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Avaliação do efeito do pentilenotetrazol e 4-aminopiridina sobre parâmetros de sobrevivência, locomotores e bioquímicos em *Drosophila melanogaster*

DISSERTAÇÃO DE MESTRADO

DEIVIDI CRISTIAN DOS SANTOS SOARES

URUGUAIANA, RS

2016

Avaliação do efeito do pentilenotetrazol e 4-aminopiridina sobre parâmetros de sobrevivência, locomotores e bioquímicos em *Drosophila melanogaster*

Por

DEIVIDI CRISTIAN DOS SANTOS SOARES

Dissertação apresentada ao Programa de Pós-Graduação em Bioquímica da Universidade Federal do Pampa, UNIPAMPA, como requisito parcial para a obtenção do título de Mestre em Bioquímica.

Orientador: Prof. Dr. Robson Luiz Puntel

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A comissão examinadora, abaixo assinada, aprova a Dissertação de Mestrado,

Avaliação do efeito do pentilenotetrazol e 4-aminopiridina sobre parâmetros de sobrevivência, locomotores e bioquímicos em *Drosophila melanogaster*

Elaborado por

Deividi Cristian dos Santos Soares

Como requisito parcial para obtenção do título de

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APRESENTAÇÃO

O presente trabalho encontra-se dividido em itens. Nos itens INTRODUÇÃO e REVISÃO BIBLIOGRÁFICA está descrito sobre os temas abordados na dissertação. Os resultados que fazem parte desta dissertação estão apresentados sob a forma de manuscrito científico, o qual se encontra alocado no item MANUSCRITO CIENTÍFICO. As seções Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas, encontram-se no respectivo manuscrito científico e representa a íntegra deste estudo.

Os itens, CONSIDERAÇÕES FINAIS E CONCLUSÕES, no final desta dissertação, apresentam interpretações e comentários gerais sobre os resultados contidos neste trabalho. As REFERÊNCIAS BIBLIOGRÁFICAS ao final da dissertação se referem às citações que aparecem nos itens INTRODUÇÃO e REVISÃO BIBLIOGRÁFICA.

LISTA DE ABREVIATURAS

AChE – Acetilcolinesterase

AVC – Acidente vascular cerebral

CAT – Catalase

EEG- Eletroencefalograma

ER- Espécies Reativas

EROs – Espécies reativas de oxigênio

ERN – Espécies reativas de nitrogênio

GABA - Ácido gama-aminobutírico

$GABA_A$ - Receptor tipo A do neurotransmissor Ácido gama-aminobutírico

$GABA_B$ – Receptor tipo B do neurotransmissor Ácido gama-aminobutírico

$GABA_C$ – Receptor tipo C do neurotransmissor Ácido gama-aminobutírico

GPx – Glutationa Peroxidase

HNO_2 – Ácido nitroso

MDA – Malondialdeído

MTT - 3- (4,5-dimetiltiazol-2-il) brometo -2,5-difeniltetrazol

NO• - Óxido nítrico

NO_2^- - Nitritos

NO_3^- - Nitratos

N_2O_3 – Óxido nitroso

NPSH – Grupos –SH não proteícos

OH•– Radical hidroxila

$O_2•^-$ – Radical ânion superóxido

$ONOO^-$ - Peroxinitritos

PC – Proteína carbonil

PTZ - Pentilenotetrazol

RO• - Radical alcoxil

RO_2• - Radical peroxil

SNC – Sistema Nervoso Central

SOD – Superóxido dismutase

TBARS – Espécies reativas ao ácido tiobarbitúrico

4AP – 4-aminopiridina

RESUMO

Dissertação de Mestrado

Programa de Pós-Graduação em Bioquímica

Universidade Federal do Pampa

Avaliação do efeito do pentilenotetrazol e 4-aminopiridina sobre parâmetros de sobrevivência, locomotores e bioquímicos em *Drosophila melanogaster*

Autor: Deividi Cristian dos Santos Soares

Orientador: Robson Luiz Puntel

Epilepsia, uma desordem neurológica crônica, pode ser experimentalmente induzida por pentilenotetrazol (PTZ) ou 4-aminopiridina (4-AP). Mais importante, ambos PTZ e 4-AP são conhecidos por serem capazes de causar o stress oxidativo, que está associada a condição epiléptica. No entanto, o efeito de PTZ e 4-AP em *Drosophila melanogaster* continua ser melhor entendido. Consequentemente, a utilização de *D. melanogaster* como modelo alternativo / complementar pode ser adequado para melhor compreender o mecanismo subjacente (s) da atividade biológica de ambas as drogas. Assim, neste estudo, investigamos o efeito de ambos PTZ e 4-AP sobre a sobrevivência, o desempenho locomotor e marcadores bioquímicos (tanto no corpo e na cabeça) em *D. melanogaster*. Moscas adultas do tipo selvagem de 7 dias de idade foram expostas, num regime dietético, com PTZ (60 mM) ou 4-AP (20 mM) durante 48 horas. Moscas alimentadas com 4-AP apresentaram maior incidência de mortalidade e um pior desempenho no teste de campo aberto em comparação com moscas não tratadas. A toxicidade de 4-AP foi associada a um aumento significativo nas espécies reativas (ER) e no conteúdo de proteína carbonilada (PC) no corpo e na cabeça. A exposição a 4-AP também resultou em um aumento significativo da catalase e na atividade da acetilcolinesterase (AChE), bem como uma diminuição significativa na redução do MTT no corpo. Por sua vez, superóxido dismutase (SOD) foi diferentemente modulada no corpo e na cabeça de moscas tratados com 4-AP. Por outro lado, a exposição ao PTZ resultou em um aumento significativo nas ER, TBARS, PC e na atividade da catalase (CAT), e uma diminuição significativa na redução do MTT no corpo. A exposição ao PTZ também foi associado a um aumento significativo na atividade da AChE no corpo e na cabeça. A

atividade da SOD foi também diferente modulada no corpo e na cabeça de moscas tratados com PTZ. Coletivamente, as nossas descobertas destacam que a 4-AP e PTZ afetaram de modo diferente a sobrevivência, a atividade locomotora e marcadores bioquímicos em *D. melanogaster*, bem como confirma a utilidade deste modelo para investigar mecanismo (s) subjacente à atividade toxicológica de PTZ ou 4-AP. Assim, as nossas observações indicam a utilização potencial deste modelo animal experimental para investigar terapias mais seguras e eficazes para o tratamento de distúrbios neurológicos, com ênfase em estratégias para neutralizar mudanças associadas à exposição a ambas as drogas.

Palavras-chave: Epilepsia; estresse oxidativo; Acetilcolinesterase; redução MTT

ABSTRACT

Dissertation of Master's Degree

Post-Graduate Course in Biochemistry

Federal University of Pampa

Effect of the pentylenetetrazole and 4-aminopyridine on parameters survival, biochemical and locomotor in *Drosophila melanogaster*

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Epilepsy, a neurological chronic disorder, could be experimentally induced by pentylenetetrazole (PTZ) or 4-aminopyridine (4-AP). Importantly, both PTZ and 4-AP are known to be able to cause oxidative stress (OS), which is associated with epileptic condition. However, the effect of PTZ and 4-AP in *Drosophila melanogaster* remains to be better understand. Accordingly, the use of *D. melanogaster* as alternative/complementary models could be suitable to better understand the subjacent mechanism(s) to the biological activity of both drugs. So, in this study we investigated the effect of both PTZ and 4-AP on survival, locomotor performance and biochemical markers (both in body and head) in *D. melanogaster*. Wild-type adult flies of 7 days old were exposed, in a dietary regimen, to PTZ (60 mM) or 4-AP (20 mM) for 48 hours. 4-AP-fed flies presented a higher incidence of mortality and a worse performance in the *open-field* test compared to non-treated flies. The 4-AP toxicity was associated to a significant increase in the reactive species (RS) and in protein carbonyl (PC) content in body and head. The 4-AP exposure also resulted in a significant increase in catalase and in acetylcholinesterase (AChE) activity, as well as a significant decrease in MTT reduction in body. In turn, superoxide dismutase (SOD) was differently modulated in body and head of 4-AP-treated flies. On the other hand, PTZ exposure resulted in a significant increase in RS, TBARS, PC and in catalase activity, and a significant decrease in MTT reduction in body. The PTZ exposure was also associated to a significant increase in AChE activity both in body and head. The SOD activity was also differently modulated in body and head of PTZ-treated flies. Collectively, our findings highlight that 4-AP and PTZ differently affected the survival, locomotor activity and biochemical markers in *D. melanogaster*, as well

as confirms the utility of this model to investigate mechanism(s) subjacent to the toxicological activity of PTZ or 4-AP. Accordingly, our observations highlight the potential use of this experimental animal model to investigate safer and more effective therapies in the treatment of neurological disorders, with emphasis in strategies to counteract changes associated to exposure to both drugs.

Keywords: Epilepsy, oxidative stress, Acetylcholinestase, MTT redution.

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SUMÁRIO

1-INTRODUÇÃO

A epilepsia é um distúrbio do sistema nervoso central caracterizado por atividade neural sincronizada anormal (Strine et al. 2005) que afeta cerca de 1% da população mundial. A epilepsia é a doença mais frequente após o AVC e tem sido considerado um grave problema de saúde pública em nível mundial (Schmidt e Sillanpaa 2012). As manifestações clínicas da epilepsia incluem episódios recorrentes de crises epilépticas, perda de consciência, perda de memória e distúrbios sensoriais (Anovadija et al. 2012).

Atualmente animais são submetidos a estímulos químicos para gerar progressivamente crises convulsivas através da aplicação pontual de fármacos (Stewart et al. 2012). Uma das drogas mais utilizadas é o pentilenotetrazol, seu mecanismo consiste no bloqueio de GABA-mediado por influxo de Cl^- associado com bloqueio de receptores $GABA_A$. Relatos demonstram um aumento na peroxidação lipídica e uma diminuição nos grupos -SH totais no cérebro de ratos sujeitos a crises induzidas por PTZ (Karami et al. 2015), uma diminuição de enzimas antioxidantes como SOD e CAT (Tambe et al. 2016) e uma atividade enzimática do complexo I mitocondrial diminuída no cérebro de camundongos (Kumar et al. 2013).

Outra droga utilizada para induzir crises é a 4-aminopiridina, que atua através do bloqueio de canais de K^+. Além disso, 4-AP aumenta a excitabilidade neuronal (Buckle e Haas 1982), aumenta a liberação de acetilcolina e glutamato (Damsma et al. 1988) e potencializa transmissão sináptica e neuromuscular (Goodman e Stone 2013). Embora existam evidências de como essas drogas agem conforme descrito anteriormente, um melhor entendimento da toxicidade de ambas as drogas ainda precisa ser melhor explorado.

A utilização do modelo de organismo invertebrado *Drosophila melanogaster* tem emergido como um potencial modelo alternativo para estudos acerca de disfunção neuronal, por conferir certas vantagens em relação a outros modelos como ciclo de vida curto, fácil manipulação, genoma sequenciado e certa similaridade neuroquímica do sistema nervoso central em relação aos humanos. No entanto, não existem dados na literatura que demonstrem os efeitos tóxicos de PTZ e 4-AP em *D. melanogaster* como modelo experimental. Com isso, este estudo foi realizado com intuito de verificar de modo comparativo os efeitos do PTZ e 4-AP sobre parâmetros locomotores e bioquímicos em moscas da fruta *D. melanogaster* a fim de melhor compreender o(s) mecanismo(s) de toxicidade pelo(s) qual(is) estas drogas agem.

2-REVISÃO BIBLIOGRÁFICA

2.1) EPILEPSIA

O termo epilepsia refere-se a descargas anormais excessivas que atingem o SNC, alterando a atividade cerebral e caracterizada por manifestações motoras, sensoriais, comportamentais e neurodegenerativas. As convulsões podem acometer apenas um grupo de neurônios (crise parcial) ou os dois hemisférios cerebrais (crise generalizada) (Guerreiro et al. 2000), podendo estar relacionados com síndromes epilépticas que iniciam com uma idade específica e associados à EEG padrões característicos. Epilepsias são classificadas em formas idiopáticas que não têm causa conhecida, exceto fatores hereditários e formas sintomáticas causadas por lesões cerebrais, como malformações, tumores ou asfixia (Scheffer and Berkovic 2003).

Crises convulsivas são sinais transitórios da ativação excessiva do SNC. Esta hiperexcitabilidade é devido à potenciação dos mecanismos excitatórios (glutamato), ou falha dos mecanismos inibitórios (GABA) do SNC (Gowers 1881). O excesso de glutamato e GABA na fenda sináptica promove uma superestimulação dos receptores, levando a um aumento além do desejado dos níveis de Ca^{2+}, Na^{+} e diminuição de Cl^{-} intracelular, que participa da ativação de proteínas como fosfolipases, endonucleases, e proteases que em excesso, que vão danificar o DNA, proteínas, e fosfolipídeos de membrana, alterando a estrutura e o funcionamento celular. Pode haver também lesão mitocondrial, através do estresse oxidativo decorrente dos níveis excessivamente elevados de íons na fenda pós-sináptica (Forman et al., 2009). As crises convulsivas podem ser de três tipos: crises tônicas (contrações mantidas durante algum tempo), clônicas (contrações intermitentes, onde os músculos são contraídos e relaxados de forma alternada) ou tônico-clônicas (Löscher 1998). Para diagnosticar epilepsias consideram-se três pontos: (1) histórico de ocorrência de pelo menos uma crise convulsiva; (2) alterações cerebrais fisiológicas e anatômicas persistentes, predispondo o paciente a futuras crises convulsivas; e (3) condições associadas como: alterações psicológicas, neurobiológicas, cognitivas, aumento de agressividade, ansiedade, déficit de atenção e memória, entre outros (Fisher et al. 2005; Lin et al. 2012).

O tratamento consiste em drogas antiepilépticas que atuam no bloqueio de canais de sódio ou cálcio ou que atuem em receptores GABAérgicos (Da Silva e Cabral 2008). Embora existam numerosas drogas antiepilépticas disponíveis, alguns problemas são relatados como incapacidade do controle das crises e limitação do uso pelos pacientes por apresentarem

efeitos adversos como ataxia, sedação e disfunções cognitivas (Bhosle, 2013). Com isso, cada vez mais é necessário desenvolver pesquisas a fim de achar terapias mais seguras e eficazes para o tratamento destas desordens.

A epilepsia é uma condição neurológica mais grave do mundo com uma incidência de cerca de 0,3%-0,5% no mundo (Lowenstein 2008). No Brasil, estima-se que mais de três milhões de pessoas têm alguma forma de epilepsia, atingindo principalmente crianças e jovens. Quanto ao impacto econômico a epilepsia é responsável por 0,5% dos gastos com doenças incapacitantes no mundo. O afastamento do trabalho, a necessidade de supervisão constante e o aumento frequente de internações são alguns dos fatores que influenciam para o aumento dos custos (Leonardi and Ustun 2002; Li and Sander 2003; Borges et al. 2004).

Os modelos experimentais de epilepsia podem ser induzidos por estimulação elétrica direta e indireta (eletrochoque, abrasamento), alteração genética (crise audiogênica em camundongo e fotossensibilidade genética) (Mello et al., 1986) ou quimicamente através da aplicação tópica ou injeção sistêmica de drogas (Stewart et al. 2010). Dentre as drogas convulsivas, historicamente usadas em modelos de roedores, incluem pentilenotetrazol (PTZ) (Giorgi et al. 1996), picrotoxina (Hamani e Mello 2002), pilocarpina (Cavalheiro 1995), cainato (Zagrean et al. 1993), cafeína (Seale et al. 1987) e 4-aminopiridina (4-AP) (Tutka et al. 2002). Estas drogas permitem estudar este sintoma, realizar triagens de fármacos que inibam estas crises e verificar alterações neuroquímicas e locomotoras decorrentes do efeito das mesmas.

2.2) PENTILENOTETRAZOL

O modelo de epilepsia induzida por pentilenotetrazol (Figura 1) é um dos modelos mais utilizados que é caracterizado por um aumento da susceptibilidade para convulsões após a injeção de doses subconvulsivas de PTZ. A administração repetida leva ao desenvolvimento de convulsões tônico-clônicas generalizadas (Macdonald e Barker 1977, 1978).

Animais têm apresentado crises convulsivas tônico-clônicas com duração de 5 min após administrações por via subcutânea ou intraperitoneal de PTZ em roedores (Brito et al. 2006). O mecanismo de ação do PTZ consiste no bloqueio sobre receptores $GABA_{A,}$ diminuindo o influxo de Cl-, diminuindo a ação inibitória de GABA no SNC (Bahcekapili et al. 2014).

O GABA é o principal neurotransmissor inibitório do sistema nervoso central (Rowley et al. 2012). Este neurotransmissor ao ser liberado pelo neurônio pré-sináptico atua em

receptores ionotrópicos ($GABA_A$ e $GABA_C$) (Laurie et al. 1992; Perfilova e Tiurenkov 2011) e metabotrópicos ($GABA_B$) (Bowery 1997). Ao ligar-se nos receptores $GABA_A$ pós-sinápticos, ocasiona influxo de Cl^- e efluxo de K^+ nos neurônios, ocorrendo uma hiperpolarização (Sieghart e Sperk 2002). Ao atingir o SNC, o PTZ liga-se a receptores $GABA_A$, inibindo a ação de GABA (Ramanjaneyulu e Ticku 1984).

Existem relatos de que espécies reativas (ER) podem estar relacionadas aos efeitos convulsivos e neurotóxicos do PTZ (Hosseini et al. 2013), pelo estresse oxidativo que esta droga causa. Estudos têm demonstrado um aumento nos níveis de MDA e redução dos -SH totais em ratos tratados com PTZ (Hale et al. 2013), afetando a compreensão espacial e memória de ratos (Zhen et al. 2014) e certas manifestações comportamentais (Loscher 2011). Aumento no conteúdo TBARS e diminuição nos níveis de NPSH também foram relatados em estresse oxidativo induzido por PTZ (Oliveira et al. 2016). Wong et al. (2010), observaram em estudos com PTZ em *zebrafish* adulto manifestações convulsivas caracterizadas por aumento na frequência de abertura opercular, elevação da atividade natatória, movimentos circulares, movimentos repetitivos, queda ao fundo do aquário e rígida extensão do corpo. Decorrente de alta reprodutibilidade e fácil observação de seus efeitos, pesquisadores o utilizam como composto potencial para testes.

Apesar da maioria dos estudos com PTZ focarem em aspectos relacionados à epilepsia (Amada et al. 2013; El-Azab e Mustafa 2012; Xu et al. 2014), não há relatos sobre a toxicidade relacionada a esta droga. Há também o fato de estes estudos serem realizados com mamíferos como modelos experimentais (Kumar et al. 2013; Ozsoy et al. 2015; Torres-Hernández et al. 2015). No entanto, para entender melhor o(s) mecanismo(s) subjacente à toxicidade dessa droga, uma estratégia é o desenvolvimento e a caracterização de novos modelos animais experimentais, nos quais seja possível fazer o rastreio de fármacos e/ou que sejam de fácil manipulação genética. Nesse contexto a *D. melanogaster* surge como um modelo útil para estudo toxicológico, pois além de apresentar estas vantagens, são conhecidos por possuir alta sensibilidade a substâncias tóxicas (Adedara et al. 2016).

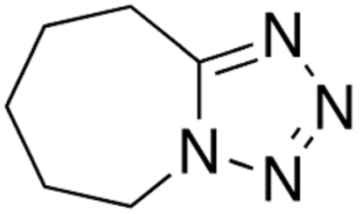

Figura1. Estrutura química do pentilenotetrazol

2.3) 4-AMINOPIRIDINA

A 4-Aminopiridina tem sido utilizada para induzir epilepsia tanto *in vitro* (Avoli et al. 2002) quanto *in vivo* (Fragoso-Veloz et al. 1990; Morales-Villagran et al. 1996). Em camundongos, a administração parenteral de 4-AP induz convulsões tônico-clonicas generalizadas e letalidade (Mihaly et al. 1990; Pasantes-Morales e Arzate 1981). 4-Aminopiridina age como bloqueador de canais de K^+, estimulando a transmissão sináptica e provocando atividade oscilatória no SNC (Luca e Singer 2013). Este fármaco atravessa a barreira hematoencefálica (Damsma et al. 1988), tem fórmula molecular $C_5H_6N_2$ e sua estrutura química é apresentada na figura 2.

A atividade dos canais de K^+ influencia em muitas funções celulares, como a excitabilidade de células neuronais e musculares, regulação do volume e proliferação celular e contribui para a aprendizagem e memória. Esses canais para íon K^+ são divididos em quatro famílias: os canais voltagem-dependentes (K_v), os cálcio-ativados (K_{Ca}), os retificadores de influxo ou *inward-rectifiers* (K_{ir}) e os que possuem dois-poros *in tandem* (K_{2P}). A 4-aminopiridina age em diferentes subtipos de canais voltagem-dependentes (K_v), porém não age nos canais cálcio-ativados (K_{Ca}) e nem os canais dois-poros *in tandem* (K_{2P}) (Goldstein et al. 2005; Gutman et al. 2005; Kubo et al. 2005; Wei et al. 2005). Em *Drosophilas* mutantes *shaker*, existem três genes relacionados a canais voltagem-dependentes homólogos aos mamíferos (*Shab, Shaw e Shal*) e que desempenham um papel importante no potencial de ação (Butler et al 1989; Salkoff et al., 1992).

Morales-Vilagran e Tapia (1996) relatam a liberação de glutamato no cérebro de ratos administrados com 4-AP. Esta liberação pode estar envolvida com o desenvolvimento e atividade epiléptica e com efeito letal da mesma. No córtex cerebral de roedores, 4-AP aumenta a liberação de acetilcolina (Golding et al. 1999). Outros resultados demonstram que a administração sistêmica e intracerebroventricular em camundongos induz a morte (Tutka et al. 2002). Além disso, 4-AP também pode afetar a composição iônica dos compartimentos extracelulares (Müller et al. 1999).

Apesar destes achados e da ampla utilização no campo geral da neurobiologia (Baraban 2007), não há dados na literatura relacionados à toxicidade de 4-AP em moscas da fruta *D. melanogaster*.

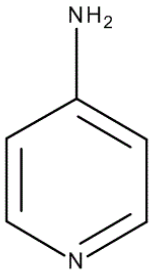

Figura 2. Estrutura química da 4-aminopiridina

2.4) ESTRESSE OXIDATIVO

Conforme descrito anteriormente evidências apontam que o estresse oxidativo, causado por PTZ e 4-AP, pode contribuir de forma independente para a progressão de doenças além de ser um importante fator no processo de muitas desordens neuronais (Patel 2004). O estresse oxidativo pode ser definido como um desequilíbro entre a produção de espécies reativas de oxigênio (ROS) e/ou especies reativas de nitrogênio (RNS) e defesas antioxidantes, que pode levar a alterações moleculares induzidas por estas reações oxidativas em biomoléculas como proteínas, lipídeos e carboidratos (Fig. 3) (Uttara et al. 2009).

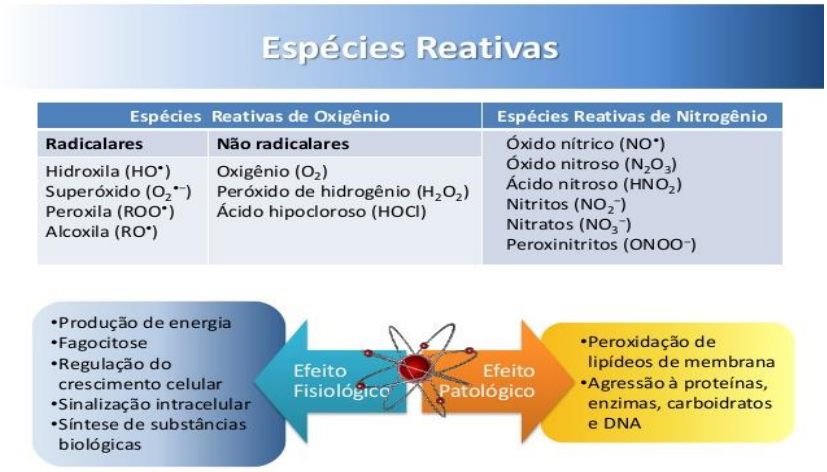

Figura 3. Fontes e respostas celulares às Espécies Reativas de Oxigênio (ERO) e/ou Nitrogênio (ERN).

Essas espécies reativas fazem parte do metabolismo humano e são observadas em diversas condições fisiológicas e tem função biológica importante, como a fagocitose, onde essas espécies são produzidas com o intuito de eliminar agentes agressores. É cada vez mais

reconhecido que as ROS/RNS tem um papel biológico duplo: em concentrações baixas ou moderadas eles desempenham um papel importante nas funções fisiológicas normais, influenciando várias vias de sinalização (Dröge 2002), mas a níveis excessivos são prejudiciais para os sistemas vivos. As principais ROS distribuem-se em dois grupos, os radicalares: hidroxila (HO•), superóxido (O2 •−), peroxila (ROO•) e alcoxila (RO•); e os não-radicalares: peróxido de hidrogênio e ácido hipocloroso. Dentre as RNS incluem-se o óxido nítrico (NO•), óxido nitroso (N2O3), ácido nitroso (HNO2), nitritos (NO2−), nitratos (NO3−) e peroxinitritos (ONOO−). Quando produzidos em excesso podem causar danos, estando frequentemente relacionados a várias doenças, tais como Alzheimer, doença de Parkinson, aterosclerose, envelhecimento precoce, entre outras (Sorg 2004).

Para evitar os danos causados pelas espécies reativas, o organismo desenvolveu vários mecanismos de defesa, isto é, potenciais de neutralização das ações dos radicais livres, chamados antioxidantes. Existem vários mecanismos de defesa antioxidantes endógenos, tanto enzimático (superóxido dismutase (SOD), catalase (CAT), etc) e não enzimático (glutationa, tocoferóis (vitamina E), ácido ascórbico (vitamina C)) (Halliwell and Gutteridge 2007). Este sistema constitui a primeira defesa endógena a agir contra ataques das espécies reativas, bloqueando a etapa de iniciação da cadeia radicular (Rover et al. 2001).

Em *Drosophilas*, foram identificados fatores de transcrição Cap ‘n’ collar isoforma-C (CnCc), homólogos a *Nrf2* em mamíferos, como um regulador central de xenobióticos (Nguyen et al 2009;. Sykiotis e Bohmann 2010). *Nrf2* (*nuclear factor-erythroid 2-related factor-2*) é um fator de regulação positivo para o elemento de resposta antioxidante (ARE), regulando a expressão de enzimas antioxidantes (Vadiveloo et al. 2013). Este fator de transcrição nuclear encontra-se inativo no citoplasma ligado a proteína *Kelch-like ECH-associated protein* 1 (*Keap*1) a qual impede sua translocação para o núcleo. Alterações no estado redox, tais como aumento na concentração de ERs, ativam este fator e promovem uma resposta transcricional no elemento de resposta antioxidante (ARE), perdendo assim sua ligação com *Keap* 1 (Wilmes et al. 2011). Logo após ser translocada para o núcleo, ela ativa genes que codificam enzimas como SOD, CAT, glutationa peroxidase (GPx), tioredoxina (Trx), heme oxigenase-1, dentre outras (De Groot et al. 2012), como demonstrado na figura 4.

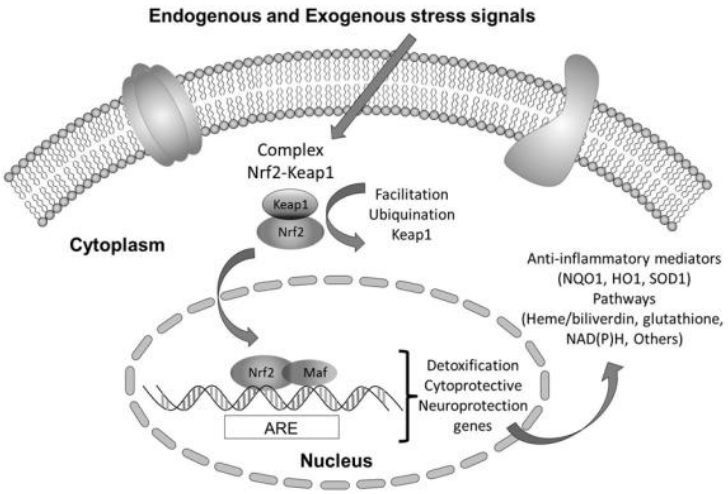

Figura 4. Mecanismo proposto responsável pela ativação da via de sinalização do *Nrf2*/ARE

Portanto, é de grande valia a caracterização e implementação de novos modelos animais que possibilitem novos achados a respeito dessas desordens e seus efeitos nestes organismos.

2.5) D. melanogaster

A *Drosophila melanogaster* é um inseto pequeno (cerca de 3mm de comprimento), apresenta o corpo dividido em cabeça, tórax e abdome e sua forma predominante selvagem (wild-tipe) apresenta olhos vermelhos e listras pretas no abdome. A mosca da fruta apresenta dimorfismo sexual, onde as fêmeas apresentam listras pretas no abdome mais espaçadas e em tamanho são maiores que os machos. Já os machos apresentam a extremidade do abdome negra devido a fusão dos segmentos terminais, um dos fatores levados em consideração para distinguir o sexo (Fig.5).

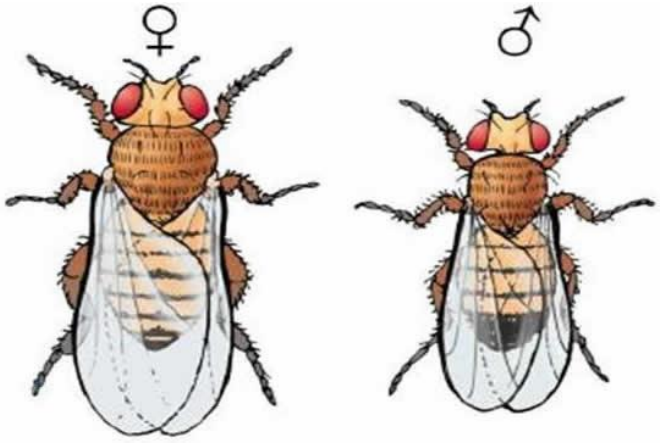

Figura 5. *Drosophila melanogaster*, diferenças entre machos e fêmeas.

A mosca tem um ciclo de vida curto, onde pode produzir centenas de proles geneticamente idênticas dentro de 10 a 12 dias a 25ºC (Pandey e Nichols 2011). Semelhante a maioria dos seres vivos, seu ciclo de vida depende de condições ambientais e seu ciclo de vida apresenta 4 fases: ovo, larva, pupa e a fase adulta (Fig.6). A expectativa média de vida das fêmeas é de 26 dias e de 33 para o macho.

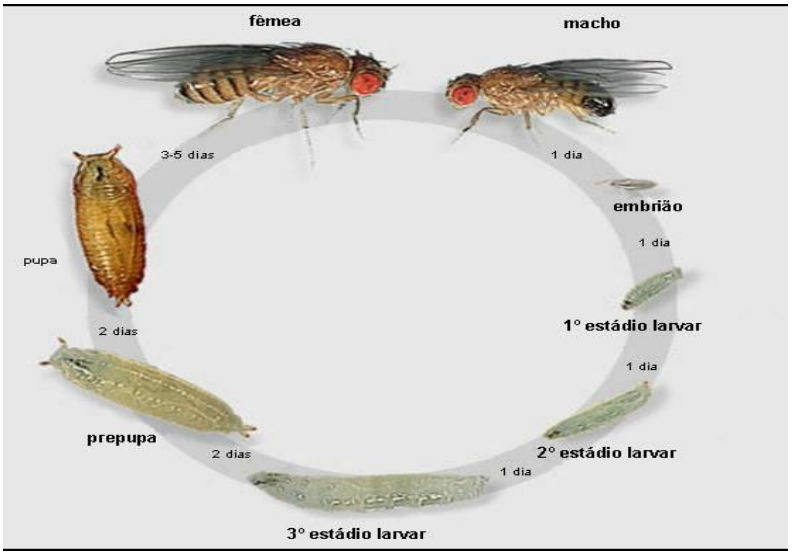

Figura 6. Ciclo de vida de *Drosophila melanogaster*

Existem várias características proeminentes da mosca da fruta, *D. melanogaster*, que o tornam um modelo experimental atraente para a investigação de desordens neuronais. A simplicidade de seu sistema nervoso, ciclo de vida curto, alta produtividade, genoma totalmente sequenciado, e capacidade de manipular sua genética contribuem para investigações a cerca de alterações na atividade neuronal durante desenvolvimento e/ou crises neste tipo de modelo experimental (Cunliffe et al. 2014).

Como descrito anteriormente, muitas características tornam este modelo atraente para estudo. Este modelo é um organismo muito sofisticado e complexo não muito diferente de organismos superiores. Possui estruturas que executam as funções equivalentes do coração, pulmão, rim, intestino, e trato reprodutivo dos mamíferos. Seu sistema nervoso é bem notável, onde mais de 100.000 neurônios formam circuitos discretos e neurópilos e que medeiam comportamentos complexos, incluindo sono, aprendizagem, memória, agressividade. Sua resposta a drogas que atuam no SNC é semelhante aos efeitos observados em mamíferos

(Andretic et al. 2008; Bainton et al. 2000; McClung e Hirsh 1998; Moore et al. 1998; Nichols et al. 2002; Rothenfluh e Heberlein 2002; Satta et al. 2003; Wolf e Heberlein 2003).

A mosca adulta codifica um pouco mais de 14.000 genes em quatro cromossomos, sendo três os que carregam a maior parte do genoma. Estima-se que 75% dos genes relacionados a doenças em humanos são ortólogos funcionais na mosca (Reiter et al. 2001; Lloyd e Taylor 2010). Atualmente, por ter esta homologia dos genes, *D. melanogaster* tem sido fundamental para identificar genes relacionados à epilepsia e funções do sistema nervoso (Pandey and Nichols 2011). Estudos recentes descrevem mutantes *temperature-sensitive* (TS) *canalopatia*, *slowpoke* e *ether-a-go-go* (Ganetzky and Wu 1986; Ganetzky 2000) e mutantes *bang-sensitive* (BS) *slamdance* e *paralytcs* (Kuebler and Tanouye 2002; Lee and Wu 2002; Reynolds et al. 2004; Tan et al. 2004) como modelos uteis para análise das crises e ação de drogas antiepilépticas.

3. JUSTIFICATIVA

A utilização da mosca da fruta *D. melanogaster* como modelo para estudo de mecanismos moleculares envolvidos em disfunções neuronais humanas têm sido descrito na literatura (Bonini and Fortini 2003; Nichols 2006). Esse modelo experimental tem sido amplamente utilizado por oferecer inúmeras vantagens como fácil manipulação, ciclo de vida curto, genoma sequenciado, sistema nervoso similar fisiologicamente aos humanos e sensibilidade a substâncias tóxicas.

No entanto, não há estudos acerca da toxicidade causada por PTZ e 4-AP em moscas da fruta selvagens, embora tenha-se idéia de como ambas agem. Assim, o presente trabalho pretende verificar os efeitos tóxicos causados por estas drogas na sobrevivência, atividade locomotora e marcadores bioquímicos, a fim de melhor compreender o(s) mecanismo(s) subjacente(s) de toxicidade das mesmas em *D. melanogaster*.

4. OBJETIVOS

4.1) OBJETIVO GERAL

Considerando o exposto, o presente estudo visa investigar as possíveis alterações locomotoras e bioquímicas induzidas pelos agentes pró-convulsivantes PTZ e 4-AP em *D. melanogaster*.

4.2) OBJETIVOS ESPECÍFICOS

- Avaliar os efeitos induzidos por PTZ e 4-AP sobre a longevidade avaliada diariamente durante 8 dias de tratamento.
- Determinar os efeitos induzidos por PTZ e 4-AP sobre os parâmetros locomotores avaliados diariamente durante 8 dias de tratamento (geotaxia negativa e open-field)
- Investigar os efeitos induzidos por PTZ e 4-AP sobre parâmetros bioquímicos (SOD,CAT, NPSH, TBARS, RS, PC, AChE, MTT) após 48 hs de tratamento.

5. RESULTADOS

5.1) MANUSCRITO CIENTÍFICO

O tratamento com pentilenotetrazol (PTZ) ou 4-aminopiridina (4-AP) afeta diferentemente a sobrevivência, a atividade locomotora e marcadores bioquímicos em *Drosophila melanogaster*

Treatment with pentylenetetrazole (PTZ) or 4-aminopyridine (4-AP) differently affects survival, locomotor activity and biochemical markers in *Drosophila melanogaster*

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Treatment with pentylenetetrazole (PTZ) or 4-aminopyridine (4-AP) differently affects survival, locomotor activity and biochemical markers in *Drosophila melanogaster*

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Abstract

The mechanism(s) by which pentylenetetrazole (PTZ) and 4-aminopyridine (4-AP) cause oxidative stress (OS), and most importantly, their effect in *Drosophila melanogaster*, needs to be better addressed. So, in this study we investigated the effect of exposure (48hs) to either PTZ (60mM) or 4-AP (20mM) on survival, locomotor performance and biochemical markers (both in body and head) in *D. melanogaster*. 4-AP-fed flies presented a higher incidence of mortality and a worse performance in the *open-field* test compared to non-treated flies. 4-AP toxicity was associated to a significantly increase in the reactive species (RS) and in protein carbonyl (PC) content in body and head. 4-AP exposure also resulted in a significantly increase in catalase and in acetylcholinesterase (AChE) activity, as well as a significant decrease in MTT reduction in body. In turn, SOD was differently modulated in body and head of 4-AP-treated flies. In turn, PTZ exposure resulted in a significantly increase in RS, TBARS, PC and in catalase activity, and a significant decrease in MTT reduction in body. PTZ exposure was also associated to a significantly increase in AChE activity both in body and head. SOD activity was also differently modulated in body and head of PTZ-treated flies. Collectively, our findings provide preliminary evidences regarding the toxicological activity of PTZ or 4-AP to flies, supporting the potential use of this model to investigate mechanism(s) subjacent to their toxicological activity, as well as, to investigate therapeutic strategies to counteract changes associated to exposure to both epileptogenic drugs.

Keywords: Epilepsy; Oxidative Stress; Acetylcholinesterase; MTT reduction; PTZ; 4-AP.

1. INTRODUCTION

Epilepsy is referred to a group of disorders that affect the central nervous system (CNS), characterized by an enduring predisposition to generate epileptic seizures and by a lot of psychological, social, neurobiological, and also cognitive consequences (Martinc et al. 2014; Mehla et al. 2009). In an attempt to mimic this disorder, and tentatively better understand the mechanism(s) involved in of the epileptic seizures episodes, were previously developed, especially in mammals, either genetic or non-genetic experimental models (Matos et al. 2012; Stewart et al. 2012; White 2002). In this scenario, chemicals such as pentylenetetrazole (PTZ) and 4-aminopyridine (4-AP) are usually used to experimentally trigger this condition.

Accordingly, PTZ is one of the convulsive agents used to cause both acute and chronic crises (Takechi et al. 2012). PTZ was previously reported to acts *via* selective blockage of $GABA_A$ receptor channel and it was also reported to lead to a decrease of GABA-mediated neurotransmission (Bambal et al. 2011). As a consequence, PTZ causes an imbalance in the excitation/inhibition, which culminates in neuronal hyperexcitability and cause a generalized seizure (Fernando and Mody 2012). Additionally, PTZ lead to a decrease in transamination reactions and seems to be associated to a decreased in glutamate content in the glutamatergic neurons (Eloqayli et al. 2003; Yudkoff et al. 2003).

In turn, 4-AP was reported to acts *via* blockage of K^+ channel (Gonzalez-Sulser et al. 2012). As a consequence, this drug was reported to affect repolarization and prolong the action potential of neurons, which leads to hyperexcitability (Laura et al. 2015). 4-AP was also reported to stimulate the release of glutamate, which culminates with the development of convulsive activity (Pena and Tapia 2000).

Of particular importance, the neurotoxicity of epileptogenic drugs seems to be also associated to oxidative stress (OS) (Brito et al. 2009; Folbergrová 2013; Martinc et al. 2014). Indeed, there is ample evidence that OS and mitochondrial dysfunction are implicated in the pathogenesis of many neuronal disorders, including epilepsy (Folbergrová and Kunz 2012; Ikonomidou and Kaindl 2011; Lin and Beal 2006; Patel 2004; Waldbaum and Patel 2010). Nevertheless, data concerning the toxicological activity of both drugs remains to be better addressed. Indeed, despite of the several reports of neurotoxic injury by epileptic drugs (Brito et al. 2009; Folbergrová 2013; Martinc et al. 2014), few data are available about their effects on other organs/tissues and the role of OS on these effects.

So, considering the necessity of better understand the mechanism(s) subjacent to the biological activity of PTZ and 4-AP, the development of simple animal models are convenient (Löscher 2011; Mohammad et al. 2009). Additionally, one of the major concerns of researchers is the reduction in the number of higher laboratory animals for research and testing due to ethical issues (for promoting the 3Rs -reduction, refinement and replacement- of laboratory animal usage in toxicity studies). Accordingly, it was previously reported that invertebrate model organisms such as *D. melanogaster* emerge as useful animal model for the study of molecular mechanisms involved in human neuronal dysfunction due to the simplicity of its nervous system, rapid life cycle, easy genetic manipulation and sequenced genome (Benton 2008; Hirth 2010; Marley and Baines 2011). Moreover, *Drosophila* has been previously demonstrated to be a useful model for elucidating the mechanisms underlying neurotoxicity associated to epilepsy, especially in epilepsy induced by genetic modifications (Jeibmann and Paulus 2009; Parker et al. 2011). However, only few data are found in the literature with respect to the toxicity associated to epileptic drugs in flies (Mohammad et al. 2009; Streit et al. 2016). Accordingly, it was previously shown that flies treated with PTZ showed locomotor deficit, which was associated to down regulation of a series of genes (Mohammad et al. 2009). Importantly, flies are well known for possess high sensitivity to toxic substances being considered as a useful model for toxicological studies (Adedara et al. 2016).

So, in the present study we assessed the toxicological effects of PTZ or 4-AP, in *D. melanogaster*, by evaluating the survival and locomotor deficit following short-term dietary regimen. Furthermore, using both the head and body region of flies, we evaluated some biochemical markers of toxicity such as reactive species (RS), TBARS, and non-protein thiol groups (NPSH) levels, protein carbonyl (PC) content, catalase, superoxide dismutase (SOD) and acetylcholinesterase (AChE) activity, as well as MTT reduction, tentatively to better understand the putative subjacent mechanism(s) of toxicity of both epileptic drugs.

2. MATERIALS AND METHODS

2.1 Chemicals

Pentylenetetrazole, 4-aminopyridine, Thiobarbituric acid (TBA), acetylthiocholine iodide, 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT), quercetin, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), 2,4-dinitrophenyl hydrazine

(DNPH), 2′,7′-Dichlorofluorescin diacetate (DCFDA) were obtained from Sigma–Aldrich (St. Louis, MO, USA). All the other chemicals were commercial products of the highest purity grade available.

2.2 Drosophila culture condition

D. melanogaster wild-type (Harwich strain) were obtained from the National Center species, Bowling Green, Ohio, USA. The flies were maintained and reared on cornmeal medium (1% w/v brewer's yeast, 1% w/v sucrose, 1% w/v powdered milk, 1% w/v agar, and 0.08% v/w methylparaben) at constant temperature and humidity (22 ± 1ºC; 60% relative humidity, respectively) under 12h dark/light cycle conditions until treatment. All the experiments were carried out with the same fly strain.

2.3 Experimental design - PTZ e 4-AP exposure

D. melanogaster (both genders) of 7 days old were divided in to three groups of 100 flies each: (1) control, (2) PTZ 60 mM, and (3) 4-AP 20 mM. Flies were exposed a diet containing PTZ and 4-AP for 48 hours for all assays, except for the analysis of survival. PTZ and 4-AP concentrations were chosen based in a pilot experiment where flies were exposed to PTZ (20 mM, 40 mM and 60 mM) and 4-AP (10 mM, 20 Mm and 40 mM), to determine the effect of both drugs on the survival and locomotor activity of flies during the experimental period. Accordingly, when treated with 4-AP flies die in a time dependent manner, regardless of 4-AP concentration, whereas PTZ exposure did not caused mortality in flies (data not shown). Based on this, we choose a higher PTZ concentration used in pilot study (60 mM) and a minimal 4-AP concentration that significantly alters locomotor activity in flies (*i.e.* 20 mM) for subsequent experiments.

2.4 *In vivo* assays

2.4.1) Survival

The flies were observed daily for the incidence of mortality. The survival rate was determined by counting the number of dead flies during 8 days, while the survivors were transferred to freshly prepared diet. The data were subsequently analyzed and plotted as percentage of live flies. For mortality response 7 independent experiments were performed,

being that 100 flies were included per group; *i.e.* 300 flies were used in each treatment repetition. Therefore 2,100 flies were used in lethality response test.

2.4.2) Negative geotaxis

Locomotor ability of flies was performed with a negative geotaxis assay as described previously by Feany and Bender (2000). In short, flies were sorted under a brief ice anesthesia and placed in a vertical glass column (length: 10 cm, diameter: 1.5 cm/ 10 flies each). After the recovery from cold exposure (approximately 15 min) the flies were gently tapped to the bottom of the column. The flies that reached the top of the column and the flies that remained at the bottom were counted separately during 6 s. The scores represent the mean of the numbers of flies at the top (ntop) as percentage of the total number of flies (ntot). This value represents the mean of ten independent experiments. The results are expressed as percentage of flies that escaped beyond a minimum distance of 6 cm in 6 s during four independent experiments. Around 10 flies per group were included for the negative geotaxis data (total of 30 flies per independent experiment; therefore 300 flies were used in negative geotaxis test).

2.4.3) *Open - field*

Open-field task was performed according to the method described by Hirth (2010). Accordingly, three flies from each group (therefore 9 flies per independent experiment) were kept in an arena divided by squares (1 cm X 1 cm) measuring 9 cm of diameter, which can be covered by petri dish. The fly's activity were recorded with a video camera and the number of squares crossed by each single fly, during a given time-window (30 s), was analyzed. This value represents the mean of ten independent experiments (90 flies were used in *open-field* test).

2.5. *Ex vivo* assays

2.5.1) Homogenized preparation

At the end of the treatment period (48 hours), flies were anesthetized in ice. Heads were separated from the body using a sharp blade/cutter. Afterward, heads and bodies were homogenized in 0.9% NaCl solution, 1:5 (flies/volume (250 µL)). The homogenates was centrifuged at 3.000 *g* for 10 min at 4°C, and the supernatant was used for biochemical

assays. All biochemical determinations were performed in duplicates in 4-9 independent experiments.

2.5.2) Determination of TBARS

The lipid peroxidation end products were quantified as thiobarbituric acid reactive substances (TBARS) according to established procedure Ohkawa et al. (1979) with some modifications. In brief, an aliquot of homogenate (100 µL) was incubated per 1 hour at 37°C in a water bath. Thereafter samples were incubated at 100 °C for 120 min in 200 µL of a medium containing equal volumes of trichloroacetic acid (10 %, w/v) and thiobarbituric acid (0.6 %, w/v) in 0.1M HCl for color development. After boiling step, 20 µL 8,1% SDS was added. The reaction product was determined at 532 nm and the results were expressed as % of control after correction by the protein content.

2.5.3) Determination of protein carbonyls (PC) content

Protein carbonyl levels were quantified using the method of Levine et al. 1990. Briefly an aliquot of sample (50 µl) was incubated per 1 hour with 200 µL 2,4-dinitrophenyl hydrazine (DNPH) in 2M HCl or with 2M HCl in white tubes. Thereafter, protein content was precipitated by adding 250 µL trichloroacetic acid (TCA 20%; 1:1 v:v). The resulting pellet was washed twice with 500 µL ethanol:ethyl acetate (1:1) and dissolved in 250 µL 2% SDS. The optical density was measured at 450 nm and the results were expressed as % of control after correction by the protein content.

2.5.4) Determination of reactive species levels (RS)

The level of intracellular reactive species (RS) generation was determined by quantifying 2′,7′- dichlorofluorescein (DCFH) oxidation according to established procedure Pérez-Severiano et al. (2004). The assay reaction mixture contained 280 µL of 10 mM TRIS (pH 7.4), 5 µL of 10 mM DCFH-DA, and 10 µL of the sample. The fluorescence emission of DCF resulting from DCFH oxidation was analyzed for 1 hour (15 min intervals) at 488 and 525 nm, excitation and emission wavelengths, respectively. The results were expressed as % of control after correction by the protein content.

2.5.5) Determination of non-protein thiol (NPSH)

The NPSH level was determined in the control and treated flies according to the method previously described by Ellman (1959). For NPSH assay, 70 µL homogenate was precipitated with 70 µL TCA 10% (1:1 v:v) followed by centrifugation at 3.000 g for 10 min at 4 ºC. The reaction system was made up of 940 µL of 1M dibasic potassium phosph buffer, 50 µL of sample, and 10 µL of 5 mM DTNB. At the end of 10 min incubation at room temperature (25 °C), the absorbance was measured at 412 nm. The results were expressed as % of control after correction by the protein content.

2.5.6) Activity of superoxide dismutase

The assay consists in the inhibition of superoxide driven oxidation of quercetin by SOD (Lushchak et al. 2005). Briefly, the reaction medium contained 200 µL of buffer (160 µL 0.02 M phosphate buffer / 0.08 mM EDTA (pH 7.4) mixed with 200 µL TEMED), 10 µL of 0.05µM quercetin and 40 µL of sample (1:5 dilution). The reaction was analyzed in time 0 and 20 min at 406 nm. The results expressed as % of control after correction by the protein content.

2.5.7) Activity of catalase

The catalase activity was measured spectrophotometrically according to the method of Aebi (1984), by monitoring the disappearance of H_2O_2. Briefly, the reaction medium contained 960 µL of 0.05M phosphate buffer (pH 7.0), 20 µL of 1M H_2O_2, and 20 µL of sample (1:5 dilution). The reaction was analyzed for 2 min (15 s intervals) at 240 nm. Results were expressed as percentage of control after correction by the protein content.

2.5.8) Activity of acetylcholinesterase (AChE)

Acetylcholinesterase activity was determined according to the method of Ellmann et al. (1961). Briefly, the assay medium consisted of 70 µL of distilled water, 100 µL of system (0.5M potassium phosphate buffer (pH 7.4) with 10 mM DTNB), 10 µL of sample, and 20 µL of 8 mM acetylthiocholine as substrate. The degradation of acetylthiocholine iodide was analyzed for 2 min (30 s intervals) at 412 nm. The results were expressed as % of control after correction by the protein content.

2.5.9) MTT reduction

Dehydrogenases activity was evaluated by using 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) reduction assay described by Hosamani and Muralidhara (2013). Briefly an aliquot of sample (50 µl) was incubated per 30 min (37 ºC) with 20 µL MTT and 5 mM succinate. Thereafter, reaction was stopped by addition of 200 µL DMSO and incubated per 30 min (37 ºC) to dissolve formazan salts. Then, the samples were centrifuged to 2000 rpm for 5 min, and the absorbance was monitored at 630 nm and 545 nm. The results were expressed as % of control after correction by the protein content.

2.5.10) Protein determination

The protein content was determined as described previously (Bradford 1976) using bovine serum albumin (BSA) as standard.

2.6) Statistical analysis

Behavior data was evaluated by nonparametric methods because Kolmogorov-Smirnov's test indicated the absence of Homogeneity of Variance. Behavior parameters were analyzed using Kruskal–Wallis (kw) followed by Dunn's multiple comparisons test when appropriate. Survival data was analyzed by ANOVA-MANOVA followed by Tukey's multiple range test when appropriate. Other results were analyzed by one-way ANOVA followed by Tukey's multiple range test when appropriate. Differences between groups were considered significant when $p < 0.05$. Data of non-parametric analysis are represented as box and whisker plots (min to max); and data of parametric analysis as means and S.E.M. Graphics were created using GraphPad Prisma 6.0.

3. RESULTS

3.1) Effect of PTZ or 4-AP on survival rate of flies

The cumulative mortality in the control and flies exposed to 4-AP or PTZ for 8 consecutive days are presented in Figure 1. Accordingly, there was no significant mortality in flies treated with PTZ (60 mM), while there was a significant ($p < 0.05$) increase in the mortality of flies exposed to 4-AP (20 mM), from day 1 until the end of experimental period (day 8) when compared with the control.

3.2) Effect of PTZ or 4-AP on locomotor performance of flies

The climbing behavior was not significantly changed by PTZ or 4-AP (Figure 2 A). In turn, in *open-field* test, Dunn's multiple comparisons test revealed that the flies treated with 4-AP had a significant decrease in the number of crossings when compared to the control group ($p < 0.05$; Figure 2B).

3.3) Effects of PTZ or 4-AP on biomarkers of OS in homogenate from head and body of flies

Data on the levels of OS markers (i.e. RS, TBARS, PC and NPSH) in flies after 48hs of treatment with 4-AP or PTZ are presented in Figures 3-6. Accordingly, flies exposed to 4-AP showed a significant increase both the RS levels, as well as, in the PC content, both in body (Figures 3A and 4A, respectively) and in head (Figures 3B and 4B, respectively), when compared to control group. In turn, PTZ exposed flies had a significant increase in the RS levels, in PC content, and also in TBARS levels in body (Figures 3A, 4A and 5A, respectively) when compared to control group. Neither PTZ nor 4-AP significantly altered the levels of NPSH (Figures 6A-B).

3.4) Effects of PTZ or 4-AP on enzyme activities in homogenate from head and body of flies

Data on the activity of enzymes (i.e. SOD, catalase and AChE) in flies after 48hs of treatment with 4-AP or PTZ are presented in Figures 7-9. Accordingly, flies exposed to 4-AP had a significant decrease in SOD activity (Figures 7A) and a significant increase in catalase activity (Figure 8A) in body, as well as a significant increase in SOD activity in head (Figure 7B), with respect to control. In turn, PTZ exposure resulted in decreased SOD activity in body (Figure 7A), increased SOD activity in head (Figure 7B), and increased catalase activity in body (Figure 8A).

Additionally, flies exposed to 4-AP had a significant increase in AChE activity in body (Figures 9A), when compared to control group. In turn, PTZ exposure resulted in increased AChE activity both in body and in head of flies (Figures 9A and 9B, respectively) with respect to control.

3.5) Effects of PTZ or 4-AP on MTT reduction in homogenate from head and body of flies

Data on the MTT reduction in flies after 48hs of treatment with 4-AP or PTZ are presented in Figure 10. Accordingly, flies exposed to both 4-AP and PTZ had a significant decrease in MTT reduction in body (Figure 10A), when compared to control group.

4. DISCUSSION

Although neuronal networks and circuits are physiologically and anatomically distinct from vertebrates (Littleton and Ganetzky 2000), *D. melanogaster* has emerged as a genetic useful model for elucidating the mechanisms underlying neurotoxicity associated to epilepsy, as well as, given its high sensitivity to toxic substances, for non-genetic toxicological studies (Adedara et al. 2016; Jeibmann and Paulus 2009; Mohammad et al. 2009; Parker et al. 2011). Of particular importance, 75% of human genes known to be associated with diseases have orthologs in flies (Pandey and Nichols 2011), which reinforces the use of this simpler model in basic research. Of note, we found here that PTZ and 4-AP treatment differently affects survival, locomotor activity and biochemical markers in *D. melanogaster*. Importantly, to the best of our knowledge, this was the first report to describe the 4-AP-induced toxicity in flies. Indeed, only flies treated with 4-AP presented significantly increase in mortality and worse performance in *open field* test (Figures 1 and 2, respectively). Moreover, our data clearly shown that in treated flies the body seems to be more sensitive both to oxidative (Figures 3-8) and non-oxidative (Figures 9 and 10) changes than head, regardless of epileptic drug.

Locomotor activity is a complex behavior that may be influenced by different neural systems in flies (Martin JR 2004). Importantly, it was previously found that loss of motor coordination and altered locomotor activities were associated to seizure-like activity in *Drosophila* (Wang et al. 2004). Consequently, locomotor behavior of flies could be used as a useful parameter in neuropsychiatric conditions (Chang et al. 2006; Mohammad et al. 2009). In line with this, changes in locomotor activity were previously reported to flies exposed to PTZ (Mohammad et al. 2009). However, we found here that in flies treated with PTZ there was no significant changes in locomotor activity (Figure 2). The differences in our findings with those previously reported may be explained, at least in part, due to different regimens of treatment/exposure. In contrast, 4-AP exposure resulted in a worse performance in *open field* (Figure 2B). To our knowledge, this is the first report to show that treatment with 4-AP result

in locomotor impairment in flies. So, considering that both PTZ and 4-AP caused similar effect in AChE activity (Figure 9), we speculate that other system, than cholinergic, must be affected by 4-AP treatment, which could be implicated in locomotor impairment reported here. Moreover, a putative energetic deficit *via* mitochondrial impairment (measured here as MTT reduction; Figure 10) also seems to be insufficient to explain our findings, once PTZ and 4-AP act in a similar way in the MTT assay. Of particular importance, MTT reduction is mediated by dehydrogenase activities, especially those from mitochondria. Therefore, MTT reduction may be understand as an indirect index of mitochondrial activity (Caughlan et al. 2004), in addition to their use as an indicator of cell viability (Sudati et al. 2013; Takahashi et al. 2002; Wang et al. 2011). Moreover, it was previously reported that the prevalence of dysfunctional mitochondrial in bang-sensitive mutants leads to impaired energy metabolism, which may be involved in the changes in locomotor activity of this flies (Fergestad et al. 2006). Surprisingly, 4-AP caused severe mortality in exposed flies, even in lower concentrations, when compared to PTZ (Figure 1). So, either the putative mechanism(s) by with 4-AP lead to flies death, as well as, mechanism(s) by which it leads to locomotor impairment needs to be better/further explored.

In spite of this, in an attempt to better understand the putative subjacent mechanism(s) of toxicity of PTZ and 4-AP, we performed a series of assays to address the involvement of OS, by using separately both head and body region of treated flies. Indeed, although little is known about the precise mechanism(s) of action of these agents in *D. melanogaster*, a growing body of evidence suggests that the exposure to both epileptogenic drugs can lead to a condition known as OS (Brito et al. 2009; Folbergrová 2013; Ilhan et al. 2005; Martinc et al. 2014). Indeed, there are evidences in the literature supporting the notion that PTZ induces changes in OS markers in the brain (both whole brain as well as in their structures) by using mammal models. Such changes include increase in TBARS and RS levels, increase in PC content, decrease in NPSH levels, decrease in SOD activity and increased catalase activity (Oliveira et al. 2016; Golechha et al. 2010; Martinc et al. 2014; Mehla et al. 2010; Oliveira et al. 2016; Ribeiro et al. 2005; Sharma et al. 2010; Xie et al. 2012; Zhen et al. 2016). In turn, 4-AP was also found to induce an increase in TBARS and RS levels in the brain of mammal (Brito et al. 2009; Folbergrová 2013). Thus, it is possible to assume that the effects mediated by epileptogenic drugs may involve OS. However, to the best of our knowledge, there are no

available data about the effects of 4-AP or PTZ on other organs/tissues and the role of OS on these effects, especially in simpler organisms.

Accordingly, we found here that PTZ exposure resulted in increase in RS (Figure 3), PC (Figure 4) and TBARS (Figure 5), which was associated to decrease in SOD (Figure 7) and increase in catalase (Figure 8), all of this in body. Additionally, SOD was found significantly increased in head of PTZ-treated flies (Figure 7). In turn, 4-AP exposure resulted in increased RS (Figure 3) and PC (Figure 4) both in body and in head, which was associated to an increased catalase (Figure 8) and decrease SOD (Figure 7) activity in body, and also increase in SOD activity in head (Figure 7). So, considering that here we have not determined concomitantly the levels and/or transcription of respective enzymes, an analysis more detailed of our results is limited. Besides, we suggest that increased of catalase activity in body, and increased SOD activity in head, may represent a compensatory response to oxidative insults. Moreover, both 4-AP and PTZ significantly decreased MTT reduction (Figure 10) without changing the NPSH levels (Figure 6) in the body of treated flies. Taken together, these data suggest a possible implication of OS on the toxicity of both epileptogenic drugs and strongly supports the notion that body is more pronounced affected by treatment by using *D. melanogaster*.

We also found that flies exposed to 4-AP had a significant increase in AChE activity in body, while PTZ exposure resulted in increased AChE activity both in body and in head of flies (Figure 9). Accordingly, AChE is an enzyme that participates in cholinergic neurotransmission and, as a consequence is involved in the regulation of locomotion (Greenspan et al. 1980). Moreover, it was previously shown that an increase in AChE expression was associated to dopaminergic neuronal death, thus supporting the notion that this enzyme plays an important role in the pathophysiology of neurodegenerative diseases (Craig et al. 2011; Zhang et al. 2013). In line with this, it was recently shown that flies exposed to manganese (at neurotoxic concentrations) or rotenone had increased AChE activity, which could lead to a decrease in acetylcholine levels in the synaptic cleft and consequently reduce cholinergic neurotransmission efficiency and impair climbing activity in the exposed flies (Adedara et al. 2016; Krishna and Muralidhara 2016). However, as pointed above, the effect of epileptogenic drugs on AChE activity are not sufficient to completely explain our findings regarding 4-AP-induced locomotor impairment, and mechanistic studies

are still necessary to better explore the mechanism(s) by which 4-AP induced locomotor deficit in our experimental conditions.

In summary, our findings highlight that 4-AP and PTZ differently affected the survival, locomotor activity and biochemical markers in *D. melanogaster* providing preliminary evidences regarding the toxicological activity of both epileptogenic drugs to flies. Moreover, our observations highlight the potential use of this model to investigate mechanism(s) subjacent to the toxicological activity, as well as, to investigate therapeutic strategies that may be promising in treatment of neurological disorders, with special emphasis in strategies to counteract both oxidative and non-oxidative changes associated to exposure to both drugs. However, additional studies about the neurological pathways involved on the (neuro)toxic role of both epileptogenic drugs are still needed.

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Conflict of interest

The authors declare that there is no conflict of interest.

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Legends

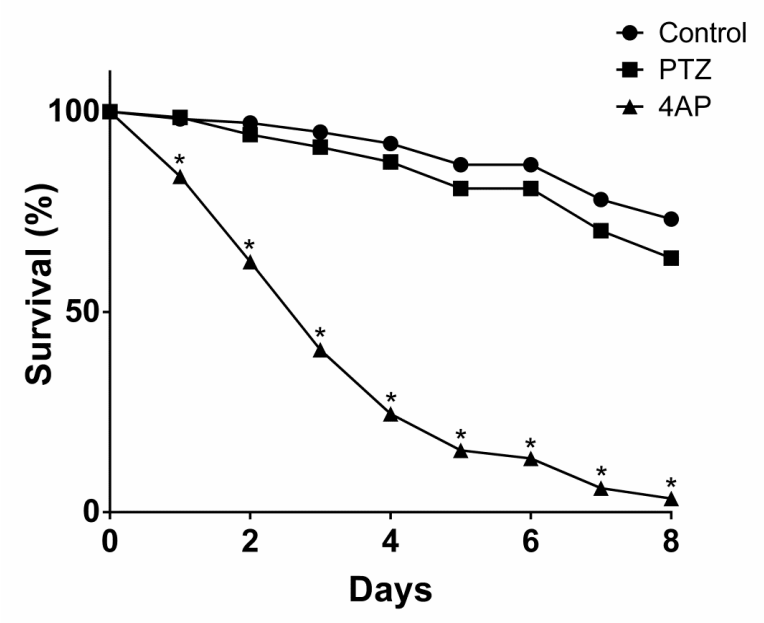

Figure 1: *Effect of 4-AP (20 mM) or PTZ (60 mM) on survival rate of treated flies.* Data were collected every 24 h for each group during 8 days. The numbers of surviving flies are represented as % of control. The total number of flies (700 per group) represents the sum of seven independent experiments. *Significant difference in relation to the control group (Tukey's multiple range test). Values are expressed as mean ± S.E.M.

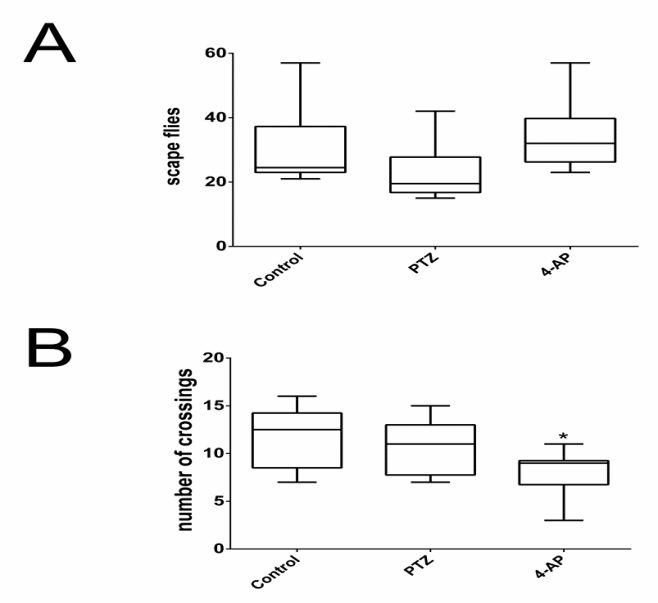

Figure 2: *Effect of 4-AP (20 mM) or PTZ (60 mM) on (A) geotaxis response (climbing) or (B) locomotor activity evaluated by open-field of treated flies.* Data were collected after 48 h of exposure. The total number of flies (100 per group in negative geotaxis; and 30 per group in *open field*) represents the sum of ten independent experiments. Values are expressed as median and range (interquartile interval). *Significant difference in relation to the control group (Kruskal–Wallis test followed Dunn' multiple comparisons test, $p < 0.05$).

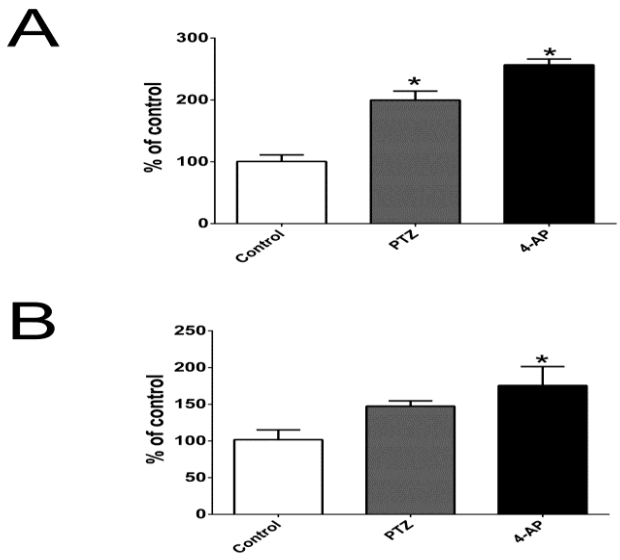

Figure 3: *Effect of 4-AP (20 mM) or PTZ (60 mM) on RS levels in homogenate of (A) body or (B) head of treated flies.* *Significant difference in relation to control group ($p < 0.05$, one-way ANOVA followed by Tukey's multiple range test). Values are expressed as mean ± S.E.M. (n = 9).

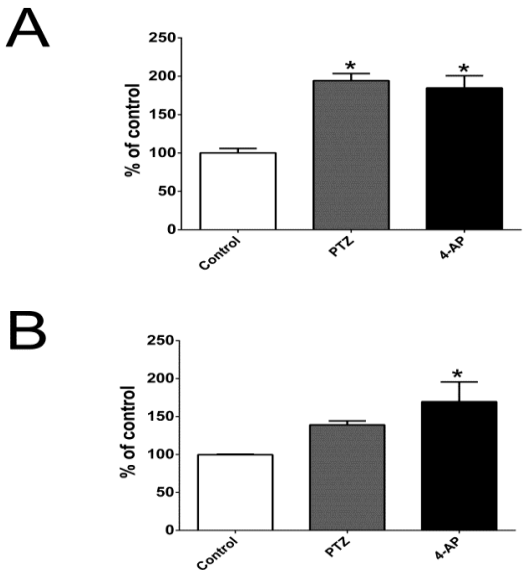

Figure 4: *Effect of 4-AP (20 mM) or PTZ (60 mM) on PC content in homogenate of (A) body or (B) head of treated flies.* *Significant difference in relation to control group ($p < 0.05$, one-way ANOVA followed by Tukey's multiple range test). Values are expressed as mean ± S.E.M. (n = 4).

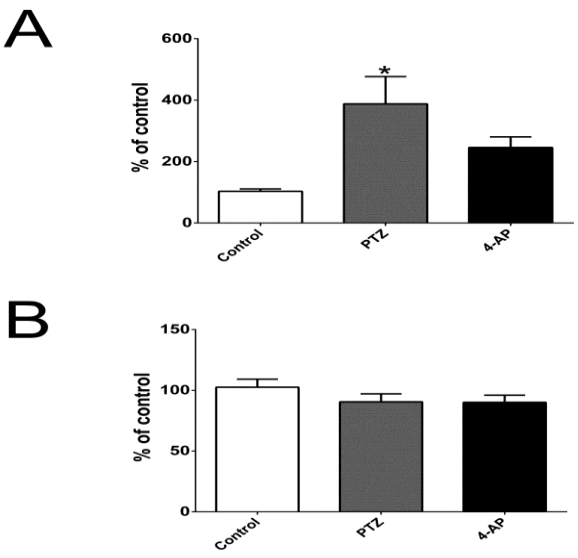

Figure 5: *Effect of 4-AP (20 mM) or PTZ (60 mM) on TBARS levels in homogenate of (A) body or (B) head of treated flies.* *Significant difference in relation to control group ($p < 0.05$, one-way ANOVA followed by Tukey's multiple range test). Values are expressed as mean ± S.E.M. (n = 5).

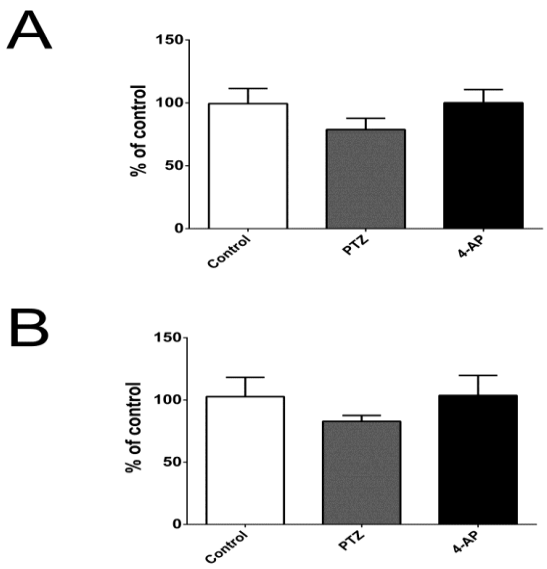

Figure 6: *Effect of 4-AP (20 mM) or PTZ (60 mM) on NPSH levels in homogenate of (A) body or (B) head of treated flies.* Values are expressed as mean ± S.E.M. (n = 5).

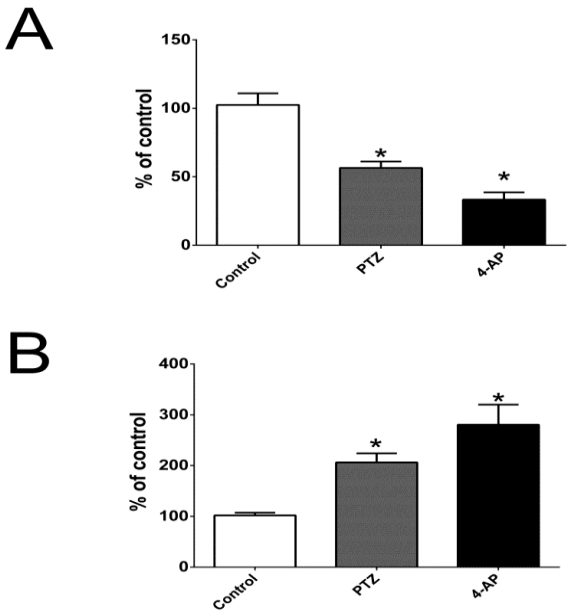

Figure 7: *Effect of 4-AP (20 mM) or PTZ (60 mM) on SOD activity in homogenate of (A) body or (B) head of treated flies.* *Significant difference in relation to control group ($p < 0.05$, one-way ANOVA followed by Tukey's multiple range test). Values are expressed as mean ± S.E.M. (n = 6).

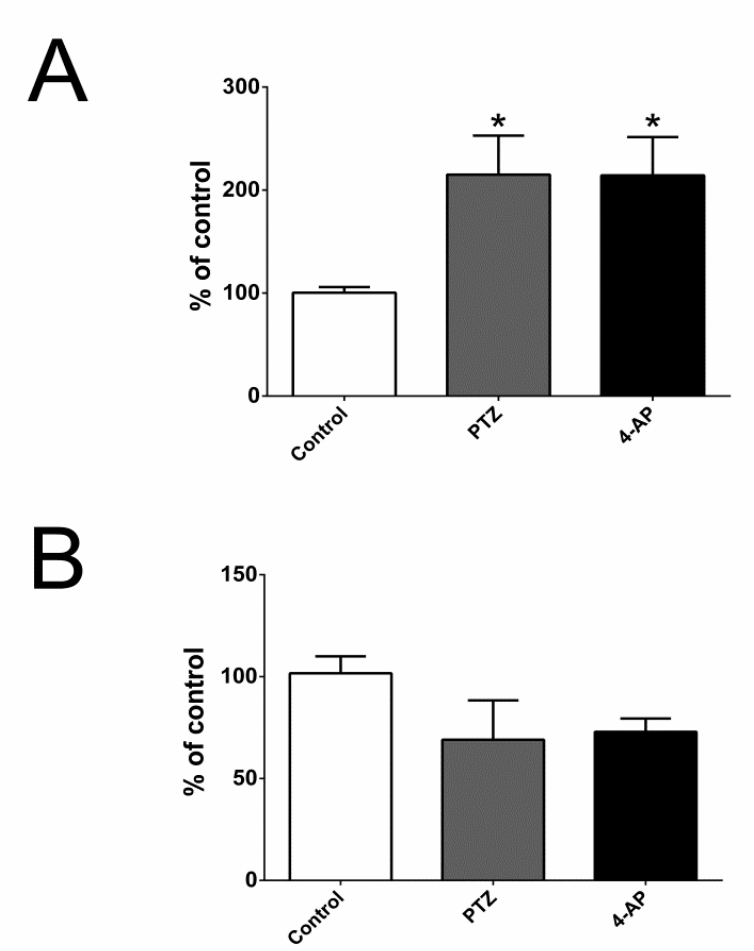

Figure 8: *Effect of 4-AP (20 mM) or PTZ (60 mM) on catalase activity in homogenate of (A) body or (B) head of treated flies.* *Significant difference in relation to control group ($p < 0.05$, one-way ANOVA followed by Tukey's multiple range test). Values are expressed as mean ± S.E.M. (n = 5).

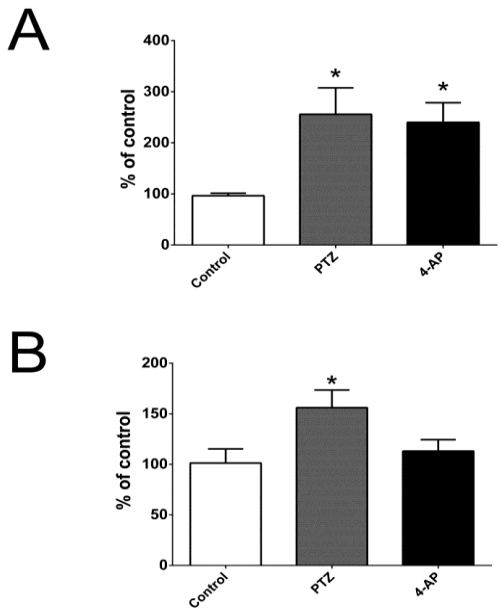

Figure 9: *Effect of 4-AP (20 mM) or PTZ (60 mM) on AChE activity in homogenate of (A) body or (B) head of treated flies.* *Significant difference in relation to control group ($p < 0.05$, one-way ANOVA followed by Tukey's multiple range test). Values are expressed as mean ± S.E.M. (n = 7).

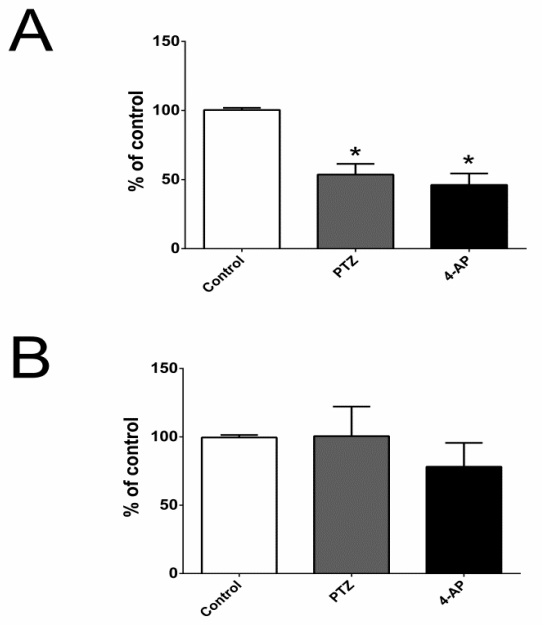

Figure 10: *Effect of 4-AP (20 mM) or PTZ (60 mM) on MTT reduction in homogenate of (A) body or (B) head of treated flies.* *Significant difference in relation to control group ($p < 0.05$, one-way ANOVA followed by Tukey's multiple range test). Values are expressed as mean ± S.E.M. (n = 6).

6. CONCLUSÕES

De acordo com os resultados apresentados nesta dissertação podemos concluir que:

- O PTZ não alterou significativamente a mortalidade e os parâmetros locomotores. Demonstrou alteração nos parâmetros bioquímicos avaliados e a maior parte observada no corpo. Houve diminuição na redução MTT e SOD e um aumento na atividade de RS, TBARS e PC e CAT. Houve também um aumento da atividade da AchE no corpo e na cabeça.

- A 4-AP causou significativa letalidade e diminuição no parâmetro locomotor *open field* em *Drosophilas* expostas. Demonstrou ainda alterar alguns parâmetros bioquímicos, tendo um aumento significativo nos ensaios de RS e PC observados no corpo e na cabeça; aumento nas atividades da CAT e AchE e diminuição na SOD e redução MTT , todas estas alterações foram observadas no corpo.

7. PERSPECTIVAS

A partir dos resultados obtidos, as perspectivas para trabalhos posteriores são:

- Investigar estratégias que possam melhor elucidar sobre o(s) mecanismos(s) de toxicidade do PTZ e 4-AP, haja vista que ainda não há um consenso sobre o mecanismo de ação exato, em *D. melanogaster* como modelo experimental por sua simplicidade.

- Propor estratégias terapêuticas que possam ser promissoras no tratamento de distúrbios neurológicos, com ênfase nas estratégias para combater alterações associadas à exposição a ambos os fármacos.

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