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CRISTINI ESCOBAR VIANA

NANOCÁPSULAS CARREGADAS DE LUTEÍNA REVERTEM COMPORTAMENTO SEMELHANTE A TRANSTORNO DO ESPECTRO AUTISTA E PROTEGEM CONTRA DANOS NEUROFISIOLÓGICOS E HEPATOTÓXICOS INDUZIDO POR ÁCIDO VALPRÓICO EM RATAS FÊMEAS

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Uruguaiana, RS, Brasil
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Tese apresentada ao Programa de Pós-graduação *Stricto Sensu* em Bioquímica, da Universidade Federal do Pampa, como requisito parcial para obtenção do título Doutora em Bioquímica.

Orientador: Dr. Gustavo Petri Guerra

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RESUMO

O Transtorno do Espectro Autista (TEA) é uma desordem neurodesenvolvimental geralmente diagnosticado na infância e com sintomas que incluem déficits de comunicação e interação social, comportamento restrito e repetitivo. Atualmente, não existe uma total compreensão sobre a patogênese molecular no TEA, desta forma, tem sido proposto um modelo experimental do TEA através da administração pré-natal de ácido valpróico (VPA) em ratos. O potencial tóxico do VPA é caracterizado por desencadear déficits comportamentais semelhante ao TEA, níveis de ansiedade e memória social prejudicadas, anormalidades neuroquímicas, distúrbios morfológicos e toxicidade hepática. Compostos bioativos como a luteína, importante carotenoide, apresentam potencial antioxidante e neuroprotetor, pois atravessa a barreira hematoencefálica, demonstrando efeitos benéficos ao sistema neurológico, metabólico e hepático. As nanocápculas carregadas de luteína proporcionam melhor biodisponibilidade deste carotenoide no organismo, potencializando seus efeitos neuroprotetor e hepatoprotetor. Neste sentido, o objetivo da presente tese foi investigar se as nanocápsulas carregadas com luteína revertem os comportamentos semelhantes ao TEA e protegem contra os danos neurofisiológicos, comportamentais e hepáticos induzido pela administração do VPA em ratas fêmeas. Foram utilizados 30 ratos Wistar adultos (90 - 120 dias de idade), como genitores. As ratas prenhes (geração F0) no 12,5º dia, foram separadas e receberam injeção intraperitoneal de VPA (600 mg / kg) ou solução salina (NaCl 0,9%, 1 mL / kg). No 21º dia, os filhotes do gênero feminino (geração F1) foram subdivididos em dois grupos e receberam nanocápsulas carregadas com luteína (5 mg / kg) ou solução salina (1 mL / kg), por via oral por 14 dias. Ao final do tratamento (15° dia), os animais passaram por testes comportamentais de déficits sociais, memória social, locomoção e ansiedade. Após os testes comportamentais o sangue, hipocampo e fígado foram coletados para análises bioquímicas. Analisamos o efeito das nanocápsulas carregadas de luteína em atenuar os déficits comportamentais, neurofisiológicos e hepatotóxicos em análises in vivo e ex vivo. Desta forma, esta tese envolveu dois principais estudos separados em dois artigos. No primeiro estudo avaliamos déficits sociais e memória social através do teste das três câmaras e atividade locomotora e ansiedade pelo teste de campo aberto. Em ambos os testes, as nanocápsulas carregadas de luteína foram capazes de reverter o déficit sociais, memória social, locomoção e ansiedade induzido

pela administração pré-natal de VPA. Ainda, analisamos por eletroforese os indicadores de estresse oxidativo e biomarcadores de apoptose no hipocampo. Os resultados demonstraram que as nanocápsulas carregadas de luteína restauraram o dano oxidativo (ROS; TBARS; SOD e Nrf2) e os níveis dos biomarcadores apoptóticos (Hsp-70; p38-MAPK; Bax e Bcl-2), os quais estão associados aos efeitos benéficos ao sistema neurológico. Destacamos também, a ação protetora das nanocápsulas carregadas de luteína na modulação dos comportamentos semelhantes ao TEA e nos prejuízos neuroquímicos induzidos por VPA em ratas. Esses resultados sugerem que as nanocápsulas carregadas com luteína possuem potencial terapêutico para o tratamento dano oxidativo vinculado à déficits comportamentais semelhantes ao TEA. No segundo estudo, analisamos o efeito metabólico de nanocápsulas carregadas de luteína nas alterações do metabolismo energético derivadas de modulações da função hepática na prole de ratas expostos ao VPA. Os resultados de estresse oxidativo e marcadores apoptóticos corroboraram com os resultados do primeiro artigo indicando que as nanocápsulas carregadas de luteína foram capazes de proteger contra os danos induzidos pela administração de VPA, tanto no tecido neurológico quanto no tecido hepático. Nas demais análises também foi possível perceber a regulação positiva da viabilidade celular, metabolismo lipídico e marcadores metabólicos no fígado e plasma proporcionados pelo tratamento com nanocápsulas carregadas com luteína. Nossos resultados mostraram que as nanocápsulas carregadas de luteína podem ser um tratamento alternativo natural para proteger contra os danos comportamentais semelhante ao TEA, déficits de memória social e ansiedade, estresse oxidativo, biomarcadores de apoptose, disfunções neuronais e hepáticas causados pela administração de VPA no período pré-natal em ratas fêmeas.

Palavras-chave: carotenoide; antioxidante; estresse oxidativo; comportamento repetitivo e restritivo; distúrbio neurodesenvolvimental; esteatose hepática.

ABSTRACT

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder usually diagnosed in childhood and with symptoms that include communication and social interaction deficits, restricted and repetitive behavior. Currently, there is no complete understanding of the molecular pathogenesis of ASD, therefore, an experimental model of ASD has been proposed through the prenatal administration of valproic acid (VPA) in rats. The toxic potential of VPA is characterized by triggering behavioral deficits similar to ASD, impaired anxiety and social memory levels, neurochemical abnormalities, morphological disturbances and liver toxicity. Bioactive compounds such as lutein, an important carotenoid, have antioxidant and neuroprotective potential, as it crosses the blood-brain barrier, demonstrating beneficial effects on the neurological, metabolic and hepatic systems. Lutein-loaded nanocapsules provide better bioavailability of this carotenoid in the body, enhancing its neuroprotective and hepatoprotective effects. In this sense, the aim of this thesis was to investigate whether lutein-loaded nanocapsules reverse ASD-like behaviors and protect against neurophysiological, behavioral and hepatic damage induced by VPA administration in female rats. Thirty adult Wistar rats (90 - 120 days old) were used as parents. Pregnant rats (F0 generation) on the 12.5th day were separated and received intraperitoneal injection of VPA (600 mg/kg) or saline solution (0.9% NaCl, 1 mL/kg). On the 21st day, female pups (F1 generation) were subdivided into two groups and received nanocapsules loaded with lutein (5 mg/kg) or saline solution (1 mL/kg), orally for 14 days. At the end of treatment (15th day), the animals underwent behavioral tests of social deficits, social memory, locomotion and anxiety. After behavioral tests, blood, hippocampus and liver were collected for biochemical analysis. We analyzed the effect of lutein-loaded nanocapsules on attenuating behavioral, neurophysiological and hepatotoxic deficits in *in vivo* and *ex vivo* analyses. Thus, this thesis involved two main studies separated into two articles. In the first study, we evaluated social deficits and social memory through the three-chamber test and locomotor activity and anxiety through the open field test. In both tests, lutein-loaded nanocapsules were able to reverse the social, memory, locomotion and anxiety deficits induced by prenatal administration of VPA. Furthermore, we analyzed by electrophoresis the indicators of oxidative stress and biomarkers of apoptosis in the hippocampus. Results demonstrated that lutein-loaded nanocapsules restored oxidative damage (ROS;

TBARS; SOD and Nrf2) and levels of apoptotic biomarkers (Hsp-70; p38-MAPK; Bax and Bcl-2), which are associated with the effects beneficial to the neurological system. We also highlight the protective action of lutein-loaded nanocapsules in modulating ASD-like behaviors and neurochemical damage induced by VPA in female rats. These results suggest that nanocapsules loaded with lutein have therapeutic potential for treating oxidative damage linked to behavioral deficits similar to ASD. In the second study, we analyzed the metabolic effect of lutein-loaded nanocapsules on changes in energy metabolism derived from modulations of liver function in the offspring of rats exposed to VPA. The results of oxidative stress and apoptotic markers corroborate the results of the first article, indicating that nanocapsules loaded with lutein were able to protect against damage induced by VPA administration, both in neurological tissue and in hepatic tissue. In the other analyses, it was also possible to perceive the positive regulation of cell viability, lipid metabolism and metabolic markers in the liver and plasma provided by the treatment with nanocapsules loaded with lutein. Our results showed that lutein-loaded nanocapsules can be a natural alternative treatment to protect against ASD-like behavioral damage, social memory and anxiety deficits, oxidative stress, apoptosis biomarkers, neuronal and liver dysfunctions caused by administration of VPA in the prenatal period in female rats.

Keywords: carotenoid; antioxidante; oxidative stress; repetitive and restrictive behavior; neurodevelopmental disorder; hepatic steatosis.

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LISTA DE ABREVIATURAS

AChE – Acetilcolinesterase
BDNF – Fator neurotrófico derivado do cérebro (do inglês *Brain-derived neurotrophic factor*)
CREB – Proteína cíclica de ligação ao elemento AMPc-resposta
CEUA – Comissão de Ética de Uso de Animais
IL-1β – Interleucina-1β
IL-6 – Interleucina-6
IFN-γ – Interferon gama
OMS – Organização Mundial da Saúde
ROS – Espécie reativa de oxigênio
TBARS - Substâncias reativas ao ácido tiobarbitúrico
SOD - superóxido dismutase
CAT - Catalase
Nrf2 - fator nuclear 2 relacionado ao fator E2
Hsp-70 - Proteína de choque térmico 70
MAPK - Proteína quinases ativadas por mitogênio p38
AKT - Proteína cinase especifíca de serina/treonina
Bcl-2 – Linfoma de células B2
Bax – Regulador de apoptose
SNC – Sistema nervoso central
TEA – Transtorno do Espectro Autista
TNF-α – Fator de necrose tumoral-α
VPA – Ácido valpróico
GR – Glutationa redutase
GSH – Glutationa reduzida
GPx – Glutationa peroxidase
GST – Glutathiona S-transferase
PCC – Proteína carbonila
ALT – Alanina aminotransferase
AST – Aspartato aminotransferase
G6Pase – Glicose-6-fosfatase
TAG – Triacilglicerol

SUMÁRIO

APRESENTAÇÃO

A presente Tese foi dividida em três partes principais. Na **parte I** encontram-se a **INTRODUÇÃO**, **REVISÃO BIBLIOGRÁFICA, OBJETIVOS e a JUSTIFICATIVA.** Na **parte II** constam o **ARTIGO CIENTÍFICO** e o **MANUSCRITO**, nestes constam as seções: *Introdução, Materiais e Métodos, Resultados, Discussão, Conclusão e Referências Bibliográticas*, estruturados de acordo com as normas da revista onde foi publicado ou submetido respectivamente.

Estes representam a íntegra deste estudo. Ainda na parte II apresento alguns **RESULTADOS PRELIMINARES** divididos em: *Introdução, Material e Métodos, e os Resultados e Discussão Preliminares e Referências Bibliográficas* de nosso tratamento com nanocápsulas carregadas com luteína. Na **parte III** encontram-se a **DISCUSSÃO GERAL** dos resultados da tese, a **CONCLUSÃO** e as **REFERÊNCIAS BIBLIOGRÁFICAS**, sendo essas últimas referentes somente às citações utilizadas na Introdução e Referências Bibliográficas da tese.

1. INTRODUÇÃO

O Transtorno do Espectro Autista (TEA) é um distúrbio do neurodesenvolvimento caracterizado por prejuízos na interação social, deficiências na comunicação e comportamento, interesses e atividades restritas, repetitivas e estereotipadas (LOMBARDO; LAI; BARON-COHEN, 2019).As proporções de gênero variam conforme os sintomas do TEA, sendo o sexo feminino subdiagnosticado pela escassez de pesquisas e conhecimento acerca de seu fenótipo (ZENER, 2019). Estima-se que a incidência e a prevalência do TEA na população mundial sejam de 1% e no Brasil está estatística se aproxima de 1,5 crianças que expressam este fenótipo, tornando o TEA um desafio individual e social significativo (DE LUCA et al., 2019; HINBEST; CHMILIAR, 2021).

A etiologia do TEA ainda permanece desconhecida, evidências científicas apontam que não há uma causa única para sua incidência, mas sim a interação de fatores genéticos e ambientais (BAI et al., 2019). Os fatores ambientais implicados no TEA compreendem a exposição pré-natal a exposição a toxinas e medicamentos, infecções durante o período gestacional, idade dos pais e possíveis complicações no parto e período neonatal (NICOLINI; FAHNESTOCK, 2018). Dentre os fármacos, Christensen *et al*., (2013) demonstraram que o ácido valpróico (VPA), um medicamento utilizado como estabilizante de humor e anticonvulsivante, possui efeitos colaterais extremos, como prejuízos no desenvolvimento neuronal dos fetos.

O VPA tem sido utilizado para indução em modelos animais do TEA quando administrado intraperitonaelmente em período pré-natal (12,5° dia embrionário) em ratas prenhes, período próximo ao fechamento do tubo neural e formação de núcleos do tronco cerebral (KERR et al., 2013). Os modelos animais de TEA induzido por VPA apresentam sensibilidade à dor reduzida, alterações comportamentais na atividade exploratória e interação social, bem como demonstram sinais tipo ansiedade e depressão (GAO et al., 2019; KONG et al., 2021). Além disso, associada as anormalidades comportamentais os biomarcadores oxidativos, inflamatórios e apoptóticos também se mostram alterados em modelo animal com TEA (HEGAZY; ALI; ELGOLY, 2015; SAGHAZADEH et al., 2019a). Ademais, o VPA é reconhecido

como potencial indutor de teratogenicidade ou toxicidade hepática, e seus mecanismos subjacentes ainda precisam ser melhor compreendidos (SARGAZI et al., 2021). Estudos emergentes demostraram que o VPA desencadeia disfunção mitocondrial, afeta a β-oxidação, aumenta concentração de subprodutos da peroxidação lipídica, diminui níveis de enzimas antioxidantes induzindo distúrbios metabólicos e desencadeando efeitos hepatotóxicos (AHANGAR et al., 2017; GUO et al., 2019; ZHOU et al., 2020; GHEENA et al., 2022).

Sabendo que a administração do VPA apresenta uma heterogeneidade de sintomas, uma alternativa viável para minimizar os efeitos adversos deste fármaco é a utilização de compostos bioativos como os carotenoides, pois expressam propriedades antioxidante, anti-inflamatório, antitumoral, antiapoptótica, além de outras bioatividades, refletindo em resultados positivos em estudos com déficit de memória, doenças neurodegenerativas, síndromes metabólicas e hepatotoxicidade (PANGRAZZI; BALASCO; BOZZI, 2020; PEREIRA et al., 2021). Dentre estes, a luteína é o segundo carotenoide com maior incidência no plasma humano, possui atividade antioxidante, neuroprotetora e anti-inflamatória (SASAKI et al., 2010). Além disso, a luteína possui a capacidade de atravessar a barreira hematoencefálica, apresentando efeito neuroprotetor contra lesões isquêmicas, reduz a apoptose induzida pelo estresse oxidativo e atua nos déficits cognitivos (SUN et al., 2014). Em seu trabalho Syamila *et al*., (2019) aponta que a luteína apresenta uma baixa solubilidade em água fator limitante para sua absorção, diminuindo seu potencial biológico e terapêutico, sendo assim, a utilização de luteína nanoencapsulada se torna uma alternativa promissora para sua administração.

Segundo Arunkumar *et al*., (2015) ao utilizarem nanocápsulas poliméricas carregadas de luteína, estes observaram que houve uma maior biodisponibilidade desta, demonstrando 5,4 vezes mais concentração de luteína no plasma de roedores do que quando administrada em sua forma livre, além de proporcionar menor degradação e melhorar sua estabilidade sendo promissora para o tratamento de doenças como o TEA. Nossa hipótese é que o tratamento com nanocápsulas carregadas de luteína contribua como alvo terapêutico no desenvolvimento de fármacos capazes de mitigar o efeito tóxico do VPA e prevenir a evolução natural de TEA. Sendo assim, nosso trabalho tem como objetivo investigar o possível efeito

protetor das nanocápsulas carregadas de luteína sobre os déficits comportamentais, bioquímicos, neuroquímicos e hepatotóxicos induzidos pela exposição pré-natal ao VPA em ratas fêmeas.

2. REVISÃO BIBLIOGRÁFICA

2.1 Transtornos Neurodesenvolvimentais

Os transtornos neurodesenvolvimentais foram citados pela primeira vez na quinta edição do Manual Estatístico e Diagnóstico dos Transtornos Mentais (DSM-5), em 2013, e alavancou pesquisas sobre etiopatogênese vinculada ao desenvolvimento neuronal, estruturando o projeto Rdoc (Research Domain Criteria) de âmbito psiquiátrico, que descreve transtornos mentais ligado ao sistema biológico (KLEIN; LIMA, 2020). Os transtornos neurodesenvolvimentais se configuram por fatores genéticos, biológicos, epigenéticos e psicológicos de um indivíduo e se caracterizam por condições neurológicas que aparecem prematuramente na infância, derivadas da insuficiência de neurotransmissores atuantes no sistema nervoso central (SNC) (STARR, 2019). Tal deficiência desencadeia déficits cognitivos complexos no organismo, envolvendo dificuldades de aquisição, manutenção ou aplicação de habilidades ou conjuntos de informações específicas ao sistema nervoso (HERNÁNDEZ et al., 2016).

Da mesma forma como estes distúrbios elucidam variados prognósticos, igualmente evidência a ampla gama de problemas neurológicos e psiquiátricos agrupados referencialmente pela quinta edição do Manual Diagnóstico e Estatístico de Transtornos Mentais (DSM-5) como: transtorno de déficit de atenção e hiperatividade (TDAH), transtorno do espectro autista (TEA), deficiência intelectual (ID), distúrbio do desenvolvimento intelectual (DDI), distúrbios da comunicação, distúrbios específicos de aprendizagem e distúrbios motores (de coordenação mental e distúrbios de tiques) (HARRIS, 2014; THAPAR ANITA, 2020).

O TDAH e o TEA são os distúrbios diagnosticados com maior incidência em crianças com idade escolar (SCANDURRA et al., 2019). O TEA possui do total de casos existentes na América do Norte 1 a cada 59 crianças (SPAIN et al., 2018) que exibem prejuízos qualitativos na comunicação e interação social, padrões estereotipados e repetitivos e desenvolvimento intelectual irregular (HULL; PETRIDES; MANDY, 2020).

Ademais, verificando estes aspectos o atual DSM-5 propôs um modelo híbrido de transtornos de personalidade incluindo uma das mudanças mais relevantes que é a possibilidade de um diagnóstico comórbido de TEA (HARRIS, 2014). Os índices e características pesquisados demonstram que os distúrbios se articulam com alta regularidade, pois 30 a 80% das crianças com TEA respondem positivamente aos mesmos critérios de análise psíquica que o TDAH, principalmente na pré-adolescência e na adolescência (SCANDURRA et al., 2019).

Portanto, uma análise conjugada desses distúrbios pode trazer opiniões ambíguas e opções terapêuticas variadas, promovendo um tratamento eficaz, porém arrisca-se exceder a intensidade do uso de drogas, além de potencializar os custos econômicos e sociais envolvidos (DESQUENNE; CAPPE, 2020). Além disso, o gerenciamento de medicamentos de outras condições psiquiátricas pode agravar a saúde mental e auxiliar no desenvolvimento de diferentes enfermidades (ROSENBERG et al., 2010).

2.2 Transtorno do Espectro Autista (TEA)

O termo “autismo” teve origem nos postulados do psiquiatra suíço Paul Eugen Bleuler em 1911, quando descreveu propriedades análogas à esquizofrenia (KANISKOV; ILIEV, 2020). Porém, a evolução do conceito dentro da psique passou por diversas transformações, sendo relacionada aos estudos epidemiológicos desde 1960 (EVANS, 2013). Em meados de 1994 o diagnóstico do autismo se baseava no DSM-4, contudo suas características ainda estavam muito agregadas a outros distúrbios psiconeuronais (WING; GOULD; GILLBERG, 2011). Já no DSM-5, 2013 a especificação do autismo se mostrou mais clara utilizando modificadores clínicos únicos incorporados à classe do TEA, como uma escala de traços artísticos (tabela 1) que se baseia no comportamento do indivíduo, no trato social, ambiental e intelectual (SUNG et al., 2018).

Tabela 1 – Os três níveis funcionais do autismo.

Os três níveis funcionais do autismo		
Nível 1 **Necessidade de pouco apoio**	**Nível 2** **Necessidade de apoio substancial**	**Nível 3** **Necessidade de apoio muito substancial**
- Dificuldade em iniciar a interação social. - Problemas de organização e planejamento podem dificultar a independência**.**	- Interação social limitada a interesses especiais estreitos. - Comportamento restritivo e repetitivo.	- Déficits graves em habilidades de comunicação social verbal e não verbal. - Grande angústia e dificuldade em mudar ações ou foco.

Fonte: Arquivo próprio (2023).

Atualmente a expressão do autismo pela Organização Mundial de Saúde (OMS) relaciona o TEA com sintomas de epilepsia, depressão, ansiedade e o TDAH (KUMMER et al., 2016), juntamente classificado em níveis de comprometimento intelectual categorizados em graus leve, moderado ou severo (GILLESPIE-SMITH et al., 2018). O quadro clínico do paciente com TEA que engloba aspectos neurológicos, comportamentais e genéticos torna imprescindível à identificação e intervenção precoce desse distúrbio (MEYZA; BLANCHARD, 2017).

O TEA abrange diferentes condições para descrever indivíduos com distúrbios neurodesenvolvimentais específicos, caracterizados por prejuízos sociocomunicativos, inflexibilidade cognitiva, comportamentos repetitivos e estereotipados, ansiedade, expressando um conjunto reduzido de interesses e atividades (LORD et al., 2020). Também demonstram variações no processamento sensorial, cognitivo e distúrbios na fala (BOURGERON, 2015). Estas características heterogêneas se expressam muito precocemente e comumente se estende por toda a vida do indivíduo (FERRI; ABEL; BRODKIN, 2018).

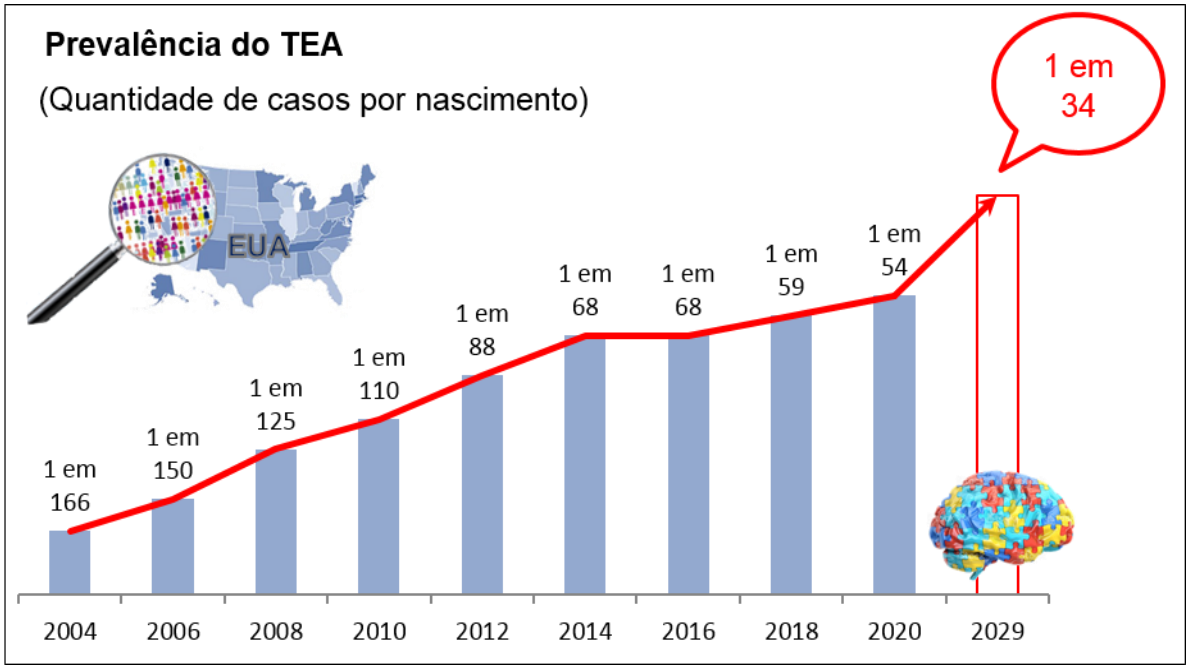
Em relação as manifestações clínicas do TEA, ocorrem alterações encefálicas nas porções do cerebelo, amígdala, hipocampo, entre outras estruturas, desencadeando anormalidades funcionais, atraso do desenvolvimento e depressão

de células Purkinje em crianças (PETER et al., 2016). Na síndrome comportamental a habilidade de elaboração interacional se manifesta de forma prejudicada, ou seja, a construção social através do diálogo muitas vezes não ocorre, havendo dificuldade qualitativa de comunicação e interação social (SUPEKAR et al., 2018). Relata-se também o comprometimento do tônus muscular vinculado à coordenação motora fina, envolvida em tarefas como manipular os talheres, a escova dental, o lápis, brinquedos, aparelho celular, dentre outras variadas funções minuciosas do cotidiano (SERDAREVIC et al., 2017).

O TEA se manifesta desde a infância e as faixas etárias de maior prevalência em diagnose são entre 5 a 8 anos de idade que correspondem às estatísticas no Brasil, dado que reforça os encontrados também nos Estados Unidos da América (EUA) (DE LIMA REIS et al., 2019). Vale destacar que a precocidade do diagnóstico é fator significativo, pois o cérebro está em fase de intensas construções neurológicas, plasticidade cerebral e amplas conexões sinápticas as quais influenciam no desenvolvimento cognitivo e sensorial da criança (ZWAIGENBAUM et al., 2015). Estima-se que 1 a cada 160 crianças possuam as características do TEA e que a maioria dos casos diagnosticados sejam em crianças do sexo masculino com razões variando de 4:1, porém na gravidade composta como geral do transtorno, não se encontra diferenças entre os gêneros (CRANE et al., 2018), conforme vários estudos que avaliaram o padrão do transtorno (CARTER et al., 2007; DICKERSON MAYES; CALHOUN, 2011; LAI et al., 2011; LAI et al., 2012).

No Brasil os dados estatísticos sobre TEA ainda não são oficiais, porém supõem-se que há aproximadamente 2 milhões de indivíduos que manifestam os sintomas deste transtorno, este número se baseia no cálculo de 200 milhões de brasileiros (FADDA; CURY, 2016). Entre os anos de 2014 a 2016 o predomínio pressuposto de TEA foi de 2,47% entre crianças e adolescentes dos EUA (CHRISTENSEN et al., 2018). Em suma, estima-se em média 3 milhões de novos casos de TEA em 2029, considerando a estatística entre 1990 – 2019 com acréscimo de 2 milhões no cenário atual nos Estados Unidos, assim como demonstra o gráfico abaixo (Fig. 1) (CHRISTENSEN et al., 2018).

Figura 1 – Ilustração da prevalência e estimativa do TEA nos EUA.



Fonte: Arquivo próprio (2020).

2.3 Fisiopatologia do TEA

Desde a década de 1940 os cientistas são os precursores na compreensão do TEA, pois seus distúrbios são incompreendidos e muitas vezes miscigenados a outras comorbidades, expressando disfunções neurológicas que desencadeiam déficits por todo o organismo (RUGGIERI; ARBERAS, 2018). Suas características morfológicas e fisiológicas são extremamente complexas e a ausência de um esclarecimento sobre suas causas, sintomas e possíveis tratamentos transforma este transtorno em um enigma clínico (FADDA; CURY, 2016). O TEA por ter etiologia multifatorial levanta várias hipóteses acerca de suas causas, que vão de aspectos biológicos, genéticos, relacional, imunológicos, neuroquímicos até as alterações ambientais (ZUCKER et al., 2017).

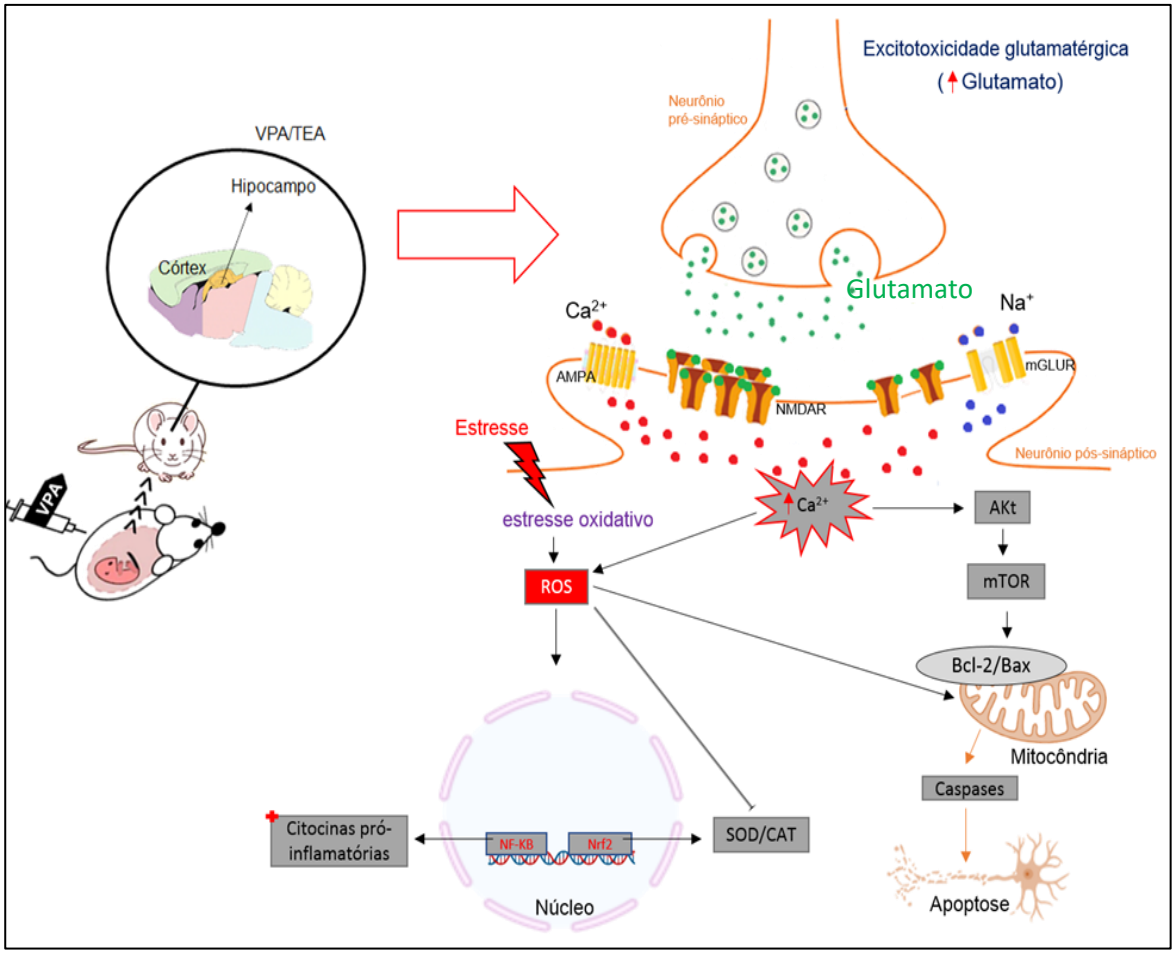
Diante destas hipóteses sobre as causas do TEA o paradigma biológico se refere à inabilidade inata do paciente em estabelecer relações interpessoais com outrem, além disso, ressaltam-se causas neurobiológicas com irregularidade na anatomia e

fisiologia no SNC (NASCIMENTO et al., 2018). No que concerne os aspectos psicanalíticos que analisam a afinidade relacional mamãe/bebê se discute duas possíveis lacunas para possível causa do TEA, como a ausência ou o excesso da função materna, influenciando diretamente na construção da criança como sujeito (GONÇALVES et al., 2017).

As anormalidades genéticas estão fortemente vinculadas ao autismo, sendo responsável por aproximadamente 50% do total de casos, onde estima-se que acometem 90% em gêmeos monozigóticos e 10% em gêmeos dizigóticos (WON; MAH; KIM, 2013). Os genes associados ao TEA, podem ser regulados pelas atividades neuronais, levando as pesquisas para a investigação da plasticidade sináptica e suas conexões (HANSEL, 2019), assim como por mutações espontâneas e aleatórias dos genes que alteram a conectividade neuronal possibilitando dificuldade na percepção de estímulos externos e sensoriais (BOURGERON, 2015). Estudo descreve desregulação de mais de 637 genes no córtex pré-frontal de roedores com administração de VPA, afetando a expressão e o comportamento do tipo autista de uma maneira ampla do genoma (ZHANG et al., 2018).

De mesmo modo, foi realizada pesquisa com verificação de imagens cerebrais onde percebe-se irregularidades díspares no cerebelo, amígdala, hipocampo, sistema límbico, entre outras estruturas cerebrais em uma amostra animal com características de TEA post-mortem e *in vitro* (FREITAS et al., 2014). Em estudo de Ozonoff *et al*. (2011) foi apontado haver um risco maior de TEA para a criança que tem irmão mais velho diagnosticado com esta patologia, na qual as investigações passadas sugeriram um risco de recorrência da doença em percentuais entre 3% e 18,7%. Distúrbios bioquímicos (Fig. 2), disfunção mitocondrial, estresse oxidativo, ativação inadequada de citocinas pró-inflamatórias e neuroinflamação são fisiopatologias do SNC associados às influências ambientais (YANG; LIU; XU, 2016). Estas respostas imunes disfuncionais afetam os processos sinápticos causando prejuízos na interação social e comunicação, perda ou ganho de apetite e alterações metabólicas (FERREIRA; FRANZOI, 2019).

Figura 2 – Ilustração do dano neurológico em modelo de TEA por VPA.



Fonte: Arquivo próprio (2021).

O dano neurológico induzido pela administração do VPA no período pré-natal, faz com que ocorra maior liberação de glutamato na fenda sináptica, o qual desencadeia aumento do influxo de cálcio no neurônio pós-sináptico. O excesso de cálcio está interligado ao estresse oxidativo (aumento de ROS), no qual ativa as vias moleculares de ativação nuclear, como o Nrf2 que desencadeia uma resposta antioxidante negativa, e o NFKB, que está ligado a liberação das citocinas pró-inflamatórias. Além disso, o aumento de cálcio nos neurônios incide sobre a via AKT/mTOR e na desregulação das proteínas pró e anti-apoptóticas, podendo causar a ação das caspases, o que leva a apoptose celular.

Corroborando, a literatura demonstra que os perfis anormais de citocinas são análogos também à depressão, transtorno bipolar e esquizofrenia (MASI et al., 2017). Para a estimulação das citocinas pro-inflamatórias (IL1β, IL6, TNF-α), proteínas agem

estimulando o processo inflamatório, sendo que estas atuam ativando o fator nuclear de transcrição NF-kβ que está envolvido diretamente com a plasticidade sináptica, desenvolvimento e neurodegeneração em neurônios e células da glia (SPISNI et al., 2020). Além disso, expressam também alterações moleculares, incluindo o funcionamento desordenado dos sistemas serotoninérgicos, dopaminérgicos, GABAérgicos e glutamatérgicos associados à interação VPA/TEA (TSUJINO et al., 2007; FUKUCHI et al., 2009; SILVESTRIN et al., 2013; GOTTFRIED et al., 2015).

Segundo Saghazadeh *et al*. (2019b) as citocinas pró-inflamatórias como a IL-6 e as anti-inflamatórios como IL-10, além de subconjunto de quimiocinas demonstraram-se elevados no Giro cingulado anterior (em inglês ACG) que é uma importante região cortical relacionada a disfunções na atividade cerebral no TEA. Estudo ressalta que comumente um número elevado de crianças com TEA já apresentaram níveis reduzidos de GSH junto a menor proporção redox de GSH/GSSG (CHAUHAN; AUDHYA; CHAUHAN, 2012). Estudo avaliou mutações no gene SHANK3, foi constatado que a mutação de uma única cópia da proteína SHANK3 (proteína sináptica) tem relação com modificações na comunicação social e comprometimento da linguagem em indivíduos com TEA (UCHINO; WAGA, 2013).

Dentre os aspectos neuroanatômicos do TEA, estudo aponta anormalidades no cerebelo apresentando um menor número de células de Purkinje e também uma maior ativação microglial e reações astrogliais em ambas camadas de células granulares e de matéria branca (PETER et al., 2016). Enquanto das modificações neuropatológicas mais relevantes em relação ao TEA está a presença de anormalidades citoarquitetônicas de regiões como o córtex cerebral, cerebelo e outros estruturas subcorticais (PALMEN et al., 2004).

O estresse oxidativo vinculado ao cérebro de indivíduos com TEA, demonstra a presença de inflamação crônica, a qual possivelmente é mediada através da ativação microglial inata e pela presença de citocinas pró-inflamatória, assim como a diminuição das concentrações de glutationa (GSH) exacerbam a resposta inflamatória, este ciclo afeta a região gastrointestinal e o SNC de indivíduos com o TEA (PARDO; VARGAS; ZIMMERMAN, 2009). Além de destaque para as alterações nos níveis de secreção de citocinas inflamatórias, aumento do estresse oxidativo,

neuroinflamação, outras vias ligadas à memória como CREB, BDNF e a desregulação da sinalização de IGF-I / PI3K / AKT / mTOR também estão envolvidas (OHJA et al., 2018).

Além do cenário neuroinflamatório descrito acima, podem ser destacadas outras vias ligadas à memória como o BDNF (do inglês-*Brain-Derived Neurotrophic Factor*) tem sua função variando de acordo com o estágio de desenvolvimento cerebral e dos constituintes neuronais, gliais ou vasculares desse tecido (SKAPER et al., 2018). É um marcador presente em quase toda a região cerebral e sua atuação mais relevante está em processos de desenvolvimento, sinaptogênese, neuroproteção e influência nas interações sinápticas de curta e longa duração que refletem nos mecanismos cognitivos e de memória (D'ALÒ et al., 2021).

Em relação ao período neonatal e o TEA, os níveis reduzidos de fatores neurotróficos, que são fatores que suportam o crescimento, sobrevivência, diferenciação de neurônios em desenvolvimento e maduros constituem-se como um fator de risco ambiental nesse período (ABDALLAH et al., 2013). Acrescentando, foi demonstrado em modelo de camundongos com infecção materna (risco ambiental pré-natal para autismo) a presença tanto de déficits na interação social e na linguagem, quanto a presença de padrões restritos e comportamentais estereotipados (MALKOVA et al., 2012). Desta forma, as pesquisas dentro da bioquímica e as análises metabólicas e de neuroimagem, como exemplo a ocorrência de alterações na neurotransmissão dopaminérgica e serotoninérgica, comprovam um diagnóstico mais preciso em indivíduos com TEA (WANG et al., 2020).

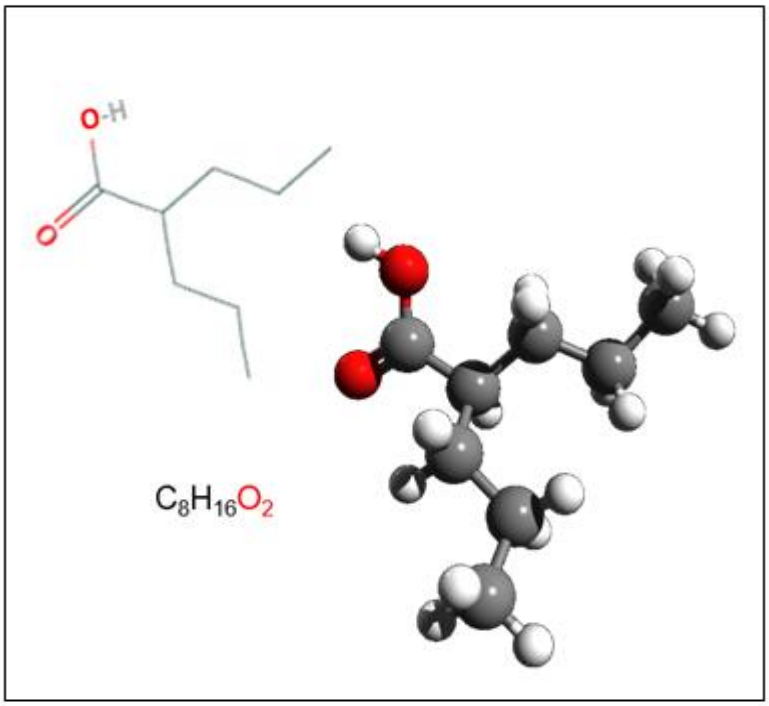
Os fatores de risco ambientais são eventos que ocorrem aos parentais que podem afetar o ciclo da gestação e de certa forma caracterizar distúrbios neurodesenvolvimentais como o TEA, iniciando prematuramente e se prolongando por toda a vida do indivíduo (WALKER et al., 2015). Os agentes ambientais podem ser divididos em grupos para facilitar o entendimento, por exemplo, os patógenos podem advir de doença contagiosa como a rubéola, também podem ser vinculados aos sintomas deste transtorno os chamados agentes associativos, que englobam a idade avançada, problemas de diabetes e hipertensão (XU et al., 2014), e igualmente danosos como os agentes químicos que advêm da poluição atmosférica e o uso ou

contato com substâncias químicas como medicamentos e seus compostos, tal qual o ácido valpróico (SCHLICKMANN; FORTUNATO, 2013). Existem evidências de que os agentes ambientais neurotóxicos atuam tanto na evolução do TEA quanto na genética em exposição precoce, pois a vulnerabilidade do sistema neuronal às exposições tóxicas de metais pesados está fortemente conectada ao desenvolvimento pré-natal (CARTER; BLIZARD, 2016).

2.4 Modelo de TEA e Toxicidade do VPA

O VPA ou valproato de sódio 2-proprilpentanoico (Fig. 3) é um ácido carboxílico de cadeia ramificada com uma estrutura química similar aos ácidos graxos de cadeia curta (NICOLINI; FAHNESTOCK, 2018).

Figura 3 – Estrutura química do ácido valpróico.



Fonte: Arquivo próprio (2020).

Se apresenta como medicamento da classe dos anticonvulsionantes indicado para crises convulsivas e epiléticas também utilizadas no tratamento de transtornos de humor, como transtorno bipolar e ciclotimia (ROULLET; LAI; FOSTER, 2013). O

VPA é utilizado para indução de modelo animal do tipo autismo há aproximadamente 14 anos, este envolve a exposição pré-natal de roedores ao VPA pois sua relação com a gestação traz prejuízos neurológicos ao embrião (MABUNGA et al., 2015). O efeito teratogênico de VPA também é observado em seres humanos causando, além de disfunção somática, disfunção do SNC descrita como síndrome do valproato fetal, que foi recentemente associada ao desenvolvimento do autismo (MUTLU-ALBAYRAK; BULUT; ÇAKSEN, 2017).

Filhotes de ratas com injeção intraperitoneal com VPA no 12,5º dia de gestação apresentam anormalidades cerebrais, semelhantes às encontradas na autópsia e em estudos de imagens cerebrais de pacientes autistas como: (1) diminuição do número de neurônios motores no oculomotor, trigêmeo, abducente e núcleos hipoglossos de nervos cranianos; (2) encurtamento da região caudal ao núcleo facial e alongamento da região rostral ao núcleo facial; (3) cerebelo menor com redução de um número de células de Purkinje tanto nos hemisférios quanto no vermis (maior no lobo posterior do que no anterior); e (4) interposição do núcleo cerebelar reduzido (correspondendo aos núcleos globosos e emboliformes em humanos) (SCHNEIDER; PRZEWŁOCKI, 2005).

De acordo com Ahmed *et al*. (2020) animais com TEA induzidos por VPA demonstram aumento do estresse oxidativo que pode estar relacionado a deficiência de Nrf2, resultando no agravamento da resposta inflamatória em doenças neurodegenerativas como Parkinson e Alzheimer. Em estudo com roedores VPA/TEA os índices da proteína Bcl-2 anti-apoptótico e Bax pró-apoptótico foram aumentados no hipocampo, indicando alterações a sobrevivência e proliferação neural (WU et al., 2017). Assim como sugere Zimmerman *et al*. (2021) que a apoptose é mediada por estresse oxidativo e inflamação, analisando os índices da proteína HSP70 elevados na etiologia do autismo, confirmando perda de neurônios e prejuízos cognitivos.

Uma característica neurobiológica do TEA é a disfunção da aprendizagem, a qual é mediada pelo mecanismo entre o tamponamento de cálcio nas células e o prejuízo funcional dos interneurônios GABAérgicos (KERCHE-SILVA; CAMPAROTO; RODRIGUES, 2020). O VPA eleva a atividade inibitória do ácido gama-aminobutírico (GABA) através de mecanismos pré-sinápticos e pós-sinápticos, resultando na

disponibilidade de GABA sináptico e facilitando as respostas mediadas por GABA (WIN-SHWE et al., 2018a). Assim o VPA é um composto com atuação na modulação pré e pós-sináptica da transmissão GABAérgica (ROMOLI et al., 2019). É observada a atuação do VPA nos receptores GABA, com maior estímulo nos receptores GABA-A e GABA-B, e com as interações de forma direta com regiões reguladoras dos benzodiazepínicos dos receptores GABA, o VPA atua retardando a extinção do potencial inibitório pós-sináptico mediante ativação dos receptores GABA-A (SCHLICKMANN; FORTUNATO, 2013).

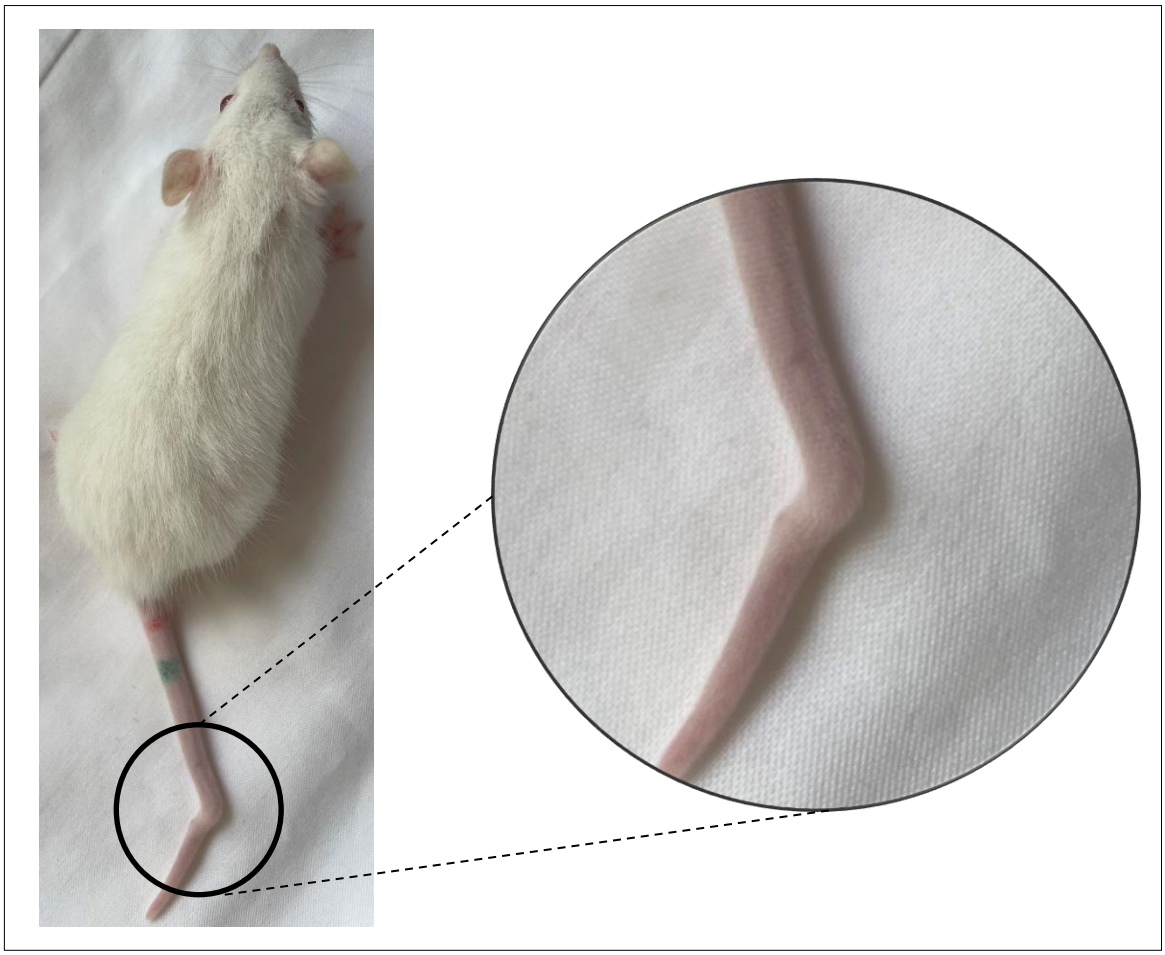
Em decorrência de uma maior transmissão seletiva mediada pelo sistema GABAérgico, pode ser visto maior concentração de GABA no cérebro com tratamento com o VPA, além de modular o sistema inibitório GABAérgico, o VPA também age na transmissão de aminoácidos excitatórios, como o glutamato (JOHNSON et al., 2018). Pode ser observado, em modelo de ratos, que o VPA diminuiu as respostas excitatórias induzidas pelo N-metil D-Aspartato (NMDA) e cainato na região cerebral do córtex pré-frontal medial (GOBBI; JANIRI, 2006). A exposição pré-natal ao inibidor da histona desacetilase (HDACi) valproato de sódio (ou ácido valpróico, VPA) foi associada a maior ocorrência de TEA em humanos (SAILER et al., 2019a).

Estudos com animais, nos últimos anos, demonstram transferência fácil e rápida de VPA através da barreira placentária, administrado tanto no período pré ou pós-natal, resulta em malformações congênitas e comprometimento comportamental, molecular e fisiológico nos fetos, aumentando o risco do estereótipo do TEA (RINALDI; SILBERBERG; MARKRAM, 2008; HARDEN, 2013; NICOLINI; FAHNESTOCK, 2018; ESHRAGHI et al., 2021).

De acordo com Bambini-Junior *et al*. (2011) ao utilizarem o modelo de indução de TEA em filhotes de ratas fêmeas com injeção intraperitoneal (ip) na dose de 600 mg/kg de VPA no 12,5° dia de gestação, observaram que os animais após seu nascimento traziam as caracterizações ao diagnóstico do TEA, malformações físicas na cauda da prole como cauda torcida e ou torta (fig. 4) em ambos os gêneros. Associado à este fenótipo, verificou-se prejuízos comportamentais e de interação social, associados aos resultados de sociabilidade e novidade social no teste das três câmaras, labirinto em Y e aprendizagem espacial no labirinto de água de Morris (MWM). A cauda torcida

também foi verificada em modelo de macaco, no qual apresentou as principais anormalidades comportamentais do TEA, incluindo interação social prejudicada, ansiedade e atividades reduzidas da rede cerebral (TU et al., 2019).

Figura 4 – Estrutura da cauda torcida ou torta característica do TEA.



Fonte: Arquivo próprio (2022).

Os transtornos comportamentais visíveis em roedores induzidos pelo VPA são de extrema relevância, visto que o diagnóstico do autismo normalmente é revelado através da análise comportamental (SCHNEIDER; PRZEWŁOCKI, 2005). Consequentemente, a caracterização em modelo animal se torna essencial para a compreensão molecular, estrutura neuroanatômica e funcional lesada, como também, são importantes para o diagnóstico e alternativas terapêuticas para a doença.

2.4.1 Hepatotoxicidade por VPA

O fígado é o principal órgão responsável pelo metabolismo de medicamentos e toxinas do organismo humano, desta forma, a lesão hepática induzida por fármacos é a causa mais comum de danos aos hepatócitos (DEVARAJ; UTHIRAPPAN, 2022). A insuficiência hepática aguda proveniente da ingesta de drogas comuns como o diclofenaco, sinvastatina, ibuprofeno, eritromicina, nimesulida, entre outros, apresenta quadro crescente em vários países do Oriente e Ocidente, porém as bases de dados confiáveis e conclusivas são escassos (GARCIA-CORTES et al., 2020; LOW et al., 2020). Medicamentos anticonvulsivos e antiepiléticos, utilizados para enxaqueca, transtornos bipolares, de humor e ansiedade como o VPA também estão relacionados à hepatotoxicidade, por possuir papel de ativação de células inflamatórias ao longo do processo de biotransformação no fígado por enzimas do citocromo P450 (JEE; SERNOSKIE; UETRECHT, 2021).

O VPA é conhecido por causar diversas reações metabólicas como a toxicidade neurológica, hepática e mitocondrial, além disso, causa síndrome do valproato fetal quando administrado no terceiro trimestre de gestação, pois atravessa a barreira placentária afetando o feto (RYBALKO et al., 2019). Ademais o uso do VPA é limitado em âmbito clínico, pois a reação farmacológica adversa mais citada é a lesão hepática, por sofrer extenso metabolismo no fígado (GAYAM et al., 2018). Além disso, a glicólise, estresse oxidativo, o metabolismo lipídico e o metabolismo de aminoácidos são desregulados pelo VPA no processo de lesão hepática (CHEN et al., 2019). De acordo com estimativas 1 em cada 10.000 pacientes que fazem uso do VPA apresentam mortalidade, principalmente se apresentam quadro de insuficiência hepática (SRIDHARAN et al., 2020).

Os metabólitos reativos do VPA associado a radicais livres induzem lesão hepática por participar ativamente da inibição da β-oxidação de ácidos graxos, indução do estresse oxidativo e polimorfismo genético de enzimas, como o citocromo P450 (CYP2E1), UDP-glucuronosiltransferases, superóxido dismutase (SOD), carbamoil-fosfato sintase, polimerase gama e glutationa S-transferases (GUO et al., 2019). Além disso, de acordo com Ma *et al*. (2020) a produção de ROS no tecido

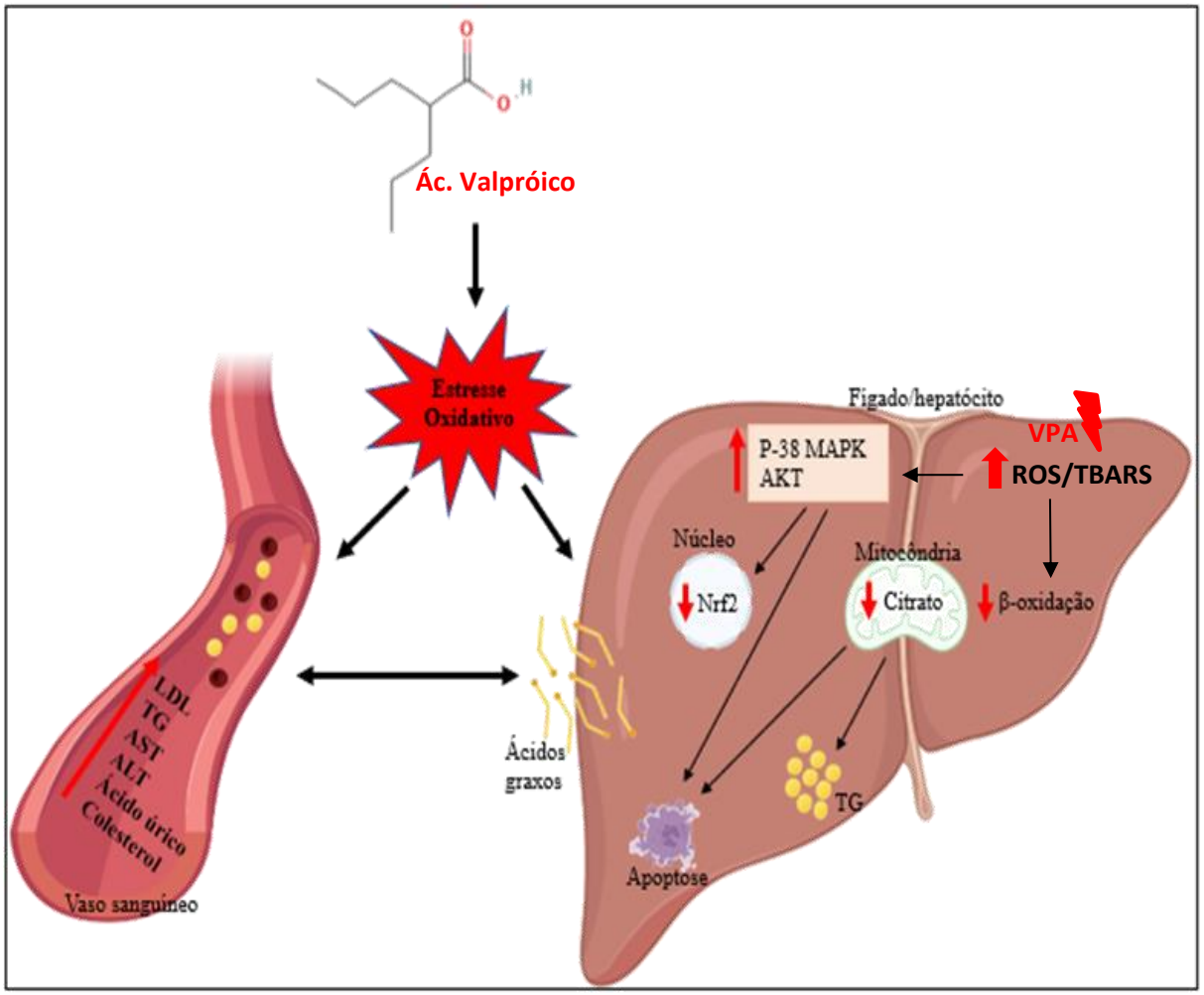
hepático se mostra aumentada devido a superexpressão da CYP2E1. Com administração do VPA ocorre maior concentração intracelular de subprodutos da peroxidação lipídica, ocasionando efeito tóxico no fígado, já que ocorre intensa peroxidação lipídica, como elucidado pelos pesquisadores Koroglu *et al*. (2021) e Ahangar *et al*. (2017) que quantificaram o malondealdeído, substância que indica peroxidação lipídica da membrana, após administração de VPA. Nas concentrações de 250 e 500 mg/kg/dia de VPA por quatorze dias, o teor de oxido nítrico, malondealdeído e glutationa reduzida tiveram sua regulação positiva, indicando conexão entre o VPA e a hepatotoxicidade em tecido hepático de modelo animal (GHEENA et al., 2022).

Em estudo experimental Ahangar *et al*. (2017) demonstrou aumento nos níveis de espécies reativas e na proteína carbonila no tecido hepático de ratos, após administração de VPA. Além de apresentar aumento na via de sinalização envolvendo AKT e MAPK levando ao quadro de apoptose em ratos com administração de VPA (XIE et al., 2010; ZHANG; ZHANG; ZHANG, 2016). Também se expressa lesão hepática pelo aumento das aminotransferases ALT e AST em pacientes com epilepsia tratados com VPA (FU et al., 2021). Estudos emergentes destacam, que a administração de VPA reduz os níveis de enzimas antioxidantes como a SOD, CAT, GPX, GSH no tecido hepático de ratos (ZHOU et al., 2020; CELIK et al., 2021).

Em estudo de caso, homem com 23 anos e histórico de TEA, fazendo uso de medicamento com VPA desenvolveu insuficiência hepática e sofreu morte em aproximadamente um ano, a biópsia no tecido hepático confirmou a presença de cirros (DEUTSCH; SPIEGEL; BURKET, 2021). Igualmente com paciente de 27 anos que fazia tratamento com VPA para problemas neurológicos como o TEA, apresentou como efeito colateral transtorno metabólico e encefalopatia aguda (GAYAM et al., 2018). Da mesma forma, mulher de 61 anos com administração de VPA por 7 dias apresentou diagnóstico de encefalopatia hepática, regulando seus níveis de amônia no sangue (GUO et al., 2017). Em estudo hospitalar foi relatado que em 20 pacientes tratados com VPA apresentaram sintomas de encefalopatia pela desregulação do metabolismo da amônia no organismo (LEWIS; TESAR; DALE, 2017). Por fim, o VPA pode interferir na atividade do fígado em seus processos metabólicos, causando hepatotoxicidade. Desse modo, demonstra-se que o VPA desencadeia inúmeras

reações bioquímicas que levam à distúrbios metabólicos e danos hepáticos (Fig. 5) em modelo animal e humanos.

Figura 5 – Ilustração do dano hepático com administração de VPA



Fonte: Arquivo próprio (2022).

O dano hepático induzido pela administração do VPA no período pré-natal em modelos de roedores, demonstra que os metabólitos reativos do VPA associados ao aumento dos radicais (ROS/TBARS) induzem o estresse oxidativo e a lesão hepática por inibir a B-oxidação pela ocorrência da diminuição da citrato sintase na mitocôndria, o qual afeta o aporte energético da célula e acúmulo de gorduras no fígado. Além disso, o estresse oxidativo incide no aumento dos marcadores de apoptose (P-38MAPK e AKT) que ativa as vias moleculares de ativação nuclear Nrf2 diminuindo a resposta antioxidante. Devido as alterações metabólicas no fígado, verifica-se

aumento de LDL, TG, colesterol total e marcadores de lesão hepática como o AST, ALT e ácido úrico na corrente sanguínea, refletindo o mau funcionamento das vias metabólicas e incidência hepática gordurosa não hepática.

2.4.2 Tratamento do TEA e Toxicidade do VPA

Devido a pluralidade dos efeitos colaterais advindos da administração do VPA e dos sintomas e mecanismos complexos da TEA as estratégias farmacológicas ainda não foram definitivas e/ou significativas, são prescritos como tratamentos os medicamentos psicoativos, antipsicóticos típicos e atípicos, as terapias comportamentais e os compostos bioativos (FADDA; CURY, 2016). A terapia cognitivo-comportamental é uma das intervenções mais utilizadas para o tratamento do TEA, porém sua atuação se baseia em perspectivas clínicas de crianças, como a reconhecer seus sentimentos e regular suas emoções, controlando a ansiedade, impulsividade e melhorar seu comportamento social (WOOD et al., 2015).

Os medicamentos antipsicóticos para o TDH ou TEA demonstram efeitos colaterais metabólicos e motores nos pacientes (CARLI et al., 2021). Dentro desses antipsicóticos os medicamentos os psicofármacos mais utilizados, aripiprazol, clozapina, ziprasidona e quetiapina, causam disfunção do metabolismo lipídico, ansiedade, tontura, náuseas, desencadeiam processos inflamatórios e apoptóticos nos sistemas neurais e hepáticos (DOYLE; MCDOUGLE, 2022).

Portanto, há uma necessidade substancial de novos tratamentos que sejam seguros e eficazes contra a síndrome metabólica e sintomas comportamentais do TEA. Neste sentido, evidências apontam para o importante papel dos compostos bioativos, que através da ação antioxidante, interfere no estresse oxidativo, formação de espécies reativas e na incidência de marcadores inflamatórios, promovendo melhora em diversas doenças neurodegenerativas como Alzheimer, Parkinson e TEA (REIN et al., 2013; SAGAR-OURIAGHLI; LIEVESLEY; SANTOSH, 2018; CHIU; VENKATAKRISHNAN; WANG, 2020; CHUGH; KAMAL-ELDIN, 2020).

2.5 Compostos Bioativos no tratamento do TEA e Hepatotoxicidade

Os compostos bioativos, também denominados fitoquímicos, são originados de plantas, não sendo considerados nutrientes essenciais, apresentam geralmente pigmentos, gosto amargo ou fragrância, os quais acredita-se que exercem papéis de proteção contra ameaças externas como a luz ultravioleta, patógenos e inclusive seres que se alimentam de plantas (OZAWA et al., 2012). Estes compostos proporcionam benefícios para a saúde e estão presentes em fontes alimentares de origem natural ou sintética, contribuem na prevenção de doenças crônicas, neurodegenerativas e hepáticas (MOHD SAIRAZI; SIRAJUDEEN, 2020). Desde as últimas duas décadas, os compostos bioativos tem sido explorados por seus efeitos gastroprotetores, hepatoprotetores, hipoglicêmicos, antioxidantes, anti-hipertensivos, anti-inflamatórios, imunomoduladores, cicatrizantes, cardioprotetores e antitumorais (GANDHI et al., 2020). Os mecanismos biológicos como o estresse oxidativo, neuroinflamação, excitotoxicidade e disfunção mitocondrial estão associados a desregulação neurológica, metabólica e hepática, portanto, estratégias de regulação desses sistemas estão relacionados os benefícios dos compostos bioativos no organismo (SINGH et al., 2019).

Na mesma linha de estudo, compostos bioativos como a melatonina demonstraram evidências de ação protetora no controle de distúrbios do sono e melhora do comportamento diurno em indivíduos com TEA (ROSSIGNOL; FRYE, 2011). De acordo com Erten (2021) o licopeno melhora as disfunções de aprendizado e memória em ratos VPA/TEA, modulando os fatores inflamatórios (IL-1α, IL-8, NF-κB, TNF-α) e os níveis cerebrais de Bcl-2, Bax e Nrf2. A ingestão de vitamina A em roedores com TEA demonstrou melhora no comportamento social no teste das três câmaras e a ansiedade verificada no campo aberto (CHENG et al., 2020). Os fitoesteróis, carotenoides, polifenóis e ácidos graxos, importantes compostos naturais advindos de plantas e animais, têm sido propostos para controlar o metabolismo lipídico e os efeitos hepatotóxicos (GANESAN; JAYACHANDRAN; XU, 2017). Assim como também, a vitamina A ou retinol que possui elevado potencial antiinflamatório e inibem a formação de espécies reativas de oxigênio atuando positivamente na

síndrome metabólica e na lesão de células hepáticas (NAIR; WEISKIRCHEN; AL-MUSHARAFI, 2014).

Ressalta-se a vitamina B6, B12 e magnésio em doses elevadas na administração em crianças com TEA auxiliam na melhora comportamental, da fala e da linguagem (MOUSAIN-BOSC et al., 2006; JAMES et al., 2009). Os compostos bioativos como os carotenoides agem positivamente aos mediadores inflamatórios, aumentando os níveis do mecanismo microRNA-CREB-BDNF e diminuindo a ação da enzima acetilcolinesterase (AChE) (WANG et al., 2016), como também, incide na progressão cognitiva e aumento da plasticidade sináptica, em processos de aprendizado e memória (ABD EL-KADER; AL-JIFFRI, 2017). A atividade reduzida da AchE, que é uma enzima responsável por modular os níveis de acetilcolina na fenda sináptica, desencadeia uma melhora dos sintomas clínicos de doenças neurodegenerativas como o Alzheimer, retardando temporariamente o declínio da função cognitiva e capacidade funcional dos indivíduos (GOMES; KOSZUOSKI, 2005). O carotenóide astaxantina possui potencial uso terapêutico em experimentação animal com TEA, pois atenuou a neuroinflamação e distúrbios comportamentais, reduzindo o estresse oxidativo no cérebro e no fígado (AL-AMIN et al., 2015).

2.5.1 Carotenoides

Dentre os fitoquímicos, está o grupo dos carotenoides que são compostos com estrutura básica C_{40} H_{56} contendo várias ligações duplas e atuando contra as espécies reativas de oxigênio (ERO). No grupo dos carotenoides estão inclusos os carotenos (composto com apenas carbono e hidrogênio) e as xantofilas (contendo átomos de oxigênio em sua estrutura química) (SILVA et al., 2010).

A classe dos carotenos é constituída por moléculas apolares, enquanto as xantofilas possuem característica polar e são subdivididas em hidroxicarotenoides (contendo um ou dois grupos hidroxila) e cetocarotenoides (presença de grupos cetona) (KEOWN; BOTHWELL; JAIN, 2014). Os carotenoides apresentam atividade antioxidante, já que são dependentes dos níveis de oxigênio, que em baixas

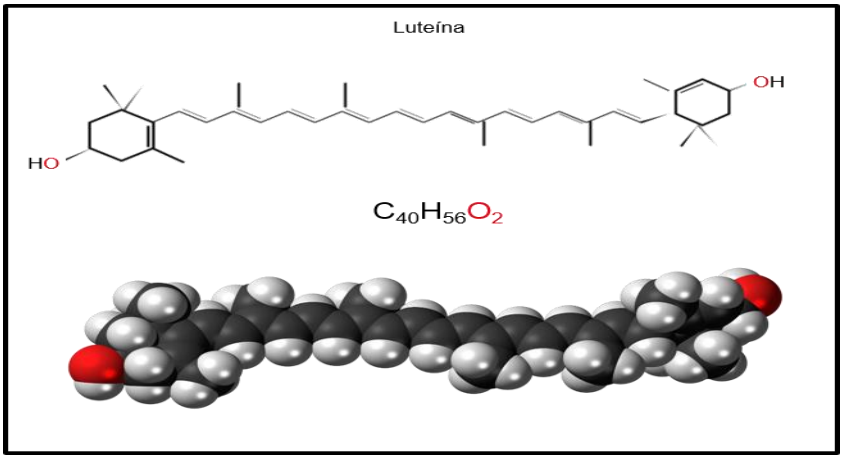
concentrações vai ocorrer atuação do tipo antioxidante e em elevadas concentrações as características são pró oxidantes (GAMMONE; RICCIONI; D'ORAZIO, 2015). As propriedades antioxidantes dos carotenoides estão associadas a inibição da cadeia de iniciação e propagação dos radicais livres, juntamente com a capacidade de supressão de oxigênio singlete (GAMMONE; RICCIONI; D'ORAZIO, 2015). Estudos citam os carotenoides na modulação de déficit de memória social e doenças neurodegenerativas, devido as suas propriedades antioxidantes e anti-inflamatórias (KAUR; CHAUHAN; SANDHIR, 2011; OBULESU; DOWLATHABAD; BRAMHACHARI, 2011).

Naturalmente, já foi apontada a presença de mais de 600 tipos de carotenoides, sendo que desses 30-50 estão presentes na dieta humana normal, e no soro humano, somente 10-15 foram identificados rotineiramente em exames, como os carotenoides luteína, zeaxantina e seus metabólitos (KHACHIK et al., 1991; VAN DE KRAATS et al., 2008). Entre os carotenoides, com destaque para a saúde humana e que tem instigado diversas pesquisas está a luteína, substância que se acumula na mácula que é a parte central da retina humana envolvida na acuidade visual (NWACHUKWU; UDENIGWE; ALUKO, 2016).

2.6 Carotenoide Luteína

A xantofila luteína (Fig. 6) (β,ε-caroteno-3,3´-diol) apresenta dois grupos terminais cíclicos (um anel β e um anel α-ionona) e a estrutura isoprenóide C_{40} básica, possui característica hidrofóbica e está presente em vegetais de folhas verde escura e também na gema do ovo, apresenta baixa biodisponibilidade, além de ser sensível ao calor, à luz, pH intestinal e oxigênio (KOTAKE-NARA; NAGAO, 2011). A luteína tem como mecanismo de ação, atuar na transferência de energia responsável por devolver o oxigênio singlete ($^1O_2$) ao seu estado basal (ROBERTS; DENNISON, 2015). O oxigênio singlete é a forma mais reativa do oxigênio molecular, seu acúmulo expressa estresse oxidativo podendo levar a morte celular, porém compostos altamente antioxidantes participam de sua regeneração ($^3O_2$) ao seu estado fundamental (PETROU et al., 2018).

Figura 6 – Estrutura química da luteína.



Fonte: Arquivo próprio (2020).

A luteína apresenta grande número de duplas ligações conjugadas, com um grupo hidroxila ligado a cada extremidade da molécula, rica em elétrons que atuam na absorção do oxigênio *singlete* e radicais livres, possui importante papel anti-inflamatório reduzindo as concentrações de IL-1β, protegendo contra a lesão hepática em modelo animal (EL-KHOLY; ELKABLAWY; EL-AGAMY, 2017). A IL-1β está relacionada ao recrutamento de células hepáticas e na ativação de suas células estreladas, as quais estão envolvidas na fibrose e progressão da esteatose hepática para esteatohepatite (DU et al., 2015). Portanto, a ligação entre o estresse oxidativo e os processos inflamatórios podem ser regulados mediante a atuação do potencial antioxidante da luteína, que apresentam relevantes propriedades anti-inflamatórias (KIM et al., 2012).

Em pesquisa com material encefálico prematuro, a contribuição de luteína para os carotenoides totais é o dobro dos valores encontrados em adultos, o que sugere a necessidade de luteína durante o desenvolvimento neuronal, estabelecendo conexões positivas entre os seus benefícios antioxidantes e anti-inflamatórios e a proteção contra doenças responsáveis por causar disfunções cognitivas como o TEA (NATARAJ et al., 2015). Os mesmos autores, descreveram que a luteína restaurou os níveis de TBARS (substâncias reativas ao ácido tiobarbitúrico), GSH (glutationa) e Gpx (glutationa peroxidase) em roedores com indução de doença de Parkinson, demonstrando potencial redutor da toxicidade neuronal mediada pelo estresse oxidativo.

Além disso, a luteína atua modulando a instabilidade dinâmica dos microtúbulos (unidades estruturais dos neurônios) e prevenindo a degradação das proteínas das vesículas sinápticas (JIA et al., 2017). Segundo Johnson *et al*. (2014) é consistente o papel da luteína a uma série de medidas cognitivas, que abrangem as funções de linguagem, aprendizagem e memória estando relacionadas a regiões específicas do cérebro. Analogamente a luteína possui a capacidade de modular o BDNF, o qual em baixos níveis, se relacionam a uma série de patologias neurodegenerativas como a doença de Huntington, doença de Alzheimer e doença de Parkinson (MATTSON; MAUDSLEY; MARTIN, 2004). Quanto a atuação da luteína, de acordo com revisão de Ola *et al*. (2015) sabe-se que este composto é capaz de atenuar os baixos níveis de BDNF, isto ocorre possivelmente por meio de sua proteção da atividade sináptica.

Segundo SASAKI *et al*. (2010) a luteína protege as células ganglionares e as células internas da retina da morte celular induzida pelo diabetes, observado por pelo menos um período de 4 meses após o início da doença. Desta forma, a luteína desempenha função na sobrevivência celular induzida por estresse oxidativo documentado em modelo animal (CHANG et al., 2013). Outra importante ação da luteína se refere ao seu potencial na supressão da ativação do STAT3 (*do inglês, signal transducers and activators of transcription*), que é ativada por citocinas inflamatórias IL-6, desta forma, este composto interrompe o ciclo inflamatório, auxiliando positivamente na condição patológica (OZAWA et al., 2012). Em consonância, no estudo de NATARAJ *et al*., (2015), estes analisaram os efeitos deletérios do Parkinson e a ação da luteína, sendo assim houve a proteção dos neurônios dopaminérgicos, favorecendo a defesa antioxidante e restringindo a disfunção mitocondrial e a apoptose celular.

2.7 Nanotecnologia e Nanocápsulas carregadas de Luteína

Nas últimas décadas, os pesquisadores concentraram-se em pesquisas na nanobiotecnologia para favorecer a eficiência da carga terapêutica no alvo específico de tratamento (GHALANDARLAKI; ALIZADEH; ASHKANI-ESFAHANI, 2014). A

nanoencapsulação apresentara vantagens em relação as abordagens convencionais, pois diminui a biodegradação da carga encapsulada, favorece a liberação controlada/sustentada dos fármacos, eleva a taxa de dissolução, a permeabilidade de substâncias insolúveis em água e a meia-vida plasmática (AHMAR et al., 2021).

A nanotecnologia trata-se de um campo tecnológico que emprega o uso de materiais em escala nanométrica com objetivo de obter produtos e processos com melhor desempenho (ZAMBRANO-ZARAGOZA et al., 2018). Para os compostos bioativos, opções como as nanocápsulas poliméricas demonstram potencial favorável de absorção, pois apresentam melhora em seu transporte, liberação, estabilidade, atua direto no tecido alvo, utiliza menor quantidade do princípio ativo e redução dos efeitos adversos (KATIYAR et al., 2015). Essa tecnologia também é utilizada para fitonutrientes e vitaminas de característica hidrofóbicas (VISHWANATHAN et al., 2014).

Compostos como a luteína tem grande importância nos processos como a nanoencapsulação, visto que a sua natureza hidrofóbica pode se agregar e formar cristalinos em uma solução aquosa, afetando a absorção intestinal e consequentemente sua biodisponibilidade (CHENG; FERRUZZI; JONES, 2019). Assim, a nanoencapsulação pode aumentar a atividade biológica e atividade farmacocinética das moléculas lipofílicas (KUMARI et al., 2010). Após o processo de encapsulação de produtos lipofílicos como a luteína, se torna evidente sua melhor biodisponibilidade ao organismo, assim como uma menor quantidade é requerida para que esta substância apresente efeito biológico satisfatório (DHIMAN et al., 2021).

De acordo com Silva *et al.* (2017) a nanoencapsulação com a matriz polimérica do Poloxamer a luteína melhorou sua solubilidade em água em mais de 43 vezes, permitindo sua melhor absorção, além disso, as nanopartículas de polivinilpirrolidona (PVP), carregadas com luteína a 10 e 1,5mg · kg $^{-1}$ aumentaram o índice de reconhecimento de objeto em camundongos, em comparativo com a luteína livre 100mg · kg $^{-1}$. Igualmente verificado por Silva de Sá *et al*. (2019) que descreveu a curva de aquecimento da luteína livre, a qual apresentou níveis de 147°C, indicando sua degradação no interior do organismo, porém com o processo de nanoencapsulação a luteína foi preservada.

Em estudo Murillo *et al*. (2016) avaliaram a biodisponibilidade da luteína no plasma, tecido adiposo, fígado e olhos em modelo de suínos da Guiné, se observou que o plasma e o fígado apresentaram maiores concentrações de luteína na forma de nanoemulsão do que em sua forma livre, com isso, foi possível relacionar que a nanoemulsão aumentou sua biodisponibilidade ao organismo proporcionando ação hepatoprotetora aos animais testados.

Em pesquisas *in vivo* com administração de luteína encapsulada em matriz de polivinilpirrolidona (PVP) observou-se melhora no índice de discriminação de objetos, demonstrando potencialização da absorção de luteína pelo organismo (DO PRADO SILVA et al., 2017). Da mesma forma em estudo de revisão, modelos de roedores com diabetes, obesidade e neurodegeneração apresentaram modulação de seus índices de estresse oxidativo, neuroinflamação e apoptose, com a absorção de luteína encapsulada (ZHANG; KONG; TAN, 2020). Na utilização de nanocápsulas de núcleo / casca de biopolímero catiônico (Quitosana @ PLGA C / SNPs) carregadas de luteína atenuou o estresse oxidativo em terapia de modelo animal com Alzheimer (DHAS; MEHTA, 2020). Em estudo com *Drosophila melanogaster*, em modelo de doença de Parkinson induzido por rotenona, foi possível observar modulação das enzimas antioxidantes SOD e catalase, elevação dos níveis de TBARS, melhora na locomoção e sobrevivência das moscas a partir do uso de nanocápsulas carregadas de luteína (FERNANDES et al., 2021).

Contudo, as nanopartículas poliméricas estão sendo mais exploradas pelo seu potencial farmacológico, pois estes compostos aumentam a biodisponibilidade e a absorção de agentes lipofílicos no organismo, além disso, facilita a entrada de fármacos ou compostos bioativos através de barreiras biológicas, maximizando o potencial terapêutico e ao mesmo tempo minimizando os efeitos colaterais (SINGH et al., 2021).

3. JUSTIFICATIVA

A prevalência de TEA parece estar aumentando globalmente, 1 em cada 160 crianças apresenta este transtorno do desenvolvimento neurológico anormal, e sua incidência se mostra superestimada no sexo masculino, porém as disfunções neurológicas e comportamentais características do TEA, acometem os dois sexos e esses déficits no sexo feminino precisam de maior atenção.

Sabendo que a administração pré-natal do VPA, indicado para o tratamento de convulsões, epilepsia, crise de ausência e transtorno bipolar está associada a desordens neuropsiquiátricas e metabólicas, teratogênicas e hepatóxicas, é imprescindível estudos que elucidem uma relação tóxica entre o VPA e a prole das fêmeas nas quais foi administrado o VPA.

Desta forma, a busca por compostos naturais com potencial terapêutico é fundamental para o tratamento e modulação dos possíveis mecanismos bioquímicos e biológicos alterados pela administração do VPA. A partir disso, as nanocápsulas carregadas de luteína possuem propriedades farmacológicas, principalmente pelo seu potencial antioxidante, já modulando vias de inflamação, oxidação e apoptose citadas na literatura. Enfatizamos a sua ação neuroprotetora e hepatoprotetora como adjuvante ao tratamento de danos causados pela administração do VPA.

Sendo assim, esta tese tem como finalidade promover embasamento na busca de estratégias terapêuticas utilizando nanocápsulas carregadas de luteína para o tratamento de déficits de memória social, ansiedade, locomoção, danos metabólicos, neurológicos e hepatotóxicos induzidos pela administração pré-natal do VPA em ratas fêmeas.

4. OBJETIVOS

4.1 Objetivo Geral

Investigar se as nanocápsulas carregadas com luteína revertem o déficit de sociabilidade, comportamento repetitivo e ansiedade, bem como possíveis mecanismos de ação, envolvendo estresse oxidativo, apoptose e alterações metabólicas, em modelo de comportamento semelhante ao TEA e em hepatotoxicidade induzidos pela exposição pré-natal de VPA em ratas fêmeas

4.2 Objetivos Específicos

- Avaliar o efeito da administração oral de nanocápsulas carregadas com luteína sobre o comportamento locomotor, autolimpeza, ansiedade e déficits de sociabilidade em modelo experimental do TEA induzido por VPA.
- Investigar os indicadores de estresse oxidativo e marcadores de apoptose no hipocampo de animais submetidos ao tratamento com nanocápsulas carregadas com luteína e expostas a administração pré-natal de VPA.
- Verificar o efeito da administração oral de nanocápsulas carregadas com luteína sobre a atividade antioxidante SOD e CAT no fígado de ratas fêmeas com administração pré-natal de VPA e tratadas com nanocápsulas carregadas com luteína.
- Investigar a relação dos marcadores lipídicos, estresse oxidativo e da apoptose com a hepatoxicidade em filhotes fêmeas induzidas pela administração pré-natal de VPA e tratadas com nanocápsulas carregadas de luteína.
- Analisar ação das nanocápsulas carregadas de luteína na associação da toxicidade do VPA no tecido neurológico e hepático em modelo comportamental do TEA e na hepatotoxicidade de ratas fêmeas induzidas pela administração pré-natal de VPA.

5. ARTIGO E MANUSCRITO CIENTÍFICO

Os resultados que fazem parte desta tese estão apresentados sob a forma de um artigo científico e um manuscrito. Os itens *Introdução, Materiais e Métodos, Resultados, Discussão e Referências*, encontram-se no próprio Artigo Científico e no Manuscrito.

Artigo:

O artigo está disposto na forma em que foi aceito para publicação em dezembro de 2022, na revista científica "Neurotoxicology".

Intitulado: *Lutein-loaded nanocapsules reverse oxidative stress, apoptosis, and autism spectrum disorder-like behaviors induced by prenatal valproic acid exposure in female rats*

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5.1 ARTIGO CIENTÍFICO

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Lutein-loaded nanoparticles reverse oxidative stress, apoptosis, and autism spectrum disorder-like behaviors induced by prenatal valproic acid exposure in female rats

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ABSTRACT

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by deficits in social interaction and repetitive behaviors. In this study, we assessed the effect of lutein-loaded nanoparticles on ASD-like behaviors induced by prenatal valproic acid (VPA) exposure in female offspring rats and the possible involvement of oxidative stress and apoptosis. Pregnant female Wistar rats received a single intraperitoneal injection of VPA (600 mg/kg), on the gestational day 12.5. The VPA-exposed female offspring rats were divided into two subgroups and received either lutein-loaded nanoparticles (5 mg/kg) or saline by oral gavage, for 14 days. The animals were submitted to the three-chamber test and open field to evaluate ASD-like behaviors. The hippocampus was removed for the determination of oxidative stress indicators (ROS; TBARS; SOD and Nrf2) and apoptosis biomarkers (Hsp-70; p38-MAPK; Bax and Bcl-2). The exposure to lutein-loaded nanoparticles reversed sociability deficit, social memory deficit, and anxiety-like and repetitive behaviors induced by VPA, and restored the oxidative stress indicators and apoptosis biomarkers in the hippocampus. This neurochemical effect must be associated with the reversal of ASD-like behaviors. These results provide evidence that lutein-loaded nanoparticles are an alternative treatment for VPA-induced behavioral damage in female rats and suggest the involvement of oxidative stress.

1. Introduction

Autism spectrum disorder (ASD) is a neurodevelopmental disorder diagnosed in early childhood. The main features of ASD include deficits in communication and social interaction, and restricted and repetitive behavior (Modabbernia et al., 2017). In addition, people with ASD develop high levels of anxiety (Kerns et al., 2015). Evidence indicates a higher prevalence of ASD in males (male 4: 1 female) (Crane et al., 2018; Gomes et al., 2015), consequently, most studies on autistic-like behavior have mainly explored the male sex and only a few studies have focused on females or gender differences (Kerr et al., 2016; Liu et al., 2018; Melancia et al., 2018). In this sense, further knowledge about the development of ASD in females is needed, once this subject is still poorly reported, as well as the development of female-specific treatments.

In both patient and animal ASD models, significant neurochemical alterations have been shown, such as an increase in reactive oxygen species, lipid peroxidation, mitochondrial dysfunction, and a decrease in the activity of antioxidant enzymes. These events cause an increase in oxidative stress followed by cell death. Therefore, the association between oxidative stress and apoptosis has emerged as a supposed

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candidate for the causes of ASD. Additionally, these neurochemical alterations observed in autistic brains often involve the hippocampus. (Bjørklund et al., 2020; Matta et al., 2019).

Considering the increase in the number of children diagnosed with ASD in recent decades and the heterogeneous behavioral symptoms, along with the lack of female-specific treatments and the current treatments, which are limited to behavioral and educational therapies associated with the use of antipsychotics that aim at controlling only some of the symptoms of the disease, the development of new therapeutic options is necessary. In this sense, research on the subject highlights the important role of bioactive compounds, such as carotenoids, which have antioxidant and anti-apoptotic properties (Pereira et al., 2021), considering that oxidative stress and apoptosis contribute to the development of ASD. Interestingly, plasma from patients with ASD has lower levels of carotenoids, when compared to normal children (Krajcovicova-Kudlackova et al., 2009).

Lutein, a fat-soluble xanthophyll carotenoid, is a possible multitarget candidate to prevent/mitigate the symptoms of ASD. Lutein is the main carotenoid found in babies' brains, accounting for more than half of the total concentration of cerebral carotenoids, and it plays an important role in neurodevelopment during the early stages of life (Fitzpatrick and Dhawan, 2014; Vishwanathan et al., 2014). Considering its ability to cross the blood-brain barrier and its preferential uptake when compared to other carotenoids, lutein, the second most prevalent carotenoid in human serum, shows a positive correlation between serum and brain concentrations (Vishwanathan et al., 2014).

Although lutein exhibits biological activities and high therapeutic potential, its use is limited by its low water solubility (Syamila et al., 2019). In fact, there are no studies on the protection promoted by lutein against ASD, even in lutein's raw form (non-encapsulated), which may be related to its low bioavailability. However, studies have shown that lutein nanoparticles have greater bioavailability than free lutein (Arunkumar et al., 2013; Bhat et al., 2020).

Therefore, the present study investigated whether lutein-loaded nanoparticles reverse VPA-induced social deficit, repetitive behavior, and anxiety in female offspring rats, as well as possible mechanisms of action, involving oxidative stress and apoptosis.

2. Materials and methods

2.1. Chemicals

Poloxamer 407 (P407, 12,000.00 g mol^{-1}, Sigma-Aldrich), Tween 80 (Dinâmica), ethanol (Vetec), and lutein (kindly gifted by Kemin S.A) were used in the preparation of lutein-loaded nanoparticles. Valproic acid (VPA) was obtained from Acros Organics (Acros Organics, NJ, USA). Lutein-loaded nanoparticles and VPA were dissolved in a saline solution (0.9% NaCl). All the other reagents used were of analytical grade.

2.2. Nanoparticles production and characterization

Nanoparticles were produced according to the method described by Silva et al. (2017) with minor modifications. Poloxamer 407 and Tween 80 were added to ethanol (120 mL in all experiments) and stirred for 5 min. Then, lutein was added and mixed for 5 min under gentle stirring. The solution was sonicated (Fisher Scientific, 120 W, 1/8′ probe) for 3 min in a pulse regime (30 s on and 10 s off) on an ice bath. Afterward, ethanol was evaporated through air circulation at 40 °C for 24 h and the resulting powder was stored at − 10 °C, protected from light. Table 1 presents the formulation used in the experiments.

The thermal properties of the nanoparticles were analyzed through Differential Scanning Calorimetry (DSC, Perkin Elmer 4000), where samples were heated in aluminum pans (0–300 °C at 20 °C.min^{-1}) under nitrogen flow (50 mL.min^{-1}). Fourier Transform Infrared spectra (FTIR; Frontier Perkin Elmer) was performed in potassium bromide (Sigma-Aldrich, spectroscopic standard) pellets, with a resolution of 2 cm^{-1} from 4500 to 400 cm^{-1} with 32 cumulative scans. Transmission electron microscopy (TEM; JEOL model JEM-1011, 100 kV) was performed to observe the nanoparticles' morphology in parlodium-covered copper grids (300 mesh). The size of the nanoparticles was determined by image analysis after measuring at least 250 particles. In the DSC and FTIR analyses, a mixture of Poloxamer 407 and lutein was obtained by manually mixing these components at the same proportion found in the nanoparticles with the objective of studying the interaction between them.

Table 1
Formulation used in the lutein-loaded nanoparticles production.

	NP1	NP2	NP3	NP4
Lutein concentration (g.g $^{-1}_{P407}$)	0.050	0.067	0.100	0.133
P407 (g)*	1.000	0.750	0.500	0.750
Lutein (g)	0.050	0.050	0.050	0.100
Tween 80 (g)* *	0.010	0.007	0.005	0.006

* Poloxamer 407. * *Concentration of 0.01 g.g P407–1.

2.3. Animals

Male and female Wistar rats (90–120 days old) were used to compose the parental couples. Twenty-eight female offspring were used for the in vivo and ex vivo assays. All the animals were housed in plastic acrylic cages and kept at constant room temperature (21 ± 1 °C) with free access to water and food under a 12:12 h light:dark cycle (lights on at 07:00 h). The behavioral tests were conducted during the light phase of the cycle (from 9:00 a.m. to 5:00 p.m.). All the experiments reported in this study were conducted according to the National and International legislation [guidelines of the Brazilian Council of Animal Experimentation (CONCEA), of the U.S. Public Health Service's Policy on Humane Care and Use of Laboratory Animals (PHS Policy) and the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) with the approval of the Ethics Committee for Animal Research of the Universidade Federal do Pampa (CEUA protocol nº 041/2018).

2.4. VPA-induced rat model of ASD

Rodents that were prenatally exposed to VPA, on the gestational day 12.5, displayed ASD-like behaviors such as social interaction deficits and anxiety, phenotypes characteristics of the human condition (Kataoka et al., 2013; Schneider et al., 2008; Schneider and Przewłocki, 2005). To obtain offspring with ASD-like behaviors, the methodology described by Singla et al. (2021), including minor modifications, was applied. Male and female rats mated overnight, and in the morning, the presence of spermatozoa in the vaginal smear was confirmed, considering that day zero of gestation (GD 0). On GD 12.5, the females were divided into two groups and received a single intraperitoneal (ip) injection of either VPA (600 mg/kg) or saline (1 mL/kg). The offspring were weaned on the post-natal day 21 (PND 21) and only the female offspring were the subjects of this study. All offspring exposed to VPA during gestation developed the character of a "twist" in the tail (Fig. 1B).

2.5. Lutein-loaded nanoparticles treatment

To investigate whether lutein-loaded nanoparticles reverse VPA-induced social deficit, repetitive behavior, and anxiety in female offspring rats, as well as the possible mechanisms of action involved, on PND 21, the VPA-exposed female offspring were randomly divided into two subgroups and received either lutein-loaded nanoparticles (5 mg/kg) or saline (1 mL/kg) by oral gavage, once a day, for 14 days (PND 21–34). Twenty-four hours after the last injection, the animals were submitted to the three-chamber and open field tests. After the behavioral

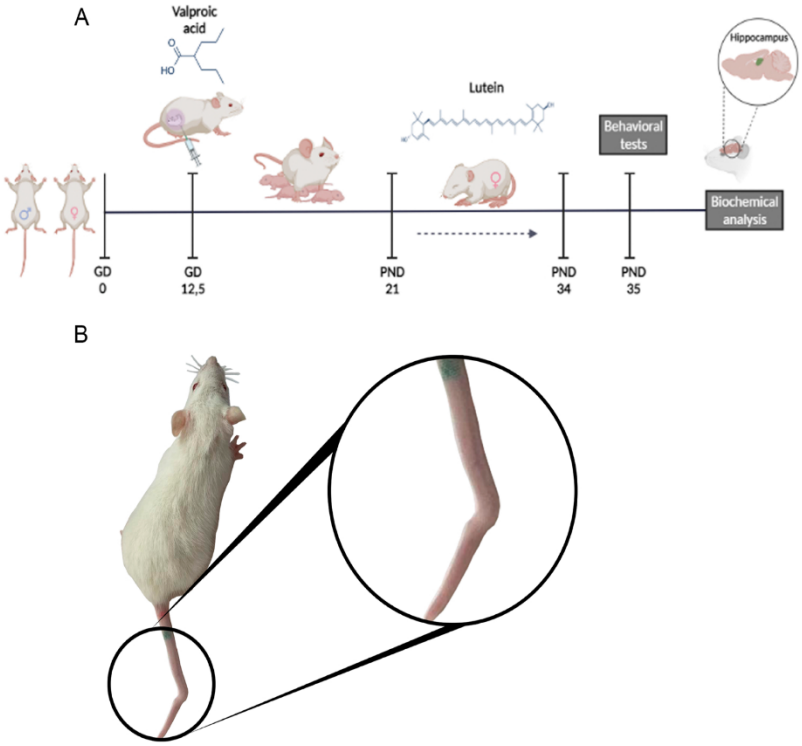

Fig. 1. A) Schematic representation of the experimental design for administration of lutein, for 14 days, in female offspring rats' exposure to VPA. B) Representative photo of crooked tail phenotype prenatal VPA-exposed female offspring.

tests, the hippocampus was removed for the determination of oxidative stress indicators (reactive oxygen species, ROS; thiobarbituric acid reactive substances, TBARS; superoxide dismutase, SOD and nuclear factor E2-related factor 2, Nrf2) and apoptosis biomarkers (Heat shock protein 70, Hsp-70; p38 mitogen-activated protein kinases, p38 MAPK; Bcl-2-associated X protein, Bax and B-cell lymphoma-2, Bcl-2). The dose and administration time were selected based on a previous study that showed lutein-loaded nanoparticles administration (5 mg/kg) does not affect memory (do Prado Silva et al., 2017). The treatment schedule is depicted in Fig. 1A.

2.6. *In vivo assays*

2.6.1. *Three-chamber test*

The three-chamber test was conducted to assess social memory, through social novelty preference, and sociability, as previously described (Castro et al., 2017; Matsuo et al., 2020). The apparatus consisted of a wooden box (60 ×40×30 cm) internally divided into three equal chambers with openings that allow the animals to freely explore the compartments. The test consisted of three sessions: habituation, sociability, and social novelty preference. In the habituation session, the animals were placed into the center chamber and allowed to explore all three chambers for 5 min. In the sociability session, a small plastic cage was placed in both side chambers and a rat that had no previous contact with the subjects (stranger 1) was placed in one of the cages. In the social novelty preference, stranger 1 remained in its cage (now called familiar rat) and another rat with no previous contact with the subjects (stranger 2) was placed in the cage that had been empty during the sociability session. In each session (sociability and social novelty), the subject-animals were placed into the center chamber and allowed to explore all three chambers for 10 min. The time spent exploring each chamber and interacting with each cage was recorded. The sociability index was then calculated, considering the difference of time spent exploring stranger 1 and the empty cage divided by the sum of time spent exploring stranger 1 and the empty cage and used as a sociability parameter (T stranger 1 - T empty cage / T stranger 1 + T empty cage). Analogously, the social novelty index was calculated (T stranger 2 - T familiar rat /T stranger 2 + T familiar rat).

2.6.2. *Open field test*

The open field, a test classically used to assess anxiety-like behavior in rodents, was performed as previously described by (Olexová et al., 2016). Immediately after the three-chamber test, the animals were placed in the corner of the open field apparatus (48 × 48 × 40 cm) facing the wall. During the 10-min open field session, the distance traveled, the time spent in the center, and the time of immobility were recorded. In addition, repetitive behaviors were assessed by monitoring the number of self-grooming.

2.7. *Ex vivo assays*

2.7.1. *Oxidative stress indicators*

Immediately after the open field test, the rats were decapitated, and the hippocampus was removed, weighed, and homogenized in a Tris–HCl (50 mM, pH 7.4) buffer. The resulting homogenate was then centrifuged at 10,000 g for 10 min at 4 °C and the supernatant fraction (S1) was used for the determination of oxidative stress indicators. The protein content was colorimetrically measured using the Bradford method (Bradford, 1976), and BSA (1 mg/mL) was used as the standard.

To estimate levels of reactive oxygen species (ROS) production in the hippocampus, S1 was diluted (1:10) in 50 mM Tris-HCl (pH 7.4) and incubated with 10 μL of 2′,7′-dichlorofluorescein diacetate (DCF-DA; 1 mM) at room temperature for 30 min. The quantification of the DCF-DA oxidation assay was monitored as a general index of oxidative stress

according to the protocol proposed by Loetchutinat et al. (2005). The fluorescence emission resulting from DCF-DA oxidation was monitored and the reading was performed in excitation at 485 nm and 530 nm emission in the spectrophotometer using an EnsPireR multimode microplate reader (Perkin Elmer, USA). The rate of DCF formation was calculated as a percentage of fluorescence treatment relative to the control group.

Lipid peroxidation was estimated by measuring thiobarbituric acid

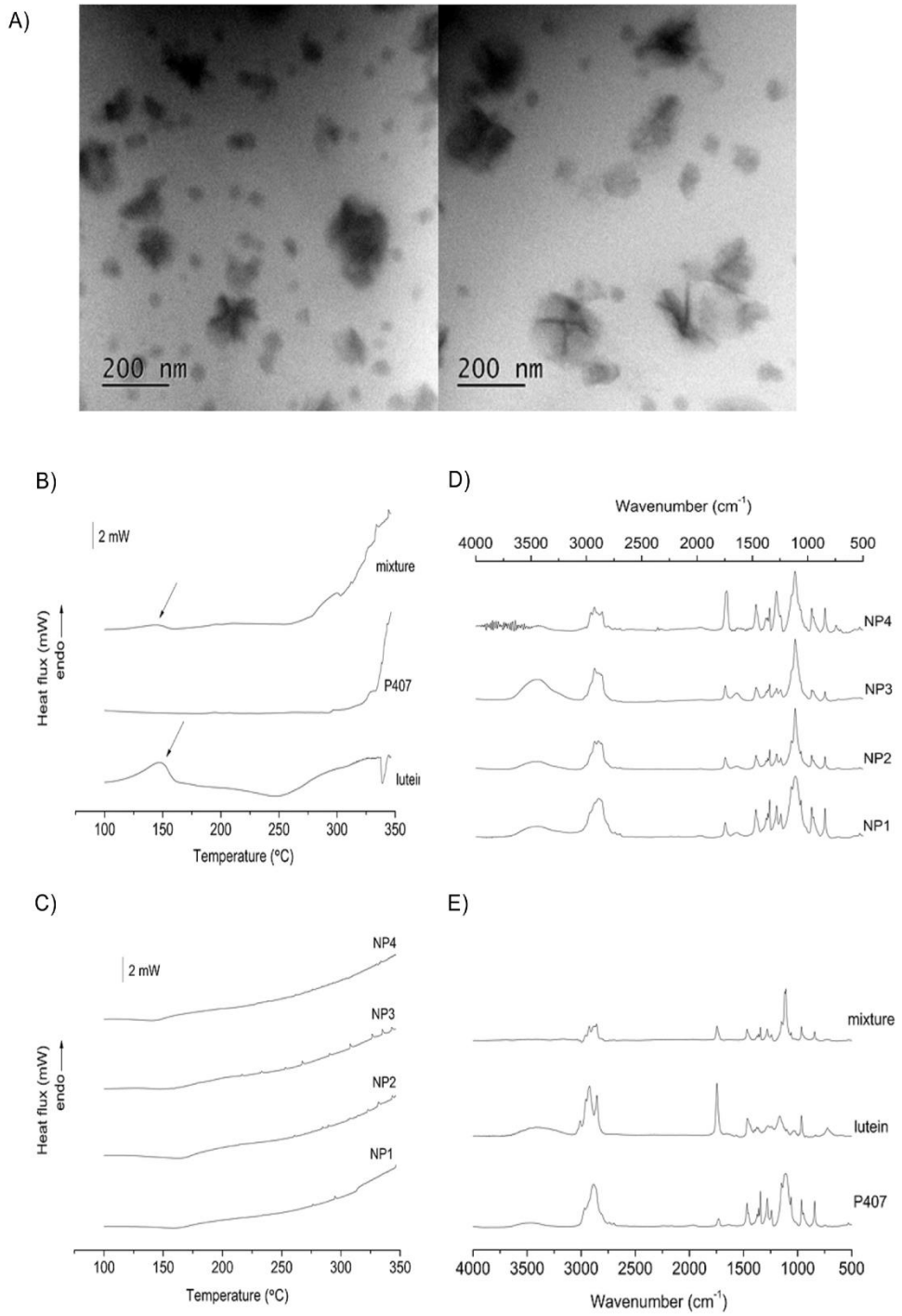

Fig. 2. Physicochemical and morphological characterization of the lutein-loaded nanoparticles A) Images of the lutein-loaded nanoparticles obtained using Transmission Electron Microscopy (formulation NP4–0.133 g.g $^{-1}_{P407}$). B) Differential Scanning Calorimetry curves of lutein, Poloxamer 407, and the mixture of these components. C) Differential Scanning Calorimetry curves of the nanoparticles obtained according to the formulations presented in Table 1. D) Infrared spectra of the nanoparticles obtained according to the formulations presented in Table 1. E) Infrared spectra of lutein, Poloxamer 407, and the mixture of these components.

reactive substance (TBARS) and was expressed in terms of the malondialdehyde (MDA) content, according to the method described by Ohkawa et al. (1979). In this method, MDA, an end product of fatty acid peroxidation, reacts with thiobarbituric acid (TBA) to form a colored complex. The absorbance was measured in the supernatant at 532 nm. The results were calculated as μmol MDA/mg of protein.

2.7.2. Western blot analysis

Western blot analysis was conducted including minor modifications, as previously described (Guerra et al., 2012). Rats were decapitated and the hippocampus was rapidly removed, dissected, and homogenized in 300 μL of ice-cold buffer (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, and 2 mM of sodium orthovanadate in 10 mM HEPES, pH 7.9), incubated on ice for 15 min, and centrifuged at 16,000 g for 45 min at 4 °C. The supernatant was reserved for posterior processing. Protein concentration was determined using the Bradford method (1976). Equivalent amounts of protein (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. Proteins were separated in 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to polyvinylidene difluoride membranes. Ponceau staining served as a loading control (Romero-Calvo et al., 2010). The membrane was blocked 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-Nrf2, anti-SOD, anti-Hsp-70, anti- p38 MAPK, anti-Bax, and anti-Bcl-2 polyclonal antibodies; 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-rabbit IgG; Santa Cruz Biotechnology, Inc.) for 10 min. Protein bands were visualized with 3,3′,5, 5′-Tetramethylbenzidine (TMB; Sigma-Aldrich). Membranes were dried, scanned, and quantified with the Scion Image PC version of the NIH image. The results were normalized by arbitrarily setting the densitometry of the control group as 100%.

2.8. Statistical analysis

The GraphPad Prism 8 software was used for statistical analysis and plotting graphs. Data were checked for normality of distribution using the Shapiro-Wilk test and homogeneity using Bartlett's test. The statistical analyses were performed by a two-way analysis of variance (ANOVA) (lutein-loaded nanoparticles versus VPA), followed by Tukey's post hoc test. Values of $P < 0.05$ were considered statistically significant. All data are expressed as the mean and S.E.M.

3. Results

3.1. Nanoparticles characterization

Thermal and spectroscopic analyses and electron microscopy imaging was carried out to characterize the components used and also the nanoparticles. Fig. 2 (A-E) presents the Transmission Electron Microscopy (TEM) images of the lutein-loaded nanoparticles (formulation NP4–0.133 g.g $^{-1}_{P407}$), the Differential Scanning Calorimetry and the Infrared spectroscopy spectra of lutein, Poloxamer 407, nanoparticles (NP1-NP4) and the lutein-Poloxamer 407 mixture. The melting of Poloxamer407 occurred at 57 °C for all samples (result not shown) while lutein presented a broad endothermic peak at 147 °C. Characteristic groups of lutein can be found at 1370 cm^{-1}, (dimethyl group) 2855 and 2921 cm^{-1} (CH_2 and CH_3 stretching vibrations), and around 3400 cm^{-1} (hydroxyl group). The CH out of the plane bending vibration was present in lutein at 963 and 826 cm ^{1} (do Prado Silva et al., 2017; Grella Miranda et al., 2020; Ranganathan et al., 2016; Silva et al., 2017). Poloxamer 407 exhibited characteristic absorption bands at 2888 cm^{-1} (C-H) and 1110 cm^{-1} (C-O) (Silva de Sá et al., 2019).

3.2. Lutein-loaded nanoparticles reversed VPA-induced social behavior deficit

Fig. 3 (B-G) shows the effect of the exposure to prenatal VPA and oral administration of lutein-loaded nanoparticles (5 mg/kg), for 14 days, on sociability and social novelty preference in the three-chamber test. The statistical analysis (two-way ANOVA) revealed a significant effect of the interaction factor (lutein-loaded nanoparticles versus VPA) on the sociability index [$F_{(1,24)} = 6.65$; $P = 0.0165$]. The post hoc comparisons demonstrated that lutein-loaded nanoparticles reversed the sociability index decrease induced by VPA in the three-chamber test (Fig. 3B).

The statistical analysis (two-way ANOVA) also revealed a significant effect for the interaction factor (lutein-loaded nanoparticles versus VPA) on the time spent in the strange 1 animal chamber [$F_{(1,24)} = 23.77$; $P < 0.0001$] and the empty chamber [$F_{(1,24)} = 15.80$; $P = 0.0006$]. The post hoc comparisons demonstrated that VPA-treated animals spent significantly less time in the strange-1 animal chamber and more time in the empty chamber, compared to the control group. However, animals treated with VPA and lutein-loaded nanoparticles spent significantly more time in the strange-1 animal chamber and less time in the empty chamber compared to the VPA group. The results suggest that lutein-loaded nanoparticles reversed the VPA-induced sociability deficit in the three-chamber test (Fig. 3 C and D).

The statistical analysis (two-way ANOVA) revealed a significant effect of the interaction factor (lutein-loaded nanoparticles versus VPA) on the social novelty preference index [$F_{(1,24)} = 6.94$; $P = 0.0145$]. The post hoc comparisons demonstrated that lutein-loaded nanoparticles reversed the social novelty preference index decrease induced by VPA in the three-chamber test (Fig. 3E). The statistical analysis (two-way ANOVA) also revealed a significant effect for the interaction factor (lutein-loaded nanoparticles versus VPA) on the time spent in the strange 2 animal chamber [$F_{(1,24)} = 27.31$; $P < 0.0001$] and the familiar animal chamber [$F_{(1,24)} = 30.69$; $P < 0.0001$]. The post hoc comparisons demonstrated that VPA-treated animals spent significantly less time in the strange-2 animal chamber and more time in the familiar animal chamber compared to the control group. However, animals treated with VPA and lutein-loaded nanoparticles spent significantly more time in the strange-2 animal chamber and less time in the familiar animal chamber compared to the VPA group. The results suggest that lutein-loaded nanoparticles reversed the VPA-induced social memory deficit in the three-chamber test (Fig. 3 F and G).

3.3. Effect of lutein-loaded nanoparticles on locomotor activity, repetitive behavior, and anxiety-like behavior induced by VPA

Fig. 4 (A-D) shows the effect of the exposure to prenatal VPA and oral administration of lutein-loaded nanoparticles (5 mg/kg), for 14 days, on the locomotor activity, anxiety-like behavior, and repetitive behavior in the open field test. The statistical analysis (two-way ANOVA) showed no significant effect of treatments on the total distance traveled [$F_{(1,24)} = 0.0028$; $P = 0.9582$] (Fig. 4A), suggesting that there was no locomotor damage to the animals. The statistical analysis (two-way ANOVA) also revealed a significant effect for the interaction factor (lutein-loaded nanoparticles versus VPA) on the time spent in the center [$F_{(1,24)} = 9.17$; $P = 0.0058$], time of immobility [$F_{(1,24)} = 8.89$; $P = 0.0065$] and grooming number [$F_{(1,24)} = 6.85$; $P = 0.0151$]. The post hoc comparisons demonstrated that VPA-treated animals spent significantly less time in the center of the apparatus, had more time of immobility, and had a higher grooming number compared to the control group. However, animals treated with VPA and lutein-loaded nanoparticles spent significantly more time in the center of the apparatus, had less time of immobility, and had a smaller grooming number compared to the VPA group. The results suggest that lutein-loaded nanoparticles reversed the anxiety-like behavior, as well as the repetitive behavior

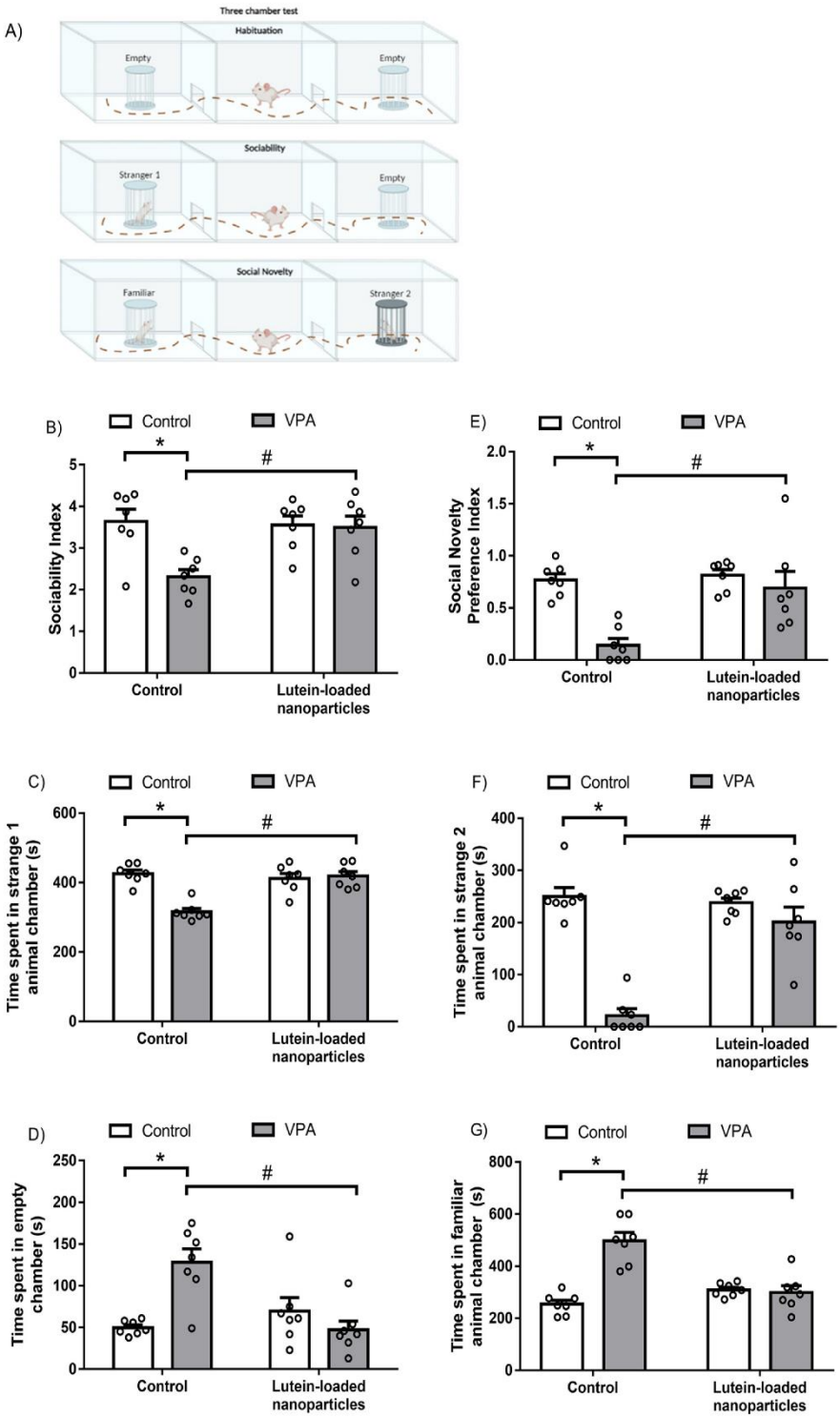

Fig. 3. A) Schematic representation of the three-chamber test used to assess sociability and social novelty preference in rodents. Exposure to prenatal VPA and oral administration of lutein-loaded nanoparticles (5 mg/kg), for 14 days, on the B) Sociability index, C) Time spent in the strange-1 animal chamber, D) Time spent in the empty chamber, E) Social novelty preference index, F) Time spent in the strange-2 animal chamber and G) Time spent in the familiar animal chamber, in the three-chamber test. Data are mean and standard error of the mean (SEM), for n = 7 animals in each group. * Indicates a significant difference ($P < 0.05$) compared to the control group. # Indicates a significant difference ($P < 0.05$) compared to the VPA group.

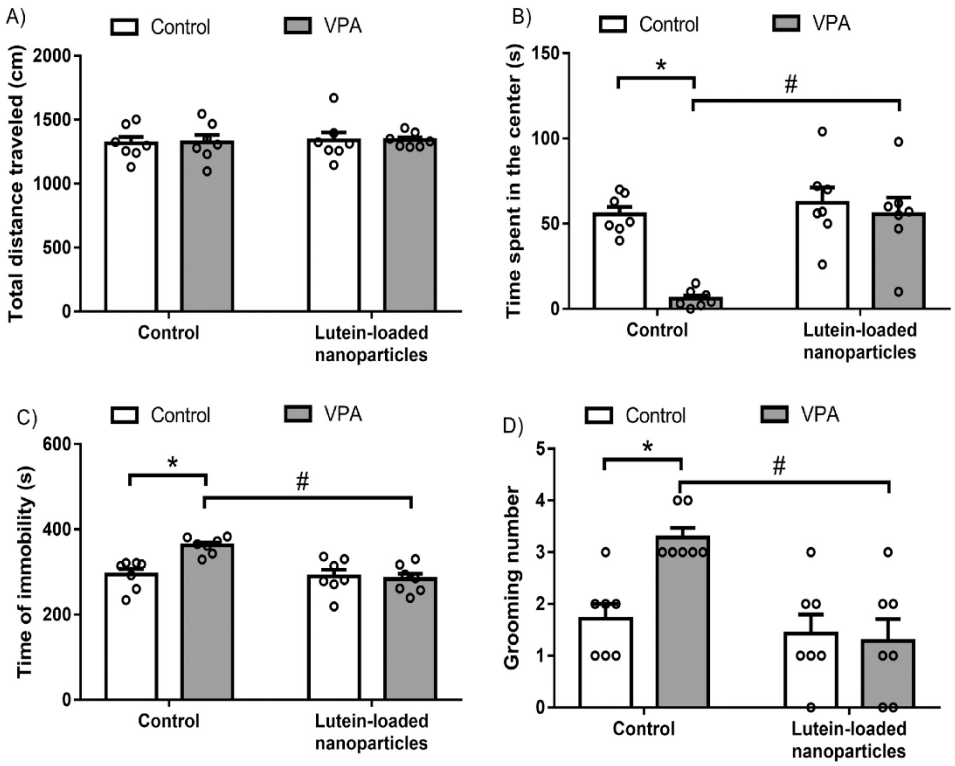

Fig. 4. Exposure to prenatal VPA and oral administration of lutein-loaded nanoparticles (5 mg/kg), for 14 days, on the A) Total distance traveled B) Time spent in the center, C) Time of immobility and D) Grooming number in the open field test. Data are mean and standard error of the mean (SEM), for n = 7 animals in each group. * Indicates a significant difference ($P < 0.05$) compared to the control group. # Indicates a significant difference ($P < 0.05$) compared to the VPA group.

induced by VPA in the open field test (Fig. 4B-D).

3.4. Lutein-loaded nanoparticles reversed VPA-induced oxidative stress indicators alterations

Fig. 5 (A and B) and 6 (A and B) show the effect of the exposure to prenatal VPA and oral administration of lutein-loaded nanoparticles (5 mg/kg), for 14 days, on oxidative stress indicators (ROS and TBARS) and western immunoblotting (Nrf2 and SOD). The statistical analysis (two-way ANOVA) revealed a significant effect for the interaction factor (lutein-loaded nanoparticles versus VPA) on the oxidative stress indicators: ROS [$F_{(1,16)} = 9.19$; $P = 0.0079$] and TBARS [$F_{(1,16)} = 6.35$; $P = 0.0227$] in the hippocampus. The post hoc comparisons demonstrated that lutein-loaded nanoparticles reversed the oxidative stress indicators increase (ROS and TBARS; Fig. 5A and B) induced by VPA in the hippocampus. The statistical analysis (two-way ANOVA) also revealed a significant effect for the interaction factor (lutein-loaded nanoparticles versus VPA) on relative intensive intensity: Nrf2 [$F_{(1,12)} = 16.06$; $P = 0.0017$] and SOD [$F_{(1,12)} = 15.09$; $P = 0.0022$] in the hippocampus. The post hoc comparisons demonstrated that lutein-loaded nanoparticles reversed the relative intensive intensity decrease of Nrf2 (Fig. 6 A) and SOD (Fig. 6B) induced by VPA in the hippocampus.

3.5. Lutein-loaded nanoparticles reversed VPA-induced apoptosis biomarkers alterations

Fig. 7 (A-D) shows the effect of the exposure to prenatal VPA and oral administration of lutein-loaded nanoparticles (5 mg/kg), for 14 days, on apoptosis biomarkers (Hsp-70, p38 MAPK, Bax, and Bcl-2). The statistical analysis (two-way ANOVA) revealed a significant effect for the interaction factor (lutein-loaded nanoparticles versus VPA) on apoptosis biomarkers: Hsp-70 [$F_{(1,12)} = 11.80$; $P = 0.0049$], p38 MAPK [$F_{(1,12)} = 9.81$; $P = 0.0086$], Bax [$F_{(1,12)} = 6.57$; $P = 0.0248$] and Bcl-2 [$F_{(1,12)} = 17.08$; $P = 0.0014$] in the hippocampus. The post hoc comparisons demonstrated that lutein-loaded nanoparticles reversed the relative intensive intensity increase of Hsp-70 (Fig. 7 A), p38 MAPK (Fig. 7B), and Bax (Fig. 7 C), as well as the relative intensive intensity decrease of Bcl-2 (Fig. 7D), induced by VPA in the hippocampus.

4. Discussion

ASD is a neurodevelopmental disorder that includes deficits in social interaction, and restricted and repetitive behavior (Modabbernia et al., 2017). Looking for candidates to prevent/mitigate the symptoms of ASD, our study evaluated the effect of lutein-loaded nanoparticles on ASD-like behaviors induced by prenatal VPA exposure in female offspring rats and the possible mechanism involved. Evidence shows that prenatal exposure of rodents to VPA induces ASD behavioral phenotypes, which are similar to patients with ASD (Kuo and Liu, 2018). Furthermore, deficits in social interaction and repetitive behavior, the major symptoms of ASD, have often been associated with anxiety (Ha et al., 2017). Our results showed that prenatal exposure to VPA induced ASD-like behaviors, such as social interaction and social memory deficits (Fig. 3), and repetitive and anxious behavior (Fig. 4) in female rats. These results confirm what was exposed by Tin-Tin Win-Shwe et al. (2018), who found a deficit of sociability and social novelty preference in both male and female exposure to VPA on day 12.5 of gestation, compared to the corresponding control group. Furthermore, increased repetitive behavior and anxiety-like behaviors in the open field test in female rats exposed to VPA have been reported (Ornoy et al., 2019; Sailer et al., 2019). In line with this view, male and female mice treated

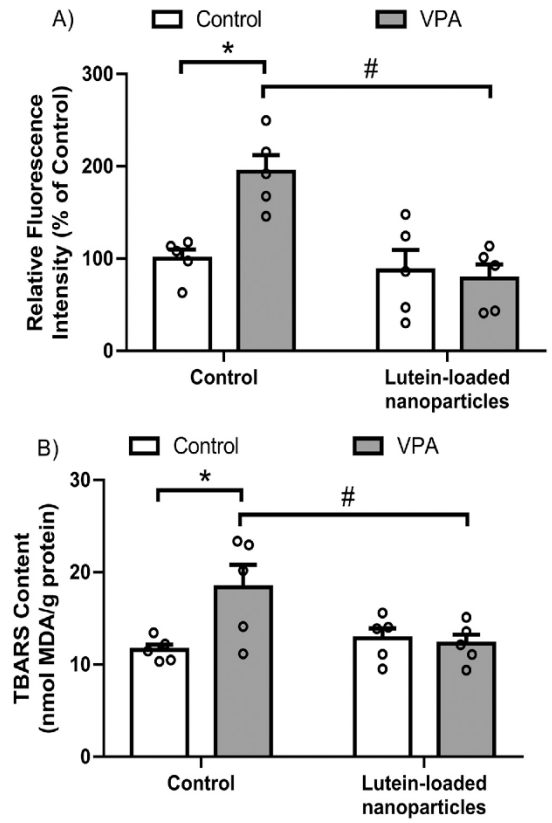

Fig. 5. Exposure to prenatal VPA and oral administration of lutein-loaded nanoparticles (5 mg/kg), for 14 days, on the oxidative stress indicators A) ROS and B) TBARS levels in the hippocampus. Data are mean and standard error of the mean (SEM), for n = 5 animals in each group. * Indicates a significant difference ($P < 0.05$) compared to the control group. # Indicates a significant difference ($P < 0.05$) compared to the VPA group.

with nicotine, in an ASD model, showed an increase in sociability in the three-chamber test and a decrease in repetitive self-grooming behavior in the open field test (Wang et al., 2015). It is also important to note that, although the VPA group spent less time in the strange animal chamber compared to the control, this group spent a long time in the strange animal chamber, corresponding to 53.5% of the total exploration time (considering all chambers). However, our results show that female offspring exposed to VPA had a social behavior deficit, confirmed by the shorter time spent in the strange animal chamber compared to the control group, regardless of the effect intensity.

In the general population, ASD is four times more often in males than in females, therefore, most studies on autistic-like behavior have mainly explored the male sex and few studies have emphasized the female as a subject. However, there are differences not only in the clinical manifestations and behavioral changes but also in the neurochemical alterations of gender-related ASD, in both humans and rodents. Studies have shown that males present ASD-like phenotypes, not observed in females. However, our results showed that prenatal exposure to VPA induced ASD-like behaviors, such as social interaction deficit and social memory deficit and repetitive and anxious behavior in female rats, which must be associated with neurochemical alterations. Therefore, gender differences in disorder characteristics might allow for missed or delayed diagnosis in females. Additionally, the aforementioned information shows the importance of developing specific studies with female subjects, allowing us to understand the disorder characteristics related to gender and the need to seek female-specific therapeutic options.

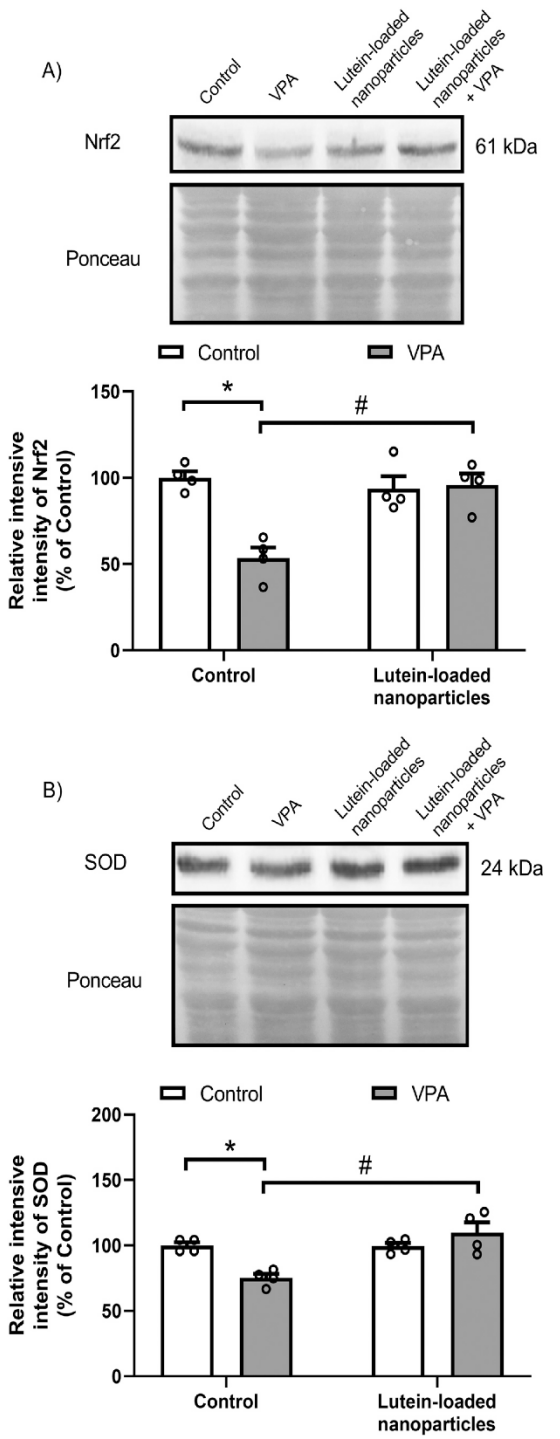

Fig. 6. Exposure to prenatal VPA and oral administration of lutein-loaded nanoparticles (5 mg/kg), for 14 days, on the representative images of Western immunoblotting and densitometry analyses of oxidative stress indicators A) Nrf2 and B) SOD in the hippocampus. The representative Ponceau-stained protein bands demonstrate equal loading. The results were normalized by arbitrarily setting the densitometry of the control group. Data are mean and standard error of the mean (SEM), for n = 4 animals in each group. * Indicates a significant difference ($P < 0.05$) compared to the control group. # Indicates a significant difference ($P < 0.05$) compared to the VPA group.

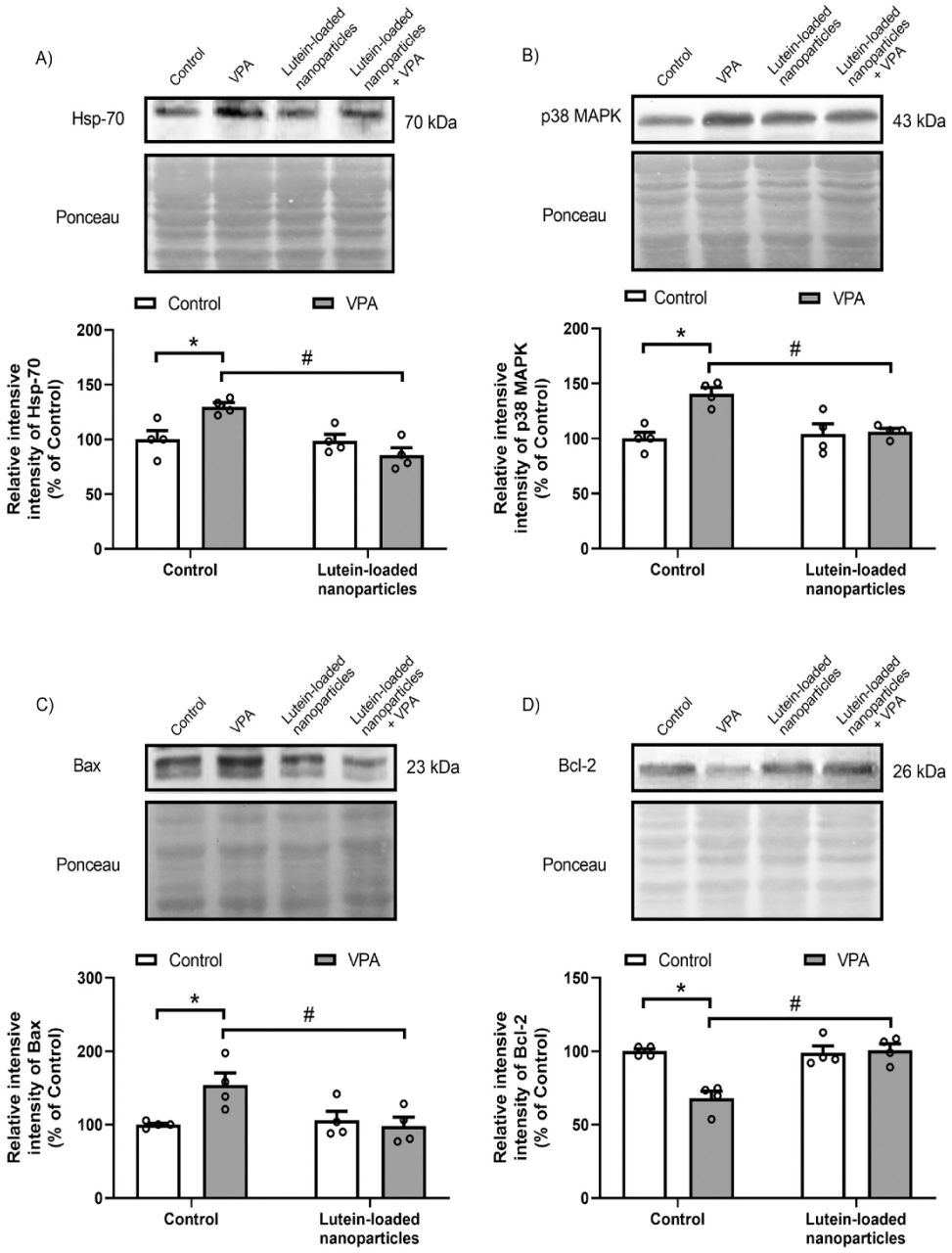

Fig. 7. Exposure to prenatal VPA and oral administration of lutein-loaded nanoparticles (5 mg/kg), for 14 days, on the representative images of Western immunoblotting and densitometry analyses of apoptosis biomarkers A) Hsp-70, B) p38 MAPK, C) Bax and D) Bcl-2 in the hippocampus. The representative Ponceau-stained protein bands demonstrate equal loading. The results were normalized by arbitrarily setting the densitometry of the control group. Data are mean and standard error of the mean (SEM), for n = 4 animals in each group. * Indicates a significant difference ($P < 0.05$) compared to the control group. # Indicates a significant difference ($P < 0.05$) compared to the VPA group.

The great finding of our study is that lutein-loaded nanoparticles reversed the behavioral damage induced by VPA. The neuroprotective effect of lutein has been described for various neurological disorders in different models and species including Huntington's disease (Binawade and Jagtap, 2013), ethanol-induced damage (Geiss et al., 2019), age-related diseases (Johnson, 2012; Kesse-Guyot et al., 2014) and Parkinson disease (Fernandes et al., 2021). Additionally, astaxanthin improved behavioral disorders in a prenatal VPA-induced mice model of autism (Al-Amin et al., 2015), and beta-carotene improved autistic-like behavior in mice (Avraham et al., 2019). However, the effect of lutein, the main carotenoid found in infants' brains, had not yet been demonstrated on ASD-like behaviors. Thus, lutein-loaded nanoparticles reversed the shorter amount of time spent in the strange animal chamber and enhanced self-grooming, as well as the less time spent in the center, suggesting that lutein-loaded nanoparticles improve the damage to sociability, social memory, repetitive behavior, and anxiety induced by

VPA in female offspring rats.

The aforementioned behavioral deficits are likely associated with low activity of antioxidant enzymes, oxidative stress, and apoptosis, which have been considered major contributing factors to ASD (Manivasagam et al., 2020). Furthermore, the increases in oxidative stress may directly induce cell death (Pangrazzi et al., 2020). Interestingly, VPA-induced oxidative stress occurrence is more pronounced in females than in males, evidenced by the increase of MDA in females only (Ornoy et al., 2019). In our study, we observed that prenatal exposure to VPA induced alterations in the oxidative stress indicators, such as increases in ROS and TBARS levels (Fig. 5), and a decrease of Nrf2 and SOD relative intensive intensity (Fig. 6), as well as changes in the apoptosis biomarkers as Hsp-70, p38 MAPK, Bax and Bcl-2 (Fig. 7) in the hippocampus of female rats. The Nrf2 relative intensive intensity decreased was associated with the decreased antioxidant enzyme SOD relative intensive intensity.

Lutein-loaded nanoparticles restored the damage induced by VPA to the neurochemical parameters assessed. This can be evidenced by the neuroprotective effect of lutein on the increases of ROS and TBARS levels, a decrease of Nrf2 and SOD relative intensive intensity, as well as increases of Hsp-70, p38 MAPK, Bax, and a decrease of Bcl-2 relative intensive intensity induced by VPA. The action of lutein as a multi-target antioxidant and anti-apoptotic bioactive compound can be the answer to the protective effect on ASD. This effect must be associated with the structural characteristics of lutein. The long carbon chain with alternating single and double bonds, which can readily accept electrons from reactive species, makes lutein act as a free radicals scavenger. Additionally, the hydroxyl group attached to each end of the molecule contributes to this property (Ahn and Kim, 2021). In this sense, a recent study has shown that lutein-loaded nanoparticles restore the decreased activity of the antioxidant enzymes SOD and catalase, as well as, the increased TBARS levels induced by rotenone in a model of Parkinson's disease in *Drosophila melanogaster* (Fernandes et al., 2021).

The increased activity of antioxidant enzymes and decreased oxidative stress may exert anti-apoptotic effects. Evidence highlights that lutein prevents the downregulation of Bcl2 expression, an anti-apoptotic protein, and the upregulation of Bax expression, a pro-apoptotic protein. Therefore, the lutein-mediated balance of Bax and Bcl-2 effectively maintains the normal apoptotic properties in neurons (Maghsoudi et al., 2021; Tan et al., 2021). Additionally, p38 MAPK is regarded as an important mediator of apoptosis, and its activation has been suppressed by lutein (Tan et al., 2021). On the other hand, Hsp-70 is responsible for protecting cells against injuries by inhibiting apoptosis. The over-expression of Hsp-70 is closely associated with oxidative stress, being an important mechanism involved in the etiology of autism (Mehta et al., 2021). An increase of Hsp-70 was observed in females exposed to VPA, this likely represents a compensatory mechanism seeking to prevent or, at least, reduce cell death in response to increased oxidative stress. Possibly, the administration of lutein counteracted the oxidative stress induced by VPA and, consequently, restored the relative intensive intensity of Hsp-70 to values close to the control group.

We also highlight that lutein-loaded nanoparticles were characterized. The particles presented an average size of (74 ± 6) nm and round morphology which is a characteristic of amorphous solid dispersions. It is worth pointing out that the mixture of Poloxamer and lutein used in the characterization analyses presented the same lutein concentration of the NP4 formulation, which is the highest concentration tested in this study. The DSC curve for the free lutein presented a broad endothermic peak at 147 °C, which is often attributed to lutein degradation (Miguel et al., 2008; Silva de Sá et al., 2019). This peak was also found in the mixture of Poloxamer and lutein but not in the nanoparticles at any of the studied concentrations. This suggests that lutein was protected from degradation at this temperature by the action of the polymeric matrix of Poloxamer. In the FTIR spectra, the attenuation of the absorption bands of lutein can be seen in comparison to the mixture of lutein and Poloxamer obtained manually. TEM, FTIR, and DSC analyses strongly suggest that Poloxamer and lutein formed a solid dispersion during the sonication step and then the amorphous solution of lutein in Poloxamer was achieved. Lutein nanoparticles were obtained using the same method as Silva et al. (2017). They demonstrated that the water solubility of lutein remarkedly increased due to the transformation into nanoparticles.

5. Conclusion

Our results support that the ability of lutein-loaded nanoparticles to inhibit oxidative stress and, consequently, to avoid apoptosis in the hippocampus of female rats must be associated with the reversal of ASD-like behaviors. The reported results promote greater knowledge about the development of ASD in females, which is still poorly reported. We believe that our study provides important insight into lutein-loaded nanoparticles as promising therapeutic agents, allowing for the identification of effective pharmacological strategies in ASD treatment in females. However, further investigation is needed to explore the mechanisms involved in the neuroprotective effects of lutein-loaded nanoparticles on ASD, as well as the possible effects in males with ASD.

CRediT authorship contribution statement

Cristini Escobar Viana: Conceptualization, Formal analysis, Investigation, Writing – original draft, Visualization. **Vandreza Cardoso Bortolotto**: Formal analysis, Investigation. **Stífani Machado Araujo**: Formal analysis, Investigation. **Mustafa Munir Mustafa Dahleh:** Formal analysis, Investigation. **Franciéle Romero Machado**: Formal analysis, Investigation. **Adson de Souza Pereira**: Formal analysis, Investigation. **Byanca Pereira Moreira de Oliveira**: Validation, Investigation. **Fernanda Vitória Leimann:** Resources, Writing – review & editing. **Odinei Hess Gonçalves:** Resources, Writing – review & editing, Visualization. **Marina Prigol:** Conceptualization, Resources, Writing – review & editing. **Gustavo Petri Guerra:** Conceptualization, Formal analysis, Resources, Writing – original draft, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

Acknowledgments

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5.2 MANUSCRITO CIENTÍFICO

Lutein-loaded nanoparticles protect against hepatotoxic damage in the offspring of female rats exposed to valproic acid

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ABSTRACT

The association between symptoms similar to autism spectrum disorder (ASD) induced by prenatal exposure to valproic acid (VPA) and possible modulations in the offspring's liver function have already been illustrated in the literature; however, possible biochemical mechanisms surrounding hepatotoxicity and how this damage occurs remain to be elucidated. In the present study, we evaluated the metabolic effect of lutein-loaded nanoparticles on energy metabolism changes derived from modulations of liver function in the offspring of VPA-exposed rats. Wistar rats of both sexes exposed to VPA (600 mg/kg) during the prenatal period were divided into two subgroups and received either lutein-loaded nanoparticles (5 mg/kg) or saline by oral gavage once daily for 14 days. At the end of the experimental design, the liver was removed for the determination of oxidative stress indicators, cell viability, metabolic markers, and apoptosis biomarkers. The plasma was used for the determination of the metabolic profile, and liver function markers. The administration of lutein-loaded nanoparticles was shown to protect against VPA-induced damage in the prenatal period in offspring rats, suggesting an antioxidant effect, by restoring the activity of antioxidant enzymes and the levels of oxidative stress markers on the liver, regulating proteins responsible for accelerating the apoptotic process. At the plasma, lutein-loaded nanoparticles reversed modulations of lipid metabolism in offspring exposed to VPA. No changes in glucose metabolism were observed in these animals. In this way, lutein-loaded nanoparticles come up as a potential multi-target compound for possible metabolic deregulations derived from VPA-exposure in offspring rats.

Keywords: Hepatotoxicity; Lutein-loaded nanoparticles; Offspring; Valproic acid.

1. Introduction

Valproic acid (VPA, 2-propylpentanoic acid) is a drug used as an antiepileptic, mood stabilizer and anticonvulsant (Lan et al., 2021). Its administration during the gestational period is potentially toxic to the offspring, presenting teratogenic effects, damage to neurodevelopment, and altering hepatic metabolism, characterizing it as a negative multi-target compound in the fetus' organism (Sargazi et al., 2021). In addition, it is considered an environmental factor for autism spectrum disorder (ASD), which is a health condition that harms development and modifies the ability of individuals to relate socially and environmentally, affecting approximately 1.5% of the population (Linnsand et al., 2021; Maenner et al., 2021).

The use of VPA as a therapy for epileptic and other neuropsychiatric disorders has demonstrated changes in lipid markers such as cholesterol and triglycerides (van Breda et al., 2018; Elnahas et al., 2021;) as well as elevated levels of intracellular reactive species (RS) in several organs, including liver tissue (Peng et al., 2022). The alteration of hepatic metabolism is one of the characteristics of VPA-induced toxicity, leading to the formation of non-alcoholic fatty liver disease, generating alterations in the cellular redox balance (Farinelli et al., 2015). Despite this, there are reports of the development of hepatic steatosis directly in the mother during the gestational period with the administration of VPA (Shafique and Winn, 2021), but there is a lack of clear and direct evidence that points to possible liver damage in the offspring since VPA has demonstrated an ability to delay the complete fetal development (Shafique and Winn, 2021). As mentioned, deregulation of cellular redox balance is suggested as a potential mechanism of toxicity for VPA (Lapehn et al., 2021), given its high potential for suppressing the nuclear factor erythroid 2–related factor 2 (Nrf2) (Zhao et al., 2022), which leads to changes in inflammatory and apoptotic biomarkers, including mitogen-activated protein kinase p38 (p38

MAPK) and protein kinase B (Akt/PKB) (Hegazy et al., 2015; Saghazadeh et al., 2019), capable of triggering tissue damage, including the liver.

Considering the increasing use of VPA, as well as its association with the gestational period, it is necessary not only to know the damage induced in the offspring but also to investigate new therapeutic targets and viable mechanisms of action that constitute alternatives for its treatment, such as bioactive compounds that have beneficial biological effects for the promotion and maintenance of health (Mitra et al., 2021). Lutein, a xanthophyll carotenoid that stimulates the immune system, is able to decrease oxidative damage and inflammatory markers, contributing to the prevention of metabolic diseases, and therefore, mitigating metabolic damage in liver tissue (Lin et al., 2015; Zheng et al., 2022). Lutein is characterized by having antioxidant, anti-inflammatory, antidiabetic, neuroprotective, and anti-apoptotic activities (Kong et al., 2021; Pereira et al., 2021). However, it has hydrophobic characteristics and its degradation is induced by light, oxygen, or heat, which limits its use and the exploitation of its biological and therapeutic activities (Syamila et al., 2019). Nevertheless, processes such as nanoencapsulation can be an alternative for compounds sensitive to oxidation and heat, improving their solubility and increasing their bioavailability and absorption (Arunkumar et al., 2013; Bhat et al., 2020).

Furthermore, considering the positive correlation between lutein's antioxidant activity, and its hepatoprotective potential, this carotenoid is a promising candidate for mitigating the liver damage induced by VPA. From this, the aim of the study was to investigate the hepatotoxic effects and metabolic changes in rat offspring caused by early exposure to VPA, associated with oral supplementation of lutein-loaded nanoparticles, a multi-target antioxidant compound.

2. Materials and Methods

2.1 Chemicals

Lutein (kindly gifted by Kemin S.A) was used in the preparation of lutein-loaded nanoparticles, which were produced according to the method described by Silva et al. (2017). Valproic acid (VPA) was obtained from Acros Organics (Acros Organics, NJ, USA). The lutein-loaded nanoparticles and VPA were dissolved in a saline solution (0.9% NaCl). All the other reagents used were analytical grade.

2.2 Animals

Male (3) and female (9) Wistar rats (90 - 120 days old) were used to composing the parental couples. The male and female offspring were used for *ex vivo* assays. All the animals were housed in a plastic acrylic cage and maintained at constant room temperature (21±1 °C) with free access to water and food under a 12:12h light:dark cycle (lights on at 07:00 h). The behavioral tests were conducted during the light phase of the cycle (from 9:00 a.m. to 5:00 p.m.). All the experiments reported in this study were conducted according to the National and International legislation (guidelines of the Brazilian Council of Animal Experimentation) (CONCEA), of the U.S. Public Health Service's Policy on Humane Care and Use of Laboratory Animals (PHS Policy) and the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978), and with the approval of the Ethics Committee for Animal Research of the Universidade Federal do Pampa (CEUA protocol nº 041/2018).

2.3 VPA-induced hepatoxicity in rat offspring

Exposure to VPA during the first two weeks of pregnancy (day 12.5) is associated with an increased risk of toxic and teratogenic effects associated with oxidative damage and apoptosis in various tissues, including the liver (Abdel-Dayem et al., 2014; Tung and Winn, 2011; Willebrords et al., 2015). Male and female rats mated overnight, in the morning the

presence of spermatozoa in the vaginal smear was confirmed and that day was considered the zero day of gestation (GD 0). On GD 12.5, the females were divided into two groups and received a single intraperitoneal (ip) injection of either VPA (600 mg/kg) or saline (1 mL/kg). The offspring were weaned on post-natal day 21 (PND 21) and only the female offspring were the subjects of this study.

2.4 Experimental design

The effect of lutein-loaded nanoparticles on hepatotoxicity induced by prenatal VPA exposure in female offspring was evaluated. On PND 21, the VPA-exposed female offspring were randomly divided into two subgroups and received either lutein-loaded nanoparticles (5 mg/kg) or saline (1 mL/kg) by oral gavage, once a day, for 14 days (PND 21-34). The liver was removed for the determination of oxidative stress indicators: reduced glutathione levels (GSH), activity of glutathione peroxidase (GPx), glutathione reductase (GR), glutathione S-transferase (GST), superoxide dismutase (SOD), catalase (CAT), and levels of reactive species (RS), thiobarbituric acid reactive substances (TBARS), protein carbonyl, Nrf2; resazurin assay for cell viability; metabolic markers: glycogen, triglyceride (TAG), alanine aminotransferase (ALT), aspartate aminotransferase (AST), total cholesterol levels, glucose-6-phosphatase (G6Pase), and activity of hexokinase and citrate synthase and apoptosis biomarkers (p38 MAPK and Akt). The plasma was used for the determination of metabolic markers: glucose, high-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL), TAG, and uric acid. The dose and administration time were selected based on a previous study, which shows that at that dose and time lutein-loaded nanoparticles administration (5 mg/kg) increases its bioavailability and absorption, in addition to its antioxidant potential used as an effective pharmacological strategy for treatments of diseases (do Prado Silva et al., 2017; Silva et al., 2017). The treatment schedule is depicted in Fig. 1.

2.5 *Ex vivo* analysis

2.5.1 Preparation of sample

The liver was removed, weighed, and homogenized in Tris–HCl (50 mM, pH 7.4) buffer. The resulting homogenate was then centrifuged at 10,000 × g for 10 min at 4ºC and the supernatant fraction (S1) was used for the determination of oxidative stress indicators, metabolic markers, apoptosis biomarkers, and cell viability. Plasma was obtained through centrifugation at 2000 × g for 10 min (hemolyzed plasma was discarded) and used for the biochemical assays of metabolic profile, and liver function markers.

2.5.2 Triglycerides, glucose, HDL-cholesterol, LDL-cholesterol, and total cholesterol levels

To measure the levels of triglyceride, glucose, HDL-cholesterol, LDL-cholesterol, and total cholesterol, a Labtest ® kit was used, following the manufacturer's instructions. Results were obtained from a standard triglyceride, glucose, HDL-cholesterol, LDL-cholesterol, and total cholesterol, respectively, with the results expressed by mg/dL.

2.5.3 Glycogen levels

To measure glycogen levels, the method of Van Handel (1965) was used, with minor modifications. For the reaction, 60 µL of KOH 30% was added to 50 µL of S1 and incubated at 100ºC for 10 minutes. Afterward, the samples were cooled at a temperature of 0-5ºC for 3 minutes, adding 50 µL of ethanol, and then they were incubated at 70ºC for 10 minutes and posteriorly cooled again to 0-5ºC. Finally, 110 µL of water and 90 µL of iodine reagent (I_2 0.1 M and KI 1.5M) were added to the samples for the detection of glycogen. The measurement

was carried out via a spectrophotometer at 460 nm, and the results were expressed as a percentage for the control/saline group.

2.5.4 **Glucose-6-phosphatase activity (G6Pase)**

For G6Pase activity, the method of Ricketts (1963) was applied. For the reaction, 150 µL of sucrose/EDTA buffer (250 mM and 1 mM, respectively) pH 7.0 was added to the supernatant, with 100 µL of S1, and the reaction was started by the addition of 50 µL of glucose-6-phosphate 50 mM and incubated immediately at 37ºC, for 30 minutes. In the end, the reaction was stopped by adding 1000 µL of trichloroacetic acid (TCA) 10%, and the samples were centrifuged at 4000 × g at 4ºC, where the pellet containing the precipitated proteins was discarded, and the supernatant containing inorganic phosphate (Pi) was used. For phosphate dosage, 200 µL of S1, 1300 µL of water, 200 µL of ammonium molybdate 2.5%, and 20 µL of ascorbic acid 2% were added. The activity of the G6Pase was obtained from the subtraction of the Pi content of the null-time blanks from each sample, and the data were expressed as nmol Pi min/mg protein.

2.5.5 **Hexokinase activity**

The hexokinase activity was verified consisting of NAD^+ reduction, based on a coupled reaction with glucose-6-phosphate dehydrogenase (BRANDSTRUP et al., 1957). The reaction mixture contained 2280 µL of Tris–magnesium chloride buffer (200 mM Tris and 20 M $MgCl_2$ - pH 8.0), 500 µL of 0.67 M glucose, 100 µL of 16 mM adenosine triphosphate (ATP), 100 µL of 6.8 mM NAD, 10 µL of 300U/mL glucose-6-phosphate dehydrogenase, and 100 µL of S1. The solution was incubated for 5 min, the absorbance was measured at 340 nm, and the results were expressed as U/mg protein.

2.5.6 **Citrate synthase activity**

For the analysis of citrate synthase activity, the method of Srere (1966) was applied, where 5 µL of S1, 20 µL of (5.5'-dithiobis (2-nitrobenzoic acid)) (DTNB) 1 mM, and 6 µL of acetyl-CoA 10 mM were added to 160 µL of water, and the first reading (basal) was performed at 412 nm in a spectrophotometer. To start the reaction, 10 µL of 10 mM oxaloacetate was added, and the reading was performed for 3 minutes, calculating the Δ by spectrophotometry at 412 nm. The results were expressed as µM/min/g tissue.

2.5.7 **ALT, AST, and uric acid**

To measure the levels of ALT, AST, and uric acid, a Labtest® kit was used, following the manufacturer's instructions. Results were obtained from a standard ALT, AST, and uric acid, respectively, with AST and ALT, represented as Units/liter, and uric acid expressed as mg/dL.

2.5.8 **Determination of Reactive Species (RS)**

To measure RS levels, the Pérez-Severiano et al. (2004) method was used. For the reaction, 34 µL of S1 was used, along with 964 µL of HEPES buffer pH 7.0, and to start the reaction, 10 µL of 2,7-dichlorofluorescein diacetate (DCFDA) 3.33 M was added, being incubated in the dark for 60 minutes. After this period, fluorescence analysis of the release of DCFH by the oxidation of DCFDA was performed, in an excitation wavelength of 488 nm and emission wavelength of 520 nm. The results were obtained and expressed as a percentage of the control DCF formation in arbitrary units (AU).

2.5.9 **Determination of thiobarbituric acid reactive substances (TBARS)**

For analysis of lipid peroxidation, the method of Ohkawa et al., (1979) was used, through TBARS, for the quantification of the malondialdehyde (MDA) released in the reaction. To measure the released MDA levels, 50 µL of S1 was added, along with 125 µL of thiobarbituric

acid (TBA) 0.8%, 12 µL of acetic acid pH 3.5 20%, 50 µL of sodium dodecyl sulfate (SDS) 8.1%, and 25 µL of water. The samples were incubated at 95ºC for 120 minutes. After cooling, the samples were analyzed via spectrophotometry at 532 nm, with the value expressed as nmol MDA/mg tissue.

2.5.10 **Protein carbonyl content (PCC)**

For the PCC analysis, the method of Levine et al. (1990) was used, with minor modifications, consisting of the detection of dinitrophenylhydrazone from the reaction of carbonyl protein and dinitrophenylhydrazine. In the analysis, 400 µL of this S1 was used, with 200 µL of 2,4-dinitrophenylhydrazine (DNPH) 10 mM, prepared from HCl 2 M. The samples were incubated in the dark for 60 minutes, being shaken every 15 minutes. After 60 minutes, 500 µL of denaturation buffer (sodium phosphate 150 mM pH 6.8 and SDS 0.1 M), 1500 µL of ethanol, and 1500 µL of hexane were added to the samples, and the samples were homogenized for 40 seconds, subsequently being centrifuged at 2400 × g at 4°C. The supernatant was discarded, and the pellet was used, being washed twice with 1000 µL of ethanol/ethyl acetate (1:1). Finally, the samples were resuspended with 1000 µL of denaturation buffer and analyzed by spectrophotometer at 370 nm. Results were reported as nmol carbonyl/mg protein.

2.5.11 **Cell viability assay**

The ability of the cell to reduce resazurin to resorufin was verified, as a fluorescence marker reaction (Franco et al., 2009). The reaction was performed by adding 180 µL of the S1, 20 µL of TRIS Buffer (pH 7.0), and 10 µL of resazurin as the reaction catalyst. The samples were incubated on a microplate for one hour, measured with a spectrophotometer at 513 nm, and the results were expressed as a percentage for the control.

2.5.12 **Superoxide dismutase (SOD) activity**

To determine the SOD activity, the method of Kostyuk & Potapovich (1984) was used, with minor modifications. For the reaction, 10 µL of S1 was used, together with 1000 µL of 0.025 M KPi/EDTA 0.1 mM pH 10, and N,N,N', N'-Tetramethylethylenediamine, and finally 50 µL of quercetin 0.15% to detect the inhibitory effect of SOD on oxidized quercetin. A spectrophotometer analysis was performed at 406 nm for 2 minutes. The enzymatic activity was expressed as U/mg protein (one unit defined as the amount of enzyme required to inhibit the rate of oxidation of quercetin by 50% at 25ºC).

2.5.13 **Catalase (CAT) activity**

For the analysis of CAT activity, the method of Aebi (1984) was used, with minor modifications. In the reaction, 50 µL of S1 was added, together with 2000 µL of 0.25 M KPi/EDTA 2.5 mM pH 7.0, Triton X-100 0.012%, and 30% of H_2O_2. The samples were analyzed via spectrophotometer at a wavelength of 240 nm for 2 minutes. The activity was expressed as U/mg protein (one unit decomposes 1 µmol H_2O_2/min at pH 7.0 and 25ºC).

2.5.14 **Glutathione reductase (GR) activity**

In the analysis of GR activity, the method of Carlberg & Mannervik (1985) was used. The reaction was based on the decay of NADPH together with the addition of GSSG, thus measuring GR activity (proportional to the consumption of NADPH). A reductase system, containing 60 mL of 0.15 M TFK/EDTA 1.5 mM pH 7.0, and NADPH 0.15 mM was used. For the analysis of GR activity, 750 µL of reductase system, and 100 µL of S1 were added. A spectrophotometer analysis was performed at a wavelength of 340 nm, at intervals of 30 seconds, for 2 minutes. Afterward, 50 µL of GSSG was added, and the spectrophotometer analysis at a wavelength of

340 nm was performed again, at intervals of 30 seconds, for 2 minutes. The Δ of both absorbances was calculated, and the results were expressed as nmol NADPH/min/mg protein.

2.5.15 **Glutathione peroxidase (GPx) activity**

For analysis of GPx activity, the method of Wendel (1981) was used, through the oxidation of GSH to oxidized glutathione (GSSG), and the dismutation of H_2O_2 in water. The measurement of GPx activity is carried out indirectly, via the decay of NADPH, which, when added to the reaction, undergoes the action of GR, forming again GSH, and the product NADP+. A reductase system was constructed using NADPH 0.15 mM, GSH 1 mM, 10 µL of GR (500 U), and 250 µL of azide 100 mM, dissolved in 20 mL of TFK buffer 100 mM pH 7.0. For the reaction 800 µL of the reductase system, 50 µL of S1, and to start the reaction, 100 µL of H_2O_2 4 mM were added to the samples. The samples were read at intervals of 30 seconds, for 2 minutes, via spectrophotometer at a wavelength of 340 nm. Results were expressed as nmol NADPH/min/mg protein.

2.5.16 **Glutathione (GSH) levels**

For the GSH levels, the method of Hissin & Hilf (1976) was adopted, with minor modifications. Briefly, 100 µL of S1 was used, with 800 µL of TFK buffer 0.1 M pH 8.0, and 100 µL of ortho-phthalaldehyde, used as a fluorophore. The samples were incubated in the dark for 15 minutes and read in a fluorescence spectrophotometer, at an excitation wavelength of 350 nm and emission wavelength of 420 nm. Results were expressed as nmol GSH/mg tissue.

2.5.17 **Glutathione-S-transferase (GST) activity**

For the analysis of GST activity, the method of Habig et al. (1974) was used. Based on the presence of GSH, a cofactor for GST, which reacts with xenobiotic compounds, detoxifying them. As xenobiotic and fluorophore, 1-chloro-2,4-dinitrobenzene (CDNB) reacts with GSH, forming 4-dinitrophenyl glutathione, determining the catalytic activity of GST. For the reaction, 30 µL of S1 was used, with 1000 µL of a system (0.25 M KPi buffer/2.5 mM EDTA pH 7.0, GSH 100 mM, and water), together with 20 µL of CDNB50 mM. The reaction was carried out by a spectrophotometer analysis at a wavelength of 340 nm, for 2 minutes. Results were expressed as nmol CDNB/min/mg protein.

2.5.18 **Western blot analysis**

Western blot analysis was conducted including minor modifications, as previously described (Guerra et al., 2012). Rats were decapitated and the liver was rapidly removed, dissected, and homogenized in 300 µL of ice-cold buffer (10 mM KCl, 2 mM $MgCl_2$, 1mM EDTA, 1 mM NaF, 10 µg/mL aprotinin, 10 mM β-glycerolphosphate, 1 mM PMSF, 1 mM DTT, and 2 mM of sodium orthovanadate in 10 mM HEPES, pH 7.9), incubated on ice for 15 min, and centrifuged at 16,000 g for 45 min at 4°C. The supernatant, denominated cytosolic fraction, was reserved for posterior processing. The pellet (P1) was resuspended in 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), incubated for 15 min on ice, and centrifuged at 16,000 X g for 45 min at 4°C. The supernatant was discarded and the pellet (P2) 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), incubated for 15 min on ice, and centrifuged at 16,000 X g for 45 min at 4^{o}C. The supernatant was considered the nuclear

fraction. The protein concentration was determined by using the Bradford method (1976). Equivalent amounts of protein (80 µg for cytosol and 20 µg for nuclear fractions) 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. Proteins were separated in 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to polyvinylidene difluoride membranes. Ponceau staining served as a loading control (data not shown) (Romero-Calvo et al., 2010). The membrane was blocked 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-p38 MAPK, anti-phospho-p38 MAPK, anti-Nrf2, anti-Akt, anti-phospho-Akt polyclonal antibodies; 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-rabbit IgG; Santa Cruz Biotechnology, Inc.) for 10 min. Protein bands were visualized with 3,3′,5,5′-Tetramethylbenzidine (TMB; Sigma-Aldrich). Membranes were dried, scanned, and quantified with the ImageJ PC version of the NIH image. The results were normalized by arbitrarily setting the densitometry of the control group as 100%.

2.5.19 **Protein levels**

The total protein levels were measured using the method of Bradford (1976), being quantified by spectrophotometry, measured at 595 nm, using a standard bovine serum albumin curve.

2.6 Statistical analysis

The GraphPad Prism 8 software was used for statistical analysis and plotting graphs. The statistical analyses were performed by a two-way analysis of variance (ANOVA) (lutein-

loaded nanoparticles versus VPA), followed by Tukey's post hoc test. Values of $P < 0.05$ were considered statistically significant. All data are expressed as the mean and S.E.M.

3. Results

3.1 Effects of oral administration of lutein-loaded nanoparticles in the offspring of female rats exposed to VPA on energetic substrates

The statistical analysis (two-way ANOVA) revealed a significant effect for the interaction factor (lutein-loaded nanoparticles versus VPA) on the energetic substrates indicators: TAG liver [$F_{(1,12)} = 15.56$, $P = 0.0019$, Fig. 2A]; Total cholesterol liver [$F_{(1,12)} = 13.54$, $P = 0.0032$, Fig. 2B]; TAG plasma [$F_{(1,12)} = 7.609$, $P = 0.0173$, Fig. 2C]; Total cholesterol plasma [$F_{(1,12)} = 12.33$, $P = 0.0043$, Fig. 2D]; LDL cholesterol [$F_{(1,11)} = 8.062$, $P = 0.0161$, Fig. 2E]; HDL cholesterol [$F_{(1,13)} = 10.41$, $P = 0.0066$, Fig. 2F]. The post hoc comparisons demonstrated that lutein-loaded nanoparticles reversed the increase in energetic substrate indicators induced by prenatal VPA exposure in female offspring in liver and blood plasma. However, the statistical analysis did not show significant differences between groups regarding glucose (Fig. 2G) and glycogen levels (Fig. 2H).

3.2 Effects of oral administration of lutein-loaded nanoparticles in the offspring of female rats exposed to VPA on metabolic enzyme activity

The statistical analysis (two-way ANOVA) did not show significant differences between groups regarding metabolic enzymes' activity: Glycose-6-phosphatase (Fig. 3A) and Hexokinase (Fig. 3B). However, the statistical analysis revealed a significant effect for the interaction factor (lutein-loaded nanoparticles versus VPA) on citrate synthase activity [$F_{(1,12)} = 13.36$, $P = 0.0033$, Fig. 3C]. The post hoc comparisons demonstrated that lutein-loaded

nanoparticles reversed the decrease in citrate synthase activity induced by prenatal VPA exposure in female offspring in the liver.

3.3 Effects of oral administration of lutein-loaded nanoparticles in the offspring of female rats exposed to VPA on liver function markers

The statistical analysis (two-way ANOVA) revealed a significant effect of the interaction factor (lutein-loaded nanoparticles versus VPA) on the liver function markers: ALT [$F_{(1,12)} = 10.50$, $P = 0.0071$, Fig. 4A]; AST [$F_{(1,12)} = 12.10$, $P = 0.0046$, Fig. 4B], and uric acid [$F_{(1,12)} = 44.25$, $P < 0.0001$, Fig. 4C]. The post hoc comparisons demonstrated that VPA exposure resulted in a marked increase in the liver function markers ALT, AST, and uric acid compared to the control group. However, the lutein-loaded nanoparticles reversed the damage induced by prenatal VPA exposure in female offspring in the liver.

3.4 Effects of oral administration of lutein-loaded nanoparticles in the offspring of female rats exposed to VPA on oxidative stress markers and cell viability

The statistical analysis (two-way ANOVA) revealed a significant effect for the interaction factor (lutein-loaded nanoparticles versus VPA) on the oxidative stress indicators: RS [$F_{(1,12)} = 13.05$, $P = 0.0036$, Fig. 5A]; TBARS [$F_{(1,12)} = 15.58$, $P = 0.0019$, Fig. 5B]; Protein carbonyl [$F_{(1,12)} = 106.2$, $P < 0.0001$, Fig. 5C] in the liver. The post hoc comparisons demonstrated that lutein-loaded nanoparticles reversed the increase in oxidative stress indicators induced by prenatal VPA exposure in female offspring in the liver. The statistical analysis also revealed a significant effect of the interaction factor (lutein-loaded nanoparticles versus VPA) on resazurin [$F_{(1,12)} = 8.77$, $P = 0.0119$, Fig. 5D]. The post hoc comparisons demonstrated that lutein-loaded nanoparticles can counteract toxicity and cellular damage by decreasing resazurin in the liver.

3.5 Effects of oral administration of lutein-loaded nanoparticles in the offspring of female rats exposed to VPA on antioxidant parameters

The statistical analysis (two-way ANOVA) revealed a significant effect of the interaction factor (lutein-loaded nanoparticles versus VPA) on the antioxidant enzymes' activity: SOD [$F_{(1,12)} = 19.05$, $P = 0.0009$, Fig. 6A], CAT [$F_{(1,12)} = 7.53$, $P = 0.0178$, Fig. 6B], GR [$F_{(1,12)} = 9.24$, $P = 0.0103$, Fig. 6C], GPx [$F_{(1,12)} = 8.73$, $P = 0.0120$, Fig. 6D], GSH [$F_{(1,13)} = 5.49$, $P = 0.0356$, Fig. 6E], and GST [$F_{(1,12)} = 10.77$, $P = 0.0066$, Fig. 6F]. The post hoc comparisons demonstrated that lutein-loaded nanoparticles reversed the decrease in antioxidant and detoxifying enzymes' activity induced by prenatal VPA exposure in female offspring in the liver.

The statistical analysis also revealed a significant effect of the interaction factor (lutein-loaded nanoparticles versus VPA) on the immunoreactivity of Nrf2 cytosolic [$F_{(1,8)} = 15.31$ $P = 0.0045$, Fig. 6G] and Nrf2 nuclear [$F_{(1,8)} = 7.89$, $P = 0.0229$, Fig. 6H]. The post hoc comparisons demonstrated that lutein-loaded nanoparticles reversed the immunoreactivity decrease of Nrf2 (cytosolic and nuclear fractions) induced by prenatal VPA exposure in female offspring in the liver.

3.6 Effects of oral administration of lutein-loaded nanoparticles in the offspring of female rats exposed to VPA the protein expression of p38 MAPK and Akt

The statistical analysis (two-way ANOVA) revealed a significant effect of the interaction factor (lutein-loaded nanoparticles versus VPA) on the immunoreactivity of Total p38 MAPK [$F_{(1,8)} = 13.42$, $P = 0.0064$, Fig. 7A], Phospho-p38 MAPK [$F_{(1,8)} = 7.05$, $P = 0.0290$, Fig. 7B] and Phospho-p38 MAPK/Total p38 MAPK ratio [$F_{(1,8)} = 20.81$, $P = 0.0018$, Fig. 7C]. The post hoc comparisons demonstrated that lutein-loaded nanoparticles reversed the

immunoreactivity increase of p38 MAPK induced by prenatal VPA exposure in female offspring in the liver.

The statistical analysis (two-way ANOVA) did not show a significant effect for the interaction factor (lutein-loaded nanoparticles versus VPA) on Total Akt immunoreactivity (Fig. 7D) and immunoreactivity of Phospho-Akt (Fig. 7E). However, it revealed a significant effect for the interaction factor (lutein-loaded nanoparticles versus VPA) on the immunoreactivity of Phospho-Akt [$F_{(1,7)} = 6.48$, $P = 0.0383$, Fig. 7E] and Phospho-Akt/Total Akt ratio [$F_{(1,13)} = 6.50$, $P = 0.03415$, Fig. 7F]. The post hoc comparisons demonstrated that lutein-loaded nanoparticles reversed the immunoreactivity increase of Akt induced by prenatal VPA exposure in female offspring in the liver.

4. Discussion

In the present study, we evaluated the effect of lutein-loaded nanoparticles in ameliorating the metabolic and hepatic impairments induced by prenatal VPA exposure in rat offspring. It is known that when VPA is injected into pregnant rats, ASD-like phenotypes are obtained (Kuo and Liu, 2018), leading to the development of changes in the energy metabolism, mainly from modulations in the hepatic (Cartocci et al., 2018b).

When administered, VPA has already had some adverse effects reported, such as teratogenic effects, however, there are not many explanations for how these damages occur (Koroglu et al., 2021) nor for whether its administration generates damages to the offspring. Based on this, in this study we demonstrate that the hepatotoxicity induced by prenatal VPA exposure occurs possibly due to dysregulation of lipid markers, leading to the accumulation of TGA and total cholesterol in the liver tissue, in a principle of formation of non-alcoholic hepatic steatosis. Furthermore, our results demonstrate that the administration of VPA during the gestational period caused dysregulations in the lipid metabolism of the offspring, with plasma

accumulation of TAG, associated with an increase in total and LDL cholesterol, with a decrease in HDL cholesterol. A possible explanation can be seen in the exposure of VPA to HepaRG cells, where due to the inhibitory properties of VPA to β-ketothiolase, changes in mitochondrial acetoacetyl-CoA thiolase were generated, causing metabolic dysregulation through modulations in the function of the electron transport chain, making the organism less efficient regarding its β-oxidation capacity (Grünig et al., 2020) . On the other hand, when verifying the association of lutein-loaded nanoparticles, we can observe a decrease in the liver damage caused by VPA in the offspring, reversing the dysregulations in the lipid metabolism.

Despite the clear changes generated by VPA in the lipid metabolism of the offspring, our results demonstrate that there are no alterations in the glucose metabolism, in which glucose and glycogen levels did not change, as well as the activity of hexokinase and G6Pase, limiting enzymes in glycolysis and gluconeogenesis, for the formation of glucose and glycogen, respectively. As found by Moreno-Sánchez et al. (2021), the expression of hexokinase II and glucose-6-phosphate dehydrogenase are increased in the VPA groups compared to the control group in HeLa cells, however, when these same markers are seen in the acetylation states, therefore, in its active catalytic form, our data are similar, without difference between groups, which helps to understand why substrates such as glucose and glycogen were not altered according to our results. Nonetheless, the citrate synthase activity, which controls the first reaction in the citric acid cycle, was decreased, causing a functional deficit in this cycle and consequently a decrease in cellular energy, since it will also affect oxidative phosphorylation and decrease energy products (NADH, $FADH_2$, and ATP), in the same way as already elucidated in HeLa cells (Moreno-Sánchez et al., 2021). On the other hand, lutein-loaded nanoparticles were able to return the normal levels of citrate synthase activity, indicating no delay in the cascade reactions of the citric acid cycle, restoring the balance of energetic reactions in offspring exposed to VPA.

In fact, the results obtained from the citrate synthase activity are in agreement with the imbalance in energetic substrates, since in VPA-exposure animals, the levels of TAG, total cholesterol (liver and plasma), and LDL cholesterol (plasma) were increased, and hepatic HDL cholesterol was decreased, as similarly found by Gong et al. (2022) in humans. A hypothesis observed in our study is that due to the low activity of citrate synthase in animals exposed to VPA during the gestational period, there is an inability to degrade TAG by β-oxidation, causing severe metabolic changes in these offspring, possibly leading to impairment in the formation of several energy equivalent substrates, such as NADH and $FADH_2$ (Zhang et al., 2014). In this sense, we can still observe that lutein-loaded nanoparticles do not allow such changes to occur, regulating citrate synthase activity, and therefore, leading to TAG degradation without further impairments. These data are similar to those found in the literature, in which lutein is able to normalize triglyceride levels in a dose-response action (Gopal et al., 2021).

Furthermore, as described by Zhu et al. (2021), acetyl-CoA molecules, formed during β-oxidation, saturate the activity of enzymes responsible for their entry into the citric acid cycle and end up being used for cholesterol formation in hepatic tissue in animals exposed to VPA, since this substance is shown to be a potential regulator of cholesterol biosynthesis in the hippocampus of mice, altering the action of HMG-CoA reductase, and allowing a greater synthesis of mevalonate from acetate (Kim et al., 2012; Cartocci et al., 2018a; Zhu et al., 2021). On the other hand, lutein-loaded nanoparticles appear to regulate cholesterol synthesis, possibly restoring the normal activity of limiting enzymes in the process of synthesizing mevalonate and other molecular equivalents and precursors (Kim et al., 2012; Soundarya Priyadharsini et al., 2018).

In the same sense, we can observe that LDL and HDL lipoproteins are altered in offspring animals exposed to VPA, indicating impairments in the transport of lipoproteins from the liver to several tissues. In the pathogenesis of ASD, changes in cholesterol and its

metabolites are also observed, which are related to tissue oxidative stress (Esposito et al., 2021). These changes are usually associated with an increase in total cholesterol, and saturated and trans fats in the liver, leading to a reduction in the amount of hepatic LDL receptors, thus preventing their return to the liver, resulting in an increase in their blood levels, which enables its accumulation in the liver and vascular endothelium, generating inflammatory and calcification processes, and forming atheromatous plaques (Xu et al., 2019). Thus, while LDL forms atheroma plaques, HDL has the function of reducing the size of the plaques, returning this cholesterol to the liver (Xu et al., 2019), however, our results show a decrease in its levels, indicating severe damage generated by exposure to VPA during the gestational period. Despite this, our data demonstrate that lutein-loaded nanoparticles are shown to be effective in regulating cholesterol levels, mainly through lutein's potential to normalize serum HDL levels, as demonstrated in monkeys supplemented with carotenoids (Jeon et al., 2018).

It is known that the aforementioned experimental model causes hepatotoxicity and teratogenicity in the offspring of animals that had VPA applied during the gestational period (Cartocci et al., 2018b), thus, in our study, this was not different, the VPA offspring animals showed an increase in the levels of AST, ALT, and uric acid, demonstrating impaired liver function, as well as liver damage and exacerbated purine metabolism, corroborating with findings in the literature (Iqbal et al., 2022). However, our data shows that lutein-loaded nanoparticles were able to reverse the damage caused by VPA in these markers, improving liver function and hepatotoxicity. Furthermore, taking into account that only these data already show us efficient mechanisms of lutein-loaded nanoparticles in improving hepatic damage by VPA, as well as cellular and energetic substrates, we went even further to try to understand how VPA can trigger possible mechanisms of oxidative stress, as well as activation of inflammation and apoptosis pathways, such as p38 MAPK and Akt, and whether lutein-loaded nanoparticles can

act to regulate possible changes induced in these mechanisms by the exposure of VPA to offspring.

In markers of oxidative stress, the prenatal offspring exposure to VPA animals had increased RS, lipid peroxidation, and protein oxidative damage, possibly associated with the free radicals generated in the liver. Consequently, VPA was shown to decrease cytosolic Nrf2, and consequently, its translocation to the cell nucleus was reduced, thus generating a decrease in the activity of antioxidant enzymes, such as SOD, CAT, GST, GPx, and GR, which led to losses in the levels of GSH, the main reducing substrate for the elimination of free radicals in the cell (Chen et al., 2013). In contrast, treatment with lutein-loaded nanoparticles in animals with VPA was able to improve and reverse losses in Nrf2 at the cytosolic level, potentiating the release of Keap1 from the Nrf2 portion, and allowing its translocation to the nucleus, besides increasing the activation of heterodimers bound to the DNA regulatory region known as ARE, and the expression of antioxidant target Nrf2 genes (SOD, CAT, GST, GPx GR), as well as their activity, which leads to an increase in GSH substrate, which is capable of eliminating free radical accumulation in the cell. Similarly, results from our research group demonstrated the lutein-loaded nanoparticles' beneficial effect on oxidative stress markers caused by Parkinson's disease model in *Drosophila melanogaster* (Fernandes et al., 2021), corroborating with our findings in the antioxidant potential of lutein.

As expected, due to impairments in the hepatic metabolism, as well as a decrease in enzymes and antioxidant substrates in hepatocytes, cell viability in prenatal offspring exposed to VPA was limited, and treatment with lutein-loaded nanoparticles was able to reverse this cell damage possibly associated with its multi-targeting ability to reverse metabolic and oxidative damage (Fernandes et al., 2021). Furthermore, the increase in apoptotic markers is in agreement with the data obtained so far, in which prenatal offspring exposed to VPA showed higher immunoreactivity of p38 MAPK and Akt. As expected, when treated with lutein-loaded

nanoparticles, p38 MAPK and Akt levels were regulated, demonstrating the anti-apoptotic action of lutein-loaded nanoparticles in the liver, which is already known to reduce inflammation, apoptosis, and oxidative stress in retinopathy (Maghsoudi et al., 2021; Viana et al., 2022), in addition to having these effects now demonstrated in liver injury.

Finally, our work helps to understand the role of lutein-loaded nanoparticles in the liver of rats exposed to VPA during the prenatal period, in which we demonstrate that the effect of VPA-induced hepatotoxicity in the offspring occurs through the modification of lipid markers, with a general impairment of the regulation of lipid metabolism, causing an increase in oxidative stress and apoptotic biomarkers, with a consequent decrease in cell viability. Furthermore, lutein-loaded nanoparticles are able to reverse this entire cascade of damage to liver cells, demonstrating their beneficial action in the regulation of energy metabolism and oxidative stress, in addition to their anti-apoptotic action in the liver of the offspring of rats exposed to VPA in the gestational period.

5. Conclusion

The results support the fact that lutein-loaded nanoparticles protected against VPA-induced damage in the prenatal period, suggesting a protective antioxidant and anti-apoptotic effect on liver tissue and blood plasma, modulating energy metabolism and oxidative stress. The currently reported data support lutein-loaded nanoparticles as a therapeutic target related to protection against VPA-induced hepatotoxic damage. However, further investigation is needed to explore the mechanisms involved in the protective effects of lutein-loaded nanoparticles against liver damage in the VPA model.

Conflict of interest: The authors declare that there are no conflicts of interest.

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Figure captions

Fig. 1. Schematic representation of the experimental design for administration of nanoparticles lutein-loaded, for 14 days, in rats' offspring exposure to VPA.

Fig. 2. Effects of prenatal offspring exposure to VPA and oral administration of lutein-loaded nanoparticles on energetic substrates in the liver and plasma blood (A) Liver TAG levels, (B) Liver total cholesterol, (C) Plasma TAG levels, (D) Plasma total cholesterol, (E) Plasma LDL cholesterol, (F) Plasma HDL cholesterol, (G) Liver Glucose levels, (H) Liver glycogen levels. Data are mean and standard error of the mean (SEM), for n = 4 animals in each group. * Indicates a significant difference ($P < 0.05$) compared to the control group. # Indicates a significant difference ($P < 0.05$) compared to the VPA group.

Fig. 3. Effects of prenatal offspring exposure to VPA and oral administration of lutein-loaded nanoparticles on metabolic enzymes in hepatic tissue. (A) G6Pase activity, B) Hexokinase activity, (C) Citrate synthase activity. Data are mean ± SEM, for n = 4 animals in each group. * Indicates a significant difference ($P < 0.05$) compared to the control group. # Indicates a significant difference ($P < 0.05$) compared to the VPA group.

Fig. 4. Effects of prenatal offspring exposure to VPA and oral administration of lutein-loaded nanoparticles on liver function. (A) ALT levels, (B) AST levels, and (C) Uric acid in the blood plasma. Data are mean ± SEM, for n = 4 animals in each group. * Indicates a significant difference ($P < 0.05$) compared to the control group. # Indicates a significant difference ($P < 0.05$) compared to the VPA group.

Fig. 5. Effects of prenatal offspring exposure to VPA and oral administration of lutein-loaded nanoparticles on oxidative stress and cell viability. (A) RS levels, (B) TBARS, (C) PCC content, (D) Cell viability. Data are mean ± SEM, for n = 4 animals in each group. * Indicates a significant difference ($P < 0.05$) compared to the control group. # Indicates a significant difference ($P < 0.05$) compared to the VPA group.

Fig. 6. Effects of prenatal offspring exposure to VPA and oral administration of lutein-loaded nanoparticles on antioxidant, detoxifying enzymes activity and immunoreactivity in hepatic tissue. (A) SOD activity, (B) CAT activity, (C) GR activity, (D) GPxactivity, (E) GSH levels, (F) GST activity, (G) Nrf2 cytosolic, (H) Nrf2 nuclear. Data are mean ± SEM, for n = 3 animals in each group. * Indicates a significant difference ($P < 0.05$) compared to the control group. # Indicates a significant difference ($P < 0.05$) compared to the VPA group.

Fig. 7. Effects of prenatal offspring exposure to VPA and oral administration of lutein-loaded nanoparticles on the representative images of Western immunoblotting and densitometry analyses of apoptosis biomarkers in hepatic tissue. (A) Total p38 MAPK, (B) Phospho-p38 MAPK, (C) Phospho-p38 MAPK/Total p38 MAPK ratio, (D) Total Akt, (E) Phospho-Akt, (F) Phospho-Akt/Total Akt ratio. The results were normalized by arbitrarily setting the densitometry of the control group. Data are mean ± SEM, for n = 3 animals in each group. * Indicates a significant difference ($P < 0.05$) compared to the control group. # Indicates a significant difference ($P < 0.05$) compared to the VPA group.

Fig. 1

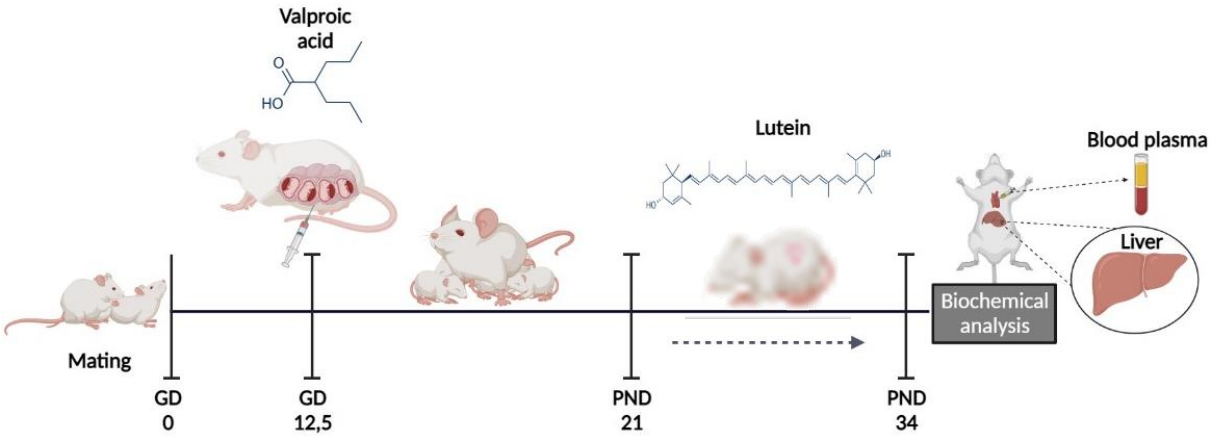

Valproic acid
Lutein
Blood plasma
Liver
Biochemical analysis
Mating
GD 0
GD 12,5
PND 21
PND 34

Fig. 2

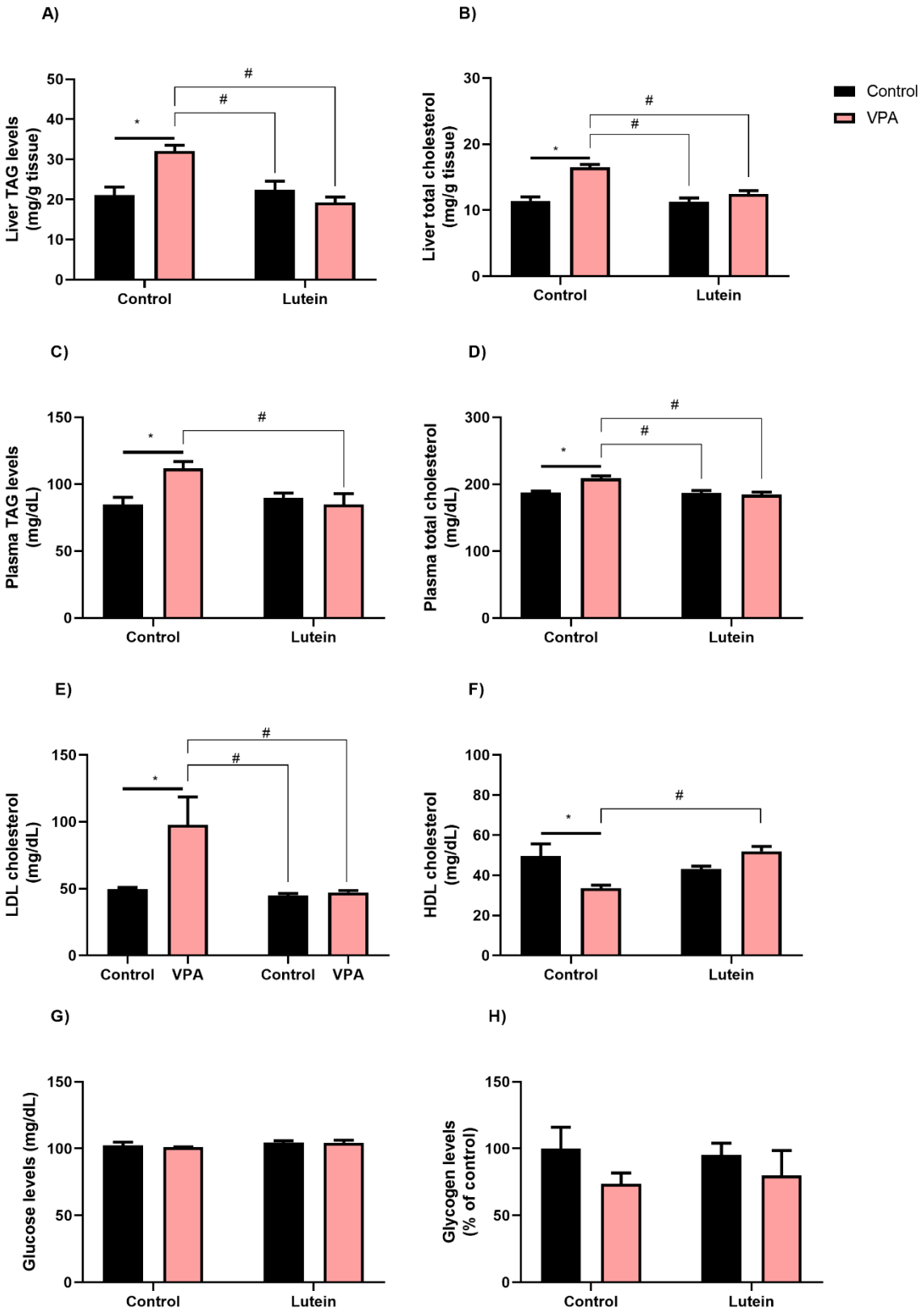

A)
Liver TAG levels (mg/g tissue)
Control
Lutein
B)
Liver total cholesterol (mg/g tissue)
Control
Lutein
Control
VPA
C)
Plasma TAG levels (mg/dL)
Control
Lutein
D)
Plasma total cholesterol (mg/dL)
Control
Lutein
E)
LDL cholesterol (mg/dL)
Control
VPA
Control
VPA
F)
HDL cholesterol (mg/dL)
Control
Lutein
G)
Glucose levels (mg/dL)
Control
Lutein
H)
Glycogen levels (% of control)
Control
Lutein

Fig. 3

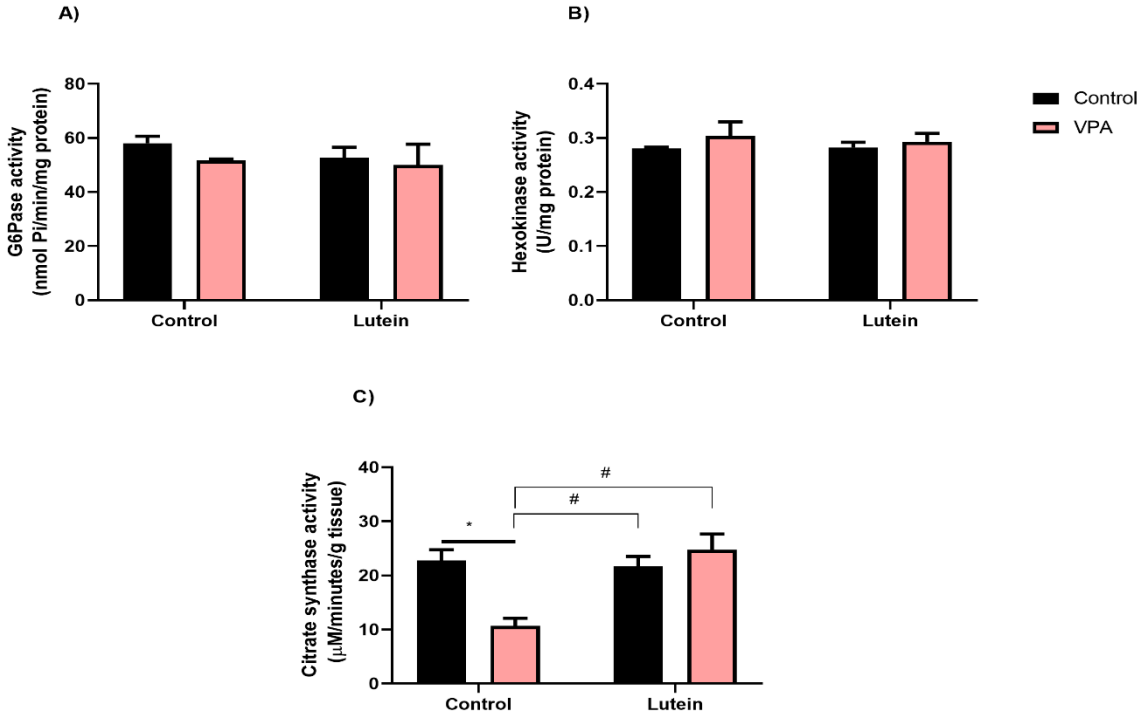

A)
G6Pase activity (nmol Pi/min/mg protein)
80
60
40
20
0
Control
Lutein
B)
Hexokinase activity (U/mg protein)
0.4
0.3
0.2
0.1
0.0
Control
Lutein
Control
VPA
C)
Citrate synthase activity (μM/minutes/g tissue)
40
30
20
10
0
*
#
#
Control
Lutein

Fig. 4

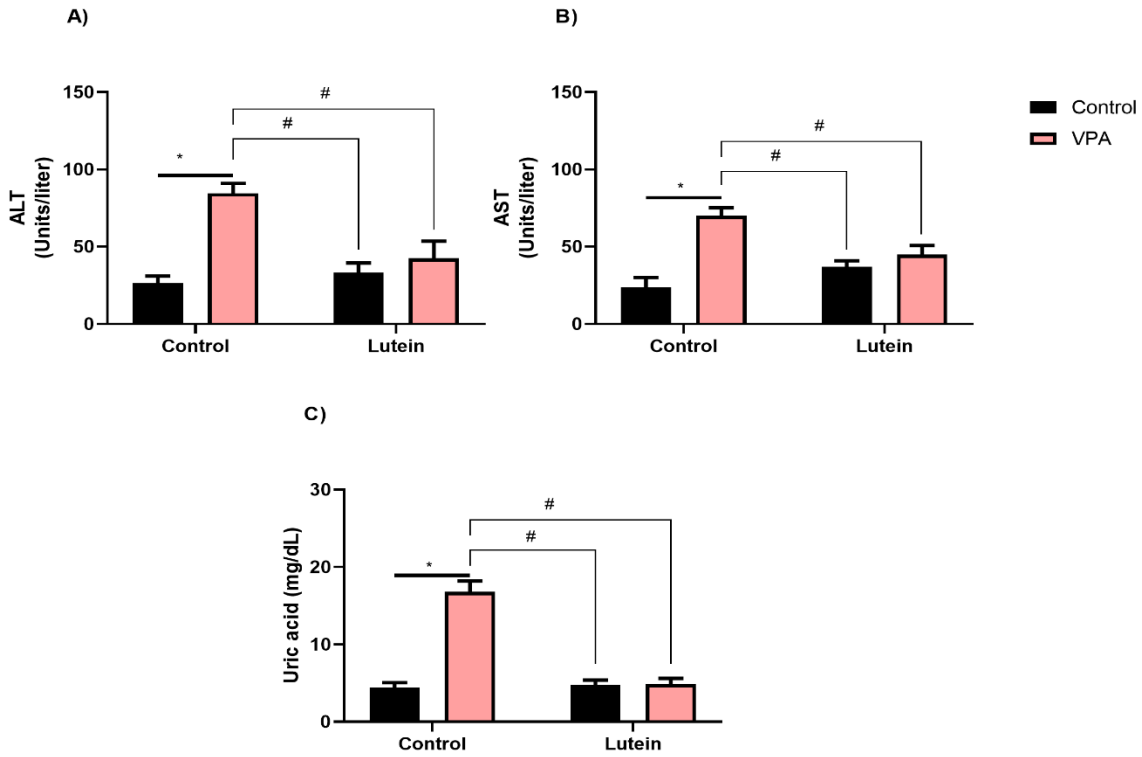

A)
ALT (Units/liter)
150
100
50
0
*
#
#
Control
Lutein
B)
AST (Units/liter)
150
100
50
0
*
#
#
Control
Lutein
Control
VPA
C)
Uric acid (mg/dL)
30
20
10
0
*
#
#
Control
Lutein

Fig. 5

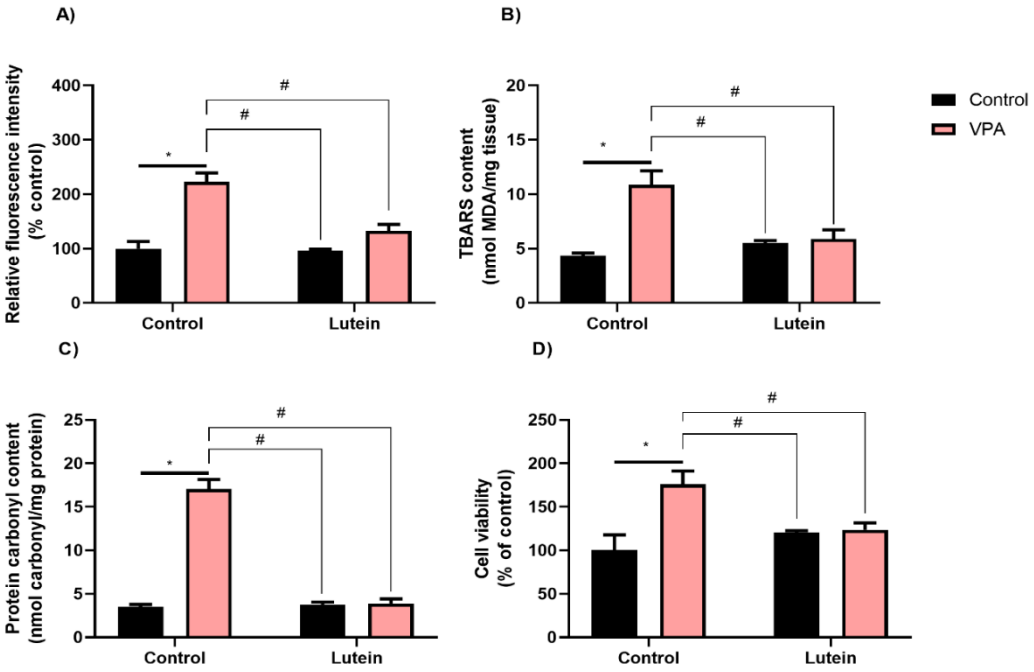

A)
Relative fluorescence intensity (% control)
400
300
200
100
0
Control
Lutein
B)
TBARS content (nmol MDA/mg tissue)
20
15
10
5
0
C)
Protein carbonyl content (nmol carbonyl/mg protein)
25
20
15
10
5
0
D)
Cell viability (% of control)
250
200
150
100
50
0
Control
VPA
*
#

Fig. 6

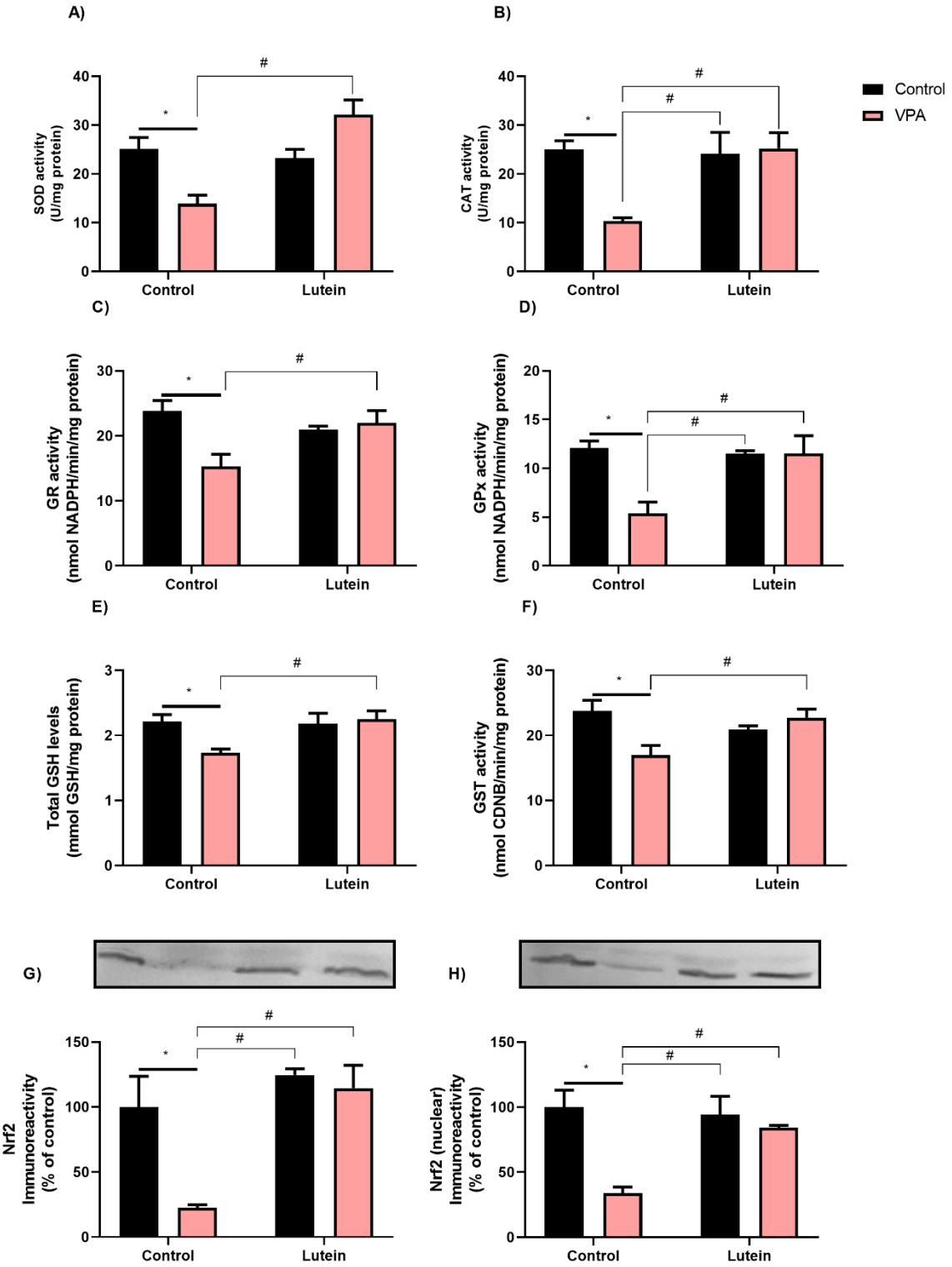
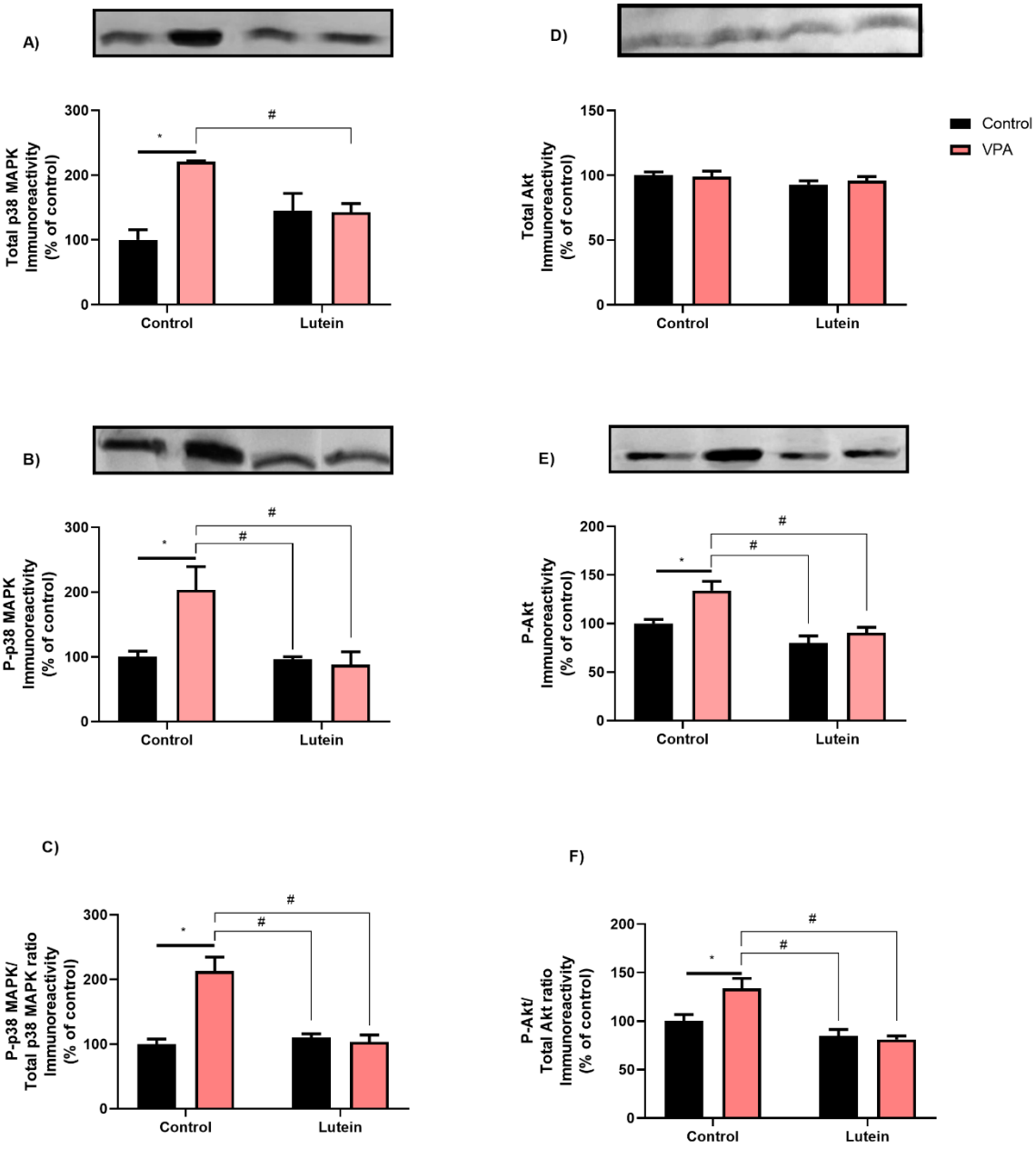
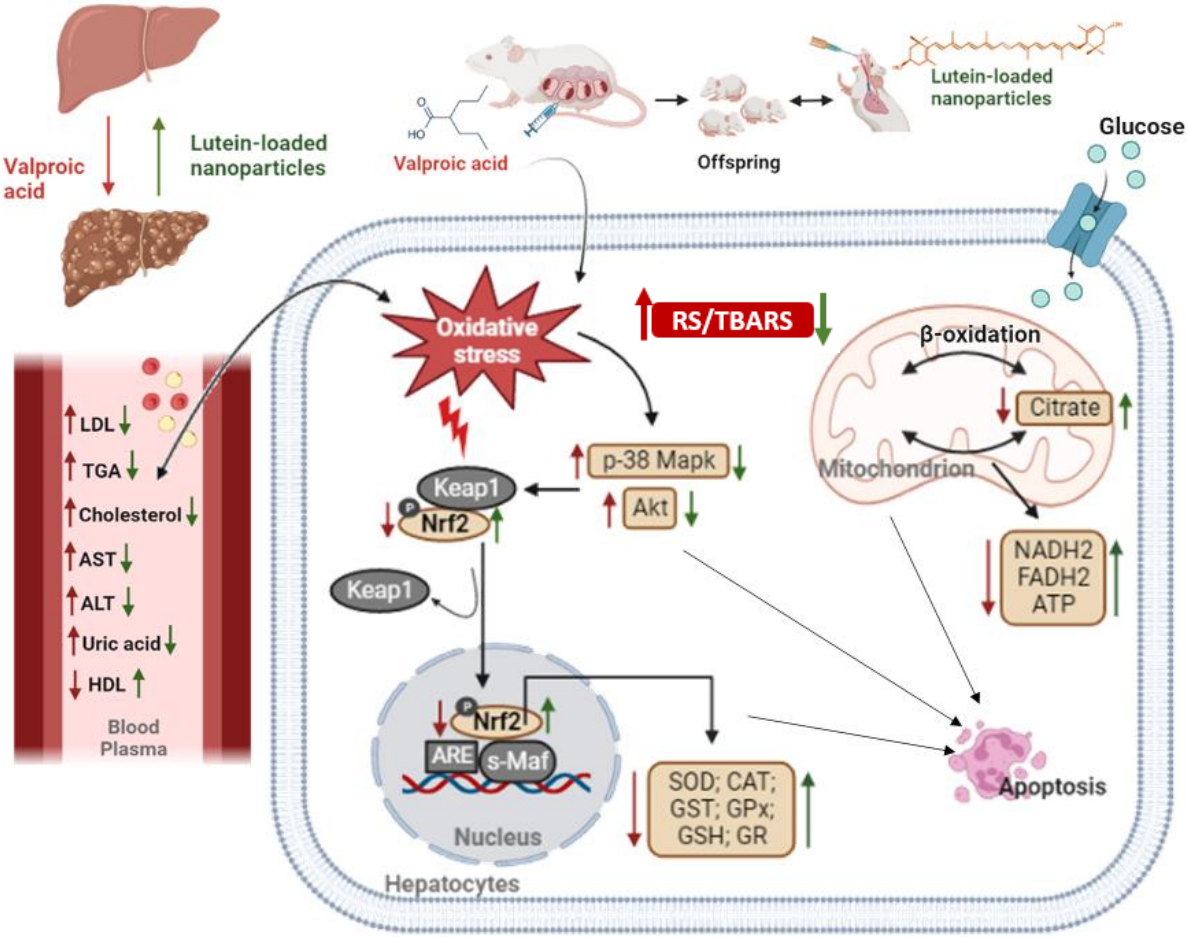

A)
B)
C)
D)
E)
F)
G)
H)
Control
VPA
SOD activity (U/mg protein)
CAT activity (U/mg protein)
GR activity (nmol NADPH/min/mg protein)
GPx activity (nmol NADPH/min/mg protein)
Total GSH levels (mmol GSH/mg protein)
GST activity (nmol CDNB/min/mg protein)
Nrf2 Immunoreactivity (% of control)
Nrf2 (nuclear) Immunoreactivity (% of control)
Control
Lutein
*
#

Fig. 7


A)
Total p38 MAPK Immunoreactivity (% of control)
300
200
100
0
Control
Lutein
*
#
D)
Total Akt Immunoreactivity (% of control)
150
100
50
0
Control
Lutein
Control
VPA
B)
P-p38 MAPK Immunoreactivity (% of control)
300
200
100
0
Control
Lutein
*
#
#
E)
P-Akt Immunoreactivity (% of control)
200
150
100
50
0
Control
Lutein
*
#
#
C)
P-p38 MAPK/ Total p38 MAPK ratio Immunoreactivity (% of control)
300
200
100
0
Control
Lutein
*
#
#
F)
P-Akt/ Total Akt ratio Immunoreactivity (% of control)
200
150
100
50
0
Control
Lutein
*
#
#

Graphical abstract



6. DISCUSSÃO

Esta tese buscou investigar o possível efeito protetor das nanocápsulas carregadas de luteína sobre os déficits comportamentais, bioquímicos, neuroquímicos e hepatóxicos induzidos pela exposição pré-natal de VPA em ratas fêmeas.

Nesta tese, que está separada em artigo e manuscrito científico, utilizamos animais do sexo feminino, pois em gravidade geral do TEA não se verifica diferença entre os gêneros masculino e feminino, assim como já foi citado por Crane *et al*. (2018).

Em um primeiro momento (Artigo), realizamos testes comportamentais padronizados para o modelo experimental utilizado, como o teste das três câmaras para analisar interação social e déficits de memória social, e campo aberto para avaliar comportamento repetitivo e do tipo ansioso. Não houveram alterações locomotoras entre os quatro grupos experimentais (grupo controle, controle/VPA, controle/nanocápsulas carregadas de luteína e VPA/ nanocápsulas carregadas de luteína. Os testes comportamentais demonstraram que a administração pré-natal do VPA induz fenótipos comportamentais de TEA em modelo animal, que se apresenta equivalente a pacientes com TEA (KUO; LIU, 2018; WIN-SHWE et al., 2018b). Assim como, estudos revelaram que em teste de campo aberto os comportamentos repetitivo e ansioso foram relevantes em ratos que foram expostos ao VPA (ORNOY et al., 2019; SAILER *et al*., 2019b).Corroborando ao nosso trabalho, estudo com ratos expostos ao VPA em período pré-natal foi verificado comportamento ansioso e normalidade na atividade locomotora geral no teste de campo aberto (HA *et al*., 2017).

Na presente tese demonstramos que a administração de VPA no período pré-natal induziu nos filhotes fêmeas da prole, déficits de interação social, memória social, comportamento repetitivo e ansioso, estes comportamentos podem estar associados ao medo, ansiedade ou compreensão prejudicada de comunicação com os animais estranhos. Em contraste, estes déficits foram atenuados pela ação das nanopartículas carregadas de luteína, provavelmente devido a sua capacidade em atravessar a barreira hematoencefálica, atingindo assim, alvos terapêuticos cerebrais (XU; LIN,

2015). Além disso, a atividade neuroprotetora da luteína já foi relatada em outros distúrbios neurológicos e em diversos modelos experimentais como a doença de Huntington (BINAWADE; JAGTAP, 2013), déficits induzidos por etanol (GEISS et al., 2019), problemas relacionados à idade (JOHNSON, 2012), doença de Parkinson (FERNANDES et al., 2021), e comportamento semelhante aos distúrbios do TEA (VIANA et al., 2022). Da mesma forma, carotenoides como a astaxantina e o beta-caroteno melhoraram os distúrbios comportamentais em um modelo de autismo induzido pela exposição prematura ao VPA (AL-AMIN et al., 2015; AVRAHAM et al., 2019). Como as nanocápsulas carregadas de luteína possuem potencial antioxidante, podem ser responsáveis pela ação ansiolítica. Como a ansiedade está relacionado ao estresse oxidativo (FEDOCE et al., 2018), podemos inferir que as nanocápsulas carregadas de luteína podem mitigar essa condição por modular o estresse oxidativo.

Em nosso estudo, percebemos que a exposição pré-natal ao VPA induziu alterações no estresse oxidativo, pois obtivemos aumento dos níveis de ROS e TBARS, diminuição da imunorreatividade de Nrf2 e consequentemente da enzima antioxidante SOD. Percebemos que estes dados interferem no desenvolvimento cerebral prematuro, ocasionando danos neuronais no hipocampo, refletidos no comportamento semelhante ao TEA. Da mesma forma, que o aumento do estresse oxidativo pode contribuir para o desenvolvimento do TEA (PANGRAZZI; BALASCO; BOZZI, 2020). Em mulheres tratadas com VPA tiveram aumento do MDA e consequentemente maior ocorrência de estresse oxidativo (ORNOY et al., 2019). Nosso estudo também obteve regulação positiva nos biomarcadores de apoptose como Hsp-70, p38 MAPK, Bax e negativa em Bcl2 no tecido hipocampal de ratas. Uma das respostas celulares ao dano oxidativo é a morte celular, os quais estão relacionados aos distúrbios no SNC, como depressão, ansiedade e TEA. Assim, o desequilíbrio celular entre o estresse oxidativo e a ação antioxidante danifica as células cerebrais (COBLEY; FIORELLO; BAILEY, 2018). Elucidando que o tecido cerebral é fortemente suscetível ao estresse oxidativo.

A ação antioxidante multialvos das nanocápsulas carregadas de luteína podem ser a resposta as modulações do estresse oxidativo, apoptose e efeito protetor ao TEA. O potencial antioxidante da luteína está associado à sua estrutura química, pois apresenta duplas ligações ao longo da cadeia de carbonos, além do grupo hidroxila

prontos para receber radicais livres (AHN; KIM, 2021). Foi analisado em estudo, que as nanopartículas carregadas com luteína modulam SOD e catalase, bem como TBARS na doença de Parkinson, induzida por rotenona em *Drosophila melanogaster* (FERNANDES et al., 2021).

As nanocápsulas carregadas de luteína estabeleceram equilíbrio entre a expressão de Bax e Bcl2, mantendo as propriedades normais apoptóticas dos neurônios. Já que Bcl2 é um regulador anti-apoptótico, enquanto Bax é um regulador pró-apoptótico (WANG et al., 2015). O equilíbrio na expressão entre a Bcl2 e Bax desempenha um papel importante na manutenção da morfologia e funcionamento celular. Nossos resultados corroboram com estudos mediados por luteína (MAGHSOUDI et al., 2021; TAN et al., 2021). Além disso, a luteína suprimiu um importante mediador de apoptose o p38 MAPK em modelo animal (TAN et al., 2021). Por outro lado, um aumento de Hsp-70, responsável por proteger as células de lesões, foi observado em fêmeas expostas ao VPA. Como cita Mehta *et al*. (2021), descrevendo que o Hsp-70 está associado ao estresse oxidativo, pré-requisito à etiologia do TEA.

Destacamos também, a caracterização das nanopartículas carregadas com luteína, as quais apresentaram tamanho médio de (74 ± 6) nm e morfologia arredondada característica, além disso, a luteína foi protegida por matriz polimérica do Poloxamer, a qual aumentou sua solubilidade. Este método foi utilizado por Silva *et al*. (2017) e administrado em nanocápsulas carregadas de luteína. Através dos dados da literatura, as nanocápsulas carregadas de luteína possuem maior potencial de solubilidade em água, o que proporciona melhor biodisponibilidade no organismo.

Em conjunto estes dados do primeiro estudo forneceram novas evidências que demonstram que o tratamento com nanocápsulas carregadas de luteína foi capaz de reverter o déficit comportamental semelhante ao TEA e os parâmetros bioquímicos induzidos pela administração pré-natal ao VPA em ratas fêmeas.

Em um segundo estudo (Manuscrito), partimos da ideia que a administração pré-natal ao VPA causa TEA, investigamos seu potencial tóxico, já que é descrito por induzir dano hepático e verificamos que a prole de ratas fêmeas apresentou

hepatotoxicidade relacionada ao efeito do VPA. Ao mesmo tempo, verificamos a possível atuação protetora aos hepatócitos pelas nanocápsulas carregadas de luteína. Neste sentido, realizamos análises bioquímicas no tecido hepático da prole e demonstramos que ocorreu hepatotoxicidade induzida pela exposição pré-natal ao VPA, iniciando sua cascata metabólica pela desregulação de marcadores lipídicos. Também foi verificado que a exposição ao VPA aumentou ROS, peroxidação lipídica e dano oxidativo proteico, possivelmente vinculados aos radicais livres em excesso no tecido hepático.

Nossos resultados demonstraram que a administração de VPA durante o período gestacional causou na prole alterações no metabolismo lipídico, aumento do TG e colesterol total e LDL, e diminuição do HDL. Estudo revela que os níveis séricos de triglicerídeos são aumentados com o uso prolongado de VPA (AKSOY et al., 2015). Estes resultados corroboram com Grünig *et al.* (2020) que explica que a exposição ao VPA reduz a capacidade de β-oxidação por alterar a acetoacetil-CoA tiolase mitocondrial desregulando a cadeia de transporte de elétrons. Da mesma forma, estudo relaciona o uso do VPA em caso de hipoadiponectinemia, com a resistência à insulina (SIDHU; SRINIVAS; SADHOTRA, 2017). O uso do VPA é indicado para epilepsia, porém em casos de mulheres obesas, seu uso deve discorrer maior atenção.

Observamos nos descendentes expostos ao VPA, alteração das lipoproteínas LDL e HDL, prejudicando transporte sanguíneo de lipoproteínas do fígado para os tecidos, também demonstrando alteração do colesterol e seus metabólitos, propiciando aos diversos tecidos estresse oxidativo, como também citado por Esposito *et al*. (2021) na patogênese do TEA. Apesar disso, Jeon *et al*. (2018) descreveram que os carotenóides são capazes de modular positivamente os níveis de colesterol e HDL em modelo de primata. Igualmente como cita Gopal *et al*. (2021), descrevendo que a luteína é capaz de normalizar os níveis de triglicerídeos. Além disso, os níveis de HDL circulantes podem ser regulados pela administração de luteína em idosos (GHASEMI et al., 2023). Essas alterações foram moduladas pelo tratamento com nanopartículas carregadas de luteína, enfatizando seu potencial antioxidante.

Em nossas análises, não houve alteração da atividade da hexoquinase e G6Pase e consequentemente nos níveis de glicose e glicogênio. Da mesma forma como citado por Moreno-Sánchez *et al*. (2021), que descreve que a administração do VPA desregulou positivamente a expressão da hexoquinase II e da glicose-6-fosfato desidrogenase. Porém, houve diminuição na atividade da citrato sintase, afetando a fosforilação oxidativa e reduzindo consequentemente os produtos energéticos. Alterações no metabolismo energético estão ligadas a distúrbios neurológicos como desordem bipolar e TEA (FEIER et al., 2013). Assim como, a insuficiência hepática induzida por VPA deve ser considerada um fator de risco por desencadear doenças mitocondriais (KRÄHENBÜHL et al., 2000).

Contudo, as nanopartículas carregadas com luteína foram capazes de modular a atividade da citrato sintase, restaurando o ciclo do ácido cítrico e reequilibrando o aporte energético nos hepatócitos na prole exposta ao VPA. Da mesma forma, estudo demonstra aumento da citrato sintase em ratos tratados com luteína (PADMANABHA; VALLIKANNAN, 2020). Han *et al.* (2015) destaca estimulação da β-oxidação de ácidos graxos e gasto de energia em modelo animal com uso de luteína. A utilização de carotenoides auxilia na elevação do gasto energético, estimulando a adiposidade em adultos de 45-65 anos de idade (HAJIZADEH-SHARAFABAD et al., 2021).

Em nosso experimento foi possível analisar hepatotoxicidade pelo aumento dos níveis de AST, ALT e ácido úrico, induzido pela administração de VPA. Igualmente como relata Cartocci *et al.* (2018) vinculando os sintomas de TEA com a teratogenicidade na prole de animais que tiveram a aplicação de VPA. Igualmente no tratamento com VPA em ratos, houve aumento no nível de ALT ou AST e uma diminuição na viabilidade celular (MA et al., 2020). Estes dados sugerem que há acúmulo de lipídios e citotoxicidade induzida pela administração do VPA. Ainda em nosso trabalho, a administração pré-natal de VPA na prole de ratas fêmeas, demonstrou aumento da imunorreatividade de p38 MAPK e Akt, que são marcadores das vias de apoptose. Resultado que corrobora com Chen *et al*. (2011) que demonstra associação do VPA com as cascatas de MAPK e Akt que são evidentes já no avanço da morte celular.

Porém, nosso tratamento com nanopartículas carregadas de luteína reverteu os níveis de AST, ALT e ácido úrico, ilustrando melhora no dano hepático, mitigando a

hepatotoxicidade induzida por VPA. Como também regulou p38 MAPK e Akt, que são marcadores das vias de apoptose, aumentados na prole pelo VPA. A luteína atenuou o dano oxidativo e a apoptose por via Akt em células (CHEN et al., 2022). O tratamento com luteína em células tumorais, afeta vias de sinalização associadas à sobrevivência celular e apoptose, incluindo a imunorreatividade de MAPK e Akt (ZHANG et al., 2022).

Como exposto até o momento, a administração pré-natal ao VPA causa hepatotoxicidade, pois desencadeia possíveis mecanismos de estresse oxidativo, bem como a ativação das vias de apoptose e o tratamento com nanopartículas carregadas de luteína foi capaz de reverter esses danos. Além disso, as enzimas antioxidantes, como SOD, CAT, GST, GPx e GR foram diminuídas na prole em exposição ao VPA, bem como a expressão do gene alvo antioxidante Nrf2 e o substrato redutor na eliminação de radicais livres GSH. Estes resultados corroboram com (CHEN et al., 2013) que elucida conexão entre doenças hepáticas e distúrbios na homeostase do GSH. Em contraste, o tratamento com nanopartículas carregadas de luteína em animais com VPA reverteu o Nrf2, aumentando o potencial antioxidante das células e aumentando o substrato GSH, mais uma vez demonstrando potencial terapêutico das nanopartículas carregadas de luteína. Assim como mostra estudo do nosso grupo de pesquisa, onde as nanopartículas carregadas de luteína foram capazes de reverter os marcadores de estresse oxidativo induzido pelo modelo da doença de Parkinson em *Drosophila melanogaster* (FERNANDES et al., 2021).

Não podemos excluir que há diversas moléculas que podem ter sido ativadas e inibidas nestes sistemas, contudo, com os resultados obtidos conseguimos demonstrar que as nanocápsulas carregadas de luteína foram capazes de reverter a cascata de danos aos hepatócitos, regulando o metabolismo energético, o estresse oxidativo, inflamação e apoptose no fígado da prole de ratos expostos ao VPA no período gestacional.

Em suma, nossos estudos possuem um papel importante, fornecendo novas evidências sobre os efeitos tóxicos na prole de ratas fêmeas induzidas pela administração do VPA, ainda, traz as nanocápsulas carregadas de luteína como uma

alternativa no tratamento do déficit de sintomas comportamentais semelhantes ao TEA e a hepatotoxicidade.

7. CONCLUSÃO

Baseado nos resultados realizados nesta tese pode-se concluir que:

Artigo

1. A administração pré-natal de VPA na prole desencadeou dano comportamental semelhante ao TEA, e as nanocápsulas carregadas de luteína conseguiram reverter esses déficits da interação social, comportamento repetitivo, memória social e ansiedade.
2. As nanocápsulas carregadas de luteína modularam os marcadores de estresse oxidativo e apoptose no hipocampo de ratas fêmeas afetadas pela administração de VPA no momento de sua gestação.
3. As nanocápsulas carregadas de luteína reverteram a atividade antioxidante SOD e CAT no hipocampo de ratas fêmeas submetidas ao modelo experimental induzido por VPA.
4. Os resultados relatados promoveram maior conhecimento sobre o desenvolvimento do TEA em ratas fêmeas, que ainda é pouco relatado.

Manuscrito

1. As nanocápsulas carregadas de luteína foram capazes de reverter as alterações gerais do metabolismo lipídico, causado pelo estresse oxidativo e biomarcadores de apoptose em ratas fêmeas induzidas pela exposição pré-natal ao VPA.
2. O aumento dos marcadores ALT e AST indicaram hepatotoxicidade induzida pela administração pré-natal de VPA na prole, e as nanocápsulas carregadas de luteína foram capazes de reverter esses marcadores de dano hepático.
3. As nanocápsulas cteína também alteraram a expressão de Nrf2 possibilitando maior proteção da atividade antioxidante da célula, a qual estava afetada pela administração do VPA na prole de ratas fêmeas.

Por fim, inferimos que as nanocápsulas carregadas de luteína podem ser utilizadas como estratégia terapêutica natural para o tratamento das consequências neurológicas e hepáticas causadas pela administração pré-natal de VPA na prole, por modular os distúrbios comportamentais semelhante ao TEA, estresse oxidativo, apoptose e metabolismo lipídico.

8. PERSPECTIVAS FUTURAS

A fim de alcançar um melhor entendimento sobre os mecanismos de ação das nanocápsulas carregadas de luteína sobre as alterações comportamentais, neurológicas e hepatotóxicas em modelo comportamental de TEA induzido pela administração de VPA em modelo animal, este trabalho terá continuidade. A seguir, algumas perspectivas futuras:

- Avaliar o efeito da administração oral de nanocápsulas carregadas de luteína sobre o comportamento locomotor, exploratório, de autolimpeza, interação e memória social após a indução do modelo comportamental de TEA em ratos machos.
- Avaliar marcadores de estresse oxidativo no córtex de animais submetidos a administração oral de nanocápsulas carregadas de luteína ao modelo comportamental de TEA induzido pela administração de VPA.
- Determinar os níveis de luteína no hipocampo e córtex cerebral de animais submetidos a administração oral de nanocápsulas carregadas de luteína em modelo comportamental de TEA.
- Avaliar a imunoreatividade do BDNF, TrkB, Akt e CREB no hipocampo e córtex de ratos machos e fêmeas submetidos a administração oral de nanocápsulas carregadas de luteína em modelo de TEA induzido por VPA.
- Avaliar o efeito da administração de ANA-12, um antagonista seletivo do receptor TrkB, sobre a possível melhora da desordem comportamental semelhante ao TEA pela administração oral de nanocápsulas carregadas de luteína em modelo de TEA induzido por VPA.

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ANEXO

Comitê de Ética no uso de Animais (CEUA)

MINISTÉRIO DA EDUCAÇÃO
FUNDAÇÃO UNIVERSIDADE FEDERAL DO PAMPA
(Lei nº 11.640, de 11 de janeiro de 2008)

Pró-Reitoria de Pesquisa, Pós-Graduação e Inovação (PROPII)

CEUA

COMISSÃO DE ÉTICA NO USO DE ANIMAIS - CEUA

Fone: (55)3911-0200. E-mail: ceua@unipampa.edu.br

CERTIFICADO DE APROVAÇÃO DE PROTOCOLO PARA USO DE ANIMAIS EM PESQUISA

Número de protocolo da CEUA: 041/2018

Titulo: Efeito de nanopartículas de luteína sobre o modelo experimental de transtorno do espectro autista induzido por ácido valpróico em ratos

Data da aprovação: 21/11/2018

Período de vigência do projeto: 21/11//2020

Pesquisadores(a): Gustavo Petri Guerra

Campus: Itaqui

Telefone: (55) 98140-9003

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CEUA

Finalidade	**( ) Ensino (X) Pesquisa**
Espécie/Linhagem/Raça	Ratos Wistar
Nº de animais	156
Peso/Idade	250 g / 90 dias (9 machos e 36 fêmeas) 50 - 60 g / 21 dias (120 machos)
Sexo	Machos e Fêmeas
Origem	Biotério Central da UFSM

Comissão de Ética em Uso de Animais

Cátia A. Siverberg

Profª. Drª. Cátia Aline Veiverberg
Coordenadora Substituta CEUA/UNIPAMPA