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AVALIAÇÃO DO EFEITO DE NANOPARTÍCULAS CARREADORAS DE LUTEÍNA SOBRE O MODELO DE TRANSTORNO DO NEURODESENVOLVIMENTO EM *Drosophila melanogaster.*

TESE DE DOUTORADO

DIENIFFER ESPINOSA JANNER

Uruguaiana, RS, Brasil
2024

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Tese apresentada ao programa de Pós-graduação *Stricto Sensu* em Bioquímica, da Universidade Federal do Pampa, como requisito para obtenção do título de doutora em Bioquímica.

Orientador: Prof. Dr. Gustavo Petri Guerra

Coorientadora: Profª. Drª. Marina Prigol

Uruguaiana, RS, Brasil

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RESUMO

Os transtornos do neurodesenvolvimento como o transtorno do espectro autista (TEA) e o transtorno do déficit de atenção com hiperatividade (TDAH), são caracterizados por alterações persistentes na comunicação e interações sociais, bem como padrões restritos e estereotipados de comportamento. A exposição a inseticidas como por exemplo a imidacloprida vem sendo utilizada para desenvolver o modelo de transtorno do neurodesenvolvimento em moscas da fruta. A luteína é amplamente conhecida por suas propriedades antiinflamatórias e antioxidantes, sendo associada a efeitos neuroprotetores. O presente estudo teve por objetivo avaliar o efeito da administração de nanopartículas carreadoras de luteína em diferentes períodos sobre o modelo experimental de transtorno do neurodesenvolvimento em *Drosophila melanogaster.* Os pares de moscas foram expostas a uma dieta contendo imidacloprida por 7 dias para indução do modelo de transtorno do neurodesenvolvimento. A administração das nanopartículas carreadoras de luteína foi realizada no período pré-concepção ou pós natal durante 24 horas. Após a progênie obtida foi submetida as avaliações comportamentais e bioquímicas. Os resultados desse trabalho estão apresentados na forma de 1 artigo científico e 1 manuscrito. Os resultados encontrados no artigo 1 mostram que o tratamento com nanopartículas carreadoras de luteína foi capaz de atenuar o dano celular e reverter o aumento do estresse oxidativo cerebral das moscas evidenciado pelos marcadores (ROS, TBARS, SOD, CAT e Nrf2), bem como resgatou imunorreatividade de Shank e consequentemente reduziu as alterações comportamentais de hiperatividade, agressividade, interação social, movimentos repetitivos e ansiedade na progênie de moscas de ambos os sexos. Já os resultados encontrados no manuscrito 1 revelam que a suplementação com nanopartículas carreadoras de luteína foi capaz de prevenir a diminuição da atividade da enzima tirosina hidroxilase (TH), assim como dos neurotransmissores dopamina (DA) e serotonina (5-HT) na cabeça das mocas, e como consequência evitou danos comportamentais como hiperatividade, ansiedade, interação social, movimentos repetitivos, aprendizagem e memória na progênie de ambos os sexos. Com base em nossos resultados podemos concluir que a administração de nanopartículas carreadoras de luteína exerceu um efeito neuroprotetor sobre os danos observados na progênie exposta a imidacloprida no

modelo de transtorno do neurodesenvolvimento. Além disso investigar o melhor período de intervenção para prevenir o surgimento desses transtornos é de extrema importância, a fim de prevenir intercorrências futuras. Assim as nanopartículas carreadoras de luteína surgem como uma possível estratégia terapêutica que pode contribuir para prevenir e/ou amenizar as alterações presentes nesses distúrbios.

Palavras-chave: Transtorno do Espectro Autista (TEA); Transtorno do Déficit de Atenção e Hiperatividade (TDAH); Estresse oxidativo; Antioxidantes, Compostos bioativos; Carotenoides; Neurotransmissores.

ABSTRACT

Neurodevelopmental disorders such as autism spectrum disorder (ASD) and attention deficit hyperactivity disorder (ADHD) are characterized by persistent changes in communication and social interactions, as well as restricted and stereotyped patterns of behavior. Exposure to insecticides such as imidacloprid has been used to develop a model of neurodevelopmental disorder in fruit flies. Lutein is widely known for its anti-inflammatory and antioxidant properties and is associated with neuroprotective effects. The present study aimed to evaluate the effect of lutein **c**arrier nanoparticles administration at different periods on the experimental model of neurodevelopment disorder in *Drosophila melanogaster*. Fly pairs were exposed to a 7-day imidacloprid diet for induction of the neurodevelopmental disorder model. Administration of the lutein carrier nanoparticles was performed in the preconceptional period or prenatal for 24 h. After the obtained offspring was subjected to behavioral and biochemical assessment. The results of that work are presented in the form of 1 scientific paper and 1 manuscript. Results found in article 1 show that treatment with lutein **c**arrier nanoparticles was able to attenuate cell damage and reverse the increase of the cerebral oxidative of flies evidenced by markers (ROS, TBARS, SOD, CAT and Nrf2), as well as rescued Shank immunoreactivity and consequently reduced behavioral changes of hyperactivity, aggressiveness, social interaction, repetitive movements and anxiety in the progeny of flies of both sexes. Already the results found in manuscript 1 reveal that supplementation with lutein **c**arrier nanoparticles was able to prevent decreased activity of enzyme tyrosine hydroxylase (TH), as did neurotransmitters dopamine (DA) and serotonin (5-HT) in the head of flies, and as a consequence it prevented behavioral damages such as hyperactivity, anxiety, social interaction, repetitive movements, learning and memory in the progeny of both sexes. Based on our results we can conclude that the administration of lutein carrier nanoparticles exerted a neuroprotective effect on the damage observed on the progeny exposed to imidacloprid in the neurodevelopment disorder model. In addition to investigating the best intervention period to prevent the emergence of these disorders is of utmost importance in order to prevent future intercourse. Thus the lutein **c**arrier nanoparticles arise as a possible therapeutic strategy that can contribute to prevent and/or soften the changes present in these disorders.

Keywords: Autism Spectrum Disorder (ASD); Attention Deficit Hyperactivity Disorder (ADHD); Oxidative stress; Antioxidant, Bioactive compounds; Carotenoids, Neurotransmitters.

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS

(Referentes a Revisão Bibliográfica)

AChE – Acetilcolinesterase

ATP – Adenosina Trifosfato

Akt – Proteína Quinase B

CAT – Catalase

CNV - Variantes no Número de Cópias

DA – Dopamina

GABA – Ácido Gama-Aminobutírico

GST – Glutationa S-Transferase

LPS – Lipopolissacarídeo

NADPH – Nicotinamida Adenina Dinucleotídeo Fosfato reduzido

ROS – Espécies Reativas de Oxigênio

SOD – Superóxido Dismutase

TBARS – Espécies Reativas ao Ácido Tiobarbitúrico

TEA – Transtorno do Espectro Autista

TDAH – Transtorno do Déficit de Atenção com Hiperatividade

TID – Transtorno Invasivo do Desenvolvimento

TH – Tirosina hidroxilase

VPA – Ácido Valpróico

5-HT – 5-hidroxitriptamina

Sumário

APRESENTAÇÃO

No item **INTRODUÇÃO** e **REVISÃO BIBLIOGRÁFICA** está descrita uma breve revisão de literatura sobre os temas abordados nesta tese, seguida pelo item **OBJETIVOS**.

A **METODOLOGIA** realizada e os **RESULTADOS** obtidos que compõem esta tese estão apresentados sob a forma de 1 artigo publicado em periódico científico e 1 manuscrito, os quais se encontram no item ARTIGO e MANUSCRITO. Neste, constam as seções: Introdução, Materiais e Métodos, Resultados, Discussão, Conclusão e Referências Bibliográficas, representando uma parte deste estudo. O artigo científico encontra-se publicado na revista “Comparative Biochemistry and Physiology C”. Já o manuscrito encontra-se estruturado de acordo com as normas da revista científica “Food and Chemical Toxicology” para a qual será submetido.

Os itens **DISCUSSÃO** e **CONCLUSÃO** encontram-se no final desta tese e apresenta interpretações e comentários gerais sobre o manuscrito contido neste trabalho.

As **REFERÊNCIAS BIBLIOGRÁFICAS** referem-se somente às citações que aparecem nos itens introdução e revisão bibliográfica.

1. INTRODUÇÃO

Os distúrbios do neurodesenvolvimento são caracterizados por condições neurológicas as quais tem ocorrência precoce, ou seja, ainda na infância, prejudicando o desenvolvimento pessoal, social e profissional dos indivíduos (AMERICAN PSYCHIATRIC ASSOCIATION, 2014). Os principais representantes desses distúrbios são o transtorno do espectro autista (TEA) e o transtorno do déficit de atenção com hiperatividade (TDAH), os quais apresentam características como comportamentos de interação social, comunicação social e não social prejudicada, movimentos repetitivos, falta de atenção e hiperatividade (JONES; KLIN, 2013; LOMBARDO; LAI; BARON-COHEN, 2019; LUKE et al., 2012). A taxa de indivíduos diagnosticados com esses transtornos aumentou significativamente nos últimos anos, um estudo recente aponta uma prevalência de aproximadamente 1 a cada 40 crianças (SCHMIDT et al., 2017).

Os distúrbios do neurodesenvolvimento apresentam contexto etiológico complexo e não totalmente compreendido, em que possivelmente combina efeitos de múltiplos genes e fatores ambientais (BAI et al., 2019; SANDIN et al., 2014). Evidências demonstram que o estresse oxidativo, assim como outros marcadores influenciam diretamente nos transtornos TEA e TDAH, desta forma o desequilíbrio da produção de antioxidantes no organismo pode contribuir para a disfunção neuronal, afetando o desenvolvimento e a função cerebral (CORONA, 2020; JOSEPH et al., 2015). Além disso, marcadores inflamatórios, metabólicos e genéticos também têm sido associados a esses transtornos, sugerindo que uma combinação de fatores biológicos desempenha um papel crucial na sua etiologia e progressão (KAPCZINSKI et al., 2008; LIU et al., 2015; MOSSA et al., 2018; SA-CARNEIRO et al., 2020).

A imidacloprida (IMI) é um pesticida neonicotinóide que atua no sistema nervoso central, como um agonista dos receptores nicotínicos de acetilcolina (FFRENCH-CONSTANT et al., 2016). A exposição pré-natal e/ou pós-natal a IMI é associada a déficits comportamentais em diferentes espécies (CROSBY et al., 2015; DUZGUNER; ERDOGAN, 2012; MENGONI GOÑALONS; FARINA, 2015; TOMIZAWA; CASIDA, 2005). Neste sentido, estudos realizados anteriormente demonstram que a exposição de *Drosophila melanogaster* ao IMI resulta em uma progênie com diferentes alterações comportamentais (JANNER et al., 2021; KIM; LEE;

PARK, 2017), evidenciando assim a utilização desse composto como ferramenta útil no desenvolvimento de modelo químico que permita a avaliação de fenótipos e vias moleculares presentes nos transtornos do neurodesenvolvimento na *Drosophila*, sendo assim uma alternativa para as avaliações com modelos genéticos já descritos.

A mosca da fruta (*Drosophila melanogaster*) é um modelo animal amplamente utilizado devido ao seu genoma condensado, o qual foi completamente sequenciado, apresentando aproximadamente 75% de similaridade genética relacionada as doenças em seres humanos (PANDEY; NICHOLS, 2011). O modelo de *Drosophila melanogaster* possui diversas vantagens práticas e genéticas, como um curto período de geração (10 dias à temperatura ambiente, sendo que as fêmeas podem depositar de 30 a 50 ovos por dia), um grande número de descendentes para análises rápidas em larga escala, (RESH; CARDÉ, 2009) e apresentam reação rápida á drogas que atuam no sistema nervoso. Além disso, evidencias demonstram que a *Drosophila melanogaster* possui inúmeros comportamentos semelhantes aos observados em humanos, como comportamentos repetitivos, de aprendizagem, memória e agressividade (ROBERTS; DAWLEY; REIGART, 2019; TAUBER; VANLANDINGHAM; ZHANG, 2011; TULLY et al., 1994).

Considerando o aumento do número de crianças diagnosticadas com distúrbios do neurodesenvolvimento ao longo das últimas décadas, que indivíduos portadores desses distúrbios podem apresentar comorbidades associadas, e o fato de que não existe uma cura, onde os tratamentos utilizados correspondem a terapias comportamentais e educacionais, associadas ao uso de antipsicóticos, que visam apenas controlar alguns sintomas-alvos da doença, o desenvolvimento de novas opções terapêuticas e a descoberta de prováveis mecanismos de ação se faz necessário. Neste sentido, evidências crescentes apontam para o importante papel dos compostos bioativos, constituintes extranutricionais presentes, principalmente, nos alimentos de origem vegetal, como a luteína, principal carotenoide encontrado no cérebro humano (OLIVEIRA et al., 2020; ZENI; CAMARGO; DALMAGRO, 2019).

A luteína é um carotenoide amplamente conhecido por suas propriedades anti-inflamatórias e antioxidantes, além da sua capacidade de atravessar a barreira hematoencefálica (BIAN et al., 2012; SIES; STAHL, 2003). Diversos estudos realizados demonstram a associação da luteína a efeitos neuroprotetores, evitando a diminuição de dopamina, redução do dano oxidativo, melhora da atividade cognitiva e memória (JOHNSON et al., 2008; NATARAJ et al., 2016; NOUCHI et al., 2020).

Além disso, nanopartículas poliméricas têm sido amplamente estudadas visando aumentar a biodisponibilidade, absorção e facilitar a entrada de drogas ou compostos bioativos através de barreiras biológicas, maximizando o potencial terapêutico e ao mesmo tempo minimizando os efeitos colaterais (REIN et al., 2013a). Estudos realizados recentemente demonstram que a administração de nanopartículas de luteína foi capaz de reduzir danos comportamentais e de memória bem como inibir danos oxidativos e apoptose em diferentes modelos de roedores (DO PRADO SILVA et al., 2017; VIANA et al., 2023), além disso também foi capaz de melhorar as barreiras antioxidantes, níveis de dopamina e a atividade da enzima acetilcolinesterase na *Drosophila melanogaster* (FERNANDES et al., 2021).

Portanto no presente estudo avaliamos o efeito da administração de nanopartículas carreadoras de luteína sobre a progênie de moscas expostas a IMI, com o intuito de elucidar os mecanismos de ação da luteína sobre os danos comportamentais e neuroquímicos ocasionados pela exposição a IMI em *Drosophila melanogaster.* Nesse viés o trabalho foi realizado com a hipótese de que as nanopartículas carreadoras de luteína, são capazes de prevenir/restaurar as modificações comportamentais e neuroquímicas ocasionadas pela exposição a IMI em ambos os sexos, desta forma as nanopartículas carreadoras de luteína podem futuramente ser utilizadas como coadjuvantes no tratamento de distúrbios do neurodesenvolvimento, visando amenizar as alterações observadas, e desvendar os mecanismos envolvidos nesses distúrbios.

2. REFERENCIAL TEÓRICO

2.1. Transtornos do neurodesenvolvimento

Os distúrbios do neurodesenvolvimento (NDDs) são desordens neurológicas, que causam impactos adversos sobre habilidades cognitivas, sociais e psicológicas na vida dos indivíduos acometidos (AMERICAN PSYCHIATRIC ASSOCIATION, 2014).O Transtorno do Espectro Autista (TEA) é um transtorno neurodesenvolvimental com início precoce, cujas principais características são as dificuldades de interação social, comunicação e reciprocidade social, apresenta ainda padrões restritos e repetitivos de comportamentos, interesses e atividades, bem como certo grau de agressividade dentre outros comportamentos, sendo o nível dessas modificações distinto entre os indivíduos autistas (DOLDUR-BALLI et al., 2022; SHARMA; GONDA; TARAZI, 2018; UEOKA et al., 2019).

Por outro lado, o Transtorno do Déficit de Atenção com Hiperatividade (TDAH) é um distúrbio do neurodesenvolvimento definido pela presença de padrões de desatenção, desorganização e/ou hiperatividade e impulsividade em níveis mais elevados do que o normal nos indivíduos (BRITES, C. 2019). Ambos os distúrbios possuem diagnóstico clínico não havendo exames laboratoriais que auxiliem no seu reconhecimento, sendo assim realizada uma avaliação comportamental do indivíduo com base em critérios previamente estabelecidos (AMERICAN PSYCHIATRIC ASSOCIATION, 2014).

2.2. Epidemiologia e Etiologia

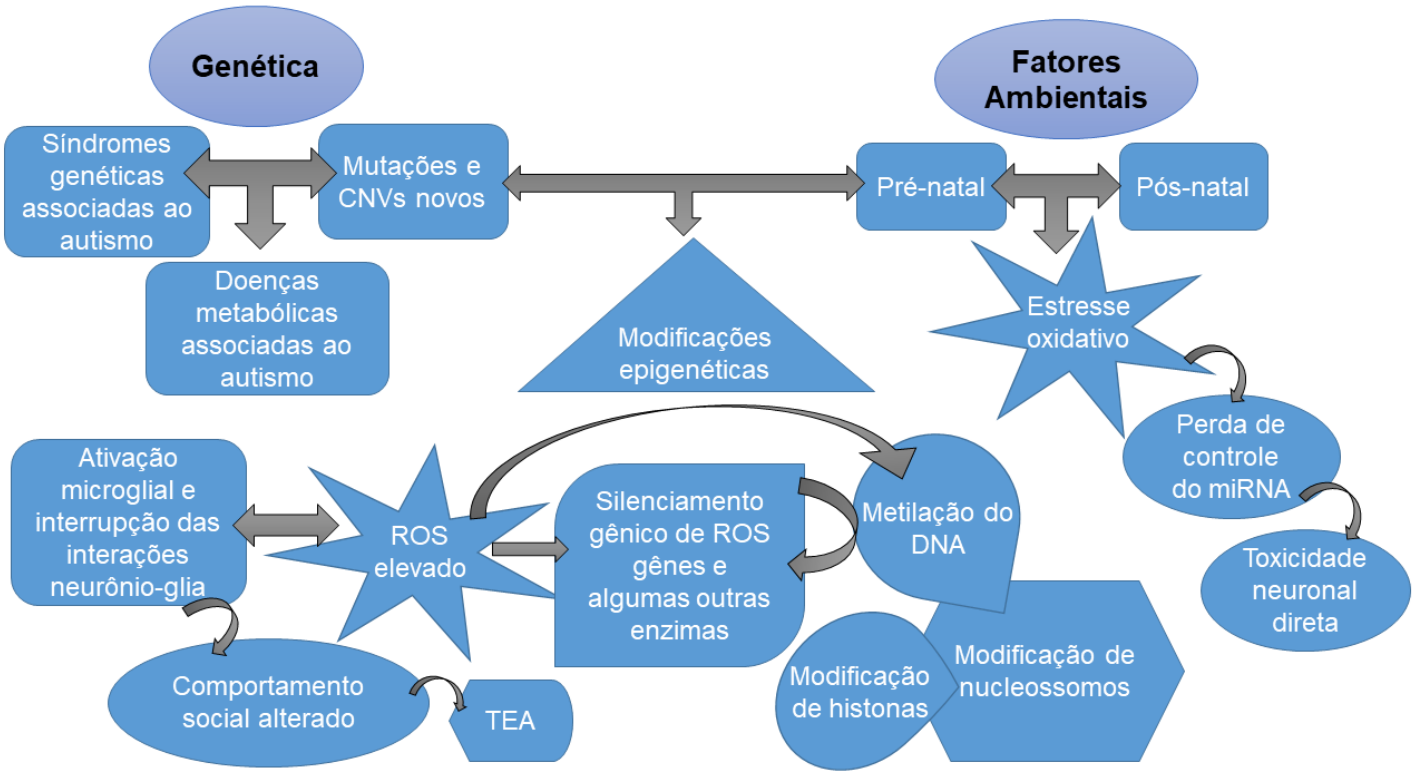
A prevalência dos transtornos do neurodesenvolvimento como o TEA e TDAH parece estar aumentando em todo o mundo, mesmo com a inexistência de dados anuais sobre a epidemiologia desses distúrbios pode-se observar que cada vez mais indivíduos são diagnosticados. De acordo com uma estudo realizado no ano de 2021 dados apontaram que a estimativa de TEA nos EUA é de 1 caso para cada 44 crianças com até 8 anos de idade (MAENNER et al., 2021). A respeito do gênero o TEA apresenta prevalência desigual, sendo aproximadamente quatro vezes mais frequente em meninos que em meninas (SCHENDEL; THORSTEINSSON, 2018), porém o sexo feminino quando afetado demonstra alterações mais severas do TEA e um alto comprometimento cognitivo (SADOCK; SADOCK, 2017).

Não há evidências de um aumento na prevalência mundial de TDAH nas últimas três décadas, sendo apontada como aproximadamente 5,29% dos indivíduos, em que este distúrbio afeta predominantemente homens com uma proporção de 4:1 em relação a mulheres (DUARTE et al., 2021; FARAONE et al., 2015; POLANCZYK et al., 2007).

A respeito da etiologia tanto do TEA quando do TDAH teorias aumentam ano após ano, sendo até o presente momento nenhuma delas comprovadas cientificamente. Embora haja uma inexistência cientifica para a razão etiológica desses distúrbios, existem inúmeros indícios que apontam para uma origem multifatorial englobando fatores genéticos, ambientais e imunológicos (BÖLTE; GIRDLER; MARSCHIK, 2019; MANDY; LAI, 2016; YOON et al., 2020). A etiologia genética é sustentada sobre tudo com base em anormalidades cromossômicas citogenéticamente visíveis, variação no número de cópias (CNV) e distúrbios de um único gene (BOURGERON, 2015; FIGUEIREDO et al., 2022).

Nesse viés as alterações epigenéticas podem promover o aumento de espécies reativas de oxigênio (ROS), levando ao comprometimento da metilação do DNA, favorecendo um mecanismo de "*feedback"* positivo dessa forma indivíduos acometidos por esses transtornos apresentam maior vulnerabilidade ao estresse oxidativo e neurotoxicidade conforme ilustrado na Figura 1 abaixo (ESSA, 2020).

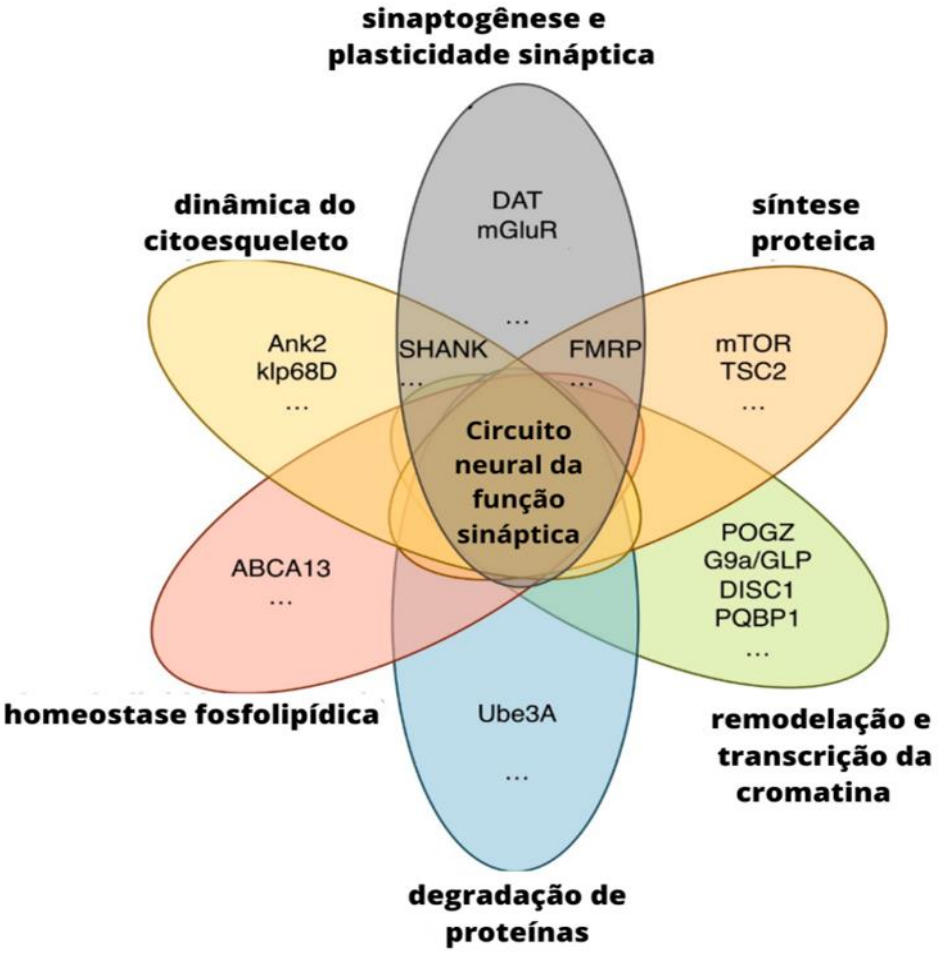
Figura 1: Diagrama envolvendo ambiente, genética e epigenética no desenvolvimento do TEA.



Fonte: Adaptado de ESSA, (2020).

Inúmeros genes de interesse foram identificados como causadores ou que aumentam o risco para o desenvolvimento de TEA, esses genes possuem múltiplas funções, incluindo remodelação e transcrição da cromatina (*POGZ*, *GLP* e *DISC1*) (BHALLA; MEHAN, 2022; ZHAO et al., 2019; ZHENG et al., 2011), síntese e degradação de proteínas (*mTOR*, *TSC2*, *UBE3A*) (WINDEN et al., 2019; WINDEN; EBRAHIMI-FAKHARI; SAHIN, 2018; ZHAO; ZHANG; YU, 2020), andaimes e dinâmica do citoesqueleto (*SHANK3*, *NBEA*, e *ANK3*) (BRUNO et al., 2021; KATO et al., 2022; KLOTH et al., 2021), bem como sinaptogênese e plasticidade sináptica (*CNTNAP2*, *Neuroligin-3,-4*, *DAT* e *mGluR*) conforme demonstrado na Figura 2 (DE JONG et al., 2021; DE LA TORRE-UBIETA et al., 2016; HEGDE et al., 2021; UEOKA et al., 2019).

Figura 2: Genes associados ao transtorno do espectro autista humano (TEA) com várias funções



Fonte: Adaptado de UEOKA et al., (2019b).

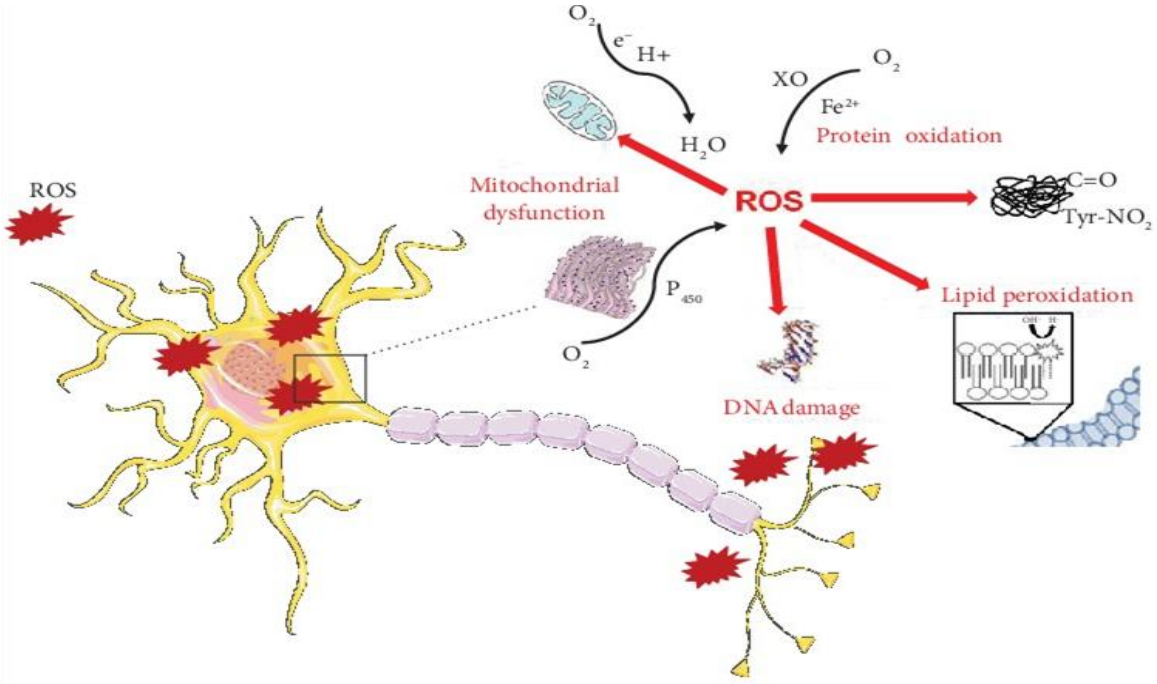
Genes de distintos sistemas de neurotransmissores que codificam transportadores, receptores ou enzimas são extremamente investigados no TDAH, tendo-se como exemplo, o DAT1 (gene do transportador de dopamina) (SHANG et al., 2016), genes dos receptores adrenérgicos α2A (ADRA2A) e α2C (ADRA2C) (CHO et al., 2008; XU et al., 2021), a COMT (gene que codifica a enzima catecol-O-metiltransferase) (FAGEERA et al., 2021), entre outros.

A respeito da contribuição ambiental para os distúrbios de TEA e TDAH apresentam-se fatores como: idade dos pais, ambiente fetal (esteroides sexuais, obesidade, diabetes, hipertensão), eventos perinatais e obstétricos (hipóxia), medicação (valproato) uso de drogas e exposição a poluentes (BÖLTE; GIRDLER; MARSCHIK, 2019).

2.3. Dano oxidative, neurotransmissores e sinaptogênese nos transtornos do neurodesenvolvimento (TEA e TDAH)

O estresse oxidativo no TEA e TDAH é atribuído a geração de radicais livres, os quais são responsáveis pela disfunção mitocondrial (ESSA, 2020). A disfunção mitocondrial possui como principais consequências: a) redução da produção de ATP, b) produção elevada de espécies reativas de oxigênio (ROS) e consequentemente danos oxidativos e c) indução da apoptose conforme ilustrado na Figura 3 (ROSSIGNOL; FRYE, 2012).

Figura 3: Dano Celular induzido pelo estresse oxidativo.



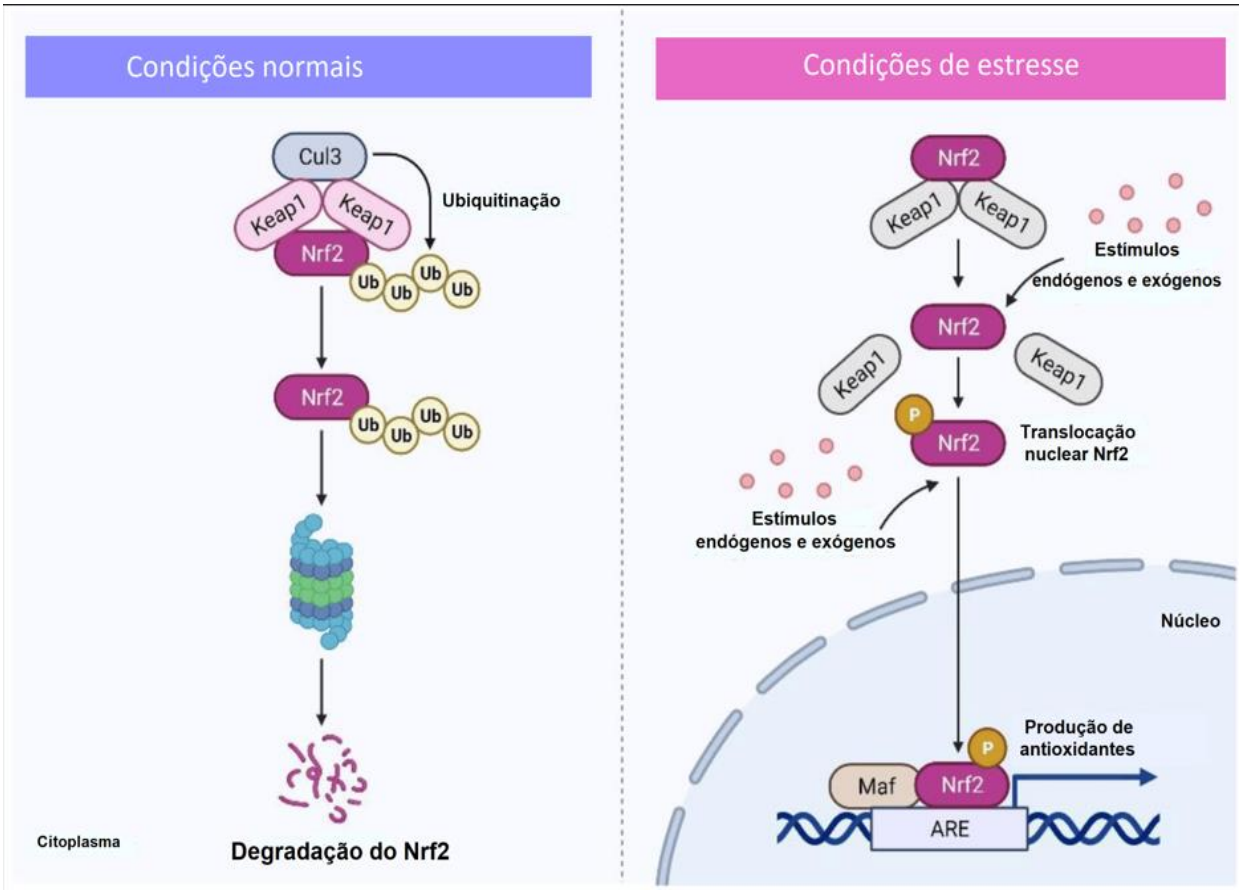
Fonte: Adaptado de HÖHN et al., (2020).

Tendo em vista que as espécies reativas têm efeitos diversos, incluindo a indução de dano oxidativo a biomoléculas e a ativação de sinalizações, dependendo dos seus níveis intracelulares, que são rigidamente regulados pela presença de antioxidantes enzimáticos e não enzimáticos (CURIESES ANDRÉS et al., 2023). O fator nuclear eritroide 2 (Nrf2), um regulador crucial, controla a expressão de diversos antioxidantes enzimáticos, influenciando sobre os níveis de ROS através de proteínas como superóxido dismutase (SOD), catalase (CAT), glutationa peroxidase (GPx) e heme oxigenase-1 (HO-1) (HAMMAD et al., 2023). Esses antioxidantes são essenciais

para manter o equilíbrio redox e a homeostase celular (ANIK et al., 2022; JOMOVA et al., 2023; SIES et al., 2022).

A produção de enzimas antioxidantes pelas células é predominantemente induzida pela ativação do Nrf2 (JOMOVA et al., 2023). Assim sob condições normais, o Nrf2 interage com a molécula Keap1 para promover a ubiquitinação do Nrf2, mediada pelo complexo E3 ligase baseado em Cullin 3 (Cul3). Como resultado, o Nrf2 é rapidamente degradado pelo proteassoma, mantendo-se em níveis baixos no citoplasma (AHSAN et al., 2022; HAMMAD et al., 2023). Já na presença de estresse, os resíduos de cisteína na Keap1 sofrem modificações, fazendo com que o Nrf2 se dissocie sendo translocado para o núcleo. Lá, ele se liga ao elemento de resposta antioxidante (ARE) no DNA, ativando genes responsáveis pela produção de novas enzimas antioxidantes e de detoxificação (AHSAN et al., 2022; COLARES et al., 2022; HAMMAD et al., 2023; WU; LU; BAI, 2019), conforme a figura 4.

Figura 4: *Sinalização de Nrf2*

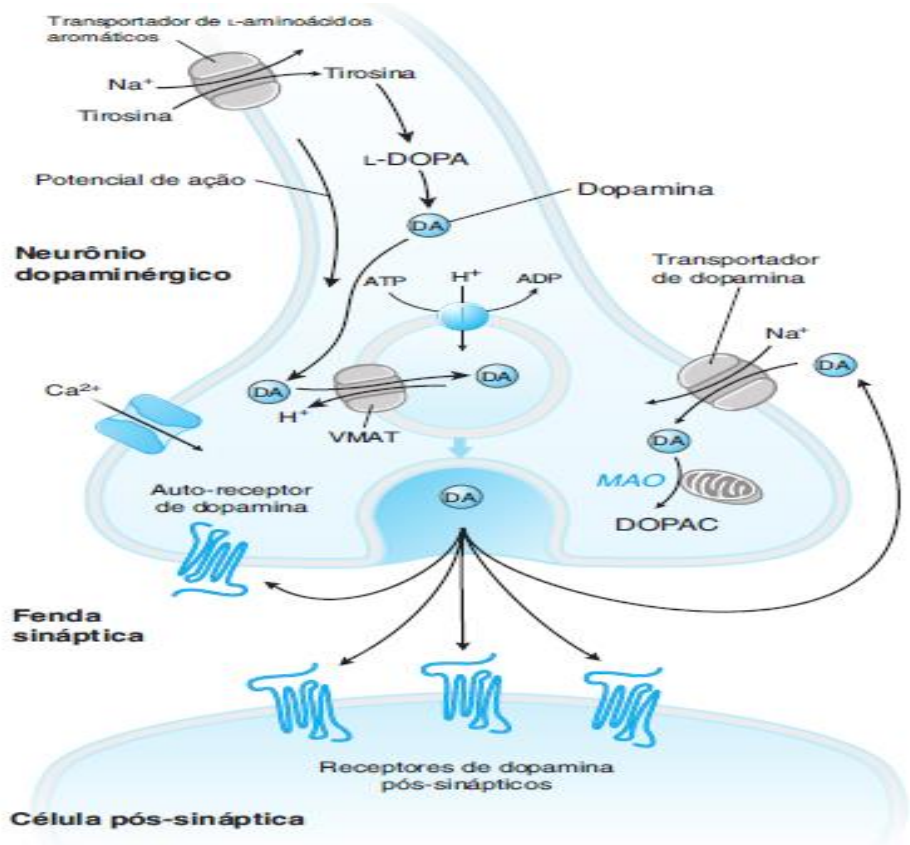


Fonte: Adaptado de HAMMAD et al. (2023).

Desta forma evidências demonstram que o estresse oxidativo, assim como outros marcadores influenciam diretamente nos transtornos TEA e TDAH, sendo esse aumento oxidativo atribuído a níveis reduzidos da proteína Nrf2 (NADEEM et al., 2020; NAPOLI et al., 2014; SCHRIER et al., 2022) bem como de BDNF (fator neurotrófico derivado do cérebro), estando a redução desse fator também implicada na disfunção dopaminérgica (KAPCZINSKI et al., 2008; LIU et al., 2015; SA-CARNEIRO et al., 2020).

A dopamina (DA) é um neuromodulador chave que desempenha inúmeras funções cognitivas, incluindo motivação, aprendizado e atenção, desta forma esse neurotransmissor é de suma importância para o sistema nervoso centra (SESCOUSSE et al., 2018). A neurotransmissão dopaminérgica acontece através de sinapses, em que a DA se liga aos receptores pós-sinápticos ou pré-sinápticos, essa ligação independentemente do receptor gera um potencial elétrico na célula pré-sináptica conforme ilustrado na Figura 5. A DA é sintetizada através conversão da L-tirosina em L-DOPA, pela ação da enzima tirosina hidroxilase (TH) nos terminais nervosos dopaminérgicos (DAUBNER; LE; WANG, 2011).

Figura 5: Neurotransmissão dopaminérgica.



Fonte: STANDAERT & GALANTER, (2009).

Em relação aos receptores DA pré-sinápticos o sinal pode excitar ou inibir a célula sináptica, já nos receptores DA pós-sinápticos o sinal se propaga para o neurônio pós-sináptico (JUÁREZ OLGUÍN et al., 2016). Após exercer sua função sináptica a DA através da ação dos transportadores de DA de alta afinidade (DAT) ou dos transportadores de monoamina da membrana plasmática de baixa afinidade é retomado no citosol pelas células pré-sinápticas (Standaert & Galanter, 2009).

A função da DA pode ser medida de diversas formas, como por exemplo, em relação a capacidade de síntese e/ou densidade do transportador, e através de alterações na disponibilidade do receptor (SESCOUSSE et al., 2018). Dado o papel importante desse sistema neurotransmissor sobre as funções cerebrais a desregulação dopaminérgica é implicada em muitos transtornos neuropsiquiátricos como na doenças de Parkinson, TDAH e TEA (DICARLO et al., 2019; ZÜRCHER et al., 2021).

A 5-hidroxitriptamina (5-HT) é um neurotransmissor monoamina produzido principalmente nos núcleos do rafe e distribuído por várias regiões do cérebro, onde exerce funções variadas em comportamentos socioemocionais, frequentemente relacionados a fenótipos semelhantes aos do TEA (ANDERSSON et al., 2021; MULLER; ANACKER; VEENSTRA-VANDERWEELE, 2016; RODNYY et al., 2024). Relatos indicam que os níveis de 5-HT encontram-se relativamente mais baixos no cérebro de indivíduos autistas (CAO et al., 2022; CHANDANA et al., 2005).

Desta forma devido ao fato de ambos os neurotransmissores DA e 5-HT interagirem com várias vias neurológicas, níveis anormais dessas monoaminas podem influenciar negativamente sobre diversos comportamentos como: movimentos repetitivos, dificuldades na interação social, hiperatividade e aprendizagem, presentes nos transtornos do neurodesenvolvimento (CAO et al., 2022; DICARLO et al., 2019).

Em contrapartida as proteínas Shank, também chamadas de proteínas associadas à sinapse ricas em prolina (ProSAPs), são proteínas estruturais chave localizadas na densidade pós-sináptica (PSD) das sinapses glutamatérgicas, onde desempenham papéis fundamentais no desenvolvimento e na função sináptica (NAISBITT et al., 1999). As três isoformas conhecidas da proteína SHANK são SHANK1 (ProSAP3; cromossomo 19q13.33), SHANK2 (ProSAP1; cromossomo 11q13.3) e SHANK3 (ProSAP2; cromossomo 22q13.3), em que por meio de seus domínios de ligação, as proteínas SHANKs interagem com receptores, canais iônicos, proteínas do citoesqueleto e outras proteínas de andaime, desempenhando um papel

crucial na integridade e composição molecular das sinapses glutamatérgicas excitatórias (VYAS et al,. 2021).

Assim deleções, duplicações ou mutações no gene SHANK3 foram frequentemente identificadas em pacientes com transtorno do neurodesenvolvimento (DURAND ET AL., 2007; BOCCUTO ET AL., 2013; LEBLOND ET AL., 2014). Essas mutações são uma das causas monogênicas mais recorrentes dos disturbios, correspondendo a pelo menos 0,69% de todos os casos. Além disso, indivíduos com mutações truncadas de SHANK3 costumam apresentar alterações comportamentais e intelectuais as quais variam de moderadas a graves (BELLOSTA P, SOLDANO A. 2019).

2.4. Tratamentos

Os transtornos do neurodesenvolvimento não apresentam cura, entretanto são realizadas intervenções terapêuticas psicológicas e educacionais com o intuito de melhorar habilidades de linguagem, comunicação, interações sociais dos indivíduos (ALMEIDA SSA, MAZETE BPGS, BRITO AR, 2018).

São realizadas algumas intervenções como: terapia ocupacional; musicoterapia e psicologia sendo o prognóstico para as estratégias psicopedagógicas mais eficiente quando realizada precocemente (ABELENDA; RODRÍGUEZ ARMENDARIZ, 2020; MUSICH; ARAGÓN-DAUD, 2022; RAMIREZ-MELENDEZ et al., 2022).

O tratamento farmacológico é utilizado apenas pra controlar as comorbidades não havendo um fármaco específico destinado para esses distúrbios, desta forma é indicado o uso de medicamentos antipsicóticos (neurolépticos), e em alguns casos faz-se o uso de dietas especiais e suplementação com vitaminas com o intuito de amenizar os efeitos desses distúrbios (ADAMS et al., 2011; NIKOLOV; JONKER; SCAHILL, 2006; SHARMA; GONDA; TARAZI, 2018). Distintas classes de medicamentos têm sido utilizadas nas intervenções farmacoterapêuticas na tentativa de controlar os sintomas que compõem os distúrbios do neurodesenvolvimento. Os fármacos empregados incluem antipsicóticos atípicos (risperidona, olanzapina, clozapina) para controlar a hiperatividade, agressividade e comportamento autolesivo; em alguns casos têm sido empregados inibidores seletivos da recaptação de serotonina (citalopram, fluoxetina, sertralina) para comportamentos repetitivos e

ansiedade; para comportamentos de hiperatividade utiliza-se o metilfenidato um psicoestimulante; já para os distúrbios do sono é faz-se o uso de mediadores do sistema nervoso central (melatonina) (BARROS NETO; BRUNONI; CYSNEIROS, 2019).

Embora os medicamentos sejam uma alternativa no tratamento dos distúrbios do neurodesenvolvimento, os mesmos apenas servem como coadjuvantes de outras intervenções como, fonoterapia e terapia ocupacional além da psicologia comportamental considerada o tratamento de primeira escolha (MASI et al., 2017).

2.5. Modelos experimentais de transtorno do neurodesenvolvimento

Em consequência da complexibilidade dos transtornos TEA e TDAH associados a comorbidades, surgem obstáculos que dificultam a compreensão das características presentes desses distúrbios, tornando-se necessário o desenvolvimento de modelos animais para auxiliar na elucidação dos mesmos (SCHLICKMANN; FORTUNATO, 2013).

A utilização de modelos experimentais permite a investigação de diversos fatores de risco, tanto genéticos quanto ambientais, possibilitando a busca de vias moleculares e mecanismos neurofisiológicos envolvidos no transtorno, em que a compreensão desses aspectos é de suma importância para o desenvolvimento de métodos de prevenção bem como terapias eficazes (KIM et al., 2016). Desta forma a validação de um modelo experimental para transtornos do neurodesenvolvimento ocorre por meio da observação de características como prejuízos comportamentais, interação social e estereotipias, além de alterações neuroquímicas as quais são visualizadas em humanos com esses distúrbios (SERVADIO et al., 2016; WÖHR; SCATTONI, 2013).

No modelo de roedores é realizada a administração de ácido valpróico (VPA) (CHALIHA et al., 2020; LIU et al., 2021a), ou lipopolissacarídeo (LPS) (XIAO et al., 2021) aplicadas por via intraperitoneal (i.p) e subcutânea em que essas são as substâncias mais utilizadas para mimetizar o modelo de TEA.

A *Drosophila melanogaster* se tornou um modelo alternativo para utilização em diversas pesquisas, pelo fato de apresentar uma ampla versatilidade. Assim inúmeros estudos utilizando moscas da fruta estão sendo realizados para investigar as funções

dos diferentes genes (MARCOGLIESE et al., 2022; MARIANO et al., 2020) e comportamentos (JANNER et al., 2021; KIM; LEE; PARK, 2017; MUSACHIO et al., 2021; SHILPA et al., 2021) associados a distúrbios do neurodesenvolvimento, entretanto a realização de modelos com exposições a produtos químicos, como por exemplo a imidacloprida (JANNER et al., 2021; KIM; LEE; PARK, 2017) ainda são pouco utilizados, sendo mais frequentemente o uso de modelos genéticos para mimetizar esses transtornos.

Em modelos genéticos do FMR1 um gene humano cuja função é codificar uma proteína chamada proteína de retardo mental X frágil, observou-se que a desregulação do ortólogo de *Drosophila melanogaster*, o dFMR1 causa morte celular (LIU et al., 2012) e supercrescimento sináptico pronunciado nos NMJs (PAN et al., 2004), alterações no metabolismo energético e função mitocondrial (WEISZ et al., 2018)

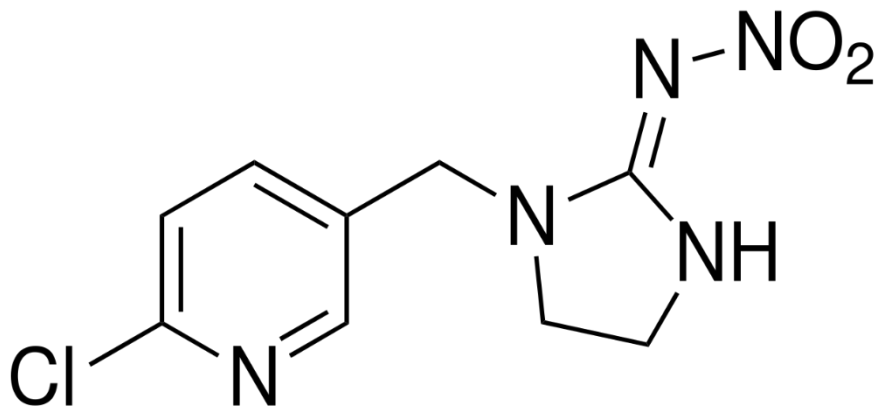
Estudos realizados com moscas knockdown para os modelos de transtorno neurodesenvolvimental vem observando alterações nos níveis dopaminérgicos além de um aumento da atividade locomotora e redução do sono nesses modelos, corroborando estudos experimentais relacionadas a esses transtornos (HARICH et al., 2020; KLEIN et al., 2020).

A utilização de produtos químicos para induzir modelos de transtornos do neurodesenvolvimento em moscas do tipo selvagem vem ganhando força nos últimos anos, servindo como uma alternativa ao uso de modelos transgênicos da *Drosophila melanogaster* (JANNER et al., 2021; KAUR et al., 2015; KIM; LEE; PARK, 2017; MUSACHIO et al., 2021).

2.5.1. Imidacloprida

A imidacloprida (IMI) é um pesticida neonicotinóide que atua no sistema nervoso central, como um agonista dos receptores nicotínicos de acetilcolina (FFRENCH-CONSTANT et al., 2016). A sua estrutura química é composta por quatro componentes estruturais distintos: um grupo heteroarilmetil ou heterociclilmetil; um ligante flexível; um anel de cinco/seis membros ou um sistema de cadeia aberta e um farmacóforo nitro/ciano (Figura 6), sendo o anel cloro-piridina da imidacloprida é essencial para fornecer fotoestabilidade (YAMAMOTO, I.; CASIDA, 1999).

Figura 6: Estrutura química da Imidacloprida.



Fonte: Adaptado de FUSETTO et al., (2017).

Em consequência da sua toxicidade seletiva para insetos, a utilização deste inseticida vem crescendo globalmente, sendo amplamente utilizado para o controle de insetos sugadores nas agriculturas, bem como injeções de solo e árvores, entre outros. Todavia para humanos aparentemente até o momento é considerado seguro (CROSBY et al., 2015; FFRENCH-CONSTANT et al., 2016; TOMIZAWA; CASIDA, 2005).

Devido ao fato dos pesticidas possuírem mecanismos de neurotoxicidade como geração de estresse oxidativo e inflamação, resultando em apoptose celular (ABDOLLAHI et al., 2004; FRANCO et al., 2009), acredita-se que a exposição a baixas concentrações de pesticidas pode promover a perda de neurônios em regiões especificas do cérebro, levando a danos cognitivos, de memória, atenção e função motora (HAYDEN et al., 2010).

No decorrer dos últimos anos tem-se observado um aumento crescente no número de estudos sobre a exposição ambiental a pesticidas durante a gestação e os primeiros anos de vida, indicando que essa exposição pode representar um risco para o surgimento de transtornos do neurodesenvolvimento (ROBERTS et al., 2007; SHELTON et al., 2014; VON EHRENSTEIN et al., 2019).

A exposição pré-natal e/ou pós-natal a IMI é associada a déficits comportamentais em diferentes espécies (CROSBY et al., 2015; DUZGUNER; ERDOGAN, 2012; MENGONI GOÑALONS; FARINA, 2015; TOMIZAWA; CASIDA,

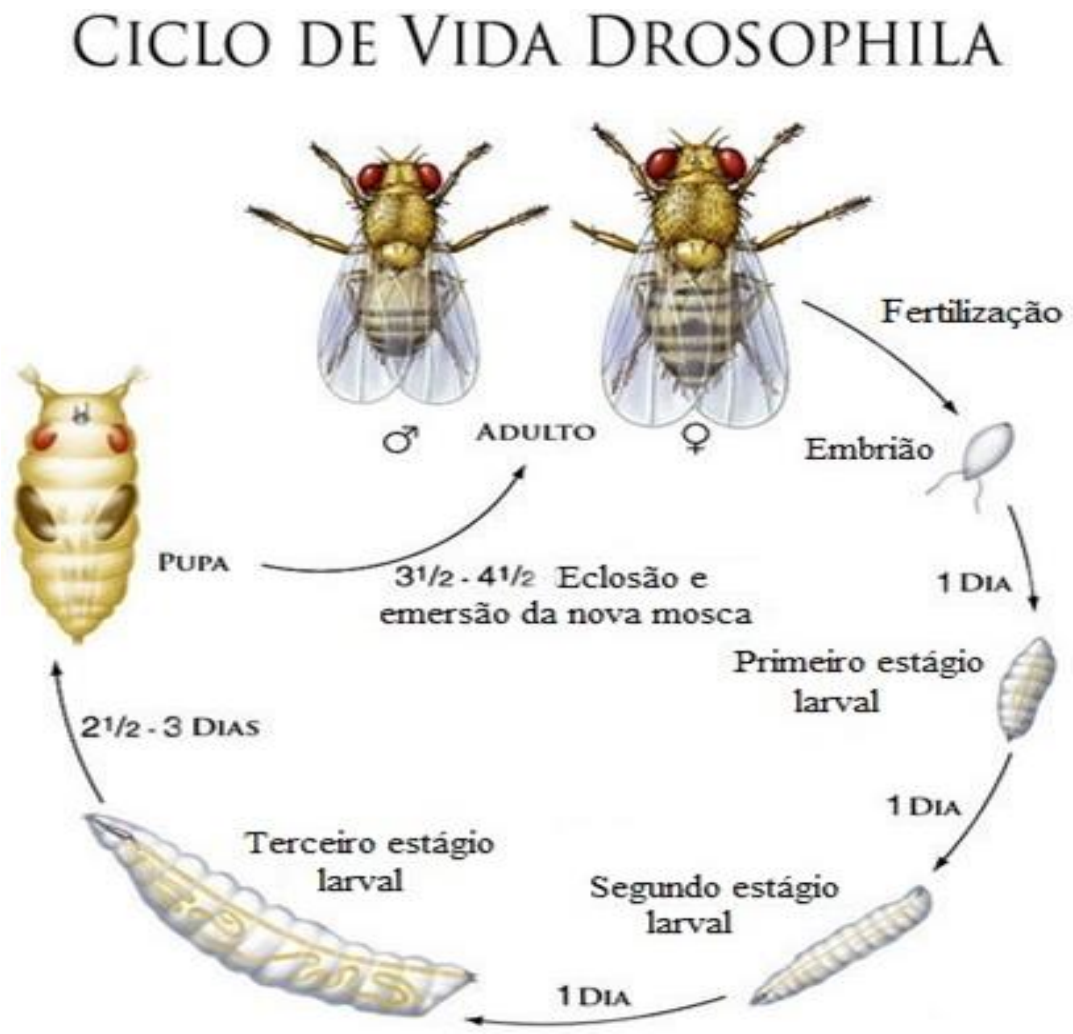
2005). Neste sentido, estudos demonstram que a exposição de *Drosophila melanogaster* a IMI resulta em uma progênie com diferentes alterações comportamentais como: interação social reduzida; aumento da agressividade; movimentos repetitivos; ansiedade e hiperatividade, evidenciando assim a utilização desse composto como ferramenta útil no desenvolvimento de um modelo químico que permita a avaliação de fenótipos e vias moleculares dos transtornos do neurodesenvolvimento em *Drosophila melanogaster*, uma alternativa para as avaliações com modelos genéticos já descritos (JANNER et al., 2021; KIM; LEE; PARK, 2017).

2.6. *Drosophila melanogaster*

A *Drosophila melanogaster* também conhecida como mosca da fruta pertence a família *Drosophilidae,* e vem sendo amplamente empregada como modelo experimental por pesquisadores, visto que a séculos estudos relacionados a parâmetros genéticos, moleculares e comportamentais relatam que a *Drosophila* apresenta alta similaridade com organismos mamíferos (HIRTH, 2010).

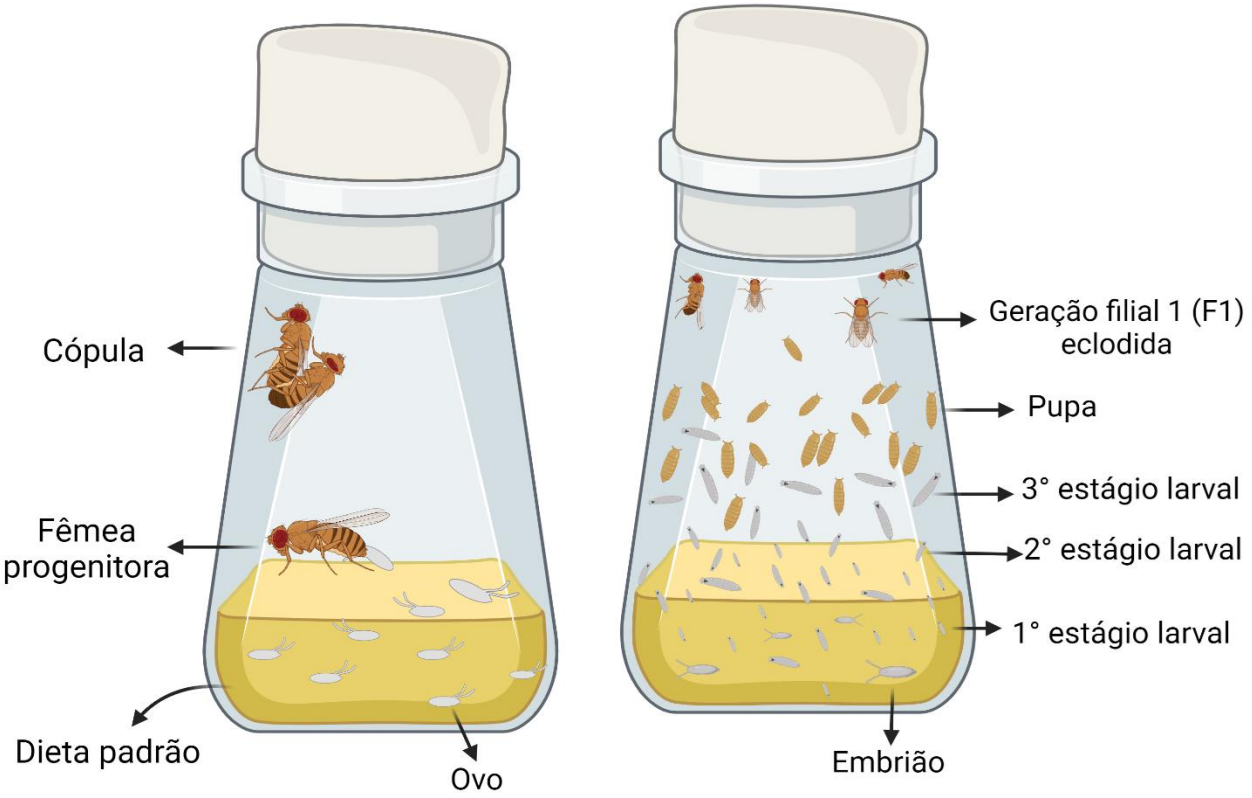
O ciclo de vida da *Drosophila melanogaster* consiste basicamente em quatro períodos: ovo, larva, pupa e mosca conforme ilustrado na Figura 7. Diferentemente dos humanos o período embrionário das moscas ocorre externo ao progenitor feminino, ou seja, o embrião se desenvolve dentro do ovo no meio em que é colocado, por aproximadamente 24 horas, na qual ao eclodir na forma de larva, passa por três estágios disponde de nutrientes do meio ao qual foi exposto. Ao final do terceiro estágio (estágio errante), a larva até então emerge do meio alimentar, sessando a alimentação, começa a produzir um muco que irá fixa-la em determinado local para iniciar o período pupal. No período pupal que demora cerca de 3-5 dias ocorre a metamorfose, que envolve a degradação de praticamente todos os tecidos larvais e a multiplicação dos discos imaginais, responsáveis pelas estruturas da mosca adulta. E por fim ocorre a eclosão da mosca que é a forma sexualmente ativa do modelo, conforme ilustrado na figura 8 (GILLETTE; TENNESSEN; REIS, 2021; KASTURE et al., 2018; LIONAKIS; KONTOYIANNIS, 2012).

Figura 7: Ciclo de vida da *Drosophila melanogaster*.



Fonte: Adaptado de BARBOSA, (2019).

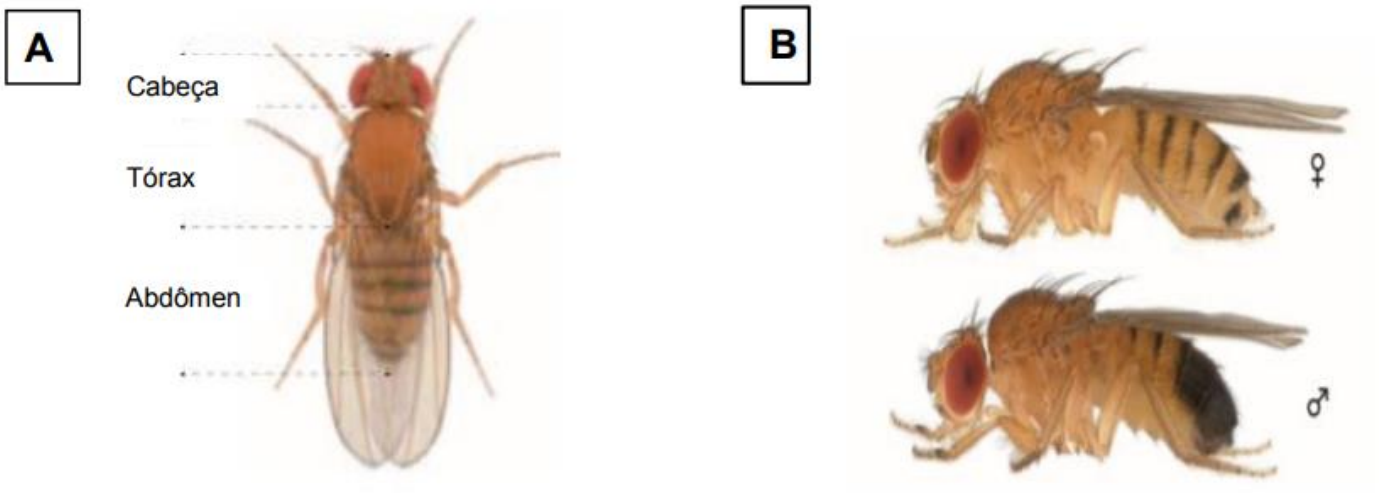
Figura 8: Desenvolvimento da *Drosophila melanogaster*.



Fonte: Arquivo próprio

Se tratando de uma espécie dimórfica, onde os machos e fêmeas são distinguidos de acordo com as características morfológicas, a mosca adulta possui o corpo dividido em três partes principais: cabeça, tórax e abdômen, ambos os sexos apresentam listras transversais no lado dorsal de cada segmento abdominal, em as moscas do sexo masculino possuem segmentos finais escuros no abdomem, e a genitália denominada epandrium é maior, mais complexa, escura e arredondada em relação a da fêmea (Chyb e Gompel, 2013), conforme demonstrado na figura 9 a seguir.

Figura 9: A) Principais estruturas do corpo B) Diferenças morfológicas da Drosophila melanogaster.

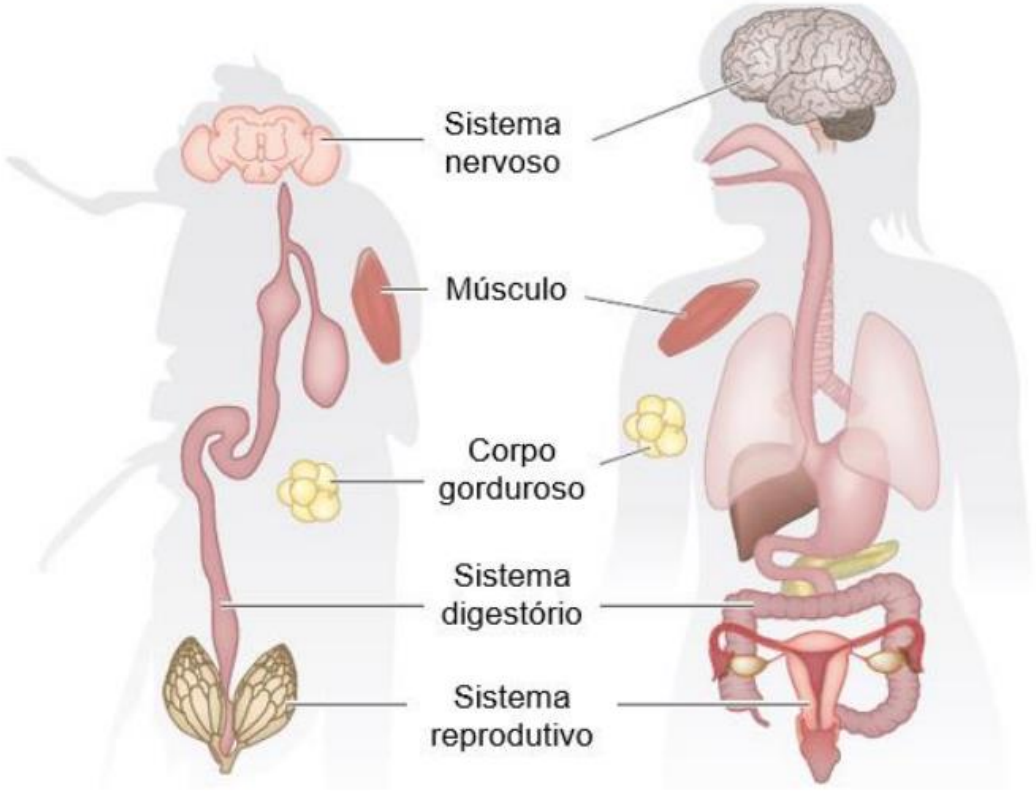


Fonte: Adaptado de CHYB & GOMPEL (2013).

Além do seu ciclo de vida rápido, baixo custo, e facilidade de manutenção em laboratório a mosca da fruta exibe comportamentos complexos tal como, caminhar, escalar, voar e agressividade, além disso, aproximadamente 75% dos genes que causa doenças em humanos apresenta um homólogo funcional na *Drosophila melanogaster* (MUÑOZ-SORIANO; PARICIO, 2011; PANCHAL; TIWARI, 2017; PANDEY; NICHOLS, 2011).

Quanto à anatomia dos órgãos, insetos e vertebrados compartilham várias vias metabólicas semelhantes, tornando-os modelos úteis para aprofundar nossa compreensão dos processos fisiológicos (DROUJININE; PERRIMON, 2016; YOON; SHIN; SHIM, 2023), como ilustrado na figura 10 abaixo.

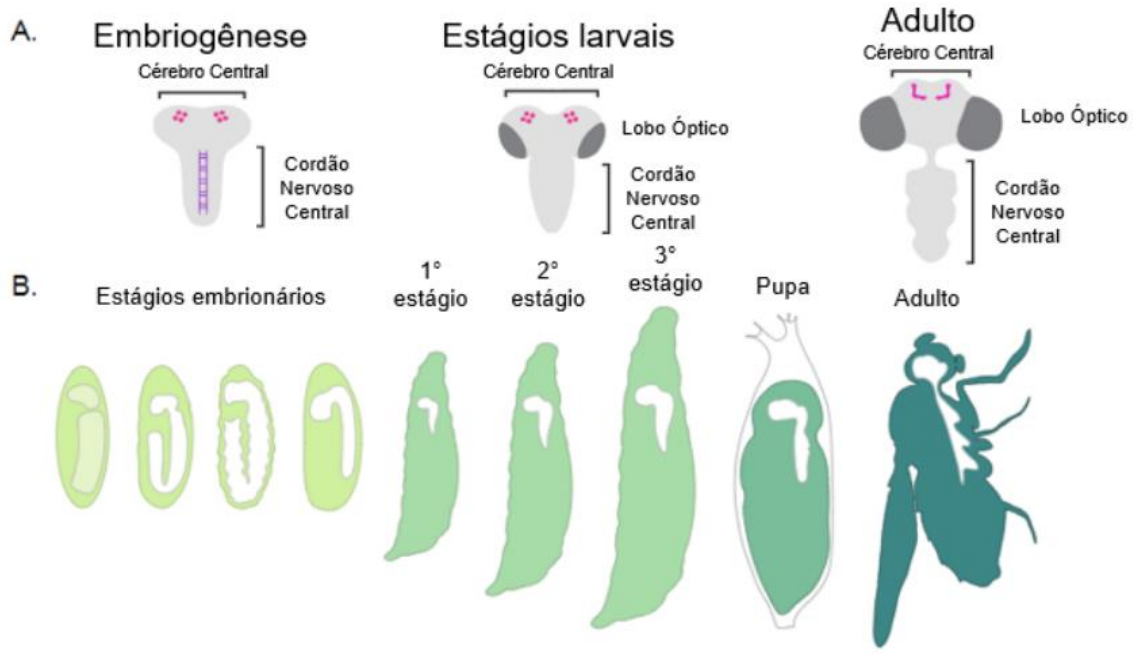
Figura 10: Relação entre os sistemas de *Drosophila melanogaster* e humanos.



Fonte: Adaptado de DROUJININE; PERRIMON, (2016).

Logo a *Drosophila* possui um sistema nervoso relativamente simples, mas com funções cerebrais que se assemelham às dos mamíferos, visto que no embrião de Drosophila, o sistema nervoso central (SNC) é formado por neurônios e células gliais, e seu desenvolvimento começa precocemente durante a fase embrionária (CREWS, 2019; PHAN et al., 2014) conforme ilustrado na figura 11 a seguir.

Figura 11: Desenvolvimento do sistema nervoso central da *Drosophila melanogaster.*



(A) Esquema do SNC embrionário da mosca no estágio larval e adulto. (B) Estágios embrionários larval, pupal e adulto. Na embriogênese, primeiramente, as regiões neurogênicas que dão origem ao cérebro e ao cordão nervoso central são mostradas em verde claro. Posteriormente, o sistema nervoso é mostrado em branco.
Fonte: Adaptado de YALONETSKAYA et al., (2018).

O desenvolvimento do SNC da *Drosophila* ocorre em duas fases distintas: a primeira fase de neurogênese acontece durante os estágios embrionários, resultando na formação do SNC larval. A segunda fase de neurogênese ocorre nos estágios larval e pupal, completando a formação do SNC que será funcional na mosca adulta (YALONETSKAYA et al., 2018). Todo o sistema nervoso é envolvido por uma camada de células gliais perineurais. Assim, semelhante ao que ocorre em vertebrados, a barreira hematoencefálica da *Drosophila* é composta por células gliais (DESALVO et al., 2011).

Ainda a *Drosophila melanogaster* apresenta sistema dopaminérgico semelhante ao de humanos, apesar das diferenças evolutivas entre os dois organismos (CICHEWICZ et al., 2017; KASTURE et al., 2018) conforme ilustrado na figura 12 abaixo.

Figura 12: Sistema dopaminérgico em *Drosophila melanogaster* e humanos.

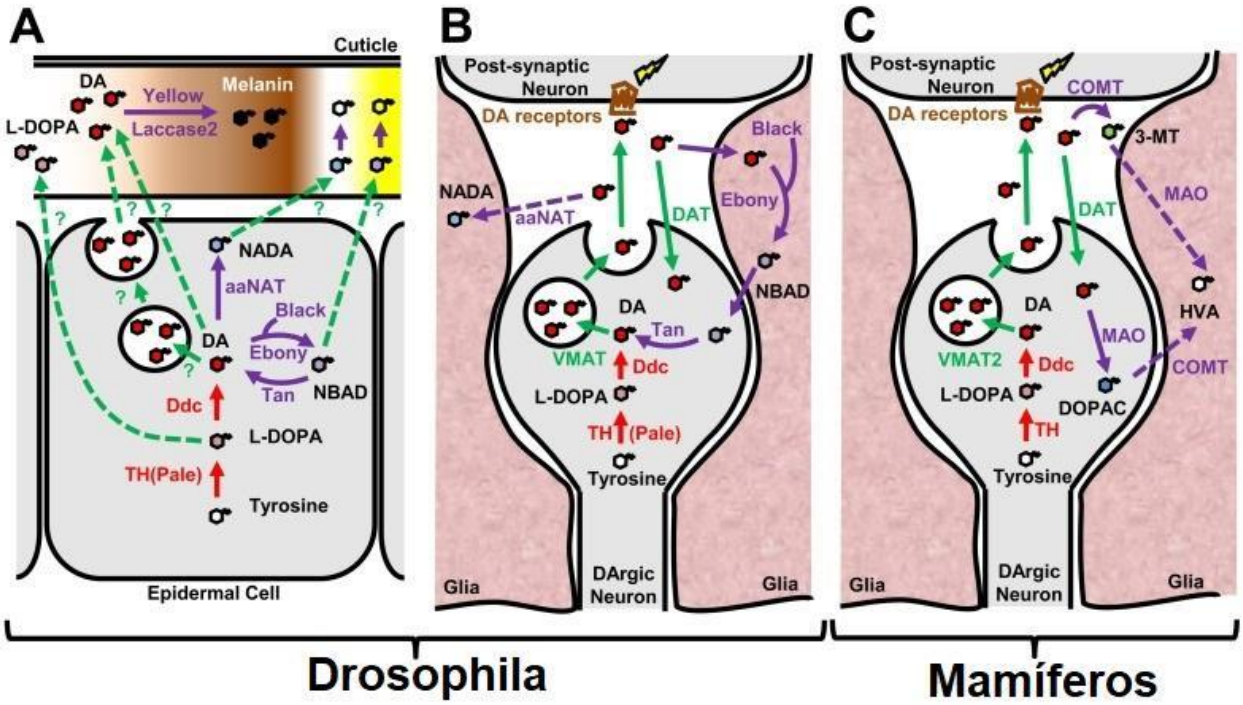


Diagrama esquemático da dinâmica e sinalização de DA em (A) cutícula de *Drosophila*, (B) cérebro de *Drosophila* e (C) cérebro de mamíferos.

Fonte: Adaptado de YAMAMOTO; SETO, (2014).

Nas moscas, assim como em mamíferos, DA é sintetizado a partir do aminoácido tirosina por meio de duas etapas enzimáticas. A primeira etapa limitante ocorre através da ação da enzima tirosina hidroxilase (TH), que é codificada pelo gene *pale (ple)* em moscas, responsável por converter a tirosina em L-3,4-dihidroxifenilalanina (L-DOPA). Na segunda etapa, a L-DOPA é convertida em DA pela ação da descarboxilase de aminoácidos aromáticos (AADC), codificada pelo gene da *DOPA descarboxilase* (*Ddc*) (YAMAMOTO; SETO, 2014). A DA não consegue atravessar a barreira hematoencefálica e é sintetizada dentro dos neurônios dopaminérgicos nos cérebros de moscas e humanos (CICHEWICZ et al., 2017). Ela é empacotada em vesículas por meio do transportador de monoamina vesicular (codificado por *Vmat*) e liberada por exocitose na sinapse, onde se liga aos receptores DA nos neurônios pós-sinápticos, bem como aos autorreceptores terminais dos neurônios DA e outros heterorreceptores terminais, desencadeando uma série de

vias de sinalização que, em última análise, modulam o comportamento (KARAM; JONES; JAVITCH, 2020; YAMAMOTO; SETO, 2014)

Assim nas últimas décadas a *Drosophila* vem sendo amplamente utilizada com a finalidade de modelar disfunções neurológicas, dentre elas a neurodegeneração. Nesse contexto foram desenvolvidos estudos com esse organismo modelo para mimetizar doenças como Parkinson (FERNANDES et al., 2021; MUÑOZ-SORIANO; PARICIO, 2011; MUSACHIO et al., 2020), Alzheimer (JALALI et al., 2021; PANCHAL; TIWARI, 2017; WANG; DAVIS, 2021), depressão (AHN et al., 2021; MOULIN et al., 2021) e, recentemente transtornos do neurodesenvolvimento (JANNER et al., 2021; KIM; LEE; PARK, 2017; MUSACHIO et al., 2021).

Nesse contexto o uso da *Drosophila melanogaster* como modelo experimental evidencia a importância da utilização de modelos alternativos, além de não ser necessária a aprovação do Comitê de Ética no Uso de Animais, também possibilita a substituição de outros organismos como, por exemplo, camundongos e ratos para estudos experimentais dessa forma a *Drosophila* têm gerado contribuições significativas para a pesquisa (MATOS et al., 2009; MCGURK; BERSON; BONINI, 2015).

2.7. Compostos bioativos

Crescentes indícios destacam o importante papel dos constituintes extranutricionais como os compostos bioativos, que estão presentes principalmente em alimentos de origem vegetal, desta forma auxiliando na manutenção da saúde e na redução do risco de desenvolver doenças esses compostos não apresentam efeitos colaterais como no caso do uso de opções farmacológicas (OLIVEIRA et al., 2020; REIN et al., 2013b).

Diversos estudos têm demonstrando que os compostos bioativos encontrados em alimentos como por exemplo: algumas vitaminas (SUNKARA; RAIZNER, 2019), flavonoides (RAHUL; SIDDIQUE, 2021), curcumina (TANG et al., 2020), piperina (HAQ et al., 2021), ácidos graxos (SIMONETTO et al., 2019) entre outros, vem sendo investigados como possíveis tratamento de várias doença como, cardiovasculares, neurodegenerativas e até mesmo para o câncer.

Os carotenoides apresentam alto potencial antioxidante, sendo capaz de combater os radicais livres formados nas células, fornecendo assim uma proteção

para o organismo muitas vezes evitando o surgimento de doenças (BEYDOUN et al., 2022; BIAN et al., 2012; GAO et al., 2011; SZTRETYE et al., 2019). Há aproximadamente setecentos carotenoides divididos em dois grupos: 1) os carotenos como por exemplo licopeno e β-caroteno (possuem hidrocarbonetos puros); e 2) as xantofilas (possuem grupos funcionais oxigenados) como a luteína e zeaxantina, sendo que destes apenas 40 carotenoides podem ser encontrados nos alimentos sendo responsáveis por fornecer coloração laranja, amarela e vermelha de frutas e vegetais (Mesquita et al., 2017).

Com relação a presença de carotenoides no organismo apenas seis estão presentes em maior quantidade no plasma sanguíneo, representando assim aproximadamente 90%, entre eles β-caroteno, α-caroteno, licopeno, luteína, zeaxantina e criptoxantina (ZHAO et al., 2006). As xantofilas luteína, zeaxantina e criptoxantina representam aproximadamente 72% do total de carotenoides no cérebro, onde a luteína é o principal componente com cerca de 34% sendo essa quantidade significativamente maior do que todos os demais carotenoides (JOHNSON et al., 2013).

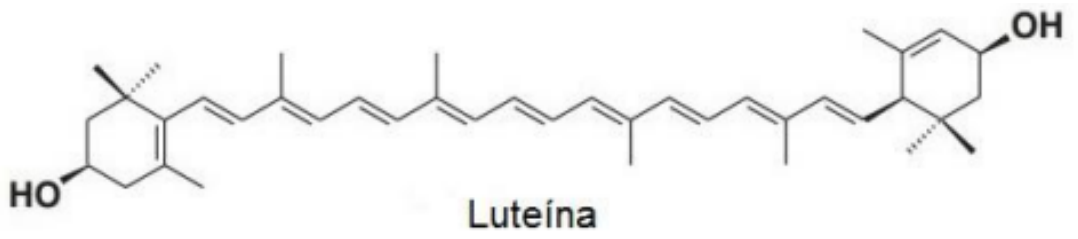
Tendo em vista que os seres humanos não são capazes de sintetizar carotenoides, assim dependendo exclusivamente de fontes alimentícias para adquirir esses componentes e desfrutar de seus efeitos benéficos, sua biodisponibilidade sempre foi alvo de interesse no meio cientifico (FERNÁNDEZ-GARCÍA et al., 2012). A biodisponibilidade dos carotenoides depende muito da matriz alimentar, como por exemplo o *status* de nutrientes do organismo hospedeiro, fatores genéticos, competição entre os nutrientes, conteúdo de lipídios e fibras alimentares entre outros fatores, desta forma alguns processos tecnológicos como aquecimento e bioencapsulação são capazes de aumentar a biodisponibilidade dos carotenoides (LIU et al., 2021b; NWACHUKWU; UDENIGWE; ALUKO, 2016).

2.7.1 Luteína

Membro das xantofilas a luteína é abundantemente encontrada nas flores de calêndula, gema de ovo e vegetais com folhas verde-escura. Com fórmula molecular $C_{40}H_{56}O_2$ possui uma longa cadeia de carbonos com ligações duplas conjugadas, grupos metil nas laterais e uma estrutura cíclica (hexenil) ligada a um grupo hidroxila nas duas extremidades da cadeia conforme demonstrado na Figura 13 a seguir. Essas

características estruturais são essenciais para controlar as funções biológicas dessa molécula (HU et al., 2012; WALLACE et al., 2015). A luteína na natureza apresenta-se como uma combinação das formas *trans* (60-90%) que demonstra maior estabilidade, e *cis* (10- 40%) responsável pela coloração menos intensa (RODRIGUES-AMAYA, 2019). Sabe-se que a luteína encontram-se acumulada na mácula, região central da retina, sendo assim também chamada de pigmento macular (NWACHUKWU; UDENIGWE; ALUKO, 2016).

Figura 13: Estrutura molecular da luteína.



Fonte: FERNÁNDEZ-GARCÍA et al., (2012).

A luteína apresenta um alto potencial antioxidante, capaz de combater os radicais livres e consequentemente impedir danos as lipoproteínas, lipídios de membrana, proteínas e DNA, assim reduzindo e/ou evitando o estresse oxidativo (KIM et al., 2008; OZAWA et al., 2012; WANG et al., 2013). Esta ação antioxidante se dá pelo fato da luteína possuir em sua estrutura grupos hidroxila em ambas extremidades da cadeia, o que torna esse composto mais hidrofílico em comparação aos carotenoides não oxigenados, melhorando assim sua interação com os lipídios (altamente oxidáveis) aumentando a proteção contra ao dano oxidativo (SUBCZYNSKI; WISNIEWSKA; WIDOMSKA, 2010).

Além da eficiência em estabilizar radicais livres a luteína apresenta atividade anti-inflamatória (AHN; KIM, 2021; DEMMIG-ADAMS et al., 2020), capacidade de atravessar a barreira hematoencefálica (JOHNSON et al., 2013; VISHWANATHAN et al., 2014), considerada o principal recurso no combate a danos oftalmológicos devido a degeneração macular relacionada a idade (CHEN et al., 2021a; LI et al., 2020; PENG et al., 2016).

Alguns estudos tem associado à luteína a diminuição de danos no fígado e intestino (DU et al., 2015; FLORES et al., 2014; LI et al., 2015; SATO et al., 2011), além de prevenir a degradação do DNA (SERPELONI et al., 2012; WANG et al., 2006) e doenças cardiovasculares (CHEN et al., 2021b; KOH et al., 2011), reduzindo também o risco de desenvolver câncer (KAVALAPPA; GOPAL; PONESAKKI, 2021; LI et al., 2018), e demonstrando efeitos neuroprotetores (MROWICKA et al., 2022; WOO et al., 2013; ZENI; CAMARGO; DALMAGRO, 2019). Desta forma a luteína exerce efeitos neuroprotetores, reduzindo dano oxidativo, e os níveis de dopamina (NATARAJ et al., 2016), também é capaz diminuir os níveis de BDNF, e promover a ativação do Nrf2 bem como de proteínas reguladoras como, por exemplo, a Akt estimulando diversas cascatas de sinalização envolvidas (SAHIN et al., 2019; SHIVARUDRAPPA; PONESAKKI, 2020; WU et al., 2015).

Há relatos de que a luteína possui envolvimento nos processos de aprendizado, e memória em doenças neurodegenerativas. Nesse viés, o consumo de uma dieta rica em fontes de luteína ou suplementação está associado às baixas taxas de declínio cognitivo e de memória decorrentes de doenças e envelhecimento (GEISS et al., 2019; JOHNSON, 2012; JOHNSON et al., 2008; KANG; ASCHERIO; GRODSTEIN, 2005; KESSE-GUYOT et al., 2012). Ainda que apresente inúmeros benefícios para a saúde humana na forma livre, a luteína possui instabilidade térmica, pode sofrer oxidação facilmente, e também apresenta baixa solubilidade em água, sendo assim pouco absorvida no trato gastrointestinal comprometendo a sua biodisponibilidade. Desta forma é necessário o emprego de técnicas capazes de estabilizar e proteger esse composto de fatores externos (DONSÌ et al., 2011; ZHAO et al., 2013).

2.8. Nanopartículas carreadoras de luteína

Nos últimos anos, vários esforços estão sendo dedicados ao avanço de tecnologias para a entrega eficaz de medicamentos (WANG et al., 2018), entre os quais os nanomateriais se destacam. O prefixo "nano", derivado da palavra latina "nanus" que significa "muito pequeno" uma vez que 1 nanômetro (nm) é igual a 10^{-9} metros (m), desta forma a nanotecnologia, atualmente está sendo aplicada em diversos setores, incluindo agricultura, controle de infecções e biomedicina (PAVELIĆ et al., 2023; RAM; VIVEK; KUMAR, 2014; ZIELIŃSKA et al., 2020).

A aplicação das nanoestruturas na medicina é impulsionada pela sua capacidade de moldagem estrutural, tamanho reduzido, biocompatibilidade, grande área de superfície e potencial para funcionalização (JEELANI et al., 2020). Desta forma a utilização de nanotecnologia para fármacos e compostos bioativos tem o propósito de aumentar a solubilidade e a biodisponibilidade desses compostos, aumentando sua biodisponibilidade e seu potencial terapêutico (ANARJAN; TAN, 2013; JOYE; MCCLEMENTS, 2013; YERRAMILLI; GHOSH, 2017).

Portanto, ao encapsular produtos lipofílicos, como a luteína, observa-se uma melhora significativa na biodisponibilidade da substância, possibilitando que uma menor quantidade seja suficiente para alcançar um efeito biológico eficaz (DHIMAN et al., 2021).

Recentemente pesquisas demonstram que a administração de nanopartículas de luteína foi capaz de melhorar os parâmetros de memória em camundongos (DO PRADO SILVA et al., 2017). Da mesma forma o estudo de VIANA et al. (2023) demonstrou a capacidade das nanopartículas carreadoras de luteína em reverter os danos comportamentais, inibir o estresse oxidativo e, consequentemente, evitar a apoptose no hipocampo de ratas no submetidas ao modelo de TEA induzido por VPA.

Além disso as nanopartículas carreadoras de luteína também apresentaram efeito benéfico sobre os níveis de dopamina, enzima acetilcolinesterase e estresse oxidativo no modelo de doença de Parkinson em *Drosophila melanogaster* (FERNANDES et al., 2021).

3. JUSTIFICATIVA

O TEA e o TDAH são transtornos do neurodesenvolvimento que afetam crianças e adolescentes, causando inúmeros impactos à vida desses indivíduos, como prejuízos escolar, familiar e social. Tendo em vista dois pontos relevantes, em que o primeiro é o aumento do número de indivíduos diagnosticados no decorrer dos últimos anos, em que aproximadamente 1 em cada 40 crianças recebem o diagnóstico para um desses transtornos, e segundo que não há cura, somente tratamentos, dentre eles psicopedagógicos e em alguns casos farmacológicos que apenas amenizam alguns sintomas característicos.

Nesse sentido surge a preocupação pela busca de compostos que proporcionem efeitos protetores afim de prevenir o desenvolvimento desses distúrbios

ou que amenizem as alterações comportamentais e neuroquímicas presentes, para assim elucidar os mecanismos envolvidos nas alterações observadas nesses transtornos. Desta forma a realização do presente estudo pode contribuir para o entendimento dos efeitos protetores das nanopartículas carreadoras de luteína bem como os possíveis mecanismos de ação, no qual o aumento da biodisponibilidade desse carotenoide, juntamente com um possível papel neuroprotetor, pode proporcionar uma estratégia farmacológica eficaz para o tratamento e/ou prevenção dos transtornos do neurodesenvolvimento do tipo TEA e TDAH.

4. OBJETIVOS

4.1. Objetivo geral

Investigar o possível efeito protetor das nanopartículas carreadoras de luteína e seu o provável mecanismo de ação sobre os déficits induzidos pelo modelo experimental de transtorno do neurodesenvolvimento em *Drosophila melanogaster*.

4.2. Objetivos específicos

- Avaliar o efeito das nanopartículas carreadoras de luteína na progênie exposta ao modelo de transtorno do neurodesenvolvimento, em ambos os sexos separadamente;
- Observar o efeito da administração de nanopartículas carreadoras de luteína durante o período pós-natal da progênie submetida ao modelo de transtorno neurodesenvolvimental,
- Avaliar o efeito das nanopartículas carreadoras de luteína sobre as alterações do comportamento locomotor, exploratório, agressividade, interação social, grooming, ansiedade, aprendizagem e memória em *Drosophila melanogaster* submetidas ao modelo experimental de transtorno do neurodesenvolvimento;
- Investigar o efeito de nanopartículas carreadoras de luteína sobre os indicadores de estresse oxidativo (SOD, CAT, ROS e TBARS e Nrf2) em *Drosophila melanogaster* submetidas ao modelo experimental de transtorno do neurodesenvolvimento;
- Avaliar o efeito de nanopartículas carreadoras de luteína sobre a proteína Shank em *Drosophila melanogaster* submetidas ao modelo experimental de transtorno do neurodesenvolvimento;
- Observar o efeito da suplementação com nanopartículas carreadoras de luteína durante o período pré-concepcional na progênie exposta ao modelo de transtorno do neurodesenvolvimento.
- Verificar o efeito de nanopartículas carreadoras de luteína sobre a viabilidade celular em *Drosophila melanogaster* submetidas ao modelo experimental de transtorno do neurodesenvolvimento;
- Investigar o efeito de nanopartículas carreadoras de luteína sobre os níveis dos neurotransmissores DA e 5HT bem como a atividade da enzima TH na cabeça de

Drosophila melanogaster submetidas ao modelo experimental de transtorno do neurodesenvolvimento.

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5. RESULTADOS

Os resultados que fazem parte desta tese estão apresentados sob a forma de 1 artigo científico e 1 manuscrito. O artigo cientifico encontra-se publicado. Já o manuscrito, encontra-se disposto conforme as normas da revista "*Food and Chemical Toxicolgy*". Os tópicos Materiais e Métodos, Resultados, Discussão e Referências encontram-se no artigo e manuscrito.

5.1 Artigo cientifico

Título: Neurodevelopmental changes in *Drosophila melanogaster* are restored by treatment with lutein-loaded nanoparticles: Positive modulation of neurochemical and behavioral parameters.

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Neurodevelopmental changes in *Drosophila melanogaster* are restored by treatment with lutein-loaded nanoparticles: Positive modulation of neurochemical and behavioral parameters

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ABSTRACT

Neurodevelopmental disorders, such as autism spectrum disorder (ASD) and attention deficit hyperactivity disorder (ADHD), are characterized by persistent changes in communication and social interaction, as well as restricted and stereotyped patterns of behavior. The complex etiology of these disorders possibly combines the effects of multiple genes and environmental factors. Hence, exposure to insecticides such as imidacloprid (IMI) has been used to replicate the changes observed in these disorders. Lutein is known for its anti-inflammatory and antioxidant properties and is associated with neuroprotective effects. Therefore, the aim of this study was to evaluate the protective effect of lutein-loaded nanoparticles, along with their mechanisms of action, on *Drosophila melanogaster* offspring exposed to IMI-induced damage. To simulate the neurodevelopmental disorder model, flies were exposed to a diet containing IMI for 7 days. Posteriorly, their offspring were exposed to a diet containing lutein-loaded nanoparticles for a period of 24 h, and male and female flies were subjected to behavioral and biochemical evaluations. Treatment with lutein-loaded nanoparticles reversed the parameters of hyperactivity, aggressiveness, social interaction, repetitive movements, and anxiety in the offspring of flies exposed to IMI. It also protected markers of oxidative stress and cell viability, in addition to preventing the reduction of Nrf2 and Shank3 immunoreactivity. These results demonstrate that the damage induced by exposure to IMI was restored through treatment with lutein-loaded nanoparticles, elucidating lutein's mechanisms of action as a therapeutic agent, which, after further studies, can become a co-adjuvant in the treatment of neurodevelopmental disorders, such as ASD and ADHD.

1. Introduction

Neurodevelopmental disorders (NDDs) are neurological conditions of early onset that cause impairment throughout the lives of individuals, with autism spectrum disorder (ASD) and attention deficit hyperactivity disorder (ADHD) being the main contributors to this group (Morris-

Abbreviations: ADHD, attention deficit hyperactivity disorder; ASD, autism spectrum disorder; CAT, catalase; IMI, imidacloprid; CDNB, 1-chloro-2-4-dinitrobenzene; DMSO, dimethyl sulfoxide; MDA, malondialdehyde; NDDs, neurodevelopmental disorders; Nrf2, erythroid nuclear factor 2-related factor 2; PSDs, postsynaptic densities; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; SOD, superoxide dismutase; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substance; TEMED, tetramethylethylenediamine.

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Rosendahl and Crocq, 2020). Individuals affected by ASD and ADHD have deficits in communication and social interaction, restricted and repetitive behaviors, as well as a tendency towards aggression and hyperactivity (American Psychiatric Association, 2013). Clinical manifestations are classified according to different degrees of cognitive and adaptive disability (Thapar et al., 2017). Nonetheless, due to ASD's multifactorial etiology, it is complex to define a specific genetic marker for its diagnosis, however, in recent years, some genes have been identified and associated with the risk of developing this condition (Rylaarsdam and Guemez-Gamboa, 2019). In this sense, alterations in Shank family proteins (Shank 1/2/3) as well as mutations in Shank genes have been identified in patients with the disorder (Wan et al., 2022), which promote cognitive changes, such as repetitive behaviors (Jaramillo et al., 2020; Kim et al., 2018; Tatavarty et al., 2020), learning difficulties (Maenner et al., 2021; Rendall et al., 2019), and social interaction deficit (Guo et al., 2019; Jaramillo et al., 2020).

Many studies have shown the involvement of oxidative stress in the pathogenesis of ASD, thus Erythroid nuclear factor 2-related factor 2 (Nrf2), responsible for activating the antioxidant response and counteracting the harmful effects of oxidative stress (Qin et al., 2020; Schrier et al., 2022), is implicated in ASD, since individuals affected by this disorder present a reduction in the Nrf2 gene and protein in the blood (Nadeem et al., 2020; Napoli et al., 2014).

Moreover, it is important to highlight that bioactive compounds have been receiving great attention due to their fundamental role in health (Almeida et al., 2021; Ayatollahi et al., 2021). Among many natural compounds, lutein is the main carotenoid present in the human brain, with anti-inflammatory and antioxidant action, in addition to the ability to cross the blood-brain barrier (Bian et al., 2012; Sies and Stahl, 2003). Several studies have demonstrated the association of lutein with neuroprotective effects, such as increasing antioxidant action (preventing increased oxidative damage) and improving cognitive and memory activity (Erdman et al., 2015; Milani et al., 2017; Nouchi et al., 2020). Despite presenting several health benefits in its free form, lutein is thermally unstable, susceptible to oxidation and has low solubility in water, resulting in limited absorption in the gastrointestinal tract and compromising its bioavailability (Steiner et al., 2018). For that reason, it is essential to use techniques that stabilize and protect the compound against external influences. However, the application of nanotechnology in drugs and bioactive compounds aims to improve solubility and bioavailability, expanding their therapeutic potential (Algan et al., 2022; Begines et al., 2020). Also, research has recently highlighted the potential of lutein in nanoparticle form, demonstrating its ability to protect against behavioral (Viana et al., 2023) and memory damage in rats (do Prado Silva et al., 2017), and to restore oxidative stress levels in *Drosophila* (Fernandes et al., 2021).

Recent studies have used imidacloprid (IMI) as a useful tool to induce a chemical model of NDDs in flies, becoming an alternative to genetic models, in addition, exposure of flies to IMI generates progeny with behavioral changes similar to those observed in individuals with these disorders (Janner et al., 2021; Kim et al., 2017). In this way, the use of *Drosophila melanogaster* allows us advantages such as experiments in different periods and generations due to its fast and short life cycle (Kasture et al., 2018; Lionakis and Kontoyiannis, 2012), in addition it allows us to evaluate behaviors such as repetitive movements, learning, memory, social interaction, aggressiveness, among others (Janner et al., 2021; Kim et al., 2017; Roberts et al., 2019; Tauber et al., 2011; Tully et al., 1994). Thus, we hypothesized that the effects of lutein-loaded nanoparticles, through its antioxidant properties, can alleviate oxidative stress by regulating the Nrf2 pathway and restoring Shank levels. Therefore, the aim of this study was to evaluate the protective effect of lutein-loaded nanoparticles, along with their mechanisms of action on *Drosophila melanogaster* offspring exposed to IMI-induced damage.

2. Materials and methods

2.1. Materials

Imidacloprid (Cas Number: 138261–41-3′) was obtained from Sigma-Aldrich (St. Louis, MO), and diluted in 0.0001 % DMSO. The lutein-loaded nanoparticles was prepared according to Freiberger et al. (2015).All the other reagents used were of analytical grade.

2.2. Obtaining and characterizing lutein-loaded nanoparticles

Polycaprolactone (average Mn 45,000 Da, Sigma-Aldrich) was used as encapsulant, and phosphatidylcholine (Sigma-Aldrich) was used as stabilizer. Distilled water was used as a continuous media in the nanoparticles production. Dichloromethane (Dinamica) was also used. Lutein was kindly gifted by Kemin S.A. Potassium bromide (KBr, Sigma-Aldrich, spectroscopic standard) was used in the Fourier spectroscopy analyses.

The lutein-loaded nanoparticles were obtained using the methodology proposed by Freiberger et al. (2015). Briefly, phosphatidylcholine (0.875 g) and PCL (0.750 g) were dissolved in dichloromethane (50 g) at 25 °C under gentle stirring. Lutein (0.750 g) was added, and the stirring was kept for 5 min. Then, water was added, and the mixture was sonicated (Fisher Scientific Sonicator, 120 W, 1/8″ titanium tip) for 3 min in a pulse regime (30 s on, 10 s off). The same procedure was carried out without the addition of lutein (blank nanoparticles). After this time, dichloromethane was allowed to evaporate for 24 h, and the nanoparticles were lyophilized. The solid was stored at 10 °C protected from light.

Nanoparticles were characterized according to the methodologies described by Silva de Sá et al. (2019), de Almeida et al. (2018) and Ramírez-Hernández et al. (2022). Fourier Transform Infrared spectra (FTIR; Frontier Perkin Elmer) was performed in KBr pellets with a resolution of 2 cm^{-1} from 4500 to 425 cm^{-1} with 32 cumulative scans. The thermal properties of the solid dispersion were analyzed by Differential Scanning Calorimetry (DSC, Instruments model Q20 TA equipment). Also, thermogravimetric analysis (TGA) was carried out in a TA Instruments model SDT Q600 equipment. For both analyses, samples were heated in aluminum pans (0 °C to 350 °C at 10 °C.min^{-1}) under nitrogen flow (20 mL.min^{-1}). X-ray diffractograms were obtained using an X-ray diffractometer (Shimadzu model LabX XRD-6000). Samples were investigated from 2° to 60° (2θ) at 5.9° min^{-1} using Kα radiation at 40 kV and 53 mA. Dynamic light scattering (DLS) was performed in a NanoDLS Brookhaven equipment at 25 °C (scattering detector at 90°, red laser, wavelength of 638 nm). Samples were diluted 1:100 in ultrapure water priorly to analysis to reduce multiple scattering. The Zeta potential was determined using a Stabino Particle Metrix PMX 400 using the same conditions described for DLS measurements. Images were obtained of uncoated nanoparticles using atomic force microscopy (Agilent Scanning Probe Microscope, model 5500). Sizes 8 × 8 μm, 4 × 4 μm, 2 × 2 μm and 1 × 1 μm were scanned in tapping mode at 1.44 ln.s^{-1} and 512 p resolution using a NSC15 cantilever (MikroMash, resonance frequency of 320 kHz and force constant of 40 N.m^{-1}). Samples were diluted at 1:100 with ultrapure water, and 300 μL were deposited on mica.

2.3. Drosophila melanogaster stock and culture

Wild *Drosophila melanogaster* (Harwich strain) of both sexes, 1 to 3 days old, obtained from LAFTAMBIO (Laboratory of Pharmacological and Toxicological Evaluations Applied to Bioactive Molecules - Unipampa Itaqui), were kept under controlled conditions of light (12 h of light/dark cycle), temperature (25 ± 1 °C), and 60 % humidity, and fed a standard diet (76.59 % cornmeal, 8.51 % wheat germ, 7.53 % sugar, 7.23 % milk in powder, 0.43 % salt and 0.08 % methylparaben).

2.4. Experimental protocol

2.4.1. Exposure to imidacloprid and treatment with lutein-loaded nanoparticles

To evaluate the effect of lutein on the behavioral and neurochemical damage induced by IMI, virgin female flies and males up to 3 days old were used to compose the parental couples. These flies were divided into 2 groups: 1) Control (standard diet only); and 2) IMI (standard diet + imidacloprid 400 ρM), where they remained for 7 days with free access to their respective diet, as well as mating and egg laying. The concentration of imidacloprid (400 ρM) in the diet was selected as it has been found to induce ASD and ADHD-like phenotypes in the offspring of *Drosophila melanogaster* (Janner et al., 2021). Following the 7-day exposure period, the adult flies were removed, and their hatched offspring (F1), which resulted from flies exposed to either the standard or the IMI diet during the gestational period, was subdivided into 2 groups and exposed to either a standard diet or a diet containing lutein-loaded nanoparticles, for 24 h. Thus, a total of 4 groups were formed: (1) Control (standard diet); (2) IMI (400 ρM); (3) lutein-loaded nanoparticles (6 μM); (4) IMI (400 ρM) + lutein-loaded nanoparticles (6 μM). Subsequently, female and male flies were separated and subjected to behavioral and biochemical evaluations, according to the treatment scheme shown in Fig. 1.

2.5. In vivo test

2.5.1. Negative geotaxis assay

To assess the climbing ability of the flies, the negative geotaxis test was performed as described by Charpentier et al. (2014), with minor modifications. The test was carried out for both sexes separately, using five flies from each group, which were individually immobilized on ice and placed separately in a vertical glass test tube with a diameter of 1.5 cm. After 10 min, the flies were gently tapped to the bottom of the tube and the time needed to ascend to the 8-cm mark on the tube wall was measured. The test was repeated five times for each fly, considering a maximum time of 120 s and an interval of 1 min between each repetition. Data were analyzed according to the average time of each fly. Five independent experiments were performed ($n = 5$).

2.5.2. Open field test

The open field test was carried out in order to assess the locomotor and exploratory activity of the flies, as previously described by Connolly (1966), with modifications by Musachio et al. (2020). Fifteen flies of both sexes were used per group, with five independent experiments being performed. Each fly was immobilized on ice and transferred to a Petri dish, which was previously divided into quadrants (1 × 1 cm). After 5 min of recovery, the number of crossings between quadrants by each fly was counted during 60 s. The test was performed in duplicate, and the mean values were calculated. Five independent experiments for males and females were performed ($n = 5$).

2.5.3. Grooming

Repetitive behavior was evaluated by observing the self-cleaning behavior of *Drosophila melanogaster*, as described by Tauber et al. (2011), with modifications. Five individual flies of both sexes were used in the test. The time for which each fly performed "self-cleaning" movements (rubbing the paws over the head, abdomen or placing one paw over the other) was recorded for 2 min. The test was performed in duplicate, and the data were analyzed according to the mean of "self-cleaning" time. For this analysis, five independent experiments were performed for female and male flies (n = 5).

2.5.4. Social interaction

The social interaction test was carried out according to the methodology of Simon et al. (2012), with adaptations by Janner et al. (2021). The test was performed for both sexes separately in order to avoid courtship activities that could interfere with the sociability of the flies. Ten flies from each experimental group were immobilized on ice and transferred to triangular chambers, and after 30 min of adaptation, an image was recorded with the aid of a digital camera. Digital images were imported into the ImageJ software (NIH, rsbweb.nih.gov/ij) and analyzed for distances (cm) from nearest neighbors. For this test, five independent experiments for each sex were performed ($n = 5$).

2.5.5. Aggressiveness test

As in the other tests, aggressiveness was evaluated for both sexes individually, using ten flies per group, which were submitted to a 90-min fastening before the beginning of the test. Pairs of flies (Female-Female and Male-Male) were then transferred to a circular combat chamber with a radius of 45 mm and a height of 12 mm containing a drop of food (sucrose). Flies were acclimated for 2 min and observed for 5 min. The following behaviors were considered aggressive encounters: leg extension from one fly to another resulting in physical contact, chasing, fast loading approach leading to direct orientation, wing raising

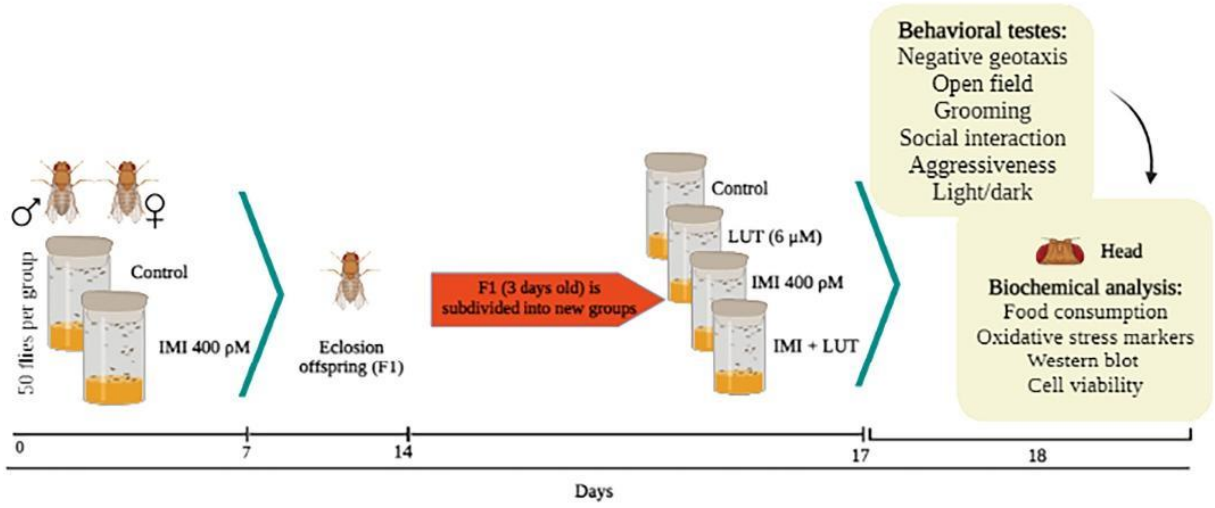


Fig. 1. Schematic representation of the experimental design.

in response to proximity/approach of the other fly. Data were recorded according to the number of encounters that characterized aggressive behavior (Araujo et al., 2018; Edwards et al., 2006). Two hundred flies were used for each sex, representing five independent experiments ($n = 5$).

2.5.6. Light/dark test

The light/dark test was performed to assess the anxious behavior of the flies as previously described by Mohammad et al. (2016), with some adaptations. The test was carried out in a box consisting of a dark compartment and a light compartment, with the two compartments being interconnected by an opening. The light compartment had a light intensity of 16 W. Five flies from each group were used for the test, which was performed individually for both sexes. At the beginning of the experiment, each fly was placed in the center of the lit box facing the opening. The test was carried out for 3 min, and a fly was considered to have entered the light or dark compartment when both forelegs were inside the compartment. The amount of time spent in the dark compartment was recorded. Five independent experiments were carried out for both sexes ($n = 5$).

2.6. Ex vivo assays

2.6.1. Homogenate preparation

Flies were cryoanesthetized after 24 h of treatment. Afterwards, the flies were decapitated for sample preparation. Each sample consisted of a homogenate of several fly heads (the number of homogenized heads depends on the protocol of each analysis). The homogenization buffer, as well as the period of centrifugation of the samples, were used according to the protocol of the analyses.

Enzyme activity results were corrected according to the protein value of the samples. Protein content was colorimetrically measured using the Bradford method (Bradford, 1976) and using bovine serum albumin (1 mg/mL) as a standard.

2.6.2. Food consumption

Food consumption was assessed according to Lushchak et al. (2011), with adaptations. Fifteen flies per group were fasted for 1 h before testing and subsequently exposed to the following experimental diets: 1) Control, 2) IMI 400 ρM, and 3) lutein-loaded nanoparticles 6 μM, with 0.5 % of the FD&C dye added Blue No1 (FCF Brilliant Blue). Flies remained on this medium for 2 h. After the feeding period, each group of flies was immediately anesthetized on ice and the flies' heads were removed. Fifteen bodies were used for homogenization in 200 μL of 20 mM HEPES, pH 7.5, centrifuged at 14,290-x g for 15 min, and the supernatant was measured in a 96-well microplate reader at 629 nm. The optical density of the homogenates from the flies that consumed the corresponding diets without the dye was used as a blank. A total of 225 flies were used in five independent experiments (n = 5). The consumption test was carried out to eliminate the hypothesis that the flies would not be consuming the diet that contained imidacloprid and the lutein-loaded nanoparticles.

2.6.3. Determination of antioxidant enzyme activities (SOD and CAT)

To evaluate the action of the lutein-loaded nanoparticles regarding the antioxidant capacity and detoxifying defense system of the offspring exposed to IMI, the activity of the superoxide dismutase (SOD) and catalase (CAT) enzymes was evaluated. Sample preparation for enzyme activity analysis was similar, following the same protocol. Therefore, 20 flies' heads were homogenized in 200 μL of 0.1 M HEPES buffer (pH 7.0) and centrifuged at 78-x g for 10 min. The supernatant was reserved for analysis. SOD activity was evaluated through the method described by Kostyuk and Potapovich (1989), with modifications by Franco et al. (2009), monitoring inhibition of quercetin auto-oxidation. The reaction mixture was composed of sodium phosphate buffer (0.025 M EDTA/0.1 mM, pH 10.0) and N, N, N, N tetramethylethylenediamine (TEMED). One mL of the mixture was combined with 10 μL of supernatant, and the reaction was started by adding 50 μL of quercetin to the cuvette for the reading in a spectrophotometer at 406 nm for 2 min. The results were corrected for the absorbance of the amount of protein present in the supernatant sample and calculated as percent inhibition of quercetin oxidation. Enzyme activity was expressed in mU/mg of protein.

To determine CAT activity, the methodology of Aebi (1984) was used. For the reading, 30 μL of supernatant were added to a quartz cuvette, with 2 mL of reaction mixture composed of potassium phosphate buffer (0.25 M/2.5 mM EDTA, pH 7.0), 30 % hydrogen peroxide (H_2O_2) and Triton X-100. The reading was carried out in a spectrophotometer with a wavelength of 240 nm for 2 min. Results were corrected according to protein concentration and expressed in mU/mg protein.

All analyses were performed for both sexes in duplicate, totaling five independent experiments each ($n = 5$).

2.6.4. Levels of reactive species

The quantification of the DCF-DA oxidation assay was monitored as a general index of oxidative stress as described by Pérez-severiano et al. (2004). Fifteen flies' heads per group were homogenized in 1000 μL of 10 mM Tris buffer, pH 7.0, and centrifuged at 1000-x g for 5 min at 4 °C. Subsequently, 34 μL of sample supernatant were added to a mixture containing 964 μL of HEPES buffer (pH 7.0) and 10 μL of 2,7-dichlorofluorescein diacetate (3.33 M; DCFDA). After one hour, the fluorescence emission resulting from the oxidation of DCF-DA was monitored. The reading was performed with an excitation of 485 nm and emission of 530 nm, with a beam of 2.5, in a spectrophotometer in an EnsPireR multimode microplate reader (Perkin Elmer, USA). The results were expressed as percentage, considering the control group. Five independent experiments for both sexes were performed ($n = 5$).

2.6.5. Determination of thiobarbituric acid reactive substances (TBARS)

Lipid peroxidation levels were evaluated by measuring thiobarbituric acid reactive substances (TBARS) as described above with modifications (Ohkawa et al., 1979). Twenty flies' heads were used for each group, being homogenized in 120 μL of 20 mM HEPES buffer (pH 7.0) and centrifuged at 80–x g for 10 min at 4 °C. The supernatant was removed and then thiobarbituric acid (0.8 % TBA, pH 3.2), acetic acid/HCl (20 %, pH 3.4) and sodium sulfate (SDS 8.1 %) were added. Afterwards, the samples were incubated for two hours at 95 °C and the absorbance was measured in a 532 nm microplate reader. The TBARS values were corrected for protein concentration and expressed as nmol MDA/mg protein. Five independent experiments for each sex were performed (n = 5).

2.6.6. Cell viability

Cell viability was measured using a method based on the ability of viable cells to reduce resazurin to resorufin, a fluorescent molecule (Franco et al., 2009). Twenty flies' heads per group were homogenized in 100 μL of 20 mM Tris buffer (pH 7.0), and centrifuged at 999-x g for 10 min at 4 °C. Then, samples were incubated on ELISA plates with 180 μL of 20 mM Tris buffer (pH 7.0) and 10 μL of resazurin for 1 h. The reading of absorbance was performed at a wavelength of 573 nm in a microplate reader. Five independent experiments were carried out for both sexes (n = 5). Data were expressed as resazurin reduction (% control).

2.6.7. Western blot analysis

Western blot analysis was performed as previously described by Guerra et al. (2012) with slight adaptations. Thirty flies were rapidly euthanized and homogenized in 300 μL of ice-cold buffer A (10 mM KCl, 2 mM $MgCl_2$, 21 mM EDTA, 1 mM NaF, 10 μg/mL aprotinin, 10 mM β-glycerolphosphate, 1 mM PMSF, 1 mM DTT, and 2 mM sodium orthovanadate in 10 mM HEPES, pH 7.9). After being homogenized, the samples were incubated at 0 °C for 15 min and then centrifuged at 16000 ×g for 45 min at 4 °C. The resulting supernatant was collected

and used for the determination of cytosolic proteins, while the pellet was resuspended to 150 μL of ice-cold buffer B (10 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM NaF, 10 μg/mL aprotinin, 10 mM β-glycerolphosphate, 1 mM PMSF, 1 mM DTT, 2 mM sodium orthovanadate, and 1 % Triton-X in 10 mM HEPES pH 7.9) and incubated at 0 °C for 15 min before being centrifuged at 16000 ×*g* for 45 min at 4 °C. Following centrifugation, the supernatant was discarded, and the pellet was resuspended in 100 μL of ice-cold buffer C (50 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM NaF, 10 μg/mL aprotinin, 10 mM β-glycerolphosphate, 1 mM PMSF, 1 mM DTT, 2 mM sodium orthovanadate, 420 mM NaCl, and 25 % glycerol in 20 mM HEPES pH 7.9). The samples were incubated at 0 °C for 15 min and then centrifuged at 16,000 ×*g* for 45 min at 4 °C, and the resulting supernatant was collected. Protein concentration was determined as described by Bradford (1976), and equivalent amounts of protein in cytosolic fractions (80 μg) were added to 0.2 volumes of concentrated loading buffer (200 mM Tris, 10 % glycerol, 2 % SDS, 2.75 mM β-mercaptoethanol and 0.04 % bromophenol blue) and boiled for 10 min. Protein separation occurred in 12 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the protein was transferred onto Amersham™ Protran® Premium Western blotting nitrocellulose membranes, using the Transfer-Blot® Turbo™ Transfer System (1.0 mA; 30 min). β-Actin staining was used as a load control. Membrane blocking was performed with 1 % BSA in 0.05 % Tween 20 in Tris-borate saline (TBS-T), then incubated overnight with specific primary antibodies diluted 1:1000 in TBS-T (anti-mouse Nrf2, anti-mouse Shank 1/2/3 (G-12) Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Blots were washed three times with TBS-T, followed by incubation with horseradish peroxidase-conjugated secondary antibody (1:5000, anti-mouse IgG-HRP; Santa Cruz Biotechnology, Inc.) for 2 h. Protein bands were developed with 3,3′,5,5′-Tetramethylbenzidine (TMB; Sigma-Aldrich). Finally, the membranes were dried, digitized, and quantified with ImageJ (NIH, Bethesda, MD, USA). The results were normalized by arbitrarily setting the densitometry of the control group to 100 %.

2.6.8. Statistical analysis

GraphPad Prism 8 software was used for statistical analyses and graphic plotting. The statistical analyses of the experiments was performed through an analysis of variance (two-way ANOVA), followed by Tukey's post hoc test. All data are expressed as mean and standard error of the mean (SEM). For the social interaction test, the Kruskal-Wallis test was used, followed by the post hoc Dunnet test, and the data were expressed as median and interquartile range. Statistically significant values of $P < 0.05$ were considered.

3. Results

3.1. Particle characterization

Fig. 2a and b shows the Transmission Electron Microscopy and the Atomic Force Microscopy images of the lutein-loaded nanoparticles. Table 1 presents the average particle size, polydispersity (PDI), and Zeta potential of the lutein-loaded nanoparticles and blank nanoparticles (no lutein added). Fig. 2c presents the particle size distribution for each sample. In the TEM images, the nanoparticles presented an irregular, non-spherical morphology, with sizes around 200 nm, while in AFM, the particles presented sizes around 150 nm. DLS number average particle size and distribution corroborated the images. Blank nanoparticles are slightly smaller than lutein-loaded nanoparticles, which was expected due to the greater number of solids in the organic phase in the latter.

Fig. 3 presents the X-ray diffractograms and FTIR spectra of the lutein, lutein-loaded nanoparticles, and blank nanoparticles (no lutein added). The diffractograms of pure polycaprolactone and phosphatidylcholine are also shown. X-Ray diffraction was used to determine if encapsulation caused changes in the crystalline structure of lutein. Lutein presented crystalline peaks between 5 and 30 °C as previously reported by other authors (Lim et al., 2021; Ma et al., 2020), revealing the existence of crystalline structures. For PCL, strong peaks were found around 21 and 24°, which are associated to the orthorhombic crystalline structures of this polymer (planes (110) and (200), respectively) (Baji et al., 2007). It is worth noting that lutein peaks were much attenuated in the nanoparticles, indicating that lutein was in an amorphous state in this form. Also, there was a small shift in the PCL peaks, probably due to the presence of lutein on the microstructure of the encapsulant polymer.

The characteristic FTIR absorption bands of lutein were found at 2930 cm^{-1} (CH_3 antisymmetric stretching), 2852 cm^{-1} (CH_2), and a broad band around 3380 cm^{-1}, attributed to the hydroxyl groups (Wu et al., 2022). PCL bands were shown at 1180 cm^{-1} (stretching vibrations

Table 1
Number average particles size, polydispersity index (PDI) and zeta potential of the blank nanoparticles (no lutein added) and the lutein-loaded nanoparticles.

	Blank nanoparticles (no lutein added)	Lutein-loaded nanoparticles
Number average particles size (nm)	58	174
PDI (−)	0.78	0.35
Zeta potential (mV)	−4.7 ± 1.2	−4.9 ± 1.1

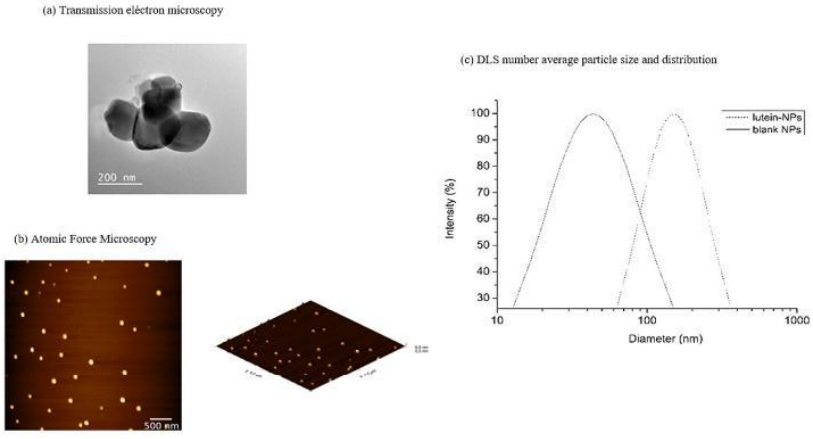
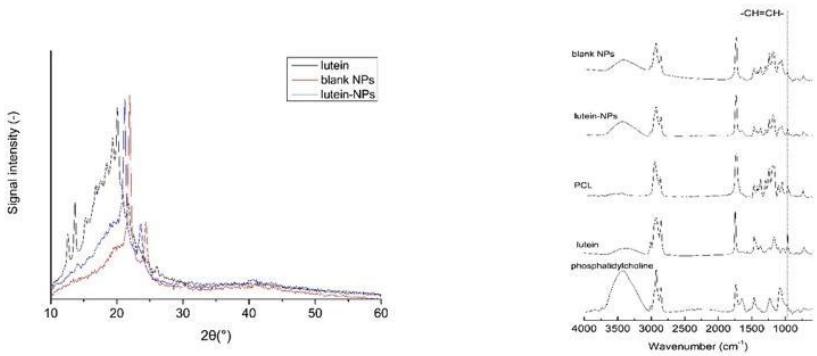


Fig. 2. Microscopy images of the lutein-loaded nanoparticles and particles size distribution of the blank nanoparticles (no lutein added) and the lutein-loaded nanoparticles. (a) Transmission electron microscopy; (b) atomic force microscopy and (c) DLS number average particle size and distribution.



(a) X-ray diffraction patterns. (b) FTIR spectra.

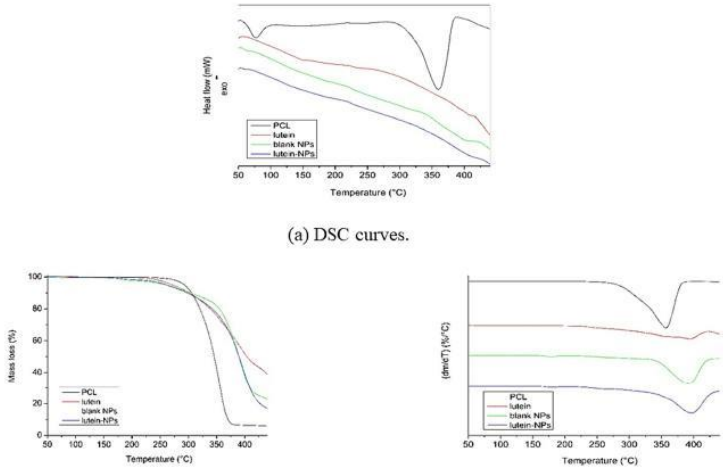
Fig. 3. X-ray diffractograms and FTIR spectra of the lutein, lutein-loaded nanoparticles and blank nanoparticles (no lutein added).

of ether groups, C–O–C), 1728 cm^{-1} (stretching vibrations of carboxyl, C═O), and 2867 cm^{-1} (symmetric stretching of C—H) (Li et al., 2018). Phosphatidylcholine presented a broad band from 3676 to 3100 cm^{-1} (attributed to the OH groups), and also characteristic bands at 3015 cm^{-1} (C═CH groups), 2930 cm^{-1} (CH_3 antisymmetric stretching), 2841 cm^{-1} (CH_2 symmetric stretching), and 1742 cm^{-1} (stretching vibrations of carboxyl, C═O) (Mohan et al., 2020). Although most lutein absorption bands were superimposed with phosphatidylcholine bands, CH_3 bands were attenuated in the lutein-loaded nanoparticles, also suggesting the encapsulation of lutein inside the polymeric matrix. This may be more evident for the trans conjugated alkene (-CH=CH-) out of plane deformation at 965 cm^{-1} (da Silva et al., 2017), which is present as a weak band in PCL but is a strong band in lutein.

Differential Scanning Calorimetry and Thermogravimetric Analysis are presented in Fig. 4. The melting temperature of PCL was found at 76.9 °C, while polymer degradation was detected at 360.8 °C, which is in agreement with the literature (Lozano-Sánchez et al., 2018). Lutein presented a small peak at 149 °C associated to its melting (Hao et al., 2022). The melting temperature was 64.0 °C and 61.1 °C for blank nanoparticles (no lutein added) and lutein-loaded nanoparticles, respectively. This may be associated to the presence of phosphatidylcholine and lutein acting as plasticizers agents of polycaprolactone. This is evidence that lutein may be located inside the polymeric matrix. The nanoparticles presented mass loss up from 140 °C, which may be associated with adsorbed water since phosphatidylcholine is hydrophilic. Mass loss at 391 and 497 °C was found in the blank nanoparticles and the lutein-loaded nanoparticles, respectively. The difference in the thermal behavior between PCL and the nanoparticles may be due to the presence of phosphatidylcholine, which presents a melting temperature around 50 °C (Zhang et al., 2012).

3.2. Behavioral tests and food consumption

Fig. 5A–F shows the effect of the lutein-loaded nanoparticles (6 μM) in the offspring of the flies exposed to IMI (400 ρM) on the climbing time, crossing number, social interaction, light/dark, aggressiveness, and grooming, respectively. The statistical analysis (two-way ANOVA) revealed a significant effect for the interaction factor (IMI versus lutein-loaded nanoparticles) in female and male flies on climbing time [$F_{(1,16)} = 9.60$; $P < 0.05$ and $F_{(1,16)} = 14.20$; $P < 0.05$], number of crossings [$F_{(1,16)} = 27.78$; $P < 0.05$ and $F_{(1,16)} = 36.26$; $P < 0.05$], nearest neighbor distance [$F_{(1,196)} = 22.77$; $P < 0.05$ and $F_{(1,196)} = 9.85$; $P < 0.05$], time of self-cleaning movements [$F_{(1,16)} = 21.67$; $P < 0.05$ and $F_{(1,16)} = 15.16$; $P < 0.05$], time spent in the dark compartment [$F_{(1,16)} = 59.77$; $P < 0.05$ and $F_{(1,16)} = 98.20$; $P < 0.05$], and aggressive events [$F_{(1,16)} = 16.8$; $P < 0.05$ and $F_{(1,16)} = 11.03$; $P < 0.05$], respectively. The



(a) DSC curves.

(b) Thermogravimetric analisys (TGA). (b) Differential Thermogravimetric analisys (DTGA).

Fig. 4. Differential scanning calorimetry and thermogravimetric analysis of PCL, lutein and lutein-loaded nanoparticles and the blank nanoparticles (no lutein added).

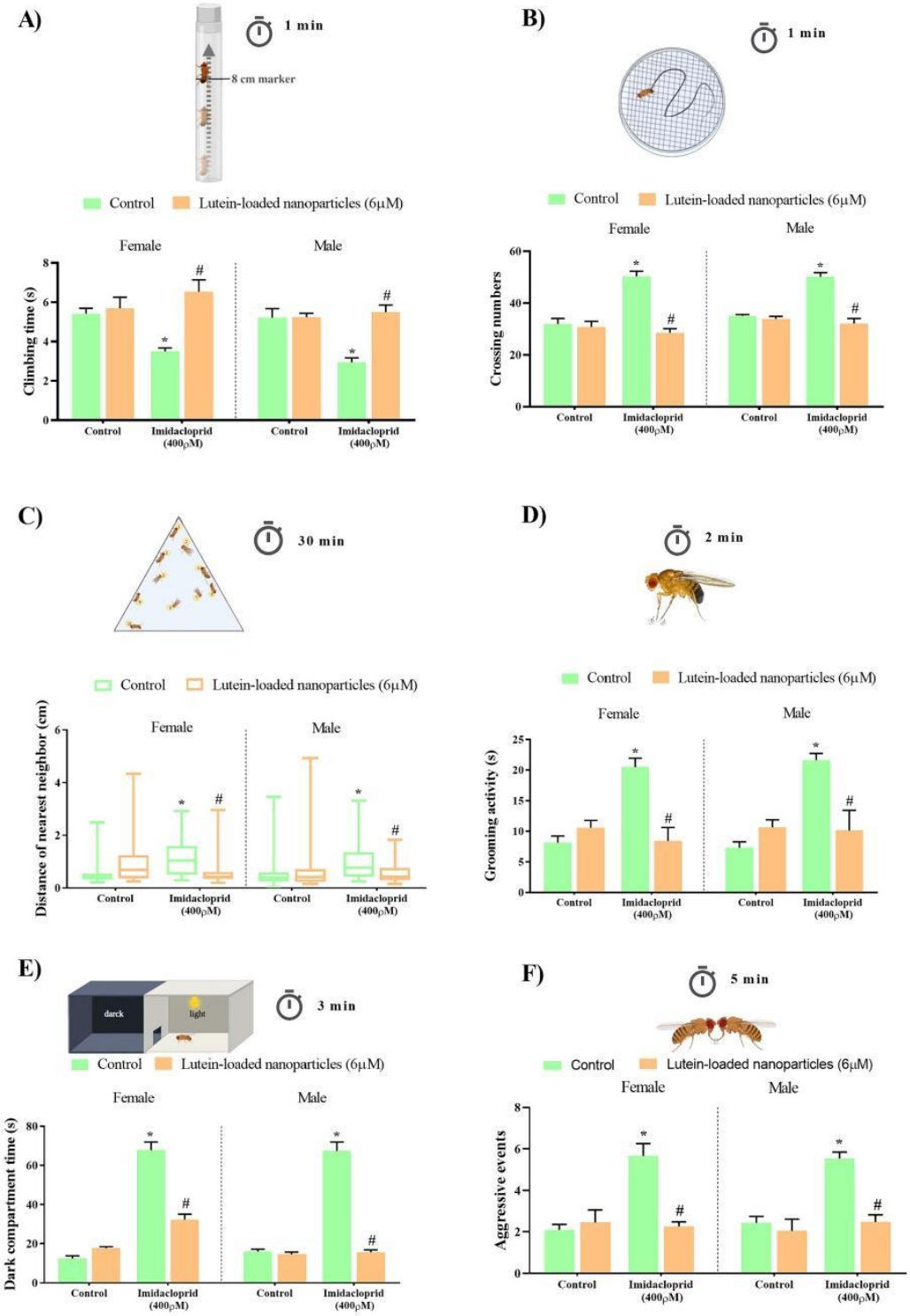


Fig. 5. Effect of treatment with lutein-loaded nanoparticles for 24 h on locomotor and exploratory activity of descendants of both sexes of *Drosophila melanogaster* exposed to IMI. (A) Negative geotaxis test; (B) open field test; (C) social interaction; (D) grooming; (E) light/dark; (F) test of aggression in the offspring of both sexes of *Drosophila melanogaster*. Data are mean ± SEM, median and interquartile range, for $n = 5$ in each group. * indicates significant difference ($P < 0.05$) in relation to the control group. # indicates a significant difference ($P < 0.05$) in relation to the IMI group.

post hoc comparisons demonstrated that the lutein-loaded nanoparticles reversed the locomotor and exploratory damage in the geotaxis (Fig. 5A) and open field tests (Fig. 5B), the social interaction deficit (decreasing the distance from the nearest fly neighbor - Fig. 5C), the increase in self-cleaning time (grooming - Fig. 5D), the increase in anxiety (since the flies spent less time in the dark compartment - Fig. 5E), as well as the increase in aggressive behavior (Fig. 5F) in the offspring of the flies exposed to IMI (400 ρM) in both sexes.

The statistical analysis did not show significant differences between groups in terms of food consumption (Fig. 6).

3.3. Antioxidant and detoxifying enzyme activity

Fig. 7A and B shows the effect of the lutein-loaded nanoparticles (6 μM) on the offspring of flies exposed to IMI (400 ρM) on the activity of antioxidant enzymes (SOD and CAT) in the *Drosophila melanogaster* head samples. The statistical analysis (two-way ANOVA) revealed a significant effect for the interaction factor (IMI versus lutein-loaded nanoparticles) in female and male flies on SOD activity [$F_{(1,16)} = 3.33$; $P < 0.05$ and $F_{(1,16)} = 19.43$; $P < 0.05$], CAT [$F_{(1,16)} = 35.09$; $P < 0.05$ and $F_{(1,16)} = 4.82$; $P < 0.05$], respectively. The post hoc comparisons demonstrated that exposure to lutein-loaded nanoparticles reversed the decreased activity of the antioxidant enzymes SOD (Fig. 7A) and CAT (Fig. 7B) in the offspring, of both sexes, of flies exposed to IMI.

3.4. Levels of reactive species and lipid peroxidation

Fig. 7C and D shows the effect of the lutein-loaded nanoparticles (6 μM) in the offspring of flies exposed to IMI (400 ρM) on oxidative stress indicators (ROS and TBARS) in the head of *Drosophila melanogaster*. The statistical analysis (two-way ANOVA) indicated a significant effect for the interaction factor (IMI versus lutein-loaded nanoparticles) on ROS levels [$F_{(1,16)} = 21.82$; $P < 0.05$] in the female flies. The post hoc comparisons demonstrated that exposure to lutein-loaded nanoparticles reversed the ROS increase in the female progeny of the flies exposed to IMI (400 ρM). The statistical analysis (two-way ANOVA) also revealed an increase in ROS levels in the male progeny of the flies exposed to IMI (400 ρM) compared to the control group, however, the lutein-loaded nanoparticles were not able to reverse this damage (Fig. 7C).

Regarding the TBARS levels, the statistical analysis (two-way ANOVA) indicated a significant effect for the interaction factor (IMI versus lutein-loaded nanoparticles) in females [$F_{(1,16)} = 60.26$; $P < 0.05$] and males [$F_{(1,16)} = 10.54$; $P < 0.05$]. The post hoc comparisons demonstrated that the lutein-loaded nanoparticles reversed the damage caused by IMI on the TBARS levels in both sexes (Fig. 7D).

3.5. Cell viability

Fig. 7E shows the effect of the lutein-loaded nanoparticles (6 μM) in the offspring of flies exposed to IMI (400 ρM) on cell viability by reducing resazurin in *Drosophila melanogaster* head. The statistical analysis (two-way ANOVA) indicated a significant effect for the interaction factor (IMI versus lutein-loaded nanoparticles) on resazurin levels in female [$F_{(1,16)} = 9.80$; $P < 0.05$] and male [$F_{(1,16)} = 17.40$; $P < 0.05$] flies. The post hoc comparisons demonstrated that lutein-loaded nanoparticles reversed the damage induced by IMI, avoiding cell viability decrease in the offspring.

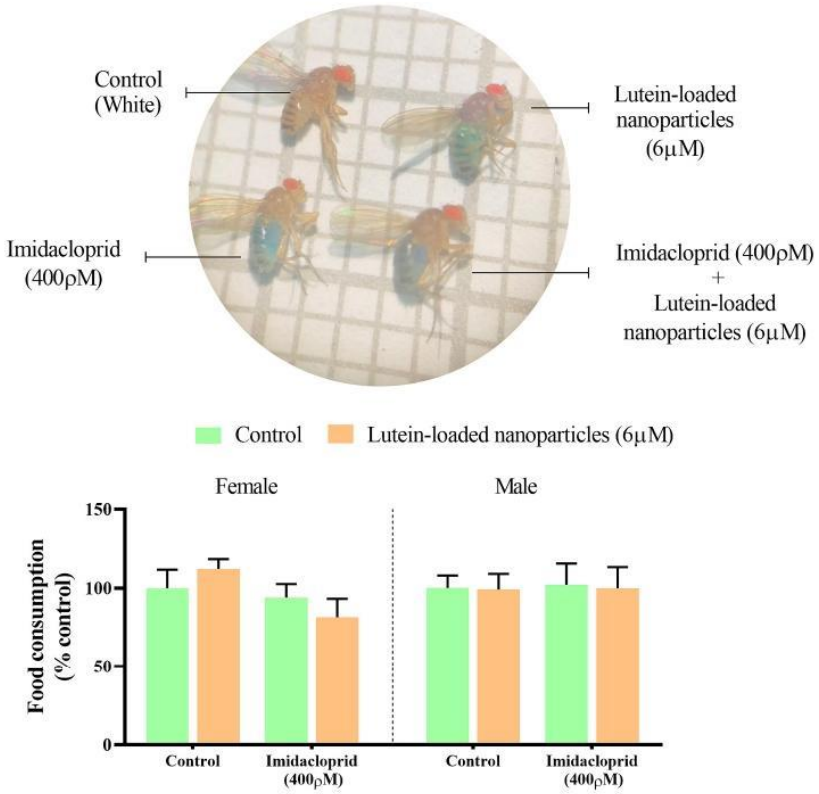


Fig. 6. Exposure to IMI (400 ρM), lutein-loaded nanoparticles (6 μM) and co-exposure to IMI and lutein-loaded nanoparticles for 24 h, on food consumption in offspring of both sexes of *Drosophila melanogaster*. Data are mean ± SEM, for n = 5 in each group.

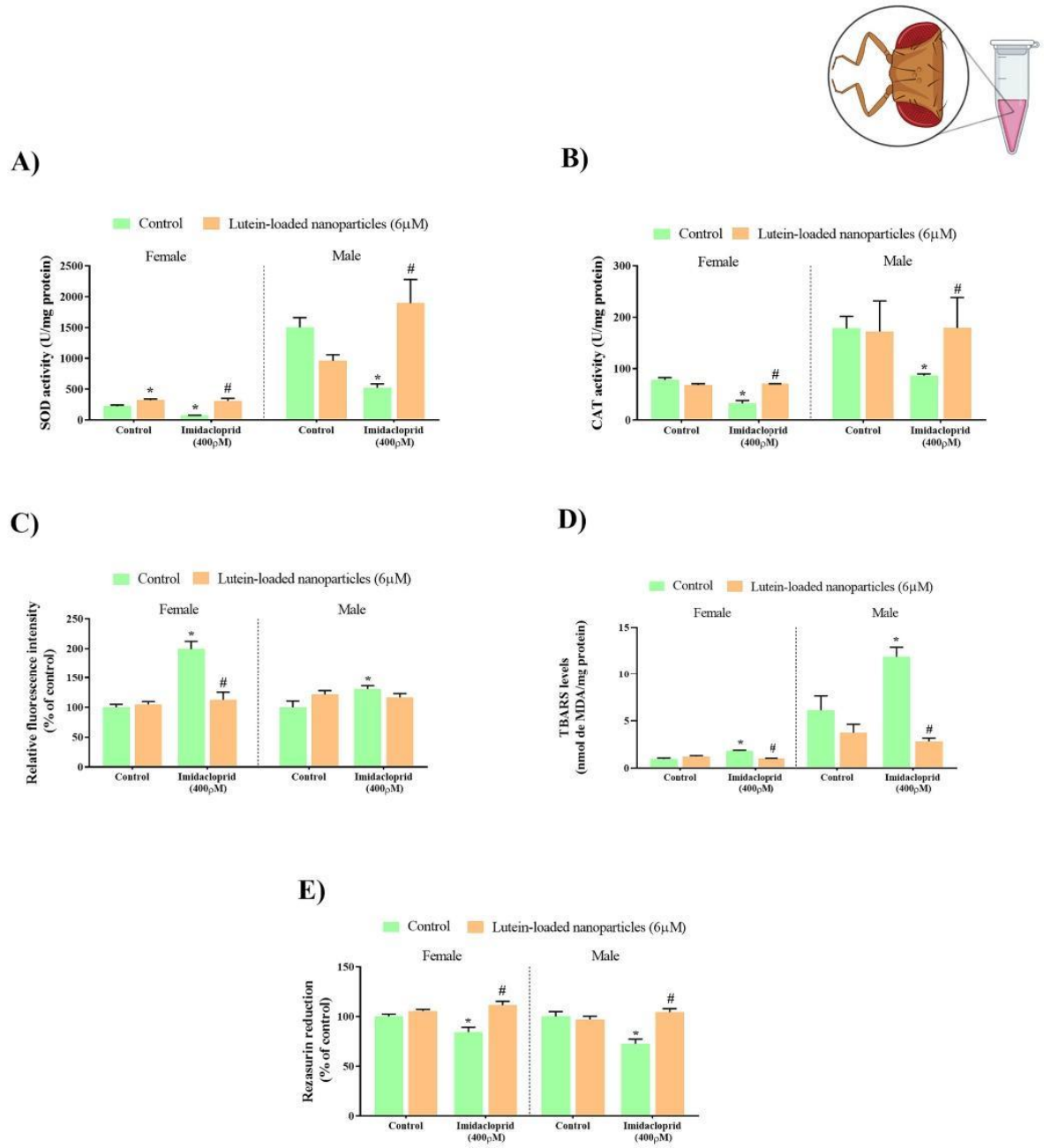


Fig. 7. Effect of treatment with lutein-loaded nanoparticles for 24 h on markers of detoxifying enzyme oxidative stress and cell viability in male and female offspring of *Drosophila melanogaster* exposed to IMI. (A) SOD activity; (B) CAT activity; (C) ROS levels; (D) TBARS and (E) cell viability. Data are mean ± SEM, median and interquartile range, for n = 5 in each group. * indicates significant difference ($P < 0.05$) compared to the control group. # indicates significant difference ($P < 0.05$) in relation to the IMI group.

3.6. Western immunoblotting

Fig. 8A–D shows the effect of the lutein-loaded nanoparticles (6 μM) on the offspring of flies exposed to IMI (400 ρM) for western immunoblotting (Nrf2 and Shank) in *Drosophila melanogaster* head samples. The statistical analysis (two-way ANOVA) indicated a significant effect for the interaction factor (IMI versus lutein-loaded nanoparticles) on Nrf2 immunoreactivity in the cytosol of females [$F_{(1,11)} = 14.96$; $P < 0.05$] and males [$F_{(1,12)} = 11,38$; $P < 0.05$]. For Shank immunoreactivity, the statistical analysis (two-way ANOVA) indicated a significant effect for the interaction factor (IMI versus lutein-loaded nanoparticles) in females [$F_{(1,10)} = 7.450$; $P < 0.05$] and males [$F_{(1,12)} = 5.513$ $P < 0.05$]. The post hoc comparisons demonstrated that lutein-loaded nanoparticles reversed the decrease in Nrf2 immunoreactivity in the cytosol in the offspring of flies exposed to IMI in both sexes (Fig. 8A and B), Furthermore, the post hoc comparisons also demonstrated that the lutein-

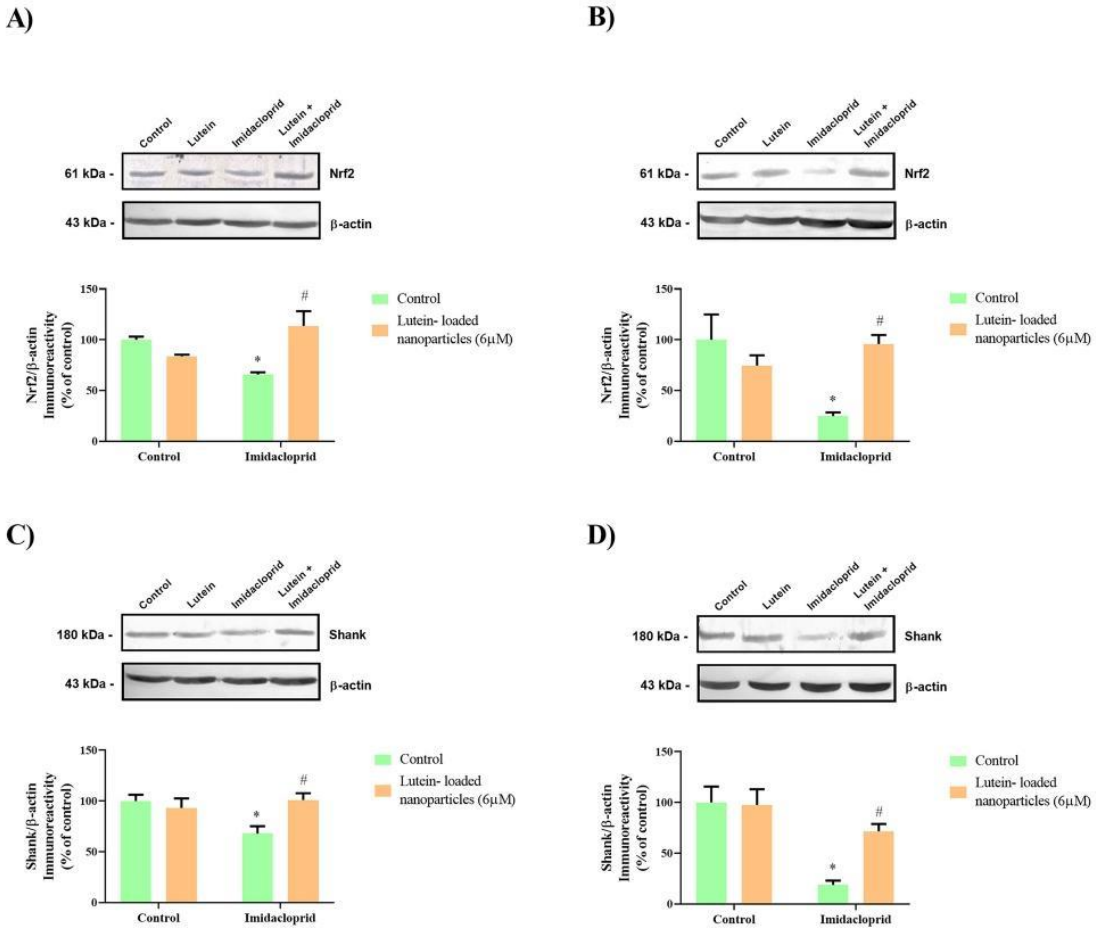


Fig. 8. Effect of treatment with lutein-loaded nanoparticles for 24 h on representative Western immunoblotting images in male and female *Drosophila melanogaster* offspring exposed to IMI. (A) relative intensive intensity of NRF2 in the cytosol of females; (B) relative intensive intensity of NRF2 in the cytosol of males; (C) relative intensive intensity of Shank in females; (D) relative intensive intensity of Shank in males. Data are mean ± SEM, median and interquartile range, for $n = 4$–6 in each group. * indicates significant difference ($P < 0.05$) in relation to the control group. # indicates a significant difference ($P < 0.05$) in relation to the IMI group.

loaded nanoparticles reversed the decrease in Shank immunoreactivity in both sexes (Fig. 8C and D).

4. Discussion

In this study, we used a model of neurodevelopmental disorder induced by exposure to IMI in the offspring of both sexes of *Drosophila melanogaster* in order to elucidate the action of lutein-loaded nanoparticles. Our results demonstrate that IMI promoted an increase in oxidative stress, leading to significant behavioral changes. However, it was possible to observe a reversal of behavioral damage associated with oxidative stress restoration through the administration of lutein-loaded nanoparticles.

Considering that ASD is more frequent in men, with a ratio of 3:1 in relation to women (Schendel and Thorsteinsson, 2018), most experimental studies focus on exploring only the male gender. Nevertheless, the evaluation of both genders is important to elucidate the possible behavioral differences and mechanisms involved in the disease in males and females, enabling a better understanding of the disorder and, therefore, the search for more specific treatments. Similarly to our previous study (Janner et al., 2021), both males and females, from the offspring of flies exposed to IMI, presented ASD-like phenotypes, including an excessive increase in activities such as locomotion and exploration, which is characteristic of hyperactivity, observed through the open field and negative geotaxis tests. In addition, the flies showed greater grooming, aggressiveness, and anxiety (assessed by the light/dark test), as well as decreased social interaction. Importantly, behavioral assessments that evaluate social interaction, grooming and aggressiveness are some of the diagnostic parameters of ASD, and the lutein-loaded nanoparticles were able to reverse these phenotypes. In recent years, numerous studies using different compounds, such as bisphenol (Musachio et al., 2021), atrazine (Figueira et al., 2017), Pb (Shilpa et al., 2021), and even IMI (Janner et al., 2021; Kim et al., 2017), have demonstrated significant behavioral changes in flies, but our work is the first to demonstrate a reversal of behavioral damage induced by a chemical agent or environmental pollutant in both sexes separately. Taking into consideration that this disorder has multifaceted causes and its diagnosis is often delayed and challenging, with few and nonspecific treatments available, the ability of lutein to reverse the established damage is extremely important.

In addition to behavioral damage, the offspring of flies exposed to IMI (400 ρM) also showed enzymatic changes, as reduced activity of antioxidant enzymes (SOD and CAT) and increased levels of ROS and TBARS, besides reduced cell viability, and changes in Shank and Nrf2

immunoreactivity. The data shows a reduction in Shank immunoreactivity, which is a candidate gene for the development of ASD and ADHD (Andrew et al., 2021; Delling and Boeckers, 2021; Tabouy et al., 2018). The Shank family of proteins are a central part of postsynaptic density (PSD), thus playing an important role in synapse formation, plasticity, glutamatergic signaling and transmission. Hence, isoform-specific mutations in Shank proteins have deleterious effects on synaptic development and plasticity (Jung and Park, 2022). In this sense, Skank's experimental models have deficiencies, such as repetitive behaviors, anxiety, and reduced sociability (Balaan et al., 2019; Vyas et al., 2021; Wan et al., 2022), which are similar phenotypes to those observed in individuals with ASD. In the present work, treatment with lutein-loaded nanoparticles was able to rescue Shank's immunoreactivity for both sexes, restoring the observed behavioral damage.

Notably, the imbalance in the number of antioxidant and oxidant molecules is one of the factors cited as a contributor to the emergence of ASD and ADHD (Campbell et al., 2019; Erten, 2021; Xie et al., 2021). Because Nrf2 plays an important role in activating the antioxidant response in the organism, our results reveal that exposure to IMI possibly resulted in a reduction of Nrf2 immunoreactivity in the cytosol in flies of both sexes when compared to the control group, since, under conditions of oxidative stress or in the presence of xenobiotics, Nrf2 is translocated from the cytoplasm to the nucleus (Guo et al., 2021), with the aim of combating the damage-inducing agent.

In this context, according to the data obtained, we strongly believe that the behavioral changes observed in the ASD and ADHD model involve changes in Shank as already described in other studies (Andrew et al., 2021; Bucher et al., 2021; Moutin et al., 2021), and consequently an increase in oxidative stress, with these changes being demonstrated in both female and male flies for a better understanding. Therefore, treatment with lutein-loaded nanoparticles attenuated the damage caused by IMI, being able to increase the immunoreactivity of Shank, restore mitochondrial damage, and reestablish the activity of antioxidant enzymes (SOD and CAT), plus increasing TBARS levels in the progeny of both sexes. As for ROS levels, treatment with lutein-loaded nanoparticles was only able to reduce ROS levels in females, however, even though it did not exert this kind of protection in males, we believe that the antioxidant action may have occurred later, as it was possible to observe a reduction in oxidative damage in the other evaluated indicators, such as TBARS. We believe that the differences observed between the sexes are associated with factors such as gender, since the level of alterations varies according to sex, where females demonstrate greater susceptibility to increased ROS when compared to males (Gomes et al., 2023; Niveditha et al., 2017; Turnell et al., 2021), and even with an increase in levels of ROS in males we observed that the increase of TBARS proves to be more determinant to cause oxidative stress damage. Moreover, we cannot ignore that lutein-loaded nanoparticles had a beneficial effect on other indicators in both sexes, demonstrating their effectiveness in reversing oxidative stress.

The improvement in the behavioral performance of flies treated with lutein-loaded nanoparticles is, most likely, related to their antioxidant and neuroprotective potential, as observed in other studies (Fernandes et al., 2021; Geiss et al., 2019; Johnson, 2014; Nataraj et al., 2016; Zeni et al., 2019). Thus, lutein-loaded nanoparticles possibly act by protecting mitochondria, which consequently reduces oxidative damage and positively regulates Nrf2, which restores the activity of antioxidant enzymes. Furthermore, treatment with lutein-loaded nanoparticles rescued Shank immunoreactivity, and consequently reduced the behavioral changes observed in both sexes.

Moreover, as expected, our results from the food consumption analysis showed no significant difference between the groups that received a standard diet (control), IMI (400 ρM), and nanoparticles with lutein (6 μM), showing that flies of both sexes ate normally regardless of the diet during the experimental period.

Thus, this research offers several contributions to the field, highlighting how our main finding is the significant changes caused by IMI, with behavioral alterations observed in flies of both sexes. Furthermore, the damage observed in females was similar to that found in males, both in behavioral and neurochemical terms, reinforcing the importance of studying both sexes. Based on these results, we believe that lutein-loaded nanoparticles may represent a promising therapeutic alternative for ASD. They have been shown to be effective in combating oxidative stress through activation of the Nrf2 pathway, helping to improve the alterations associated with antioxidant/oxidant imbalance at both the neurochemical and behavioral levels. Furthermore, the nanoparticles enhance the beneficial action of lutein by increasing its bioavailability.

5. Conclusion

Flies exposed to IMI during the prenatal period developed phenotypes similar to those observed in ASD and ADHD. The results support the hypothesis that chemical induction of the neurodevelopmental disorder model in *Drosophila melanogaster* is related to changes in Shank immunoreactivity, along with increased oxidative stress caused by mitochondrial dysfunction. Treatment with lutein-loaded nanoparticles revealed the protective role of this compound against changes promoted by IMI in both sexes separately, providing relevant elucidations for the use of bioactive compounds as possible therapeutic agents, as well as bases for effective pharmacological strategies in the treatment of neurodevelopmental disorders.

Ethics approval

Not applicable.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

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CRediT authorship contribution statement

Dieniffer Espinosa Janner: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Márcia Rósula Poetini:** Methodology, Investigation, Data curation. **Elize Aparecida Santos Musachio:** Investigation, Data curation, Conceptualization. **Nathalie Savedra Gomes Chaves:** Investigation, Formal analysis, Data curation, Conceptualization. **Luana Barreto Meichtry:** Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Eliana Jardim Fernandes:** Investigation, Formal analysis, Data curation, Conceptualization. **Mustafa Munir Dahleh Mustafa:** Investigation. **Amarilis Santos De Carvalho:** Investigation. **Odinei Hess Gonçalves:** Investigation. **Fernanda Vitória Leimann:** Investigation. **Rilton Alves de Freitas:** Investigation. **Marina Prigol:** Supervision, Resources, Methodology, Funding acquisition, Data curation, Conceptualization.

Gustavo Petri Guerra: Writing – original draft, Supervision, Resources, Project administration, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

Data availability statements

Datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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5.2 Manuscrito

Título: Modulation of Dopamine, Serotonin, and Behavior by Lutein Carrier Nanoparticles in a *Drosophila melanogaster* Model of Neurodevelopmental Disorders.

Será submetido ao periódico: Food and Chemical Toxicology

Modulation of Dopamine, Serotonin, and Behavior by Lutein Carrier Nanoparticles in a *Drosophila melanogaster* Model of Neurodevelopmental Disorders

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Abstract:

Considering that woman's health during pregnancy is crucial to well-being as much maternal and fetal as well as the child's future, supplementation with antioxidant compounds has emerged as a promising strategy to prevent the development of future diseases. Given this context, the study aimed to evaluate the effect of lutein carrier nanoparticles supplementation during the preconception period on the offspring of *Drosophila melanogaster* subjected to a neurodevelopmental disorder model. Female flies, were exposed to either a standard diet or a diet containing NPs LUT (6 µM) for 24 hours. Following this period, the flies were transferred to new experimental vials, and eighteen males were added, resulting in a total of 53 flies per experimental group. The male and female flies were then subdivided into two groups and exposed to either a standard diet or imidacloprid (IMI), for 7 days, to induce the neurodevelopmental disorder model, creating four experimental groups: 1) Control; 2) IMI; 3) NPs LUT; 4) NPs LUT + IMI. The hatched offspring were then used for behavioral and biochemical evaluations. Our results showed that supplementation with lutein carrier nanoparticles was able to prevent decreased activity of enzyme tyrosine hydroxylase (TH), as did neurotransmitters dopamine (DA) and serotonin (5-HT) in the head of flies, and as a consequence it prevented behavioral damages such as hyperactivity, anxiety, social interaction, repetitive movements, learning and memory in the progeny of both sexes. Thus, these findings highlight the relevance of preconception supplementation with lutein carrier nanoparticles as an effective approach to prevent the emergence of symptoms associated with neuropsychiatric disorders, paving the way for future research aimed at investigating the best intervention period to prevent ASD and ADHD-type disorders.

Keywords: Neurotransmitters, Monoamines, Supplementation, Preconception.

1. Introduction

Nutritional health during the pre-conception period, which precedes pregnancy, is an extremely important topic that has garnered increasing interest in recent years (Dean et al., 2014; Li et al., 2019; Stephenson et al., 2018; Teshome et al., 2020). Given that a woman's health leading up to pregnancy plays a fundamental role in both maternal and future fetal and child well-being, supplementation with compounds that possess antioxidant properties has emerged as a potential strategy to prevent the onset of future diseases (Harding et al., 2017; Ochiai and Kuroda, 2020; Pitkin, 2007; Rizki et al., 2021).

Therefore, the use of bioactive compounds such as lutein has gained prominence in the scientific community, with studies indicating that this compound exerts a neuroprotective effect in various experimental models of disease (Mrowicka et al., 2022; Nataraj et al., 2016; Zeni et al., 2019). Additionally, lutein carrier nanoparticles supplementation has been associated with reduced rates of cognitive and memory impairment in rats (Viana et al., 2023), as well as the restoration of oxidative stress biomarkers and of neurotransmitter dopamine in *Drosophila melanogaster* (Fernandes et al., 2021; Janner et al., 2024).

These findings highlight the crucial role of oxidative stress and neurotransmitter balance in cognitive function, particularly given that dopamine (DA) and serotonin (5-HT) are essential for the development and function of the central nervous system (Loula and Monteiro, 2022). Therefore, dysfunctions in these neurotransmitters lead to behavioral changes strongly associated with neurodevelopmental disorders, such as autism spectrum disorder (ASD) and attention deficit hyperactivity disorder (ADHD), which often appear early in childhood (Al-Amin et al., 2015; Campbell et al., 2019; Martin et al., 2024; Morris-Rosendahl and Crocq, 2020). These disorders have a multifactorial origin, involving both genetic and environmental factors. Although genetic factors are not currently modifiable, reducing or eliminating environmental risks could potentially lower the probability of ASD manifestation. Studies indicate that environmental factors contribute significantly, accounting for approximately 40 to 50% of the risk (Deng et al., 2015; Kim and Leventhal, 2015; Modabbernia et al., 2017).

This perspective is further supported by several studies highlighting different factors that influence fetal neurodevelopment and the typical characteristics of ASD- and ADHD-type disorders. However, the influence of factors affecting parents before

pregnancy is still poorly understood. Although studies indicate that maternal nutrition is linked to children's cognitive abilities (Borge et al., 2017; Crider et al., 2022; Freedman et al., 2018; Tan et al., 2020), questions about the optimal timing for adding supplements to the diet, whether before or during pregnancy, remain unanswered.

Given this context, the study aimed to evaluate the effect of lutein carrier nanoparticles supplementation during the preconception period on the offspring of *Drosophila melanogaster* subjected to a neurodevelopmental disorder model.

2. Materials and methods

2.1. *Drosophila melanogaster* stock

Drosophila melanogaster of the Harwich lineage were obtained from LAFTAMBIO (Laboratory of Pharmacological and Toxicological Assessments Applied to Bioactive Molecules - Unipampa Itaqui). The flies were fed a standard laboratory diet based on corn flour, wheat germ, sugar, powdered milk, salt and antifungal methylparaben, maintained under controlled conditions of light (12 hours of light/dark cycle), temperature and humidity (25 ± 1°C and 60% relative humidity).

2.2. Reagents

Imidacloprid (CAS Number: 138261-41-3) was sourced from Sigma-Aldrich (St. Louis, MO) and diluted in 0.0001% DMSO. The lutein carrier nanoparticles were prepared following the method described by Freiberger et al. (2015). All other reagents used were of analytical grade.

2.3. Lutein carrier nanoparticles

The present study utilized lutein carrier nanoparticles previously used by our group. Therefore, data regarding the characterization and selection of concentration are available in our earlier study (Janner et al., 2024).

2.4. Experimental protocol

The concentration of lutein carrier nanoparticles (NPs LUT) used was 6 µM, as determined in our previous study (Janner et al., 2024). Imidacloprid (CAS Number: 138261-41-3) was obtained from Sigma-Aldrich (St. Louis, MO) and diluted in 0.001% DMSO. Thirty-five female flies, up to 3 days old, were exposed to either a standard diet or a diet containing NPs LUT (6 µM) for 24 hours. Following this period, the flies were transferred to new experimental vials, and eighteen males were added, resulting in a total of 53 flies per experimental group with a 5:3 female-to-male ratio. The male and female flies were then subdivided into two groups and exposed to either a standard diet or imidacloprid (IMI), for 7 days, to induce the neurodevelopmental disorder model, creating four experimental groups: 1) Control (females pre-exposed to a standard diet + standard diet); 2) IMI (females pre-exposed to a standard diet + imidacloprid 400 µM); 3) NPs LUT (females pre-exposed to NPs LUT 6 µM + standard diet); 4) NPs LUT + IMI (females pre-exposed to NPs LUT 6 µM + imidacloprid 400 µM). The flies were maintained with ad libitum feeding, mating, and egg laying. After the exposure period, the parents were removed, and the experimental vials were preserved for hatching the offspring (F1). The hatched offspring were then used for behavioral and biochemical evaluations. The experimental protocol is illustrated in Figure 1.

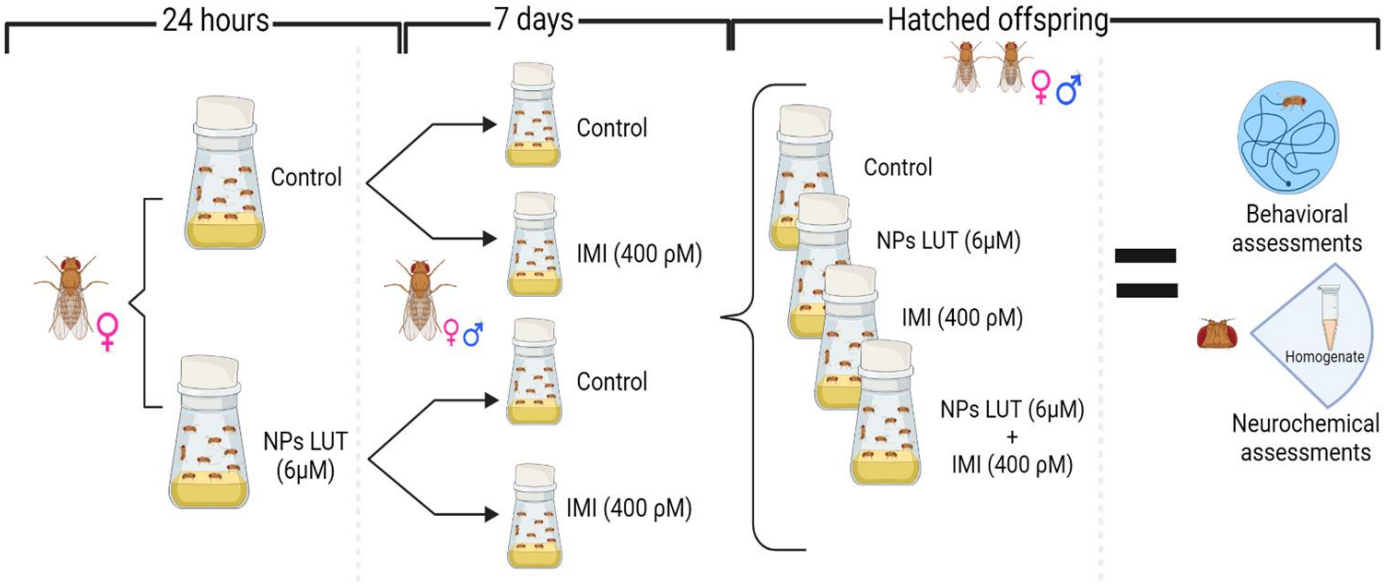

Figure 1: Schematic representation of the experimental design.

2.5. *In vivo* test

2.5.1. Negative geotaxis assay

The negative geotaxis test was performed to evaluate possible behavioral changes similar to hyperactivity in *Drosophila*. Since the use of negative geotaxis is recognized for exhibiting a characteristic hyperactivity present in neurological and neurodevelopmental disorders (Ruhela et al., 2019). The test was carried out with 25 female and male flies from each experimental group, and the time needed to reach 8 cm of the tube was counted (Ternes et al., 2014). The test was repeated five times for each fly, considering a maximum time of 120 seconds and an interval of 1 minute between each repetition. Data were analyzed according to the average time of each fly. Five independent experiments were performed (n = 5) and the results expressed as climbing time (s).

2.5.2. Anxiety-like behavior

The open field test was used to evaluate anxiety-like behavior in *Drosophila* as described by Palacios-Muñoz et al. (2022) with modifications. In a customized circular acrylic arena (8.5 x 8.5 cm), the flies were evaluated individually and their behavior was filmed for 5 minutes. Five flies per experimental group were evaluated and the time spent in the center of the apparatus was recorded individually for each fly. Five independent experiments were performed for males and females (n = 5) and data were expressed as time in center (s).

2.5.3. Repetition behavior

Repetitive behavior was performed as described by Tauber et al. (2011), with adaptations. Five flies of both sexes from each experimental group were evaluated individually in a transparent polycarbonate petri dish (9 mm in diameter). The test consists of observing "self-cleaning movements" such as rubbing the paws on the head, abdomen or placing one paw over the other. The test was performed in duplicate and the data was analyzed according to the average “self-cleaning” time observed over 2 minutes. For this analysis, we performed five independent experiments (n = 5) and data expressed as grooming activity (s).

2.5.4 Social interaction

The social interaction test was carried out according to the methodology of Simon et al. (2012), with adaptations by Janner et al. (2021). The test was performed on female and male flies separately in order to avoid courtship activities that could interfere with the sociability of the flies. Ten flies of the same sex from each experimental group were cryoanesthetized and transferred to the triangular chambers, and after 30 minutes of adaptation, an image was recorded with the aid of a digital camera. Digital images were imported into ImageJ software (NIH, rsbweb.nih.gov/ij) and analyzed for distances (cm) from the nearest neighboring fly. For this test, five independent experiments were carried out for each sex (n = 5) and the data expressed as distance from the nearest neighboring fly (cm).

2.5.5. Aversive phototaxic suppression (aps)

The APS test was performed as described by (Le Bourg; Buecher, 2002; Seugnet et al., 2009) with slight modifications. Flies of both sexes from each experimental group were individually transferred to tubes in the dark and tested for phototaxis (light stimulation) in order to classify them for the experimental test. Flies that did not have phototaxis were discarded from the test. Then the flies were individually placed in the dark tube for one minute to adapt. Afterwards, the flies were transferred to the experimental apparatus, which consisted of a dark tube connected to another transparent tube illuminated by a 150W lamp. A filter paper moistened with 100 µl of 10-1M quinine hydrochloride solution was added to the transparent tube, used as negative reinforcement due to the repellent action of flies. apparatus. Then, the flies were trained nine times, and the time it took the fly to reach the illuminated tube was recorded for 1 minute. Twenty-four hours after training, the flies were placed back in the apparatus to perform the test, in which the aversive compound was not used. To do this, the flies had 10 seconds to choose between the lit or dark side of the platform. The presence of the fly on the dark side at the end of this time demonstrates that the memory was consolidated. The test was performed 5 times per fly. In total, five independent experiments were carried out, totaling fifteen flies per experimental group

for each sex (n= 5). The data were expressed as learning index and approval percentage (%).

2.6. *Ex vivo* assays

2.6.1. Quantification of DA, 5-HT using HPLC-DAD

The determination of dopamine and serotonin levels was performed according to the previous protocol (Bianchini et al., 2019) with modifications. Thirty heads were homogenized in NaCl (0.9%) and 0.5M HCl (96:1) solution, and centrifuged for 1 min at 10,000 rpm at (4°C). After, 200µl of the supernatant was collected and diluted in 800µl of homogenization solution. After dilution, the samples were filtered through 0.22 µm PTFE filters and stored at -80°C until use. DA and 5-HT standards were prepared in the homogenization solution, forming a standard curve with concentrations of 0.1, 0.5, 1, 2.5, 5, 7.5 and 10 mg/L^{-1}. Chromatographic analysis were performed in an Thermo Scientific Dionex UltiMate 3000 Series, equipped with Autosampler Column Compartment ACC-3000, Diode Array Detector - DAD and a GL Sciences HPLC Column Inertsil C8-3 5 µm 4.6 x 150 mm. The absorbance was evaluated in 200 nm, temperature of the column was maintained at 25°C, injection volume was 40 µL, mobile phase flow rate 0.5 mL/min, in a isocratic mode containing metanol and ultrapure water (12:88 v/v). The running time of 6 min was adopted, with acquisition between 2.3 min and 6 min. Results were expressed as µg/mg of protein. Five independent experiments were performed (30 fly heads per group).

2.6.2. Tyrosine hydroxylase activity

Tyrosine hydroxylase (TH) activity was monitored as described by Vermeer et al. (2013), with adaptations from Figueira et al. (2017). The offspring of treated flies were cryoanesthetized and quickly decapitated. Thus, twenty fly heads per group were homogenized in 250 µL of Tris-HCl buffer (0.05 M, pH 7.2) and centrifuged at 13,000 g for 5 min at 4 °C. An aliquot of the supernatant (100 µL) was added to a mixture (100 µL) composed of 100 mM HEPES buffer, 100 µM tyrosine, and 200 µM sodium periodate. The reading was carried out in a spectrophotometer at 475 nm for 1 hour at 25 °C. The results were expressed in nm/min/mg. For this analysis, five independent experiments were carried out (n = 5).

2.7. Statistical analysis

GraphPad Prism 8 software was used for statistical analyses and graphical plotting. Data normality was verified using the Shapiro–Wilk test and homoscedasticity using the Bartlett test. Statistical analysis of the experiments was performed using analysis of variance (two-way ANOVA), followed by Tukey's *post hoc* test for normally and homogeneously distributed data. All data are expressed as mean and standard error of the mean (SEM). Statistical analysis of social interaction behavior was performed by the Scheirer–Ray–Hare extension of the Kruskal–Wallis test (nonparametric two-way ANOVA), and data were expressed as median and interquartile range. Statistical analysis of learning behavior was performed using repeated measures ANOVA (two-way RM ANOVA). Differences between groups were considered significant when $p < 0.05$.

3. Results

3.1. Protective effect of lutein carrier nanoparticles on the negative geotaxis activity in *Drosophila* offspring

Figure 2 (A-B) shows the protective effect of lutein carrier nanoparticles (6 µM) in the offspring of the both sexes flies exposed to IMI (400 ρM) on climbing time. Statistical analysis (two-way ANOVA) revealed a significant effect for the interaction factor (NPs LUT *versus* IMI) in female [$F_{(1,16)} = 17.83$; $P < 0.05$] and male [$F_{(1,16)} = 5.136$; $P < 0.05$] on flies climbing time. *Post hoc* comparisons demonstrated that lutein carrier nanoparticles prevent locomotor damage in geotaxis (Fig 2A and B) in the offspring of flies exposed to IMI (400 ρM) in both sexes.

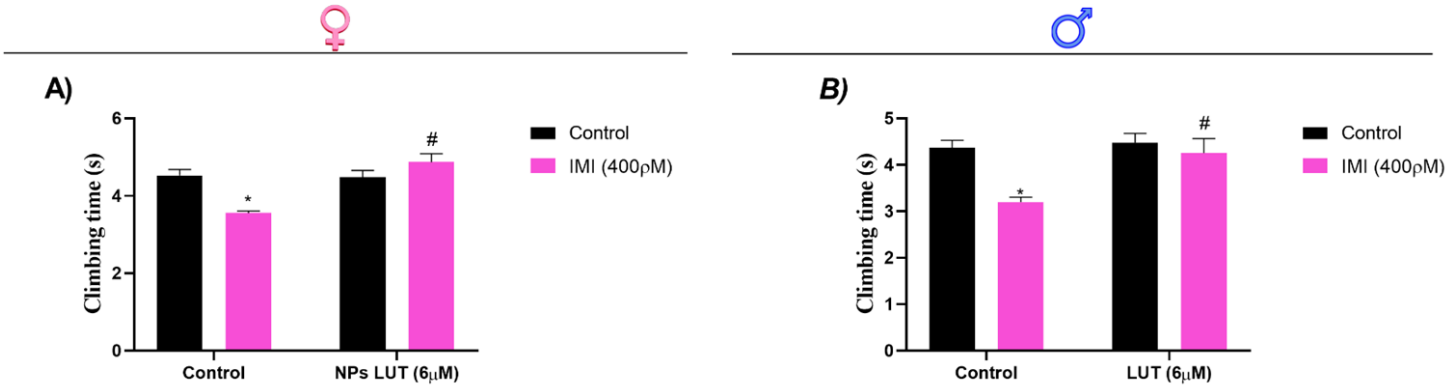

Figure 2: Effect of 24-hour preconception supplementation with NPs LUT on the locomotor activity of female and male *Drosophila melanogaster* offspring exposed to IMI. (A) Negative geotaxis test in females and (B) Negative geotaxis test in males. Data are presented as mean ± SEM, with n = 5 in each group. *Indicates a significant difference ($P < 0.05$) compared with the control group. #Indicates a significant difference ($P < 0.05$) compared with the IMI group.

3.2. Lutein carrier nanoparticles prevent anxiety-like behavior in *Drosophila*

Figure 3 (A-D) shows the protective effect of lutein carrier nanoparticles (6 µM) in the offspring of the both sexes flies exposed to IMI (400 ρM) on anxiety behavior. Statistical analysis (two-way ANOVA) revealed a significant effect for the interaction factor (NPs LUT *versus* IMI) for females [$F_{(1,16)} = 14.09$; $P < 0.05$] and males [$F_{(1,16)} = 9.108$; $P < 0.05$] on the time spent in the center of the apparatus. Regarding the time spent in the periphery (edges of the apparatus), statistical analysis (two-way ANOVA) indicated a significant effect for the interaction factor (NPs LUT *versus* IMI) females [$F_{(1,12)} = 6.620$; $P < 0.05$] and males [$F_{(1,12)} = 12.01$; $P < 0.05$]. *Post hoc* comparisons demonstrated that lutein carrier nanoparticles prevent anxiety-like behaviors in IMI-exposed offspring in both sexes (Fig 3 A-D).

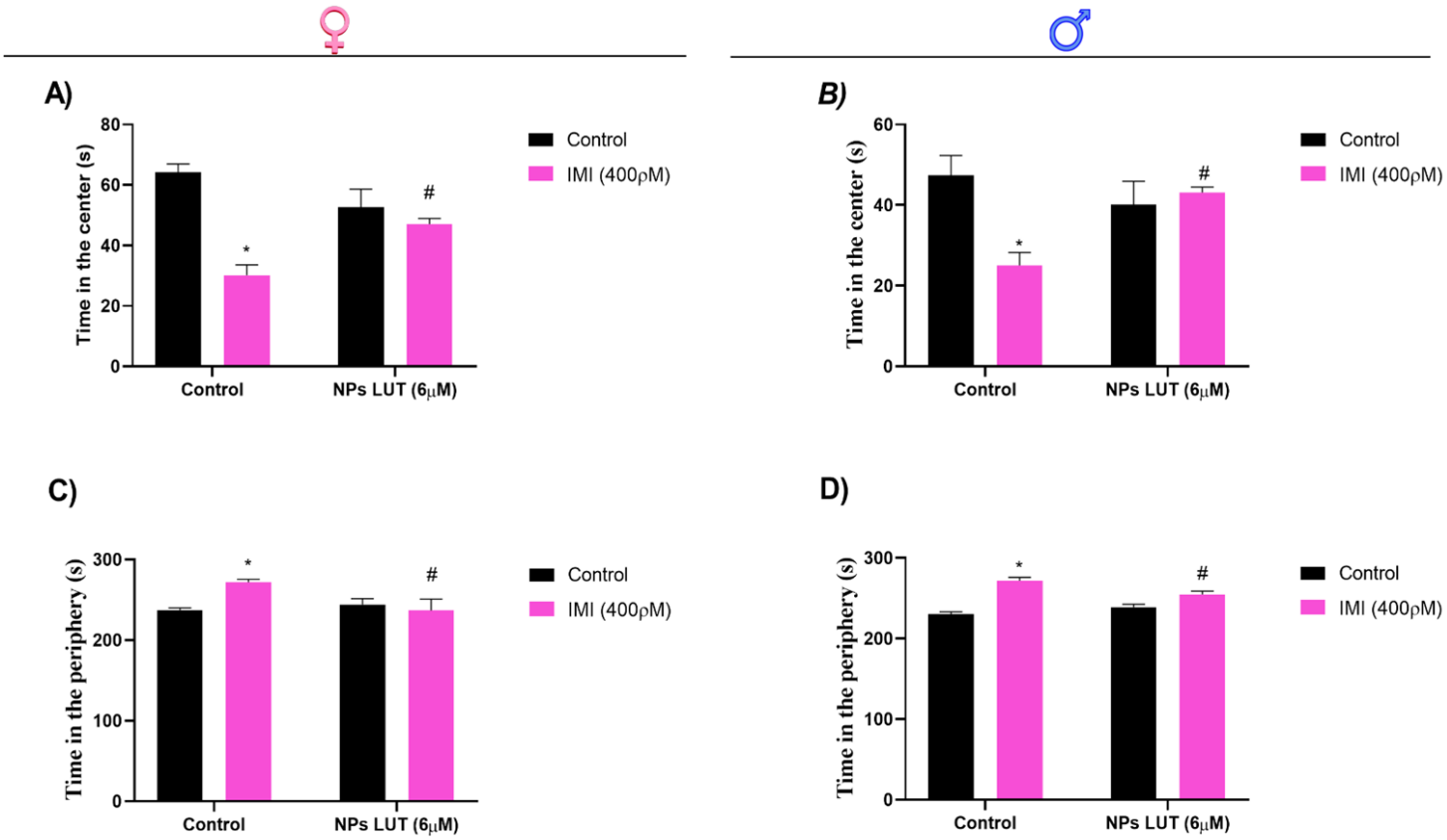

Figure 3: Effect of 24-hour preconception supplementation with NPs LUT on anxiety-like behavior of female and male *Drosophila melanogaster* offspring exposed to IMI. (A) Time in the center of the apparatus - females; (B) Time in the center of the apparatus - males; (C) Time in the periphery of the apparatus - females, and (D) Time in the periphery of the apparatus - males. Data are presented as mean ± SEM, with n = 5 in each group. *Indicates a significant difference ($P < 0.05$) compared with the control group. #Indicates a significant difference ($P < 0.05$) compared with the IMI group.

3.3. Lutein carrier nanoparticles supplementation attenuates damage in social interaction and repetitive movements in *Drosophila* offspring

Figure 4 (A-D) shows the protective effect of lutein carrier nanoparticles (6 µM) on the offspring of flies of both sexes exposed to IMI (400 ρM) on social interaction and grooming behavior. Scheirer-Ray-Hare statistical analysis (two-way nonparametric ANOVA) showed a significant interaction (NPs LUT *versus* IMI) in females [$H_{(1)} = 6.556$; $P < 0.025$] and males [$H_{(1)} = 23.80$; $P < 0.001$], demonstrating

that lutein carrier nanoparticles prevent social interaction deficits (decreasing the distance to the nearest flying neighbor - Fig 4A and B).

In addition, statistical analysis (two-way ANOVA) revealed a significant effect of the interaction factor (NPs LUT *versus* IMI) for females [$F_{(1,16)} = 15.28$; $P < 0.05$] and males [$F_{(1,16)} = 9.782$; $P < 0.05$] in the time of self-grooming movements. *Post hoc* comparisons demonstrated that lutein carrier nanoparticles decreased the time of self-grooming (Fig 4C and D), compared to offspring of flies exposed to IMI (400 ρM) in both sexes.

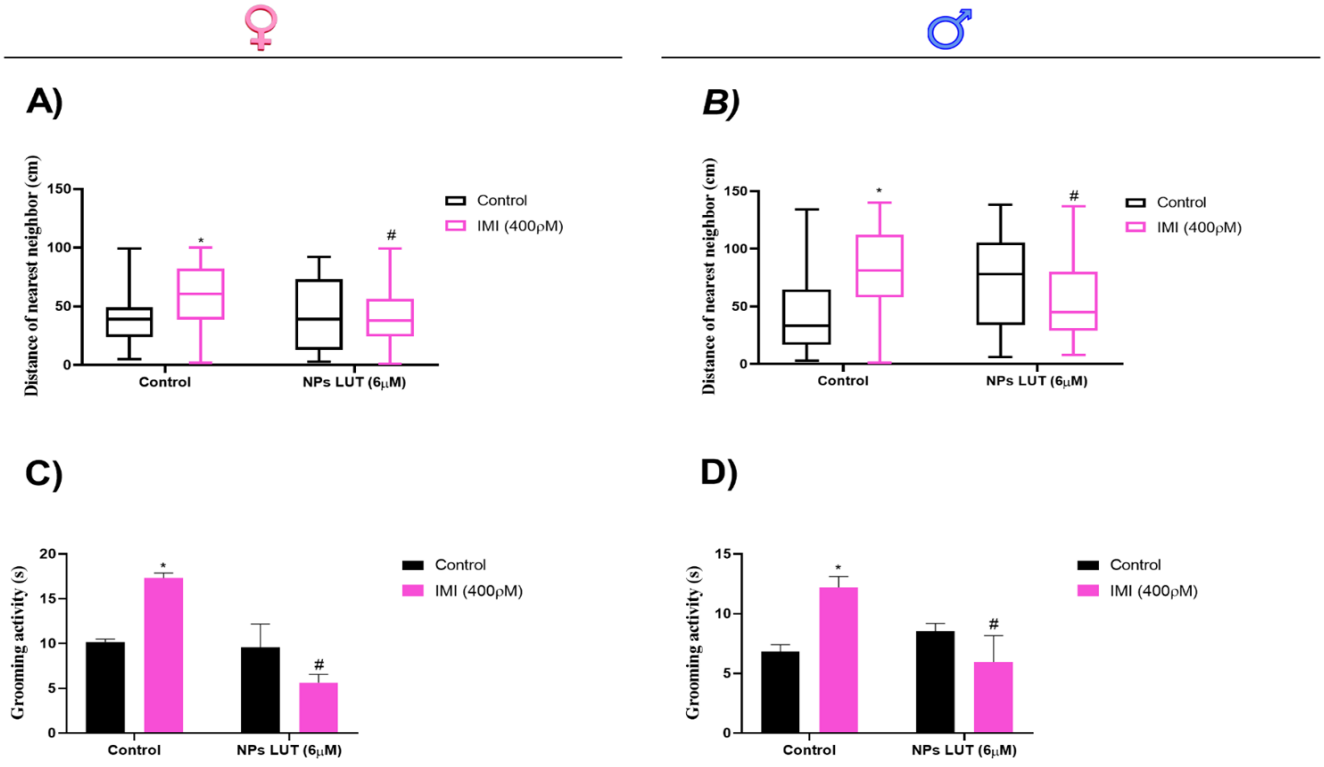

Figure 4: Effect of 24-hour preconception supplementation with NPs LUT on social interaction behavior and repetitive movements in female and male *Drosophila melanogaster* offspring exposed to IMI. (A) Social interaction - females; (B) Social interaction - males; (C) Grooming - females, and (D) Grooming - males. Data are mean ± SEM, social interaction (Scheirer-Ray-Hare) the data are expressed as the median and interquartile interval, for n = 5 in each group. *Indicates a significant difference ($P < 0.05$) compared with the control group. #Indicates a significant difference ($P < 0.05$) compared with the IMI group.

3.4. Lutein carrier nanoparticle supplementation prevents learning and memory impairment in *Drosophila* offspring exposed to IMI.

Figure 5 (A-D) shows the protective effect of lutein carrier nanoparticles (6 µM) in the offspring of the both sexes flies exposed to IMI (400 ρM) on learning and memory parameters. Statistical analysis (two-way ANOVA) revealed a significant effect of the interaction factor (NPs LUT *versus* IMI) for females [$F_{(24,504)} = 2.570$; $P < 0.05$] and males [$F_{(24,504)} = 1.671$; $P < 0.05$] on the learning index, as well as, long-term memory for females [$F_{(1,56)} = 12.99$; $P < 0.05$] and males [$F_{(1,56)} = 8.813$; $P < 0.05$]. *Post hoc* comparisons demonstrated that lutein carrier nanoparticles prevent learning deficits (Fig 5A and B), as well as improve long-term memory (Fig 5C and D), in offspring of both sexes of flies exposed to IMI (400 ρM).

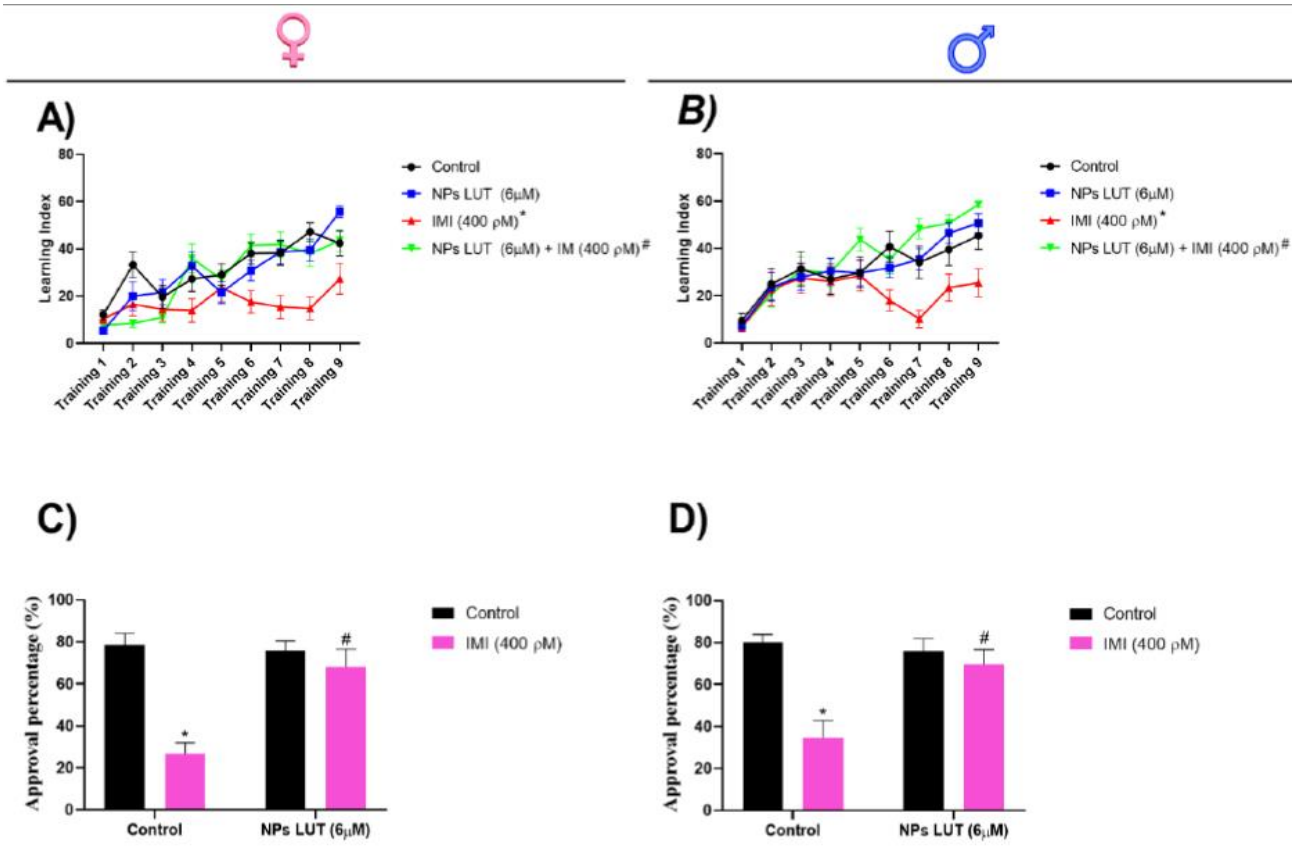

Figure 5: Effect of 24-hour preconception supplementation with NPs LUT on learning and memory in female and male *Drosophila melanogaster* offspring exposed to IMI. (A) Learning - females; (B) Learning - males; (C) Memory - females, and (D) Memory - males. Data are presented as mean ± SEM, with n = 5 in each group. *Indicates a significant difference ($P < 0.05$) compared with the control group. $^{\#}$Indicates a significant difference ($P < 0.05$) compared with the IMI group.

3.5. Lutein carrier nanoparticles supplementation preserves the integrity of DA and 5-HT neurotransmitters in addition to TH enzyme activity in *Drosophila* offspring exposed to IMI.

Figure 6 (A-F) shows the protective effect of lutein carrier nanoparticles (6 µM) in the offspring of the both sexes flies exposed to IMI (400 ρM) on the activity of the enzyme TH (tyrosine hydroxylase) and in the levels of dopamine (DA) and serotonin (5-HT). Statistical analysis (two-way ANOVA) revealed a significant effect of the interaction factor (NPs LUT *versus* IMI) for females and males on TH enzyme activity [$F_{(1,12)} = 59.21$; $P < 0.05$ and $F_{(1,12)} = 27.77$; $P < 0.05$] as well as in the levels of DA [$F_{(1,16)} = 15.18$; $P < 0.05$ and $F_{(1,16)} = 5.329$; $P < 0.05$] and 5-HT neutrotransmitters [$F_{(1,16)} = 7.825$; $P < 0.05$ and $F_{(1,16)} = 22.11$; $P < 0.05$] respectively. *Post hoc* comparisons demonstrated that lutein carrier nanoparticles prevent changes in neurotransmission (Fig 6A-F) in offspring of both sexes of flies exposed to IMI (400 ρM).

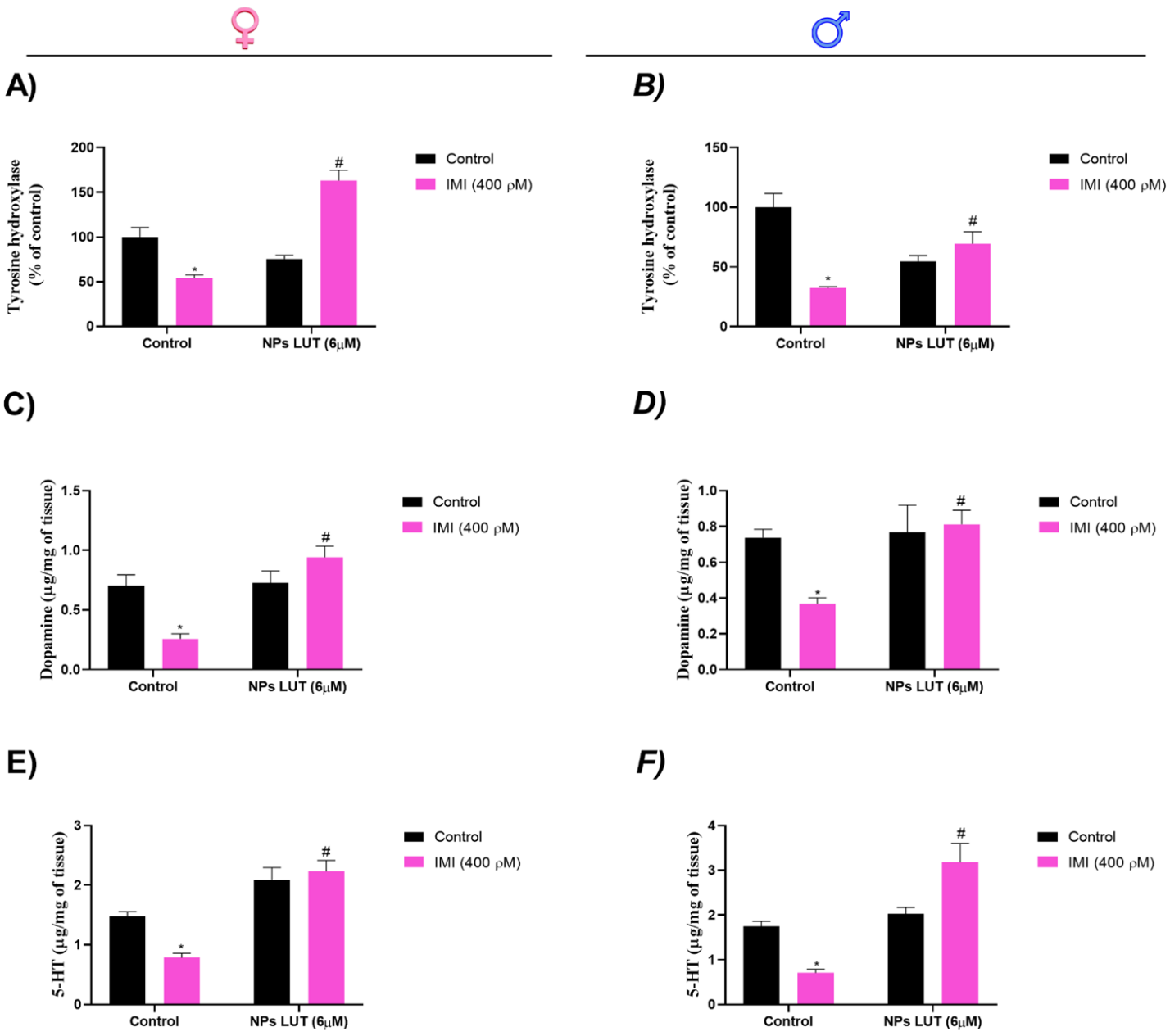

Figure 6: Effect of 24-hour preconception supplementation with NPs LUT on activity of the enzyme tyrosine hydroxylase (TH), and neurotransmitter level in female and male *Drosophila melanogaster* offspring exposed to IMI. (A) Tyrosine hydroxylase - females; (B) Tyrosine hydroxylase - males; (C) Dopamine – females; (D) Dopamine – males; (E) 5-HT – females and (F) 5-HT - males. Data are presented as mean ± SEM, with n = 5 in each group. *Indicates a significant difference ($P < 0.05$) compared with the control group. #Indicates a significant difference ($P < 0.05$) compared with the IMI group.

4. Discussion

In the present study, we investigated the effect of supplementation with lutein carrier nanoparticles during the preconception period on offspring on *Drosophila melanogaster* subjected to the neurodevelopmental disorder model through exposure to IMI.

Our findings indicate that the offspring of flies exposed to IMI exhibit phenotypes similar to the neurodevelopmental disorder. These include increased hyperactivity, anxiety-like behavior, increased repetitive movements, and reduced social interaction and learning, observed in both female and male flies. These results are in line with our previous research, which demonstrated similar behavioral changes in flies directly exposed to IMI, accompanied by a reduction in dopamine levels (Janner et al., 2021) and an increase in oxidative stress (Janner et al., 2024, 2021). Likewise, in the present study, the descendants of IMI-exposed flies also showed significant alterations in the levels of the neurotransmitters DA and 5-HT, as well as in the activity of the TH enzyme.

Imidacloprid, being a partial agonist of nicotinic acetylcholine receptors (nAChRs), exerts a biphasic effect on the binding sites of these receptors, where higher affinity stimulates and lower affinity blocks their action (Pyakurel et al., 2018). Since nAChRs are widely distributed throughout the central nervous system and are involved in behaviors related to neurodevelopmental disorders, imidacloprid's interaction with these receptors may influence such behaviors (Wang et al., 2015; Perry et al., 2001).

Considering DA synthesis, where the TH enzyme catalyzes the conversion of L-tyrosine to L-dihydroxyphenylalanine (L-DOPA), a rate-limiting step in DA production, our data show that TH enzyme activity was reduced in the progeny of flies exposed to IMI, in both females and males. DA and serotonin (5-HT) are crucial neurotransmitters that regulate a wide range of behaviors, including hyperactivity, anxiety, sociability, learning, and memory (DeGroot et al., 2020; Martin et al., 2024; Yang et al., 2023). It was observed that the levels of these monoamines (DA and 5-HT) were dysregulated in both female and male offspring of flies exposed to IMI, compared to the control group. Given that dopamine release is regulated by the stimulation of nAChRs, it is important to emphasize the hypothesis that prolonged exposure to imidacloprid may block the activation of these receptors, as demonstrated in our previous study (Janner et al., 2021). This blockade would lead to a reduction in

dopamine levels, which, in turn, would affect serotonin release. Our data, therefore, support findings in the literature that show reduced levels of DA and 5-HT, as well as decreased activity of the rate-limiting enzyme TH, in both patient studies and animal models. These changes are associated with the development of symptoms similar to those seen in neurodevelopmental disorders (Campbell et al., 2019; Hara et al., 2015; Nguyen et al., 2018; Sahu et al., 2021). Therefore, although most studies have focused on analyzing these neurotransmitters individually, the DA and serotonin 5-HT systems have interconnected targets and functions and can interact in complex ways to influence behavior (Martin et al., 2024). Studies show that DA and 5-HT are involved in general neurotransmission rather than being limited to the specific actions of just one neurotransmitter, thus highlighting the need for integrated approaches to better understand the influence of these neurotransmitters in the regulation of behavior and cognitive functions (Cabana-Domínguez et al., 2022; De Rubeis et al., 2014; Fu et al., 2023; Jones and Raghanti, 2021).

However, supplementation with lutein carrier nanoparticles during the preconception period effectively prevented the onset of behavior-related impairments in the offspring of both sexes. Specifically, we did not observe an increase in hyperactivity in the negative geotaxis test, as well as in anxiety. Similarly, repetitive movements and social interactions in the supplemented offspring were not adversely affected. Additionally, the learning rate in the progeny of supplemented flies improved progressively during training, demonstrating the protective effect of lutein carrier nanoparticles. Furthermore, supplementation with lutein carrier nanoparticles during the preconception period was able to preserve monoamine levels. Therefore, we hypothesize that supplementation with lutein carrier nanoparticles reduces the damage caused by prolonged exposure to imidacloprid through the activation of nAChRs. Given that lutein's antioxidant and neuroprotective properties can shield neurons from synaptic dysfunction induced by the blockade of these receptors, it may help restore dopamine levels, contributing to neurotransmission balance. This, in turn, minimizes the impact on serotonin release, promoting the recovery of neurochemical health. Since 5-HT and DA are directly related to behavior, maintaining the levels of these neurotransmitters may serve as a protective mechanism against neurobehavioral alterations.

Therefore, based on previous studies, we emphasize the critical importance of supplementation with various compounds, as it has been shown to reduce

complications during both the pre- and postnatal periods (Cetin et al., 2010; Noventa et al., 2016; Xing et al., 2022). The World Health Organization (WHO) recommends that women supplement with folic acid daily, starting at least 4 weeks before pregnancy (Mao et al., 2020). In light of this, the use of additional compounds, such as lutein carrier nanoparticles, should be considered, as they may contribute to maternal health during conception, support proper fetal development, and reduce the incidence of gestational and postnatal complications.

In this context, whenever possible, preventing damage is considerably more crucial than reversing damage that has already occurred, as evidenced in a previous study conducted by our group (Janner et al., 2024). Thus, prevention not only reduces immediate risks, but also protects future development and overall integrity by preventing the emergence and intensification of complications, thus providing a more solid basis for growth and evolution. Therefore, it is crucial to consider the timing of intervention, as the effectiveness of supplementation heavily depends on when it is administered. The development of the nervous system is particularly susceptible to external influences at various stages of life (Chang et al., 2021; Gluckman et al., 2007; Koletzko, 2005).

In the present study, we observed that supplementation with lutein carrier nanoparticles during the preconception period plays a protective role against the changes induced by IMI in the neurodevelopmental disorder model. This supplementation effectively prevented the manifestation of behavioral changes and neurochemical damage, such as monoaminergic dysregulation. We therefore believe that preconception supplementation offers a crucial window of opportunity to optimize the intrauterine environment, laying the foundation for a healthy pregnancy and successful infant development.

5. Conclusion

Our findings demonstrate that preconception supplementation with lutein carrier nanoparticles has the ability to prevent phenotypes similar to those observed in the neurodevelopmental disorder model in female and male flies. Specifically, we observed that the nanoparticles were able to prevent changes in tyrosine hydroxylase enzyme activity, as well as in the regulation of dopamine and serotonin levels. Thus, these findings highlight the relevance of preconception supplementation with lutein

carrier nanoparticles as an effective approach to prevent the emergence of symptoms associated with neuropsychiatric disorders, paving the way for future research aimed at investigating the best intervention period to prevent ASD and ADHD-type disorders.

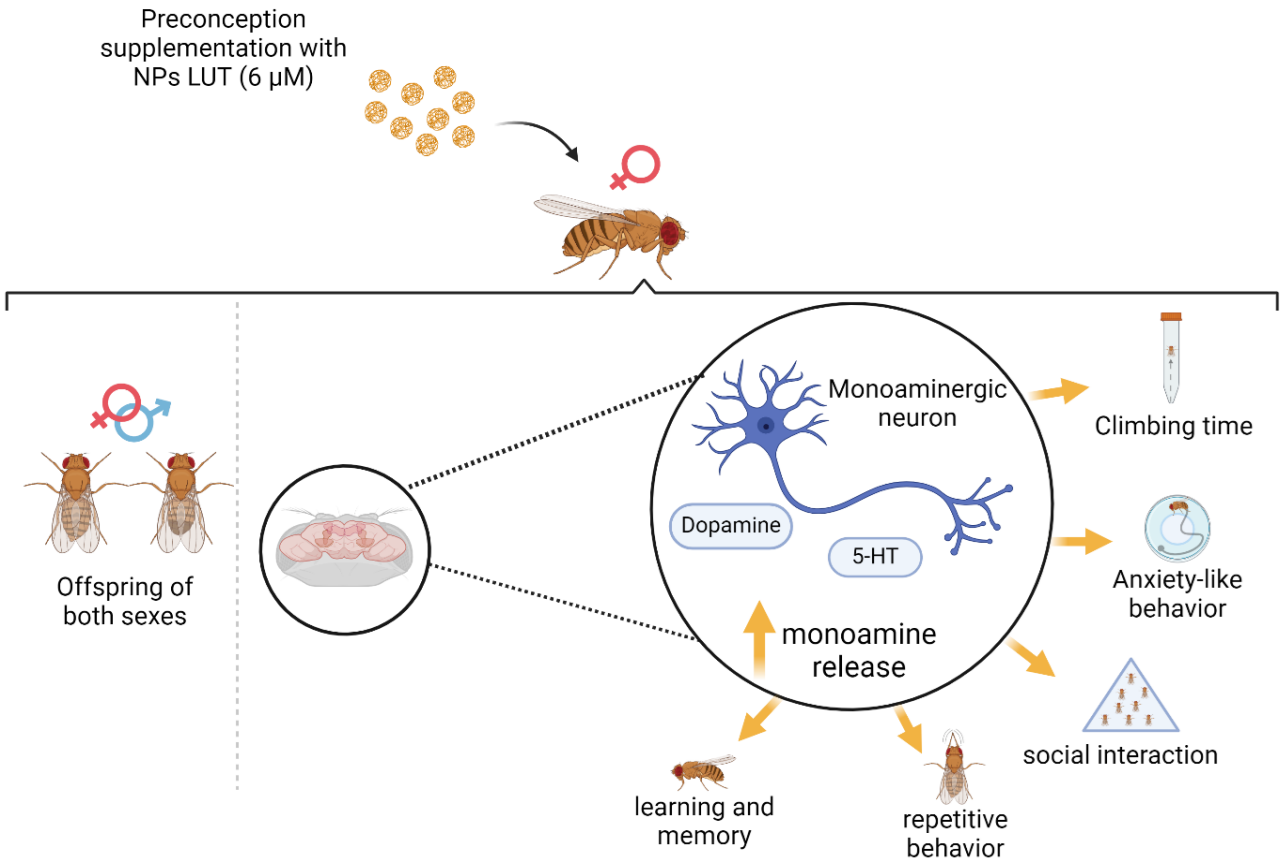

Figure 7: *Graphical abstract*

Conflict of interest:

The authors declare that there are no conflicts of interest.

Data availability statements:

Datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request

Ethics approval:

Not applicable.

Consent to participate:

Not applicable.

Consent for publication:

Not applicable.

Author Contribution Statement:

Dieniffer Espinosa Janner: Conceptualization, Formal analysis, Investigation, Writing - Original Draft, Visualization Frâncelly Marquez de Figueiredo: Formal analysis, Investigation; Andriele de Moura Brinck: Formal analysis, Investigation; Elize Aparecida Santos Musachio: Formal analysis, Investigation; Luana Barreto Meichtry: Formal analysis, Investigation; Eliana Jardim Fernandes: Formal analysis, Investigation; Pamela Piardi de Almeida: Formal analysis, Investigation; Carlos Borges Filho: Formal analysis, Investigation; Magali Kemmerich: Formal analysis, Investigation; Odinei Hess Gonçalves: Resources, Writing - Review & Editing; Fernanda Vitória Leimann: Resources, Review & Editing; Rilton Alves de Freitas: Resources, Review & Editing: Amarilis Santos De Carvalho: Resources, Review & Editing; Marina Prigol: Conceptualization, Resources, Writing - Review & Editing;

Gustavo Petri Guerra: Conceptualization, Formal analysis, Resources, Writing - Original Draft, Supervision, Project administration.

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6. DISCUSSÃO

A presente tese é composta por 2 trabalhos, onde avaliamos os efeitos da administração de nanopartículas carreadoras de luteína na progênie de ambos os sexos submetidas ao modelo experimental de transtorno do neurodesenvolvimento através da exposição a IMI.

Apesar da prevalência dos distúrbios do tipo TEA e TDAH ser maior em indivíduos do sexo masculino (SCHENDEL; THORSTEINSSON, 2018), nosso estudo investigou alterações em ambos os gêneros, visto que, é de extrema importância compreender melhor as alterações comportamentais e neuroquímicas, auxiliando pesquisas voltadas a tratamentos mais específicos para os transtornos do neurodesenvolvimento.

Nossos resultados demonstram que a progênie submetida ao modelo de transtorno do neurodesenvolvimento através da exposição a IMI (400 ρM) apresentou danos comportamentais como: locomoção e exploração elevadas, caracterizando hiperatividade, além de um aumento significativo nos movimentos repetitivos, agressividade e ansiedade, bem como uma redução da interação social. Assim tais avaliações comportamentais na *Drosophila melanogaster* já são bem consolidadas e servem como parâmetros para validar inúmeros modelos experimentais como os disturbios do tipo TEA e TDAH (JANNER et al., 2021; KIM; LEE; PARK, 2017; MUSACHIO et al., 2021).

Dessa forma, baseado no fato de que a imidacloprida atua como um agonista parcial dos receptores nicotínicos de acetilcolina (nAChRs), resultando em um efeito bifásico nos locais de ligação: em que uma afinidade elevada estimula esses receptores, enquanto uma afinidade reduzida os bloqueia. Os nAChRs, que estão amplamente distribuídos no sistema nervoso central, estão envolvidos em comportamentos relacionados ao autismo (Pyakurel et al., 2018; Wang et al., 2015; Perry et al., 2001).

Assim em nossa pesquisa a progênie exposta a IMI apresentou uma redução na imunorreatividade da proteína Shank, a qual é um gene candidato para o desenvolvimento de TEA e TDAH (ANDREW et al., 2021; DELLING; BOECKERS, 2021). Além disso foi possível observar danos oxidativos como redução das enzimas antioxidantes SOD e CAT, aumento dos níveis de ROS e TBARS e redução da

viabilidade celular e da imunorreatividade de Nrf2 nas moscas de ambos os sexos. Nesse contexto, diante dos dados coletados, acreditamos que as mudanças comportamentais observadas nos modelos de transtornos do neurodesenvolvimento estão ligadas a alterações no gene Shank, como já reportado em outros estudos (ANDREW et al., 2021; BUCHER et al., 2021; MOUTIN et al., 2021), e a um consequente aumento do estresse oxidativo, onde no presente estudo essas mudanças foram evidenciadas em ambos os sexos, visando um entendimento mais aprofundado.

Desta forma a administração de nanopartículas carreadoras de luteína durante 24 horas, no periodo pós-natal foi capaz de atenuar os efeitos na progênie de moscas expostas a IMI em todos os marcadores invetigados em nosso estudo. Portanto, as nanopartículas carreadoras de luteína possivelmente atuam protegendo as mitocôndrias, o que por sua vez, reduz o dano oxidativo e regula positivamente o Nrf2, restaurando a atividade das enzimas antioxidantes. Somado a isso, o tratamento com nanopartículas carreadoras de luteína resgatou a imunorreatividade de Shank e, consequentemente, reduziu as mudanças comportamentais observadas na progênie de moscas de ambos os sexos.

Ainda, foi possível observar que a suplementação com nanopartículas carreadoras de luteína durante o periodo pré-concepção foi capaz de prevenir alterações comportamentais promovidas pela indução do modelo de transtorno do neurodesenvolvimento através da exposição a IMI em moscas. Desta forma a progênie de moscas onde as fêmeas foram suplementadas não desenvolveram alterações de comportamento como hiperatividade, ansiedade, movimentos repetitivos e de interação social, também não apresentaram defeitos na aprendizagem e memória avaliados.

Adicionalmente a suplementação com as nanopartículas carreadoras de luteína exerceram papel preventivo diante dos neurotransmissores, uma vez que 5HT e DA estão diretamente relacionados ao comportamento, desta forma preservar os níveis desses neurotransmissores pode ser um possível mecanismo de proteção contra alterações comportamentais. Desta forma, embora a maioria dos estudos se concentre em analisar esses neurotransmissores de forma isolada, os dois sistemas possuem alvos e funções interligados e podem interagir de maneira complexa para influenciar o comportamento, visto que estudos demonstram o envolvimento da DA e 5-HT na transmissão geral e não especificamente em apenas um dos

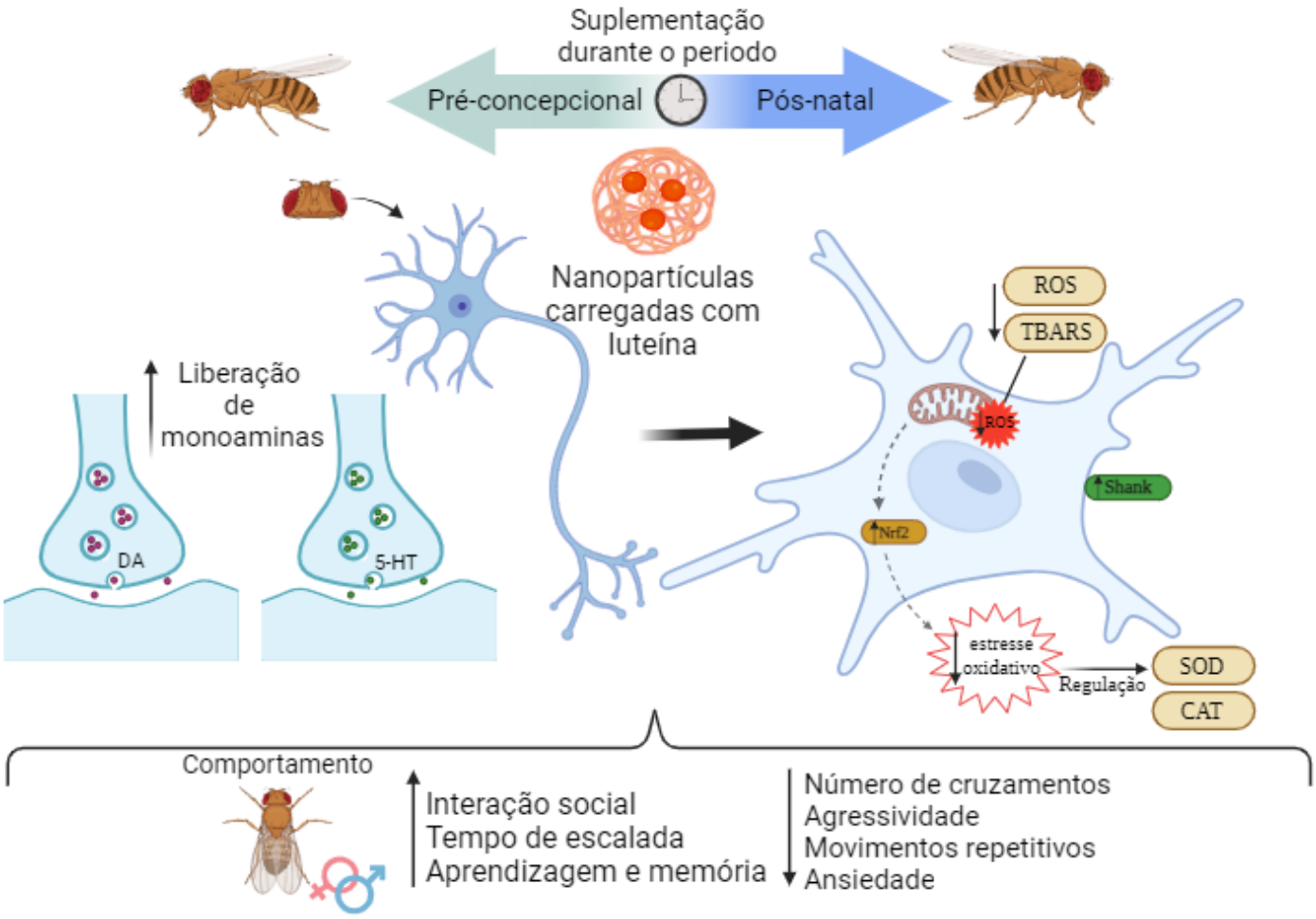
neurotransmissores em questão (CABANA-DOMÍNGUEZ et al., 2022; DE RUBEIS et al., 2014; FU et al., 2023).

Portanto, acretidamos que a suplementação com nanopartículas carreadoras de luteína pode auxiliar na redução dos danos causados pela exposição prolongada à imidacloprida, a qual bloqueia a ativação dos receptores nicotínicos de acetilcolina (nAChRs). A luteína possui propriedades antioxidantes e neuroprotetoras que podem proteger os neurônios contra o estresse oxidativo e a disfunção sináptica induzidos pelo bloqueio dos nAChRs. Ao melhorar a função neuronal e reduzir o estresse oxidativo, a luteína pode contribuir para a restauração dos níveis de dopamina, colaborando para o equilíbrio da neurotransmissão e, assim, minimizando o impacto na liberação de serotonina. Esse efeito restaurador pode ajudar a preservar a saúde cerebral e a função neuroquímica normal, mitigando os danos causados pela exposição à imidacloprida.

Somado a isso, nosso estudo é pioneiro em investigar as alterações em ambos os sexos de forma separada, a fim de elucidar os possíveis mecanismos envolvidos a fim de auxiliar na escolha da melhor opção de intervenção para cada gênero. Assim em nossa pesquisa foi possível verificar que tanto o tratamento pós-natal quanto a suplementação no período pré-concepção com nanopartículas carreadoras de luteína demonstram efeito benéfico mitigando e/ou prevenindo danos comportamentais, aumento de estresse oxidativo (SOD, CAT, ROS, TBARS, Nrf2), redução da viabilidade celular e da proteína Shank, bem como a desregulação a atividade da enzima TH e níveis dos neurotransmissores DA e 5-HT.

Logo acreditamos que a administração de nanopartículas carreadoras de luteína durante o período pré-concepcional ou pós-natal oferece uma janela de oportunidade para investigar o melhor estágio para implementar opções terapêuticas que contribuam para uma gravidez saudável e um desenvolvimento infantil bem-sucedido, assim minimizando a predisposição e/ou fenótipos dos transtornos do neurodesenvolvimento, conforme demonstrado na figura 14 a seguir.

Figura 14: Correlação dos resultados obtidos na tese.



Fonte: Arquivo próprio.

7. CONCLUSÃO

Diante dos resultados apresentados nessa tese conclui-se que:

A administração de nanopartículas carreadoras de luteína exerce efeitos preventivos e restaurador diante das alterações promovidas na progênie de moscas submetidas ao modelo de transtorno do neurodesenvolvimento induzido pela exposição a IMI.

As nanopartículas carreadoras de luteína também foram capazes de reverter totalmente ou parcialmente o dano sobre os marcadores de estresse oxidativo (SOD, CAT, ROS e TBARS e Nrf2), viabilidade celular e imunorreatividade da proteína Shank.

Também a suplementação durante o periodo pré-concepção preveniu a desregulação dos neurotransmissores DA e 5-HT, e a atividade da enzima TH na progênie exposta a imidacloprida.

Em virtude disso nossos resultados sugerem um papel positivo das nanopartículas carreadoras de luteína frente os danos induzidos pela exposição a imidacloprida em *Drosophila melanogaster* de ambos os sexos. Fornecendo um maior entendimento diante das alterações observadas em moscas de ambos os sexos, além disso salientamos a importância de investigar o melhor periodo de vida para realizar intervenções terapêuticas a fim de prevenir ou tratar os fenótipos observados nos distúrbios do neurodesenvolvimento.

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