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A EXPOSIÇÃO AO BISFENOL A INDUZ À ALTERAÇÕES PARKINSONIANAS EM *Drosophila melanogaster* - CARACTERIZAÇÃO DE UM NOVO MODELO EXPERIMENTAL

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

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RESUMO

A constante exposição ao Bisfenol A (BFA) pode estar atribuída ao desenvolvimento de doenças neurodegenerativas. Neste sentindo, a doença de Parkinson é uma doença degenerativa, na qual a patogênese ainda não está totalmente esclarecida, e atualmente vem se atribuindo também ao estresse oxidativo causado por toxicantes ambientais. Portanto, o objetivo deste trabalho foi elucidar se a exposição ao BFA estaria envolvida com o desenvolvimento de alterações tipo parkinsonianas em *Drosophila melanogaster*. Moscas adultas foram expostas por sete dias à duas concentrações de BFA (0.5mM e 1mM), acrescentados à dieta, além de um grupo controle no qual recebeu apenas a dieta sem o BFA. Ao final do sétimo dia, foram realizados testes comportamentais de geotaxia negativa, campo aberto e de equilíbrio. Também foram analisados biomarcadores de estresse oxidativo, níveis de dopamina e mortalidade. Foi observado que o BFA induziu estresse oxidativo a nível cerebral, caracterizado pelo aumento nos níveis de malondialdeído e de espécies reativas, e reduziu a atividade das enzimas antioxidantes superóxido dismutase e catalase, e da enzima detoxificante glutationa-S-transferase. Observou-se também a diminuição da atividade metabólica mitocondrial e celular, redução da atividade da acetilcolinesterase e redução dos níveis de dopamina. Acredita-se que essas alterações estejam associadas à diminuição da atividade locomotora observada nos comportamentos de geotaxia negativa, campo aberto e equilíbrio, e também ao aumento da mortalidade das moscas expostas ao BFA. Em síntese, o BFA induziu à características Parkinsonianas em *Drosophila melanogaster*, sugerindo um novo modelo viável para estudos futuros.

Palavras-chave: estresse oxidativo, mosca, Parkinson, toxicante ambiental, Bisfenol A

ABSTRACT

Constant exposure to Bisphenol A (BPA) may be attributed to the development of neurodegenerative diseases in young people. In this sense, Parkinson's disease is a degenerative alteration, in which the pathogenesis is not well known and attributed to the oxidative stress caused by environmental toxifiers. Therefore, the objective of this study was to elucidate whether exposure to BPA would be involved in the development of parkinsonian alterations in a *Drosophila melanogaster* model, and thus to establish a new model for future studies. Adult flies were exposed for seven days at two concentrations of BPA (0.5mM and 1mM) in the diet, in addition to a control group that received the diet without BPA. At the end of the seventh day, behavioral tests of negative geotaxis, open field and equilibrium test were performed. Biomarkers of oxidative stress, dopamine levels and mortality were also analyzed. It was observed that BPA induced oxidative stress at the cerebral level characterized by increased levels of malondialdehyde and reactive species, and reduced activity of antioxidant enzymes superoxide dismutase and catalase, and detoxifying enzyme glutathione-S-transferase. There was also a decrease in mitochondrial and cellular metabolic activity, reduction of acetylcholinesterase activity and reduction of dopamine levels. These changes can be associated with decreased locomotor activity observed in negative geotaxis, open field and equilibrium behaviors, as well as increased mortality of flies exposed to BPA. In summary, the BPA induced the Parkinsonian characteristics in *Drosophila melanogaster*, suggesting a new viable model for future studies.

Key words: environmental toxifier, fly, oxidative stress, Parkinson, Bisphenol A

LISTA DE SIGLAS E ABREVEATURAS

BFA - Bisfenol A

BADGE- Éter diglicidílico de Bisfenol A

Cat- Catalase

DA- Dopamina

DP- Doença de Parkinson

GST- Glutatione-S-transferase

HPLC-DAD- Cromatografia Líquida de Alta Performance com Detector de Matriz de Diodo

LOAEL- (Lowest Observed Adverse Effect Level) Nível Mais Baixo de Efeito Adverso Observado

DP- Doença de Parkinson

EROs- Espécies reativas de oxigênio

SOD - Superóxido dismutase

MDA- Malondialdeído

PVC- Policloreto de vinila

UGT- uridina 5'-difosfo-glucuronosiltransferase

1. INTRODUÇÃO

O Bisfenol A (BFA) é o nome dado ao composto 2, 2-bis (4-hidroxifenol) (GORE et al., 2015), um fenol sintético amplamente utilizado na fabricação de policarbonato e resinas epóxi (JALAL et al., 2018). O policarbonato compõe o material de utensílios de cozinha termo resistentes, ou seja, para uso em fornos ou microondas, garrafas e copos plásticos. Já a resina epóxi está presente em revestimentos de encanamentos e latas de bebidas e de alimentos (GEENS et al., 2012; VANDENBERG et al., 2009).

Tais itens são submetidos a processos de lavagem, aquecimento e contato com variações do pH do meio, sendo esse bastante ácido ou básico, e essas condições predispõe a hidrólise das ligações éster da molécula, liberando monômeros de BFA e contaminando alimentos e bebidas (BAE et al., 2002; VANDENBERG et al., 2009). A lixiviação faz com que esses itens destinados ao contato direto com alimentos sejam importantes meios de exposição humana a toxicantes (HAIGHTON et al., 2002), sendo a exposição por via oral o principal meio de contato.

A exposição contínua da população torna-se uma questão preocupante (WHO/UNEP, 2013), visto que muitos estudos mostram que o BFA é um potente indutor de estresse oxidativo (MASUO e ISHIDO, 2011; OOE et al., 2005; QIU et al., 2016; SHIRANI et al., 2019). O estresse oxidativo é uma condição fisiopatológica associada ao desenvolvimento de diversas doenças neurológicas, destacando-se como um importante evento dos principais fatores da patogênese da Doença de Parkinson (OOE et al., 2005; TOLLESON e FANG, 2013).

Grande parte dos estudos sobre a neurotoxicidade do BFA são realizados em vertebrados, e avaliam de forma isolada alguns fatores que podem contribuir para o desenvolvimento da DP (ISHIDO e MASUO, 2014; ISHIDO et al., 2007) porém, esses estudos acabam apenas citando o BFA como um possível indutor a DP. Já os estudos com invertebrados elucidam os efeitos genotóxicos, neurodesenvolvimentais e reprodutivos causados pela exposição ao BFA (ANET et al., 2019; ATLI e ÜNLÜ 2012; ATLI, 2013; KAUR et al., 2015).

Nesse sentido, a mosca *Drosophila melanogaster* tem se destacado como uma alternativa eficiente para investigar a etiologia e aspectos iniciais de doenças neurológicas (SHULMAN e FEANY, 2003). A mosca compartilha muitas características estruturais e funcionais semelhantes aos mamíferos, o que facilita a observação do mecanismo de ação tóxica de diversos xenobióticos, tais como o BFA (NAGOSHI, 2018).

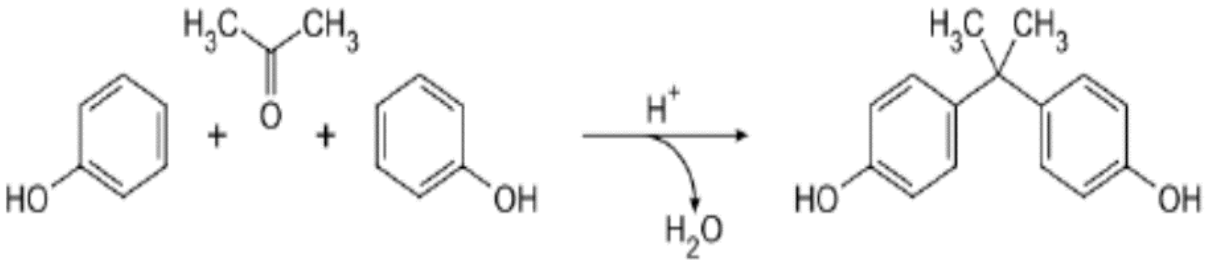
Então, considerando que a etiologia da DP não está totalmente esclarecida, sendo também condicionada a fatores extrínsecos como a exposição a toxicantes ambientais (CHIN-CHAN et al., 2015; JONES e MILLER, 2008), e a existência de resultados inconclusivos quanto a indução da DP pelo BFA, esse trabalho objetivou utilizar a mosca *Drosophila melanogaster* para elucidar os mecanismos envolvidos no desenvolvimento de alterações Parkinsonianas induzida por BFA.

2. REFERENCIAL TEÓRICO

2.1. Bisfenol A

Bisfenol A é o nome atribuído ao monômero 2,2-bis (4-hidroxi-fenil) propano, utilizado amplamente pela indústria de plásticos e resinas. Foi descrito pela primeira vez em 1891 pelo químico russo Alexander P. Dianin. Em 1905 foi sintetizado por Thomas Zincke na universidade de Marburg (Alemanha), através da condensação de duas moléculas de fenol e uma molécula de acetona na presença de um catalisador ácido (MICHAŁOWICZ, 2014; HUANG et al., 2017), conforme a Figura 1.

FIGURA 1: Síntese do Bisfenol A

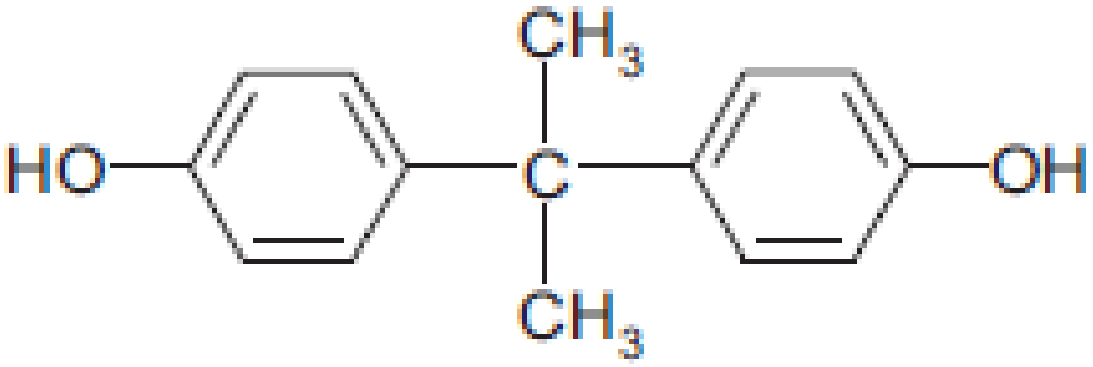


Fonte: (BESERRA, 2012)

A estrutura química do BFA, observada na Figura 2, é constituída por dois anéis fenólicos unidos por uma ponte de metil com um grupamento hidroxila diretamente ligado a extremidade de cada anel, proporcionando ao composto uma boa reatividade (MICHALOWICZ, 2014). O BFA é fisicamente sólido e de cor branca em temperatura

ambiente, seu peso molecular é 228,29 g/mol, ponto de fusão a 156˚C e de ebulição a 220˚C. Possui solubilidade maior em gordura e menor em água, de acordo com coeficiente água/octanol expressa na forma logarítima de 3,32 (log P= 3,32) (MICHALOWICZ, 2014).

FIGURA 2: Estrutura química do Bisfenol A



Fonte: (RUBIN, 2011)

Em 1930, Edward Charles Dodds constatou propriedades estrogênicas no BFA (RUBIN, 2011), que não chegou a ser comercializado como finalidade clínica (VOGEL, 2009), sendo rapidamente substituído por um estrogênio análogo mais potente, o dietilestilbestrol (RUBIN, 2011). Em 1940, foi fabricada a primeira resina comercial a base de BFA. As resinas epoxídicas são utilizadas em proteção de equipamentos metálicos, tubulações e latas de alimentos, selantes dentários e tampas de garrafas (USMAN e AHMAD., 2016).

Em 1957, o químico Hermann Schnell, descobriu que o processo de polimerização do BFA formava uma resina rígida e transparente, conhecida hoje como como policarbonato (RUBIN, 2011). O BFA possui também outras aplicações, porém menos significativas, como na produção de policloreto de vinila (PVC), selantes e resinas dentárias, papel térmico, poliuretano, poliamida e retardante de chamas tetrabromobisfenol-A (BESERRA et al., 2012).

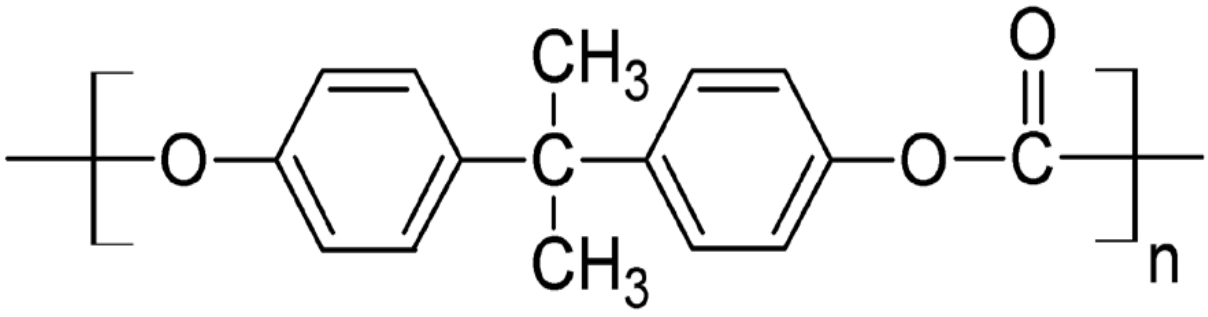
Considerada um reservatório geoquímico, a atmosfera é o local onde ocorre a interação de vários compostos químicos com os oceanos, terra e organismos vivos. Uma importante questão mundial é a origem, transporte e destino de componentes químicos, para que não ocorra a contaminação de forma indiscriminada do meio ambiente (FU e KAWAMURA, 2010). A detecção de BFA em amostras de aerossois de regiões urbanas, rurais, marinhas e polares, indica a onipresença deste monômero na atmosfera, que pode ser liberado

através da produção industrial, queima de lixos domésticos e eletrônicos a céu aberto (FU e NAWAMURA, 2010).

No Brasil, a empresa Rhodia é a única fabricante de BFA, gerando aproximadamente 25 mil toneladas por ano. Sendo de uso exclusicamente comercial, essa substância é destinada a produção de policarbonato e resina epoxi (BRUGNERA, 2009). Já a nível mundial, são produzidos anualmente aproxidadamente 8 milhões de toneladas de BFA, e estima-se que no mínimo 100 toneladas sejam liberadas na atmosfera (VANDENBERG et al., 2007). Devido a baixa adsorção de umidade e estabilidade térmica dos polímeros sintéticos de BFA (HUANG et al., 2017), 90% da produção deste monômero é destinado a fabricação de polímeros sintéticos, incluindo resinas epóxi e policarbonatos (GEENS et al., 2012).

Cerca de 80% da produção de BFA é destinada fabricação de policarbonato (VANDENBERG et al., 2007 e BRIGNON e GOUZY, 2010). Como pode ser observado na Figura 3, o policarbonato é um polímero de cadeia linear, sintetizado pela reação de transesterificação entre um composto aromático hidroxilado, BFA e carbonato de difenila (BERNARDO et al., 2015; PEREZ, 2012). No entanto, a maioria dos policarbonatos comerciais são produzidos pela reação do BFA com cloreto de carbonila, por atribuir menor custo à indústria (JORGE, 2012).

FIGURA 3. Estrutura química do policarbonato



Fonte: (BERNARDO et al., 2015)

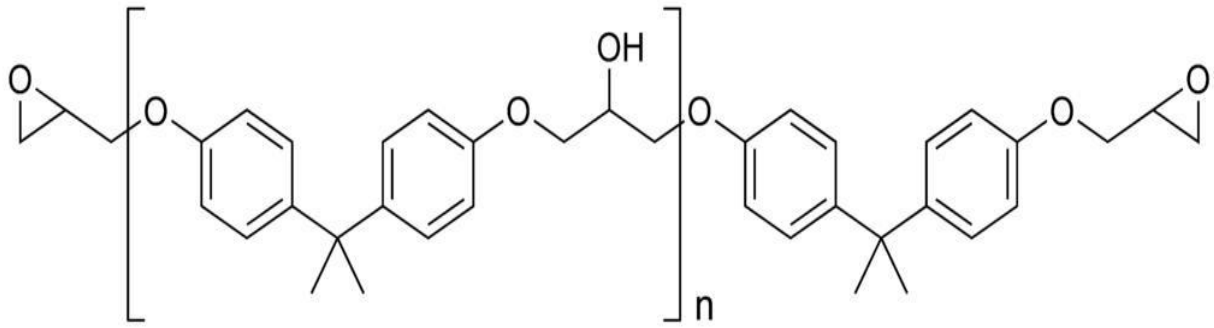
A presença do carbonato na estrutura molecular confere maior rigidez e durabilidade. A transparência, leveza e resistência a variações de temperatura de -40 a 145 °C, são características do policarbonato, e fazem com que este seja utilizado na fabricação de

recipientes retornáveis e reutilizáveis (JORGE, 2012) como garrafas plásticas, embalagens, pratos, copos e recepientes (GEENS et al., 2012).

A segunda maior utilização do BFA, aproximadamente 18% da sua produção total é utilizado como precursor de resina epóxi (VANDENBERG et al., 2007; BRIGNON e GOUZY, 2010; HOEKSTRA e SIMONEAU, 2011). Resinas epoxídicas são utilizadas principalmente no revestimento interno de latas de bebidas e alimentos para proteger contra ferrugem e corrosão (VANDERNBERG et al., 2007; RUDEL et al., 2011). Além disso, possui propriedades mecânicas e de isolamento elétrico, consituíndo uma gama de materiais poliméricos e termoendurecíveis (JORGE, 2012).

A síntese desta resina requer a condensação de BFA com epicloridrina para criar o éter diglicidílico de BFA, conhecido como BADGE (VANDENBERG et al., 2007). Como pode ser observado na Figura 4, em sua estrutura química, a resina epóxi apresenta grupamentos epóxi, que são grupos constituídos por átomos de oxigênio ligados a dois átomos de carbono.

FIGURA 4: Estrutura química da resina epóxi



Fonte: (BERNARDO et al., 2015)

As resinas epóxis estão presentes em revestimentos de superfícies internas e externas de tubulações e rede de água, para evitar que metais, especialmente chumbo e cobre, sejam transferidos à água potável (LANE et al., 2014). A resina também é utilizada no interior de latas de alimentos e bebidas. Após o enlatamento esses produtos são submetidos à altas temperatura para esterilização, e também possuem pH interno extremamente ácido ou alcalino, para que haja o controle de proliferação microbiana. Esses fatores fazem com que ocorra a aceleração da hidrólise das ligações ésteres da molécula, aumentando a lixiviação de BFA para os alimentos

mesmo que a polimerização tenha sido realizada completamente (BAE et al., 2002; WELSHONS et al., 2006; VANDENBERG et al., 2007; TALSNESS et al., 2009).

No que se refere a toxicidade, evidencia-se elevado potencial de exposição, pois o BFA encontra-se presente em todos os compartimentos ambientais como água, ar e solo, tornando a população humana constantemente exposta ao BFA (KANG et al., 2006). Porém seu maior meio de exposição são os alimentos e bebidas contaminados (KANG et al., 2006), e assim a exposição oral a principal via de acesso ao organismo humano.

A ingestão humana média diária de BFA é <1 µg/kg de peso corporal/dia (KANG et al., 2006), porém alguns estudos mostram que mesmo em menores quantidades o BFA desempenha efeito disruptor do sistema endócrino (CHITRA et al., 2003; KAUR et al., 2017). Ainda, aproximadamente 95% do BFA ingerido é absorvido rapidamente pelo trato gastrointestinal, metabolizado no fígado pela ação das enzimas UGT (processo de glicuronidação), para tornar a molécula mais polar, facilitando sua eliminação do organismo. A maior quantidade de BFA é conjugado com o ácido glicurônico gerando glicuronídeo de BFA, e em menor proporção é conjugado com sulfato, gerando sulfato de BFA (MATSUMOTO et al., 2002). Essa biotransformação ocorre apenas quando a via de exposição é oral, e não cutânea ou respiratória (NUNES, 2014).

Após ser conjugado, o glicuronídeo de BFA e sulfato de BFA formados possuem elevada solubilidade em água, assim são rapidamente depurados do sangue pelos rins e excretado na urina. Porém, mesmo sendo conjugado para ser eliminado do organismo, uma parte do BFA livre acumula-se de forma generalizada no organismo (SHIRANI et al., 2019). Mesmo havendo exposição à baixas dosagens, o BFA acumula-se no tecido adiposo e em membranas, criando um potencial de exposição a longo prazo (OOA et al., 2005). Alguns estudos ainda destacam a formação de um outro subproduto, o 5-hidroxi-BFA (BFA catecol), convertido em BFA-o-quinona , presente em reações redox e na geração de EROs (JAEG et al., 2004; YOSHIHARA et al., 2004).

O BFA é classificado como um disruptor endócrino (BAE et al., 2002; WELSHONS et al., 2003). Os disruptores endócrinos são toxicantes ambientais encontrados em diversos produtos, e que mesmo em baixa dosagem exercem efeitos sobre os hormônios naturais (MASUO e ISHIDO, 2011). Interferem na síntese, secreção e ação de hormônios responsáveis pela regulação da homeostase, alterando a função normal dos sistemas imunológico, nervoso e reprodutivo de humanos e animais (CRISP et al.,1989).

Portanto, o BFA é capaz de interferir com o sistema endócrino, alterando os níveis de esteróides endógenos (FRYE et al., 2012). A desregulação endócrina ocorre em resposta a

efeitos denominados agonista ou antagonista. A ação agonista é quando o disruptor mimetiza a ação do hormônio natural, e mesmo assim gera uma resposta. Já na ação antagonista, o disruptor se liga ao receptor, bloqueando a ligação do hormônio natural, não gerando nenhum sinal para ser propagado (BIRKETT e LESTER, 2003).

O BFA é capaz de produzir danos multidirecionais em animais e possivelmente em humanos (LANDOLFI et al., 2017), pois apresenta alta capacidade oxidativa (MOON et al., 2012; QIU et al., 2016). Estudos mostraram que a exposição do BFA, mesmo em doses abaixo do permitido pela legislação que é de 50mg/kg de peso corporal/dia, aumentou demasiadamente a produção de EROs, ocasionando a inibição de enzimas antioxidantes e detoxificantes (QIU et al., 2015).

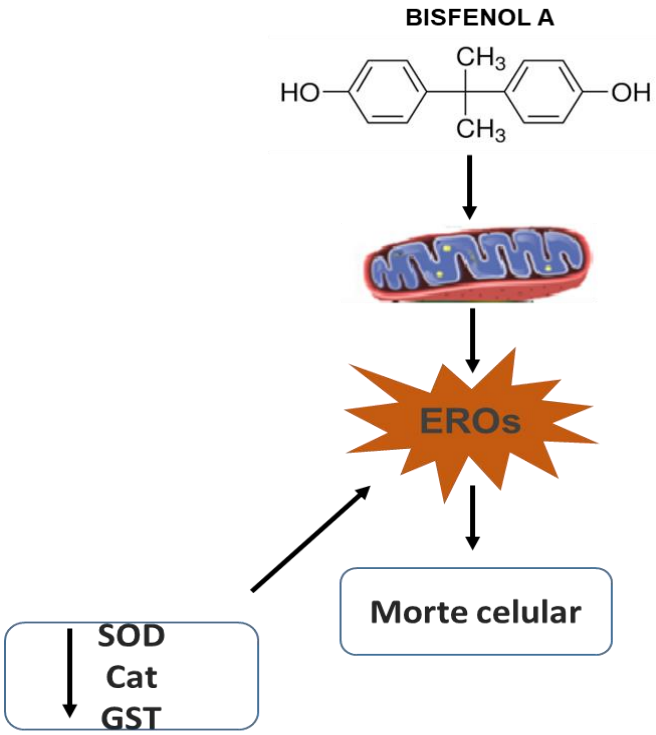
Vários estudos enfatizam que o BFA tem efeito prejudicial às células, devido ao aumento do estresse oxidativo e disfunção mitocondrial em organismos expostos (ANJUM et al., 2011; CHITRA et al., 2003; HASSAN et al., 2012; MAHMOUDI et al., 2015). O aumento da produção de EROs, faz com que ocorra a oxidação exacerbada dos ácidos graxos poli-insaturados que compõem a membrana celular. Assim, em consequência ocorre a ruptura da membrana, e a geração de compostos tóxicos como malondialdeído (MDA), um marcador de estresse oxidativo. (YIIN e LIN, 1995; ADONAYLO e OTEIZA, 1999).

O BFA apresenta um potencial de exposição prolongado, mesmo quando ocorre a exposição a baixa dosagem, devido ao acúmulo no tecido adiposo e em membranas (OOA et al., 2005), e ainda pode ser detectado em vários tecidos e no soro sanguíneo (IKEZUKI et al., 2002; LANDOLFI et al., 2017). O BFA também está relacionado ao desenvolvimento de várias doenças, sejam elas neurodesenvolvimentais (JARDIM et al., 2017; KAUR et al., 2015), reprodutivas (AKINTUNDE et al., 2019; KAUR et al., 2017) ou metabólicas, como resistência à insulina e síndrome metabólica (CAPOROSSI e PAPALEO, 2017; JAHAN et al., 2016). Também pode causar danos em órgãos como fígado e rim (QIN et al., 2012; SHIRANI et al., 2019) e cérebro.

O dano mitocondrial causado pela exposição ao BFA é bastante expressivo devido a sua estrutura química, que proporciona um potencial de partição aumentado em membranas mitocondriais, na qual a composição estrutural é um pouco diferente da membrana celular, contendo mais proteína hidrofóbica no interior ao invés de hidrofílica polar (LAW et al., 1986; NUNEZ et al., 2001). Assim, como pode ser observado na Figura 6, o comprometimento mitocondrial pode ocorrer pelo aumento na produção de EROs (MOON et al., 2012), redução da atividade de enzimas mitocondriais e inibição do complexo I da cadeia de transferência de

elétrons (KHAN et al., 2016; OOE et al., 2005), resultando na morte de células, e assim dano em vários órgãos (SHIRANI et al., 2019).

FIGURA 6: Produção de espécies reativas de oxigênio na mitocôndria



Fonte: (SHIRANI et al., 2019) com adaptações.

O desenvolvimento de doenças neurodesenvolvimentais pela exposição ao BFA passou a ser bastante investigada (KAUR et al., 2015; JARDIM et al., 2017). A exposição ao BFA durante o período pré e pós-natal pode afetar o neurodesenvolvimento (GOLUB et al., 2010). A neurotoxicidade nesses dois períodos críticos, ocorre porque a barreira hematoencefálica ainda não está totalmente formada, facilitando a partição e acúmulo desta substância no sistema nervoso (FAVERO et al., 2006). O BFA transpassa a placenta, e ao entrar em contato com o feto, pode causar efeitos danosos muito maiores devida a baixa concentração endógena da enzima uridina 5'-difosfo-glucuronosiltransferase (UGT). Assim, o BFA livre prossui um grande efeito oxidante que pode danificar o sistema neurológico que ainda está em desenvolvimento nesse período intrauterino.

O BFA também pode causar problemas neurológicos se a criança for exposta nos primeiros anos de idade (BESERRA, 2012). Assim, alguns países como Canadá, França e

Dinamarca, adotaram medidas preventivas, proibindo o uso de BFA em embalagens para uso infantil, como mamadeiras. No Brasil, como forma de proteção, a Agência Nacional de Vigilância Sanitária (ANVISA), através da Resolução RDC n. 41/2011, que entrou em vigor em 2012, também proibiu a venda, fabricação e importação de itens destinados a alimentação de crianças, contendo BFA (ANVISA, 2015; EFSA, 2010).

Observa-se o aumento nas pesquisas nas quais examinam os mecanismos pletóricos subjacentes à exposição ao BFA (SCHUG et al., 2013). O estresse oxidativo é uma condição fisiopatológica que está relacionada ao desenvolvimento de diversas doenças neurológicas, como a DP (KAUR et al., 2018; OOE et al., 2005). A neurodegeneração na DP está apoiada a fatores multifatoriais (DAKHEEL et al., 2014; SCHENK et al., 2017), como o danos oxidativos em neurônios dopaminérgicos (JONES e MILLER, 2008).

Há estudos que mostram que o BFA, assim como outros produtos pertencentes a classe dos bifenilos policlorados, atuam interrompendo de forma direta o sistema dopaminérgico, levando ao desenvolvimento de distúrbios neurodegenerativos com sintomas característicos da DP, semelhantes à ação de pesticidas como rotenona, paraquat, organoclorados, e metais pesados (JONES e MILLER, 2008). Um dos fatores correspondentes a esse dano foi relatado por Tando et al. (2007), na qual observaram que a exposição ao BFA diminuiu os neurônios positivos para tirosina hidroxilase na substância negra de camundongos fêmeas, e reduziu o número de receptores funcionais de dopamina, os D3 (MIZUO et al., 2004), e induziu morte celular por apoptose na substância negra (ISHIDO and MASUO, 2014).

Assim, as alterações no sistema neurológico de adultos devem ser melhor estudadas considerando que este composto induz alterações moleculares e comportamentais similares a drogas usadas em modelos experimentais de doença de Parkinson.

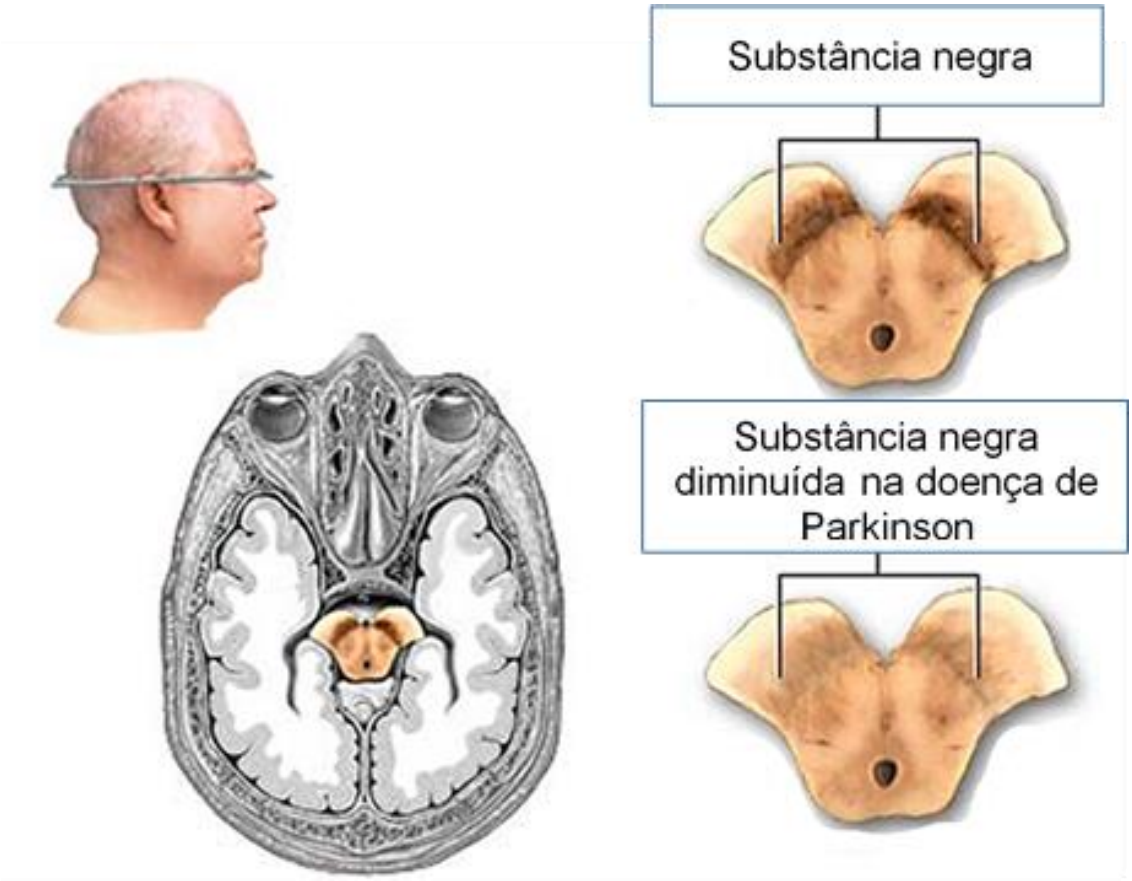
2.1. Doença de Parkinson

A doença de Parkinson (DP) é a segunda doença neurodegenerativa mais frequente no mundo, acometendo de 7 à 10 milhões de pessoas, sendo que aproximadamente 1% dessa população é afetada aos 60 anos de idade (NAGOSHI, 2018), e apenas 15% dos pacientes com DP têm uma história familiar e não mais do que 10% dos casos têm herança mendeliana (KALIA e LANG, 2015; SCHULTE e GASSER, 2011).

A DP é caracterizada pela degeneração progressiva e seletiva dos neurônios dopaminérgicos na substância negra do cérebro, como pode ser observado na Figura 9

(EBRAHIMI et al., 2017). No início da DP já se tem uma perda expressiva de aproximadamente 60% dos neurônios dopaminérgicos da substância negra (DAUER e PRZEDBORSKI, 2003), podendo evoluir conforme a progressão, para a perda de mais de 80% dos neurônios dopaminérgicos, (HEENA et al., 2015). A presença de estruturas conhecidas como corpos de Lewy, (agregados proteicos, compostos basicamente por proteínas como a α-sinucleína, ubiquitina e sinfilina-1) também é outro fator relacionado a perda de neuronal (BRAAK et al., 2003).

FIGURA 9: Degeneração da substância negra na doença de Parkinson



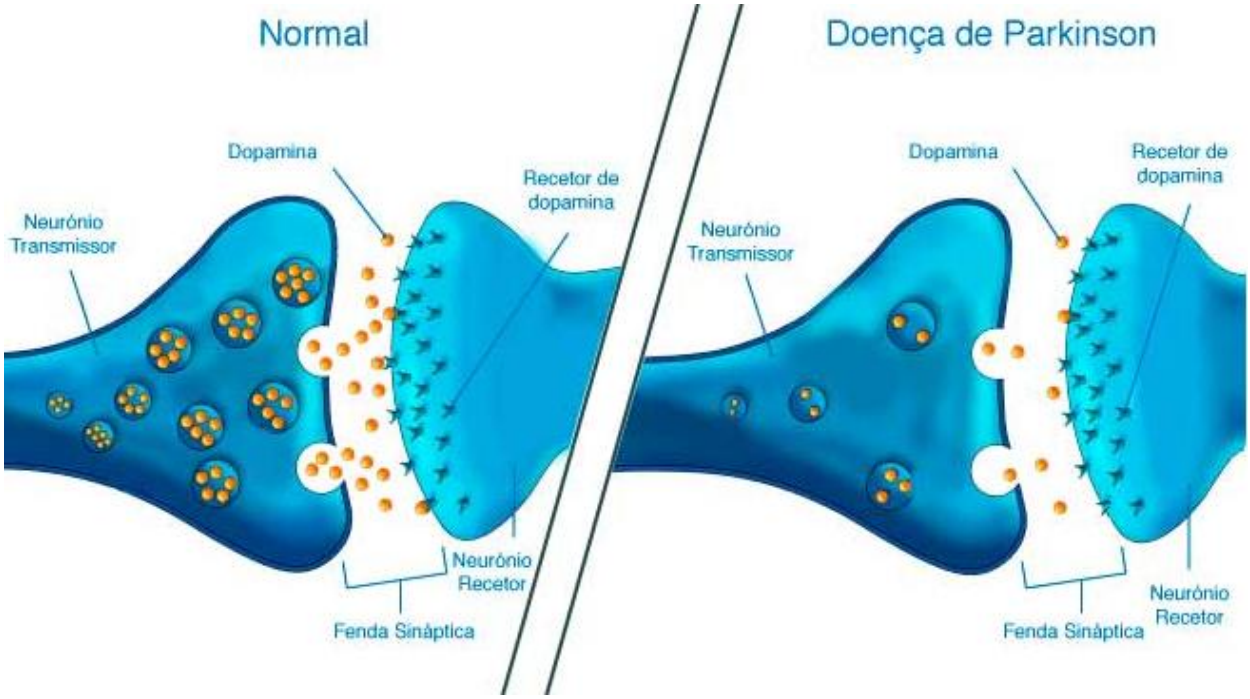
Fonte: https://museudinamicointerdisciplinar.wordpress.com/tag/dopamina (adaptado)

Portanto, devido à perda neuronal, evidencia-se diminuição nos níveis de dopamina na DP, como pode ser observado na figura 10. A degeneração dos neurônios dopaminérgicos está associada a alterações na atividade motora (BRAAK et al., 2003). Assim, a DP apresenta como principais características sintomáticas motoras, alterações como bradicinesia, tremor em repouso, enrigecimento muscular, perda dos reflexos posturais, e congelamento dos movimentos (JANKOVIC, 2008), descritos pela primeira vez em 1817, por James Parkinson. A DP também

desenvolve e alterações comportamentais não motoras como ansiedade generalizada, ataques de pânico e fobias sociais (KULISEVSKY et al., 2008; LAUTERBACH, 2005).

Além disso, também é observado em pacientes com DP a má conformação da α-sinucleína (apresenta conformação amilóide β-cruzada) acumulando-se nos corpos de Lewy (OLANOW e BRUNDIN, 2013).

FIGURA 10: Representação da diminuição nos níveis de dopamina em neurônio afetado na DP



Fonte: https://www.saudebemestar.pt/pt/medicina/neurologia/doenca-de-parkinson

Ainda não se sabe ao certo o que desencadeia o início da DP, porém há uma forte vertente que indica fatores genéticos e ambientais, ou eles combinados entre si como peça chave no desenvolvimento desta patologia (OLANOW e BRUNDIN, 2013). A neurodegeneração na DP responde a causas multifatoriais como, disfunção mitocondrial, estresse oxidativo, neuroinflamação e degradação proteica (MUÑOZ-SORIANO e PARICIO, 2011; Al DAKHEEL et al., 2014; SCHENK et al., 2017).

2.2. *Drosophila melanogaster*

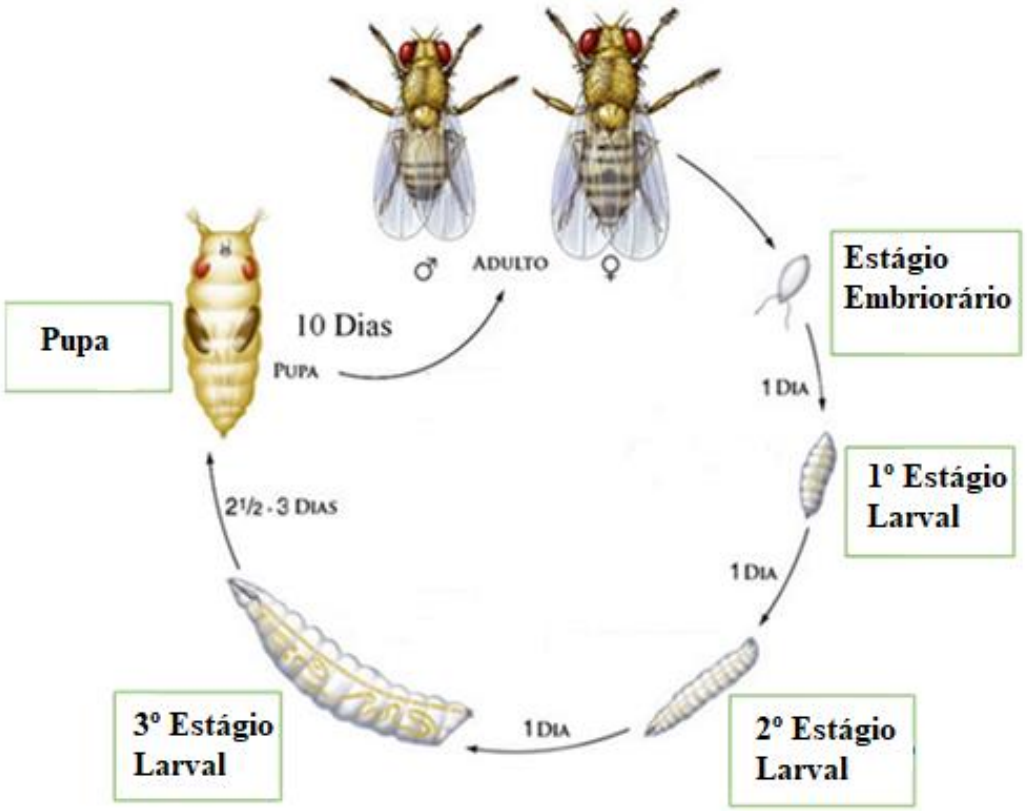
A mosca *Drosophila melanogaster* é um artrópode ecdisozoário, pertencente a subespécie dos *Drosophilidae*, são insetos dípteros presentes em todo planeta terra (ADOLT et al., 2000; PETERSON et al., 2004). Possui aproximadamente 75% de homologia genética a humanos, e conserva um terço dos genes envolvidos em diversas doenças (REITER et al., 2001), assim apresentando alto potencial para ser utilizada como modelo de doença humana (PANCHAL and TIWARI, 2017).

A busca por modelos alternativos está em ascensão, e neste sentido, a *Drosophila melanogaster*, vem sendo cada vez mais investigada e utilizada em diversas pesquisar (BONILLA-RAMIREZ et al., 2011). Esse organismo modelo apresenta alguns benefícios como baixo custo, fácil manuseio e manutenção laboral, reprodução rápida, gerando a prole geneticamente idêntica, e curto ciclo de vida, que varia de 40 a 120 dias, dependendo da dieta ofertada (PIPER et al., 2005; PLETCHER et al., 2005). Além disso, é livre de impedimentos éticos, não sendo necessário aprovação do comitê de ética para que as pesquisas ocorram.

Uma única fêmea pode colocar de 30 à 50 ovos por dia (BARNES et al., 2008). Após a fertilização, inicia-se o ciclo de desenvolvimento da mosca, na qual é composto por quatro estágios: embrião (ovo), larva, pupa e mosca (Figura 8). As larvas combinam movimentos de locomoção para alimentar-se por sucessivas extensões e retrações de seus ganchos bucais, (SHAVER et al., 2000). O estágio larval também é dividido em 3 estágios, 1º, 2º e 3º, sendo este último a fase mais intensa de consumo, para garantir estoque energético para o período de pupação. Enquanto pupa, permanece fixa e imóvel em superfície rígida e lisa, e posteriormente as características larvais são substituídas por estrutura de mosca adulta (SHAVER et al., 2000).

As moscas apresentam respostas fisiológicas à insultos externos comparáveis aos seres humanos (CARMONA et al., 2013), sendo possível avaliar os efeitos mutagênicos, genotóxicos, morfológicos e desenvolvimentais responsivos à vários xenobióticos, sendo um ótimo sistema utilizável para a realização de ensaios rápido de detecção dos efeitos de adversos toxicantes (ATLI, 2013).

FIGURA 8: Ciclo do desenvolvimento de *Drosophila melanogaster*



Fonte: https://dendroterra.jimdo.com/art%C3%ADculos/droshophila-alimento-vivo

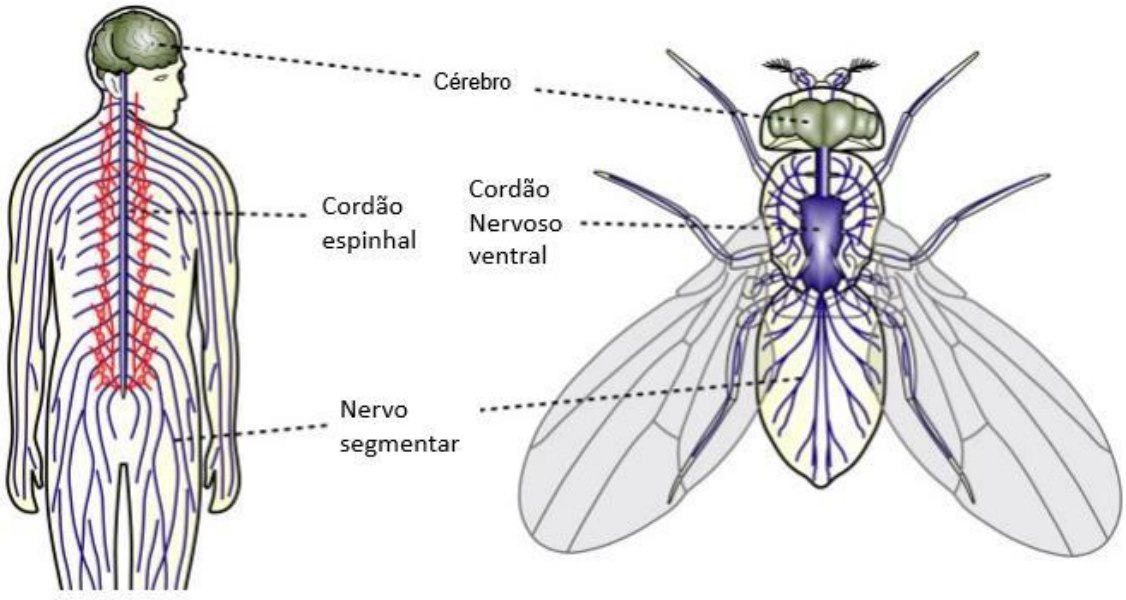
A *Drosophila melanogaster* está sendo amplamente utilizada em estudos que implicam processos genéticos, celulares e moleculares à serviço do desenvolvimento da neurociência (VAN ALPHEN e VAN SWINDEREN, 2013). Nesse sentido, vale ressaltar que a mosca preserva muitas características estruturais e funcionais semelhantes aos mamíferos, o que torna fácil a observação do mecanismo de ação tóxica a nível cerebral (NAGOSHI, 2018).

Segundo Kaur et al. (2015), a mosca é um modelo bastante eficiente para estudos comportamentais e de doenças neurológicas, destacando-se em pesquisas relacionadas a progressão de doenças neurodegenerativas e disfunção neuronais em geral, como por exemplo a doença de Parkinson (PODDIGHE et al., 2013).

O sistema nervoso (SN) da *Drosophila melanogaster* e de humanos derivam da mesma origem evolutiva, assim apresentam similaridade neurobiológica (HIRTH e REICHERT,

1999), como o compartilhamento de sinalização de classes neurais, e de neurotransmissores (KANE, 2011). O SN da mosca é constituído por um cérebro, dispondo de aproximadamente 100.000 neurônios, um cordão nervoso ventral (que se equivale a medula espinhal) e sistema nervoso periférico (SHIN et al., 2018), conforme pode ser observado na Figura 9. O principais neurotransmissores observados nas mosca são: dopamina, serotonina, histamina e octopamina (MONASTIRIOTI, 1999).

FIGURA 9: Analogia entre a composição do sistema nervoso humano e de moscas.



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

A octopamina e tiramina são neurotransmissores característicos em artrópodes, desempenham função semelhante à epinefrina e norepinefrina em humanos (ROEDER, 2004). Porém, as moscas adultas apresentam neurotransmissão de dopamina e serotonina semelhantes a mamíferos, e ambas são reguladas por transportadores homólogos aos humanos (PORZGEN et al., 2001). A síntese de dopamina também é conservada entre moscas e humanos, sendo detectadas no cérebro de moscas durante a fase larval e adulta (MONASTIRIOTI, 1999).

Assim como em humanos, a *Drosophila melanogaster* exibe comportamentos dependentes de dopamina (NAGOSHI, 2018). A locomoção é controlada pela dopamina, e no entanto, o comprometimento do neurônios dopaminérgicos acaba afetando a locomoção das moscas (HIRTH, 2012), sendo essa, uma situação comparável a DP (MONASTIRIOTI, 1999)

Além de reproduzir várias doenças humanas, a *Drosophila melanogaster* proporciona muitos insights sobre DP (PANDEY e NICHOLS, 2011). Além disso, as moscas já estão bem estabelecido na literatura quanto ao desenvolvimento de DP pela exposição a toxicantes ambientais como paraquat e rotenona, por modificações genéticas, ou pela combinação entre esses dois fatores (FENGER e BENDER, 2000). Assim, esse modelo torna-se eficaz para executar experimentos que avaliem o desenvolvimento DP pela exposição ao BFA.

3. OBJETIVOS

3.1. Objetivo geral

Avaliar se a exposição ao Bisfenol A causa alterações tipo Parkinsonianas em *Drosophila melanogaster*

3.2. Objetivos específicos

- Avaliar as alterações comportamentais de *Drosophila melanogaster* induzidas pela exposição ao Bisfenol A e atividade da enzima acetilcolinesterase;

- Avaliar o potencial oxidante a nível cerebral do Bisfenol A por meio de biomarcadores de estresse oxidativo;

- Dosar os níveis de dopamina;

- Verificar a correlação entre os níveis de dopamina com o estresse oxidativo e comportamentos de geotaxia negativa, campo aberto e de equilíbrio em *Drosophila melanogaster*.

2. MANUSCRITO

BISPHENOL EXPOSURE IS INVOLVED IN THE DEVELOPMENT OF PARKINSON LIKE DISEASE IN *Drosophila melanogaster*

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BISPHENOL A EXPOSURE IS INVOLVED IN THE DEVELOPMENT OF PARKINSON LIKE DISEASE IN *Drosophila melanogaster*

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

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ABSTRACT

The pathogenesis of Parkinson's disease has not yet been fully clarified but its cause is known to be multifactorial. One of these factors is the oxidative stress induced by exposure to environmental toxifiers. We studied in adult *Drosophila melanogaster* the effect of Bisphenol A (BPA) at concentrations of 0.5 mM and 1 mM, (the concentration of 1 mM corresponding to Lowest Observed Adverse Effect Level (LOAEL) for humans). The BPA induced oxidative stress was established by increased levels of malondialdehyde, reactive species, and decreased activity of antioxidant enzymes superoxide dismutase and catalase, and detoxificant enzyme glutathione-S-transferase. Associated with oxidative stress, there was a reduction of acetylcholinesterase activity and reduction of dopamine levels, which are related to the decreased locomotion activity as observed in negative geotaxis, open field and equilibrium behaviors. Oxidative stress also impaired mitochondrial and cellular metabolic activity in the head causing an increase in the mortality of flies exposed to both BPA concentrations. Therefore, the BPA induced Parkinsonian-like changes in flies and it is possible that the oxidative stress is closed related with this effect, providing new insights for future studies.

Key words: environmental toxifier, fly, oxidative stress, Parkinson, Bisfenol A

1. INTRODUCTION

Bisphenol A (BPA) (Figure 1) is a synthetic phenol widely used in polycarbonate manufacturing and epoxy resins (Jalal et al., 2018). Thus, the BPA is present in kitchen utensils, packaging and heat-resistant plastic bottles and inner coating of canned food (Geens et al., 2012; Vandenberg et al., 2007). When these items are subjected to washing processes, heating and contact with pH acidic or basic, occur the hydrolysis of ester bonds of the molecule, thus BPA monomers are released to the environment and contaminate foods and beverages (Bae et al., 2002; Vandenberg et al., 2007).

Continuous exposure to BPA is directly related to changes in the central nervous system (Inadera, 2015). Among the target neurological systems, the dopaminergic system is strongly affected by exposure to BPA (Jones and Miller, 2008). In rodent studies, decreased nigra was observed due to the degeneration of dopaminergic neurons, reduction of tyrosine hydroxylase (TH) activity and dopamine (DA) transporters (Ishido et al., 2007). In this sense, it is worth noting that changes in the dopaminergic system result in debilitating behavioral disorders such as Parkinson's disease (PD) (Jones and Miller, 2008), characterized by the progressive loss of dopaminergic neurons (Ebrahimi et al., 2017).

The dopaminergic neurodegeneration has the oxidative stress as one importante mediator (Jones and Miller, 2008). Qiu et al., (2016) found that there was induction to oxidative stress by prolonged exposure to low doses of BPA. The oxidative stress caused by BPA also compromised mitochondrial activity and reduced the activity of complex I (NADH ubiquinone oxidoreductase) (Ooe et al., 2005), and decreased mitochondrial enzyme activity (Khan et al., 2016). Even though many results have evaluated, even in isolation, some factors that may contribute to the development of PD by exposure to BPA in rodents (Jones and Miller, 2008; Ooe et al., 2005), but the results are still inconclusive, but suggest that BPA is an important risk factor.

It is important to emphasize the characterization of new experimental models of degenerative diseases, and it is fundamental to understand more about the appearance and progression of these diseases. *Drosophila melanogaster* is an alternative model valid for studies related to PD (Poddighe et al., 2013), because it has a structure and neurological function similar to mammals, but ordered in a simpler way, which makes it easier to observe the mechanism of action of xenobiotics in the development and progression of the disease (Nagoshi, 2018). In addition *Drosophila melanogaster* exhibit behaviors dependent of DA, controlled by dopaminergic neurons, widely distributed in the brain (Chen et al., 2014; Nagoshi,

2018). The fly is also feasible for studies related to oxidative stress, due to its biochemical and metabolic similarity to humans (Doran et al., 2017). Therefore, the objective of this study was to show the mechanisms involved in the development of Parkinson-like disease in *Drosophila melanogaster* induced by BPA, thus characterizing and establishing a model for future studies on a neurotoxicity of bisphenol A.

2. MATERIALS AND METHODS

Chemicals

The BPA (> 99% purity; Sigma Aldrich, St. Louis, MO, USA) was diluted in 0.1% of Dimethylsulfoxide (DMSO), according to the methodology of Kaur et al. (2015). The DMSO and other analytical grade reagents used in this work came from the laboratory of the Federal University of Pampa, Campus Itaqui.

Drosophila melanogaster stock and culture

It was used *Drosophila melanogaster* of Harwich strain, wild type, obtained from the National Species Center (Bowling Green, Ohio, USA). The flies were kept in glass flasks, under controlled temperature of 25 ± 1°C, humidity of 60-70% and circadian cycle light/dark of 12 hours, fed with standard food composed of corn flour (76,59%), wheat germ (8,51%), sugar (7,23%), milk powder (7,23%), salt (0,43%) and antifungal methylparaben.

Experimental Protocol

Flies of both sexes, aged between one and two days were divided into three groups, containing 50 flies each. The control group received only 0,1% DMSO. In order that the exposure was orally, the BPA was incorporated in food in concentrations of 0.5mM and 1mM (Figure 2). The highest concentration (1mM) corresponds to the Lowest Observed Adverse Effect Level (LOAEL) of 50 mg/kg body/day for humans (Kaur et al., 2015).

In vivo assays

2.1 Survival assessment

The influence of BPA on the survival of flies was valued. For this, the daily count (1x at the end of each day) of the number of live flies was performed during the seven days of

treatment of the control groups, BPA 0.5 and 1 mM. Each group consisted of 50 adult flies of both sexes added randomly to the treatment. There was no exchange of food during these seven days to exclude the risk of death by the manipulation process. Three independent experiments were performed

Behavioral tests

After 7 days of treatment, the flies of both sexes were submitted to behavioral tests. The tests were conducted in a acclimatized environment (25 ± 1°C), at times between 10AM to 4PM, to avoid changes in an behavior, because according to Kaur et al. (2015), the circadian rhythm is related to changes in performance and variations in fly behavior.

2.2 Negative geotaxis

The negative geotaxis test corresponds to the innate escape response in flies. It is widely used in studies related to the development of neurodegenerative diseases, because it is sensitive to deficits in motor coordination and muscle tone (Chen et al., 2014) . It was used the method described by Jimenez-Del-Rio et al. (2010), with some adaptations.

The test was performed on 15 flies from each group, from four randomized experiments. The flies were temporarily immobilized with ice to be individually transferred to 1.5cm diameter tubes. After 10 minutes of recovery, the tubes (one per time), in an upright position, were lightly beaten on a flat platform so that the flies remained in the tube base. Then the timer was quickly triggered beginning the count of the time spent by each fly to reach the height of 8cm, measured from the base of the tube. Each fly had the time maximum of 120 seconds to scroll the apparatus until to reaches 8cm of height. The test was repeated five times per fly, and the average of the times was calculated individually for the statistics analysis.

2.3 Open field test

Through the open field test, it is possible to evaluate the exploratory and locomotor capacity of the flies. The test was performed according to the methodology established by Hirth, (2010), with some adaptations. Fifteen flies from each group were used, from four independent experiments, totaling the application of the test in 60 flies per group. After mild anesthesia on ice, the flies were placed separately in polycarbonate petri dish (6 mm diameter). In the cover of the plate there were squares measuring 1 cm each slightly marked, but without interference

in the fly behavior. The locomotor and exploratory activity was monitored for 60 seconds, by counting the quadrants traversed by each fly. The test was performed in duplicate and the mean values were calculated.

2.4 Test of motor coordination

It was performed some modifications in the methodology described by Iliadi et al. (2018), however the test is effective for measure motor coordination and locomotion of *Drosophila melanogaster*. Fifteen of both sexes were anesthetized with ice to enable the cutting of its wings, a procedure performed 3 days before the test. The test apparatus consists of an acrylic box, measuring (28cm long, 11.8cm wide and 2.4cm high), filled with water (23 to 27°C) up to a distance of 5 mm from the transparent nylon line (0.6 mm), which was secured between two platforms submerged in water. They were individually overlaid on the platform with a brush, and it was timed when the fly passed the red zone (located 1cm after the platforms). The maximum time for locomotion of 13cm was 60 seconds. The test was performed in triplicate, where the average rate observed (seconds) was used to access certain distance (mm).

Ex vivo assays

Homogenized preparation

The flies were first anesthetized and euthanized on ice after 7 days of treatment. Subsequently, the flies were decapitated and all analyzes were performed only on head tissue samples in duplicate (n=3-5), except for AChE activity, which was measured on head and body of flies.

2.5 Determination of acetylcholinesterase (AChE) activity

Twenty heads and 20 bodies of each group were homogenized in 200µL and 800µL of HEPES buffer (20mM, pH 7.0) respectively and centrifuged at 1000 G for 10 minutes, according Ellman et al. (1961), with some adaptations. A mixture containing 0.25M KPi buffer (pH 8.0) and 5,5'-dithiobis (2-nitrobenzoic acid) (5mM DTNB) was prepared. At the time of analysis, the supernatant sample heads (15µL) was added sequentially to 935µL of the mixture and 50µL of acetylthiocholine (AcSCh) (7, 25µM) and 50µL of sample were used in the body

samples to 900µL of mixture and 50µL of acetylthiocholine (AcSCh) (7.25µM). The AChE activity was determined spectrophotometrically at 412nm for 2 minutes, expressed as µmol AcSCh/h/mg protein.

2.6 Determination of reactive species levels

The oxidation of DCFDA as free radical generation index and oxidative stress were measured without supernatant of the samples, according to Pérez-Severiano et al. (2004). Twenty heads of flies were homogenized in 1mL of Tris buffer (10mM, pH 7.0) and centrifuged at 3570 G for 5 minutes. An aliquot of 34µL sample supernatant was added in a mixture containing 964µL HEPES buffer (pH 7.0) and 10µL 2,7-dichloroflurescein diacetate (3,33M; DCFDA). Therefore, after 1 hour of incubation, the emission of fluorescence from the DCFH resulting from the oxidation of DCFDA was monitored in a spectrophotometer in an excitation wavelength of 488nm and an emission wavelength of 520nm. The results obtained were expressed as a percentage of the control. The average of three independent experiments was used totaling 60 flies for each group.

2.7 Determination of thiobarbituric acid reactive substances

Considered a marker of oxidative stress, malonildialdehyde (MDA) is a final product resulting the lipid peroxidation. Its content in supernatants was measured according to Ohkawa et al. (1979) with some modifications. Fifteen fly heads were homogenized in 90µL of 0.1M HEPES buffer (pH 7.0) and centrifuged at 1000 G for 10 minutes. The supernatant were transferred (50µL) to assay tubes with solution containing 125µL of thiobarbituric acid (TBA, 8%), 12µL of acetic acid, 50µL of Sodium dodecyl sulfate (1.2% SDS) and 25µL of water, taken to incubation in a water bath at 95°C for 2 hours. After cooling in room temperature, 200µL it was transferred to acrylic microplates for spectrophotometer reading at 532nm.

2.8 Antioxidant and detoxifying enzymes activities

To evaluate the action of BPA on the antioxidant and detoxifying defense system, the activity of enzymes catalase (Cat), SOD (superoxide dismutase) and Glutathione-S-transferase (GST) was evaluated.

The preparation of the samples to analyze the activity of both enzymes was similar, following the same protocol. Therefore, 20 heads of fly were homogenized in 200µL of 0.1 M HEPES buffer (pH 7.0), and centrifuged at 14000 G for 30 minutes. The supernatant was reserved to carry out analyzes.

2.8.1 Determination of superoxide dismutase activity

The activity of SOD was evaluated by the method described by (Kostyuk and Potapovicha (1989), with modifications by Franco et al. (2009), by monitoring the inhibitory effect of SOD on quercetin oxidation. To produce the analysis samples, an aliquot of 10µL of the supernatant was diluted in 90µL of HEPES buffer. The reaction solution comprised sodium phosphate buffer (0.025M/0.1mM EDTA, pH 10) and N, N, N-tetramethylethylenediamine (TEMED) was added (1mL) along with 10µL of diluted sample and 50µL of quercetin in the cuvette for spectrophotometer reading at 406nm for 2 minutes. The results were corrected for the absorbance of the amount of protein present in the supernatant sample, calculated as percentage inhibition of the oxidation of quercetin.

2.8.2 Determination of catalase activity

The activity of Cat was determined according to the methodology of Aebi (1987) with some modifications made by Paula et al. (2012), which consists in the enzyme's ability to degrade H_2O_2. It was added 30µL of supernatant to a quartz cuvette along with 2mL reaction mixture composed of 0.25M KPi buffer/2.5mM EDTA pH 7.0, and Triton H_2O_2. The reading was performed in a spectrophotometer at wave length of 412nm for 2 minutes. The analysis was performed in 5 independent experiments, with samples in duplicate, and the results were corrected according to the protein concentration. The final result was expressed in Cat unit per mg protein (U/mg).

2.8.3 Glutatione-S-transferase activity

The GST activity was determined according to the method described by Habig et al. (1974), with some adaptations. The technique consists in the catalytic action of GST in the conjugation reaction of 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione (GSH) to obtain a thioether (4-dinitrophenyl glutathione).

To perform the reading in a spectrophotometer at 340nm for 2 minutes, it was added to an acrylic cuvette 30µL of supernatant, 1000µL of mix (0.25M KPi buffer/2.5mM EDTA pH

7.0, 100mM GSH and distilled water) and finally, 20µL of the CDNB primer substrate (50mM). The results were corrected by value of the protein and expressed in milli units of enzyme activity/mg protein (mU/ mg protein).

2.9 Evaluation of mitochondrial metabolic activity by the reduction of MTT (3-4,5-dimethylthiazol-2-yl] -2,5-diphenyltetrazolium bromide)

The methodology was performed according to Hosamani et al. (2010), with some modifications. Therefore, it was homogenized approximately 100 heads of flies in ice Tris-sucrose (0.25M, pH 7.4) buffer containing Mannitol (220mM), Sucrose (68mM); KCl (10mM), HEPES (10mM) and BSA (0.1%). The homogenate was centrifuged at 3570 G for 5 minutes. The supernatant was removed and the homogenate was centrifuged again to obtain the pellet. It was added 200µL of suspension buffer (Tris 0.25M sucrose, pH 7.4 without BSA) to sediment. After 3 hours on ice, the samples were incubated with MTT for 30 minutes at 37ºC and centrifuged at 10000 G, for 5 minutes. Upon removing the supernatant, 200µL of DMSO was added and the sample were re-incubated for 30 minutes at 37°C and centrifuged at 10000 G for 5 minutes. Finally, 150µL of the supernatant was transferred to microplates for spectrophotometer reading at 540nm. The results were expressed as a percentage of the control (n= 4-6).

2.10 Evaluation of cellular metabolic activity by the Resazurin reduction method

Cell viability was assessed according to Franco et al. (2009). The method consists in the ability of cells to reduce resazurin, forming resorufin that is a fluorescent molecule. Samples for analysis were obtained by homogenizing 10 fly heads in 100mL of 20mM Tris buffer (pH 7.0), and centrifuging at 3570 G for 10 minutes. The supernatant was removed, and 20µL of the sample was added to an acrylic microplate along with 180µL of 20mM Tris (pH 7.0) and 10µL of resazurin. Every 1 hour, for the total of 4 hours the readings were carried out at 573nm. The value of the last reading was considered as the final result and was expressed as a percentage of control.

2.11 Determination of Dopamine levels

Dopamine levels were determined through High Performance Liquid Chromatography with a Diode Matrix Detector (HPLC-DAD) equipped with a quaternary pump and automatic sampler, as described by Bianchini et al. (2016). For the preparation of samples, 30 head of flies were homogenized in 288μL of 0.9% NaCl and 12μL of 0.5M HCl for 1 minute. Subsequently, this homogenized solution was centrifuged for 10 minutes at 10000 G at -4°C. After centrifugation, the supernatants were filtered with 0.22μm syringe filters. Subsequently, 200μL of sample were injected into the HPLC system by the automatic sampling device. Ultrapure water and methanol (12.5%), pH 2.5, were used in the mobile phase. The flow was maintained at 0.8 mL /min and detection was performed at 198 nm.

2.12 Determination of protein concentration

The determination of protein concentration in replicate fly head samples were made according to the method of Bradford (1976), using bovine serum albumin as standard.

2.13 Statistical analysis

It was used the GraphPad Prism software, version 6 (San Diego, CA, USA) to perform the statistical analysis. Fly survival rates during the 7 days of treatment were determined by the log-rank test (Mantel-Cox). The results were analyzed by means of one-way analysis (ANOVA), using Bonferroni test for multiple comparisons. Correlation analyzes were also performed using the Pearson correlation coefficient. Values of probability less than 0.05 (p <0.05) were considered statistically significant.

3. RESULTS

3.1 Survival assessment

Adult flies were exposed for 7 days at two concentrations of BPA (0.5mM and 1mM) had a decrease in survival rate, when compared to the control group ($p < 0.0001$, Figure 3).

3.2 Behavioral tests

All behavioral tests are shown in Figure 4. In the negative geotaxis test BPA increased the flap climbing time in both groups (0.5mM and 1mM) in comparison to the control group (Fig. 4 A p <0.0006; F = 18.98). In the open field test even at a lower dose (0.5mM) the BPA decreased the locomotion within of arena, being similar to the group exposed to the highest concentration of BPA (1mM), which had a marked decrease compared to the control group (0.5mM BPA) (Fig. 4 B p <0.0025; F = 19.15). In the test that evaluated the locomotion and motor cordenation BPA in the two concentrations affected the locomotion and balance of the flies in comparison to the control group (Fig. 4 C p <0.0001; F= 55.74).

3.3 AChE activity

In the head samples there was a decrease in the activity of the AChE enzyme in the two groups exposed to BPA (0.5mM and 1mM), when compared to the control group (Fig. 5 A p <0.0007; F = 18.08), however the body samples. AChE activity did not change between groups (Fig. 5 B p <0.2738; F = 1.620).

3.4 Oxidative stress and inhibition of detoxifying and antioxidant protection system by exposure to BPA

It is possible to observe the significant increase in the production of reactive species in the heads of the flies in the group that were exposed to 1mM BPA (Fig. 6 p <0.0005; F = 20.16), whereas in the group exposed to the lowest concentration of BPA 0.5mM) had no statistically significant increase of reactive species when compared to control. In relation to TBARS analysis (Fig. 7 p <0.0001; F = 119.9), the BPA increased lipid peroxidation, in both tested concentrations.

BPA decreased the activity of the antioxidant enzymes SOD (Fig. 8 A p < 0.0005; F = 20.16) and Cat (Fig. 8 B p <0.0001; F = 50.47) in the two concentrations. There was also a reduction in the activity of the detoxifying enzyme GST (Fig. 8 C p <0.0065; F = 9.282) in both groups with BPA when compared to the control group.

3.5 Mitochondrial and cellular metabolic activity

There was a decrease in mitochondrial metabolic activity in two groups treated with BPA, when compared to the control group (Fig. 9 p <0.0001; F = 39.41). There was also a decrease

in cell viability of the groups exposed to BPA at both concentrations compared to the group (Fig. 10 p <0.0001; F = 64.22).

3.6 Depletion of the levels of dopamine (DA) induced BPA

Exposition to BPA at higher concentration (1mM) decreased DA levels in the head of flies (Fig. 11 p <0.0275; F = 4.922), whereas the lower concentration of BPA (0.5mM) did not change the DA levels. Decreases in dopamine levels correlate with decreased locomotion and fly balance (Table 1 p <0.05), increased oxidative stress at brain level (Table 2 p <0.05).

4. DISCUSSION

Environmental pollutants can cause various behavioral and biological changes in flies. The induction of oxidative stress mediated by BPA in *Drosophila melanogaster* can be one of the possible mechanisms for the development of various diseases. In this study we observed that BPA promoted oxidative stress in flies by increasing lipid peroxidation and free radicals levels, in addition to inhibiting antioxidant enzymes, causing a mitochondrial and cellular metabolic imbalance. There was a decrease in dopamine levels and a decrease in acetylcholinesterase activity, which are related to changes in locomotor behavior, similar to PD. These biochemical changes induced by BPA are factors that influenced the survival of the flies during the 7 days of treatment. Thus, the groups that were exposed to BPA had an increase in mortality, when compared to the control.

It was observed that there was an increased production of reactive species (RS) in the head of flies in the group exposed to the highest concentration of BPA (1mM), corresponding to LOAEL for humans. The group that was exposed to the lowest concentration of BPA (0.5mM) had no statistical difference in relation to the control, however, a tendency to increase RS production was observed. This result is concomitant to the increase in lipid peroxidation, since BPA at both concentrations triggered the increase in MDA levels. As TBARS is a broad indicator of lipid peroxidation (Kabuto et al., 2003), the results indicate that exposure to BPA caused damage to the membranes of brain tissue due to the increase in the production of reactive species.

BPA also damaged the detoxifying and antioxidant system of *Drosophila melanogaster*. This compost at both concentrations decreased the activity of GST, a phase II detoxifying enzyme that solubilizes conjugated substances in phase I (Chahine and O'Donnell, 2011). The

phase I system conjugates the BPA converting into reactive species. Thus, our result indicates that reduction in GST activity promotes non-neutralization of these reactive species generated in the phase I conjugation, contributing to oxidative stress at the brain level. The antioxidant enzymes SOD and Cat also had decreased activity in both groups that received BPA. SOD catalyzes the conversion of the superoxide anion radical into H_2O_2. The H_2O_2 generated in the cells or from the action of SOD are converted by Cat into reactive species more soluble in water (Fridovich, 1997). Thus, the decrease in the activity of SOD and Cat reflect in the increase of the ROS and LPO levels, similar to a study by Anet et al., (2019), and emphasize that BPA is an inducer of oxidative stress.

Oxidative stress and disruption of the electron transport chain causes mitochondrial damage (Khan et al., 2016). It can be indicated by the MTT reduction assay that is mediated by dehydrogenase enzymes present in the mitochondria (Caughlan et al., 2004); therefore, the dehydrogenases inactivation decreases the MTT reduction, thus characterizing the mitochondrial damage. BPA was able to reduce the metabolism of MTT, at both concentrations, by asserting mitochondrial damage. The chemical structure of BPA confers greater affinity to mitochondrial membranes that are constituted in his internal by hydrophobic proteins rather than hydrophilic (Law et al., 1986; Nunez et al., 2001) .Thus, BPA accumulates in the mitochondrial membrane inhibiting the complex I of respiratory chain (Ooa et al., 2005; Khan et al., 2016), interfering in electron transport and increasing the production of reactive oxygen species, which may be involved in the immobilization of mitochondrial enzymes. Mitochondrial damage can lead to cell death (Shirani et al., 2019). Thus, through the resazurin reduction assay, as an indicator of cell damage, also mediated by dehydrogenases, it is possible to observe that BPA even at low doses decreased neuronal cell function.

Oxidative stress is also a primary mediator of dopaminergic neurodegeneration (Jones and Miller, 2008). Dopaminergic neurons are extremely vulnerable to degeneration, and the death of these neurons is related to the development of PD (Ishido and Masuo, 2014). In a study by Ishido et al. (2007), it was found that BPA decreased dopamine levels, tyrosine hydroxylase activity and dopamine transporters, and death of dopaminergic neurons. In this study, we observed that the group exposed to 1mM of BPA had their dopamine levels reduced in comparison to the control group. The decrease in dopamine correlated with all the oxidative stress markers mentioned above. Thus, we observed that the decrease in dopamine is related to decrease in the activities of antioxidant enzymes (SOD and Cat) and detoxifying (GST), and increasing levels of RS and MDA.

The decrease in dopamine is also related to the behavior characteristic of PD, such as tremor, loss of balance (Dauer and Przedborski, 2003). The open-field and negative geotaxis tests are widely used to assess deficits in motor activity, whereas the psychomotor test evaluates the balance considering fine motor coordination in fly (Iliadi et al., 2018). In the negative geotaxis test the flies increased the climbing time in the open field test, less quadrants were observed within 1 minute when compared to the control group and in the equilibrium test, the flies also had a reduction of this ability when exposed to BPA. All these results are also correlated with a decrease in DA levels. Any substance that causes interference in the neurotransmission components of dopamine may alter behaviors that are essential for the locomotion and survival of flies (Figueira et al., 2017). Thus, in our study, BPA induced in addition to the decrease in dopamine levels and impairment of locomotion, the increase in fly mortality. In already consolidated models of PD in *Drosophila melanogaster*, such as rotenone, it is also observed the decrease of dopamine levels related to increased mortality and reduction of locomotion and balance (Couto et al., 2019).

Another neurotransmitter directly related to locomotor regulation is acetylcholine, and neurodegeneration of dopaminergic neurons is directly related to the development of neurodegenerative diseases (Craig et al., 2011). There was a decrease in AChE activity at the brain level in the groups exposed to BPA at both concentrations. However, in the body samples, there was no significant difference between the 0.5mM and 1mM BPA when compared to the control. Some studies evidence the inhibition of AChE activity by the action of free radicals (Tsakiris et al., 2015; Wyse et al., 2004). In our results, the oxidative stress induced by the BPA at the cerebral level decreased the activity of the enzyme AChE.

The environmental risk factor for PD development consists of xenobiotics that cause oxidative damage, mitochondrial dysfunction and death of dopaminergic neurons, such as MPTP, paraquat and rotenona (Aryal and Lee, 2019). Through this study, the possible involvement of BPA in the development of Parkinson like disease in *Drosophila melanogaster* was evident. It is possible to observe clearly that even in a lower concentration the BPA is able to deplete the redox, cholinergic, and dopaminergic system that leads to changes in the behavior of flies and increase mortality. We demonstrated the possible mechanisms involved in the development of Parkinson like disease in *Drosophila melanogaster*, and thus we designed a new model for future studies related to the pathogenesis of this disease. We believe that this work can serve as a warning for the constant use of BPA, and spur interest in new endorsements and future studies.

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FIGURES CAPTIONS

Fig. 1 Chemical structure of Bisphenol A (BPA);

Fig. 2 Schematic diagram of the experimental treatment protocol.

Fig.3 Evaluation of survival rate of flies exposed to Bisphenol A (BPA) for 7 days. The total number of flies represents the sum of 3 independent experiments. Mortality was determined by comparing the survival curves of the Mantel-Cox log-rank tests and multiple comparisons were corrected using the Bonferroni test.

Fig. 4 Evaluation of the spontaneous locomotor activity of flies exposed to Bisphenol A (BPA) for 7 days, through A) Negative geotaxis, B) Open field and C) Balance test. Significance was determined by one-way ANOVA with Bonferroni test. *Significant difference in relation to the control group; (p <0.05).

Fig. 5 Changes induced by exposure to Bisphenol A (BPA), acetylcholinesterase activity in A) head and B) body of adult *Drosophila melanogaster*. Significance was determined by one way analysis of variance (ANOVA) followed by the Bonferroni test. *Significant difference in relation to the control group; (p <0.05).

Fig. 6 Production levels of reactive species (RS) induced by exposure to Bisphenol A (BPA) in *Drosophila melanogaster* head samples. Significance was determined by one-way analysis of variance (ANOVA) followed by the Bonferroni test. Results were expressed as the percentage (%) of the control group. * Significant difference in relation to the control group, (p <0.05).

Fig. 7 Levels of lipid peroxidation (LPO) induced by exposure to Bisphenol A (BPA) in adult *Drosophila melanogaster* head samples. Significance was determined by one-way analysis of variance (ANOVA) followed by the Bonferroni test. * Significant difference in relation to the control group; (p <0.05).

Fig. 8 Action of Bisphenol A (BPA) on the activity of antioxidant enzymes A) Superoxide dismutase (SOD) and B) Catalase (Cat), and detoxifier C) glutathione-S-transferase (GST). The result corresponds to analyzes performed on adult *Drosophila melanogaster* head samples. Significance was determined by one-way analysis of variance (ANOVA) followed by the Bonferroni test. * Significant difference in relation to the control group, (p <0.05).

Fig. 9 Mitochondrial metabolic viability assessed by the MTT reduction method in head homogenate of adult flies exposed for 7 days to Bisphenol A (BPA). Significance was determined by ANOVA one-way analysis of variance using the Bonferroni test. The results were expressed as percentage (%) of the control group. * Significant difference in relation to the control group, (p <0.05).

Fig. 10 Cell viability in homogenate of head samples from flies exposed to Bisphenol A (BPA), evaluated by the rezaruzin reduction assay. Significance was determined by ANOVA one-way

ANOVA with Bonferroni test. Results were expressed as the percentage (%) of the control group. * Significant difference in relation to the control group (p <0.05).

Fig.11 Change in dopamine levels in head homogenate of adult flies exposed for 7 days to Bisphenol A (BPA). Significance was determined by ANOVA one-way analysis of variance using the Bonferroni test. * Significant difference in relation to the control group (p <0.05).

Table 1 Effects of Bisphenol A (BPA) on *Pearson's* correlation analysis (r) between Dopamine and locomotor and balance behavior. * p <0.05 is considered significant.

Table 2 Effects of Bisphenol A (BPA) on *Pearson's* correlation analysis (r) between Dopamine and oxidative stress markers. * p <0.05 is considered significant.

FIGURES

Fig. 1

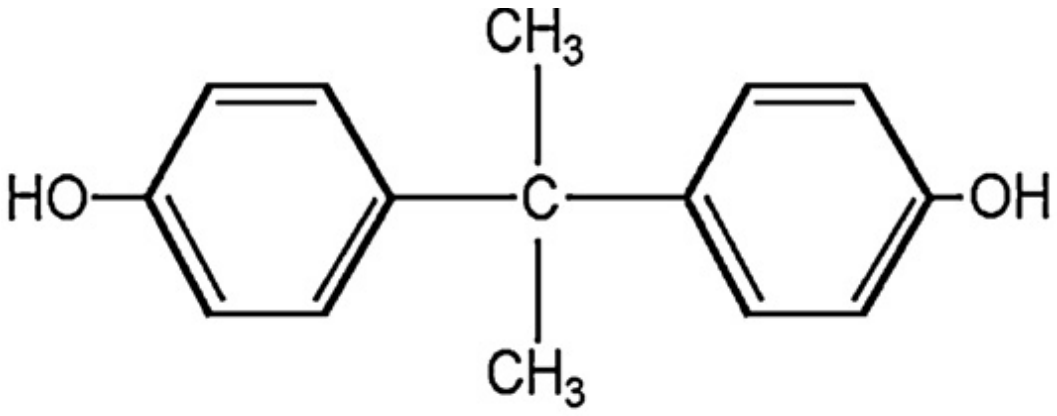

Fig. 2

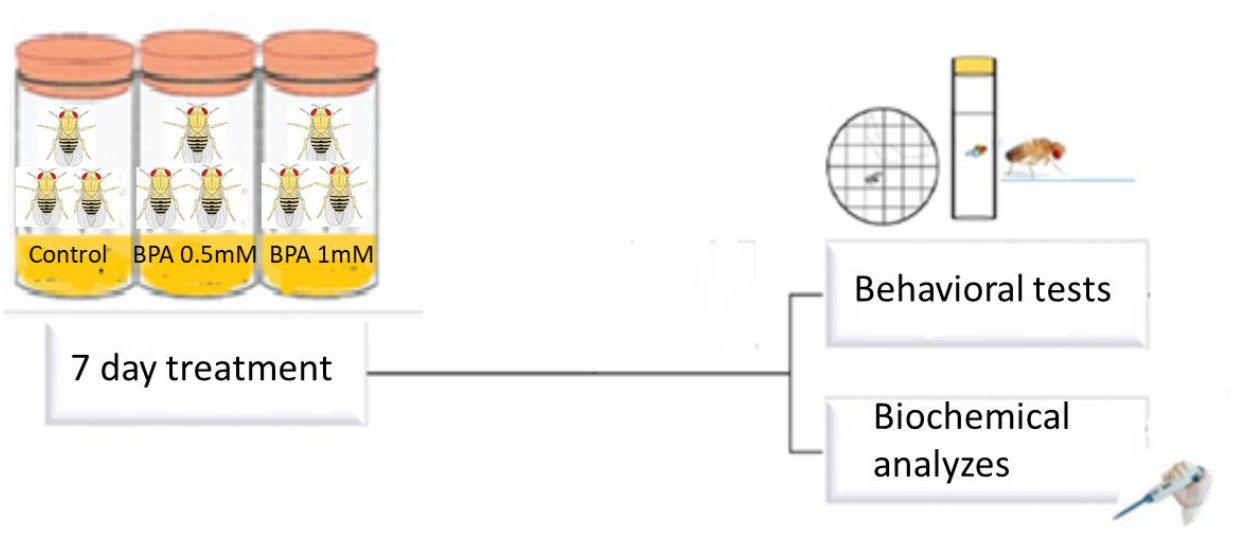

Fig. 3

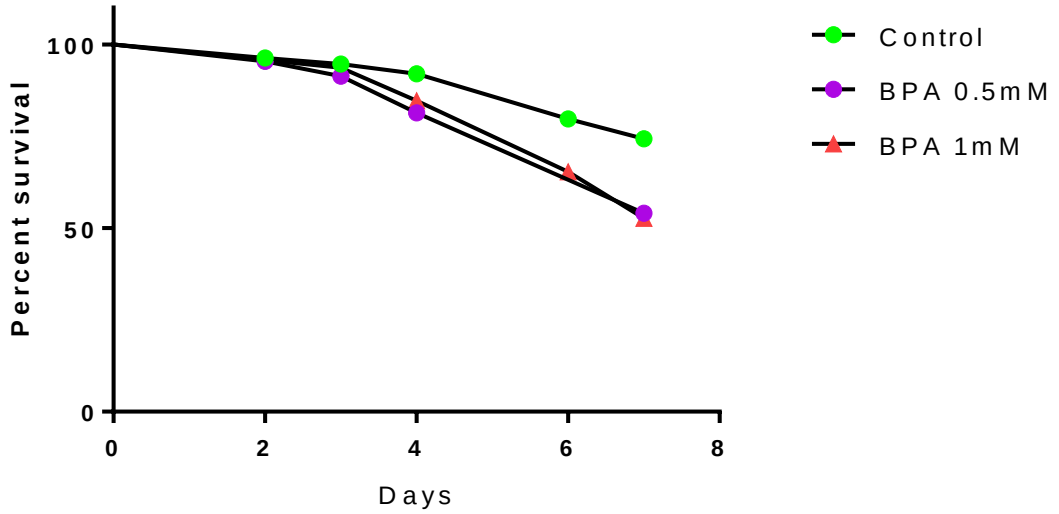
Percent survival
100
50
0
0
2
4
6
8
Days
Control
BPA 0.5mM
BPA 1mM

Fig. 4

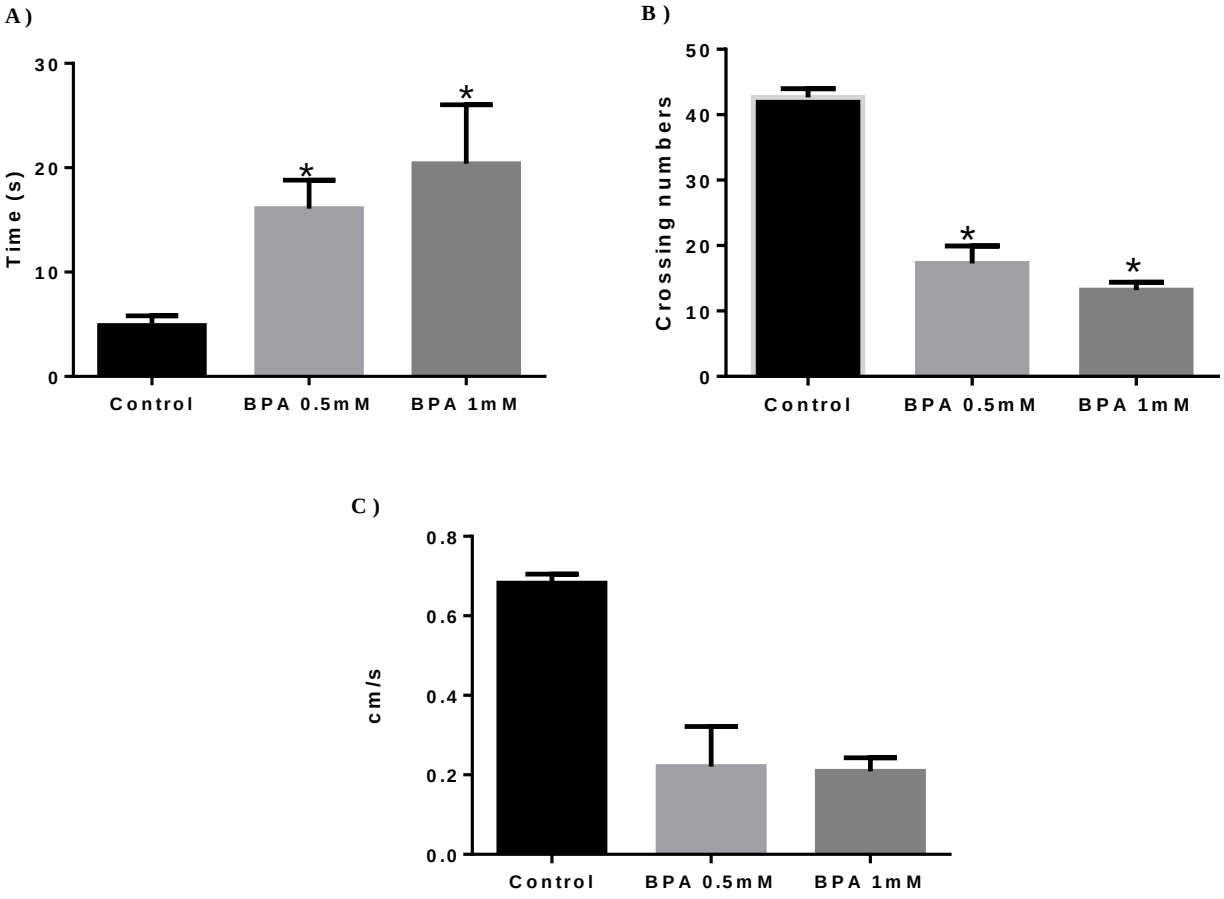
A)
Time (s)
30
20
10
0
Control
BPA 0.5mM
BPA 1mM
B)
Crossing numbers
50
40
30
20
10
0
Control
BPA 0.5mM
BPA 1mM
C)
cm/s
0.8
0.6
0.4
0.2
0.0
Control
BPA 0.5mM
BPA 1mM

Fig. 5

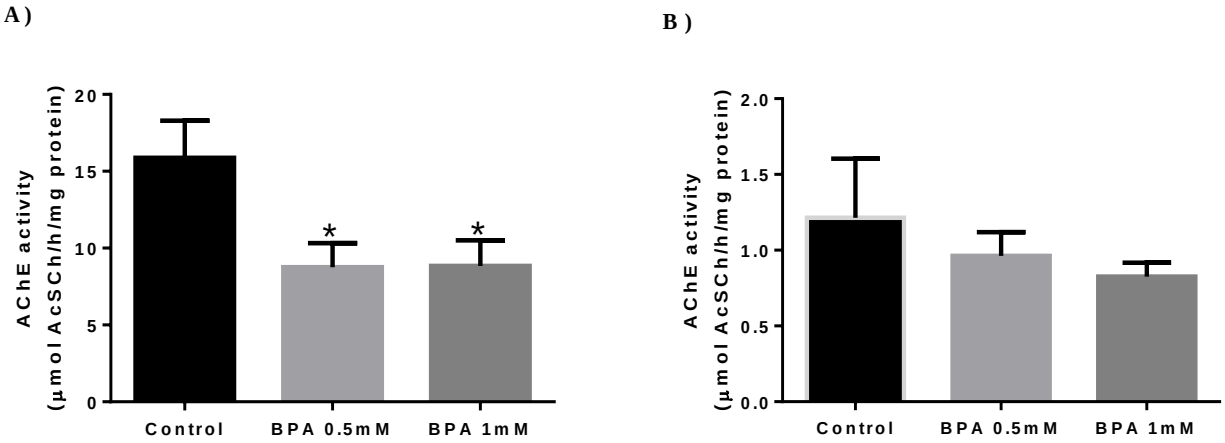
A)
AChE activity
(µmol AcSCh/h/mg protein)
20
15
10
5
0
*
*
Control
BPA 0.5mM
BPA 1mM
B)
AChE activity
(µmol AcSCh/h/mg protein)
2.0
1.5
1.0
0.5
0.0
Control
BPA 0.5mM
BPA 1mM

Fig. 6

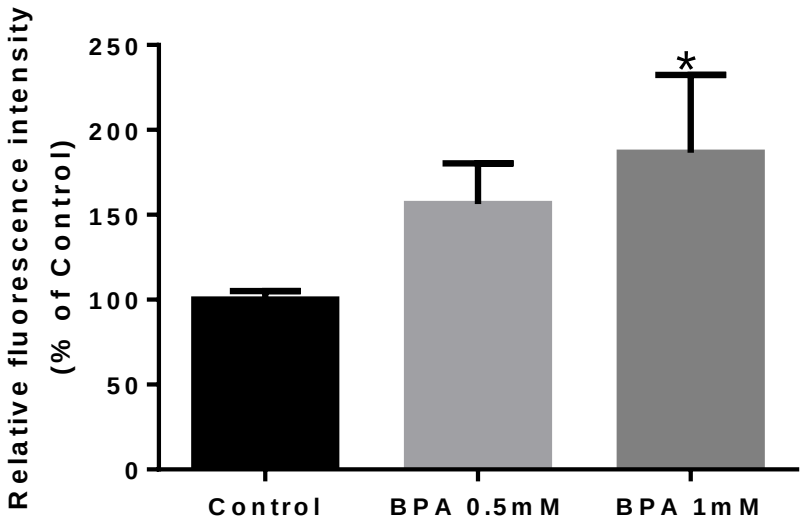
Relative fluorescence intensity
(% of Control)
250
200
150
100
50
0
*
Control
BPA 0.5mM
BPA 1mM

Fig. 7

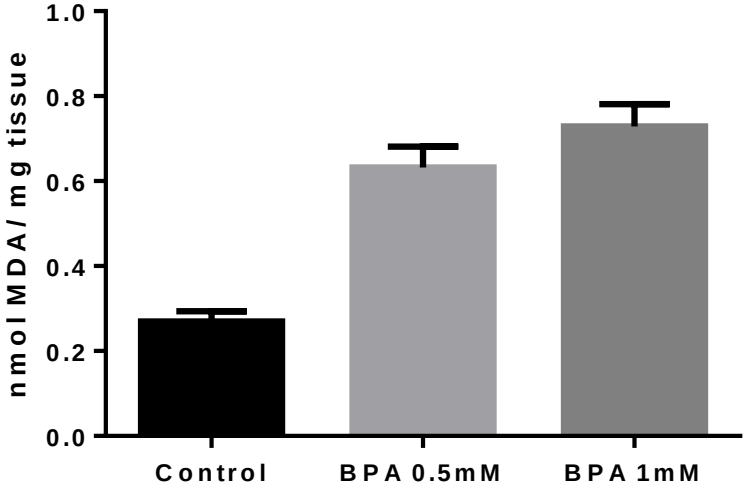
nmol MDA/ mg tissue
1.0
0.8
0.6
0.4
0.2
0.0
Control
BPA 0.5mM
BPA 1mM

Fig. 8

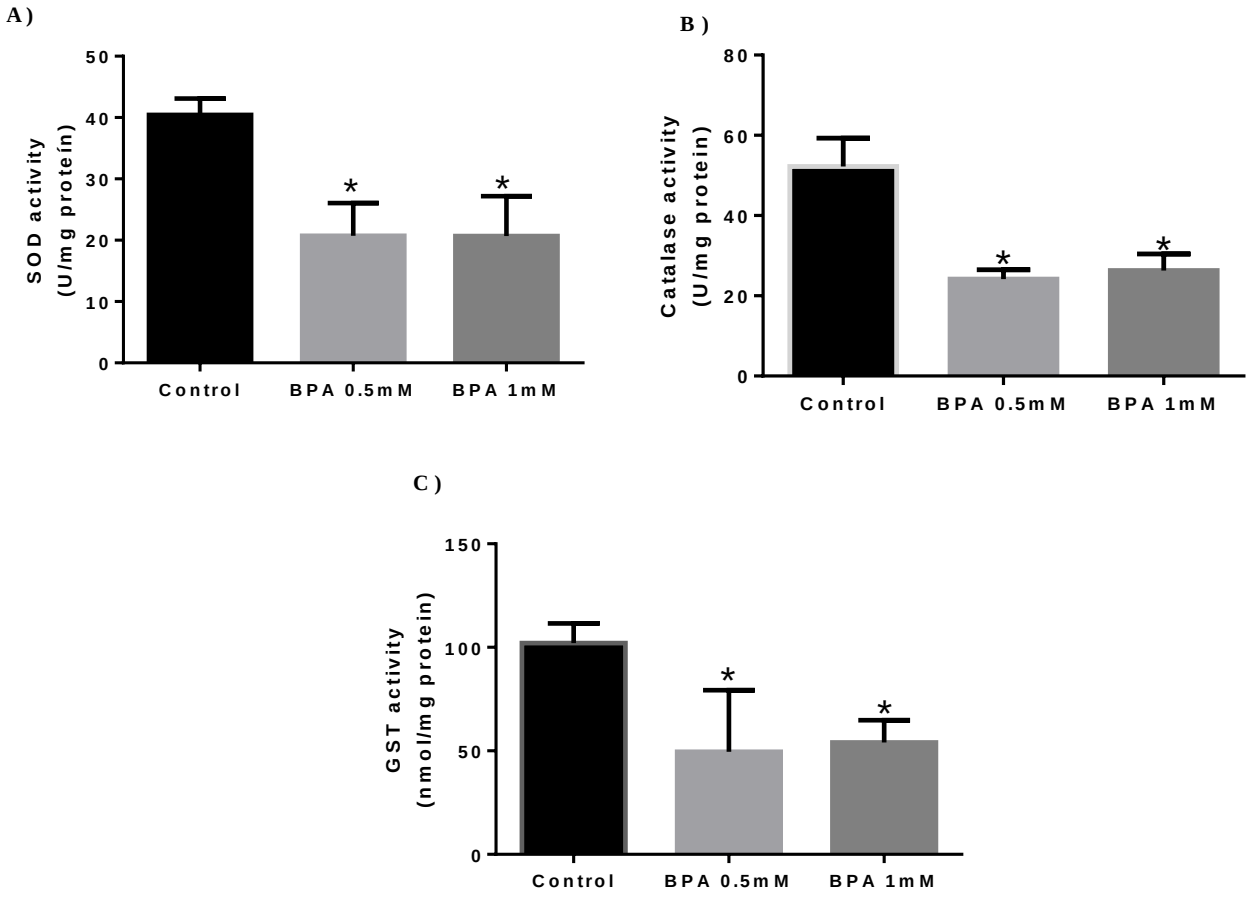
A)
SOD activity (U/mg protein)
50
40
30
20
10
0
*
*
Control
BPA 0.5mM
BPA 1mM
B)
Catalase activity (U/mg protein)
80
60
40
20
0
*
*
Control
BPA 0.5mM
BPA 1mM
C)
GST activity (nmol/mg protein)
150
100
50
0
*
*
Control
BPA 0.5mM
BPA 1mM

Fig. 9

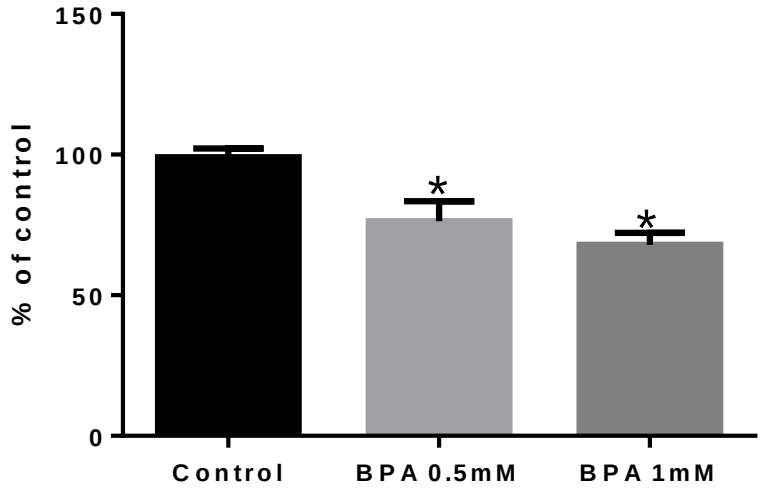
150
100
50
0
% of control
*
*
Control
BPA 0.5mM
BPA 1mM

Fig. 10

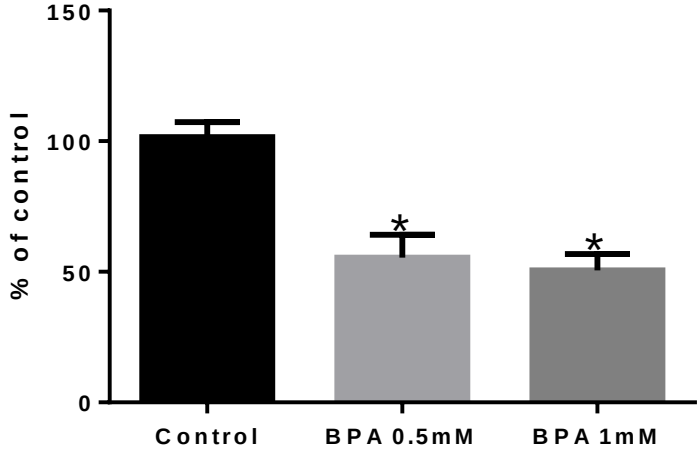
150
100
50
0
% of control
*
*
Control
BPA 0.5mM
BPA 1mM

Fig. 11

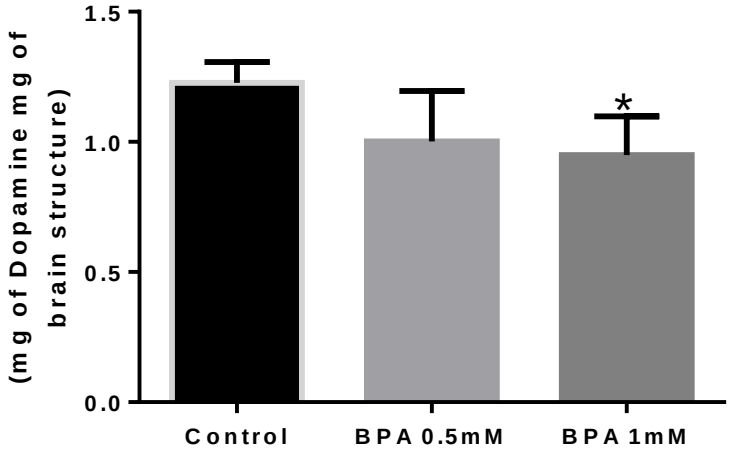

Table 1

Dopamine (DA) and behavioral analysis	***r***	***p***	***N***
DA x Open Field	0.7852	0.0025*	12
DA x Negative geotaxis	-0.8329	0.0008*	12
DA x Equilibrist Test	0.8115	0.0079*	9

Table 2

Dopamine (DA) and oxidative stress markers	***r***	***p***	***N***
DA x Reactive species	-0.8664	0.0025*	9
DA x malonildialdehyde	0.8227	0.0010*	12
DA x Superoxide dismutase	0.9103	0.0001*	12
DA x Catalase	0.8497	0.0005*	12
DA x Glutathione-S-transferase	0.9040	0.0001*	12

5. CONCLUSÃO

Através dos resultados deste trabalho, podemos concluir que houve de fato um dano a nível cerebral induzidas pela exposição ao Bisfenol A em *Drosophila melanogaster* adultas. As alterações comportamentais e bioquímicas foram semelhantes à características parkinsonianas relatadas na literatura, também utilizando moscas, porém induzidos com outros toxicantes ambientais.

Assim concluímos, que através deste estudo, caracterizamos e desenvolvemos um novo modelo experimental de doença de Parkinson, para auxiliar em pesquisas futuras para conhecimento mais abrangente sobre esta patologia.

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