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Tássia Limana da Silveira

ÁCIDO QUINOLÍNICO E NEURODEGENERAÇÃO GLUTAMATÉRGICA EM *Caenorhabditis elegans*

Santa Maria, RS

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

Orientador: Professor Dr. Félix Alexandre Antunes Soares

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(Paulo Freire)

RESUMO

ÁCIDO QUINOLÍNICO E NEURODEGENERAÇÃO GLUTAMATÉRGIA EM *Caenorhabditis elegans*

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O ácido quinolínico (QUIN) é uma neurotoxina endógena que atua como agonista do receptor N-metil-D-aspartato (NMDAR) gerando uma cascata tóxica, que pode levar à neurodegeneração. A ação de QUIN em *Caenorhabditis elegans*, bem como de outras neurotoxinas que permitem o estudo dos distúrbios do sistema glutamatérgico, ainda não foram totalmente elucidadas. Os efeitos de QUIN em parâmetros toxicológicos e comportamentais em animais mutantes VM487 (*nmr-1*) e VC2623 (*nmr-2*) e selvagem Bristol N2 (WT) foram realizados para avaliar se QUIN poderia ser usado como uma neurotoxina em *C. elegans*. O QUIN reduziu a sobrevivência de vermes WT de uma forma dose dependente. Uma concentração não letal de QUIN (20 mM) aumentou os níveis de espécies reativas de oxigênio (ROS) de uma maneira dependente de NMR-1/NMDAR, ativou o fator de transcrição DAF-16/FOXO e aumentou a expressão de proteínas como a superóxido dismutase-3, Glutationa S-transferase-4 e a proteína de choque térmico-16.2. O QUIN não alterou os parâmetros comportamentais motores, mas alterou o comportamento sensorial em animais WT e VM487 (*nmr-1*). Notavelmente, o efeito de QUIN nos parâmetros comportamentais sensoriais pode ocorrer, pelo menos em parte, secundário ao aumento de ROS. No entanto, o comportamento de resposta ao toque indica um mecanismo de ação que pode ser independente da geração de ROS diretamente. Além disso, doses não letais de QUIN podem ter desencadeado uma possível neurodegeneração no sistema glutamatérgicos considerando a relação entre os dados comportamentais e as medidas de GFP-neuronal. Nossos achados indicam que o *C. elegans* tem um mecanismo de ação do QUIN similar ao que encontramos em organismos como os mamíferos, o que indica que o modelo alternativo pode ser útil para estudos com o sistema glutamatérgico. Assim, o *C. elegans* pode ser usado mais especificamente em doenças que possam ter entre suas etiologias a excitotoxicidade glutamatérgica assim como em mamíferos.

Palavras chaves: neurotransmissão glutamatérgica, neurodegeneração, NMDA, QUIN, excitotoxicidade.

ABSTRACT

QUINOLINIC ACID AND GLUTAMATERGIC NEURODEGENERATION IN *Caenorhabditis elegans*

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Quinolinic acid (QUIN) is an endogenous neurotoxin that acts as an N-methyl-D-aspartate receptor (NMDAR) agonist generating a toxic cascade, which can lead to neurodegeneration. The action of QUIN in *Caenorhabditis elegans* and the neurotoxins that allows the study of glutamatergic system disorders have not been carefully addressed. The effects of QUIN on toxicological and behavioral parameters in VM487 (*nmr-1*) and VC2623 (*nmr-2*) mutants strains, as well as in wild-type (WT) animals were performed to evaluate whether QUIN could be used as a neurotoxin in C. elegans. QUIN reduced survival of WT worms in a dose-dependent manner. A sublethal concentration of QUIN (20 mM) increased reactive oxygen species (ROS) levels in a nmr-1/NMDAR-dependent manner, activated the DAF-16/FOXO transcription factor, and increased expression of the proteins, as the superoxide dismutase-3, Glutathione S-transferase-4, and heat shock protein-16.2. QUIN did not change motor behavioral parameters but altered the sensory behavior in WT and VM487 (*nmr-1*) worms. Notably, the effect of QUIN on the sensory behavioral parameters might occur, at least in part, secondary to increased ROS. However, the touch response behavior indicates a mechanism of action independent of ROS generation. In addition, the non-lethal concentration of QUIN can have unleashed possible neurodegeneration in the glutamatergic system considering the relation between the behavioral data and the GFP-neuronal measures. Our findings indicate that *C. elegans* have a QUIN mechanism like that found in organisms like mammals, indicating that it can be useful to studies with the glutamatergic system. Thus, the *C. elegans* can be used more specifically in diseases that have among their etiologies the glutamatergic excitotoxicity as in mammals.

Keywords: Glutamatergic neurotransmission, Neurodegeneration; NMDA; QUIN; Excitotoxicity.

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LISTA DE ABREVIATURAS E SIGLAS

·NO	Óxido nítrico
3-HANA	Ácido 3-hidroxiantranílico
3-HK	3-hidroxiquinurenina
Ach	Acetilcolina
AMPA	Ácido α-amino-3-hidróxi-5-metil-4-isoxazol propiônico
ATP	Adenosina trifostato
DNA	Ácido desoxirribonucleico
ERNs	Espécies reativa de nitrogênio
EROs	Espécies reativa de oxigênio
GABA	Ácido γ-aminobutírico
GFP	Do inglês proteína verde fluorescente – Green Fluorescent Protein
Glu	Glutamato
GST	Glutationa S-transferase
HSP	Do inglês proteína de choque térmico – Heat Shock Protein
iGluRs	Receptores de ionotrópicos de glutamato
KYN	Quinurenina
KYNA	Ácido quinurênico
mGluRs	Receptores metabotrópicos de glutamato
NAD+	Coenzima nicotinamida adenina dinucleotídeo
NMDA	N-metil-D-aspartato
NMDAR	Receptor N-metil-D-aspartato
O_2^-	Ânion superóxido
$OH^{\cdot}$	Radical hidroxil
QPRTase	Fosforibosiltransferase de ácido quinolínico
QUIN	Ácido 2,3-piridinodicarboxílico
SOD	Superóxido dismutase
UMPS	Enzima monofosfato fosfatil transferase de uridina

SUMÁRIO

APRESENTAÇÃO

Essa dissertação traz uma breve introdução que trata sobre o sistema glutamatérgico, enfocando no seu funcionamento geral e em condições patológicas envolvendo o ácido quinolínico em mamíferos; bem como sobre o nematoide *Caenorhabditis elegans* e o funcionamento do seu organismo, focando na sua utilização como modelo de estudos envolvendo neurotoxicidade.

Posteriormente apresentamos os objetivos, os materiais e métodos utilizados, os resultados obtidos, bem como a discussão.

Os materiais e métodos, os resultados e discussão encontram-se no formato de artigo científico publicado na revista Neurotoxicology.

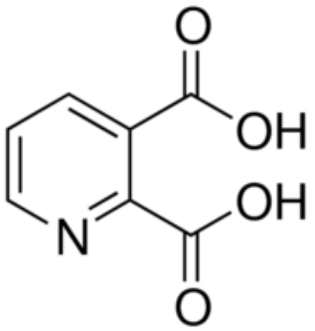
Após, apresentamos uma conclusão que contém interpretações e comentários acerca dos resultados obtidos.

Por fim, a seção Referências refere-se às citações mencionadas ao longo da introdução.

1. INTRODUÇÃO

O ácido 2,3-piridinodicarboxílico (Fig. 1), amplamente conhecido como ácido quinolínico (QUIN) apresenta em sua estrutura um ácido dicarboxílico com uma estrutura de piridina. O QUIN é um metabolito da degradação do aminoácido triptofano da via das quinureninas, via esta que é responsável pela síntese da coenzima nicotinamida adenina dinucleotídeo (NAD+) e de vários intermediários neuroativos além do QUIN, como a quinurenina (KYN), o ácido quinurênico (KYNA), a 3-hidroxiquinurenina (3-HK), e o ácido 3-hidroxiantranílico (3-HANA). Dentre eles, o QUIN atua no sistema nervoso central como agonista dos receptores N-metil-D-aspartato (NMDAR), preferencialmente nas subunidades dos receptores NR2A e NR2B, ativados por glutamato, em mamíferos (Chen and Guillemin 2009, Chen, Meininger et al. 2009, Schwarcz, Guidetti et al. 2010).

Figura 1: Estrutura molecular do Ácido Quinolínico



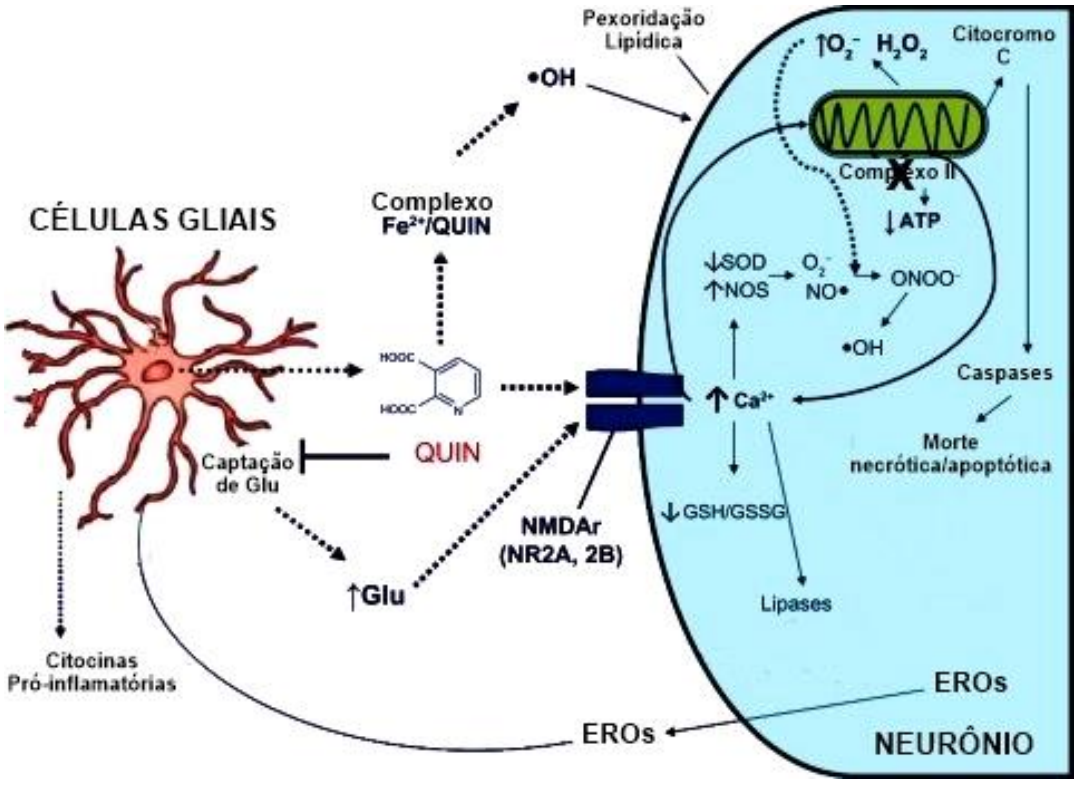
Fonte: https://www.sigmaaldrich.com/

O QUIN é produzido principalmente pelas células da micróglia e pelos macrófagos ativados (Braidy, Grant et al. 2009). As células da micróglia possuem função de defesa inicial contra insultos exógenos ou endógenos, desempenhando um papel determinante na proteção imunológica do sistema nervoso central (Kremlev, Roberts et al. 2004). Contudo, em condições patológicas, o QUIN foi descrito por estar presente no cérebro em concentrações maiores do que em homeostasia, atuando como uma potente neurotoxina endógena, a qual pode levar a disfunção neuronal e a morte celular por diferentes mecanismos (Guillemin 2012).

Sabe-se que altas concentrações de QUIN estão envolvidas em muitas condições neuropatológicas, como doença de Alzheimer (Guillemin and Brew 2002) e doença de Huntington (Finkbeiner, Cuervo et al. 2006). Dentre os mecanismos de toxicidade do QUIN (Fig. 2) está a hiperestimulação dos receptores NMDA (Schwarcz and Kohler 1983), o aumento

da liberação de glutamato (Glu) por neurônios, bem como a redução de sua captação pelos astrócitos (Tavares, Tasca et al. 2000, Tavares, Tasca et al. 2002). Além disso, sabe-se que o QUIN pode desregular o ciclo glutamato-glutamina por inibir a atividade da enzima glutamina-sintetase, levando a um aumento na concentração de glutamato dentro dos astrócitos bem como na fenda sináptica (Ting, Brew et al. 2009). O QUIN também pode induzir a síntese de oxido nítrico em neurônios, por aumentar a atividade da enzima oxido nítrico sintase, podendo fosforilar proteínas da estrutura celular desestabilizando o citoesqueleto das células (Perez-De La Cruz, 2012)

Figura 2: Representação esquemática dos mecanismos clássicos pelos quais QUIN induz toxicidade



Fonte: Adaptado e traduzido de (Perez-De La Cruz, Carrillo-Mora et al. 2012)
Legenda: QUIN pode causar danos extracelular e intracelular. Extracelularmente, QUIN pode reduzir a captação de glutamato, como também formar um complexo com o Fe^{2+} que é capaz de gerar espécies reativas de oxigênio que podem peroxidar as membranas das células. Já intracelularmente QUIN por atuar hiperestimulando os receptores de glutamato do tipo NMDA, auxilia no aumento do influxo de íons como o cálcio, inibe o complexo II da cadeia transportadora de elétrons reduzindo a produção de ATP e aumentando a geração de espécies reativas como O_2^-, $NO^{\cdot}$, $^{\cdot}OH$, H_2O_2; reduzindo as defesas antioxidantes como a SOD, a razão GSH/GSSG, ativando, por fim, a via das caspases sinalizando para a morte celular.

Como consequência dos mecanismos de toxicidade do QUIN, ocorre aumento do influxo de cálcio bem como aumento dos seus níveis citosólicos, depleção de ATP por toxicidade sinérgica com outras excitotoxinas (Bordelon, Chesselet et al. 1997) e formação de

radicais livres como espécies reativas de oxigênio (EROs) (Perez-De La Cruz, Konigsberg et al. 2007) e de nitrogênio (ERNs) (Seminotti, Amaral et al. 2016). Essas EROs/ ERNs, que incluem o ânion superóxido (O_2^-), o radical hidroxil ($OH^•$), o peróxido de hidrogênio (H_2O_2) e o óxido nítrico (•NO) (Halliwell and Gutteridge 1999), através dos seus elétrons desemparelhados oxidam outras moléculas, causando danos nas células.

A excitotoxicidade gerada pelo QUIN que leva ao aumento da geração de EROs também está associada à redução da atividade do sistema de defesas antioxidantes, resultando em dano oxidativo (Braidy, Grant et al. 2009). Geralmente, as espécies reativas podem ser neutralizadas por enzimas antioxidantes como a superóxido dismutase e a catalase, como também por antioxidantes não enzimáticos como glutationa e vitamina C. O sistema antioxidante, que faz a detoxificação de EROs, pode atuar através de diferentes mecanismos (Kurutas 2016). Entre eles, ocorre a ativação dos fatores de transcrição necessários para síntese de enzimas antioxidantes como a superóxido dismutase (SOD) e a glutationa S-transferase (GST), ou de chaperonas (proteínas de choque térmico do inglês HSP - heat shock proteins) que são proteínas que auxiliam no dobramento de proteínas recém-sintetizadas (Kubicova, Hadacek et al. 2013) para reparar e realizar a manutenção da homeostase celular. Estudos anteriores sugerem que a toxicidade QUIN também está associada à redução na transcrição de genes relacionados às defesas antioxidantes (Tasset, Perez-De La Cruz et al. 2010). Contudo, quando ocorre um desbalanço entre a produção de espécies reativas e a detoxificação das mesmas pelas defesas antioxidantes do organismo, pode gerar o estresse oxidativo, que é conhecido por colaborar com a toxicidade do QUIN (Rodriguez-Martinez, Camacho et al. 2000, Santamaria, Salvatierra-Sanchez et al. 2003, Perez-De La Cruz, Carrillo-Mora et al. 2012).

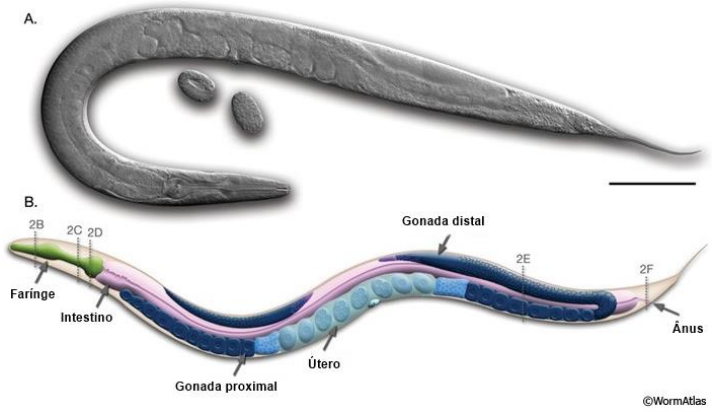
Apesar das concentrações do QUIN estarem aumentadas em situações patológicas, seus eventos tóxicos podem ser desencadeados mesmo em baixas concentrações (Perez-De La Cruz, Carrillo-Mora et al. 2012). Estudos anteriores mostraram que o QUIN pode oxidar o DNA, gerar disfunção mitocondrial (Kalonia, Kumar et al. 2010) e peroxidação lipídica através da sua capacidade de formar complexos pró-oxidantes com o Ferro II (Platenik, Stopka et al. 2001), podendo dessa forma causar eventos oxidativos parcialmente independentes do NMDAR (Perez-De La Cruz, Carrillo-Mora et al. 2012).

Apesar do sistema glutamatérgico e QUIN terem sido abordados em diversos estudos, ainda existem muitas questões a serem respondidas, envolvendo principalmente o efeito do QUIN como potencializador de toxicidade em patologias como a epilepsia, síndrome da imunodeficiência adquirida e doenças neurodegenerativas. Ainda, estudos recentes apontam

que uma das características encontradas nas doenças neurodegenerativas é a super-regulação da via das quinureninas onde o QUIN pode ser produzido em maiores quantidades (Castellano-Gonzalez, Jacobs et al. 2019). Além disso, QUIN é utilizado em roedores como um modelo de neurotoxina que possibilita estudos no sistema glutamatérgico, sendo necessário para busca de novos tratamentos para doenças que envolvam sua neurotoxicidade. Contudo, nos últimos tempos têm-se tentado reduzir a utilização de roedores em pesquisas, buscando modelos alternativos que possibilitem estudos para preencher as lacunas existentes.

O nematoide *Caenorhabditis elegans* é um modelo experimental alternativo (Fig. 3) muito utilizado, pois apresenta fácil cultivo, rápido desenvolvimento de 3-4 dias de ovo até a fase adulta, de curto ciclo de vida (Fig. 4), vivendo cerca de 20-30 dias e de reprodução numerosa de aproximadamente 300 ovos por animal. Os nematoides se encontram em dois sexos, hermafrodita (mais abundante) e machos, sendo este o que apresenta maior número de células, por ter em sua cauda uma espícula copulatória utilizada para reprodução (Riddle *et al.*, 1997; Nass e Blakely, 2003).

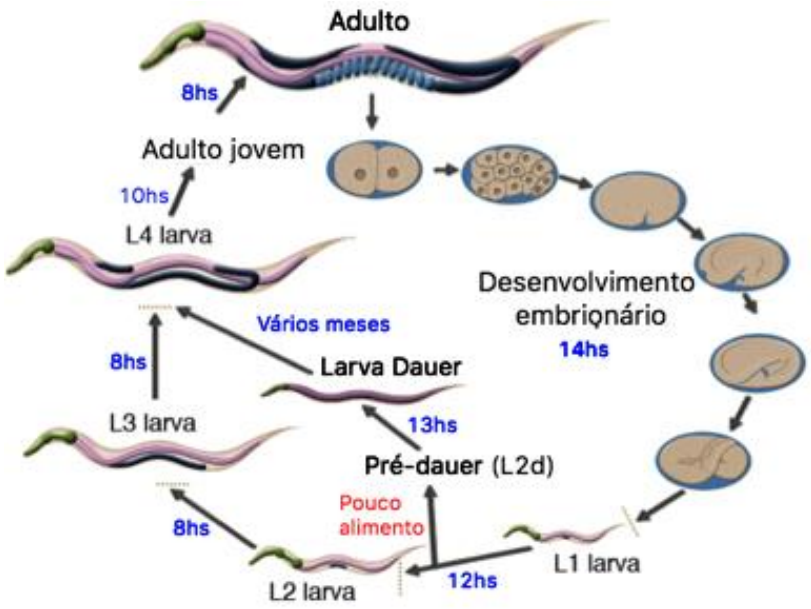
Figura 3: Anatomia de um animal hemafrodita de *C. elegans*



A) Representação real e **B)** esquemática das estruturas anatômicas do nematoide *C. elegans*
Fonte: Traduzido de http://www.wormatlas.org/

Em *C. elegans* cerca de 60-80% dos seus genes apresentam ortólogos no genoma humano (Kaletta and Hengartner 2006), os quais sintetizam proteínas que participam de diversas vias metabólicas, permitindo que estudos relacionados a essas vias possam ser feitos. Além disso, pode-se realizar múltiplas alterações genéticas em *C. elegans*, auxiliando na avaliação da toxicidade e alvos de ação de diferentes compostos (Arantes, Peres et al. 2016, Du, Guan et al. 2018), como também a avaliação de moléculas com potencial neuroprotetor em modelos de doenças neurodegenerativas (Zamberlan, Arantes et al. 2014).

Figura 4: Representação gráfica do ciclo de vida do nematoide *C. elegans* na temperatura de 22º C.



Fonte: Adaptado e traduzido de ttp://www.sfu.ca/biology/faculty/hutter/hutterlab/research/Celegans.html

Além disso, *C. elegans* possui diversos processos celulares e mecanismos moleculares que modulam respostas de estresse altamente conservadas, homologas a mamíferos. Um exemplo disso é a via do fator de transcrição DAF-16 que é homologa a via de sinalização do tipo insulina IGF-FoxO em mamíferos (Fontana, Partridge et al. 2010, Kenyon 2010). Essa via é responsável por uma regulação chave do crescimento, metabolismo, respostas ao estresse, controle do ciclo celular e longevidade em *C. elegans*. Em situações de homeostase, a proteína DAF-16 encontra-se fosforilada no citoplasma. Já em situações de estresse como falta de alimento, situações de luta/fuga, a DAF-16 é desfosforilada e migra para o núcleo ativando a transcrição de diversos genes responsáveis pela resposta contra estressores, como a SOD-3, catalase e HSP-16.2.

Além disso, a morfologia transparente do *C. elegans* permite a utilização de marcadores fluorescentes como a proteína verde fluorescente no genoma do nematoide (do inglês GFP – Green Fluorescente Protein) para estudos de expressão de moléculas/proteínas *in vivo* (Brenner 1974, Li, Kim et al. 1999). Um exemplo dessa tecnologia é o caso da proteína DAF-16 que quando ligada a GFP possibilita a visualização da localização da mesma em microscopia de fluorescência, auxiliando na análise de ativação ou não desta via (Murphy and Hu 2013). Também é possível a utilização da GFP ligada a proteínas, auxiliando na expressão de enzimas

antioxidantes como o superóxido dismutase -3 (SOD-3) (Wang and Wink 2016), bem como na marcação de neurônios, possibilitando a avaliação da viabilidade desses neurônios, como também neurodegeneração (de Carlos Caceres, Porto et al. 2018).

O simples sistema nervoso de *C. elegans* é formado por uma rede de neurônios que se estendem ao longo de todo o animal, contendo 302 neurônios em animais hermafroditas e 385 em machos, que se concentram na região da cabeça formando um anel nervoso onde ocorre a grande parte das sinapses e ainda, nos machos, encontram-se dispostos próximo da espícula copulatória. Apresentam um conjunto de neurotransmissores, entre eles acetilcolina (ACh), ácido γ-aminobutírico (GABA), Glu, óxido nítrico, serotonina e outras monoaminas (Brownlee and Fairweather 1999, Li, Kim et al. 1999).

Dentre os neurotransmissores, sabe-se que a neurotransmissão glutamatérgica não está totalmente esclarecida. Estudos de mapeamento de neurônios que expressam receptores de glutamato mostraram que os nematoides apresentam 3 receptores metabotrópicos de Glu (mGluRs) que são receptores acoplados à proteína G e 10 subunidades de receptores de ionotrópicos de Glu (iGluRs) que apresentam canais iônicos de cloreto controlados por Glu (Brockie, Madsen et al. 2001, Brockie and Maricq 2003). Entre os mGluRs sabe-se que existem os MGL-1, MGL-2 e MGL-3, apesar de não se conhecer muito sobre seu papel no sistema glutamatérgico do *C. elegans.* Já em relação aos iGluRs, são conhecidas duas subunidades do tipo NMDA a NMR-1 e NMR-2 que pertencem a subfamílias NR1 e NR2A de mamíferos, respectivamente, e as subunidades não-NMDA a GLR-1 - GLR-8 semelhantes às subfamílias do ácido α-amino-3-hidróxi-5-metil-4-isoxazol propiônico (AMPA) ou kainato (Brockie, Mellem et al. 2001, Brockie and Maricq 2003), os quais são os focos da maioria dos estudos envolvendo sistema glutamatérgico. Além disso, os nematoides apresentam transportadores de Glu que se encontram distribuídos de maneira distinta ao longo do seu corpo cilíndrico. Como por exemplo o transportador GLT-1 localizado principalmente nos músculos da cabeça nos animais adultos, o GLT-3 expresso no canal celular excretor e fracamente expresso na faringe, o GLT-4 que é expresso em neurônios pré-sinápticos, neurônios de dupla função (Pré e Pós-sinápticos) mas não em neurônios pós-sinápticos, e o transportador vesicular de glutamato EAT-4 que se encontra em neurônios (Mano, Straud et al. 2007, Lee, Jung et al. 2008).

Assim como em mamíferos, a neurotransmissão glutamatérgica em *C. elegans* está envolvida em grande parte das sinapses, atuando nos mais diversos comportamentos. Um desses comportamentos é a resposta a estímulos mecanossensoriais que é controlado por neurônios sensoriais como o neurônio ASH localizado no gânglio lateral da cabeça do

nematoide e que possui neurotransmissão glutamatérgica expressando os iGluR tanto NMDA, quanto não-NMDA (Brockie, Madsen et al. 2001). A resposta ao toque e a resposta a alguns químicos como o 1-octanol também é mediada por neurônios ASH, sendo modulada prioritariamente por Glu.

Os neurônios sensoriais que apresentam neurotransmissão glutamatérgica podem interagir com interneurônios sensoriais, modulando os mais diversos comportamentos em *C. elegans* como a resposta à estímulos sensoriais, estímulos químicos (Baidya, Genovez et al. 2014) ou mecanossensoriais (Maricq, Peckol et al. 1995), também modula a memória associativa (Kano, Brockie et al. 2008), parâmetros locomotores como locomoção espontânea (Zheng, Brockie et al. 1999), movimento para frente e para trás e também número de reversões do animal (Brockie, Mellem et al. 2001), entre outros. Os batimentos faríngeos e a locomoção também são controlados por Glu, sendo que no primeiro comportamento a presença de glutamato nos neurônios M3 promove sua repolarização, induzindo o relaxamento do músculo, consequentemente ocorre a redução da contração do musculo da faringe (Dent, Davis et al. 1997). Já na locomoção, sabe-se que pelo menos 5 pares de interneurônios auxiliam em seu controle (Chalfie, Sulston et al. 1985), bem como neurônios motores e sensoriais (Tolstenkov, Van der Auwera et al. 2018), os quais expressam iGluRs apresentando assim neurotransmissão glutamatérgica. Apesar do comportamento locomotor ser formado por uma rede neuronal complexa, sabe-se que a despolarização desses interneurônios ativa o movimento de deslocamento para frente, e que a hiperpolarização inativa esses interneurônios que resulta no comando inverso (movimento para trás). Essa regulação também pode ocorrer independentemente dos neurônios sensoriais, modulando de diferente maneiras vários outros comportamentos, e também pode contar com a participação de mecanismos secundários, envolvendo em ambas as situações o iGluR não-NMDA GLT-1 (Wicks, Roehrig et al. 1996, Zheng, Brockie et al. 1999, Thapliyal, Ravindranath et al. 2018).

As alterações comportamentais podem vir acompanhadas de problemas na fisiologia e função dos neurônios que as controlam, levando a neurodegeneração. Essa morte neuronal além de perturbar a rede de conexões de neurônios, pode ainda levar a danos irreparáveis dentro dos organismos. Sabe-se que em *C. elegans* a neurotransmissão glutamatérgica exacerbada, bem como toda sua cascata de eventos tóxicos, como o aumento do cálcio intracelular, a geração de espécies reativas e a ativação da via apoptótica são os principais contribuintes para eventos neurodegenerativos (Choudhary, Mandelkow et al. 2018, Sarasija, Laboy et al. 2018). Apesar de não se conhecer neuropatologias endógenas em *C. elegans*, a modificação genética

possibilita a mimetização de diversas patologias como o modelo para doenças de Alzheimer (Leiteritz, Dilberger et al. 2018), Huntington (Boasquivis, Silva et al. 2018) e Parkinson (Tsai, Tsai et al. 2017); auxiliando na elucidação do funcionamento de vias metabólicas, locais onde algumas drogas agem, mapeamento do desenvolvimento das proteinopatias, etc.

Além de receptores e transportadores envolvidos na neurotransmissão glutamatérgica, sabe-se que *C. elegans* também possui células gliais, contudo sua função não possibilita uma adequada comparação com a de mamíferos, pois os nematoides na natureza ocupam nichos diferentes e também possuem um sistema nervoso reduzido (Stout, Verkhratsky et al. 2014). Apesar disso, algumas rotas genéticas para desenvolvimento glial parecem ser compartilhadas entre glia de mamíferos e de *C. elegans* (Yoshimura, Murray et al. 2008), bem como o papel na regulação da morfologia sensorial e modulação da atividade neuronal (Stout, Verkhratsky et al. 2014). Ainda que em *C. elegans* existam células da glia com algumas rotas semelhantes, não são conhecidos estudos relacionando as células da glia com a metabolização do triptofano para a produção de NAD^+, como ocorre em mamíferos. Sabe-se que a síntese de novo de NAD^+ é conservada em *C. elegans* apresentando todas as enzimas da via das quinureninas, com exceção da fosforibosiltransferase de ácido quinolínico (QPRTase), enzima que em mamíferos faz a síntese de NAD^+ a partir de QUIN (Vrablik, Huang et al. 2009). Nos nematoides, a síntese de novo de NAD^+ depende das concentrações de intermediários dessa via como o QUIN. Contudo a enzima monofosfato fosfatil transferase de uridina (UMPS), que atua na biossíntese da pirimidina, é quem realizada a biossíntese de NAD+ no lugar da QPRTase não codificada (McReynolds, Wang et al. 2017).

Como em *C. elegans*, não há evidências da ação de QUIN gerada de maneira endógena ou mesmo pouco se sabe sobre os efeitos desta, ou outras neurotoxinas nos vermes, para que seja possível o estudo de lesões do sistema glutamatérgico, bem como tratamentos para patologias geradas pela excitotoxicidade de QUIN em um modelo alternativo. Desta forma, nesse estudo foram avaliados os efeitos do QUIN sobre parâmetros comportamentais e bioquímicos em *Caenorhabditis elegans.*

2. OBJETIVOS

2.1. OBJETIVO GERAL

Avaliar o efeito do QUIN em parâmetros bioquímicos e comportamentais em *C. elegans* a fim de estabelecer um novo modelo para estudos envolvendo neurotoxicidade glutamatérgica.

2.2. OBJETIVOS ESPECÍFICOS

- Investigar os efeitos do QUIN em diferentes parâmetros comportamentais em *C. elegans;*
- Verificar a geração das espécies reativas e a ativação das enzimas do sistema antioxidante após exposição ao QUIN *in vivo* em *C. elegans*;
- Avaliar da viabilidade neuronal em *C. elegans* após exposição ao QUIN.

3. JUSTIFICATIVA

O sistema glutamatérgico é um dos sistemas mais importantes nos mamíferos e está presente na grande maioria das sinapses, sendo os distúrbios relacionados com esse sistema os responsáveis por desencadear patologias severas. O QUIN é utilizado como modelo de neurotoxina em roedores, possibilitando estudos para a busca de novos tratamentos envolvendo patologias no sistema glutamatérgico.

Além disso, visando reduzir a utilização de roedores nas pesquisas, e uma vez que o modelo alternativo *C. elegans* já é amplamente utilizado na pesquisa de neurobiologia, não se conhecendo nenhuma neurotoxina que possibilite o estudo de patologias relacionadas com o sistema glutamatérgico em doenças neurológicas, é de grande importância a avaliação da relação e ação do QUIN com o sistema glutamatérgico no início e no desenvolvimento de doenças neurológicas, em *C. elegans*.

4. MATERIAIS E MÉTODOS, RESULTADOS E DISCUSSÃO

Os materiais e métodos, bem como os resultados e discussão encontram-se abaixo no formato de artigo científico publicado em abril de 2018 na revista Neurotoxicology.

4.1. ARTIGO

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Full Length Article

Quinolinic acid and glutamatergic neurodegeneration in *Caenorhabditis elegans*

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ABSTRACT

Quinolinic acid (QUIN) is an endogenous neurotoxin that acts as an N-methyl-D-aspartate receptor (NMDAR) agonist generating a toxic cascade, which can lead to neurodegeneration. The action of QUIN in *Caenorhabditis elegans* and the neurotoxins that allow the study of glutamatergic system disorders have not been carefully addressed. The effects of QUIN on toxicological and behavioral parameters in VM487 and VC2623 transgenic, as well as wild-type (WT) animals were performed to evaluate whether QUIN could be used as a neurotoxin in *C. elegans*. QUIN reduced survival of WT worms in a dose-dependent manner. A sublethal dose of QUIN (20 mM) increased reactive oxygen species (ROS) levels in an *nmr-1*/NMDAR-dependent manner, activated the DAF-16/FOXO transcription factor, and increased expression of the antioxidant enzymes, superoxide dismutase-3, glutathione S-transferase-4, and heat shock protein-16.2. QUIN did not change motor behavioral parameters, but altered the sensory behavior in N2 and VM487 worms. Notably, the effect of QUIN on the sensory behavioral parameters might occur, at least in part, secondary to increased ROS. However, the touch response behavior indicates a mechanism of action that is independent of ROS generation. In addition, non-lethal doses of QUIN triggered neurodegeneration in glutamatergic neurons. Our findings indicate that *C. elegans* might be useful as a model for studies of QUIN as a glutamatergic neurotoxin in rodent models.

1. Introduction

2,3-Pyridine dicarboxylic acid, widely known as quinolinic acid (QUIN), is a metabolite of tryptophan in the kynurenine pathway, which acts as an *N*-methyl-D-aspartate receptor (NMDAR) agonist, preferentially on the NR2A and NR2B receptor subunits (Chen and Guillemin, 2009; Schwarcz et al., 2010; Chen et al., 2009). QUIN is produced mostly by microglia and activated macrophages in the brain (Braidy et al., 2009) under pathological conditions and has been described as a potent endogenous neurotoxin. The mechanisms of action of QUIN take different forms causing excitotoxicity by elevating cytosolic calcium concentrations, depleting ATP, and forming free radicals (Perez-De La Cruz et al., 2007). QUIN plays a role in several neuropathological conditions, such as Alzheimer's disease (Guillemin and Brew, 2002), Huntington's disease (Finkbeiner et al., 2006), and neurodegenerative disorders (Schwarcz et al., 2012).

The toxic events triggered by QUIN occur even at low concentrations (Perez-De La Cruz et al., 2012). Moreover, excitotoxicity generated by QUIN is associated with increased formation of reactive oxygen species (ROS) and reduced activity of antioxidant defense systems, resulting in oxidative damage (Braidy et al., 2009). Previous studies have shown that QUIN disrupts DNA and promotes lipid peroxidation. Secondary to its ability to form complexes with Fe(II), QUIN may cause oxidative events partially independent of NMDAR (Hamanaka and Chandel, 2010). Furthermore, ROS can be detoxified through different mechanisms (Kurutas, 2016). Among them, activation of transcription factors required for antioxidant enzyme synthesis and chaperones. Previous studies have suggested that the toxicity of QUIN is associated with a reduction in the transcription of genes related to antioxidant defenses (Tasset et al., 2010).

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The nematode *Caenorhabditis elegans* is an invaluable experimental model, as it is easy to culture, develops and reproduces rapidly, has a simple nervous system, and is easy to genetically manipulate. In addition, its transparent morphology allows the use of fluorescent markers for studies on various molecules. *C. elegans* has cellular processes and molecular mechanisms that modulate the stress response, which are highly conserved in mammals (Tasset et al., 2010; Li et al., 1999).

The *C. elegans* nervous system is comprised of a set of neurotransmitters, such as acetylcholine (ACh), γ-aminobutyric acid, glutamate, nitric oxide, serotonin, and other monoamines (Li et al., 1999; Brownlee and Fairweather, 1999; Nathoo et al., 2001). Glutamatergic neurotransmission has been partially elucidated in *C. elegans*. Studies that have mapped neurons expressing glutamate receptors have revealed 10 ionotropic glutamate receptor subunits (Brockie et al., 2001a; Brockie and Maricq, 2003). Among them, eight are in the non-NMDA class (GLR-1–GLR-8), which are similar to AMPA or kainate subfamilies, and two are in the NMDA class (NMR-1 and NMR-2), which are homologous to the NR1 and NR2A subfamilies, respectively in mammals (Brockie and Maricq, 2003; Brockie et al., 2001b). There is no evidence of QUIN production in *C. elegans* astrocytes. Furthermore, there is no pharmacological model that induces glutamatergic neurotoxicity in this worm. Thus, the aim of this study was to investigate QUIN as a neurotoxin in *C. elegans* and establish a new model to allow the study of diseases associated with glutamatergic neurotoxicity.

There is no evidence of a neurotoxin acting on the *C. elegans* glutamatergic system. In our study, we evaluated the toxicity generated by QUIN in *C. elegans* and the mechanisms involved in this process to contribute to new models and allow a further understanding of neurodegenerative disorders.

2. Materials and methods

2.1. C. elegans *strains, maintenance and treatment*

The wild type strain N2 and mutant strains CL2070 (*dvls70* [*hsp-16.2p*::GFP + rol-6(*su1006*)]), TJ356 (*zls356* [*daf-16p::daf-16a/b*::GFP + rol-6(*su1006*)]), CF1553 (*muIs84* [(*pAd76*) *sod-3p*::GFP + rol-6(*su1006*)]), VM487 [*nmr-1*(*ak4*)], VC2623 [*nmr-2*(*ok3324*)], CL2166 (*dvIs19* [(*pAF15*) *gst-4p*::GFP::NLS] and OH438 (*otIs117* [*unc-33p*::GFP + *unc-4*(+)] pan-neuronal GFP) were obtained from the Caenorhabditis Genetics Center (CGC, University of Minnesota, Minneapolis, MN, USA). The CL2070, TJ356, CF1553 and OH438 has GFP fused with promotor region for heat shock protein 16-2, transcription factor DAF-16/FOXO, superoxide dismutase 3 and pan-neuronal, respectively. The VM487 and VC2623 does not contain the *nmr-1* and *nmr-2* subunits of the NMDA ionotropic glutamate receptors gifts in C. elegans, respectively.

For all worms, age-synchronized eggs were obtained by isolating embryos from gravid hermaphrodites using bleaching solution (1% NaOCl, 0.25 M NaOH), washed three times and stored overnight in M9 to obtain all animals in stage L1. The L1 population was transferred to NGM (Nematode Growth Medium) previously seeded with *Escherichia coli* OP50 as food source, at 20 °C, until reaching the young adult stage, about 40 h (Brenner, 1974).

Worms at young adult stage (after approximately 40 h of L1 stage) were exposed acutely to QUIN at final concentrations different in plate (5, 10, 20, 50, 100 and 200 mM) or control, during periods 1 h in plates containing NGM and OP50 culture medium (O. D.600 = 1.0) as predicted in previous assays, where it was added only in the preparation of the culture medium. The QUIN-concentration were added onto the NGM, previously seeded with OP50, 1 h before exposing the worms. Subsequently, the worms were washed three times and the analyses were performed.

2.2. Survival

After exposure to different concentrations of QUIN, the worms were transferred to news plates containing NGM seeded with and OP50 in the absence of QUIN during 24 h to recovery. About 100 worms were analyzed per group per experiment and the number of survivors was quantified. This assay was repeated four times in duplicate.

2.3. Measurement of reactive oxygen species (ROS)

Intra-worm ROS generation was measured in *C. elegans* wild-type and transgenic strains using CM-H_2DCFDA, as previously described (Sakaue et al., 2010) with minor modifications (Zamberlan et al., 2014). Briefly, the worms were washed with M9 buffer three times and transferred to micro tubes. After, 10 μL of 2 mM CM-H_2DCFDA were added to the Eppendorf tubes containing 1000 worms in 990 μL M9 (20 μM CM-H_2DCFDA - final concentration) and incubated for 2 h. The worms were washed and transferred to 96-well plates (100 worms per well). The fluorescence intensity was measured with a plate reader in SpectraMax i3 (Excitation: 488 nm; Emission: 510 nm).

2.4. Subcellular Daf-16 localization

About 50 worms per group were transferred to a microscopic slide in M9 buffer. Fluorescence images was acquired with an OLYMPUS® FLUOVIEW FV10i Confocal Microscope housed in air-conditioned rooms (20 °C). Localization DAF-16::GFP in animals was classified in three category: as total localization in the cytoplasm, partially in the nucleus (intermediate) or located totally in the nucleus. Four assays was performed and 10 worms for group were randomly picked for evaluation. The worms exposed to heat stress at 35 °C for 1 h on NGM plates with OP50 was used as a positive control.

2.5. Quantitation of SOD-3, GST-4, HSP-16.2 and pan-neuronal expression

Approximately 50 worms in M9 buffer were transferred to microscopic slides and 10 uL of 10 mM sodium azide was added as a paralyzing agent were used to better visualize the whole animal. The positive control utilized in strains are worms exposed to heat stress at 35 °C for 1 h on NGM plates with OP50. The expression of HSP-16.2, SOD-3 and GST-4 (Chaudhuri et al., 2016; Wang and Wink, 2016) was measured by quantifying the fluorescence of the reporter protein GFP, according to fluorescence specificity location for each transgenic strain. In the pan-neuronal strain neuronal integrity was evaluated through the quantification and evaluation of the neurons located in the region of the head of the animals, where there is presence of the majority of the neurons that express glutamate receptors of type NMDA. The integrity of the neurons present in the ventral nerve cord of the animals was also analyzed by observing the presence or absence of neuron fluorescence.

The data are expressing in % AFU of Ctrl refers to the amount of arbitrary fluorescence units (AFU) present as a percentage of the control values (non-QUIN-treated animal), considering the mean of the control values as 100%. The intensity of fluorescence of each worm in each image was analyzed using ImageJ2X (ImageJ2X software; Rawak Software, Inc., Stuttgart, Germany). From each group, 20 worms were randomly picked to measure the mean pixel density.

2.6. Behavior analysis

After 1 h-QUIN exposure, the worm behavior worms were evaluated in the same exposure plates. Behaviors of locomotion

and pharyngeal beating of approximately 20 animals, in each plate, were evaluated using optical microscopy and later classified in: animals as having standard behavior (pharyngeal pumps and locomotion unchanged) and non-standard behavior (paralysis or absence of pharyngeal pumps). The locomotion unchanged were considered ability to traverse the plaque in search of food by the presence of movement/locomotion of the animal, and non-standard behavior were animals no present movement of the body musculature even after stimulated with brush bristles (Link et al., 2003). Animals that had omega turns and cork screws were not considered abnormal. The pharyngeal pumps were classified according to the presence or absence of contraction of the pharyngeal muscle.

The assay was repeated four days independently, by duplicate and approximately 10 worms of each group were evaluated in each assay. In addition, worms that presented standard behavior were submitted to an additional behavioral task, nose touch response, in order to test potential effects related with on the glutamatergic system.

2.6.1. Pharyngeal pumping

The number of pharyngeal contractions during a 10-sec interval in triplicate was measured (Huang et al., 2004). The assay was performed at three independent times with 10 worms per group and results are shown as pumping/minute.

2.6.2. Defecation cycle

The defecation cycle length was assessed by observing the duration between the pBoc steps (posterior body muscle contraction) of two consecutive defecations. Ten animals per group were scored for three consecutive cycles (three successive pBoc steps) per assay. Experiments were performed at three independent times.

2.6.3. Body bends

Well-fed worms were transferred to food-free NGM plate and allowed to freely move. After 30 s adaptation, they were scored for the number of body-bends generated in 1-min interval. A body bend was defined as change in the direction of propagation along the y axis, assuming the worm was traveling along the x axis (Chalfie et al., 1985). Twenty worms of each group were evaluated were repeated three times by duplicate.

2.6.4. Touch response

Nematode touch response was performed by touching gently the head-region of the animal with a bristle brush. The backward was considered as a positive response a touch-sensitive in the opposite direction to the stimulated site in total ten touches and as non-response animals were considered that did not present any adverse reaction when stimulated, as previously described (Wang and Wink, 2016). Four assays were realized at different times, they mean different synchronization and days and 10 worms analyzed for each experiment. 10-second resting period between trials.

2.7. Statistical analysis

Statistical analysis was performed using *GraphPad* (Version 5.0, San Diego, CA). Significance was assessed using *t*-tests, or one-way analysis of variance (ANOVA), followed by Newman-Keuls Test, or two-way ANOVA, followed by Bonferroni or Tukey Test for *post hoc* comparison. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. QUIN induces toxicity in wild-type C. elegans

We first investigated whether QUIN was toxic to wild-type (N2) worms. Thus, the percentage of worms that survived exposure to QUIN was determined. Wild-type worms treated with QUIN exhibited a significant decrease in survival (11.3%, 17.7%, and 20.3% decrease for 50, 100, and 200 mM QUIN, respectively) when compared to control worms ($p < 0.01$; Fig. 1).

3.2. QUIN increases ROS generation in an NMR-1R-dependent manner

As shown in Fig. 2, wild-type (N2) worms exposed to 20 mM QUIN exhibited a prominent increase in DCF fluorescence (78%), whereas untreated worms exhibited basal fluorescence levels that corresponded to the physiological production of ROS. Upon exposure to 20 mM QUIN, the VM487 transgenic worms had indistinguishable levels of ROS compared to those in the untreated control. Analogous to the wild-type, ROS levels increased in VC2623 knockout worms after exposure to 20 mM QUIN ($p < 0.05$).

3.3. QUIN partially induces activation of DAF-16/FOXO

Fig. 3A shows localization of the DAF-16::GFP protein and the percentage of worms in each category. The DAF-16::GFP protein was located in the cytoplasm under normal control culture conditions (96.4%). However, the DAF-16::GFP protein was located entirely in the nucleus during exposure to high temperature (35 °C;

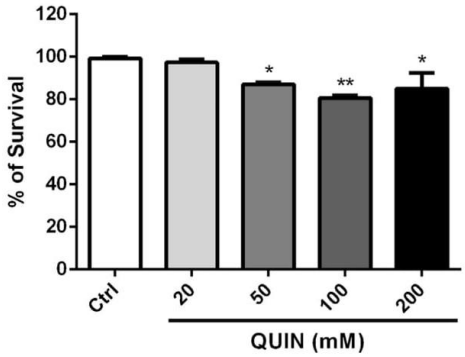

Fig. 1. Effects of QUIN on survival assay of wild-type (N2) worms. Data are expressed as percentage of living worms derived from four independent assays of 100 worms per group in each experiment (n = 4 experiments per group). Error bars represent as means ± S.E.M. *$p < 0.01$ and **$p < 0.001$ compared to Ctrl (One-Way ANOVA followed by post-hoc Bonferroni).

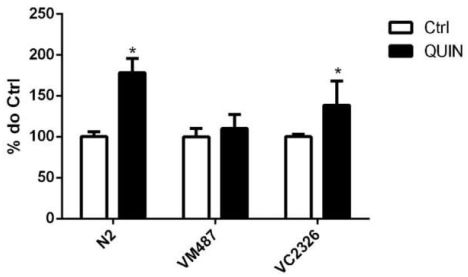

Fig. 2. Effect of QUIN on reactive oxygen species (ROS) production. Data are expressed as percentage of the arbitrary fluorescence units (AFU) of Ctrl of each strain derived from 5 independent assays (n = 5). **Levels of basal ROS production in wild-type (N2), VM487** (*nmr-1*) and VC2623 (*nmr-2*) mutants treated or not with the QUIN 20 mM. Error bars represent as means ± S.E.M. *$p < 0.05$ compared to untreated group (Test-t).

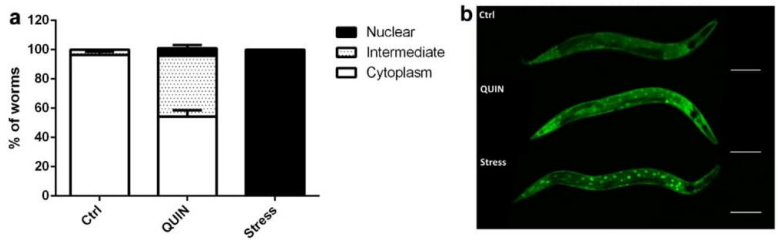

Fig. 3. Effect of QUIN in migration of daf-16 protein. A) Effect of QUIN (20 mM) and thermal stress (35 °C) on DAF-16 subcellular localization in the transgenic *C. elegans* TJ356 (*daf-16*::GFP) strain. B) Cytosolic, intermediate and nuclear localization. Data are expressed as percent of worms exhibiting cytosolic, intermediate or nuclear pattern of location, derived from five independent assays with 10 worms per group in each experiment (n = 5). Error bars represent as means ± S.E.M (Two-way ANOVA followed by post-hoc Bonferroni).

nuclear localization in 100% of the worms). After the QUIN-treatment, the DAF-16 protein partially migrated to the nucleus (54.2% cytoplasmic localization), and significant DAF-16 activation and migration to the nucleus was observed ($p < 0.01$, Fig. 3). The DAF-16::GFP protein was localized in the cytoplasmic, intermediate, and nuclear categories (Fig. 3B).

3.4. QUIN induces SOD-3, GST-4, and HSP-16.2 expression

Animals treated with QUIN showed increased GFP-labeled SOD-3, HSP-16.2/GFP, and GST-4/GFP fluorescence (30.6%, 12.2%, and 46.5% respectively) compared to that in control animals ($p < 0.01$, Fig. 4A–C).

3.5. QUIN induces behavioral deficits

Significant behavioral deficits in locomotion and pharyngeal contraction were observed in wild-type worms treated with 20 mM QUIN ($p < 0.05$, 46.4%) compared to that in the control group (Fig. 5A). Knockout animals of the *nmr-1* subunit of the ionotropic glutamate receptor NMDA type (VM487) exposed to various concentrations of QUIN demonstrated no significant difference in behavioral phenotype (Fig. 5B). The worms that changed the number of pharyngeal beats and locomotion were grouped for later evaluation because they presented with low survival.

The number of pharyngeal beats, duration of defecation cycles, and body bend frequency in the worms that presented standard behavior were not different between the QUIN-treated N2 and VM487 knockout worms (data not shown), compared to those in untreated worms. However, a reduction in the touch response was noted in the N2 and VM487 strains treated with QUIN compared with their respective controls ($p < 0.05$, Fig. 6A, B).

3.6. QUIN induces neurodegeneration

Reduced neuronal fluorescence at the anterior region of the head was observed in 25% of pan-neuronal/GFP animals treated with 20 mM QUIN compared to the mean of all arbitrary fluorescence unit values obtained in the QUIN-untreated control animals. ($^*p < 0.05$, Fig. 7A, B). In contrast, no significant change in fluorescence was observed in the ventral cord neurons (Fig. 7C).

4. Discussion

In this study, we evaluated the adverse effects of the glutamatergic neurotoxin QUIN in the nematode *C. elegans*. Our data indicate that QUIN adversely affected survival of *C. elegans* and increased ROS generation in an nmr-1-dependent manner. QUIN caused behavioral disturbances involving the glutamatergic system. In addition, QUIN caused neuron-specific neurodegeneration in *C. elegans*.

The QUIN treatment resulted in 12.28%, 18.62%, and 21.28% decreased survival of worms exposed to the 50, 100 and 200 mM QUIN doses, respectively (Fig. 1). QUIN is known to exert neurotoxic effects at nanomolar concentrations in rats and humans (Chen et al., 2009). However, the response to neurotoxicity-triggering molecules in *C. elegans* is only visible when the worm is exposed to high concentrations (Martinez-Finley et al., 2013; Perez-Severiano et al., 2004). These worms can absorb compounds through the cuticle, in addition to ingesting them orally. The cuticle acts as a highly impermeable barrier, making it difficult to pass external agents into the animal (Page and Johnstone, 2007). It is likely that the lethality associated with QUIN exposure in the worms reflects the nature of their cuticle, as only the highest QUIN concentrations tested decreased survival.

Pathologies related to excitotoxicity often develop by hyper-stimulation of glutamatergic membrane receptors, which increase the glutamate concentration in the synaptic cleft (Lin et al., 2012). Excitotoxicity, ATP depletion, and oxidative stress are toxic events that commonly lead to neuronal death in several neurodegenerative diseases (Uttara et al., 2009). The toxic cascades triggered in the glutamatergic system by QUIN involve formation of ROS and nitrogen reactive species, which cause oxidative damage as part of the neurodegenerative process. It is well known that ROS generated by QUIN is due to activation of NMDAR, and that QUIN causes excitotoxicity characteristic of excess activation of the glutamatergic system (Chen et al., 2009; Perez-De La Cruz et al., 2012). In this regard, *C. elegans* expresses the *nmr-1* and *nmr-2* subunits of the NMDA-like receptors, which belong to the subfamilies NR1 and NR2A, respectively (Brockie and Maricq, 2003; Brockie et al., 2001b; Baidya et al., 2014; Kano et al., 2008).

Our data demonstrate that wild-type worms treated with 20 mM QUIN presented with increased ROS levels (Fig. 2). This effect has already been demonstrated in different brain regions of rats exposed to QUIN (Dobrachinski et al., 2012). However, when the QUIN evaluation was performed in *nmr-1* transgenic worms, no increase in ROS levels was observed. In contrast, an increase in ROS was also observed in the transgenic *nmr-2* treated worms after exposure to 20 mM QUIN, which resembled the levels observed in wild-type animals.

Previous studies in hippocampal cell cultures exposed to Aβ oligomers (inducer of Alzheimer's disease) also show that increased ROS occurs in a manner dependent on activation of NMDA-type receptors (De Felice et al., 2007), specifically the NR1 receptor homologous to *nmr-1* in *C. elegans*. Thus, our data suggest that QUIN increased ROS levels in *C. elegans* by stimulating nmr-1/NMDA receptors, which were toxic to the worms. Therefore,

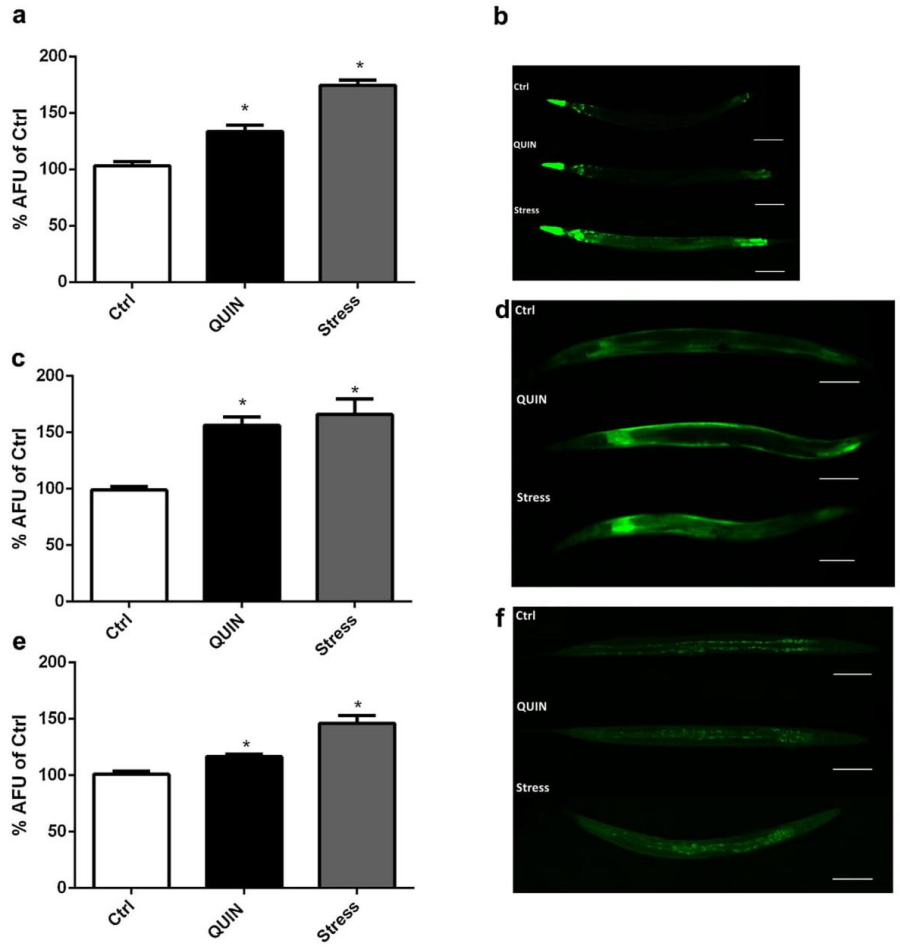

Fig. 4. Effect of QUIN (20 mM) and thermal stress (35 °C) on expression of antioxidant enzymes. (A) Quantification of SOD-3 expression and (B) representative images of the transgenic strain CF1553. (C) Quantification of GST-4 expression and (D) representative images of the transgenic strain CL2166. (E) Quantification of HSP-16.2 expression and (F) representative images of the transgenic strain CL2070. Data are expressed as percentage of the arbitrary fluorescence units (AFU) of control derived from three independent assays with 5 worms per group in each experiment (n = 3). Error bars represent as means ± S.E.M. *$p < 0.01$ compared to ctrl untreated group (One-way ANOVA followed by post-hoc Bonferroni).

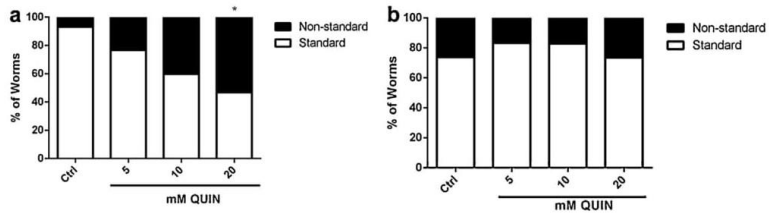

Fig. 5. Effect QUIN on behavioral analysis in wild-type (N2) and transgenic VM487(*nmr-1*) strain. Data are expressed as percent of worms with standard or altered behavior in N2 (A) and VM487 (B) derived from three independent assays with 20 worms per group in each experiment (n = 5). *$p < 0.05$ compared to non-standard behavior control (One-way ANOVA, followed by post-hoc Tukey).

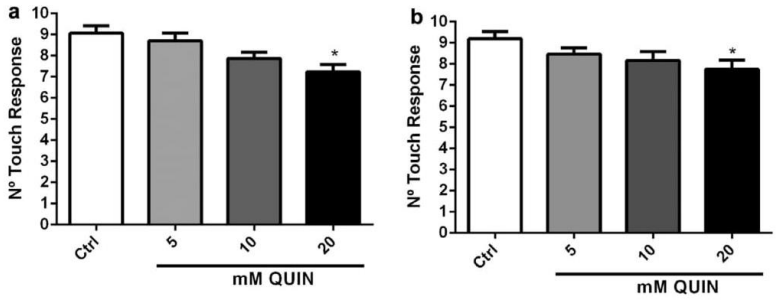

Fig. 6. Effect of QUIN on touch response behavior assays in the N2 and VM487 strains. Touch response in N2 (A) and in VM487 (B) worms. Data are derived from three independent assays with 5 worms per group in each experiment (n = 15). Error bars represent as means ± S.E.M. *p < 0.05 compared to control for each behavior evaluated. (One-way ANOVA followed by post-hoc Bonferroni).

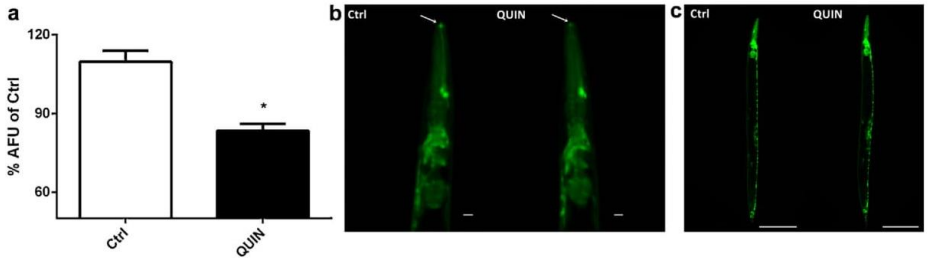

Fig. 7. Effect QUIN on neurodegeneration in the OH438 (pan-neuronal::GFP) strain. The OH438 strain shows all GFP-labeled neurons. A) Quantification of the neurons head fluorescence, indicated neurodegeneration by reducing fluorescence, in the dates expressed as percentage of the arbitrary fluorescence units (AFU) of Ctrl. B) Images the previous region of the worm head. The arrows indicate reduction GFP-fluorescence in neurons. C) GFP-labeled ventral nerve cord without morphological alteration in QUIN-treated worms. Data are derived from three independent assays with 10 worms per group at each experiment (n = 30, *p < 0.020).

C. elegans is an invaluable model to evaluate oxidative stress generated by QUIN, analogous to other mammals. We established that acute treatment with 20 mM QUIN increased ROS generation in wild-type *C. elegans* dependent on the *nmr-1* receptor.

In turn, DAF-16 is responsible for regulating metabolism, resistance to stress, and longevity *in C. elegans* (DiLoreto and Murphy, 2015). The DAF-16/FOXO transcription factor is known to play a key role in oxidative and heat stress resistance, ageing, and other biological functions. DAF-16/FOXO is a target of insulin/insulin-like growth factor signaling in *C. elegans*, which when reduced or absent, results in dephosphorylated DAF-16/FOXO translocating to the nucleus (Kenyon, 2005; Kenyon, 2010). We determined whether QUIN was able to affect the location of DAF-16 in the TJ356 transgenic worm strain. We observed partial migration of DAF-16 to the nucleus in *C. elegans* (Fig. 3). Our findings corroborate the hypothesis that QUIN acts as a pro-oxidant compound. We also observed increased expression of SOD-3, GST-4, and HSP-16.2 (Fig. 4), which are directly related to stress resistance by reducing insulin-like signaling and increasing lifespan (Oh et al., 2005; Sanchez-Blanco and Kim, 2011; Leiers et al., 2003; Rea et al., 2005). These enzymes, which are also transcriptional targets of DAF-16 activation, reflect the stress response when elevated. Thus, our data demonstrate that the generation of ROS triggered by QUIN induced activation of daf-16 and its subsequent translocation to the nucleus and increased transcription of these antioxidant enzymes.

We observed paralysis and the absence of pharyngeal contractions in a large number of the treated worms when evaluating the general behavior of the N2 worms in plates containing QUIN. This effect of QUIN was not observed in the VM487 strain. We tested the standard behavior of worms (N2 and VM487), such as pharyngeal pumping, duration of defecation cycle, body bends, and the nose touch response to evaluate if QUIN altered a more specific glutamatergic behavior. As expected, we showed that QUIN did not change behaviors related with muscular contraction (pharyngeal pumping, duration of defecation cycle, or body bends). This result is not surprising, as the main neurotransmitter that drives these behaviors is acetylcholine ACh (Winnier et al., 1999; Hallam et al., 2000; McKay et al., 2004). In addition, we speculate that the absence of a toxic effect on motor behavior may reflect additional factors, such as the low concentrations of QUIN used and the low density of NMDA receptors in the *C. elegans* nervous system (Maricq et al., 1995). In contrast, QUIN was able to reduce the touch response in worms independent of the *nmr-1* NMDA receptor. The touch response is mediated by sensory neurons, such as ASH. ASH neurons are located in the lateral ganglia, one in each side of the head where they express glutamate, the neurotransmitter responsible for sensory responses in *C. elegans*. Although glutamate NMDA receptors are not directly related to the mechanical sensory response, non-NMDA glutamate type receptors, such as *glr-1*, are mainly responsible for regulating this behavior (Brockie et al., 2001b; Chen and Diamond, 2002; Kass et al., 2001; Mellem et al., 2002). Thus, changes in the glr-1 receptor result in worms that are defective in the touch response task (Maricq et al., 1995; Hart et al., 1995). Considering these observations, our data suggest that the mechanism of action of the QUIN-induced reduction in the touch response may represent an nmr-1 independent pathway that is not likely caused by an increase in ROS levels.

A classic toxic effect induced by QUIN is glutamate-mediated neurotoxicity, with ensuing neurodegeneration (Pierozan et al., 2016). This occurs once QUIN triggers activation of the NMDA receptor, leading to intracellular increases in Ca^{2+} levels. The increase in Ca^{2+} is dependent on NMR-1 hyperactivation and may be caused by QUIN secondary to direct phosphorylation of NMDA receptor subunits via a cAMP/PKA-dependent pathway (Pierozan et al., 2016). Here, we observed reduced GFP fluorescence in neurons of the anterior region of *C. elegans* treated with QUIN, with no changes detected in the ventral nerve cord (Fig. 7C). As pan-neuronal::GFP labels a variety of neurons, we evaluated the main region associated with loss of GFP fluorescence. The mechano-sensory ASH neurons, as well as interneurons responsible for synodal movement and locomotion against mechanical stimuli (AVA, AVB, AVD, AVE, and PVC) are located in the anterior region of the worm's head (Chalfie et al., 1985). These neurons predominantly express glutamate (Brockie and Maricq, 2003). Therefore, we suggest that the neurodegeneration observed upon QUIN exposure was likely associated with the demise of glutamatergic neurons, and may account for the reduced touch response behavior associated with QUIN exposure. Taken together, our findings indicate that QUIN acts on glutamatergic neurons in the anterior region, affecting the touch response in *C. elegans* by interfering with glutamatergic neurotransmission.

In conclusion, the present study demonstrated the toxicity of a known neurotoxin in worms and corroborated that analogous mechanisms are involved in the neurotoxic effects of QUIN to those inherent in cell cultures and rodents models. Specifically, we found that QUIN caused mortality, changed behavioral patterns, and increased the generation of ROS in a manner consistent with glutamatergic dysfunction in *C. elegans*. These results suggest that QUIN could be a useful tool for understanding NMDA-associated pathologies or involvement of the glutamatergic system in disorders of the nervous system (Kotlar et al., 2017).

Conflict of interest

The authors declare that they have no conflict of interest.

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5. CONCLUSÃO

Em conclusão, o presente estudo demonstrou a toxicidade em *C. elegans* do QUIN, uma neurotoxina amplamente conhecida, e corroborou na elucidação de que os mecanismos neurotóxicos do QUIN em roedores e em cultivo celulares são análogos aos que foram visualizados no nematoide *C. elegans*. O fato de tal mecanismo de QUIN atuando como uma neurotoxina em organismos tão distintos, nematoide e mamíferos, ter se mantido ao longo da evolução pode ter ocorrido pela importância deste metabólito em diferentes vias de sobrevivência, desde a produção de nucleotídeos até a sinalização de neuropatologias.

Especificamente, descobrimos que o QUIN causou mortalidade, modificou padrões comportamentais e aumentou a geração de EROS de uma maneira consistente com a disfunção glutamatérgica em *C. elegans*. Esses resultados sugerem que o QUIN pode ser uma ferramenta útil para entender as patologias associadas ao NMDA ou ao envolvimento do sistema glutamatérgico em distúrbios do sistema nervoso em *C. elegans*.

Além disso, a utilização de QUIN em *C. elegans* como modelo de neurotoxicidade do sistema glutamatérgico traz vantagens como por exemplo possibilitar avaliações com modificações genéticas para avaliar diferentes vias metabólicas como também a realização de experimentos mais robustos, os quais não são possíveis de executar em mamíferos.

6. PERSPECTIVAS

Com base nos dados encontrados na presente dissertação, tem-se como perspectivas continuar a investigação dos efeitos de QUIN no nematoide *C. elegans*, bem como o teste de moléculas que tenham potencial para proteger frente a toxicidade de QUIN.

- Avaliar a toxicidade do QUIN na produção de ovos de *C. elegans*;
- Investigar os efeitos do QUIN em comportamentos de resposta sensorial em *C. elegans;*
- Avaliar a viabilidade de neurônios glutamatérgicos de *C. elegans* após a exposição ao QUIN;
- Avaliar a produção de ATP em *C. elegans* após a exposição do QUIN;
- Investigar os efeitos do QUIN nas enzimas Ca^{2+}ATPase, Na^{2+}, K^{+} ATPase em *C. elegans;*
- Avaliar o potencial de membrana após a exposição ao QUIN em *C. elegans.*

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