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**ASPECTOS MOLECULARES E COMPORTAMENTAIS ASSOCIADOS AO
POTENCIAL NEUROPROTETOR DO 7-CLORO-4-(FENILSELANIL) QUINOLINA
EM UM MODELO MIMÉTICO À DOENÇA DE PARKINSON EM *Drosophila
melanogaster***

TESE DE DOUTORADO

Shanda de Freitas Couto

URUGUAIANA, RS, BRASIL

2019

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Tese apresentada ao Programa de Pós-graduação *Stricto sensu* em Bioquímica da Universidade Federal do Pampa, como requisito para a obtenção do Título de Doutor em Bioquímica.

Orientadora: Dra. Marina Prigol

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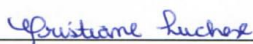
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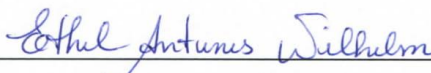
Prof. Dra. Marina Prigol (UNIPAMPA)

(Orientadora)



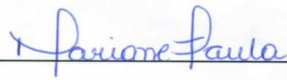
Prof. Dra. Cristiane Luchese

(UFPeI)



Prof. Dra. Ethel Antunes Wilhelm

(UFPeI)



Prof. Dra. Mariane Trindade de Paula
(IFFar)



Prof. Dra. Francieli Weber Santos Cibir
(UNIPAMPA)

URUGUAIANA, RS, BRASIL

2019

Dedico essa tese a meus pais que, com muito amor e dedicação, não mediram esforços para que eu pudesse ir em busca de todos os meus sonhos.

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“Um sonho que se sonha só, é só um sonho que se sonha só, mas sonho que se sonha junto é realidade” (Raul Seixas)

*“Que as coisas são inatingíveis? Ora! Isso não é motivo para não querê-las.
Que tristes seriam os caminhos sem a presença distante das estrelas”*

(Mario Quintana)

PARTE I

RESUMO

A doença de Parkinson (DP) está associada à múltiplos fatores, incluindo comprometimento mitocondrial, apoptose e estresse oxidativo, que culminam com a perda de neurônios dopaminérgicos, e consequente deficiência de dopamina. Clinicamente é caracterizada por déficits locomotores, no entanto, evidencia-se a presença de sintomas psicomotores e não-motores, cuja terapia farmacológica tradicional é ineficaz e associada a efeitos colaterais, tornando imprescindível o desenvolvimento de novas estratégias terapêuticas para a DP. Diante disso, avaliou-se os aspectos moleculares e comportamentais associados ao potencial neuroprotetor de 7-cloro-4-(fenilselanil) quinolina (4-PSQ) em um modelo mimético à DP induzida por rotenona (ROT) em *Drosophila melanogaster*. As moscas adultas foram divididas em grupos: (1) controle, (2) 4-PSQ 25 μ M, (3) ROT 500 μ M, (4) 4-PSQ 25 μ M+ ROT 500 μ M, expostas concomitantemente ao 4-PSQ e a ROT por 7 dias, de acordo com cada grupo. Foram realizadas análises de sobrevivência/mortalidade e testes comportamentais, visando avaliar as alterações motoras relacionadas a atividade de escalada, capacidade exploratória e atividade locomotora espontânea; e alterações psicomotoras e não-motoras envolvendo aprendizagem, memória e comportamento tipo ansiedade. Ainda, foram avaliados os níveis de espécies reativas (ERs), peroxidação lipídica (LPO), atividade da superóxido dismutase (SOD), catalase (CAT), e níveis de dopamina na região da cabeça; e a atividade da acetilcolinesterase (AChE) e níveis de selênio nas regiões da cabeça e corpo das moscas. Observou-se um aumento na mortalidade das moscas do grupo ROT, enquanto no grupo tratado concomitantemente com o 4-PSQ uma melhor sobrevivência. As moscas expostas à ROT exibiram fenótipo similar a DP, com comprometimento locomotor, déficits na atividade de escalada, capacidade exploratória, e atividade locomotora espontânea; além de prejuízos psicomotores e não-motores, incluindo danos na aprendizagem, na memória, e comportamento semelhante a ansiedade; em contrapartida o 4-PSQ foi capaz de prevenir tais deficiências comportamentais nas moscas. A exposição a ROT culminou em um aumento nos níveis de ERs e LPO, paralelamente a diminuição na atividade das enzimas antioxidantes (SOD e CAT), enquanto o tratamento com 4-PSQ preveniu o dano oxidativo e melhorou as defesas antioxidantes. Ainda, verificamos uma redução nos níveis de dopamina na cabeça das moscas e, de forma inédita, nos níveis de selênio (cabeça/corpo) com a exposição a ROT; em contrapartida o 4-PSQ restabeleceu os níveis de dopamina e selênio, sendo ainda observada correlação positiva entre esses dois parâmetros. Ademais, foi verificado um aumento na atividade da AChE com a exposição a ROT, enquanto que o 4-PSQ preveniu o aumento na atividade dessa enzima. Nossos achados evidenciam o 4-PSQ como uma molécula multi-alvo, atuando no sistema dopaminérgico por diferentes mecanismos, reduzindo danos oxidativos, melhorando as defesas antioxidantes e exercendo ação anticolinesterásica, fatores que juntos podem proteger os neurônios dopaminérgicos, e consequentemente prevenir a mortalidade e déficits motores, psicomotores e não motores em *Drosophila melanogaster*; ações correlacionadas com a presença de selênio em sua estrutura. Portanto, o 4-PSQ foi capaz de prevenir as alterações

moleculares e comportamentais nesse modelo, devido à sua ação multi-alvo, e principalmente por sua eficácia como antioxidante e inibidor da AChE, características que potencializam seu efeito terapêutico na DP.

Palavras-chave: selenoquinolina, antioxidante, anticolinesterase, dopaminérgico, paradigmas comportamentais, neurotoxicidade.

ABSTRACT

Parkinson's disease (PD) is associated with multiple factors, including mitochondrial impairment, apoptosis and oxidative stress, culminating in the loss of dopaminergic neurons and consequent dopamine deficiency. Clinically it is characterized by locomotor deficits, however, it is evidenced the presence of psychomotor and non-motor symptoms, whose traditional pharmacological therapy is ineffective and associated with side effects, making it essential to develop new therapeutic strategies for PD. Therefore, we evaluated the molecular and behavioral aspects associated with the neuroprotective potential of 7-chloro-4-(phenylselanyl) quinoline (4-PSQ) in a mitotic model for PD induced by rotenone (ROT) in *Drosophila melanogaster*. Adult flies were divided into groups: (1) control, (2) 4-PSQ 25 μ M, (3) ROT 500 μ M, (4) 4-PSQ 25 μ M + 500 μ M ROT, concomitantly exposed to 4-PSQ and ROT for 7 days, according to each group. Survival / mortality and behavioral tests were performed to evaluate the motor alterations related to climbing activity, exploratory capacity and spontaneous locomotor activity; and psychomotor and non-motor alterations, involving learning, memory and anxiety-like behavior. The levels of reactive species (RS), lipid peroxidation (LPO), superoxide dismutase activity (SOD), catalase (CAT), and dopamine levels in the head region were also evaluated; and acetylcholinesterase (AChE) activity and selenium levels in the head and body regions of flies. An increase in mortality of the ROT group flies was observed, while in the group treated concomitantly with the 4-PSQ a better survival. Flies exposed to ROT exhibited similar phenotype to PD, with locomotor impairment, deficits in climbing activity, exploratory capacity, and spontaneous locomotor activity; in addition to psychomotor and non-motor damage, including damage to learning, memory, and anxiety-like behavior; in contrast, 4-PSQ was able to prevent such behavioral deficiencies in flies. Exposure to ROT culminated in an increase in RS and LPO levels, in parallel with a decrease in the activity of antioxidant enzymes (SOD and CAT), while 4-PSQ treatment prevented oxidative damage and improved antioxidant defenses. Also, we observed a reduction in dopamine levels in the head of flies and, unprecedentedly, in selenium levels (head / body) with exposure to ROT; in contrast, 4-PSQ reestablished dopamine and selenium levels, and a positive correlation was observed between these two parameters. In addition, an increase in AChE activity was observed with ROT exposure, whereas 4-PSQ prevented the increase in the activity of this enzyme. Our findings evidence 4-PSQ as a multi-target molecule, acting in the dopaminergic system by different mechanisms, reducing oxidative damages, improving antioxidant defenses and exerting anticholinesterase action, factors that together can protect dopaminergic neurons, and consequently prevent mortality and motor deficits, psychomotor and non-motor in *Drosophila melanogaster*, actions correlated with the presence of selenium in its structure. Therefore, 4-PSQ was able to prevent molecular and behavioral alterations in this model, due to its multi-target action, and mainly for its effectiveness as antioxidant and AChE inhibitor, characteristics that potentiate its therapeutic effect in PD.

Keywords: selenoquinoline, antioxidant, anticholinesterase, dopaminergic, behavioral paradigms, neurotoxicity.

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Revisão Bibliográfica

4-PSQ - 7-cloro-4-(fenilselanil) quinolina

6-OHDA – 6-hidroxidopamina

AChE – acetilcolinesterase

APS – Aversive Phototaxis Suppression Assay

ATP – adenosina trifosfato

CAT – catalase

COMT – catecol-o-metil transferase

COX-2 – ciclo-oxigenase-2

DRIs – Dietary Reference Intakes

DNA – ácido desoxirribonucleico

DP – Doença de Parkinson

EROs – espécies reativas de oxigênio

ERs – espécies reativas

FADH₂ – flavina adenina

FMN – flavina mononucleótido

GSH – glutationa reduzida

GST – glutationa S-transferase

GPX – glutationa peroxidase

L-DOPA – levodopa

LPO – peroxidação lipídica

MA – metanfetamina

MAO – monoamino oxidase

MAO-B – monoamino oxidase B

MPTP – 1-metil-4-fenil-1,2,3,6-tetra-hidropiridina

NADH – nicotinamida adenina

ROT – rotenona

SOD – superóxido dismutase

LISTA DE ABREVIATURAS

Artigo 1

4-PSQ - 7-chloro-4-(phenylselanyl) quinoline

ROT – rotenone

PD – Parkinson's disease

GC/MS – gas chromatography with mass spectrometry

CRM – certified reference material

ERM – European Reference Materials

ICP-MS – inductively coupled plasma mass spectrometry

RSD – relative standard deviation

HPLC – high performance liquid chromatography

AChE – acetylcholinesterase

AcSCh – acetylthiocholine

RS – reactive species

LPO – lipid peroxidation

TBARS - thiobarbituric acid reactive substance

SOD – superoxide dismutase

CAT – catalase

PSH – proteic thiol

NPSH – non-proteic thiol

BBB – blood–brain barrier

LISTA DE ABREVIATURAS

Manuscrito 1

4-PSQ - 7-chloro-4-(phenylselanyl) quinoline

APS – Aversive Phototaxis Suppression Assay

AChE – acetylcholinesterase

AcSCh – acetylthiocholine

DTNB – 5 mM 5,5'-dithiobis (2-nitrobenzoic acid)

PC0 – 0 hr post conditioning

PC6 – 6 hr post conditioning

PD – Parkinson's disease

ROT – rotenone

APRESENTAÇÃO

A presente tese foi dividida em três partes principais. Na parte I encontram-se os itens RESUMO, INTRODUÇÃO e DESENVOLVIMENTO, sendo nesse último apresentado o referencial teórico sobre os temas trabalhados do decorrer dessa tese, a justificativa e objetivos do referido trabalho.

A metodologia realizada e os resultados obtidos que fazem parte da tese estão apresentados sob a forma de um artigo científico e um manuscrito, nos itens ARTIGO CIENTÍFICO e MANUSCRITO, que apresentam as seções: Introdução, Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas, apresentados na parte II desse trabalho.

Os itens DISCUSSÃO e CONCLUSÃO, inclusos na parte III, ao final desta tese, constam os principais achados e interpretações referente ao artigo e manuscrito presentes nesse trabalho. As REFERÊNCIAS dizem respeito às citações que aparecem nos itens INTRODUCAO, DESENVOLVIMENTO e DISCUSÃO apresentados na referida tese. No item PERSPECTIVAS, estão expostos possíveis objetivos e/ou estudos para dar continuidade a este trabalho.

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1 INTRODUÇÃO

A doença de Parkinson (DP), é a segunda doença neurodegenerativa mais comum, e a primeira no que se refere aos distúrbios do movimento neurológico, atingindo 1 a 2% em indivíduos acima de 60 anos no mundo (SANDERS e GREENAMYRE, 2013; SUDATI et al., 2013; REEVE e TURNBULL, 2014; STAVELEY, 2015; HELLEY et al., 2017; SONG et al., 2017). Sua fisiopatologia aponta como principal marca patológica o dano nos neurônios dopaminérgicos na região da substância negra compacta do mesencéfalo, com uma neurotransmissão decrescente na região do estriado (ZAITONE et al., 2012; STAVELEY, 2015; SIDDIQUE et al., 2016; EBRAHIMI et al., 2017). Além da perda de neurônios dopaminérgicos, outra característica patológica evidenciada é a formação de corpos de Lewy, que são agregados da proteína α -sinucleína, nos neurônios dopaminérgicos remanescentes, relacionados grandemente aos sintomas psicomotores da DP (KUMARAN e COOKSON, 2015; BENSKEY et al., 2016; HELLEY et al., 2017; SONG et al., 2017)

Nesse sentido, a degeneração seletiva dos neurônios dopaminérgicos leva a deficiência de dopamina, tendo como consequência um conjunto de sintomas caracterizados principalmente por distúrbios motores, tais como tremor de repouso, rigidez muscular, bradicinesia, e instabilidade postural (SANDERS e GREENAMYRE, 2013; STAVELEY, 2015; EBRAHIMI et al., 2017). No entanto também tem sido descrita a ocorrência de sintomas não motores, que incluem disfunção cognitiva, alterações de humor (depressão e ansiedade), dor, disfunção sensorial, disautonomia, distúrbios do sono, perda de olfato e constipação, que podem surgir em estágios iniciais mesmo antes do diagnóstico de DP, e são exacerbados com a progressão da doença (ALVES et al., 2008; CHEN e MARSH, 2014; MUNHOZ, et al., 2015; RANA et al., 2015; SONG et al., 2017).

Além disso, o processo da neurodegeneração na DP parece estar apoiado por diversos fatores, incluindo comprometimento mitocondrial, agregação anormal de proteínas, apoptose e estresse oxidativo (MUÑOZ-SORIANO e PARICIO, 2011; SANDERS e GREENAMYRE, 2013; STAVELEY, 2015). No entanto, ainda existem muitas lacunas sobre o papel do estresse oxidativo na etiologia dessa patologia, porém, estudos apontam o dano oxidativo como significativo na patogênese da DP

(BOTELLA et al., 2008; SANDERS e GREENAMYRE, 2013). Ainda, a literatura aponta múltiplas disfunções para além do sistema dopaminérgico, incluindo déficit colinérgico, serotoninérgico e noradrenérgico (HASEGAWA et al., 2017; HELLEY et al., 2017).

Estudos experimentais utilizam o pesticida rotenona (ROT) para a indução de modelo tipo DP em animais, visto que tal substância química reproduz características importantes da doença, tanto em *Drosophila melanogaster* como em roedores (TANNER et al., 2011; MANJUNATH e MURALIDHARA 2013; ARAUJO et al., 2015). Esse pesticida atua principalmente trazendo prejuízos para a função mitocondrial (MANJUNATH e MURALIDHARA 2013; SANDERS e GREENAMYRE, 2013; ARAUJO et al., 2015), e está associado a morte dos neurônios dopaminérgicos e aumento da produção espécies reativas (ERs), que conseqüentemente levam ao estresse oxidativo (PERFEITO e REGO, 2012; MANJUNATH e MURALIDHARA 2013; SANDERS e GREENAMYRE, 2013).

Ainda, tem ganhado destaque os estudos com modelos animais alternativos, tais como os que utilizam a *Drosophila melanogaster*, sendo um importante instrumento genético na elucidação da base biológica das doenças humanas, entre essas as neurodegenerativas (ARAUJO et al., 2015; STAVELEY, 2015; SONG et al., 2017). Ademais, no que se refere a DP esse modelo tem sido utilizado devido a homologia entre cinco dos seis genes relacionados a essa patologia (WHITWORTH et al., 2006; ARAUJO et al., 2015) e tem sido utilizado em estudos relacionados a caracterização dos papéis do selênio na biologia e na medicina, emergindo na investigação de parâmetros no metabolismo do selênio (MARTIN-ROMERO, 2001).

Ainda, acrescida a problemática da DP, aponta-se a terapia farmacológica tradicional, que baseada na utilização de moduladores dopaminérgicos, atua principalmente frente aos sintomas motores, sendo ineficaz para os sintomas não motores, além provocar importantes efeitos colaterais importante, apresentar eficácia limitada ao uso prolongado e ineficiência no controle da progressão da doença (HASEGAWA et al., 2017; HELLEY et al., 2017; SONG et al., 2017; INOUE et al., 2018). Nesse panorama, torna-se imprescindível o desenvolvimento de novas estratégias terapêuticas mais abrangentes para DP, que possam atuar não somente frente aos sintomas motores e no sistema dopaminérgico.

Nessa perspectiva, a busca por compostos naturais e/ou sintéticos com ação antioxidante tem aumentado notavelmente. Dentre as várias aplicações terapêuticas dos antioxidantes, ressalta-se a ação neuroprotetora (HALLIWELL 2006; HALLIWELL, 2011; SANDERS e GREENAMYRE, 2013). Por isso, compostos químicos de síntese relativamente simples, que apresentem atividade antioxidante, têm ganhado papel importante nas pesquisas em busca de agentes terapêuticos.

Neste sentido, os compostos orgânicos de selênio destacam-se como interessante recurso de novas substâncias sintéticas com potencial terapêutico, devido às suas atividades farmacológicas (NOGUEIRA E ROCHA, 2011). Aliado a isso, o estudo de compostos de selênio combinados à quinolinas tem demonstrado resultados promissores (SAVEGNAGO et al., 2013; PINZ et al., 2016; DUARTE et al., 2017; SILVA et al., 2017; PINZ et al., 2018; VOGT et al., 2018, VOSS et al., 2018). Recentemente o composto 7-cloro-4-(fenilselanil) quinolina (4-PSQ) tem se destacado devido as suas ações farmacológicas, caracterizada como uma droga multi-alvo, principalmente devido a sua ação antioxidante (SAVEGNAGO et al., 2013; PINZ et al., 2016; DUARTE et al., 2017; SILVA et al., 2017; VOGT et al., 2018), anti-inflamatória (PINZ et al., 2016; SILVA et al., 2017; VOSS et al., 2018), anticolinesterásica (PINZ et al., 2018), anti-noceptiva (PINZ et al., 2016; SILVA et al., 2017) e ansiolítica (REIS et al., 2017; PINZ et al., 2018), sem apresentar efeitos toxicológicos ou distúrbios locomotores em modelos com roedores (SAVEGNAGO et al., 2013; PINZ et al., 2016; REIS et al., 2017). Tais fatores nos levaram a considerar o 4-PSQ como uma potencial droga a ser testada em um modelo de DP induzido pela neurotoxina ROT.

Diante do exposto, o presente investigou os aspectos moleculares e comportamentais associados ao potencial neuroprotetor do composto 4-PSQ em um modelo mimético à DP induzida por ROT em *Drosophila melanogaster*.

2 REFERENCIAL TEÓRICO

2.1 Doença de Parkinson

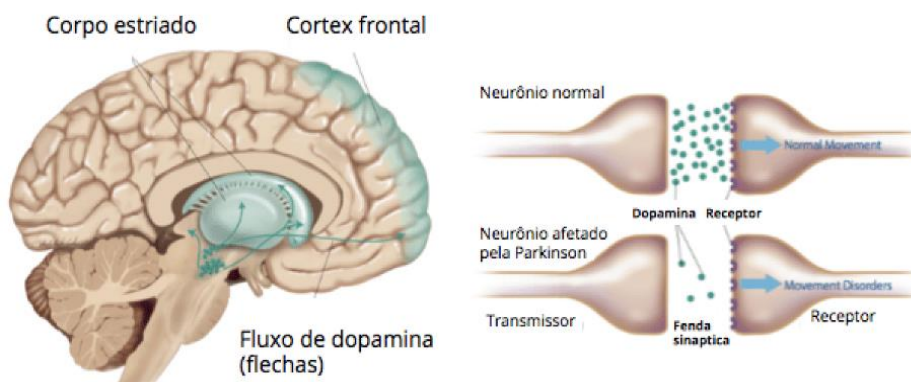
Atualmente observa-se uma expressiva modificação do perfil demográfico no Brasil e no mundo, caracterizando o envelhecimento da população (BOVOLENTA e FELÍCIO, 2016). Essa situação acarreta grande impacto nas questões de saúde pública e qualidade de vida dos indivíduos, devido a incidência de doenças associadas ao processo do envelhecimento, tais como as doenças neurodegenerativas, sendo as principais o Alzheimer e a DP (STAVELEY, 2015; BOVOLENTA e FELÍCIO, 2016).

A DP é a segunda doença neurodegenerativa mais comum e com longa sobrevida atualmente, e que tem como consequências prejuízos em relação a capacidade funcional, qualidade de vida, e aspectos econômicos (STAVELEY, 2015; BOVOLENTA e FELÍCIO, 2016). Estimativas apontam prevalência de 1 - 2% da população mundial, com idade superior a 60 anos com essa patologia (REEVE e TURNBULL, 2014; SUDATI et al., 2013; SONG et al., 2017). Projeções internacionais indicam um crescimento substancial, baseadas em estudos de prevalência específica para a idade entre as nações mais populosas no mundo, o número de pessoas acima dos 50 anos com DP passaria de 4,1 milhões em 2005 para 8,7 milhões em 2030 (DORSEY et. al., 2007). No que se refere a existência de dados fidedignos da realidade brasileira, um fator complicador é que no Brasil a notificação da DP não é compulsória, assim dados de estimativas apontam prevalências superiores a 3% na população maior de 70 anos. Ainda, tais estimativas internacionais indicaram que aproximadamente 220 mil pessoas seriam acometidas por esse problema no Brasil e direcionam para um aumento superior ao dobro desse número entre os anos de 2005 e 2030. (DORSEY et. al., 2007; PETERNELLA e MARCON, 2009; BOVOLENTA e FELÍCIO, 2016). Dados preocupantes, considerando a distribuição universal da doença, independente de grupos étnicos ou classes socioeconômicas, e sua relação direta com o envelhecimento da população (WORLD HEALTH ORGANIZATION, 2006; TANNER et al, 2011). Nesse contexto, tais doenças neurodegenerativas de início tardio representam uma grande preocupação de saúde pública, uma vez que a

idade média da população mundial aumenta constantemente (BOVOLENTA e FELÍCIO, 2016).

No que se refere a fisiopatologia, a DP é caracterizada por uma degeneração neuronal seletiva, na região do mesencéfalo, chamada de substância negra compacta. Tais neurônios contêm o neurotransmissor dopamina, e suas fibras nervosas projetadas para o estriado (DAUER e PRZEDBORSKI, 2003; WANG et al., 2015; SONG et al., 2017). Assim, devido à perda neuronal, evidencia-se diminuição nos níveis de dopamina na DP (Figura 1), com conseqüentes manifestações locomotoras e clínicas aos portadores da doença. Estimativas indicam a existência de uma perda superior a 80% das células produtoras de dopamina na maioria dos pacientes com DP (HEE NA et al., 2014), sendo que já no início das manifestações clínicas pode ser observada uma perda de aproximadamente 60% dos neurônios dopaminérgicos da substância negra e uma redução da dopamina no estriado de cerca de 80% (DAUER e PRZEDBORSKI, 2003; VIVES-BAUZA et al., 2009). Ainda, outro fator intimamente relacionado a perda de neuronal é a presença de estruturas conhecidas como corpos de Lewy, que são agregados proteicos, compostos basicamente por proteínas como a α -sinucleína, ubiquitina e sinfilina-1, entre outras (BRAAK et al., 2003; PERFEITO e REGO, 2012; STAVELEY, 2015). No entanto, a neuropatologia subjacente a DP não está restrita somente ao sistema dopaminérgico, e podem incluir áreas que não envolvam apenas o controle motor, tais como em outros núcleos do tronco cerebral (por exemplo, núcleo motor dorsal do vago), córtex cerebral, e ainda estender-se ao sistema autonômico periférico, como os gânglios simpáticos, neurônios simpáticos eferentes cardíacos e o plexo mioentérico do trato gastrointestinal, repercutindo assim em outros órgãos e sistemas (BRAAK, 2003; BRASIL, 2010).

Figura 1 - Representação da diminuição nos níveis de dopamina em neurônio afetado na DP.



Fonte: <https://www.dicavida.com.br/doenca-de-parkinson/>

Clinicamente, os pacientes com DP exibem sintomas motores e não motores (HASEGAWA et al., 2017; SONG et al., 2017). A literatura evidencia como os principais sintomas as alterações motoras, que se reflete pela locomoção prejudicada, caracterizada por sintomas clássicos, como a lentidão de movimentos, tremores em repouso, rigidez e instabilidade postural (WORLD HEALTH ORGANIZATION, 2006). Tais sintomas decorrem da associação existente entre a dopamina e a atividade motora, sendo que a condição patológica de perda dos neurônios dopaminérgicos, como ocorre na DP, tem como consequência essas importantes alterações locomotoras (BRAAK et al., 2003). Ainda a presença de processo neurodegenerativo na DP, além do sistema nigroestriatal e de neurônios dopaminérgicos, explica as repercussões não-motoras, tais como demência, depressão, ansiedade, transtornos de personalidade, dificuldades de sono, dor, alterações na cognição, disfunção sensorial, disautonomia, constipação, alterações no órgão olfativo e o tronco encefálico, que são frequentemente observados no início da doença (BRAAK et al., 2003; LEES et al., 2009; WINNER et al., 2011; HAUSER e HASTINGS, 2013; CHEN e MARSH, 2014; MUNHOZ et al., 2015; SONG et al., 2017).

Em relação as possíveis causas, a maioria dos casos de DP é idiopática (90-95%), e a interferência de alguns fatores ambientais tem sido investigado, tais como o uso ocupacional de herbicidas e pesticidas (ROT e 1-metil-4-fenil-1,2,3,6-tetra-

hidropiridina – MPTP), a exposição a solventes orgânicos, monóxido de carbono e dissulfeto de carbono; e, em geral, a industrialização, o ambiente rural, toxinas derivadas de plantas, infecções bacterianas e virais (PEREIRA e GARRET, 2010; SCHAPIRA e JENNER, 2011; TANNER et al. 2011; WANG et al., 2015; HASEGAWA et al., 2017; HELEY et al., 2017). Ainda, acredita-se que sua ocorrência esteja relacionada a interação entre fatores ambientais e genéticos, contribuindo para o desenvolvimento da neurodegeneração; associada ainda ao processo de envelhecimento (CHINTA et al., 2010; PEREIRA e GARRET, 2010). Estudos apontam que DP pode estar associada à disfunção mitocondrial devido a alterações em diversas rotas, incluindo a geração de radicais livres, inflamação, crise bioenergética e disfunção do complexo I da cadeia respiratória mitocondrial (SANDERS e GREENAMYRE, 2013; EBRAHIMI et al., 2017). Além disso, indícios apontam que as inclusões de α -sinucleína e a regulação Ciclo-oxigenase-2 (COX-2) contribuem para a disfunção mitocondrial e neuroinflamação na DP. Evidências indicam que o aumento da expressão de COX-2 possibilitaria a oxidação da dopamina, que aciona o estresse oxidativo e à acumulação α -sinucleína, apontando o dano oxidativo como um fator importante na progressão da DP (MUÑOZ-SORIANO e PARICIO, 2011; GUPTA e BENZEROUAL, 2013; SANDERS e GREENAMYRE, 2013; STAVELEY, 2015; WANG et al., 2015).

Assim, danos no ácido desoxirribonucleico (ADN) e o estresse oxidativo podem estar envolvidos na fisiopatologia dessa degeneração neuronal (DIAS et al., 2013; SANDERS e GREENAMYRE, 2013; FISCHER e MAIER, 2015; KHATRI e JUVEKAR, 2016; JIANG et al., 2017). Nesse sentido, associa-se ainda o envelhecimento, que devido as falhas nos processos celulares que ocorrem no envelhecer, proporcionando uma maior vulnerabilidade a danos dopaminérgicos (OBESO et al., 2010; HWANG, 2013; SANDERS e GREENAMYRE, 2013; KHATRI e JUVEKAR, 2016).

Também são descritas causas familiares, relacionada a fatores genéticos, que frequentemente tem início precoce, por volta dos 45 anos de idade, e envolvem mutação em vários genes que levam a degeneração neuronal (LESS et al., 2009; HWANG 2013; BEILINA e COOKSON, 2016). No entanto a literatura atual aponta, que apenas 30% dos casos familiares e 3 a 5% dos casos esporádicos de DP pode estar diretamente ligada a mutações monogênicas, enquanto os demais casos são

classificados como idiopáticos, com etiologia desconhecida (Hernandez et al., 2016; HELLEY et al., 2017). Ainda a literatura refere que estão implicados na DP idiopática causas atribuídos a outros genes associados ao risco, a exposições ambientais e a interações gene-ambiente, fatores que tornam a DP um distúrbio multifatorial com etiologia complexa (HELLEY et al., 2017). Ademais, recentemente dentro dessa multifatorialidade as hipóteses etiológicas impulsionam cada vez mais a ideia de uma interação entre a predisposição genética e os fatores de risco ambientais para o início da cascata patológica, e apontam como característica importante a disfunção monoaminérgica múltipla, incluindo o déficit de sistemas dopaminérgicos, colinérgicos, serotoninérgicos e noradrenérgicos (HASEGAWA et al., 2017; HELLEY et al., 2017).

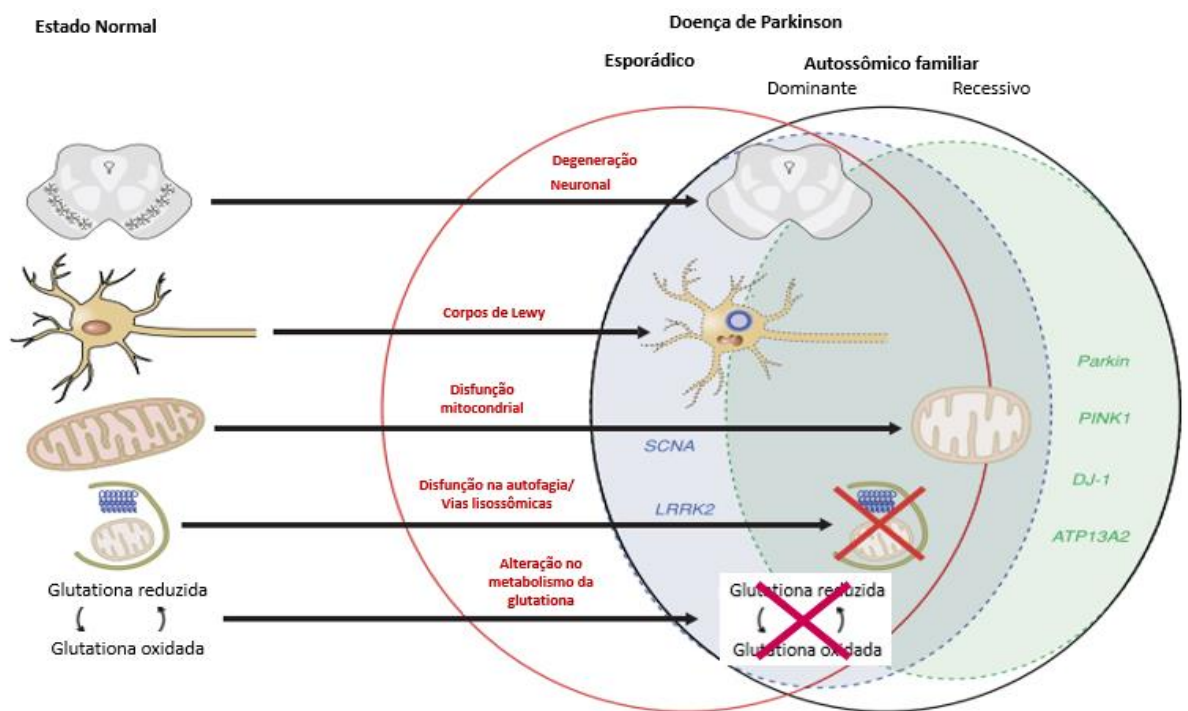
No que se refere a relação com o sistema colinérgico, no estriado, acetilcolina e dopamina interagem fortemente e desempenham um papel importante no controle motor normal (JENSEN et al, 2011). Assim, um desequilíbrio da transmissão desses dois neurotransmissores no sistema nigro-estriatal é a base patogênica das desordens extrapiramidais, como no caso da DP. Ainda, estudos apontam o envolvimento da acetilcolinesterase (AChE), enzima que hidrolisa a acetilcolina, na patogênese do modelo de DP através de neurotoxina via apoptose neuronal (ZHANG, et al., 2013). Assim, especificamente uma deficiência ou inibição da acetilcolinesterase poderia diminuir a apoptose e proteger os neurônios dopaminérgicos no modelo de neurotoxina da DP (ZHANG, et al., 2013).

2.2 Aspectos moleculares na Doença de Parkinson

A etiologia das doenças neurodegenerativas ainda é em parte desconhecida, porém, atualmente o envolvimento do estresse oxidativo é bem aceito entre os pesquisadores (ANGELINE et al., 2012; SANDERS e GREENAMYRE, 2013; HASEGAWA et al., 2017; HELLEY et al., 2017) e o sistema nigroestriatal parece ser particularmente sensível ao dano oxidativo causado por diferentes mecanismos e agentes (MORETTO e COLOSIO, 2011; HASEGAWA et al., 2017). Evidências de estudos bioquímicos, toxicológicos, celulares e genéticos tem descrito hipóteses de

várias cascatas patogênicas envolvendo mecanismos moleculares na degeneração neuronal na DP (Figura 2), incluindo estresse oxidativo, estresse do retículo endoplasmático, disfunção mitocondrial, agregação anormal de proteína (principalmente α -sinucleína), disfunção da ubiquitina-proteassoma, neuroinflamação, comprometimento do sistema de autofagia (via lisossômica), depleção de tióis, excitotoxicidade glutamatérgica, alterações no transporte de membrana e apoptose; os quais tem relação com o estresse oxidativo (LEES et. al., 2009; CORTI et al., 2011; SANDERS e GREENAMYRE, 2013; SUDATI et al., 2013; WANG et al., 2015; BENSKEY et al., 2016; HASEGAWA et al., 2017; HELLEY et al., 2017).

Figura 2 - Representação esquemática de alguns fatores envolvidos na neurodegeneração dopaminérgica na DP.



Fonte: Adaptado de Corti et al. (2011, pag. 1196).

No que se refere ao tecido nervoso, observa-se uma maior susceptibilidade ao dano oxidativo devido a alguns fatores específicos, tais como o elevado consumo de

O₂, comparativamente a outros tecidos corporais, que pode resultar em um aumento na formação de O₂ e outras ERs (HALLIWELL 2006; HALLIWELL 2011). Ainda, cita-se outros fatores, como a baixa quantidade de defesas antioxidantes, elevadas concentrações de lipídeos polinsaturados (substratos para lipoperoxidação) e a presença de ferro em altas concentrações em algumas regiões do cérebro, possibilitando aumento da peroxidação lipídica e autoxidação de neurotransmissores, pela formação de radical hidroxila na reação de Fenton (HALLIWELL, 2006; RAJENDRAN et al., 2014).

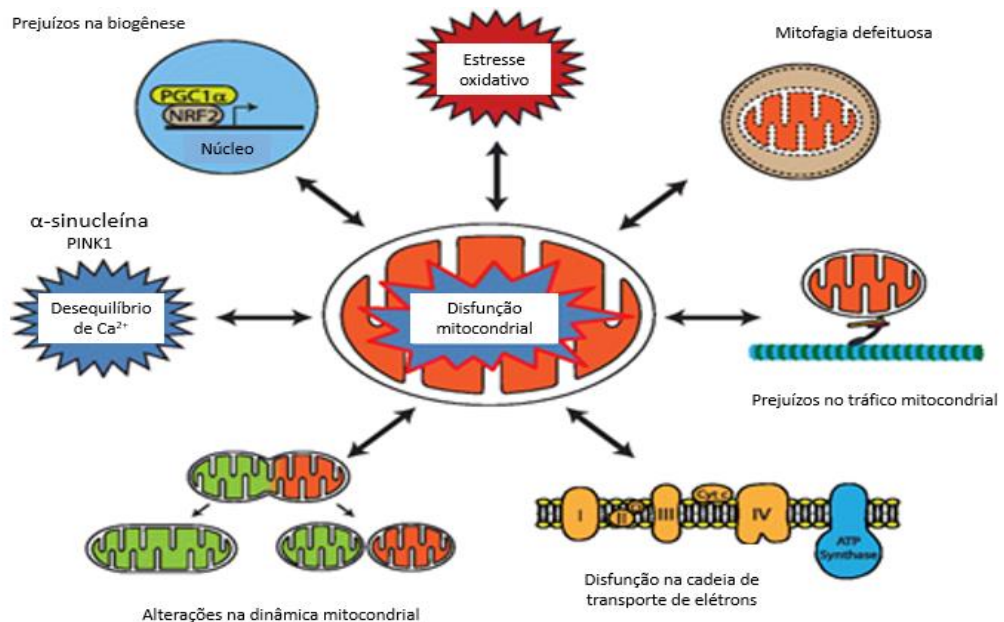
Assim, o estresse oxidativo tem papel fundamental na neurodegeneração dopaminérgica na DP, sendo um agente importante no comprometimento da integridade dos neurônios (HALLIWELL e GUTTERIDGE, 2007; SUDATI et al., 2013). No entanto os mecanismos exatos que desencadeiam o estresse oxidativo na neurodegeneração precisam ainda ser mais esclarecidos. Evidências apontam entre os principais fatores para o dano oxidativo, a disfunção mitocondrial (complexo I), a alteração no metabolismo da dopamina, agregação da proteína α -sinucleína e apoptose (DIAS et al., 2013; FISCHER e MAIER, 2015; KHATRI e JUVEKAR, 2016; JIANG et al., 2017).

No que se refere a disfunção mitocondrial, que ocorre no complexo I, considera-se como um importante fator na geração das ERs. As mitocôndrias são responsáveis pela produção de adenosina trifosfato (ATP), pelo processo de respiração e fosforilação oxidativa. Na síntese de ATP ocorre o acoplamento das reações redox e de fosforilação através do processo de fosforilização oxidativa na membrana interna de mitocôndrias. Durante este processo, ocorre o transporte dos elétrons do NADH (nicotinamida adenina) ou FADH₂ (flavina adenina), através da cadeia transportadora de elétrons, por complexos I-IV, criando um gradiente de prótons através da membrana interna mitocondrial (SUBRAMANIAM e CHESSELET, 2013). Nesse processo, a principal porta de entrada para os elétrons é o complexo I, que catalisa a transferência de elétrons do NADH para a ubiquinona, envolvendo uma flavina mononucleótido (FMN) e sete agrupamentos de ferro-enxofre. A atividade desse complexo é acoplada ao bombeamento de quatro prótons através da membrana interna mitocondrial, formando assim, um potencial de membrana eletroquímico (WINKLHOFER e HAASS, 2010). Evidências apontam que disfunções nesse sistema

nas mitocôndrias são a primeira fonte de geração de ERs, que pode então contribuir de forma expressiva para o dano oxidativo (MURPHY, 2009).

Nesse sentido, uma desregulação mitocondrial pode estabelecer um círculo vicioso de estresse oxidativo e insuficiência bioenergética da célula, e assim, com essa desregulação e/ou inibição interrompe-se o transporte de elétrons, que além de provocar um aumento na produção de ERs, tem como consequência a diminuição na geração de ATP; o aumento intracelular da concentração de cálcio; a excitotoxicidade e danos celulares relacionados com o aumento de óxido nítrico. E ainda, relacionado a isso pode ocorrer a abertura do poro de transição permeável mitocondrial, possibilitando a liberação de citocromo C, que desencadeia vias de sinalização direcionadas à morte celular por apoptose. Contudo, estes eventos citados demonstram ser um mecanismo contribuinte para a morte dos neurônios dopaminérgicos na DP (Figura 3) (PERFEITO e REGO, 2012; SUBRAMANIAM e CHESSELET, 2013; HASEGAWA et al., 2017; PARK et al., 2018).

Figura 3 - Esquema dos caminhos representativos da disfunção mitocondrial na fisiopatologia da DP.

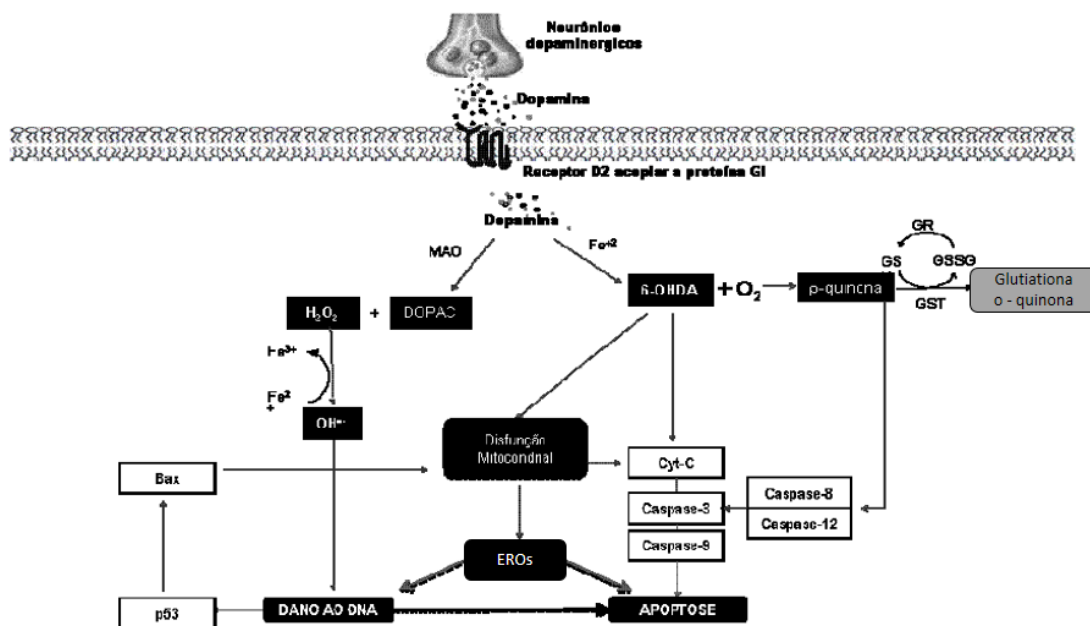


Fonte: Adaptado de PARK et al. (2018, pag. 3)

Outro fator na gênese dos danos oxidativos diz respeito as alterações no metabolismo da dopamina. O sistema nervoso central é dotado de neurônios dopaminérgicos, localizados na região do mesencéfalo, mais especificamente na porção compacta do cérebro, chamada de substância negra. Tais neurônios desempenham papéis importantes em múltiplas funções cerebrais, que vão desde questões relacionadas ao movimento voluntário, o afeto, a recompensa, o sono, a atenção, memória e aprendizagem, até funções fisiológicas na regulação do olfato, hormônios, funções cardiovasculares, renais e sistema imunológico, entre outros, e ainda são a principal fonte de dopamina nos mamíferos (CHINTA e ANDERSEN, 2005). A dopamina é uma catecolamina sintetizada pela ação da tirosina hidroxilase, que hidroxila o aminoácido L-tirosina no precursor L-DOPA (3,4-dihidroxifenilalanina), que será descarboxilado em dopamina (CHINTA e ANDERSEN, 2005; TIEU, 2011; STAVELEY, 2015;).

Com o processo de envelhecimento a auto-oxidação da dopamina aumenta, o que se relaciona com a toxicidade intracelular, e processos degenerativos no cérebro. A auto-oxidação da dopamina ocorre em presença de íons Fe^{2+} livres e H_2O_2 , promovendo a hidroxilação da dopamina na posição 6, formando a 6-hidroxidopamina, convertida em torno de 20% a *orto*-quinona. O sistema mais efetivo para detoxificação da *orto*-quinona no cérebro é através da enzima glutathione S-transferase (GST), a qual utiliza glutathione reduzida (GSH) para formar um complexo estável com a *orto*-quinona. Ainda, a dopamina pode sofrer oxidação enzimática, através da ação das enzimas monoamino oxidase (MAO) e catecol-o-metil transferase (COMT). No processo de oxidação enzimática a dopamina oxidada pela enzima MAO, forma ácido 3,4 diidroxifenilacético e H_2O_2 , capaz de gerar OH^{\bullet} através da reação com íons de ferro. Essa via (Figura 4) ocorre especialmente nos gânglios da base, onde existem altas concentrações de dopamina, oxigênio e ferro (ANDREAZZA, 2008). Tais transportadores de dopamina medeiam à captação de dopamina, sendo importantes alvos para ação de vários compostos farmacologicamente ativos e toxinas ambientais (CHEN e REITH, 2000). A perda de neurônios dopaminérgicos, que são ricos em neuromelanina e ferro, razão pela qual aparece escurecida em cortes histológicos, resulta em despigmentação da substância negra (DAUER e PRZEDBORSKI, 2003).

Figura 4 - Rota de metabolização da dopamina via MAO e produção de radicais livres.



Fonte: Adaptado de Andrezza (2008, p.128)

Na neurofisiologia da DP, se observa microscopicamente o aparecimento de inclusões intracelulares denominadas corpos de Lewy, que são inclusões citoplasmáticas nos neurônios da substância negra (HASEGAWA et al., 2017). Os corpos de *Lewy*, são estruturas esféricas, compostas por numerosos agregados de proteínas, principalmente de agregados de α -sinucleína filamentosa (α SYN) hiperfosforilados, incluindo ainda a parkina, ubiquitina e neurofilamentos (STAVELEY, 2015; BENSKEY et al, 2016). Tais estruturas perturbam o funcionamento normal dos neurônios, interrompendo a ação de neurotransmissores, tais como dopamina e acetilcolina (BENSKEY et al, 2016). Nesse sentido, uma hipótese que tem sido evidenciada remete ao fato de que um acúmulo de proteínas agregadas, mal dobradas, nos tecidos nervosos não representaria apenas marcas patológicas, mas também poderia desencadear uma série complexa de eventos nocivos que resultam em degeneração neuronal (HASEGAWA et al., 2017).

Ainda, ao longo dos anos percebe-se avanços em relação a compreensão dos processos bioquímicos e fatores genéticos na DP (HASEGAWA et al., 2017). Nesse sentido, embora DP apresente múltiplos fatores causais associados, que ainda

precisam ser melhor esclarecidos, têm chamado a atenção dos pesquisadores, algumas formas de DP familiar, com envolvimento genético na sua patogênese (COULOM e BIRMAN, 2004; WHITWORTH et al., 2006). Nesse sentido, são descritos pelo menos seis genes como causa da DP familiar: SNC/ α -sinucleína – PARK1, UCH-L1 – PARK5 e Dardarin/ LRRK2- PARK8, os quais apresentam padrão de herança autossômico dominante; e parkin – PARK2, PINK1 – PARK6 e DJ1-PARK-7 (COULOM e BIRMAN, 2004; GASSER, et al., 2006; STAVELEY, 2015). Ainda, mutações no gene PARK1 levam a formação de agregados de α -sinucleína, enquanto mutações no PARK2 levam a deficiência de parkina e degeneração nigroestrial, sem a presença de corpos de Lewy, e quando ocorrem no PARK7, as mutações são indicadoras de alterações oxidativas (COULOM e BIRMAN, 2004; GASSER, et al., 2006; TANNER et al., 2011; STAVELEY, 2015).

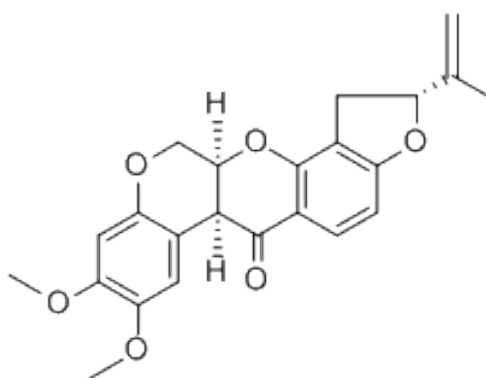
No que se refere a agregação da α -sinucleína, em condições fisiológicas, essa relaciona-se com a entrega, acúmulo e controle da função mitocondrial nas sinapses, estando envolvida na liberação de neurotransmissores (NAKAMURA et al., 2011). Porém, quando ocorrem mutações no gene PARK1, verifica-se a substituições do aminoácido alanina na proteína α -sinucleína por treonina (A53T) e o impossibilidade de degradação proteica, contribuindo para a produção de oligômeros (protofibrilas) nos neurônios. Essa mutação, aumenta a agregação proteica intracelular (corpos de Lewy), onde ocorre o aprisionamento de mitocôndrias pela α -sinucleína, levando à disfunção mitocondrial e falhas no controle de qualidade bioenergética com geração de ERs, impedindo a função dos neurônios dopaminérgicas na DP (FOLLMER e NETTO, 2013; STAVELEY, 2015). Dessa forma, evidências relacionam tais mutações genéticas a prejuízos mitocondriais e na resposta ao estresse oxidativo, apontados como fatores importantes na patogênese da DP (STAVELEY, 2015).

2.3 Rotenona versus Doença de Parkinson

A ROT (Figura 5) é uma substância química, pertencente ao grupo dos inseticidas orgânicos de origem natural, os rotenóides, que ocorre naturalmente nas raízes e talos de algumas plantas (TANNER et al.; 2011). Em 1848 passou a ser

utilizada como inseticida agrícola, quando foi aplicada às plantas, objetivando o controle de lagartas em plantações (WARE, 1989; WORLD HEALTH ORGANIZATION, 1992), posteriormente utilizada como inseticida, pesticida e piscicida, sendo obtida a partir das raízes de certas plantas subtropicais pertencentes ao gênero *Lonchocarpus* ou *Derris elliptica* (WORLD HEALTH ORGANIZATION, 1992).

Figura 5 - Estrutura química da rotenona (ROT).



A ROT é uma substância química inodora, incolor e cristalina, com ponto de fusão de 165-166°C, e solúvel em acetona, dissulfeto de carbono, etil, acetato e clorofórmio, menos solúvel em éter e em água à temperatura ambiente, e as soluções são prontamente oxidadas na presença de luz e álcalis, em produtos com propriedade inseticidas reduzidas (WORLD HEALTH ORGANIZATION, 1992). Ainda, é uma substância com elevada capacidade lipofílica, sendo que sua toxicidade se deve a essa característica, que possibilita sua travessia facilmente pela barreira hematoencefálica e membranas biológicas, sem necessidade de receptor ou transportador (COULOM e BIRMAN, 2004; TIEU, 2011).

Este pesticida é capaz de apresentar atividade neurotóxica para modelo animal de DP, possibilitando a investigação da ação desse no estresse oxidativo, apoptose, disfunção mitocondrial, inflamação e comprometimento das vias de degradação de proteínas (BOVÉ e PERIER, 2012; SUBRAMANIAM e CHESSELET, 2013; SALA et al., 2016). Assim, a ROT tem sido utilizada para indução da DP, *in vitro* e *in vivo*, estando atualmente consolidado tanto para modelos com roedores como para *Drosophila melanogaster* (COULOM e BIRMAN, 2004; MANJUNATH e

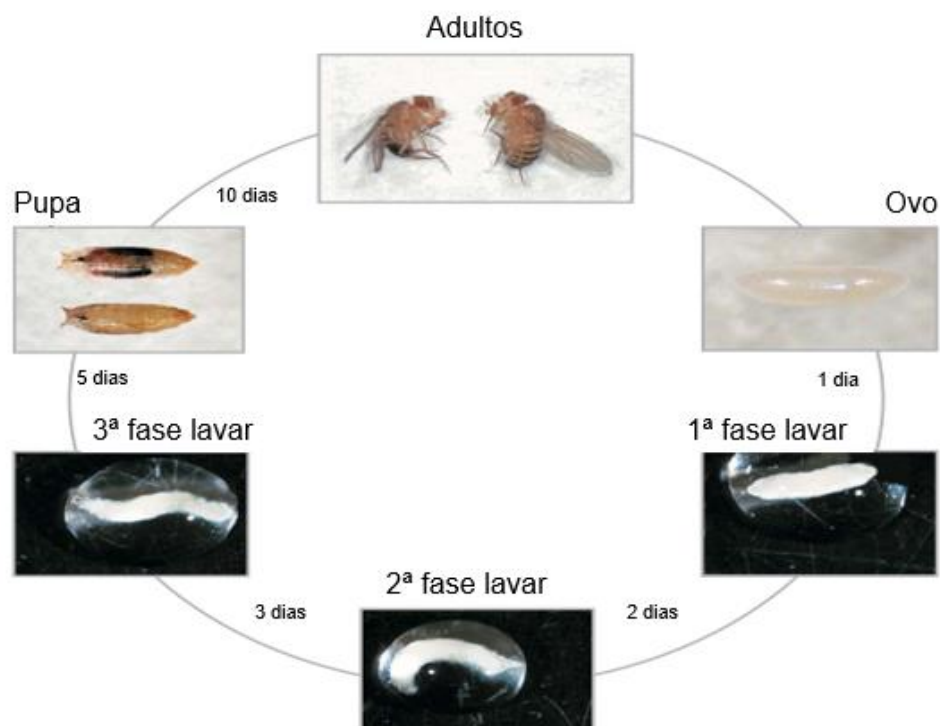
MURALIDHARA, 2013; SUDATI et al., 2013; ARNHOLD et al., 2016). Seu potencial neurotóxico, pode estar relacionado à inibição específica de alta afinidade da NADH desidrogenase no complexo I mitocondrial, com consequente aumento na geração de ERs e interrupção da fosforilação oxidativa mitocondrial, e assim redução nos níveis de ATP, e morte celular por apoptose ou necrose, através da despolarização devido a abertura do poro de transição de permeabilidade mitocondrial, ocorrendo a liberação de fatores pró-apoptóticos que eventualmente causam a morte do neurônio (TIEU, 2011; SUBRAMANIAM e CHESSELET, 2013; ARNHOLD et al., 2016). Dessa forma, quando inibidores do complexo mitocondrial I, tais como ROT e MPTP são usados, espécies reativas de oxigênio (EROs) são formadas, e cascatas apoptóticas ou citotóxicas são ativadas, levando à morte de neurônios dopaminérgicos e lesões no sistema nigrostriatal consistentes com DP (CORTI et al., 2011). Ainda, evidências apontam danos oxidativos nos ácidos nucleicos, lipídios e proteínas, no cérebro e nos tecidos periféricos de animais, em modelos de indução de DP utilizando a ROT (MANJUNATH e MURALIDHARA 2013; SANDERS e GREENAMYRE, 2013).

2.4 *Drosophila melanogaster* na Doença de Parkinson

Na última década, estudos com modelos animais alternativos tem sido cada vez mais explorados, tais como os que utilizam *Drosophila melanogaster*, sendo este um dos animais de laboratório mais estudados em toda a história da investigação científica (BELLEN et al., 2010; STAVELEY, 2015). Com mais de um século de pesquisas intensivas sobre a biologia da mosca da fruta, existe uma grande quantidade de conhecimento sobre desenvolvimento e recursos genéticos, moleculares e celulares desse invertebrado (STAVELEY, 2015; PROKOP, 2016). Ainda, esse modelo destaca-se principalmente por ser economicamente viável e acessível, principalmente no que diz respeito ao seu manejo, pois a criação dos estoques de moscas é relativamente simples e econômica, e ainda contam com interessante cruzamento genético, pela geração de um grande número de progênes geneticamente homogêneas (STAVELEY, 2015; PROKOP, 2016). Outras vantagens do estabelecimento da *Drosophila melanogaster* como organismo modelo para estudos experimentais deve-se o seu curto ciclo de vida (Figura 6) e a possibilidade

de utilização de um maior número de indivíduos, facilidades apontadas quando comparadas a outros modelos mais complexo, por exemplo com roedores (BELLEN et al., 2010). Ainda a *Drosophila melanogaster* tem ciclo de vida diploide, e com grande potencial reprodutivo, com dezenas de descendentes resultantes de uma única fecundação, e em média demora um dia entre a fertilização e o aparecimento das larvas. As larvas passam por vários estágios até formar pupa (6 dias), e em 5 dias ocorre a eclosão, e após 12 horas dessa os indivíduos se tornam férteis. Assim, esse modelo além de apresentar uma dinâmica reprodutiva relativamente simples, com um curto ciclo de geração (em torno de 10 dias), apresentam uma expectativa média de vida de cerca de 60 dias, dependendo das condições de temperatura e umidade do ambiente (STAVELEY, 2015; PROKOP, 2016). Dessa forma são modelos ideais para experimentos de larga escala ou alto rendimento, como um importante pré-requisito para obter uma compreensão conceitual mais profunda dos processos biológicos (PROKOP, 2016).

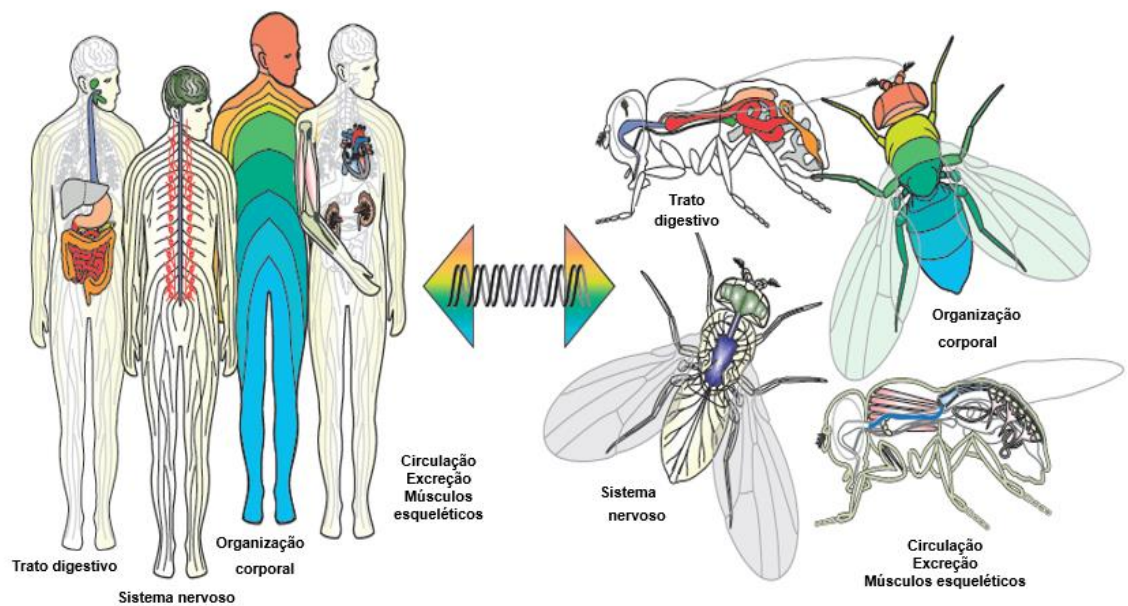
Figura 6 - Representação do ciclo de vida da *Drosophila melanogaster*.



Fonte: http://darwin2009.cienciaviva.pt/img/upload/EC_pdf_CicloVida.pdf

Além disso esse modelo alternativo traz como vantagem a similaridade com aos órgãos e sistemas dos mamíferos, compartilhando princípios fundamentais de sua organização e funções, como as do cérebro, coração, pulmão, rim, intestino e sistema reprodutor (Figura 7) (PROKOP, 2016). Assim, muitos processos bioquímicos, celulares e fisiológicos são similares, o que permite uma grande gama de comparações entre mosca e humanos.

Figura 7 - Ilustração da analogia dos órgãos e sistemas entre humanos e *Drosophila melanogaster*.

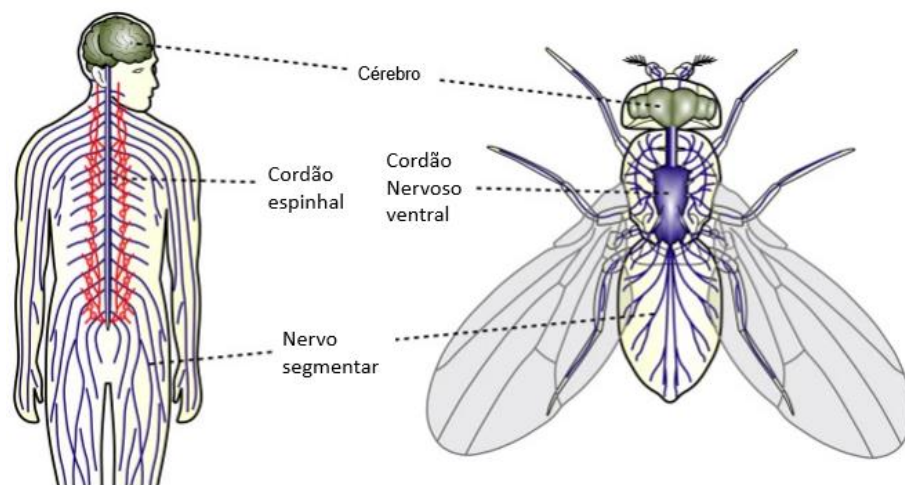


Fonte: Adaptado de Prokop (2016, pag. 11).

No que se refere a neurofisiologia, as moscas apresentam um intrincado sistema nervoso e exibem relação com comportamentos bastante complexos (STAVELEY, 2015; PROKOP, 2016). O cérebro da mosca é altamente especializado e subdividido em centros funcionais de visão, olfato, coordenação motora, aprendizado e muitos outros comportamentos (Figura 8 e 9). Ainda esse sistema neuronal com mais de 100.000 neurônios em circuitos discretos e fibras nervosas relacionadas a comportamentos complexos, tais como: ritmo circadiano, sono, visão, olfato, aprendizagem, memória, alimentação, agressividade, adestramento, coordenação motora e trajetória de vôo, e ainda aspectos biológicos, tais como a

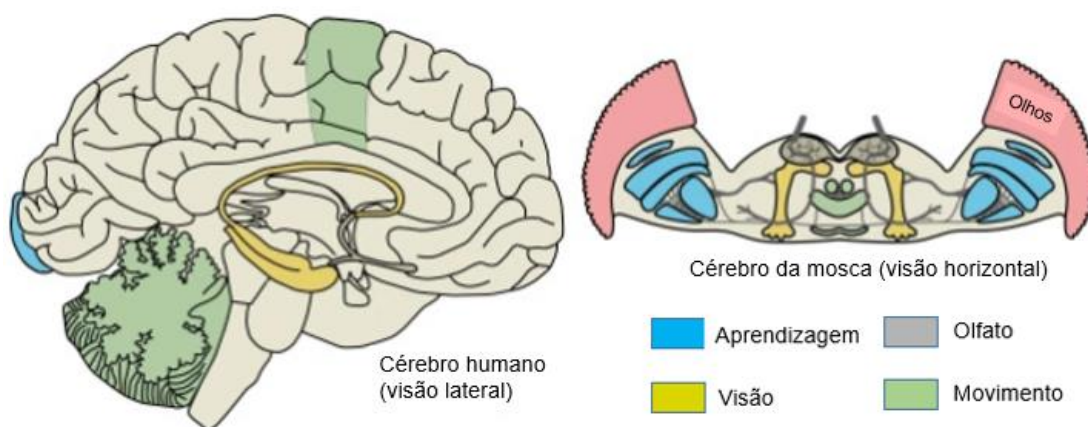
conectividade da sinalização celular neuronal, e morte celular; a regulação da expressão gênica, o tráfego de membrana, o citoesqueleto (PROKOP, 2016). Assim, na *Drosophila melanogaster* a compreensão molecular e celular do desenvolvimento do sistema nervoso é muito complexa, variando de mecanismos que sustentam o padrão neural inicial até o desenvolvimento sináptico e plasticidade no cérebro maduro (BROADIE et al., 2011). Tais fatores apontam esse modelo como promissor, pois devido a essa similaridade, é possível avaliar os caminhos relacionados ao funcionamento do comportamento motor, psicomotor e não-motor, se estabelecendo como organismo útil na avaliação dos mecanismos moleculares de doenças neurodegenerativas.

Figura 8 - Ilustração da analogia da organização do sistema nervoso: cérebro, medula espinhal/ ventral e nervos segmentares entre humanos e *Drosophila melanogaster*.



Fonte: <https://droso4schools.wordpress.com/organs/>.

Figura 9 - Ilustração da analogia da organização do cérebro em centros funcionais entre humanos e *Drosophila melanogaster*.



Fonte: <https://droso4schools.wordpress.com/organs/>.

Nesse sentido, esse modelo é bem caracterizado e apresenta sofisticada ferramenta genética e diversos comportamentos, que podem ser úteis para a compreensão de várias doenças humanas, e tem sido utilizado em diferentes áreas da neurociência, destacando-se ao estudo das doenças neurodegenerativas, tais como a DP (HIRTH 2010; HOSAMANI e MURALIDHARA, 2013; MANJUNATH e MURALIDHARA 2013; STAVELEY, 2015; KHATRI e JUVEKAR, 2016; SONG et al., 2017). Nesse sentido, a *Drosophila melanogaster* apresenta em seu genoma homólogos de 77% dos genes humanos relacionados com as doenças neurológicas e mais de 85% de genes associados com as deficiências cognitivas (STAVELEY, 2015; PROKOP, 2016). Também possui similaridade com os sistemas humanos no que diz respeito a processos celulares fundamentais e vias de sinalização (MANJUNATH e MURALIDHARA 2013; STAVELEY, 2015). E destaca-se o fato de apresentar analogia entre o mecanismo de ação de drogas, tornando-se um modelo útil para a compreensão da patogênese, progressão e para o rastreamento de possíveis agentes terapêuticos no tratamento de doenças neurodegenerativas, incluindo DP (MUÑOZ-SORIANO e PARICIO, 2011; TIEU, 2011; STAVELEY, 2015; KHATRI, 2016).

No que se refere ao estudo da DP, tal modelo apresentou grande interesse dos pesquisadores devido a homologia existente com os humanos entre cinco dos seis genes relacionados a essa patologia (WHITWORTH et al., 2006; HOSAMANI e

MURALIDHARA, 2013; ARAUJO et al., 2015; STAVELEY, 2015). Ainda, o sistema nervoso central da *Drosophila melanogaster* é composto por seis conjuntos de neurônios dopaminérgicos, localizados em cada hemisfério do cérebro da mosca adulta, e também contém transportadores de dopamina e vesículas de transporte de monoamina para a manutenção da homeostase da dopamina (UENO e KUME, 2014). Outro traço semelhante entre moscas e mamíferos é a suscetibilidade dos neurônios dopaminérgicos a certas neurotoxinas, como é o caso da ROT, que em ambos é considerada como um inibidor do complexo I relacionado a degeneração de neurônios dopaminérgicos (COULOM e BIRMAN, 2004; STAVELEY, 2015). E assim como nos humanos, as moscas apresentam sistema dopaminérgico cujos os neurônios estão mais proeminentemente envolvidos no controle locomotor, no aprendizado e memória (COULOM e BIRMAN, 2004; RIEMENSBERGER et al., 2013). Nesse sentido torna-se um modelo alternativo para mimetizar a DP, visto que reproduz com analogia os fenótipos físicos e comportamentais relacionados a DP, tais como a neurodegeneração dopaminérgica e o declínio da função locomotora (MANJUNATH e MURALIDHARA 2013; ARAUJO et al., 2015; STAVELEY, 2015). Ainda, *Drosophila melanogaster* tem se estabelecido também como modelo no estudo dos mecanismos moleculares e aspectos comportamentais significativos da DP, sendo assim ferramenta útil para investigação rápida de potenciais fármacos para tratamento da DP (HOSAMANI e MURALIDHARA, 2013; MANJUNATH e MURALIDHARA 2013; SUDDATI et al., 2013; STAVELEY, 2015).

Nas doenças neurodegenerativas e no estudo do envelhecimento observam-se muitas mudanças comportamentais nos humanos, entre elas o declínio da habilidade motora, olfativa, de memória e de ritmo circadiano, alterações essas também preservadas em modelos de DP com *Drosophila melanogaster*, seja induzido através de neurotoxinas ou por manipulação genética (JONES e GROTEWIEL, 2011; STAVELEY, 2015). A DP traz como marca patológica mais proeminente as alterações motoras, tais como perda de controle motor, lentidão de movimento, rigidez, instabilidades posturais e tremor de repouso, em humanos; similarmente no modelo com *Drosophila melanogaster* também são descritas alterações motoras importantes, que mimetizam os prejuízos motores da DP, tais como déficit locomotor na atividade de escalada, redução na capacidade exploratória e atividade locomotora espontânea,

avaliada pelos testes de geotaxia negativa e ensaio de campo aberto (“open field”), respectivamente (COULOM e BIRMAN, 2004; HIRTH 2010; STAVELEY, 2015).

Outra característica importante nas doenças neurodegenerativas cita-se o importante comprometimento psicomotor, evidenciado muitas vezes em fase relativamente precoces do desenvolvimento de doenças como a DP em humanos (ILADI et al., 2016; SONG et al., 2017). Esses comportamentos psicomotores são comandados por uma tênue relação entre atividades motoras e funções cognitivas (ILADI et al., 2016). Além da disfunção cognitiva, observam-se outros sintomas não-motores da DP, tais como as alterações cognitivas, de humor (depressão e ansiedade), algia, disfunção sensorial, disautonomia, distúrbio olfatório e constipação (ALVES et al., 2008; CHEN e MARSH, 2014; MUNHOZ et al., 2015; RANA, et al., 2015; SONG et al., 2017). Nesse contexto, também tem sido identificadas alterações psicomotoras e não-motoras, compatíveis com as relacionadas a DP em modelos que mimetizam essa patologia em *Drosophila melanogaster* (STAVELEY, 2015; HASEWAGA, 2017; HELLEY et al., 2017). Para tanto são evidenciados alguns testes comportamentais com *Drosophila melanogaster* que vem ao encontro do estudo de tais comportamentos psicomotores e não-motores, através da avaliação da atividade locomotora das moscas com o envolvimento de habilidade cognitivas, tais como: o teste do equilíbrio (“*Equilibrist test*”) que avalia atividade psicomotora e aprendizagem (ILADI et al. 2016; ILADI et al., 2018); o ensaio de supressão de fototaxia aversiva (“*Aversive Phototaxis Suppression Assay- APS*”), para avaliação da aprendizagem e habilidades de memória a curto prazo (ALI et al. 2011); e o teste de claro/escuro o qual investiga comportamentos tipo ansiedade nesse modelo (NECKAMEYER e NIETO, 2015).

Nesse sentido, esse modelo alternativo está na vanguarda entre os estudos experimentais no que se refere a compreensão e busca por novas possibilidades terapêuticas na DP, devido a sua capacidade de modelagem da doença, e em possibilitar fenótipos físicos e comportamentais para uma investigação detalhada na DP (STAVELEY, 2015).

2.5 Estratégias terapêuticas na Doença de Parkinson

Na década de 60, após a identificação das alterações patológicas e bioquímicas no cérebro de pacientes com DP, surgiu o primeiro tratamento com sucesso através de um precursor dopaminérgico o 3,4-dihidroxi-fenilalanina ou levodopa (L-DOPA), sendo considerado o maior avanço terapêutico na DP (BRASIL, 2010). Inicialmente esse medicamento mostrou benefícios clínicos para praticamente todos os pacientes, suprimindo alguns dos sintomas motores e compensando a perda de células dopaminérgicas, e conseqüentemente aumentando a síntese de dopamina nos terminais remanescentes (PEREZ-PARDO et al., 2017). Atualmente o tratamento tradicional da DP ainda está baseado na terapia dopaminérgica, visando reverter os efeitos da depleção de dopamina induzida pela destruição da via nigroestriatal (JANKOVIC, 2002; BRASIL, 2010). No entanto, essa terapia não previne a degeneração neuronal dopaminérgica, apresenta baixa eficiência sobre os sintomas não motores, e ainda tem como conseqüência uma série de efeitos colaterais (LEE e KOH, 2015; PEREZ-PARDO et al., 2017). Assim, ao longo dos anos, os pacientes passam a apresentar sintomas motores resistentes à L-DOPA (comprometimento da fala, postura e equilíbrio), e sinais não motores resistentes ao L-DOPA (comprometimento do humor, sono e cognição) (LAKKAPPA et al., 2019). Ainda, são observados efeitos colaterais relacionados ao medicamento, tais como alucinações, demência, depressão, problema de deglutição, distúrbio do sono, constipação e fadiga, distúrbios que podem ser melhorados pela alteração do tratamento medicamentoso (LANG, 2009; OLANOW et al., 2009; WINNER et al., 2011; STAVELEY, 2015; SONG et al., 2017; LAKKAPPA et al., 2019). Ainda, a literatura aponta que tais complicações relacionadas a resistência medicamentosa podem ocorrer devido as lesões não dopaminérgicas, não abordadas por essa terapia tradicional (AGID 1991; WINNER et al., 2011; STAVELEY, 2015).

Ainda, considerando a relação entre o papel do estriado nas vias motoras cerebrais, e que uma redução da modulação da dopamina estriatal conseqüentemente reflete em alterações motoras, pode-se prever que aumentando a estimulação dopaminérgica, ou reduzindo a estimulação colinérgica, ou glutamatérgica, poderiam ser melhorados os sintomas da DP (LANG, 2009; OLANOW et al., 2009; BRASIL,

2010). Assim, tem sido apontado na terapêutica farmacológica algumas opções de intervenção sintomática, entre eles: L-DOPA *standard* ou com formulações de liberação controlada, em associação com inibidor da L-DOPA descarboxilase; agonistas dopaminérgicos; inibidores da monoamino oxidase B (MAO-B); inibidores da COMT; anticolinérgicos e antiglutamatérgicos (LANG, 2009; OLANOW et al., 2009; BRASIL, 2010).

Assim, devido a multiplicidade de fatores envolvidos na DP, a ascendente progressão da doença, a exacerbação das manifestações clínicas (motoras e não motoras), somadas aos efeitos colaterais precoces e tardios da intervenção farmacológica, constata-se a complexidade do tratamento dessa patologia. Nesse sentido, enfatiza-se a necessidade de desenvolvimento de novas terapias efetivas que possam apresentar efeitos farmacológicos sobre os principais sintomas, produzir melhora funcional com o mínimo de efeitos adversos, evitar o aparecimento de complicações futuras e a progressão da doença, e ainda que possam agir também frente as alterações não motoras (BRASIL, 2010). Assim, a busca por compostos multi-alvos que possam atuar em vias dopaminérgicas e não-dopaminérgicas, melhorando as funções motoras, psicomotoras e não-motoras, sem riscos de complicações associadas com a L-DOPA, são de extrema necessidade na terapêutica da DP.

2.6 Selênio e Doenças Neurodegenerativas

O selênio é um elemento traço essencial para a saúde humana, desempenhando múltiplas funções biológicas e de grande importância para a manutenção das funções neurológicas (NOGUEIRA E ROCHA, 2010; ELLWANGER et al., 2015; SOLOVYEV, 2015). Esse elemento quando usado em níveis apropriados, é um importante agente antioxidante e, portanto, está envolvido no combate ao estresse oxidativo (FERGUSON et al., 2012; HART et al., 2013), apresentando valoroso efeito neuroprotetor (NOGUEIRA et al., 2004; GLASER et al., 2010; SANTAMARÍA et al., 2013; ELLWANGER et al., 2015). Além disso, um possível papel do selênio nas vias de sinalização cerebral pode ser assumido, visto que está

envolvido em diversas funções do sistema nervoso central, como o desempenho motor, coordenação, memória e cognição (CADET, 1986; SOLOVYEV, 2015). Nesse sentido, o selênio também foi identificado como desempenhando um papel em vários distúrbios neurodegenerativos, como a doença de Alzheimer, DP, esclerose lateral amiotrófica e epilepsia, entre outros (BENTON, 2002; NOGUEIRA E ROCHA, 2011).

Na DP, os neurônios dopaminérgicos são conhecidos por serem vulneráveis ao estresse oxidativo e a inibição da captação de glutamato relacionada com a oxidação da dopamina através de radicais livres (CADET, 1986; SOLOVYEV, 2015). Ainda, estudos sugerem a implicação das selenoproteínas na preservação da substância nigroestriatal (ELLWANGER et al., 2015; SOLOVYEV, 2015). Por exemplo, a substância negra e putâmen, parte do corpo estriado, apresentam concentrações mais elevadas de selênio em comparação com outras regiões do cérebro. Ademais, observa-se uma diminuição no total de glutatona na substância negra de pacientes com DP, demonstrando um possível envolvimento das enzimas glutatona peroxidase (GPx) selênio dependente (CANNON e GREENAMYRE, 2010; SOLOVYEV, 2015).

Ainda, estudos em diferentes modelos apontam uma relação entre uma possível deficiência de selênio associada à DP, tanto em modelos experimentais com roedores (IMAM e ALI, 2000; KIM, et al.; 2000; KHAN, 2010; ELLWANGER, et al., 2016), como em modelos celulares (LOPES, 2012) e com *Drosophila melanogaster* (MARTIN-ROMERO, 2001). Além disso, respostas clínicas positivas obtidas no tratamento de doenças neurodegenerativas com selênio e outros antioxidantes, tem fornecido provas substanciais sobre o papel importante dos radicais livres e estresse oxidativo em processos patológicos (ELLWANGER et al., 2015; SOLOVYEV, 2015). Outras evidências apontam aumento nos danos aos terminais dopaminérgicos em ratos com deficiência de selênio (SOLOVYEV, 2015); e redução de danos, em modelo de DP induzida por paraquat, em ratos que receberam suplementação de selênio (ELLWANGER et al., 2015). Outro estudo verificou que a suplementação de selênio reverte o comprometimento da neurotransmissão dopaminérgica induzida por MPTP (KHAN, 2010). Imam e Ali (2000) mostraram aumento do dano nos terminais dopaminérgicos de camundongos com deficiência de selênio em um modelo PD com metanfetamina (MA). Além disso, Kim et al. (2000), em um estudo de PD induzida por

MA, demonstraram que o tratamento com MA produz uma perda de células DA em camundongos e que a deficiência prolongada de selênio potencializa o comprometimento do sistema de desintoxicação antioxidante principalmente em células dopaminérgicas. Já outro estudo usando um modelo *in vitro* com neuroblastoma humano, apontou baixa citotoxicidade e alto poder antioxidante de três tipos diferentes organosselenetos sintéticos, e que o tratamento prévio das células com esses compostos promove neuroproteção significativa contra a toxicidade por 6-hidroxi-dopamina (6-OHDA), diretamente relacionadas às suas propriedades antioxidantes, sendo essas comparadas ao disseleneto de difenila [(PhSe)₂] (LOPES, et al., 2012). Enquanto que em *Drosophila melanogaster* Martin-Romero et. al, (2001) verificaram influência da suplementação com selênio dietético na melhora do tempo de vida, processo que pode envolver o sistema de selenoproteínas, previamente identificado nesse modelo.

No entanto, embora o uso de selênio no tratamento da DP precoce tenha sido sugerido na década de 1980, sua aplicação no tratamento dietético de pacientes com DP tem sido pouco explorada (ELLWANGER et al., 2015). Esse fato provavelmente esteja relacionado a resultados contraditórios, devido aos efeitos pró-oxidantes e antioxidantes do selênio (NOGUEIRA E ROCHA, 2010; ALEXANDER, 2015), sobre os benefícios de uma possível utilização do selênio a níveis acima das recomendações nutricionais. Atualmente a dose diária recomendada de acordo com as DRIs (*Dietary Reference Intakes – DRIs*), estipulada é de 55 µg/dia para homens e mulheres adultos e saudáveis (INSTITUTE OF MEDICINE, 2006), o que corresponde à quantidade estimada necessária para a maximização da atividade da GPx no plasma (FOOD AND NUTRITION BOARD INSTITUTE, 2000).

Além disso, nas últimas décadas tem sido destacada importantes ações farmacológicas de compostos orgânicos de selênio (NOGUEIRA et al., 2004; NOGUEIRA e ROCHA, 2010; NOGUEIRA e ROCHA, 2011; NOGUEIRA e ROCHA, 2012). Em relação a neuroproteção, estudos com o disseleneto de difenila [(PhSe)₂] tem demonstrado resultados relevantes na proteção do sistema dopaminérgico de camundongos (BURGER et al., 2006; SAVEGNAGO et al., 2007; FIGUEIRA et al. 2015) e na reversão de deficiências motoras em um modelo de DP induzida por 6-OHDA em roedores (SAMPAIO et al., 2017). Por outro lado, estudos apontam que, de

forma contraditória, ações farmacológicas de alguns compostos de selênio podem estar associadas aos efeitos tóxicos do composto, e que poderia explicar pelo menos alguns dos efeitos antioxidantes (NOGUEIRA E ROCHA, 2010; NOGUEIRA E ROCHA, 2011; NOGUEIRA E ROCHA, 2012; WILHELM, et al., 2017). No entanto a utilização de compostos de selênio, dentro da janela terapêutica tem sido apontada como opção segura em estudos com animais (NOGUEIRA et al., 2004; NOGUEIRA e ROCHA, 2010; NOGUEIRA e ROCHA, 2011; NOGUEIRA e ROCHA, 2012). Ainda, a literatura aponta que os efeitos neuroprotetores e/ ou neurotóxicos do selênio dependem muito das espécies químicas do elemento (NOGUEIRA e ROCHA, 2010). Resultados importantes foram relatados com a utilização do ebselen, um composto sintético selenoorgânico que apresentou eficácia limítrofe e relativo sucesso em estudos com animais (NOGUEIRA e ROCHA, 2010; NOGUEIRA e ROCHA, 2011; NOGUEIRA e ROCHA, 2012). Ainda, Nogueira e Rocha, (2010), em um estudo analisando a química farmacológica do disseleneto de difenila $[(\text{PhSe})_2]$ e de compostos relacionados, apontam que doses farmacológicas eficazes poderiam estar associadas a respostas tóxicas sutis, mas que, no entanto, o potencial terapêutico parece ser superior aos efeitos tóxicos.

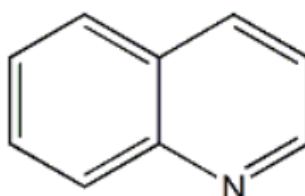
2.7 Compostos derivados de quinolinas associados a orgânicos de selênio

Nas últimas décadas, a busca por compostos como novas alternativas terapêuticas, principalmente baseada em uma ação antioxidante tem aumentado notavelmente (NOGUEIRA e ROCHA, 2011). Dentre as várias aplicações terapêuticas dos antioxidantes, ressalta-se a ação neuroprotetora, podendo auxiliar no controle dos insultos oxidativos (HALLIWELL, 2006; HALLIWELL 2011). Por isso, os compostos químicos, que possam ser sintetizados de maneira simples, e que desenvolvam ação antioxidante, ganham destaque nas pesquisas recentes na procura por inovações farmacológicas. Nesta direção, os compostos orgânicos de selênio emergem entre as novas substâncias sintéticas, com expressivo potencial terapêutico, associado às suas atividades farmacológicas (NOGUEIRA e ROCHA, 2011). Nesse sentido, tem sido amplamente estudada a associação entre compostos de selênio combinados à

quinolinas, que tem demonstrado resultados importantes (SAVEGNAGO et al., 2013; PINZ et al., 2016; REIS et al., 2017; PINZ et al., 2018).

As quinolinas (Figura 10) são estruturas químicas aromáticas heterocíclicas contendo nitrogênio na sua estrutura química, consideradas bases terciárias fracas, apresentando fórmula molecular C_9H_7N e peso molecular de 129,16 g/mol, e isenta de toxicidade para humanos por via oral, absorção e inalação (MARELLA et al., 2012).

Figura 10 - Estrutura química da quinolina.



Os núcleos quinolínicos ocorrem em vários compostos naturais, como nos alcaloides extraídos da árvore Cinchona, na qual a quinina é o principal constituinte, e em substâncias farmacologicamente ativas, o que justifica a sua ampla gama de atividade biológica (MARELLA et al., 2012). A partir de sua identificação, as quinolinas começaram a ser produzidas sinteticamente, e destacadas devido a suas importantes propriedades anti-inflamatória, antimalárica, antitumoral, anticarcinogênica, anti-hipertensiva, anti-asmática, anti-histamínica, anti-parasitária, cardiotônica, anticonvulsiva, antidepressiva e antinociceptiva (BHASIN et al., 2010; MARELLA et al., 2012; GRUPTA e MISHRA, 2016; CHANQUIA et al., 2019). Associada a essas valorosas ações farmacológicas, destaca-se o fato que tais compostos não foram relacionados a toxicidade gástrica, hepática e renal em roedores (SANTOS et al., 2015).

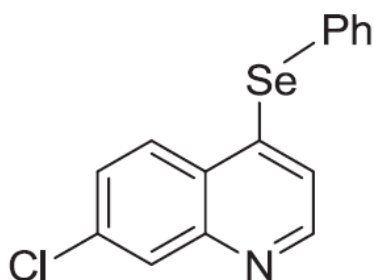
Essas propriedades farmacológicas são de extrema relevância, principalmente no que se refere ao potencial antioxidante, o qual pode contribuir para o tratamento de doenças neurodegenerativas que tem o estresse oxidativo envolvido na sua patogenia. Ainda, quando associadas suas propriedades com as do selênio podem trazer possíveis benefícios em diversas patologias, tais como nas doenças neurodegenerativas. Ainda, literatura aponta os compostos orgânicos de selênio como

potentes protetores neuronais, pela expressão de selenoproteínas, que participam da regulação do estado redox e na defesa antioxidante (SCHWEIZER et al., 2004), o que poderia ser potencializado pela associação com as quinolinas, fato tornaria diferenciado o potencial desse composto frente a outros compostos orgânicos de selênio. Nesse contexto evidências tem destacado um novo derivado de quinolina com um substituinte organosselênio, o 7-cloro-4-(fenilselanil) quinolina (4-PSQ), devido ao seu potencial efeito terapêutico (SAVEGNAGO et al., 2013; PINZ et al., 2016; DUARTE et al., 2017; SILVA et al., 2017; PINZ et al., 2018; VOGT et al., 2018, VOSS et al., 2018).

2.8 Composto 7-cloro-4-(fenilselanil) quinolina (4-PSQ)

Atualmente, diante do aumento das doenças neurodegenerativas e da problemática referente aos efeitos colaterais da terapêutica medicamentosa existente, se faz necessário o estudo contínuo de compostos com potenciais para o tratamento de tais patologias. Nesse contexto, o composto 4-PSQ (Figura 11), um derivado de quinolina contendo selênio, emerge como um composto com achados importantes através de estudos recentes (SAVEGNAGO et al., 2013; PINZ et al., 2016; DUARTE et al., 2017; SILVA et al., 2017; REIS et al., 2017; PINZ et al., 2018; VOGT et al., 2018, VOSS et al., 2018).

Figura 11 - Estrutura química do 7-cloro-4-(fenilselanil) quinolina (4-PSQ).



Fonte: SAVEGNAGO et. al., 2013.

Em um primeiro momento o 4-PSQ revelou potencial antioxidante, sem efeitos toxicológicos *in vitro* (SAVEGNAGO et al., 2013). Posteriormente, outros estudos com modelos roedores confirmaram ações antioxidantes do 4-PSQ contra o aumento do estresse oxidativo (PINZ et al., 2016, SILVA et al., 2017; PINZ et al., 2018; VOGT et al., 2018) e peroxidação lipídica (SILVA et al., 2017; PINZ et al., 2018). Em estudos posteriores, os pesquisadores desse composto evidenciaram efeitos anti-nociceptivo, anti-inflamatório e ansiolítico, sem efeitos colaterais, tais como distúrbios locomotores, toxicidade renal e hepática (PINZ et al., 2016; REIS et al., 2017; SILVA et al., 2017; PINZ et al., 2018; VOGT et al., 2018). No que se refere ao potencial antioxidante, os pesquisadores salientam que o 4-PSQ pode atuar em diferentes linhas de defesa antioxidante, protegendo contra danos oxidativos cerebrais, e sugeriram que esse composto apresente atuação similar a outros compostos organosselênio, como o disseleneto de difenila [(PhSe)₂] e ebselen (VOGT et al., 2018). Ainda, tais autores apontam que o 4-PSQ poderia agir, em um ambiente rico em tiol, formando um radical selenolado, que pode reagir com H₂O₂ formando ácido selênico, que posteriormente reage com grupo tiol (RSH) para formar sulfeto de selenila, transformando-se novamente em selenolato (VOGT et al., 2018).

No que se refere a ação neuroprotetora, foi verificado resultados importantes em relação as ações antioxidantes (PINZ et al., 2018; VOGT et al., 2018) e anticolinesterásica (PINZ et al., 2018). Na avaliação da atividade do 4-PSQ contra o estresse oxidativo induzido pelo nitroprussiato de sódio (SNP) em cérebros roedores, Vogt et al., 2018 demonstraram que o 4-PSQ foi capaz de evitar o aumento da peroxidação lipídica e os níveis de proteína carbonila, bem como a diminuição nos níveis de tióis não proteicos, e atividade da catalase, glutathione-S-transferase e de δ-aminolevulínico (δ-ALA-D). Ainda, tais autores comprovaram a importância do grupo fenilselenil, presente na posição 4 da estrutura da quinolina, ao efeito antioxidante do composto (VOGT et al., 2018). No estudo de Pinz et al., 2018 o 4-PSQ foi testado em modelo de DA, induzida pelo peptídeo β-amilóide (Aβ) em camundongos, sendo verificado sua eficácia na proteção contra problemas de aprendizagem, memória e ansiedade, sendo essas ações associadas ao efeito farmacológico anticolinesterásico e antioxidante do composto (PINZ et al., 2018). Ainda, em relação ao efeito ansiolítico, Reis et al., (2017) apontaram que o 4-PSQ reduziu a captação de glutamato no cérebro e protegeu contra a ansiedade induzida pelo cainato em camundongos. Ainda

foi apontada a contribuição dos sistemas glutamatérgico, serotoninérgico e nitrérgico aos efeitos antinoceptivo e anti-inflamatório do 4-PSQ (SILVA et al., 2017).

Assim, esse novo composto, associa a versatilidade das propriedades das quinolinas as do selênio, com ações correlacionadas as propriedades antioxidante e anticolinesterásica (PINZ et al., 2016; PINZ et al., 2018). Assim, os efeitos farmacológicos desse composto surgem como uma opção dentro da investigação por alternativas terapêuticas para a DP, corroborando com a proposta da terapêutica farmacológica na DP, a qual visa a redução da progressão da doença, e/ ou a busca por compostos neuroprotetores, e que sejam capazes de controlar e/ou melhorar os sintomas motores e não-motores com o mínimo possível de efeitos colaterais (BRASIL, 2010). Nesse sentido, diante do escopo de estudos recentes que evidenciam suas múltiplas propriedades farmacológicas, principalmente ao potencial antioxidante e anticolinesterásico, aliada a problemática da DP, torna-se relevante a investigação dos possíveis efeitos do composto 4-PSQ nessa patologia.

3 JUSTIFICATIVA

Diante do aumento do envelhecimento da população e a ocorrência de doenças neurodegenerativas, como a DP, e ainda da reduzida eficácia da terapêutica farmacológica para essa patologia, se faz necessária a busca por novos compostos que possam apresentar efeito protetor na neurodegeneração. Neste sentido, um novo composto derivado de quinolina contendo selênio, o 4-PSQ, tem sido estudado recentemente, devido suas propriedades biológicas promissoras. Assim, esse trabalho visa avaliar os possíveis efeitos neuroprotetores do 4-PSQ sob os aspectos moleculares e comportamentais em um modelo mimético a DP através da exposição a ROT em *Drosophila melanogaster*.

4 OBJETIVOS

4.1 Objetivo Geral

Investigar os aspectos moleculares e comportamentais associados ao potencial neuroprotetor do composto 7-cloro-4(fenilselanil) quinolina (4-PSQ) em um modelo mimético à DP induzida por ROT em *Drosophila melanogaster*

4.2 Objetivos Específicos

- Verificar a taxa de sobrevivência e mortalidade em *Drosophila melanogaster* após exposição a ROT e ao tratamento com 4-PSQ;
- Avaliar as alterações motoras, relacionadas a atividade de escalada, a capacidade exploratória e atividade locomotora espontânea, em *Drosophila melanogaster* posteriormente a exposição à ROT e ao tratamento com o 4-PSQ;
- Investigar as alterações psicomotoras e não motoras, que envolvem a aprendizagem, memória e ansiedade, em *Drosophila melanogaster* expostas à ROT e ao tratamento com o 4-PSQ concomitantemente;
- Avaliar a atividade da enzima AChE, nas regiões do corpo e cabeça, em *Drosophila melanogaster* expostas a ROT e ao 4-PSQ;
- Avaliar o estresse oxidativo e a atividade das enzimas antioxidantes nas moscas após exposição a ROT e ao tratamento com 4-PSQ;
- Determinar os níveis de dopamina na cabeça das moscas após a exposição a ROT e concomitantemente ao 4-PSQ;
- Determinar os níveis de selênio na cabeça e corpo em *Drosophila melanogaster* expostas a ROT e tratadas concomitantemente com 4-PSQ.

PARTE II

5 ARTIGO CIENTÍFICO E MANUSCRITO

A metodologia realizada e os resultados que fazem parte dessa tese de doutorado estão apresentados na forma de um artigo científico e de um manuscrito. As seções Materiais e Métodos, Resultados, Discussão e Referências encontram-se no corpo do artigo e do manuscrito. Posteriormente, serão apresentadas as seções de Conclusões e Perspectivas Futuras.

Artigo 1: 7-cloro-4-(fenilselanil) quinolina previne a depleção de dopamina em um modelo de *Drosophila melanogaster* na doença de Parkinson

Manuscrito 1: Eficácia do 7-cloro-4-(fenilselanil) quinolina em evitar déficits de comportamento psicomotor e não motor em um modelo mimético a doença de Parkinson em *Drosophila melanogaster*

ARTIGO 1

7-chloro-4-(phenylselanyl) quinoline prevents dopamine depletion in a *Drosophila melanogaster* model of Parkinson's-like disease

Shanda de Freitas Couto, Stéfani Machado Araujo, Vandrezza Cardoso Bortolotto,
Marcia Rósula Poetini, Franciane Cabral Pinheiro, Elize Aparecida Santos Musachio,
Luana Barreto Meichtry, Manoela do Sacramento, Diego Alves, Diogo La Rosa
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7-chloro-4-(phenylselanyl) quinoline prevents dopamine depletion in a *Drosophila melanogaster* model of Parkinson's-like disease

Shanda de Freitas Couto^{a,b}, Stéfani Machado Araujo^a, Vandrezza Cardoso Bortolotto^a, Marcia Rósula Poetini^a, Franciane Cabral Pinheiro^a, Elize Aparecida Santos Musachio^a, Luana Barreto Meichtry^a, Manoela do Sacramento^c, Diego Alves^c, Diogo La Rosa Novo^d, Márcia Foster Mesko^d, Marina Prigol^{a,b,*}

^a Laboratório de Avaliações Farmacológicas e Toxicológicas Aplicadas às Moléculas Bioativas, LaftamBio Pampa, Universidade Federal do Pampa, Campus Itaqui, Rua Luiz Joaquim de Sá Britto, s/n - Bairro: Promorar, Itaqui, Rio Grande do Sul, CEP 97650-000, Brazil

^b Department of Nutrition, Universidade Federal do Pampa, Campus Itaqui, Rua Luiz Joaquim de Sá Britto, s/n - Bairro: Promorar, Itaqui, Rio Grande do Sul, CEP 97650-000, Brazil

^c Laboratório de Síntese Orgânica Limpa, LASOL, Centro de Ciências Químicas, Farmacêuticas e de Alimentos (CCQFA), Universidade Federal de Pelotas, Campus Universitário, S/N - Prédio/Bloco: 30 e 32, Capão do Leão, Rio Grande do Sul, CEP 96160-000, Brazil

^d Centro de Ciências Químicas, Farmacêuticas e de Alimentos, Universidade Federal de Pelotas, Campus Universitário, S/N - Prédio/Bloco: 30 e 32, Capão do Leão, Rio Grande do Sul, CEP 96160-000, Brazil

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ABSTRACT

Neurodegeneration in Parkinson's disease appears to be caused by multiple factors, including oxidative damage and an increase in acetylcholinesterase expression that can culminate in loss of dopaminergic neurons. A selenium-containing quinoline derivative, 7-chloro-4-(phenylselanyl) quinoline (4-PSQ), shows important pharmacological actions mainly attributed to its antioxidant and anticholinesterase properties. Thus, this study investigated the neuroprotective effect of 4-PSQ in a model of Parkinson's-like disease induced by rotenone (ROT) in *Drosophila melanogaster* and verified whether these effects are related to selenium levels. Adult flies were divided into: [1] control, [2] 4-PSQ (25 µM), [3] ROT (500 µM), and [4] 4-PSQ (25 µM) + ROT (500 µM) groups and exposed to a diet containing ROT and/or 4-PSQ for 7 days, according to their respective groups. Survival, behavioral, and *ex vivo* analyses were performed. Dopamine levels, reactive species levels (RS), lipid peroxidation (LPO), superoxide dismutase (SOD) and catalase (CAT) activity, and proteic thiol (PSH) and non-proteic thiol (NPSH) content in the head region were analyzed, while acetylcholinesterase (AChE) activity and selenium levels in the head and body regions were analyzed. 4-PSQ was able to reverse the ROT-induced deficits in flies, reestablish dopamine and selenium levels, reverse cholinergic deficits, improve motor function, and ameliorate mortality. Furthermore, 4-PSQ also reduced RS levels and LPO, and restored the activities of the antioxidant enzymes, SOD and CAT. Interestingly, a positive relationship between dopamine and selenium levels could be seen. Our results demonstrate the neuroprotective effect of 4-PSQ, and we suggest that the compound may act *via* different mechanisms, such as improving antioxidant defenses and consequently reducing oxidative damages, as well as having an anticholinesterase action, which together can prevent dopamine depletion, as these actions were correlated with the presence of selenium in the 4-PSQ molecule.

Abbreviations: 4-PSQ, 7-chloro-4-(phenylselanyl) quinoline; ROT, rotenone; PD, Parkinson's disease; GC/MS, gas chromatography with mass spectrometry; CRM, certified reference material; ERM, European Reference Materials; ICP-MS, inductively coupled plasma mass spectrometry; RSD, relative standard deviation; LOD, limit of detection; HPLC, high performance liquid chromatography; AChE, acetylcholinesterase; AcSch, acetylthiocholine; RS, reactive species; LPO, lipid peroxidation; TBARS, thiobarbituric acid reactive substance; SOD, superoxide dismutase; CAT, catalase; PSH, proteic thiol; NPSH, non-proteic thiol; BBB, blood-brain barrier

* Corresponding author at: Universidade Federal do Pampa, Campus Itaqui, Rua Luiz Joaquim de Sá Britto, s/n - Bairro: Promorar, Itaqui, Rio Grande do Sul, CEP 97650-000, Brazil.

E-mail addresses: marinaprigol@gmail.com, marinaprigol@unipampa.edu.br (M. Prigol).

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1. Introduction

Parkinson's disease (PD) is a neurodegenerative disturbance that presents with progressive and selective loss of nigrostriatal dopaminergic neurons, resulting in a deficiency of dopamine (DA) in the striatum [1,2]. The decreased viability of dopaminergic neurons leads to the onset of symptoms such as rigidity, bradykinesia, resting tremor, and postural instability [1,3,4]. Studies suggest possible mechanisms for neurodegeneration in PD, such as mitochondrial impairment, abnormal protein aggregation, apoptosis and oxidative stress, factors that may culminate in neuronal injury [1,3,5]. Recently, a possible relationship between an increase in acetylcholinesterase expression in the process of cellular apoptosis in a neurotoxin model of PD has been highlighted [6].

Therefore, molecules capable of preventing oxidative stress and inhibiting the acetylcholinesterase enzyme could protect dopaminergic neurons, suggesting a potential novel treatment for PD. In this sense, organic selenium compounds stand out as a new prospect among synthetic substances with therapeutic potential due to their pharmacological activities [7–10]. In addition, studies point important actions of quinoline compounds in conjunction with selenium organics [11–13]. Recently, 7-chloro-4-(phenylselenanyl) quinoline (4-PSQ) has been highlighted due to its pharmacological actions, characterized as a multi-target drug, mainly due to its antioxidant [11–16], anti-inflammatory [12,17], and anticholinesterase actions [14,18], which led us to consider its use as a potential drug to be tested in a model of PD induced by the neurotoxin, rotenone (ROT).

Drosophila melanogaster, like vertebrates, have dopaminergic neurons of the central nervous system that are involved in locomotor control [3,19,20]. Additionally, fly models have shown strong PD related phenotypes, including mitochondrial dysfunction, reactive oxygen species production, loss of dopaminergic neurons and reduced locomotion [19,20].

Based on this, we aimed to evaluate the neuroprotective effects of 4-PSQ, in a model of Parkinson's-like disease induced by rotenone in *Drosophila melanogaster*.

2. Materials and methods

2.1. Chemicals

4-PSQ (Fig. 1) was prepared and characterized according to the method previously described by Savegnago et al. [11]. Analysis of the ^1H NMR and ^{13}C NMR spectra showed analytical and spectroscopic data in full agreement with its assigned structure. The chemical purity of the 4-PSQ (99.9%) was determined using gas chromatography combined with mass spectrometry (GC/MS). ROT was purchased from Sigma-Aldrich (St. Louis, MO, USA) and other reagents were of analytical grade from our laboratory in the Campus of UNIPAMPA.

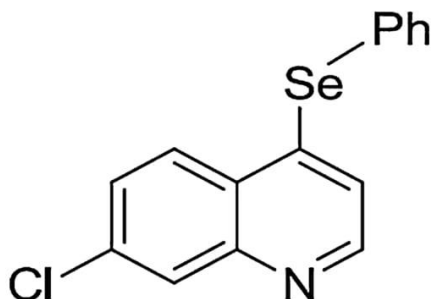


Fig. 1. Chemical structure of 7-chloro-4-(phenylselenanyl) quinoline (4-PSQ).

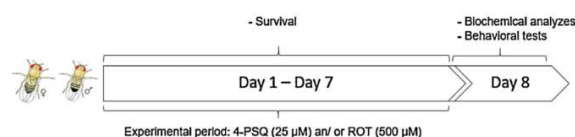


Fig. 2. Schedule of the experimental protocol.

2.2. *Drosophila melanogaster* stock and culture

The experiments were carried out using *Drosophila melanogaster* (Harwich strain) obtained from the National Species Center (Bowling Green, Ohio, USA). The flies were reared and maintained for about 4 days in an incubator (BOD) at a controlled temperature environment of $25 \pm 1^\circ\text{C}$ and 60–70% humidity with a 12-hour light/12-hour dark cycle. The flies were kept in glass bottles with 10 mL of standard food comprising corn flour (76.59%), wheat germ (8.51%), sugar (7.23%), powdered milk (7.23%), salt (0.43%), an antifungal agent (Nipagin), and a pinch of dry yeast.

3. Experimental protocol

3.1. ROT exposure and 4-PSQ treatment

The experiment protocol (Fig. 2) was performed with flies of both sexes, aged 1–4 days, divided into four groups consisting of 50 flies each: [1] control group, [2] 4-PSQ (25 μM) group, [3] ROT (500 μM) group, and [4] 4-PSQ (25 μM) + ROT (500 μM) group. Flies were concomitantly exposed to a diet containing ROT and/or 4-PSQ for 7 days, according to their respective groups.

ROT (500 μM) was dissolved in ethanol at a concentration similar to that used by Hosamani and Muralidhara [21], Hosamani et al. [22], and Araujo et al. [23] in their published studies with *Drosophila melanogaster*. 4-PSQ (25 μM) was dissolved in ethanol, and this concentration was chosen based on a pilot experiment where flies were exposed to 4-PSQ at various concentrations (12.5 μM , 25 μM , 50 μM , and 100 μM) to determine the effect of 4-PSQ alone on the survival of flies during the experimental period of 7 days. A concentration of 25 μM was considered satisfactory when assessing mortality and the negative geotaxis behavioral test in flies, because it was effective and did not cause toxicity to the flies.

4-PSQ and ROT were placed into the flies' feed at a final concentration of 25 μM and 500 μM , respectively. The diet treatment consisted of 1% w/v yeast beer, 2% w/v sucrose, 1% w/v milk powder, 1% agar w/v, and 0.08% w/v nipagin. The control group additionally received the same vehicle as that of the ROT and 4-PSQ groups, and the final concentration of vehicle (ethanol) in medium was 0.5%.

4. In vivo assays

4.1. Survival rate

The flies were observed daily and survival was assessed by counting the number of live flies per day until the end of the experimental period of 7 days. For the evaluation of survival data, 500 flies were included in each group, with the total number of flies representing the sum of ten independent experiments (50 flies/each replicated treatment).

4.2. Negative geotaxis

Negative geotaxis is a widely used behavioral test to evaluate locomotor performance. In this study, the control and treated flies performed the negative geotaxis test after 7 days of treatment [23,24]. Briefly, 15 flies from each group were separately immobilized and anesthetized using ice and placed individually in a 1.5-cm diameter glass test tube. After a 10-minute recovery period, the test was begun.

Regarding the analysis, we measured the time spent by each fly to reach a height of 8 cm measured from the bottom of the test tube, in a maximum evaluation time of 120 s. The test was repeated five times for each fly and data analyzed according to the averaged time. Three independent experiments were performed, using a total of 45 flies per group.

4.3. Open field test

To evaluate the behavior and exploratory activities of the flies, an open field test of the control and treated groups was performed as per Hirth (2010) [25]. In this test 15 flies from each group were kept in a petri dish divided into squares measuring one centimeter each. They were kept there for a 10-minute recovery period before beginning the test. The exploratory activity and movement of flies were evaluated by counting the number of squares crossed/explored by each fly for a 60-second period. Furthermore, the open field trial was evaluated using the velocity (cm/s) maintained during this exploratory activity. For this analysis, we performed four independent experiments, with a total of 60 flies per group.

5. Ex vivo assays

5.1. Homogenized preparation

For biochemical analysis, the flies were first euthanized on ice. The head and body regions were then carefully separated and individually homogenized in ice-cold HEPES buffer (20 mM, pH 7.0), 1:10 (flies/volume μL), for 2 min immediately after centrifugation of the samples according to the desired analysis. The supernatant from the samples was then removed and used for the biochemical assays. The present study examined only the head region of the flies in all *ex vivo* assays, except for determination of selenium concentration and acetylcholinesterase activity, which were analyzed in the head and body regions. All biochemical determinations were performed in duplicate. For each behavioral or biochemical analysis, at least three independent experiments were performed.

5.2. Determination of Selenium concentration

Head and body masses of flies were measured using an analytical balance (AUY220, Shimadzu, Philippines), with a maximum load of 220 g and a resolution of 0.0001 g. In the digestion process, the head (around 4 mg) or body (around 25 mg) of flies were transferred to microtubes (2 mL), 250 μL of HNO_3 were added and were heated in a water bath (Thermomix BM - B, Braun Biotech International, Germany) at $\pm 95^\circ\text{C}$ for 1 h. After cooling, the digests were diluted with water up to 2 mL. Nitric acid (Merck, Germany) used in this study was purified using a sub-boiling system (Duopur, Milestone, Italy), and ultrapure water (18 $\text{M}\Omega\text{ cm}$) was obtained from a purification system (Mega Up, Megapurity, South Korea). Six independent experiments for each group of head and body were performed.

The determination of Se in the head and body was performed using an inductively coupled plasma mass spectrometer (NexION 300X, Perkin-Elmer, Canada), equipped with a concentric nebulizer (Meinhard Associates, USA), cyclonic spray chamber (Glass Expansion Inc., Australia), and quartz torch with a quartz injector tube (2 mm i.d.). Instrumental performance was optimized following previous work published in the literature [26,27]. The Se-82 isotope was measured based on reported studies in the literature [26,27]. External calibration was performed using reference solutions of Se (0.01–10 $\mu\text{g L}^{-1}$), which were prepared by sequential dilution of a stock solution (1000 mg L^{-1} , Merck) in 5% HNO_3 . The same monoelemental stock standard solution was used in recovery tests to evaluate the accuracy of the determination step. High-purity argon (99.998%, White Martins, Brazil) was used for plasma generation, nebulization, and as an auxiliary gas in the

determination step. Results were expressed considering the flies weight (ng of Se per gram of head or body weight).

In order to evaluate the accuracy of the analytical method, the digestion of a certified reference material (CRM) of fish muscle (BB422) from European Reference Materials (ERM) was performed under the same conditions as for the fly samples and Se was determined by inductively coupled plasma mass spectrometry (ICP-MS). The obtained concentration of Se ($1.20 \pm 0.08 \mu\text{g g}^{-1}$) for ERM was in agreement ($P < 0.05$) with the reference value for Se ($1.33 \pm 0.13 \mu\text{g g}^{-1}$). The relative standard deviation (RSD) was lower than 7%. Thus, the analytical method used in this work presented suitable accuracy and precision for Se determination. The limit of detection (LOD) was calculated from the mean of the blank values plus three times the standard deviation obtained for ten replicates of blank. The sample mass, the final volume of digests and, when necessary, the dilution factor were taken into account. Limits of detection for the determination of Se in the head and body were 124 ng g^{-1} and 21 ng g^{-1} , respectively; different values were obtained in part because of different sample masses used in the sample preparation method (median mass for the head was 4 mg and for the body was 25 mg).

5.3. Determination of DA levels

DA levels were determined using high performance liquid chromatography (HPLC) according to the procedure proposed by Dalpiaz et al. [28]. In the preparation of the sample, the heads of 20 flies per treatment group were homogenized in 100 μL of sodium phosphate buffer (0.1 M, pH 7.4) with 1 mM EDTA, and then centrifuged at 17857 rpm for 10 min at 4°C , forming a supernatant that was evaluated using HPLC. Three to five independent experiments (20 flies per group) were performed.

5.4. Determination of acetylcholinesterase (AChE) activity

The AChE activity in the head and body regions was determined through a homogenized sample of a total of 20 flies from each group in 200 μL of 20 mM HEPES buffer (pH 7.0) and 20 bodies in 800 μL of 20 mM HEPES buffer (pH 7.0) and centrifuged at 1000 rpm for 5 min at 4°C , according to Ellmann et al. [29]. The reaction was prepared with 0.25 M KPi buffer (pH 8.0) and 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB 5 mM), and this was added to the supernatant sample solution (50 μL) and to acetylthiocholine (AcSch) (7.25 mM). The reaction was then monitored for 2 min at 412 nm. The AChE activity is expressed as $\mu\text{mol AcSch/h/mg protein}$. The total of 20 flies per group represents the sum of three and five independent experiments performed for head and body samples, respectively.

5.5. Determination of reactive species levels

The level of reactive species (RS) was determined using a spectrofluorometric method, according to Pérez-Severiano et al. [30]. Therefore, 10 flies per group were euthanized on ice, and the heads were homogenized in 500 μL 10 mM Tris buffer (pH 7). Afterwards, the head homogenate was centrifuged at 3570 rpm for 5 min at 4°C and the supernatant was removed, and used for assay quantification of 2',7'-dichlorofluorescein diacetate (DCF-DA) oxidation. The fluorescence emission of DCF resulting from DCF-DA oxidation was monitored after 1 h at an excitation wavelength of 488 nm and an emission wavelength of 520 nm using a fluorescence spectrometer. The results were obtained and are expressed as a percentage of the control DCF formation in arbitrary units (AU). The mean of three to four independent experiments was used (30–40 flies for each group).

5.6. Determination of thiobarbituric acid reactive substances

Lipid peroxidation (LPO) and products in the head region were

obtained using thiobarbituric acid reactive substance (TBARS) according to the protocol proposed by Ohkawa et al. [31] with minor modifications. Briefly, the heads of 12 flies from each treatment group were homogenized in 500 μ L 0.1 M phosphate buffer (pH 7.0) and centrifuged at 1000 rpm for 5 min (4 °C). After centrifugation, the supernatant was incubated in 0.45 M acetic acid/HCl buffer (pH 3.4), 0.28% thiobarbituric acid, and 1.2% SDS at 95 °C for 60 min. The absorbance at 532 nm was then measured. The results of TBARS were normalized based on the protein concentration of the respective fly head regions and are expressed as nmol malondialdehyde (MDA)/mg protein. For this analysis, we performed four to five independent experiments (a total of 36 to 60 flies for each group, respectively).

6. Antioxidant enzymes activities

6.1. Determination of superoxide dismutase activity

The superoxide dismutase (SOD) activity in the head of flies was assayed by evaluating the inhibition of quercetin auto-oxidation, according to the procedure of Kostyuk and Potapovich [32], and modifications as made by Franco et al. [33]. The heads of 20 flies in each group were homogenized and centrifuged (at 14000 rpm for 30 min at 4 °C). SOD was then measured. The reaction mixture contained sodium phosphate buffer (0.025 M/EDTA 0.1 mM, pH 10), *N,N,N,N*-tetramethylethylenediamine (TEMED), and 10 μ L of the head tissue sample. The reaction was started by adding 0.15% quercetin dissolved in dimethyl formamide and monitored for 2 min at 406 nm. Results represent the mean of three independent experiments with a total of 20 flies in each group. The enzymatic activity is expressed as U/mg protein (one unit is defined as the amount of enzyme required to inhibit the rate of autooxidation of quercetin by 50% at 26 °C).

6.2. Determination of catalase activity

The catalase (CAT) activity in the fly head was spectrophotometrically determined according to Aebi [34] with modifications as per Paula et al. [24]. A total of 20 fly heads per group were homogenized and centrifuged (at 14000 rpm for 30 min at 4 °C). The reaction mixture contained phosphate buffer (0.25 M/EDTA 2.5 mM, pH 7.0), hydrogen peroxide (10 mM), Triton X-100 (0.012%), and 30 μ L head sample; the reactions were evaluated at 240 nm for 2 min. Enzyme activity is expressed as U/mg protein (1 U decomposes 1 μ mol H_2O_2 /min at pH 7 and 25 °C) and was determined from three to four independent experiments (20 flies per group).

6.3. Determination of thiol content

The determination of protein (PSH) and non-protein (NPSH) thiol

content was estimated based on spectrophotometry, as per Ellmann et al. [35]. To determine thiol content, 10 head of flies per group were homogenized, and later added to 0.5 M perchloric acid and centrifuged (at 10,000 rpm for 5 min at 4 °C). For evaluation of non-protein thiol, the supernatant was used, and the pellet reserved for later analyses of non-protein thiol. Next, DTNB 5 mM was added; the mixture was then kept for 15 min in the dark, and read at 412 nm using a spectrophotometer. For evaluation of non-protein thiol measures, the pellet was resuspended in Tris/HCl 0.5 M with pH 8.0, after the supernatant had been removed. Next, 5 mM DTNB was added and the mixture was kept for 15 min (temperature and light suitable) and read at 412 nm using a spectrophotometer. Results represent the mean of three independent experiments (10 flies in each group) and are shown as a percentage of the control group.

6.4. Determination of protein concentration

The protein concentration in the head and body regions was evaluated using the method of Bradford (1976) [36] with standard bovine serum albumin.

6.5. Statistical analysis

Differences in lifespan were analyzed by comparing the survival curves by log-rank (Mantel-Cox) test. The results of other analyses were performed by one-way analysis of variance (ANOVA), followed by Newman-Keuls test or two-way ANOVA (4-PSQ \times ROT) followed by Bonferroni *post hoc* test where appropriate. Pearson's correlation coefficient was used to examine the correlation between the analyzed parameters. GraphPad Prism, version 6 (San Diego, CA, USA) was used for all statistical analyses and their respective figures (2–8). The results of descriptive statistical data are presented as mean(s) \pm standard error (SE) of the mean. Probability values less than 0.05 ($p < 0.05$) were considered statistically significant.

7. Results

7.1. Effect of 4-PSQ on the survival rate of *Drosophila melanogaster*

The exposure of adult flies, for an experimental period of 7 days, to ROT decreased the survival rates compared with those in the control group. The survival rates of the 4-PSQ and 4-PSQ + ROT groups were similar, and all groups had greater survival when compared to the ROT-alone group, evidencing the effectiveness of 4-PSQ in preventing ROT-induced mortality ($p < 0.05$; Fig. 3).

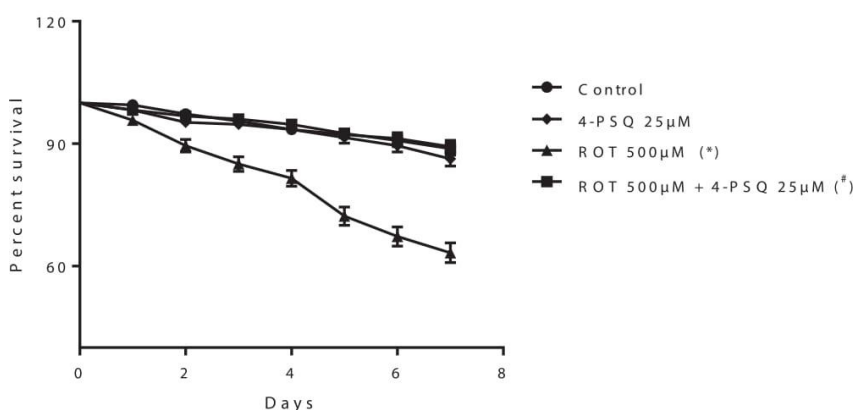


Fig. 3. Effect of 7-chloro-4-(phenylselanyl) quinoline (4-PSQ) on the survival rate of flies exposed to rotenone (ROT). Data were collected every 24 h for each group over 7 days. The total number of flies (500 per group) represents the sum of ten independent experiments. Lifespan measurement was determined by comparing the survival curves from Mantel-Cox log-rank tests and multiple comparisons were corrected using the Bonferroni test. * Significant difference from the control group; # significant difference between ROT and ROT + 4-PSQ ($p < 0.05$).

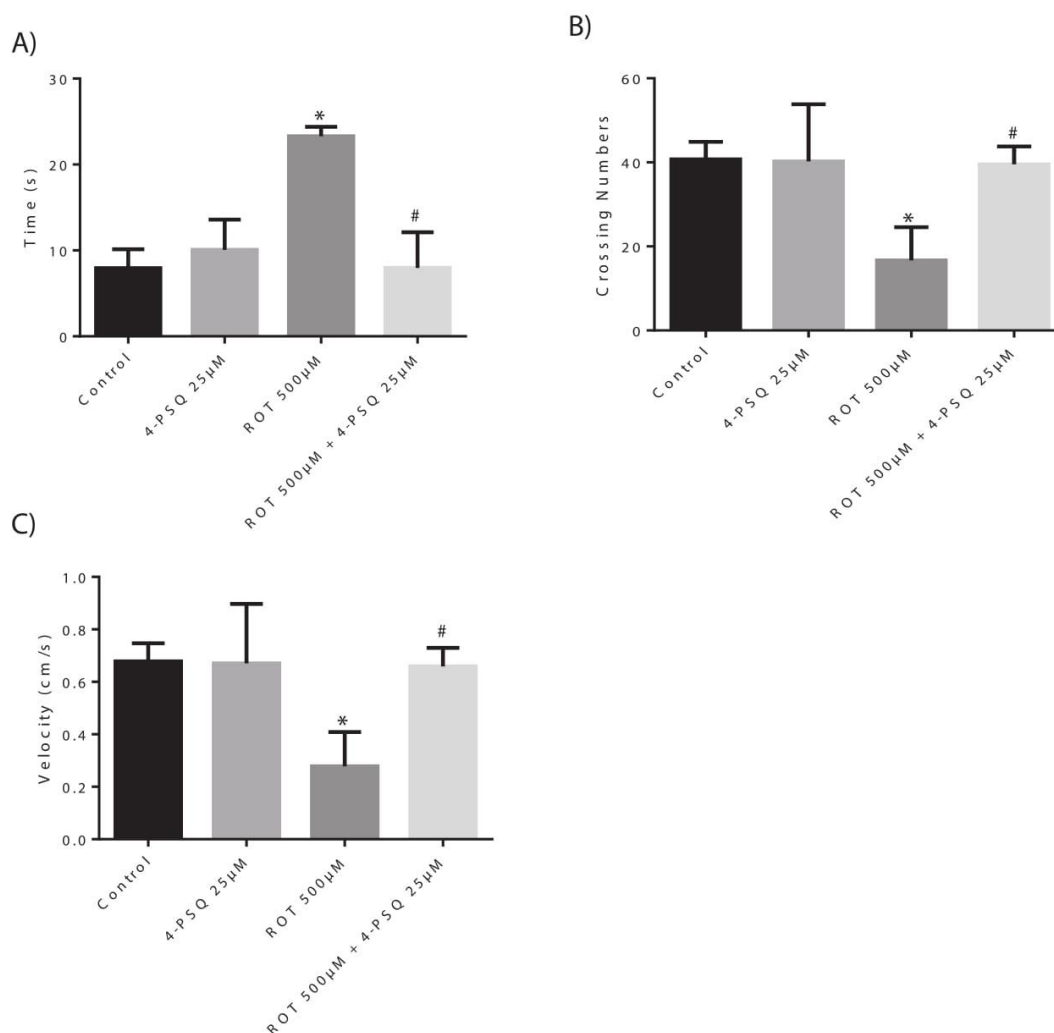


Fig. 4. Effect of 7-chloro-4-(phenylselanyl) quinoline (4-PSQ) on geotaxis response (climbing) and exploratory activities of flies exposed to rotenone (ROT) over 7 days. (A) Negative geotaxis assay; (B) open-field, and (C) velocity (cm/s) on open field. Fifteen flies per group were included for the negative geotaxis and open-field tests (total of 45 flies). These values represent the sum of three or four independent experiments. Values are mean \pm SE, and significance determined by two-way analysis of variance (ANOVA) followed by Newman-Keuls test. * Significant difference from the control group; # significant difference between ROT and ROT + 4-PSQ ($p < 0.05$).

7.2. Locomotor performance

Figs. 3A and 2 B shows the consequences of treatments on locomotor behavior and exploratory activities. The two-way ANOVA of the locomotor behavior of the flies showed a 4PSQ \times ROT interaction ($F_{(1,8)} = 25.68$, $p = 0.0010$; two-way ANOVA). *Post hoc* comparisons showed that the exposure to ROT compromised the locomotor behavior of the flies, verified by the decrease (approximately 194%) in the climbing rate compared with that in the control group ($p < 0.05$). Treatment with 4-PSQ prevented significantly the negative effects on climbing performance induced by ROT (Fig. 4A).

Fig. 3B and C shows the effect of treatment on exploratory activity. The two-way ANOVA revealed a 4-PSQ \times ROT interaction ($F_{(1,12)} = 7.723$, $p = 0.0167$; two-way ANOVA; Fig. 4B and C). In the open field test, there was a reduction in the exploratory activity (approximately 59%) and velocity of exploration (approximately 59%) of flies in the ROT group relative to the control group ($p < 0.05$), characterizing a locomotor injury. 4-PSQ was effective in preventing the

locomotor deficit and increasing the velocity of exploration of the flies.

7.3. Selenium levels

The two-way ANOVA revealed a significant interaction between 4-PSQ and ROT in the selenium levels in the head of flies [$F_{(1,2)} = 72.12$; $p < 0.0001$; Fig. 5A]. In relation to the body, the two-way ANOVA demonstrated a significant main effect of 4-PSQ [$F_{(1,2)} = 169.3$; $p < 0.0001$; Fig. 5B] and ROT [$F_{(1,2)} = 58.30$; $p < 0.0001$; Fig. 4B] on the levels of selenium. *Post hoc* analysis showed that flies exposed to ROT had reduced levels of selenium in the head (approximately 80%) and body (approximately 88%) compared to those in the control group. Additionally, flies treated only with 4-PSQ had higher selenium levels in the head and body regions (383% and 270%, respectively) than the control group. There was an increase in selenium levels in the head (763%) and body (1353%) of flies in the group treated with 4-PSQ + ROT when compared with the levels in the ROT group ($p < 0.05$), evidencing the compound's ability to restore selenium

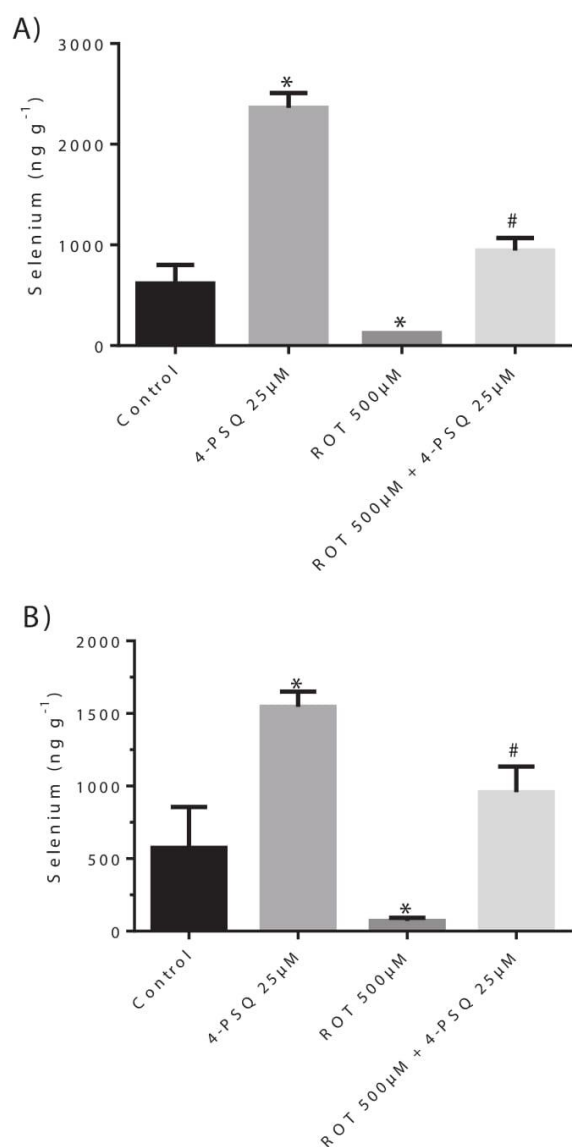


Fig. 5. Effect of 7-chloro-4-(phenylselenanyl) quinoline (4-PSQ) on rotenone (ROT)-induced alterations in selenium levels in the head (4A) and body (4B) of adult *Drosophila melanogaster*. Values are mean \pm SE. Significance determined by two-way analysis of variance (ANOVA) followed by Newman-Keuls *post hoc* test. * Significant difference from the control group; # significant difference between ROT and ROT + 4-PSQ ($p < 0.05$).

levels in the head and body of flies.

7.4. DA levels

Fig. 6 illustrates the effects of treatments when analyzing the DA levels in the head of flies. Two-way ANOVA revealed an interaction between 4-PSQ and ROT [$F_{(1,13)} = 4.857$; $p = 0.0462$; Fig. 5]. The *post hoc* test showed that flies exposed to ROT had reduced levels of DA (approximately 33%) in the head compared to that of the control group. However, the data from the group treated with 4-PSQ and ROT simultaneously demonstrated that the 4-PSQ protected against the effect of ROT, since it increased DA levels in the head of flies ($p < 0.05$).

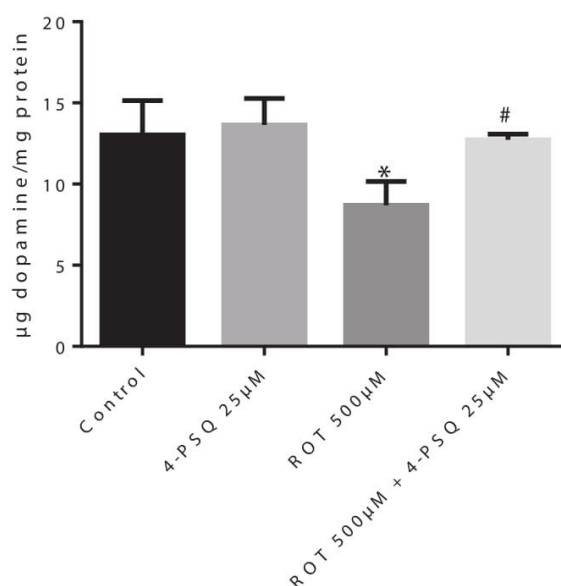


Fig. 6. Effect of 7-day treatment with 7-chloro-4-(phenylselenanyl) quinoline (4-PSQ) on rotenone (ROT)-induced alterations in dopamine levels in the homogenized head region of adult *Drosophila melanogaster*. Values are mean \pm SE ($n = 20$ flies per replicate, three to five replicates used). Significance determined by two-way analysis of variance (ANOVA) followed by Newman-Keuls *post hoc* test. * Significant difference from the control group; # significant difference between ROT and ROT + 4-PSQ ($p < 0.05$).

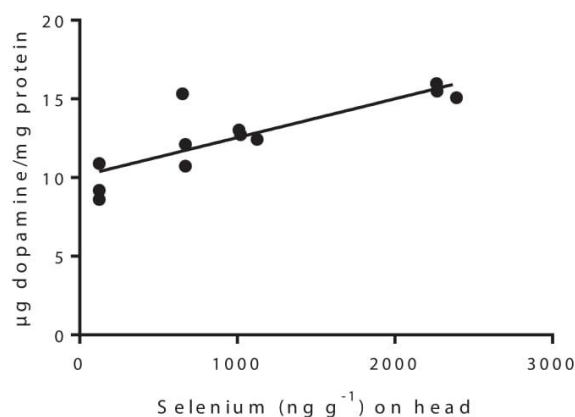


Fig. 7. Effects of treatment with 7-chloro-4-(phenylselenanyl) quinoline (4-PSQ) on correlation between selenium levels and dopamine (DA) levels (B) in the head of flies. Pearson's correlation analysis was considered significant difference when $p < 0.05$.

In the Pearson's correlation analysis, we observed a significant positive correlation between DA and selenium levels in the head of flies ($r = 0.8342$, $p = 0.0007$, $n = 12$; Fig. 7).

7.5. AChE activity

The two-way ANOVA revealed a significant interaction between 4-PSQ and ROT in the head [$F_{(1,8)} = 15.12$; $p = 0.0046$] and body [$F_{(1,16)} = 33.99$; $p < 0.0001$]. *Post hoc* analysis showed that the flies exposed to ROT had a significant increase in AChE activity in the head (approximately 60%) and body (approximately 51%), while additional treatment with 4-PSQ prevented against an increase in AChE activity in

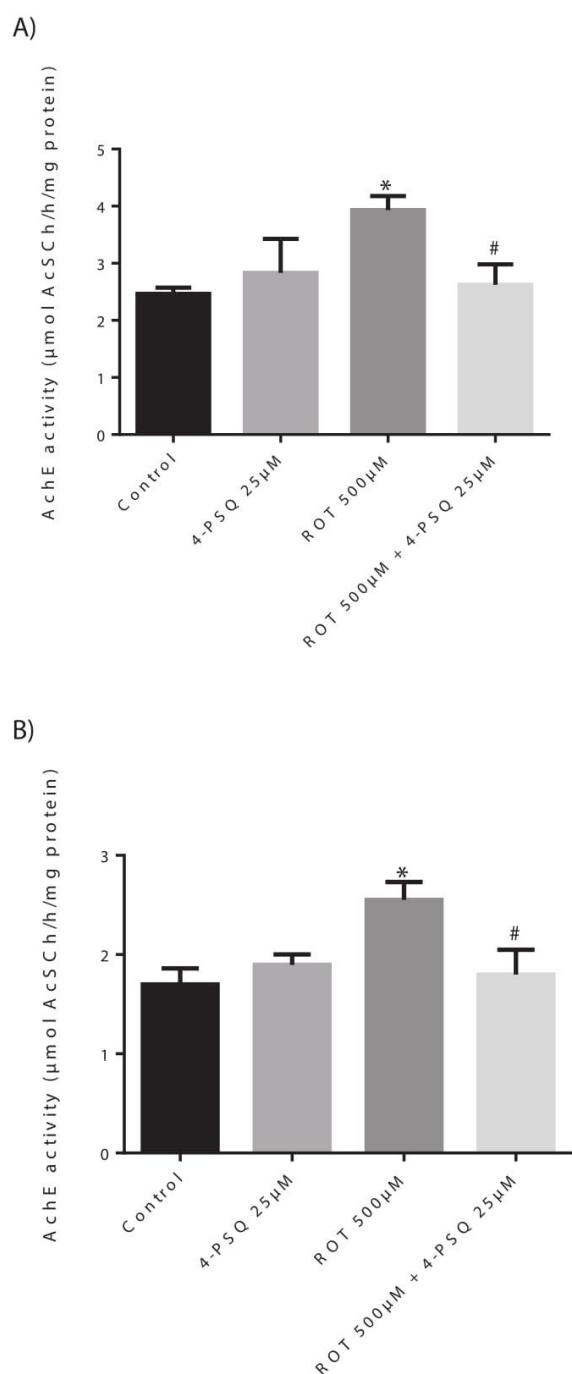


Fig. 8. Effect of 7-chloro-4-(phenylselenanyl) quinoline (4-PSQ) on rotenone (ROT)-induced alterations in AChE activity ($\mu\text{mol}/\text{min}/\text{mg}$ protein) in the head (A) and body (B) regions of adult *Drosophila melanogaster*. Values are mean \pm SE ($n = 20$ flies per replicate, three and five replicates used, respectively). Significance determined by two-way analysis of variance (ANOVA) followed by Newman-Keuls test. * Significant difference from the control group; # significant difference between ROT and ROT + 4-PSQ ($p < 0.05$).

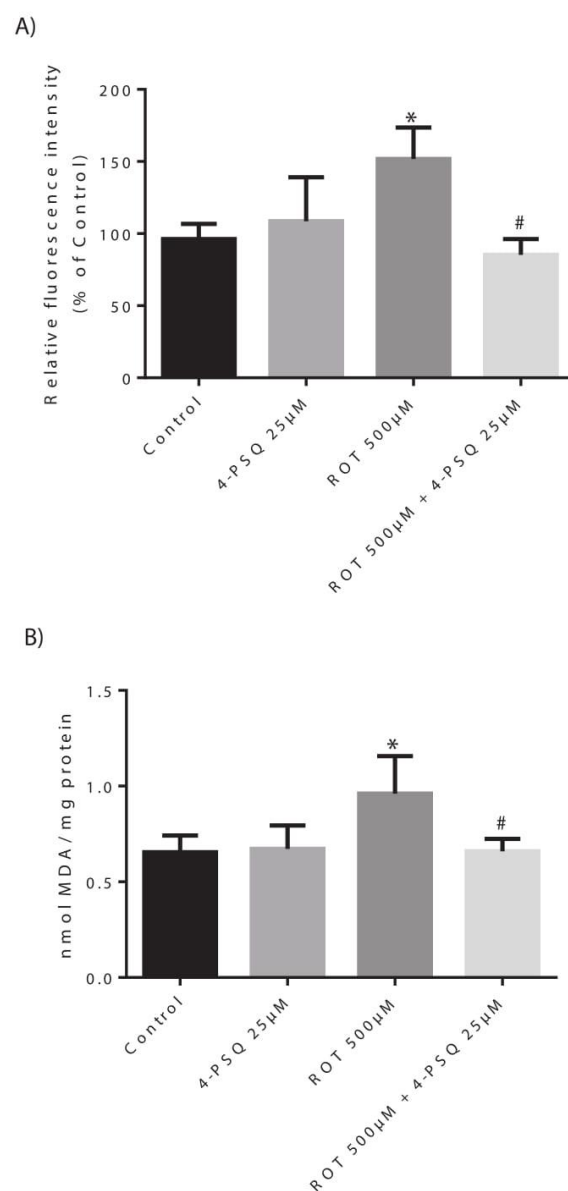


Fig. 9. Effect of 7-day treatment with 7-chloro-4-(phenylselenanyl) quinoline (4-PSQ) for 7 days on rotenone (ROT)-induced alterations in markers of endogenous oxidative stress in the head region of adult *Drosophila melanogaster*. (A) Reactive species levels (RS) and (B) levels of lipid peroxidation (LPO) in the head region of adult *Drosophila melanogaster*. Values are mean \pm SE ($n = 10$ and 12 flies, respectively, per replicate, three to five replicates used). Significance determined by two-way analysis of variance (ANOVA) followed by Newman-Keuls test. * Significant difference from the control group; # significant difference between ROT and ROT + 4-PSQ ($p < 0.05$).

the head and body of flies (Fig. 8A and B, $p < 0.05$).

7.6. Biomarkers of oxidative stress

Fig. 9A shows the RS levels. The two-way ANOVA showed a significant interaction between 4-PSQ and ROT [$F_{(1,9)} = 13.17$; $p = 0.0055$] in the RS levels. *Post hoc* analysis revealed that flies exposed to ROT had significantly increase in the production of RS (about

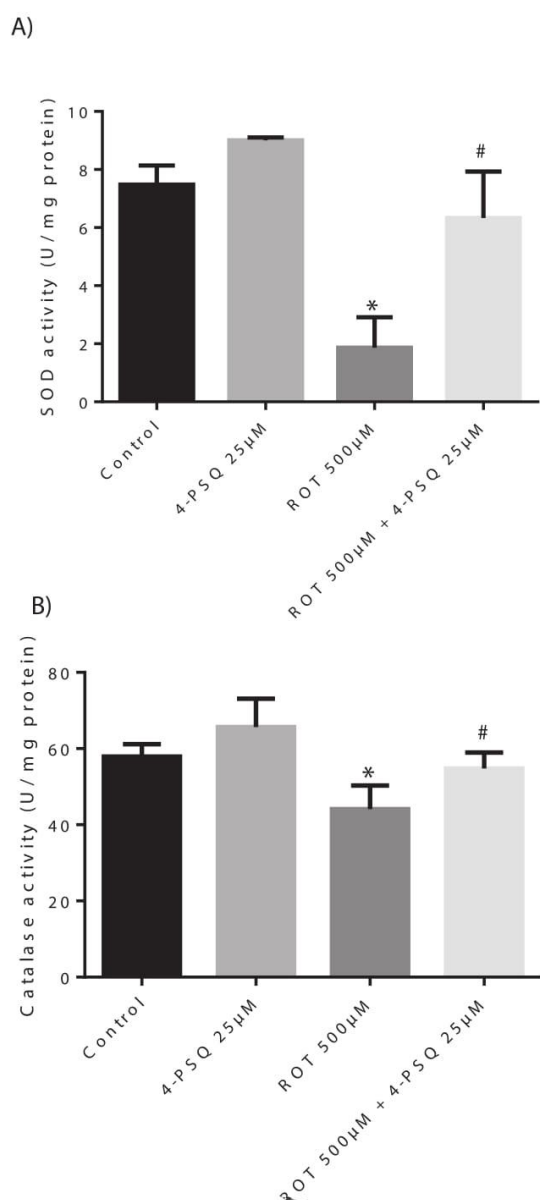


Fig. 10. Effect of 7-day treatment with 7-chloro-4-(phenylselanyl) quinoline (4-PSQ) for 7 days on alterations induced by rotenone (ROT) on the activities of antioxidant enzymes in the homogenized head regions of adult *Drosophila melanogaster*. (A) Superoxide dismutase (SOD) and (B) catalase (CAT). Values are mean \pm SE ($n = 20$ flies per replicate, three to four replicates used). Significance determined by two-way analysis of variance (ANOVA) followed by Newman-Keuls test. * Significant difference from the control group; # significant difference between ROT and ROT+ 4-PSQ ($p < 0.05$).

58%) when compared with the control group ($p < 0.05$). Additionally, there was a significant decrease in the production of RS in the group exposed to ROT and treated with 4-PSQ when compared with the untreated group.

Fig. 9B evidenced the effects the treatment LPO and products in the head region. In the two-away analysis it was possible to verify an interaction between 4-PSQ and ROT [$F_{(1,14)} = 6.560$, $p = 0,0226$; Fig. 8B] in the LPO. We observed increased in LPO (about 48%) in flies

exposed to ROT, while the treatment with 4-PSQ was effective in reducing LPO ($p < 0.05$).

7.7. Antioxidant defenses

The Fig. 10A and B shows the results of enzymatic activity of SOD and CAT. Two-way ANOVA revealed a significant interaction between 4-PSQ and ROT for SOD activity [4-PSQ \times ROT interaction $F_{(1,8)} = 6.365$, $p = 0.0356$], but no significant interaction for CAT activity [4-PSQ \times ROT interaction $F_{(1,9)} = 0.2221$, $p = 0.6486$]. Post-hoc analysis revealed inhibition of SOD (about 75%) and CAT (about 24%) activities in the ROT compared to the control group. Furthermore, 4-PSQ was successful in preventing the inhibition of SOD and CAT activities (Fig. 9A and B; $p < 0.05$).

7.8. Thiol determination

Two-way ANOVA revealed no significant interaction between 4-PSQ and ROT for PSH [4-PSQ \times ROT interaction $F_{(1,8)} = 0.1962$, $p = 0.6695$] or NPSH [4-PSQ \times ROT interaction $F_{(1,8)} = 0.4131$, $p = 0.5384$]. Similarly, in the *post hoc* analysis, there were no statistical differences between groups with regard to the PSH and NPSH in the head regions (data not shown).

7.9. Effects of 4-PSQ on correlation between DA levels and behavioral and biochemical parameters

In the Pearson's correlation analysis (Table 1), DA levels in the head were significantly negatively correlated with negative geotaxis ($r = -0.8336$, $p = 0.0008$), AChE activity in the head ($r = -0.8118$, $p = 0.0013$) and body ($r = -0.8731$, $p < 0.0001$), RS levels ($r = -0.8487$, $p = 0.0002$), and LPO ($r = -0.8943$, $p < 0.0001$). Furthermore, the correlation analysis found a significant positive correlation between DA levels and survival ($r = 0.8739$, $p = 0.0002$), crossing numbers on the open field test ($r = 0.9050$, $p < 0.0001$), velocity of open field exploration ($r = 0.9051$, $p < 0.0001$), and SOD ($r = 0.8232$, $p = 0.0010$) and CAT ($r = 0.9172$, $p < 0.0001$) activity. No significant correlation was observed between DA levels and PSH ($r = -0.2641$, $p = 0.4068$) or NPSH ($r = -0.3386$, $p = 0.2816$).

8. Discussion

The current study investigated the effects of 4-PSQ in a model of ROT-induced PD in *Drosophila melanogaster*. Our results demonstrate

Table 1

Effects of 7-chloro-4-(phenylselanyl) quinoline (4-PSQ) on correlation between dopamine (DA) levels in the head of flies with behavioral or biochemical parameters.

DA and behavioural and biochemical parameters	r	p	n
DA x Survival	0.8739	0.0002 *	12
DA x Negative geotaxis	-0.8336	0.0008 *	12
DA x Test Open Field	0.9050	< 0.0001 *	15
DA x Velocity of Open Field	0.9051	< 0.0001 *	15
DA x AChE in head	-0.8118	0.0013 *	12
DA x AChE in body	-0.8731	< 0.0001 *	17
DA x RS	-0.8487	0.0002 *	13
DA x LPO	-0.8943	< 0.0001 *	17
DA x SOD	0.8232	0.0010 *	12
DA x CAT	0.9172	< 0.0001 *	14
DA x PSH	-0.2641	=0.4068	12
DA x NPSH	-0.3386	=0.2816	12

Acetylcholinesterase (AChE); Reactive species (RS); Lipid peroxidation (LPO); Superoxide dismutase (SOD); Catalase (CAT); Proteic thiol (PSH); Non-proteic thiol (NPSH).

* $p < 0,05$ is considered significant.

the ability of 4-PSQ to prevent the damage caused by this model, and its relation to the levels of selenium in the head of flies. We observed that treatment with 4-PSQ increased selenium levels, as well as prevented oxidative stress, restored antioxidant defenses, and inhibited the action of AChE. These effects can be related to the restored DA levels observed in the head of flies and, consequently the prevention of locomotor impairments and the improvement in the survival of flies exposed to ROT.

Interestingly, the results of the study indicate that treatment with 4-PSQ protects against dopaminergic damage caused by ROT in the head of flies. An increasing number of recent studies have shown that ROT exposure produces similar behavioral and biochemical changes as observed in PD and other neurological diseases [1,37,38]. Regarding the characterization of PD induced by exposure to ROT, our results corroborate with other studies that found DA depletion in *Drosophila melanogaster* [21–23]. The toxicity caused by ROT can lead to signs and symptoms similar to those observed for PD; this is associated with mitochondrial dysfunction due to complex I inhibition, which decreases electron transport chain antioxidant levels, and increases iron levels, and impairs dopaminergic neurons that may be more susceptible to oxidative stress than other neurons in the brain because they contain DA [39]. Surprisingly, an unprecedented and very important finding is that selenium levels are reduced in the group treated with ROT when compared with those in the control group. One hypothesis for this finding would be a compensatory mechanism in which, due to the oxidative stress provoked by ROT, there would be an increase in the demand for selenium in the brain for the production of selenoprotein to combat oxidative insults [40,41]. However, treatment with 4-PSQ was able to reestablish selenium levels in both the head and body of the flies. Consistent with our findings, Khera et al. [42] in a study with placental trophoblast cells verified that cells treated with selenium are more resistant to mitochondrial oxidative stress stimulated by ROT. Ellwanger et al. [8], observed bradykinesia and DNA damage reduction in a paraquat PD model in rats receiving selenium in the form of selenite in drinking water. Another study verified that selenium supplementation reverses the impairment of dopaminergic neurotransmission induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine [43]. However, none of these studies measured selenium levels. Therefore, we first verified that selenium levels are decreased in the head of flies in this model of Parkinson's disease.

4-PSQ reduced the depletion of DA caused by ROT, demonstrating its potential to increase levels of DA and its neuroprotective effect, evidenced also by the association between the increase in dopamine levels as the levels of selenium increased in the head of flies. Moreover, evidence indicates the involvement of selenium in dopaminergic protection pathways, possibly due to the high level of reactive oxygen species production during dopamine cycling and autooxidation [44]. It is well documented that 4-PSQ has neuroprotective actions such as those reported by Duarte et al. [14] *in vitro* and Vogt et al. [16] *in vivo*. In this study, the 4-PSQ reduced the depletion of DA, demonstrating the involvement of the dopaminergic system in the neuroprotective effect of this new selenium-containing quinoline compound, and thus may be a possible candidate for the treatment of PD. We believe that 4-PSQ has a mechanism of action similar to that of another organoselenium compound that is well-described in the literature, diphenyl diselenide [(PhSe)₂]. Studies conducted by Burger et al. [45], Savegnago et al. [46], and Figueira et al. [47] using (PhSe)₂ noted that the pharmacological effects of the compound mainly related to the dopaminergic system of mice. Additionally, a recent study conducted by Sampaio et al. [7] demonstrated that treatment with (PhSe)₂ reverses some motor impairments in a 6-hydroxydopamine (6-OHDA) model of PD in rats.

In this study, flies exposed to ROT exhibited increased mortality and locomotor impairment with a phenotype similar to PD. Other studies with ROT also showed high mortality and locomotor deficits in flies exposed to this pesticide, characterizing the model of ROT-induced PD

in flies [19,21–23]. In this sense, the reduction in locomotor behavior and deficit in the negative geotaxis and open field tests indicate that the PD model was successfully induced in the flies exposed to ROT. A mitochondrial deficiency caused by ROT can bring about damage to the locomotor system as it contributes to energy deficits of the ambulatory and flight muscles that are rich in mitochondria and have a high energy requirement, affecting locomotor and exploratory behaviors due to inhibition of electron transport chain complex I, caused by this bioenergetic crisis [1,21]. In the present study, we found that flies treated with 4-PSQ exhibited enhanced survival rates against ROT treatment in the co-exposure paradigm showed the ability of 4-PSQ in promoting survival pathways and abrogating locomotor impairments. Ellwanger et al. [8], in a study of an experimental model of PD induced by paraquat in rodents pointed that the use of selenium may contribute to the maintenance of locomotor skills. In studies with rodents, the compound 4-PSQ *per se* did not affect the behavior of animals [12,18,48]. Corroborating our findings regarding the neural protection exercised by 4-PSQ, recently its neuroprotective action against anxiety and learning and memory impairment caused by amyloid β -peptide (A β) was reported after evaluation using behavioral tests in a murine Alzheimer disease model [18].

Acetylcholine is the primary excitatory neurotransmitter in the central nervous system that plays a pivotal role in the regulation of motor function and locomotion [49,50]. AChE has an important role in the function of cholinergic synapses in the nervous system (central and peripheral), and became an attractive target for the development of new drugs [51]. Evidence suggests a significant role for the degeneration of cholinergic neurons in the pathophysiology of neurodegenerative diseases [52] and the involvement of AChE in the pathogenesis of PD, in which the increased expression of AChE is proposed to induce neuronal cell death [6]. Nonetheless, there are indications that dopaminergic deficits are associated with deterioration of the base ganglion circuit dynamics, resulting in overactivation of the cholinergic system, leading to motor and cognitive impairments [53]. AChE activity was also evaluated in this study, and an increase in AChE activity was found in the head and body of flies exposed to ROT, resulting in changes in locomotor activity with respect to the control group, corroborating the behavioral data and compatible with symptoms of PD. Similar to the present study, Rao et al. [54] verified an increase in AChE activity in a ROT-induced model of PD. In addition, Soares et al. [55] reported that paraquat, another agent similar to ROT and with an herbicidal action, affects the cholinergic system specifically by activating AChE; they suggested that this may be related to the fact that DA depletion caused by paraquat blocks auto-inhibition of acetylcholine release through muscarinic auto-receptors, leading to an excess of acetylcholine in the synaptic cleft and an increase in AChE activity [53]. Several previous reports showed that increased AChE activity is associated with increased neuronal oxidative and nitrosative stress, and alterations in energy metabolism in invertebrates and vertebrates [6].

We found that 4-PSQ was effective in protecting against the increase in AChE activity caused by ROT in the head and body regions, suggesting its efficacy in protecting cholinergic function at the central and peripheral level. It is an important result that molecules capable of inhibiting the enzyme acetylcholinesterase could protect the dopaminergic neurons arising as a possibility in the treatment of PD, since an increase in acetylcholinesterase expression in the process of cellular apoptosis in neurotoxin-induced models of PD has been observed [6]. Studies using 4-PSQ reported an inhibitory effect on cerebral AChE activity *in vitro* [14,56] and *in vivo*, while also having an effect on the brain structure of mice [18]. Duarte et al. [14] verified this effect only *in vitro*; however, they suggested that due to the fact these selenium-containing quinolone derivatives inhibit AChE activity and improve cognitive enhancement, this compound may be a potential therapeutic agent for the treatment of neurodegenerative disorders. More recently, Pinz et al. [18] in their study of a murine Alzheimer's disease model showed the inhibitory effect of 4-PSQ on AChE. Nevertheless, our data

corroborate those of other studies using other selenium compounds, such as a study that found dietary supplementation with (PhSe)₂ decreased AChE activity and improved climbing performance in flies exposed to manganese [57].

Numerous molecular pathways have been proposed for the development of PD, including among them oxidative stress and ROS generation [4]. Indeed, the increased incidence of age-associated diseases, such as PD, reflects the generation of free radicals and associated oxidative stress enhanced in certain regions of the brain during aging [58]. Evidence suggests that ROT-induced neurotoxicity can be attributed to the specific sensitivity of dopaminergic neurons to RS and oxidative damage [37,59]. Moreover, signs of oxidative damage have been detected frequently in dopaminergic neurons from PD patients and animal models, suggesting an implication of oxidative stress in this disease [37,59]. Similarly, in our study, we observed a significant increase in the production of RS and LPO in the ROT treatment group.

Recently, the antioxidant hypothesis of PD has become widely accepted and the potency of various neuroprotective agents in neurotoxin-based models has been demonstrated [2,19,60]. The inhibition of free radicals by antioxidant compounds offers beneficial effects against dopaminergic neurotoxicity. However, many currently available antioxidants cannot readily penetrate the blood–brain barrier (BBB) after systemic administration, pointing to the need to search for novel BBB-compatible antioxidant therapies [43,61]. Thus, an important result here is that 4-PSQ penetrated the BBB after administration since we observed an increase in selenium levels in the head of flies, consequently reversing increases in RS and LPO in the head of flies. These results indicate the potential of 4-PSQ to attenuate oxidative damage while maintaining cellular homeostasis. Following this, we speculate that the protective effect of 4-PSQ in this model of PD can also be attributed to its ability to abrogate oxidative stress. Several different strategies have been proposed to fight oxidative stress in PD, including molecules that can promote endogenous mechanisms to buffer free radicals, such as selenium [60]. Studies of the pesticides ROT [38,62] and Paraquat [8], which have been related to the development of PD, have pointed out that selenium can act as a shield against the oxidative stress caused by such pesticides. Our findings are also consistent with other studies that found potential antioxidant actions of 4-PSQ in *in vitro* and *ex vivo* rodent models against the increase in RS [12,13,16] and LPO [16,56]. 4-PSQ can act on different antioxidant defense lines, protecting against cerebral oxidative damage, suggesting that 4-PSQ acts in a manner similar to other organoselenium compounds [16]. This proposition can be supported by the results of Vogt et al. [16] who found that the phenylselenenyl substituent group in the quinoline structure is important for the antioxidant effect of this compound at the brain level. From the results presented in this study, we suggest that 4-PSQ, through its antioxidant action, acts to reduce the production of oxidative stress in neuronal cells.

To better understand the underlying mechanisms of the protective activity of 4-PSQ against ROT-induced oxidative damage, we assessed the activities of antioxidant enzymes (SOD and CAT) in the head of flies. In the present study, there was a significant decrease in SOD and CAT activities in the head regions of ROT-exposed flies. The protective role of SOD and CAT in the cells is related to the elimination of deleterious O₂ and H₂O₂ [50]. Our results evidently point to a compromised antioxidant defense system that can impair cellular function in the head regions of flies exposed to ROT. However, 4-PSQ reversed this inhibition of SOD and CAT activities caused by ROT exposure. Thus, 4-PSQ prevents ROT-associated depletion of the antioxidant defense system through the increased enzyme activity in the treated flies. The marked reduction in RS and increase in SOD and CAT activity after treatment with 4-PSQ revealed its potential antioxidant effect against ROT-induced oxidative stress. Recently, the antioxidant effect of 4-PSQ against cerebral stress was demonstrated, in addition to reducing RS and LPO and increasing CAT and glutathione-S-transferase activities [16]. Furthermore, in a study of the action of 4-PSQ on a model of Alzheimer's

disease, the authors point to the multi-target component of this compound, since it is not only an AChE inhibitor in the cerebral cortex and hippocampus, but also reduces LPO levels in the cortex of mice [18]. Thus, our results confirm the antioxidant potential of 4-PSQ, increasing the activity of antioxidant enzymes in the fight against oxidative injury.

The thiol level is an indirect oxidative stress biomarker and thus is indicative of chemical changes in thiol groups of proteins and peptides [50]. In the evaluation of non-enzymatic antioxidant defenses, the main ones are PSH and NPSH, no differences were observed between groups, consistent with the results of other studies of ROT-induced PD [22,23]. However, a study with 4-PSQ that aimed to evaluate the effect of the compound at the brain level in mice showed that 4-PSQ protected completely against the reduction in the levels of PSH and NPSH, evidencing the potential of the compound to act at the level of non-enzymatic protection against oxidative insults [14,16].

Furthermore, we can observe a correlation between the levels of DA and some of the evaluated parameters. Thus, we verified that according to a decrease in DA levels there is an increase in the time spent and velocity in the negative geotaxis test, an increase in AChE activity, and in the oxidative stress parameters (RS and LPO), or *vice versa*, while a decrease in dopamine levels would be correlated to a decrease in fly survival, activity, and exploratory behavior, and activity of antioxidant enzymes (SOD and CAT), or *vice versa*. Thus, a strong correlation can be observed between dopamine levels and fly survival, behavioral parameters, oxidative stress, and antioxidant defenses. These factors demonstrate the adequate establishment of the ROT-induced model of PD in *Drosophila melanogaster*, corroborating other studies in the literature [21–23]. Nonetheless, a positive correlation between DA and selenium levels that is, a relationship between the decrease in DA and selenium levels in the head of flies, was found. These data clearly demonstrate the relationship between AChE activity and oxidative stress on dopaminergic and behavioral changes in this PD model, as well as reinforcing the antioxidant and anticholinesterase action of 4-PSQ associated with its neuroprotective effect in our work, and the relation with selenium levels.

In summary, our results indicate that 4-PSQ can be considered a multi-target molecule, acting in the dopaminergic system through different mechanisms: reducing oxidative damages, improving antioxidant defenses, and exercising an anticholinesterase action, factors which together can protect dopaminergic neurons, preventing dopamine depletion, and consequently reversing the behavioral motor deficits in *Drosophila melanogaster*. Nevertheless, we found that these actions of 4-PSQ are mainly correlated with the presence of selenium in its structure. Therefore, 4-PSQ highlights an important target in the search for compounds with therapeutic potential in the neurodegenerative diseases, such as PD.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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MANUSCRITO 1

Effectiveness of 7-chloro-4-(phenylselanyl) quinoline in avoid psychomotor and non-motor behaviors deficits in a *Drosophila melanogaster* Parkinson disease mimetic model

Shanda de Freitas Couto, Stéfani Machado Araujo, Vandrezza Cardoso Bortolotto, Mustafa Munir Mustafa Daleh; Elize Aparecida Santos Musachio, Franciane Cabral Pinheiro, Leugim Corteze Romio, Manoela do Sacramento, Diego Alves, Marina Prigol

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Effectiveness of 7-chloro-4-(phenylselanyl) quinoline in avoid deficits on psychomotor and non-motor behaviors in *Drosophila melanogaster* Parkinson disease mimetic model

Short title:

4-PSQ avoid deficits on psychomotor and non-motor behaviors in a model of Parkinson's-like disease

Shanda de Freitas Couto^{a,b}, Stífani Machado Araujo^a, Vandrezza Cardoso Bortolotto^a, Mustafa Munir Mustafa Dahleh^a; Elize Aparecida Santos Musachio^a, Franciane Cabral Pinheiro^a, Leugim Corteze Romio^c, Manoela do Sacramento^d, Diego Alves^d, Marina Prigol^{a,b*}

^aLaboratório de Avaliações Farmacológicas e Toxicológicas Aplicadas às Moléculas Bioativas - LaftamBio Pampa - Universidade Federal do Pampa - Campus Itaqui - Rua Luiz Joaquim de Sá Britto, s/n - Bairro: Promorar, Itaqui, Rio Grande do Sul, Brasil, CEP 97650-000.

^bDepartment of Nutrition - Universidade Federal do Pampa - Campus Itaqui - Rua Luiz Joaquim de Sá Britto, s/n - Bairro: Promorar, Itaqui, Rio Grande do Sul, Brasil, CEP 97650-000.

^cDepartment of Mathematics - Universidade Federal do Pampa - Campus Itaqui - Rua Luiz Joaquim de Sá Britto, s/n - Bairro: Promorar, Itaqui, Rio Grande do Sul, Brasil, CEP 97650-000.

^dLaboratório de Síntese Orgânica Limpa - LASOL, Centro de Ciências Químicas, Farmacêuticas e de Alimentos (CCQFA) - Universidade Federal de Pelotas – Campus Universitário, S/N - Prédio/Bloco: 30 e 32, Capão do Leão, Rio Grande do Sul, Brasil, CEP 96160-000.

Conflict of Interest: The authors have nothing to disclose and no conflicts of interest to report.

*Corresponding author:

Marina Prigol

Universidade Federal do Pampa, Campus Itaqui

Rua Luiz Joaquim de Sá Britto, s/n - Bairro: Promorar, Itaqui, Rio Grande do Sul, Brasil,
CEP 97650-000

Phone number: +55 55 996447424

E-mail address: marinaprigol@gmail.com (M. Prigol)

Highlights

- 4-PSQ restores ROT-induced mortality and psychomotor deficit in flies
- 4-PSQ improves the learning and short-term memory in flies
- 4-PSQ prevents anxiety-like behaviors in flies
- 4-PSQ prevents the increase activity acetylcholinesterase in flies
- Neuroprotection of 4-PSQ is correlated with its multi-target action

Abstract

Parkinson's disease (PD) presents a symptomatology of motor and non-motor symptoms with relevant impact in the ability and patient's quality of life. It is necessary to study more about the involvement of psychomotor and non-motor symptoms like anxiety, learning and memory related to PD. The present study evaluated the effects of 7-chloro-4-(phenylselanyl) quinoline (4-PSQ) in the psychomotor and non-motor alterations in a mimetic model for PD induced by rotenone (ROT) in *Drosophila melanogaster*. It was used flies of both sexes and with 1 to 4 days of age, divided into four groups: [1] control group, [2] 4-PSQ (25 μ M) group, [3] ROT (500 μ M) group, and [4] 4-PSQ (25 μ M) + ROT (500 μ M) group, exposed to a diet with ROT and/or 4-PSQ for 7 days, according with each group. Were analyzed the mortality rate; behavioral tests: Open field and equilibrist test, Aversive Phototaxis Suppression Assay and Light/Dark Exploration Test; and Acetylcholinesterase (AChE) activity (head and body of flies). The flies exposed to ROT presented damages in the spontaneous locomotor activity, learning and memory impairment, and anxiety type behavior and it was observed an increase AChE activity in the head and body of flies exposed to ROT. Treatment with 4-PSQ proved to be effective in the prevention of locomotor, memory and learning deficits, and anxiety type behavior in this mimetic model of PD in *Drosophila melanogaster*. And it is possibly due its remarkable anticholinesterase activity associated with other targets of this multi target compound.

Keywords: selenoquinoline, anticholinesterase, learning, short-term memory, anxiety, behavioral paradigms.

1. Introduction

Neurodegenerative diseases associated with aging, such as Parkinson's disease (PD), have a symptomatology with a relevant impact on the patients [1,2]. Commonly described are a set of symptoms, mainly motor signals such as bradykinesia, rest tremor, stiffness and postural instability, resulting in loss of dopaminergic neurons, with consequent deficiency of the neurotransmitter dopamine [1-3]. However, in addition to motor symptoms, non-motor symptoms have also been prominent, associated with significant disability and loss of quality of life [1-5, 6].

The non-motor symptoms include mood changes (depression and anxiety), cognitive and sensory impairment, pain, and dysautonomia, which may arise in early stages prior to the clinical diagnosis of PD and exacerbate with the progression of the disease [7, 8]. And it is unanimous in the literature that the progressive and diffuse distribution of degeneration of nerve cells at any stage of the disease advances to cortical and subcortical areas and is not restricted to the motor system alone [7]. However, little is yet known about the exact mechanism of non-motor symptoms, being implicated in dopaminergic neurodegeneration, with noradrenergic and cholinergic deficits [1, 7].

In this sense, it is necessary a better understanding about the involvement of psychomotor and non-motor symptoms, such as anxiety, learning and memory related to the development of PD. Currently *Drosophila melanogaster* has been highlighted as an alternative model in the field of neurodegenerative diseases, because as in humans, it has dopaminergic system implied in locomotion, feeding, sleep/circadian rhythm, learning and memory [3, 9, 10, 11]. Furthermore, the fly has been used as a model human conditions similar to PD, including mitochondrial damage, oxidative stress, protein aggregation, reduction of dopaminergic neurons, locomotive and cognitive deficits [1, 3,12-17].

In this context, researchers have been engaged in the investigation of compounds with neuroprotective therapeutic potential. Recently a study demonstrated multi-target effects of 7-chloro-4-(phenylselanyl) quinoline (4-PSQ), a quinoline derivative containing selenium compound, in a PD model induced by the pesticide rotenone (ROT) in *Drosophila melanogaster*, protecting dopaminergic and cholinergic function, correlated to the prevention of locomotor deficits and selenium levels in flies

[18]. Previous studies have already proven the therapeutic potential of this compound through its antioxidant action [19-23], anti-inflammatory [20, 24], anticholinesterase [25] and anxiolytic action [25, 26] in rodents.

In view of these important effects in a model like of DP, we consider the opportunity to investigate the effects of 4-PSQ on psychomotor, and non-motor behaviors, such as locomotor performance, and no motor behaviors such as learning, memory and anxiety in a mimetic model for PD induced by ROT in *Drosophila melanogaster*.

2. Materials and Methods

2.1. *Drosophila melanogaster* stocks and drug exposure

Wild-type *Drosophila melanogaster* Harwich strains were obtained from the National Species Center (Bowling Green, Ohio, USA). Flies stocks were reared and maintained in glass containers with 10 mL of standard food, keep in an incubator (BOD), with controlled temperature environment of $25 \pm 1^\circ\text{C}$ and 60–70% humidity with on a 12-hour light/12-hour dark cycle. The standard food consists in corn flour (76.59%), wheat germ (8.51%), sugar (7.23%), powdered milk (7.23%), salt (0.43%), an antifungal agent (Nipagin®) and a pinch of dry yeast.

The compound 4-PSQ (Fig. 1) was prepared according the method described by Savegnago et al. [19]. The ROT was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and reagents used were the most efficient analytical grade in our laboratory.

2.2. Experimental Protocol

The experiments (Fig. 2) were carried out with flies of both sexes and with 1 to 4 days of age, divided into four groups of 50 flies each: [1] control group, [2] 4-PSQ (25 μM) group, [3] ROT (500 μM) group, and [4] 4-PSQ (25 μM) + ROT (500 μM) group. In this protocol the flies were exposed to a diet with ROT and/or 4-PSQ for 7 days, according to their respective groups. Therefore, the ROT and 4-PSQ were placed into the flies' feed at final concentration of 500 μM to ROT described by Hosamani and Muralidhara [27], Hosamani et al. [28], and Araujo et al. [29]; and 4-PSQ 25 μM according to Couto et al. [18], both dissolved in ethanol. The diet treatment was

composed of: 2% w/v sucrose, 2% agar w/v, 1% w/v milk powder and 0.08% w/v Nipagin®. The control group received the same vehicle as that of the ROT and 4-PSQ groups, and the final concentration of vehicle (ethanol) in medium it was 0.5%.

2.3. *In vivo* Assays

2.3.1. Mortality rate

For this analyze flies were observed daily and counted the number of dead flies per day until the end of the 7 days of the experimental period. It was used 250 flies in each group, and the total number of flies representing the sum of five independent experiments (50 flies/each group).

2.3.2. Behavioral tests

2.3.2.1. Open field and e Equilibrism test

For evaluation the spontaneous locomotor activity, psychomotor activity and learning it was used two tests performed according Iladi et al. [30] and Iladi et al. [31] with some adaptations. For these behaviors the flies were anesthetized on ice and their wings were removed for an interval of 3 hours, recommended according to the aforementioned authors, which it was performed from 16 to 19 hours. The wings were cut 3 days before the tests, this being a recovery period.

The spontaneous locomotor activity was performed by filming the displacement of the fly for a period of 10 minutes. Circular acrylic plates were used, with 52 mm in diameter and 7 mm in height. Individually each fly was placed on the plate, and left acclimatizing for 30 minutes. Subsequently each plate was filmed during 10 minutes, being carried out evaluations of 15 flies for each group. The videos were analyzed using the software idTracker [32], a multitracking algorithm that extracts a characteristic fingerprint of animal in a video recording, allowing the automatic identification and tracking of animals. In this, the path of the flies that moved freely inside the arena it was tracked for 10 minutes of test, thus providing a database with the trajectories traversed, and this data of each fly separately was calculated and analyzed using Offline numerical analysis software (GNU Octave) and statistical software (Prism, GraphPad). The total distance traveled in 10 minutes, the displacement velocity and the percentage of time in locomotion activity were obtained in this test. To reduce any effects due to nonspecific sudden movements and/or

oscillations, it was considered a minimum movement speed of 2mm/s, so that the fly was not discarded from the test.

For psychomotor evaluation and learning an appliance was constructed using a rectangular acrylic plate (simulating a swimming pool), that presents two platforms submersed, with small parts superiors of 5 mm on the surface of the water. Between the two platforms a transparent fishing line (0.6 mm) of 15 cm it was tied, and 1 cm (red zone) between the platform and the beginning of the zone of counting of the distance covered (counted in 13 cm), also 1 cm after the end of the evaluation mark and between the platform. The learning protocol consisted of three sessions, each separated by a day. Each session consisted of three consecutive trials (no interval). To start the test each, fly individually was placed in the red zone and then started counting the time, using a timer, and the distance traveled, both annotated. In the "equilibrant test" the walking speed of each group was evaluated and a total of 15 flies per group were analyzed in both tests.

2.3.2.2. Aversive Phototaxis Suppression Assay (APS Assay)

The evaluation of learning and short memory skills was performed through the APS assay proposed by Ali et al. [33]. Positive phototactic flies were used in the test, which were trained to associate light with a bitter aversive taste, and subsequently tested: 0 hr post conditioning (PC0) and 6 hr post conditioning (PC6), evaluating learning and memory, respectively. This test, according to Ali et al. [33] was originally adapted by Le Bourg and Buecher [34] describing the positive phototactic behavior of flies after being trained to associate light with aversive stimuli, such as the bitter taste of quinine.

For the test, a T-maze-style apparatus was constructed that consists of two independent chambers, the "lighted" chamber and a "dark" chamber, being connected to one another by an aperture. Each chamber was constructed with two 13mL plastic centrifuge tubes (approximate measures recommended by Ali et al. [33]). The light intensity in the light compartment it was 16 W.

First a single fly was placed in the dark chamber of the apparatus, and left 30 seconds to acclimatize, and subsequently, light was turned on at the extremity of the illuminated chamber. So individually each fly was validated to be fit to the test, being

placed in the apparatus at dark part, and evaluated its phototaxis and mobility from the dark chamber to the light (without quinine) in a maximum time of 10 seconds (being inappropriate when time exceeds 10 seconds, and like this discarded from the evaluation). The failure of phototaxis indicates problems in the visual system and flies with negative phototaxis need to be excluded from the test [33]. When the fly was considered positively phototactic, the training it was then initiated.

The training consisted of: the fly was placed back into the darkroom (lightly tapping the tube down), and then given 30 seconds of acclimatization. Meanwhile on the other extremity it was replaced with another light compartment (tube) containing quinine solution (0,1M) in a filter paper. Then the light was lit at the end of the quinine side, and the fly was allowed to enter the illuminated chamber, leaving the time of 1 minute for the tube to be explored. After 1 minute the fly was played back to the darkroom, a total of 9 trainings were performed for each fly.

According to Ali et al. [33], wild flies start avoiding the lit chamber after 3 to 5 training tests. Similar behavior was also observed in the test, then to get around this characteristic and follow up the training, we chose to use the maximum time of 1 minute so that the fly could move towards the light compartment. If the fly remained in the dark compartment, for this maximum time stipulated, then it was after this time, lightly beaten the tube and started the count of the next training until completing a total of 9 training sessions. On the other hand, if the fly within that time exited from the dark to the light was then reset the timer, and counted 1 minutes for exposure and exploration in the chamber with quinine, following the protocol.

Immediately after the training, five tests were performed, each test consisted in putting the fly in the dark compartment, and given a time of 10 seconds for evaluation, so that the fly leaves from the dark part to the light. The number of times within 5 tests in which the fly remained on the dark side, understood as a task learned through aversive training, it was considered for the approval rate and it was described as PC0 (0 h after conditioning). After the acquisition phase, the flies are able to associate the light area with the aversive taste of quinine and begin to avoid this compartment. On the other hand, flies with compromised learning fail to make such an association, and tend to move into the light side.

Then, for the evaluation of the short-term memory, the second test (in the same way as in PC0) 6 hours after conditioning (PC6) was performed and the number of times the fly avoids entering the lighted bottle is registered approval rate, thus, their ability to remember the task learned was evaluated, being considered an indicator of short-term memory. For this test a total of 15 flies were used in each group.

2.3.2.3. Light/Dark Exploration Test

The Light/Dark test was performed according to Neckameyer and Nieto [35] with some adaptations. The apparatus for evaluating the light/dark test had a light compartment made of transparent material, and a dark compartment, coated with black material, being connected to one another by a hole. Each compartment with 8 cm in length, 4,5 cm width and 2 cm height, and a 16W bulb was used in the light side. In the test each fly was placed in the center of the illuminated box, and evaluated the behavior for 3 minutes, being considered entry from one compartment to another when both front legs moved to the other side. For the analyzes, the amount of time spent in each side was transformed into percentage in the light/dark compartment, and it was also evaluated the number of times it entered the dark box. For the test, five flies from each group were used in three different experiments (15 total flies per group).

2.4. Ex vivo assays

2.4.1. Acetylcholinesterase (AChE) activity

The evaluation of AChE activity was carried out in the head and body regions of the flies. 10 fly heads of each group were homogenized in 100 μ l of 20 mM HEPES buffer (pH 7.0) and 10 bodies in 400 μ L of 20 mM HEPES buffer (pH 7.0) and centrifuged at 1000 rpm for 10 min at 4°C, according to Ellmann et al. [36]. The reaction was prepared with 0.25 M KPi buffer (pH 8.0) and 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB 5 mM), and this was added to the supernatant solution sample (50 μ L) and to acetylthiocholine (AcSCh) (7.25 mM), and the reaction was monitored in a spectrophotometer for 2 min at 412 nm. The AChE activity was expressed as μ mol AcSCh/h/mg protein. The total of 10 flies per group represents the sum of three and four independent experiments were performed for head and body samples, respectively.

2.5. Protein concentration

The protein concentration in the head and body regions was evaluated using the method of Bradford [37] with standard bovine serum albumin.

2.6. Statistical analysis

The results of differences in rate mortality were determined by comparing the mortality curves by log-rank (Mantel-Cox) test. The results of open field test and an analysis of the equilibrist test were executed with two-way ANOVA (ROT and 4-PSQ) by Kruskal–Wallis (kw) followed by Dunn's multiple comparisons test when appropriate. Other analyses were performed by one-way analysis of variance (ANOVA), followed by Newman-Keuls test or two-way ANOVA (ROT and 4-PSQ) followed hoc test by Bonferroni or Tukey's multiple range test when appropriate. For videos analysis was performed with the program idTracker [32] and processed the data with Octave (GNU Octave). GraphPad Prism version 6 (San Diego, CA, USA) was used for all statistical analyses. The results of descriptive statistical data are presented as mean(s) \pm standard error (SE) of the mean, and median. Probability values less than 0.05 ($p < 0.05$) were considered as statistically significant.

3. Results

3.1. Effect of 4-PSQ on the mortality rate of *Drosophila melanogaster*

The Fig. 3 shows the mortality rate of *Drosophila melanogaster* exposed for 7 days to ROT and treated with 4-PSQ. We observed an increase in mortality rates of the ROT-group compared with the control group. While the mortality in the 4-PSQ and 4-PSQ + ROT groups were similar, being smaller when compared to the ROT-alone group. This data proves that the 4-PSQ were able in prevent ROT-induced mortality ($p < 0.05$; Fig. 3).

3.2. Effect of 4-PSQ on spontaneous locomotor activity, psychomotor activity and learning

Fig. 4 illustrate the effect of treatments when analyzing the locomotors deficits. According to Dunn's multiple comparison test we observed that the ROT-exposed flies had a decrease in the total distance traveled (kw = - 19.53, $p = 0.0062$, Fig 4A), in the walking velocity (kw = - 29.63, $p < 0,0001$, Fig. 4B) and in the percentage of activity

($kw = -15.15$, $p = 0.0360$, Fig. 4C). In the present study, the 4-PSQ treatment was able to revert the effect of ROT on such parameters (total distance: $kw = 16.63$, $p = 0.0429$, Fig. 4A; walking velocity: $kw = 16.93$, $p = 0.0360$, Fig. 4B; and percentage of activity: $kw = 17.46$, $p = 0.0137$, Fig. 4C). No significant differences were observed when 4-PSQ alone it was compared to the control group (total distance: $kw = -6.429$, $p = 0.999$, Fig. 4A; walking velocity: $kw = -13.76$, $p = 0.1882$, Fig. 4B; percentage of activity: $kw = -8.140$, $p = 0.8837$, Fig. 4C).

The evaluation of equilibrist test verified during the 9 experiments carried out in 3 days and 3 consecutive times each day is described in Fig. 5 trial number 1 to 9 are represented by T1 to T9, describing the results of the three days together, with 1 to 3 referring to the first day, 4 to 6 to the second day, and 7 to 9 the sections of the third day). The two-way ANOVA, with Tukey's multiple comparisons test revealed a significant interaction between ROT and 4-PSQ in some trial numbers [$F_{(24, 504)} = 1.635$; $p = 0.0301$]. When comparing the results among the four groups of the experimental protocol (Fig. 5A) we found significant differences between the ROT group compared to the control group in some trial numbers (1, 2, 3, 6 and 9). And in trials 2, 3, 5 and 6, statistically significant differences were observed between the ROT and ROT + 4-PSQ groups. Thus, we observed a difference in walking speed in only a few points, and observed the ability of 4-PSQ to reverse the deficit in walking speed induced by ROT in T2, T3 and T6.

However, when analyzed the walking speed within each group, through one-way ANOVA (Tukey's multiple comparisons test), there was no improvement in its performance, except in group 4-PSQ that there was a significant difference in the displacement of trial numbers in the 3 to 4 (T3 to T4, in the Fig 5B – 5E; $p < 0.05$). Two-way ANOVA (Tukey's multiple comparisons test), revealed no significant interaction between trial numbers within each group (control [T1 to T9 interaction $F_{(8, 126)} = 1.505$; $p = 0.1618$]; ROT [T1 to T9 interaction $F_{(8, 129)} = 1.488$; $p = 0.1676$]; and ROT+ 4-PSQ [T1 to T9 interaction $F_{(8, 126)} = 1.257$; $p = 0.2720$]. Except for the 4-PSQ group, there is a difference between T3 and T4 showing an increase in walking from one attempt to another (4-PSQ [T1 to T9 interaction $F_{(8, 126)} = 5.874$; $p < 0.0001$]).

These data point out important deficiencies in the group exposed with ROT alone in relation to walking speed. However, there was a poor relation between learning outcomes within the same group over T1 until the end of T9.

3.3. Effect of 4-PSQ on learning and memory in association aversive to light

Fig. 6A shows the results of the initial time in the quinine aversion test (PC0), which is characterized as the learning assessment test. In the two-way analysis it was possible to verify an interaction between ROT and 4-PSQ [$F_{(1,55)} = 20.16$; $p < 0.0001$] in the average pass rate in the PC0. In this sense, it was observed that the flies at the average pass rate in the ROT group were lower (about 60.1%), pointed that flies were not as able to associate light with bitter taste, when compared to the control group. While the treatment with 4-PSQ was effective in improve the learning rate ($p < 0.05$).

Fig. 6B evidenced the effects the treatment in the short-term memory (test 6 hours post conditioning (PC6)). Two-way analysis showed an interaction between ROT and 4-PSQ [$F_{(1,55)} = 22.91$; $p < 0.0001$] in the average pass rate in PC6. We observed decreased average pass rate (about 57.8%) in flies exposed to ROT, flies were unable to associate light with bitter taste, as well as do not remember the association light with bitter taste, as shown by a decline in PC6 passage rate in this group compared at the control. While the treatment with 4-PSQ was effective in improve the short-term memory ($p < 0.05$).

The Fig. 6C showed the differences between PC0 e PC6 within each group, being verified through “t test” that there was no statistically significant difference between PC0 and PC6 time individually, within each group ($p > 0.05$ for the control groups, 4-PSQ, ROT and ROT + 4-PSQ).

Together the results show that within the groups the flies learned the task for which they were trained, and that after 6 hours they maintained the memory of this aversive behavior to quinine. However, with the comparison between the 4 groups of the experimental protocol it can be seen in the ROT group a lower rate of learning (correct association between light with bitter taste) and maintenance of the same patterns learned in the PC6 evaluation, that is not remembering the association light with bitter taste (quinine).

3.4. Effect of 4-PSQ on Anxiety-like behavior

The Fig. 7A and 7B showed the percentage of time in the light or dark compartment in which the fly remained for time of 3 minutes. Two-way ANOVA (for Bonferroni multiples comparing Test) pointed a significant interaction between ROT and 4-PSQ (Fig. 7A [$F_{(1,8)} = 32.35; p = 0.0005$] and Fig. 7B [$F_{(1,8)} = 32.35; p = 0.0005$]). The post-hoc analysis pointed that flies exposed to ROT remained more time in dark compartment, with an increase of approximately 363.2% of the time in this compartment compared to the control, and a decreased percentage of time in lit compartment (about 34.6%) too compared with control ($p < 0,05$). Whereas treatment with 4-PSQ were able reverse this behavior, increasing time of permanence of flies in the lit compartment and decreasing in the dark compartment, corroborating with phototoxic behavior of flies ($p < 0.05$).

The Fig. 7C presented the time in seconds that flies remained in the dark compartment, being observed that on average the flies of the ROT group remained about 72.2 seconds in the dark chamber, an increase of 78.4%, while the controls remained about 15.6 seconds in the dark compartment ($p < 0.05$). Two-way ANOVA pointed a significant interaction between ROT and 4-PSQ (Fig. 7C [$F_{(1,4)} = 114.1; p = 0.0004$]).

Still, to complement the data, Fig. 7D shows the average number of entries in the dark compartment between the groups. It was observed interaction between ROT x 4-PSQ [$F_{(1,4)} = 18.06; p = 0.0132$] with respect to the number of entrances in the dark. We can observe a greater number of entries in the dark chamber in the ROT group (900%) when compared to the control group ($p < 0.005$). While the treatment with 4-PSQ prevented the increase in the number of entries in the dark compartment ($p < 0.005$).

3.5. Effect of 4-PSQ on AChE activity

Two-way ANOVA (Bonferroni test) revealed a significant interaction between ROT and 4-PSQ in the AChE activity in head [$F_{(1,9)} = 12.05, p = 0.0070$] and body [$F_{(1,10)} = 11.11, p = 0.0076$]. Post-hoc analysis pointed that the flies exposed to ROT presented a significant increase in AChE activity in the head (about 122.4%) and body

(about 138.8%). Concomitant treatment with 4-PSQ prevented increasing of AChE activity in the head and body of flies (Fig. 8 A and B, $p < 0.05$).

4. Discussion

The compound 4-PSQ has been outstanding in studies with rodents [19-26] and currently in the model of *Drosophila melanogaster* [18], showing important antioxidant [18-22, 24-25] and anticholinesterase actions [25]. In our previous study [18], in a mimetic model for PD in *Drosophila melanogaster*, were identified important actions of 4-PSQ in the prevention of dopaminergic and cholinergic deficits, in the improvement of antioxidant mechanisms, in the prevention of mortality, and in the optimization of behavioral activities in animals studied. In order to give continuity to this theme, the present data complement the evaluation of 4-PSQ as a potential therapeutic treatment for PD, investigating the possible effects of 4-PSQ under psychomotor and in the non-motor behavior in model like PD induced by ROT in *Drosophila melanogaster*. In this sense we performed the evaluation of behavioral tests and the evaluation of AChE activity, an important enzyme used to estimate deficits related to locomotor performance in neurodegenerative diseases. We verified that 4-PSQ treatment was able to prevent locomotor, psycomotor and non-motor deficits in behavioral tests. Also, for the first time in fly, an anxiolytic potential of 4-PSQ was verified in a light/dark assay protocol. And, the anticholinesterase activity of the compound was observed, preventing the increase on AChE activity evidenced by the use of ROT.

In this study an increased mortality rate was observed with ROT exposure, and a preventive effect of 4-PSQ was demonstrated against this parameter. Similarly, our previous study verified the effectiveness of 4-PSQ in protect these changes to mortality in flies, due to its antioxidant effect, justified by the presence of selenium in your structure [18]. In the literature it is well established the damages caused by the ROT in mortality and in the behavioral parameters on *Drosophila melanogaster* models aiming the study PD [2, 27-29, 38, 39]. And important damage to mitochondria is attributed to ROT, reflecting in a bioenergetics crisis, which is related to detrimental results in the survival and behavior of flies [40]. In this sense, our study sought to take a step forward in the evaluation of 4-PSQ, not only of mortality and psychomotor factors, characterizing the model, also aiming to evaluate possible non-motor effects.

The dopaminergic system of the insect and vertebrates shares a similarity in about the involvement with learning and memory or locomotion control [3]. Experimental studies with *Drosophila melanogaster* have used ROT pesticide, to reproduce mimetic phenotypes to PD in flies, especially about locomotive injuries [18, 27-29]. Among the main damages observed is related to locomotor behavior, however, due to this close relationship with cognitive deficits, it is necessary to investigate through behavioral tests able to make a more complete collection of mimetic symptoms to PD. In this regard, we verified damages in the spontaneous locomotor activity evaluated through the open field test in the flies exposed to ROT, with decrease in the distance traveled, walking velocity e percent of activity over time in a performance for a period of 10 minutes in flies that had their wings removed. Emphasizing that unlike our previous work, in this case we evaluated the motor performance associated with the activity of only the muscles of the flies legs (since in this work the wings of the flies were removed). Importantly, all of movement disturbances caused by ROT were ameliorated by 4-PSQ. Our first study that evaluated the effects of the 4-PSQ compound on *Drosophila melanogaster* evidenced the protective effects against the ROT- induced toxicity in flies in the behavioral parameters, by negative geotaxis and open field test for time of 1 minutes [18]. This study pointed to a strong correlation between the decrease in dopamine levels and locomotor deficits in the flies and show an association in the increase in dopamine levels with the increase in selenium levels, as a possible involvement of selenium in the mechanism to improve locomotor damages with the treatment with 4-PSQ in flies [18]. Importantly, studies with rodents and fly have pointed out that this compound alone does not cause detrimental effects on behavior [18, 20, 25, 26].

Still, neurodegenerative diseases present within their main characteristics the psychomotor impairment and are often evidenced in relatively early ways in the development of such diseases [6, 31]. These psychomotor behaviors are governed by a tenuous relationship between motor activities and cognitive functions and being involved several general symptom categories such as sensory perception, cognition, emotion, and movement [31, 41, 42]. In this context, psychomotor dysfunctions may reflect in different domains, such gross and fine motor activity and body movements and speech and motor response time [42]. The open field is a test previously been validated in *Drosophila melanogaster*, however its capacity is largely based on the

evaluation of general defects in locomotion, not being sufficient to understand the damages fine-tune movements and/or motor coordination [43]. Thus, aiming at the evaluation of such psychomotor characteristics, like defects in fine motor movements including balance, gait, coordination, and movement slowing. In this sense Iladi et al. [31] purposed the equilibrist test that can be used to measure fine-tune motor movements, motor coordination, and psychomotor learning in wild type *Drosophila melanogaster*.

In PD the motor learning encompasses the formation and retention of motor skills and, in particular, the achievement and the use of automaticity in daily routines, which are both impaired in this pathology [44, 45]. The motor learning features different neural networks and neurotransmitters systems depending on the type of task and on the stage of learning, with involvement of the dopaminergic and cholinergic pathways [44, 45]. Our findings have shown diminished performance in walking speed on the balance test in ROT group, whereas the treatment with 4-PSQ increased the performance of the flies in some parameters of the test. However, we did not observe differences regarding learning in the evaluation of each group individually demonstrated by similar results in walking speed in the nine learning attempts, characterizing an inexpressive relation of learning in our findings by this test. Corroborating with the reported by Iladi et al. [31] in their test, we also verified the flies with damage spent a lot of time balancing at a specific point on the line, climbing upside down, and eventually falling down into the water, while some flies attempted to move along the line, after a few attempts. However, we observed that at first all the flies by being without wings tried to escape by jumping, and because of this we believe that these results may be related to exploratory characteristic and an attempt to escape of a restricted space (freedom reflex). Still, another factor that may have been limiting in this test and influenced it was the exhausting manipulation of the flies, since each day they were placed in the apparatus 3 times, and there were times when they jumped in the water, which could be considered a stressful factor of this test in the fly.

The progressive loss of dopaminergic inputs to the striatum leads to impaired motor control in the PD is considered a central symptom of the pathology [1, 4, 45]. However, evidence that learning and reward processing are also compromised in PD confirm with the proposed role of dopaminergic signals in learning [45, 46]. In PD,

learning that depends on attentional resources and cognitive strategies is usually slower, being considered a deficit in the retention of new abilities present in the early stages of PD, and that has little effect with drug treatment for dopaminergic pathways [44]. Therefore, to better elucidate the learning related issues we decided to carry out the APS assay, described by Ali et al. [31], which aims to investigate learning and short-term memory in answer aversive to light. According to Seugnet et al. [16], the APS is a sensitive test to measure deficits in fly models of human conditions associated with cognitive deficits, which we can think about to be useful for cognitive evaluation in PD. Unlike the results of the previous test (concerning learning by the equilibrist test), it was possible to observe that the flies from each group learned the task satisfactorily, except for the ROT group, in which presented a lower aversive light/quinine approval rate. While treatment with 4-PSQ concomitantly with ROT exposure enabled an improvement in the learning rate. Similarly, in the short-term memory evaluation, we observed that flies from the other groups (with the exception of ROT) maintained their percentage of approval, showing an efficient memory of aversion light/quinine as a negative reinforce in the APS. However, the ROT group presented a lower approval rate in the first and second tests, evidencing possible learning and memory deficits in this group. In addition, our results are in line with data from a recent study in which 4-PSQ has been shown to promote improvements in learning and memory in rodents subjected to memory impairment caused by the amyloid β peptide ($A\beta$) model in Alzheimer's disease [25].

The anxiety disorders in the PD are recognized as a non-motor and psychiatric comorbidities that occur in idiopathic patients, which lead to impairments in quality of life [47, 48]. The clinically significant anxiety symptoms reach in between 20% and 50% of PD patients, and evidence pointed a frequent coexistence of anxiety and depression in patients with PD. Still, the difficulty of diagnosis and treatment of these disorders in PD contribute to increased damages in areas of cognitive, functional, motor and social performance [48, 49]. In PD the anxiety is related to a combination of medical, neurochemical and psychosocial phenomena and has been considered as an early non-motor phenotype of PD [48]. And evidence suggests involvement of degeneration of subcortical nucleus and ascending dopamine, norepinephrine and serotonin pathways within the basal ganglia-frontal circuits in the symptoms of anxiety in the patients with PD [48, 50]. Thus, study of compounds with a potential anxiolytic effect

such as 4-PSQ is necessary. In this sense in a study conducted by Pinz et al. [25] reported protective effect this compound on the anxiogenic action and learning and memory impairment in a model Alzheimer's disease induced by amyloid β -peptide ($A\beta$) in rodents and relate the pharmacological effect of the compound with anti-cholinesterase and antioxidant action, and with cholesterol metabolism. Reis et al. [26] verified an ansiolitic action of 4-PSQ, based on the effect on the glutamatergic system in anxiety like behavior in mice. The light/dark test considers the positive phototaxis behavior of the fly, which under normal conditions, during daytime, tends to move towards the light [51]. In this sense, flies with anxiety-like behavior tend to remain on the dark side of the test, reflecting a conflict between exploratory instinct and a new environment and escape from a possible predator [51]. Similarly, our results pointed to a greater permanence and number of entrances of the flies exposed to ROT in the dark compartment and a smaller percentage of time in the light compartment compared to the control. While treatment with 4-PSQ was able to reverse this behavior, demonstrating the potential anxiolytic effect of this compound also in behavioral tests in *Drosophila melanogaster* exposed to mimetic model of DP. In our previous study [18] it was observed that exposure to ROT promotes a reduction in dopamine levels, a factor that may possibly explain the anxiety type behavior in the fly, verified in the present study. Thus, by preventing against this decrease in dopamine levels the 4-PSQ could exert anxiolytic action in this model of PD in flies. The literature indicates that the relationship between anxiety and dopaminergic transmission is complex and needs to be better clarified, however it suggests that in PD the anxiety may be associated with abnormalities in dopaminergic transmission [48, 50] and with reduced levels of dopamine uptake in the striatum [52].

The most effective pharmacological therapy in PD is through of reference treatment (use of levodopa), however the continued use of this dopaminergic therapy is associated with a decline in therapeutic efficacy and long-term side effects, such as motor fluctuations, dyskinesia and neuro-psychiatric disorders [53, 54]. While treatment with dopaminergic agonists, highly effective in treating the early stages of the disease, however does not significantly prevent the progression of the disease [6, 55]. In this sense it becomes important to study new therapies aimed at non-dopaminergic systems within the nuclei of the base that go beyond the damaged nigrostriatal pathways [53, 54], such as agents that can act in cholinergic system, in the

nicotinic and muscarinic cholinergic receptors [56]. Studies indicate that anticholinesterase drugs improve the cognitive function of patients with dementia in Parkinson's disease, and it has been considered an important therapy for the management of non-motor symptoms of PD [57]. In the present study, we verified an increase in the activity of AChE in flies exposed at ROT, in which the increase in the body corroborates with our findings of neuromotor evaluation, with the worsening in the spontaneous locomotor activity, fine-tuning motor movements and motor coordination. While the effects of increased AChE activity on the head of flies could have a consequence on the effects on psychomotor learning, short-term memory in aversive to light tests, and on the anxiety test carried out. In this direction, we again verified the anticholinesteric effect of 4-PSQ compound at central and peripheral level, preventing the increase in AChE activity, results endorsed by reversing behavioral deficit, induced by toxicity to ROT in this model with flies. In our previous study we verified a protective effect of 4-PSQ on the increase of AChE and dopaminergic deficits, and the correlation between an increase in dopamine levels and a decrease in the activity of this enzyme, evidencing that 4-PSQ act in the dopaminergic system through different mechanisms reducing oxidative damages, improving antioxidant defenses, and exercising an anticholinesterase action, consequently protecting dopaminergic neurons, preventing dopamine depletion, and reversing the behavioral motor deficits in *Drosophila melanogaster* [18]. In this sense, we think that possibly similar mechanisms may explain our results regarding psychomotor and non-motor behavior, especially in what refers to the involvement of the anti-cholinesterase action of compound 4-PSQ in the prevention of such behaviors in this mimetic model to PD in flies.

Recently studies have pointed to the multi-targeting action of selenium compounds [18, 26, 58], as verified by Couto et. al., [18] that evidenced the ability of 4-PSQ to act in the cholinergic and dopaminergic system, being effective in reducing mortality and modulating oxidative stress, factors that may be related to the results about the psychomotor and non-motor deficit evidenced in the present study. Therefore, the 4-PSQ highlighting in the search for new multi-target compounds that can act simultaneously in several places and different routes, by potentializing its effect in the alterations in this model of PD.

5. Conclusion

Promising findings from the present study support a broader understanding of PD-related changes. In an unprecedented way, the present study was able to show, through the set of experimental paradigms, besides motor alterations the presence of non-motor alterations in this model of PD by ROT exposure in *Drosophila melanogaster*. Thus, our results indicate that 4-PSQ exerted a preventive effect about evaluations regarding behavioral tests on spontaneous locomotor activity, learning, memory and anxiety. Therefore, possibly due to its multi-target action and its efficacy as an AChE inhibitor 4-PSQ was effective in the prevention of motor and non-motor alterations, characteristic that potentiate its therapeutic effect in the prevention of symptoms and the appearance complications related to DP.

Conflict of interest

The authors declare no conflict of interest.

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Figures captions:

Fig. 1. Chemical structure of 7-chloro-4- (phenylselanyl) quinoline (4-PSQ).

Fig. 2. Schedule of the experimental treatment protocol of the rotenone (ROT) exposure and 7-chloro-4- (phenylselanyl) quinoline (4-PSQ) treatment and mortality analysis, behavioral tests and Acetylcholinesterase (AChE) activity.

Fig. 3. Effect of 7-chloro-4- (phenylselanyl) quinoline (4-PSQ) on the mortality rate of flies exposed to rotenone (ROT) every 24 h for each group over 7 days. The total number of flies (250 per group) represents the sum of 5 independent experiments. Mortality rate measurement was determined by comparing the survival curves from Mantel-Cox log-rank tests and multiple comparisons were corrected using the Bonferroni test. *Significant difference from the control group; #significant difference between ROT and ROT + 4-PSQ ($p < 0.05$).

Fig. 4. Effect of 7-chloro-4- (phenylselanyl) quinoline (4-PSQ) on spontaneous locomotor activity of flies exposed to rotenone (ROT) over 7 days through the open field 10 min video-tracking. (A) Total distance traveled; (B) Walking velocity; (C) Percent of activity over time. Fifteen flies per group were included per group for all tests. Values are expressed as median and range (interquartile interval). *Significant difference in relation to the control group; #significant difference between ROT and ROT + 4-PSQ (Kruskal–Wallis test followed Dunn multiple comparisons test, $p < 0.05$).

Fig. 5. Effect of 7-chloro-4- (phenylselanyl) quinoline (4-PSQ) on psychomotor activity and learning of flies exposed to rotenone (ROT) over 7 days through the equilibrist test. Fig. 5A: mean of the walking speed for groups of treatments according to trial numbers (T1 to T9). Tukey's multiple comparisons test (two-way ANOVA) show differences between groups in the mean of walking speed for every trial. Vertical bars denote 0.95 confidence intervals. Current effect represents the interaction effect between four groups and number of trials, and also, shown are the comparisons between first and last trial. *Significant difference in relation to the control group; #significant difference between ROT and ROT + 4-PSQ ($p < 0,05$). Fig. 5B through 5E (control, 4-PSQ, ROT and ROT+ 4-PSQ, respectively): mean of the walking speed separately for each of the groups to trial numbers (T1 to T9), through one-way ANOVA Tukey's multiple comparisons test. *Significant difference ($p < 0,05$). Fig. 5F assesses

the difference between the four treatment groups through the median analysis, for Kruskal–Wallis test followed Dunn multiple comparisons test. Fifteen flies per group were included per group for all tests. Values are expressed as median and range (interquartile interval). *Significant difference in relation to the control group; #significant difference between ROT and ROT + 4-PSQ with $p < 0.05$).

Fig. 6. Effect of 7-chloro-4- (phenylselanyl) quinoline (4-PSQ) on aversive phototaxis suppression assay, for evaluation of learning and short-term memory of flies exposed to rotenone (ROT) over 7 days. The Fig. 6A showed the results of 0 hr post conditioning (PC0) (learning) and Fig. 6B of 6 hr post conditioning (PC6) (memory), by one-way ANOVA followed by Newman-Keuls test or two-way ANOVA (ROT and 4-PSQ) followed hoc test by Bonferroni. Fig. 6C showed the comparison within each group between PC0 and PC6 (control, 4-PSQ, ROT and ROT+4-PSQ) by “T test”, and still pointed the difference between the four treatments, through Tukey’s multiple comparison test ($p < 0,05$). Values are mean \pm SE and were included fifteen flies per group for all tests. *Significant difference in relation to the control group; #significant difference between ROT and ROT + 4-PSQ with $p < 0.05$).

Fig. 7. Effect of 7-chloro-4- (phenylselanyl) quinoline (4-PSQ) on anxiety-like behavior of flies exposed to rotenone (ROT) over 7 days through the Light/Dark Exploration Test. (A) Percentage of time in the lit compartment; (B) percentage of time in the dark compartment; (C) Percent of activity over time; (D) The average number of entries in the dark compartment between the groups. Values are mean \pm SE ($n = 6$ flies per replicate, three replicates). Significance determined by two-way analysis of variance (ANOVA with Bonferroni test) followed by Newman-Keuls test (one-way ANOVA). *Significant difference from the control group; #significant difference between ROT and ROT + 4-PSQ ($p < 0.05$).

Fig. 8. Effect of 7-chloro-4- (phenylselanyl) quinoline (4-PSQ) on rotenone (ROT)-induced alterations in AChE activity ($\mu\text{mol}/\text{min}/\text{mg}$ protein) in the head (A) and body (B) regions of adult *Drosophila melanogaster*. Values are mean \pm SE ($n = 10$ flies per replicate, three and four replicates used, respectively). Significance determined by two-way analysis of variance (ANOVA with Bonferroni test) followed by Newman-Keuls test (one-way ANOVA). *Significant difference from the control group; #significant difference between ROT and ROT + 4-PSQ ($p < 0.05$).

Figures

Fig. 1

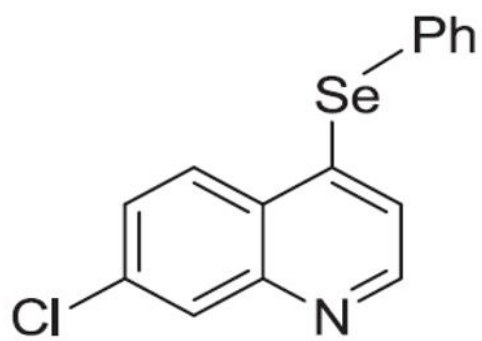


Fig. 2

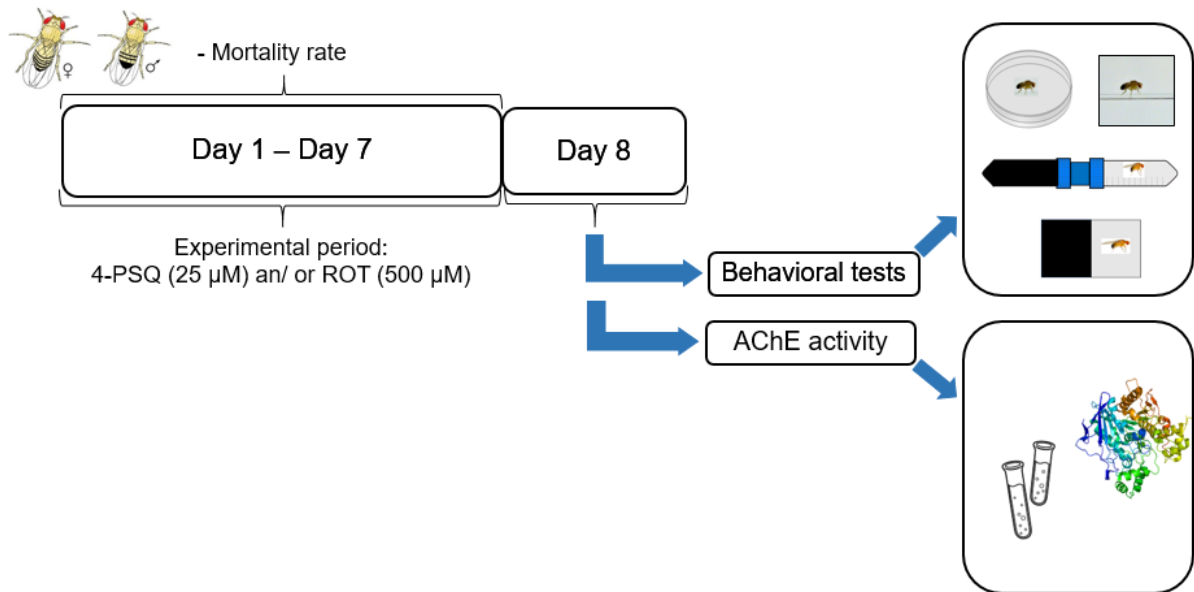


Fig. 3

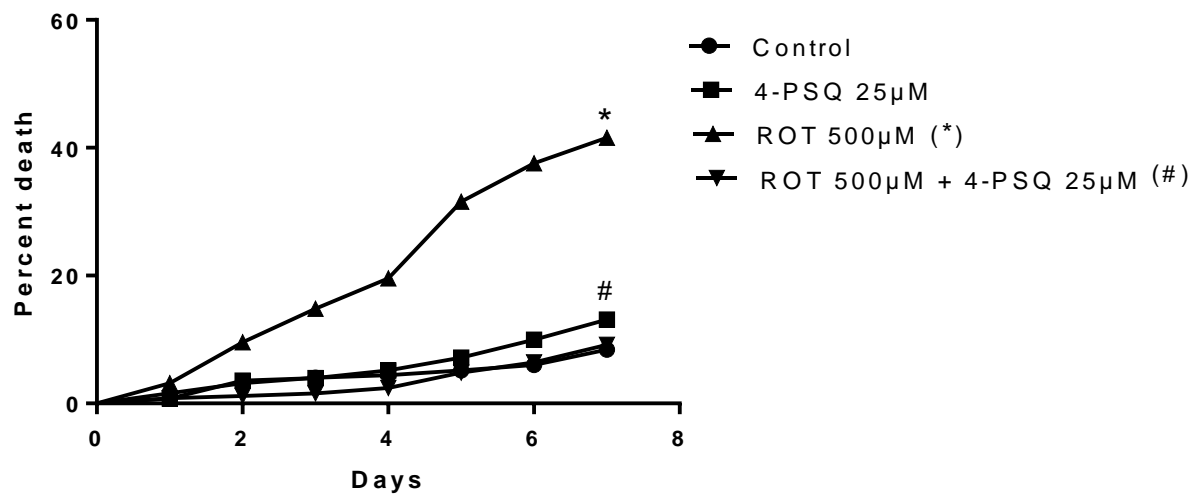
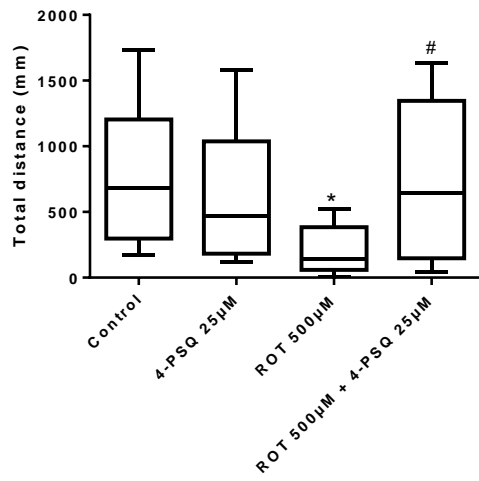
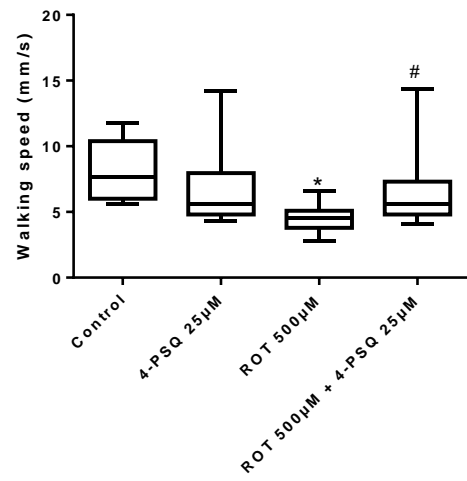


Fig. 4

A)



B)



C)

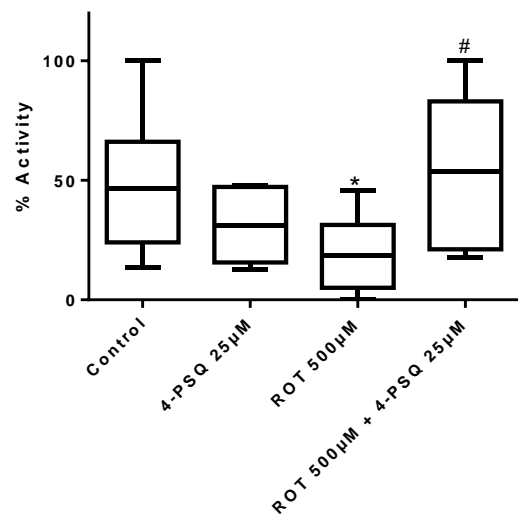
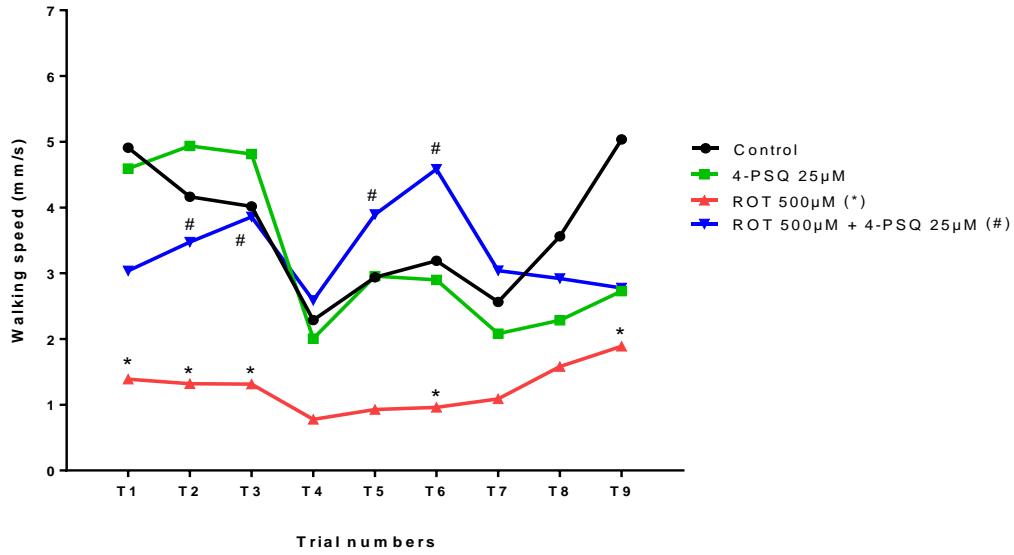
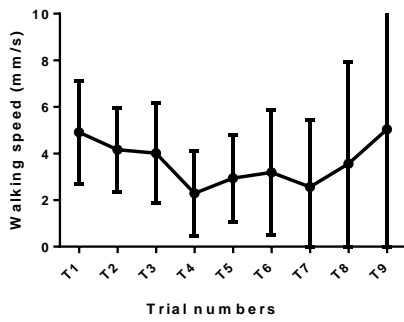


Fig. 5

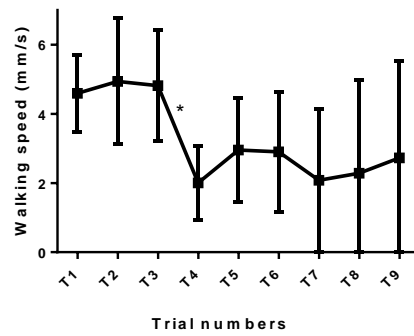
A)



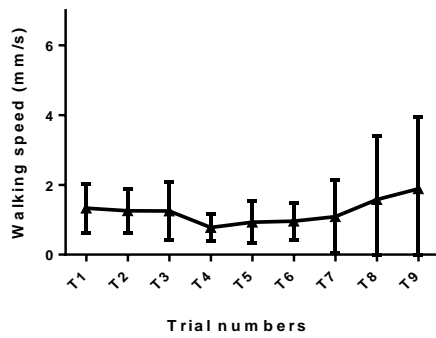
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E)

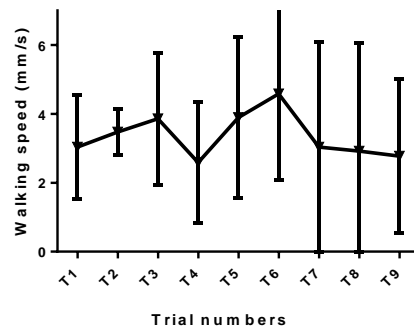
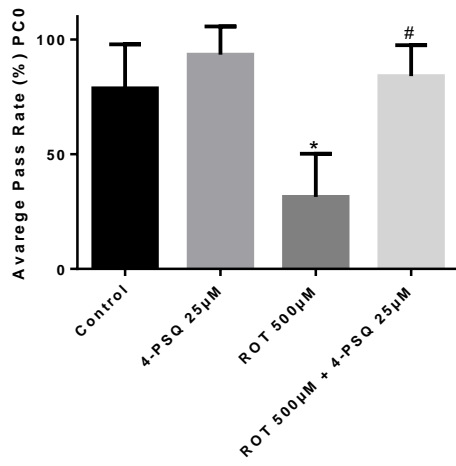
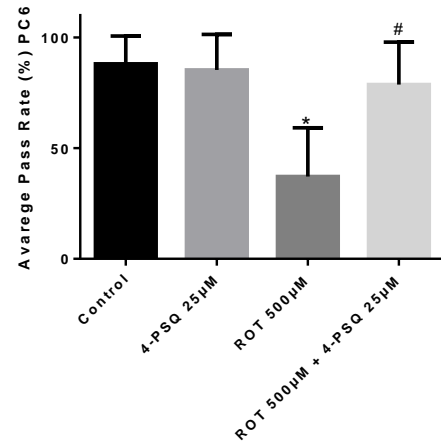


Fig. 6

A)



B)



C)

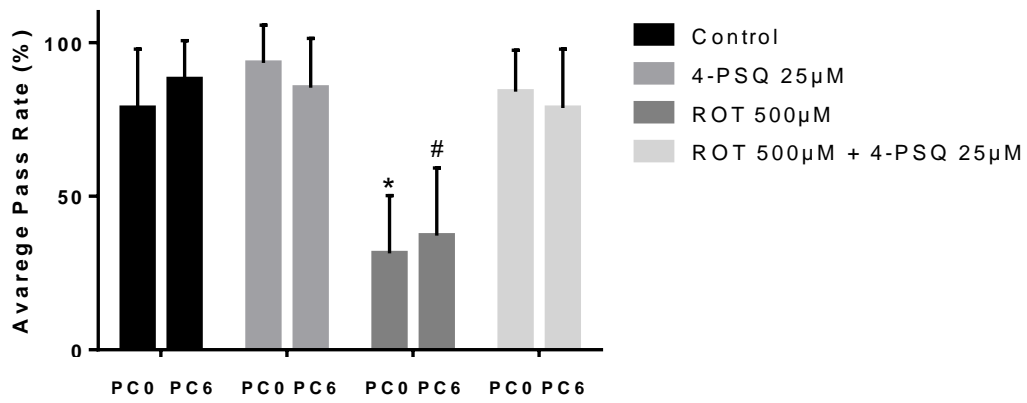
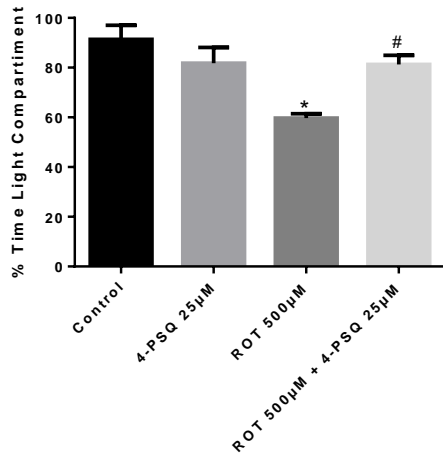
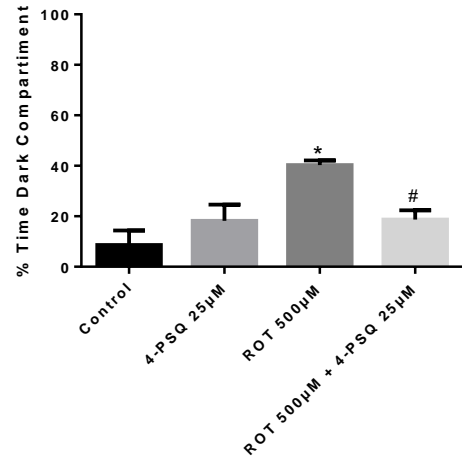


Fig. 7

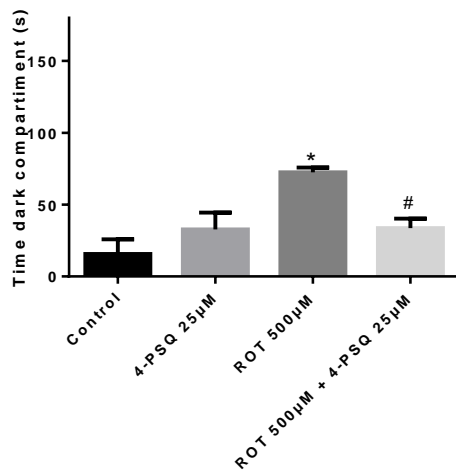
A)



B)



C)



D)

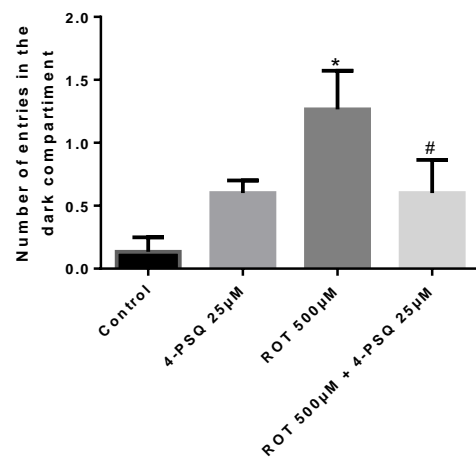
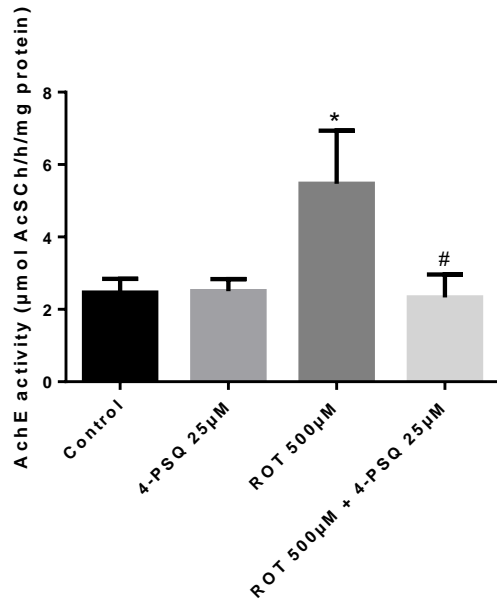
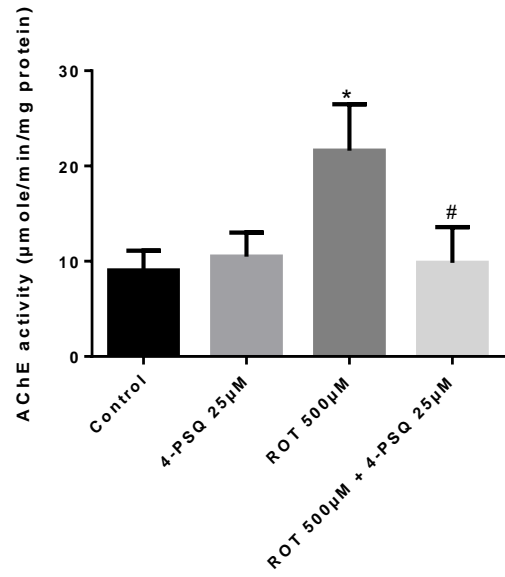


Fig. 8

A)



B)



PARTE III

6 DISCUSSÃO

O presente estudo evidenciou o efeito neuroprotetor do 4-PSQ frente as alterações moleculares e comportamentais induzidas pela neurotoxina ROT em um modelo mimético à DP com *Drosophila melanogaster*. Nossos resultados são pioneiros em demonstrar a capacidade do 4-PSQ na prevenção dos danos causados nesse modelo, e em evidenciar sua relação com os níveis de selênio na cabeça das moscas. Em nosso estudo, constatamos que o tratamento com 4-PSQ foi capaz de aumentar os níveis de selênio; prevenir as alterações motoras, psicomotoras e não motoras; melhorar a sobrevivência; combater o estresse oxidativo e fortalecer as defesas antioxidantes; e modular a atividade da AChE. Ainda, em conjunto nossos achados permitiram inferir que tais efeitos, principalmente os antioxidantes e anticolinesterásico, estariam relacionados aos níveis de dopamina restaurados na cabeça de moscas e, conseqüentemente, na prevenção de deficiências locomotoras, psicomotoras e não motoras, e na melhora da sobrevivência de moscas expostas à ROT.

Em um primeiro momento, podemos observar através dos resultados nos testes comportamentais (geotaxia e *open field*) importantes déficits locomotores no que se refere a atividade de escalada e capacidade exploratória das moscas, ao serem expostas a esse pesticida, determinando o total desenvolvimento desse modelo de DP nas moscas expostas à ROT. Evidências indicam que a exposição à ROT associada à DP é caracterizada pela sua neurotoxicidade e expressa pelas altas taxas de déficits locomotores observados nos paradigmas comportamentais (COULOM e BIRMAN, 2004; TANNER et al., 2011; ARNHOLD et al., 2016; SALA et al., 2016). Assim, as moscas com danos locomotores têm tendência a uma menor atividade de escalada e exploratória, refletida em dificuldade na coordenação das patas, principal expressão fenotípica da DP (KHATRI e JUVEKAR, 2016), como consequência da exigência de alta energia dos músculos do ambulatório e de vôo, que são ricos em mitocôndrias (HOSAMANI e MURALIDHARA, 2009). A ROT pode levar a sinais e sintomas semelhantes de DP, devido a sua relação com a disfunção mitocondrial, pela inibição do complexo I, que diminuiu os níveis de antioxidantes na cadeia de transporte

de elétrons e aumenta os níveis de ferro, acrescido a excessiva vulnerabilidade dos neurônios dopaminérgicos ao estresse oxidativo, e pela auto-oxidação da dopamina (CICCHETTI et al., 2009; HOSAMANI e MURALIDHARA, 2009; HOSAMANI e MURALIDHARA, 2013; KHATRI e JUVEKAR, 2016). Tais fatores podem comprometer também a sobrevivência das moscas, identificado pelo aumento na mortalidade com a exposição ao pesticida. Por outro lado, constatamos que o tratamento com o 4-PSQ apresentou-se eficaz na proteção frente aos danos comportamentais e a mortalidade em *Drosophila melanogaster*.

Assim, a hipótese oxidante tem sido largamente associada a DP, e por esse motivo, direcionado a investigação de possíveis agentes neuroprotetores, em modelos com neurotoxina, através de compostos que possam ser capazes de contribuir na proteção dos neurônios dopaminérgicos (COULOM e BIRMAN, 2004; MANJUNATH e MURALIDHARA, 2013). Nessa perspectiva, verificamos que o 4-PSQ foi capaz de reduzir o estresse oxidativo e a peroxidação lipídica provocados pela exposição a ROT. A partir disso, consideramos que as ações do 4-PSQ possam estar atribuídas à sua capacidade de combater o estresse oxidativo, corroborando com estudos que apontam seu potencial antioxidante *in vitro* e em modelos com roedores (SAVEGNAGO et al., 2013; PINZ et al., 2016; REIS et al., 2017; SILVA et al., 2017; PINZ et al., 2018; VOGT et al., 2018). Por outro lado, também observamos um aumento nas defesas antioxidante pelo tratamento com o 4-PSQ, protegendo contra o a diminuição na atividade de enzimas antioxidantes, tais como a CAT e SOD, nas regiões de cabeça das moscas expostas a ROT. Assim nossos dados vão de encontro com a literatura, a qual propõe que neurodegeneração devido a exposição a ROT ocorre por mecanismos relacionados a geração de ERs e atividade prejudicada de enzimas antioxidantes, tendo como consequência o dano neuronal que predispõe aos sintomas da DP (HALLIWELL e GUTTERIDGE, 2007; MANJUNATH e MURALIDHARA 2013; ADEDARA et al., 2015; JIANG et al., 2017).

Dessa forma os dados do presente estudo evidenciam que o composto 4PSQ, além de melhorar as atividades de enzimas antioxidantes foi capaz de reduzir marcadores de estresse oxidativo, em decorrência da ação da neurotoxina. Nossos dados corroboram com outros estudos que demonstraram resultados importantes em relação ao seu potencial farmacológico e antioxidante do 4-PSQ (PINZ et al., 2016, SILVA et al., 2017; PINZ et al., 2018; VOGT et al., 2018). Assim, este efeito

neuroprotetor possivelmente atrelado a característica antioxidante desse composto derivado da quinolina com substituinte organosselênio, sendo destacado por Vogt et al. (2018) que o efeito do 4-PSQ está relacionado a presença do grupo fenilselenil na estrutura da quinolina. Vários estudos apontam para os benefícios de compostos de selênio em doses e/ ou concentrações adequadas, dentro da janela terapêutica existente na farmacologia (NOGUEIRA et al., 2004; NOGUEIRA e ROCHA, 2010; NOGUEIRA e ROCHA, 2011; NOGUEIRA e ROCHA, 2012;). E diante da premissa dos possíveis efeitos contraditórios do selênio, no que diz respeito aos efeitos pró-oxidantes e antioxidantes, procuramos ser cautelosos ao usar uma concentração do 4-PSQ que não causasse toxicidade, a partir de dados de um estudo piloto aplicando testes de sobrevivência e comportamentais, e considerando as peculiaridades do modelo com *Drosophila melanogaster*. Para a determinação da concentração a ser utilizada no presente estudo, as moscas foram expostas a 4-PSQ em quatro concentrações no estudo piloto: 12,5 µM, 25 µM, 50 µM e 100 µM, para determinar o efeito de 4-PSQ sozinho na sobrevivência e comportamento das moscas durante o período experimental de 7 dias. A concentração de 25 µM foi considerada satisfatória, posteriormente a avaliação da mortalidade e de testes comportamentais, pelo fato de se mostrar eficaz sem causar toxicidade às moscas. Tais resultados corroboram com os realizados através de testes comportamentais em modelos de roedores, em que o 4-PSQ não exerceu comprometimento nos comportamentos exploratórios e locomotores, e ainda não apresentou toxicidade gástrica, hepática e renal (PINZ et al., 2016; REIS et al., 2017; PINZ et al., 2018).

Ademais, outra importante ação evidenciada no presente trabalho, deve-se ao potencial efeito neuroprotetor do 4-PSQ sobre os neurônios dopaminérgicos. No nosso artigo 1 podemos observar uma reversão nos níveis de dopamina pelo tratamento com o 4-PSQ, protegendo os níveis de dopamina contra a ação deletéria da ROT. A ROT promove inibição do complexo I mitocondrial, a diminuição das defesas antioxidantes na cadeia de transporte de elétrons e aumenta os níveis de ferro, prejudicando os neurônios dopaminérgicos, e assim refletindo negativamente nos níveis de dopamina (CICCHETTI et al., 2009). Similarmente outros estudos com *Drosophila melanogaster* evidenciaram importante prejuízos na quantidade de dopamina, associada a presença de alterações comportamentais e bioquímicas semelhantes às observadas na DP com a exposição a neurotoxina ROT (HOSAMANI

e MURALIDHARA, 2009; HOSAMANI et al., 2010; ARAUJO et al., 2015). Consideramos que esse efeito sobre o sistema dopaminérgico esteja fortemente relacionado a ação antioxidante desse composto, na proteção da integridade e funções dos neurônios dopaminérgicos. Ainda, podemos verificar uma forte correlação positiva entre os níveis de dopamina e os níveis de selênio, relacionando a neuroproteção, conferida ao 4-PSQ, a um possível papel do selênio presente na sua estrutura.

Nesse panorama, um achado inédito do presente trabalho se refere a diminuição nos níveis de selênio com a exposição ROT, devido provavelmente a um mecanismo compensatório, que para combater os insultos oxidativos causados pela ROT, há o aumento da demanda de selênio no cérebro para produção de selenoproteína (PILLAI et al., 2014; COMINETTI et al., 2017). De forma importante verificamos que tratamento com 4-PSQ restabeleceu os níveis de selênio na cabeça e no corpo das moscas. Corroborando com tais achados Khera et al. (2017) com a utilização de ROT em modelos celulares, Ellwanger et al. (2015) com paraquat e Khan (2010), com MPTP, ambos em roedores, verificaram efeitos protetores do selênio frente aos danos provocados por tais neurotoxinas. Além disso, outro resultado inovador diz respeito ao efeito neuroprotetor na restauração nos níveis de dopamina, e a sua forte associação positiva conforme o aumento nos níveis de selênio na cabeça das moscas. Para tanto, possivelmente a proteção exercida pelo 4-PSQ possa ser apoiada por mecanismos parecidos a outros compostos orgânicos de selênio, tais como disseleneto de difenila $[(\text{PhSe})_2]$, cujos efeitos farmacológicos estão relacionados principalmente a proteção dopaminérgica (BURGER et al. 2006; SAVEGNAGO et al., 2007; FIGUEIRA et al.; 2015; SAMPAIO et al., 2017).

Recentemente tem sido apontado que um desequilíbrio da transmissão de acetilcolina e dopamina no sistema nigroestriatal estaria relacionada as desordens extrapiramidais, como na DP (DAGAEV et al., 2004; JENSEN et al, 2011; HASEGAWA et al., 2017). Esses dois neurotransmissores, na região do estriado, interagem fortemente e desempenham um papel fundamental na regulação da função motora e de locomoção, e por esse fato tem sido descrito o envolvimento da neurodegeneração colinérgica na fisiopatologia de patologias como a DP (CRAIG et al., 2011; JENSEN et al., 2011; KIM e LEE, 2013; ADEDARA et al., 2015). Nessa direção, a via da apoptose surge entre os fatores que predispõe a morte neuronal na DP, estando o

aumento na expressão da AChE relacionado com a apoptose neuronal e em modelos com neurotoxina (ZHANG, et al., 2013). Assim, poderíamos considerar que uma modulação da AChE reduziria a apoptose, podendo protegendo os neurônios dopaminérgicos (ZHANG, et al., 2013). E ainda, outra hipótese levantada seria que danos no sistema dopaminérgico estariam associados a problemas no circuito ganglionar basal, culminando em superativação do sistema colinérgico, tendo como consequência déficits motores e cognitivos (AOSAKI, et al., 2010). Nesse sentido, o presente estudo demonstrou um possível potencial terapêutico do 4-PSQ, pela modulação da atividade da AChE na cabeça e no corpo das moscas que foram expostas a ROT e tratadas com 4-PSQ, concomitantemente. Ainda verificamos uma forte correlação negativa entre a atividade da AChE (cabeça e corpo) com os níveis de dopamina na cabeça das moscas, ou seja, conforme diminui os níveis de dopamina existe um aumento na atividade da AChE, ou vice-versa. Ainda, podemos demonstrar que uma diminuição nos níveis de dopamina estava relacionada além do aumento da atividade da AChE, ao aumento dos danos comportamentais, aumento nos insultos oxidativos, diminuição das defesas anti-oxidantes, e diminuição nos níveis de selênio. Achados que nos direcionam relacionar a ação antioxidante e anticolinesterásica como importante na proteção dos neurônios dopaminérgicos, e assim na manutenção dos níveis de dopamina pelo 4-PSQ nesse modelo mimético a DP.

A acetilcolina é o neurotransmissor que desempenha um papel importante nas funções cognitivas, tais como a aprendizagem e memória, e também está envolvido nas funções motoras, sendo especialmente utilizado na junção neuromuscular (ARAÚJO et al., 2016). A AChE é essencial para o funcionamento das sinapses colinérgicas no sistema nervoso central e periférico, através da hidrólise da acetilcolina, tornando essa enzima alvo na busca de novas drogas (ARAÚJO et al., 2016). AChE quando bloqueada é incapaz de hidrolisar a acetilcolina, assim, essa tende a permanecer ativa na fenda sináptica por um maior período, potencializando a transmissão colinérgica (ARAÚJO et al., 2016). Dessa forma, a atividade da AChE é um importante parâmetro para avaliação além da função locomotora e cognitiva, e para identificação de déficits associados a doenças neurodegenerativas (AOSAKI et al., 2010). Ainda é apontado que um aumento na atividade da AChE e dos déficits dopaminérgicos relacionam-se à degeneração do sistema neuronal colinérgico, refletida nos danos motores e cognitivos na DP (ZHANG et al., 2013; PICCIOTTO et

al., 2012; CRAIG et al., 2011). Nesse sentido, com a caracterização exitosa do modelo de DP em *Drosophila melanogaster* e dispendo de achados importantes, no que se refere ao potencial antioxidante, anticolinesterásico e na proteção dopaminérgica, que apontaram para uma provável ação multi-alvo desse composto, descrito no artigo 1, vislumbramos a necessidade do entendimento da atuação do 4-PSQ sobre sintomas psicomotores e não motores relacionados a DP. Assim, buscamos através de alguns paradigmas comportamentais (descritos no manuscrito 1) avaliar o efeito do 4-PSQ frente aos prejuízos promovidos pela ROT em comportamentos locomotores relacionadas a habilidades cognitivas tais como a aprendizagem, memória e ansiedade, que são sintomas frequentemente identificados já em fases precoces da doença, de diagnóstico negligenciado e de difícil tratamento.

Primeiramente, a fim de caracterizar o modelo repetimos no manuscrito 1 as avaliações referentes a mortalidade e ensaio de campo aberto. Porém, nesse segundo momento, realizamos tal teste com algumas variações, sendo utilizadas moscas com as asas cortadas (devidamente recuperadas antes da realização do teste), cuja avaliação diz respeito ao desempenho dos músculos das patas das moscas, expostas ao teste por 10 minutos para exercerem a sua inata atividade exploratória espontânea. E de maneira similar aos resultados do artigo 1, podemos comprovar os efeitos do composto 4-PSQ em melhorar a taxa de mortalidade e o desempenho na atividade locomotora espontânea em moscas que foram expostas a ROT e ao 4-PSQ concomitantemente. A proteção exercida pelo 4-PSQ nesses quesitos deve-se provavelmente as importantes ações anteriormente identificadas na prevenção de déficits dopaminérgicos, colinérgicos, e no sistema antioxidante (artigo1).

Ainda, a literatura aponta que nas patologias neurodegenerativas as alterações psicomotoras são marcas importantes, e que apresentam relação estreita com funções cognitivas, refletidas em sintomas não motores (KUMMER et al., 2009; BUYUKDURA et al., 2011; FAURHOLT-JEPSEN et al., 2012; ILADI et al., 2016;). Nesse sentido, realizamos o teste do equilíbrio ("*Equilibrist test*") (ILADI et al. 2016; ILADI et al., 2018), a fim de avaliar os movimentos motores finos, coordenação motora e aprendizado psicomotor nas moscas. Foi possível verificar um menor desempenho na velocidade de caminhada no teste de equilíbrio no grupo exposto somente a ROT, enquanto o tratamento com o 4-PSQ foi capaz de melhorar desempenho das moscas em alguns parâmetros em alguns pontos ao longo do teste. Na avaliação ao longo dos

nove testes, comparando as moscas dentro do mesmo grupo, não observamos melhora no seu desempenho. No entanto, algumas limitações do teste devem ser consideradas, principalmente no que diz respeito a excessiva manipulação das moscas, pelo fato de muitas caírem na água, e pelo próprio comportamento exploratório da mosca em que, na tentativa de fuga de um espaço restrito, tendiam a pular para a água, com exceção do grupo ROT que permaneciam mais tempo paradas ou acabavam caindo na água antes do final do teste. Assim, devido às limitações e dificuldade enfrentadas nesse teste realizamos a busca por outro tipo de aparato que possibilitasse avaliar de maneira mais efetiva a relação entre comprometimento da aprendizagem e DP.

Para tanto, realizamos o Ensaio de supressão de fototaxia aversiva (APS), descrito por Ali et al., (2016), que é um teste preconizado devido sua sensibilidade na avaliação de déficits cognitivos em modelos miméticos a patologias humanas (SEUGNET et al., 2009). Através desse teste avaliamos a aprendizagem e a memória de curto prazo em resposta aversiva à luz, com associação a quinina (substância aversiva). Através de um protocolo de treinamento para associação da luz ao sabor amargo da quinina, foi possível avaliar a aprendizagem, e em um segundo momento a memória, através da repetição do teste 6 horas após o teste inicial. Consideramos esse teste positivo, visto que há uma menor manipulação e assim menores efeitos estressores comparados ao teste anterior. Nossos resultados mostram a eficácia do 4-PSQ em melhorar a aprendizagem e memória a curto prazo das moscas que foram expostas a ROT. Nossos resultados vão de encontro a evidências que apontam que distúrbios em diferentes redes neurais e neurotransmissores, como nas vias dopaminérgica e colinérgica, podem comprometer a aprendizagem e memória (FOERDE et al., 2015; MARINELLI et al., 2017), assim a atuação do 4-PSQ nesses sistemas possibilitaria a prevenção de tais distúrbios de aprendizagem e memória.

E por fim, outra alteração não motora importante avaliada foi a ansiedade, que além de apresentar elevada prevalência em humanos representa uma condição de difícil diagnóstico e tratamento, contribuindo para o declínio cognitivo na DP (HANNA e CRONIN-GOLOMB, 2011; PONTONE et al., 2011; CHEN e MARSH, 2014). Nesse sentido utilizamos o teste claro/escuro para reproduzir um modelo tipo a ansiedade, que é caracterizada quando a mosca contraria seu comportamento normal de fototaxia (direcionando-se à luz), e tendem a permanecer no lado escuro do teste. Assim,

nossos achados apontam a eficácia do 4-PSQ em reverter as alterações provocadas pela ROT, reduzindo o tempo de permanência das moscas tratadas com o 4-PSQ no lado escuro. Assim, propõe-se um efeito ansiolítico do 4-PSQ nesse paradigma comportamental em *Drosophila melanogaster*. Esse efeito ansiolítico do 4-PSQ já havia sido descrito no estudo de Pinz et al., (2018) em um modelo de doença de Alzheimer em roedores, apontando efeito ansiolítico, melhora da aprendizagem e memória, relacionando ainda tais efeitos as ações anticolinesterásica e antioxidante do composto.

Nesse sentido, de forma mais abrangente nossos resultados nos permitem relacionar o aumento na atividade da AChE aos déficits motores, psicomotores e não motores presentes nas moscas expostas na ROT. Em nível periférico, um aumento na atividade da AChE resulta em dificuldade na atividade locomotora espontânea, coordenação motora, ajuste fino dos movimentos motores. Por outro lado, um aumento dessa enzima na cabeça, relaciona-se com alterações na aprendizagem psicomotora, memória e em comportamentos tipo ansiedade. Assim, em conjunto nossos resultados mostram que 4-PSQ foi eficaz na prevenção de déficits motores, psicomotores e não motores avaliados, bem como na prevenção do aumento da atividade da AChE em moscas expostas ao pesticida ROT. Ademais, tais resultados vêm de encontro com evidências que apontam que a neuropatologia subjacente à DP abrange outras áreas do cérebro, para além do sistema dopaminérgico, incluindo múltiplas disfunções nos sistemas colinérgico, serotoninérgico e noradrenérgico; fatores que endossam a grande variedade de sintomas psicomotores e não-motores na DP (HASEGAWA et al., 2017; HELLEY et al., 2017). No entanto, a terapia farmacológica tradicional através da L-DOPA atende apenas ao sistema dopaminérgico, e assim o restabelecimento principalmente dos sintomas motores, não abrangendo os sintomas não motores, além de estar associada com efeitos colaterais importantes, eficácia e tolerabilidade diminuída a longo prazo, e não ser eficaz no combate a progressão da doença (SALAMONE, 2010; LEE e KOH, 2015; PEREZ-PARDO et al., 2017). Assim, o 4-PSQ vem de encontro a essa problemática, visto que podemos sugerir que o efeito inibitório da AChE pelo 4-PSQ, poderia auxiliar na proteção dopaminérgica e assim exercer efeito sobre as melhorias nas alterações comportamentais, sejam elas motoras, psicomotoras ou não-motoras.

Portanto, nossos resultados mostram que o composto 4-PSQ através de sua ação no sistema dopaminérgico, colinérgico, no estresse oxidativo, mortalidade e parâmetros comportamentais (motores, psicomotores e não-motores) pode ser considerado um composto multi-alvo, característica que torna o 4-PSQ um possível candidato no preenchimento dessa lacuna do tratamento terapêutico. No entanto, evidencia-se a necessidade de continuidade nos estudos com esse composto quinolínic com organosselênio, devido seus valorosos efeitos farmacológicos, tais como as ações antioxidante e anticolinesterásica.

7 CONCLUSÕES

Achados promissores do presente estudo auxiliam para um conhecimento mais abrangente de alterações relacionadas a DP. Assim, através das alterações na sobrevivência, paradigmas comportamentais, e alterações bioquímicas induzidas pela exposição a ROT, podemos constatar a importância da utilização de tal modelo para pesquisas que busquem inovações terapêuticas, tendo como alvo as doenças neurodegenerativas. E diante da gama de sintomas motores, psicomotores e não motores apresentados pelos pacientes com DP, torna-se cada vez mais necessário considerarmos a investigação de compostos com ação mais abrangente, com o mínimo possível de efeitos colaterais, e capaz de controlar a progressão da doença.

Nesse panorama o composto 4-PSQ destaca-se como um novo composto quinolínico contendo selênio, com grande potencial terapêutico, por atuar frente a diferentes linhas de proteção, melhorando a sobrevivência, os déficits comportamentais (locomotores, aprendizagem, memória e ansiedade) e as alterações bioquímicas. Ainda, tais achados nos permitiram comprovar importantes ações na prevenção do estresse oxidativo, na inibição da enzima AChE, e na proteção dos neurônios dopaminérgicos. Nessa perspectiva, podemos destacar o 4-PSQ como uma droga multi-alvo, cujas propriedades farmacológicas decorrem principalmente da presença do selênio na sua estrutura.

Nesse sentido, diante de resultados tão promissores, se faz necessária a continuação dos estudos com o 4-PSQ vislumbrando um potencial tratamento para DP, e fim de estabelecer com maiores detalhes os mecanismos de ação envolvidos nas diversas alterações associadas a problemática da DP.

8 PERSPECTIVAS

O presente trabalho evidenciou relevantes achados dos efeitos neuroprotetores do 4-PSQ sobre as alterações moleculares e comportamentais nesse modelo de DP induzido pela ROT. Diante de tais resultados promissores, percebe-se necessária a continuidade na investigação dos efeitos 4-PSQ, a fim de elucidar outras possíveis ações desse composto, assim como obter uma maior compreensão dos mecanismos de ação envolvidos na neuroproteção exercida pelo 4-PSQ. Para tanto, pretende-se como perspectivas futuras responder aos seguintes objetivos:

- Investigar através de teste comportamental a alteração não-motora tipo depressão, e verificar os efeitos do 4-PSQ frente a uma possível associação entre depressão e ansiedade nesse modelo mimético a DP, em *Drosophila melanogaster*;
- Investigar o mecanismo anticolinesterásico do 4-PSQ através da cinética enzimática da AChE;
- Avaliar a viabilidade celular e mitocondrial na cabeça das moscas após a exposição a ROT e ao 4-PSQ;
- Analisar a atividade dos complexos mitocondriais I e III na cabeça das moscas após a exposição a ROT e ao 4-PSQ;
- Avaliar o envolvimento da neuroinflamação nesse modelo mimético a DP em *Drosophila melanogaster* pela exposição a ROT, e verificar os efeitos neuroprotetores do tratamento com 4-PSQ;
- Avaliar a eficácia do 4-PSQ frente a outras alterações não motoras, induzidas pela exposição a ROT, e de repercussão em sistemas, tais como no olfatório e digestivo, que são frequentemente relacionadas a DP.

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