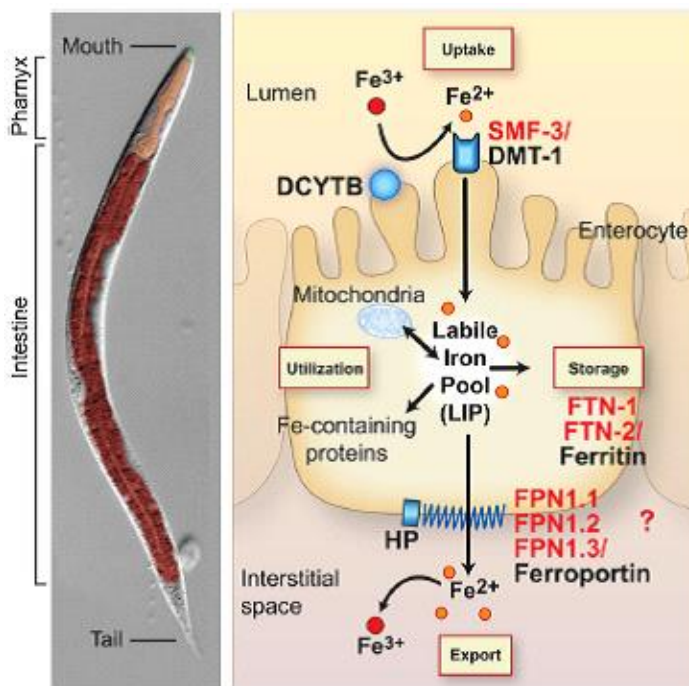


Figura II: *C. elegans* expressa genes ortólogos para DMT1 (SMF-3), ferritina (FTN-1, FTN-2) e ferroportina (FPN-1.1, FPN-1.2, FPN-1.3). SMF-3 é o principal transportador de Fe (II) no intestino do *C. elegans*. Fonte: Anderson & Leibold (2014)

2.2. Estresse oxidativo e Fe

O oxigênio apresenta um papel essencial em nosso organismo, mas também um papel tóxico (Marks, 2006). Durante a respiração mitocondrial, o oxigênio molecular é reduzido em água pelas células, para a formação de energia, produzindo concomitantemente pequenas quantidades de radicais livres (KUMAR, 2012). Através de processos enzimáticos e não enzimáticos que ocorrem normalmente na célula, o oxigênio aceita elétrons livres e se transforma em radicais de oxigênio altamente reativos: H_2O_2 , O_2^- .

As espécies reativas de oxigênio (ERO) possuem um papel importante em seres vivos, um exemplo de suas funções no organismo é na resposta imune a infecções, além disso desempenham um papel importante na sinalização intracelular (RAY; HUANG; TSUJI, 2012). Por outro lado, podem ser extremamente tóxicos, danificando lipídios, proteínas, DNA e RNA celulares, levando a várias formas de lesão celular. Porém, as células possuem sistemas de defesa (proteínas quelantes de metais, enzimas de defesa, antioxidantes) para prevenir lesões causadas por ERO.

Um desequilíbrio entre a taxa de geração e a capacidade de remoção celular de radicais livres causa um estresse oxidativo, que pode ser causa direta de uma patologia ou estar associado a uma forma de perpetuar o dano celular causado por outro processo patológico (MARKS, 2006). Metais de transição, como ferro ou cobre, podem doar ou aceitar elétrons livres durante reações intracelulares, catalisando a formação de radicais livres, como na reação de Fenton (Figura I). Visto que a maior parte do Fe intracelular está na forma férrica (Fe^{3+}) ele primeiro precisa ser reduzido para a forma ferrosa (Fe^{2+}) para participar da reação de Fenton (KUMAR, 2012). No estado metabólico normal, o superóxido favorece a oxidação de Fe^{2+} a Fe^{3+} . No entanto, se a concentração intracelular de superóxido é elevada, a reação favorece a redução de Fe^{3+} a Fe^{2+} , perpetuando a reação de Fenton e formando mais radicais livres (CASTELLANI et al., 2007).

2.3. Ferro e neurodegeneração

Uma classificação clara de neurodegeneração pode basear-se nas principais alterações neuropatológicas, ou seja, presença de componentes proteicos anormais que

se acumulam no cérebro, levando à perda neuronal, dependentes da idade (FERNANDEZ, 2007).

Um mecanismo comum no desenvolvimento de processos neurodegenerativos é a presença de alteração na conformação de proteínas (por oxidação protéica ou dano oxidativo de RNA), gerando estruturas intermediárias, que formam oligômeros solúveis (considerados os mais tóxicos), que posteriormente agregam-se, formando protofibrilas e por fim fibrilas que são consideradas marcadores de neurodegeneração. Tanto a agregação proteica como o estresse oxidativo, presentes nessas patologias, estão associados com a presença de metais (CASTELLANI et al., 2007; MATTSON, 2007; MOLINA-HOLGADO et al., 2007). Segundo La Spada e Ranum (2010) as doenças neurodegenerativas têm se tornado cada vez mais prevalentes em relação às gerações passadas e por isso são de grande importância epidemiológica. A neurodegeneração é o componente principal do Mal de Parkinson e do Mal de Alzheimer, assim como de outras doenças nas quais os neurônios motores estão envolvidos (ESPOSITO e CUZZOCREA, 2012). Estudos epidemiológicos indicam que tais doenças possuem origem genética e ambiental (PENG e ANDRESEN, 2010; ALTAMURA e MUCKENTHALER 2009), em relação aos fatores ambientais, diversos estudos demonstram que desequilíbrios na homeostase do Fe são um evento importante na fisiopatogenia da doença de Parkinson e da doença de Alzheimer (BARTZOKIS et al., 1997; GORELL et al., 1997; JELLINGER, 1999; JOHNSTONE; MILWARD, 2010; JOMOVA; VALKO, 2011b; MESQUITA et al., 2012).

O Fe pode ser extremamente tóxico devido a sua capacidade redox, formando ERO, tais como o radical hidroxila (OH). A formação de OH[•] pode danificar diferentes biomoléculas e iniciar um ciclo vicioso de danos celulares (FARINA et al., 2013). Além disso, o par redox Fe²⁺/Fe³⁺ serve como um iniciador in vivo de reações citotóxicas, particularmente, peroxidação lipídica (RYAN et al., 1990; WELCH et al., 2002). No que diz respeito à neurodegeneração, uma vasta quantidade de dados indica que o Fe é um importante fator etiológico associado a indução do estresse oxidativo e morte celular em situações patológicas (GORELL et al., 1997; MESQUITA et al., 2012). Quando ocorre um excesso ou desregulação na homeostase do Fe em áreas cerebrais relevantes, o dano oxidativo induzido por Fe aumenta consideravelmente, levando a processos neurodegenerativos com consequente morte neuronal (SAYRE et al., 2005; BERG; YODIM, 2006).

Tem sido proposto Fe que poderia ser um fator primário e unificador envolvido

na progressão de diferentes doenças neurodegenerativas crônicas, como a doença de Parkinson, a doença de Alzheimer e a doença de Huntington (KELL, 2010). No entanto, a relação entre a deposição de Fe e a neurodegeneração ainda não foi claramente estabelecida. Assim, em algumas doenças, a deposição de Fe pode ser a consequência e não a causa da neurodegeneração. A deposição de Fe foi observada apenas em regiões específicas do cérebro em pacientes com doenças crônico-degenerativas (SIAN-HULSMANN et al., 2011; KUMAR et al., 2012; ROSAS et al., 2012). Os núcleos basais representam um local preferencial de deposição de Fe nas doenças neurodegenerativas (GREGORY; HAYFLICK, 2011; AKATSU et al., 2012)

2.4. *Caenorhabditis elegans*

Apesar do Fe ser um metal bastante estudado, pouco se sabe sobre quais os grupos de neurônios especificamente afetados pela sua toxicidade. Sendo os modelos utilizados roedores e outros mamíferos muito complexos, particularmente em relação ao sistema nervoso, modelos mais simples, que reproduzam os efeitos observados em modelos superiores e que estejam em acordo com a política 3R (reduzir, refinar, reusar) têm sido cada vez mais valorizados pela comunidade científica. Na literatura estão presentes diversos estudos sobre neurodegeneração causada por metais utilizando *Caenorhabditis elegans* como modelo experimental (HU et al., 2008; BENEDETTO et al., 2010; VANDUYN et al., 2010)

O *C. elegans*, é um nematoide de vida livre que habita a parte líquida do solo, alimentando-se de micróbios, especialmente bactéria (RIDDLE, D. L. et al., 1997). Em 1965, Sydney Brenner (1974) estabeleceu seu uso como um modelo para estudar desenvolvimento e comportamento animal por motivos hoje bem conhecidos: o rápido ciclo de vida (Figura III) e de reprodução, a grande capacidade reprodutiva, o pequeno tamanho (até 1,5mm quando adulto), a transparência, o fácil cultivo e manuseio no laboratório e grande potencial para análises gênicas (BRENNER, 1974). Estima-se que 60%-80% dos genes humanos possuem homólogos em *C. elegans* (KALETTA; HENGARTNER, 2006). Sendo este o ser vivo mais utilizado para estudos de biologia do desenvolvimento, genética, envelhecimento e de ecotoxicologia (SCHIERENBERG; WOOD, 1985). Devido ao pequeno genoma, com o avanço de novas tecnologias, foi possível clona-lo e mapeá-lo inteiramente, revelando a alta homologia com o genoma humano, também foi possível desenvolver técnicas de marcação de proteínas, microcirurgias laser e o RNA interferente (WHITE et al., 1986).

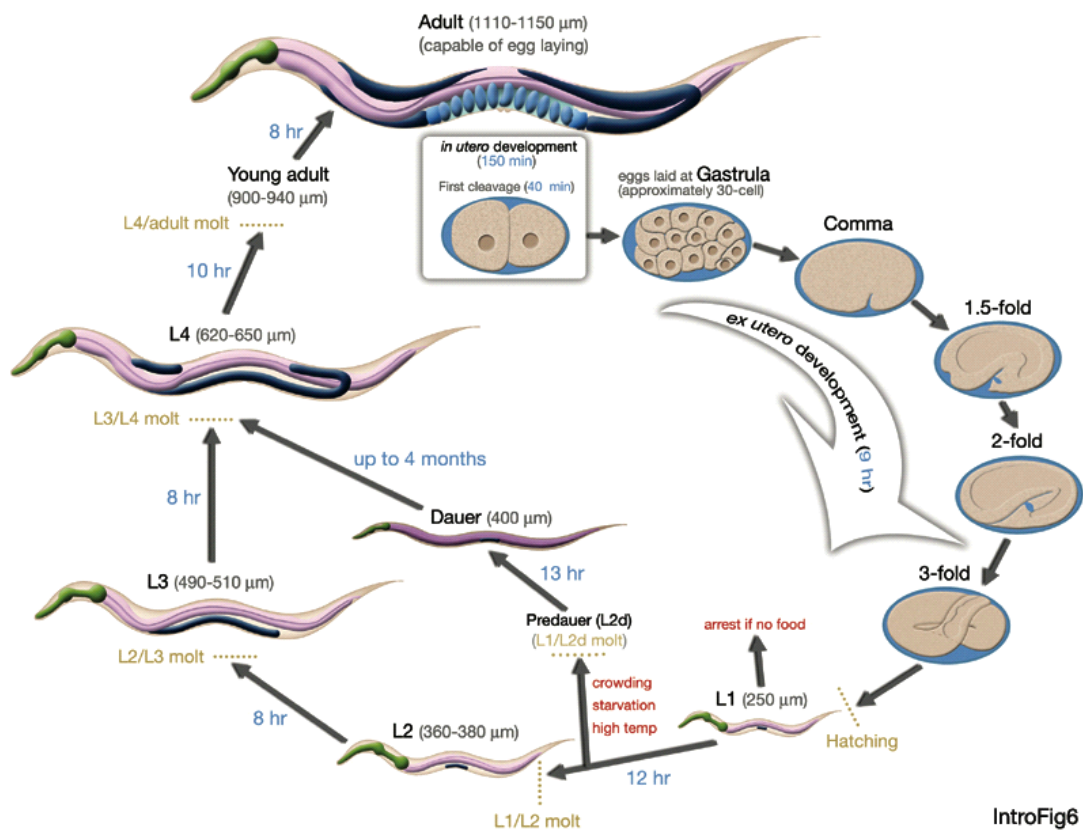


Figura III Ciclo de vida do *C. elegans*. Fonte: Wormatlas

Em contraste com os estudos com células livres e cultura de células, o *C. elegans* permite a investigação dentro do contexto de um organismo completo, com diferentes células funcionando em consonância com diferentes sistemas (KALETTA; HENGARTNER, 2006).

Além disso, trabalhando com proteína fluorescente extraída de águas-vivas, Martin Chalfie (1994), construiu cepas de *C. elegans* expressando proteínas marcadas com este fluoróforo, possibilitando a visualização in vivo de neurônios (Figura IV), *trafficking* de proteínas, atividade enzimática e migração de fatores de transcrição, por exemplo. Essa marcação com GFP proporcionaria a observação in vivo de neurodegeneração após a exposição a um agente neurotóxico como o Fe.

Com 302 neurônios bem caracterizados (Hobert, 2005), o *C. elegans* proporciona um modelo in vivo para estudos de mecanismos de danos neuronais. Portanto defeitos na locomoção refletem problemas na rede neuronal.

Estudos prévios demonstram que a exposição aguda ao manganês causa degeneração específica de neurônios dopaminérgicos, efeito que pode ser constatado utilizando esse modelo (BENEDETTO et al., 2010). Similarmente, neurônios dopaminérgicos também são degenerados na presença de metilmercúrio, entretanto tal efeito só é observado em animais nos quais o gene que codifica o fator de transcrição SKN-1 sofreu uma mutação tipo deleção (VANDUYN et al., 2010). Já foi reportado que o Fe causa danos reprodutivos e aumenta a mortalidade de animais expostos a 200 μM por 36 horas em *C. elegans*, (HU et al., 2008), entretanto, ainda não há evidências concretas na literatura de quais neurônios são afetados pela exposição ao Fe.

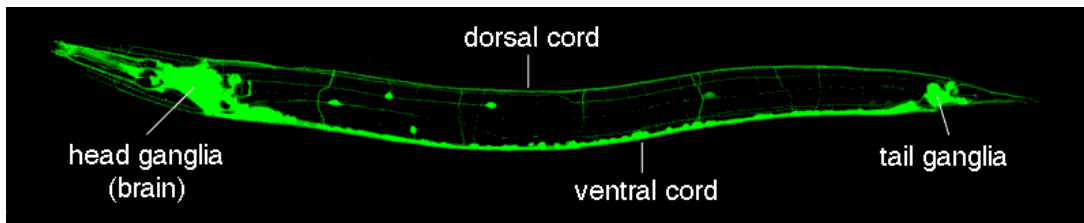


Figura IV: Sistema nervoso do *C. elegans*, todos os neurônios marcados com GFP. Fonte: Simon Fraser University

3. JUSTIFICATIVA

O Fe tem sido descrito como um elemento importante na patogênese de algumas doenças neurodegenerativas. O entendimento do seu metabolismo e das disfunções relacionadas ao estresse oxidativo é fundamental para desvendar a fisiopatologia de doenças neurodegenerativas, como Doença de Parkinson e Demência de Alzheimer, cada vez mais prevalentes no nosso meio devido ao aumento da expectativa de vida. Sendo assim o uso de *C. elegans*, especialmente de mutantes que expressam GFP em várias classes de neurônios, poderia contribuir para a investigação de neurotoxicidade induzida por Fe e para validar um modelo no qual se possa procurar alvos terapêuticos que sejam mais efetivos do que os que são hoje utilizados, os quais envolvem o uso de agentes quelantes que muitas vezes pode causar toxicidade como a desferroxamina.

4. OBJETIVOS

4.1. Objetivo geral

Avaliar os efeitos neurotóxicos do ferro *in vivo* utilizando *C. elegans* através de parâmetros como comportamento, postura de ovos, longevidade, estresse oxidativo.

4.2. Objetivos específicos

Determinar a dose letal 50% do Fe (na forma de sulfato ferroso, FeSO_4) utilizando uma exposição aguda (30 minutos) em *C. elegans*;

Verificar possíveis efeitos do Fe sobre parâmetros como: postura de ovos e longevidade;

Avaliar alterações comportamentais causadas pela toxicidade induzida por Fe em *C. elegans*;

Quantificar, através de cepas fluorescentes, os níveis de superóxido dismutase e catalase
Determinar, *in vivo*, a toxicidade neuronal do Fe utilizando microscopia de fluorescência;

Mensurar os níveis de peroxidação lipídica pelo método de TBARS

Quantificar os níveis de ferro absorvidos pelos animais expostos ao metal

5. ARTIGO CIENTÍFICO

Os resultados que fazem parte desta dissertação estão apresentados sob a forma de artigo científico. As seções *Materiais e Métodos*, *Resultados*, *Discussão dos Resultados* e *Referências Bibliográficas* encontram-se no próprio manuscrito. O manuscrito está apresentado da mesma forma que foi submetido à Revista **Neurotoxicology**.

Oxidative and neuronal damage induced by acute iron toxicity in *C. elegans*

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Abstract

Iron (Fe) is an important metal to the organism homeostasis and exists abundantly in the environment. Moderate levels of Fe obtained from food are necessary for normal cell physiology; however, abnormally high levels of Fe(II)/Fe(III) can be cytotoxic effects via reducing H₂O₂ to the highly cytotoxic hydroxyl radical (OH•) (Fenton catalysis). Consequently leading to oxidative stress. Fe is ubiquitous toxicant in the environment and also widely used in food products, however its effects to the nervous system is not well understood. Seeking for alter native experimental models that may substitute and offer new possibilities to assay xenobiotics toxicity, the nematode *Caenorhabditis elegans* has been found favorable as a valuable bioindicator organism. Hence, this study evaluated the toxic effects of Fe using *C. elegans* and investigating different parameters in order to contribute to the investigation of Fe-induced toxicity and to validate this model aiming, ultimately, the search for therapeutic targets that are more effective than those currently used. Our study depicted that the Fe LD₅₀ in acute exposure (30min) was 1.2 mM, as we verified that worms can uptake this metal. Furthermore, sublethal Fe concentrations decreased significantly the worms lifespan

and brood size compared to non-exposed worms. We also observed that animals exposed to Fe showed decreased locomotor activity and decreased mechanic sensitivity, which suggests possible dysfunction of the nervous system. In agreement, we found cholinergic and dopaminergic alterations in the worms. In summary, we suggest that iron leads to damage of certain neurons, which could be the cause of altered behavior and of the defects of reproduction.

Keywords: iron toxicity, oxidative stress, locomotion behavior, acute exposure, *Caenorhabditis elegans*

1. INTRODUCTION

Iron (Fe) is an important metal to aerobic cell physiology and exists abundantly in the environment. As a micronutrient, iron plays a fundamental role as a component of mitochondrial respiratory chain complexes (FARINA et al., 2013). Consequently, iron deficiency can be associated with abnormal neuroembryogenesis, myelination and metabolism of biogenic amines. Abnormally high levels of Fe can cause toxic effects in many organisms (HU et al., 2008); through acute intoxication in children or chronic exposure, especially to environmental contamination. Iron is also a structural component of catalase and oxygen storing or transporting proteins (myoglobin and hemoglobin). Optimistically, all the absorbed iron is bound to storage or transporting proteins and the levels of intra and extracellular free iron is very low. Indeed, even low levels of free Fe can cause toxic effects in different types of cells (HU et al., 2008). In accordance, a vast amount of literature data indicates that Fe is an important etiologic factor associated with oxidative stress induction and cell demise in pathological situations (JOHNSTONE; MILWARD, 2010; JOMOVA; VALKO, 2011a; MESQUITA et al., 2012; WU, J.; DING; SUN, 2012). Iron-induced oxidative stress is thought to be involved in the pathogenesis of some neurodegenerative diseases, such as

Parkinson and Manganis(CORDOVA et al., 2013; FARINA et al., 2013) because iron deposition has been observed in brain regions disrupted in these chronic degenerative diseases (BARTZOKIS et al., 1997; PFEFFERBAUM et al., 2009; SCHIPPER, 2012)

It is known that iron accumulation causes free radical damage through the Fenton reaction (AISEN; ENNS; WESSLING-RESNICK, 2001; FRAGA; OTEIZA, 2002), as Fe²⁺ reduces hydrogen peroxide to the highly-cytotoxic hydroxyl radical (OH•) (Fenton catalysis). Mitochondria is the main site of superoxide production and an important site of Fe metabolism. Hence, the continue Fe influx renders this organelle susceptible to the oxidative effects of Fe. In mitochondria, Fe can trigger different cell death pathways and lead to ferroptosis, a form of cell death very different from necrosis or apoptosis(DIXON et al., 2012). In this context, it is very important to evaluate the acute toxicity of Fe and oxidative stress-related mechanisms. Several studies show *Caenorhabditis elegans*, a free-living soil nematode, as a model for toxicity testing as potential alternative method to substitute for animal models (COLBERT; BARGMANN, 1997; ANDERSON, G. L.; COLE; WILLIAMS, 2004; WANG, D. Y.; WANG, 2008; GUO; YANG; WANG, 2009; LI et al., 2009; WU, Q. et al., 2013). *Caenorhabditis elegans* is well-characterized animal at the genetic, physiological, molecular, and developmental levels (RIDDLE, D. L., BLUMENTHAL, T., MEYER, B.J., PRIESS, J.R, 1997). It was the first multicellular organism to have its genome completely sequenced, which has been found to have a high level of conservation with the vertebrate genome (BRENNER, 1974; HOPE, 1999; BETTINGER et al., 2004).

The worm advantages in relation to invertebrate models include short life cycle, small size, easy cultivation and behavioral assessment. The nervous system of the *C. elegans* is composed of 302 neurons (WHITE et al., 1986) which can be specifically marked with green fluorescent protein (GFP) and observed under fluorescence microscopy. In this study, we evaluated the toxic effects of Fe using *C. elegans* by assessing different endpoints of toxicity. Indeed, our goal was to contribute to better understand iron-induced neurotoxicity as well as to validate this model to assist the research of environmental toxicology and for developing new therapeutic targets that could be more effective than those currently used such as deferoxamine, which has properties that diminish its usefulness as its acute (WHITTEN et al., 1965; WESTLIN, 1966) and chronic (OLIVIERI et al., 1986) toxicity limiting the dose that can be safely used and it has a very short plasma half-life (SUMMERS et al., 1979). We hypothesized that Fe may be able to cause changes in egg laying and longevity, locomotion, and even

lead to oxidative stress and neurodegeneration even in a short time exposure to iron(II) (III).

2. MATERIALS AND METHODS

Chemicals and Strains

All *C. elegans* used were originally obtained from the Caenorhabditis Genetics Center (CGC). They were maintained on nematode growth medium (NGM) plates seeded with *Escherichia coli* OP50 at 20°C (BRENNER, 1974). Gravid *C. elegans* were washed off the plates into centrifuge tubes and were lysed with a bleaching mixture (0.45N NaOH, 2% HOCl) to obtain a synchronous populations of L1 (first larval stage) after \pm 14 hours, when eggs hatched (AL-REFAIE et al., 1995). Iron sulfate (FeSO₄), bacto-agar, bacto-peptona and other reagents were obtained from Sigma Aldrich.

Fe exposure

In the absence of bacteria, one thousand and five hundred L1 worms previously synchronized were exposed for 30 min to Fe concentrations of 0.05; 0.1; 0.5; 1.0; 1.5 and 2.0 mM in liquid media containing 0.5% NaCl. After exposure, worms were washed 3 times with saline 0.05% and then transferred to NGM recovery plates inoculated with *Escherichia coli*/OP50 to posterior assays.

LD50 determination

To determine the lethal concentration of FeSO₄, worms were exposed as described above and the alive worms were counted 24 hours after exposure. The lethality was evaluated by normalizing the data as percentage of control. Three replicates were performed.

Iron levels measurement by GFAAS

After iron exposure (triplicates), samples with 10.000 worms were treated as described above. After six washes with saline 0.05% worms were frozen and 24hs later they were dried at 80°C for 4h. Then it was added 250 µL of nitric acid (P.A) and samples were left in water bath at 70° C for 1h. The Fe levels were quantified by Graphite Furnace Atomic Absorption Spectrometry (GFAAS).

Reproduction

To evaluate the iron effects on reproduction, three worms from each Fe treatment were transferred separately to new NGM/OP50 plates every day. The eggs were counted daily until the end of the reproductive period (four days).

Life span

Twenty L4 worms from each group were transferred to new NGM/OP50 plates every day and scored until all animals were dead. The nematodes were considered dead if they did not respond to a mechanical stimulus using a small wire (WU, Q. et al., 2012). All the experiments were made in duplicates and repeated at least three times.

SOD and CAT quantification

The GFP expressing strains GA800,wuIs151[(ctl-1+ctl-2+ctl-3;myo-2::GFP)] and CF1553,muIs84([Psod-3::GFP]) worms were treated at the L1 stage. Worms were placed in NGM/OP50 and 48h after they were washed with M9 buffer. The worms were then transferred to microtubes. The Escherichia coli was washed until exhaustion (three dilutions of 1:10). Worms were placed into 200 µL of M9 buffer in a well of 96-well plate. Total GFP fluorescence was measured using 485 nm excitation and 530 nm emission filters in a plate reader (CHAMELEON™V Hidex Model 425-106). All experiments were made in duplicates and repeated at least five times.

Lipid peroxidation – Thiobarbituric acid assay

Worms treated at the L1 stage were placed in NGM/OP50 and 48h after they were washed with M9 buffer and then transferred to microtubes. The *Escherichia coli* was washed until exhaustion, the worms were frozen 2x and sonicated 2x por 10s on ice at 100% amplitude. We incubated 60 μ L of sample with 60 μ L of phosphoric acid (0,1%), 60 μ L of TBA (0,6%) and 60 μ L of SDS (0,6%) for 1.5h at 100°C as described by Okawa (1979). Absorbance measurements were performed in 96-well plate (532nm). The data was corrected by protein content that was measured as described by Bradford, 1974.

Head thrash frequency

The assays were performed 48 hours after iron exposure. Individual worms were transferred to a NGM/*E. coli* free plate. After 1 min recovery period, the head thrashes were counted for 1 min. A thrash was defined as a change in the direction of bending at the mid body (HU et al., 2008). We analyzed five worms from each group considered the mean number of head thrashes per experiment. Each experiment was repeated 3 times.

Nose touch

The method was performed as previously described (MURAKAMI; MURAKAMI, 2005) and worms were analyzed 48hs after Fe exposure. Three basic movements, forward sinusoidal movement (forward turns), reversal movement (backward turns), and turns in which nematodes change direction (Omega/U turns) of bodies in a 30-s interval were measured. In Omega turns, a young adult nematode's head touches the tail and its shape looks similar to the shape of Greek letter Omega, whereas the angle of the body bend is typically $> 90^\circ$ in U turns (WANG, D.; XING, 2008). We analyzed 5 worms of each group at every experiment and calculated the average per experiment. Each experiment was repeated 3 times.

Egg-laying induced by Levamisole

After iron exposure, worms were transferred to plates with levamisole (1mM), an anthelmintic which functions as a cholinergic agonist, for 30 minutes. The occurrence of whole-worm hypercontraction and laid eggs were counted (ESTEVEZ et al., 2012). Worms with functional neurotransmitter release should be sensitive to the substance and become paralysed due to muscle hypercontraction (ESTEVEZ et al., 2012). Resistance to levamisole can typically indicate a post-synaptic defect.

Dopaminergic neurons images

BY200 (Pdat-1::GFP) L4 later exposed animal were transferred to agarose (2%) pads in M9 with 22.5 μ M of levamisole. Image acquisitions were carried out with an epifluorescence microscope in an appropriate room (20-22°C).

Statistical analysis

All statistical analysis and figures were generated with GraphPad Prism (GraphPad Software Inc.). We used a sigmoidal dose-response model with a top constraint at 100% to draw the curves and determine the LD50 value reported in the graph. Statistical analysis of significance was carried out by one-way ANOVA (for more than 2 groups) followed by Tukey post-hoc test when appropriated. For longevity, a repeated measure ANOVA was used in order to analyze the whole curve. It was considered significant when $p \leq 0.05$. Error bars represent standard error of mean (SEM). For the quantification of neurons fluorescence we used the software ImageJ and then transferred the data to GraphPad.

RESULTS

Iron acute exposure in *C. elegans* decreases worms survival and reproduction

The concentration response curve analysis indicated that the LC₅₀ for FeSO₄ was 1.2 mM for N2 *C. elegans* (Figure 1). After this assay, we selected the concentrations of 0.05mM, 0.1mM and 0.5mM of FeSO₄ to study the iron toxicity. All the tested concentration of Fe significantly reduced worms lifespan compared to control (Figure 2, p<0.05). In addition, we observed that animals exposed acutely to iron show decrease in total brood size (Figure 3, p<0.05).

Fe levels increased in a concentration-dependent manner

The Fe was measured to verify whether worms were absorbing the metal as concentrations were increased. Figure 4 shows that Fe deposition in *C. elegans* increased as a function of iron concentration with a plateau reached at about 1mM of the metal.

Iron exposure is associated with oxidative stress in *C. elegans*

Typically, antioxidant enzymes play a detoxifying role in the normal homeostasis for redox balance. CAT levels were significantly decreased at L4 stage at the 0.05 mM concentration as well SOD levels (Table 1). In agreement, worms exposed to Fe depicted a higher lipid peroxidation in comparison to untreated worms (Figure 5).

Changes in nematodes behavior can indicate neuronal damage

Figure 8 shows that there was a significant decrease in locomotion (Figure 6A, p<0.05) and in the nose touches necessary to desensibilize the mechanic sensory neurons (Figures 6B and C, p<0.05) 48hs after acute iron exposure. In the levamisole paralysis assay iron also showed decrease the muscular contraction as seen by the egg liberation (Figure 7).

Dopaminergic neurons are sensitive to iron acute exposure

Here, we investigated possible dopaminergic neurons damage using a transgenic strain BY200 (*dat-1::GFP(vtIs1)*) which expresses the green fluorescent protein in dopaminergic neurons. After acute exposure to Fe a dose-dependent neuronal damage was observed, the degeneration is observed by discontinued and punctuated GFP fluorescence (Figure 8).

DISCUSSION

Fe is an essential micronutrient for different types of living cells, when present bound to specific iron proteins. However, even small concentrations of free iron can cause several cytotoxic effects. Fe is needed for mammals homeostasis (recommended daily allowance for humans is 10-15mg Fe and at higher levels (20-30 mg Fe) maybe it come toxic (HALLIWELL; GUTTERIDGE, 1984; HUSTER, 2010). Several experimental studies on Fe toxicity have been investigated in mice, rats, and humans (LU; HAYASHI; AWAI, 1989; ZHANG et al., 2006), however these models are complex and have long life span. In the current work, we analyzed toxicological endpoints to evaluate Fe toxicity in *C. elegans*. Remarkably, we found that acute Fe exposure in worms caused alterations in survival, life span and reproduction; furthermore, animals showed decrease in motor function and in mechanic sensitivity, which strongly suggest that Fe caused neuronal damage via induction of oxidative stress.

In the present study, we used acute oral exposure to Fe in first larval staged worms. It has been shown that L1 worms are more sensitive to the reproductive toxicity than young adult nematodes, and may be more sensitive to reproductive and all others endpoints of toxicity (GUO; YANG; WANG, 2009). It seems likely that nematodes cuticle should protect them from the exposure of toxicants. Thus, oral exposure might be the best way for Fe absorption and its toxicity, in the liquid the nematodes are forced to swim and drink the dissolved iron continuously (HU et al., 2008). We confirmed Fe absorption in the treated worms by GFAAS quantification, and the total content of iron in the worms increased to a maximal value about 1 mM of FeSO₄. This may indicate that the worms can regulate the intake of iron through feeding or alterations in the divalent metal transporters or metallothioneins.

When exposed to stress, nematodes can alter reproductive performance, life cycle, and other properties (SWAIN et al., 2004). We observed that worms showed a decreased egg-laying following Fe exposure, which may indicate delayed development of animal gonads, as demonstrated in other studies with other metals/compounds (HU et al., 2008; ABDELHAMID; WU, 2012).

Taking into account that the reproductive system is closely linked to longevity in *C. elegans* (WANG, D.; XING, 2008) we verified that lifespan was also reduced in Fe-exposed worms when compared to controls. In this line, lifespan is regulated by the prooxidant/antioxidant homeostasis in worms. Fe is a classic inducer of reactive oxygen species in vitro and in vivo, via the Fenton reaction. Fe (II) is oxidized by H₂O₂ to Fe(III) generating the highly reactive hydroxyl radical OH· (HALLIWELL; GUTTERIDGE, 1984; GUTTERIDGE; HALLIWELL, 2000). The iron-catalyzed Fenton reaction is a major source of OH· in biological systems (FRIDOVICH, 1978; KEYSER; GORT; IMLAY, 1995) though other transition metals (e.g. copper) can also catalyze this reaction. In agreement, we observed that iron increased lipid peroxidation production, reduced lifespan and also affected reproduction.

The fidelity of the metabolic redox reactions (BABCOCK & WIKSTROM, 1992) and the sequestering of iron in ferritin and transferrin (REIF, 1992; THEIL 1987) generally minimize the burden from reactive oxygen species. Active oxygen species produced by iron/H₂O₂ are also removed by superoxide dismutases (SODs), catalases and peroxidases that catalyze the reduction of H₂O₂ by organic reducers such as glutathione, ascorbate, and cytochrome *c*. The major source of protection would appear to be SOD. Since superoxide dismutation generates H₂O₂, the detoxifying effect of SOD is most likely a result of preventing the accumulation of free Fe (II) and peroxynitrite production. The activity of catalase is essential to reduce H₂O₂ produced by SOD. In our experimental design, both SOD and catalase levels were reduced, contributing to iron-induced oxidative stress.

Because of environmental insults, an organism's behavior can change. In invertebrates, changes in movement are the most common endpoints for determining behavioral effects after toxic exposures (ANDERSON, G. L.; COLE; WILLIAMS, 2004). In *C. elegans* neuronal functions once committed directly reflect changes in behavior, locomotion and mechanosensation were impaired in worms exposed to Fe, even though worms were recovering for 48h off exposure.

Indeed, we have found that dopaminergic neurons marked with GFP had decreased fluorescence after Fe exposure, indicating some disturbance in these neurons. Furthermore, we also observed the functioning of cholinergic neurons in the presence of levamisole, which is an acetylcholine receptor agonist. A reduction in the number of eggs indirectly indicates a possible disruption in the cholinergic system (MAHONEY; LUO; NONET, 2006). Exposure to the antihelminthic levamisole has been reported in *C. elegans* to cause rapid paralysis due to hyper-contraction of body wall muscles (LEWIS et al., 1980) and to induce egg-laying due to contraction of vulval muscles in wild-type (WT) animals (TRENT et al., 1983). Notably, Fe exposure still caused reduction in egg-laying, indicating that cholinergic neurons may also be affected by this metal.

Some studies show that Fe is involved in age-related diseases like Alzheimer's and Parkinson's (CASTELLANI et al., 2007; ALTAMURA; MUCKENTHALER, 2009; LIU et al., 2011; WAN et al., 2011). These diseases have as characteristics the neurodegeneration and accumulation of Fe, as observed in the post-mortem analysis of patients (SCHIPPER, 2012; BARTZOKIS, 1997; PFEFFERBAUM, 2009).

Taken together, our results show that Fe toxicity can cause severe damage in neuronal function, as demonstrated by behavioral alterations in *C. elegans* and neurons images. Furthermore, our work shows that the nematode is a valuable model to study Fe toxicity in depth in order to understand this metal toxicity, which is widely ingested by the population and still considered of low toxicity.

CONCLUSIONS

Our data suggest that the Fe exposure results in multiple biological defects in *C. elegans*, including reproductive and motor impairment, what could be related to oxidative stress and neuronal damage. In this line, more studies are interesting especially to know if these effects could occur to low concentrations of Fe in chronic exposure and the role of iron in neurodegenerative diseases.

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CONFLIC OF INTEREST DISCLOSURE

There was no conflict of interest in the preparation of this manuscript.

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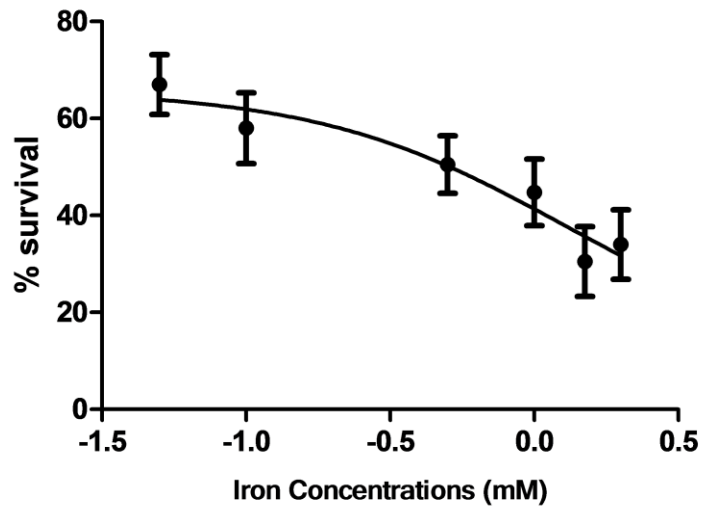


Figure 1: Concentration-dependent response curve for LC50 determination after acute exposure (30 min). The lethality was evaluated by the endpoint of percentage of survival animals. Three replicates were performed.

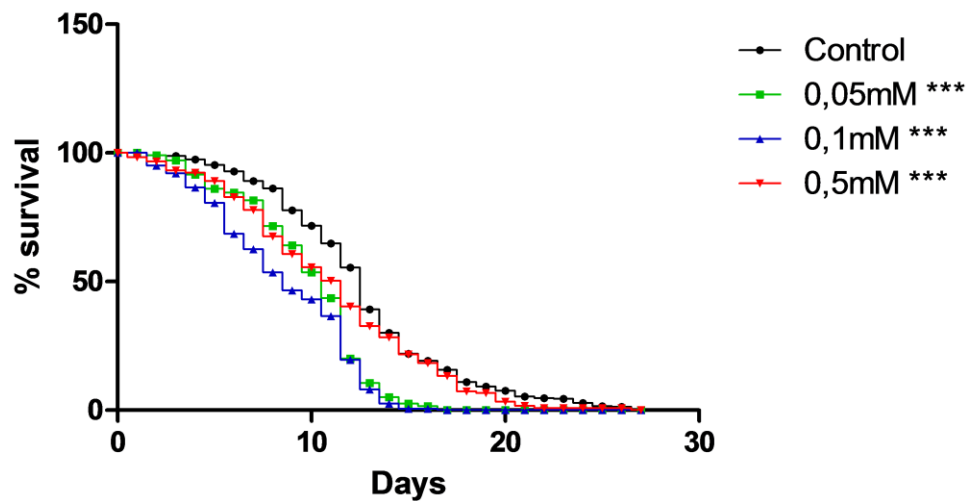


Figure 2: Iron decreases life span in *C. elegans*. Twenty aged L4 (exposed to Fe when at L1 stage) from each group were transferred to a new NGM/OP50 every day and scored until all animals were dead. All concentrations decreased lifespan compared to control (n=3). *** indicates statistical difference from control group by one-way ANOVA following by Tukey's post-hoc test ($p < 0.0001$).

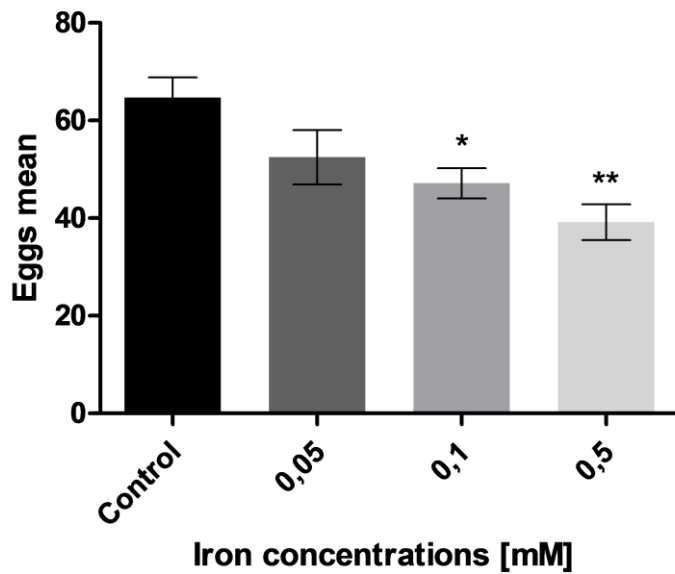


Figure 3: Iron effects on *C. elegans* brood size. The experiments were performed by scoring for four days the eggs layed by individual worms until the end of reproductive period. Each bar represents mean \pm S.E.M (n=3). * indicates statistical difference from control group by One way ANOVA followed by Tukey's post-test (p<0.05).

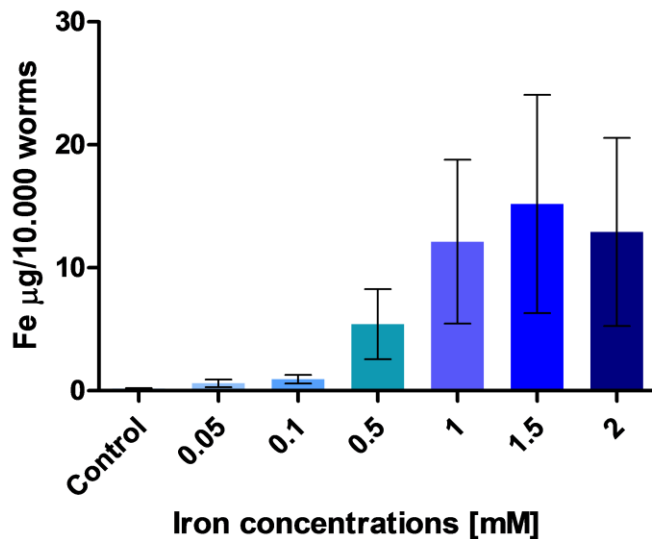


Figure 4: Iron levels in *C. elegans* after acute exposure (30 minutes). The Fe levels were determinate by graphite Graphite Furnace Atomic Absorption Spectrometry (GFAAS). Each bar represents mean (n=3 independent experiments)

Antioxidant enzyme levels	Groups			
	Control	0.05mM	0.1mM	0.5mM
SOD	369,3±15,59	299,3±13,80	213,7±14,18	74,17±30,93**
CAT	297,5±23,12	376,0±31,76	310,5±15,95*	167,0±21,86*
SOD/CAT	1,262±0,1416	0,8047±0,06127	0,6950±0,07585*	0,4523±0,1757**

Table 1. Enzyme levels measured by GFP strains after iron exposure

* (p<0.005), ** (p<0.01) indicates statistical difference from control group by On- Way ANOVA followed by Tukey's post-hoc test.

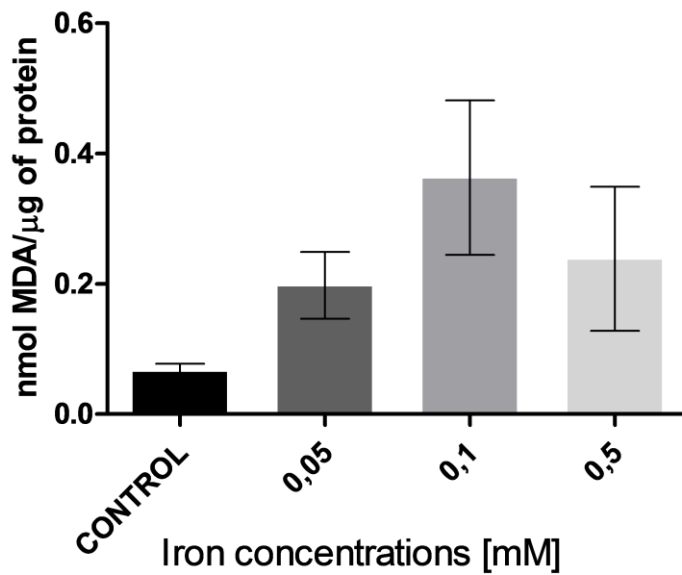
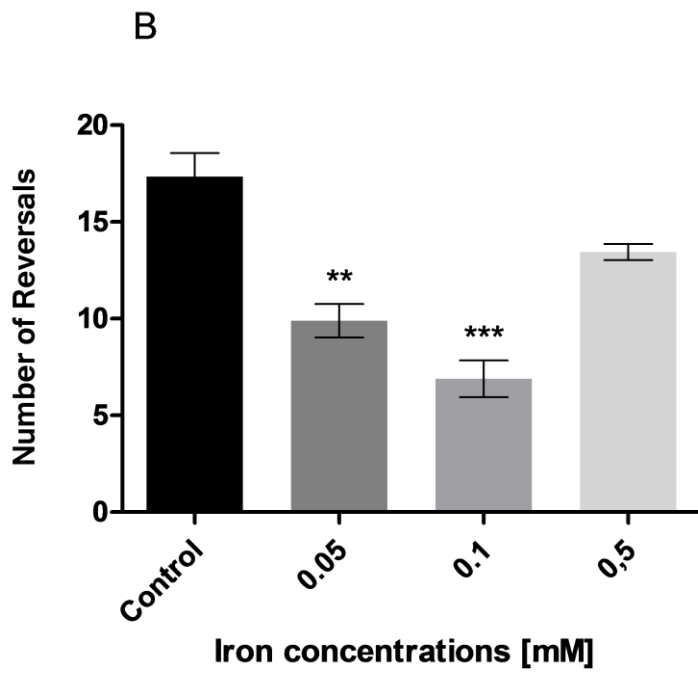
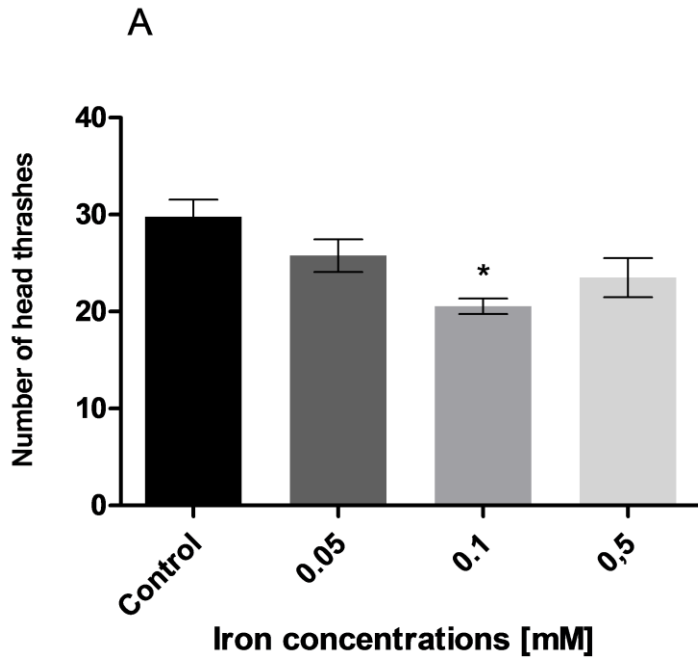


Figure 5: Lipid peroxidation assessed by TBARS in L4 worms. Statistical analysis was performed by On- Way ANOVA (n=4).



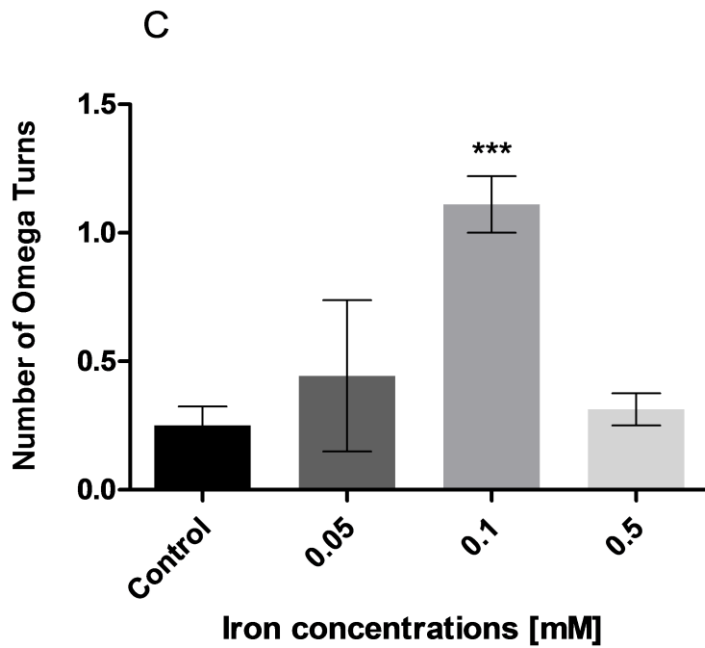


Figure 6: Acute exposure to Fe promotes a decrease in *C. elegans* basic movements. A) Head thrash frequency. B) Reverses by nose touch. C) Omega turns by nose touch. Each bar represents mean \pm S.E.M (n=5). * indicates statistical difference from control group by one-way ANOVA following by Tukey's post-hoc test ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.0001$).

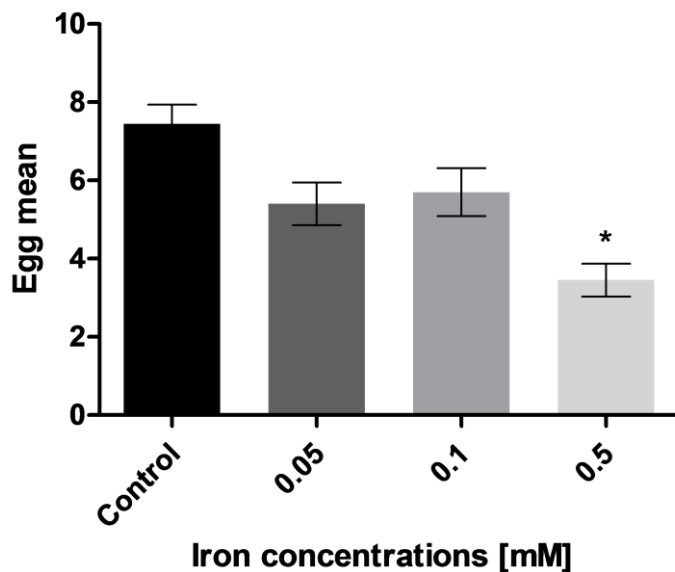


Figure 7: Levamisole induced behavior. When treated with the levamisole, both mock- and exposed adults were observed to hypercontract and lay eggs. Each bar represents mean \pm S.E.M (n=3). * indicates statistical difference from control group by One way ANOVA followed by Tukey's post-test (p<0.05).

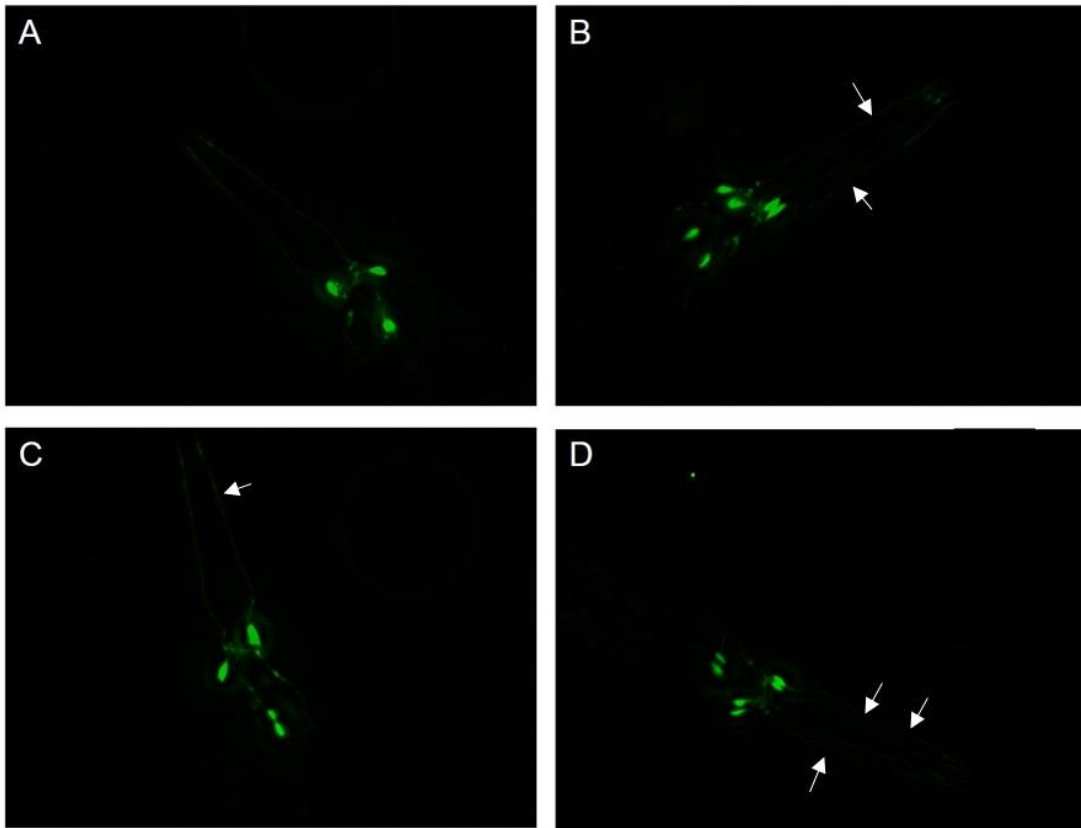


Figure 8: Dopaminergic neurons in alive L4 *C. elegans* (Pdat-1::GFP) following Fe treatment. Morphological changes were evident with all Fe concentrations. (A) Control, (B) 0.05mM, (C) 0.1mM and (D) 0.5 mM.

6. CONCLUSÕES

- A DL50 para o FeSO₄ em *C. elegans* do tipo selvagem (N2) foi de 1,2mM
- As doses sub letais de FeSO₄ causaram uma diminuição na reprodução dos *C. elegans* bem como diminui a vida dos vermes
- O comportamento do *C. elegans* é alterado quando expostos ao ferro indicando um possível dano neuronal.
- A exposição ao ferro é capaz de alterar a expressão de enzimas como SOD e Catalase
- A exposição ao ferro promove em *C. elegans* da cepa transgênica BY200 a degeneração dos neurônios dopaminérgicos.

Juntos, nossos resultados contribuem para elucidar os efeitos tóxicos do ferro e validar o modelo *C. elegans* para estudos envolvendo neurodegeneração, mostrando que o ferro tem efeitos neurotóxicos em *C. elegans*.

7. PERSPECTIVAS

Tendo em vista os resultados obtidos neste trabalho, as perspectivas para trabalhos posteriores são:

- verificar outros neurônios que podem ser afetados pelo ferro utilizando cepas de *C. elegans* transgênicas
- verificar o papel do ferro em cepas que mimetizam doenças neurodegenerativas como: Parkinson, Alzheimer e Huntington
- investigar os mecanismos pelo qual o ferro age para levar a neurodegeneração, usando cepas nocaute para os genes *smf-1*, *smf-3* e *smf-3*, avaliar a expressão de ferritina e o papel do ferro no fator de transcrição DAF-16

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