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EXPOSIÇÃO CRÔNICA AO HERBICIDA PARAQUAT PROMOVE DESEQUILÍBRIO NO SISTEMA ANTIOXIDANTE E ALTERAÇÕES COMPORTAMENTAIS EM PEIXE ZEBRA (*Danio rerio*)

DISSERTAÇÃO DE MESTRADO

Mauro Eugênio Medina Nunes

Santa Maria, RS, Brasil.
2015

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Dissertação apresentada ao Programa de Pós-Graduação em Ciências Biológicas, Área de Concentração em Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para a obtenção do grau de

Mestre em Bioquímica Toxicológica.

Orientadora: Profª. Drª. Vania Lucia Loro

Co-orientador: Prof. Dr. Denis Broock Rosemberg

Santa Maria, RS, Brasil.

2015

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elaborada por
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como requisito parcial para obtenção do grau de
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COMISSÃO EXAMINADORA

_____

Vania Lucia Loro, Dr[a].

(Presidente/Orientadora)

_____

Denis Broock Rosemberg, Dr.

(Co-orientador)

_____

Juliano Zanette, Dr. (FURG)

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Sara Marchesan de Oliveira, Dr[a]. (UFSM)

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DEDICATÓRIA

Dedico esta dissertação:

Aos meus pais Eugênio e Terezinha, ao meu irmão Franklin e minha namorada Renata, pessoas que para mim sempre foram fonte de motivação e inspiração.

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RESUMO

Dissertação de Mestrado
Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica
Universidade Federal de Santa Maria, RS, Brasil.

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AUTOR: Mauro Eugênio Medina Nunes
ORIENTADOR: Vania Lucia Loro
CO-ORIENTADOR: Denis Broock Rosemberg
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Na última década, tem se investigado padrões comportamentais que reflitam as alterações fisiológicas e bioquímicas de organismos modelo promissores, como o peixe zebra (*Danio rerio*). O peixe zebra tem se mostrado uma útil ferramenta para diferentes estudos dose e resposta de drogas, e em estudos de biomonitoramento. A exposição ao herbicida paraquat (PQ), um comum contaminante ambiental, é considerada um fator de risco para o desenvolvimento de doenças neurodegenerativas, como a doença de Parkinson. No cérebro o PQ atua sobre os neurônios dopaminérgicos induzindo o estado de estresse oxidativo através do seu ciclo redox sobre a fosfato de nicotinamida adenina dinucleotídeo (NADPH) e também sobre as mitocôndrias, induzindo disfunção mitocondrial e morte desses neurônios. Desse modo, o objetivo desse estudo foi avaliar parâmetros comportamentais e alterações bioquímicas em cérebros de peixe zebra em um modelo de tratamento crônico com PQ. PQ (20 mg/kg; grupo tratado) ou salina (0.9%; grupo controle) foram administrados por via intraperitoneal, uma injeção a cada 3 dias durante 16 dias, totalizando seis injeções em peixe zebra adulto de ambos os sexos. Peixes zebra tratados com PQ mostraram um caráter menos ansioso em relação ao grupo controle. O comportamento menos ansioso foi determinado pela diminuição em comportamentos de defesa, como diminuição tempo de permanência na parte inferior do tanque, latência para a primeira entrada seção superior e número e duração de episódios de avaliação de risco no ensaio ao novo tanque e teste claro-escuro. Além disso, os animais tratados apresentaram um caráter mais agressivo, aumentando o tempo e duração de episódios agressivos no teste de agressão induzida pelo espelho. No entanto, não foram observadas alterações nos parâmetros locomotores e motores. Além disso, o tratamento com PQ induziu dano cerebral através da diminuição da viabilidade mitocondrial. O tratamento com PQ induziu também o aumento da atividade de biomarcadores do sistema de defesa antioxidande, como catalase (CAT), glutationa peroxidase (GPx), e níveis de tióis não proteicos (NPSH), assim como na glutationa-S-tranferase (GST). Entretanto, não houve alterações nos níveis de espécies reativas de oxigênio (EROS) e danos oxidativos em lipídios (TBARS). Nós demonstramos pela primeira vez que PQ induziu a um comportamento mais agressivo e diminuição de parâmetros não motores relacionados a comportamentos de defesa. Estes dados sugerem que animais expostos a PQ estejam mais suscetíveis a predação, uma vez que se expõem mais ao perigo. O aumento da agressividade pode influenciar o comportamento social do cardume. As alterações comportamentais podem ser produto dos danos cerebrais demonstrados pela diminuição da viabilidade de células encefálicas.

Palavras-chave: paraquat, peixe zebra, comportamento, agressividade, sistema antioxidante.

ABSTRACT

Dissertation of Master's Degree
Post-Graduate Course in Biological Science - Toxicological Biochemistry
Federal University of Santa Maria, RS, Brazil

CHRONIC EXPOSURE TO HERBICIDE PARAQUAT PROMOTES IMBALANCE IN THE ANTIOXIDANT SYSTEM AND BEHAVIORAL CHANGES IN ZEBRAFISH (*Danio rerio*)

AUTHOR: Mauro Eugênio Medina Nunes
ADVISOR: Vania Lucia Loro
CO-ADVISOR: Denis Broock Rosemberg
DATE AND PLACE OF THE DEFENSE: Santa Maria, August, 20, 2015

In the last decade, it has been investigated behavioral patterns that may to reflect the physiological and biochemical changes in promising experimental models. Zebrafish (*Danio rerio*) is an useful tool for studying dose-response effects when exposed to contaminants and biomonitoring studies. The exposure to herbicide paraquat (PQ), a common environmental contaminant, is considered a risk factor for the development of neurodegenerative diseases such as Parkinson's disease. In the brain the PQ acts on the dopaminergic neurons by inducing a state of oxidative stress through its redox cycle on the nicotinamide adenine dinucleotide phosphate (NADPH) and also on the mitochondria, leading mitochondrial dysfunction and death of these neurons. Thus, the aim of this study was to evaluate behavioral parameters and biochemical changes in zebrafish brain chronically treated PQ. PQ (20 mg/kg; treated group) or saline (0.9%; control group) were administrated intraperitoneally, one injection every 3 days for 16 days totalized six injections in adult zebrafish of both sexes. Zebrafish treated with PQ showed a less anxious character in relation to control group, due the decrease in defense behaviors, such as time spend in bottom section, latency to first entry to upper section and number and duration of risk assessment episodes in novel tank and light-dark test. Additionally, treated animals showed a more aggressive character, increasing the time and duration of the aggressive episodes in the inclined mirror-image stimulus. However, alterations in locomotor and motor patterns were not observed. In addition, PQ induced brain damage due decrease of ± 10% of the mitochondrial viability. Treatment with PQ also induced the increase of antioxidant defense system activity, as biomarkers catalase (CAT) glutathione peroxidase (GPx), and non-protein thiols (NPSH) levels, as well as the glutathione-S-transferase (GST). However, there were no changes in the levels of reactive oxygen species (ROS) and oxidative damage in lipids (TBARS). We demonstrated for the first time that PQ induced an increase in aggressive behavior, decreasing non-motors patterns associated to defense behaviors. We suggest that fish exposed to PQ are more susceptible to predation, since it seems to be more exposed to danger. The increase in aggression can influence the social behavior of the fish group. Behavioral changes may be associated to brain damage observed by decreased mitochondrial viability in zebrafish brain.

Key-words: paraquat, zebrafish, behavior, aggression, antioxidant system.

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INTRODUÇÃO

LISTA DE ILUSTRAÇÕES

MANUSCRITO

LISTA DE ABREVIAÇÕES

AChE – Acetilcolinesterase

ANVISA – Agência Nacional de Vigilância Sanitária

CAT - Catalase

cm – Centímetros

DP – Doença de Parkinson

e^- - Elétron

EROS - Espécies Reativas ao Oxigênio

Fe_2^+ - Ferro Ferroso

GPx – Glutationa Peroxidase

GSH - Glutationa Reduzida

GST - Glutationa-S-Transferase

H_2O_2 – Peróxido de Hidrogênio

HAPs - Hidrocarbonetos Aromáticos Policíclicos

IDA – Ingestão Diária aceitável

LAT-1 - Transportadores de Aminoácidos do Sistema L

NADPH – Fosfato de Nicotinamida Adenina Dinucleotídeo

ND – Neurônios Dopaminérgicos

$O_2^{\bullet -}$ - Ânion Superóxido

OH – Radical Hidroxila

PQ – Paraquat

PQ^+ - paraquat monocátion

PQ^{2+} - paraquat dicátion

SOD - Superóxido Dismutase

TBARS - Substâncias Reativas ao Ácido Tiobarbitúrico

SUMÁRIO

APRESENTAÇÃO

Esta dissertação está descrita na seguinte forma: primeiramente são apresentados a introdução e os objetivos. A seguir, os resultados e a discussão são apresentados no item manuscrito. O item conclusão encontrado no final desta dissertação, apresenta interpretações gerais sobre o manuscrito contido nesta dissertação. As referências bibliográficas apresentadas no final da dissertação referem-se somente as citações que aparecem no item introdução.

1. INTRODUÇÃO

Visando o aumento da produtividade, as atividades agrícolas em geral tem utilizado uma carga cada vez maior de agroquímicos para combater pragas, plantas daninhas, fungos e bactérias (PRIMEL et al., 2005). Os agroquímicos são uns dos principais contaminantes responsáveis pela degradação dos recursos hídricos (TOMITA; BEYRUTH, 2002). Contudo, alguns estudos demonstram que apenas uma pequena percentagem de agroquímicos atingem os recursos hídricos superficiais, devido à sua baixa solubilidade em água e também pelo fator de diluição (HIGASHI, 1991). Entretanto, isto não descarta a possibilidade que concentrações maiores possam ser depositadas nos recursos de forma não pontuais, na qual os xenobióticos são lançados de forma difusa ou indireta nos recursos hídricos devido a processos de escoamento, drenagem e lixiviação (CEREJEIRA et al., 2003). A contaminação também pode ocorrer pelo transporte atmosférico devido à volatilização dos compostos presentes nos agroquímicos e pela formação de poeira do solo contaminado (COOPER, 1993). Estes tipos de contaminações são difíceis de serem avaliadas, pois o grau de contaminação pode variar conforme o clima, uma vez que as chuvas podem tanto diluir a contração de um contaminante presente na água como também podem transportar mais contaminantes para os recursos hídricos através da lixiviação. Estas características acompanhadas do uso indiscriminado de agroquímicos ao longo do tempo têm desencadeado a acumulação e bioacumulação de compostos químicos nocivos nos ecossistemas aquáticos (BITTENCOURT, 2004).

Dentro da classe geral de agroquímicos, os herbicidas têm chamado a atenção devido a sua facilidade de lixiviação até corpos de água, além de representar mais de 50% de todos os agrotóxicos usados nos Estados Unidos e na Europa (TEKEL; KOVACICOVÁ, 1993; TOMITA; BEYRUTH, 2002). Dentre os herbicidas mais perigosos encontra-se o paraquat (N, N'-dimetil-4, 4, 4'-bipiridínio dicloreto; PQ) conhecido comercialmente como Gramoxone® (disponível em solução contendo 20% do princípio ativo). O PQ é um herbicida de contato e não seletivo largamente utilizado na agricultura, em várias culturas como: fumo, algodão, arroz, café, cana-de-açúcar, feijão, maça, soja, uva, abacaxi (ANVISA, 2007). Quando aplicado na lavoura, o PQ é depositado à superfície dos vegetais sofrendo degradação fotoquímica, a qual dá origem a compostos menos tóxicos. Assim que atinge o solo, o PQ é rapidamente absorvido pelos minerais argilosos presentes no solo se tornando biologicamente inerte. Enquanto isso, o PQ livre é degradado lentamente por uma série de microrganismos (taxa de degradação de 5 a 10% por ano) (HONORÉ et al., 1994).

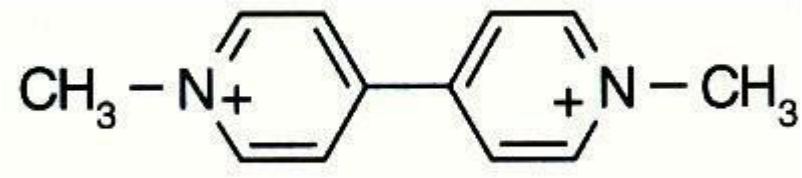

FIGURA 1 - Estrutura química do PQ (ANVISA, 2007).

Segundo a ANVISA (2007) o PQ (Figura 1) pertence ao grupo químico bipiridílio, com classificação toxicológica I (Extremamente Tóxico), conhecido pela sua utilização para suicídio. A ingestão diária aceitável (IDA) é de 0,004 mg/kg em humanos e em modelos animais o PQ apresenta uma dose letal 50% (DL50) em média de 35 mg/kg (HONORÉ et al., 1994). Os efeitos tóxicos do PQ são relacionados com seus efeitos pró-oxidativos, tanto em células vegetais e animais (FRANCO et al., 2009). Em células vegetais, o PQ é absorvido na forma de paraquat dicátion (PQ^{2+}) agindo sobre o Fotossistema I dos cloroplastos. Os elétrons (e^-) livres do Fotossistema I reagem com íon do PQ resultando na forma de radical livre (paraquat radical monocation; PQ^+). O oxigênio rapidamente reconverte esse radical e nesse processo produz superóxido ($O_2^{\bullet-}$), o qual é altamente reativo e provoca danos oxidativos em biomoléculas e estruturas celulares. Além disso, nesse processo o íon de PQ livre então se recicla, produzindo maiores quantidades de $O_2^{\bullet-}$ até que o suprimento de elétrons livres cesse (MARTINS, 2013). Em células animais, o PQ tem sido demonstrado como indutor de morte celular em uma variedade de tecidos (DINIS-OLIVEIRA et al., 2008). Células animais também absorvem o PQ na forma de PQ^{2+}, o qual é reduzido PQ^+, após reagir com fosfato de nicotinamida adenina dinucleotídeo (NADPH) sofrendo uma redução por ação da enzima NADPH-citocromo P450 redutase. O radical PQ^+ reage rapidamente com oxigênio para gerar o ânion radical superóxido ($O_2^{\bullet-}$), o qual sofre dismutação não enzimática formando peroxido de hidrogênio (H_2O_2). Este na presença de ferro ferroso (Fe_2^+), pela reação de Fenton, é capaz de formar superoxido ($^{\bullet}OH$), o qual é altamente reativo as biomoléculas, oxidando-as (DINIS-OLIVEIRA et al., 2006).

O PQ tem surgido como uma neurotoxina através de evidências de danos significativos no tecido encefálico observado em indivíduos expostos a doses letais desse herbicida (GRANT et al., 1980). Além disso, estudos epidemiológicos tem relatado a

exposição ao herbicida PQ como fator de risco para o desenvolvimento da doença de Parkinson (PD) (LIOU et al., 1997). No tecido encefálico, o PQ atravessa barreira hematoencefálica através de um transportador de aminoácido neutro, tais como transportadores de aminoácidos do sistema L (LAT-1) (SHIMIZU et al., 2001), atuando de maneira seletiva sobre os neurônios dopaminérgicos (ND) (MANNING-BOG et al., 2003). Em células neuronais há um número menor de enzimas do citocromo P450 do que em outros tecidos, no entanto há outras enzimas capazes de iniciar o ciclo redox do PQ (conforme descrito acima), as quais foram identificadas em microssomos, membrana plasmática, e em componentes citosólicos que incluem sintase de óxido nítrico, NADPH-oxidase e tioredoxina redutase (BONNEH-BARKAY et al., 2005). Além disso, o PQ também pode interagir com as mitocôndrias, sendo transportado para a membrana interna da mitocôndria por transportadores dependentes de potencial. Uma vez dentro da mitocôndria o PQ atua sobre o complexo III da cadeia respiratória promovendo a produção de espécies reativas de oxigênio (EROS), tais como: $O_2^{\bullet-}$ e H_2O_2. Na mitocôndria as EROS são formadas através do ciclo redox dependente do potencial de membrana mitocondrial (CASTELLO et al., 2007).

Flutuações nos níveis de EROS podem desempenhar importantes funções de regulação, mas quando presentes em altas quantidades podem causar graves danos à ácidos nucléicos, proteínas e lipídios (FINKEL; HOLBROOK, 2000). O estresse ocasionado pela desregulação redox celular é provocado pela produção excessiva de EROS, tais como $O_2^{\bullet-}$, H_2O_2 e radical hidroxila ($^{\cdot}OH$). O aumento de EROS junto com a ineficiência nos sistemas de defesa antioxidante, representado por enzimas e componentes não-enzimáticos configuram o estado de estresse oxidativo. O sistema de defesas antioxidantes incluem enzimas como superóxido dismutase (SOD), que dismuta o $O_2^{\bullet-}$ em H_2O_2, e também enzimas como a catalase (CAT) e glutationa peroxidase (GPx), que se neutralizam o H_2O_2, impedindo a formação de $^{\cdot}OH$ pela reação de Fenton (COSSU et al., 2000). Além dessas enzimas, as diferentes isoformas da enzima glutationa-S-transferase (GST) são importantes na detoxificação de xenobióticos e também atuam junto com o sistema de defesa antioxidante (FONSECA et al., 2010). As isoformas de GSTs são uma família de enzimas de fase-II que conjugam compostos electrofílicos (hidrocarbonetos aromáticos policíclicos - HAPs) com glutationa (GSH) e participam na proteção celular contra os efeitos tóxicos de uma variedade de xenobióticos e subprodutos metabólicos oxidados (HAYES et al., 2005). Outro fator que contribui para o estado de estresse oxidativo é a ocorrência de baixos níveis de cofatores dessas enzimas como glutationa (GSH) (BERRY et al., 2010). Danos oxidativos podem ser avaliados pela peroxidação lipídica, um dos principais contribuintes para a perda da função da

célula no estado de estresse oxidativo e frequentemente determinado pelos níveis de malondialdeído (MDA), um marcador de dano oxidativo de membranas celulares, (HERMES-LIMA et al., 1995)

Estudos têm demonstrado efeitos pró-oxidantes de agroquímicos em modelos experimentais. Danos oxidativos sobre moléculas e estruturas celulares são associados à fisiopatologia de muitas doenças neurodegenerativas. Desse modo, a exposição crônica a agroquímicos é considerada um fator de risco para desenvolvimento dessas doenças (BALDERESCH et al., 2003). O PQ tem sido utilizado como indutor de parkinsonismo em modelos animais. Devido a sua especificidade com o mesmo transportador aminoácido neutro utilizado por L-valina e L-dopa nos ND (MANNING-BOG et al., 2003). A morte dos ND é a principal patologia da doença de Parkinson (DP), levando às alterações locomotoras característicos da doença como tremor em repouso, bradicinesia e rigidez muscular (OBESO et al., 2010; SPILLANTINI et al.,1997). A DP também apresenta sinais não motores, relacionados com perturbações psiquiátricas e cognitivas, como comprometimento do sono, ansiedade, depressão, cognição prejudicada e demência (CHAUDHURI et al., 2006; BRAAK et al., 2004). Os mecanismos patológicos que levam a perda dos ND ainda não são totalmente compreendidos, no entanto evidências apontam a disfunção mitocondrial, disfunção ubiquitina-proteassoma, alterações na homeostase do cálcio e estresse oxidativo como os principais fatores (SCHÜLE et al., 2010). No entanto, ainda não são totalmente esclarecidos os efeitos da exposição crônica à PQ tanto em ecossistemas aquáticos e também como fator de risco para o desenvolvimento de DP.

Organismos modelo para estudos *in vivo* de doenças neurodegenerativas vêm sendo propostos recentemente (TORRÃO et al., 2012). Dentre esses modelos animais, destacam-se peixes como *Danio rerio* (Fig. 2), também conhecido por peixe zebra ou “paulistinha”, um pequeno teleósteo de 3-4 centímetros (cm) pertencente à família *Cyprinidae*. O estudo dessa espécie começou no final da década de 60 por George Streisinger através de técnicas de mutagênese (GRUNWALD; EISEN, 2002). Este espécie foi de grande valia para o avanço no conhecimento da embriogênese e ciclo de vida dos vertebrados, devido à presença de ovos translúcidos, abundante prole e rápido desenvolvimento (DAHM; GEISLER, 2006). Além disso, o instituto Sanger através de técnicas de sequenciamento do genoma do peixe zebra identificou genes evolutivamente conservados entre esta espécie com os genes de mamíferos, apresentado 70% de genes homólogos aos humanos (STERN; ZON, 2003). Outras características, como anatomia básica do encéfalo, sistemas de neurotransmissores similares aos de mamíferos (PANULA et al., 2006) e atributos práticos como fácil manejo, pequeno

espaço requerido, baixo custo de manutenção, alta reprodutividade, pequeno porte e baixo custo para triagens em larga escala (LITTLETON; HOVE, 2013), fazem com que o peixe zebra seja atraente para estudos de laboratório. As vantagens no uso desse modelo animal assemelham-se as oferecidas pelos modelos de culturas de células, mosca da fruta (*Drosophila melanogaster*) e *Caenorhabditis elegans*. No entanto, proporcionam maior complexidade nas interações bioquímicas e maior similaridade com mamíferos, se aproximando do modelo de roedores, mas oferecendo vantagens na manutenção e nos custos. Por apresentar um tamanho relativamente pequeno e fácil absorção de compostos adicionados diretamente à água ou por via intraperitoneal, a quantidade dos reagentes a serem testados passa a ser significativamente menor. Desse modo, otimizando o uso das drogas de estudo e também com uma menor produção de resíduos (GOLDSMITH, 2004). Pode-se destacar, com isso, que o peixe zebra é um animal que combina a relevância de ser um vertebrado com a escala de um invertebrado.

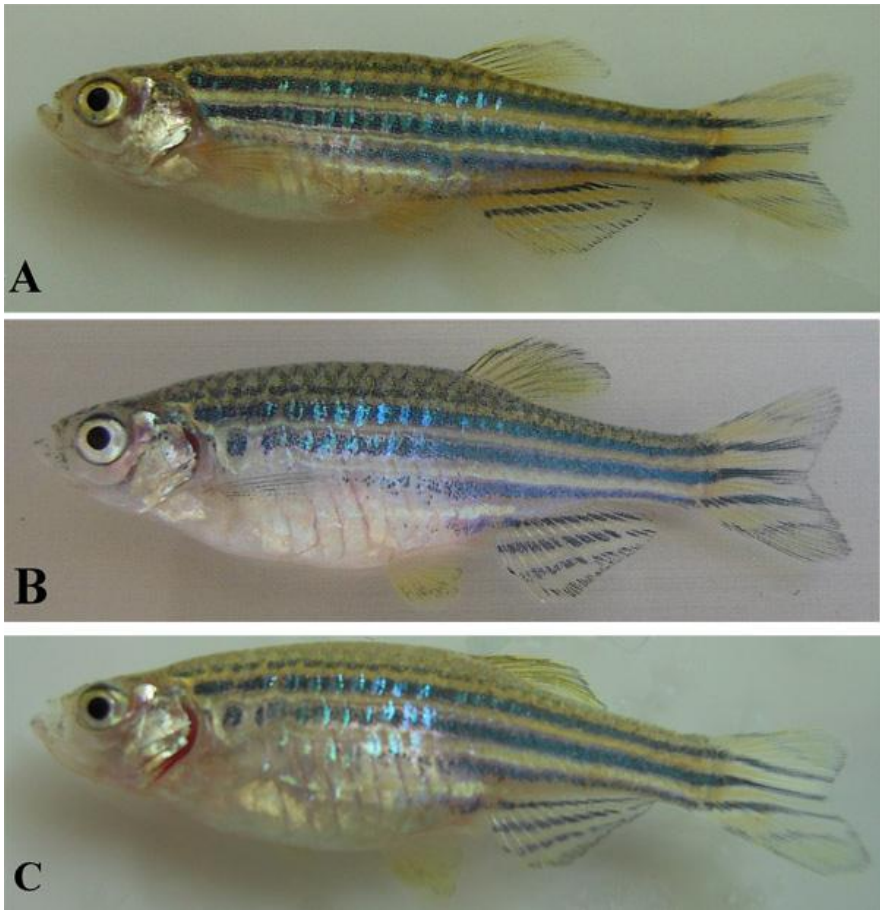

FIGURA 2 – Exemplares de *Danio rerio* tipo selvagem adulto macho (A) e fêmea (B, C) (AVDESH et al., 2012).

A utilização do peixe zebra como modelo animal vem se expandido para várias áreas do conhecimento, como bioquímica, neurociência, farmacologia e biologia do comportamento

(MAXIMINO et al., 2010; RICO et al., 2011; KALUEFF et al., 2013). Peixe zebra tem se mostrado útil em pesquisas biomédicas relacionadas a doenças humanas e em estudos relacionados com as bases moleculares da neurobiologia (VASCOTTO et al., 1997; GUO, 2004). Nesse sentido, a identificação dos principais sistemas de neurotransmissão existentes em humanos já foi realizada em peixe zebra (RICO et al., 2011). Na última década, foram desenvolvidos estudos avaliando características comportamentais do peixe zebra (CACHAT et al., 2010; MAXIMINO et al., 2010; PIATO et al., 2011; BLASER, ROSEMBERG, 2012; GERLAI et al., 2000). O peixe zebra adulto apresenta um repertório comportamental bastante complexo, onde a exposição a agentes estressores pode evocar medo ou comportamento tipo-ansiedade facilmente quantificáveis através de exploração reduzida, aumento da escototaxia (aversão a ambientes claros), geotaxia (resposta de mergulho), tigmotaxia (preferência pela periferia do tanque), congelamento (imobilidade junto a aumento da frequência respiratória), avaliação de risco (entrada parcial no compartimento claro e rápido retorno para o compartimento escuro), movimentos erráticos (repentinas mudanças bruscas de velocidade e direção do nado), preferência por coespecíficos e/ou agressividade (EGAN et al., 2009; MAXIMINO et al., 2010; ROSEMBERG et al., 2011; BLASER, ROSEMBERG, 2012; KALUEFF et al., 2013).

Alterações comportamentais podem estar relacionada com a inibição da enzima acetilcolinesterase (AChE), cuja ação é crucial na propagação do impulso nervoso. A AChE inativa a ação do neurotransmissor acetilcolina (ACh) hidrolisando-o em acetato e colina. O neurotransmissor ACh é bastante difundido por todo o sistema nervoso. No sistema nervoso periférico, essa enzima é liberada por todos os neurônios motores do sistema nervoso somático e também no sistema nervoso autônomo. A inibição da AChE leva à perda da homeostase colinérgica, podendo causar inúmeros distúrbios neuroquímicos, neurocomportamentais e neuromorfológicos. Dentre as alterações provocadas por esses compostos estão: mudança na conformação dos receptores colinérgicos e na densidade dos mesmos, citotoxidade, vacuolização citoplasmática, aumento no espaço intercelular, apoptose, alteração em cascatas de sinalização celular, diminuição no número de células cerebrais, na comunicação sináptica, proliferação da célula da glia, comprometimento na inervação colinérgica em diferentes áreas cerebrais. Tais modificações podem refletir em desordens neuropsiquiátricas não motoras, tais como alterações locomotoras, ansiedade, depressão, perda de memória e déficits de aprendizagem (EYER, 1995).

Ao longo da última década, o peixe zebra tem sido utilizado com sucesso em pesquisas que abordam os efeitos de drogas nas mais diversas respostas comportamentais. BRETAUD et al. (2004) e BORTOLOTTO et al. (2014) avaliaram os efeitos de diferentes concentrações de PQ em peixe zebra visando um novo modelo para o estudo da DP. O

método de exposição crônica por imersão não mostrou alterações comportamentais e nem em parâmetros bioquímicos em adultos ou larvas de peixe zebra (BRAUTAD et al., 2004). No entanto, quando PQ foi administrado por via intraperitoneal e em concentrações maiores do que as utilizadas no método por imersão, significativas alterações comportamentais e bioquímicas foram observadas (BORTOLOTTO et al., 2014). Entretanto, parâmetros comportamentais como agressividade e tipo ansiedade ainda não foram totalmente elucidados nesse modelo de parkinsonismo, assim como respostas do sistema de defesa antioxidante e viabilidade celular. Com base nos resultados, pretendemos avaliar as alterações em parâmetros comportamentais e resposta do sistema de defesa antioxidante e danos em células no tecido encefálico, além dos efeitos pró-oxidativo do PQ sobre membranas celulares em encéfalo de peixe zebra tratatos cronicamente com PQ. Corroborando com o modelo já proposto de parkinsonismo, peixe zebra-PQ, e também estabelecendo marcadores de intoxicação por este herbicida.

2. OBJETIVOS

2.1 Objetivo Geral

Avaliar parâmetros comportamentais e bioquímicos de peixes zebra tratados cronicamente com paraquat por via intraperitoneal.

2.2 Objetivos específicos

- Avaliar alterações nos parâmetros comportamentais motores e não motores em peixe zebra após tratamento com PQ.
- Avaliar a viabilidade celular do tecido encefálico de peixes zebra
- Avaliar a possível peroxidação lipídica
- Determinar a atividade das enzimas catalase, glutationa peroxidase, glutationa-S-transferase e acetilcolinesterase
- Determinar os níveis de EROS e tióis não proteicos (NPSH)

3. MANUSCRITO

Tratamento crônico com herbicida paraquat induz danos cerebrais, aumento das defesas antioxidantes e alterações comportamentais no modelo experimental de peixe zebra

Chronic treatment with paraquat herbicide induces brain injury, increase antioxidant defenses and behavior alterations in experimental model zebrafish

Mauro Nunes, Talise Müller, Marcos Braga, Vanessa Quadros, Aline Marins, Cíntia Rodrigues, Charlene Menezes, Denis Rosemberg, Vania Lucia Loro*.

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Chronic treatment with paraquat herbicide induces brain injury, increase antioxidant defenses and behavior alterations in experimental model zebrafish

Mauro Nunes, Talise Müller, Marcos Braga, Vanessa Quadros, Aline Marins, Cíntia Rodrigues, Charlene Menezes, Denis Rosemberg, Vania Lucia Loro*.

Corresponding author:
*Drª. Vania Lucia Loro, Pós-Doctor
Biochemistry and Molecular Biology Department
Federal University of Santa Maria
97105.900 - Santa Maria, RS, Brazil
Phone: 55-55 3220-9456 Fax: 55-55-3220-8240
e-mail: vania.loro@gmail.com

ABSTRACT

The exposure to herbicide paraquat (PQ) is a risk factor for the development of neurodegenerative diseases, due to its ability to induce changes in dopaminergic system and mitochondrial dysfunction, leading to redox imbalance. The aim of this study was to evaluate the actions of PQ in behavioral functions of adult zebrafish and its influence on oxidative stress biomarkers and mitochondrial viability in brain samples. PQ (20 mg/kg) was administered intraperitonially with six injections for 16 days (one injection every 3 days). PQ-treated group showed a significant decrease in time spent in bottom section and a shorter latency to enter the top area in the novel tank test. Moreover, PQ-exposed fish showed a significant decreased in the number and duration of risk assessment episodes in the light-dark test, as well as an increase in the agonistic behavior in the mirror-image stimulus (MIS) test. PQ induced brain damage by decreasing mitochondrial viability. Concerning the antioxidant defense system, PQ increased catalase (CAT), glutatione peroxidase (GPx) and the non-protein sulfydryl content (NPSH), but no increases in ROS formation and lipid peroxidation were detected. We demonstrated, for the first time, that PQ induced an increase in aggressive behavior, decreasing nonmotors patterns associated to defense behaviors. We suggest that fish exposed to PQ in natural environment may be more susceptible to predation, since it more exposes to danger. The alterations in defense and aggressive behavior may be related with decrease in neuronal cells viability.

Keywords: paraquat · zebrafish · aggression · anxiety · chronic exposure.

1. INTRODUCTION

The aquatic ecosystems are one of the main targets of agrochemicals, due to its susceptibility to accumulate agrochemical residues [1]. The herbicide paraquat (1,1′-dimethyl-4,4′-bipyridinium dichloride; PQ) is commonly used in agriculture presenting a potential toxicity in several species, including humans [2-4].

PQ acts mainly in dopaminergic neurons (DN), due to its specificity with the same neutral amino acid transporter used by L-valine and L-dopa [5]. Additionally, DN are sensitive to oxidative stress triggered by producing superoxide anions through redox cycling induced by PQ [6]. PQ is absorbed as paraquat dication (PQ^{2+}) and crosses the inner mitochondrial membrane by a dependent-potential carrier [7]. In the mitochondrial matrix, PQ^{2+} is reduced to PQ radical monocation (PQ^{+}) by the complex I of the respiratory chain of mitochondria in mammals. The PQ^{+} radical reacts rapidly with oxygen to generate the anion superoxide ($O_2^{\bullet-}$), which undergoes nonenzymatic dismutation to form hydrogen peroxide (H_2O_2). In the presence of ferrous iron (Fe^{2+}), the hydroxyl radical ($^{\bullet}OH$) may be formed by Fenton's reaction, which is highly reactive to biomolecules [8].

The interactions between PQ and DN have been used in experimental models of Parkinson [8]. Moreover, the redox imbalance triggered by PQ, due to mitochondrial dysfunction, may be associated with neuronal disorder [9]. Several studies associate the neurodegenerative diseases development with redox imbalance, since brain is highly vulnerable to oxidative stress due to its high O_2 consumption, its modest antioxidant defenses and its lipid-rich constitution [10-11]. In rats chronically treated with PQ, significant alterations in anxiety-like phenotypes [12-13], loss of olfactory discrimination, locomotor and motor deficits were observed [14]. The anxiety responseof mice in the light-dark box is accompanied by significantly high levels of intracellular ROS in blood cells [15]. Bortolotto et al. [16] and Bretaud et al. [17] proposed a model that mimics some phenotypes observed in PD for adult zebrafish (*Danio rerio*) exposed chronically to PQ.

The zebrafish is a prominent model used in neuroscience studies, presenting evolutionary conserved brain functions and neurotransmitter systems [18]. In addition, other advantageous features such as low-cost, easy maintenance, and abundant offspring [20-24] make zebrafish an alternative and complementary vertebrate model. This species is a suitable organism to neurobehavioral studies due to its well-described behavior patterns [19]. However, behavioral parameters such as aggressiveness and anxiety-like have not been fully elucidated in this model of parkinsonism, as well as the response of antioxidant defense

system and cell viability. Therefore, the goal of present study was to investigate the possible effects of chronic treatetment with PQ in motor and nonmotor patterns, and the relation between brain damage and biomarkers with behavior changes in zebrafish model. In order to establish biomarkers for future biomonitoring studies of aquatic environments contaminated with this herbicide.

2. MATERIALS AND METHODS

2.1 Animals Management

We used adult zebrafish (4–6 months-old) of short fin (SF) wild-type of both sexes. The fish were obtained from a commercial supplier (Hobby Aquários, RS, Brazil) weighing 0.5 ± 0.1 grams (g) and measuring 3.0 ± 1.0 centimeters (cm) of length. The fish were maintained in aerated and temperature-regulated (27 ± 1 ºC) water in 40 liters (L) aquaria under constant mechanical, biological, and chemical filtration containing at a density of three animals per liter. Illumination was provided by ceiling-mounted fluorescent light tubes set under a light/dark photoperiod cycle of 14/10 hours (h) (lights on at 7:00 am). All animals used in this study were experimentally naïve and fed alcon BASIC™ Flakes (Alcon, Brazil) twice daily. Before treatment, fish were acclimated for 15 days in aquarium of 35 L with partitions filled with non-chlorinated water treated with AquaSafe™ (Tetra, VA, USA). This aquarium allowed the maintenance of 16 animals individually separated, kept in the same aerated and heated home-tank water. Moreover, fish were able to maintain direct visual contact with others to minimize the stress of isolation, allowing the identification and observation of each animal. Animal experimentation in this study fully adhered to the National Institute of Health Guide for Care and Use of Laboratory and the protocols were approved by the Ethics Commission on Animal Use of the Federal University of Santa Maria under process number 046/2014.

2.2 Experimental Groups

After the acclimation period, fish were separated into two experimental groups: PQ-treated group (PQ) and untreated group (control, CTL). Each group were consisted by 60 animals and a total of 120 animals were used. CTL group was treated with only saline

solution 0.9 %. PQ group was treated with 20 mg/kg of PQ (Methyl viologen dichloride hydrate; Sigma®) diluted in saline solution 0.9 %.

2.3 Drug Administration

PQ and saline were administrated intraperitoneally, one injection every 3 days for 16 days totalizing six injections as described by Bortolotto et al. [16]. For each injection a total volume of 10 µL was applied. Before each injection, the zebrafish were anesthetized 0.1 g/L tricaine (3-amino benzoic acidethylester; Fluka). The fish were kept in aquarium with partitions as described above during the treatment period.

2.4 Neurological Behavioral Measurements

The behavioral tests were performed twenty-four hours after the last injection between 10:00 am and 4:00 pm. Was used a number of 25 animals in each behavioral test. The apparatuses were filled with nonchlorinated water (27 ± 1 ºC) and the experimental procedures were performed on a stable surface with all environmental distractions kept to a minimum. The behavioral activities of zebrafish were recorded for a single session of 300 seconds (s) except for the observation novel tank test (Experiment 1), in which the behavior was analyzed during the exposure period (360 s). The swimming location of zebrafish was recorded using a webcam connected to a laptop at a rate of 30 frames/s using appropriate video-tracking software (ANY-maze™, Stoelting CO, USA). After, the animals were carefully removed from their aquarium and euthanized as described below.

2.4.1 Experiment 1: novel tank test

Locomotor and exploratory activities were analyzed in the novel tank test, which may reflect habituation to novelty stress [20,25,26]. After the treatment period, zebrafish (*n* =25 in each group) were individually placed in a novel apparatus filled with 2 L water and their swimming behavior was recorded. The apparatus was virtually divided in two horizontal sections (bottom and top) to assess the vertical exploration by the following endpoints: number of entries and time spent (s) in bottom area, latency (s) to enter the top, number of entries and time spent (s) in top area. Distance travelled (m), maximum speed (m/s) and absolute turn angle (º) were used to measure locomotor and motor patterns. For measuring of

fear/anxiety-related behaviors we determined the number and duration (s) of freezing bouts as well as the number and duration (s) of erratic movements.

2.4.2 Experiment 2: light-dark test

The light-dark test was carried out based in the method described elsewhere by Maximino et al. [27]. The test apparatus consisted in tank (15 × 10 × 25 cm, height × depth × length) divided into two equally sized dark and lit areas and filled with 2 L water. In this test, we used 25 animals per group and each animal was placed initially at the lit area. During the 300 s of trial, the number of entries and time spent (s) in lit area, number and duration (s) of freezing bouts, latency (s) to enter the dark area and number of risk assessments events were measured. Risk assessments was defined as a partial entry in the white compartment (i.e. the pectoral fin does not cross the midline) associated to a fast return to the dark compartment [27-28].

2.4.3 Experiment 3: Aggression test

The aggression test was performed using the inclined mirror-image stimulus (MIS) [29]. Fish (*n* =25 in each group) were individually netted into a small experimental tank (15 × 10 × 25 cm, height × depth × length). The apparatus was filled with 2 L water. A mirror was placed inclined at 22.5° to the back wall of the tank so that the left vertical edge of the mirror was touching the side of the tank and the right edge was further away. Thus, when the experimental fish swam to the left side of the tank their mirror image appeared closer to them. Fish were able to explore both compartments for 5 min and the following behaviors were determined: number of entries and time spent (s) in each section (A1, A2, A3 and A4) (figure 3C) and number and duration (s) of attacks. Entry to the left segment (A1) indicated preference for proximity to the “opponent',' whereas entry to the right segment (A4) implied avoidance. In addition, the amount of time the fish spent with aggressive display, or attack behavior, was also measured and analyzed as aggression. Aggressive display was defined as a posture during in which the fish erects its dorsal, caudal, pectoral, and anal fins, usually associated with undulating body movements and attacks. Attack behavior is a characteristic short bout of fast swimming directed towards the opponent when fish open the mouth and bite the image [29].

2.5 BIOCHEMICAL PARAMETERS

2.5.1 Tissue preparation

Twenty four hours after the last injection, zebrafish were anesthetized with 0.25g/L tricaine [30] and euthanized by punching the spinal cord behind the opercula, and brains were dissected out in ice. Brains were further washed with 150 mM saline solution, packed in microtubes and kept at -80 ºC for posteriors assays.

2.5.2 Determination of acetylcholinesterase activity (AChE)

The rate of hydrolysis of acetylthiocholine iodide (0.88 mM) was determined in a final volume of 300 µL, with 33 µL of 100 mM phosphate buffer, pH 7.5 mixed to 2.0 mM 5,5'-dithionitrobis 2-nitrobenzoic acid (DTNB). Samples (n=10 per group) containing 5 µg of protein and the reaction medium specified above were preincubated for 10 min at 25 °C. The hydrolysis of acetylthiocholine iodide was monitored in a microplate reader by the formation of thiolate dianion of DTNB at 412 nm for 2–3 min (intervals of 30 s) [31]. Controls without the homogenate preparation were performed in order to determine the non-enzymatic hydrolysis of the substrate. AChE activity was expressed as µmol thiocholine (SCh)/h/mg of protein.

2.5.3 Measurement of formazan production

The mitochondrial viability was evaluated by 2,3,5-triphenyltetrazolium chloride (TTC) staining that quantifies the level of formazan produced by mitochondrial dehydrogenase activities in living tissues [32]. After the treatment, the animals (n=7 per group) were anesthetized and euthanized as described above, and brains were removed. The protocol used was the same as described previously by Braga et al. [33]. Results were expressed in absorbance per tissue dry weight (g), which were normalized as a percentage in relation to the control.

2.5.4 Lipid peroxidation estimation assay

Lipid peroxidation was estimated by thiobarbituric acid-reactive substance (TBARS) production, which is widely performed for measurement of lipid redox state [34]. Briefly, two zebrafish brains were homogenized in 0.3 mL phosphate buffer saline (PBS) pH 7.4, containing: NaCl (137 mM), Na_2HPO_4 (10.1 mM), and KH_2PO_4 (1.76 mM). The homogenate (n=11 per group) was initially centrifuged (700 × *g* for 5 min, 4º C). Then, supernatant samples (80–100 µg protein) were mixed with 600 µL of 15% trichloroacetic acid (TCA) and centrifuged (10.000 × *g*, 10 min). Supernatants (100 µL) were mixed with 100 µL of 0.67% thiobarbituric acid (TBA, 4,6-Dihydroxypyrimidine-2- thiol) and heated at 100 °C for 30 min. TBARS levels were determined by the absorbance at 532 nm using malondialdehyde (MDA) reaction with TBA. MDA was used as standard and results were expressed as nmol MDA/mg of protein.

2.5.5 Reactive oxygen species (ROS)

The ROS levels were measured using the fluorescent dye 2,7-dichlorofluorescein diacetate (DCFDA) as described by Ali et al. [35]. A whole zebrafish brain (n=7 per group) was homogeneized in 50 mM Tris HCl (pH 7.5) buffer and centrifuged (3.000 × *g*, 10 min at 4 ºC). Supernatants (0.3–0.5 mg of protein) were mixed with 0.1 mM 2',7'-dichlorofluorescein-diacetate (DCFH-DA). ROS levels were determined by fluoresce at emission (570 nm) and excitation (545 nm) using dichlorofluorescein (DCF) as standard. Results were expressed as µmol DCF/mg protein.

2.5.6 Antioxidant enzymes

Antioxidant enzymes measurements were considered n=7 per group. Brains were homogenized in 20 mM potassium phosphate buffer, pH 7.5 (10 brains/mL buffer) and centrifuged at 10.000×g for 10 min at 4 ºC. Catalase (CAT) activity was assessed by measuring the rate of decrease in H_2O_2 absorbance at 240 nm by ultraviolet spectrophotometry [36]. The assay mixture was consisted by 2 mL potassium phosphate buffer (50 mM, pH 7.0), 0.05 mL H_2O_2 (0.3 M) and 0.02 mL homogenate (20 – 30 µg of protein). The results were expressed as nmol/min/mg of protein. Brain glutathione peroxidase (GPx) activity was assessed by ultraviolet spectrophotometry following the rate of NADPH

oxidation at 340 nm by the coupled reaction with glutathione reductase [37]. The assay mixture consisted of potassium phosphate buffer (100 mM, pH 7.0), 1 mM NaN_3, 1 mM reduced glutathione (GSH), 0.15 mM NADPH and homogenized tissue (5 µL, 40 – 60 µg of protein). The reaction was started by the addition of 30 µL 0.4 mM H_2O_2 totalizing a final volume of the reaction mixture of 300 µL in a microplate reader. The specific activity was determined using the extinction coefficient of 6.22 mM/cm and expressed as nmol/min/mg of protein. Glutathione S-transferase (GST) activity was analyzed according to Habig et al. [38]. The assay mixture contained 1mM 1-chloro-2, 4-dinitrobenzene (CDNB) in ethanol, 10 mM GSH, 20mM potassium phosphate buffer (pH 6.5), and 50 µl of the tissue homogenates (40 – 60 µg of protein). Enzyme activity was calculated from the changes in absorbance at 340 nm using a molar extinction coefficient of 9.6mM/cm. One unit GST activity was defined as the amount of enzyme required to catalyze the conjugate on of 1mol CDNB with GSH/min at 25 ºC. The activity was expressed as µmol GS-DNB/min/mg protein. All experiments were performed in triplicate.

2.5.7 Non-protein thiols levels (NPSH)

Two zebrafish brains were homogenized in 250 µL Tris HCl 50 mM (pH 7.5) followed by centrifugation at 700 × g for 5 min at 4 ºC. An aliquot of supernatant (100 µL) was further mixed with 100 µL of 10% trichloroacetic acid (TCA) and centrifuged (3.000 × *g*, 10 min at 4 ºC). Supernatants (60 – 80 µg of protein) were mixed with DTNB (0.01 M dissolved in ethanol) and the intense yellow color developed was measured at 412 nm after 1 h [39]. Results were expressed as nmol SH/ mg of protein.

2.5.8 Protein determination

Protein was determined by the Coomassie blue method using bovine serum albumin as standard. Absorbance of samples was measured at 595 nm [40]. All experiments were performed in duplicate.

2.6 STATISTICS

Normality of data and homogeneity of the variances were analyzed by Shapiro-Wilk and Kolmogorov–Smirnov tests, respectively. Data were expressed as mean ± standard error

of the mean (S.E.M.) and analyzed by unpaired Student's *t* test. The significance level was set at $p \leq 0.05$.

3. RESULTS

After PQ exposure, fish were individually placed in the behavioral apparatuses. In the novel tank test (Figure 1), PQ did not significantly alter total distance travelled, transitions to upper section and absolute turn angle in comparison to untreated group (Figure 1A). Although zebrafish exposed to PQ did not present significant changes in the number and duration of freezing, a decrease in the duration of erratic movements was observed (Figure 1B). Considering the vertical activity (Figure 1C), PQ decreased the time spent ($p<0.01$) in bottom area. A significant decrease in the latency to enter the top ($p<0.01$) was observed in fish exposed to PQ, which also spent more time in the top area ($p<0.0001$).

In the light-dark test, no significant differences in transitions and in the time spent in lit area were detected (Figure 2A). Exposure to PQ induced a significant decrease in the number (p>0,001) and time ($p<0.01$) of risk assessment episodes (Figure 2B). The number and duration of freezing and of erratic movement episodes remained unchanged (Figure 2B).

In the MIS test, PQ-exposed fish showed a significant increase in the number ($p<0.0001$), time ($p<0.001$), and average duration ($p<0.0001$) of aggressive episodes (Figure 3A). In terms of exploration, animals exposed to PQ spent more time in A1 section (closest to the mirror) ($p <0.01$) and less time in A4 section (farthest to the mirror) ($p<0.001$). Additionally, CTL fish performed more entries in the A2 section when compared to PQ group ($p < 0.01$). When the PQ-exposed animals were in the proximal sections of the test apparatus (A1 and A2), the number and duration of attack to the opponent image were higher than the values obtained from CTL ($p< 0.001$). No significant alterations in aggression-related parameters were observed in A2 section (Figure 3C). The construction of representative ethograms confirmed the main alterations in behavioral profile of zebrafish after PQ exposure in the novel tank (Figure 1D), light-dark (Figure 2C), and in the MIS tests (Figure 3D).

No significant difference in AChE activity between groups was observed (Figure 4A). A significant decrease in TTC staining was observed in PQ group when compared to CTL ($p< 0.01$) (90.8% of the absorbance of the control group, respectively) (Figure 4A). The ROS formation did not significantly change in both groups (Figure 4B). However, PQ group demonstrated a significant decrease in TBARS levels when compared to CTL ($p<0.0001$) (Figure 4B). Additionally, fish treated with PQ presented a significant increase in CAT and

GPx activities when compared to CTL control group ($p<0.01$) (Figure 4C). Fish exposed to PQ also showed a significant increase in GST activity ($p<0.0001$) (Figure 4C).

4. DISCUSSION

The increase in agrochemical use have been triggered several studies of effects of these contaminants effects in environment and human health. Among the agrochemicals most studies, the exposure to herbicide PQ have been associated with changes in behavior, biochemical and neurochemical patterns in rodents, fish and *Drosophila* models [3-6,8,12-16, 41-45]. The analysis of the behavioral profile is an effective method to characterize the effects of different compounds on swimming activity of fish [20]. Bretaud et al. [17] showed no difference in behavior and neurochemical patterns in adults zebrafish exposed to 5 mg/L of PQ for 4 weeks via immersion. When adults male zebrafish were treated with PQ (10 and 20 mg/kg) by intraperitoneal injection, according to the chronic treatment model described by Bortolotto et al. [16], alterations in locomotor and neurochemical patterns may be observed. In the present study, the results clearly demonstrate, for the first time, that zebrafish exposed to repeated i.p. injections of the hercide PQ exhibit alterations in non-motor patterns, such as increase in aggressive behavior and decrease in defense patterns behaviors.

Alterations in non-motor behaviors may be usually associated with psychiatric disorders [46-48] and neurodegenerative disease [49-50]. The adult zebrafish presents a complex behavioral repertoire andexposure to stressors can evoke fear or standard anxiety-like through behavior easily quantifiable such as, reduced operation, increased escototaxia, geotaxia, freezing, risk assessment and erratic movements [25]. We analyzed non-motor patterns associate with anxiety in the novel tank and in the light-dark tests. In the novel tank test, fish did not show alterations between PQ and CTL group in locomotor patterns. However, anxiety-like behavior, such as increase in time spent and a decrease latency to enter in upper half, showing a decrease in patterns anxiety-like in zebrafish treated with PQ. This character is reinforced by a decrease in the number and duration of risk assessment episodes in PQ group related to CTL observed in light-dark test. In both tests other patterns related to anxiety-like behavior, such as freezing and erratic movements were analyzed. In these patterns no alterations was observed between groups, only a tendency to decrease in the number and time of freezing was observed in PQ group in the novel tank test. In contrast with datas showed by Bortolotto et al. [16], that showed decrease in turn angle, transitions and mean velocity in adult male zebrafish treated with PQ (20 mg/kg) for 16 days by i.p. injection

in relation to control group, our data no showed alterations in these locomotor patterns. Moreover, the decrease in anxiety behavior in the PQ group also differ of the results showed by Bortolotto et al. [16], which there was not significant difference between groups. The difference in the data of Bortolotto et al. [16] with our can be partly explained by the differences in the genotypes of the strains and differences between sexes used in each experiment.

In the literature, rats treated with PQ (10 mg/kg) weekly for a month also presented after of the first injection a decrease in anxiety-like behavior [16]. In rodents, alterations in anxiety-like behavior can be triggered by reduction of vesicular monoamine transporter that preceded depressive symptoms [51]. Other studies demonstrated a relation between depletion in serotonin levels with exaggerated aggression and decreased anxiety behaviors in human [52-54], monkey [55] and mice [56]. Millan et al. [57] demonstrated that serotonergic neurons may modulate the dopaminergic and noradrenergic activity in the frontal cortex. In our study, we observed similar results in these non-motor patterns, raising the hypothesis of possible damage effects of PQ treatment on serotonergic neurons. Moreover, PQ group showed a significant decrease in brain viability. However, more studies are needed to confirm this hypothesis.

Considering the differences showed with the same PQ test, our set of tests showed that pattern of behavior on fish exposed to PQ at present study is different and pointed out some aspects at first time. For example the test of mirror (MIS) recorded that PQ exposure fish exhibited aggressive behavior. Fish spend more time in section close to the mirror, confirming the hypothesis that PQ exposure is able to induce aggressive behavior. The behavior results suggest that animals were left with a significant decrease in defensive behavior´s, which could change animals exposed to PQ in the natural environment. When the fish do not stay long time in the background and make less risk assessment may be less protected in the natural environment and this would bring many ecological consequences for fish species. The exposure PQ consequences including the reduction in assessment risk, another point observed in the present study was the increased aggressiveness that can influence the social behavior of fish group.

The significant increase in GST activity and decrease of TBARS levels also may also have been related with elimination of lipid peroxide by-products. In mammals, the GST-pi isoform conjugates unsaturated aldehydes produced during the lipid peroxidation process, and its regulation is coordinated with other antioxidant defenses [58]. After the conjugation with GSH, the lipid peroxide by-products may be eliminated from the cell by a GS-X pump

transporter [59]. In a lepidopteron insect, a purified GST omega homolog has been shown to conjugate the lipid peroxide product 4-hydroxynonenal [60]. Citosolic, mitochondrial and microsomal GST classes may act as important oxidative stress defenses, preventing lipid peroxidation [61].. Moreover, the increase of the GPx and CAT activity together with significant increase GST activity and NPSH levels, suggest a compensatory mechanism in response to oxidant effects triggered by exposure to PQ, which are reported in the literature [2-4,7]. The increase in activity of antioxidants defenses represented by enzymes and non-enzyme antioxidants and other antioxidant defenses not analyzed in this study were involved in the protection against lipid peroxidation and no alterations in ROS levels. However, brain injury can be observer in PQ group, these damage may be associated with the PQ effects about mitochondria brain cell's. PQ is absorbed as PQ^{2+} and crosses the inner mitochondrial membrane by a dependent-potential carrier [7]. Castello et al. [62] proposed that PQ^{2+} acts in complex III of the electron transport chain (ETC) promoting the increase in ROS, however mitochondrial H_2O_2 production induced by PQ^{2+} in the brain is an early event that may later initiate other cellular events, such as nonspecific ETC inactivation, microgliosis, and NADPH oxidase activation. Thus, is possible that the mitochondrial injuries will be first damage effects of the PQ.

The modulation of antioxidant defenses due to PQ exposure contributes to the non-occurrence of ROS and also to absence of lipid damage, despite the decrease in mitochondrial viability. In conclusion, the present study proposed that zebrafish chronically treated with paraquat modules alterations in nonmotor behavior patterns, as anxyet-like decrease and aggression increase, without changes in locomotor and motor behavior. In fact, the behavioral changes could indicate ecological importance of behavior test to risk assessment in fish populations.

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FIGURES

Figure 1

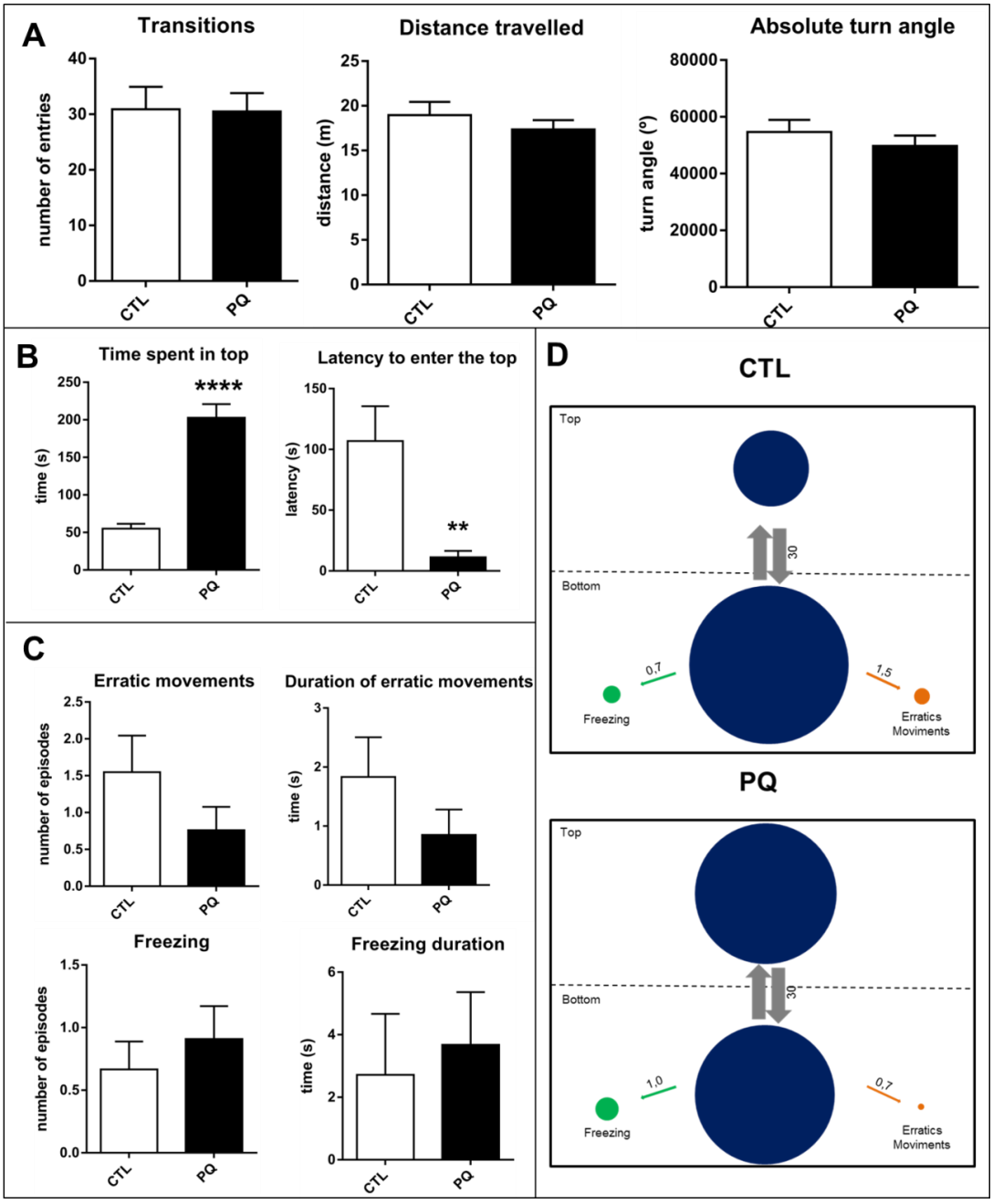

Figure 1. Effects triggered by PQ in locomotion and exploration in the novel tank test in comparison to CTL. **(A)** Locomotor and motor activities represented by the distance travelled, transitions and absolute turn angle. **(B)** Vertical exploration measured by time spent in top areas and latency to enter the top. **(C)** Number and duration of freezing bouts and erratic movements. **(D)** Rrepresentative ethograms were generated based on frenquecies and transitions between each individual behavioral activity. The diameter of each circle corresponds to the frequency of each individual behavioral activity, whereas the arrow width and direction reflect the frequency of transitions between these behaviors. Data are expressed as mean ± SEM (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, CTL = control; PQ = paraquat).

Figure 2

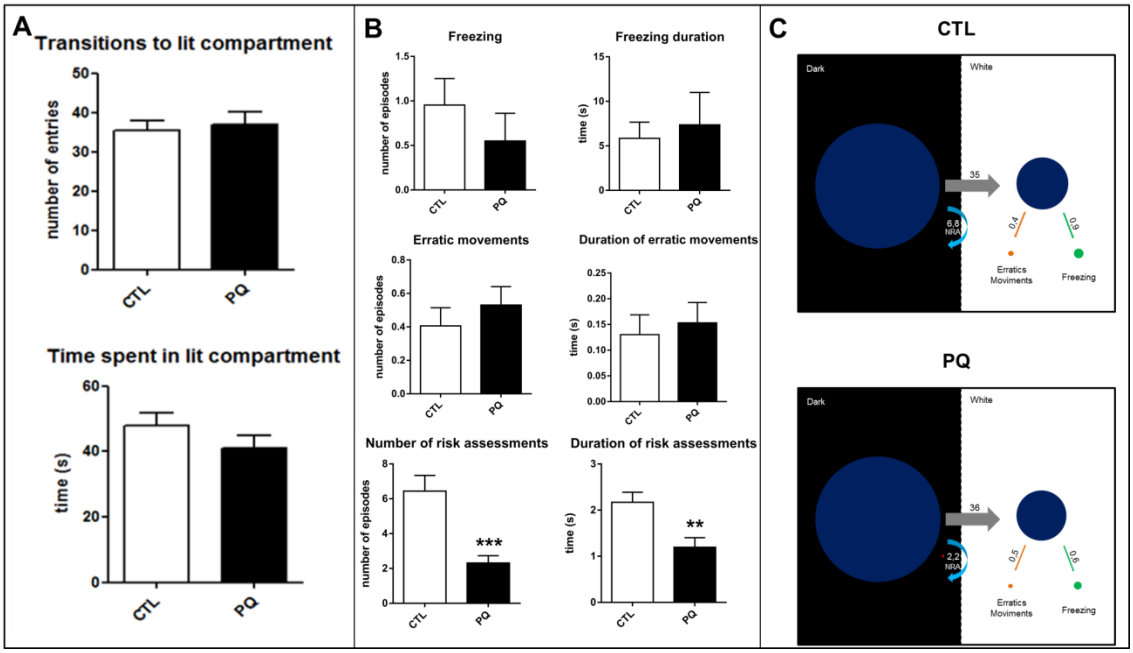

Figure 2. Effects of PQ on behavioral pattern in the light-dark test. **(A)** Transitions and time spent in lit compartment. **(B)** Number and duration of freezing, erratic movements and risk assessments. **(C)** Representative ethograms were generated based on frequencies and transitions between each individual behavioral activity. The diameter of each circle corresponds to the frequency of each individual behavioral activity, whereas the arrow width and direction reflect the frequency of transitions between these behaviors. Data are expressed as mean ± SEM (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, CTL = control; PQ = paraquat).

Figure 3

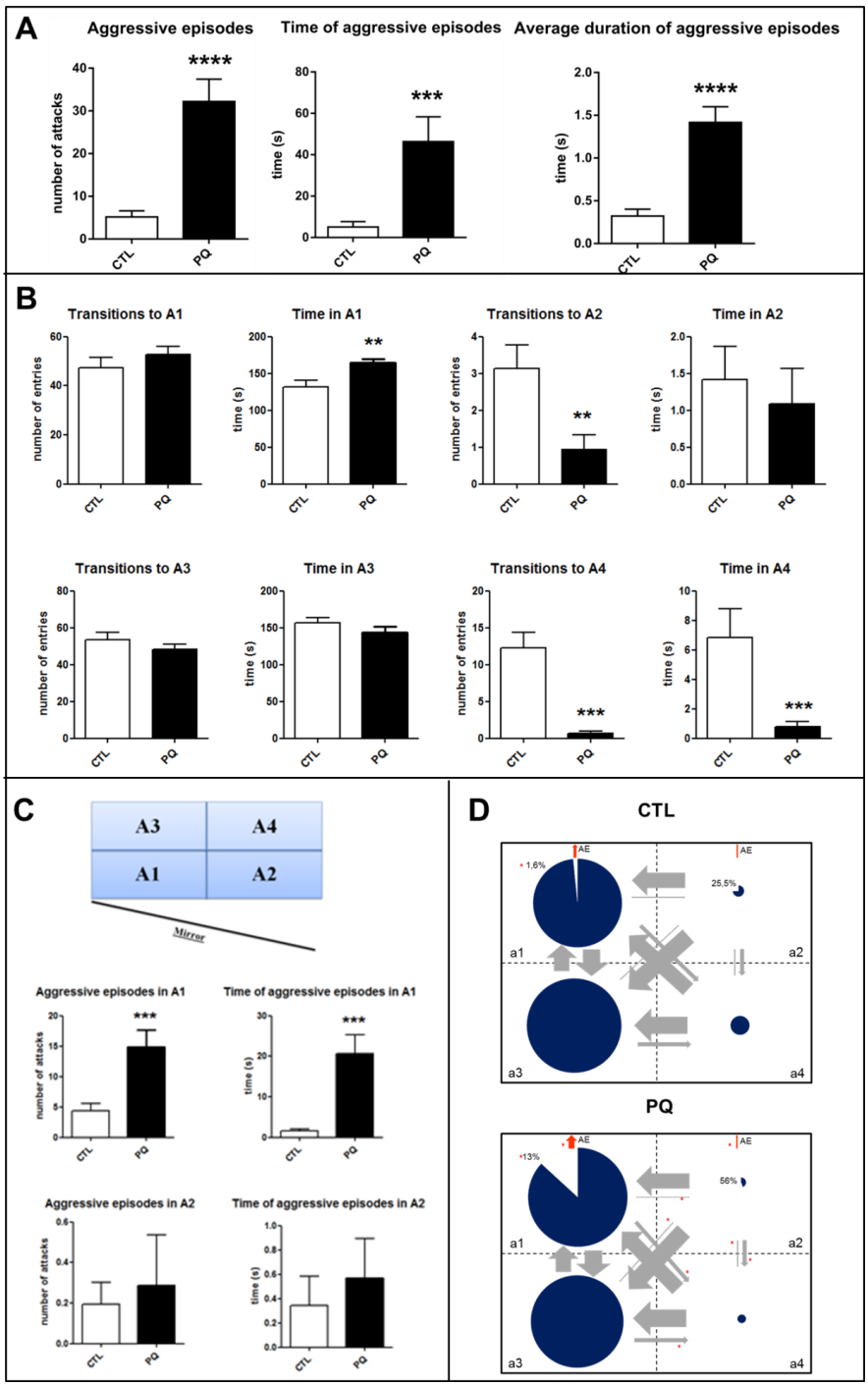

Figure 3. Effects triggered by PQ in aggression pattern in the MIS test in comparison to control groups. **(A)** Number, time and average duration of aggressive episodes. **(B)** Transition and time spend in four sections of the apparatus. **(C)** Schematic representation of the apparatus sections in relation to mirror, number and time of aggressive episodes in A1 and A2 sections. **(D)** Rrepresentative ethograms were generated based on frenquecies and transitions between each individual behavioral activity. The diameter of each circle corresponds to the frequency of each individual behavioral activity, whereas the arrow width and direction reflect the frequency of transitions between these behaviors. Data are expressed as mean ± SEM and (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, CTL = control; PQ = paraquat).

Figure 4

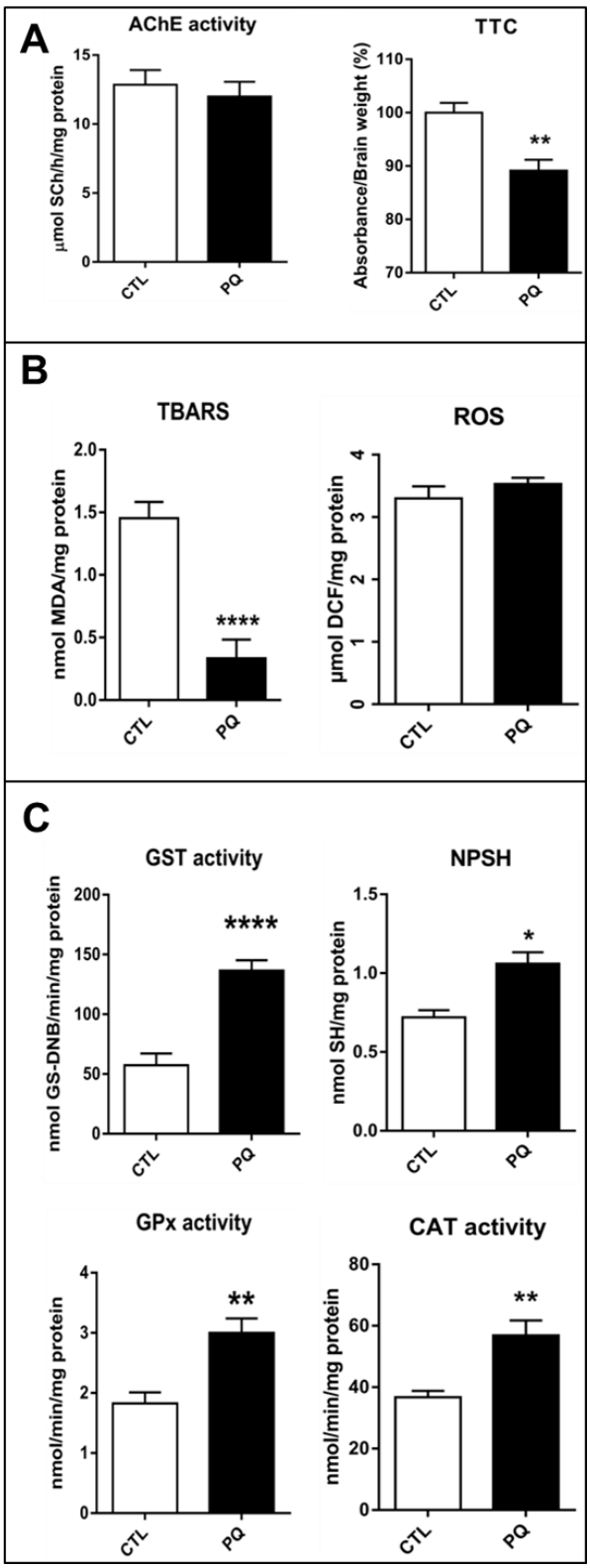

Figure 4. Effects of PQ in biochemical parameters of the zebrafish brain. **(A)** AChE activity and brain damage by measurement of formazan production (TCC). **(B)** TBARS and ROS levels. **(C)** CAT, GPx and GST activity and NPSH levels. Data are expressed as mean ± SEM (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, CTL = control; PQ = paraquat).

4. CONCLUSÕES

- A diminuição de comportamentos defensivos dos peixes zebras tratados com PQ sugere que peixes expostos a esse herbicida tentem a se expor mais ao perigo em ambientes naturais.

- O aumento do comportamento agressivo dos peixes zebras tratados com PQ indica que peixes expostos a esse herbicida pode influenciar as interações sociais entre o cardume.

- As alterações nos comportamentos agressivos e defensivos dos peixes zebra tratados com PQ demonstraram que o tratamento crônico com esse herbicida modula parâmetros comportamentais não motores.

- A não alteração de parâmetros locomotores e motores demonstra que a concentração de 20 mg/kg ou o tempo de exposição não sejam suficientes para causar danos que levem a alteração desses parâmetros.

- Os danos cerebrais observados pela diminuição na viabilidade mitocondrial das células cerebrais do grupo PQ pode estar relacionada com as alterações em parâmetros comportamentais não motores.

- A ausência de alterações na atividade da AChE indica que as alterações comportamentais observadas não são resultado do possível déficit da atividade dessa enzima.

- O aumento do sistema de defesa antioxidante enzimático e não enzimático juntamente com a não elevação nos níveis de EROS e de TBARS nos peixes tratados com PQ demonstra uma eficaz resposta do sistema antioxidante aos efeitos pró-oxidativos do PQ.

- A ativação do sistema da GST nos peixes zebras tratados com paraquat mostrou uma resposta de forma positiva na tentativa de eliminar o herbicida no cérebro e/ou na eliminação de metabólitos oriundos da peroxidação lipídica.

Os mecanismos de ação do herbicida PQ ainda são totalmente esclarecidos, assim como seus efeitos a nível comportamental. Esse trabalho mostrou pela primeira vez alterações não motoras do PQ em comportamentos defensivos e de agressividade no promissor modelo experimental peixe zebra. As elevações de marcadores do sistema de defesa antioxidante demonstrou uma resposta eficaz em combater os efeitos pró-oxidativos do PQ. No entanto, foi possível observar significativos danos em células do tecido encefálico, através da diminuição da viabilidade celular. Com nossos dados é possível especular uma possível relação entre os danos observados com as alterações comportamentais. Os dados obtidos também podem ser implicados no modelo experimental de indução de parkinsonismo em peixe zebra, corroborando com o desenvolvimento de uma ferramenta para futuros estudos de estratégias terapêuticas no tratamento da doença de Parkinson. E adicionalmente, gerando marcadores de intoxicação por PQ.

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