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Programa de Pós-Graduação em Biotecnologia



Tese

O uso de um selenilindol na busca pela felicidade

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O uso de um selenilindol na busca pela felicidade

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Para meus pais e meu irmão, os quais me deixaram voar desde cedo, mas nunca saíram do meu lado.

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“People are usually afraid of change because they fear the unknown. But the single greatest constant of history is that everything changes.”

— Yuval Noah Harari

“I hope that in this year to come, you make mistakes. Because if you are making mistakes, then you are making new things, trying new things, learning, living, pushing yourself, changing yourself, changing your world. You're doing things you've never done before, and more importantly, you're doing something.”

— Neil Gaiman

Resumo

CASARIL, Angela Maria. **O uso de um selenilindol na busca pela felicidade.** 2020. 276f. Tese (Doutorado) - Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

A depressão é o transtorno psiquiátrico mais prevalente no mundo, afetando aproximadamente 300 milhões de pessoas. A fisiopatologia desse transtorno ainda não foi completamente elucidada, possivelmente por ser multifatorial e heterogênea. No entanto, evidências sugerem que a disfunção na neurotransmissão monoaminérgica e glutamatérgica, hiperativação do eixo hipotálamo-pituitária-adrenal, inflamação e estresse oxidativo estão associados com as alterações comportamentais observadas nos pacientes. Apesar da relevância socioeconômica, 40% dos pacientes depressivos não apresentam melhora sintomatológica com os medicamentos disponíveis. Dessa forma, considerando o caráter multifatorial da depressão e a necessidade de tratamentos mais eficientes, torna-se clara a importância da procura por moléculas multialvo com efeito antidepressivo. Estudos iniciais realizados pelo nosso grupo de pesquisa reportaram que o composto 3-[(4-clorofenil)selenil]-1-metil-1*H*-indol (CMI) apresentou efeito antioxidante e protegeu camundongos contra o comportamento tipo-depressivo induzido pela inflamação. Essa molécula de caráter híbrido, combina os efeitos farmacológicos do átomo de selênio com o grupo indólico. Assim, a necessidade de fármacos mais eficientes para a depressão aliada ao promissor efeito do CMI nos impulsionou a aprofundar o conhecimento a respeito da capacidade do CMI em reverter o comportamento tipo-depressivo em diferentes modelos animais. No capítulo 1 desta tese, descreveu-se o potencial do CMI em neutralizar oxidantes derivados da inflamação. No capítulo 2, explorou-se a capacidade do CMI em reverter o comportamento tipo-depressivo em camundongos submetidos ao estresse agudo de restrição, e sua capacidade de reverter a neuroinflamação e o estresse oxidativo nos córtex pré-frontais e hipocampus dos camundongos. No capítulo 3, investigou-se a capacidade do CMI em reverter o comportamento tipo-depressivo e tipo-ansiosogênico induzido pela inflamação aguda, através da modulação do sistema serotoninérgico. No capítulo 4, observou-se que o CMI reverteu o comportamento tipo-depressivo e o déficit cognitivo em camundongos portadores de tumor mamário, através da modulação do estresse nitro-oxidativo e da neuroinflamação. No capítulo 5, constatou-se que o efeito tipo-antidepressivo e tipo-ansiolítico do CMI depende da ativação da via do BDNF/mTOR. No capítulo 6, descobriu-se que o CMI melhorou o comportamento tipo-depressivo, tipo-ansiosogênico e o prejuízo cognitivo em camundongos que sobreviveram a sepse, através da modulação de parâmetros sanguíneos, estresse oxidativo periférico, neuroinflamação e estresse nitro-oxidativo central. Em conjunto, nossos resultados descrevem o potencial farmacológico do CMI e reforçam a relevância da neuroinflamação e do estresse nitro-oxidativo como alvos para o tratamento da depressão. Dessa forma, objetiva-se contribuir com o desenvolvimento de um novo adjuvante terapêutico capaz de melhorar a qualidade de vida de pacientes depressivos e colaborar com a divulgação científica e a quebra do estigma de que a depressão é uma doença puramente mental.

Palavras-chave: Depressão, selênio, modelos animais, estresse oxidativo, inflamação.

Abstract

CASARIL, Angela Maria. **The use of a senalyindole in the pursuit of happiness.** 2020. 276f. Tese (Doutorado) - Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

Depression is the most prevalent psychiatric disorder in the world, affecting approximately 300 million people. The pathophysiology of this disorder has not been fully elucidated, possibly because it is highly heterogenic and multifactorial. However, evidences suggest that a dysfunction in monoaminergic and glutamatergic neurotransmission, hyperactivity of the hypothalamus-pituitary-adrenal axis, inflammation, and oxidative stress are associated with behavioral changes observed in patients. Despite the socioeconomic relevance, 40% of depressed patients do not show symptomatic improvement with the drug treatments available today. Thus, considering the multifactorial nature of depression and the need for more efficient treatments, the importance of looking for multi-target molecules with antidepressant effect becomes clear. Initial studies carried out by our research group reported that the compound 3-[(4-chlorophenyl)selenyl]-1-methyl-1*H*-indole (CMI) has antioxidant effect and protects mice against the depressive-like behavior induced by inflammation. This hybrid molecule combines the pharmacological effects of the selenium atom with the indole group. Thus, the need for more efficient drugs for depression combined with the promising effects of CMI prompted us to dive into the CMI's ability to reverse depression-like behavior in different animal models. In chapter 1 of this thesis, we deepened our knowledge about the potential of CMI to neutralize inflammation-derived oxidants. In chapter 2, we observed that the CMI reversed the depressive-like behavior in mice subjected to acute restraint stress, while also reversed neuroinflammation and oxidative stress. In chapter 3, we faced the ability of CMI to reverse depressive- and anxiogenic-like behavior induced by acute inflammation, through the modulation of the serotonergic system. In chapter 4, we observed that CMI reversed depressive-like behavior and cognitive impairment in mice with breast tumors, through the modulation of nitro-oxidative stress and neuroinflammation. In chapter 5, we advanced the knowledge about the involvement of the BDNF/mTOR pathway in the antidepressant- and anxiolytic-like effect of CMI. In chapter 6, we investigated the ability of CMI to improve the depression-like and anxiogenic-like behavior and cognitive impairment in mice that survived sepsis, through modulation of blood parameters, peripheral oxidative stress, neuroinflammation and central nitro-oxidative stress. Together, our results describe the pharmacological potential of CMI and reinforce the relevance of neuroinflammation and nitro-oxidative stress as targets for the treatment of depression. Thus, we aimed to contribute to the development of a new therapeutic adjuvant capable of improving the quality of life of depressed patients and to collaborate with the science communication to help breaking the stigmatization that depression is a purely mental illness.

Keywords: Depression, selenium, animal models, oxidative stress, inflammation.

Lista de Figuras

Figura 1. Representação esquemática dos sintomas do transtorno de depressão maior (TDM) de acordo com o DSM-V	24
Figura 2. Representação esquemática da prevalência de depressão na população mundial (em milhões)	25
Figura 3. Prevalência de auto relato de depressão na população brasileira de adultos (≥ 18 anos), segundo unidades federativas	26
Figura 4. Heterogeneidade da depressão e influências na susceptibilidade à doença	27
Figura 5. Rota de biossíntese das catecolaminas	29
Figura 6. Rota de biossíntese da serotonina	30
Figura 7. Neurotransmissão monoaminérgica	31
Figura 8. Representação do eixo hipotálamo-pituitária-adrenal (HPA)	36
Figura 9. Neurotransmissão glutamatérgica e suporte neurotrófico no córtex pré-frontal de indivíduos saudáveis	43
Figura 10. Neurotransmissão glutamatérgica e suporte neurotrófico no córtex pré-frontal de pacientes depressivos	45
Figura 11. Propagação da resposta imune periférica ao sistema nervoso central em situação de estresse	50
Figura 12. Potenciais mecanismos responsáveis pelos efeitos de citocinas pró-inflamatórias no cérebro durante a depressão	52
Figura 13. Principais espécies reativas e enzimas antioxidantes intracelulares	58
Figura 14. Estrutura de alguns compostos orgânicos de selênio estudados pelo nosso grupo de pesquisa	71
Figura 15. Estrutura do núcleo indólico e moléculas relacionadas	72
Figura 16. Resumo do mecanismo de ação responsável pelo efeito tipo-antidepressivo do CMI avaliado nesta tese	90

Lista de Tabelas

Tabela 1. Principais classes de antidepressivos que agem modulando a neurotransmissão monoaminérgica	63
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Lista de Abreviaturas

4-HNE – 4-hidroxinonenal

5-HTP – 5-hidroxitriptofano

ACTH – Hormônio adrenocorticotrófico (*adrenocorticotropin hormone*)

AIM2 – Receptores tipo AIM2 (*AIM2-like receptors*)

Akt – Proteína quinase B

AMPA-R – Ácido alfa-amino-3-hidroxi-metil-5-4-isoxazolpropiónico (*alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor*)

AP-1 – Proteína ativadora-1 (*activator protein-1*)

APA – Associação Americana de Psiquiatria (*American Psychiatric Association*)

BBB – Barreira cérebro-sangue (*blood-brain barrier*)

BDNF – fator neurotrófico derivado do cérebro (*brain derived neurotrophic fator*)

BH4 – Tetraidrobiopterina

CAMKII – Proteína quinase II dependente de Ca^{2+} /calmodulina (*Ca²⁺/calmodulin 13xtracelul protein kinase II*)

cAMP – AMP cíclico

CAT – Catalase

CCL2 – Ligante C-C de quimiocina 2

CLRs – Receptores de lectina tipo C (*C-type lectin receptors*)

CMI – 3-[(4-cloforenil)selenil]-1-metil-1*H*-indol

COX-2 – ciclooxigenase 2

CREB – Proteína de ligação responsiva ao AMP cíclico (*cyclic-AMP-response-element binding protein*)

CRH – Hormônio liberador de corticotrofina (*corticotropin releasing hormone*)

CRP – Proteína C reativa

CTE – Cadeia transportadora de elétrons

DAMPs – Padrões moleculares associados ao perigo (*damage-associated molecular patern*)

DSM – Manual Diagnóstico e Estatístico de Transtornos Mentais (*Diagnostic and Statistical Manual of Mental Disorders*)

EAAT – Transportadores de aminoácidos excitatórios (*excitatory amino acid transportes*)

EAR – Estresse agudo de restrição

eEF2 – Fator de alongamento da tradução (*eukaryotic translation elongation factor 2*)
ERK – Quinases regulada por sinal extracelular (*extracellular signal-regulated*)
ERRO/N – Espécies reativas de oxigênio/nitrogênio
EUA – Estados Unidos da América
FDA – *Food and Drug Administration*
GABA – Ácido gama-aminobutírico (*gamma aminobutyric acid*)
GABA_A-R – Receptores de GABA do tipo A
GPx – Glutathione peroxidase
GR – Receptores de glicocorticoide (*glucocorticoid receptors*)
GRE – Elementos de resposta aos glicocorticoides (*glucocorticoid-response elements*)
GRIP – Proteína de interação com GR (*GR interacting protein*)
GSH – Glutathione reduzida
GSK-3 β – Glicogênio sintase quinase 3 beta (*glycogen synthase kinase 3 beta*)
GSSG – Glutathione oxidada
GWAS – Associação em genoma completo (*genome wide association study*)
HDAC – Histona deacetilases (*histone deacetylases*)
HMGB1 – Proteínas de alta mobilidade do grupo de caixa 1 (*high-mobility group box 1*)
HPA – Hipotálamo-hipófise-adrenal (*hypothalamic-pituitary-adrenal*)
HSP – Proteínas de choque térmico (*heat shock proteins*)
I3C – Indol-3-carbinol
IDO – Indoleamina 2,3-dioxigenase
IFN γ – Interferon gama
IL – Interleucina
iNOS – Óxido nítrico sintase induzível
IRF3 – Fator de regulação de interferon 3 (*interferon regulatory factor 3*)
JNK – Jun quinase (*Jun kinase*)
KA – Ácido quinurênico (*kynurenic acid*)
L-DOPA – 3,4-diidroxifenilalanina
LTD – Depressão de longa duração (*long-term depression*)
LTP – Potenciação de longa duração (*long-term potentiation*)
MAOIs – Inibidores das monoaminas oxidases (*monoamine oxidase inhibitors*)
MAOs – Monoaminas oxidases

MAPK p38 – Proteína quinase ativada por mitógeno p38 (*mitogen-activated protein kinase p38*)

MCP-1 – Proteína quimioatrativa de monócito 1 (*monocyte chemoattractant protein 1*)

MDA – Malondialdeído

MPO – Mieloperoxidase

mtDNA – DNA mitocondrial

mTORC1 – Complexo 1 do alvo mamífero da rapamicina (*mammalian target of rapamycin complex 1*)

NADPH – Dinucleotídeo de nicotinamida e adenina

NFκB – Fator nuclear kappa B (*nuclear factor kappa B*)

NK – *Natural killer*

NLRP3 – NLR proteína 3 (*NOD-like receptor protein 3*)

NLRs – Receptores do tipo NOD (*NOD-like receptors*)

NMDA-R – Receptores N-metil-D-aspartato (*N-methyl-D-aspartate receptor*)

NOX – NADPH oxidase

Nrf2 – Fator nuclear eritroide 2 relacionado ao fator 2 (*nuclear factor erythroid 2-related factor 2*)

OMS – Organização Mundial da Saúde

PAMPs – Padrões moleculares associados a patógenos (*pathogen-associated molecular patterns*)

PCP – Fenciclidina (*phencyclidine*)

PE2 – Prostaglandina E2 (PE2)

PI3K – Fosfatidilinositol 3-quinase (*phosphatidylinositol 3-kinase*)

PKA – Proteína quinase A

PLC – Fosfolipase C (*phospholipase C*)

PRR – Receptores de reconhecimento padrão (*pattern recognition receptors*)

PSD-95 – Proteína de densidade pós-sináptica 95 (*postsynaptic density protein 95*)

pTEFb – Fator positivo b de alongamento de transcrição (*positive transcription elongation factor*)

PVN – Núcleo paraventricular (*paraventricular nucleus*)

QA – Ácido quinolínico (*quinolinic acid*)

REDD1 – Fator regulador do desenvolvimento e resposta de dano no DNA 1 (*regulated in development and DNA damage responses 1*)

RIG-I – Receptores do tipo RIG-I (*RIG-I-like receptors*)

Sec – Selenocisteína

SelP – Selenoproteína P

SERT – Transportador de serotonina

SOD – Superóxido dismutase

SSRIs – Inibidores seletivos da recaptação de serotonina (*selective serotonin reuptake inhibitors*)

TCA – Antidepressivos tricíclicos (*tricyclic antidepressants*)

TDM – Transtorno de depressão maior

TGF- β – Fator transformador de crescimento beta (*transforming growth factor beta*)

TLRs – Receptores do tipo Toll (*Toll-like receptors*)

TNF – Teste do nado forçado

TrkB – Receptor de tropomiosina quinase B (*tropomyosin receptor kinase B*)

tRNA – RNA transportador

TrxR – Tiorredoxina redutase

TSC – Teste de suspensão da cauda

VDCC – Canal de cálcio dependente de voltagem (*voltage-gated calcium channel*)

Sumário

1 INTRODUÇÃO GERAL	18
2 REVISÃO BIBLIOGRÁFICA	21
2.1 Da melancolia ao transtorno de depressão maior: classificação, diagnóstico e prevalência	21
2.2 Feeling blue: a etiologia da depressão	28
2.2.1 Disfunção da neurotransmissão mononoaminérgica	29
2.2.2 Hiperativação do eixo hipotálamo-pituitária-adrenal	33
2.2.3 Prejuízo na neuroplasticidade sináptica	39
2.2.4 Ativação da resposta inflamatória	46
2.2.4.1 Estabelecimento do estresse oxidativo.....	57
2.3 De agentes tuberculostáticos à anestésicos gerais: tratamentos farmacológicos para a depressão	60
2.4 Da Deusa da lua à compostos bioativos: atividade farmacológica de compostos orgânicos de selênio	67
2.4.1 3-[(4-cloforenil)selenil]-1-metil-1 <i>H</i> -indol	71
3 HIPÓTESE E OBJETIVOS	74
3.1 Hipótese	74
3.2 Objetivo geral	74
3.3 Objetivos específicos	74
4 CAPÍTULOS	75
4.1 Capítulo 1 – Selenium-containing indolyl compounds: kinetics of reaction with inflammation-associated oxidants and protective effect against oxidation of extracellular matrix proteins	75
4.2 Capítulo 2 – The selenium-containing compound 3-[(4-chlorophenyl)selenyl]-1-methyl-1 <i>H</i> -indole reverses depressive-like behavior induced by acute restraint stress in mice: modulation of oxido-nitrosative stress and inflammatory pathway	87

4.3 Capítulo 3 – Depression- and anxiogenic-like behaviors induced by lipopolysaccharide in mice are reversed by a selenium-containing indolyl compound: Behavioral, neurochemical and computational insights involving the serotonergic system	101
4.4 Capítulo 4 – The antioxidant and immunomodulatory compound 3-[(4-chlorophenyl)selenyl]-1-methyl-1 <i>H</i> -indole attenuates depression-like behavior and cognitive impairment developed in a mouse model of breast tumor	114
4.5 Capítulo 5 – The modulation of the BDNF-mTOR pathway is involved in the antidepressant- and anxiolytic-like effect of a selenium-containing compound in corticosterone-treated mice	128
4.6 Capítulo 6 – The antioxidant and immunomodulatory compound 3-[(4-chlorophenyl)selenyl]-1-methyl-1 <i>H</i> -indole attenuates depression-like behavior and cognitive impairment developed in a mouse model of breast tumor	154
5 DISCUSSÃO GERAL E PERSPECTIVAS	189
6 CONCLUSÃO GERAL	195
7 REFERÊNCIAS	196
8 ANEXOS	220
Anexo A – Demais artigos publicados durante o período de doutoramento cujos temas se relacionam a esta tese	220
Anexo B – Demais artigos publicados durante o período de doutoramento cujos temas não se relacionam a esta tese	255
Anexo C – Patentes depositadas durante o período de doutoramento	259
Anexo D – Aprovações da Comissão de Ética em Experimentação Animal..	261
Anexo E – Outras informações relevantes	266
Anexo F – Autorização para o reuso dos artigos científicos	267

1 INTRODUÇÃO GERAL

Perder uma pessoa amada, ser demitido de um emprego, passar pelo divórcio, ficar doente e outras situações difíceis podem levar uma pessoa a se sentir triste, sozinha, sem energia e desmotivada. Esses sentimentos são reações normais aos estressores encontrados durante a vida, sendo que a maioria das pessoas se sente triste e desmotivada em inúmeros momentos. No entanto, no caso de indivíduos clinicamente diagnosticados com depressão, esses sentimentos de tristeza, solidão, culpa, desmotivação e fadiga são muito mais intensos e persistentes, podendo acompanhar a pessoa por anos.

A palavra depressão tem origem latina, do verbo *deprimere*, que significa pressionar. O escritor F. Scott Fitzgerald frequentemente se descobria “...odiando a noite, quando não podia dormir, e odiando o dia, que levava à noite” (Fitzgerald, 1956). A escritora J.K. Rowling personificou a depressão na forma dos dementadores da saga *Harry Potter*. Para ela, “*depressão não é tristeza. Eu conheço tristeza. Tristeza é chorar e sentir. Mas é essa fria ausência de sentimento - esse sentimento realmente oco. Isso é o que os dementadores são. Se puder, o dementador se alimentará de você o tempo suficiente para reduzi-lo a algo como ele mesmo... sem alma e mal. Você ficará com as piores experiências de sua vida. Eles congelam seu interior*” (Entwistle, 2016).

Apesar de ser abordada em diversos domínios da arte, discussões casuais sobre depressão frequentemente são curtas, pois, terminam com a frase clássica de que “*depressão é só coisa da sua cabeça*”, ou terminam em um silêncio constrangedor quando ninguém sabe como responder. Isso se chama estigma. A estigmatização moderna da depressão e outras doenças mentais não é explícita, mas é evidente que muitas vezes se prefere não falar sobre o assunto (Bullmore, 2018). A depressão é estigmatizada pelo que não é feito ou dito: é comum que se exerça um tipo de quarentena, separando as experiências da pessoa deprimida da conversa em questão, deixando que ele/ela supere a doença, se recomponha, e volte ao grupo quando tenha se resolvido (Bullmore, 2018). Por ter sido segregada para o domínio mental, a depressão é vista como um sinal de falha pessoal: se a depressão for puramente mental, então a pessoa é responsável por ter a doença, do mesmo jeito que ela é responsável por suas decisões e pensamentos. Isso não poderia estar mais errado.

Atualmente, sabe-se que a depressão é uma doença multifatorial e heterogênea, sendo influenciada por fatores que incluem a resposta imune pró-inflamatória, estresse e traumas na infância e adolescência, microbiota intestinal, vulnerabilidade genética, alterações metabólicas e disfunção do eixo hipotálamo-pituitária-adrenal. Apesar do reconhecimento da influência desses diversos sistemas na depressão, a patofisiologia dessa doença ainda não foi completamente elucidada, o que reflete a relativa ineficiência dos antidepressivos disponíveis atualmente. Com a descoberta acidental de que moléculas com ação antidepressiva aumentavam a disponibilidade de neurotransmissores monoaminérgicos na fenda sináptica, a pesquisa e o desenvolvimento de antidepressivos focou em moléculas com esse mecanismo de ação. Infelizmente, moduladores da neurotransmissão monoaminérgica não são eficazes para 40% dos pacientes e apresentam inúmeros efeitos adversos (Institute for Quality and Efficiency in Health Care, 2017).

De fato, alterações na resposta ao estresse, inflamação e estresse oxidativo tem sido frequentemente encontradas em alguns subgrupos de pacientes depressivos. Portanto, a busca por novas moléculas que possuam atividade antidepressiva se justifica através da necessidade de abordagens multialvo, que sejam capazes de abranger os casos em que os antidepressivos atuais possuem baixa eficiência. Dessa forma, torna-se clara a importância da busca por novas moléculas com ação antidepressiva, que sejam capazes de modular a resposta ao estresse, inflamação e estresse oxidativo, culminando com a melhora do humor dos pacientes.

Nesse sentido, os compostos orgânicos de selênio têm recebido bastante atenção por apresentarem diversas propriedades farmacológicas, como a ação antioxidante, antidepressiva e neuroprotetora (Nogueira e Rocha, 2011). O interesse por esses compostos vem do fato de que o selênio se comporta tanto como um agente antioxidante quanto como um agente anti-inflamatório (Rayman, 2000). Além disso, demonstrou-se previamente que pacientes depressivos apresentam uma redução nos níveis plasmáticos de selênio (Mertens et al., 2015) e que a suplementação com selênio melhora o humor negativo e os sintomas depressivos em pacientes adultos jovens (Conner et al., 2015). Notavelmente, o composto orgânico de selênio ebselen está em ensaios clínicos de fase II para o tratamento de desordem bipolar (Clinicaltrials.gov, ID: NCT03013400).

Além do selênio, outra característica interessante de moléculas bioativas é a presença da porção indol. Estudos apontam que essa é a estrutura heterocíclica mais abundante na natureza que apresenta ação farmacológica, sendo encontrada, por exemplo, no triptofano, serotonina e melatonina (Kaushik et al., 2013). Dentre os efeitos biológicos dos indóis, destaca-se a ação antioxidante, neuroprotetora e anti-inflamatória (Guerra et al., 2011).

Nesse sentido, a síntese de compostos contendo selênio e indol em sua estrutura é uma área de pesquisa bastante interessante, uma vez que a combinação de duas ou mais porções bioativas em uma única molécula é uma estratégia relevante para o desenvolvimento de novos compostos com atividade farmacológica promissora (Martinez et al., 2015). Diante disso, o Grupo de Pesquisa em Neurobiotecnologia em parceria com o Laboratório de Síntese Orgânica Limpa da Universidade Federal de Pelotas sintetizaram o 3-[(4-cloforenil)selenil]-1-metil-1*H*-indol (CMI), um composto orgânico de selênio contendo um núcleo indólico, pertencente a classe dos selenilindoís (Vieira et al., 2015).

O primeiro estudo investigando o perfil farmacológico do CMI foi realizado em 2017, aonde demonstrou-se que essa molécula apresenta atividade antioxidante *in vitro* (Vieira et al., 2017). Considerando-se o envolvimento do estresse oxidativo na depressão e sua relação com o processo inflamatório, avaliou-se então a capacidade do CMI em proteger contra o comportamento tipo-depressivo induzido por inflamação em camundongos (Casaril et al., 2017). Esse estudo mostrou que apenas uma administração de CMI 30 minutos antes do desafio inflamatório protege contra o comportamento tipo-depressivo e contra o aumento nos níveis de espécies reativas, peroxidação lipídica e expressão citocinas pro-inflamatórias nos córtex pré-frontais e hipocampus dos animais, sem apresentar toxicidade aguda (Casaril et al., 2017).

Esses estudos iniciais sobre o CMI nos impulsionaram a avançar na compreensão do potencial tipo-antidepressivo dessa molécula em diferentes modelos animais através do uso de ferramentas biotecnológicas aliadas à química medicinal. Regidos por essa motivação, este trabalho visou contribuir com os ensaios iniciais para o desenvolvimento de um novo adjuvante terapêutico capaz de melhorar a qualidade de vida de pacientes depressivos.

2 REVISÃO BIBLIOGRÁFICA

2.1 Da melancolia ao transtorno de depressão maior: classificação, diagnóstico e prevalência

Diferentemente de outras doenças psiquiátricas, a descoberta da depressão não é creditada a um único indivíduo. A primeira descrição do que conhecemos hoje por depressão é datada do segundo milênio a.C. na Mesopotâmia (Reynolds e Wilson, 2013). Nos textos babilônicos, a depressão que conhecemos hoje era discutida como uma condição espiritual grave, desencadeada pela raiva de um deus ou deusa particular ao indivíduo afetado, a qual requeria a realização de rituais e os serviços de um padre (Reynolds e Wilson, 2013). As descrições babilônicas, notáveis pela objetividade e ausência de sentimentos e pensamentos subjetivos, relatam que o indivíduo era afetado por fraqueza, insônia, frustrações pessoais e perda de pessoal, colheitas e renda (Reynolds e Wilson, 2013). Diversas culturas acreditavam que a depressão era causada por demônios e espíritos malignos, por isso, métodos de espancamento, restrição física, isolamento e fome eram usados na tentativa de expulsar os demônios.

Foi apenas em torno de 400 a.C. que o médico e filósofo grego Hipócrates atribuiu uma causa fisiológica para os sintomas que eram observados. De acordo com a teoria humoral de Hipócrates, a vida era mantida pelo equilíbrio entre quatro humores: sangue, fleuma, bílis amarela e bílis negra. O excesso da bílis negra era responsável por causar tristeza e medo, as quais eram descritas como sintomas da *melancolia* (Telles-Correia e Marques, 2015). O tratamento da melancolia, de acordo com Hipócrates, era baseado na dieta, exercícios, laxantes e sangria.

Galen (129 – 216 d.C.), seguindo a teoria humoral de Hipócrates, descreveu quatro tipos de comportamento resultantes do excesso dos humores: melancólico (bílis negra), otimista (sangue), colérico (bílis amarela) e fleumático (fleuma) (Telles-Correia e Marques, 2015). Para Galen, a etiologia da melancolia não se restringia à bílis negra, mas incluía bílis amarela, deficiência na dieta, supressão do fluxo menstrual e alterações emocionais (Ban, 2014). De acordo com Galen, pacientes melancólicos também apresentavam ideias fixas e excêntricas, como os “pacientes que pensam ter se tornado uma espécie de caracol para que então eles possam escapar de todos para evitar ter sua concha esmagada, enquanto outros temem que

Atlas, que apoia o mundo, possa se cansar e desaparecer, entre outras ideias imaginárias” (Telles-Correia e Marques, 2015).

Ainda no século XVII a teoria humoral de Hipócrates era aceita. Notavelmente, o clérigo e melancólico Robert Burton (1577 – 1640) escreveu *The Anatomy of Melancholy* (1628), no qual o acúmulo de bÍlis negra responsável pela melancolia era consequência de uma variedade de fatores externos. Em adição ao desequilíbrio dos humores, Burton descreveu a melancolia como uma epidemia crescente e alarmante em resposta aos eventos políticos e religiosos não resolvidos de sua época (Edwards, 2010).

Apenas no século XVIII a teoria humoral começou a ser abandonada. Philippe Pinel (1745 – 1826) utilizou uma etiologia descritiva mais rigorosa para classificar os transtornos mentais em quatro grupos principais: melancolia, mania, idiotia e demência (Telles-Correia e Marques, 2015). Pinel descreveu que na melancolia, os pacientes eram oprimidos por uma ideia exclusiva, interminável em suas palavras, que parecia absorver todas as suas outras faculdades.

Por sua vez, o início do século XIX foi caracterizado pelo aumento da insatisfação com o termo melancolia. Assim, novos termos foram introduzidos para identificar as “insanidades emocionais”, como, por exemplo *tristimania* (Benjamin Rush, 1745 – 1815), *lipemania* (Jean-Dominique-Etienne Esquirol, 1772 – 1840) e *insanidade afetiva ou patética* (Henry Maudsley, 1835 – 1918) (Davison, 2006). No entanto, essas opções também não foram universalmente aceitas.

Foi a partir do trabalho do psiquiatra alemão Emil Kraepelin (1855 – 1926) que o termo *estado depressivo* (*depressão*) passou a ser usado para incluir vários tipos de melancolia (Davison, 2006). Através de observações clínicas cuidadosas e análises longitudinais do curso de doenças mentais, Kraepelin diferenciou as desordens mentais e a *dementia praecox* (demência precoce, posteriormente chamada de esquizofrenia), na chamada “dicotomia kraepeliniana”. Seguindo essa terminologia, Adolf Meyer (1866 – 1950) argumentou que o termo *depressão* deveria ser usado, ao defender que a doença seria uma reação a fatores psicológicos, sociais e biológicos. Meyer enfatizou a singularidade de pacientes e caracterizou a depressão como uma reação aos estresses atuais e aos desajustes anteriores (Davison, 2006). Finalmente, em 1950, Karl Leonhard (1904 – 1988) diferenciou depressão bipolar (exibida em manifestações multiformes, mudando continuamente dentro e ao longo dos episódios) e depressão unipolar, a qual exibe uma sintomatologia simples e fixa

que retorna em um curso periódico inalterado (Ban, 2014). Atualmente, a palavra *melancolia* é usada para descrever a característica da depressão associada com o risco aumentado de suicídio e a completa ausência na capacidade de sentir prazer (American Psychiatry Association, 2013).

Evidentemente, durante a história, diversos estudiosos vêm contribuindo com a descrição de sintomas e possíveis causas da depressão, favorecendo assim sua classificação e diagnóstico. Em 1952 foi criado o Manual Diagnóstico e Estatístico de Transtornos Mentais (DSM, do inglês *Diagnostic and Statistical Manual of Mental Disorders*) pela Associação Americana de Psiquiatria (APA, do inglês *American Psychiatric Association*), a fim de que profissionais da saúde tenham uma linguagem comum ao realizar o diagnóstico de doenças mentais.

Em sua quinta edição lançada em 2013, o DSM classifica as desordens depressivas (depressão) em (i) distúrbio da desregulação do humor, (ii) transtorno depressivo (incluindo episódio depressivo maior), (iii) transtorno depressivo persistente (distímia), (iv) distúrbio disfórico pré-menstrual, (v) depressão induzida por substância, (vi) transtorno depressivo devido a outra condição médica, (vii) outros transtornos depressivos especificados e (viii) outros transtornos depressivos não-especificados (American Psychiatric Association, 2013). Comum a todos esses distúrbios são a presença de tristeza, irritação e sensação de vazio, acompanhado por mudanças somáticas e cognitivas que afetam significativamente a rotina do indivíduo. Duração, tempo e etiologia presumida são os fatores que diferenciam esses subtipos de desordens depressivas.

O transtorno de depressão maior (TDM) representa a condição clássica das desordens depressivas, sendo caracterizado por episódios depressivos com pelo menos duas semanas de duração que podem persistir por até um ano, envolvendo alterações afetivas, cognitivas e neurovegetativas (Figura 1) (American Psychiatric Association, 2013). O diagnóstico do TDM depende da gravidade do episódio atual, da presença/ausência de características psicóticas, do estado de remissão e se o episódio é único ou recorrente (American Psychiatric Association, 2013).

De acordo com o DSM-V, os critérios de diagnóstico do TDM envolvem: (1) Presença de cinco ou mais dos seguintes sintomas durante pelo menos duas semanas, sendo que pelo menos um dos sintomas é o humor deprimido ou perda de interesse, ou prazer: (i) humor deprimido na maior parte do dia, aproximadamente todos os dias, como indicado por autoavaliação (sentimento de tristeza, vazio,

desesperança) ou por observações feitas por outros (episódios de choro); (ii) redução significativa na capacidade de sentir interesse ou prazer em todas (ou quase todas) as atividades durante a maior parte do dia, aproximadamente todos os dias (como indicado por autoavaliação ou por observações feitas por outros); (iii) significativa perda de peso quando não fazendo dieta, ou ganho de peso, ou redução, ou aumento do apetite aproximadamente todos os dias; (iv) insônia ou hipersonia aproximadamente todos os dias; (v) agitação ou retardo psicomotor aproximadamente todos os dias (observável por outros); (vi) fadiga ou perda de energia aproximadamente todos os dias; (vii) sentimentos de inutilidade ou culpa excessiva, ou inapropriada (as quais podem ser delusões) aproximadamente todos os dias (não apenas autoavaliação ou culpa por estar doente); (viii) reduzida capacidade de pensamento ou concentração, ou indecisão aproximadamente todos os dias (como indicado por autoavaliação ou por observações feitas por outros); (ix) pensamentos recorrentes de morte (não apenas medo de morrer), recorrentes idealizações suicidas sem um plano específico, ou tentativa suicida, ou um plano específico para cometer suicídio. (2) Os sintomas causam sofrimento clinicamente significativo ou prejuízo no funcionamento social, ocupacional ou em outras áreas importantes do funcionamento. (3) O episódio não é atribuído ao efeito fisiológico de substâncias ou outras condições médicas.

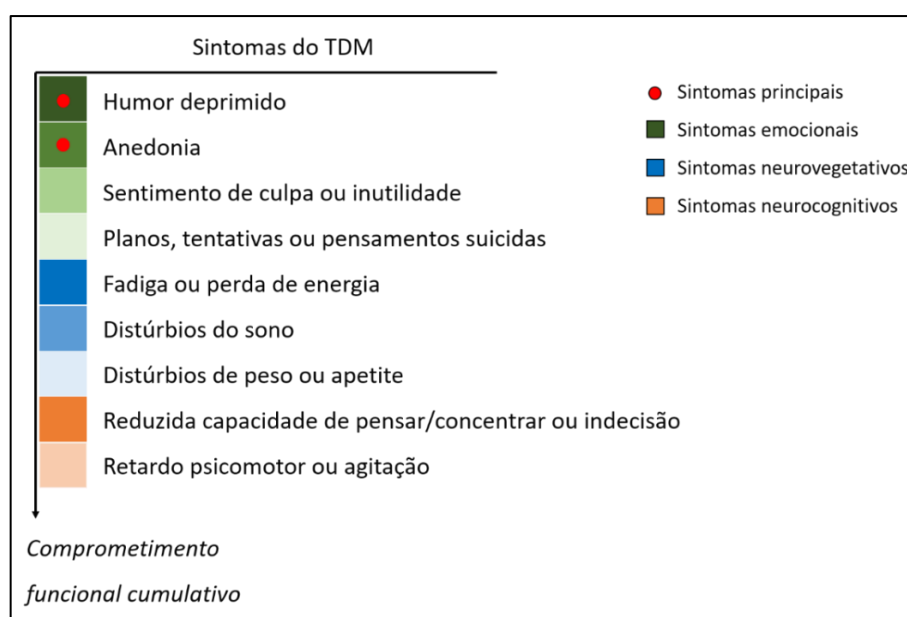


Figura 1. Representação esquemática dos sintomas do transtorno de depressão maior (TDM) de acordo com o DSM-V. Os sintomas do TDM podem ser agrupados em emocionais, neurovegetativos e neurocognitivos. Fonte: adaptado de Malhi e Mann (2018).

Dados da Organização Mundial da Saúde (OMS) reportam que a prevalência global do TDM aumentou aproximadamente 13% durante o período de 2007 – 2017, afetando mais de 300 milhões de pessoas (Figura 2) (James et al., 2018). O TDM pode aparecer pela primeira vez em qualquer idade, mas é mais prevalente em adultos (18 – 64 anos) e tem o início marcadamente aumentado com a puberdade (em torno dos 20 anos), além de apresentar maior prevalência em mulheres do que em homens (American Psychiatric Association, 2013). De acordo com a OMS, a depressão é a principal causa de incapacidade ao redor do mundo, sendo responsável por 7,5% de todos os anos vividos com incapacidade em 2015 (World Health Organization, 2017). De acordo com o Fórum Econômico Mundial (*World Economic Forum*), o custo global de doenças mentais foi de aproximadamente 2,5 trilhões de dólares em 2010, com um aumento estimado para mais de 6 trilhões de dólares em 2030 (Bloom, et al., 2011; Insel, 2011). Nos casos mais graves, a depressão pode levar ao suicídio, sendo responsável por 800,000 casos de suicídio por ano (World Health Organization, 2017).

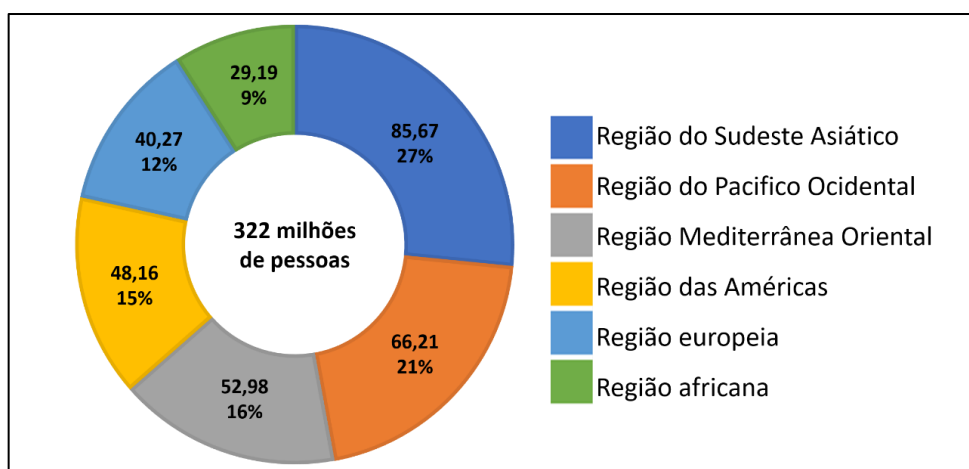


Figura 2. Representação esquemática da prevalência de depressão na população mundial (em milhões). Dados obtidos da Organização Mundial da Saúde, 2015. Fonte: Angela Maria Casaril.

Aproximadamente 15% dos casos de depressão no mundo (48,16 milhões de pessoas) acometem a população residente na região das Américas, sendo que o Brasil é o segundo país com maior incidência da doença (5,8% da população), ficando atrás apenas dos Estados Unidos da América (EUA) (5,9% da população) (World Health Organization, 2017). No Brasil, estudos de base populacional sobre a prevalência da depressão e transtornos mentais, em geral, são relativamente escassos. A Pesquisa Nacional de Saúde de 2013, mostrou que a prevalência do TDM

é maior em mulheres, em indivíduos com idade entre 40 e 59 anos ou 80 anos ou mais, indivíduos residentes em áreas urbanas, com menor nível educacional, fumantes e entre indivíduos com hipertensão, diabetes e doenças cardíacas (cor da pele, estado civil e abuso de álcool não foram associados à depressão) (Munhoz et al., 2016). Os dados também mostraram que a menor prevalência do TDM foi observada na região Norte, enquanto a maior prevalência foi encontrada na região Sul (Figura 3) (Stopa et al., 2015). Entre as unidades federativas, as maiores prevalências de depressão foram no Rio Grande do Sul (13,2%), em Santa Catarina (12,9%) e no Paraná (11,7%), e as menores foram observadas no Pará (1,6%), no Amazonas (2,7%) e no Amapá (3,4%). Em relação às capitais, Florianópolis (11,3%), Natal (10,7%) e Porto Alegre (10,6%) foram as capitais que apresentaram as maiores prevalências, e Porto Velho (1,2%), São Luís (2,4%) e Manaus (2,7%) as que apresentam as menores prevalências (Stopa et al., 2015).

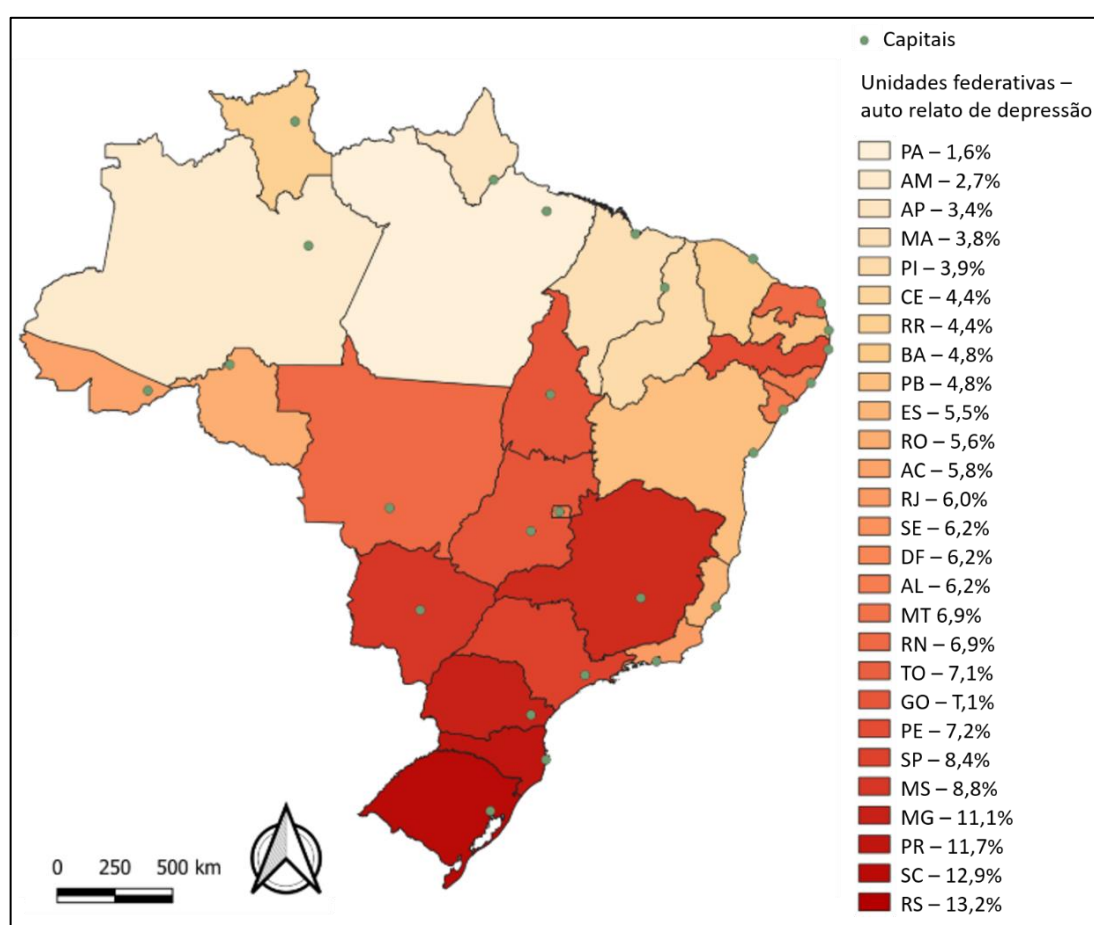


Figura 3. Prevalência de auto relato de depressão na população brasileira de adultos (≥ 18 anos), segundo unidades federativas. Dados obtidos da Pesquisa Nacional de Saúde, 2013. Fonte: adaptado de Diego Montenegro (2019).

Notavelmente, a susceptibilidade à depressão é influenciada por uma variedade de fatores de risco ambientais, genéticos e endócrinos (Figura 4) (Duman et al., 2016).

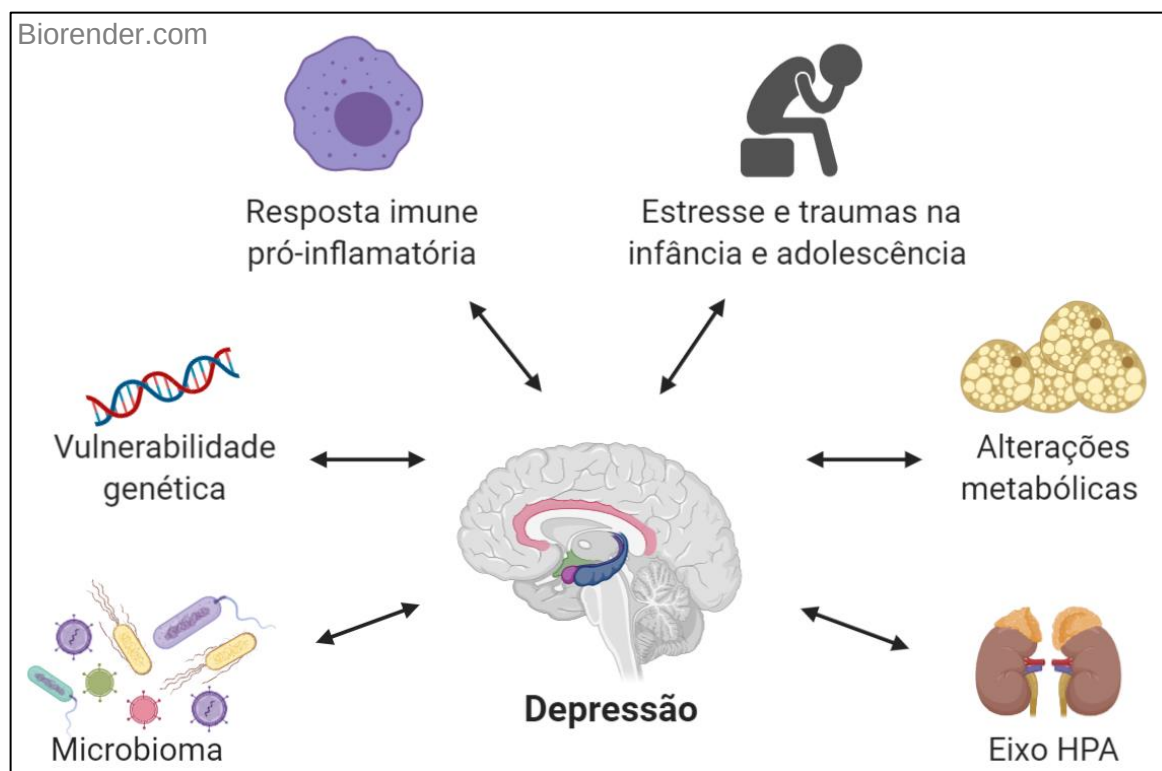


Figura 4. Heterogeneidade da depressão e influências na susceptibilidade à doença. A heterogeneidade da depressão resulta de mais de um determinante patológico. Notavelmente, essas influências incluem o microbiota, vulnerabilidade genética, resposta imune pro-inflamatória, estresse e traumas na infância e adolescência, alterações metabólicas e disfunção do eixo HPA. Fonte: Angela Maria Casaril.

Por exemplo, eventos estressantes na vida podem desencadear a depressão, mas é possível que indivíduos geneticamente susceptíveis sejam mais propensos a desenvolverem depressão ao lidarem com os eventos estressantes da vida, dificultando a determinação da direção dos efeitos. Por isso, a vulnerabilidade genética e a influência ambiental provavelmente contribuem em conjunto para a complexidade e heterogeneidade da depressão (Duman et al., 2016). A disfunção do eixo hipotálamo-pituitária-adrenal (HPA, do inglês *hypothalamic-pituitary-adrenal*), fatores neurotróficos, microbioma, citocinas inflamatórias, situações estressantes e alterações metabólicas podem levar a alterações nos neurotransmissores, sinalização intracelular, transcrição e tradução gênica que contribuem para a manifestação dos

sintomas depressivos (Duman et al., 2016). Consoante a isso, um estudo de associação em genoma completo (GWAS, do inglês *genome-wide association study*) de mais de 60,000 casos encontrou associações significativas entre a depressão e o sistema imune, sinalização neuronal, densidade sináptica e a função de histonas (Network and Pathway Analysis Subgroup of Psychiatric Genomics Consortium, 2015). A depressão como uma doença hereditária é observada em apenas 35 – 40% dos casos, mas provavelmente também é influenciada por fatores ambientais (Sullivan et al., 2000).

Por outro lado, alguns fatores aumentam a resiliência e aumentam a capacidade de evitar os efeitos prejudiciais do estresse traumático ou crônico (Russo et al., 2012). A resiliência à depressão pode ser o resultado da ausência de respostas negativas observadas em indivíduos suscetíveis ou dos mecanismos adaptativos que promovem humor e emoções normais (Duman et al., 2016).

Apesar do impacto social e econômico da depressão, a sua ainda não foi elucidada, e diferentes hipóteses têm sido postuladas na tentativa de se encontrar um fator causal para essa desordem.

2.2 Feeling blue: a etiologia da depressão

Ao contrário de outras áreas da medicina, os últimos 35 anos não trouxeram nenhum avanço concreto para elucidar a etiologia da depressão, possivelmente por ela ainda ser predominantemente discutida como uma doença de caráter exclusivamente mental (Bullmore, 2018). De acordo com o DSM-V, o diagnóstico do TDM seria explicitamente excluído se o paciente fosse diagnosticado com alguma outra doença. No entanto, a depressão pode se manifestar como uma consequência das alterações fisiológicas decorrentes de outra doença, como, por exemplo, artrite reumatoide, câncer e diabetes. Hoje sabemos que, assim como as outras desordens psiquiátricas, a etiologia da depressão é notavelmente complexa, envolvendo fatores neuroendócrinos, neuroimunológicos genéticos, epigenéticos e psicossociais (Bienvenu et al., 2011; Li et al., 2011; Nestler, 2014). Essa complexidade tem um impacto direto na acurácia do diagnóstico da depressão e seus subtipos, no entendimento de sua patofisiologia (Krishnan e Nestler, 2008) e na descoberta e escolha de estratégias terapêuticas eficientes (Alexander e Preskorn, 2014).

2.2.1 Disfunção da neurotransmissão monoaminérgica

As catecolaminas são um grupo de moléculas fisiologicamente ativas, estruturalmente caracterizadas por possuírem um anel catecol e um grupo amina, agindo como neurotransmissores e hormônios. As principais catecolaminas são a dopamina, noradrenalina e adrenalina (Figura 5). Enquanto a dopamina desempenha papel importante no controle emocional, motivação, formação de memória, modulação endócrina e controle motor, a adrenalina e noradrenalina estão envolvidas principalmente com a regulação de funções viscerais (resposta de luta-ou-fuga) através do sistema nervoso simpático e consolidação de memórias de eventos emocionais (Bear et al., 2017).

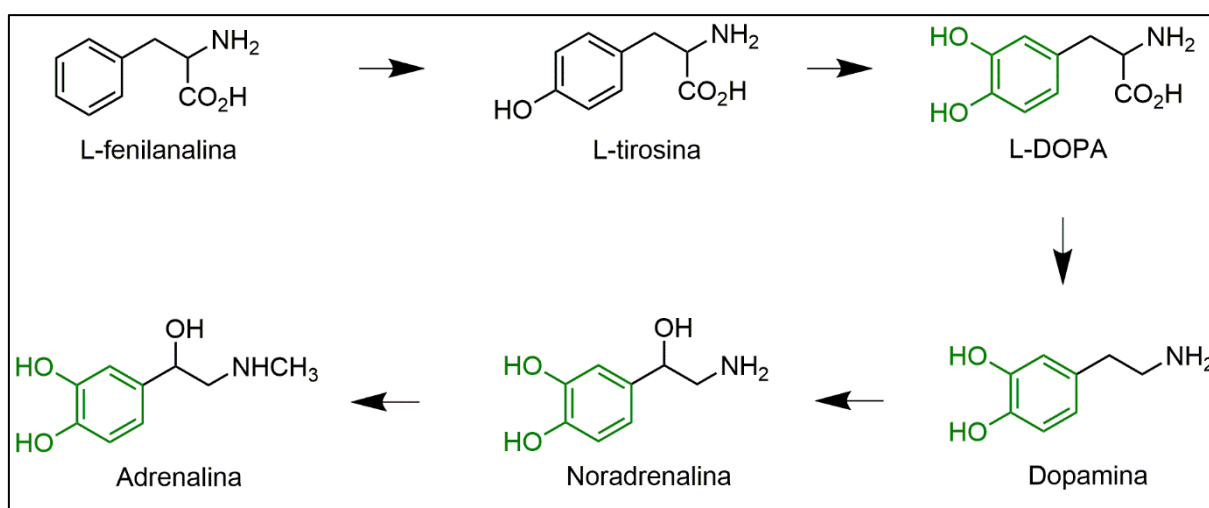


Figura 5. Rota de biossíntese das catecolaminas. As catecolaminas são derivadas do aminoácido L-fenilalanina, o qual é convertido em L-tirosina pela enzima fenilalanina 4-hidroxilase. A L-tirosina é convertida em 3,4-diidroxifenilalanina (L-DOPA) pela enzima tirosina 3-hidroxilase. A L-DOPA é convertida em dopamina pela enzima L-DOPA descarboxilase. Adicionalmente, a dopamina pode ser convertida em norepinefrina pela enzima dopamina beta-hidroxilase, enquanto a noradrenalina pode ser convertida em adrenalina pela enzima feniletanolamina N-metiltransferase. Grupo catecol representado em verde. Fonte: Angela Maria Casaril

Similar às catecolaminas, a serotonina (ou 5-hidroxitriptamina) compartilha a presença de um grupo amina em sua estrutura, sem possuir o grupo catecol. Sintetizada a partir do aminoácido triptofano, a serotonina é caracterizada estruturalmente pela presença do anel indólico (Figura 6). Neurônios serotoninérgicos são relativamente poucos, mas inervam grande parte do sistema nervoso central,

sendo importantes na regulação do humor, cognição, comportamento emocional, sono e aprendizado (Bear et al., 2017).

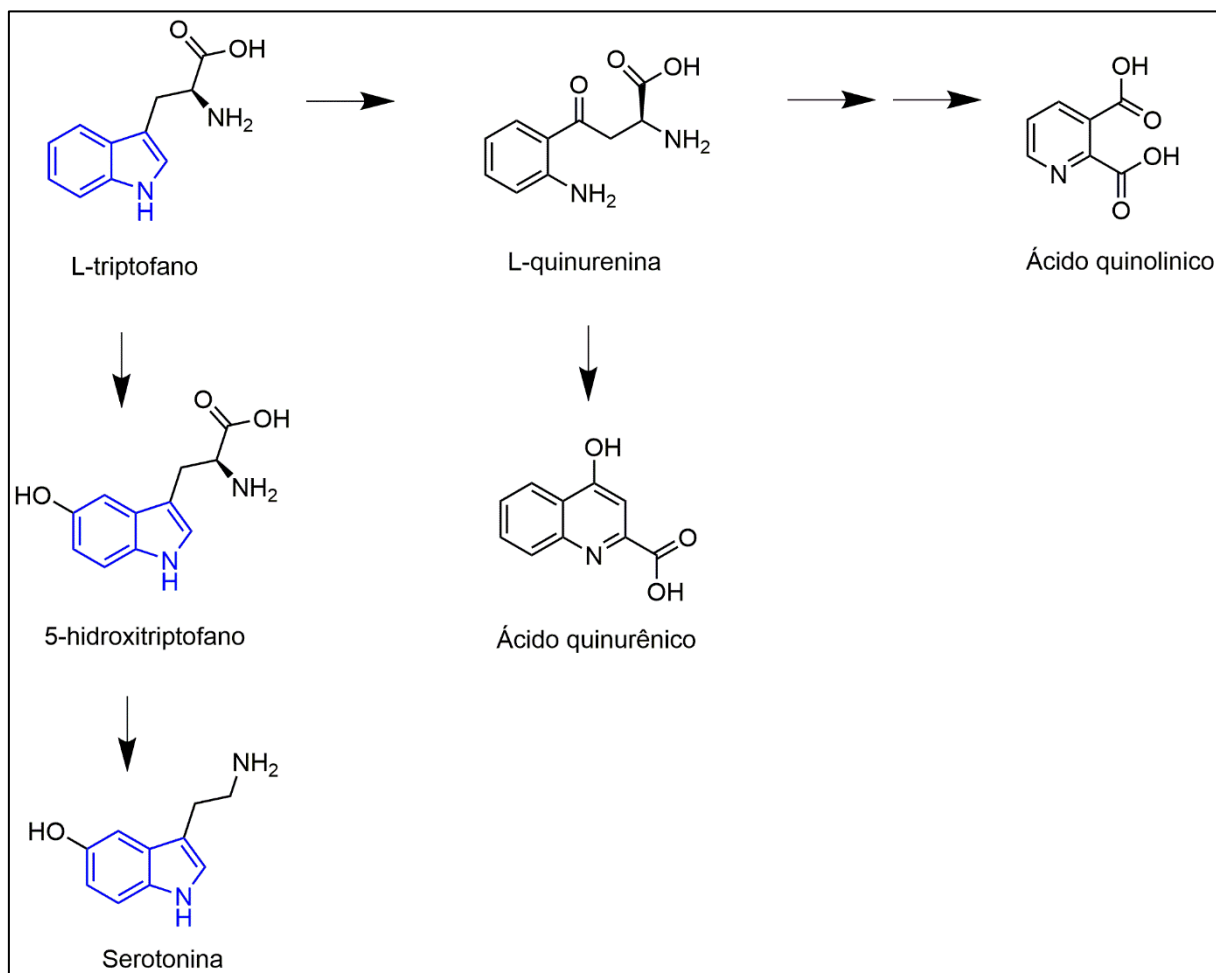


Figura 6. Rota de biossíntese da serotonina. O triptofano é o aminoácido precursor da síntese de serotonina. O triptofano é convertido em 5-hidroxitriptofano (5-HTP) pela enzima triptofano hidroxilase. O 5-HTP é então convertido em serotonina pela enzima 5-HTP descarboxilase. Vale destacar que o triptofano pode ser convertido em quinurenina quando metabolizado pela enzima indoleamina 2,3-dioxigenase (IDO). Por sua vez, a quinurenina pode gerar ácido quinolínico ou ácido quinurênico. Núcleo indólico representado em azul. Fonte: Angela Maria Casaril.

Por compartilharem a presença de um grupo amina em sua estrutura, a serotonina, dopamina e noradrenalina são classificadas como neurotransmissores monoaminérgicos. Uma vez que não há uma enzima extracelular de degradação rápida para o sistema monoaminérgico, a ação desses neurotransmissores na fenda sináptica é terminada pela sua captação seletiva de volta para o terminal axonal através de transportadores (Bear et al., 2017). Uma vez dentro do terminal axonal, as monoaminas podem ser transportadas novamente para as vesículas sinápticas para

serem reutilizadas, ou podem ser degradadas enzimaticamente pela ação das monoaminas oxidases (MAOs), flavoproteínas localizadas na membrana mitocondrial externa (Figura 7) (Bear et al., 2017).

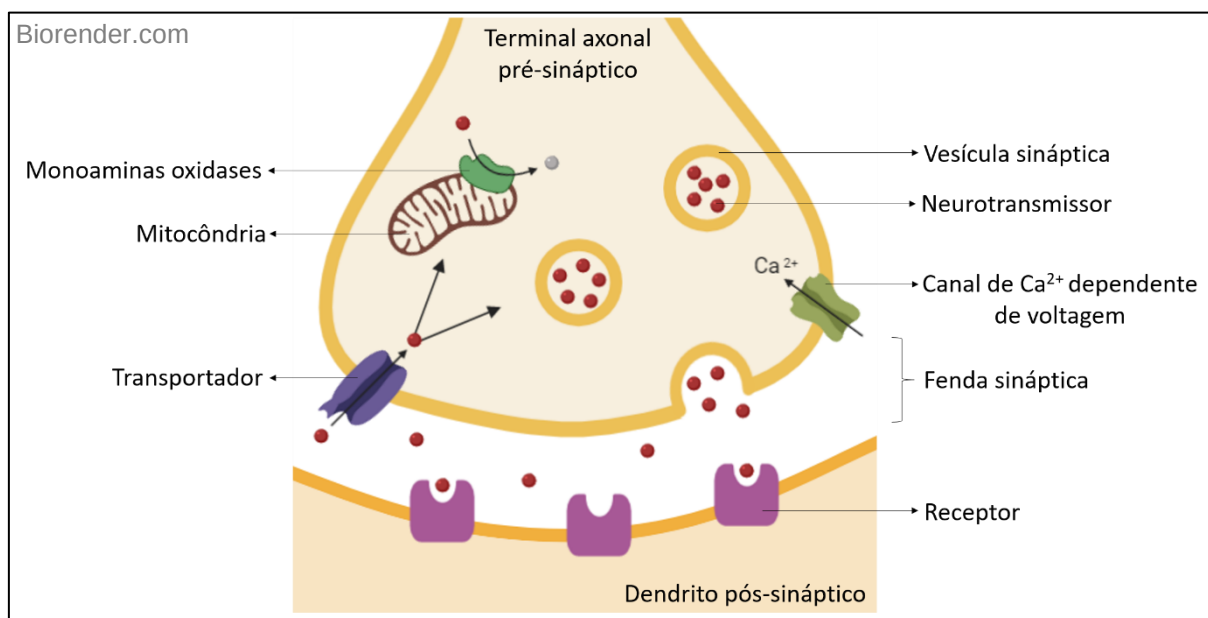


Figura 7. Neurotransmissão monoaminérgica. Após sintetizadas, as monoaminas são internalizadas e armazenadas em vesículas sinápticas. A chegada de um potencial de ação no terminal axonal ativa os canais de cálcio (Ca^{2+}) dependentes de voltagem, desencadeando a liberação dos neurotransmissores das vesículas para a fenda sináptica por um processo de exocitose. Quando liberados, os neurotransmissores monoaminérgicos podem se ligar aos receptores nos neurônios pós-sinápticos para desencadear uma resposta fisiológica, ou podem ser recaptados para o neurônio pré-sináptico através de transportadores. Uma vez recaptados, os neurotransmissores monoaminérgicos podem novamente ser internalizados nas vesículas sinápticas, ou podem ser degradados pela ação enzimática das monoaminas oxidases, localizadas na membrana mitocondrial externa. Fonte: Angela Maria Casaril.

Em 1965, Joseph Schildkraut propôs a primeira hipótese para explicar a etiologia da depressão. A hipótese catecolaminérgica de distúrbios afetivos, como foi chamada, baseou-se nas observações de que (i) a reserpina induzia depressão em humanos por causar a depleção de noradrenalina, (ii) as anfetaminas agiam como estimulantes por aumentar a liberação de noradrenalina, (iii) os medicamentos inibidores das MAOs (MAOIs, do inglês *monoamine oxidases inhibitors*) eram antidepressivos por aumentar as catecolaminas no cérebro, (iv) e de que a imipramina agia como antidepressivo por aumentar a ação da noradrenalina (Schildkraut et al., 1965). Na mesma época, a hipótese serotoninérgica da depressão começou a ser

desenvolvida. Em 1963, Alec Coppen demonstrou que a administração de triptofano em animais com comportamento tipo-depressivo aumentavam os efeitos terapêuticos dos MAOIs (Coppen et al., 1963). Um ano depois, Herman Praag sugeriu a relação entre a inibição das MAOs e a ação antidepressiva e entre a disfunção serotoninérgica e alguns tipos de depressão (López-Muñoz e Alamo, 2009; Van Praag et al., 1964).

Nesse sentido, a hipótese monoaminérgica propõe que pacientes depressivos apresentam redução na disponibilidade dos neurotransmissores monoaminérgico na fenda sináptica. De fato, tem sido reportado que pacientes com os tipos mais severos de depressão apresentam baixos níveis plasmáticos de triptofano (Anderson et al., 1990). Entretanto, estudos recentes têm fornecido evidências de que a depleção de monoaminas é muito simplista para explicar o surgimento da depressão. Por exemplo, a depleção de monoaminas em pacientes saudáveis não produz sintomas depressivos (Salomon et al., 1997), a depleção de triptofano não aumenta os sintomas depressivos em pacientes depressivos não medicados (Berman et al., 2002) e os MAOIs e os inibidores seletivos da recaptação de serotonina (SSRIs, do inglês *selective serotonin reuptake inhibitors*) aumentam rapidamente a transmissão monoaminérgica, mas necessitam de semanas para melhorar o humor dos pacientes (Krishnan e Nestler, 2008).

Assim, uma revisão da hipótese monoaminérgica sugere que a depleção de monoaminas está presente no contexto de estressores ou que essa depleção exerce um papel modulador e influencia outros sistemas neurobiológicos (como a sinalização intracelular e outros neurotransmissores e neuropeptídios) (Charney, 1998; Heninger et al., 1996). Atualmente, sabe-se que o uso de antidepressivos acarreta alterações na neuroplasticidade sináptica, através da influência nos processos de transcrição e tradução de proteínas envolvidas com a plasticidade celular (Krishnan e Nestler, 2008; Pittenger e Duman, 2008). Por exemplo, ensaios pré-clínicos e clínicos demonstraram que antidepressivos administrados cronicamente aumentam a expressão do fator de transcrição proteína de ligação responsiva ao AMP cíclico (cAMP) (CREB, do inglês *cyclic-AMP-response-element binding protein*), o qual faz parte da via de sinalização de diversos receptores serotoninérgicos e outros receptores acoplados a proteína G (Krishnan e Nestler, 2008; Pittenger e Duman, 2008). Adicionalmente, tem sido proposto que o bloqueio simultâneo de autoreceptores serotoninérgicos 5HT_{1A} e dos transportadores serotoninérgicos (SERT, do inglês *serotonin transporter*) acelera o tempo de ação dos efeitos antidepressivos, quando comparado ao uso dos SSRIs

sozinhos (Artigas et al., 1994; Maes et al., 1999; Starr et al., 2007). O bloqueio dos receptores 5HT_{2A} e 5HT_{2C} também desencadeia efeitos tipo-antidepressivos (Millan, 2005; Zaniowska et al., 2010), podendo agir de forma sinérgica com os SSRIs (Marek et al., 2003). Similarmente, o antagonismo dos receptores 5HT₃ tem sido associado com o rápido efeito de ação de moléculas antidepressivas (Alam et al., 2013; Bétry et al., 2013).

Notavelmente, a prescrição dos antidepressivos moduladores de monoaminas é realizada sem a confirmação de que os pacientes apresentam níveis reduzidos desses neurotransmissores. Embora a hipótese monoaminérgica seja a base para a maioria dos antidepressivos disponíveis atualmente, as observações de que as monoaminas podem ser coadjuvantes na fisiopatologia da depressão impulsionou a pesquisa de novas hipóteses para estabelecer um fator causal para essa doença.

2.2.2 Disfunção do eixo hipotálamo-pituitária-adrenal

Como resultado do trabalho de Board e colaboradores, a década de 50 também foi marcada pela observação inicial de que pacientes depressivos apresentavam elevados níveis plasmáticos de cortisol (Board et al., 1956). Subsequentemente, diversos outros estudos deram suporte a essa observação (Bunney et al., 1965; Butler e Besser, 1968; Carroll et al., 1976; Gibbons, 1964; Gold et al., 1986; Sachar, 1967, 1970; Sachar et al., 1973), aumentando o interesse sobre a influência do estresse e do sistema endócrino na fisiopatologia da depressão.

Independente do contexto ambiental, a sobrevivência é a prioridade fundamental de todos os organismos, dependendo grandemente da capacidade de adaptação às mais diversas ameaças à homeostase ou ao bem-estar. Essas ameaças (físicas ou psicológicas e reais ou percebidas) são chamadas de estressores e dão início a “resposta ao estresse”. A resposta ao estresse evoluiu como um processo adaptativo que envolve a resposta integrada de diferentes sistemas fisiológicos aos estressores, caracterizada pelo comportamento de esquiva, aumento da vigilância e alerta, ativação do sistema nervoso simpático e liberação de cortisol pelas glândulas adrenais (Bear et al., 2017). Embora o sistema nervoso autonômico também esteja envolvido, o eixo HPA representa o principal sistema regulatório da resposta ao estresse (Herman et al., 2016; Selye, 1936).

O eixo HPA consiste em um sistema de retroalimentação (*feedback*) que inclui primariamente o hipotálamo, a hipófise e as glândulas adrenais, mas que também é

fortemente regulado pelo hipocampo e amígdala (Figura 8). A ativação do eixo HPA é iniciada via neurotransmissão excitatória a nível do núcleo paraventricular (PVN, do inglês *paraventricular nucleus*) do hipotálamo (Herman et al., 2016). Estressores físicos (como hipovolemia, dor, inflamação, desequilíbrio de fluídos e desequilíbrio metabólico) desencadeiam a resposta ao estresse através de neurônios ascendentes ou órgãos circunventriculares, os quais enviam projeções diretas ao PVN (Herman, 2003; Herman et al., 2016). Esse tipo de ativação do eixo HPA caracteriza a chamada “resposta reativa”, uma vez que há um estímulo que sinaliza para uma ameaça direta à homeostase do organismo (os chamados “estressores sistêmicos”) (Jankord e Herman, 2008). A ativação do eixo HPA também pode ocorrer na ausência de uma ameaça clara, servindo para alertar o organismo sobre uma futura ameaça à homeostase. Essas respostas antecipatórias são iniciadas através da comparação de um estímulo ambiental com memórias (por exemplo, experiências prévias com um estímulo doloroso) ou com sistemas inatos (por exemplo, o instinto em sentir medo de predadores), caracterizando os “estressores psicogênicos” (Jankord e Herman, 2008). Essas respostas antecipatórias dependem fortemente do sistema límbico (especialmente amígdala e hipocampo) e suas conexões indiretas com o PVN (Herman, 2003).

Seguido por um estressor sistêmico ou psicogênico, o PVN do hipotálamo libera o hormônio liberador de corticotrofina (CRH, do inglês *corticotropin releasing hormone*), o qual interage com receptores CRH tipo 1 acoplados à proteína G na hipófise anterior, para estimular a produção e liberação do hormônio adrenocorticotrófico (ACTH, do inglês *adrenocorticotropin hormone*). O ACTH é transportado pela circulação sanguínea para o córtex da adrenal e interage com receptores de melanocortina 2 para estimular a biossíntese de colesterol, e consequentemente, a produção e liberação de glicocorticoides pela zona fasciculada do córtex da adrenal. Os principais produtos finais da ativação do eixo HPA são o cortisol em humanos e a corticosterona em roedores. Fisiologicamente, a atividade do eixo HPA é caracterizada pelo ritmo circadiano e pelo padrão de liberação de glicocorticoides em picos, com um pico de produção a cada 1-2 horas (Young et al., 2004).

Devido ao seu caráter lipofílico, o cortisol é transportado na corrente sanguínea ligado às proteínas carreadoras (especialmente a transcortina). O cortisol desencadeia seus efeitos através da interação com dois tipos principais de receptores

intracelulares, os receptores mineralocorticoides (MR, do inglês *mineralocorticoid receptors*) e os receptores de glicocorticoide (GR, do inglês *glucocorticoid receptor*), amplamente distribuídos pelo organismo.

Dentre os inúmeros efeitos do cortisol, vale destacar sua capacidade de mobilizar reservas energéticas (estimulo da glicólise, lipólise e proteólise) e sua atividade anti-inflamatória/imunossupressora (Cruz-Topete e Cidlowski, 2015). A ligação do cortisol aos GR causa alterações conformacionais que permitem a dissociação de proteínas de choque térmico (HSP, do inglês *heat shock proteins*) seguido da translocação do complexo GR-cortisol para o núcleo. Esse complexo interage com elementos de resposta ao glicocorticoide (GRE, do inglês *glucocorticoid-response elements*), regulando a expressão de inúmeros genes (Gravanis e Margioris, 2001). O efeito anti-inflamatório do complexo GR-cortisol decorre, principalmente, da sua capacidade de reduzir a expressão dos fatores de transcrição pró-inflamatórios proteína ativadora-1 (AP-1, do inglês *activator protein-1*) e fator nuclear kappa B (NFkB, do inglês *nuclear fator kappa B*), os quais são ativados após a exposição do organismo a um patógeno. O complexo GR-cortisol pode reduzir atividade da AP-1 através da interação física com o c-Jun (uma das subunidades da AP-1), reprimindo assim a atividade da proteína e a transcrição de genes inflamatórios (Busillo e Cidlowski, 2013). A redução na atividade do NFkB por parte do complexo GR-cortisol decorre por inúmeros mecanismos (Cruz-Topete e Cidlowski, 2015): (i) GR-cortisol interage fisicamente com a subunidade p65 do NFkB; (ii) GR-cortisol recruta a proteína de interação com GR (GRIP, do inglês *GR interacting protein*) que bloqueia a formação do heterodímero de NFkB com o fator de regulação de interferon 3 (IRF3, do inglês *interferon regulatory factor 3*); (iii) GR-cortisol previne a fosforilação e ativação da RNA polimerase II através do bloqueio da interação com o fator positivo b de alongamento de transcrição (pTEFb, do inglês *positive transcription elongation factor*); (iv) GR-cortisol recruta histona deacetilases (HDAC, do inglês *histone deacetylases*) que atuam como correpessoras da subunidade p65 do NFkB; (v) GR-cortisol previne a interação do NFkB com a proteína de ligação ao CREB; (vi) GR-cortisol interage com a p53 para alterar a atividade transcricional do NFkB. Em adição, glicocorticoides podem reduzir a expressão de moléculas de adesão (as quais previnem o rolamento, adesão e extravasamento de neutrófilos aos sítios de inflamação) e bloquear a produção e citocinas derivadas de células T (Cruz-Topete e Cidlowski, 2015). No sistema nervoso central, o cortisol regula a sobrevivência

neuronal, neurogênese, aquisição de memória e potenciação de longa duração (Anacker et al., 2011; Pavlides e McEwen, 1999).

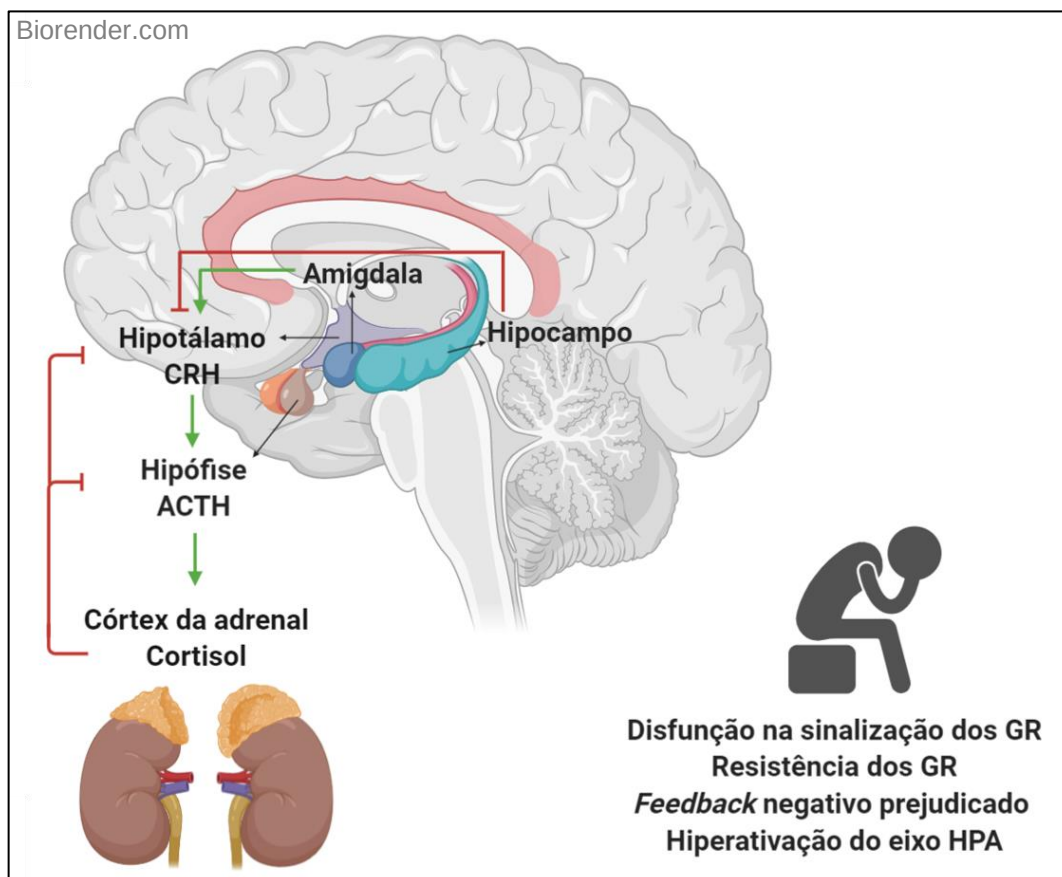


Figura 8. Representação do eixo hipotálamo-pituitária-adrenal (HPA). Em resposta a estressores físicos ou psicogênicos, o PVN do hipotálamo produz CRH que age na hipófise para estimular a liberação de ACTH. Liberado na corrente sanguínea, o ACTH estimula o córtex da adrenal a produzir cortisol. Através de um mecanismo de *feedback* negativo, o cortisol reduz a liberação de CRH e ACTH, consequentemente reduzindo a resposta ao estresse. Na depressão, a disfunção da sinalização e resistência dos GR impede o *feedback* negativo, acarretando na hiperativação do eixo HPA e hipercortisolemia periférica. Enquanto que a amígdala está envolvida com a ativação do eixo HPA frente a uma ameaça, a sinalização de GR no hipocampo tem papel crucial no *feedback* negativo do eixo HPA. Fonte: Angela Maria Casaril.

Diante do potente efeito catabólico e imunossupressor do cortisol, um mecanismo de *feedback* negativo sob a resposta secretória do CRH e ACTH limita a duração da exposição do organismo ao cortisol. Tanto os MR quanto os GR estão envolvidos com o *feedback* do cortisol (de Kloet, 1991), mas há diferenças na distribuição, ocupação e propriedades de ligação desses dois receptores que afetam suas funções fisiológicas. No cérebro, os MR são altamente expressos no sistema

límbico enquanto o GR é encontrado tanto em estruturas corticais quanto subcorticais, mas com uma distribuição preferencial no córtex pré-frontal (Patel et al., 2000). O cortisol se liga aos MR com uma afinidade de 6 a 10 vezes maior do que com os GR (de Kloet et al., 1999). Consequentemente, em níveis basais, 90% dos MR estão ocupados pelo cortisol, enquanto apenas 10% dos GR são ocupados. Dessa forma, apenas em condições de alta secreção de cortisol (como durante os picos circadianos e durante o estresse) os MR se tornam saturados e a ocupação do GR aumenta para 67 – 74% (Reul e Kloet, 1985). Quando os níveis de cortisol estão muito elevados, a ativação dos GR no hipocampo e hipotálamo promove a inibição da liberação de CRH (e subsequentemente de ACTH) (Bear et al., 2017). Assim, os GR são imprescindíveis para a regulação do eixo HPA em situações de excesso de cortisol, e o prejuízo nesse sistema de auto regulação tem sido reconhecido como um fator causal para a depressão.

Os prejuízos na função do eixo HPA em pacientes depressivos se manifestam pela hipersecreção de CRH e ACTH (Holsboer et al., 1987; Nemeroff et al., 1984; Rubin et al., 1987) e a presença elevados níveis plasmáticos (Carroll, 1982) e salivares (Vreeburg et al., 2009) de cortisol. Adicionalmente, a disfunção dos GR tem sido observada em diferentes níveis em pacientes depressivos. Estruturalmente, observou-se a redução na expressão do mRNA de GR em amostras de cérebro *post-mortem* (Webster et al., 2002). Funcionalmente, pacientes depressivos não respondem à supressão pela dexametasona (Rush et al., 1996). Corroborando com esses dados, sintomas depressivos são encontrados em 50 – 70% dos pacientes portadores da doença de Cushing, uma síndrome resultante da produção excessiva de glicocorticoides pelas glândulas adrenais (Sonino e Fava, 1998). Dessa forma, embora sejam observados elevados níveis de cortisol em pacientes depressivos, a sinalização dos GR parece estar prejudicada, acarretando no prejuízo do *feedback* negativo para inibir a resposta ao estresse e evitar a hiperativação do eixo HPA, caracterizando a chamada “resistência aos glicocorticoides” (Anacker et al., 2011).

Um dos principais mecanismos responsáveis pela disfunção dos GR e pela resistência aos glicocorticoides vistas na depressão é a ativação da resposta imune. Citocinas pró-inflamatórias podem diminuir a função dos GR por reduzirem a afinidade dos GR pelo cortisol (Maddock e Pariante, 2001; Pariante et al., 1999). Além disso, mostrou-se que a interleucina 1 (IL-1) bloqueia diretamente a translocação dos GR do citoplasma para o núcleo (Pariante et al., 1999) através do estímulo da via de

580 sinalização da proteína quinase ativada por mitógeno p38 (MAPK p38, do inglês
581 *mitogen-activated protein kinase p38*) e fosforilação dos GR (Wang et al., 2004).
582 Adicionalmente, a ativação de quinases regulada por sinal extracelular (ERK, do inglês
583 *extracellular signal-regulated kinases*) por citocinas pró-inflamatórias acarreta na
584 fosforilação de cofatores dos GR, inibindo sua função (Rogatsky et al., 1998).

585 Um segundo possível mecanismo envolvido na resistência dos GR na
586 depressão é a redução na atividade da proteína quinase A (PKA), a qual está
587 envolvida na fosforilação e ativação dos GR (Pace et al., 2007). Agonistas da PKA
588 aumentam os níveis de mRNA, a transcrição e a função dos GR (Penuelas et al.,
589 1998). Inibidores da fosfodiesterase tipo 4 (como o rolipram, conhecidos por aumentar
590 a atividade da PKA), não só aumentam a função dos GR quando administrados
591 sozinhos, mas também potencializam a capacidade dos antidepressivos em aumentar
592 a função dos GR (Miller et al., 2002). Adicionalmente, estudos demonstraram que a
593 via de sinalização do cAMP/PKA age como mediadora do efeito psicotrópico de
594 antidepressivos, possivelmente levando ao aumento da neurogênese (Duman et al.,
595 2001; Rasenick et al., 1996). Corroborando com essas observações, um decréscimo
596 na atividade da PKA foi observado *in vitro* em fibroblastos de pacientes depressivos
597 (Manier et al., 2000).

598 Um terceiro mecanismo que explicaria a função reduzida dos GR na depressão
599 é pelo decréscimo na entrada de cortisol no cérebro, devido a atividade anormal do
600 sistema de transporte de esteroides da membrana celular. O cortisol plasmático não
601 pode entrar no cérebro por difusão passiva pois seu acesso é limitado pelos
602 transportadores de esteroides, como a glicoproteína P, os quais promovem o efluxo
603 do cortisol do cérebro (de Kloet et al., 1998). Dessa forma, tem sido sugerido que a
604 hipercortisolemia periférica é uma resposta compensatória adaptativa a
605 hipocortisolemia central (Pariante 2003): a baixa concentração de cortisol no cérebro
606 impede que o *feedback* negativo ocorra, acarretando na hiperativação do eixo HPA,
607 que quando ocorre cronicamente, leva à hipercortisolemia central. Consoante a isso,
608 estudos *in vitro* demonstraram que os antidepressivos convencionais inibem
609 diretamente a atividade desses transportadores de esteroides (Weiss et al., 2003).

610 Notavelmente, um dos principais efeitos da hipercortisolemia central é a
611 redução da proliferação neuronal na zona subgranular do hipocampo, levando a
612 atrofia e redução do volume hipocampal vistos na depressão (Krishnan e Nestler,
613 2008; McEwen, 2007). Evidências sugerem que elevados níveis de cortisol reduzem

os efeitos do fator neurotrófico derivado do cérebro (BDNF, do inglês *brain derived neurotrophic fator*) (Smith et al., 1995), reduzem a arborização dendrítica (Wooley et al., 1990) e acarretam na morte celular (Sapolsky, 1992) no hipocampo.

Diante da influência do eixo HPA na depressão, novas estratégias terapêuticas para o tratamento dessa doença estão sendo testadas. O antagonista do receptor CRH do tipo 1, R121919, mostrou-se promissor no tratamento da depressão (Zobel et al., 2000), mas foi posteriormente retirado dos ensaios clínicos devido à hepatotoxicidade. A mifepristona (RU486), um antagonista dos GR, mostrou-se eficaz no tratamento da depressão psicótica em ensaios clínicos (Flores et al., 2006), uma vez que o bloqueio dos GR pode “reiniciar” o eixo HPA (Belanoff et al 2002). Apesar de promissores, os medicamentos moduladores do eixo HPA ainda não se tornaram antidepressivos aprovados clinicamente (Aubry, 2013; Malhi e Mann, 2018).

2.2.3 Prejuízo na neuroplasticidade sináptica

Em adição à hiperativação do eixo HPA em pacientes com depressão, a disfunção da neurotransmissão glutamatérgica e prejuízos na neuroplasticidade sináptica vem se destacando como possíveis mecanismos envolvidos com a patofisiologia da depressão. A primeira observação da “hipótese glutamatérgica” é datada do início dos anos 90, com a demonstração de que antagonistas dos receptores N-metil-D-aspartato (NMDA-R, do inglês *N-methyl-D-aspartate receptor*) possuíam efeito tipo-antidepressivo (Trullas e Skolnick, 1990). Com a aquisição de mais dados pré-clínicos e clínicos, essa hipótese foi reformulada para integrar observações de diversas áreas, incluindo a sinalização intracelular e mecanismos de expressão gênica, mecanismos neurotróficos, neurogênese, plasticidade sináptica e remodelamento de circuitos neuronais, criando a chamada “hipótese da neuroplasticidade” (Pittenger e Duman, 2008; Racagni e Popoli, 2008; Sanacora et al., 2008).

A plasticidade sináptica representa uma das principais funções do cérebro, a habilidade de detectar, avaliar e armazenar informações complexas e realizar respostas adaptativas subsequentes ao estímulo relacionado (Holtmaat e Svoboda, 2009). A sinaptogênese é regulada por uma complexa interação de cascatas intracelulares, e a disfunção em uma dessas vias, como por exemplo, a perda do suporte neurotrófico, tem sido implicada na patofisiologia da depressão (Duman e Aghajanian, 2012).

Vale destacar que a maioria das alterações na neuroplasticidade sináptica tem sido observadas em neurônios que pertencem ao sistema de neurotransmissão glutamatérgica (Figura 9), ao invés dos neurônios monoaminérgicos (Sanacora et al., 2008). O glutamato é responsável pela maioria das sinapses excitatórias rápidas no cérebro, com um papel crucial na plasticidade sináptica, humor, aprendizado e memória.

O glutamato é sintetizado *de novo* a partir da glicose via ciclo de Crebs e alfa-cetoglutarato, ou pode ser reciclado através do ciclo glutamato/glutamina. O glutamato é então transportado e armazenado no interior de vesículas sinápticas até ser liberado por exocitose. Quando liberado, o glutamato se liga à diferentes receptores, incluindo os receptores metabotrópicos acoplados à proteína G e os receptores ionotrópicos NMDA-R, ácido alfa-amino-3-hidroxi-metil-5-4-isoxazolpropiónico (AMPA-R, do inglês *alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor*) e cainato. Os AMPA-R e NMDA-R são fundamentais para a plasticidade sináptica dependente de atividade, principalmente através da potenciação de longa duração (LTP, do inglês *long-term potentiation*) (Morris et al., 1986). Nesse processo, os AMPA-R rapidamente conduzem a corrente de despolarização pela membrana, levando ao influxo de Ca^{2+} pelos NMDA-R e subsequente ativação da proteína quinase II dependente de Ca^{2+} /calmodulina (CAMKII, do inglês *Ca²⁺/calmodulin dependent protein kinase II*) e outros segundo-mensageiros *downstream* que culminam com a incorporação de AMPA-R na membrana.

A sinalização adequada dos NMDA-R promove a sobrevivência celular e suporte neurotrófico. A ativação moderada dos NMDA-R sinápticos promove o influxo de Ca^{2+} e ativa vias de sinalização que favorecem a neuroproteção, incluindo as vias de sinalização das MAPK Ras-Raf-MEK e do CREB, resultando no aumento da expressão do BDNF (Murrough et al., 2017). Por outro lado, a ativação dos NMDA-R extra sinápticos induz vias de sinalização pro-apoptóticas, que, consequentemente, podem contribuir com a apoptose neuronal (Hardingham e Bading, 2010). A excitotoxicidade glutamatérgica pode ser consequência da ativação dos NMDA-R extra sinápticos devido ao prejuízo da remoção do glutamato da fenda sináptica. Uma vez que não há enzimas sinápticas responsáveis pela eliminação do glutamato, sua remoção depende da entrada nas células gliais através dos transportadores de aminoácidos excitatórios (EAAT, do inglês *excitatory amino acid transporters*). De fato, a redução no número e densidade de células gliais (Hamidi et al., 2004) e expressão

reduzida dos EAAT (Choudary et al., 2005) foram encontradas em pacientes depressivos. A redução no número e densidade de células gliais em pacientes com desordens do humor são um dos achados mais consistentes na pesquisa psiquiátrica (Sanacora et al., 2008).

De acordo com a observação de que o estresse é um dos principais fatores de risco para a depressão, o estresse crônico afeta a liberação e recaptação de glutamato no córtex pré-frontal por reduzir a disponibilidade de AMPA-R e NMDA-R, reduzir a densidade e o diâmetro sináptico, reduzir o tamanho e arborização dendrítica e diminuir o fluxo glutamato/glutamina (Popoli et al., 2012). Recentes estudos *post-mortem* também demonstraram redução no número de sinapses (Kang et al., 2012) e nos níveis de glutamato (Moriguchi et al., 2018) no córtex pré-frontal de pacientes depressivos, o que explicaria a reduzida plasticidade sináptica observada nessa região cerebral. Estudos pré-clínicos deram suporte a essa observação ao confirmar que a exposição ao estresse crônico causa alterações na densidade de espinhas dendríticas e arborização dendrítica, remodelamento de sinapses, atrofia neuronal, perda de células gliais e redução na neurogênese, especialmente no córtex pré-frontal e no hipocampo (Duman e Aghajanian 2012; Gould et al., 1997; Morrison e Baxter, 2012). Vale destacar que, enquanto a exposição crônica ao cortisol promove a perda das espinhas dendríticas (Liston e Gan, 2011), a exposição aguda ao cortisol promove a interação dos GR com o receptor de tropomiosina quinase B (TrkB, do inglês *tropomyosin receptor kinase B*) para induzir a adaptação de espinhas dendríticas, indicando a complexa interação entre a sinalização dos GR e TrkB-BDNF (Jeanneteau et al., 2008). Enquanto que o estresse agudo aumenta a liberação de glutamato e a sinalização apropriada, o estresse crônico leva a alterações mal adaptativas na sinapse glutamatérgica. Essas alterações incluem a redução no fluxo de conversão de glutamato/glutamina (reduzindo a concentração de glutamato) (Banasr et al., 2010), redução na recaptação de glutamato pelas células gliais e aumento na ativação dos NMDA-R extra sinápticos, potencialmente contribuindo com a excitotoxicidade, perda sináptica e ativação de vias intracelulares pró-apoptóticas (Figura 9) (Murrough et al., 2017). Adicionalmente, estudos têm demonstrado que pacientes depressivos apresentam redução nos níveis do neurotransmissor ácido gama-aminobutírico (GABA, do inglês *gamma aminobutyric acid*) e déficits na função dos receptores de GABA (GABA-R) (Fee et al., 2017; Godfrey et al., 2018). Consoante a isso, estudos pré-clínicos demonstraram que o estresse crônico reduz a síntese de GABA no córtex

pré-frontal e outras regiões corticais (Ghosal et al., 2017; Fee et al., 2017). O GABA é o principal neurotransmissor inibitório do sistema nervoso central, participando do controle da neurotransmissão glutamatérgica. Assim, o decréscimo na neurotransmissão GABAérgica contribui com o aumento da excitotoxicidade glutamatérgica vista em pacientes depressivos (Duman et al., 2009). Como consequência, essas alterações explicariam a redução no volume do córtex pré-frontal e hipocampo (Kemptom et al., 2011), alterações nos níveis de glutamato (Yuksel e Ongur, 2010) e na conectividade dos circuitos neurais (Kaiser et al., 2015) observadas na depressão.

Um dos principais fatores neurotróficos envolvidos com a plasticidade sináptica dependente da neurotransmissão glutamatérgica é o BDNF. O BDNF é expresso em altas concentrações no cérebro de adultos, especialmente no córtex pré-frontal, hipocampo, amígdala e hipotálamo, as quais são regiões amplamente envolvidas com a regulação do humor (Friedman e Greene, 1999). O BDNF é sintetizado como um precursor pró-BDNF de 32 kDa que sofre processamento proteolítico (via furina, proconvertases, plasmina e metaloproteinases) para originar a forma madura do BDNF de 14 kDa (Lessmann e Brigadski, 2009). A forma madura do BDNF sinaliza via TrkB, recrutando as vias de sinalização das MAPK Ras-Raf-MEK, fosfolipase C (PLC, do inglês *phospholipase C*) e fosfatidilinositol 3-quinase (PI3K, do inglês *phosphatidylinositol 3-kinase*) (Chao, 2003) (Figura 9). O pró-BDNF se liga ao receptor de neurotrofina p75 (p75NTR, do inglês *p75 neurotrophin receptor*) e modula as vias de sinalização do NFκB, RhoGTPase e Jun quinase (JNK, do inglês *Jun kinase*) (Mizui et al., 2016). O pró-BDNF e o BDNF maduro desencadeiam efeitos biológicos opostos, o chamado “yin e yang” das neurotrofinas (Lu et al., 2005). Enquanto o pró-BDNF aumenta a apoptose, induz a depressão de longa duração (LTD, do inglês *long-term depression*) e reduz as espinhas dendríticas, o BDNF maduro promove a sobrevivência neuronal, LTP, neurogênese e formação de espinhas dendríticas (Lu et al., 2005).

Diversas observações clínicas sugerem o prejuízo do suporte neurotrófico na depressão. Os níveis de BDNF maduro foram encontrados reduzidos em amostras *post-mortem* de pacientes depressivos (Guilloux et al., 2012; Thompson Ray et al., 2011; Tripp et al., 2012) e em vítimas de suicídio que sofriam de depressão severa (Chen et al., 2001; Dwivedi et al., 2009). Em adição ao BDNF, os níveis do TrkB também foram encontrados reduzidos em pacientes depressivos (Tripp et al., 2012).

A sinalização do BDNF envolve a ativação de diversos alvos *downstream* que influenciam na função neuronal (Figura 9). Um dos principais alvos é a ativação do complexo 1 do alvo mamífero da rapamicina (mTORC1, do inglês *mammalian target of rapamycin complex 1*) (Watson e Baar, 2014).

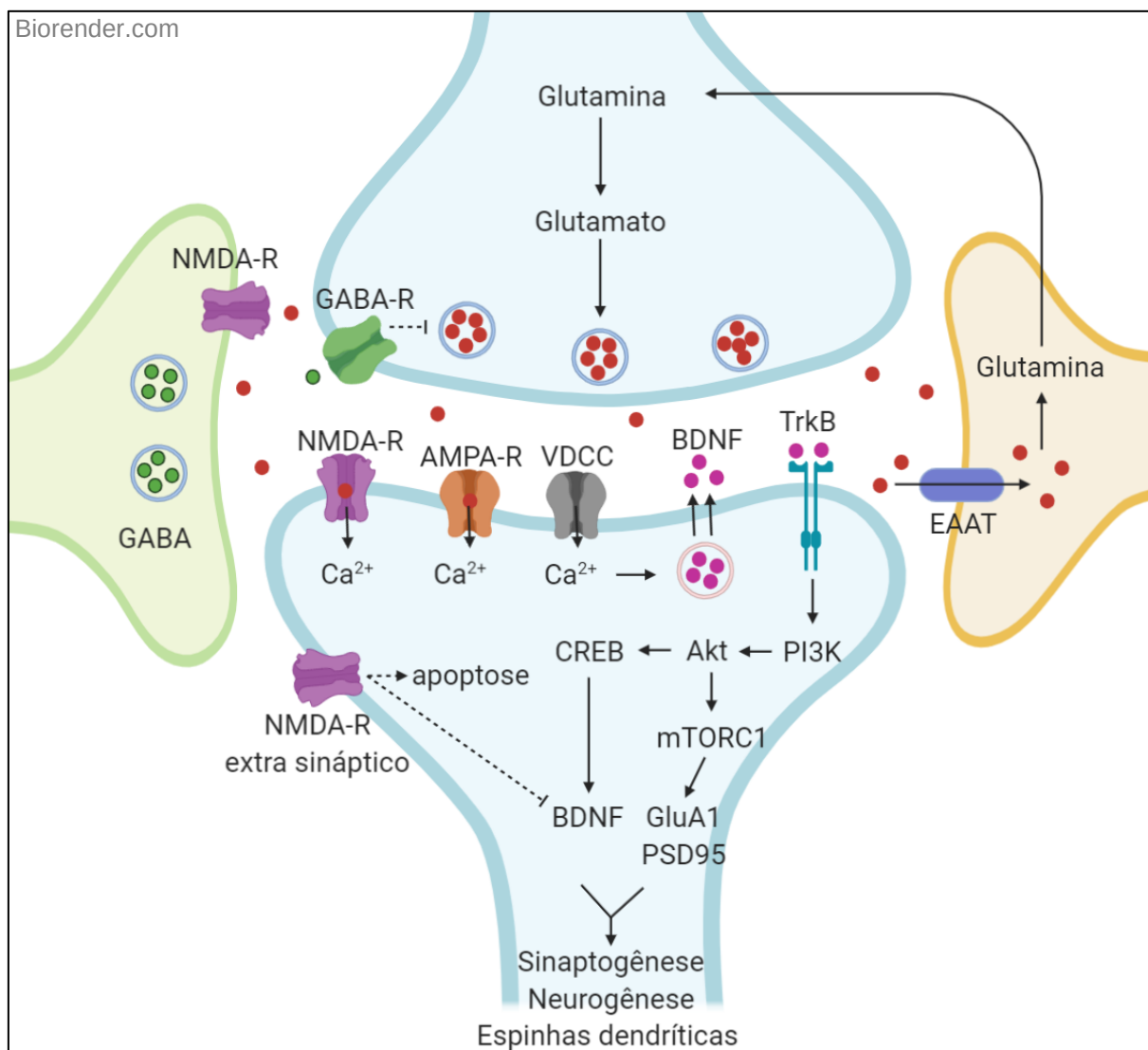


Figura 9. Neurotransmissão glutamatergica e suporte neurotrófico no córtex pré-frontal de indivíduos saudáveis. A neurotransmissão glutamatergica é controlada rigorosamente com a ajuda de células gliais e da neurotransmissão GABAérgica. A glutamina é convertida à glutamato, o qual é armazenado em vesículas sinápticas até ser liberado na fenda sináptica. A remoção do glutamato da fenda sináptica ocorre pela ação dos EAAT presentes predominantemente em células gliais. O glutamato é convertido em glutamina, a qual é transportada ao neurônio pré-sináptico para dar origem ao glutamato. A despolarização da membrana do neurônio pré-sináptico promove a exocitose do glutamato para a fenda sináptica. Vários receptores de glutamato estão presentes em neurônios pós-sinápticos, incluindo os AMPA-R e NMDA-R. O efeito do glutamato é determinado pelo subtipo e localização do receptor. A ativação moderada dos NMDA-R e AMPA-R sinápticos promove o influxo de Ca^{2+} que estimula a

liberação de BDNF. Através da sinalização via receptores TrkB/PI3K/Akt, o BDNF induz a atividade do mTORC1 e CREB, os quais induzem a expressão de proteínas envolvidas com a plasticidade sináptica. Fonte: Angela Maria Casaril.

A via do mTORC1 é regulada pela sinalização de neurotrofinas, demanda energética, e sistema endócrino e imune, e, portanto, está envolvida com inúmeros fatores de susceptibilidade à depressão (Watson e Baar, 2014). Nesse sentido, o mTORC1 serve como um sensor neuronal da demanda pela síntese de novas proteínas e sinaptogênese (Duman et al., 2016). De fato, a expressão e função do mTORC1 é reduzida no córtex pré-frontal de pacientes depressivos, contribuindo com o decréscimo na síntese de proteínas sinápticas nessa estrutura (Jernigan et al., 2011). Estudos *post-mortem* demonstraram que a expressão do fator regulador do desenvolvimento e resposta de dano no DNA 1 (REDD1, do inglês *regulated in development and DNA damage responses 1*), um regulador negativo do mTORC1, é aumentada no córtex pré-frontal de pacientes com depressão e estudo pré-clínicos suportam a observação de que o estresse crônico reduz a sinalização do mTORC1 (Ota et al., 2014). Por outro lado, antidepressivos com um início de ação rápido, como a cetamina e escetamina, estimulam a sinalização do mTORC1 no córtex pré-frontal (Li et al., 2010; Maeng et al., 2008).

O envolvimento do glutamato e do BDNF na etiologia da depressão (Figura 10) ganhou mais força com a aprovação da escetamina, um antagonista dos NMDA-R, para o tratamento da depressão resistente aos tratamentos (Food and Drug Administration, 2019a). O mecanismo pelo qual o antagonismo dos NMDA-R no córtex pré-frontal leva à indução do mTORC1 e sinaptogênese ocorre através de vias indiretas. A sinalização do mTORC1 induzida pela escetamina depende da transmissão glutamatérgica e ativação dos AMPA-R (Li et al., 2010; Maeng et al., 2008). Por ser um antagonista dos NMDA-R, acredita-se que a escetamina bloqueia os NMDA-R extra sinápticos e os NMDA-R em interneurônios GABAérgicos, aumentando a liberação de GABA e glutamato (Homayoun e Moghaddam, 2007; Stone et al., 2012). O bloqueio dos NMDA-R nos interneurônios GABAérgicos é transitório, resultando no pico de liberação de glutamato que leva à liberação de BDNF-dependente de atividade e efeitos duradouros nas sinapses glutamatérgica e GABAérgicas. Nesse sentido, a liberação do BDNF-dependente de atividade, que aumenta tanto a função sináptica do glutamato e do GABA, pode ser o evento chave

para mediar os rápidos efeitos da escetamina (Duman, 2018). O aumento na liberação de glutamato favorece a ligação aos AMPA-R, uma vez que os NMDA-R (sinápticos e extra sinápticos) estão bloqueados, indicando que o efeito antidepressivo da escetamina é o aumento da neurotransmissão glutamatérgica dependente dos AMPA-R. A ligação do glutamato aos AMPA-R causa a despolarização da membrana neuronal e ativação de canais de Ca^{2+} dependentes de voltagem, liberação do BDNF e estimulação da via do TrkB-Akt. Como resultado, ocorre a ativação da sinalização do mTORC1, levando ao aumento na síntese de proteínas necessárias para a maturação e formação de sinapses, como por exemplo a GluA1 e a proteína de densidade pós-sináptica 95 (PSD-95, do inglês *postsynaptic density protein 95*) (Duman et al., 2016).

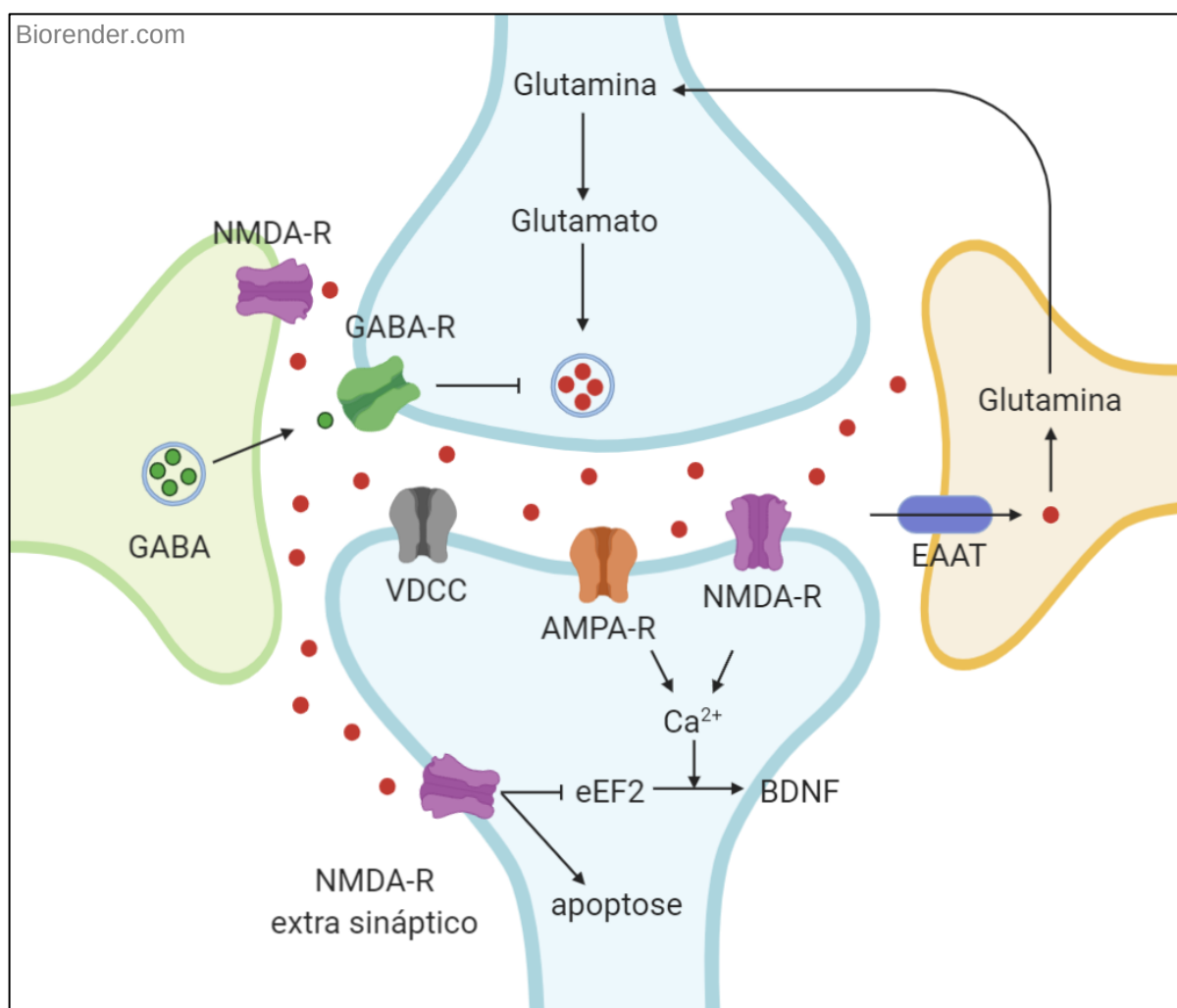


Figura 10. Neurotransmissão glutamatérgica e suporte neurotrófico no córtex pré-frontal de pacientes depressivos. O estresse crônico e a depressão acarretam na redução no fluxo de conversão de glutamato/glutamina (reduzindo a concentração de glutamato), redução na recaptura de glutamato

pelos EAAT nas células gliais, redução na neurotransmissão inibitória mediada pelo GABA e aumento na ativação dos NMDA-R extra sinápticos, contribuindo com a excitotoxicidade, perda sináptica (através da menor sinalização de neurotrofinas) e ativação de vias intracelulares pró-apoptóticas. Essas alterações culminam com a atrofia celular e redução no volume do córtex pré-frontal e perda da conectividade dos circuitos neurais. O antidepressivo escetamina, por sua vez, bloqueia os NMDA-R extra sinápticos e os NMDA-R em interneurônios GABAérgicos (transientemente; 30 a 60 min após a administração), acarretando na maior liberação de glutamato pré-sináptico na fenda sináptica e sinalização via AMPA-R para promover a LTP, plasticidade sináptica e homeostase da neurotransmissão glutamatérgica e GABAérgica (uma vez que os NMDA-R extra-sinápticos também estão bloqueados pela escetamina). VDCC: canal de cálcio dependente de voltagem (do inglês, *voltage-gated calcium channel*). eEF2: fator de alongamento da tradução (do inglês, *eukaryotic translation elongation factor 2*). Fonte: Angela Maria Casaril.

Vale destacar que, diferentemente dos antagonistas dos NMDA-R, a administração aguda ou crônica de antidepressivos convencionais (como a fluoxetina) não aumenta a sinalização do mTORC1 (Li et al., 2010). Evidentemente, a sinalização do mTORC1 e a sinaptogênese são importantes para o tratamento rápido e eficaz da depressão, abrindo caminho para o desenvolvimento racional de novos antidepressivos com mecanismos de ação mais específicos.

Em termos de adaptação, a significância evolutiva das alterações na plasticidade sináptica em resposta ao estresse não é clara (Duman et al., 2016). Uma hipótese é que os NMDA-R extra sinápticos funcionam como sensores neurais para estresse oxidativo ou alterações metabólicas, resultando na regressão de espinhas dendríticas que protegem a viabilidade neuronal (Popoli et al., 2012). Possivelmente o estresse crônico, combinado com fatores genéticos, resulta em alterações adaptativas de curta duração (como mobilização da glicose e ativação do sistema imune) que podem ter consequências deletérias a longo prazo para as sinapses, para o cérebro e para a saúde do organismo em geral (Duman et al., 2016).

2.2.4 Ativação da resposta inflamatória

Por volta dos anos 90, quando as descobertas acidentais sobre os antidepressivos nos anos 50 renderam a aprovação do Prozac® para o tratamento da depressão, alguns artigos reportando alterações no sistema imune nos pacientes depressivos foram publicados sem muita notoriedade (Dantzer e Kelley, 1989; Maes, 1995; Smith, 1991). Dantzer e Kelley (1989) propuseram que a observação antiga de que o estresse exacerba a progressão de doenças físicas através dos efeitos

imunossupressores mediados pelo cortisol deveria ser revisada, uma vez que estudos davam suporte a ideia de que a depressão (Tecoma e Huey, 1985) e diferentes estressores (Ader, 1981) alteravam a atividade de linfócitos e macrófagos. Começava a se tornar claro que as conexões recíprocas entre o cérebro e o sistema imune desempenhavam papéis importantes na resposta coordenada à infecção e inflamação. Em 1991, Smith propôs a “teoria dos macrófagos na depressão”, a qual sugeria que a ativação do sistema imune periférico contribuía com os sintomas depressivos (Smith, 1991). Essa teoria fornecia uma explicação para a associação significativa da depressão com casos de doença coronária, artrite reumatoide, acidente vascular encefálico e outras doenças em que ocorria a ativação de macrófagos (Smith, 1991).

Até recentemente, o cérebro era considerado um órgão imunoprivilegiado que era protegido do sistema imune periférico pela barreira cérebro-sangue (BBB, do inglês *blood-brain barrier*). No entanto, hoje sabe-se que o cérebro é diretamente influenciado pelas células imunes, citocinas periféricas e glicocorticoides, os quais acessam o cérebro e influenciam inúmeros circuitos neurais relacionados com a depressão.

O sistema imune inato, através de diferentes tipos celulares e componentes solúveis, defende o organismo contra potenciais agentes patogênicos e durante a carcinogênese. Juntamente com a capacidade de ativar o sistema imune adaptativo, uma das funções cruciais do sistema imune inato é a sua capacidade de discriminar entre o “próprio e o não-próprio” através do reconhecimento de padrões moleculares associados à patógenos (PAMPs, do inglês *pathogen-associated molecular patterns*) (Medzhitov, 2009). Em casos de trauma ou dano tecidual, padrões moleculares associados ao perigo (DAMPs, do inglês *damage-associated molecular patterns*) liberados por células mortas ou danificadas também podem ativar a resposta imune inata. Dentre os principais DAMPs, pode-se citar o ATP, DNA mitocondrial (mtDNA), HSP e proteínas de alta mobilidade do grupo de caixa 1 (HMGB1, do inglês *high-mobility group box 1*), enquanto um dos principais PAMPs é o lipopolissacarídeo (LPS) de bactérias Gram-negativas. PAMPs e DAMPs se ligam à diversos receptores de reconhecimento padrão (PRRs, do inglês *pattern recognition receptors*) expressos no citosol ou nas membranas de células imunes inatas, como os receptores do tipo Toll (TLRs, do inglês *Toll-like receptors*), receptores de lectina tipo C (CLRs, do inglês *C-type lectin receptors*), receptores tipo AIM2 (ALRs, do inglês *AIM2-like receptors*),

receptores do tipo RIG-I (RLRs, do inglês *RIG-I-like receptors*) e receptores do tipo NOD (NLRs, do inglês *NOD-like receptors*). Subsequentemente, os PRRs ativados desencadeiam uma cascata de sinalização, resultando na liberação de citocinas e outros fatores solúveis pelas células mieloides que orquestram o recrutamento de leucócitos para sítios de inflamação (Iwasaki e Medzhitov, 2015). Notavelmente, alterações na contagem de células mieloides, quimiotaxia, fagocitose, produção de óxido nítrico (NO^{*}), expressão de citocinas e função efetora de células T são encontradas alteradas em pacientes depressivos e podem desempenhar importantes papéis patofisiológicos envolvidos com alguns subtipos de depressão (Ahmetspahic et al., 2018).

O envolvimento do sistema imune na patogênese da depressão é evidenciado por dados identificando o aumento de citocinas inflamatórias, receptores de citocinas, quimiocinas e proteínas de fase aguda em alguns pacientes depressivos (Köhler et al., 2017). Uma das principais evidências para o envolvimento de citocinas no comportamento depressivo é a observação de que uma porção significativa de pacientes que recebem interferon alfa (IFN- α) como tratamento para a hepatite C ou que recebem interleucina (IL)-2 para o tratamento do câncer sofrem de sintomas depressivos como um efeito adverso (Bonaccorso et al., 2002; Renault et al., 1987; Walker et al., 1997). Vale destacar que uma meta-análise recente de 82 estudos encontrou que pacientes com depressão apresentam níveis periféricos mais elevados de fator de necrose tumoral alfa (TNF- α), IL-6, IL-10, IL-13, IL-18, IL-12, receptor solúvel de IL-2, ligante C-C de quimiocina 2 (CCL2) e antagonista do receptor IL-1 quando comparados com controles saudáveis (Köhler et al., 2017). Uma análise *post-mortem* do cérebro de indivíduos depressivos vítimas de suicídio encontrou que esses pacientes apresentavam aumento nos níveis proteicos e na expressão gênica dos receptores TLR3 e TLR4, contribuindo com a ideia do envolvimento da neuroinflamação em alguns casos de depressão (Pandey et al., 2012). Consoante a isso, a chamada “resposta doentia” (*sickness behavior*) é desencadeada pela inflamação causada por infecção ou dano tecidual e leva ao comportamento de anedonia, fadiga, isolamento social e irritabilidade, os quais são frequentemente encontrados em alguns subtipos de pacientes com depressão (Dantzer et al., 2008). No entanto, é fundamental destacar que a depressão não deve ser vista como uma doença inflamatória *per se*, mas, que em alguns pacientes, o processo inflamatório parece contribuir significativamente com o desenvolvimento e manutenção de

sintomas depressivos (Raison e Miller, 2013). Adicionalmente, vale destacar que a inflamação é associada principalmente com os sintomas somáticos (fadiga, motivação reduzida, alterações no sono e apetite) ao invés dos sintomas psicológicos (humor deprimido e pensamentos suicidas) (Iob et al., 2019; Jokela et al., 2016). Diante disso torna-se importante a identificação do subgrupo de pacientes depressivos que exibem um perfil inflamatório aumentado e que podem ser menos responsivos aos antidepressivos que modulam a neurotransmissão monoaminérgica.

Um princípio central da inflamação associada à depressão é a capacidade de sinais inflamatórios periféricos serem traduzidos para o cérebro, afetando o comportamento (Holmes et al., 2018). As citocinas são um grupo heterogêneo de proteínas de 6 a 70 kDa que são produzidas por células imunocompetentes com a finalidade de regular as respostas imunes, reparar tecidos danificados e restaurar a homeostase (Haroon et al., 2012). Devido ao alto peso molecular, as citocinas não atravessam rapidamente a BBB. Diferentes vias estão envolvidas com a passagem de citocinas para o cérebro (Figura 11): (i) passagem através de regiões permeáveis da BBB, como os órgãos circunventriculares (os quais são altamente vascularizados e possuem capilares fenestrados), ou passagem através da ativação de células localizadas ao longo da vasculatura cerebral (incluindo células endoteliais e macrófagos perivasculares, os quais liberam citocinas e outros mediadores inflamatórios no cérebro) (a chamada “rota humoral”) (Cao et al., 1997; Ericsson et al., 1994) – uma vez que as citocinas ativam as células microgliais nessas regiões, elas liberam prostaglandina E2 (PGE2) que rapidamente transmite o sinal inflamatório pelo cérebro (Maier, 2003); (ii) ligação à receptores de citocinas associados à fibras nervosas periféricas aferentes (como o nervo vago), o qual então transmite os sinais de citocinas para algumas regiões cerebrais, como o núcleo do trato solitário e o hipotálamo (a chamada “rota neural”) (Ericsson et al., 1994); (iii) recrutamento de células ativadas, como monócitos e macrófagos da periferia para o cérebro, as quais passam a produzir citocinas (a chamada “rota celular”) – esse recrutamento é mediado pela liberação microglial da proteína quimiotrática de monócito 1 (MCP-1, do inglês *monocyte chemoattractant protein 1*) (D'Mello et al., 2009).

Uma vez no cérebro, uma vasta rede de células gliais são responsáveis pela propagação da neuroinflamação. As principais células gliais relacionadas com a depressão induzida pela inflamação são a microglia e astrócitos (De Leo et al., 2006). Em condições normais, a microglia inspeciona o meio celular do cérebro, respondendo

à moléculas imunes, como as citocinas, DAMPs e PAMPs. Vale destacar que a resposta imune no cérebro em condições fisiológicas é extremamente importante para a neurogênese, LTP e plasticidade sináptica (Yirminia e Goshen, 2011). Em condições patológicas, no entanto, a microglia adota um estado ativo, altamente pró-inflamatório liberando mais citocinas. Em adição a essa inspeção imune, a microglia é ativamente envolvida com a neuroplasticidade, aprendizado e desenvolvimento neuronal (Wu et al., 2015). Os astrócitos fornecem nutrientes e suporte para células neurais, equilibram os níveis extracelulares de ferro, liberam gliotransmissores, ATP e glutamato, tendo influência direta na manutenção, função e plasticidade neuronal (Zhang et al., 2013).

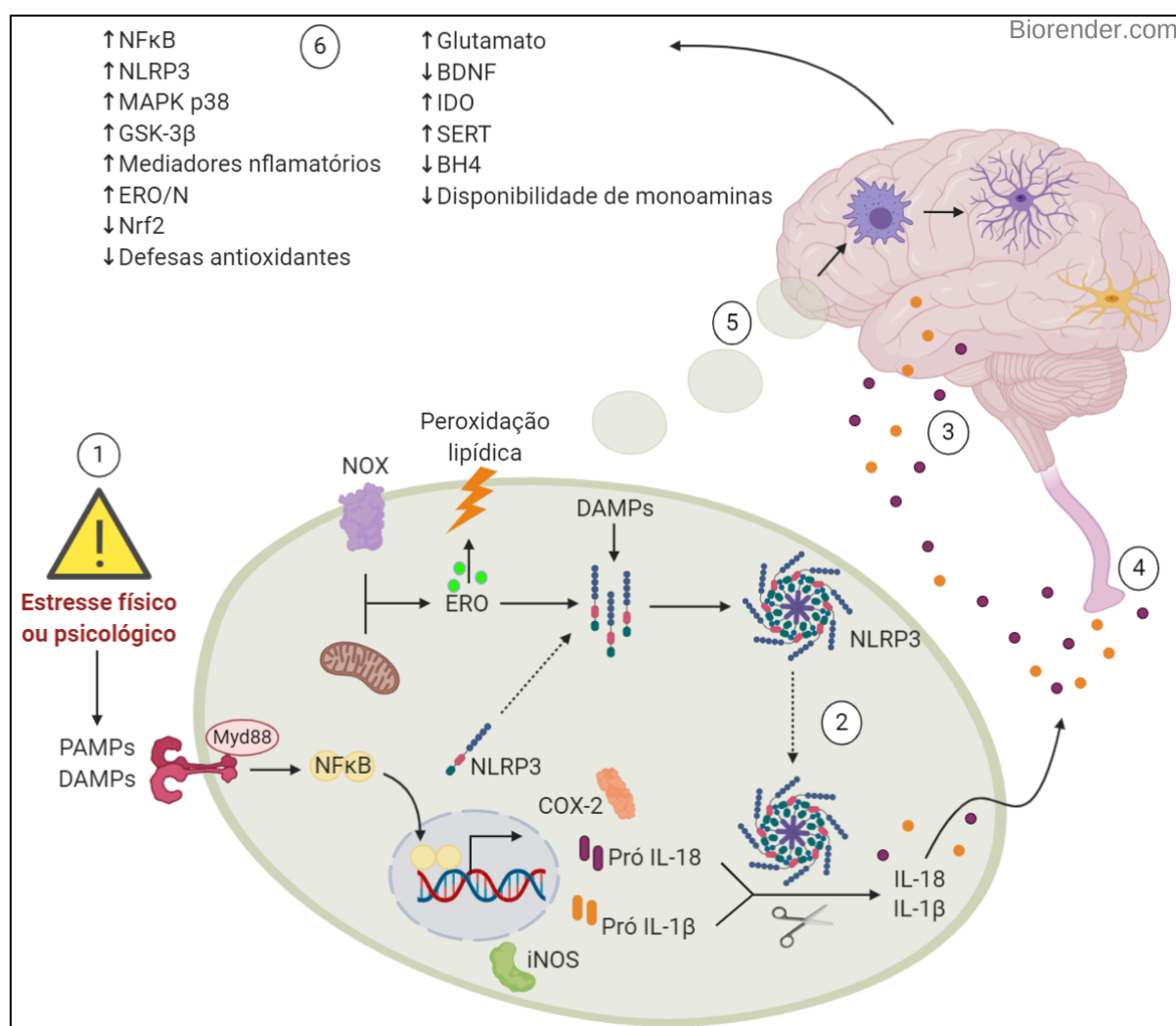


Figura 11. Propagação da resposta imune periférica ao sistema nervoso central em situação de estresse. 1) Estressores físicos ou psicológicos podem levar a liberação de padrões moleculares associados a patógenos (PAMPs) e ao perigo (DAMPs) que sinalizam via receptor tipo Toll 4 (TLR4) para ativar o fator de transcrição nuclear kappa B (NFκB). A translocação do NFκB do citosol para o núcleo induz a expressão da óxido nítrico sintase induzível (iNOS), ciclooxigenase 2 (COX-2), pró-

interleucina 18 (IL-18) e pró-interleucina 1 β (IL-1 β) e da subunidade do inflamassoma NLRP3. A cadeia transportadora de elétrons mitocondrial e a enzima NADPH oxidase (NOX) são responsáveis pela produção de espécies reativas de oxigênio (ERO) que, em conjunto com outros DAMPs, induzem a oligomerização e montagem do inflamassoma NLRP3. 2) O NLRP3 é responsável pela clivagem da pró-IL18 e pró-IL-1 β em suas formas solúveis, as quais são então liberadas da célula. A sinalização das citocinas chega ao sistema nervoso central através de três mecanismos principais. 3) Pela via humoral, as citocinas entram no sistema nervoso central através da passagem por regiões permeáveis da barreira cérebro-sangue (BBB). Adicionalmente, células endoteliais e macrófagos perivasculares podem secretar citocinas que entram no sistema nervoso central. 4) Pela via neural, as citocinas e PAMPs podem ativar nervos aferentes, como o nervo vago, uma vez que ele expressa receptores de citocinas e do tipo Toll. Esses nervos aferentes periféricos transmitem sinais do sistema imune periférico para diversas regiões cerebrais relacionadas com o humor. 5) Pela via celular, monócitos periféricos ativados podem infiltrar a BBB e produzir citocinas pró-inflamatórias. 6) Uma vez no sistema nervoso central, as citocinas agem por inúmeros mecanismos para contribuir com o estabelecimento do comportamento depressivo. Fonte: Angela Maria Casaril.

Quando ativadas por um estímulo inflamatório, a microglia produz e libera citocinas pro-inflamatórias (especialmente IL-1 β , IL-6 e TNF- α) através da ativação do NF κ B ou do inflamassoma NLR proteína 3 (NLRP3, do inglês *NOD-like receptor protein 3*). A relação entre a produção de citocinas e as alterações comportamentais e neurofisiológicas encontradas em pacientes depressivos ocorre, principalmente, através de alterações no metabolismo das monoaminas, evidenciado pela ativação da enzima IDO, MAPK p38, modulação da tetraidrobiopterina (BH4) e do metabolismo do glutamato/neurogênese (Haroon et al., 2012). A influência das citocinas no sistema endócrino também contribui para as alterações comportamentais vistas em pacientes depressivos (Haroon et al., 2012). Um resumo do mecanismo de ação das citocinas no cérebro durante a depressão está representado na Figura 12 (Felger e Lotrich, 2013).

A IDO é uma enzima expressa em diferentes tipos células, incluindo macrófagos, células dendríticas, microglia, astrócitos e neurônios (Huang et al., 2010). A IDO é ativada por diversas citocinas, como TNF- α , IL-1 e IL-6 através do estímulo de vias de sinalização, incluindo a via da MAPK p38 e NF κ B (Fujigaki et al., 2006). Relevante para o metabolismo da serotonina, a ativação da IDO em células imune periféricas (macrófagos e células dendríticas) ou em células do cérebro (microglia, astrócitos e neurônios) leva a produção de quinurenina, a qual é convertida à ácido quinurênico (KA, do inglês *kynurenic acid*) nos astrócitos ou ácido quinolínico (QA, do

inglês *quinolinic acid*) na microglia ou macrófagos infiltrados (Haroon et al., 2012). Através do bloqueio de receptores de acetilcolina, o KA pode reduzir a liberação de glutamato e dopamina, impactando negativamente a cognição (Schwarcz e Pellicciari, 2002). Por outro lado, o QA age como agonista dos NMDA-R, levando a liberação de glutamato (Schwarcz e Pellicciari, 2002). O QA também foi associado com a indução da peroxidação lipídica e estresse oxidativo (Schwarcz e Pellicciari, 2002), conjuntamente contribuindo com a excitotoxicidade no cérebro.

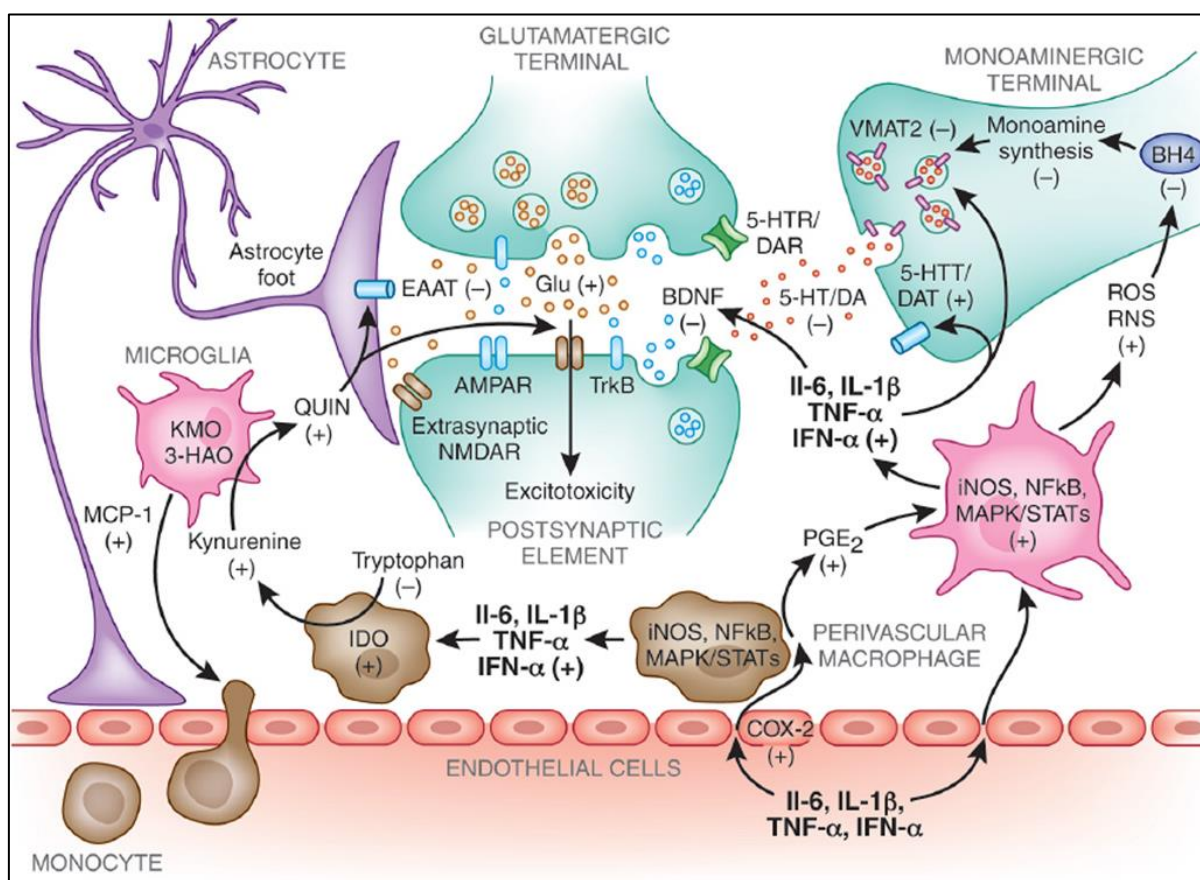


Figura 12. Potenciais mecanismos responsáveis pelos efeitos de citocinas pro-inflamatórias no cérebro durante a depressão. Citocinas periféricas podem acessar o sistema nervoso central e aumentar a produção de mediadores inflamatórios locais como a ciclooxigenase 2 (COX-2), prostaglandina E2 (PGE2), óxido nítrico (NO^{*}), citocinas e quimiocinas por células endoteliais, macrófagos perivasculares e microglia. A produção da proteína quimioatrativa de monócito (MCP-1) recruta células imunes periféricas para o cérebro para produzirem ainda mais citocinas e mediadores inflamatórios. Citocinas inflamatórias estão associadas com o aumento do estresse oxidativo e geração de espécies reativas de oxigênio e nitrogênio (ERO/N). O aumento de ERO/N contribui com a oxidação da tetraidrobiopterina (BH4), um cofator necessário para a síntese das monoaminas. Além disso, evidências indicam que as citocinas e suas vias de sinalização, como a da proteína quinase ativada por mitógeno p38 (MAPK p38) podem reduzir a expressão e a função do transportador vesicular de monoaminas 2 (VMAT2) e/ou

aumentar a expressão ou função dos transportadores de serotonina e dopamina (5-HTT/DAT). As citocinas também reduzem a sinalização do fator neurotrófico derivado do cérebro (BDNF) e interferem com o seu receptor de tropomiosina quinase (TrkB), influenciando negativamente a neurogênese e neuroplasticidade. Por fim, as citocinas inflamatórias podem afetar a sinalização do glutamato (Glu) pela ativação da enzima indoleamina-2,3-dioxigenase (IDO), a qual cataboliza o aminoácido triptofano (precursor da serotonina) em quinurenina. A quinurenina é então metabolizada em ácido quinurênico e ácido quinolínico (QUIN). O ácido quinurênico pode agir como antagonista dos receptores colinérgicos e reduzir a liberação de glutamato e dopamina, impactando negativamente a cognição. O QUIN age como agonista dos receptores N-metil-D-aspartato (NMDA-R), aumentando a liberação de glutamato e inibindo a recaptação de glutamato pelos astrócitos via transportadores de aminoácidos excitatórios (EAAT), permitindo o maior acesso do Glu aos NMDA-R extra sinápticos e contribuindo com a excitotoxicidade. Fonte: Felger e Lotrich (2013).

Outra via que pode influenciar o metabolismo de monoaminas é a sinalização via MAPK p38, a qual aumenta a expressão e função dos SERT (Zhu et al., 2006). Por exemplo, o tratamento de sinaptossomas do estriado e mesencéfalo de camundongos com IL-1 β e TNF- α leva a um aumento tempo- e dose-dependente na recaptação de serotonina, o qual pode ser revertido pelo tratamento com o SB203580, um antagonista da MAPK p38 (Zhu et al., 2006).

As citocinas também podem influenciar o metabolismo monoaminérgico através da modulação da BH4. A BH4 é um cofator enzimático essencial para as enzimas triptofano hidroxilase e tirosina hidroxilase, as quais são enzimas limitantes na síntese de serotonina e dopamina/noradrenalina, respectivamente (Neurauter et al., 2008). A BH4 também é um cofator para a enzima NO $^{\circ}$ sintase, a qual converte arginina em NO $^{\circ}$ (Neurauter et al., 2008). A BH4 é altamente sensível à oxidação não-enzimática, a qual leva a degradação da BH4 para diidrobiopterina (Neurauter et al., 2008). De fato, a neuroinflamação é associada com o aumento na produção de NO $^{\circ}$ (Zielasek e Hartung, 1996), sugerindo que as citocinas influenciam a BH4 através do NO $^{\circ}$. Isso indica que o estresse oxidativo pode ser um mecanismo pelo qual as citocinas e a neuroinflamação reduzem a disponibilidade de monoaminas em regiões cerebrais (Haroon et al., 2012).

Além das monoaminas, as citocinas também influenciam no metabolismo do glutamato e, conseqüentemente, a neurogênese. Citocinas inflamatórias podem reduzir a expressão de transportadores de glutamato em células gliais e aumentar a liberação de glutamato pelos astrócitos (Ida et al, 2008; Tilleux e Hermans, 2007; Volterra e Meldolesi, 2005). Vale destacar que o glutamato liberado pelos astrócitos

tem acesso preferencial aos NMDA-R extra sinápticos, os quais estão envolvidos com a excitotoxicidade e redução na sinalização do BDNF (Hardingham et al, 2002; Haydon e Carmignoto, 2006). O TNF- α e a IL-1 também podem induzir os astrócitos e a microglia a liberarem espécies reativas de oxigênio (ERO) e nitrogênio (ERN), as quais podem amplificar o estresse oxidativo, ao mesmo tempo que estimulam a liberação de glutamato e contribuem com a excitotoxicidade (Ida et al, 2008; Matute et al, 2006). Citocinas inflamatórias também influenciam a fosforilação do TrkB, interferindo ainda mais com a sinalização do BDNF (Cortese et al., 2011). De fato, os efeitos inibitórios da IL-1 na neurogênese são mediados pela ativação do NF κ B (Koo e Duman, 2008).

Com relação ao eixo HPA, as citocinas pro-inflamatórias podem reduzir a função e a expressão dos GR (Pace et al., 2007), contribuindo com a resistência aos glicocorticoides. Por exemplo, demonstrou-se que o INF- α inibe a função do GR por romper a interação GR-DNA (Smoak e Cidlowski, 2004). A IL-1, por sua vez, inibe a translocação do GR do citoplasma para o núcleo através da ativação da MAPK p38 (Pariante et al., 1999; Engler et al., 2008). Notavelmente, as alterações na translocação do GR que levam a resistência aos glicocorticoides induzidas por estresse são mediadas pela IL-1 (Engler et al., 2008). O potente efeito anti-inflamatório dos glicocorticoides explica a observação pré-clínica de que a resistência aos glicocorticoides em decorrência do estresse é associada com o aumento na letalidade em resposta a administração de endotoxina (Quan et al., 2001). No entanto, a exposição crônica às citocinas inflamatórias aumenta a expressão do GR- β , uma isoforma do GR que não se liga aos glicocorticoides (Kino et al., 2009).

Diante da noção de que a inflamação periférica pode se propagar para o cérebro, a identificação das possíveis fontes de inflamação periférica em pacientes depressivos ajudaria no entendimento da patofisiologia da depressão. Uma das principais fontes de inflamação intimamente ligada com a depressão é o estresse (Haroon et al., 2012). Estressores físicos e psicológicos podem ativar células imunes periféricas e no sistema nervoso central para liberar citocinas que influenciam nos neurotransmissores e no comportamento (Koo e Duman, 2008; Maier e Watkins, 1998). A nível celular, acredita-se que o estresse libera DAMPs – através da chamada “inflamação estéril” – que são detectados pelo sistema imune (Maslanik et al., 2013; Fleshner, 2013). Juntamente com os PAMPs, os DAMPs podem estimular o inflamassoma NLRP3, o qual cliva os precursores imaturos da IL-1 β e IL-18 formando

1096 suas formas solúveis (Iwata et al., 2012; Maslanik et al., 2013). Esse aumento na
1097 liberação de IL-1 β pode induzir a produção de outras citocinas inflamatórias que são
1098 liberadas durante o estresse. Portanto, os DAMPs e o NLRP3 podem servir como o
1099 principal *link* pelo qual o estresse crônico ou psicológico se traduz para a liberação de
1100 sinais que promovem a atividade inflamatória, contribuindo com o estabelecimento da
1101 depressão (Figura 11) (Iwata et al., 2012). Apesar de bem estabelecido em estudos
1102 pré-clínicos (Kauffmann et al., 2017), apenas um ensaio clínico reportou que a
1103 expressão de caspase-1 e NLRP3 e os níveis proteicos de NLRP3 estão elevados em
1104 células mononucleares circulantes em pacientes depressivos comparados à
1105 indivíduos não depressivos (Alcocer-Gómez et al., 2014).

1106 Outra fonte de citocinas inflamatórias é o intestino, e bastante atenção tem sido
1107 dada a influência da microbiota sob as respostas imunes de um organismo (Cryan e
1108 Dinan, 2012; Lee e Mazmanian, 2010; Valles-Colomer et al., 2019). Estima-se que
1109 nosso corpo contenha aproximadamente 100 trilhões de microrganismos, um número
1110 dez vezes maior do que o número de células humanas (Lee e Mazmanian, 2010).
1111 Acredita-se que a composição da microbiota intestinal e sua translocação do intestino
1112 para o corpo, o chamado “intestino hiper permeável” (ou do inglês, *leaky gut*) no
1113 contexto do estresse pode contribuir com a inflamação e o comportamento depressivo
1114 relacionado (Haroon et al., 2012). Diferentes mecanismos estão envolvidos com a
1115 interação entre o microbioma intestinal e a sinalização cerebral, incluindo a ativação
1116 de neurônios sensoriais aferentes, modulação da via da quinurenina, metabolitos de
1117 microrganismos (como ácidos graxos de cadeia curta) e neurotransmissores
1118 derivados de microrganismos (Cryan e Dinan, 2012). Portanto, microrganismos e seus
1119 metabolitos podem entrar na circulação sistêmica e interagir com PRRs
1120 (especialmente os TLRs) para exacerbar a resposta imune (Lucas e Maes, 2013).
1121 Além disso, o revestimento intestinal também abriga células imunes, incluindo células
1122 dendríticas e células T (Coombes e Powrie, 2008; Fagarasan et al., 2010), e pode
1123 servir como fonte de citocinas circulantes. Dessa forma, a interação entre a microbiota
1124 e a mucosa intestinal regulam a produção de diversas citocinas pró-inflamatórias e
1125 quimiocinas, incluindo a IL-8, IL-10 e o fator transformador de crescimento beta (TGF-
1126 β , do inglês *transforming growth factor beta*) (Cryan e Dinan, 2012). Em camundongos,
1127 a manipulação da microbiota intestinal altera o comportamento e o conteúdo de BDNF
1128 no hipocampo, independente de mudanças em citocinas circulantes periféricas e
1129 hormônios endócrinos, indicando uma possível interação direta entre o intestino e o

cérebro que pode alterar o comportamento (Bercik et al., 2011). Vale destacar que, no momento, há um ensaio clínico avaliando o comportamento depressivo e a microbiota de 40 pessoas depressivas antes e depois de receberem um único transplante fecal (através de capsulas pela via oral) (Cinicaltrials.gov, ID: NCT03281044).

A descoberta de que a depressão está envolvida com a ativação do sistema imune e estresse fornece uma nova perspectiva sobre porque os genes de vulnerabilidade para depressão podem ter sido retidos no pool genético humano, apesar do impacto negativo da depressão na morbidade e mortalidade. Acredita-se que o *sickness behavior* induzido pela inflamação evoluiu como um mecanismo protetor para prevenir que patógenos se espalhassem por diferentes hospedeiros (Hart, 1998). No entanto, uma teoria mais recente sugere que a interação constante dos nossos ancestrais com patógenos no ambiente e estressores (incluindo estresse psicológico) direcionaram a vantagem evolutiva para um fenótipo mais inflamatório, e, portanto, os alelos para genes associados com a inflamação e depressão são comuns no *pool* gênico moderno (Raison e Miller, 2013). Apesar de representar um risco de depressão, essas variantes alélicas podem ter melhorado simultaneamente a aptidão geral, aumentando a resistência ao ataque de patógenos antes do advento do saneamento moderno e dos antibióticos (Maier et al., 1998).

O interesse pelo uso de agentes anti-inflamatórios para o tratamento de uma subpopulação de pacientes depressivos tem crescido diante da importância do sistema imune para a progressão da doença. Uma meta-análise recente revelou que o uso de anti-inflamatórios não-esteroidais, ácidos graxos ômega-3, estatinas e minociclina mostraram significativo efeito antidepressivo (Bai et al., 2019). Corroborando com esses dados, outra meta-análise recente indicou que anti-inflamatórios não-esteroidais, inibidores de citocinas, estatinas, glicocorticoides e minociclina mostraram efeito antidepressivo (Köhler-Forsberg et al., 2019). Esses resultados estão de acordo com outra recente meta-análise demonstrando um efeito antidepressivo significativo da terapia anti-citocinas (Kappelmann et al., 2016). Outros possíveis moduladores da inflamação também foram sugeridos como antidepressivos, incluindo a IL-10 (Asadullah et al., 2000), canabinoides (Hill e Gorzalka, 2009) e moduladores da microbiota intestinal (Bravo et al., 2011). Apesar disso, até o momento, as intervenções terapêuticas anti-inflamatórias estão em ensaios pré-clínicos ou em fases iniciais de ensaios clínicos.

2.2.4.1 Estabelecimento do estresse oxidativo

Além da influência nos sistemas de neurotransmissores, eixo HPA e neurogênese, a ativação dos TLRs e a sinalização de citocinas também pode aumentar o estresse oxidativo observado no cérebro de pacientes depressivos, especialmente através da ativação da via do NFκB (Madrigal et al., 2002).

O estresse oxidativo é caracterizado como uma condição de desequilíbrio em que há a produção excessiva de ERO/N e/ou a redução nas defesas antioxidantes enzimáticas e não enzimáticas (Figura 13) (Halliwell e Gutteridge, 1995). Os antioxidantes são classificados como moléculas que, quando presentes em baixas concentrações, são capazes de prevenir a oxidação de diferentes substratos, como proteínas, lipídios e DNA (Halliwell e Gutteridge, 1995).

A produção de ERO/N ocorre fisiologicamente no organismo, especialmente na cadeia transportadora de elétrons (CTE), desempenhando funções importantes na sinalização celular, defesa contra infecções, reações de detoxificação e apoptose (Ghosh et al., 1997; Johnson et al., 1996; Maes et al., 2011). Na CTE, a formação de espécies reativas se inicia com a geração do radical ânion superóxido ($O_2^{\cdot-}$), o qual também pode ser formado por ação do complexo do fosfato de dinucleotídeo de nicotinamida e adenina (NADPH) oxidase (NOX). O $O_2^{\cdot-}$ pode reagir com o $NO^{\cdot}$ formado pela enzima óxido nítrico sintase induzível (iNOS) e formar peroxinitrito ($ONOO^{\cdot-}$). A enzima superóxido dismutase (SOD) é responsável por dismutar o $O_2^{\cdot-}$, formando peróxido de hidrogênio (H_2O_2). O H_2O_2 não é um radical livre, mas sim uma espécie reativa capaz de reagir com íons metálicos (Fe^{2+} e Cu^+ , na chamada reação de Fenton) e formar o radical hidroxil ($OH^{\cdot}$), o mais danoso às biomoléculas. A decomposição do H_2O_2 ocorre pela ação das enzimas catalase (CAT) e glutathione peroxidase (GPx). A enzima GPx possui selênio (Se) no sítio ativo e oxida a glutathione reduzida (GSH) à glutathione oxidada (GSSG). A reciclagem da GSSG ocorre pela ação da glutathione redutase. Adicionalmente, a enzima mieloperoxidase (MPO) pode utilizar o H_2O_2 para produzir ácido hipocloroso (HOCl, na presença de íons cloro), ácido hipobromoso (HOBr, na presença de íons bromo) e ácido hipotiocianoso (HOSCN, na presença de íons hipotiocianato), também danosos às biomoléculas. Quando em excesso, os oxidantes produzidos podem levar à peroxidação lipídica, oxidação de proteínas, danos ao DNA e depleção de antioxidantes. Dentre os antioxidantes não-enzimáticos, pode-se citar o tripeptídeo GSH, vitamina E (α -tocoferol), vitamina C (ácido ascórbico), flavonoides e micronutrientes, como o zinco

(Zn) e o Se (Maes et al., 2011; Moylan et al., 2014; Kim et al., 2015). Caso as ERO/N não forem mantidas em níveis fisiológicos, elas podem levar a oxidação de proteínas (formando proteínas carboniladas), lipídeos (gerando o malondialdeído (MDA) ou o 4-hidroxinonenal (4-HNE)) e DNA (gerando a 8-hidroxiguanosina).

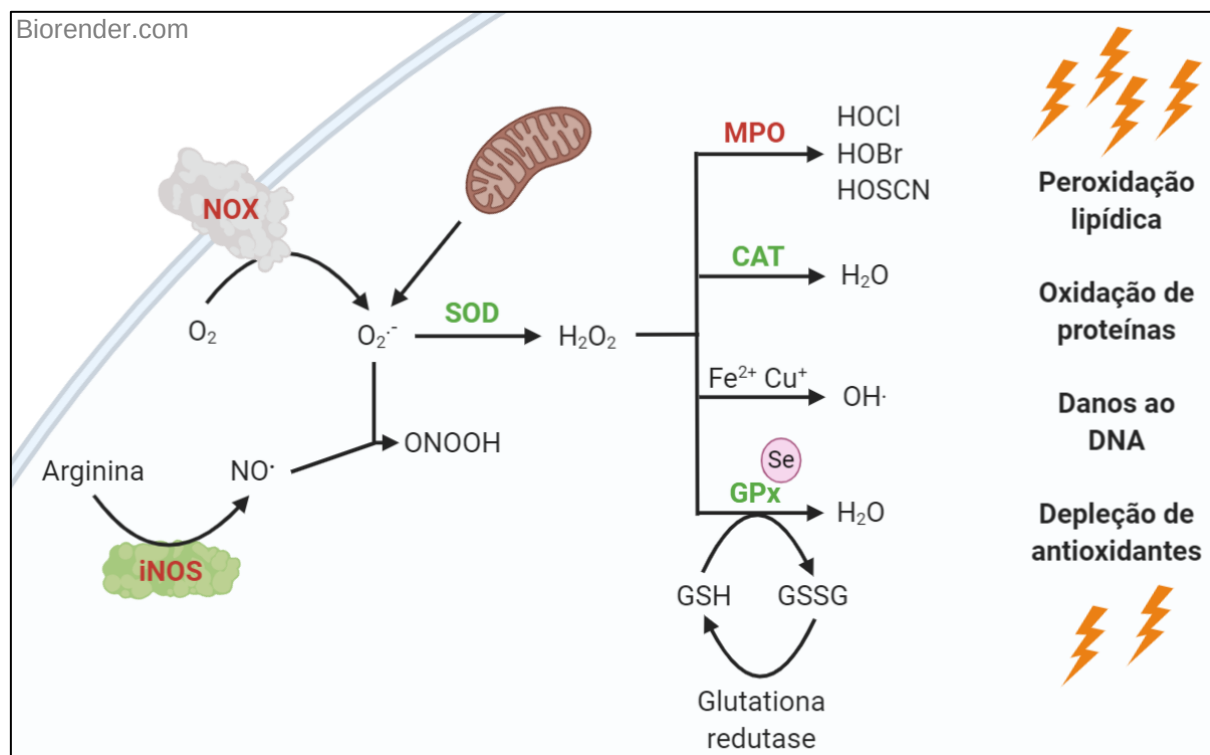


Figura 13. Principais espécies reativas e enzimas antioxidantes intracelulares. A primeira espécie reativa formada na cadeia transportadora de elétrons é o radical ânion superóxido ($O_2^{\cdot-}$), o qual também pode ser formado por ação das enzimas NADPH oxidases (NOX). O $O_2^{\cdot-}$ pode reagir com o óxido nítrico ($NO^{\cdot}$) formado pela enzima óxido nítrico sintase induzível (iNOS) e formar peroxinitrito (ONOO $^{\cdot}$). A enzima superóxido dismutase (SOD) é responsável por dismutar o $O_2^{\cdot-}$, formando peróxido de hidrogênio (H_2O_2). O H_2O_2 pode reagir com íons metálicos (Fe^{2+} e Cu^+ , na chamada reação de Fenton) e formar o radical hidroxil ($OH^{\cdot}$), o mais danoso às biomoléculas. A decomposição do H_2O_2 ocorre pela ação das enzimas catalase (CAT) e glutatona peroxidase (GPx). A enzima GPx possui selênio (Se) no sítio ativo e oxida a glutatona reduzida (GSH) à glutatona oxidada (GSSG). A reciclagem da GSSG ocorre pela ação da glutatona redutase. Adicionalmente, a enzima mieloperoxidase (MPO) pode utilizar o H_2O_2 para produzir ácido hipocloroso (HOCl), ácido hipobromoso (HOBr) e ácido hipotiocianoso (HOSCN), também danosos às biomoléculas. Quando em excesso, os oxidantes produzidos podem levar à peroxidação lipídica, oxidação de proteínas, danos ao DNA e depleção de antioxidantes. Fonte: Angela Maria Casaril.

A ativação de vias inflamatórias e o estresse oxidativo estão intrinsicamente relacionados (Figuras 11 e 12). O reconhecimento de PAMPs e DAMPs pelos TLRs

resulta na ativação da via do fator de transcrição NFκB, o qual induz a expressão de citocinas inflamatórias e do NLRP3. A ativação do NFκB também resulta na expressão de enzimas produtoras de espécies reativas e mediadores inflamatórios, como a iNOS e a ciclooxigenase 2 (COX-2), respectivamente. O NO[•] produzido pela iNOS pode reagir com o O₂^{•-} para formar o radical livre ONOO⁻ (Beckman e Koppenol, 1996). As prostaglandinas formadas pela COX-2 contribuem com a propagação da neuroinflamação e reduzem a atividade do BDNF (Hein e O'Banion, 2009; Rage et al., 2006). Além de contribuir com a neurogênese, o BDNF tem papel importante na ativação do fator nuclear eritroide 2 relacionado ao fator 2 (Nrf2, do inglês *nuclear factor erythroid 2-related factor 2*) (Ishii et al., 2018), responsável por induzir a expressão de genes antioxidantes, incluindo a SOD e CAT (Kensler et al., 2007). Em alguns tipos celulares, como macrófagos, neutrófilos, células epiteliais e microglia, as citocinas (IL-1 e TNF) ativam a produção de O₂^{•-} via ativação das NOX. De fato, um estudo recente demonstrou que espécies reativas derivadas do NOX1 oxidam e reduzem a atividade de NMDA-R, levando a redução na expressão de BDNF associada ao desenvolvimento do comportamento tipo-depressivo (Ibi et al., 2017). As espécies reativas também podem induzir a ativação da via do NFκB, MAPK p38 e servir como segundo sinal para induzir a montagem do complexo NLRP3. Além disso, DAMPs derivados da peroxidação lipídica (como o MDA) podem ativar o complexo TLR2/TLR4, desencadeando uma resposta imune e oxidativa (Lucas e Maes, 2013). Consequentemente, a depressão parece ser acompanhada por um ciclo vicioso entre a ativação de TLRs, sinalização de citocinas e atividade de espécies reativas.

Vale destacar que a sinalização do TLR4 via PAMPs e DAMPs e o aumento de cortisol podem resultar na ativação da proteína glicogênio sintase quinase 3-beta (GSK-3β, do inglês *glycogen synthase kinase 3 beta*) (Dobarro et al., 2013; Joep et al., 2017). Dentre suas inúmeras funções, a ativação da GSK-3β por fosforilação no resíduo de tirosina 216 previne a translocação do Nrf2 do citosol para o núcleo, contribuindo com a redução na expressão das defesas antioxidantes (Kensler et al., 2017). Adicionalmente, a GSK-3β promove a ativação do NFκB, aumentando a resposta pro-inflamatória no sistema nervoso central, especialmente na microglia e astrócitos (Joep et al., 2017).

Um desequilíbrio no sistema redox do organismo tem sido observado em pacientes depressivos. Estudos clínicos e pré-clínicos têm demonstrado que a depressão é associada com o aumento na peroxidação lipídica (evidenciada pelo

aumento dos níveis de MDA), dano ao DNA (evidenciada pelo aumento dos níveis de 8-hidroxi-guanosina), peroxidação do ácido araquidônico (evidenciada pelo aumento da 8-iso-prostaglandina F₂) e encurtamento de telômeros (Dimopoulos et al., 2008; Forlenza e Miller, 2006; Shalev et al., 2014; Yager et al., 2010). Esses efeitos podem ser decorrência dos níveis reduzidos de vitamina E (Maes et al., 2000) e C (Khanzode et al., 2003), níveis reduzidos de triptofano e tirosina (Maes et al., 2000), e níveis reduzidos de SOD e GPx (Maes et al., 2011). De fato, o cérebro é particularmente vulnerável aos danos causados pelo estresse oxidativo devido à alta concentração de lipídios, alta utilização de oxigênio (e consequentemente maior produção de espécies reativas) e insuficientes mecanismos de defesa antioxidante (Pandya et al., 2013). Dessa forma, as observações de que o estresse oxidativo está envolvido com a depressão fornece novos alvos terapêuticos para a doença, além da possibilidade de identificação de biomarcadores periféricos que refletem o estado redox de pacientes depressivos.

2.3 De agentes tuberculostáticos à anestésicos gerais: tratamentos farmacológicos para a depressão

Durante a história, o tratamento para a depressão (ou melancolia) acompanhou a evolução nas hipóteses que descreviam sua etiologia. Quando classificada como uma doença espiritual, rituais eram empregados a fim de eliminar os espíritos malignos responsáveis pela doença. Quando Hipócrates postulou que a melancolia era causada pelo excesso de bÍlis negra, a cura vinha por meio da sangria e melhoras na dieta, na tentativa de equilibrar os humores. Quando a depressão foi descrita como uma reação a fatores psicológicos, sociais e biológicos, empregou-se tratamentos psicológicos baseados fortemente na psicanálise desenvolvida por Sigmund Freud. Enquanto a psicanálise considerava a depressão como uma manifestação sintomatológica de certos conflitos pessoais internos e dominava o ramo da psiquiatria, uma descoberta inesperada na década de 50 revolucionou o tratamento de doenças psiquiátricas.

Enquanto desenvolviam novos agentes anti-tuberculose derivados da isoniazida, Herbert Fox e John Gibas sintetizaram a iproniazida, um inibidor das MAOs (Zeller et al., 1952), que apresentou efeitos promissores no tratamento de pacientes com tuberculose (López-Muñoz e Álamo, 2016). Entretanto, um estudo clínico realizado em 1952 reportou que o tratamento com a iproniazida desencadeava

inúmeros “efeitos adversos” em pacientes com tuberculose, incluindo euforia, psicoestimulação, aumento do apetite e melhora na qualidade do sono (Selikoff et al., 1952). Interessado nesses efeitos, o psiquiatra Nathan Kline liderou o primeiro estudo clínico para testar a iproniazida em pacientes depressivos sem tuberculose. Em 1957, eles reportaram que dos 24 pacientes tratados com iproniazida por cinco semanas, 18 mostraram melhoras de humor e sociabilidade, propondo assim o termo *energizador psíquico* para se referir ao efeito da iproniazida (Loomer et al., 1957). Apesar da falta de um grupo controle e do fato de que a maioria dos pacientes eram esquizofrênicos ao invés de depressivos, 400,000 pacientes depressivos foram tratados com a iproniazida menos de um ano depois, abrindo caminho para a primeira classe de antidepressivos, posteriormente chamada de MAOIs (López-Muñoz e Álamo, 2016). As MAOs constituem um sistema enzimático localizado na membrana mitocondrial, cuja função é catalisar a desaminação oxidativa de amins biogênicas (como a serotonina e catecolaminas) e amins simpatomiméticas (como a tiramina). Dessa forma, o efeito antidepressivo de MAOIs decorre da redução da oxidação das amins biogênicas. A descoberta do efeito antidepressivo da iproniazida revolucionou o campo de substâncias psicoativas nos anos 50 ao demonstrar que sintomas psicológicos não necessitariam exclusivamente de tratamento psicológico, podendo ser tratados com intervenções medicamentosas.

Nos anos subsequentes, a iproniazida deu lugar a outros MAOIs mais potentes, como a isocarboxiazida, mebanazina e feniprazina. Entretanto, casos de hepatotoxicidade, nefrotoxicidade, hipertensão e morte foram reportados após o uso de MAOIs, o que impulsionou a retirada desses medicamentos do mercado (López-Muñoz e Álamo, 2016). Atualmente, os MAOIs são considerados como medicamento de segunda escolha, sendo indicados em casos de ineficácia de outros antidepressivos (depressão refratária) (Ban, 2001).

Os anos 50 também foram marcados pela descoberta acidental do efeito antidepressivo da imipramina. Como resultado da identificação do efeito antipsicótico da clorpromazina, a pesquisa por moléculas mais potentes se intensificou. Em 1956, Roland Kuhn observou que o composto G22355 (um derivado da prometazina, posteriormente chamado de imipramina) não possuiu efeito antipsicótico em pacientes com esquizofrenia, mas melhorou significativamente o humor e sociabilidade de pacientes depressivos (López-Muñoz e Álamo, 2016). Kuhn também relatou a ausência de efeitos adversos graves nos 500 pacientes tratados com imipramina, o

que foi uma melhora considerável quando comparado aos MAOIs (Hillhouse e Porter, 2015). Em 1959 a imipramina (Tofranil®) foi aprovada para o tratamento do TDM, sendo classificada como uma molécula pertencente a classe dos antidepressivos tricíclicos (TCAs, do inglês *tricyclic antidepressants*). A classificação dos TCAs baseou-se na estrutura molecular composta por três anéis benzênicos, uma vez que o mecanismo de ação era desconhecido no momento da descoberta. O mecanismo de ação dos TCAs é bastante heterogêneo, sendo que essas moléculas podem atuar na inibição de transportadores pré-sinápticos de recaptação de noradrenalina e serotonina, bloqueio de receptores muscarínicos pós-sinápticos e bloqueio de receptores de histamina H1 pós-sinápticos. Acredita-se que a inibição da recaptação de noradrenalina e serotonina seja responsável pelos efeitos terapêuticos dos TCAs, enquanto o antagonismo dos receptores adrenérgicos, muscarínicos e histaminérgicos contribui principalmente para os efeitos colaterais de tontura, comprometimento da memória e sonolência, respectivamente (Hillhouse e Porter, 2015).

Embora o efeito antidepressivo da iproniazida e da imipramina tenha sido descoberto acidentalmente através de observações clínicas, acreditava-se que seus efeitos dependiam da modulação catecolaminérgica, especialmente da noradrenalina e adrenalina. Entretanto, na década de 60, começaram a surgir evidências indicando o envolvimento da serotonina na depressão. Por exemplo, o efeito antidepressivo de MAOIs aparentava ser potencializado quando esses medicamentos eram combinados com triptofano (Coppen et al., 1963). Além disso, um estudo *post-mortem* revelou que pacientes depressivos que cometeram suicídio apresentavam concentrações reduzidas de serotonina quando comparado a vítimas de morte repentina (Shaw et al., 1967). Essas observações sugeriram que a serotonina poderia estar envolvida com a depressão tanto quanto a noradrenalina. Como resultado, a empresa farmacêutica Eli Lilly começou a desenvolver ligantes seletivos para os transportadores de serotonina para inibir a sua recaptação e, assim, aumentar as concentrações de serotonina na fenda sináptica, resultando no estímulo de seus receptores pós-sinápticos. Em 1974, David Wong e colaboradores publicaram o primeiro artigo sobre o composto LY110140 (posteriormente chamado de fluoxetina), um dos SSRIs (Wong et al., 1974). Finalmente em 1987, mais de 16 anos após a publicação original, a fluoxetina foi aprovada pelo *Food and Drug Administration* (FDA) e lançada ao mercado sob o nome comercial Prozac®. Em 2002 (um ano após o término da exclusividade de venda pela

Eli Lilly), o Prozac® havia sido prescrito para mais de 40 milhões de pessoas ao redor do mundo, com um total de vendas de 22 bilhões de dólares – o que o tornou conhecido como um medicamento inovador para o tratamento da depressão (Wong et al., 2005). Desde a introdução da fluoxetina no mercado, vários outros SSRIs foram aprovados pelo FDA, como por exemplo a sertralina (Zoloft®), citalopram (Celexa®), paroxetina (Paxil®) e escitalopram (Lexapro®).

Até 2018, aproximadamente 30 anos após o lançamento da fluoxetina, diversos novos antidepressivos foram desenvolvidos. Entretanto, eles ainda apresentam como mecanismo de ação a modulação da neurotransmissão monoaminérgica (Tabela 1). De um modo geral, esses antidepressivos possuem eficácia limitada, sendo que aproximadamente 40% dos pacientes que fazem uso da medicação não apresentam melhoras na sintomatologia, possivelmente devido à complexidade e heterogeneidade da depressão (Institute for Quality and Efficiency in Health Care, 2017). Adicionalmente, mesmo pacientes com melhoras nos sintomas apresentam alta taxa de descontinuação do tratamento, o que pode estar relacionado com a demora para o início dos efeitos farmacológicos (que varia entre 4 a 6 semanas), e a presença de inúmeros efeitos adversos, como boca seca, sudorese, tontura, náusea e sonolência/insônia (Cipriani et al., 2018; Gartlehner et al., 2017).

Tabela 1. Principais classes de antidepressivos que agem modulando a neurotransmissão monoaminérgica.

Classe	Mecanismo de ação	Exemplos
Inibidores das enzimas monoaminas oxidases	Inibem as enzimas monoaminas oxidases A e B (MAO-A e MAO-B), evitando a degradação das monoaminas ¹	Fenelzina (Nardil®)
Antidepressivos tricíclicos	Possuem mecanismo de ação bastante heterogêneo, modulando a neurotransmissão serotoninérgica, dopaminérgica, noradrenergica, adrenérgica, histaminérgica e colinérgica ¹	Imipramina (Tofranil®)

Inibidores seletivos da recaptação de serotonina	Inibem seletivamente a recaptação de serotonina através do bloqueio dos transportadores ²	Fluoxetina (Prozac®)
Inibidores da recaptação de dopamina e noradrenalina	Inibem a recaptação de dopamina e noradrenalina através do bloqueio dos transportadores ²	Bupropiona (Wellbutrin®)
Inibidores seletivos da recaptação de noradrenalina	Inibem seletivamente a recaptação de noradrenalina através do bloqueio dos transportadores ¹	Reboxetina (Edronax®)
Inibidores da recaptação de serotonina e noradrenalina	Em baixas doses inibem a recaptação de serotonina. Em altas doses inibem a recaptação de serotonina e noradrenalina ²	Venlafaxina (Effexor®)
Antidepressivos multimodais	Inibem a recaptação de serotonina, dopamina e noradrenalina, agem como agonista dos receptores 5-HT _{1A} , agonista parcial dos receptores 5-HT _{1B} e antagonista dos receptores 5-HT ₃ e 5-HT ₇ ¹	Vortioxetina (Brintellix®)

¹ Fava e Kendler (2000); ² Hillhouse e Porter, 2015.

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Apesar das principais classes de antidepressivos utilizadas atualmente modularem o sistema de neurotransmissão monoaminérgico, outros efeitos farmacológicos secundários também foram reportados, como por exemplo a modulação das vias oxidativas, inflamatórias e hipercortisolemia. Diversos estudos vêm demonstrando que os antidepressivos reduzem a produção de TNF- α , IL-12 e interferon gama (IFN γ), além de reduzirem os níveis de cortisol circulantes em pacientes com depressão (Maes et al., 2012; Manthey et al., 2011; Pariante et al., 2004). Tais observações enfatizam o envolvimento de diferentes vias de sinalização celular no efeito farmacológico de antidepressivos, sugerindo ainda o envolvimento do sistema imune, endócrino e antioxidante na etiologia da depressão.

É possível que uma nova revolução no ramo da psiquiatria tenha começado em 2019, com a liberação da escetamina (Spravato®) contra a depressão resistente aos

tratamentos convencionais (Food and Drug Administration, 2019a) e da brexanolona (Zulresso®) para o tratamento da depressão pós-parto (Food and Drug Administration, 2019b).

A escetamina é o S-enantiômero da cetamina, um anestésico dissociativo com propriedades alucinógenas derivado da fenciclidina (PCP, do inglês *phencyclidine*). Desde os anos 70 a mistura racêmica de cetamina (Ketalar®) foi aprovada para a indução e manutenção da anestesia através da administração intramuscular ou intravenosa (Salvadore e Singh, 2013), atuando como um antagonista dos NMDA-R. Nos anos 90, a cetamina se tornou uma droga recreacional popular, passando a ser conhecida como *Special K*. Como resultado do aumento do uso recreacional, a cetamina passou a ser classificada como um não-narcótico nível III sob o estatuto de controle de substâncias (*Controlled Substances Act*) dos EUA (Drug Enforcement Administration, 2013).

Diversos estudos clínicos tem demonstrado que uma única dose sub-psicotomimética de cetamina produz uma rápida resposta antidepressiva em pacientes (Berman et al., 2000; Zarate et al., 2006). Entretanto, a principal limitação do seu uso como um antidepressivo é a administração pela via endovenosa, reduzindo sua aplicabilidade na população. Por sua vez, a escetamina apresenta maior afinidade pelos NMDA-R do que a cetamina (Popova et al., 2019) e foi desenvolvida como uma formulação intranasal, apresentando efeito antidepressivo de início rápido e persistente por mais de dois meses seguindo uma baixa frequência de dosagem (Daly et al., 2018). Entretanto, diversos efeitos adversos foram experienciados por pacientes tratados com escetamina nos ensaios clínicos, como por exemplo sensação de dissociação, tontura, náuseas, sedação, vertigem, ansiedade, letargia e aumento da pressão sanguínea (Food and Drug Administration, 2019a).

A rápida resposta dos pacientes depressivos ao tratamento com cetamina e escetamina sugere um mecanismo que resulta em rápidas alterações na função e plasticidade sináptica, possivelmente dependente do antagonismo dos NMDA-R, aumento da sinalização dos AMPA-R (Duman et al., 2016) via mTORC1, ERKs e Akt (Zhou et al., 2014) e aumento de sinapses GABAérgicas no córtex pré-frontal (Ghosal et al., 2017). Vale destacar que, diferentemente dos antagonistas dos NMDA-R, a administração aguda ou crônica de antidepressivos convencionais (como a fluoxetina) não aumenta a sinalização do mTORC1 (Li et al., 2010).

Além da escetamina, em 2019 o FDA aprovou pela primeira vez um medicamento destinado ao tratamento da depressão pós-parto (Food and Drug Administration, 2019b). A brexanolona é uma formulação exógena da alopregnalona, um esteroide neuroativo endógeno. A alopregnalona é o principal metabolito da progesterona, e seus níveis aumentam rapidamente durante a gravidez, com um pico no terceiro semestre (Luisi et al., 2000). A queda nos níveis de alopregnalona após o parto tem sido hipotetizada como um dos principais fatores que desencadeiam a depressão pós-parto. O mecanismo de ação da brexanolona não está completamente elucidado, mas sabe-se que a alopregnalona modula a excitabilidade neuronal através da modulação alostérica dos GABA-R do tipo A, aumentando sua função (Paul e Purdy, 1992). Quando administrada como uma infusão intravenosa contínua durante 60 horas, a brexanolona reduziu os sintomas depressivos em mulheres com depressão pós-parto moderada a severa (Meltzer-Brody et al., 2018). Entretanto, pacientes tratados com brexanolona apresentaram diversos efeitos adversos, incluindo boca seca, perda de consciência e sonolência, e em alguns casos, pensamentos suicidas (Food and Drug Administration, 2019b).

Vale especular que a liberação da escetamina e da brexanolona pelo FDA inicia uma nova revolução na psiquiatria no sentido de que, finalmente, será considerado que diferentes tipos de depressão requerem diferentes tipos de tratamento. Além da heterogeneidade de sua etiologia, os sintomas depressivos não se manifestam igualmente nas pessoas, e por isso, não há um tratamento universal para essa doença. Atualmente, recomenda-se que a depressão seja tratada com antidepressivos, psicoterapia (principalmente a terapia cognitiva-comportamental, terapia de solução de problemas e terapia interpessoal) ou uma combinação de ambos (National Institute of Mental Health, 2015). Entretanto, devido a eficácia limitada dos antidepressivos disponíveis, a busca por novas moléculas antidepressivas mais eficientes, com menos efeitos adversos e com início de ação rápido se faz necessária. No entanto, vale destacar que há uma crise no desenvolvimento de novos tratamentos para doenças mentais. As indústrias vêm abandonando a pesquisa e desenvolvimento de medicamentos para doenças psiquiátricas devido à falta de conhecimento sobre a patofisiologia das desordens e à falta de alvos validados, deixando a academia com o papel crucial de buscar novas estratégias terapêuticas para o tratamento de doenças psiquiátricas (Conn e Roth, 2008).

2.4 Da Deusa da lua à compostos bioativos: atividade farmacológica de compostos orgânicos de selênio

O selênio (Se) foi descoberto em 1817 pelo químico Jöns Jacob Berzelius, o qual nomeou o elemento em homenagem a Deusa Grega da lua, Selene (Arner 2010). Juntamente com o oxigênio, enxofre, telúrio, polônio e livermório, o Se faz parte do grupo dos calcogênios na tabela periódica, pertencentes ao grupo 16. Fisiologicamente, o Se é um micronutriente essencial, obtido na dieta, que pode ser encontrado em castanhas, salmão, atum, carne e cereais. O Se é incorporado em 25 selenoproteínas humanas na forma de selenocisteína (Sec), a qual é codificada pelo códon UGA (Hatfield e Gladyshev, 2002; Kryukov et al., 2003; Kudva et al., 2015). A exata função biológica das selenoproteínas ainda não está completamente elucidada, mas sabe-se que essas proteínas são essências para a vida, uma vez que modelos animais transgênicos com deleção no gene que codifica o RNA transportador (tRNA) necessário para a síntese da Sec resulta em morte embrionária (Bosl et al., 1997; Reeves e Hoffmann, 2009). Além disso, não há nenhuma evidência concreta de que o Se está envolvido com funções fisiológicas importantes em fungos e plantas, o que o torna bastante complexo e intrigante (Nogueira e Rocha, 2011).

Atualmente é bem estabelecido que o Se é imprescindível para a manutenção da homeostase em humanos (Rayman et al., 2000). Isso pode ser exemplificado pela doença de Keshan, uma cardiomiopatia endêmica na China que ocorre devido a deficiência do Se na alimentação da população, a qual pode ser completamente prevenida através suplementação com esse micronutriente (Zhou et al., 2003). Embora a deficiência de Se em humanos seja rara, evidências indicam que alterações nas concentrações de Se no organismo podem influenciar diversos mecanismos biológicos, como a resposta imune, neurodegeneração, câncer e doenças cardiovasculares (Gromadzinska et al., 2008; Hoffmann e Berry, 2008; Roman et al., 2014). Nesse sentido, recomenda-se a ingestão diária de aproximadamente 45 µg de Se para adultos, para que as funções biológicas do organismo sejam adequadamente desempenhadas, respeitando o limite de ingestão de 400 µg/dia para evitar a selenose (Christensen et al., 2015; Instituite of Medicine, 2000).

O Se se comporta tanto como um agente antioxidante quanto como um agente anti-inflamatório (Rayman, 2000). Isso ocorre pois, o Se, atuando via GPx, pode: (i) reduzir o H₂O₂ e hidroperóxidos lipídicos, bloqueando a propagação de radicais livres e ERO/N; (ii) reduzir intermediários hidroperóxidos da via das COX e lipoxigenases

para diminuir a formação de prostaglandinas e leucotrienos; e (iii) modular a chamada “explosão respiratória” (*respiratory burst*) através da remoção de H_2O_2 e redução da formação de O_2^- (Spallholz et al., 1990). Dessa forma, acredita-se que condições associadas com o aumento do estresse oxidativo e inflamação podem ser influenciadas pelos níveis de Se, o que pode ser o caso da depressão.

Apesar das particularidades, grande parte das selenoproteínas são classificadas como agentes antioxidantes, cuja função é atenuar o dano celular causado pelo excesso das ERO/N. Dentre essas proteínas, pode-se citar a GPx, a tiorredoxina redutase (TrxR) e a selenoproteína P (SelP), as quais tem sido amplamente estudadas devido suas funções na regulação do estado redox das células (Kudva et al., 2015). Sabe-se que a SelP, por exemplo, é essencial para a sobrevivência e função neuronal, seja por agir como um antioxidante direto (prevenindo o estresse oxidativo), ou fornecendo Se para a síntese de outras enzimas antioxidantes, como a GPx e TrxR (Bellinger et al., 2008).

Notavelmente, a deficiência de Se é acompanhada pelo prejuízo na imunocompetência, tanto na imunidade celular quanto na função de células B (Rayman, 2000). Por outro lado, a suplementação com Se tem efeito imunoestimulante, incluindo o aumento na proliferação de células T ativadas (expansão clonal), o aumento da função citotóxica dos linfócitos e aumento na atividade de células *natural killer* (NK) (Kiremidhian-Schumacer et al., 1994). Acredita-se que isso ocorra, pois, a suplementação com Se aumenta a expressão dos receptores para a IL-2 na superfície de linfócitos ativados e células NK. A sinalização da IL-2 é crucial para a expansão clonal e diferenciação em células T citotóxicas (Kiremidhian-Schumacer et al., 1994). Adicionalmente, células T ativadas apresentam aumento na atividade da selenofosfato sintetase, uma enzima envolvida com a síntese de Sec (Guimaraes et al., 1996), evidenciando a importância de selenoproteínas para o controle da resposta imune. Consoante a isso, um estudo recente identificou que o aumento nos níveis plasmáticos de Se está associado com a redução na concentração sérica da proteína C reativa (CRP, do inglês *C-reactive protein*) em humanos (Yuan et al., 2020).

Diversos estudos também apontam para a importância do Se no cérebro. Em condições de deficiência de Se, o fornecimento desse micronutriente é destinado preferencialmente ao cérebro do que aos outros órgãos, sugerindo que o Se exerce funções de grande importância no sistema nervoso central (Finley e Penland, 1998;

Benton e Cook, 1991). Além disso, baixas concentrações de CAT são encontradas no cérebro, então a remoção de peróxidos depende grandemente da atividade da GPx (Rayman, 2000). Relevante à depressão, a atividade da GPx é menor em pacientes depressivos (Maes et al., 2011), baixos níveis de Se foram associados com depressão, ansiedade e hostilidade (Haekes e Hornbostel, 1996) e a suplementação com Se é capaz de melhorar o humor negativo e os sintomas depressivos em pacientes adultos jovens (Conner et al., 2015). Adicionalmente, os níveis de SeP foram encontrados reduzidos em condições inflamatórias, como a sepse (Mertens et al., 2015). Em conjunto, essas observações sugerem que as funções biológicas do Se podem estar intrinsicamente relacionadas com o estresse oxidativo e a inflamação observados na depressão (Reeves e Hofmann, 2009).

O Se consumido em alimentos e suplementos existe em várias formas orgânicas e inorgânicas, incluindo selenometionina (fontes e suplementos vegetais e animais), Sec (principalmente fonte animais), selenato e selenito (principalmente suplementos), sendo que a biodisponibilidade do Se depende da forma ingerida. Por exemplo, a selenometionina é mais eficaz no aumento do status aparente de Se porque é incorporada não especificamente às proteínas no lugar da metionina. No entanto, a selenometionina não possui atividade catalítica e deve ser catabolizada para um precursor inorgânico antes de entrar no *pool* de Se disponível. Por sua vez, o selenito ou o selenato rapidamente fornecem selenofosfato, o precursor da Sec. Apesar disso, as formas orgânicas são frequentemente preferidas em intervenções, em parte porque apresentam menor toxicidade aguda (Standing Committee on the Scientific Evaluation of Dietary Reference Intakes of the Food and Nutrition Board, 2000). Além disso, o interesse no uso de compostos orgânicos de Se como possíveis estratégias terapêuticas também apresenta a vantagem de que essas moléculas podem agir como antioxidantes diretos ou miméticos à GPx.

Embora o primeiro composto orgânico de Se tenha sido sintetizado em 1847 por Wöhler e Siemens (Nogueira e Rocha, 2011), apenas no início dos anos 70 os compostos orgânicos de Se se tornaram versáteis na química orgânica (Sharpless e Lauer, 1973). Para que um composto orgânico de Se atue como um antioxidante, ele deve mostrar nucleofilicidade necessária para atividade mimética à GPx, atividade neutralizadora de radicais livres e baixa toxicidade (Nogueira e Rocha, 2011).

Sabendo-se da importância biológica do Se, diversos grupos de pesquisa têm desenvolvido compostos orgânicos contendo Se na tentativa de contribuir com o

tratamento de diversas patologias. Dentre os efeitos farmacológicos apresentados por compostos orgânicos de Se, vale destacar as atividades antioxidante (Luchese et al., 2020), anti-inflamatória (Araujo et al., 2020), antidepressiva (Martins et al., 2020), mimética à GPx (Ibrahim et al., 2015) e neuroprotetora (Zborowski et al., 2019).

Dentre os compostos orgânicos de Se melhor caracterizados, é possível citar o ebselen (2-fenil-1,2-benziosselenazol-3(2H)-1). As atividades antioxidante, anti-inflamatória, anticâncer, citoprotetora e estabilizadora de humor foram elucidadas *in vitro* e *in vivo* (Imai et al., 2001; Nakamura et al., 2002). Devido a esses potenciais efeitos farmacológicos, o ebselen encontra-se em ensaios clínicos de fase II para o tratamento do transtorno bipolar, como um adicional ao tratamento de estabilização de humor, uma vez que apresenta atividade similar ao lítio (Clinicaltrials.gov, ID: NCT03013400; Masaki et al., 2016).

Diante dos promissores efeitos farmacológicos dos compostos orgânicos contendo Se, o Grupo de Pesquisa em Neurobiotecnologia da Universidade Federal de Pelotas têm reportado consideráveis efeitos *in vitro* e *in vivo* de alguns compostos orgânicos contendo Se.

O 3-((4-metoxifenil)selenil)-2-fenilimidazo[1,2-a]piridina (Figura 14) é um composto orgânico de Se que possui atividade tipo-antidepressiva, tipo-ansiolítica, anti-inflamatória e antioxidante em modelos animais de comportamento tipo-depressivo e tipo-ansioso induzidos por LPS e estresse (Domingues et al., 2018, 2019). Por sua vez, o (octilseleno)-xilofuranosídeo (Figura 14) possui efeito tipo-antidepressivo por modular a neurotransmissão monoaminérgica (Brod et al., 2016) e vias de sinalização intracelular (Brod et al., 2017). A modulação do sistema monoaminérgico também está envolvida com a atividade anti-inflamatória e antinociceptiva do 1,2-bis-(4-metoxifeniselenil) estireno (Figura 14) (Anversa et al., 2018).

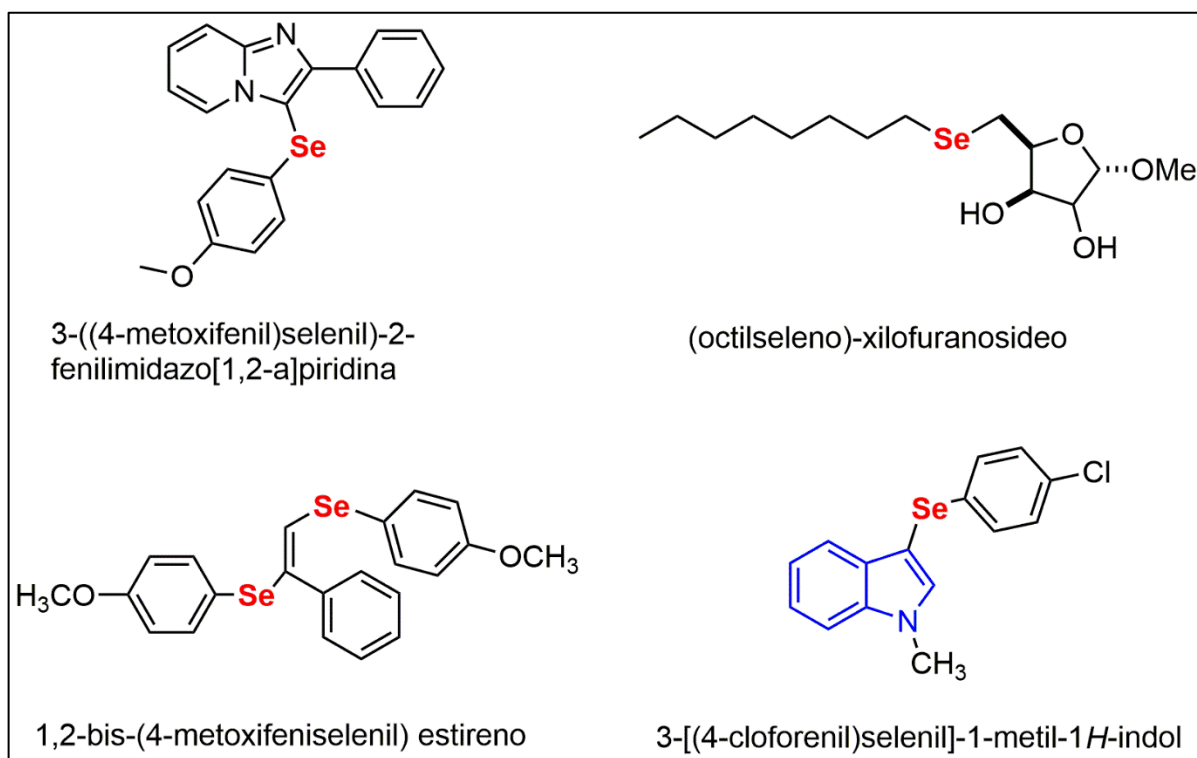


Figura 14. Estrutura de alguns compostos orgânicos de selênio estudados pelo nosso grupo de pesquisa. Vale destacar o composto 3-[(4-cloforenil)selenil]-1-metil-1*H*-indol, alvo deste trabalho.

2.4.1 3-[(4-cloforenil)selenil]-1-metil-1*H*-indol

Além dos compostos orgânicos de Se, outra classe de moléculas com propriedades biológicas interessantes são os indóis. Acredita-se que essa é a estrutura heterocíclica mais abundante na natureza com ação biológica, sendo encontradas por exemplo na estrutura do aminoácido triptofano, do hormônio melatonina e do neurotransmissor serotonina (Figura 15) (Kaushik et al., 2013).

As moléculas contendo a porção indol tem sido empregadas como agentes terapêuticos em diversas desordens neurodegenerativas (Klein et al., 2014) e inflamatórias (Guerra et al., 2011), além de apresentarem ação antioxidante (Karaaslan et al., 2013), antitumoral (Shaveta, 2014) e antiproliferativa (Spallarossa et al., 2015). Vale destacar que o anti-inflamatório não esteroide indometacina (Indocid®) e a estatina fluvastatina (Lescol®) são medicamentos disponíveis comercialmente e apresentam o núcleo indólico em sua estrutura.

Estudos recentes reportaram que o indol-3-carbinol (I3C) (Figura 15) possui efeito anti-inflamatório por inibir a produção de NO[•] e TNF-α em macrófagos estimulados com LPS (Tsai et al., 2010). Notavelmente, o anti-inflamatório não

1607 esteroideal indometacina (Figura 15) também se caracteriza pela presença do núcleo
1608 indólico em sua estrutura.

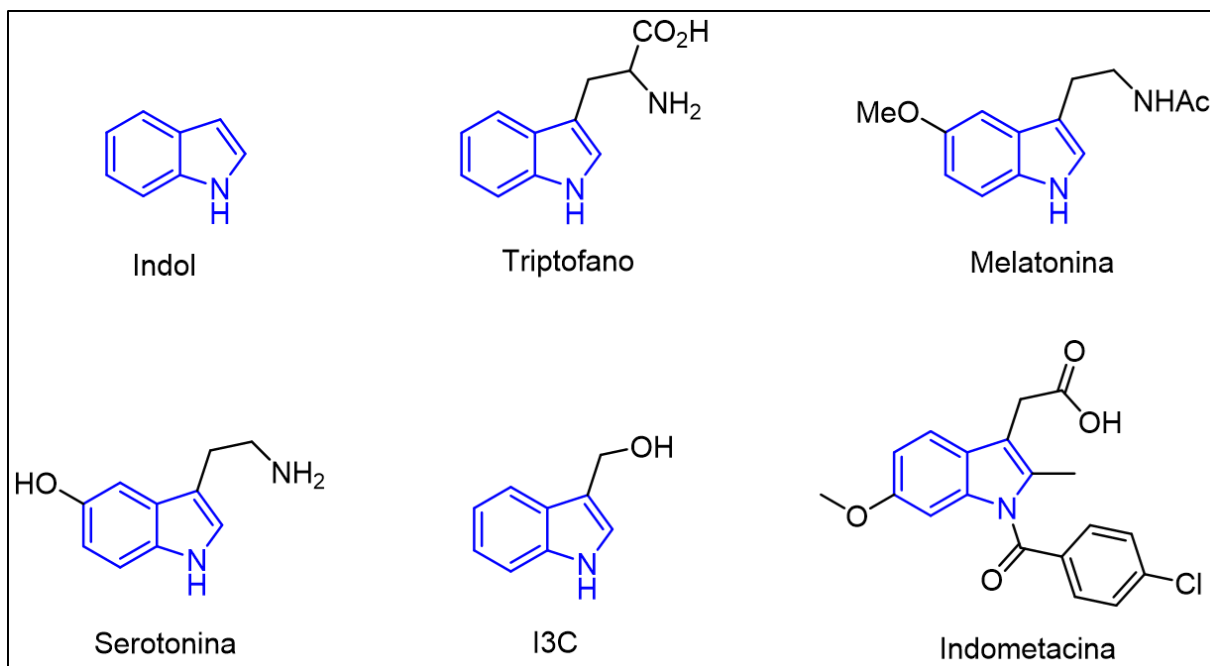


Figura 15. Estrutura do núcleo indólico e moléculas relacionadas.

Nesse sentido, a síntese de compostos contendo Se e indóis em sua estrutura é uma área de pesquisa bastante interessante, já que a combinação de duas ou mais porções bioativas em uma única molécula é uma estratégia relevante para o desenvolvimento de novos compostos com atividade farmacológica (Martinez et al., 2015). Diante disso, o Grupo de Pesquisa em Neurobiotecnologia em parceria com o Laboratório de Síntese Orgânica Limpa da UFPel sintetizaram o 3-[(4-cloforenil)selenil]-1-metil-1*H*-indol (CMI, Figura 14), um composto orgânico de Se contendo um núcleo indólico (Vieira et al., 2015).

O primeiro estudo investigando o perfil farmacológico do CMI foi realizado em 2017, aonde mostrou-se que ele apresenta atividade antioxidante *in vitro* (Vieira et al., 2017). Considerando o envolvimento do estresse oxidativo na depressão, avaliou-se então a capacidade do CMI em proteger contra o comportamento tipo-depressivo induzido por LPS em camundongos. Observou-se apenas uma administração de CMI 30 minutos antes do desafio com LPS preveniu o aumento no tempo de imobilidade do teste de suspensão da cauda (TSC) e teste do nado forçado (TNF) e protegeu contra o aumento nos níveis de espécies reativas, peroxidação lipídica e expressão de IL-6, IL-1 β e IL-4 no córtex pré-frontal e hipocampo dos animais (Casaril et al.,

2017). Adicionalmente, o estudo demonstrou a segurança no uso do composto, uma vez que o CMI não alterou os níveis plasmáticos de ureia, creatinina, aspartato aminotransferase e alanina aminotransferase e não inibiu a enzima sulfidrídica delta-aminolevulinato desidratase (sensível a agentes tóxicos) no rim, fígado e cérebro de camundongos (Casaril et al., 2017).

Esses estudos iniciais sobre o CMI nos impulsionaram a seguir investigando o potencial terapêutico dessa molécula em diferentes modelos animais de comportamento tipo-depressivo, a fim de contribuir com o desenvolvimento de um novo adjuvante terapêutico capaz de melhorar a qualidade de vida de pacientes depressivos.

3 HIPÓTESE E OBJETIVOS

3.1 Hipótese

Com base no caráter multifatorial da depressão e a capacidade do CMI em proteger contra o comportamento tipo-depressivo, hipotetizou-se que o CMI seria capaz de reverter o comportamento tipo-depressivo e suas comorbidades que se manifestam em animais submetidos ao estresse agudo de restrição (EAR), desafiados com LPS, portadores de tumor mamário, suplementados com corticosterona e sobreviventes à sepse.

3.2 Objetivo geral

Investigar os mecanismos moleculares envolvidos com o efeito tipo-antidepressivo do CMI.

3.3 Objetivos específicos

- Aprofundar o conhecimento a respeito dos mecanismos responsáveis pelo efeito antioxidante do CMI (capítulo 1).
- Avaliar a capacidade do CMI em reverter o comportamento tipo-depressivo em camundongos submetidos ao EAR através da modulação do estresse nitro-oxidativo e neuroinflamação (capítulo 2).
- Investigar a capacidade do CMI em reverter o comportamento tipo-depressivo e tipo-ansioso em animais desafiados com LPS, através da modulação da via serotoninérgica (capítulo 3).
- Avaliar a capacidade do CMI em reverter o comportamento tipo-depressivo e o déficit cognitivo em camundongos portadores de tumor mamário, através da modulação da neuroinflamação e estresse oxidativo (capítulo 4).
- Investigar a capacidade do CMI em reverter o comportamento tipo-depressivo e tipo-ansiosogênico em camundongos suplementados com corticosterona, através da modulação da via do BDNF/mTOR (capítulo 5).
- Investigar a capacidade do CMI em reverter o comportamento tipo-depressivo, tipo-ansiosogênico e o déficit cognitivo em camundongos sobreviventes à sepse, através da modulação da resposta imune e do estresse oxidativo periférico e central (capítulo 6).

4 CAPÍTULOS

4.1 Capítulo 1 – Selenium-containing indolyl compounds: kinetics of reaction with inflammation-associated oxidants and protective effect against oxidation of extracellular matrix proteins

Artigo publicado na revista *Free Radical Biology and Medicine*

Uma gama de oxidantes é gerada em condições fisiológicas e patológicas *in vivo*, desempenhando importantes funções de sinalização celular, apoptose e controle de patógenos. No entanto, sua produção excessiva está relacionada com a inflamação aguda e crônica, aterosclerose, câncer, doenças neurodegenerativas e depressão. Neste trabalho, queríamos investigar a capacidade do CMI em neutralizar oxidantes derivados da inflamação. Através da análise de cinética de reação, nosso estudo demonstrou que o CMI interage diretamente com o H_2O_2 , HOCl e ONOO^- . O CMI reage mais rapidamente com o HOCl do que os antioxidantes Trolox, taurina e β -caroteno, e reage mais rapidamente com o ONOO^- do que a N-acetil metionina e cisteína. Similarmente, a velocidade de reação do CMI com o H_2O_2 é maior do que a velocidade de reação da cisteína e metionina com o H_2O_2 . De um modo geral, o CMI tem uma alta reatividade com oxidantes-derivados da inflamação, com uma constante de velocidade (k) similar ou maior do que aquelas para as cadeias proteicas laterais (como a cisteína e metionina). Em conjunto, essas observações indicam que o CMI se apresenta como um promissor antioxidante. Esses resultados nos impulsionaram a seguir investigando o potencial farmacológico do CMI *in vivo*, o qual pode representar uma nova estratégia terapêutica para doenças associadas à inflamação e ao estresse oxidativo, como a depressão.



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Original article

Selenium-containing indolyl compounds: Kinetics of reaction with inflammation-associated oxidants and protective effect against oxidation of extracellular matrix proteins



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ABSTRACT

Activated white blood cells generate multiple oxidants in response to invading pathogens. Thus, hypochlorous acid (HOCl) is generated via the reaction of myeloperoxidase (from neutrophils and monocytes) with hydrogen peroxide, and peroxynitrous acid (ONOOH), a potent oxidizing and nitrating agent is formed from superoxide radicals and nitric oxide, generated by stimulated macrophages. Excessive or misplaced production of these oxidants has been linked to multiple human pathologies, including cardiovascular disease. Atherosclerosis is characterized by chronic inflammation and the presence of oxidized materials, including extracellular matrix (ECM) proteins, within the artery wall. Here we investigated the potential of selenium-containing indoles to afford protection against these oxidants, by determining rate constants (*k*) for their reaction, and quantifying the extent of damage on isolated ECM proteins and ECM generated by human coronary artery endothelial cells (HCAECs). The novel selenocompounds examined react with HOCl with k $0.2\text{--}1.0 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$, and ONOOH with k $4.5\text{--}8.6 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$. Reaction with H_2O_2 is considerably slower ($k < 0.25 \text{ M}^{-1} \text{ s}^{-1}$). The seleno-compound 2-phenyl-3-(phenylselenanyl)imidazo[1,2-*a*]pyridine provided protection to human serum albumin (HSA) against HOCl-mediated damage (as assessed by SDS-PAGE) and damage to isolated matrix proteins induced by ONOOH, with a concomitant decrease in the levels of the biomarker 3-nitrotyrosine. Structural damage and generation of 3-nitroTyr on HCAEC-ECM were also reduced. These data demonstrate that the novel selenium-containing compounds show high reactivity with oxidants and may modulate oxidative and nitrosative damage at sites of inflammation, contributing to a reduction in tissue dysfunction and atherogenesis.

1. Introduction

A wide range of oxidants are intentionally generated in biological systems during both physiologic and pathological processes [1]. Activation of white blood cells results in the generation of superoxide radicals ($\text{O}_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) from NADPH oxidases (NOX) enzymes [2], and the release of the enzyme myeloperoxidase (MPO) from neutrophils and monocytes [3]. H_2O_2 can induce two electron oxidation reactions (with thiols or thioethers) or react with redox-active metal ions (mainly iron and copper) via single electron

transfer to give radicals [1]. In the presence of physiological concentrations of chloride (Cl^-), MPO utilizes H_2O_2 to generate the potent oxidant hypochlorous acid (HOCl) [3–5]. HOCl reacts with many biological targets [5], and particularly rapidly with sulfur- and selenium-containing species [6–8], but proteins are often major targets due to their high abundance and reactivity [9–11].

$\text{O}_2^{\cdot-}$ and H_2O_2 are also generated by multiple other cell types including activated macrophages, along with nitric oxide ($\text{NO}^\cdot$), which is generated from arginine by nitric oxide synthase enzymes (e.g. the inducible form, iNOS, present in multiple cells and especially

Abbreviations: EC-ECM, endothelial cell-derived extracellular matrix; ECM, extracellular matrix; HCAEC, human coronary artery endothelial cells; EHS, Engelbreth-Holm-Swarm tumour cells; FN, fibronectin; HOCl, the physiological mixture of hypochlorous acid and its anion OCl^- ; HOX, hypochlorous acid; HRP, horseradish peroxidase; HSA, human serum albumin; LN, laminin; mAb, monoclonal antibody; 3-nitroTyr, 3-nitrotyrosine; ONOOH, the physiological mixture of peroxynitrous acid and its anion ONOO^- ; pAb, polyclonal antibody; PL, perlecan

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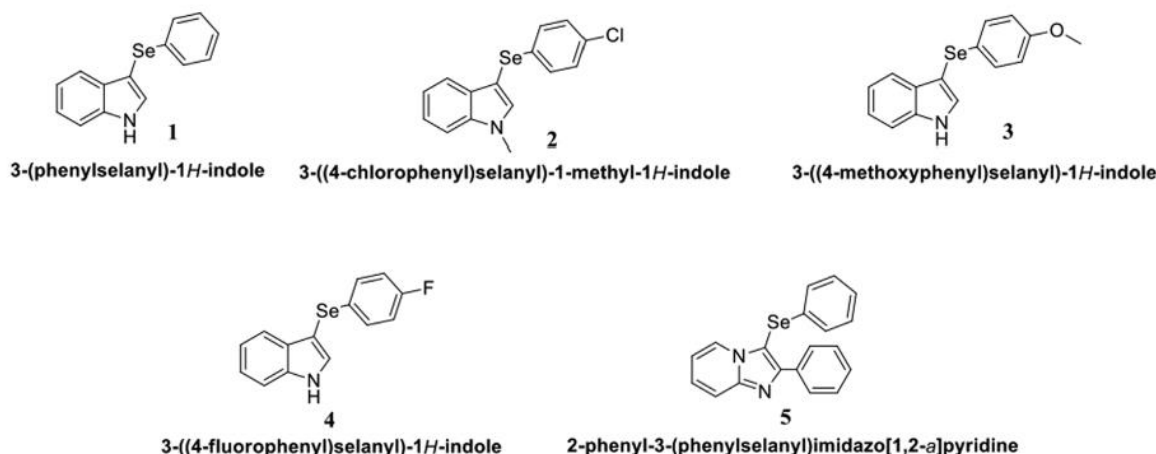


Fig. 1. Structures and chemical names of the selenocompounds investigated.

macrophages) [1,12]. Diffusion-controlled reaction of $O_2^{\cdot-}$ with NO[•] radicals produces the potent oxidant peroxynitrous acid (ONOOH) [13,14], which is in equilibrium with its anion form (ONOO⁻) at physiological pH values (this mixture is designated ONOOH from hereon, as the acid form is the more reactive species) [12,15]. ONOOH modifies a wide variety of biomolecules, including lipids [16], proteins [17,18], nucleic acids [19], carbohydrates [20], thioethers (methionine) [21] and thiols [22] either through one- or two-electron reactions. Reaction of ONOOH with tyrosine (Tyr) residues, both free and on proteins, generates nitrated products including 3-nitrotyrosine (3-nitroTyr), a long-lived and readily detectable marker of reactive nitrogen species [23].

These oxidant-generating systems play an important role in killing invading pathogens *in vivo* [24], but excessive or misplaced generation has been associated with multiple human pathologies characterized by acute or chronic inflammation, including cardiovascular disease (atherosclerosis), rheumatoid arthritis, asthma, cystic fibrosis, some forms of cancer and neurodegenerative conditions [1]. Considerable evidence supports the presence of oxidized materials (e.g. chlorinated, hydroxylated, carbonyl-containing and nitrated compounds) at elevated levels on proteins in human atherosclerotic lesions (e.g. [23,25–27]). Modifications have been detected on both cellular and extracellular proteins including lipoproteins and extracellular matrix (ECM) components [28–31]. ECM materials have been suggested to be major targets [31], due the high abundance of these materials (e.g. elastin comprises 30–60% of the dry mass of many arteries [32]), their long half-lives (which results in the accumulation of damage) and the relatively poor antioxidant defences and repair mechanisms present in extracellular compartments [33]. In particular, recent studies have provided evidence for modification to the major extracellular proteoglycan perlecan (PL) [34], the glycoprotein fibronectin (FN) [35], and laminin (LN) isoforms [36], as well as other components of the ECM [37,38]. Modification of these materials is associated with structural and functional changes, including altered ECM assembly, modified cell binding, proliferation and survival, changes in the binding of cytokines, growth factors and enzymes, altered enzyme activity, decreased atherosclerotic lesion stability and modified angiogenesis [38]. Furthermore, modified ECM materials have been shown to alter the expression of genes in cells exposed to modified ECM [38].

In contrast to the high levels of low-molecular-mass protective agents present within cells (e.g. GSH levels of 2–10 mM [39]), the extracellular compartment is poorly protected (cf. low-molecular-mass thiol concentrations of < 25 μ M in plasma [40]) and these protective mechanisms may be easily overwhelmed [1]. Consequently, supplementation of these defences by use of synthetic compounds may represent a promising pharmacological approach to minimize oxidative damage, as long as the added compounds are bioavailable and react

with very high rate constants with biologically-relevant oxidants. It has been reported that seleno-containing compounds react more rapidly with some oxidants than their sulfur analogues [8,41–43], as a result of the greater nucleophilicity and more favourable redox properties of selenium compared to sulfur [43–46]. Selenium is an essential trace element, and in the form of selenocysteine (Sec), plays a central role in the activity of several important protective enzymes, including glutathione peroxidase (GPx), thioredoxin reductase (TrxR), some isoforms of methionine sulfoxide reductases (Msrs) and selenoprotein P [46,47].

An increasing body of evidence supports the hypothesis that selenium-containing molecules modulate the activity of biologically-relevant oxidants and protect against inflammation-induced damage [46,47]. The synthetic organoselenium compound ebselen, and the amino acid selenomethionine (SeMet) react more rapidly with ONOOH than their sulfur analogues [41]. Furthermore, other low-molecular-mass selenium-containing compounds (selenols, selenides and, to a less extent, diselenides) also react with ONOOH, HOCl and secondary oxidants (e.g. chloramines) with high rate constants [8,41,48,49] and can protect both isolated and plasma proteins from oxidative damage [8,41,48]. Evidence has also been presented for recycling (repair) of the oxidized selenium species (e.g. the selenoxides generated from selenides) by both low-molecular-mass thiols (e.g. GSH) and also enzymatic systems [49–51]. These data are compatible with a catalytic activity of these materials in removing oxidant species.

In the light of these data, the aim of the studies presented here was to investigate the kinetics of oxidation of novel 3-(arylselanyl)-1-*H*-indoles and 3-(phenylselanyl)imidazo[1,2-*a*]pyridine derivatives against a range of biologically-important oxidants (HOCl, ONOOH and H_2O_2) and to determine whether these compounds can protect purified human serum albumin (HSA), isolated matrix proteins (FN, LN, PL) and native ECM synthesized by human coronary artery endothelial cells (HCAECs), against oxidative damage.

2. Materials and methods

2.1. Materials

Four 3-arylselanylindoles (1–4) and 2-phenyl-3-(phenylselanyl)imidazo[1,2-*a*]pyridine (5) (Fig. 1) were synthesized and purified at the Universidade Federal de Pelotas, Brazil as described previously [52]. All of these selenium-containing compounds were dissolved in dimethyl sulfoxide (DMSO) and then diluted into the relevant reaction systems. Control incubations had the corresponding concentration of vehicle (DMSO) added. All other chemicals were purchased from Sigma-Aldrich unless stated otherwise.

The concentration of human plasma-derived FN was calculated using a protein molecular mass of 450 kDa. The concentration of

murine LN-111, isolated from Engelbreth-Holm Swarm (EHS) cells, was calculated using a protein molecular mass of 722 kDa [36]. PL was isolated and purified as described previously [34], with the concentration calculated using a core protein molecular mass of 470 kDa. HCAEC-ECM was obtained as described previously [38]. All concentrations reported are final values unless stated otherwise.

HOCl solutions were prepared daily by dilution of a concentrated stock solution (ca. 2 M in 0.1 M NaOH) into 10 mM phosphate buffer, pH 7.4 [53]. UV/visible spectroscopy was used to quantify HOCl using an extinction coefficient at 292 nm of $350 \text{ M}^{-1} \text{ cm}^{-1}$ [54]. Stock solutions of H_2O_2 were prepared from concentrated commercial solutions using Milli-Q water. ONOOH was synthesized and stock solutions prepared in 0.1 M NaOH as described previously [55], with the concentrations determined using its extinction coefficient at 302 nm [56].

2.2. Determination of rate constants for reaction of selenocompounds with HOCl using competition kinetics with Fmoc-Met

The kinetics of the reactions of HOCl (6 μM) with the selenocompounds 1–5 (“scavenger”; 5–50 μM) were investigated in competition with Fmoc-Met (5 μM) at 21 °C, in which the conversion of Fmoc-Met to the Fmoc-Met sulfoxide is quantified, in the absence and presence of a putative scavenger [6,8]. The yields of Fmoc-Met sulfoxide generated in the presence of increasing concentration of the selenocompounds ($\text{yield}_{\text{scavenger}}$) were determined by UHPLC and compared to the maximal yield in the absence of the added selenocompound ($\text{yield}_{\text{max}}$). Using a competition kinetic analysis, the yields are related by Eq. (1), and rearrangement of this equation results in the linear form ($y = ax + b$), given in Eq. (2).

$$\frac{\text{yield}_{\text{scavenger}}}{\text{yield}_{\text{max}} - \text{yield}_{\text{scavenger}}} = \frac{k_{\text{FmocMet}} [\text{FmocMet}]}{k_{\text{scavenger}} [\text{scavenger}]} \quad (1)$$

$$\frac{\text{yield}_{\text{max}} [\text{FmocMet}]}{\text{yield}_{\text{scavenger}}} = \frac{k_{\text{scavenger}} [\text{scavenger}]}{k_{\text{FmocMet}}} + [\text{FmocMet}] \quad (2)$$

From a plot of $[\text{Fmoc-Met}] \cdot \text{yield}_{\text{max}}/\text{yield}_{\text{scavenger}}$ against increasing concentrations of the scavenger ($[\text{scavenger}]$), the slope of the corresponding line allows determination of the value of second-order rate constant ($k_{\text{scavenger}}$) for reaction of HOCl with the selenocompound using the previously reported value for $k_{\text{Fmoc-Met}}$ ($1.5 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$) [6] with the y-intercept equal to $[\text{Fmoc-Met}]$.

Quantification of Fmoc-Met and Fmoc-Met sulfoxide were carried out on a Shimadzu UHPLC system (SPD-M20A) with samples separated on a reverse phase LC column (Kinetex® Phenomenex 2.6 μm EVO C18 100 Å, 150 mm $\times$ 3.0 mm) at 40 °C with a solvent flow rate of 0.8 mL min^{-1} coupled with fluorescence detection. The mobile phase comprised of a gradient of solvent A [MeOH (20%), THF (2.5%), 1 M NaOAc, pH 5.3 (5%) and H_2O (72.5%)] and solvent B [MeOH (80%), THF (2.5%), 1 M NaOAc, pH 5.3 (5%) and H_2O (12.5%)], programmed as follows: 75% solvent B and 25% solvent A at 0 min, increasing to 87.5% solvent B over 5 min, followed by a further increase to 100% solvent B over the next 0.5 min and a washing step with 100% solvent B for 2.5 min, before returning to 75% solvent B over the next 0.5 min with 3.5 min of re-equilibrating preceding the next injection. 6 μL of samples were injected for each run. The eluent was monitored by fluorescence detection (Shimadzu RF-20AXS detector; λ_{ex} 265 nm; λ_{em} 310 nm), with peak areas determined using Lab Solutions 5.32 SP1 software (Shimadzu) and compared to authentic standards as required. Using these conditions, Fmoc-Met sulfoxide was detected at a retention time of 1.28 min, and Fmoc-Met at 1.56 min.

2.3. Determination of rate constants for reaction of selenocompounds with ONOOH and H_2O_2 by competition kinetics using coumarin boronic acid (CBA)

Rate constants for reaction of the selenocompounds 1–5 with ONOOH and H_2O_2 were determined by competition kinetics using the reaction between CBA (0.5 mM, dissolved in DMSO) and ONOOH or H_2O_2 as the standard “clock” reaction as reported previously [57], with minor modifications. ONOOH and H_2O_2 convert (non-fluorescent) CBA to the fluorescent product 7-hydroxycoumarin (COH), with the concentration of the later determined by fluorescence measurements in the absence ($\text{yield}_{\text{max}}$) and presence of increasing concentrations of the selenocompounds ($\text{yield}_{\text{scavenger}}$). Stock solutions of ONOOH (1 mM) and H_2O_2 (0.5 mM) were prepared as described above and added directly to the samples (final concentrations: 50 μM ONOOH, 25 μM H_2O_2). The selenocompounds were prepared as stock solutions (0.5 mM in DMSO, with the corresponding amount of DMSO added to the control incubations). The fluorescence yields were recorded with a SpectraMax i3 plate reader 30 min after ONOOH (λ_{ex} 323 nm and λ_{em} 455 nm) or H_2O_2 (λ_{ex} 350 nm; λ_{em} 450 nm) addition, and also after 1, 2, 3, 4 and 5 h at 21 °C. The kinetic analysis was carried out in a similar manner to that described above, using the linear expression:

$$\frac{\text{yield}_{\text{max}} [\text{CBA}]}{\text{yield}_{\text{scavenger}}} = \frac{k_{\text{scavenger}} [\text{scavenger}]}{k_{\text{CBA}}} + [\text{CBA}] \quad (3)$$

From a plot of $[\text{CBA}] \cdot \text{yield}_{\text{max}}/\text{yield}_{\text{scavenger}}$ against increasing concentrations of the scavenger ($[\text{scavenger}]$), the slope of the corresponding line allows determination of the value of k for reaction of ONOOH or H_2O_2 with the selenocompounds, using the established rate constants $k_{(\text{CBA} + \text{ONOOH})}$ $1.1 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ and $k_{(\text{CBA} + \text{H}_2\text{O}_2)}$ $1.5 \text{ M}^{-1} \text{ s}^{-1}$ reported previously [57,58], with a set y-intercept equal to $[\text{CBA}]$.

2.4. Protein oxidation

The protective effect of selenocompounds 1–5 against HOCl-mediated damage was assessed using human serum albumin (HSA) as the target. HSA (7.5 μM in 10 mM phosphate buffer, pH 7.4) was treated with various amounts of HOCl (10-, 30-, 40-, 50-, 60-, 70-, 80-, 90-, 100-fold molar excess compared to protein concentration), for 5 min at 21 °C, and then subjected to SDS-PAGE with Coomassie Blue staining, to determine conditions under which significant protein damage was occurring. Subsequent experiments used 75- and 100-fold molar excesses of HOCl over protein to test possible protective effects of the organoselenium compounds, with 2-, 4-, and 8-fold molar excess of the compounds used compared to the protein concentration. The incubated samples were then submitted to SDS-PAGE analysis with Coomassie Blue staining. Pierce unstained protein standards (116–14.4 kDa) were used as reference.

To test the protective effect of selenocompound 5 against ONOOH-mediated damage to ECM proteins, FN (0.5 μM), LN (1 μM), PL (225 nM) and HCAEC-ECM were diluted in 100 mM phosphate buffer, pH 7.4 and then incubated with ONOOH at the stated concentrations with or without added selenocompound at 0, 2-, 4- and 8-fold molar excess over the protein concentration, for 20 min at 21 °C, before analysis by SDS-PAGE with Coomassie Blue staining, or Western blotting. In all cases representative data from multiple gels are presented. None of the parent selenocompounds affected the isolated proteins or extracellular matrix preparations studied here in the absence of added oxidant (data not shown). Samples for electrophoresis were prepared using NuPAGE® Sample Buffer (20x) and NuPAGE® Sample Reducing Agent (10x) according to the manufacturer's instructions (Invitrogen).

SDS-PAGE was carried out on 1 mm NuPAGE® NOVEX 4–12% Bis-Tris gels with NuPAGE® MES SDS Running Buffer 1X (for HSA) or 1 mm NuPAGE® NOVEX 3–8% Tris-Acetate gels with NuPAGE® Tris-Acetate SDS Running Buffer 1X (for FN, LN, PL and ECM) at 160 V for 1 h. Following electrophoretic separation, proteins were visualized using Coomassie R-250, or submitted to Western blotting. HiMark™ pre-stained protein standards (31–460 kDa) was used as reference.

For Coomassie Blue staining, the gels were fixed and stained using 0.1% (w/v) Coomassie R-250 in 40% (v/v) methanol and 10% (v/v) acetic acid followed by overnight incubation at 21 °C on an orbital shaker. The staining solution was then removed and the gels were de-stained using 10% (v/v) ethanol in water. Gels were then scanned using a flatbed scanner.

For Western blotting, proteins were electroblotted onto nitrocellulose membranes using an iBlot® transfer apparatus (Invitrogen). The membranes were blocked with 1% (w/v) bovine serum albumin (BSA) in PBS with Tween 20 (PBST) for 1 h, and then incubated with a primary antibody diluted in blocking solution overnight at 4 °C. Then, membranes were rinsed once for 5 min with PBST before incubation with an appropriate secondary HRP-conjugated antibody (1:5000 dilution) for 1 h with HRP-conjugated anti-rat (A10549; Invitrogen), anti-rabbit (NA934; GE) or anti-mouse (sc-2005; Santa Cruz, Dallas, TX, USA) IgG antibodies. Unbound HRP was removed by washing the membranes 4 times for 15 min with PBST followed by transfer to PBS, and then immune complexes were detected using Western Lightning Plus ECL reagent (NEL104001EA; Perkin Elmer, Waltham, MA, USA). Chemiluminescence images were obtained using the GeneSys software (Syngene) and a G: Box Chemi XR5 acquisition system. In some cases, re-probing of the membrane with multiple primary antibodies was performed, with the immune complexes stripped off the membrane by incubating with Restore™ Western Blot Stripping Buffer (Thermo Scientific) with stirring for 15 min and then re-probed as described above. In order to eliminate false positives, the membranes were also probed with only the secondary antibody and the detection of immune complexes performed as described above. The antibodies and the dilutions used, were: mouse anti-3-nitroTyr monoclonal antibody (clone HM11; Invitrogen; 1:1000 dilution), mouse anti-FN (clone A17, ab26254; Abcam, Cambridge, UK; 1:5000 dilution), rat monoclonal anti-laminin-111 antibody (clone AL-2; R & D Systems, Minneapolis, MN, USA; 1:15000 dilution), rabbit polyclonal anti-laminin (Ab11575; Abcam, Cambridge, MA; 1:2000); mouse anti-perlecan protein core (clone 5D7-2E4; Merck Millipore; 1:1000 dilution).

2.5. Statistics

All rate constants reported for 3-(arylselanyl)-1-*H*-indoles and 2-phenyl-3-(phenylselanyl)imidazo[1,2-*a*] pyridine were derived from more than 3 independent experiments, using at least three different substrate concentrations. Data are expressed as mean ± standard deviation. Graphics were made using Graph Pad Prism 5.0 (GraphPad, LaJolla, CA, USA) and in some cases, the error bars are smaller than the symbols.

3. Results

3.1. Determination of rate constants for the reaction of HOCl, ONOOH and H₂O₂ with selenocompounds

Reaction of HOCl (6 μM) with selenocompounds 1–5 (5–50 μM; see Fig. 1 for structures) was investigated using an adaption of previously established competition kinetics approach, with Fmoc-Met (5 μM) as the competing target [6]. A decrease in the yield of Fmoc-Met sulfoxide, which is generated on oxidation of Fmoc-Met, was detected with increasing concentrations of the selenocompounds present in the reaction mixture. Determination of the maximal yield in the absence of added scavenger ($yield_{max}$) and those in the presence of the selenocompounds

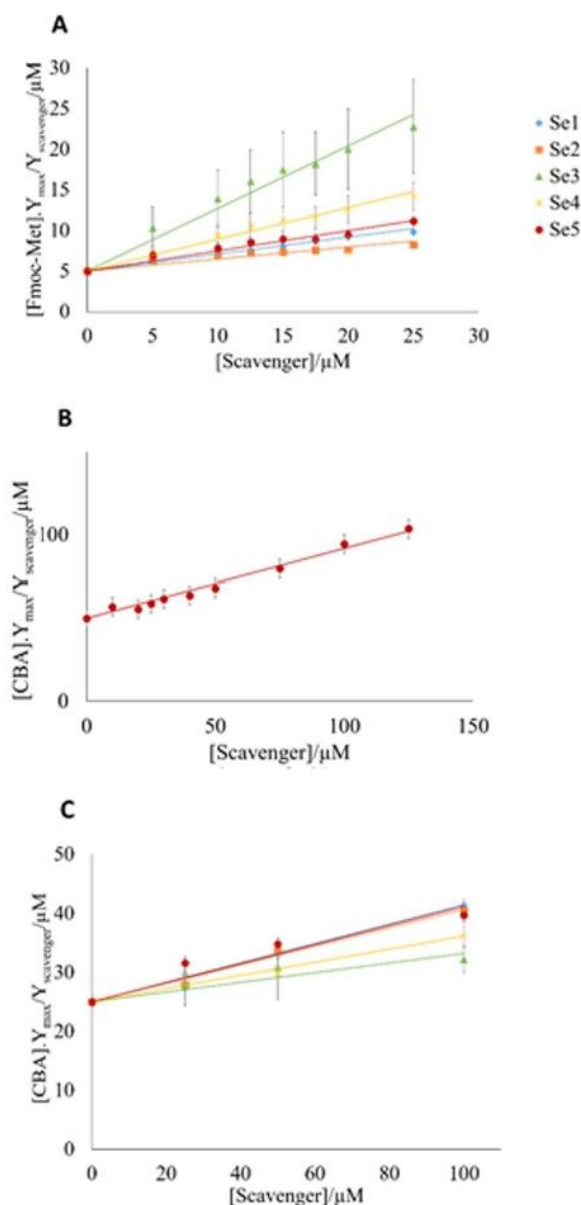


Fig. 2. Kinetic data for the reaction of selenocompounds with (A) HOCl, (B) ONOOH and (C) H₂O₂. (B) Only data for selenocompound 5 are shown for clarity, as data for the other compounds overlay the selenocompound 5 ones (see Supplementary data for graph for all compounds). Reactions were carried out at pH 7.4 and 21 °C in 0.1 M phosphate buffer. Rate constants for the HOCl reactions were determined by competition against Fmoc-Met using UPLC with fluorescence detection to quantify Fmoc-Met sulfoxide yields. Slope = $k_{(HOCl + selenocompounds)}/k_{(HOCl + Fmoc-Met)}$ with $k_{(HOCl + Fmoc-Met)} = 1.5 \times 10^8 M^{-1} s^{-1}$. ONOOH and H₂O₂ rate constants were determined competition kinetics using oxidation of CBA to COH, with fluorescence detection to quantify COH yields. Slope = $k_{(peroxynitrite + selenocompounds)}/k_{(CBA + peroxynitrite)} = 1.1 \times 10^6 M^{-1} s^{-1}$ and $k_{(H_2O_2 + selenocompounds)}/k_{(CBA + H_2O_2)} = 1.5 M^{-1} s^{-1}$. Errors bars for all points are expressed as standard error of the mean ($n = 3$); in some cases, the error bars are smaller than the symbols.

($yield_{scavenger}$) and analysis as outlined in the Materials and methods, gave plots (Fig. 2A) from which the rate constant for reaction of HOCl with the selenocompounds could be determined using $k_{(HOCl + Fmoc-Met)} = 1.5 \times 10^8 M^{-1} s^{-1}$ [6]. The resulting kinetic data are collected in Table 1.

The rate constants for reaction of ONOOH (50 μM) and H₂O₂ (25 μM) with these selenocompounds were also investigated using a competition kinetics approach, with CBA as the substrate for oxidation. The conversion of CBA to COH in the absence and presence of the selenium-containing compounds was monitored by fluorescence

Table 1

Summary of the second order rate constants for reaction of HOCl, ONOOH and H₂O₂ with selenocompounds and related sulfur-containing molecules ^a.

Compound	k (HOCl) M ⁻¹ s ⁻¹	k (ONOOH) M ⁻¹ s ⁻¹	k (H ₂ O ₂) M ⁻¹ s ⁻¹
1	$2.9 (\pm 0.2) \times 10^7$	$4.5 (\pm 0.5) \times 10^5$	$0.2 (\pm 0.02)$
2	$1.8 (\pm 0.3) \times 10^7$	$8.6 (\pm 2) \times 10^5$	$0.2 (\pm 0.02)$
3	$1.0 (\pm 0.3) \times 10^8$	$5.4 (\pm 1) \times 10^5$	$0.1 (\pm 0.04)$
4	$4.8 (\pm 0.9) \times 10^7$	$5.6 (\pm 1) \times 10^5$	$0.2 (\pm 0.01)$
5	$3.2 (\pm 0.9) \times 10^7$	$4.9 (\pm 0.2) \times 10^5$	$0.15 (\pm 0.06)$
Cysteine	3.6×10^{8b}	5.9×10^{3d}	$2.9 (\pm 0.1)^g$
Methionine	3.4×10^{7b}	1.6×10^{2e}	0.009^h
			0.004^i
Selenocysteine	ND	$6.6 (\pm 0.3) \times 10^{5f}$	ND
Selenomethionine	$3.2 (\pm 0.1) \times 10^{8c}$	$2.5 (\pm 0.1) \times 10^{3f}$	ND

^a Values were determined by UPLC (using the “clock” reaction of HOCl with Fmoc-Met, which has k 1.5×10^8 M⁻¹ s⁻¹ [6]), stopped flow (for HOCl and ONOOH) or competition kinetic assay with coumarin boronic acid (for H₂O₂ and ONOOH).

^b From [6].

^c From [8].

^d From [22].

^e From [21].

^f From [41].

^g From [69].

^h From [78] at pH 4.5.

ⁱ From [68]. ND: not determined.

detection of COH. A decrease in the yield of COH was detected with increasing concentrations of added selenocompounds in the reaction mixtures. The graphs obtained by plotting [CBA]·(yield_{max}/yield_{scavenger}) (see Materials and methods) against the concentration of the selenocompounds gave a slope from which the rate constant for the reaction of ONOOH (Fig. 2B and Supplementary Fig. 1) or H₂O₂ (Fig. 2C) with the selenocompounds could be calculated, using the previously established rate constants for reaction of CBA with these oxidants $k_{(CBA+ONOOH)}$ 1.1×10^6 M⁻¹ s⁻¹ and $k_{(CBA+H_2O_2)}$ 1.5 M⁻¹ s⁻¹ [57,58]. The resulting kinetic data are compiled in Table 1.

3.2. Potential protection of model isolated proteins against protein damage induced by HOCl by selenocompounds

The kinetic data reported in Table 1 indicate that the tested selenocompounds react very rapidly with HOCl, with rate constants similar to those for the most reactive (Cys) residues present on proteins, and with rate constants considerably higher than for reaction with all other protein side-chains [9,10,59]. These data therefore suggest that these materials may afford protection to protein exposed to HOCl, if the selenocompounds are present at similar or greater molar concentrations; this was tested using the model protein HSA, by using SDS-PAGE to examine proteins modification. HSA (7.5 μM) was treated with various molar excesses of HOCl (10-, 30-, 40-, 50-, 60-, 70-, 80-, 90-, 100-fold) and then subject to electrophoresis on reducing gels. Significant loss of the parent band (Fig. 3A), as indicated by Coomassie Blue staining, was observed with HOCl: HSA molar ratios of > 60:1 in line with previous data [53,60]. On the basis of these data, 75- and 100-fold molar excesses of HOCl to HSA were chosen to test potential protective effects of the selenocompounds. The presence of increasing concentrations of compounds 1–5 (at 2-, 4-, and 8-fold molar excesses compared to HSA) afforded significant protection of HSA, as assessed by loss of the parent band, with significant differences detected with a 2-fold, or greater, molar excess of selenocompound over HSA (Fig. 3B and Supplementary Fig. 2). Further studies were carried out exclusively with compound 5 due to the availability of this species.

3.3. Potential protection of isolated human plasma fibronectin (FN) against ONOOH-induced protein damage by selenocompound 5

Further information on the potential protective effects of selenocompound 5 was obtained using isolated ECM proteins, and the mixture

of these species present in ECM laid down by human coronary artery endothelial cells (HCAEC). These materials have been shown to be damaged by ONOOH both in vitro and in vivo [34–36,38], and the extent of damage induced by ONOOH can be readily characterized using antibodies specific to defined native protein epitopes, and the (long-lived) modification product 3-nitroTyr.

Modification to isolated human derived FN (0.5 μM in 0.1 M phosphate buffer, pH 7.4) induced by increasing concentrations of ONOOH were assessed by SDS-PAGE followed by protein staining with Coomassie Blue, or Western blotting using antibodies against native FN (monoclonal antibody A17, which recognises a cell-binding region on FN that contains a Arg-Gly-Asp (RGD) sequence,) or 3-nitroTyr (monoclonal antibody HM11). The protein gels revealed a detrimental effect of ONOOH on FN structure, with a loss of staining intensity of the parent protein band with increasing oxidant concentration; this was most marked at the highest ONOOH concentration (Supplementary Fig. 3A). The loss of the parent band was accompanied by the detection of cross-linked aggregates of ~480 kDa and higher masses, at 10-fold or greater excesses of oxidant. Complementary data consistent with the above results were detected using the anti-FN antibody in Western blotting experiments, though these changes were detected at lower (equimolar) levels of ONOOH to protein (Supplementary Fig. 3B). These changes were accompanied by increased levels of 3-nitroTyr (Supplementary Fig. 3C), which was detected at 10-fold or greater molar excesses of ONOOH, with significant staining of higher mass aggregates (~460 kDa and greater) detected with higher oxidant concentrations.

Based on these data, the protective effect of selenocompound 5, at 2-, 4- and 8-fold molar excesses over FN, was examined against damage mediated by 100- and 500-fold excesses of ONOOH. Coomassie Blue staining revealed that the presence of selenocompound 5 diminished the loss of the parent FN band, and decreased the intensity of the aggregate bands, resulting from exposure to ONOOH, with these effects occurring in a concentration-dependent manner with higher levels of selenocompound 5 (Fig. 4A). Complementary data were obtained from Western blot analysis, with the loss of epitope recognition and formation of aggregates induced by the 100- and 500-fold molar excesses of ONOOH, decreased by selenocompound 5 (Fig. 4B). The levels of 3-nitroTyr on both the parent protein and aggregate species, were also decreased in a dose-dependent manner with increasing concentrations of selenocompound 5 (Fig. 4C).

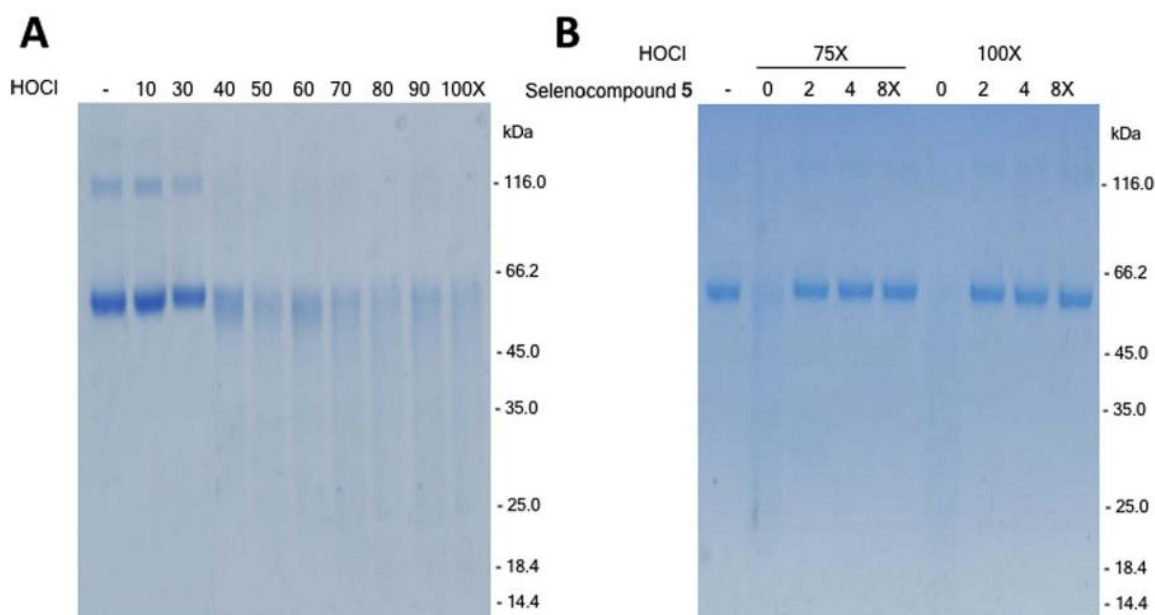


Fig. 3. SDS/PAGE gel of HSA treated with HOCl in the presence or absence of selenocompounds. (A) HSA (7.5 μM) was treated with HOCl at the molar ratios indicated. Reactions were carried out in 10 mM phosphate buffer (pH 7.4) for 5 min at 21 °C. (B): HSA (7.5 μM) treated with 75- and 100-fold molar excesses of HOCl in the presence of 0, 2-, 4-, and 8-fold molar excesses of selenocompound 5 compared to protein concentration. The gels were developed with Coomassie Blue. Position of molecular mass markers are shown for reference.

3.4. Selenocompound 5 protects laminin (LN) and perlecan (PL) against ONOOH-induced damage

In order to examine whether protection by selenocompound 5 is unique to FN, or is common to multiple ECM proteins, the potential role of selenocompound 5 in modulating damage to LN and PL was examined. LN (1 μM) was exposed to increasing molar ratios of ONOOH for 20 min at 21 °C in either the presence or absence of selenocompound 5, in a similar manner to that described above for FN. The LN chains were then separated by SDS-PAGE, blotted on to membranes and probed with an anti-LN mAb by Western blotting. Two sets of bands were detected at ~460 (α1-chain) and ~220 kDa (β1- and γ1-chains) of LN-111 (Supplementary Fig. 4A). Exposure to increasing concentrations of ONOOH resulted in a significant loss of epitope recognition of the parent α1-, β1- and γ1-chains, and an increased level of high-molecular-mass aggregates (> 460 kDa) detected as a smear at the top of the gel. Significant formation of 3-nitroTyr was also detected, with this occurring in a concentration-dependent manner starting with the lowest concentration of oxidant employed (Supplementary Fig. 4B). This product was detected on the monomer α1-, β1- and γ1-chains, as well as on fragments with lower mass (~170, ~75, ~55 and ~35 kDa) and high-molecular-mass aggregates (> 460 kDa).

Inclusion of selenocompound 5 at increasing concentrations, with a 100-fold molar excess of ONOOH, resulted in an increase in the recognition of the α1-chain and to a lesser extent the β1- and γ1-chains (Fig. 4D). No significant protection was detected with a 500-fold molar excess of oxidant. This may reflect the higher molecular mass (and hence greater amount of protein target) of LN compared to FN. However, selenocompound 5 did significantly decrease the extent of 3-nitroTyr formation, in a concentration-dependent manner, induced by both the 100- and 500-fold molar excesses of ONOOH (Fig. 4E). These data are consistent with selenocompound 5 decreasing ONOOH-mediated damage to LN in a similar manner to that observed with FN.

To determine whether protection extends to proteoglycans where the glycosaminoglycan chains may also be targets [20,34,37,61], studies were carried out with perlecan (PL), the major proteoglycan of many basement membranes [62]. Western blot analysis using a protein core antibody (5D7-2E4) revealed that ONOOH-treatment of PL resulted in broader and smeared bands when compared to native PL

(Supplementary Fig. 5A), and the formation of higher mass aggregates. This treatment also resulted in dose-dependent 3-nitroTyr formation on both the parent protein and aggregated materials (Supplementary Fig. 5B). Damage was most noticeable with the highest molar excess (1000-fold) of ONOOH employed, consistent with the high molecular mass of the intact proteoglycan. Selenocompound 5, at increasing concentrations, resulted in a dose-dependent increase in recognition of the core protein by the 5D7-2E4 antibody (Fig. 4F), and a decreased level of 3-nitroTyr formation (Fig. 4G).

3.5. Selenocompound 5 protects the complex ECM generated by HCAEC cells against ONOOH-induced damage

Native ECM synthesized by HCAECs in vitro (EC-ECM) was exposed to increasing concentrations of ONOOH (100–500 μM) in the absence or presence of selenocompound 5, followed by examination of binding of antibodies specific to particular epitopes. In line with a previous report [38], 100 μM or higher concentrations of ONOOH gave rise to a significant loss of antibody recognition of FN (Supplementary Fig. 6A) and LN (Supplementary Fig. 6B), when compared to native EC-ECM. ONOOH treatment resulted in a marked increase in recognition of the EC-ECM by the anti-3-nitroTyr antibody (Supplementary Fig. 6C), consistent with significant generation of 3-nitroTyr on the ECM proteins, with the yield increasing at higher concentrations of ONOOH.

The protective effect of selenocompound 5 against such damage was assessed by incubating EC-ECM with 2, 4 and 8 μM of this compound and 100 or 500 μM of ONOOH. The loss of recognition of the FN epitope induced by 100 μM ONOOH was prevented by selenocompound 5 in a concentration-dependent manner (Fig. 4H). With 8 μM of selenocompound 5 the antibody reactivity to the cell-binding regions of the laminin isoforms present in the EC-ECM treated with 100 μM of ONOOH was maintained (Fig. 4I). However, at this concentration, selenocompound 5 did not prevent the loss of FN and LN antibody recognition when 500 μM ONOOH was employed. In addition, it was observed that the increased 3-nitroTyr formation induced by 100 and 500 μM ONOOH was prevented by selenocompound 5, in a concentration-dependent manner (Fig. 4J).

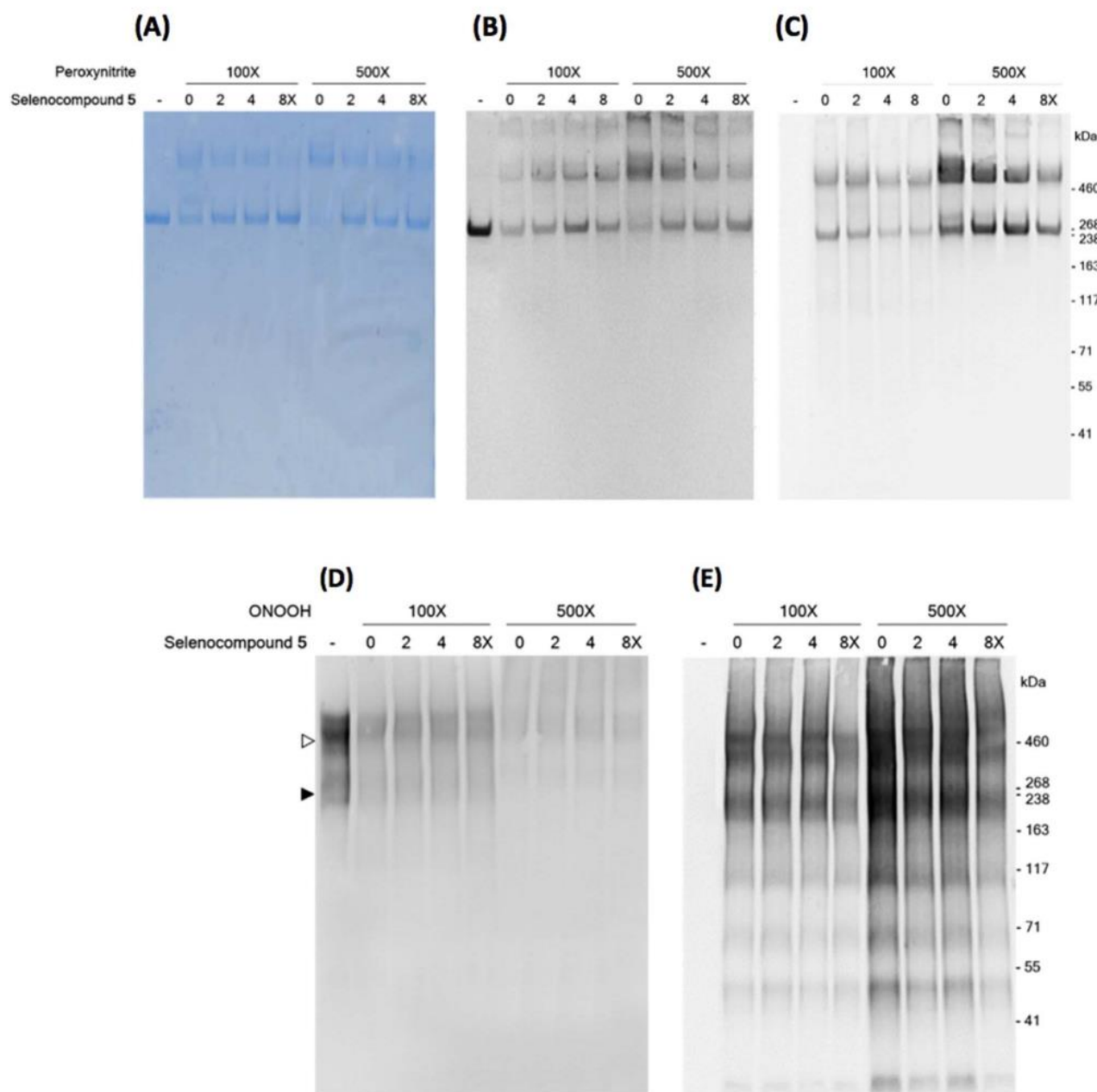


Fig. 4. Modification of isolated matrix components (FN, panels A-C; LN, panels D-E; and PL, panels F-G) and matrix proteins present in native HCAEC-ECM (panels H-J) induced by ONOOH and protective effect of selenocompound 5 against such damage. In all cases reactions were carried out for 20 min at 21 °C in 0.1 M phosphate buffer (pH 7.4). Panels (A-C): FN (0.5 μM) was treated with 100- and 500-fold molar excesses of ONOOH in the presence of 0, 2-, 4- and 8-fold molar ratios of selenocompound 5 compared to the protein concentration. Modifications on FN were detected using either (A) Coomassie staining, or by Western blotting and probing for the presence of (B) FN epitopes (mAb A17) or (C) 3-nitroTyr (HM11). Panels (D-E): LN (1 μM) was treated with 100- and 500-fold molar excess of ONOOH relative to protein, in the presence of 0, 2-, 4- and 8-fold molar excesses of selenocompound 5 relative to protein. Modifications of LN were detected by Western blotting using antibodies against (D) LN epitopes (AL-2) and (E) 3-nitroTyr (HM11); ▷ LN α-chain, ► LN β- and γ- chain. Panels (F-G): PL (225 nM) was treated with a 1000-fold molar excess of ONOOH in the presence of 0, 2-, 4- and 8-fold molar excesses of compound selenocompound 5, relative to the protein concentration. Modifications to PL were detected by Western blotting using antibodies against (F) the PL protein core (mAb 5D7-2E4) and (G) 3-nitroTyr (HM11). Panels (H-J): Matrix proteins present in native HCAEC-ECM were treated with 100 or 500 μM ONOOH in the presence of the indicated concentrations of selenocompound 5. Modification of ECM proteins was detected by Western blotting and detection with primary antibodies against (H) FN cell binding epitopes (mAb A17), (I) LN pAb (Ab11575) and (J) 3-nitroTyr (HM11). The position of molecular mass markers are shown for reference on each panel.,.

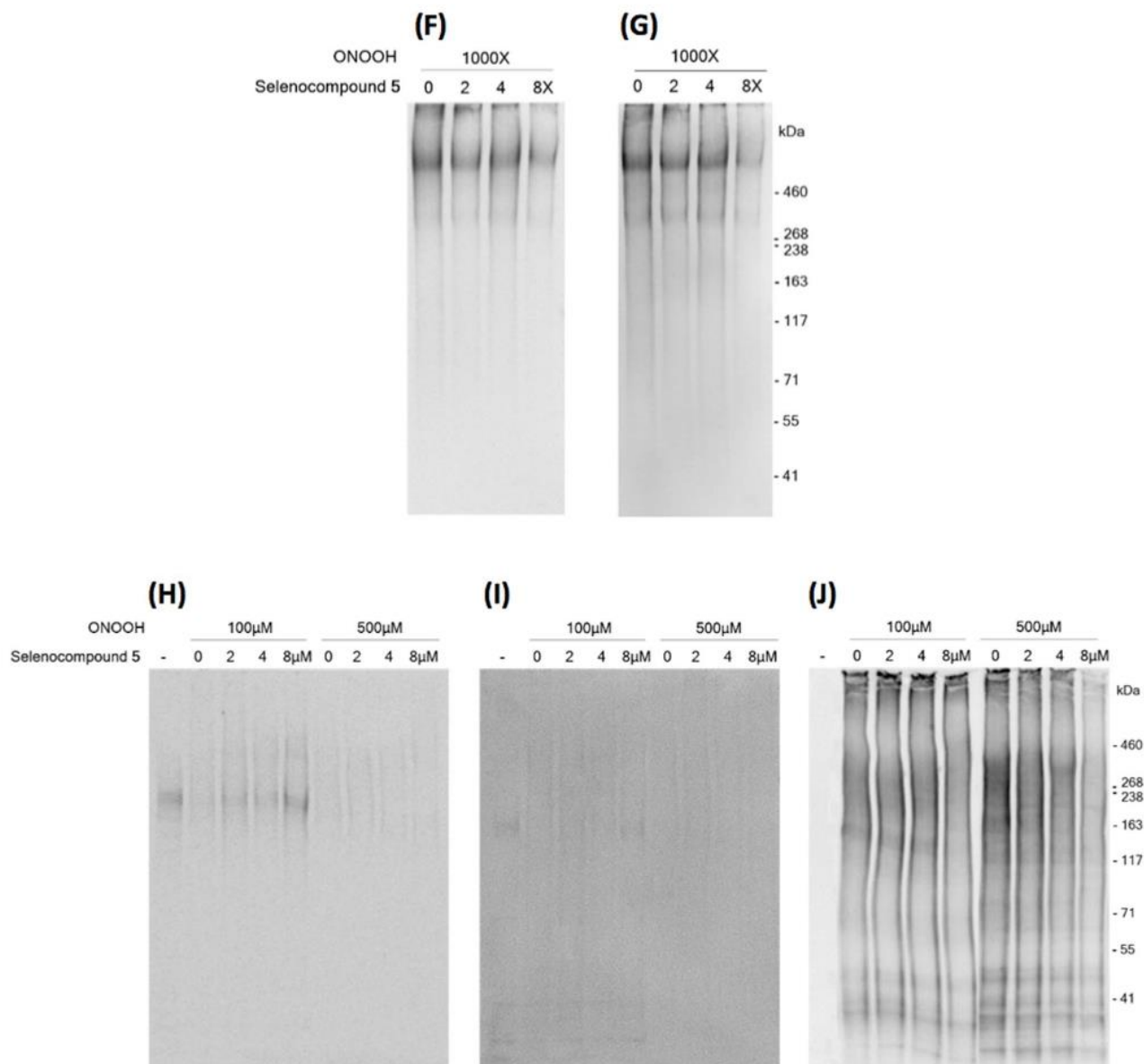


Fig. 4. (continued)

4. Discussion

Biological systems are exposed to a wide range of radical and non-radical oxidants including HOCl, H_2O_2 and ONOOH [1]. These oxidants can damage proteins, which are a major component of many biological systems, as a result of the high rate constants for reaction of some of these species (e.g. HOCl and ONOOH) with amino acid side chains and the backbone of proteins [63,64]. As a consequence, a large number of human pathologies have been linked with excessive oxidant production and/or a reduced rate or extent of damage removal/repair [1]. Determination of rate constants for oxidation of putative scavengers with these oxidants may therefore provide valuable insights into the therapeutic potential of new molecules. As sulfur-containing amino acids (Cys, Met and cystine) are major targets for many oxidants [63,64], there is considerable interest in the identification of species that have similar or greater reactivity, as such compounds might behave as competitive scavengers. Selenium-containing compounds have gained considerable attention, as selenium lies below sulfur in the periodic table, and is both a better nucleophile and more readily oxidized [45,46]. Furthermore, when compared to the corresponding sulfur-

containing compounds, the oxidation products arising from seleno-compounds are typically more readily reduced, allowing some of these species to show *catalytic* (greater than stoichiometric) activity in removing oxidants (e.g. [49,50]).

The two-electron oxidant HOCl is generated at high concentrations by the heme enzyme myeloperoxidase, that is released by activated neutrophils, monocytes and some tissue macrophages [5]. Cys and Met side-chains on proteins show very high reactivity with HOCl, when compared to other protein side-chains, DNA bases, lipids and most antioxidants, making these species particularly susceptible to modification [9,10,59]. The data presented here show that seleno-compounds 1–5 react with high rate constants with HOCl, with values similar to those for the thioether function of Met [6]. These seleno-compounds also react with HOCl faster than the antioxidants Trolox C (a water-soluble analogue of α -tocopherol; k $1.3 \times 10^3 M^{-1} s^{-1}$ [65]), β -carotene ($2.3 \times 10^4 M^{-1} s^{-1}$ [59]), taurine ($4.8 \times 10^5 M^{-1} s^{-1}$ [59]), a proposed antioxidant defence system against halogenated oxidants [66]), and with a similar rate constant to ascorbate ($6 \times 10^6 M^{-1} s^{-1}$) [67]. However, the values for 1–5 are slower than those for Cys, selenocysteine and selenomethionine [6,8], suggesting that

whilst these selenocompounds may afford protection to Met and other amino acid side-chains, significant oxidation will still occur at Cys.

The less reactive oxidants ONOOH and H_2O_2 show different behaviour from HOCl. The kinetic data show that each selenium compound reacts rapidly with ONOOH, but more slowly with H_2O_2 . In general, these selenocompounds react with ONOOH with k values that are approximately 100-fold higher than those of *N*-acetyl methionine ($k\ 2.8 \times 10^3\ \text{M}^{-1}\ \text{s}^{-1}$ [21]) and cysteine ($k\ 5.9 \times 10^3\ \text{M}^{-1}\ \text{s}^{-1}$ [22]). These data are in line with the rate enhancement data reported previously for selenocysteine ($6.6 \times 10^5\ \text{M}^{-1}\ \text{s}^{-1}$) and selenomethionine ($2.5 \times 10^3\ \text{M}^{-1}\ \text{s}^{-1}$) compared to Cys and Met [41]. Interestingly, the values for these novel selenocompounds are higher than for selenomethionine, and on par with selenocysteine, which may be of significance at sites where ONOOH is generated. The higher reactivity of these indolyl selenides compared to selenomethionine, which has alkyl substituents on the selenium atom, may reflect the conjugation of the selenium atom lone pairs with the neighbouring aromatic ring, and therefore an increased ease of oxidation of these species compared to selenomethionine. Using the CBA assay, data have also been obtained for reaction with H_2O_2 . The k values for selenocompounds 1–5 are much lower than those for HOCl and ONOOH, but these values are significantly higher than those for Met [68], but lower than those for Cys [69].

These selenocompounds are also likely to undergo rapid reaction with radicals (see data reviewed in [43]), but this has not been investigated here. Radicals such $\text{HO}^\bullet$ react with most biological targets with k values close to the diffusion limit ($10^9 - 10^{10}\ \text{M}^{-1}\ \text{s}^{-1}$), and the values for selenocompounds 1–5 would be expected to be of a similar magnitude (see [43]), suggesting that high concentrations of these selenocompounds would be required to provide significant protection against radical-mediated damage. As a consequence, the studies reported here have been concentrated on reactions of two-electron oxidants where there is a greater difference between the rate constants for reaction of the oxidant with the selenocompound compared to the biological targets.

As proteins are major targets for HOCl [11], the selenocompounds were examined as potential protective agents against HOCl-mediated oxidation. HSA was chosen as a test protein due to its high abundance in plasma and other extracellular fluids. Previous studies have reported dose-dependent changes in amino acid composition, structure and conformation of HSA (and the related protein BSA) on exposure to HOCl [53,60,70,71]. All the tested selenocompounds provided significant protection against 75- and 100-fold molar excesses of HOCl. Even the lowest concentration of the selenium species tested (15 μM , compared to 7.5 μM protein) afforded significant protection, as determined by staining of the parent protein band on SDS-PAGE gels, suggesting that these compounds are potent protective agents. This inhibition of damage with sub-stoichiometric levels of selenocompounds is of particular interest, and inconsistent with simple stoichiometric reaction. This may be due to rapid recycling of the parent (which is unlikely in these simple chemical systems) or “over-oxidation” (i.e. greater than one mole of oxidant consumed per mole selenocompound) of the initial reaction product which is likely to be the selenoxide. Further studies characterizing the products of these reactions are required to elucidate this point. Interestingly, and consistent with this argument, these selenocompounds afford protection at lower molar ratios than those reported previously for “stoichiometric” protective agents such as Trolox, GSH and ascorbic acid, which also afford protection against HOCl-induced BSA fragmentation [53].

These selenocompounds react rapidly with ONOOH, and hence might afford protection against damage induced by this species at sites of inflammation [12]. Both ONOOH and ONOCO_2^- , formed on reaction of $\text{ONO}^\bullet$ with CO_2 , have been shown to induce structural and functional modifications to extracellular proteins both in human atherosclerotic lesions and in vitro [33–36,38]. Like HOCl, ONOOH causes significant protein damage, and reacts rapidly with Cys and Met residues [17,72]. However, ONOOH also induces damage to multiple

other residues, including Tyr, Trp and some disulfide (cystine) residues [17,72,73]. Some of these reactions (e.g. nitration) occur via radical (one-electron) reactions, and whether the protection observed here against protein nitration arises from direct removal of the nitrating species (e.g. $\text{HO}^\bullet$ and $\text{NO}_2^\bullet$) or the precursor (ONOOH) is not resolved and requires further study. Within cells, reaction with Cys residues is likely to predominate due to the high abundance of these residues on proteins and in GSH. However, external to cells (i.e. in plasma and the extracellular matrix) other targets are likely to be quantitatively important, due to the low abundance of Cys and Met in extracellular proteins. Evidence has been presented for an accumulation of damage on ECM proteins due to their high abundance, the low levels of protective enzyme outside cells, the relatively low rate of turnover of many ECM proteins, and a paucity of extracellular repair systems [33]. Therefore, compounds that react rapidly with ONOOH may represent a useful pharmacological approach against atherosclerosis and other inflammatory conditions. This was tested with selenocompound 5 as an example of this family of selenocompounds; studies were not carried out with the other compounds, but it is expected that these might behave similarly, though this requires investigation.

Significant dose-dependent ONOOH-mediated damage to FN, LN and PL was detected in line with previous reports [34–36]. These reactions modify the protein composition, structure and function, as evidenced by decreased antibody recognition of specific native protein epitopes, and the generation of high levels of 3-nitroTyr, a marker of ONOOH-mediated damage [12]. Selenocompound 5 provided significant protection to each isolated ECM protein, and the complex EC-ECM mixture, though the level of protection differed. With isolated FN, selenocompound 5 provided protection against damage induced by both 100- and 500-fold molar excesses of ONOOH, with increased recognition of the cell-binding epitope by a specific monoclonal antibody detected with increasing concentrations of selenocompound 5. This was accompanied by a decrease in Tyr nitration. Thus, selenocompound 5 may provide protection against decreased cell adhesion to FN induced by ONOOH. Protection by selenocompound 5 was also observed with isolated LN with moderate (100-fold), but not high (500-fold) molar excesses of ONOOH. The absence of observable protection against loss of native protein epitopes with the high oxidant dose is not altogether surprising, given the high molecular mass of this protein (and hence a high abundance of potential targets) and the excess of ONOOH over the concentration of selenocompound 5 employed (molar ratios of protein: oxidant: selenocompound of 1: 500: 8, and therefore a 62.5-fold excess of ONOOH over selenocompound 5). However, despite these ratios, significant protection by selenocompound 5 against Tyr nitration was detected with a 500-fold excess of ONOOH. As some of these Tyr residues (e.g. the laminin $\beta 1$ chain Tyr residue present in the peptide YIGSR) are critically involved in endothelial cell adhesion, angiogenesis and spreading [74], and are modified by ONOOH to give 3-nitroTyr [36], selenocompound 5 may provide protection against the reduced cell adhesion to ONOOH-treated laminin [36]. Further work is needed to confirm this suggestion.

PL is also involved in adhesion of cells to vascular basement membranes, and their subsequent differentiation and proliferation, with some of the functions being dependent on the core protein, rather than the glycosaminoglycan chains, of this proteoglycan [75]. ONOOH has been shown previously to induce damage to both the protein core and the three attached heparan sulfate glycosaminoglycan chains [34]. The current data indicates that selenocompound 5 provides limited protection against core protein damage as evidenced by the detection of more defined (less smeared) parent protein bands, and less aggregated material, recognised by the antibody 5D7-2E4. This effect was most obvious with the highest concentration of selenocompound 5, suggesting that relatively high levels of selenocompound 5 are required to compete for the oxidant when compared to the large number of side-chains present on the PL core protein. This effect was accompanied by a dose-dependent decrease in 3-nitroTyr levels.

This protection against ONOOH-mediated damage prompted further studies on the ECM generated by HCAEC. ONOOH induced changes to this matrix in a dose-dependent manner, with a reduced recognition of FN and LN, and increased levels of 3-nitroTyr, detected by Western blotting, as reported previously [38]. No PL was detected in these ECM preparations (data not shown) using the 5D7-2E4 antibody, in contrast to a previous report [38]. Selenocompound 5 prevented the loss of reactivity against FN induced by a 100-fold molar excess of ONOOH, in a concentration-dependent manner. The addition of 8 μ M selenocompound 5 also maintained the antibody reactivity of the cell-binding regions of laminins in this ECM when treated with 100 μ M ONOOH, however this protection was incomplete. Selenocompound 5 did not protect against the loss of antibody recognition of FN and LN epitopes in the ECM exposed to a 500-fold molar excess of ONOOH, though less 3-nitroTyr formation was detected. These differences between the purified proteins and HCAEC-ECM are not surprising, given that the latter is more complex, with multiple potential targets, and has a 3-dimensional structure that may limit both oxidant accessibility to particular sites, and antibody access to specific epitopes. The nitration pattern observed for HCAEC-ECM is in accordance with previous data [38], where at least one of the major bands detected has a similar molecular mass to parent LN. As the antibodies used to detect FN and LN recognize cell-binding epitopes, these data are consistent with significant modification of the cell-binding capacity of ONOOH-modified HCAEC-ECM [38]. The protection afforded by selenocompound 5 may indicate that this compound could prevent loss of cell adhesion induced by ONOOH. Such an action may be significant, as endothelial cell dysfunction, cell loss and poor re-endothelialisation are early markers of damage to the artery wall, and the development of atherosclerosis [76,77]. Further studies with selenocompound 5 may therefore be warranted in models of atherosclerosis.

Taking together, these data suggest that these selenocompounds have high reactivity with inflammation-associated oxidants, with second order rate constants (k) that are equal or greater in magnitude than those for protein side-chains, with the exception of the reaction of HOCl with Cys and the rare amino acid selenocysteine (Sec). The ability of the compounds to protect HSA against HOCl-mediated damage may reflect these high values, even at the low levels of the selenocompounds employed. Furthermore, selenocompound 5 protected purified ECM proteins, and ECM laid down by HCAEC, against damage induced by ONOOH, suggesting that this compound may limit the loss of cell adhesion and matrix damage induced by ONOOH both in vitro and in vivo. These positive data suggest that the efficacy of these selenium-containing compounds should be examined in in vivo models, as they may represent new avenues for the treatment of diseases associated with oxidative damage and inflammation.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.freeradbiomed.2017.10.344>.

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4.2 Capítulo 2 – The selenium-containing compound 3-((4-chlorophenyl)selanyl)-1-methyl-1*H*-indole reverses depressive-like behavior induced by acute restraint stress in mice: modulation of oxido-nitrosative stress and inflammatory pathway

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No capítulo anterior, demonstramos o potencial antioxidante *in vitro* do CMI. Neste estudo, queríamos investigar se o efeito antioxidante contribuiria com a melhora no comportamento tipo-depressivo de camundongos submetidos ao EAR. O EAR, com duração de quatro horas, é um modelo animal que promove a liberação de PAMPs e DAMPs que sinalizam via TLR4 para induzir a produção de citocinas inflamatórias e ERO/N, as quais contribuem com a resistência dos GR e a hiperativação do eixo HPA. Neste trabalho nós demonstramos que o tratamento com CMI (i) reduziu os níveis plasmáticos de corticosterona; (ii) regulou a atividade da SOD e CAT nos córtex pré-frontais e hipocampus de camundongos estressados; (iii) reverteu o aumento nos níveis de ERO/N e peroxidação lipídica nos córtex pré-frontais e hipocampus de camundongos estressados; e (iv) normalizou a expressão de *GR*, *NFκB*, *iNOS*, *TNF-α*, *IDO*, *GSK-3β* e *BDNF* nos córtex pré-frontais e hipocampus de camundongos submetidos ao EAR. Em conjunto, nossos dados demonstraram que a administração do CMI, uma molécula com efeito antioxidante e anti-inflamatório, reverteu o comportamento tipo-depressivo induzido por estresse em camundongos. Esses estudos reforçam o estresse como um possível fator causal para a depressão, e enfatiza a modulação do estresse nitro-oxidativo e vias inflamatórias como possíveis estratégias terapêuticas para a depressão.



The selenium-containing compound 3-((4-chlorophenyl)selenyl)-1-methyl-1*H*-indole reverses depressive-like behavior induced by acute restraint stress in mice: modulation of oxido-nitrosative stress and inflammatory pathway

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Abstract

Rationale and objectives Stress-induced alterations in oxidative and inflammatory parameters have been implicated in the pathophysiology of mood disorders. Based on the antioxidant and anti-inflammatory properties of the selenium-containing compound 3-((4-chlorophenyl)selenyl)-1-methyl-1*H*-indole (CMI), we assessed its ability to reverse depression-like behavioral alterations, neuroinflammation, and oxidative imbalance induced by acute restraint stress.

Methods Mice submitted to restraint for 240 min received CMI (1 or 10 mg/kg, orally) 10 min after the end of the stress induction. Behavioral and biochemical tests were carried out after further 30 min.

Results Restraint-induced depression-like behavior in the tail suspension test (TST), splash test, and new object exploration test was reversed by CMI. None of the treatments evoked locomotor alteration. In addition, CMI abrogated restraint-induced increases in plasma levels of corticosterone and in markers of oxidative stress and impaired superoxide dismutase and catalase activity in the prefrontal cortex (PFC) and hippocampus (HC). CMI also blocked stress-induced downregulation of mRNA levels of glucocorticoid receptor and brain-derived neurotrophic factor and upregulation of nuclear factor kappa B, inducible nitric oxide synthase, tumor necrosis alpha, indoleamine-2,3-dioxygenase, and glycogen synthase kinase 3 beta in PFC and HC.

Conclusions These preclinical results indicate that administration of selenium-containing compounds might help to treat depression associated with inflammation and oxidative stress.

Keywords Antidepressant · Selenium · Acute restraint stress · Oxidative stress · Neuroinflammation

Angela Maria Casaril and Micaela Domingues contributed equally to this work.

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Introduction

Exposure to stress induces behavioral, emotional, and cognitive modifications that can increase the risk of progression of neuropsychiatric disorders such as major depressive disorder (Joëls et al. 2006). Although glucocorticoids (GCs) released by the adrenal cortex in response to stress are claimed to have immunosuppressive and anti-inflammatory properties, recent data indicate they can also have pro-inflammatory influence on the immune system (Elenkov 2008).

Stress can activate the innate immune system through the activation of Toll-like receptor 4 (TLR4) by damage-associated molecular patterns (DAMPs) that are generated from insults to tissues, or by pathogen-associated molecular patterns (PAMPs), which can be a result of bacterial translocation. Activated TLR4 promotes the activation of glycogen synthase kinase 3 beta (GSK-3 β) (Joep et al. 2017), which can also be activated by increases in cortisol (Dobarro et al. 2013). Among its many actions, activation of GSK-3 β by phosphorylation at tyrosine 216 residue prevents the translocation of nuclear factor erythroid 2-related factor 2 (Nrf2) from the cytosol to the nucleus (Kensler et al. 2007). Consequently, Nrf2 does not bind to the antioxidant response element (ARE), thereby reducing the transcription of antioxidant enzymes, such as heme oxygenase-1 (HO-1), superoxide dismutase (SOD), and catalase (CAT) (Kensler et al. 2007). Additionally, GSK-3 β promotes the activation of transcription factors in the inflammatory response, such as nuclear factor kappa B (NF- κ B), thereby increasing the production of pro-inflammatory cytokines in the central nervous system, especially by microglia and astrocytes (Joep et al. 2017). Pro-inflammatory cytokines are also responsible for the activation of the enzymes indoleamine-2,3-dioxygenase (IDO) (Schwarcz and Pellicciari 2002) and inducible nitric oxide synthase (iNOS), further contributing to impaired neurotransmission and oxidative imbalance by increased reactive species (RS) generation. Excessive formation of free radicals and/or defects in the antioxidant defense can damage lipids, proteins, and nucleic acids, which in turn are implicated in major depressive disorder (Maes et al. 2011), highlighting the importance of the antioxidant balance in the central nervous system.

Selenium is an essential trace element for human health and exerts most of its action as integral constituent of selenoproteins that are widely implicated in redox signaling (Brigelius-Flohé and Flohé 2017). In turn, selenium-containing organic compounds have emerged as promising biologically active molecules (Casaril et al. 2015; Domingues et al. 2018; Singh et al. 2016; Masaki et al. 2016; Kil et al. 2017; Gandin et al. 2018; Sudati et al. 2018). Recently, Vieira et al. (2015) reported that the synthetic organoselenium compound 3-((4-chlorophenyl)selenanyl)-1-methyl-1*H*-indole (CMI) has in vitro antioxidant activity, while Casaril et al. (2017b) showed that it potently inhibits inflammation-associated oxidative stress (i.e., hypochlorous acid, peroxynitrite, and hydrogen peroxide). In addition, Birmann et al. (2018) have demonstrated that the antinociceptive effect of CMI is mediated by the monoaminergic, opioidergic, and adenosinergic systems, pointing out the promising role of CMI in modulating different pathways for the treatment of pain and inflammation. Further studies revealed that CMI prevents the depressive-like behavior induced by lipopolysaccharide (LPS) in mice (Casaril et al. 2017a).

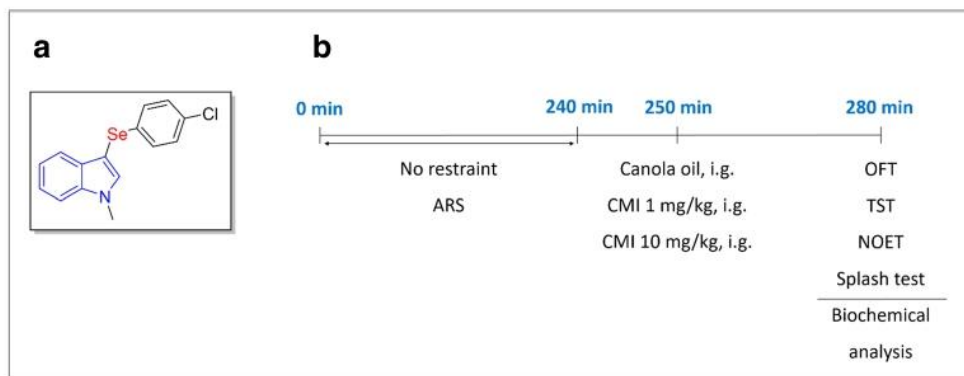
In light of the potential antioxidant and anti-inflammatory profile of CMI, we hypothesized that this organoselenium compound should be able to counteract stress-induced depression-like behavior in mice. We used for this purpose a model of acute restraint stress in mice that had already been validated (Pesarico et al. 2015; Thakare et al. 2016; Surkin et al. 2018). We show here that CMI has antidepressant properties in this model that are associated with downregulation of stress-induced inflammation and oxidative stress.

Materials and methods

Drugs and reagents

CMI (Fig. 1A) was prepared and characterized at the Laboratory of Clean Organic Synthesis at the Federal University of Pelotas, according to Vieira et al. (2015). The RNA extraction reagent was purchased from Ambion (Life

Fig. 1 (A) Chemical structure of 3-((4-chlorophenyl)selenanyl)-1-methyl-1*H*-indole (CMI) and (B) experimental design performed in this study. ARS, acute restraint stress; OFT, open field test; NOET, new object exploration test; TST, tail suspension test



Technology, USA). The oligonucleotides were synthesized by Exxtend Biotecnologia Ltda (Campinas, Brazil). All other chemicals were of analytical grade and were obtained from Servylab and WF Científica (Brazil). CMI was dissolved in canola oil (a non-polar and inert substance) and administered intragastrically (i.g.) at a constant volume of 10 ml/kg body weight.

Animals

Behavioral experiments were performed in Swiss male mice (25–30 g; 10–13-week-old), maintained at 22–25 °C and 40–60% relative humidity with free access to water and food, under a 12:12-h light/dark cycle (lights on at 7:00 a.m.). The studies were performed in accordance with protocols approved by the Committee on the Care and Use of Experimental Animal Resources at the Federal University of Pelotas, Brazil (4034-2017).

Acute restraint stress

The physical restraint was performed in mice as previously reported by Pesarico et al. (2015), with minor modifications. Briefly, mice were subjected to immobilization for 240 min using an individual rodent restraint device made of Plexiglas fenestrate, restraining all physical movement and causing minimum pain. Mice were deprived of food and water during the physical stress. After the restraint stress, mice were put back in their home cage and received canola oil or CMI 10 min later. They were submitted to behavioral testing 30 min later and euthanized immediately after.

Experimental design

Animals were randomly divided into six experimental groups ($n = 6/\text{group}$) and the physical stress was performed for 240 min (Fig. 1B). The behavioral tasks—open field test (OFT), tail suspension test (TST), new object exploration test (NOET), and splash test—were carried out 280 min after the beginning of the acute restraint stress (ARS) protocol, and the vehicle or CMI (1 or 10 mg/kg, orally) was given 10 min after the physical restraint (Pesarico et al. 2015). Previous data also support the 30-min pre-treatment to evaluate the antidepressant-like activity of CMI (Casaril et al. 2017a) and other selenium-containing compounds (Martinez et al. 2014; Pinto Brod et al. 2016). All the observations were done by an observer blinded to the treatment and the behavioral tests were scored manually. Following the behavioral assessment, mice were anesthetized (inhalation of isoflurane) before blood collection by cardiac puncture (Parasuraman et al. 2010). After that, mice were killed by cervical dislocation, followed by brain removal (residual blood and blood cells can be present

in the samples) and isolation of the prefrontal cortex (PFC) and total hippocampus (HC) for analysis (Sunkin et al. 2013).

Behavioral tests

Open field test

The open field test (OFT) was carried out before the other behavioral tests (Walsh and Cummins 1976) to assess the possible effect of the treatments on the locomotor activity. Mice were placed individually in the center of a box ($30 \times 30 \times 15$ cm) divided into nine quadrants of equal areas, and observed for 5 min to report their locomotor (scored by the number of segments crossed with the four paws) and exploratory activities (expressed by the number of time the mice stood on rear limbs).

Tail suspension test

The total duration of immobility in the tail suspension test (TST) was measured according to the method described by Steru et al. (1985). Mice that were both acoustically and visually isolated from each other were suspended 50 cm above the floor by adhesive tape placed approximately 1 cm from the tip of their tail. During the first 2 min of habituation, the latency time to immobility was recorded (i.e., time for the first immobility episode), and during the last 4 min, the immobility duration (defined as the absence of the escape attempt behavior) was observed.

New object exploration test

For this test, mice were allowed to explore a novel object for 15 min in an arena 18×25 cm² surrounded by plastic walls (Strekalova et al. 2004). The object had a complex texture surface (artificial flower, $2.5 \times 2.5 \times 4$ cm³) and it was fixed to the center of the arena. The total duration of time spent exploring the object was scored, and at the end of each test session, the arena was cleaned with a 70% ethanol solution.

Splash test

The grooming behavior of mice was observed as a measurement of motivational and self-care activities, which are considered to be deficient in depressive patients. A 10% sucrose solution was squirted on the dorsal coat of each mice and the grooming activity (including nose/face grooming, head washing, and body grooming) was recorded for 5 min (Freitas et al. 2013).

Biochemical evaluation

Tissue processing

The PFC and HC were separated in two hemispheres in order to submit each sample to all biochemical determinations (Casaril et al. 2017a). The right hemispheres were immersed in TRIzol, maintained at -80°C , and were submitted to the quantitative real-time polymerase chain reaction (qRT-PCR). The left hemispheres were homogenized in 50 mM Tris-HCl, pH 7.4 (1:10, w/v). The homogenate was centrifuged at 2500g for 10 min at 4°C , and the supernatant fraction was used for the determination of reactive oxygen species (ROS) formation, thiobarbituric acid reactive species (TBARS) levels, nitric oxide metabolites (NO_x), catalase (CAT), and superoxide dismutase (SOD) activities.

Plasma corticosterone assay

Determination of plasma corticosterone levels was performed according to Zenker and Bernstein (1958). Briefly, aliquots of plasma were incubated with chloroform and centrifuged for 5 min at 2500 rpm, followed by addition of 0.1 M NaOH and another round of centrifugation. After the addition of the fluorescence reagent (H_2SO_4 and ethanol 50%), samples were centrifuged (5 min at 10,000g) and incubated at room temperature for 2 h. Fluorescence intensity emission, corresponding to plasma corticosterone levels, was recorded at 540 nm (with 247-nm excitation) and corticosterone levels were expressed as nanogram per milliliter.

Determination of the reactive oxygen species formation

Quantification of reactive oxygen species (ROS) levels in the PFC and HC of mice was performed according to Loetchutinat et al. (2005). Briefly, aliquots of the homogenate supernatant were incubated with 1 mM dichloro-dihydro-fluorescein diacetate (DCHF-DA) and 10 mM Tris-HCl pH 7.4. The oxidation of DCFH-DA to fluorescent dichlorofluorescein (DCF) is measured for the detection of intracellular ROS. The DCF fluorescence intensity emission was recorded at 520 nm (with 480-nm excitation) and ROS levels were expressed as arbitrary units (AU) of fluorescence.

Thiobarbituric acid reactive species assay

Lipid peroxidation in the PFC and HC was measured by the formation of thiobarbituric acid reactive species (TBARS) during an acid-heating reaction, as described by Ohkawa et al. (1979). An aliquot of the homogenate supernatant was incubated with 8.1% SDS, 0.8% TBA, and acetic acid/HCl (pH 3.4) at 95°C during 2 h. Malondialdehyde (MDA) was

used as a biomarker of lipid peroxidation. Absorbance was measured at 532 nm, and the results were expressed as nanomoles of MDA per gram of tissue.

Nitric oxide in neural tissue

The total nitric oxide (NO) metabolites, NO_x , in the PFC and HC of mice were determined by the Griess reaction as an indicator of nitrate/nitrite production (Lima-Junior et al. 2013). Briefly, aliquots of the homogenate were incubated with equal volume of the Griess reagent for 5 min at 25°C , and the nitrite concentration was determined by measuring the optical density at 550 nm in reference to a standard curve of NaNO_2 solution. Results were expressed as nanomoles of NO_x per gram of tissue.

Evaluation of superoxide dismutase activity

The measurement of superoxide dismutase (SOD) activity is based on the capacity of SOD to inhibit autoxidation of adrenaline to adrenochrome, as described by Misra and Fridovich (1972). The color reaction was detected spectrophotometrically at 480 nm and the enzymatic activity was expressed as units per milligram of protein.

Evaluation of catalase activity

Catalase (CAT) activity was assessed spectrophotometrically by the method described by Aebi (1984), which involves monitoring the disappearance of H_2O_2 in the presence of S_1 at 240 nm. Enzymatic activity was expressed in units per milligram of protein (1 U decomposes 1 μmol of H_2O_2 per minute at pH 7 at 25°C).

Quantitative real-time polymerase chain reaction

Total mRNA was extracted in the HC and PFC right hemispheres using TRIzol (Invitrogen™, Carlsbad, USA) followed by DNase treatment with DNA-free® kit (Ambion™, USA) and mRNA quantification. The cDNA synthesis was performed using High Capacity cDNA Reverse Transcription kit (Applied Biosystems™, UK) according to the manufacturer's protocol. The amplification was made with UltraSYBR Mix (COWIN Bioscience Co., Beijing, China) using the Stratagene Mx3005P and the oligonucleotides were obtained from Exxtend Biotecnologia Ltda, Campinas, Brazil. Gene expressions were normalized using GAPDH as a reference gene and the conditions for the reaction involved 95°C for 15 s, 60°C for 60 s, and 72°C for 30 s. The $2^{-\Delta\Delta\text{CT}}$ (delta-delta comparative threshold) method was used to normalize the fold change in gene expressions. The following genes were analyzed: glucocorticoid receptor (GR; fwd 5'-ACA GCA ACG

GGA CCA CCT C-3', rev 5'-ATG GCA TCC CGA AGC TTC-3'), nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B; fwd 5'-GCT TTC GCA GGA GCA TTA AC-3', rev 5'-CCG AAG CAG GAG CTA TCA AC-3'), inducible nitric oxide synthase (iNOS; fwd 5'-GTG GTG ACA AGC ACA TTT GG-3', rev 5'-AAG GCC AAA CAC AGC ATA CC-3'), tumor necrosis factor alpha (TNF- α ; fwd 5'-CAT CTT CTC AAA ATT CGA GTG ACA A-3', rev 5'-TGG GAG TAG ACA AGG TAC AAC CC-3'), indoleamine-2,3-dioxygenase (IDO; fwd 5'-AAT CAA AGC AAT CCC CAC TG-3', rev 5'-AAA AAC GTG TCT GGG TCC AC-3'), glycogen synthase kinase 3 beta (GSK-3 β ; fwd 5'-CGG GAC CCA AAT GTC AAA CT-3', rev 5'-TCC GAG CAT GTG GAG GGA TA-3'), brain-derived neurotrophic factor (BDNF; fwd 5'-CCA TAA GGA CGC GGA CTT GTA C-3', rev 5'-AGA CAT GTT TGC GGC ATC CAG G-3'), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; fwd 5'-AGG TCG GTG TGA ACG GAT TTG-3', rev 5'-TGT AGA CCA TGT AGT TGA GGT CA-3').

Protein determination

Protein concentration was measured according to the method of Lowry et al. (1951) using serum bovine albumin as a standard.

Statistical analysis

All experimental data are presented as mean $\pm$ standard error of the mean (SEM). Comparisons between stress exposure and treatment were performed by two-way analysis of variance (ANOVA). When ANOVA revealed a significant main effect, Tukey's post hoc test was used for between-group comparisons. Pearson's correlation analysis was performed to investigate any possible relationship between the immobility time in the TST and neurochemical data. Probability values less than 0.05 ($p < 0.05$) were considered statistically significant. The statistical analysis was accomplished using GraphPad Prism version 7.0 for Windows, GraphPad Software (San Diego, CA, USA).

Results

CMI abrogates restraint-induced depressive-like behavior without altering locomotor activity

The exploratory and locomotor activities of mice submitted to the experimental protocol of this study were assessed in order to confirm the absence of false positive results in the other behavioral tests. Figure 2 A and B

show that none of the treatments altered locomotor and exploratory activities in the OFT, respectively. A two-way ANOVA revealed no significant ARS $\times$ CMI interaction ($F_{(2,30)} = 0.65$, $p = 0.53$) for the number of crossings and no ARS $\times$ CMI interaction ($F_{(2,30)} = 0.82$, $p = 0.45$) for the number of rearings.

The TST is widely used to measure depression-like behavior in rodents, while assessing the antidepressant-like response from several pharmacological classes of drugs (Browne and Lucki 2013). As depicted in Fig. 2C, mice submitted to physical stress showed increased duration of immobility when compared to the vehicle-treated group. Noteworthy, this depressive-like behavior was reversed by the administration of CMI independently of the dose (ARS $\times$ CMI interaction; $F_{(2,30)} = 16.70$, $p < 0.001$). Administration of CMI in non-stressed mice did not influence immobility time.

Corroborating with this result, stressed mice showed reduced latency for the first immobility episode, when compared to the vehicle-treated group (Fig. 2D). On the contrary, treatment with CMI at both doses was able to reverse the decreased latencies (ARS $\times$ CMI interaction; $F_{(2,30)} = 15.4$, $p < 0.001$). Administration of CMI to non-stressed mice did not influence latency time.

Regarding the new object exploration test (NOET) (Fig. 2D), a two-way ANOVA of the exploratory time revealed a significant ARS $\times$ CMI interaction ($F_{(2,30)} = 13.20$, $p < 0.001$). Concerning self-care and grooming (Fig. 2E), a two-way ANOVA revealed a significant ARS $\times$ CMI interaction ($F_{(2,30)} = 7.06$, $p = 0.003$). Administration of CMI to non-stressed mice did not influence these behaviors.

Increased circulating glucocorticoid levels induced by ARS were restored by treatment with CMI

To document the involvement of the HPA axis in our experiments, plasma levels of corticosterone were measured in mice submitted to ARS. The results presented in Fig. 2G show that the restraint-induced increase in corticosterone levels was significantly reversed by CMI (ARS $\times$ CMI interaction; $F_{(2,30)} = 23.90$, $p < 0.001$) while the administration of CMI to non-stressed mice had no effect.

Acute treatment with CMI reversed oxidative stress in the PFC and HC of stressed mice

As depicted in Fig. 3 A and B, the increased ROS production elicited by ARS was significantly abolished by CMI in the PFC (ARS $\times$ CMI interaction; $F_{(2,30)} = 16.00$, $p < 0.001$) and HC (ARS $\times$ CMI interaction; $F_{(2,30)} = 13.50$, $p < 0.001$) of mice. Similarly, CMI

Fig. 2 Effect of CMI (1 and 10 mg/kg; i.g.) on behavioral tasks and corticosterone levels in mice submitted to the ARS (240 min). (A) Number of crossings and (B) number of rearings evaluated in the OFT. (C) Immobility time and (D) latency time on the TST. (E) Exploratory time in the NOET. (F) Grooming time in the splash test. (G) Circulating levels of corticosterone. Data are expressed as mean $\pm$ SEM of six independent animals. $^{##}p < 0.01$ and $^{###}p < 0.001$ when compared to non-stressed vehicle group. $^{***}p < 0.001$ when compared to stressed mice

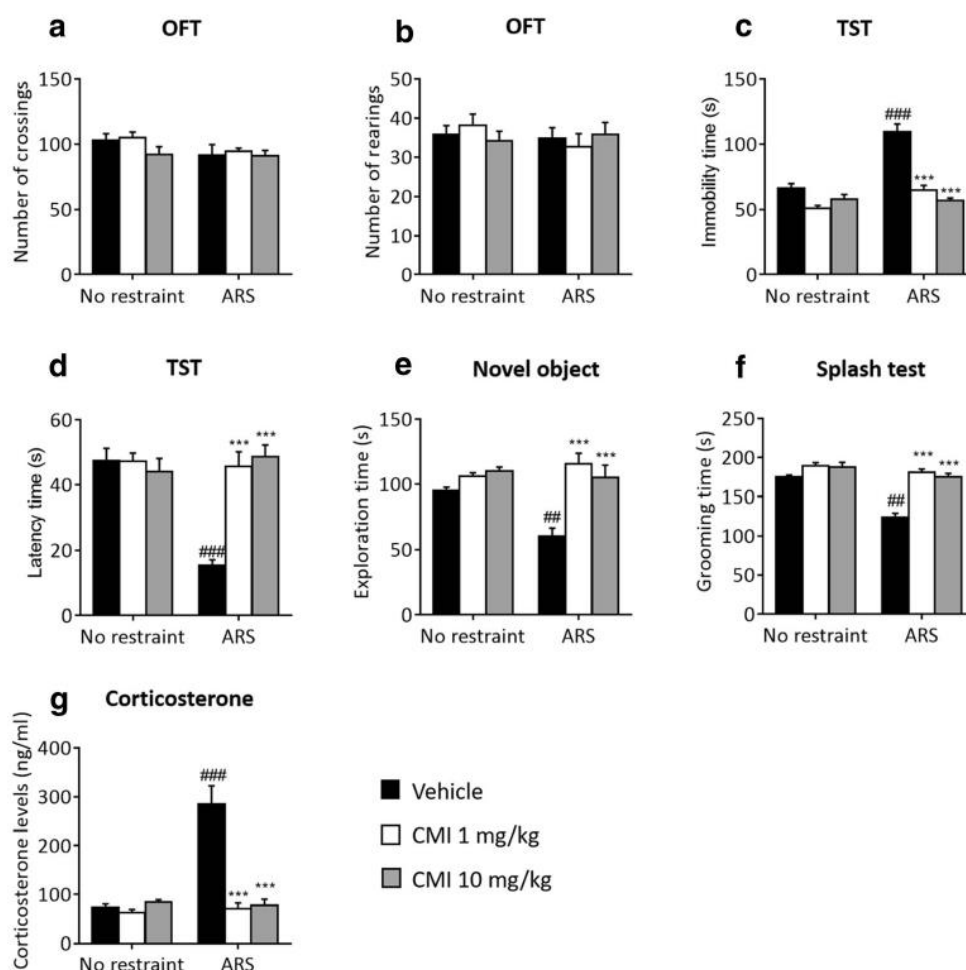


Fig. 3 Effect of CMI (1 and 10 mg/kg; i.g.) on markers of oxidative stress in mice submitted to the ARS (240 min). ROS levels in (A) PFC and (B) HC; TBARS levels in (C) PFC and (D) HC; NO_x levels in (E) PFC and (F) HC. Data are expressed as mean $\pm$ SEM of six independent animals. $^{###}p < 0.001$ when compared to non-stressed vehicle group. $^{**}p < 0.01$ and $^{***}p < 0.001$ when compared to stressed mice

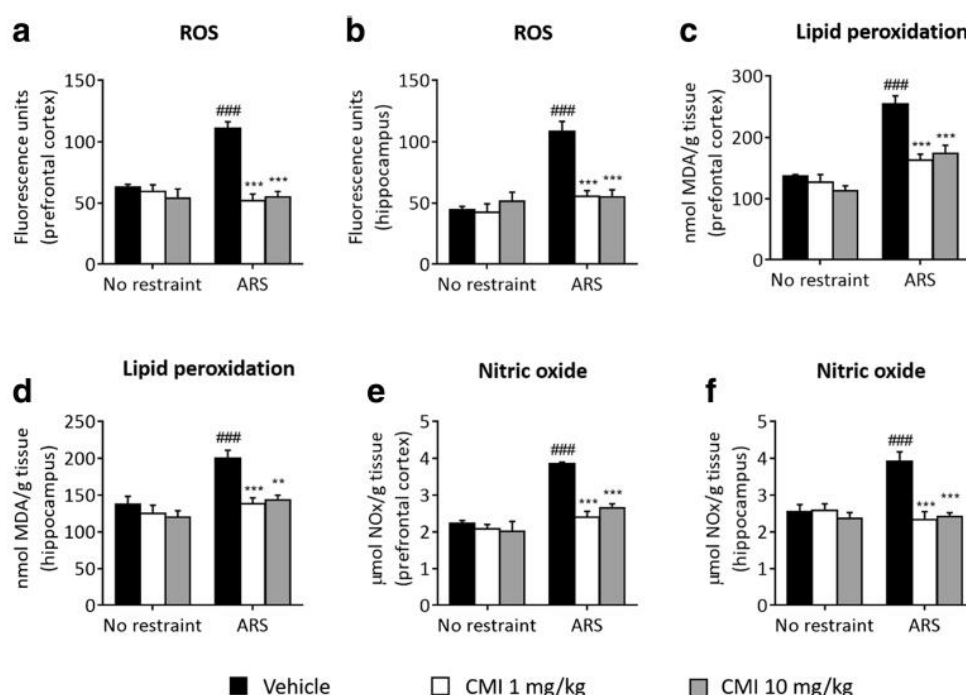
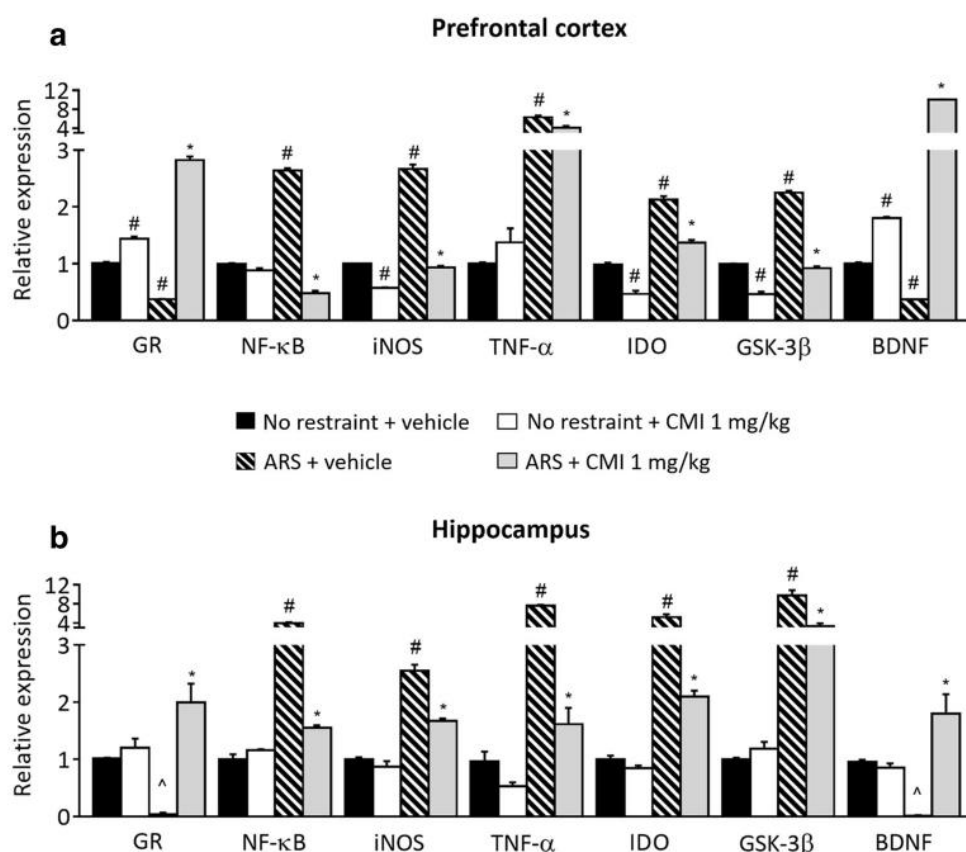


Fig. 5 Effect of CMI (1 mg/kg, i.g.) in the mRNA expression of target genes in mice submitted to the ARS (240 min). Expression of GR, NF- κ B, iNOS, TNF- α , IDO, GSK-3 β , and BDNF in (A) PFC and (B) HC of mice. Data are expressed as mean $\pm$ SEM of six independent animals. $^{\wedge}p < 0.01$ and $^{\#}p < 0.001$ when compared to non-stressed + vehicle. $***p < 0.001$ when compared to ARS + vehicle



$p < 0.001$; ARS $\times$ CMI interaction in the HC; $F_{(1,20)} = 25.60$, $p < 0.001$). Administration of CMI to non-stressed mice downregulated the expression of GSK-3 β in the PFC ($p < 0.001$) when compared to the control group, while no alteration was found in the HC.

The two-way ANOVA on BDNF mRNA expression revealed a significant ARS $\times$ CMI interaction in the PFC ($F_{(1,20)} = 12.895.00$, $p < 0.001$; Fig. 5A) and HC ($F_{(1,20)} = 28.90$, $p < 0.001$; Fig. 5B). Administration of CMI to non-stressed mice downregulated the expression of IDO in the PFC ($p < 0.001$) when compared to the control group, while no alteration was found in the HC.

Correlations between behavioral and biochemical effects in the PFC and HC of mice

Considering that the administration of CMI reduced the increased immobility time in the TST (Fig. 2C) and improved several biochemical endpoints altered by restraint (Figs. 3, 4, and 5), we analyzed if these effects were correlated using Pearson's correlation analysis (Table 1). The results demonstrated significant positive correlation between the immobility time and ROS formation, NO generation, TBARS levels, SOD activity, and NF- κ B, iNOS, TNF- α , IDO, and GSK-

3 β expressions in the PFC and HC of mice. A significant negative correlation was found between the immobility time and CAT activity in the HC and GR and BDNF expressions in the PFC and HC of mice. No significant correlation was found between the immobility time and CAT activity in the PFC.

Discussion

The present study shows for the first time that a single administration of CMI is able to reverse the behavioral response to restraint in the TST, splash test, and NOET by a mechanism dependent, at least in part, on the modulation of oxidative stress and neuroinflammation. Indeed, we observed that CMI regulated the SOD and CAT activities in the PFC and HC of stressed mice, alongside with a decrease in plasma levels of corticosterone. Additionally, we reported here that CMI reversed the increases in TBARS, ROS, and NO $_x$ formation in the PFC and HC of mice submitted to ARS. Noteworthy, CMI was also able to restore the mRNA expression of GR, NF- κ B, iNOS, TNF- α , IDO, GSK-3 β , and BDNF in the PFC and HC of mice. A summary of these results is shown in Table 2. Thus, the beneficial effects of CMI on behavior are associated with its capacity to modulate

Table 1 Pearson's correlation between the immobility time (s) in the TST and biochemical endpoints in the PFC and HC of restrained mice

Immobility time TST			Immobility time TST		
RS formation	PFC	0.889***	NF-κB expression	PFC	0.897***
	HC	0.798***		HC	0.858***
NO _x generation	PFC	0.814***	iNOS expression	PFC	0.891***
	HC	0.740***		HC	0.796**
TBARS levels	PFC	0.647**	TNF-α expression	PFC	0.684*
	HC	0.909***		HC	0.857***
SOD activity	PFC	0.660**	IDO expression	PFC	0.833***
	HC	0.733***		HC	0.8171***
CAT activity	PFC	−0.001 ^{ns}	GSK-3β expression	PFC	0.898***
	HC	−0.826***		HC	0.768**
GR expression	PFC	−0.885***	BDNF expression	PFC	−0.892***
	HC	−0.886***		HC	−0.810**

^{ns} non-significant, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$

oxidative stress and neuroinflammation caused by immobilization stress. The hypothesis of a causal role of oxidative stress and neuroinflammation in the behavioral effects of restraint is supported by the significant correlations between the biochemical endpoints and immobility time in the TST.

Despite several reports having examined the ability of promising drugs to protect behavioral and biochemical modifications induced by ARS (Moretti et al. 2013; Freitas et al. 2014; Ai et al. 2017), few positive results have been obtained so far (Pesarico et al. 2015). Therefore, our study was designed to help to fulfill this gap and if possible increase the range of potential treatments for major depressive disorder. We show here that CMI has clearly the ability to treat stress-induced depression. This effect is independent of the dose at least in the range of doses tested in the present experiments. This lack of a dose-response effect has already been documented for other selenium-containing compounds (Pinto

Brod et al. 2016; Domingues et al. 2018) and neuroprotective agents (Rosa et al. 2018).

The ARS is a widely used animal model to induce depressive-like behavior (Thakare et al. 2016; Surkin et al. 2018) akin to major depressive disorder symptoms, partially by targeting the brain antioxidant and inflammatory systems (Buynitsky and Mostofsky 2009; Spiers et al. 2016; Jope et al. 2017; Surkin et al. 2018). Indeed, the establishment of brain oxidative stress as a result of increased activity of nicotinamide adenosine dinucleotide phosphate (NADPH) oxidase (NOX) (Schiavone et al. 2009) and/or mitochondrial impairment (Jevtić et al. 2016) has been linked to neuropathogenetic alterations induced by different types of stress in rodents. In addition, stress procedure has been shown to cause alterations in amyloid beta (Aβ) release, contributing to the induction of depressive-like behavior in rodents (Morgese et al. 2017). The depressive-like behaviors induced by Aβ occur via impact on

Table 2 Summary of the main neurochemical findings in the PFC and HC of mice treated with CMI

	Prefrontal cortex			Hippocampus		
	ARS + vehicle	ARS + CMI	NS + CMI	ARS + vehicle	ARS + CMI	NS + CMI
RS	↑	↓	=	↑	↓	=
NO _x	↑	↓	=	↑	↓	=
TBARS	↑	↓	=	↑	↓	=
SOD	↑	↓	=	↑	↓	=
CAT	=	=	=	↓	↑	=
GR	↓	↑	↑	↓	↑	↑
NF-κB	↑	↓	=	↑	↓	=
iNOS	↑	↓	↓	↑	↓	=
TNF-α	↑	↓	=	↑	↓	=
IDO	↑	↓	↓	↑	↓	↓
GSK-3β	↑	↓	↓	↑	↓	=
BDNF	↓	↑	↑	↓	↑	=

NS, non-stressed mice

serotonergic and neurotrophin levels, HPA axis activation, and microglial TLR4 signaling (Justice et al. 2015; Morgese et al. 2014; Ledo et al. 2016). In the present study, we focused on the ability of CMI to counteract depressive-like behaviors via modulation of oxidative alterations and neuroinflammation, opening a window for future investigations related to mitochondrial function and A β signaling.

Hyperactivity of the HPA axis is the most common alteration found in patients with major depressive disorder (Stetler and Miller 2011). Increased levels of circulating corticosterone with concentrations in the range of those reported in other studies (Spiers et al. 2016) confirm the effectiveness of the ARS protocol employed in the present study. Additionally, the downregulation of GR mRNA expression in the PFC and HC of mice submitted to ARS could contribute to the reduced negative feedback loop that controls GC secretion. In accordance with this interpretation, we observed that the acute administration of CMI reversed the increasing plasmatic levels of corticosterone and upregulated the GR mRNA expression in the PFC and HC of mice submitted to ARS. These findings, together with the significant negative correlation with immobility time in the TST, show the ability of CMI to modulate hyperactivity of the HPA axis, an effect that could play a role in its antidepressant properties.

Stress is known to activate intracellular pathways involved in increasing free radical production. GC can induce neuronal oxidative stress by enhancing mitochondrial respiration and oxidative phosphorylation (You et al. 2009). NO is a neuromodulator able to control the release of corticosterone (Rettori et al. 2009), thus further influencing the stress response (Gadek-Michalska et al. 2016), including in the model of ARS (Chen et al. 2016; Surkin et al. 2018). Overall, RS produced during the stress promote lipid peroxidation, as evidenced by increased MDA formation (Maes et al. 2011; Niki 2012), that in fact is the major consequence of the oxidative stress in the brain (Niki 2012). In line with this, the present study showed a higher production of ROS, TBARS, and NO $_x$ and decreased mRNA expression of iNOS in the PFC and HC of mice submitted to the ARS, when compared to the non-stressed control. Interestingly, these oxidative alterations in mice subjected to ARS were reversed by a single dose of CMI. Considering that CMI was already capable of preventing oxidative alterations in LPS-challenged mice (Casaril et al. 2017a), this antioxidant effect of CMI is perhaps not surprising, but still very promising in terms of recovery from acute stressors. Since behavioral disturbances may be a reflection of increased oxidative alterations in the brains of stressed mice, we propose that the blockade of depression-like behavior by CMI treatment is mediated by its ability to decrease ROS, NO $_x$, and TBARS generation, a hypothesis that is supported by the significant correlation among these variables.

Further analysis of oxidative stress endpoints confirmed alterations of SOD and CAT activities in the PFC and HC of mice submitted to the ARS protocol. We found significant increases in the SOD activity in the PFC and HC and a reduced CAT activity in the HC of mice submitted to the ARS. Previous studies have shown contradictory results about the activity of antioxidant enzymes in major depressive disorder (Selek et al. 2008; Kotan et al. 2011; Lukic et al. 2014; Tsai and Huang 2016). The reduced CAT activity in the HC of stressed and increased SOD activity observed in the present study are in agreement with data previously reported by others (Moretti et al. 2013; Freitas et al. 2014; Pesarico et al. 2015; Tsai and Huang 2016). Increased SOD activity accompanied by reduced CAT activity is an indicator of a pro-oxidative state, since SOD converts superoxide anion to hydrogen peroxide (H $_2$ O $_2$), but CAT does not metabolize H $_2$ O $_2$ to water (Halliwell 2007). This excessive production of H $_2$ O $_2$ may favor the Fenton reaction and the generation of hydroxyl radical, which in turn triggers lipid peroxidation. In this way, SOD and CAT activities in stressed mice treated with CMI (1 and 10 mg/kg) were restored to values similar to those in the control group, pointing out the ability of CMI to normalize oxidative alterations in depressive mice. This effect of CMI could help to explain its ability to reverse the increased expression of NF- κ B. H $_2$ O $_2$ formed in the brain can rapidly diffuse to extracellular spaces, affecting neighboring cells and contributing to oxidative stress and activation of NF- κ B. Therefore, the restoration of SOD and CAT activities after CMI administration could result in less H $_2$ O $_2$ available to diffuse and influence the oxidative pathways. Another possible explanation for the ability of CMI to reduce the oxidative imbalance in the PFC and HC of mice submitted to ARS is related to the downregulation of the GSK-3 β mRNA expression, which in turn could increase the synthesis of antioxidant enzymes (such as SOD and CAT) mediated by Nrf $_2$.

In addition to this antioxidant effect, decreased GSK-3 β signaling by CMI could reduce the expression of pro-inflammatory cytokines (such as TNF- α) through the reduction of NF- κ B activation, explaining the downregulation of IDO mRNA in the PFC and HC after CMI treatment. Reduction of GSK-3 β signaling may also increase BDNF expression by facilitating the cyclic AMP response element-binding protein (CREB) activity (Grimes and Jope 2001). It is important to note that stress can increase pro-inflammatory cytokines (García-Bueno et al. 2008), and the main consequence of a dysregulation of cytokine levels in the brain is the establishment of neuroinflammation and oxidative and nitrosative stress that could affect neurogenesis (Dantzer et al. 2008; Stepanichev et al. 2014). The antioxidant activity of CMI alongside with its ability to downregulate the expression of NF- κ B and TNF- α induced by physical stress and IL-1 β and IL-6 induced by LPS (Casaril et al. 2017a) and to

upregulate BDNF mRNA indicates that CMI may be able to prevent impairment of neurogenesis. In addition, the reduction of pro-inflammatory cytokine expression evoked by the CMI treatment could account for the decreased corticosterone levels on stressed mice. Additionally, mitochondrial ROS (mtROS) can induce inflammatory cytokine responses (notably IL-1 β and IL-6) and antioxidants that act within mitochondria, such as melatonin and indole-3-carboxylic acid, can reduce IL-1 β and IL-6 levels via downregulation of NF- κ B expression in endothelial cells (Lowes et al. 2011). In this regard, downregulation of NF- κ B, IL-1 β , and IL-6 mRNA by CMI could contribute to its antioxidant profile. Nonetheless, more studies are warranted to confirm the effect of CMI on mtROS and on the reduction in adult neurogenesis that takes place in response to inflammatory stimuli or environmental stress.

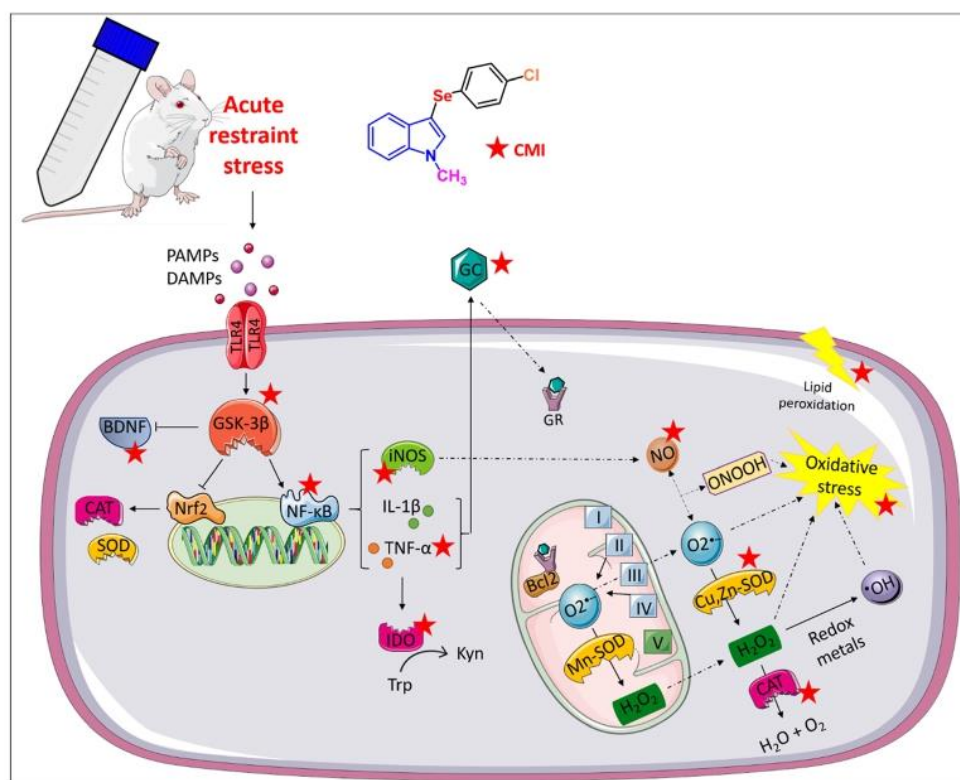
Alongside with the well-known effects of physical stress in the brain antioxidant and inflammatory systems, new evidences have suggested that increased intestinal permeability and consequently translocation of intestinal bacteria may influence TLR4 signaling pathway activation after acute restraint stress exposure (Gárate et al. 2013, 2014; MacDowell et al. 2015). Indeed, Gárate et al. (2014) have demonstrated that bacterial translocation is partially responsible for the physical stress-induced TLR4 mRNA upregulation and protein expression in rat brain frontal cortices. Therefore, the results presented here

agree with previous evidence (Casaril et al. 2017b) suggesting that, by modulating the effects of inflammatory and oxidative mediators produced after TLR4 activation by DAMPs and PAMPs in an acute stress response, CMI can reverse the depression-like behaviors in mice (Fig. 6).

Conclusion

The inhibition of GSK-3 β (Jope et al. 2017; Rosa et al. 2018; Rana and Singh 2018) and IDO (Felger and Lotrich 2013; Felger and Treadway 2017) and the reduction of neuroinflammation and oxidative stress (Maes et al. 2011; Felger and Lotrich 2013) have already been shown to represent novel treatment strategies to major depressive disorders beyond the classical therapies aimed at monoaminergic neurotransmission. Altogether, our data demonstrated that the oral administration of a selenium-containing compound with anti-oxidative and anti-inflammatory properties is able to reverse stress-induced increases in depression-like behavior. These data support the antidepressant properties of this type of compound and clearly encourage further studies to elucidate the pathways underlying these properties. Nevertheless, although we provided evidence of the involvement of the TLR4-NF- κ B and Nrf2-GSK-3 β pathways in the antidepressant-like effect of CMI through evaluation of

Fig. 6 Summary of the effects of acute restraint stress (ARS) on brain oxidative and inflammatory pathways and possible targets of CMI. Administration of CMI after ARS downregulated the mRNA expression of GSK-3 β , NF- κ B, iNOS, and IDO in prefrontal cortex (PFC) and hippocampus (HC), while increased the expression of GR and BDNF in those brain structures. Treatment with CMI reversed the increased generation of RS, NO $_x$ metabolites, and lipid peroxidation in PFC and HC of mice. CMI also modulated the activity of SOD and CAT in PFC and HC and the plasmatic levels of corticosterone. Together, these effects contributed to improve the behavioral phenotype altered by ARS in mice



mRNA expression, the present study is not sufficient to provide direct evidence supporting this hypothesis, which is the major limitation of this study. Therefore, the role of each of these targets deserves further studies to determine their relation to the antidepressant-like effect of CMI.

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Contributions AMC and MD performed the experiments and the analysis of data and wrote the manuscript. SRB, DAL, and MS performed the experiments. AMC, MD, and LS designed the project. NBP and EJJ synthesized the compound CMI. TC, FKS, LS, and CWN supervised the experiments. RD revised the scientific content of the manuscript and provided valuable intellectual insights. All authors critically reviewed the content and approved the final version for publication.

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Compliance with ethical standards

The studies were performed in accordance with protocols approved by the Committee on the Care and Use of Experimental Animal Resources at the Federal University of Pelotas, Brazil (4034-2017).

Conflict of interest The authors declare they have no conflict of interest.

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4.3 Capítulo 3 – Depression- and anxiogenic-like behaviors induced by lipopolysaccharide in mice are reversed by a selenium-containing indolyl compound: Behavioral, neurochemical and computational insights involving the serotonergic system

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No capítulo anterior, investigamos a capacidade do CMI em reverter o comportamento tipo-depressivo induzido por EAR. Neste trabalho, aprofundamos nosso conhecimento a respeito do efeito tipo-antidepressivo do CMI em camundongos desafiados com LPS. O LPS é uma endotoxina de bactérias Gram-negativas que sinaliza via TLR4 para induzir a expressão de citocinas inflamatórias e ERO/N, as quais podem influenciar a neurotransmissão serotoninérgica. De fato, a relação entre o sistema serotoninérgico e desordens psiquiátricas em decorrência da comunicação entre o sistema imune e o cérebro tem sido amplamente investigada. Diante disso, nesse trabalho nós investigamos se o CMI seria capaz de melhorar o comportamento tipo-depressivo e tipo-ansiosogênico induzido por LPS. Através do uso de antagonistas, observamos que os efeitos tipo-antidepressivo e tipo-ansiolítico do CMI dependem da modulação dos receptores serotoninérgicos 5-HT_{1A}, 5-HT_{2A/2C} e 5HT₃. Em adição, o tratamento com CMI reverteu o aumento na expressão da *IDO* e citocinas pro-inflamatórias nos córtex pré-frontais e hipocampus de camundongos desafiados com LPS. Uma análise *in silico* contribuiu com a elucidação do mecanismo de ação do CMI, indicando sua possível interação com o sitio ativo da *IDO* e do transportador de serotonina, SERT. Assim, propusemos que o perfil multialvo do CMI contribuiu para o efeito terapêutico na comorbidade entre depressão e ansiedade.



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Depression- and anxiogenic-like behaviors induced by lipopolysaccharide in mice are reversed by a selenium-containing indolyl compound: Behavioral, neurochemical and computational insights involving the serotonergic system

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ABSTRACT

Major depression and anxiety are highly incapacitating psychiatric disorders often present simultaneously, and the causal relationship between these disorders and inflammation are under extensive investigation. The treatment for this comorbidity still relies on drugs acting on the serotonergic neurotransmission, but the modulation of immune-inflammatory pathways has attained an increasing interest in the drug discovery. We have previously demonstrated that the selenoorganic compound 3-[(4-chlorophenyl)selenyl]-1-methyl-1*H*-indole (CMI) possess antioxidant, anti-inflammatory, antinociceptive and antidepressant-like effect in mice. Considering these pharmacological properties and the structural similarities between tryptophan, serotonin and CMI, the aim of the present study was to investigate whether CMI ameliorates depression- and anxiogenic-like behavior induced by lipopolysaccharide (LPS) in Swiss male mice by modulating the serotonergic system and reducing neuroinflammation. The administration of CMI (1 mg/kg, i.g) reversed the behavioral deficits induced by LPS (0.83 mg/kg, i.p) in the tail suspension test, splash test and elevated plus maze. The pre-treatment of mice with WAY100635 (5-HT_{1A} receptor antagonist), ketanserin (5-HT_{2A/2C} receptor antagonist) and ondansetron (5-HT₃ receptor antagonist) prevented the antidepressant- and anxiolytic-like effect elicited by CMI treatment after the LPS challenge. The administration of CMI also counteracted the increased expression of pro-inflammatory cytokines and indoleamine 2,3-dioxygenase (IDO) in the prefrontal cortex and hippocampus of mice challenged with LPS. Additionally, a molecular docking analysis showed that CMI binds to the active site of the serotonin transporter and IDO. These findings suggest that CMI reversed behavioral and biochemical alterations in the depression-anxiety comorbidity induced by LPS, possibly by modulation of neuroinflammatory mediators and the serotonergic system.

1. Introduction

According to the World Health Organization, 4.4% of the global population suffer from major depressive disorder (MDD) and 3.6% from anxiety disorder (World Health Organization, 2017). These conditions are often presented simultaneously, and patients with anxious depression are more resistant to treatments and tend to have more severe depressive symptoms, such as fatigue and worthlessness (Fava et al.,

2008; Goldberg et al., 2014).

Despite the high social impact and economic burden caused by MDD and anxiety, the current therapy to treat this comorbidity is still limited. Benzodiazepines are anxiolytics that present a high risk of abuse with long-term use and have limited efficacy treating depressive symptoms (Hoffman and Mathew, 2008). Selective serotonin (5-hydroxytryptamine; 5-HT) reuptake inhibitors (SSRIs) are the first line of treatment for MDD and anxiety disorders (Griebel and Holmes, 2013).

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However, SSRIs exhibit modest efficacy and considerable adverse effects (including nausea, diarrhea, insomnia, cognitive impairment, sexual dysfunction, and sleep disturbances), leading to the discontinuation of treatment (Lader, 2007; Shelton, 2018). It has been proposed that the simultaneously blocked of 5-HT_{1A} autoreceptors and 5-HT transporters (SERT) increase the time for onset of antidepressant action when compared to SSRI alone (Artigas et al., 1994; Maes et al., 1999; Starr et al., 2007). Downregulation of 5-HT_{2A} has also been shown to produce antidepressant-like activities (Zaniewska et al., 2010) and can act synergistically with SSRIs (Marek et al., 2003). The blockade of 5-HT_{2C} receptors parallels with the onset of action of SSRIs, while activation of these receptors induces sleep disturbances and motor impairment (Millan, 2005). Similarly, the antagonism of the 5-HT₃ receptors has been associated with the faster onset of action of antidepressant drugs (Alam et al., 2013; Bétry et al., 2013).

It has been acknowledged that the modulation of the central serotonergic system may involve the activation of systemic immune-inflammatory pathways (Couch et al., 2013; Felger, 2018; Miller et al., 2013). Environmental challenges (such as diet and lifestyle factors), psychosocial stress and medical illness contribute to the increased inflammation found in patients with mood and anxiety disorders. Pro-inflammatory cytokines, such as interleukin-1 beta (IL-1 β) and tumor necrosis factor-alpha (TNF- α), induce the activity of the enzyme indoleamine 2,3-dioxygenase (IDO), which reduces the availability of tryptophan disrupting the synthesis of central 5-HT (Wichers and Maes, 2004). Additionally, IL-1 β can upregulate the 5-HT transporter (SERT) causing a decrease of extracellular 5-HT (Ramamoorthy et al., 1995) and antidepressant drugs are known to reverse the inflammation-induced depression, corroborating the idea that cytokine production may directly affect the 5-HT system (Horikawa et al., 2003). Indeed, the relationship between the serotonergic system and psychiatric disorders as a function of immune-to-brain communications has been investigated for years and provide a valid ground for the development of improved therapies (Miller et al., 2017). Several lines of evidence suggest that inflammation plays a role in depression and anxiety (Felger, 2018; Mattei and Notter, 2019; Singhal et al., 2014), as the action of inflammatory cytokines ultimately leads to altered neurocircuits in the brain. In pre-clinical studies, depression-like behavior can be induced by acute inflammation by the administration of cytokine inducers, such as lipopolysaccharide (LPS) (O'Connor et al., 2009).

Recently, it has been reported that the selenium-containing compound 3-[(4-chlorophenyl)selenanyl]-1-methyl-1*H*-indole (CMI) possesses antioxidant activity (Vieira et al., 2015) and it is able to protect extracellular matrix proteins against damage induced by inflammation-derived oxidants (Casaril et al., 2017b). The administration of CMI has also been shown to prevent the depression-like behavior induced by systemic LPS injection and reversed the depression-like behavior elicited by acute restraint stress in mice by targeting oxidative alterations, neuroinflammation and corticosterone levels (Casaril et al., 2019). In addition, the antinociceptive effect of CMI involves the modulation of 5-HT_{1A}, 5-HT_{2A/2C}, and 5-HT₃ receptors (Birmann et al., 2018). Importantly, no signs of hepatic and renal toxicity were found after CMI treatment (Casaril et al., 2017a). The structure of CMI may be responsible for some of these effects: the indole nucleus found in this selenoorganic compound is also present in naturally occurring bioactive compounds, such as tryptophan, melatonin, serotonin, and lysergic acid. In light with this, based on (i) antioxidant, anti-inflammatory and antidepressant-like effect of CMI; (ii) the structural similarity between CMI, tryptophan and serotonin; and (iii) the involvement of the serotonergic system in the antinociceptive effect of CMI, we hypothesize that CMI would be able to counteract the behavioral and biochemical alterations induced by acute inflammation in mice.

In this study, our goal was to evaluate whether CMI reverses the depression- and anxiogenic-like behaviors induced by LPS in mice and whether the 5-HT_{1A}, 5-HT_{2A/2C}, and 5-HT₃ receptors and decreased neuroinflammation are involved in its effect. A depth understanding of

the mechanism of action of CMI may enable us to characterize CMI as a promising complementary therapy for the comorbidity between MDD and anxiety disorders.

2. Materials and methods

2.1. Animals

Adult male Swiss mice (25–30 g) provided by the Animal Facility of the Federal University of Pelotas (UFPEL) were used. Mice were housed in groups of six animals in standard polypropylene cages, with wood shavings litter, in a temperature (22 $\pm$ 1 °C)- and humidity (45–55%)-controlled environment with a 12/12-h light/dark cycle (light on at 07:00 a.m.). Food and water were available *ad libitum*. Six to eight animals were randomly allocated into each experimental group and all behavioral tests were carried out between 09:00 a.m. and 03:00 p.m. All procedures were approved by the Committee on the Care and Use of Experimental Animal Resources at the Federal University of Pelotas, Brazil (8331-2017) and comply with the Animal Research: Reporting in Vivo Experiments (ARRIVE) guidelines, items 5 to 13 (Kilkenny et al., 2010).

2.2. Drugs

LPS from *E. coli* (L-3129, serotype 0127:B8), ketanserin, ondansetron and WAY100635 were purchased from Sigma Chemical Co, USA. CMI (Fig. 1A) was prepared and characterized at the Laboratory of Clean Organic Synthesis at the Federal University of Pelotas (Vieira et al., 2015). All other chemicals were of analytical grade and obtained from standard commercial suppliers. LPS was diluted in saline and administered intraperitoneally (i.p.) at a dose of 0.83 mg/kg. CMI was dissolved in canola oil (a non-polar and inert substance) and administered intragastrically (i.g.) at 1 mg/kg. WAY100635 (0.1 mg/kg, subcutaneously (s.c.); a 5-HT_{1A} receptor antagonist), ketanserin (1 mg/kg, i.p.; a 5-HT_{2A/2C} receptor antagonist) and ondansetron (1 mg/kg, i.p.; a 5-HT₃ receptor antagonist) were diluted in saline (Martinez et al., 2014; Pinto Brod et al., 2016). Observers blind to the drug treatments recorded all behavioral tests and analyzed the results.

2.3. Experimental design

The experimental design is depicted in Fig. 1. In the first part of the study (Fig. 1B), we addressed the ability of CMI to reverse the depression- and anxiogenic-like behaviors induced by LPS. For that, mice were divided into four groups (n = 6 animals/group): (1) saline + canola oil; (2) saline + CMI; (3) LPS + canola oil; (4) LPS + CMI. The behavioral tests were carried out in the following order: open field test, elevated plus maze test, marble burying test, tail suspension test, and splash test. Fifteen minutes after the last behavioral test, mice were euthanized by an overdose of isoflurane inhalation followed by removal of the prefrontal cortex (PFC) and hippocampus (HC) for determination of IL-1 β , TNF- α , and IDO mRNA expression. In this sense, the brain regions were collected within 90–120 min after CMI administration. The dose and the time of CMI administration were selected based on our previous studies (Birmann et al., 2018; Casaril et al., 2017a, 2019) and preliminary data (data not shown). The administration of CMI 1, 5 and 10 mg/kg (i.g.) after LPS (0.83 mg/kg; i.p.) had similar effects in the immobility time in the tail suspension test (TST) and number of entries in the open arms in the elevated plus maze test (EPM); therefore, the dose of 1 mg/kg was selected for the present study. Additionally, 1 mg/kg of CMI reversed behavioral and biochemical alterations induced by acute restraint stress in mice (Casaril et al., 2019).

The second cohort of mice (Fig. 1C) was used to address the involvement of the serotonergic system in the antidepressant- and anxiolytic-like effects of CMI in LPS-challenged mice. Mice were separated in eight groups (n = 8 animals/each) and received WAY100635,

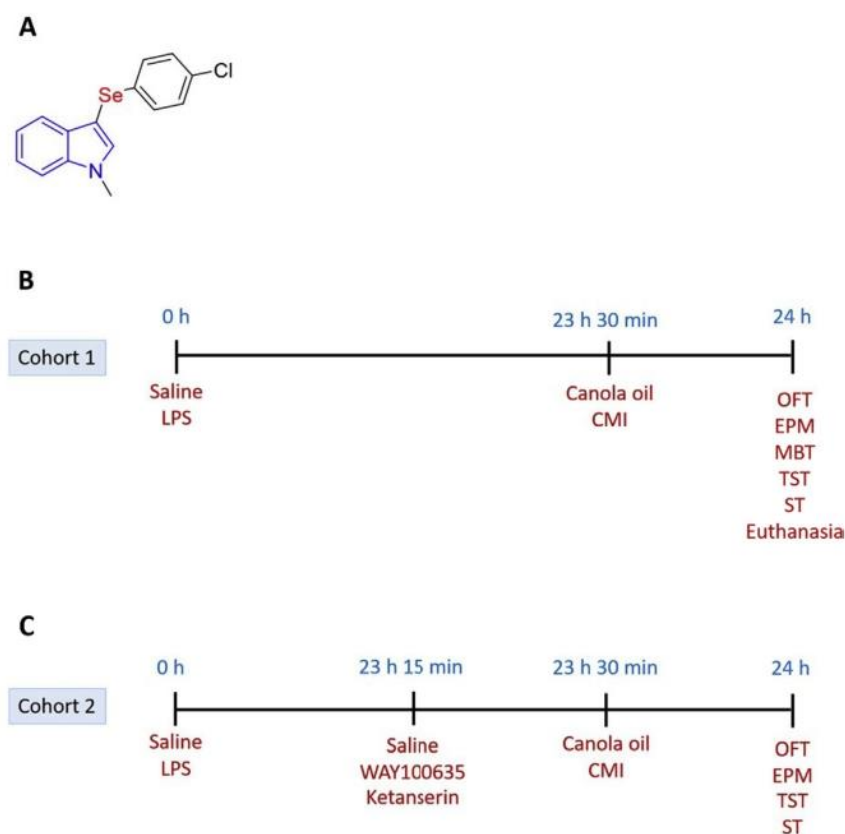


Fig. 1. (A) Chemical structure of 3-((4-chlorophenyl)selanyl)-1-methyl-1H-indole (CMI) and (B and C) experimental design performed in this study. LPS: lipopolysaccharide. OFT: open field test. TST: tail suspension test. ST: splash test. EPM: elevated plus maze. MBT: marbles burying test.

ketanserin, ondansetron, or vehicle (saline) 23 h and 15 min after LPS or vehicle (saline). After 15 min, they were treated with CMI or vehicle (canola oil), followed by behavioral analysis 30 min later (Martinez et al., 2014). This schedule was used to maintain the behavioral tests 24 h after the LPS-challenge and 30 min after CMI treatment. All mice were submitted to the same sequence of behavioral tests 30 min after CMI administration (open field test, elevated plus maze, tail suspension test, and splash test). A crossover design was not used in the present study since we do not know if a second administration of CMI would result in a different behavioral outcome, a topic that deserves further investigation. Additionally, we used four behavioral tests to characterize the involvement of the serotonergic system in the anti-depressant- and anxiolytic-like effect of CMI for the first time. In this sense, mice were submitted just once to the behavioral tests in an attempt to avoid possible interferences due to the stress imposed by the testing session.

2.4. Behavioral tests

2.4.1. Open field test (OFT)

To address the possible effects of CMI on locomotor activity, mice were evaluated in the open-field paradigm (Walsh and Cummins, 1976). Mice were individually placed in the center of a box (30 × 30 × 15 cm) with the floor divided into 9 equal squares. The number of rectangles crossed by the animals with its four paws (crossing) and rising of the front paws (rearing) was registered during a period of 5 min. The number of crossing and rearing was considered as indicative of locomotor activity and exploratory behavior, respectively.

2.4.2. Tail suspension test (TST)

The depression-like behavior was characterized by the total immobility time induced by the tail suspension test (Steru et al., 1985).

Mice were suspended 50 cm above the floor and the immobility time was recorded during a 4 min period of a 6 min session. Mice were considered immobile only when they hung passively and completely motionless.

2.4.3. Splash test

This test consists in spraying a 10% sucrose solution on the dorsal coat of the mice in their home cage (Taksande et al., 2013). The sucrose solution dirties the coat and induces grooming behavior (including nose/face grooming, head washing, and body grooming), which was observed during 5 min. The time spent grooming is an index of self-care and motivational behavior.

2.4.4. Elevated plus maze

The anxiolytic-like behavior of mice was evaluated in the elevated plus maze test. The elevated plus maze consists of a plus-shaped apparatus with two open and two enclosed arms (arm length: 100 cm; arm width: 10 cm) connected by a central platform elevated 40 cm above the floor (Boulle et al., 2014). For the test, the mouse was placed in the center of the apparatus, and the number of entries and time spent in the open arms were scored manually in a 5 min session.

2.4.5. Marble burying test

The marble burying test was also used to address the anxiolytic-like behavior of mice. For the marble burying test, eight glass marbles (ø 1 cm) were evenly spaced on regular bedding in exact copies of the home cages (Broekkamp et al., 1986). Each mouse was allocated to a marble-containing cage and allowed 30 min to explore. After returning the animals to their home cages, the marbles were counted. A marble was considered buried when 2/3 or more of its size was covered with the bedding.

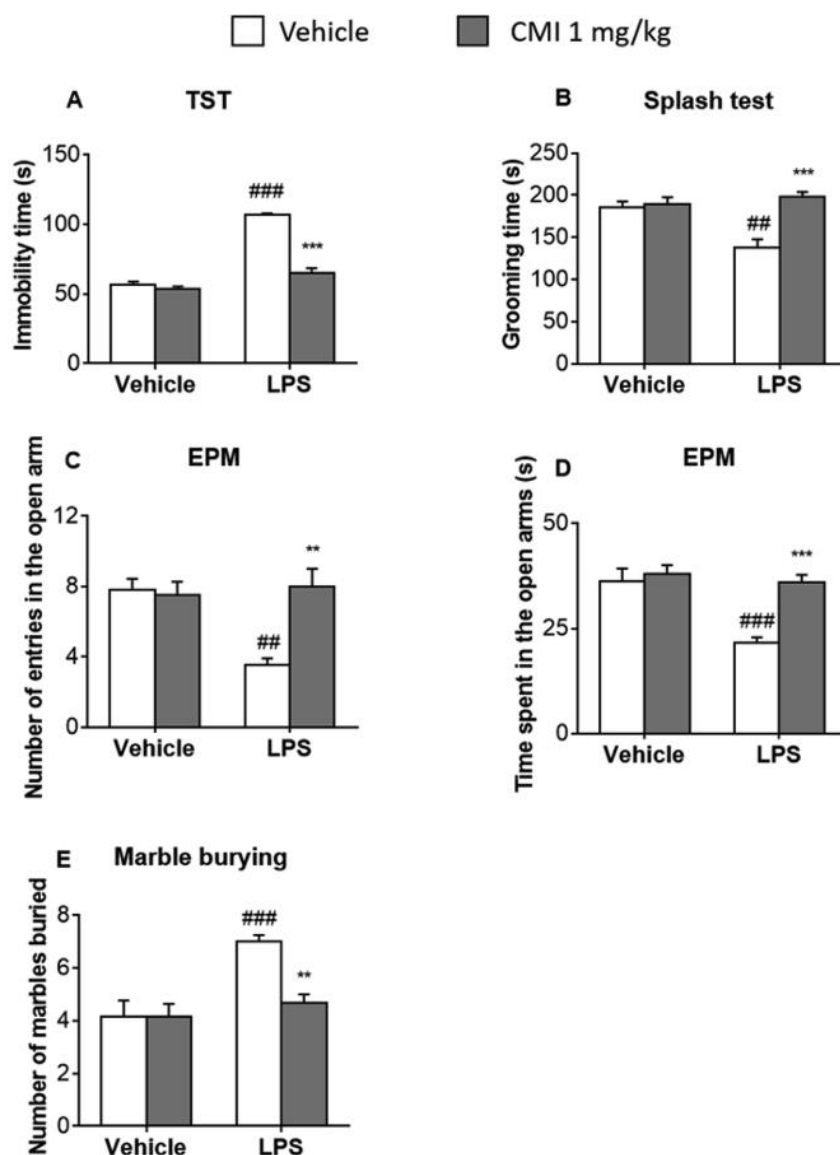


Fig. 2. Effect of CMI (1 mg/kg, i.g.) on behavioral tasks of mice challenged with LPS. (A) Immobility time in the tail suspension test, (B) grooming time in the splash test, (C) number of entries and (D) time spent in the open arms of the elevated plus maze apparatus and (E) number of marbles buried. Data are expressed as mean $\pm$ SEM of six independent animals. (##) $p < 0.01$ and (###) $p < 0.001$ when compared to control group. (**) $p < 0.01$ and (***) $p < 0.001$ when compared to LPS-treated group. LPS: lipopolysaccharide. CMI: 3-((4-chlorophenyl)selenanyl)-1-methyl-1H-indole.

2.5. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total mRNA was extracted in PFC and HC of mice as previously described (Casaril et al., 2017a; Domingues et al., 2019). The primer sequences for interleukin 1-beta (IL-1 β ; fwd 5'-GCT GAA AGC TCT CCA CCT CAA TG-3', rev 5'-TGT CGT TGC TTG GTT CTC CTT G -3'), tumor necrosis factor-alpha (TNF- α ; fwd 5'-CAT CTT CTC AAA ATT CGA GTG ACA A-3', rev 5'-TGG GAG TAG ACA AGG TAC AAC CC-3'), indoleamine-2,3-dioxygenase (IDO; fwd 5'-AAT CAA AGC AAT CCC CAC TG-3', rev 5'-AAA AAC GTG TCT GGG TCC AC-3'), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; fwd 5'-AGG TCG GTG TGA ACG GAT TTG-3', rev 5'-TGT AGA CCA TGT AGT TGA GGT CA-3') were obtained from Exxtend Biotecnologia Ltda, Campinas, Brazil. The $2^{-\Delta\Delta CT}$ (Delta-Delta Comparative Threshold) method was used to normalize the fold change in gene expressions.

2.6. Molecular docking

The type of interaction of CMI with the serotonin transporter (SERT,

PDB: 5I6X) and indoleamine 2,3-dioxygenase (IDO, PDB: 5WHR) was predicted using the software Autodock Vina 1.1.2 (Trott and Olson, 2010). The crystal structure of the proteins was retrieved from the Protein Data Bank (<http://www.pdb.org/pdb/>) and prepared by CHIMERA 1.5.3, including removal of ligands (Pettersen et al., 2004). A grid box size covering the residues in the active site of the proteins was implemented by Autodock Tools 1.5.6 (Morris et al., 2009). CMI was designed and optimized in the software Avogadro 1.1.1. (Hanwell et al., 2012). Docking poses of the investigated compound were visualized using Accelrys Discovery Studio 3.5.

2.7. Statistical analysis

All experimental results are given as a mean $\pm$ standard error of the mean (SEM). Behavioral and biochemical comparisons were performed by two-way analyses of variances (ANOVAs) (LPS vs vehicle $\times$ CMI vs vehicle). Three-way ANOVAs (LPS vs vehicle $\times$ CMI vs vehicle $\times$ serotonergic antagonist vs vehicle) were performed for measures of serotonergic antagonists influence on behavioral outcomes.

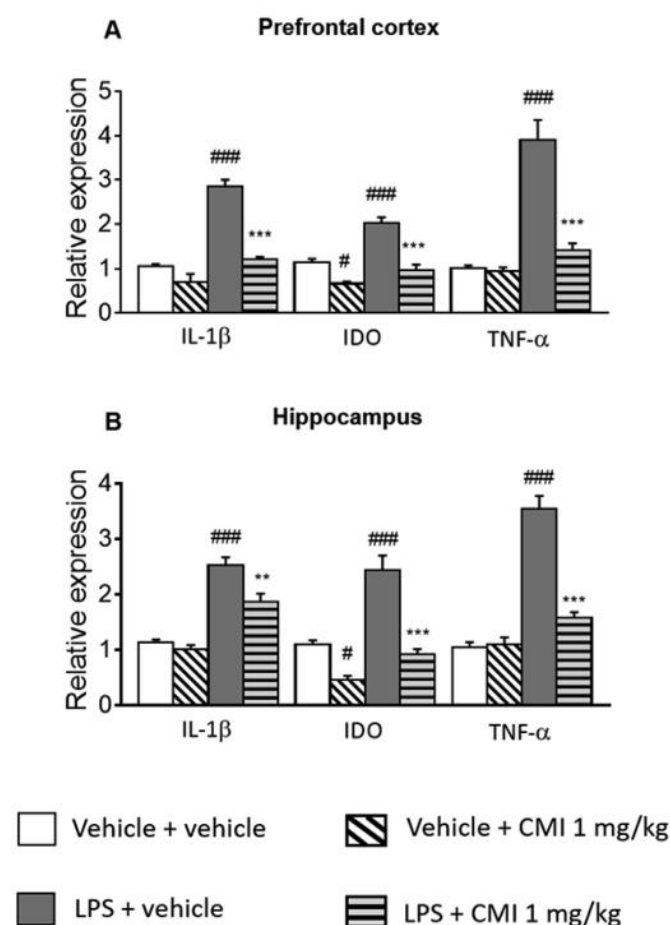


Fig. 3. Effect of CMI (1 mg/kg, i.g.) in the mRNA expression of target genes. Expression of IL- β , TNF- α and IDO in PFC (A) and HC (B) of LPS-challenged mice. Data are expressed as mean $\pm$ SEM of six independent animals. (#) $p < 0.05$ and (###) $p < 0.001$ when compared to control group. (**) $p < 0.01$ and (***) $p < 0.001$ when compared to LPS-treated group. LPS: lipopolysaccharide. CMI: 3-((4-chlorophenyl)selenyl)-1-methyl-1*H*-indole. PFC: prefrontal cortex. HC: hippocampus.

When ANOVA revealed a significant interaction, the Tukey's multiple comparison tests was employed. Pearson's correlation analysis was used to investigate any possible relationship between behavioral and neurochemical data. A value of $p < 0.05$ was considered significant. The analysis were performed using the software Graph Pad Prism version 7.0 for Windows.

3. Results

3.1. Acute administration of CMI reversed LPS-induced depression- and anxiogenic-like behavior without evoking locomotor alteration

In the present study, neither LPS challenge nor CMI treatment changed the number of crossings and rearings in the OFT (data not shown). A two-way ANOVA revealed no significant LPS $\times$ CMI interaction ($F_{(1, 20)} = 0.17$, $p = 0.68$) for the number of crossings and for the number of rearing ($F_{(1, 20)} = 0.71$, $p = 0.41$). Additionally, the administration of CMI in control mice did not alter the behavioral outcomes in the present study.

The results depicted in Fig. 2A show that CMI treatment reversed the increased immobility time induced by LPS in the TST, as compared to the LPS group (LPS $\times$ CMI interaction $F_{(1, 20)} = 61.20$, $p < 0.001$). Fig. 2B shows the effect of CMI administration after LPS challenge on grooming time in the splash test, and a statistically significant LPS $\times$ CMI interaction ($F_{(1, 20)} = 12.70$, $p = 0.002$) was found.

The effect of CMI in the anxiolytic-like behavior induced by LPS in the elevated plus maze is depicted in Fig. 2C and D. A two-way ANOVA showed a statistically significant LPS $\times$ CMI interaction for the number of entries ($F_{(1, 20)} = 11.00$, $p = 0.003$) and for the time spent in the open arms ($F_{(1, 20)} = 8.25$, $p = 0.009$). Similarly, as depicted in Fig. 2E, the treatment with CMI reversed the increased number of marbles buried (LPS $\times$ CMI interaction $F_{(1, 20)} = 7.10$, $p = 0.01$).

3.2. The reversion of LPS-induced IL-1 β , TNF- α and IDO mRNA upregulation may be responsible for the antidepressant- and anxiolytic-like effects of CMI

A two-way ANOVA of IL-1 β mRNA expression revealed a significant LPS $\times$ CMI interaction in the PFC ($F_{(1, 20)} = 23.30$, $p < 0.001$) (Fig. 3A) and HC ($F_{(1, 20)} = 5.43$, $p = 0.03$) (Fig. 3B) of mice, while the administration of CMI in control mice did not influence the IL-1 β expression in the PFC and HC. Similarly, a two-way ANOVA of TNF- α mRNA expression revealed a significant LPS $\times$ CMI interaction in the PFC ($F_{(1, 20)} = 24.50$, $p < 0.001$) (Fig. 3A) and HC ($F_{(1, 20)} = 45.90$, $p < 0.001$) (Fig. 3B) of mice and the administration of CMI in control mice did not influence the expression of TNF- α in both brain structures. A two-way ANOVA also revealed a significant LPS $\times$ CMI interaction between the mRNA levels of IDO in the PFC ($F_{(1, 20)} = 8.25$, $p = 0.009$) (Fig. 3A) and HC ($F_{(1, 20)} = 8.32$, $p = 0.009$) of mice (Fig. 3B). In this case, administration of CMI in control mice also reduced the expression of IDO in PFC ($p = 0.02$) and HC ($p = 0.03$).

3.3. Significant correlations were found between behavioral variables and mRNA expression in PFC and HC of mice

Bearing in mind the administration of CMI reversed the behavioral alterations induced by LPS and counteracted the increased mRNA expression of IL-1 β , TNF- α and IDO in the PFC and HC of mice, we

Table 1

Pearson's correlation between the behavioral variables and mRNA expression of selected genes in the PFC and HC of mice treated with CMI after challenge with LPS.

		Immobility time	Grooming time	Number of entries in the open arms	Time spent in the open arms	Number of marbles buried
IL-1 β	PFC	0.918***	-0.821**	-0.725**	-0.843***	0.821**
	HC	0.587*	-0.462	-0.488	-0.650*	0.428
TNF- α	PFC	0.813**	-0.586*	-0.573	-0.777**	0.617*
	HC	0.877***	-0.701*	-0.702*	-0.887***	0.759**
IDO	PFC	0.901***	-0.779**	-0.740**	-0.804**	0.857***
	HC	0.831***	-0.780**	-0.765**	-0.777**	0.815**

LPS: lipopolysaccharide. CMI: 3-((4-chlorophenyl)selenyl)-1-methyl-1*H*-indole. PFC: prefrontal cortex. HC: hippocampus. IL-1 β : interleukin-1beta. TNF- α : tumor necrosis factor-alpha. IDO: indoleamine 2,3-dioxygenase. (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$.

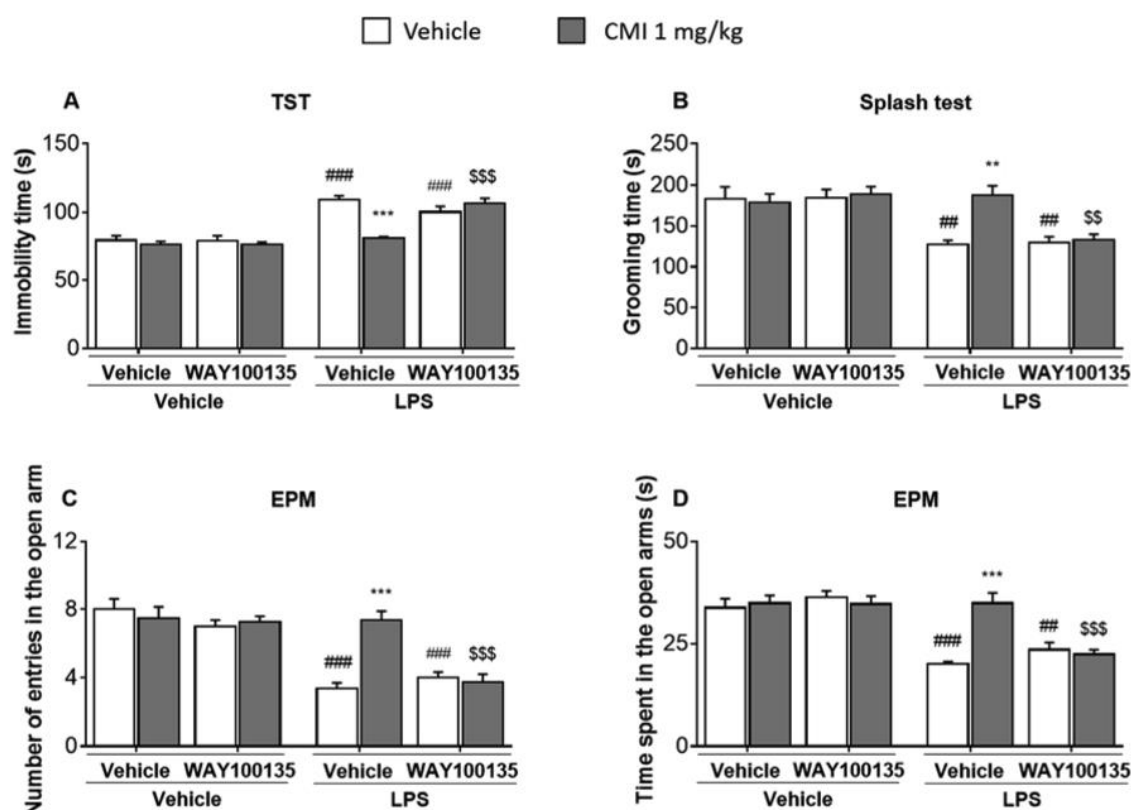


Fig. 4. Effect of WAY100635 pre-treatment on the behavioral effects of CMI in LPS-challenged mice. (A) Immobility time in the tail suspension test, (B) grooming time in the splash test, (C) number of entries and (D) time spent in the open arms of the elevated plus maze apparatus. Data are expressed as mean $\pm$ SEM of six independent animals. (##) $p < 0.01$ and (###) $p < 0.001$ when compared to control group. (**) $p < 0.01$ and (***) $p < 0.001$ when compared to LPS-treated group. (\$\$) $p < 0.01$ and (\$\$\$) $p < 0.001$ when compared to LPS-challenged mice treated with CMI. LPS: lipopolysaccharide. CMI: 3-((4-chlorophenyl)selenyl)-1-methyl-1H-indole.

analyzed if these effects were linked using Pearson's correlation analysis (Table 1). The results demonstrated a significant positive correlation between the immobility time in the TST and IL-1 β , TNF- α , and IDO expressions in PFC and HC. A significant negative correlation was found between the grooming time in the splash test and the expression of IL-1 β (in PFC), TNF- α , and IDO (in PFC and HC). Regarding the number of entries in the open arms of the EPM apparatus, a significant negative correlation was found with the expression of IL-1 β (in PFC), TNF- α (in HC), and IDO (in both PFC and HC). Similarly, we found a significant negative correlation between the time spent in the open arms of the elevated plus maze apparatus and the expression of IL-1 β , TNF- α and IDO in PFC and HC. Moreover, we found a significant positive correlation between the number of marbles buried and the expression of IL-1 β , in PFC, and TNF- α and IDO in PFC and HC. On the other hand, no significant correlation was found between the expression of IL-1 β in HC and grooming time, the number of entries and the time spent in the open arms of the elevated plus maze test and the number of marbles buried. In addition, we did not find a significant correlation between TNF- α expression in the PFC and the number of entries in the elevated plus maze test.

3.4. The antidepressant- and anxiolytic-like effects of CMI in LPS-challenged mice may be mediated by 5-HT_{1A} receptor

The involvement of the 5-HT_{1A} receptor in the antidepressant- and anxiolytic-like effects of CMI after the LPS challenge is depicted in Fig. 4. A three-way ANOVA showed a statistically significant WAY100135 $\times$ LPS $\times$ CMI interaction for the immobility time in the TST ($F_{(7, 56)} = 14.64$, $p < 0.001$) (Fig. 4A) and the grooming time in the splash test ($F_{(7, 56)} = 5.50$, $p = 0.02$) (Fig. 4B). A three-way ANOVA

also showed a statistically significant WAY100135 $\times$ LPS $\times$ CMI interaction for the number of entries ($F_{(7, 56)} = 13.90$, $p < 0.001$) (Fig. 4C) and the time spent in the open arms ($F_{(7, 56)} = 6.57$, $p = 0.01$) (Fig. 4D) of the elevated plus maze apparatus. Neither three-way ANOVA nor post-hoc comparison showed statistically significant effects of the treatments in the number of crossings and rearings in the OFT (data not shown).

3.5. The 5-HT_{2A/2C} receptor may be involved in the antidepressant- and anxiolytic-like effects of CMI in LPS-challenged mice

Fig. 5 shows the involvement of the 5-HT_{2A/2C} receptors in the antidepressant- and anxiolytic-like effects of CMI after the LPS challenge. A three-way ANOVA showed a statistically significant ketanserin $\times$ LPS $\times$ CMI interaction for the immobility time in the TST ($F_{(7, 56)} = 31.26$, $p < 0.001$) (Fig. 5A), the grooming time in the splash test ($F_{(7, 56)} = 19.56$, $p < 0.001$) (Fig. 5B) and the number of entries ($F_{(7, 56)} = 10.73$, $p = 0.002$) (Fig. 5C) and time spent ($F_{(1, 1)} = 12.86$, $p < 0.001$) (Fig. 5D) in the open arms of the elevated plus maze apparatus. None of the experimental groups promoted alterations in the number of crossings and rearings in the OFT (data not shown).

3.6. The interaction with the 5-HT₃ receptor may contribute to the antidepressant- and anxiolytic-like effects of CMI in LPS-challenged mice

Fig. 6 shows the modulation of the 5-HT₃ receptor by CMI treatment after the LPS challenge. A three-way ANOVA showed a statistically significant ondansetron $\times$ LPS $\times$ CMI interaction for the immobility time in the TST ($F_{(7, 56)} = 40.95$, $p < 0.001$; (Fig. 6A) and the grooming time in the splash test ($F_{(7, 56)} = 8.68$, $p = 0.005$) (Fig. 6B). A

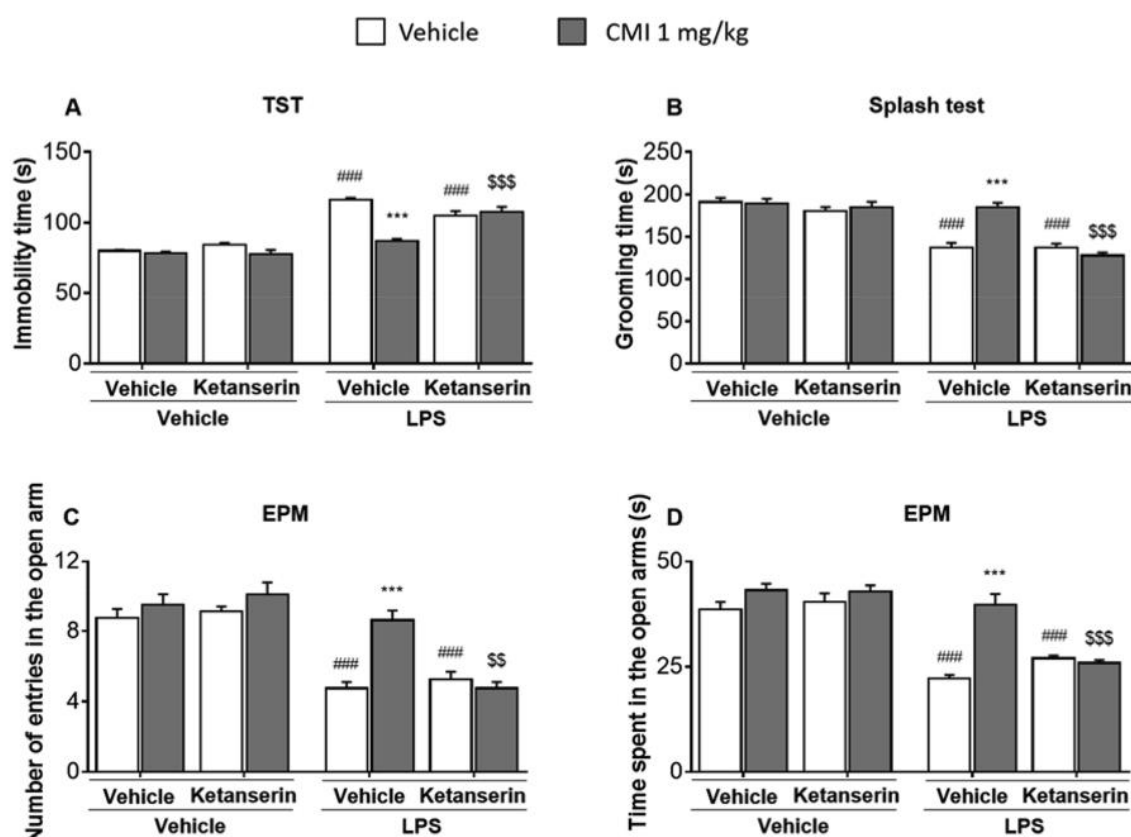


Fig. 5. Effect of ketanserin pre-treatment on the behavioral effects of CMI in LPS-challenged mice. (A) Immobility time in the tail suspension test, (B) grooming time in the splash test, (C) number of entries and (D) time spent in the open arms of the elevated plus maze apparatus. Data are expressed as mean $\pm$ SEM of six independent animals. (###) $p < 0.001$ when compared to control group. (***) $p < 0.001$ when compared to LPS-treated group. (\$\$) $p < 0.01$ and (\$\$\$\$) $p < 0.001$ when compared to LPS-challenged mice treated with CMI. LPS: lipopolysaccharide. CMI: 3-((4-chlorophenyl)selanyl)-1-methyl-1H-indole.

three-way ANOVA also showed a statistically significant ondansetron $\times$ LPS $\times$ CMI interaction for the number of entries ($F_{(1,1)} = 10.36$, $p = 0.002$) (Fig. 6C) and the time spent in the open arms ($F_{(7,56)} = 26.02$, $p < 0.001$) (Fig. 6D) of the elevated plus maze apparatus. Of note, none of the treatments evoked psychomotor alterations in OFT (data not shown).

3.7. The modulation of SERT and IDO may account for the biological effects of CMI

The best binding positions of CMI with SERT and IDO are depicted in Figs. 7 and 8, respectively. Significant interactions were formed between CMI and the residues E332 (3.59 Å), F335 (3.73 Å) and V501 (3.76 Å) in the active site of SERT (Fig. 7). As well, other residues such as R104, I172, F341, E493, T497, G498, F556 played important roles for the interaction between CMI and SERT (binding free energy (ΔG) of -7.9 kcal/mol). Fig. 8 shows the best binding mode of CMI with IDO (ΔG of -8.3 kcal/mol), which involved close interactions with the residues S167 (3.60 Å) and A264 (3.85 Å). In addition, the residues Y126, C129, P163, V166, V170, F214, I217, L234, S263, F270, R343, H346 and T379 also contributed to the interaction of CMI with the active site of IDO.

4. Discussion

In the present study, we provided the first evidence that CMI reverses depression- and anxiogenic-like effects elicited by LPS in mice, depending on the modulation of 5-HT_{1A}, 5-HT_{2A/2C}, and 5-HT₃ receptors. In addition, CMI treatment reversed LPS-induced increased expression of pro-inflammatory cytokines and IDO in the PFC and HC of

mice. The molecular docking approach showed that CMI interacts with the active site of SERT and IDO. Considering the use of multiple behavioral assays, combined with *ex vivo* and *in silico* investigation, our study provides solid evidence of the ability of CMI to counteract the depression-anxiety comorbidity in male mice.

Peripheral administration of LPS has been widely used to induce depression- and anxiogenic-like alterations in rodents, especially by inducing neuroinflammation (Domingues et al., 2018; O'Connor et al., 2009). With this in mind, we showed here that a single administration of CMI reversed the behavioral impairment induced by LPS. Additionally, we found significant correlations suggesting that the improved behavioral outcomes after CMI treatment may involve the reduction of neuroinflammation by downregulating the mRNA expression of IL-1 β , TNF- α , and IDO in PFC and HC of mice challenged with LPS. It has been acknowledged that the activation of IDO by pro-inflammatory cytokines forms a link between immune function and altered serotonergic neurotransmission, since it reduces the availability of tryptophan that could be used for the synthesis of 5-HT, therefore, contributing to the establishment of depressed mood and anxiety (O'Connor et al., 2009). In light with this, we propose here that the reversion of the neuroinflammation by CMI may be involved in its antidepressant- and anxiolytic-like effects, corroborating with previous findings by our research group (Casaril et al., 2017a, 2019).

Through a molecular docking investigation, we supported the modulation of IDO as a possible mechanism of action of CMI. We showed that CMI interacted with IDO binding site (-8.3 kcal/mol) in a manner similar to the substrate tryptophan (Lewis-Ballester et al., 2017) and to epacadostat, the most advanced IDO inhibitor in clinical trials (Yue et al., 2017). Both tryptophan and CMI interacted with the protein matrix via various hydrophobic and polar interactions, possibly

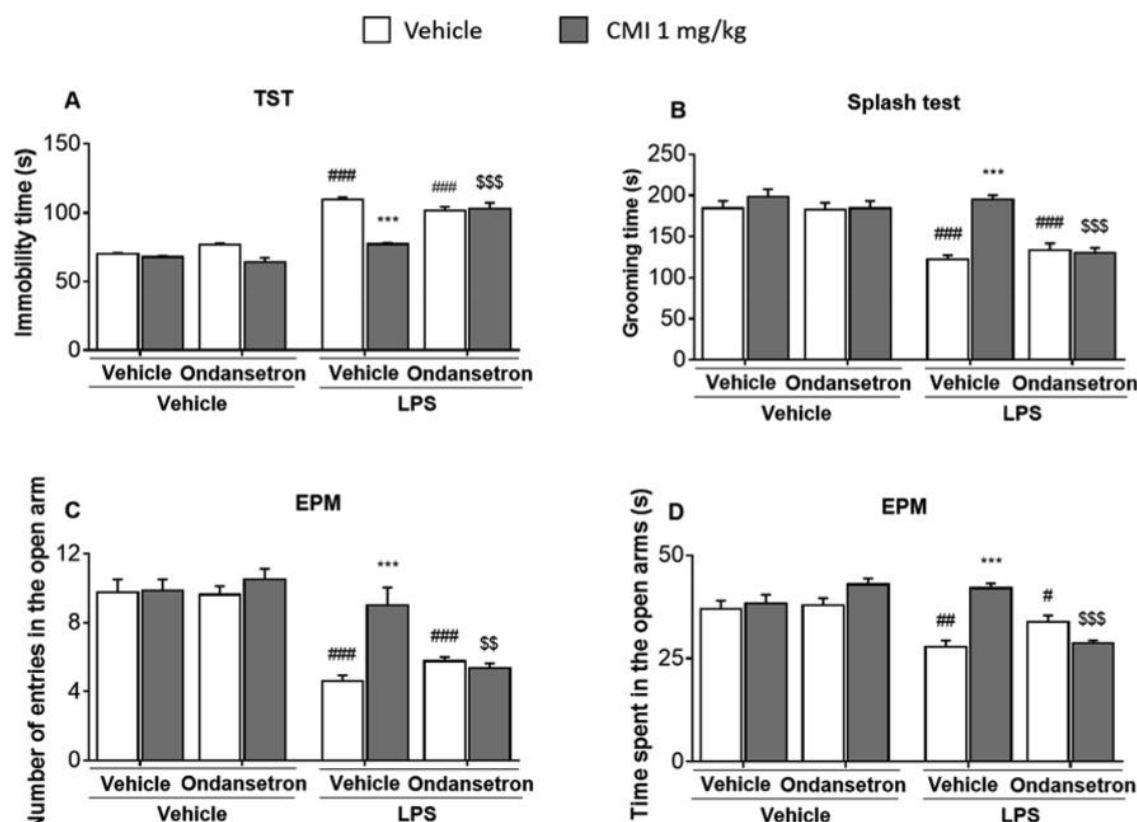


Fig. 6. Effect of ondansetron pre-treatment on the behavioral effects of CMI in LPS-challenged mice. (A) Immobility time in the tail suspension test, (B) grooming time in the splash test, (C) number of entries and (D) time spent in the open arms of the elevated plus maze apparatus. Data are expressed as mean $\pm$ SEM of six independent animals. (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$ when compared to control group. (###) $p < 0.01$ and (###) $p < 0.001$ when compared to LPS-challenged mice treated with CMI. LPS: lipopolysaccharide. CMI: 3-((4-chlorophenyl)selanyl)-1-methyl-1H-indole.

because these molecules share the structural feature of having the indole nucleus. Particularly, the indole nucleus of the tryptophan and CMI interacts with A264 and S167 in the active site of IDO, which belongs to the so-called “A-site” (Röhrig et al., 2015). Of note, the residue H346 involved in the putative binding mode of CMI is critical for the dioxygenase activity of IDO (Lewis-Ballester et al., 2009). These findings indicate that CMI is able to downregulate the IDO mRNA expression whilst it could also directly inhibit the enzyme activity and indirectly downregulate the expression of its inducing cytokines, reversing the depression- and anxiogenic-like behavior induced by LPS.

Likewise, IL-1 β and TNF- α p38 MAPK-linked pathways are implicated in the neuronal SERT expression (Zhu et al., 2010), which would reduce the availability of 5-HT in the synapse. This observation prompted us to investigate if CMI would also inhibit SERT, since MDD and anxiety are associated with dysregulation of this transporter. The molecular docking analysis revealed that CMI interacted with the central site of SERT with a ΔG of -7.9 kcal/mol. The involvement of residues I172 and F341 may characterize CMI as a high-affinity molecule since these amino acids are important for the high-affinity interaction of (S)-citalopram and paroxetine with SERT (Coleman et al., 2016), which are among the widely used SSRI for the treatment MDD and anxiety. Interestingly, the fluorophenyl group of paroxetine and the chlorophenyl group of CMI interacted with F335, T497, and V501, suggesting the importance of the halophenyl group in the development of therapeutic drugs. It is worth highlighting that the residues R104, A331, and F556, belonging to the allosteric site of SERT, participate in the binding mode of CMI. Binding to both orthosteric and allosteric binding sites of SERT leads to higher extracellular 5-HT levels in vivo and faster 5-HT_{1A} autoreceptor desensitization promoting greater

efficacy and/or a faster onset of action (Sanchez, 2006), which can support the antidepressant- and anxiolytic-like effect of CMI in LPS-challenged mice.

It is worth highlighting that SSRIs are still the first line of treatment of MDD and anxiety (Griebel and Holmes, 2013), however, improved outcomes of this treatment have been obtained with simultaneous blockage of 5-HT_{1A} autoreceptors (Artigas et al., 1994; Maes et al., 1999; Starr et al., 2007). These protein G-coupled receptors regulate the serotonergic tone and limit the SSRIs-induced initial increase in 5-HT extracellular levels (Hervás et al., 2000), which can delay the therapeutic response (Artigas et al., 1996). Of note, Ceglia et al. (2004) have shown that in addition to binding SERT, (S)-citalopram desensitizes the 5-HT_{1A} receptors, regulating the release of 5-HT in the prefrontal cortex. Here, we used a dose of WAY10635 that preferentially blocks 5-HT_{1A} autoreceptors (Ago et al., 2003) to investigate their involvement in the activity of CMI. Interestingly, it was observed that the pretreatment with WAY10635 prevented the antidepressant- and anxiolytic-like effects elicited by CMI in the TST, ST and EPM test, without evoking psychomotor alterations. Therefore, we suggest that the simultaneous interaction with SERT and 5-HT_{1A} may account for the neuropharmacological effect of CMI against the depression-anxiety comorbidity induced by LPS, which could explain the improved behavior observed 30 min after the administration of this organoselenium compound.

Similarly, the antagonism of 5-HT_{2A} and 5-HT_{2C} receptors elicits antidepressant and anxiolytic effects. For example, the antagonism of 5-HT_{2A} receptors enhances the antidepressant effect of SSRI (Celada et al., 2004). Clinical data have shown the rapid (1–2 days) antidepressant effect of risperidone, a potent 5-HT_{2A} receptor antagonist (Ostroff and

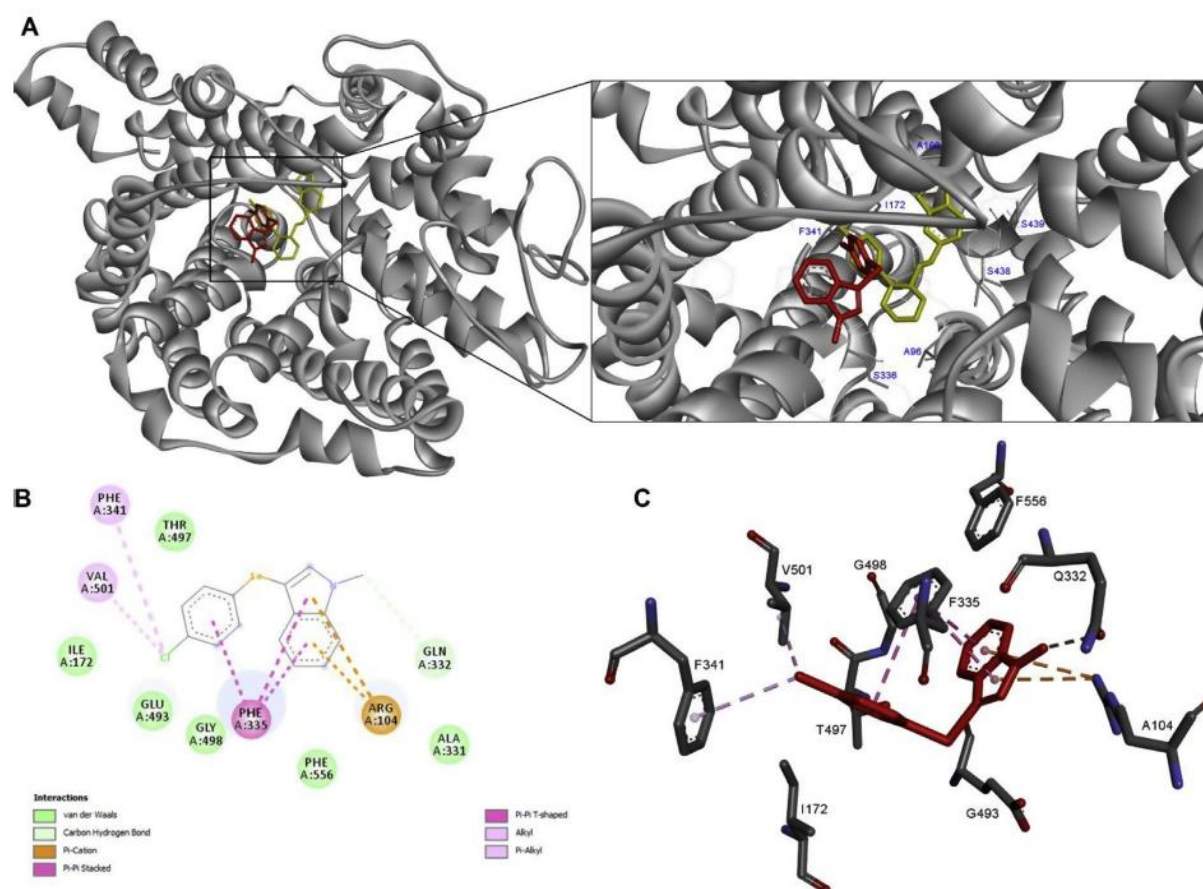


Fig. 7. Molecular docking of CMI in the binding pocket of SERT. (A) View of CMI (red) and the co-crystallized ligand, Paroxetine (yellow), bound to the X-ray crystallographic structure of SERT. (B) 2D and (C) 3D visualization of CMI interacting with amino acids residues in the active site of SERT.

Nelson, 1999). Administration of olanzapine, a 5-HT_{2A} and 5-HT_{2C} receptor antagonist, augmented the antidepressant effect of fluoxetine, with enhanced efficacy and faster onset of action (Shelton et al., 2001). Mirtazapine, which is a 5-HT₂ and 5-HT₃ antagonist, is particularly effective in anxious patients, exerting rapid and sustained effect (Alam et al., 2013). On the other hand, the activation of 5-HT_{2C} receptors induces motor impairment and sleep disturbances in patients (Millan, 2005), and the anxiogenic effects of 5-HT_{2C} receptors agonists are reduced by genetic deletion of these receptors in mice (Das and Tecott, 1996). In the present study, the pretreatment of mice with the 5-HT_{2A/2C} antagonist ketanserin blocked the antidepressant- and anxiolytic-like effects elicited by CMI in LPS-challenged mice, without evoking psycholocomotor alterations. Considering that the antagonism of 5-HT_{2A} and 5-HT_{2C} receptors has antidepressant and anxiolytic effects, we suggest that the modulation of these receptors is possibly involved in the improved behavior observed in LPS-challenged mice treated with CMI. It is possible that the modulation of the 5-HT_{2C} receptor is responsible for the anxiolytic-like effect of CMI; however, more studies are required to confirm the antagonist nature of CMI towards these receptors.

The antagonism of 5-HT₃ receptors has an antidepressant effect, as evidenced with the drugs mirtazapine (Alam et al., 2013) and vortioxetine (Mørk et al., 2013). The 5-HT₃ receptors are the only serotonergic receptors belonging to ligand-gated ion channel superfamily, and 5-HT₃ antagonists have been shown to alleviate behavioral abnormalities in pre-clinical studies (Devadoss et al., 2010; Gupta et al., 2014), also showing anxiolytic activity in clinical trials (Lecrubier et al., 1993; Smith et al., 1999). Here we reported that the 5-HT₃ receptors are also involved in the antidepressant- and anxiolytic-like effects of CMI in LPS-challenged mice, since the blockade of this receptor subtype

abolished the behavioral effects elicited by CMI. Indeed, the antagonism of 5-HT₃ receptors has been implicated in a faster onset of action of antidepressant and anxiolytic drugs (Alam et al., 2013), which in our case could account for the observed effects of CMI 30 min after its administration. Due to its nature, the activation of 5-HT₃ receptors leads to an influx of calcium (Ca²⁺) ions in the neurons. Ca²⁺ transiently stimulates neuronal nitric oxide synthase (nNOS) mediated nitric oxide (NO) synthesis (Lipton et al., 1993), and residually elevated intracellular Ca²⁺ may increase NO levels within the brain. The central nervous system has a modest antioxidant defense system (Cobley and Riorello, 2018), and both depression and anxiety are characterized by increased oxidative stress (Salim, 2014). Considering the likelihood of CMI in antagonizing 5-HT₃ receptors (therefore decreasing the influx of Ca²⁺), it is possible to speculate that CMI also reduces oxidative stress in the brain, which contributes to the antidepressant- and anxiolytic-like effects following its administration. Our previous works support this hypothesis, since the same dose of CMI used in the present study also reduces the levels of reactive species, lipid peroxidation and nitric oxide in the prefrontal cortex and hippocampus of mice challenged with LPS (Casaril et al., 2017a) and submitted to an acute restraint stress protocol (Casaril et al., 2019).

In conclusion, we used behavioral, biochemical and computational analysis to suggest the involvement of the serotonergic system in the antidepressant- and anxiolytic-like effects of CMI. Our data indicate that the effect of CMI in LPS-challenged mice may rely on its ability to modulate the 5-HT_{1A}, 5-HT_{2A/2C}, and 5-HT₃ receptors. Additionally, the behavioral effects of CMI may be dependent on the downregulation of IL-1 β , TNF- α and IDO expression in the PFC and HC, alongside with the inhibition of IDO and SERT activities. It is important to mention that the dose of antagonists used here did not evoke behavioral alterations

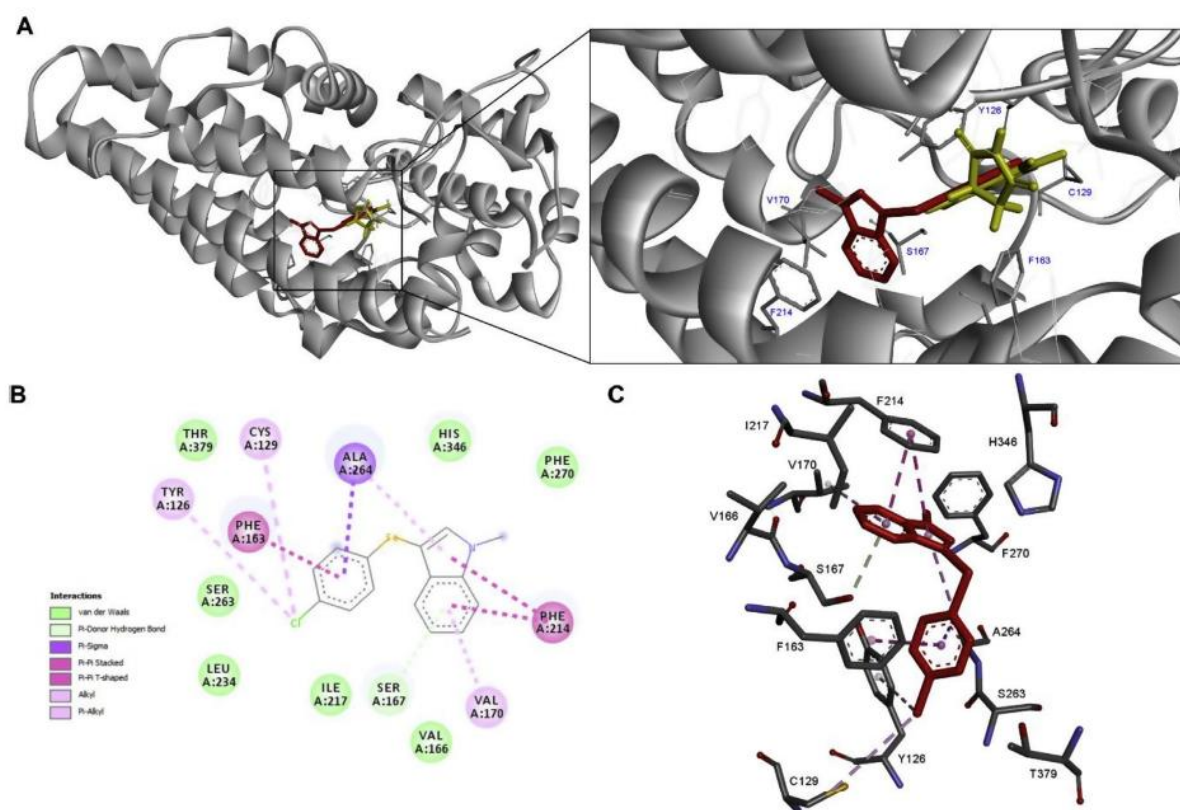


Fig. 8. Molecular docking of CMI in the binding pocket of IDO. (A) View of CMI (red) and the co-crystallized ligand, PF-06840003 (yellow), bound to the X-ray crystallographic structure of IDO. (B) 2D and (C) 3D visualization of CMI interacting with amino acids residues in the active site of IDO.

in mice, which allowed us to study the CMI effects. The simultaneous interaction of CMI with different pharmacological targets in the serotonergic system might have greater therapeutic efficacy than modulating each target alone. Therefore, the pharmacological activity of CMI may be useful in tailoring treatments for the depression-anxiety comorbidity. However, the present study has some limitations and, therefore, (i) the radioligand-binding analysis, (ii) the determination of extracellular 5-HT concentration, (iii) the investigation of the long-lasting effects of CMI, and (iv) the evaluation of the antidepressant-like effect of CMI in female mice should be considered for future research.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jpsychires.2019.05.006>.

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4.4 Capítulo 4 – The antioxidant and immunomodulatory compound 3-[(4-chlorophenyl)selenyl]-1-methyl-1*H*-indole attenuates depression-like behavior and cognitive impairment developed in a mouse model of breast tumor

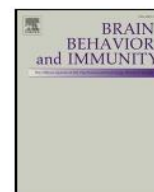
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Nos capítulos anteriores, investigamos a capacidade antioxidante *in vitro* e o efeito tipo-antidepressivo do CMI em modelos agudos de indução do comportamento tipo-depressivo. Neste trabalho, impulsionados pela prevalência de sintomas depressivos em aproximadamente 20 – 30% das mulheres com câncer da mama, investigamos a capacidade do CMI em melhorar o comportamento tipo-depressivo e a cognição de camundongos fêmeas portadoras de tumor mamário. Observamos que, quando administrado durante 7 dias, o CMI (i) reverteu o comportamento tipo-depressivo e o prejuízo cognitivo em camundongos portadores de tumor; (ii) modulou a atividade da enzima SOD, os níveis de ERO, NO[•] e peroxidação lipídica nos córtex pré-frontais e hipocampus de camundongos portadores de tumor e; (iii) modulou a expressão do *NFκB*, *IL-1β*, *TNF-α*, *IL-10*, *IDO*, *COX-2*, *iNOS*, *Nrf2* e *BDNF* nos córtex pré-frontais e hipocampus de camundongos portadores de tumor. Adicionalmente, estudos *in silico* demonstraram o potencial do CMI em interagir com o sitio ativo das enzimas iNOS e COX-2, contribuindo com os efeitos observados. Em conjunto, esses dados demonstram o potencial farmacológico do CMI em um modelo de depressão que se manifesta em decorrência de uma condição crônica, nesse caso, o câncer de mama. Uma vez que o CMI não influenciou o crescimento tumoral, ele não deve ser tratado como um agente quimioterápico, mas sim como um adjuvante terapêutico para melhorar os sintomas depressivos e cognitivos. O uso de adjuvantes a quimioterapia tem sido amplamente reconhecido como um importante componente do tratamento multimodal, e a descoberta de novas drogas para tratar a depressão e o declínio cognitivo induzidos por câncer seriam um notável avanço para a melhora na qualidade de vida de pacientes com câncer.



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The antioxidant and immunomodulatory compound 3-[(4-chlorophenyl)selenanyl]-1-methyl-1H-indole attenuates depression-like behavior and cognitive impairment developed in a mouse model of breast tumor

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ABSTRACT

Psychiatric alterations are often found in patients with breast cancer even before the initiation of adjuvant therapy, resulting in a poor quality of life. It has become accepted that neuroinflammation and oxidative stress are involved in the pathophysiology of depression and cognitive impairment. Herein, we tested the hypothesis that treatment with the antioxidant and immunomodulatory selenium-containing compound 3-[(4-chlorophenyl)selenanyl]-1-methyl-1H-indole (CMI) could attenuate behavioral and neurochemical alterations in a mammary (4T1) tumor model. Female BALB/c mice were subcutaneously inoculated with 4T1 cancer cells (1×10^5 cells/mice) or PBS. From days 14 to 20, mice received daily gavage with canola oil or CMI. On day 21, mice were submitted to behavioral tests followed by euthanasia. We found that CMI did not alter tumor growth, body weight, and body temperature in tumor-bearing mice. Importantly, treatment with CMI abrogated tumor-induced depression-like behavior and cognitive impairment. By the time CMI improved the behavioral alterations, it had reduced tumor-induced neuroinflammation (altered expression of *NFκB*, *IL-1β*, *TNF-α*, *IL-10*, *IDO*, and *COX-2*) and oxidative stress (altered expression of *iNOS* and *Nrf2*, and levels of reactive species, nitric oxide, lipid peroxidation, and superoxide dismutase activity) in the prefrontal cortices and hippocampi of mice. A molecular docking approach suggested the ability of CMI to inhibit the activity of *iNOS* and *COX-2*. Together, our results indicate that CMI treatment may attenuate depression and cognitive impairment in 4T1 tumor-bearing mice, and be a groundbreaking strategy for the treatment of cancer-related psychiatric symptoms to improve the quality of life of cancer patients.

1. Introduction

Breast cancer is the most frequent cancer in women and one of the three most common malignancies worldwide, along with lung and colon cancer (Harbeck and Gnant, 2016). Although the mortality from breast cancer has decreased in developed countries (Harbeck and Gnant, 2016), the disease and the treatment distress result in psychiatric comorbidity and behavioral symptoms, such as disturbances in sleep, energy, mood, and cognition (Bower, 2008; Schwarz et al., 2008). In particular, approximately 20–30% of women experience depressive symptoms during the course of breast cancer (Bower, 2008),

and cognitive impairment has been reported in women with breast carcinoma before the initiation of adjuvant therapy (Cimprich et al., 2010; Wefel et al., 2004). Unfortunately, these behavioral alterations found in cancer patients are often under-reported and undertreated (Bower, 2008).

Cancer cells release pro-inflammatory cytokines that signal through cognate cytokine receptors (Weinberg, 2007). Damage-associated molecular patterns (DAMPs) are also released by cancer cells, stimulating the immune system through a broad family of receptors, including the Toll-like receptors (TLRs) (Krysko et al., 2013). The brain monitors peripheral immune responses through the activation of several

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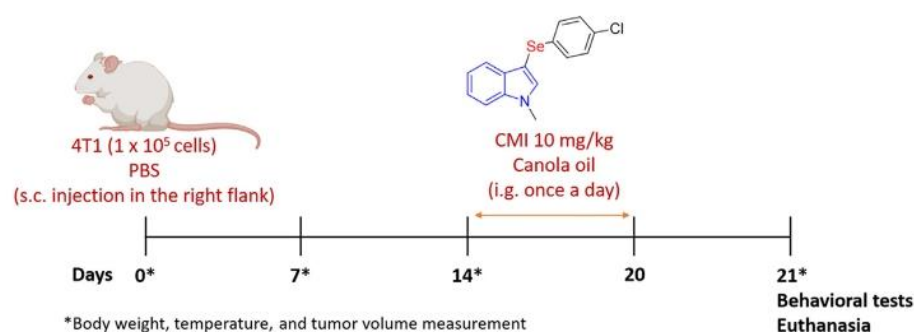


Fig. 1. Representation of the experimental protocol. On day 0, mice received a subcutaneous injection of 1×10^5 4T1 tumor cells or PBS in the right flank. On day 7, the tumors became palpable. From day 14 until day 20, mice were treated once a day with CMI 10 mg/kg or canola oil via oral gavage. Twenty-four hours after the last administration of CMI (day 21), mice were submitted to the behavioral tests followed by euthanasia. Body weight, temperature, and tumor measurement were performed on days 0, 7, 14 and 21.

communication pathways that ultimately lead to the production of pro-inflammatory cytokines by microglia (Dantzer et al., 2010). Importantly, the activation of these immune-to-brain communication pathways is associated with the presence of sickness behavior (Dantzer et al., 2010) and depressive symptoms (Felger and Lotrich, 2013; Miller and Raison, 2016).

One of the consequences of cytokine signaling and TLR activation is increased oxidative stress in the brain through the activation of nuclear factor kappa-light-chain-enhancers of activated B cells (NFκB) (Madrigal et al., 2002). Activated NFκB induce the expression of inflammatory cytokines, reactive species (RS)-producing enzymes, and pro-inflammatory enzymes, such as inducible nitric oxide (NO[•]) synthase (iNOS) and cyclooxygenase-2 (COX-2), respectively. The iNOS activation leads to production of NO[•], which can react with radical anion superoxide to produce the unstable radical peroxynitrite (Beckman and Koppenol, 1996). Prostaglandins (PG) formed by COX-2 reduce the activity of brain-derived neurotrophic factor (BDNF) (Hein and O'Banion, 2009; Rage et al., 2006). BDNF plays an important role in the activation of nuclear factor erythroid 2 (NFE2)-related factor 2 (Nrf2) (Ishii et al., 2018). The suppression of the Nrf2-antioxidant responsive element (ARE) pathway impairs redox homeostasis by arresting the expression of antioxidant genes, such as catalase (CAT) and superoxide dismutase (SOD) (Kensler et al., 2007). In addition, inflammatory cytokines are required for the induction of indoleamine-2,3-dioxygenase (IDO) (Von Bergwelt-Baildon et al., 2006), a critical molecular mediator of inflammation-induced depression (O'Connor et al., 2009). The overproduction of RS and/or the impaired antioxidant defense in the brain can lead to oxidative stress and damage to lipids, proteins, and nucleic acids, contributing to the establishment of depression (Maes et al., 2011).

Although immune-to-brain communication has been increasingly explored in the context of cancer, there is not enough investigation regarding possible therapies for the psychiatric symptoms that arise during tumor progression. Therefore, bearing in mind the prevalence of psychiatric symptoms in cancer patients and the role of pro-inflammatory signaling and oxidative stress in depression, we hypothesized that the compound 3-[(4-chlorophenyl)selenyl]-1-methyl-1H-indole (CMI) would be able to reverse depression-like behavior and cognitive impairment in a heterotopic mouse model of mammary carcinoma.

CMI is an antioxidant selenium-containing compound (Vieira et al., 2017) that modulates central oxidative stress and inflammation to reverse depression- and anxiolytic-like behaviors induced by lipopolysaccharide (Casaril et al., 2017a, 2019b) and acute restraint stress (Casaril et al., 2019a) in mice. In addition, CMI potentially inhibits inflammation-associated oxidants, such as hypochlorous acid (HOCl), peroxynitrite, and hydrogen peroxide (H₂O₂) (Casaril et al., 2017b), highlighting its antioxidant and immunomodulatory profile.

In light of this, we performed a series of experiments to examine whether CMI would ameliorate depression-like behavior, cognitive impairment, neuroinflammation, and oxidative stress that arise in a mouse model of mammary carcinoma. Studying the antidepressant-like

effect of CMI in tumor-bearing mice provides clues for a novel adjuvant therapeutic strategy targeting the antioxidant system and neuroinflammation for the management of cancer-related psychiatric symptoms.

2. Materials and methods

2.1. Animals

Female BALB/c mice (5–6 weeks old) were obtained from the Central Animal Facility of the Federal University of Pelotas (Rio Grande do Sul, Brazil). The animals were housed in cages with free access to food and water in a room maintained at 25 ± 1 °C with a relative humidity of $50 \pm 5\%$. They were kept on a 12 h light/12 h dark cycle (the lights were turned on every day at 7:00 a.m.). The Committee on the Care and Use of Experimental Animal Resources from the Federal University of Pelotas approved the protocols used in the present study (CEEA 8373), and the animals were cared for in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011). Mice were randomly assigned to each experimental group ($n = 6$ –8 mice/group).

2.2. Drugs

CMI (Fig. 1) was prepared and characterized at the Laboratory of Clean Organic Synthesis at the Federal University of Pelotas (Vieira et al., 2015). CMI was dissolved in canola oil (a non-polar and inert substance) and administered intragastrically (i.g.) at a dose of 10 mg/kg and at a constant volume of 10 mL/kg body weight, as has been previously reported by our group (Casaril et al., 2017a).

The RNA extraction reagent, DNA-freeTM kit, TRIzolTM, and High Capacity cDNA Reverse Transcription kit were obtained from Life Technologies (Carlsbad, USA). The UltraSYBR Mix was obtained from COWIN Bioscience Co. (Beijing, China). The oligonucleotides were synthesized by Exxtend Biotecnologia, Ltda (Campinas, Brazil). All other chemicals were of analytical grade and were obtained from Servylab and WF Científica (Brazil).

2.3. Tumor cell line

Murine 4T1 mammary carcinoma cells are derived from a spontaneously arising mammary tumor syngenic to BALB/c mice (Pulaski and Ostrand-Rosenberg, 2001). The cells were obtained from Rio de Janeiro Cell Bank (PABCAM, Federal University of Rio de Janeiro, Brazil). The carcinoma cells were cultured in RPMI-1640 medium modified to contain 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4500 mg/L glucose, and 1500 mg/L sodium bicarbonate as well as fetal bovine serum at a final concentration of 10% at 37 °C and 5% CO₂ in a humidified incubator. The cells were re-suspended in PBS for injection.

2.4. Experimental protocol

Mice were injected with 50 μ L of tumor cell suspension (1×10^5 cells/mice) into the right flank subcutaneous space (Pulaski and Ostrand-Rosenberg, 2001). Control mice were injected with the same volume of PBS. This tumor site was selected instead of the orthotopic site to avoid movement constraints that could interfere with the behavioral evaluations (Nashed et al., 2015). The day tumors were injected was considered to be day 0. Once tumors became palpable on day 7, tumor size was monitored weekly using a digital caliper, and the body weight and body temperature (measured with a self-calibrating thermometer that measures the infrared heat generated by the eardrum and surrounding tissues) were recorded at the same time. The overall health status was initially monitored every 3 days, and then daily during CMI administration. Treatment with CMI (10 mg/kg, i.g.) or canola oil was initiated at day 14 and continued until day 20. Twenty-four hours after the last administration of CMI or canola oil, mice were submitted to the behavioral tests followed by euthanasia. During necropsy, a gross visual examination of the brain, liver, lungs, heart, and kidneys was conducted for the identification of macroscopic metastases. A diagram of the experimental design is shown in Fig. 1.

In a pilot experiment, we treated 4T1 tumor-bearing mice over 7 days (from days 14 to 20 after tumor implantation) with CMI at 1 or 10 mg/kg, and we observed that only 10 mg/kg of CMI reversed the tumor-induced depression-like behavior on day 21 (data not shown). The doses selected for the pilot test elicited antidepressant-like effects in acute animal models of depression (Casaril et al., 2019a,b). The schedule of CMI treatment (7 days) was selected based on a previous study conducted by our group, in which we demonstrated that a 7-day treatment with an organoselenium compound from the same chemical class as CMI exerted antidepressant-like effects on a chronic mice model of depression (Bampi et al., 2019).

2.5. Behavioral tests

2.5.1. Open-field test (OFT)

To assess the possible effects of tumor and CMI treatment on locomotor activity, mice were evaluated in the open-field paradigm (Walsh and Cummins, 1976). Mice were individually placed in an open wooden box (30 $\times$ 30 $\times$ 15 cm) with the floor divided into 9 quadrants of equal area and observed for 5 min. The number of quadrants crossed by the animal with its four paws (crossings) was indicative of locomotor activity, and the number of rearings indicated exploratory behavior.

2.5.2. Tail suspension test (TST)

The TST was performed according to the method described by Steru et al. (1985). Mice were suspended 50 cm above the floor by adhesive tape placed approximately 1 cm from the tip of the tail. During the first 2 min of habituation, the latency time to immobility was recorded (i.e., time for the first immobility episode), and during the last 4 min, the immobility time (defined as the absence of the escape attempt behavior) was observed.

2.5.3. Forced swimming test (FST)

The FST was performed according to the method described by Porsolt et al. (1978). Mice were placed in an open cylindrical container (diameter 10 cm, height 25 cm), containing 19 cm of water (depth) at 25 ± 1 °C. During the first 2 min of habituation, the latency time to immobility was recorded (i.e., time for the first immobility episode), and during the last 4 min, the immobility time (defined as the absence of the escape attempt behavior) was observed. Each mouse was judged to be immobile when it ceased struggling and remained floating motionless in the water, making only those movements necessary to keep its head above water.

2.5.4. Splash test

The grooming behavior of mice was observed as a measurement of motivational and self-care activities, which can be impaired in depressive patients. A 10% sucrose solution was sprayed on the dorsal coat of each mouse, and the grooming activity (nose/face/body grooming) was recorded for 5 min (Freitas et al., 2013).

2.5.5. Novel object recognition (NOR) test

The NOR test was performed in the same open field apparatus, as previously described (Rosa et al., 2003). Plastic objects with different shapes and different primary colors that were the same size were used. The apparatus and the objects were cleaned after each animal testing with 70% ethanol solution. The animals were randomly selected to perform the behavioral task and the studies were conducted in a blinded manner. The task consisted of habituation (10 min), training (10 min), and testing sessions (5 min). In the first session (habituation), mice were allowed to freely explore the arena without any object, and then returned to their home cage. After 24 h, the training session took place, and animals were exposed to two equal objects (object A). Exploration time corresponded to each animal's nose either touching or getting close to the object (< 2 cm) was recorded with two stop watches (climbing or sitting on the object was not considered exploration). The test session was carried out 24 h after the training session in order to assess long-term memory. Mice were placed back in the behavioral chamber and one of the familiar objects (object A) was replaced by a novel object (object B). The time spent exploring the familiar and the novel objects was recorded. The discrimination index was calculated following the formula: $Object\ recognition\ (index) = \left(\frac{T_{novel} - T_{familiar}}{T_{novel} + T_{familiar}} \right) \times 100$.

2.5.6. Y-maze

Short-term working memory was assessed by recording spontaneous alternation behavior in a Y-maze (Sarter et al., 1988). The Y-maze is a three-arm horizontal maze (40 cm long and 3 cm wide, with walls 12 cm high) in which the three arms are symmetrically separated at 120°. Each mouse was placed at the distal end of one arm and allowed to explore the apparatus during an 8 min session. The sequence of arm entries was recorded manually. Alternation was defined as successive entries into the three arms on overlapping triplet sets. The percentage of spontaneous alternation behavior was calculated according to the following equation: $(\%)\ alternation = \frac{(number\ of\ alternations)}{(total\ arm\ entries - 2)} \times 100$.

2.6. Biochemical determinations

2.6.1. Sample collection

After the behavioral tests, mice were euthanized by an overdose of isoflurane inhalation followed by cervical dislocation. The brains were dissected, and the whole prefrontal cortex and hippocampus were collected and kept at -80 °C until the analysis of gene expression. For the biochemical assays, the brain samples were homogenized in 50 mM Tris-HCl, pH 7.4 (1:10, w/v), and the homogenate was centrifuged at 2500 g for 10 min at 4 °C. The supernatant fraction was used to assess the levels of reactive species (RS), NO[•] metabolites (NO_x), lipid peroxidation, and the total content of sulphhydryl groups as well as SOD and CAT activities.

2.6.2. Gene expression

Total mRNA was extracted from the hippocampus and the prefrontal cortex using TRIzol followed by DNase treatment with the DNA-free[®] kit and mRNA quantification. The cDNA synthesis was performed using a High Capacity cDNA Reverse Transcription kit according to the manufacturer's protocol. The amplification was made with the UltraSYBR Mix using the Stratagene Mx3005P. Gene expressions were normalized using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a reference gene, and the conditions for the reaction involved 95 °C

for 15 s, 60 °C for 60 s, and 72 °C for 30 s. The $2^{\Delta\Delta CT}$ (delta-delta comparative threshold) method was used to normalize the fold change in gene expressions. The following genes were analyzed: *NfκB* (fwd 5'-GCT TTC GCA GGA GCA TTA AC-3', rev 5'-CCG AAG CAG GAG CTA TCA AC-3'), interleukin 1 beta (*IL-1β*; fwd 5'-GCT GAA AGC TCT CCA CCT CAA TG-3', rev 5'-TGT CGT TGC TTG GTT CTC CTT G-3'), tumor necrosis factor alpha (*TNF-α*; fwd 5'-CAT CTT CTC AAA ATT CGA GTG ACA A-3', rev 5'-TGG GAG TAG ACA AGG TAC AAC CC-3'), interleukin 10 (*IL-10*; fwd 5'-CCA AGC CTT ATC GGA AAT G-3', rev 5'-CCT GAG GGT CTT CAG C-3'), *IDO* (fwd 5'-AAT CAA AGC AAT CCC CAC TG-3', rev 5'-AAA AAC GTG TCT GGG TCC AC-3'), *COX-2* (fwd 5'-CAG ACA TAA ACT GCG CCT T-3', rev 5'-GAT ACA CCT CTC CAC CAA TGA CC-3'), *iNOS* (fwd 5'-GTG GTG ACA AGC ACA TTT GG-3', rev 5'-AAG GCC AAA CAC AGC ATA CC-3'), *Nrf2* (fwd 5'-GTC TTC ACT GCC CCT CAT C-3', rev 5'-TCG GGA ATG GAA AAT AGC TCC-3'), *BDNF* (fwd 5'-CCA TAA GGA CGC GGA CTT GTA C-3', rev 5'-AGA CAT GTT TGC GGC ATC CAG G-3'), and *GAPDH* (fwd 5'-AGG TCG GTG TGA ACG GAT TTG-3', rev 5'-TGT AGA CCA TGT AGT TGA GGT CA-3').

2.6.3. Reactive species levels

The levels of RS were determined according to Loetchutin et al. (2005). Aliquots of the supernatant were incubated with 1 mM dichloro-dihydro-fluorescein diacetate (DCFH-DA) and 10 mM Tris-HCl at pH 7.4. The oxidation of DCFH-DA to the fluorescent form dichloro-fluorescein (DCF) was measured for the detection of intracellular RS. The DCF fluorescence was recorded at 520 nm emission and 480 nm excitation. The RS levels were expressed as fluorescence units.

2.6.4. Nitric oxide levels

Aliquots of the supernatant were incubated with equal volume of Griess reagent for 5 min at 25 °C, and the nitrite concentration was determined by measuring the absorbance at 550 nm (Lima-Junior et al., 2013). A standard curve of sodium nitrite was used. Results were expressed as micromoles of NO_x per gram of tissue (μmol NO_x/g tissue).

2.6.5. Lipid peroxidation

Lipid peroxidation was measured by the formation of thiobarbituric acid reactive species (TBARS) (Ohkawa et al., 1979). Aliquots of the supernatant were incubated with 8.1% SDS, 0.8% TBA, and acetic acid/HCl (pH 3.4) at 95 °C for 2 h. Malondialdehyde (MDA) was used as a biomarker of lipid peroxidation. Absorbance was measured at 532 nm, and the results were expressed as nanomoles of MDA per gram of tissue (nmol MDA/g tissue).

2.6.6. Total content of sulfhydryl groups

The concentration of sulfhydryl groups (SH groups) was determined using 0.2 mmol/L 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) (Ellman, 1959). Total sulfhydryl group content values were expressed as micromoles of SH groups per gram of tissue (μmol SH/g of tissue).

2.6.7. Superoxide dismutase (SOD) activity

The activity of SOD was measured as a reflection of the capacity for SOD to inhibit the autooxidation of adrenaline to adrenochrome in tissue (Misra and Fridovich, 1972). The color reaction was measured spectrophotometrically at 480 nm and the enzymatic activity was expressed as units of SOD per milligram of protein (U SOD/mg prot).

2.6.8. Catalase (CAT) activity

The CAT activity was measured spectrophotometrically by monitoring the disappearance of the substrate H₂O₂ in the presence of tissue at 240 nm (Aebi, 1984). The enzymatic activity was expressed as units of CAT per milligram of protein (U CAT/mg prot).

2.6.9. Protein determination

Protein concentration was measured according to the method of Lowry et al. (1951) using serum bovine albumin as standard.

2.7. Molecular docking

The possible interaction of CMI with the enzymes iNOS (PDB: 3E7G) and COX-2 (PDB: 5KIR) was predicted by the software Autodock Vina 1.1.2 (Trott and Olson, 2010). The crystal structure of the proteins was retrieved from the Protein Data Bank (<http://www.pdb.org/pdb/>) and optimized with CHIMERA 1.5.3. The grid box size covering the residues in the active sites of proteins was implemented by Autodock Tools 1.5.6. CMI was designed and optimized in the software Avogadro 1.1.1. The docking poses of CMI with the active site of the enzymes were visualized using Accelrys Discovery Studio 3.5.

2.8. Statistical analysis

All experimental data are presented as mean ± standard error of the mean (SEM). A two (± 4T1) by two (± CMI) analysis of variance (ANOVA) was used for the analysis of behavioral and neurochemical experiments. When an ANOVA revealed significant interactions, Tukey's *post hoc* test was used for between-group comparisons. A two-way ANOVA with time as a repeated measure was used for the analysis of tumor burden in 4T1 tumor-bearing mice with and without CMI treatment and for the analysis of body weight and body temperature between the four experimental groups. All the aforementioned analysis were performed using GraphPad Prism version 7.0 (CA, USA). Additionally, the tumor burden was a covariate in the analysis of body weight variation over time using a one-way analysis of covariance (ANCOVA) with IBM SPSS software version 20 (NY, USA). Probability values < 0.05 (*p* < 0.05) were considered statistically significant for all the analyses.

3. Results

3.1. Effects of CMI on body weight, body temperature, and tumor growth in 4T1 tumor-bearing mice

To assess the effects of CMI treatment on depression-like behavior and cognitive impairment in 4T1 tumor-bearing mice, we initially assessed body weights, tumor growth, and body temperatures of mice. The body weights (Fig. 2A) and body temperatures (Fig. 2B) of tumor-bearing mice with or without CMI treatment did not differ from control mice throughout the experiment. Additionally, the tumor burden was a covariant in the analysis of body weight variation over time, and we confirmed that 4T1 tumor-bearing mice did not develop cachexia by day 21 (*p* = 0.091). CMI treatment to non-tumor-bearing mice did not alter body weight and temperature.

As expected, we observed that tumors arose in all animals that received 4T1 tumor cell injections in the right flank, and no tumor was found in animals without the injection of 4T1 cells (Fig. 2C). The tumors were initially measured on day 7 (tumor average size: 104.15 mm³) and the tumor growth was consistent among the animals until day 21. CMI treatment started on day 14 (4T1 group tumor average size: 95.30 mm³; 4T1 + CMI group tumor average size: 113 mm³), but it did not affect the tumor growth up to day 21 (4T1 group tumor average size: 279.00 mm³; 4T1 + CMI group tumor average size: 314 mm³). A two-way ANOVA (with time as a repeated measure) comparing the tumor burden of 4T1 tumor-bearing mice with or without CMI treatment revealed no significant interaction (*F*_(3,42) = 0.788, *p* = 0.51), indicating that treatment with CMI did not influence tumor growth throughout the experiment. No mortality was observed during the study. No macroscopic metastasis manifestations were found by gross visual examinations of the brains, livers, lungs, hearts, and kidneys of tumor-bearing mice with and without CMI treatment.

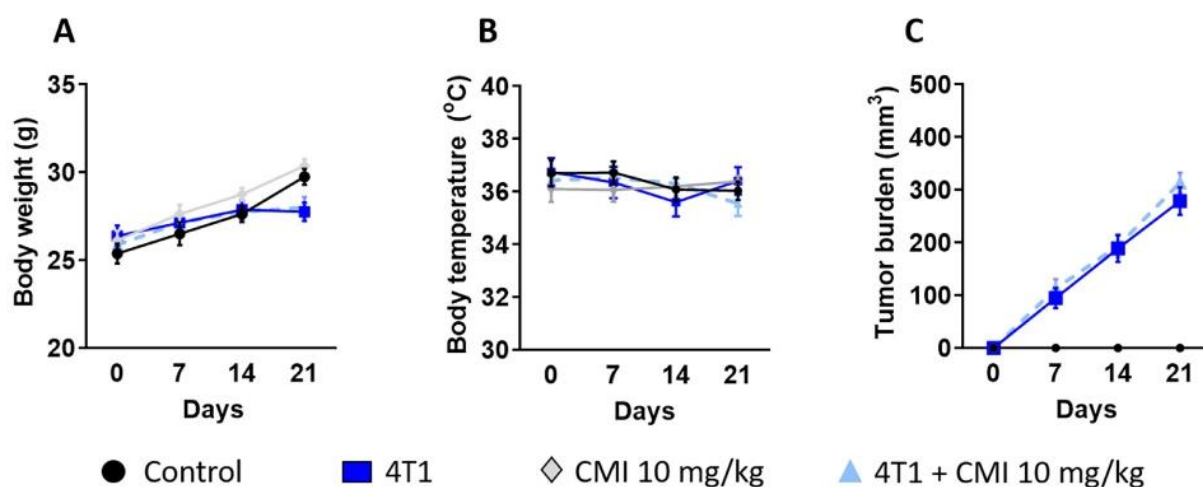


Fig. 2. Effect of CMI treatment on body weight and tumor growth in 4T1 tumor-bearing mice. (A) Body weight, (B) body temperature, and (C) tumor burden were measured every 7 days.

3.2. The depression-like behavior in 4T1 tumor-bearing mice was attenuated by CMI treatment

After confirming the tumor presence, we wanted to address whether 4T1 tumor-bearing mice would exhibit depression-like behavior and if CMI treatment would be able to block it. We initially conducted the OFT to characterize the locomotor and exploratory profile of mice. The number of crossings and rearings were similar among mice from all experimental groups (Fig. 3A), excluding the possibility of any psychostimulant effect that would alter the behavior in the subsequent analysis.

Next, to investigate the antidepressant-like effect of CMI in tumor-bearing mice, the TST, FST, and splash test were conducted. As depicted in Fig. 3B, tumor-bearing mice presented increased immobility time and decreased latency time in the TST, and these effects were diminished by treatment with CMI. A two-way ANOVA revealed a significant 4T1 $\times$ CMI interaction for immobility ($F_{(1,28)} = 28.30$, $p < 0.001$) and latency ($F_{(1,28)} = 17.30$, $p < 0.001$) time in the TST. Similarly, treatment with CMI attenuated the increased immobility time and the decreased the latency time in the FST induced by 4T1 tumor cells injection (Fig. 3C). A two-way ANOVA revealed a significant 4T1 $\times$ CMI interaction for immobility ($F_{(1,28)} = 4.86$, $p = 0.04$) and latency ($F_{(1,28)} = 10.10$, $p = 0.004$) time in the FST. Additionally, 4T1 tumor-bearing mice spent less time grooming in the splash test when compared to control mice, and CMI treatment increased the grooming time in tumor-bearing mice (4T1 $\times$ CMI interaction: $F_{(1,28)} = 11.00$, $p = 0.003$; Fig. 3D). Of note, treatment with CMI in non-tumor-bearing mice did not alter these parameters. These data indicate that the depression-like behavior displayed by tumor-bearing mice could be attenuated by CMI treatment.

3.3. Cognitive impairment in 4T1-tumor bearing mice was attenuated by CMI treatment

Bearing in mind that cognitive dysfunction may be a primary mediator of functional impairment in major depression (McIntyre et al., 2013) and cancer (Janelsins et al., 2014), we investigated if treatment with CMI would block cognitive impairment in 4T1 tumor-bearing mice. As depicted in Fig. 3E, tumor-bearing mice had a lower object recognition index when compared to control mice, and CMI treatment eliminated the impaired recognition memory (4T1 $\times$ CMI interaction: $F_{(1,28)} = 6.50$, $p = 0.02$). Similarly, CMI treatment blocked the tumor-induced working memory impairment (Fig. 3F), as evidenced by the increased percentage of alternations in the Y maze when compared to tumor-bearing mice that were not treated with CMI (4T1 $\times$ CMI

interaction: $F_{(1,28)} = 8.38$, $p = 0.007$). The treatment of non-tumor-bearing mice with CMI did not alter the object recognition index and the percentage of alternations in the Y maze. Overall, CMI treatment could improve cognitive dysfunction developed in mice carrying a peripheral tumor.

3.4. CMI treatment attenuated neuroinflammation and oxidative stress in the prefrontal cortices of mice

Even though the central nervous system (CNS) is still considered immune-privileged, it is now understood that the CNS communicates extensively with the immune system (Louveau et al., 2015). In this sense, we investigated whether CMI treatment would abrogate the neuroinflammatory status in the prefrontal cortices of mice carrying peripheral tumors (Fig. 4A). We confirmed that the prefrontal cortices of tumor-bearing mice had increased mRNA expression of *NFκB*, *IL-1β*, *TNF-α*, *IDO*, and *COX-2*. Interestingly, CMI treatment abolished these alterations, and a two-way ANOVA revealed significant 4T1 $\times$ CMI interactions for the relative expression of *NFκB* ($F_{(1,20)} = 35.60$, $p < 0.001$), *IL-1β* ($F_{(1,20)} = 31.20$, $p < 0.001$), *TNF-α* ($F_{(1,20)} = 17.50$, $p < 0.001$), *IDO* ($F_{(1,20)} = 7.60$, $p = 0.01$), and *COX-2* ($F_{(1,20)} = 6.05$, $p = 0.02$). Neither the tumor presence nor the CMI treatment altered *IL-10* and *BDNF* expression in the prefrontal cortices of mice.

To determine whether the antioxidant compound CMI could modulate oxidative stress in the prefrontal cortices of tumor-bearing mice, we initially analyzed the expression of the NO $^{\circ}$ -producing enzyme iNOS and the expression of the transcription factor *Nrf2* (Fig. 4A). Tumor presence increased the expression of iNOS and decreased the expression of *Nrf2*. CMI treatment abrogated these alterations, and a two-way ANOVA revealed a significant 4T1 $\times$ CMI interaction for the relative expression of iNOS ($F_{(1,20)} = 92.50$, $p < 0.001$) and *Nrf2* ($F_{(1,20)} = 4.17$, $p = 0.05$). Next, we quantified the levels of RS, NO $_x$, lipid peroxidation, and total sulfhydryl groups. Tumor presence increased the levels of RS (Fig. 4B), NO $_x$ (Fig. 4C), and lipid peroxidation (Fig. 4D) in the prefrontal cortices of mice, while the treatment of tumor-bearing mice with CMI blocked these alterations. A two-way ANOVA revealed a significant 4T1 $\times$ CMI interaction for the levels of RS ($F_{(1,28)} = 9.92$, $p = 0.004$), NO $_x$ ($F_{(1,28)} = 7.30$, $p = 0.003$), and lipid peroxidation ($F_{(1,28)} = 24.90$, $p < 0.001$) in the prefrontal cortices of mice. On the other hand, neither the tumor inoculation nor the CMI treatment altered the total contents of sulfhydryl groups (Fig. 4E) or CAT activity (Fig. 4G) in the prefrontal cortices of mice. We also observed that the prefrontal cortices of tumor-bearing mice had increased SOD activities (Fig. 4F), which was abolished by CMI treatment

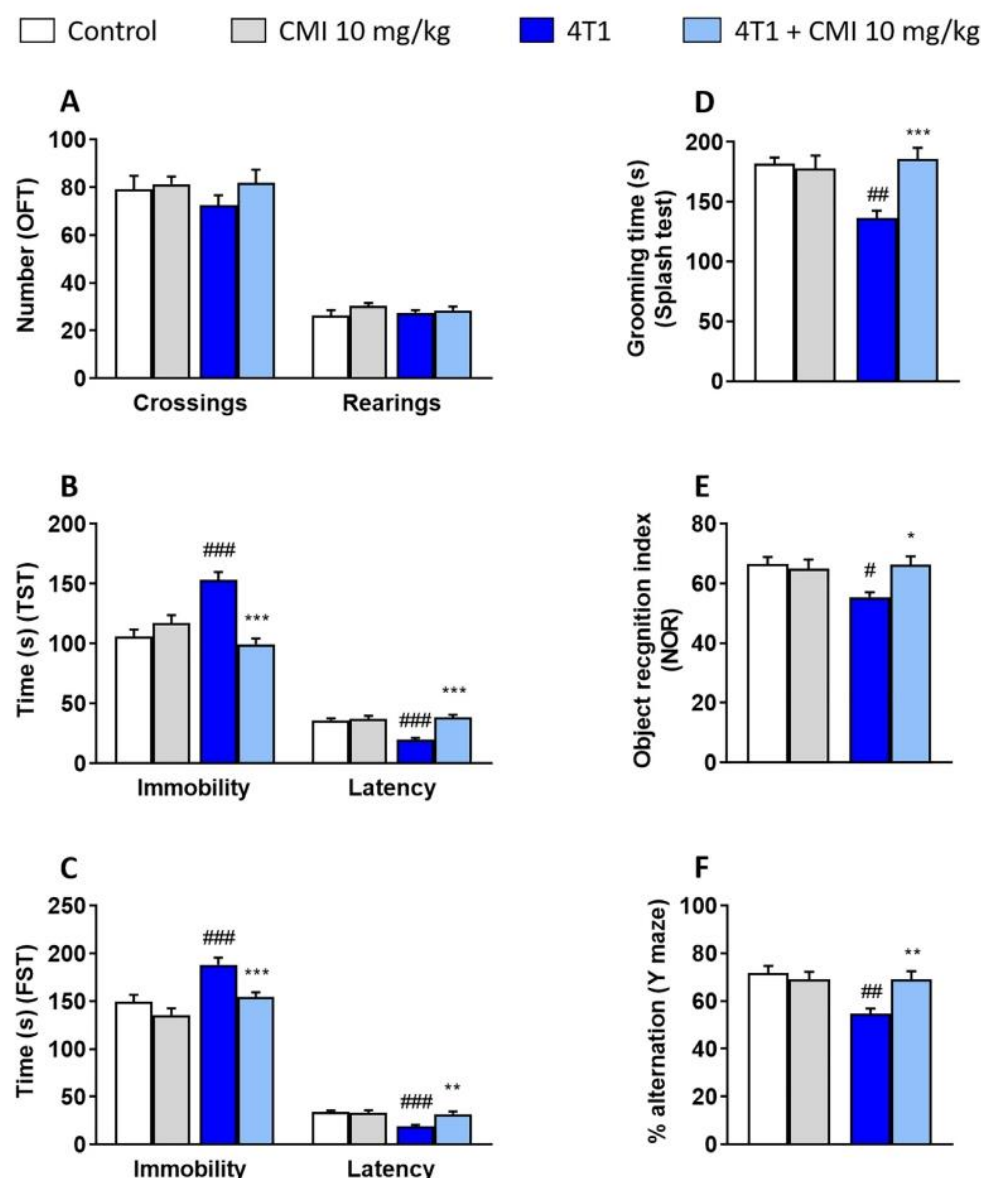


Fig. 3. Effect of CMI treatment on depression-like behavior and cognitive impairment in 4T1 tumor-bearing mice. (A) Number of crossings and rearings in the open field test (OFT). (B) Immobility and latency time in the tail suspension test (TST). (C) Immobility and latency time in the forced swimming test (FST). (D) Grooming time in the splash test. (E) Object recognition index in the novel object recognition (NOR) test. (F) Percentage of alternation in the Y maze. Data are expressed as mean $\pm$ SEM of eight independent animals/group. # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$ when compared to the control group. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ when compared to 4T1 tumor-bearing mice.

(4T1 $\times$ CMI interaction: $F_{(1,28)} = 6.02$, $p = 0.02$). The administration of CMI in non-tumor-bearing mice did not alter these parameters.

3.5. CMI treatment attenuated neuroinflammation and oxidative stress in the hippocampi of mice

The effects of neuroinflammation and oxidative stress in the hippocampus have been extensively investigated due to the critical role this limbic structure plays in the regulation of cognitive processes, involving emotions, learning, and memory. In this sense, we addressed whether CMI treatment would attenuate hippocampal alterations induced by the presence of a peripheral tumor in mice. Initially, we confirmed that 4T1 tumor-bearing mice had increased expression of *NFκB*, *IL-1β*, *TNF-α*, *IDO*, *COX-2*, and *iNOS*, while the expression of *IL-10*, *Nrf2*, and *BDNF* were lower than in the control group (Fig. 5A). Importantly, CMI treatment decreased the expression of these inflammatory markers, while increasing the expression of *IL-10*, *Nrf2*, and *BDNF*. A two-way ANOVA revealed a significant 4T1 $\times$ CMI interaction for the expression of *NFκB* ($F_{(1,20)} = 8.98$, $p = 0.007$), *IL-1β* ($F_{(1,20)} = 186.00$, $p < 0.001$), *TNF-α* ($F_{(1,20)} = 128.00$, $p < 0.001$), *IL-10* ($F_{(1,20)} = 17.10$, $p < 0.001$), *IDO* ($F_{(1,20)} = 36.70$, $p < 0.001$),

COX-2 ($F_{(1,20)} = 150.00$, $p < 0.001$), *iNOS* ($F_{(1,20)} = 17.50$, $p < 0.001$), *Nrf2* ($F_{(1,20)} = 6.57$, $p = 0.02$), and *BDNF* ($F_{(1,20)} = 128.00$, $p < 0.001$) in the hippocampi of mice.

Additionally, the tumor presence increased the hippocampal levels of RS (Fig. 5B), NO_x (Fig. 5C), lipid peroxidation (Fig. 5D), and SOD activity (Fig. 5F), which were attenuated by the CMI treatment. A two-way ANOVA revealed a significant 4T1 $\times$ CMI interaction for the hippocampal levels of RS ($F_{(1,28)} = 11.40$, $p = 0.002$), NO_x ($F_{(1,28)} = 10.90$, $p = 0.003$), lipid peroxidation ($F_{(1,28)} = 45.50$, $p < 0.001$), and SOD activity ($F_{(1,28)} = 4.55$, $p = 0.04$). On the other hand, neither the tumor inoculation nor the CMI treatment altered the total contents of sulfhydryl groups (Fig. 5E) or the CAT activity (Fig. 5F). The administration of CMI in non-tumor-bearing mice did not alter these parameters.

3.6. CMI interacts with the active site of *iNOS* and *COX-2*

The ability of CMI to block the upregulation of *iNOS* and *COX-2* expression in tumor-bearing mice prompted us to investigate if CMI would also interact with the active sites of these enzymes, as part of its antidepressant-like effect. The optimal binding sites for CMI with *iNOS*

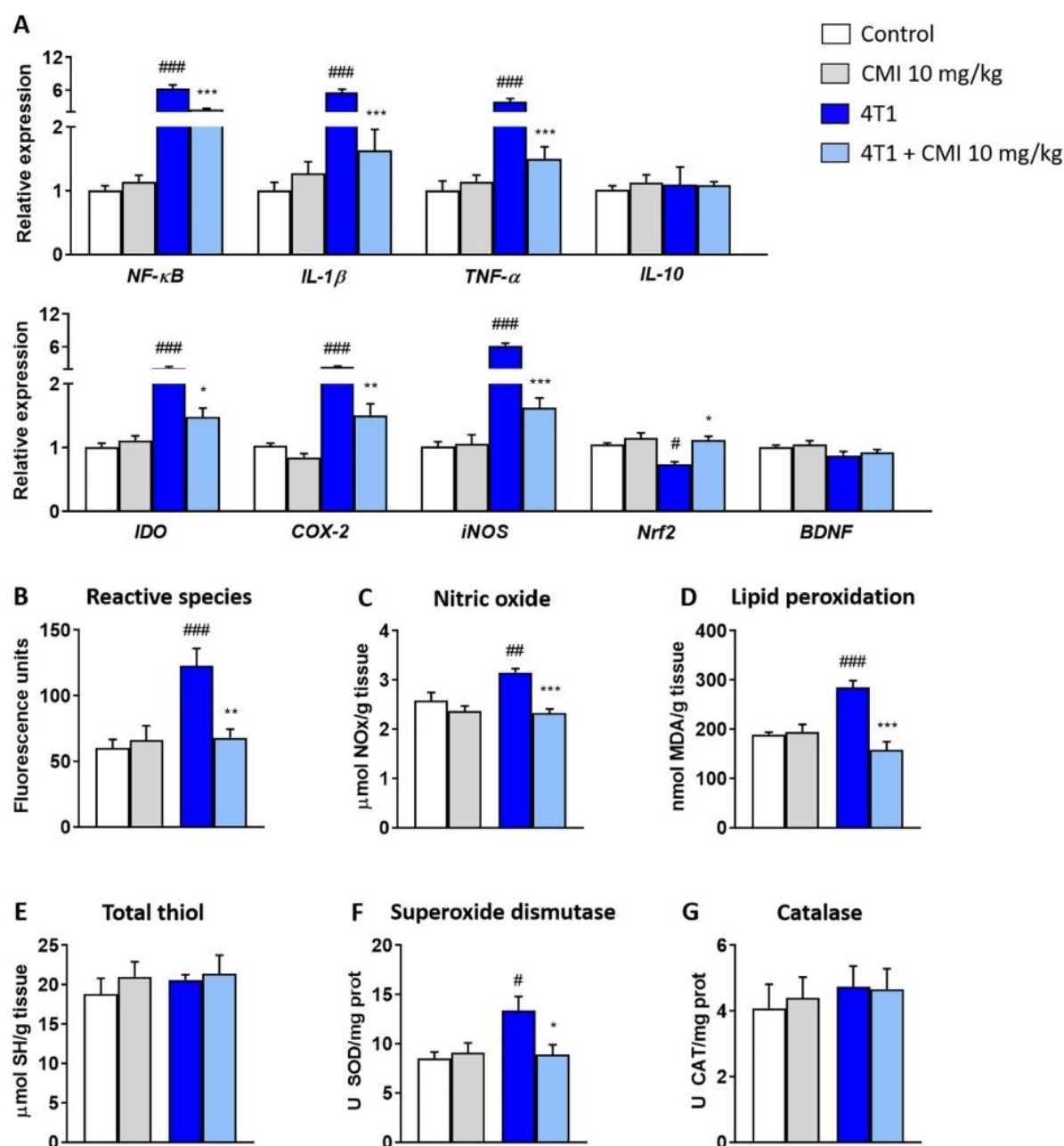


Fig. 4. Effect of CMI on markers of oxidative stress and neuroinflammation in the prefrontal cortices of 4T1 tumor-bearing mice. (A) Relative expression of *NFκB*, *IL-1β*, *TNF-α*, *IL-10*, *IDO*, *COX-2*, *iNOS*, *Nrf2*, and *BDNF*. Levels of (B) reactive species (RS), (C) nitric oxide metabolites (NO_x), (D) lipid peroxidation, and (E) sulfhydryl groups. Activity of (F) superoxide dismutase and (G) catalase. Data are expressed as mean $\pm$ SEM of six to eight independent animals/group. ##p < 0.01 and ###p < 0.001 when compared to the control group. *p < 0.05, **p < 0.01, and ***p < 0.001 when compared to 4T1 tumor-bearing mice.

and COX-2 are depicted in Fig. 6 and Fig. 7, respectively. Significant interactions were formed between CMI and the residues TRP194, CYS200, LEU209, PRO350, VAL352, and PHE369 in the active site of iNOS (Fig. 6). In addition, other residues such as ALA197, ILE244, SER242, ALA351, ASN370, GLY371, and TYR489 played important roles for the interaction between CMI and iNOS (binding free energy (Δ_G) of -9.2 kcal/mol). Fig. 7 shows the best binding position of CMI with COX-2 (Δ_G of -6.8 kcal/mol), which involved a carbon-hydrogen bond with HIS90 and close interactions with the residues LEU352, SER353, LEU384, TRP387, MET522, and VAL523. In addition, the residues GLN192, VAL349, SER350, TYR355, ARG513, ALA516, ILE517, PHE518, and GLY526 also contributed to the interaction of CMI with the active site of COX-2.

4. Discussion

In the present study, CMI treatment attenuated depressive-like behavior and impaired cognition in 4T1 tumor-bearing mice, as evidenced by the modulation of neuroinflammation and oxidative stress in the prefrontal cortices and hippocampi of mice. Whilst numerous observations were reported in accordance with our previous studies on the antidepressant-like effect of CMI in mice (Casaril et al., 2017a; Casaril et al., 2019a, 2019b), the present study was the first to report that CMI treatment ameliorated central nervous system alterations caused by a peripheral chronic disease. Moreover, this study reported that CMI treatment attenuated neuroinflammation and oxidative stress that arose in a mouse model of a mammary tumor, highlighting the significance of

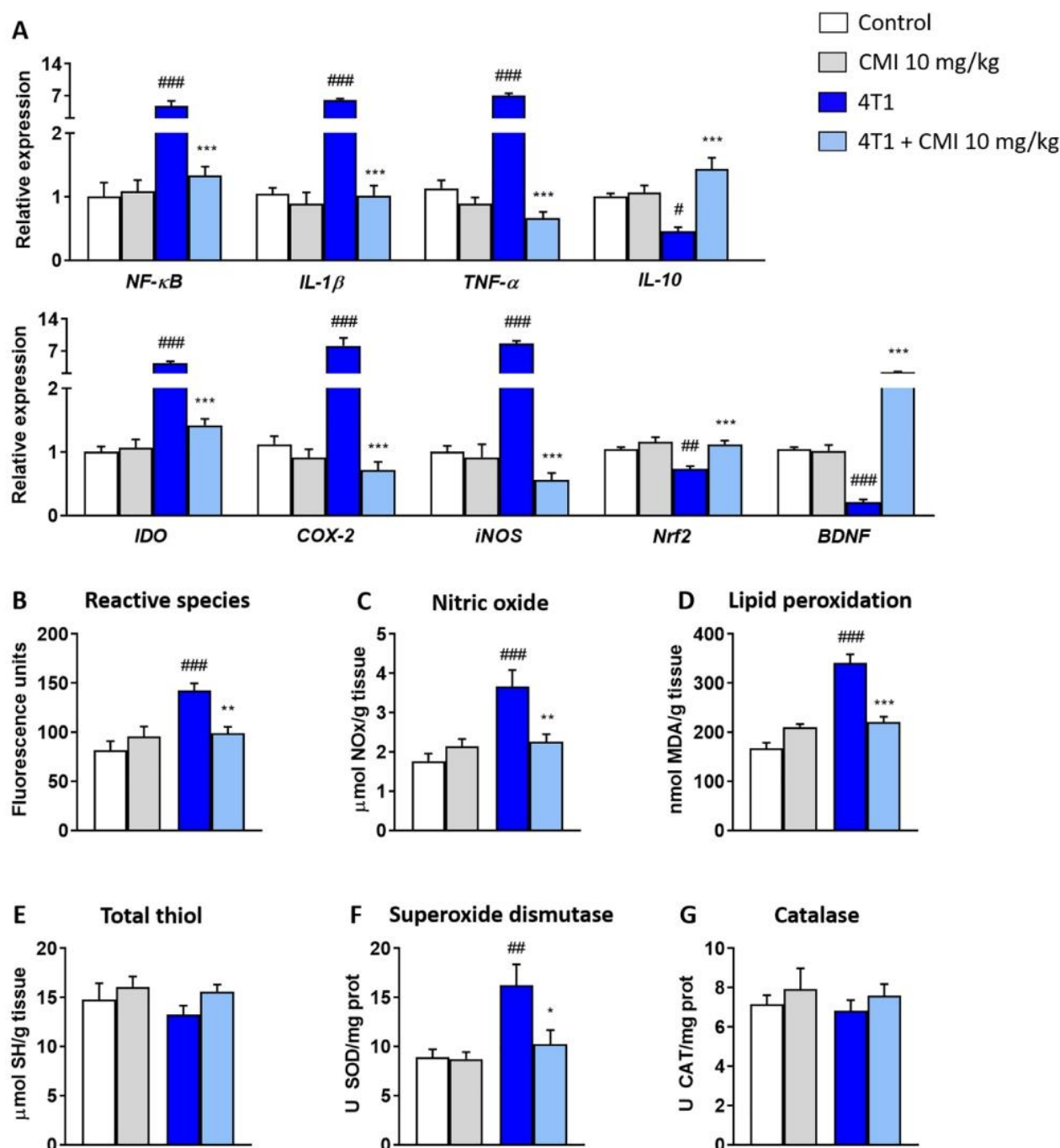


Fig. 5. Effect of CMI on markers of oxidative stress and neuroinflammation in hippocampi of 4T1 tumor-bearing mice. (A) Relative expression of *NFκB*, *IL-1β*, *TNF-α*, *IL-10*, *IDO*, *COX-2*, *iNOS*, *Nrf2*, and *BDNF*. Levels of (B) reactive species (RS), (C) nitric oxide metabolites (NO_x), (D) lipid peroxidation, and (E) sulfhydryl groups. Activity of (F) superoxide dismutase and (G) catalase. Data are expressed as mean $\pm$ SEM of six to eight independent animals/group. # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$ when compared to the control group. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ when compared to 4T1 tumor-bearing mice.

the immunomodulatory and antioxidant effects of CMI. Lastly, this study emphasized the involvement of peripheral-to-central communication pathways shared between the brain and the periphery, especially in the context of cancer-related depression and impaired cognition. A summary of our findings is presented in Fig. 8.

Our data confirmed previous findings showing that 4T1 tumor-bearing mice presented depression-like behavior (Nashed et al., 2015) and cognitive impairment (Walker et al., 2018). Although there are many studies evaluating behavioral alterations in tumor-bearing mice, few studies have evaluated the behavior of mice with mammary

carcinoma, despite the high incidence of psychiatric disorder symptoms in breast cancer patients. In addition, our work differs from previous studies in that treatment with a novel molecule (CMI) ameliorated behavioral alterations caused by peripheral tumors. Repeated administration of CMI at 10 mg/kg (orally) once a day for one week improved behaviors in the FST, TST, splash test, NOR test, and Y maze after tumor inoculation.

Malignant cells and resident cells within the tumor microenvironment (such as, dendritic cells, tumor-associated macrophages, and mesenchymal stem cells) release growth factors, pro-inflammatory

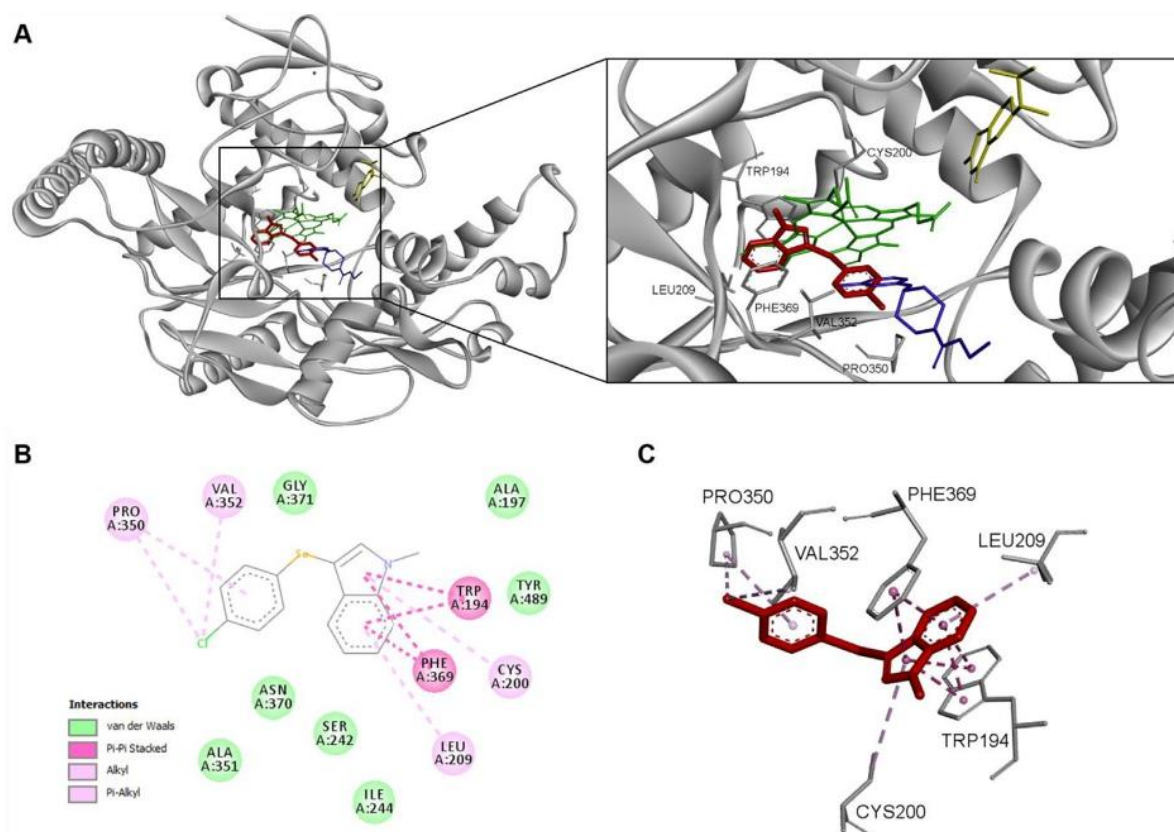


Fig. 6. Molecular docking of CMI in the binding pocket of Hs iNOS (PDB: 3E7G), with a free binding energy (ΔG) of -9.2 Kcal/mol (RMSD 0.83). (A) View of CMI (red), the co-crystallized ligand AR-C95791 (blue), the heme group (green), and the co-factor tetrahydrobiopterin (yellow) bound to the X-ray crystallographic structure of Hs iNOS. (B) 2D and (C) 3D visualization of CMI interacting with amino acids residues in the active site of Hs iNOS. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

cytokines, and DAMPs that influence tumor development (Lim et al., 2018). These molecules can trigger immune-to-brain communication signaling pathways in the central nervous system, altering behavior in both clinical and animal models. Signaling through DAMPs and cytokine receptors activate the NF κ B pathway, which induces the production of pro-inflammatory cytokines (such as, IL-1 β and TNF- α) and second messengers (such as, COX-2 and iNOS). Here, we showed that the administration of CMI downregulated the expression of NF κ B, IL-1 β , TNF- α , COX-2, and iNOS in the prefrontal cortices and hippocampi of tumor-bearing mice, reducing the depressive-like behavior and cognitive impairment.

The administration of CMI also blocked tumor-induced IL-10 downregulation in the hippocampi of mice, while none of the treatments altered the expression of IL-10 in the prefrontal cortices. The behavioral effects of IL-10 may be a consequence of a downregulation of pro-inflammatory cytokines (Moore et al., 2001) or increased cell survival (Roque et al., 2009). Indeed, it has been reported that depression-like behavior induced by stress (Duan et al., 2019) and sterile inflammation (Bampi et al., 2020) downregulated IL-10 expression in the prefrontal cortices and hippocampi of mice. Hence, it is unclear why the mammary tumor presence reduced the expression of IL-10 exclusively in the hippocampi. It has been reported that mammary tumors induce central pro-inflammatory cytokine expression without altering the IL-10 mRNA expression in the prefrontal cortices and hippocampi of mice (Walker II et al., 2017).

Activated NF κ B and pro-inflammatory cytokines can induce COX-2 expression, increasing PG generation. Notably, PGE2 signaling was shown to reduce the activity-dependent BDNF transcription necessary for long-term memory formation (Hein and O'Banion, 2009; Rage et al.,

2006). Additionally, COX-2 inhibitors improve memory deficits via downregulation of COX-2 expression and upregulation of the BDNF signaling pathway (Chen et al., 2017; Yang and Gao, 2017). Here, we showed that CMI treatment in 4T1 tumor-bearing mice downregulated the expression of COX-2 in the prefrontal cortices and hippocampi, while increasing the expression of BDNF in the hippocampi. Importantly, CMI may not only modulate the expression of COX-2, but also its activity. As revealed by the molecular docking investigation, CMI interacts with the residues in the active site of COX-2 in a similar manner to the inhibitor Rofecoxib (Orlando and Malkowski, 2016). Altogether, it is plausible that CMI improved cognition and depression-like behavior by a mechanism dependent, at least in part, on the downregulation of COX-2 and upregulation of BDNF, especially in the hippocampi of tumor-bearing mice.

Pro-inflammatory cytokines can activate IDO, which diverts available tryptophan away from serotonin synthesis and towards the generation of neurotoxic metabolites. Here we showed that the CMI treatment stopped the increased expression of IDO in the prefrontal cortices and hippocampi of tumor-bearing mice. Since the activation of IDO has been proposed to account for the depressogenic action of cytokines (Miller, 2009), it is possible that the modulation of IDO expression accounted for the antidepressant-like effect of CMI in 4T1 tumor-bearing mice. Recently, a molecular docking analysis revealed that CMI might interact with the active site of IDO, similar to the inhibitor Epacadostat (Casaril et al., 2019b).

The enzyme iNOS can also be activated through the NF κ B signaling pathway (Aktan, 2004). The NO $^{\cdot}$ that is generated can interact with radical anion superoxide (O $_2^{\cdot-}$) to produce peroxynitrite (Beckman and Koppenol, 1996). Several lines of research have suggested the

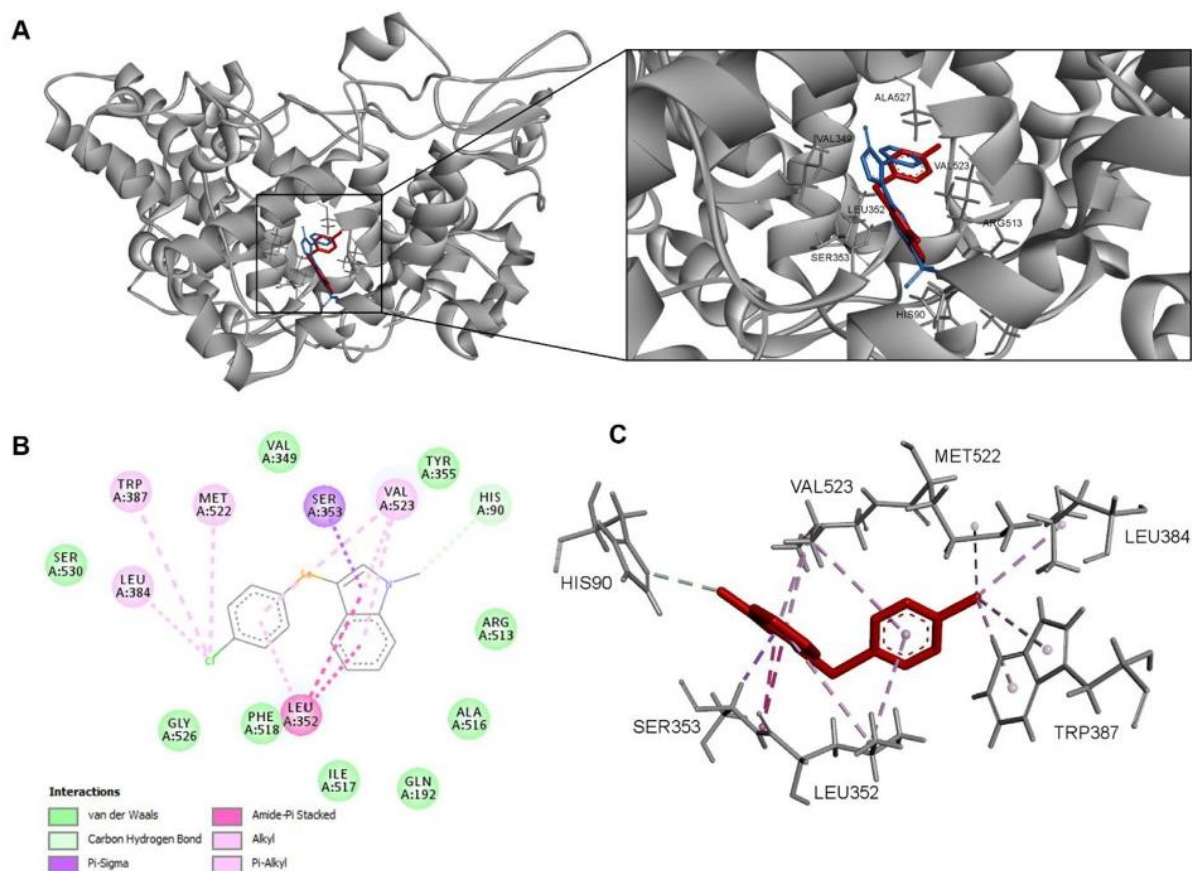


Fig. 7. Molecular docking of CMI in the binding pocket of Hs COX-2 (PDB: 5KIR), with a free binding energy (ΔG) of -6.8 Kcal/mol (RMSD 2.1). (A) View of CMI (red) and the co-crystallized ligand Rofecoxib (blue) bound to the X-ray crystallographic structure of Hs COX-2. (B) 2D and (C) 3D visualization of CMI interacting with amino acids residues in the active site of Hs COX-2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

importance of $\text{NO}^{\bullet}$ in depression. For example, higher levels of $\text{NO}^{\bullet}$ are found in depressed patients (Suzuki et al., 2001), and inhibitors of iNOS enhance the cellular levels of serotonin and dopamine (Wegener et al., 2000). In addition to the antidepressant-like and pro-cognitive effects of CMI administration in tumor-bearing mice, CMI downregulated iNOS expression and NO_x levels in the prefrontal cortices and hippocampi. In agreement, we recently showed that CMI rapidly reacted with peroxynitrous acid (second-order rate constant of $8.6 (\pm 2) \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$) (Casaril et al., 2017b), suggesting that CMI may also undergo rapid reaction with this inflammation-associated oxidant. On top of that, it is possible that the reduced levels of NO_x by CMI treatment in tumor-bearing mice were also a consequence of its ability to inhibit the activity of iNOS. With a molecular docking approach, we showed that CMI binds to iNOS active site and shares some residues with the inhibitor AR-C95791 (Garcin et al., 2008), the heme group, and the co-factor tetrahydrobiopterin. These results highlight the role of CMI as an antioxidant molecule and suggest that the behavioral effects of CMI may be a consequence of its antioxidant effect.

Reinforcing the antioxidant potential of CMI in a model of chronic disease, we observed that the expression of *Nrf2* in the prefrontal cortices and hippocampi of tumor-bearing mice was upregulated in response to CMI treatment. In addition, the prefrontal cortices and hippocampi of tumor-bearing mice exhibited increased SOD activity, which was blocked by treatment with CMI. Intriguingly, neither the tumor inoculation nor the CMI treatment altered CAT activity. *Nrf2* is the main driver of the cellular response to oxidative stress and induces the expression of antioxidant enzymes. Here, increased SOD activity may indicate a pro-oxidative state, since it can promote the

accumulation of H_2O_2 (Halliwell, 2007). Given the importance of scavenging biological oxidants, we have recently reported that CMI directly interacted with H_2O_2 and HOCl (Casaril et al., 2017b). Therefore, the antioxidant effect of CMI may rely on its ability to influence the expression of *Nrf2*, to modulate the activity of antioxidant enzymes, and to react directly with inflammation-associated oxidants. Consequently, we also found that treatment with CMI abrogated the tumor-induced increased levels of RS and lipid peroxidation in the prefrontal cortices and hippocampi of mice. Interestingly, the total content of sulfhydryl groups (especially glutathione, the main thiol in animal cells) was not altered by the tumor presence or by CMI treatment, possibly due to the lack of sensitivity of the method used here. Although the antioxidant potential of CMI has been previously demonstrated using acute insults (Casaril et al., 2017a; Casaril et al., 2019a, 2019b), this was the first study characterizing the antioxidant effect of CMI in a mouse model of chronic neurochemical alterations, thus offering more credibility to the antioxidant effect of CMI.

However, limitations of this study must be acknowledged. We injected tumor cells in the right flank of mice to avoid possible movement constraints due to tumor growth, bearing in mind that mice with heterotopic mammary tumors presented depression-like behavior without impaired locomotion (Nashed et al., 2015). Nonetheless, mice with 4T1 cells injected in the orthotopic site also exhibited behavioral alterations, such as cognitive impairment (Walker et al., 2018) and increased nociception (Lofgren et al., 2018), but with altered locomotion. Indeed, the orthotopic injection of 4T1 cells is widely used to study the immunological and neurochemical alterations induced by mammary tumors in mice (Liu et al., 2015; Rogers et al., 2018), having the obvious

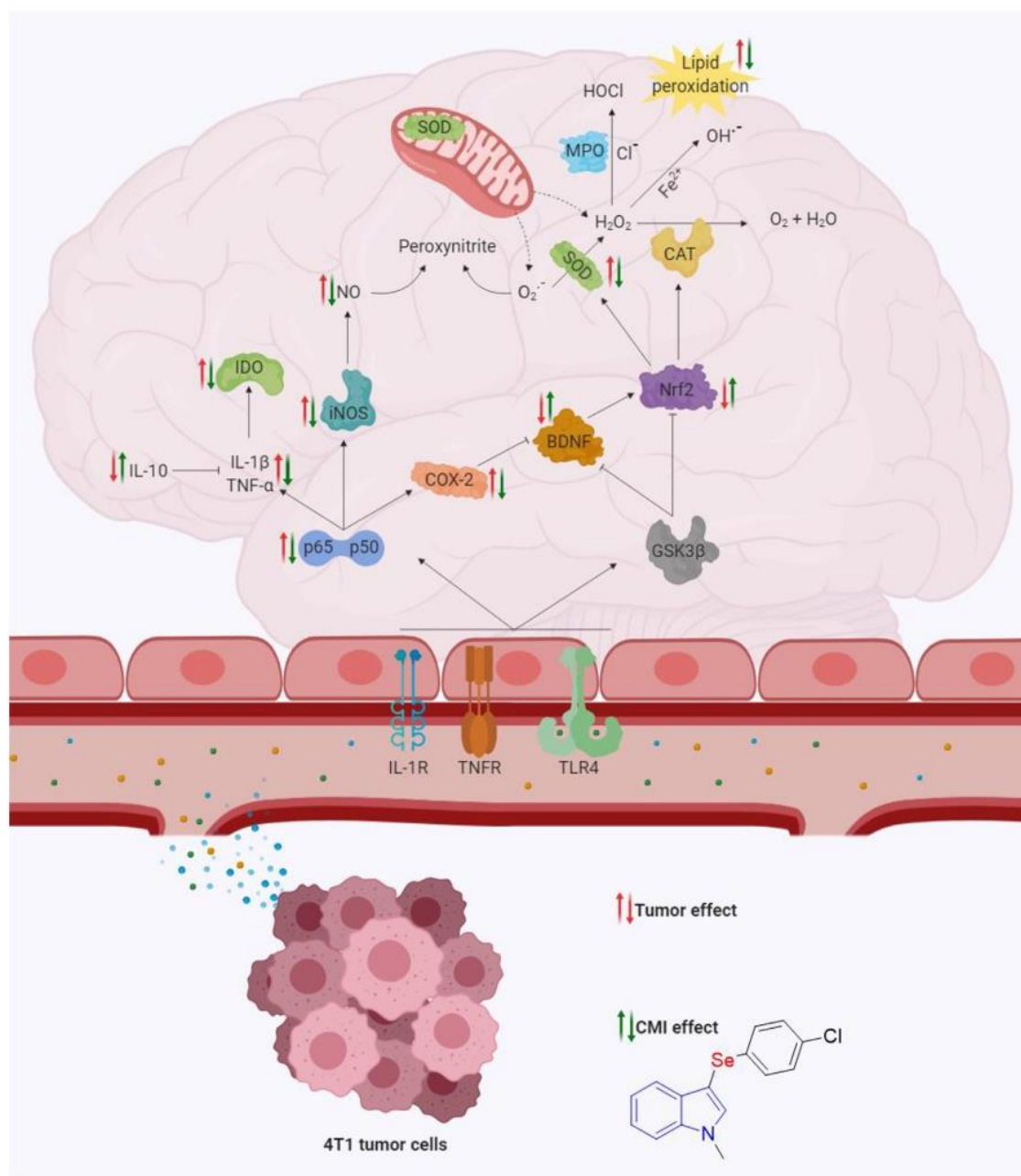


Fig. 8. Overview of the neurochemical alterations found in the prefrontal cortices and hippocampi of 4T1-tumor bearing mice treated with CMI. Administration of CMI in tumor-bearing mice downregulated the expression of *NFκB*, *IL-1β*, *TNF-α*, *IDO*, *iNOS*, and *COX-2*, while increased the expression of *IL-10*, *BDNF*, and *Nrf2*. Treatment with CMI also attenuated the increased level of RS, NO_x, lipid peroxidation, and SOD activity in the brain of tumor-bearing mice. MPO: myeloperoxidase.

advantage of mimicking the tumor microenvironment. Moreover, it is worth highlighting that 4T1 tumor cells can spontaneously metastasize to distant organs (Pulaski and Ostrand-Rosenberg, 2001). Here, we only checked for macroscopic metastases by a gross visual examination of brain, liver, lungs, heart, and kidneys at necropsy, and no sign of metastases was found. The identification of metastases in distant organs appears to be variable, with some authors reporting the arise of metastases more than 4 weeks after mammary tumor cell inoculation (Liu et al., 2013; Tao et al., 2008), while others reported metastases on day 14 (Pulaski and Ostrand-Rosenberg, 2001). Finally, the ability of CMI treatment to modulate oxidative and inflammatory signaling pathways in the brain of tumor-bearing mice warrants further investigation in terms of protein detection and transcription factors activation.

Collectively, we showed here that CMI attenuated depression-like

behavior, cognitive impairment, neuroinflammation, and oxidative stress in a mouse model of mammary carcinoma. The antioxidant supplementation has mixed effects on tumor growth and proliferation, with studies showing beneficial (Wang et al., 2013) or detrimental (Assi et al., 2016) effects. However, the goal of our study was not to find a molecule able to influence the tumor growth, but rather to characterize a molecule able to improve the behavioral alterations induced by the presence of the tumor. CMI had no effect on tumor growth, so it should not be addressed as a chemotherapeutic drug, but as an adjuvant therapy to improve depressive and cognitive symptoms. The use of adjuvant chemotherapy is widely recognized as an important component of multimodal treatment, and finding drugs to treat cancer-induced depression and cognitive impairment would be a noteworthy advance towards improving the quality of life of cancer patients.

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.

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4.5 Capítulo 5 – The modulation of the BDNF-mTOR pathway is involved in the antidepressant- and anxiolytic-like effect of a selenium-containing compound in corticosterone-treated mice

Manuscrito preparado de acordo com as normas da revista *Molecular Neurobiology*

Nos capítulos anteriores, focamos no envolvimento do estresse nitro-oxidativo, neuroinflamação, e sistema serotoninérgico no efeito tipo-antidepressivo do CMI. Neste trabalho, investigamos se a via do mTOR/BDNF estaria envolvida com a rápida ação farmacológica do CMI em camundongos tratados cronicamente com corticosterona. Através do uso do antagonista rapamicina, mostramos que o efeito tipo-antidepressivo e tipo-ansiolítico do CMI é dependente da sinalização do mTOR. Adicionalmente, observamos que o CMI reverteu o aumento nos níveis de marcadores oxidativos no plasma e nos hipocampus de animais tratados com corticosterona. Corroborando com esses dados, o CMI restaurou a expressão dos *GR*, *BDNF*, *sinaptofisina*, *GSK-3 β* , *Nrf2* e *IDO* no hipocampo de animais estressados. Em conjunto, esses dados mostraram que uma única administração de CMI desencadeou efeito tipo-antidepressivo e tipo-ansiolítico em animais estressados através da modulação da via do mTOR/BDNF, neuroinflamação e estresse oxidativo periférico e central.

The modulation of BDNF-mTOR pathway is involved in the antidepressant- and anxiolytic-like effect of a selenium-containing compound in corticosterone-treated mice

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Abstract

Major depressive disorder is the most prevalent psychiatric disorder and is often accompanied by anxiety disorders. Both disorders are heterogenic and multifactorial and have been widely investigated intrinsic to chronic stress. However, the current treatment still relies mostly on the monoaminergic neurotransmission, which prompts the research for new multitarget drugs. In this sense, we have previously demonstrated the antioxidant, anti-inflammatory, antinociceptive, and antidepressant-like effects of the organoselenium compound 3-[(4-chlorophenyl)selenanyl]-1-methyl-1*H*-indole (CMI). Considering these properties of CMI, the aim of the present study was to deepen into the acknowledgment about the molecular mechanism of action of CMI in reversing the depressive- and anxiogenic-like behavior induced by repeated corticosterone administration during 14 days in Swiss male mice. A single administration of CMI (1 mg/kg, i.g.) reversed the behavioral alterations induced by corticosterone in the open field test, splash test, and elevated plus maze. The pre-treatment with rapamycin (inhibitor of the mammalian target of rapamycin, mTOR) prevented the antidepressant- and anxiolytic-like effect elicited by CMI in corticosterone-treated mice. Moreover, the administration of CMI restored the expression levels of glucocorticoid receptor, brain-derived neurotrophic factor, synaptophysin, glycogen synthase kinase 3 beta, nuclear factor erythroid 2-related factor 2, and indoleamine 2,3-dioxygenase in the hippocampus of stressed mice. Additionally, the antioxidant effect of CMI was evident by its ability to decrease the levels of reactive species and lipid peroxidation in the plasma of corticosterone-treated mice. These findings indicate the CMI ability to reverse behavioral and biochemical alterations in the depression-anxiety comorbidity induced by corticosterone, possibly by the modulation of mTOR signaling pathway.

Keywords: Organoselenium, antidepressant, anxiolytic, corticosterone, mTOR.

1906 **Abbreviations**

1907	<i>AKT</i>	protein kinase B
1908	<i>AMPA</i>	α -amino-3-hydroxyl-5-methyl-4-isoxazolepropionic acid
1909	<i>BDNF</i>	brain-derived neurotrophic factor
1910	<i>CNS</i>	central nervous system
1911	<i>DAMPs</i>	danger associated molecular patterns
1912	<i>DMSO</i>	dimethyl sulfoxide
1913	<i>EPM</i>	elevated plus maze
1914	<i>GR</i>	glucocorticoid receptor
1915	<i>GSK3-β</i>	glycogen synthase kinase 3 beta
1916	<i>HC</i>	hippocampus
1917	<i>HO-1</i>	heme-oxygenase 1
1918	<i>HPA</i>	hypothalamic-pituitary-adrenal axis
1919	<i>ICV</i>	intracerebroventricular
1920	<i>IDO</i>	indoleamine 2,3-dioxygenase
1921	<i>IG</i>	intragastrically
1922	<i>iNOS</i>	inducible nitric oxide synthase
1923	<i>LPS</i>	lipopolysaccharide
1924	<i>MDA</i>	malondialdehyde
1925	<i>MDD</i>	major depressive disorder
1926	<i>mTOR</i>	mammalian target of rapamycin
1927	<i>NFκB</i>	factor nuclear kappa B
1928	<i>NMDA</i>	N-methyl-D-aspartate
1929	<i>Nrf₂</i>	nuclear factor erythroid 2-related factor 2
1930	<i>OFT</i>	open field test
1931	<i>PFC</i>	pre-frontal cortex
1932	<i>PI3K</i>	phosphoinositide 3-kinase
1933	<i>qRT-PCR</i>	quantitative real-time polymerase chain reaction
1934	<i>RS</i>	reactive species
1935	<i>TBARS</i>	thiobarbituric acid reactive species
1936	<i>TNF-α</i>	tumor necrosis factor alpha
1937	<i>TrkB</i>	tropomyosin receptor kinase B

1. Introduction

Major depressive disorder (MDD) is the most common neuropsychiatric disorder, affecting 322 million people worldwide. It is the leading cause of disability and the major contributor to deaths by suicide. MDD is often accompanied by other disorders, such as anxiety, with affects 59% of depressed patients [1, 2]. MDD and anxiety are complex and multifactorial disorders that have chronic stress as a risk factor. Chronic exposure to stress leads to several peripheral and central alterations, such as inflammation, nitro-oxidative stress, and reduction of neurotrophic factors [3–6].

Despite the social and economic relevance of MDD, the symptomatology of 40% of the patients does not improve with the current treatments [7], emphasizing the urgent need to find better molecules to treat MDD. In this sense, we have recently showed that the 3-[(4-chlorophenyl)selenanyl]-1-mehtyl-1*H*-indole (CMI) [8], a synthetic organoselenium compound, (i) has *in vitro* and *in vivo* antioxidant activity [9, 10], (ii) presents antidepressant-like and anxiolytic effects in animal models induced by LPS and ARS [11, 12], and (iii) modulates de depression-like behavior and nociception induced by chronic pain [13, 14]. However, the exact mechanism of action of CMI is not fully elucidated.

The mTOR complex is ubiquitously expressed in the brain and modulates neuronal activity, growth factors, energy, nutrient levels, and synaptic plasticity [15]. Changes in mTOR signaling are involved in several disorders, such Alzheimer's disease, Parkinson's disease, and MDD [16–18]. The PI3K/Akt/GSK-3 β /mTOR signaling pathway has been implicated in the integration of synaptic neurotransmission and in the fast-acting antidepressant effects of novel drugs, such as ketamine and creatine [19–21]. The mTOR-mediated antidepressant-like effect is mainly related to the activation of TrkB by BDNF and to the modulation of the glutamatergic system by the activation of AMPA receptors and the inhibition of extra-synaptic NMDA receptor [22]. Furthermore, the activation of mTOR increases the transcription and the translation of proteins critically involved in the formation of new synapsis, such as synapsin I [23].

In turn, GSK-3 β is involved in the pathogenesis of various psychiatric diseases, such Alzheimer's disease, bipolar disorder, and MDD [24]. This enzyme, which is found upregulated in response to neuroinflammation, oxidative stress, and hypercortisolemia, phosphorylates the GR leading to glucocorticoid resistance [9, 25,

26]. Resistant GR inhibits the negative feedback of the HPA axis, contributing to its hyperactivation [27]. However, once the PI3K/Akt pathway is activated, the Akt phosphorylates and inhibits the GSK-3 β activity, and as a consequence, anxiety and depression-like behaviors are attenuated in mice [28, 29].

Additionally, activated GSK-3 β prevents the translocation of Nrf2 to the nucleus and, as a consequence, the expression of HO-1 [30]. Dysfunction in Nrf2 expression leads to redox imbalance and neuropsychiatric and neurodegenerative disorders [31–33]. In addition to the inflammation that arises from DAMPs released by stress, activation of GSK-3 β increases the activation of NF κ B and, thereafter, the expression of pro-inflammatory cytokines [24]. Pro-inflammatory cytokines, primarily TNF- α , activate IDO and iNOS, contributing to the reduction of the serotonergic transmission and impaired redox homeostasis [34, 35]. Interestingly, the signalling pathways activated by stress result in the excessive production of reactive species (RS) and/or decreased antioxidant defences and are similar to the pathways altered in depressive patients.

In light of the above, (i) considering the urgent need to find better molecules to treat MDD and anxiety, (ii) the relevance of the PI3K/Akt/GSK-3 β /mTOR pathway to the antidepressant-like effect of fast-acting drugs, and (iii) the biological properties of CMI previously reported, the aim of the present study was to evaluate the antidepressant-like and anxiolytic-like effects of CMI in corticosterone-treated mice. Furthermore, we wanted to dive into the possible mechanism of action of CMI based on the molecular pathways that are activated by fast-acting antidepressant drugs.

2. Materials and methods

2.1. Animals

Male Swiss mice (newly weaned), maintained at 22-25°C with water and food *ad libitum*, under a 12:12h light/dark cycle, were provided by the Animal Facility of the Federal University of Pelotas (UFPe) (Pelotas, Brazil). The studies were performed in accordance with protocols approved by the Committee on the Care and Use of Experimental Animal Resources at UFPe (2208-2018).

2.2. Drugs

CMI (Figure 1A) was synthesized at the Laboratory of Clean Organic Synthesis at Federal University of Pelotas [8], dissolved in canola oil (non-polar substance), and

administered intragastrically (i.g.) at a dose of 1 mg/kg body weight (constant volume of 10 ml/kg). Corticosterone was purchased from Sigma Aldrich (St Louis, Missouri, USA), dissolved in distilled water with 2% Tween 80 and 0.2% DMSO and administered i.g. at 20 mg/kg/day [36]. Rapamycin, an inhibitor of mTOR was dissolved in 100% DMSO and administered by intracerebroventricular route (i.c.v) at 0.2 nmol/site at a volume of 3µL per mouse [36]. I.c.v. injections were performed using the “free hand” method under isoflurane anesthesia according to the procedures described by Kaster et al. [37]. The RNA extraction reagents were purchased from Ambion (Life Technology, USA) and oligonucleotides were synthesized by Exxtend Biotecnologia Ltda (Campinas, Brazil). Other chemicals were of analytical grade and obtained from standard commercial suppliers.

2.3. Experimental design

2.3.1. Experiment 1 – Antidepressant- and anxiolytic-like effects of CMI

Animals were randomly assigned to four experimental groups (n=8-9 mice/group). Two groups received corticosterone (20 mg/kg) orally once a day, whereas the other two groups received the vehicle. The administration lasted 14 days, and in the 15th day, CMI (1 mg/kg) or its vehicle were administered (Figure 1B). The behavioral tests were performed 30 minutes after CMI or vehicle administration, aligned with previous data supporting the effect of organoselenium compounds and CMI after this time [9, 38, 39]. The depressive-like behavior of mice was assessed in the splash test and the locomotor activity was evaluated in the open field test (OFT). The anxiogenic-like behavior was assessed with the elevated plus maze (EPM) and OFT. All observations were done by observers blinded to the treatments. After the behavioral evaluations, mice were anesthetized (inhalation of isoflurane) for blood collection by cardiac puncture. Following that, mice were euthanized by overdose of isoflurane inhalation following by confirmation by cervical dislocation. Finally, the brains were removed and the hippocampi (HC) were isolated for analysis.

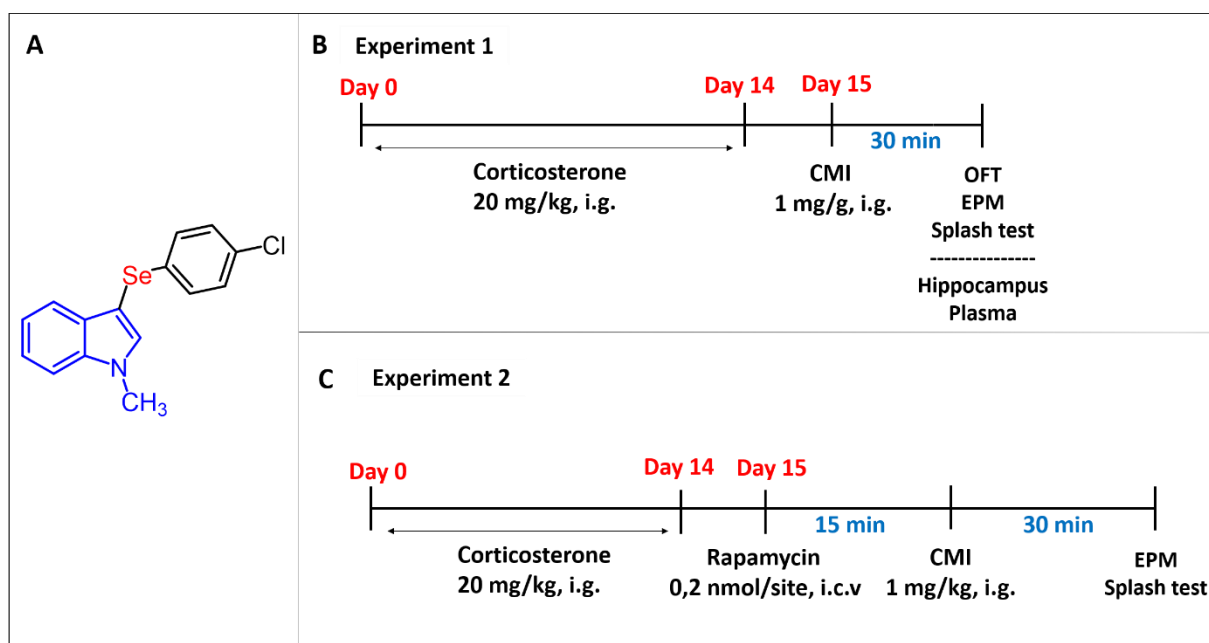


Figure 1. Schematic diagram of experimental design. CMI chemical structure (A), experimental design for experiment 1 (B) and for experiment 2 (C). CMI: 3-[(4-chlorophenyl)selanyl]-1-methyl-1H-indole; i.g.: intragastrically; OFT: open field test; EPM: elevated plus maze; RS: reactive species assay; TBARS: thiobarbituric acid reactive species assay; CORT: corticosterone assay; qRT-PCR: quantitative real-time polymerase chain reaction.

2.3.2. Experiment 2 – Involvement of the mTOR signaling in the antidepressant- and anxiolytic-like effects of CMI

Animals were randomly assigned to eight experimental groups (n=6 mice/group). Four groups received corticosterone (20 mg/kg) orally once a day, whereas the other four groups received vehicle. The administration lasted 14 days. In the 15th day, mice were anesthetized with isoflurane and received i.c.v injection of rapamycin (0.2 nmol/site) or its vehicle. After 15 min, CMI (1 mg/kg) or its vehicle were administrated and after 30 minutes the behavioral tests were performed (Figure 1C). After the behavioral analysis, mice were euthanized by overdose of isoflurane following by confirmation by cervical dislocation

2.4. Behavioral tests

2.4.1. Open field test (OFT)

The OFT was performed in order to assess the locomotion of mice [40]. Animals were placed individually in the center of a box (30 x 30 x 15 cm) divided into nine quadrants with equal areas. During 5 min, the locomotion (number of quadrants

crossed with the four paws), the exploratory behavior (the number of rearings), and the anxiogenic-like behavior (the number of groomings and entries in the central square) were assessed.

2.4.2. *Elevated plus maze (EPM)*

The EPM was carried out to assess the anxiolytic-like behavior of mice [41]. The apparatus is plus-shaped with two open and two enclosed arms (arm length: 100 cm; arm width: 10 cm) elevated 40 cm above the floor. For the test, each animal was placed in the center of the apparatus and the number of head dips, number of entries and time spent in the open arms were scored manually during 5 min.

2.4.3. *Splash test*

The splash test was carried out [42] to assess the depressive-like behavior. Briefly, a 10% sucrose solution was squirted on the dorsal coat of each mouse placed on a polypropylene box and the grooming time was recorded manually during 5 min (nose/face grooming, head washing and body grooming). The grooming behavior is a measurement of motivational and self-care activities, which are decreased in mice with depression-like behavior.

2.5. **Biochemical evaluation**

2.5.1. *Tissue processing*

The HC were separated in two hemispheres in order to perform all biochemical determinations. The right hemispheres were immersed in TRIzol and maintained at -80°C for the quantitative real time polymerase chain reaction (qRT-PCR). The blood was collected in heparinized tubes, centrifuged for 10 min at 12.000 *g* and 4 °C and the plasma fraction was used for determination of RS and thiobarbituric acid reactive species (TBARS) levels.

2.5.2. *Determination of the reactive species (RS) formation*

For the quantification of RS levels in the plasma of mice [43], aliquots of plasma were incubated with 1 mM dichloro-dihydro-fluorescein diacetate (DCHF-DA) and 10 mM Tris-HCl pH 7.4. In the presence of RS, DCFH-DA is oxidized to the fluorescent form dichlorofluorescein (DCF). The fluorescence intensity emission is measured at 520 nm (with 480 nm excitation). The RS levels were expressed as fluorescence units.

2092

2093 *2.5.3. Thiobarbituric acid reactive species (TBARS) assay*

2094 Aliquot of plasma were incubated with 8.1% SDS, 0.8% Thiobarbituric acid and
 2095 acetic acid/HCl (pH 3.4) during 2 h at 95°C [44]. Malondialdehyde (MDA) was used as
 2096 biomarker of lipid peroxidation. Absorbance was measured by spectrophotometry at
 2097 532 nm and the results were expressed as nmol MDA/mL.

2098

2099 *2.5.4. Quantitative real-time polymerase chain reaction (qRT-PCR)*

2100 Total mRNA was extracted in the HC right hemispheres using TRIzol
 2101 (Invitrogen™, Carlsbad, USA) followed by DNase treatment with DNA-free® kit
 2102 (Ambion™, USA) and mRNA quantification. The cDNA was synthesized using High
 2103 Capacity cDNA Reverse Transcription kit (Applied Biosystems™, UK) according to the
 2104 manufacturer's protocol. The amplification was made with UltraSYBR Mix (COWIN
 2105 Bioscience Co., Beijing, China) using the Stratagene Mx3005P and the
 2106 oligonucleotides were obtained from Exxtend Biotecnologia Ltda, Campinas, Brazil.
 2107 Gene expressions were normalized using GAPDH as a reference gene and the
 2108 conditions for the reaction involved 95°C for 15 s, 60 °C for 60 s and 72 °C for 30 s.
 2109 The $2\Delta\Delta CT$ (Delta-Delta Comparative Threshold) method was used to normalize the
 2110 fold change in gene expressions. The following genes were analyzed: *GR* (fwd 5'-ACA
 2111 GCA ACG GGA CCA CCT C-3', rev 5'-ATG GCA TCC CGA AGC TTC -3'), *GSK-3 β*
 2112 (fwd 5'-CGG GAC CCA AAT GTC AAA CT-3', rev 5'-TCC GAG CAT GTG GAG GGA
 2113 TA-3'), *BDNF* (fwd 5'-CCA TAA GGA CGC GGA CTT GTA C-3', rev 5'-AGA CAT GTT
 2114 TGC GGC ATC CAG G-3'), *Nrf2* (fwd 5'-GTC TTC ACT GCC CCT CAT C-3', rev 5'-
 2115 TCG GGA ATG GAA AAT AGC TCC-3'), *synaptophysin* (fwd 5'-TGT GTT TGC CTT
 2116 CCT CTA CTC-3', rev 5'-TCA GTG GCC ATC TTC ACA TC-3'), and *GAPDH* (fwd 5'-
 2117 AGG TCG GTG TGA ACG GAT TTG-3', rev 5'-TGT AGA CCA TGT AGT TGA GGT
 2118 CA-3').

2119

2120 **2.6. Statistical analysis**

2121 All data are presented as mean $\pm$ standard error of the mean (SEM).
 2122 Comparisons in Experiment 1 between corticosterone and treatment groups were
 2123 analyzed by two-way analysis of variance (ANOVA). The Tukey post-hoc test was
 2124 performed for between-group comparisons when ANOVA revealed significant
 2125 differences. Comparisons in Experiment 2 between corticosterone, rapamycin and

treatment groups were analyzed by three-way ANOVA. The Newman-Keuls post-hoc test was performed for between-group comparisons when ANOVA revealed significant differences. Values equal or less than 0.05 ($p \leq 0.05$) were considered statistically significant. The statistical analyzes were performed using Graph Pad Prism version 7.0 for Windows, Graph Pad Software (San Diego, CA, USA).

3. Results

3.1. A single administration of CMI reversed corticosterone-induced depression- and anxiogenic-like behavior without evoking locomotor alteration

In the present study, neither corticosterone administration nor CMI treatment changed the number of crossings and rearings in the OFT (Fig. 2A and 2B). The two-way ANOVA revealed no significant corticosterone $\times$ CMI interaction for the number of crossings ($F_{(1,24)} = 2.11$, $p = 0.16$) and for the number of rearings ($F_{(1,23)} = 0.12$, $p = 0.71$).

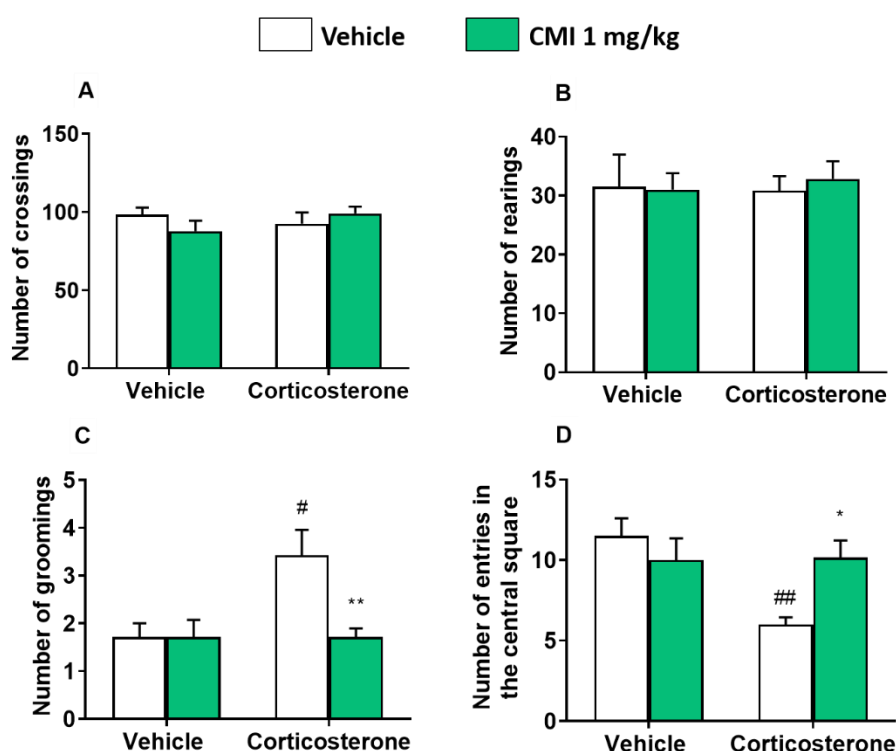


Figure 2. Effect of CMI (1 mg/kg, i.g.) on behavioral tasks in the OFT. (A) number of crossings, (B) number of rearing, (C) number of groomings, and (D) number of entries in the central square. Data were analysed by a two-way ANOVA followed by Tukey post hoc test and are expressed as mean $\pm$ SEM of

6 – 7 independent animals. (#) $p < 0.05$ and (###) $p < 0.01$ when compared to control group. (*) $p < 0.05$ and (**) $p < 0.01$ when compared to corticosterone group.

The anxiolytic-like effect of CMI treatment is depicted in Fig. 2C and 2D. A two-way ANOVA revealed statistically significant corticosterone $\times$ CMI interaction for the number of groomings in the OFT (Fig. 2C; $F_{(1,24)} = 5.61$, $p = 0.03$). The post hoc analysis showed that CMI decreased the number of groomings in corticosterone-treated mice ($p < 0.01$). A single administration of CMI reversed the reduced number of entries in the central square induced by corticosterone administration (Fig. 2D), as observed in the statistically significant corticosterone $\times$ CMI interaction ($F_{(1,20)} = 7.50$, $p = 0.01$) and in the post hoc analysis ($p < 0.05$).

The effect of CMI in the depressive-like behavior induced by corticosterone administration in the splash test is depicted in Fig. 3A. The two-way ANOVA showed a statistically significant corticosterone $\times$ CMI interaction for the grooming time ($F_{(1,20)} = 7.38$, $p = 0.01$). A post hoc analysis showed that CMI reversed the decreased grooming time in corticosterone-treated mice ($p < 0.01$).

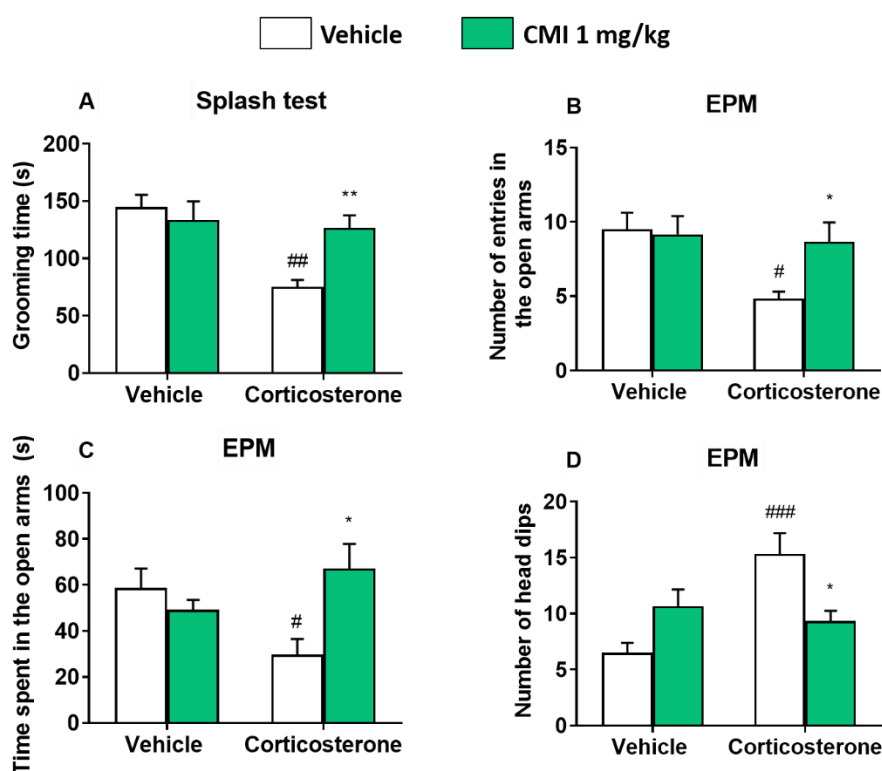


Figure 3. Effect of CMI (1 mg/kg, i.g.) in the splash test and elevated plus maze. (A) grooming time in the splash test, (B) number of entries in the open arms, (C) time spent in the open arms, and (D) number

of head dips in the elevated plus maze apparatus. Data were analysed by a two-way ANOVA followed by Tukey post hoc test and are expressed as mean $\pm$ SEM of 6 – 7 independent animals (#) $p \leq 0.05$, (##) $p < 0.01$ and (###) $p < 0.001$ when compared to control group. (*) $p < 0.05$ and (**) $p < 0.01$ when compared to corticosterone group.

A two-way ANOVA revealed a statistically significant corticosterone $\times$ CMI interaction for the number of entries in the open arms (Fig. 3B; $F_{(1,20)} = 4.25$, $p \leq 0.01$), for the time spent in the open arms (Fig. 3C; $F_{(1,20)} = 8.79$, $p = 0.008$) and for the number of head dips (Fig. 3D; $F_{(1,20)} = 14.13$, $p = 0.001$) in the EPM apparatus. The post hoc analysis showed that CMI increased the number of entries in the open arms ($p \leq 0.05$) and the time spent in the open arms ($p < 0.01$) while decreased the number of head dips ($p \leq 0.05$) in corticosterone-treated mice.

3.2. The antidepressant- and anxiolytic-like effects of CMI in corticosterone treated mice may be dependent on mTOR signaling

The antidepressant- and anxiolytic-like effect of CMI may be dependent on mTOR signaling (Fig. 4), since a single administration of CMI in corticosterone-treated mice, in the presence of rapamycin (mTOR inhibitor), did not elicit the antidepressant- and anxiolytic-like effects previously observed. The results regarding the splash test are depicted in Fig. 4A. A three-way ANOVA showed a statistically significant corticosterone $\times$ CMI $\times$ rapamycin interaction for the grooming time ($F_{(1,40)} = 5.78$, $p = 0.02$). The post hoc analysis showed that corticosterone-treatment induced depressive-like behavior in mice in the absence ($p < 0.01$) and in the presence ($p \leq 0.05$) of rapamycin. However, CMI did not exert the antidepressant-like effect in the presence of rapamycin while this effect was observed in the absence of rapamycin ($p \leq 0.05$).

The results regarding the anxiogenic-like behavior evaluated in the EPM are depicted in Fig. 4B, 4C and 4D. The three-way ANOVA did not reveal a significant corticosterone $\times$ CMI $\times$ rapamycin interaction for the number of entries in the open arms (Fig. 4B), though a significant main effect was found for corticosterone ($F_{(1,40)} = 24.7$, $p = 0.0001$), CMI administration ($F_{(1,40)} = 6.93$, $p = 0.0120$), and corticosterone $\times$ CMI interaction ($F_{(1,40)} = 8.20$, $p = 0.0072$). The post hoc analysis showed that corticosterone administration induced anxiogenic-behavior in mice in the absence ($p <$

0.01) and in the presence ($p < 0.01$) of rapamycin, but CMI treatment was only effective in the absence of rapamycin ($p \leq 0.05$).

The three-way ANOVA revealed no significant interaction for the time spent in the open arms of the EPM apparatus (Fig. 4C), however a main effect for corticosterone was found ($F_{(1,40)} = 6.40$, $p = 0.0072$). The post hoc analysis did not show any significant difference. For the number of head dips (Fig. 4D), no significant interaction was found, however, main effects were found for the corticosterone treatment ($F_{(1,40)} = 13.90$, $p = 0.0006$), CMI administration ($F_{(1,40)} = 9.76$, $p = 0.0033$), rapamycin $\times$ CMI interaction ($F_{(1,40)} = 6.34$, $p = 0.0159$), and corticosterone $\times$ CMI interaction ($F_{(1,40)} = 7.04$, $p = 0.0114$). The post hoc analysis revealed that corticosterone-treatment induced anxiogenic-behavior in mice in the absence ($p \leq 0.05$) and in the presence ($p \leq 0.05$) of rapamycin. A single administration of CMI elicited anxiolytic-like effect in the absence of rapamycin ($p < 0.001$), an effect that was blocked by rapamycin.

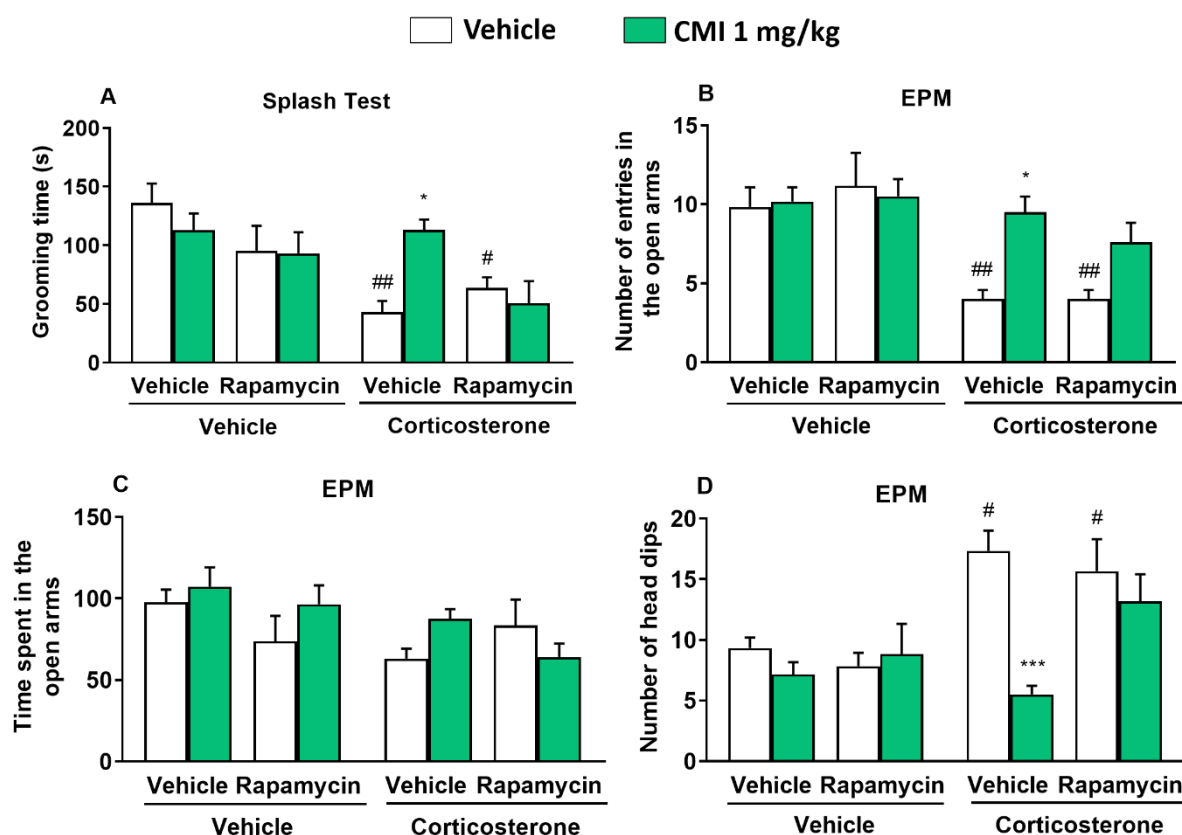


Figure 4. Effect of CMI (1 mg/kg, i.g.) and rapamycin (0,2 nmol/site, i.c.v.) on behavioral tasks of splash test and EPM test. (A) grooming time in the splash test, (B) number of entries in the open arms, (C) time spent in the open arms, and (D) number of head dips in the EPM apparatus. Data were analysed by a

three-way ANOVA followed by Newman-Keuls post hoc test and are expressed as mean $\pm$ SEM of 6 independent animals (#) $p \leq 0.05$ and (###) $p < 0.001$ when compared to control group (vehicle of corticosterone – vehicle of rapamycin – vehicle of CMI). (*) $p \leq 0.05$ and (***) $p < 0.001$ when compared to corticosterone – rapamycin group.

3.3. CMI ameliorated the increased oxidative stress markers in the plasma of corticosterone-treated mice

The two-way ANOVA revealed that CMI treatment ameliorated the increased levels oxidative stress markers induce by corticosterone administration in plasma of mice. As depicted in Fig. 5A, RS were increased in corticosterone treated mice while a single administration of CMI reversed these levels (corticosterone $\times$ CMI interaction, $F_{(1,20)} = 6.03$, $p = 0.02$). The two-way ANOVA did not reveal a statistically significant corticosterone $\times$ CMI interaction for TBARS levels, but a main effect was found for the CMI administration ($F_{(1,21)} = 30.57$, $p < 0.001$) and for the corticosterone-treatment ($F_{(1,21)} = 52.39$, $p < 0.001$). A post hoc analysis showed that CMI *per se* exerted antioxidant effect *in vivo* ($p < 0.001$) and that corticosterone-treatment increased TBARS levels ($p < 0.01$). Additionally, CMI decreased the TBARS levels induced by corticosterone ($p < 0.001$).

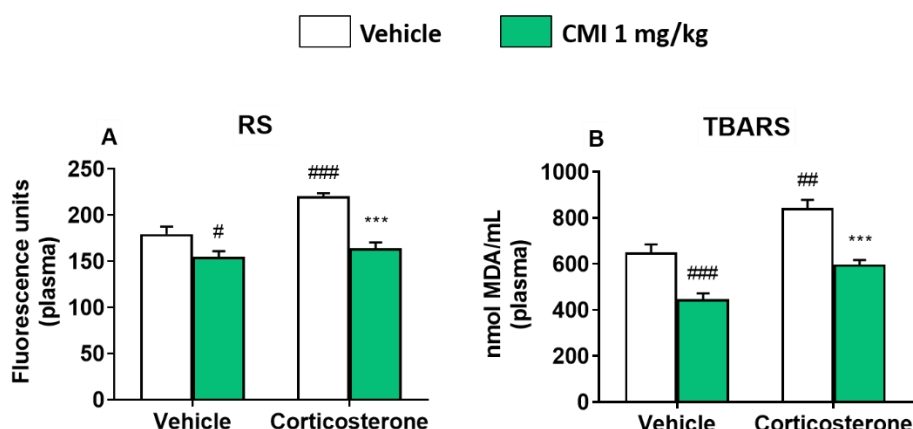


Figure 5. Effect of CMI (1 mg/kg, i.g.) on the levels of (A) RS and (B) TBARS. Data were analysed by a two-way ANOVA followed by Newman-Keuls post hoc test and are expressed as mean $\pm$ SEM of 5 – 6 independent animals (#) $p \leq 0.05$, (##) $p < 0.01$ and (###) $p < 0.001$ when compared to control group. (***) $p < 0.001$ when compared to corticosterone group.

3.4. The modulation of GR, BDNF, synaptophysin, GSK3- β , Nrf2, and IDO mRNA expression may be involved in the antidepressant- and anxiolytic-like effects of CMI

The effects of corticosterone and CMI treatment on gene expression are depicted in Fig. 6. Corticosterone administration downregulated GR expression in the HC when compared to the control group. A two-way ANOVA revealed a statistically significant corticosterone $\times$ CMI interaction for the GR expression in the HC of mice ($F_{(1,20)} = 7.18, p = 0.01$). Corticosterone treatment decreased GR expression ($p < 0.001$) when compared to the control group and CMI reversed it ($p < 0.001$).

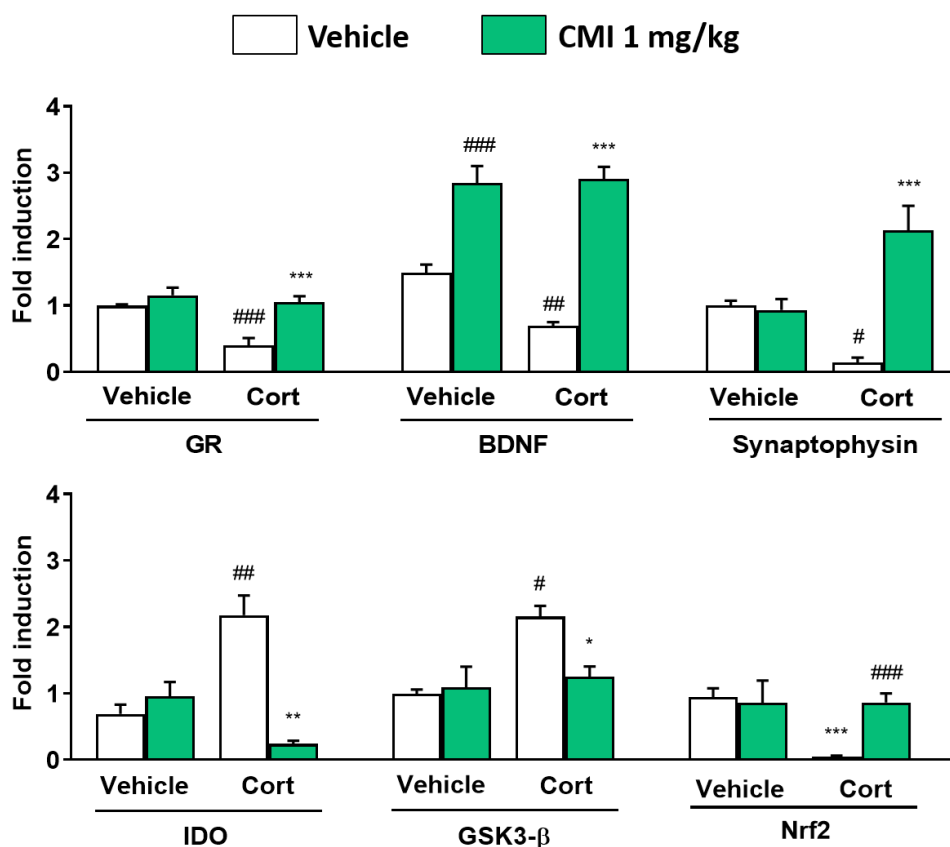


Figure 6. Effect of CMI (1 mg/kg, i.g.) on the expression of selected genes. Data were analysed by a two-way ANOVA followed by Newman-Keuls post hoc test and are expressed as mean $\pm$ SEM of 5 – 6 independent animals (#) $p \leq 0.05$, (##) $p < 0.01$ and (###) $p < 0.001$ when compared to control group. (***) $p < 0.001$ when compared to corticosterone group. Cort: corticosterone; GR: glucocorticoid receptor; BDNF: brain derived neurotrophic factor; GSK3- β : glycogen synthase kinase 3 beta; Nrf2: nuclear factor erythroid 2-related factor 2; IDO: indoleamine 2,3-dioxygenase.

A two-way ANOVA revealed a significant corticosterone $\times$ CMI for the BDNF mRNA in HC of mice interaction ($F_{(1,20)} = 6.37, p = 0.02$). A post hoc analysis showed

that CMI administration in non-stressed mice increased *BDNF* expression ($p < 0.001$). Corticosterone administration decreased *BDNF* expression ($p < 0.01$) when compared to the control group and CMI reversed this decrease ($p < 0.001$).

The two-way ANOVA showed a statistically significant corticosterone $\times$ CMI interaction for *synaptophysin* expression ($F_{(1,20)} = 24.70$, $p < 0.001$). The post hoc analysis revealed that corticosterone treatment downregulated *synaptophysin* expression in the HC of corticosterone-treated mice ($p \leq 0.05$) and a single administration of CMI reversed it ($p < 0.001$).

The two-way ANOVA showed a significant corticosterone $\times$ CMI interaction ($F_{(1,20)} = 6.66$, $p = 0.02$) for the *GSK3- β* levels in the HC of mice. The post hoc test indicated that corticosterone-treated mice showed upregulated *GSK3- β* ($p < 0.01$), while CMI treatment reversed it ($p < 0.01$).

HC from stressed mice presented downregulated *Nrf2* expression ($p \leq 0.05$) and a single administration of CMI ($p \leq 0.05$) abolished this effect (corticosterone $\times$ CMI; $F_{(1,20)} = 5.35$, $p = 0.03$).

Further, a two-way ANOVA revealed a statistically significant corticosterone $\times$ CMI interaction for *IDO* expression ($F_{(1,20)} = 31.48$, $p < 0.001$). Corticosterone-treated mice showed upregulated *IDO* expression in the HC ($p < 0.001$) and the single administration of CMI reversed this effect ($p < 0.001$).

4. Discussion

The present study showed for the first time that a single administration of CMI was able to reverse the behavioral response to corticosterone administration in the OFT, splash test, and EPM test, and that these effects may be dependent on the mTOR signaling (Fig. 7). Of note, the antidepressant- and anxiolytic-like effects of CMI may also depend on the modulation of peripheral and central oxidative stress and neuroinflammation. Here, we reported that pre-treatment of mice with the mTOR inhibitor rapamycin blocked the antidepressant- and anxiolytic-like effects of CMI in corticosterone-treated mice. Additionally, we observed that CMI reversed the increased levels of oxidative stress biomarkers by decreasing RS and TBARS levels in the plasma of corticosterone-treated mice. Corroborating with the results, CMI was also able to restore the mRNA expression of *GR*, *BDNF*, *synaptophysin*, *GSK3- β* , *Nrf2*, and *IDO* in the HC of stressed mice.

Administration of corticosterone is a validated preclinical model of depression. In addition to inducing behavioral alterations, corticosterone also induces neurochemical alterations. Indeed, decreased hippocampal neurogenesis has been found in corticosterone-treated mice and in depressive patients [45, 46]. The behavioral improvement following CMI administration may depend on the modulation of neurogenesis. It has been acknowledged that BDNF is crucial for neuronal integrity and survival [47] and for synaptic plasticity through TrkB pathway [48, 49]. BDNF expression is dependent on GC-binding to GR [50] and high levels of circulant GC leads to GR resistance [27], and, consequently, decreases *BDNF* expression. BDNF-binding to TrkB activates the mTOR signaling that results in the translation of several proteins involved with the synaptogenesis, including synaptophysin [51]. At the time CMI presented antidepressant- and anxiolytic-like effects, is also increased the expression of *GR*, *BDNF*, and *synaptophysin* expression in the HC of corticosterone-treated mice. Therefore, it is possible that the behavioral effects of CMI may be a consequence of its ability to improve neurogenesis, synaptogenesis, and synaptic plasticity processes. Nonetheless, more studies are warranted to support this hypothesis.

Alongside with the above-mentioned mechanisms, high levels of circulating GC induces an inflammatory response through activation of TLR4, which promotes the activation of GSK3- β [12, 24]. Once activated, GSK3- β prevents the translocation of Nrf2 to the nucleus, thus, reducing the transcription of antioxidant enzymes, such as HO-1 [30]. Moreover, GSK3- β also promotes the translocation of NFkB to the nucleus, thereby, increasing the inflammatory response by upregulating the expression of pro-inflammatory cytokines, such as TNF- α and IL-1 β [24]. The increased levels of pro-inflammatory cytokines can induce the activation of iNOS, the production of RS, and the activation of IDO, further contributing to oxidative stress, neuroinflammation, and impaired neurotransmission [52]. It is known that activated GSK3- β phosphorylates GR, high levels of pro-inflammatory cytokines can suppress GR through activation of p38 MAPK, and increased concentrations of RS can oxidize GR [26]. Altogether, these alterations in the GR function and signaling can contribute to GR resistant and, consequently, hyperactivation of HPA axis through inhibition of the negative feedback.

In this study, we reported that the repeated administration of corticosterone upregulated the expression of GSK3- β and *IDO*, and downregulated the expression of Nrf2 in the HC. In addition, it also increased the levels of RS and TBARS in the plasma

of mice. Inasmuch, a single administration of CMI restored the expression levels of *GSK3-β*, *IDO*, and *Nrf₂*, and reduced the levels of RS and TBARS in the plasma of corticosterone-treated mice. These observations corroborate with the antioxidant activity of this organoselenium compound previously described [9, 11, 12].

The mTOR is a serine/threonine kinase classified in two different protein complexes – TORC1 and TORC2. Rapamycin allosterically inhibits the TORC1 activity by blocking interactions with regulatory proteins and conformational changes [22, 53]. The mTOR signaling pathway in the CNS is related to several main physiological processes such as neurogenesis, synaptogenesis, and synaptic plasticity [22]. The mTOR signaling pathway is found impaired in depressive patients and this pathway can be inactivated by *GSK3-β* [22, 54]. The modulation of mTOR pathway is intrinsic to the fast action new antidepressants, such as esketamine [55, 56].

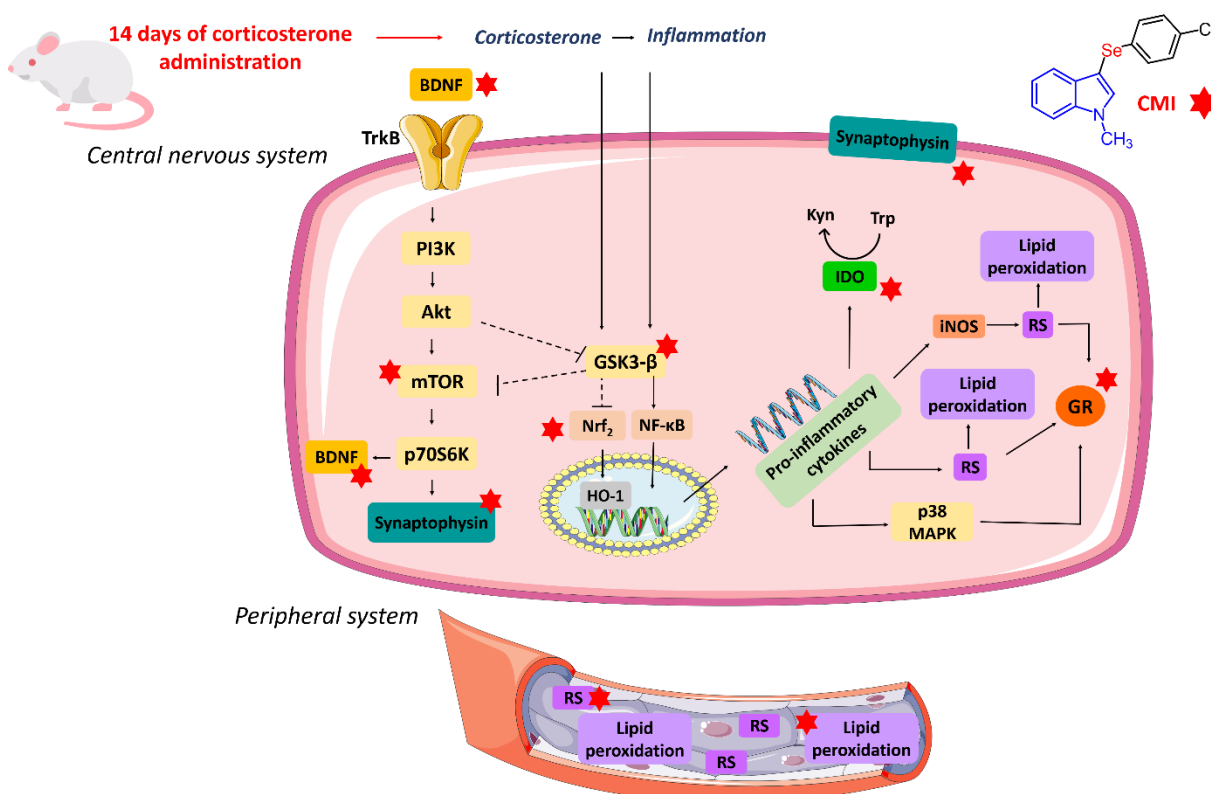


Figure 7. Summary of the effects of CMI treatment in corticosterone-treated mice. A single administration of CMI after 14 days of corticosterone administration downregulated the mRNA expression of *GSK3-β* and *IDO* and upregulated the mRNA expression of *GR*, *BDNF*, *synaptophysin*, and *Nrf₂*. Furthermore, the pre-treatment of rapamycin prevented the effects of CMI on behavioral alterations. Altogether, these results indicate that the mechanism of action of CMI may depend on mTOR signaling pathway.

5. Conclusions

In this study, we gathered evidence that the antidepressant- and anxiolytic-like effects of CMI may be dependent, at least in part, on the mTOR signaling pathway. Probably, through the mTOR signaling and consequently modulation of oxidative stress and inflammatory pathways, CMI is able to reverse depressive- and anxiogenic-like behaviors induced by corticosterone treatment in mice. Meantime, CMI downregulated *GSK3-β* and *IDO* expression and upregulated *GR*, *BDNF*, *synaptophysin*, and *Nrf2* mRNA in the HC and reduced RS and lipid peroxidation levels in the plasma of corticosterone-treated mice. However, more studies are needed to delve into the molecular mechanisms responsible for the multitarget effects of CMI.

Ethical approval

All procedures were approved by the Committee on the Care and Use of Experimental Animal Resources at the Federal University of Pelotas, Brazil (2208-2018) and comply with the Animal Research: Reporting in Vivo Experiments (ARRIVE) guidelines, items 5 to 13 [57].

Author contribution

AMC and DAL designed the study and protocol, performed the experiments, analysed the data and wrote the manuscript. MD, TAS, PTB, and MSS performed the experiments and contributed to the manuscript writing. BV and EJJ synthesized CMI. FKS, TC and LS supervised the experiments and revised the scientific content of the manuscript. All authors critically reviewed the content and approved the final version for publication.

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4.6 Capítulo 6 – The selenocompound 3-[(4-chlorophenyl)selenyl]-1-methyl-1*H*-indole ameliorates long-lasting affective, cognitive, and biochemical alterations in endotoxin-induced sepsis survivor mice

Manuscrito preparado de acordo com as normas da revista *Brain, Behavior and Immunity*

Neste capítulo, investigamos se o CMI seria capaz de melhorar as alterações comportamentais presentes em camundongos que sobreviveram à sepse. Sabe-se que aproximadamente 60% dos pacientes que sobrevivem à sepse apresentam alterações psicológicas de longa duração, e, apesar disso, pouco se tem estudado sobre possíveis estratégias terapêuticas para o controle desses sintomas. Na tentativa de preencher essa lacuna, nós mostramos neste trabalho que o CMI melhorou o comportamento tipo-depressivo, tipo-ansioso e o prejuízo cognitivo presentes 30 dias após a indução da sepse (através da administração intraperitoneal de LPS na dose de 5 mg/kg). Além disso, o CMI (i) reduziu a permeabilidade da BBB avaliada através do extravasamento do corante Evans blue; (ii) diminuiu alterações oxidativas periféricas, avaliadas através dos níveis de ERO em neutrófilos, linfócitos e monócitos; (iii) normalizou o número de neutrófilos circulantes; (iv) reduziu os níveis plasmáticos de AST, ALT, ureia e creatinina; (v) reduziu os níveis de ERO/N e TBARS em córtex pré-frontais e hipocampus; e (vi) restaurou a expressão do *NFκB*, *TNF-α*, *IL-1β*, *iNOS*, *COX-2* e *BDNF* em córtex pré-frontais e hipocampus de camundongos que sobreviveram a sepse. Com este trabalho abrangente que analisa parâmetros comportamentais e bioquímicos, o CMI surge como uma estratégia promissora para melhorar as alterações comportamentais duradouras que se manifestam em pacientes que sobreviveram a sepse, a fim de melhorar sua qualidade de vida.

2599 **The selenocompound 3-[(4-chlorophenyl)selanyl]-1-methyl-1*H*-indole**
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Abstract

Only in the last decade the long-term consequences of sepsis started to be studied and even less attention has been given to the treatment of psychological symptoms of sepsis survivors. It is estimated that 60% of sepsis survivors have psychological disturbances, including depression, anxiety, and cognitive impairment. Although the causative factors remain largely poorly understood, blood-brain barrier (BBB) disturbances, neuroinflammation, and oxidative stress have been investigated. Therefore, we sought to explore if the immunomodulatory and antioxidant selenocompound 3-[(4-chlorophenyl)selenanyl]-1-methyl-1*H*-indole would be able to ameliorate long-term behavioral and biochemical alterations in sepsis survivors male Swiss mice. CMI treatment (1 mg/kg, given orally for seven consecutive days) attenuated depression- and anxiogenic-like behavior and cognitive impairment present one month after the induction of sepsis (lipopolysaccharide, 5 mg/kg intraperitoneally). Meantime, CMI treatment modulated the number of neutrophils and levels of reactive species in neutrophils, lymphocytes, and monocytes. In addition, peripheral markers of liver and kidneys dysfunction (AST, ALT, urea, and creatinine) were reduced after CMI treatment. Noteworthy, CMI ameliorated BBB dysfunction induced by sepsis, modulating the expression of inflammation-associated genes (*NFκB*, *IL-1β*, *TNF-α*, *IDO*, *COX-2*, *iNOS*, and *BDNF*) and markers of oxidative stress (reactive species, nitric oxide, and lipid peroxidation levels) in the prefrontal cortices and hippocampi of mice. Through this comprehensive study analyzing behavioral and biochemical parameters, CMI arises as a promising strategy aimed to manage the long-lasting behavioral alterations of sepsis survivors to improve their quality of life.

Keywords: Selenocompound; sepsis; lipopolysaccharide; behavior; oxidative stress; inflammation.

1. Introduction

Sepsis is defined as a life-threatening organ dysfunction characterized by a systemic inflammatory response (SIRS) to a microbial infection, with SIRS defined by the presence of at least two of the following parameters: tachycardia, tachypnoea, pyrexia or hypothermia, and leukocytosis, leukopenia, or neutrophilia (Bone et al., 1992; Levy et al., 2003). It has been acknowledged that sepsis is the quintessential medical disorder of the 21st century (Deutschman and Tracey, 2014), with 31.5 million cases and 5.3 million deaths being reported annually (Fleischmann et al., 2016). Nonetheless, owing to fast recognition and improved therapeutic and supportive care, over 1.3 million patients survive sepsis in the United States alone each year (Stevenson et al., 2014), but often experience persistent physical, mental, and cognitive symptoms, which are described as post-intensive care syndrome (PICS) (Needham et al., 2012). It is estimated that 60% of sepsis survivors have psychological disturbances (Nelson et al., 2004), including depression, anxiety (Davydow et al., 2013), and cognitive impairment (Iwashyna et al., 2010).

Although the causative factors remain largely poorly understood, blood-brain barrier (BBB) disturbances (Nwafor et al., 2019), neuroinflammation (Anderson et al., 2015; Huang et al., 2019), and oxidative stress (Berg et al., 2011; Denstaedt et al., 2018) have been investigated in the behavioral consequences of sepsis. During peripheral inflammation, cytokines and other immunoactive substances released from circulating immune cells of peripheral blood, astrocytes, microglia, and leukocytes contribute to a dysfunctional BBB (Banks, 2015). This facilitates the trafficking of peripheral immune cell and cytokines into the brain, which collectively promote neuroinflammation and oxidative stress (Andonegui et al., 2018; Singer et al., 2016). Mechanistically, pathogen-associated molecular patterns (PAMPs), danger-associated molecular patterns (DAMPs), and cytokines activate Toll-like receptors (TLRs) and cytokine receptors in endothelial cells, microglia, and astrocytes to activate the nuclear factor kappa-light-chain-enhancer of activated B cells (*NFκB*) (Madrigal et al., 2002). Activated *NFκB* translocates to the nucleus to promote the expression of inflammatory cytokines, inducible nitric oxide (NO[•]) synthase (*iNOS*), and cyclooxygenase-2 (*COX-2*). Inflammatory cytokines further induce indoleamine-2,3-dioxygenase (*IDO*), contributing to neurotoxicity (Wichers et al., 2005) and depression-like behavior (O'Connor et al., 2009). NO[•] is produced following *iNOS* activation, and together with other RS (e.g. hydrogen peroxide (H₂O₂) and anion

superoxide ($O_2^{\cdot-}$)), lead to proteins, lipids, and nucleic acids damage (Halliwell, 2007). In addition, proinflammatory cytokines and prostaglandins (PG) formed by COX-2 reduce the activity of brain-derived neurotrophic factor (BDNF), contributing to impaired neurogenesis and cognition (Hein and O'Banion, 2009; Tong et al., 2008). Indeed, neuroinflammation and oxidative stress have been implicated in the pathophysiology of depression (Bakunina et al., 2015), anxiety (Bouayed et al., 2009), and cognitive decline (Agostinho et al., 2010).

Only in the last decade the long-term consequences of sepsis started to be studied (Hotchkiss et al., 2016), and even less attention has been given to the treatment of psychological symptoms of sepsis survivors. We have recently showed that the selenocompound 3-[(4-chlorophenyl)selenyl]-1-methyl-1*H*-indole (CMI) presents antioxidant activity *in vitro* (Vieira et al., 2017) and modulates central oxidative stress and inflammation to reverse depression- and anxiolytic-like behaviors and cognitive impairment induced by lipopolysaccharide (LPS) (Casaril et al., 2017a; Casaril et al., 2019b), acute restraint stress (Casaril et al., 2019a), and breast tumor (Casaril et al., 2020) in mice. In addition, CMI potently inhibits inflammation-associated oxidants, such as hypochlorous acid (HOCl), peroxynitrite ($ONOO^-$), and H_2O_2 , protecting extracellular matrix (ECM) proteins against oxidative damage (Casaril et al., 2017b). Consequently, in the present study, we hypothesized that the immunomodulatory and antioxidant compound CMI would be able to ameliorate long-lasting behavioral and biochemical alterations in post-septic mice. Exploring the effects of CMI may shed some light into the development of a novel adjuvant therapeutic strategy targeting neuroinflammation and oxidative stress to improve the quality of life of sepsis survivors.

2. Materials and Methods

2.1. Reagents

LPS from *E. coli* (L-3129, serotype 0127:B8) was purchased from Sigma Chemical Co, USA. CMI (Fig. 1B) was prepared and characterized at the Laboratory of Clean Organic Synthesis at the Federal University of Pelotas (Vieira et al., 2015). The RNA extraction reagent was obtained from Ambion (Life Technology, USA). The oligonucleotides were synthesized by Exxtend Biotecnologia, Ltda (Campinas-SP, Brazil). All other chemicals were of analytical grade and obtained from standard commercial suppliers. LPS was diluted in saline and administered intraperitoneally

(i.p.) at a dose of 5 mg/kg. CMI was dissolved in canola oil and administered intragastrically (i.g.) at 1 mg/kg. Observers blind to the drug treatments recorded all behavioral tests and analyzed the results.

2.2. Animals

Adult male Swiss mice (25-30 g) provided by the Animal Facility of the Federal University of Pelotas were used. Only male mice were used because males are more susceptible to sepsis than females (Angele et al., 2014). Mice were housed in temperature (22 ± 1 °C)- and humidity (45–55%)-controlled environment with a 12/12-h light/dark cycle (light on at 07:00 a.m.). Food and water were available *ad libitum*. Six animals were randomly allocated into each experimental group and all behavioral tests were carried out between 09:00 a.m. and 05:00 p.m. All procedures were approved by the Committee on the Care and Use of Experimental Animal Resources at the Federal University of Pelotas, Brazil (8331) and comply with The Animal Research: Reporting in Vivo Experiments (ARRIVE) guidelines, items 5 to 13 (Kilkenny et al., 2010).

2.3. Experimental design

Experiment 1: The first cohort of mice was used to confirm LPS-induced sepsis in Swiss male mice (Fig. 1A). After the LPS 5 mg/kg (or vehicle) injection, the overall health of mice was monitored every two hours, and the observations were used to classify the sepsis severity (murine sepsis score). The body temperature was assessed 2 and 24 h after the LPS injection. After the last body temperature measurement, mice were euthanized for the collection of peripheral blood for cell count and for determination of biomarkers of liver and kidney dysfunction. Hence, we validated that our protocol induced sepsis in mice by using four independent measurements: sepsis score, body temperature, blood cell count, and organ dysfunction.

Experiment 2: The second cohort of mice was used to investigate the effects of CMI on LPS-induced sepsis (Fig. 1B). One day prior to the LPS injection, mice were exposed to the two-bottle paradigm in order to assess the sucrose preference at baseline. The day mice received the LPS (or vehicle) injection was considered as day 0. After the LPS challenge, the overall health of mice was monitored every two hours for the murine sepsis score. From day 24 to day 30, mice were treated with CMI (or

canola oil). On day 28, mice were submitted to the open field test (OFT), which was also used as the habituation period for the novel object recognition test (NORT). Next, mice were submitted to the marble burying test, followed by access to the two-bottles paradigm for the sucrose preference test. On day 29, mice were placed in the open field apparatus for the training phase of the NORT, followed by the TST and FST. On day 30, mice were evaluated in the NORT, followed by the Y-maze test and the elevated plus-maze (EPM) test. After the behavioral testing, mice were euthanized for the removal of peripheral blood, prefrontal cortices, and hippocampi.

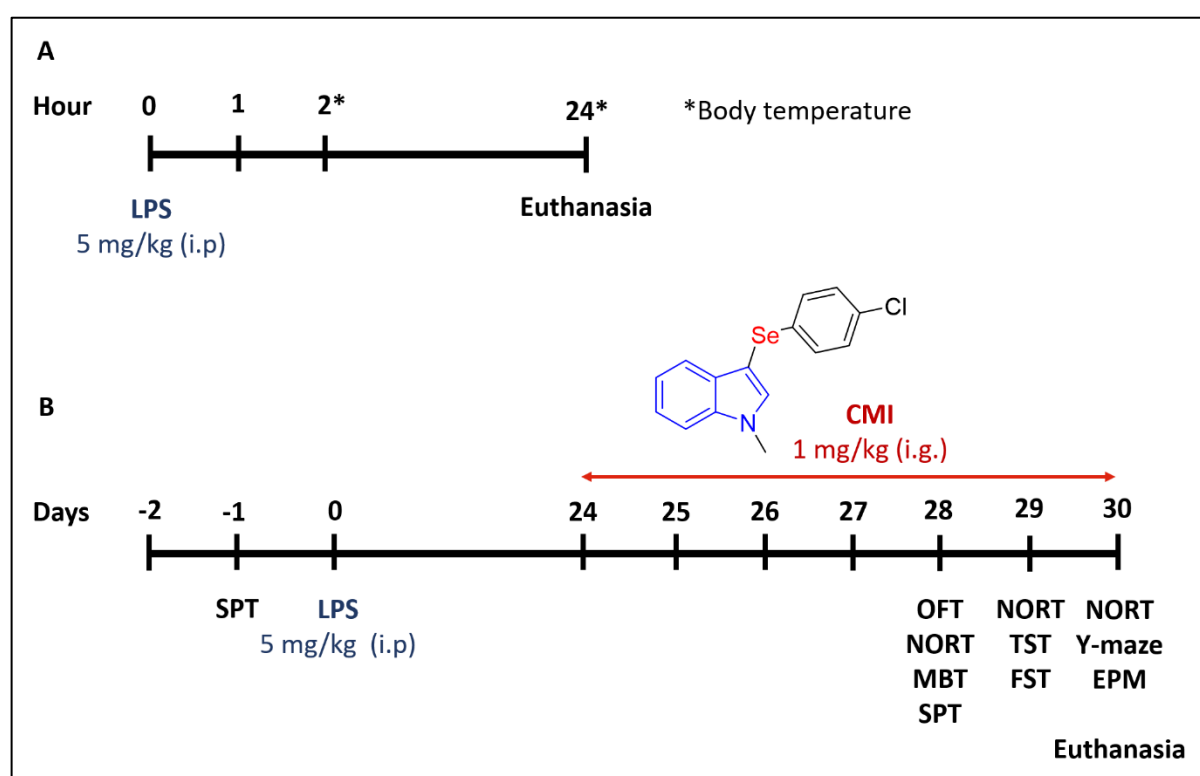


Figure 1. Experimental design of LPS-induced sepsis model. (A) Experimental timeline used for experiment 1 to validate the LPS-induced sepsis. (B) Experimental timeline used for experiment 2 to analyse the effects of CMI on the long-lasting effects of LPS-induced sepsis. SPT: sucrose preference test. OFT: open field test. NORT: novel object recognition test. MBT: marble burying test. TST: tail suspension test. FST: forced swimming test. EPM: elevated plus maze test. CMI: 3-[(4-chlorophenyl)selenyl]-1-methyl-1*H*-indole.

It was previously reported that 5 mg/kg of LPS induces long-lasting behavioral and neurochemical alterations in mice (Anderson et al., 2015; Qin et al., 2007). Based on our previous studies, the behavioral tests were conducted 30 min after the administration of CMI and a maximum of three behavioral tests/day were performed

(Bampi et al., 2019; Casaril et al., 2017a). CMI was administered for 7 days as this schedule of administration was used to test the antidepressant-like, immunomodulatory, and antioxidant effects of CMI on tumor bearing mice (Casaril et al., 2020). Mortality occurred in approximately 10% of animals up to 7 days following induction of sepsis.

2.4. Murine sepsis score

The murine sepsis score was performed according to (Shrum et al., 2014) and it was used to evaluate the clinical condition of mice to validate the experiment. Mice were carefully analyzed for seven variables: appearance, level of consciousness, activity, response to a stimulus, eyes, respiration rate, and respiration quality. Each of these variables received a score between 0 and 4. Mice were euthanized if the murine sepsis score at any given time point was greater than 21, or if the points ascribed to respiratory rate or quality increased by more than 3.

2.5. Behavioral tests

2.5.1. Locomotor activity

The locomotor activity was assessed in the open field test (OFT) (Walsh and Cummins 1976). The apparatus consisted of a wooden box (30 × 30 × 15 cm) divided into nine quadrants of equal areas. The number of squares crossed with all paws (crossings), as well as the number of rearing episodes, were registered during a 5-min session.

2.5.2. Depression-like behavior

2.5.2.1. Tail suspension test (TST)

Mice that were both acoustically and visually isolated from each other were suspended 50 cm above the floor by adhesive tape placed approximately 1 cm from the tip of their tail. During the last 4 min of a 6 min session, the immobility time (defined as the absence of the escape attempt behavior) was observed. A decrease in immobility time is indicative of an antidepressant-like effect (Steru et al., 1985).

2.5.2.2. Forced swimming test (FST)

Mice were individually forced to swim in an open cylindrical container (diameter: 10 cm, height: 25 cm) filled with water (depth: 19 cm) maintained at 25 ± 1 °C. The

total duration of immobility was recorded during the last 4 min of a 6 min session. Each mouse was judged to be immobile when it ceased struggling and remained floating motionless in the water, making only those movements necessary to keep its head above water. A decrease in immobility time is indicative of an antidepressant-like effect (Porsolt et al., 1977).

2.5.2.3. Sucrose preference test

Sucrose preference was assessed by providing mice access to two identical water bottles, one containing normal drinking water and the other containing a 1% sucrose solution. Mice rapidly developed a preference for the sweetened solution as measured by weighing the bottles (percent sucrose preference = $[\text{sucrose consumed}] / [\text{total sucrose} + \text{water consumed}] \times 100$). Preference was established over a period of 24 h, one day prior to the LPS injection. The influence of the treatments over the sucrose preference was evaluated by giving mice access to the two-bottle paradigm again on day 29, and the bottles were weighed on day 30.

2.5.3. Anxiety-like behavior

2.5.3.1. Elevated plus-maze (EPM)

The anxiolytic-like behavior of mice was evaluated in the elevated plus-maze test. The elevated plus-maze consists of a plus-shaped apparatus with two open and two enclosed arms (arm's length: 100 cm; arm's width: 10 cm) connected by a central platform elevated 40 cm above the floor (Boulle et al., 2014). For the test, each mouse was placed in the center of the apparatus, and the number of entries and time spent in the open arms were scored manually in a 5 min session.

2.5.3.2. Marble burying

For the marble burying test, eight glass marbles ($\varnothing$ 1 cm) were evenly spaced on regular bedding (Broekkamp et al., 1986). Each mouse was allocated to a marble-containing cage and allowed 30 min to explore. After returning the animals to their home cages, the marbles were counted. A marble was considered buried when 2/3 or more of its size was covered with the bedding.

2.5.4. Cognition

2.5.4.1. Novel object recognition test (NORT)

The NORT was performed in the same open field apparatus, as previously described (Rosa et al., 2003). Plastic objects with different shapes and composed only by primary different colors but with the same size were used. The apparatus and the objects were cleaned after each animal testing with a 70% ethanol solution. The task consisted of habituation (10 min), training (10 min), and testing sessions (5 min). In the first session (day 28), mice were allowed to freely explore the arena without any object, and then returned to their home cage. After 24 h, the training session took place, and animals were exposed to two equal objects (object A). The exploration time corresponding to an animal's nose touch or getting close the object (a distance of less than 2 cm) was recorded with two stopwatches (climbing or sitting on the object was not consider exploration). The test session was carried out 24 h after the training session (day 30), in order to assess long-term memory. Mice were placed back in the behavioral chamber and one of the familiar objects (object A) was replaced by a novel object (object B). The time spent exploring the familiar and the novel objects was recorded. The discrimination index was calculated following the formula: Object recognition (index) = $[(T_{\text{novel}} / (T_{\text{novel}} + T_{\text{familiar}}))] \times 100$.

2.5.4.2. Y-maze

Short-term working memory was assessed by recording spontaneous alteration behavior in Y-maze (Sarter et al., 1988). Y-maze is a three-arm horizontal maze (40 cm long and 3 cm wide with walls 12 cm high) in which the three arms are symmetrically separated at 120°. Each mouse was placed at the distal end of one arm and allowed to explore the apparatus during an 8 min session. The sequence of arm entries was recorded manually. The alteration was defined as successive entries into the three arms on overlapping triplet sets. The percentage of spontaneous alternation behavior was calculated according to the following equation: (%) alteration = $[(\text{number of alternations}) / (\text{total arm entries} - 2)] \times 100$.

2.6. Biochemical determinations

Following the behavioral assessment, mice were anesthetized (inhalation of isoflurane) before blood collection by cardiac puncture. After that, mice were killed by cervical dislocation followed by brain removal. The blood was collected in fetal bovine serum (FBS) for the flow cytometer analysis and in heparinized tubes for the determination of markers of liver and kidney dysfunction. The prefrontal cortices and

hippocampi were separated into two hemispheres in order to submit each sample to all biochemical determinations (Casaril et al., 2017a). The right hemispheres were immersed in TRIzol, maintained at -80 °C and were submitted to the quantitative real-time polymerase chain reaction (qRT-PCR). The left hemispheres were homogenized in 50 mM Tris-HCl, pH 7.4 (1:10, w/v). The homogenate was centrifuged at $2500 \times g$ for 10 min at 4 °C, and the supernatant fraction was used for the determination of the levels of reactive oxygen species (RS), nitric oxide metabolites (NO_x), and lipid peroxidation. A different set of mice was used for the determination of Evans blue dye extravasation.

2.6.1. Peripheral measurements

2.6.1.1. Markers of liver and kidney dysfunction

The levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), urea, and creatinine were determined by commercially available kits according to the manufacture's instruction (LABTEST).

2.6.1.2. Blood cell count

Blood cell count was performed using ethylenediaminetetraacetic acid (EDTA) as anticoagulant. Erythrocyte and leukocyte containers were analyzed by the electronic counter Sysmex pocH-100iV Diff™. Differential leukocyte and cell morphology evaluations were performed on Romanowski dye-stained blood smears.

2.6.1.3. Detection of RS via flow cytometry

Attune® Acoustic Focusing Flow Cytometer (Applied Biosystems) was used to detect the cells population. The removal of cell debris and other cellular types was based on the FSC x SSC scatter plots and they were eliminated by staining cells with Hoechst 33342 at concentration of 16.2 µM. A total of 20.000 nucleated cells per sample with flow of 200 event/s were analyzed. Only viable cells (quantified by propidium iodide, PI) were used in the study.

The levels of RS were determined by using the fluorescent dye 2',7'-dichlorodihydro-fluorescein diacetate (DCFH-DA; at a final concentration of 1 µM), which emits green fluorescence when oxidized by intracellular RS. The median intensity of green fluorescence for viable cells was used.

2.6.2. Neurochemical measurements

2.6.2.1. Assessment of blood brain barrier (BBB) integrity

The Evans blue extravasation method was used to evaluate BBB integrity. Briefly, Evans blue dye 2% (in saline) was given i.p. at 4 ml/kg 3 h before euthanasia. Mice were perfused with normal saline to wash residual dye from the blood vessels. Brain samples (excluding the cerebellum) were collected and homogenized in 50% trichloroacetic acid. Samples were centrifuged at $10000 \times g$ for 10 min and the supernatant was diluted 1:3 v/v with 95% ethanol. The infiltrated Evans blue dye was quantified at 680 nm (with 620 nm excitation). Data were expressed as $\mu\text{g}/\text{mg}$ of tissue.

2.6.2.2. Gene expression

Total mRNA was extracted from the prefrontal cortices and hippocampi right hemispheres using TRIzol (Invitrogen™, Carlsbad, USA) followed by DNase treatment with DNA-free® kit (Ambion™, USA) and mRNA quantification. The cDNA synthesis was performed using High Capacity cDNA Reverse Transcription kit (Applied Biosystems™, UK) according to the manufacturer's protocol. The amplification was made with UltraSYBR Mix (COWIN Bioscience Co., Beijing, China) using the Stratagene Mx3005P and the oligonucleotides were obtained from Exxtend Biotechnologia Ltda, Campinas, Brazil. Gene expressions were normalized using glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as a reference gene and the conditions for the reaction involved 95°C for 15 s, 60 °C for 60 s and 72 °C for 30 s. The $2^{\Delta\Delta\text{CT}}$ (Delta-Delta Comparative Threshold) method was used to normalize the fold change in gene expressions. The following genes were analyzed: *NF-κB* (fwd 5'-GCT TTC GCA GGA GCA TTA AC-3', rev 5'-CCG AAG CAG GAG CTA TCA AC-3'), interleukin-1 beta (*IL-1β*; fwd 5'-GCT GAA AGC TCT CCA CCT CAA TG-3', rev 5'-TGT CGT TGC TTG GTT CTC CTT G-3'), tumor necrosis factor alpha (*TNF-α*; fwd 5'-CAT CTT CTC AAA ATT CGA GTG ACA A-3', rev 5'-TGG GAG TAG ACA AGG TAC AAC CC-3'), *IDO* (fwd 5'-AAT CAA AGC AAT CCC CAC TG-3', rev 5'-AAA AAC GTG TCT GGG TCC AC-3'), *COX-2* (fwd 5'-CAG ACA TAA ACT GCG CCT T -3', rev 5'-GAT ACA CCT CTC CAC CAA TGA CC -3'), *iNOS* (fwd 5'-GTG GTG ACA AGC ACA TTT GG-3', rev 5'-AAG GCC AAA CAC AGC ATA CC-3'), *BDNF* (fwd 5'-CCA TAA GGA CGC GGA CTT GTA C-3', rev 5'-AGA CAT GTT TGC GGC ATC CAG G-3'), and *GAPDH* (fwd 5'-AGG TCG GTG TGA ACG GAT TTG-3', rev 5'-TGT AGA CCA TGT AGT TGA GGT CA-3').

2.6.2.3. *Reactive oxygen species (RS) levels*

For the determination of RS levels in the prefrontal cortices and hippocampi of mice, aliquots of the homogenate supernatant were incubated with 1 mM DCHF-DA and 10 mM Tris-HCl pH 7.4. The oxidation of DCFH-DA to fluorescent dichlorofluorescein (DCF) is measured for the detection of intracellular RS (Loetchutin et al., 2005). The DCF fluorescence intensity emission was recorded at 520 nm (with 480 nm excitation) and RS levels were expressed as fluorescence units.

2.6.2.4. *Metabolites of nitric oxide (NO_x)*

The levels of the nitric oxide (NO) metabolites, NO_x, were determined by the Griess reaction as an indicator of nitrate/nitrite production (Lima-Junior et al. 2013). Briefly, aliquots of the homogenate were incubated with an equal volume of Griess reagent for 5 min at 25°C, and the nitrite concentration was determined by measuring the optical density at 550 nm in reference to a standard curve of NaNO₂ solution. Results were expressed as μmol NO_x/g tissue.

2.6.2.5. *Lipid peroxidation*

The lipid peroxidation in the prefrontal cortices and hippocampi of mice was measured by the formation of thiobarbituric acid reactive species (TBARS) during an acid-heating reaction (Ohkawa et al., 1979). Aliquots of the homogenate supernatant were incubated with 8.1% SDS, 0.8% TBA and acetic acid/HCl (pH 3.4) at 95 °C for 2 h. Malondialdehyde (MDA) was used as a biomarker of lipid peroxidation. Absorbance was measured at 532 nm, and the results were expressed as nmol MDA/g tissue.

2.7. *Statistical analysis*

All experimental data are presented as mean ± standard error of the mean (SEM). A two-way ANOVA with time as a repeated measure and Sidak's post hoc test were used to analyze the body temperature in experiment 1. An unpaired *t*-test was used to analyze the levels of AST, ALT, urea, creatinine, total leukocyte count, and percentage of cells in experiment 1. A two (± LPS) by two (± treatment) analysis of variance (ANOVA) was used for the analysis of experiment 2 analyzing the effects of CMI on LPS-induced sepsis. When ANOVA revealed a significant interaction, Tukey's post hoc test was used for between-group comparisons. Probability values less than 0.05 ($p \leq 0.05$) were considered statistically significant. The statistical analysis was

accomplished using GraphPad Prism version 7.0 for Windows, GraphPad Software (San Diego, CA, USA).

3. Results

3.1. LPS-treated mice showed increased murine sepsis score, organ dysfunction, and altered blood cell count 24 h after the injection

Initially, we wanted to confirm that 5 mg/kg of LPS would induce sepsis in Swiss male mice (experiment 1). First of all, we classified each LPS-treated mouse according to the murine sepsis score (Shrum et al., 2014) over a period of 24 h after the injection (Figure 2A). The severity of the disease gradually increased during the observed period, and at 24 h, all the variables had at least a score of 2. A two-way ANOVA with time as a repeated measure indicated a significant interaction for the variation of body temperature ($F_{(1,8)} = 6.33$, $p = 0.04$; Figure 2B). LPS-treated mice presented hypothermia at 2 ($p = 0.004$) and 24 h ($p < 0.001$) after the injection.

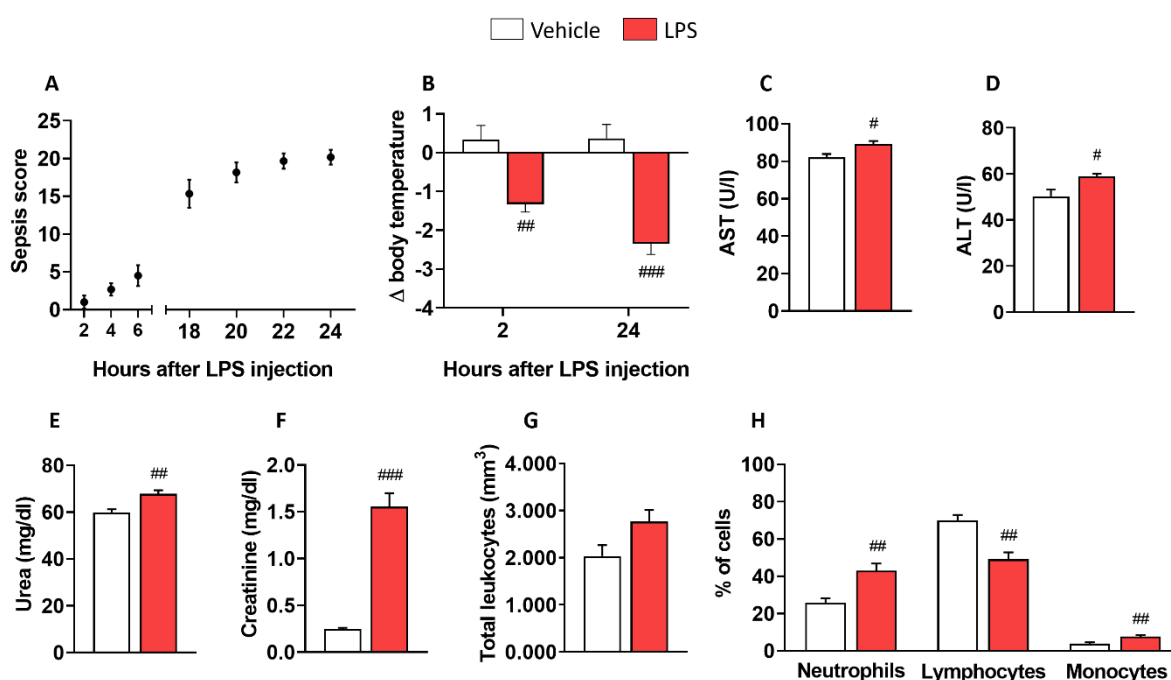


Figure 2. LPS induced sepsis in Swiss male mice. (A) Sepsis score. (B) Variation of body temperature 2 and 24 h after LPS injection. Peripheral levels of (C) AST, (D) ALT, (E) urea, and (F) creatinine 24 h after LPS injection. (G) Total number of leukocytes and (H) percentage of neutrophils, lymphocytes, and monocytes 24 h after LPS injection. Data are expressed as mean $\pm$ SEM of six independent animals/group. # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$ when compared to vehicle-treated mice.

Since sepsis is associated with organ dysfunction (Hotchkiss et al., 2016), we examined whether LPS injection would damage liver and kidneys. The levels of AST

($p = 0.01$; Figure 2C), ALT ($p = 0.02$; Figure 2D), urea ($p = 0.003$; Figure 2E), and creatinine ($p < 0.001$; Figure 2F) increased after LPS injection when compared to control mice.

The assessment of leukocyte numbers in the peripheral blood of LPS-treated mice was also used to validate LPS-induced sepsis. The number of total leukocytes (Figure 2G) was not changed after LPS injection ($p = 0.06$). On the other hand, LPS increased the percentage of neutrophils ($p = 0.004$) and monocytes ($p = 0.009$), while decreased the percentage of lymphocytes ($p = 0.001$) (Figure 2H).

3.2. CMI treatment attenuated long-lasting behavioral changes in post-septic mice

Next, we examined on a battery of behavioral tests whether CMI would ameliorate the long-lasting behavioral changes of animals that had previously undergone sepsis. Initially, we used the murine sepsis score to confirm the induction of sepsis in this set of mice (Figure 3A). The severity of the disease gradually increased during the observed period, and at 24 h, all the variables had at least a score of 2.

With the OFT, we observed that none of the treatments altered the number of crossings ($F_{(1,20)} = 0.048$, $p = 0.83$) and rearings ($F_{(1,20)} = 0.004$, $p = 0.95$) (data not shown). Regarding the depression-like behavior, a two-way ANOVA revealed a significant interaction for the immobility time in the TST ($F_{(1,20)} = 7.60$, $p = 0.01$; Figure 3B) and FST ($F_{(1,20)} = 11.30$, $p = 0.003$; Figure 3C). Further post-hoc analysis revealed a significant increase in the immobility time in the TST ($p = 0.001$) and FST ($p < 0.02$) in post-septic mice, an effect that was significantly abolished by CMI treatment (TST, $p = 0.009$; FST, $p = 0.01$). With respect to the sucrose preference test, a two-way ANOVA revealed a significant interaction for the percentage of sucrose preference ($F_{(1,20)} = 4.47$, $p = 0.05$; Figure 3D). A post-hoc analysis revealed that post-septic mice had reduced sucrose preference ($p < 0.001$) when compared to control mice, an effect that was ameliorated by CMI treatment ($p = 0.002$), highlighting the antidepressant-like effect of CMI in post-septic mice.

Regarding the anxiogenic-like behavior, a two-way ANOVA revealed a significant interaction for the number of entries ($F_{(1,20)} = 7.16$, $p = 0.01$; Figure 3E) and the time spent ($F_{(1,20)} = 9.89$, $p = 0.005$; Figure 3F) in the open arms of the apparatus. Subsequent post-hoc analysis revealed that post-septic mice made significantly fewer entries ($p = 0.01$) and spent less time ($p = 0.006$) in the open arms. CMI treatment was

able to significantly increase the number of entries ($p = 0.01$) and the time spent ($p = 0.005$) in the open arms of the EPM. Moreover, a two-way ANOVA revealed a significant interaction for the number of marbles buried ($F_{(1,20)} = 38.83$, $p < 0.001$; Figure 3G). Post-septic mice buried more marbles than control mice ($p < 0.001$), an effect that was ameliorated by CMI treatment ($p < 0.001$).

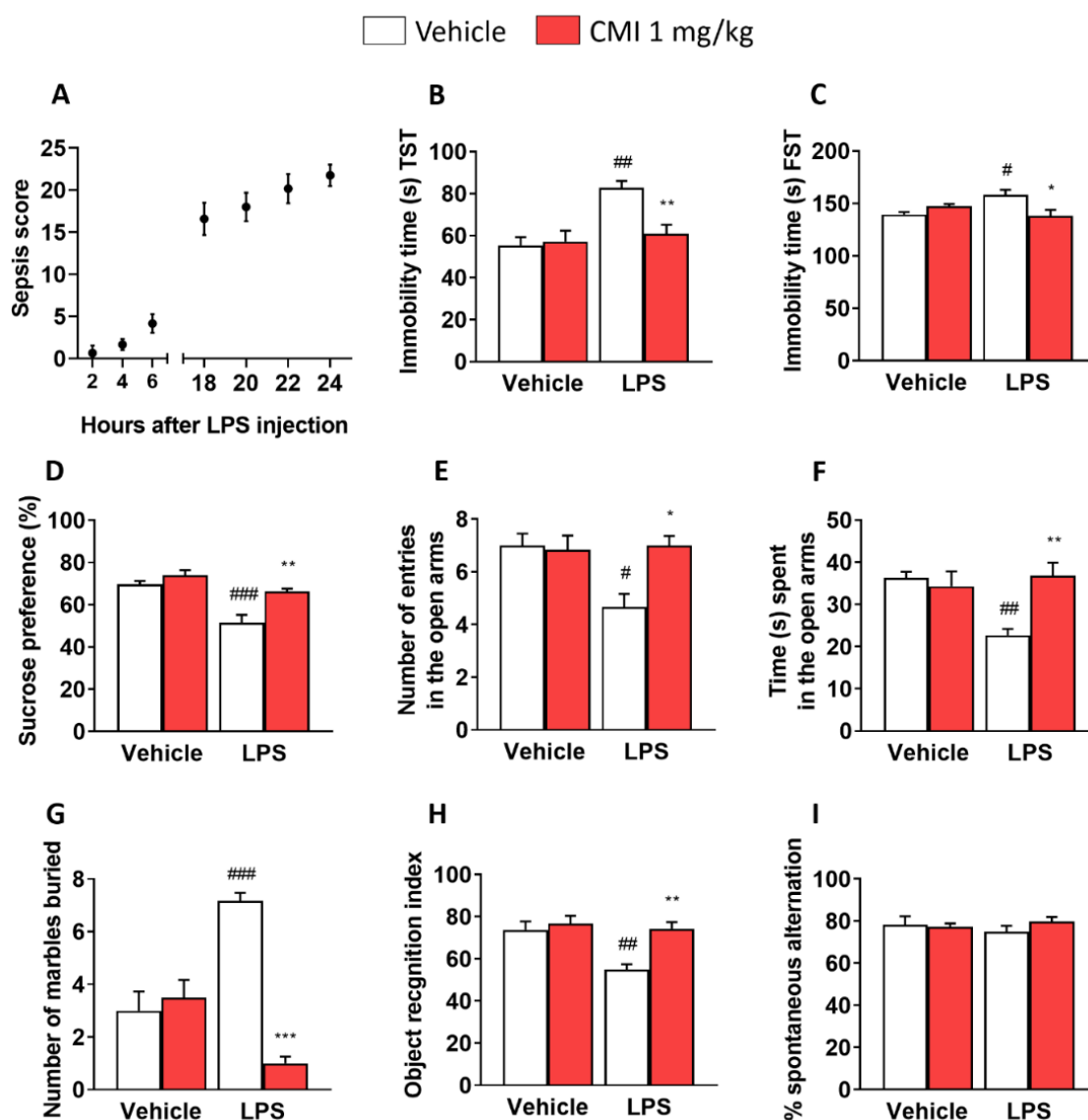


Figure 3. CMI treatment mitigated behavioral alterations in post-septic mice. (A) Sepsis score. (B) Immobility time in the tail suspension test (TST). (C) Immobility time in the forced swimming test (FST). (D) Percentage of sucrose preference. (E) Number of entries in the open arms of the elevated plus-maze (EPM). (F) Time spent in the open arms of the elevated plus-maze (EPM). (G) Number of marbles buried. (H) Object recognition index in the novel object recognition test (NORT). (I) Percentage of spontaneous alternation in the Y-maze. Data are expressed as mean $\pm$ SEM of six independent animals/group. # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$ when compared to vehicle-treated mice. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ when compared to LPS-treated mice.

With respect to cognition, a two-way ANOVA revealed a significant interaction for the object recognition index ($F_{(1,20)} = 5.76$, $p = 0.03$; Figure 3H). A post-hoc analysis revealed that post-septic mice spent less time exploring the new object ($p = 0.004$), an effect that was counteracted by CMI treatment ($p = 0.004$). Regarding the working memory assessed by the Y-maze, none of the treatments altered the percentage of spontaneous alterations ($F_{(1,20)} = 1.10$, $p = 0.31$; Figure 3I).

3.3. CMI treatment ameliorated long-lasting alterations in the blood of post-septic mice

We next performed experiments in which we examined whether peripheral alterations induced by LPS would be maintained for 30 days and if CMI treatment would be able to ameliorate them (Figure 4). A two-way ANOVA did not indicate a significant interaction for the number of total leukocytes ($F_{(1,20)} = 3.75$, $p = 0.07$) of mice, but indicated a main effect of CMI treatment ($F_{(1,20)} = 6.37$, $p = 0.02$) (Figure 4A). Regarding the cell type, a two-way ANOVA revealed a significant interaction for the percentage of neutrophils ($F_{(1,20)} = 5.36$, $p = 0.03$). The post-hoc analysis indicated that post-septic mice had increased percentage of neutrophils ($p = 0.006$), which was decreased by treatment with CMI ($p = 0.007$). No significant alterations were found for the percentage of lymphocytes ($F_{(1,20)} = 3.17$, $p = 0.09$) and monocytes ($F_{(1,20)} = 0.14$, $p = 0.71$) in the blood.

With respect to oxidative alterations in blood, a two-way ANOVA indicated a significant interaction for the levels of RS (Figure 4C) in neutrophils ($F_{(1,20)} = 10.37$, $p = 0.004$), lymphocytes ($F_{(1,20)} = 5.18$, $p = 0.03$), and monocytes ($F_{(1,20)} = 6.09$, $p = 0.02$). Further post-hoc analysis indicated that neutrophils ($p = 0.002$), lymphocytes ($p = 0.02$), and monocytes ($p = 0.01$) of post-septic mice had increased levels of RS, which were decreased by CMI treatment (neutrophils, $p = 0.003$; lymphocytes, $p = 0.04$; monocytes, $p = 0.01$).

Regarding markers of liver dysfunction, a two-way ANOVA revealed a significant interaction for the levels of AST ($F_{(1,20)} = 4.48$, $p = 0.05$; Figure 4D) and ALT ($F_{(1,20)} = 15.60$, $p < 0.001$; Figure 4E) in the blood. A post-hoc analysis indicated that post-septic mice presented increased levels of AST ($p = 0.03$) and ALT ($p = 0.007$), which decreased with CMI treatment (AST, $p = 0.005$; ALT, $p = 0.01$).

As shown in Figure 4F and Figure 4G, a two-way ANOVA revealed a significant interaction for the levels of urea ($F_{(1,20)} = 5.38$, $p = 0.03$) and creatinine ($F_{(1,20)} = 7.42$,

p = 0.01), respectively, which are markers of kidneys dysfunction. A post-hoc analysis indicated that post-septic mice presented increased levels of urea (p = 0.007) and creatinine (p < 0.001), which decreased with CMI treatment (urea, p = 0.002; creatinine, p = 0.02).

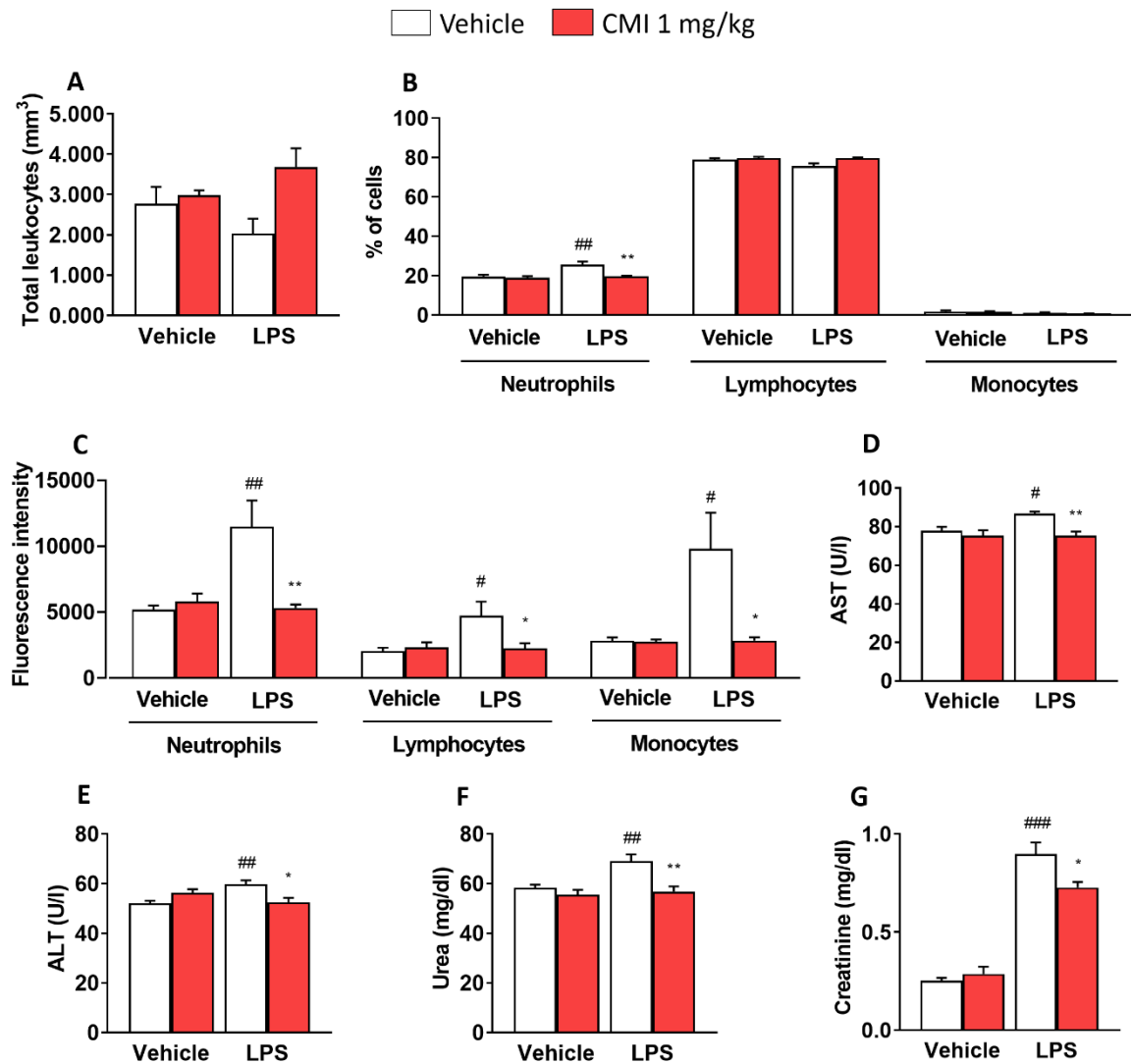


Figure 4. CMI treatment ameliorated peripheral alterations in post-septic mice. (A) Total number of leukocytes. (B) Percentage of neutrophils, lymphocytes, and monocytes. (C) ROS levels on leukocytes. (D) Lipid peroxidation on leukocytes. Peripheral levels of (E) AST, (F) ALT, (G) urea, and (H) creatinine. Data are expressed as mean $\pm$ SEM of six independent animals/group. #p < 0.05, ##p < 0.01, and ###p < 0.001 when compared to vehicle-treated mice. *p < 0.05, **p < 0.01, and ***p < 0.001 when compared to LPS-treated mice.

3.4. CMI treatment mitigated long-lasting neuroinflammation associated with LPS-induced sepsis and ameliorated BBB integrity

Considering that systemic inflammation can alter BBB permeability, we investigated the BBB integrity in sepsis survivors (Figure 5A). A two-way ANOVA revealed a significant interaction for the concentration of Evans blue dye in the brain of mice ($F_{(1,20)} = 5.07$, $p = 0.04$). A post-hoc analysis indicated that sepsis induced loss of BBB integrity ($p = 0.010$), which was ameliorated by CMI treatment ($p = 0.04$).

To examine the neuroinflammatory processes that occur in post-septic brain, we analyzed the relative expression of pro-inflammatory markers in the prefrontal cortices and hippocampi of mice. A two-way ANOVA revealed a significant interaction for the mRNA expression of *NF- κ B* in the prefrontal cortices ($F_{(1,20)} = 5.71$, $p = 0.03$) and hippocampi ($F_{(1,20)} = 10.20$, $p = 0.005$) of mice (Figure 5B). Further post-hoc analysis indicated that post-septic mice had increased *NF- κ B* expression in the prefrontal cortices ($p = 0.04$) and hippocampi ($p = 0.01$), which was reversed by CMI treatment (prefrontal cortices, $p < 0.01$; hippocampi, $p < 0.001$).

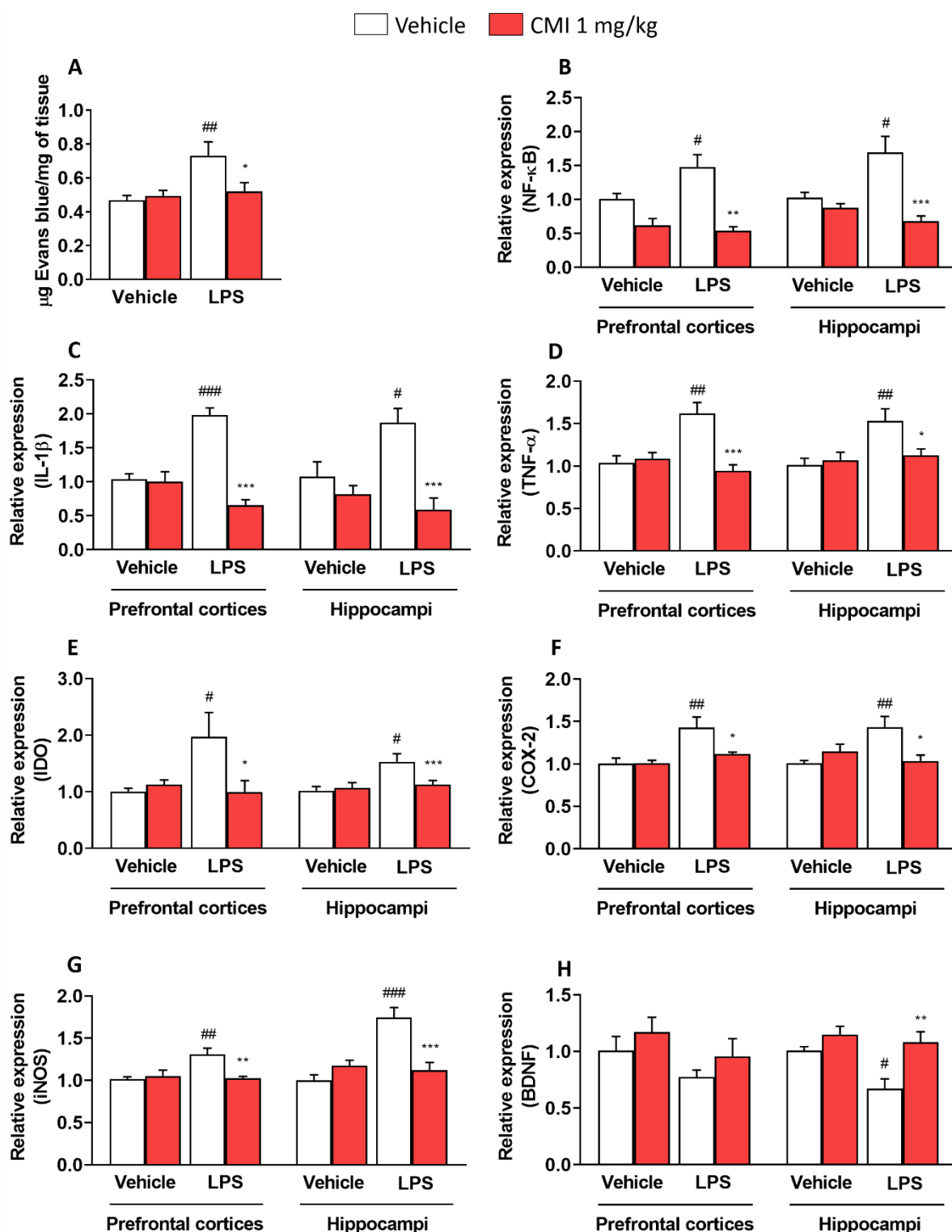
As shown in Figure 5C, a two-way ANOVA revealed a significant interaction for the mRNA expression of *IL-1 β* in the prefrontal cortices ($F_{(1,20)} = 37.40$, $p < 0.001$) and hippocampi ($F_{(1,20)} = 7.57$, $p = 0.01$) of mice. A post-hoc analysis indicated that the *IL-1 β* expression was increased in the prefrontal cortices ($p < 0.001$) and hippocampi ($p = 0.03$) of post-septic mice, whilst treatment with CMI reduced it (prefrontal cortices, $p < 0.001$; hippocampi, $p < 0.001$).

A significant interaction was found for the mRNA expression of *TNF- α* in the prefrontal cortices ($F_{(1,20)} = 15.40$, $p < 0.001$) and hippocampi ($F_{(1,20)} = 5.13$, $p = 0.03$) of mice (Figure 5D). Further post-hoc analysis indicated that post-septic mice had increased *TNF- α* expression in the prefrontal cortices ($p = 0.001$) and hippocampi ($p = 0.009$), which was reversed by CMI treatment (prefrontal cortices, $p < 0.001$; hippocampi, $p < 0.05$).

A two-way ANOVA revealed a significant interaction for the mRNA expression of *IDO* in the prefrontal cortices ($F_{(1,20)} = 5.13$, $p = 0.03$) and hippocampi ($F_{(1,20)} = 11.30$, $p = 0.005$) of mice (Figure 5E). Further post-hoc analysis indicated that post-septic mice had increased *IDO* expression in the prefrontal cortices ($p = 0.05$) and hippocampi ($p = 0.04$), which was reversed by CMI treatment (prefrontal cortices, $p = 0.04$; hippocampi, $p < 0.001$).

A significant interaction was found for the mRNA expression of *COX-2* in the prefrontal cortices ($F_{(1,20)} = 4.75$, $p = 0.04$) and hippocampi ($F_{(1,20)} = 10.10$, $p = 0.005$) of mice (Figure 5F). Further post-hoc analysis indicated that post-septic mice had

3123 increased *COX-2* expression in the prefrontal cortices ($p = 0.003$) and hippocampi (p
 3124 $= 0.01$), which was reversed by CMI treatment (prefrontal cortices, $p = 0.03$;
 3125 hippocampi, $p = 0.02$).



3126
 3127 **Figure 5.** CMI treatment ameliorated BBB disruption and attenuated neuroinflammation in post-septic
 3128 mice. (A) Extravasation of Evans blue in the whole brain of mice. Relative expression of (B) NF-κB, (C)
 3129 IL-1β, (D) TNF-α, (E) IDO, (F) COX-2, (G) iNOS, and (H) BDNF in the prefrontal cortices and hippocampi

of mice. Data are expressed as mean $\pm$ SEM of six independent animals/group. # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$ when compared to vehicle-treated mice. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ when compared to LPS-treated mice.

As shown in Figure 5G, a two-way ANOVA revealed a significant interaction for the mRNA expression of *iNOS* in the prefrontal cortices ($F_{(1,20)} = 8.98$, $p = 0.007$) and hippocampi ($F_{(1,20)} = 21.50$, $p < 0.001$) of mice. A post-hoc analysis indicated that *iNOS* expression was increased in the prefrontal cortices ($p = 0.004$) and hippocampi ($p < 0.001$) of post-septic mice, whilst treatment with CMI reduced it (prefrontal cortices, $p = 0.007$; hippocampi, $p < 0.001$).

Due to the influence of inflammation on neurotrophic factors, we also assessed the expression of *BDNF*. As shown in Figure 5H, a two-way ANOVA revealed a significant interaction for the mRNA expression of *BDNF* in the hippocampi of mice ($F_{(1,20)} = 3.24$, $p = 0.09$). A post-hoc analysis indicated that *BDNF* expression was increased in the hippocampi ($p = 0.02$) of post-septic mice, whilst treatment with CMI reduced it ($p = 0.005$). The *BDNF* expression was not altered in the prefrontal cortices of mice ($F_{(1,20)} = 0.006$, $p = 0.94$).

3.5. CMI treatment attenuated long-lasting oxidative alterations in the prefrontal cortices and hippocampi of post-septic mice

To verify whether the prefrontal cortices and hippocampi of post-septic mice would present oxidative alterations that could be reduced by CMI treatment, we analyzed the levels of RS, NO_x metabolites, and TBARS (Figure 6). A two-way ANOVA revealed a significant interaction for the RS levels in the prefrontal cortices ($F_{(1,20)} = 48.40$, $p < 0.001$) and hippocampi ($F_{(1,20)} = 15.80$, $p < 0.001$) of mice (Figure 6A). Further post-hoc analysis indicated that post-septic mice had increased RS levels in the prefrontal cortices ($p < 0.001$) and hippocampi ($p = 0.001$), which was reversed by CMI treatment (prefrontal cortices, $p < 0.001$; hippocampi, $p < 0.001$).

As shown in Figure 6B, a two-way ANOVA revealed a significant interaction for the NO_x levels in the prefrontal cortices ($F_{(1,20)} = 34.90$, $p < 0.001$) and hippocampi ($F_{(1,20)} = 13.80$, $p = 0.001$) of mice. A post-hoc analysis indicated that NO_x levels were increased in the prefrontal cortices ($p = 0.003$) and hippocampi ($p < 0.001$) of post-septic mice, whilst treatment with CMI reduced it (prefrontal cortices, $p < 0.001$; hippocampi, $p < 0.001$).

A significant interaction was found for the TBARS levels in the prefrontal cortices ($F_{(1,20)} = 55.90$, $p < 0.001$) and hippocampi ($F_{(1,20)} = 11.10$, $p = 0.003$) of mice (Figure 6C). Further post-hoc analysis indicated that post-septic mice had increased TBARS levels in the prefrontal cortices ($p < 0.001$) and hippocampi ($p = 0.001$), which was reversed by CMI treatment (prefrontal cortices, $p < 0.001$; hippocampi, $p = 0.005$).

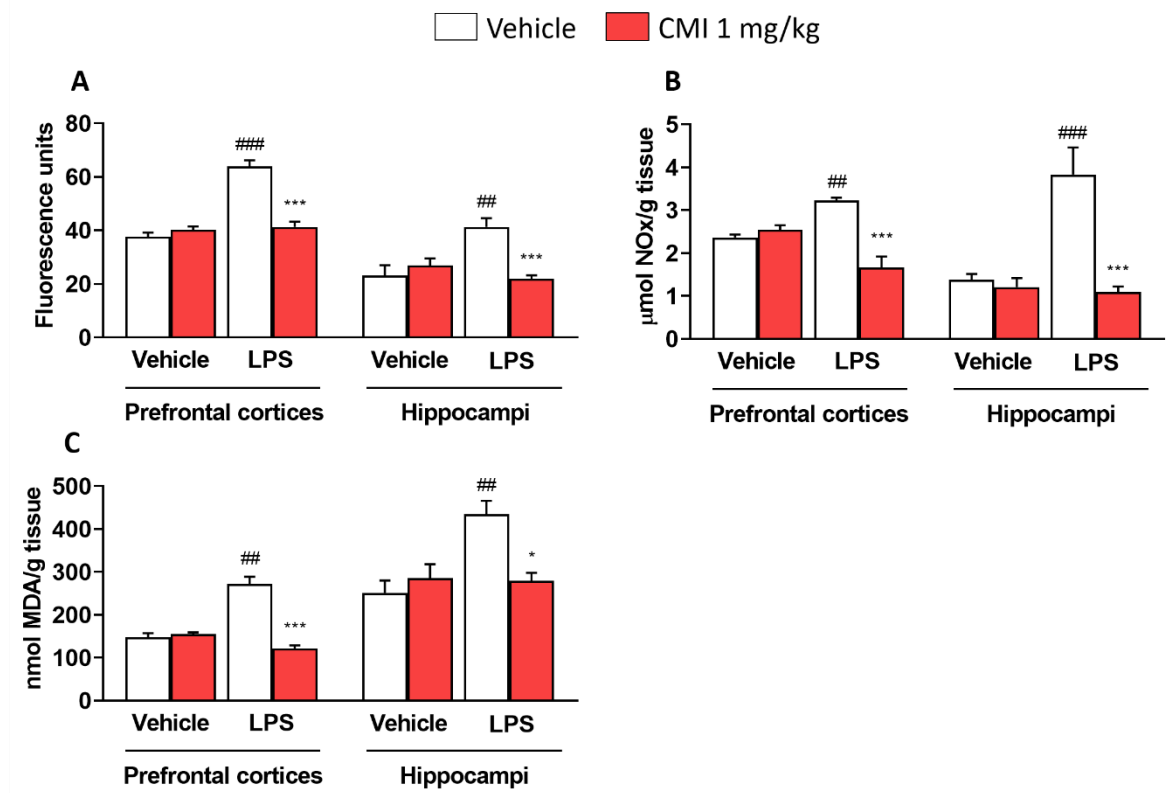


Figure 6. CMI treatment reduced oxidative stress in post-septic mice. (A) ROS, (B) NOX, and (C) lipid peroxidation levels in pre-frontal cortices and hippocampi of mice. Data are expressed as mean $\pm$ SEM of six independent animals/group. ## $p < 0.01$ and ### $p < 0.001$ when compared to vehicle-treated mice. * $p < 0.05$ and *** $p < 0.001$ when compared to LPS-treated mice.

4. Discussion

The results presented here provide compelling evidence that CMI treatment for 7 days was able to improve long-term behavioral and biochemical alterations present in mice that survived sepsis. Here, we showed that CMI was effective in reducing depression- and anxiogenic-like behavior and cognitive impairment in post-septic mice. Meantime, CMI treatment modulated the number of neutrophils, the levels of RS in neutrophils, lymphocytes, and monocytes, and reduced the levels of peripheral markers of liver and kidneys dysfunction (AST, ALT, urea, and creatinine). Noteworthy, CMI ameliorated BBB dysfunction induced by sepsis and modulated the expression of

inflammation-associated genes (*NFκB*, *IL-1β*, *TNF-α*, *IDO*, *COX-2*, *iNOS*, and *BDNF*) and markers of oxidative stress (RS, NO_x, and MDA levels) in the prefrontal cortices and hippocampi of mice. Additionally, our study strengthens the use of LPS to induce sepsis and highlights the presence of long-term alterations in sepsis survivor mice. A summary of the findings is presented in Figure 7.

Since most laboratory animals are relatively resistant to endotoxin, we conducted a preliminary investigation to see if the endotoxin injection would induce sepsis under our experimental conditions in Swiss male mice. Overall, LPS 5 mg/kg increased the murine sepsis score over 24 h, caused hypothermia 2 and 24 h after the injection, increased the circulating levels of AST, ALT, urea, and creatinine, increased the percentage of neutrophils and monocytes, and decreased the percentage of lymphocytes. Together, these observations characterized the septic condition (Bone et al., 1992; Hotchkiss et al., 2016; Levy et al., 2003; Shrum et al., 2014) and allowed us to explore the ability of CMI to modulate long-lasting alterations in mice that survived sepsis. It is important to highlight that the aim of this study was not to find a therapeutic strategy to improve sepsis survival, but to find an approach that would improve the long-term psychological impairment post-sepsis.

The long-term consequences of sepsis only recently started to be studied (Hotchkiss et al., 2016) and not enough attention has been given to the investigation of therapeutic approaches for the management of psychological symptoms post-sepsis. Whilst several studies have focused on strategies to treat cognitive deficits in post-septic mice (Michels et al., 2014; Huang et al., 2019; Xu et al., 2019), our study provides a comprehensive examination of the effects of CMI on cognitive impairment and affective disorders in sepsis survivors mice. Repeated administration of CMI at 1 mg/kg (orally) once a day for one-week ameliorated behavioral alterations found in the TST, FST, sucrose preference test, EPM test, marble burying test, and NORT that were present one month after the induction of sepsis. The assessment of the behavioral response of mice one month after LPS injection presents as an advantage over the investigations conducted up to 10 days after sepsis, such as in the case of cecal ligation and puncture (CLP) method. Indeed, a single injection of LPS (0.5 mg/kg – 5 mg/kg) induced cognitive deficits and microglial alterations up to two months later (Kondo et al., 2011; Weberpals et al., 2009).

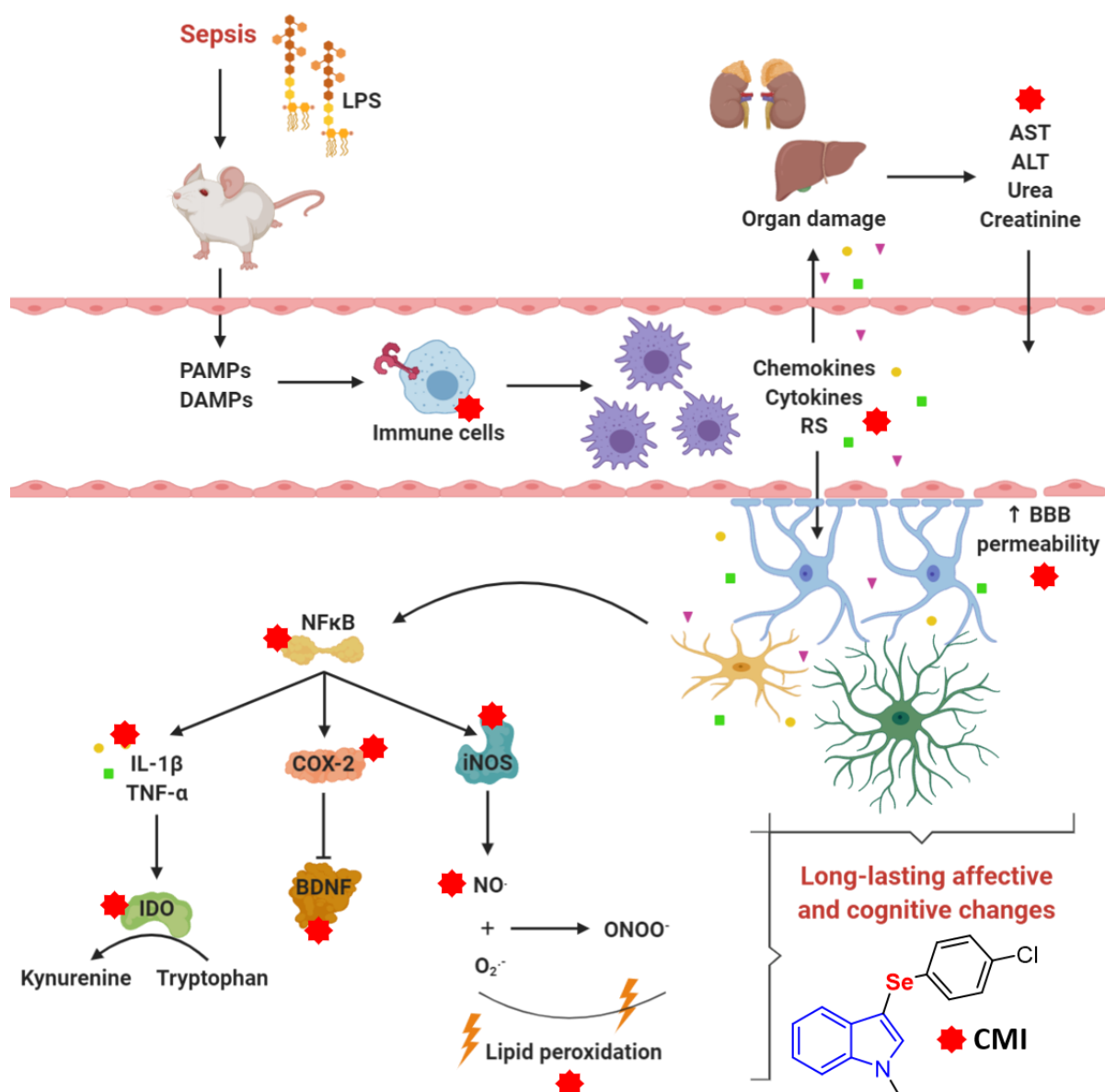


Figure 7. Overview of the alterations found in post-septic mice treated with CMI. Administration of CMI in mice that survived sepsis ameliorated the depressive- and anxiogenic-like behavior and cognitive impairment. CMI treatment modulated the number of neutrophils, the levels of RS in neutrophils, lymphocytes, and monocytes, and reduced the plasma levels of AST, ALT, urea, and creatinine. CMI also reduced BBB permeability and modulated the expression of *NFκB*, *IL-1β*, *TNF-α*, *IDO*, *COX-2*, *iNOS*, and *BDNF* and reduced the levels of RS, NO_x , and lipid peroxidation in the prefrontal cortices and hippocampi of mice.

Although infection is the triggering event in sepsis, the abnormal immune response often remains after successful treatment of the infection (van der Poll et al., 2017). Here, CMI treatment reduced the elevated percentage of circulating neutrophils that were identified one month after the induction of sepsis. Neutrophils are the key cell subpopulation against invading pathogens, responsible for the release of cytokines

and RS at sites distal to the infection, leading to multiorgan failure (Brown et al., 2006). In agreement, post-septic mice presented increased circulating levels of AST, ALT, urea, and creatinine, which decreased after CMI treatment, suggesting that the selenocompound may alleviate liver and kidneys damage induced by sepsis. This outcome may be mediated by the antioxidant and immunomodulatory effects of CMI, since inflammation and oxidative stress are present in peripheral organs after sepsis (Zheng and Zhu, 2016); however, histopathological studies would help to confirm the ability of CMI to improve organ function. Additionally, circulating neutrophils, lymphocytes, and monocytes of post-septic mice showed increased levels of RS, which were reduced by CMI treatment. RS generated by neutrophils, especially O_2^- , H_2O_2 and NO^* are part of their microbicidal activity (Winterbourn et al., 2016). However, these data should be interpreted with caution as RS are important signaling molecules for cell activation, proliferation, and cytokine production (Winterbourn et al., 2016). Even though the results are interesting as they show long-lasting peripheral alterations in mice that survived sepsis, a more detailed analysis of the immune cell functions should be conducted.

Brain function in humans is significantly impaired in both early and late sepsis (Gofton and Young, 2012). Systemic infection initiates a hyperinflammatory response that stimulates the production of immune cells, cytokines, and inflammatory mediators. These inflammatory molecules initiate a cascade of events that lead to disruptive and non-disruptive BBB changes, including immune cell infiltration, increased BBB permeability, activation of cerebral cytokines, and enhanced neuroinflammation (Nwafor et al., 2019; Varatharaj and Galea, 2017). Here, we observed that CMI treatment for one-week reduced BBB permeability to Evans blue dye, suggesting that the selenocompound may alleviate long-lasting BBB changes observed in post-septic mice.

Improvement in BBB function may decrease the entry of cytokines and other inflammatory mediators from periphery into the brain, consequently, reducing neuroinflammation. Here, we observed that at the time CMI improved BBB permeability, it also downregulated the expression of inflammatory mediators (e.g. *NFκB*, *IL-1β*, *TNF-α*, *iNOS*, *COX-2*, and *IDO*) in the prefrontal cortices and hippocampi of mice one month after the induction of sepsis. Indeed, *NFκB*-induced *IL-1β* and *TNF-α* signaling is involved in inflammation-induced depression, anxiety, and impaired cognition. Noteworthy, pro-inflammatory cytokines can activate IDO and increase the

expression of the serotonin transporter (SERT), thus impairing the serotonergic neurotransmission and playing a part in the establishment of depressive behavior (Felger and Lotrich, 2013). Through an *in silico* approach, we previously showed that CMI may interact with the active site of IDO and SERT (Casaril et al., 2019b), and therefore, we can speculate that this could also be involved in the pro-cognitive, antidepressant- and anxiolytic-like effect of CMI. Moreover, in the periphery, it has been reported that IDO-mediated tryptophan catabolism is increased in septic patients and is associated with dysregulated immune responses, increased lymphocyte apoptosis and increased IL-6 levels (Darcy et al., 2011). Further studies should be conducted to investigate whether CMI modulates peripheral IDO to restore the immune function.

Secondary to neuroinflammation during sepsis, increased metabolism and energy demand by brain cells can induce oxidative stress (van Gool et al., 2010). Indeed, persistent endothelial and glial cell activation enhances the generation of RS, and together with inflammatory cytokines, feed a vicious cycle of BBB changes, neuroinflammation, and oxidative stress. This vicious cycle causes an impaired oxidative metabolism and lipid peroxidation of cerebrovasculature and brain parenchyma, which likely continues after sepsis recovery (Berg et al., 2011). Since sustained production of RS after recovery may be another mechanism that contributes to long-term psychological impairment post sepsis (Nwafor et al., 2019), decrease RS, NO_x, and TBARS levels in the prefrontal cortices and hippocampi may help to explain the ability of CMI to ameliorate depression- and anxiogenic-like behaviour and cognitive impairment in post-septic mice.

Current literature indicates that COX-2-derived PG and NO^{*} synthesized by cerebrovascular endothelium, microglia, and astrocytes are central for the effects of LPS on BBB integrity (Iwase et al., 2000). Moreover, PG signaling was shown to reduce the activity-dependent *BDNF* transcription necessary for long-term memory formation (Hein and O'Banion, 2009). Here, we observed that CMI downregulated the expression of COX-2 and *iNOS* and decreased the levels of NO_x in the prefrontal cortices and hippocampi while upregulated the expression of *BDNF* in the hippocampi. In agreement, we previously showed that CMI may modulate the activity of COX-2 and *iNOS* by interacting with the active site of these enzymes (Casaril et al., 2020). Together, the ability of CMI to modulate the expression and function of these enzymes,

aligned with the effects of CMI on the *BDNF* expression may contribute to the improvement of the BBB permeability and the behavior of post-septic mice.

Nonetheless, this study has some limitations. Here, we used the toxemia model characterized by systemic injection of a high dose of LPS to mimic inflammation induced by a bacterial infection without the intricacy related to pathogen replication (Anderson et al., 2015; Quatrini et al., 2017). Another advantage is that LPS-treated mice did not need to be treated with antibiotics, so the behavioral and biochemical improvements were a consequence of CMI treatment. However, we are aware that the gold standard to study sepsis is the CLP method and, therefore, the effects of CMI should be confirmed in mice with CLP-induced sepsis. Moreover, more studies should be conducted to analyse microglia and astrocytes activation and cell energy metabolism in post-septic mice.

Taken together, our results provide compelling evidence that CMI ameliorated depression- and anxiogenic-like behavior, cognitive impairment, BBB permeability, peripheral oxidative alterations, organ dysfunction, neuroinflammation and central oxido-nitrosative stress in endotoxin-induced sepsis survivor mice. These results further emphasize the importance of the anti-inflammatory and antioxidant systems in the brain and acquaint that multiple targets are required for adequate therapeutic efficacy of treatments against psychological symptoms in sepsis survivors. Therefore, through this comprehensive study analyzing behavioral and biochemical parameters, CMI arises as a promising strategy aimed to manage the long-lasting behavioral alterations of sepsis survivors to improve their quality of life.

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Conflicts of interest

The authors declare that they have no conflict of interests.

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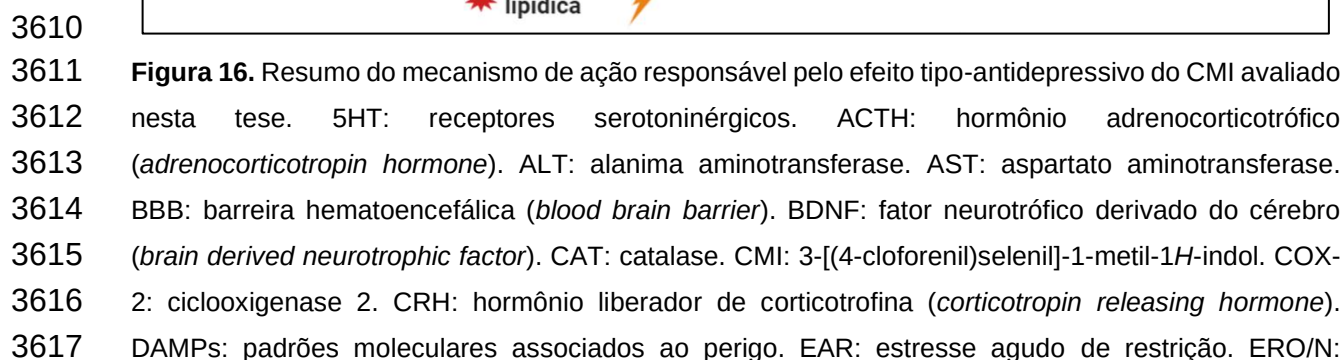
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5 DISCUSSÃO GERAL E PERSPECTIVAS

A presente tese foi planejada e executada para caracterizar o efeito tipo-antidepressivo de um novo composto orgânico de selênio, o CMI (Figura 16). A proposta não teria sido cumprida com êxito sem a integração do conhecimento de diversas áreas, notavelmente a biotecnologia, bioquímica, imunologia, neurociências, farmacologia, química e psicologia, enfatizando a importância de abordagens multialvo para o avanço científico. Pontualmente, nesta tese investigamos (i) o mecanismo antioxidante do CMI; (ii) o efeito tipo-antidepressivo, antioxidante e imunomodulador do CMI em animais submetidos ao EAR; (iii) o envolvimento do sistema serotoninérgico no efeito tipo-antidepressivo e tipo-ansiolítico do CMI; (iv) o efeito tipo-antidepressivo, antioxidante e imunomodulador do CMI em animais portadores de tumor mamário; (v) o envolvimento da via do BDNF-mTOR no efeito tipo-antidepressivo e tipo-ansiolítico do CMI; e (vi) a capacidade do CMI em reverter alterações afetivas e cognitivas, estresse oxidativo e neuroinflamação em animais que sobreviveram a sepse. Além da contribuição científica, esperamos que esta tese contribua com a divulgação da ciência e com a quebra do estigma de que a depressão é uma doença puramente mental.

A depressão é uma doença multifatorial e heterogênea, sendo influenciada por fatores que incluem a resposta imune pró-inflamatória, estresse e traumas na infância e adolescência, microbiota intestinal, vulnerabilidade genética, alterações metabólicas e disfunção do eixo HPA. Nesse sentido, a capacidade tipo-antidepressiva do CMI foi avaliada em cinco modelos animais a fim de termos uma compreensão abrangente sobre seus efeitos em diferentes condições patológicas em que a depressão é presente. O primeiro estudo investigando o perfil farmacológico do CMI foi realizado em 2017, aonde mostrou-se que ele apresenta atividade antioxidante *in vitro* (Vieira et al., 2017). Considerando o envolvimento do estresse oxidativo na depressão e sua relação com o processo inflamatório, avaliou-se então a capacidade do CMI em proteger contra o comportamento tipo-depressivo induzido por inflamação em camundongos (Casaril et al., 2017). Esse estudo mostrou que apenas uma administração de CMI 30 minutos antes do desafio inflamatório protege contra o comportamento tipo-depressivo e contra o aumento nos níveis de ERO, peroxidação lipídica e expressão citocinas pró-inflamatórias nos córtex pré-frontais e hipocampus dos animais, sem apresentar toxicidade aguda na dose de 300 mg/kg (Casaril et al.,



espécies reativas de oxigênio/nitrogênio. GPx: glutathione peroxidase. GR: receptor de glicocorticoide (*glucocorticoid receptor*). GSK-3 β : glicogênio sintase quinase 3 beta (*glycogen synthase kinase 3 beta*). IDO: indoleamina 2,3-dioxigenase. IL-1 β : interleucina 1 beta. iNOS: óxido nítrico sintase induzível. LPS: lipopolissacarídeo. MPO: mieloperoxidase. mTOR: alvo mamífero da rapamicina (*mammalian target of rapamycin*). NF κ B: fator nuclear kappa B (*nuclear factor kappa B*). NMDA-R: receptores N-metil-D-aspartato (*N-methyl-D-aspartate*). Nrf2: fator nuclear eritroide relacionado ao fator 2 (*nuclear factor erythroid 2-related factor 2*). PAMPs: padrões moleculares associados a patógenos. SERT: transportador de serotonina (*serotonin transporter*). SOD: superóxido dismutase. TNF- α : fator de necrose tumoral alfa (*tumor factor necrosis alpha*). TLR4: receptores do tipo Toll 4 (*toll-like receptors 4*). Fonte: Angela Maria Casaril.

A exposição de um organismo a diferentes estressores, físicos ou psicológicos, como o estresse, inflamação, ou tumor, acarreta na liberação de PAMPs e DAMPs na corrente sanguínea. Essas moléculas, através da interação com os TLRs presentes em células imunes, induzem a produção de quimiocinas, citocinas e ERO/N, as quais podem levar a peroxidação lipídica e a danos em órgãos longe do sítio de infecção. Nesta tese, demonstramos a capacidade do CMI em modular o número de neutrófilos circulantes e os níveis plasmáticos de ERO/N, peroxidação lipídica, AST, ALT, ureia e creatinina. Esses dados sugerem que o CMI pode apresentar um efeito sistêmico que contribui com a melhora comportamental observada nos camundongos.

Citocinas e ERO/N também podem aumentar a permeabilidade da BBB, culminando com a entrada de citocinas e mediadores inflamatórios para o sistema nervoso central (Banks, 2015). Adicionalmente, PAMPs e DAMPs podem sinalizar via TLR4 localizados na membrana de células endoteliais da BBB para ativar as vias de sinalização do NF κ B e GSK-3 β . A ativação do NF κ B via TLR4 ou citocinas induz a expressão de genes envolvidos com a resposta neuroinflamatória, como por exemplo *iNOS*, *COX-2* e mais citocinas inflamatórias. A IL-1 β e o TNF- α , por sua vez, podem diminuir a função dos GR por reduzirem a afinidade dos GR pelo cortisol (Pariante et al., 1999; Maddock e Pariante, 2001) ou por bloquearem a translocação dos GR do citoplasma para o núcleo (Pariante et al., 1999). Esse prejuízo na sinalização dos GR resulta na redução do *feedback* negativo do eixo HPA, resultando no aumento dos níveis circulantes de corticosterona. Na presente tese, demonstramos que o CMI reduziu a expressão do NF κ B, IL-1 β e TNF- α e aumentou a expressão de GR nos córtex pré-frontais e hipocampus de camundongos, além de reduzir os níveis plasmáticos de corticosterona.

No sistema nervoso central, as citocinas inflamatórias influenciam a neurotransmissão serotoninérgica em diferentes níveis. Por exemplo, as citocinas podem aumentar a expressão e função dos SERT e ativar a enzima IDO, que em conjunto, contribuem com a menor disponibilidade de serotonina (Felger e Lotrich, 2013). Nesta tese, observamos que o CMI reduziu a expressão da IDO e agiu através da interação com os receptores 5HT_{1A}, 5HT_{2A/2C} e 5HT₃. Adicionalmente, demonstramos que o CMI possivelmente atua como um antagonista dos SERT e da IDO, uma vez que interage com o sítio ativo dessas proteínas de uma maneira similar aos inibidores paroxetina e epacadostat, respectivamente. Uma vez que a estrutura do CMI, do triptofano e da serotonina compartilham a presença do núcleo indólico, é possível que esses efeitos do CMI sejam consequência dessa semelhança estrutural. A inibição da IDO também é importante pois sua ativação está relacionada com o aumento dos níveis de quinurenina, um metabólito tóxico que, através da interação com os NMDA-R, pode aumentar a excitotoxicidade glutamatérgica.

Além do aumento na sinalização de citocinas inflamatórias, a ativação do NFκB induz a expressão da enzima *iNOS*, responsável pela produção de NO[•], que ao interagir com o O₂⁻ leva a formação de ONOO⁻. Nesta tese, demonstramos que o CMI reduziu a expressão da *iNOS* e os níveis de metabólitos de NO[•] nos córtex pré-frontais e hipocampus de camundongos, além de atuar como um antioxidante por interagir diretamente com o ONOO⁻ *in vitro*. Adicionalmente, é possível que o CMI module a atividade da *iNOS* por se ligar ao seu sítio ativo, de uma maneira similar ao inibidor AR-C95791.

A expressão da COX-2 também pode ser aumentada pela ativação do NFκB. As prostaglandinas formadas pela COX-2 contribuem com a propagação da neuroinflamação e reduzem a atividade do BDNF (Hein e O'Banion, 2009; Rage et al., 2006). Os dados obtidos nesta tese indicam que o CMI reduziu a expressão de COX-2 nos córtex pré-frontais e hipocampus de camundongos, além de possivelmente modular a atividade da COX-2 por interagir com o seu sítio ativo de uma maneira similar ao inibidor rofecoxibe.

A atividade do BDNF também pode ser reduzida pela ativação da GSK-3β, e vice-versa (Mai et al., 2002). A sinalização do BDNF envolve a ativação do mTOR para influenciar a sobrevivência neuronal (Watson e Baar, 2014). O mTOR serve como um sensor neuronal da demanda pela síntese de novas proteínas e sinaptogênese (Duman et al., 2016), induzindo a expressão de proteínas como a *sinaptofisina* e *PSD-*

95 que contribuem com o aumento da neurogênese e sinaptogênese. Nesta tese, demonstramos que o CMI aumentou a expressão do *BDNF* e da *sinaptofisina* nos córtex pré-frontais e hipocampus de camundongos. Adicionalmente, a melhora comportamental causada pelo tratamento com CMI depende da ativação da via do mTOR, uma vez que a inibição dessa via pela rapamicina bloqueou os efeitos do CMI. Vale destacar que, diferentemente dos antagonistas dos NMDA-R, a administração aguda ou crônica de antidepressivos convencionais (como a fluoxetina) não aumenta a sinalização do mTORC1 (Li et al., 2010).

Cabe ressaltar que a ativação da GSK-3 β também previne a translocação do Nrf2 do citosol para o núcleo (Kensler et al., 2017), enquanto que o BDNF tem papel importante na ativação Nrf2 (Ishii et al., 2018). A ativação do Nrf2 induz a expressão de enzimas antioxidantes, como a SOD e CAT, contribuindo com o equilíbrio redox e evitando o estresse oxidativo. Nesta tese, demonstramos que o CMI aumentou a expressão do *Nrf2* e modulou a atividade da SOD e CAT nos córtex pré-frontais e hipocampus de camundongos, contribuindo com a redução na peroxidação lipídica. Adicionalmente, o CMI agiu como um antioxidante por neutralizar diretamente o H₂O₂ e o HOCl *in vitro*.

De fato, a patofisiologia da depressão não foi completamente elucidada, e as alterações bioquímicas e comportamentais encontradas em pacientes depressivos são altamente heterogêneas. Adicionalmente, o TDM frequentemente apresenta-se em pacientes acometidos por outras doenças, como o câncer, doenças neurodegenerativas, ansiedade, doença de Cushing, sepse e doenças inflamatórias. Em conjunto, essa complexidade dificulta os estudos pré-clínicos para a descoberta de novas moléculas antidepressivas. Para transpor essas dificuldades, utilizamos nesta tese diferentes modelos animais a fim de obtermos uma visão mais abrangente sobre o efeito tipo-antidepressivo do CMI. Através da indução do comportamento tipo-depressivo por modelos agudos e crônicos, observamos que diferentes regimes de tratamento com CMI apresentaram efeito tipo-antidepressivo, tipo-ansiogênico, e pró-cognitivo através da modulação, principalmente, de vias nitro-oxidativas e inflamatórias.

Através do uso de ferramentas de biotecnologia, os últimos anos tem sido palco de avanços consideráveis no tratamento de diversas doenças. Infelizmente, estratégias biotecnológicas ainda não renderam resultados concretos para o tratamento da depressão, possivelmente pois a etiologia da doença não está

completamente elucidada e ainda não há um biomarcador confiável que auxilie no diagnóstico/tratamento da depressão. No entanto, tem-se observado que inibidores do TNF- α melhoram o humor depressivo em pacientes com outras doenças. Por exemplo, pacientes com artrite reumatóide e psoríase que recebem etanercept, uma proteína quimérica antagonista do TNF- α , reportaram redução nos sintomas depressivos (Gelfand et al., 2008; Kekow et al., 2011). Similarmente, pacientes com a doença de Crohn que receberam infusões de infliximab, um anticorpo monoclonal quimérico inibidor do TNF- α , experienciaram redução nos sintomas depressivos, o qual foi associado com a redução em citocinas pró-inflamatórias (Guloksuz et al., 2013). Esses resultados são de grande importância para reforçar a relevância do sistema imune na fisiologia da depressão. Entretanto, ainda não há nenhum medicamento modulador da inflamação aprovado para o tratamento da depressão. Diante disso, esta tese combina o uso de ferramentas biotecnológicas aliada a química medicinal para contribuir com o desenvolvimento de uma nova molécula com atividade biológica. A capacidade do CMI em modular a expressão de fatores de transcrição, citocinas inflamatórias, enzimas envolvidas com a inflamação, neurotrofinas, oxidantes derivados da inflamação e a atividade de enzimas antioxidantes dão suporte ao potencial terapêutico dessa molécula. Esperamos que esta tese sirva como base para estudos mais aprofundados a respeito do mecanismo de ação do CMI e, que a longo prazo, esses resultados contribuam para o surgimento de um novo produto terapêutico capaz de melhorar a qualidade de vida dos pacientes depressivos.

Nos últimos anos, diversos estudos tem reportado a relação do estresse nitro-oxidativo e da inflamação com os sintomas depressivos, e ainda assim, os antidepressivos disponíveis atualmente atuam através da modulação dos sistemas de neurotransmissores. Além disso, a crise no investimento de novos tratamentos para desordens psiquiátricas (com as indústrias abandonando a pesquisa e o desenvolvimento de medicamentos contra esses distúrbios) deixa a academia com o papel crucial de buscar novas estratégias para o tratamento de desordens psiquiátricas (Conn e Roth, 2008). Nesse sentido, torna-se importante que estudos pré-clínicos considerem o envolvimento de outras vias bioquímicas ao investir na pesquisa e no desenvolvimento de novos agentes antidepressivos mais eficazes e com menos efeitos adversos. A presente tese veio de encontro à essa proposta.

6 CONCLUSÃO GERAL

Na presente tese, validamos a nossa hipótese inicial de que o CMI seria capaz de reverter o comportamento tipo-depressivo (e suas comorbidades) que se manifestam em animais submetidos ao EAR, desafiados com LPS, portadores de tumor mamário, suplementados com corticosterona e sobreviventes à sepse. Adicionalmente, alcançamos os objetivos propostos, ao elucidarmos o envolvimento do estresse nitro-oxidativo, inflamação, neurotransmissão serotoninérgica e via do mTOR/BDNF no efeito tipo-antidepressivo do CMI. Os efeitos farmacológicos promissores do CMI enfatizam a importância de abordagens multialvo para o tratamento de desordens heterogêneas e multifatoriais, como a depressão.

Por fim, esperamos que a presente tese contribua com a divulgação científica, tanto para aproximar a sociedade das pesquisas realizadas na academia, quanto para ressaltar que a depressão é uma desordem grave e altamente incapacitante, e por isso, deve ser abordada com respeito e investimento científico.

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8 ANEXOS

Anexo A – Demais artigos publicados durante o período de doutoramento cujos temas se relacionam a presente tese

Bampi, S.R., **Casaril, A.M.**, Domingues, M., Lourenço, D.A., Pesarico, A.P., Vieira, B.,
Begnini, K.R., Seixas, F.K., Collares, T., Lenardão, E.J., Savegnago, L., 2020.
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Depression-like behavior, hyperglycemia, oxidative stress, and neuroinflammation presented in diabetic mice are reversed by the administration of 1-methyl-3-(phenylselanyl)-1*H*-indole

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ABSTRACT

Oxidative stress and neuroinflammation are found both in diabetes mellitus and major depressive disorder (MDD). In addition to damage in peripheral organs, such as liver and kidney, diabetic patients have a higher risk of developing depression. In this sense, the objective of the present study was to characterize the antidepressant-like effect of a selenium-containing compound, the 1-methyl-3-(phenylselanyl)-1*H*-indole (MFSeI), in streptozotocin (STZ)-induced diabetic mice. STZ (200 mg/kg, i.p.) was used to induce diabetes mellitus type I, and after seven days, the administration of MFSeI (10 mg/kg, i.g.) was initiated and followed for the next 14 days. Twenty-four hours after the last administration of MFSeI, the behavioral tests were performed, followed by euthanasia. The treatment with MFSeI was able to reverse the hyperglycemia induced by STZ. MFSeI also decreased the plasma levels of biomarkers of liver and kidney damage. Importantly, MFSeI reversed the depression-like behavior induced by STZ in the tail suspension test and forced swimming test without promoting locomotor alterations. Furthermore, MFSeI reversed the increased levels of reactive species and lipid peroxidation in the prefrontal cortex (PFC), hippocampus (HC), liver, and kidney of STZ-treated mice. Treatment with MFSeI also decreased the expression of tumor necrosis factor- α , inducible nitric oxide synthase and indoleamine 2,3-dioxygenase, while increasing the expression of interleukin-10, insulin receptor substrate-1 and glucose transport-4 in the PFC and HC of mice. Taken together, the results indicate the effectiveness of MFSeI against depression-like behavior and central and peripheral complications caused by diabetes in mice.

1. Introduction

Diabetes mellitus and major depressive disorder (MDD) are two of the major health problems worldwide, with an incidence expected to increase in the next years (WHO, 2017; Rowley et al., 2017). Diabetes mellitus is a chronic metabolic disorder characterized by a chronic hyperglycemic status due to defects in insulin secretion and/or insulin action (WHO, 2018). Besides the consequences of chronic high blood glucose to the functioning of the main organs (including liver, kidneys, and brain), previous studies have suggested that approximately 30% of diabetic patients develop depression and the depressive symptoms are more severe in diabetic patients (Moulton et al., 2015). In fact, there is

growing interest regarding the bidirectional interaction between diabetes mellitus and MDD since it is frequently found that these conditions co-occur. It has been reported that 11% of patients with diabetes met the criteria for comorbid MDD (Anderson et al., 2001), while depressed adults have a 37% increased risk for developing diabetes mellitus (Knol et al., 2006). Both diabetes mellitus and MDD are characterized by increased oxidative stress (Réus et al., 2019), activation of the immune system (Herder et al., 2018), decreased brain monoamine levels (Roopan and Larsen, 2017), and altered neuronal synaptic plasticity (Ho et al., 2013).

Regardless of what condition comes first, both diabetes mellitus and MDD should be targeted with effective treatments. It has been shown

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that diabetes-induced depression behavior may be reversed by treatment with antidepressant and hypoglycemic drugs (Deuschle, 2013; Gupta et al., 2014) and that treatment-resistant depression may be alleviated by treatment with insulin-sensitizing agents (Lin et al., 2015). However, hypoglycemic drugs can elicit hypoglycemia, which is responsible for more than 95,000 visits to the emergency department in the USA (Moheet and Seaquist, 2014). In addition, the long-term use of antidepressants presents several adverse effects and may increase the risk of diabetes (Andersohn et al., 2009; Barnard et al., 2013). These constraints of the currently available medications prompted us to investigate the effects of a novel selenium-containing compound.

Selenium is an essential micronutrient for human health with important functions in maintaining antioxidant defenses (Brigelius-Flohé and Flohé, 2017). The administration of synthetic organoselenium compound has presented several beneficial effects in rodents, such as the antioxidant (Casaril et al., 2017b), antidepressant-like (Domingues et al., 2018, 2019), antigenotoxic (Abu-El-Zahab et al., 2019), neuroprotective, and antiapoptotic effects (Martini et al., 2019). These potential effects encouraged our group to synthesize and study a class of selenium and indole-containing compounds. We have previously shown that compounds of this class present antioxidant (Casaril et al., 2017b; Vieira et al., 2017) and antidepressant-like effect (Casaril et al., 2019a,b). Particularly, the compound 1-methyl-3-(phenylselenanyl)-1H-indole (MFSeI) was shown to have antioxidant and antidepressant-like effects in a mouse model of depression-like behavior induced by a sub-diabetogenic dose of streptozotocin (STZ) (Bampi et al., 2019).

In light of the biological effects of MFSeI and the concerns about diabetes mellitus and MDD, in this current study, we investigated the antidepressant-like, antioxidant, and anti-inflammatory effects of MFSeI in STZ-induced diabetes in mice. Since MFSeI was able to counteract the effects of a sub-diabetogenic dose of STZ, we hypothesized that the compound would be able to ameliorate the behavioral and neurochemical alterations induced by STZ in a mouse model of diabetes. With this mechanistic study, we aimed to understand how a multi-target intervention can target the depressive consequences of diabetes.

2. Materials and methods

2.1. Animals

The behavioral experiments were carried out using adult male Swiss mice ($n = 6$ mice/group), 2-month-old (weight of 25–35 g). The animals were kept in a separate animal room in a temperature-controlled environment (22–25 °C) and a time-controlled lighting system (12:12 h light/dark) with lights turned on every day at 7.00 a.m. Mice were housed in cages with free access to tap water and commercial food. All manipulations were carried out between 09.00 a.m. and 04.00 p.m. The present experimental study was approved by the Institutional Ethics Committee on Care and Use of Experimental Animal Resources from the Federal University of Pelotas, Brazil and registered under the number of 6407-2016.

2.2. Drugs

The 1-methyl-3-(phenylselenanyl)-1H-indole (MFSeI; Fig. 1A) was prepared and characterized at the Laboratory of Clean Organic Synthesis (LASOL) at Federal University of Pelotas according to a previously described method (Vieira et al., 2017). The MFSeI was dissolved in canola oil and administered at the dose of 10 mg/kg intragastrically (i.g.). Fluoxetine hydrochloride (Pfizer, Brazil) was diluted in saline solution (0.9%) at the dose of 10 mg/kg (i.g.) (Bampi et al., 2019) and Humulin N insulin (Lilly) was administered at 1 U/kg through subcutaneous route (s.c.). Oral administration of fluoxetine and subcutaneous insulin were chosen to mimic the procedures done in the clinic. STZ was purchased from Sigma-Aldrich (St Louis USA) and injected once intraperitoneally (i.p.) at a dose of 200 mg/kg. STZ was dissolved in 0.1 N pH 4.5 sodium citrate buffer (Castillo-Gomez et al., 2015).

2.3. Experimental design

A diagram of the experimental design is depicted in Fig. 1B. On the first day of the experiment, mice were rendered diabetic by a single i.p. injection of STZ (200 mg/kg body weight). Another group of mice was used as a control and received an i.p. injection of sodium citrate buffer (vehicle solution). To successfully induce diabetes using an STZ solution, we administered the solution within 30 min of preparation, storing it on ice and in the dark.

STZ-induced diabetic mice received a 5% glucose solution instead of water for 24 h after the injection of STZ in order to prevent death from hypoglycemic shock. On the third day after the STZ injection, the animals were weighed and the blood glucose was measured using a portable glucometer Accu-Check Nano Performa (Roche, Brazil). The blood drops were obtained by tail tip puncture and mice with glycemia higher than 250 mg/dl were considered diabetic. The blood glucose levels were measured again at the end of the experiment (day 21).

MFSeI and fluoxetine were administered at the dose of 10 mg/kg (i.g.) once a day during 14 days, starting 7 days after the STZ administration. The dose of MFSeI was chosen based on our previous study (Bampi et al., 2019). All drugs were administered at a volume of 10 ml/kg of body weight.

On the last day of the experiment, 24 h after the last administration of MFSeI and fluoxetine, the behavioral tests were carried out on 1 day at the following sequence: open field test (OFT), tail suspension test (TST) and forced swimming test (FST). The mice were immediately euthanized after completion of the behavioral tests and the hippocampus, prefrontal cortex, liver and kidney samples were excised and stored at -80°C for the analyses.

Mice were randomly divided into seven groups: Group I: sodium citrate buffer + canola oil; Group II: STZ + canola oil; Group III: STZ + MFSeI 10 mg/kg; Group IV: STZ + Fluoxetine 10 mg/kg; Group V: STZ + insulin 1 U/kg; Group VI: sodium citrate buffer + MFSeI 10 mg/kg; Group VII: sodium citrate buffer + Fluoxetine 10 mg/kg.

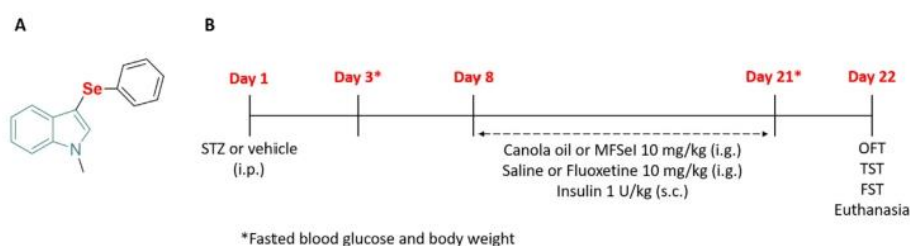


Fig. 1. MFSeI treatment presented antidepressant-like effect and restored blood glucose levels, oxidative stress, and neuroinflammation in STZ-induced diabetic mice. (A) Chemical structure of 1-methyl-3-(phenylselenanyl)-1H-indole (MFSeI) and (B) schematic representation of the timeline followed in the present study. STZ: streptozotocin. OFT: open field test. TST: tail suspension test. FST: forced swimming test.

Table 1
Primers used for the analysis of gene expression.

Gene	Sequence 5'-3'
GAPDH	F- AGGTCGGTGTGAACGGATTG R-TGTAGACCATGTAGTTGAGGTCA
IRS-1	F- CGATGGCTTCTCAGACGT R- CAGCCGCTTGTGTTGTTG
GLUT-4	F- GGGCTGTGAGTGAGTGCTTTC R- CAGCGAGGCAAGGCTAGA
TNF- α	F- CATCTTCTCAAAATTCGAGTGACAA R- TGGGAGTAGACAAGGTACAACCC
IL-10	F- CCAAGCCTTATCGGAAATG R- CCTGAGGCTCTCAGC
iNOS	F- GTGGTGACAAGCACATTGG R- AAGGCCAAACACAGCATA
IDO	F- AATCAAAGCAATCCCCACTG R- AAAAACGTGTCTGGGTCCAC

GAPDH: glyceraldehyde-3-phosphate dehydrogenase; IRS-1: insulin receptor substance; GLUT-4: glucose transporter-4; TNF- α : tumor necrosis factor- α ; IL-10: interleukin-10; iNOS: inducible nitric oxide synthase; IDO: indoleamine-2,3-dioxygenase.

2.4. Behavioral tests

2.4.1. Open field test (OFT)

The OFT was used to evaluate the locomotor and exploratory activities. Each animal was individually placed at the center of a box (30 × 30 × 15 cm) divided into nine quadrants of equal areas and observed for 5 min. The number of quadrants crossed with the four paws (locomotor activity) and the number of rearing on the hind limbs (exploratory activity) was observed (Walsh and Cummins, 1976).

2.4.2. Tail suspension test (TST)

The antidepressant-like effect of MFSeI was assessed by the TST according to the method described by Steru et al. (1985). Mice were suspended 50 cm above the floor by adhesive tape placed approximately 1 cm from the tip of their tail. The total immobility time was recorded during the last 4 min of a 6 min session (2 min for adaptation). Mice were considered immobile only when they hung passively and completely motionless.

2.4.3. Forced swimming test (FST)

The FST was also carried out to evaluate the antidepressant-like effect of MFSeI (Porsolt et al., 1977). The mice were individually placed in a cylinder (15 cm diameter, 25 cm height) containing 20 cm of water maintained at 25 ± 1 °C. After 2 min of habituation, the immobility time (in seconds) of the animals was observed for 4 min. Immobility was assigned when no additional activity was observed other than that required to keep the mice head above the water.

2.5. Biochemical determinations

2.5.1. Tissue preparation

After the behavioral tests, on the 22nd day, the animals were anesthetized by isoflurane inhalation for blood collection by heart puncture in tubes containing heparin. Plasma was obtained by centrifugation at 2500 × g for 10 min and utilized for the determination of triglycerides, total cholesterol, and biomarkers of hepatic and renal toxicity. Next, the animals were killed by cervical dislocation and the liver, kidney, prefrontal cortex, and hippocampus were quickly removed, weighed and homogenized in 50 mM Tris/HCl, pH 7.4 (1/10, weight/volume). The homogenized tissues were centrifuged at 4000 × g for 10 min at 4 °C and the supernatant was used for the determination of reactive species (RS) and thiobarbituric acid reactive species (TBARS) levels. Another group of animals was euthanized for the removal of the prefrontal cortex and hippocampus, which were stored in TRIzol reagent at −80 °C for the analysis of gene expression.

2.5.2. Plasma biochemical parameters

The plasma levels of triglycerides, total cholesterol, aspartate aminotransferase (AST), alanine aminotransferase (ALT), urea and creatinine were determined by commercially available kits according to the manufacturer's instruction (LABTEST).

2.5.3. Reactive species (RS) quantification

The levels of RS were determined by a spectrofluorimetric method, using the dichlorofluorescein diacetate (DCFH-DA) assay (Loetchutinat et al., 2005). An aliquot of the supernatant was incubated with 1 mM DCFH-DA. The oxidation of DCFH-DA to the fluorescent for dichlorofluorescein (DCF) is measured for the detection of intracellular RS. The DCF fluorescence intensity emission was recorded at 520 nm (with 480 nm excitation) 15 min after the addition of DCFH-DA to the medium. RS levels were expressed as fluorescence units.

2.5.4. Thiobarbituric acid reactive substances (TBARS) assay

The evaluation of lipid peroxidation was estimated using the method described by Ohkawa et al. (1979). An aliquot of the supernatant was incubated with 0.8% thiobarbituric acid (TBA), acetic acid buffer pH 3.4, and 8.1% sodium dodecyl sulfate (SDS) at 95 °C for 1 h. The colorimetric reaction was measured at 532 nm. TBARS levels were expressed as nmol MDA/g tissue.

2.5.5. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total mRNA was extracted from the prefrontal cortex (PFC) and hippocampus (HC) of mice using TRIzol (Invitrogen™, Carlsbad, USA), followed by total RNA quantification. The cDNA synthesis was performed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems™, UK) according to the manufacturer's protocol. The amplification was made with SYBR Green PCR Master Mix (Applied Biosystems, UK) using the Stratagene Mx3005P. The sequence of primers used is indicated in Table 1. The GAPDH gene was used to normalize the reactions. The conditions for the reaction included 95 °C for 15 s, 60 °C for 60 s and 72 °C for 30 s. The 2^{ΔΔCT} (Delta-Delta Comparative Threshold) method was used to normalize the fold change in gene expression.

2.6. Statistical analysis

The statistical analysis was performed using the GraphPad Prism 7.0 software. The data were analyzed by one-way analysis of variance (ANOVA), followed by the Newman-Keuls *post hoc* test and are presented as the mean ± standard error of the mean (SEM). The data were considered statistically significant when *p* < 0.05. Pearson's correlation coefficient was used for correlation analysis.

3. Results

3.1. MFSeI treatment decreased blood glucose, triglycerides, and creatinine levels in STZ-induced diabetic mice

Fig. 2A shows that three days after the STZ administration the mice became diabetic, as indicated by their plasma glucose levels higher than 250 mg/dl ($F_{6,35} = 150.00$; *p* < 0.001). On day 21 (Fig. 2B), STZ-treated mice still presented high glucose levels (average of 592 mg/dl), validating the use of STZ as a diabetes mellitus-inducer in the present work ($F_{6,35} = 767.00$; *p* < 0.001). The treatment with MFSeI significantly decreased the glucose levels (average of 494 mg/dl; *p* < 0.001), but not as strong as the administration of insulin (average of 158 mg/dl; *p* < 0.001). Treatment with fluoxetine did not decrease the STZ-induced glucose levels. Administration of MFSeI and fluoxetine without the induction of diabetes mellitus did not alter these parameters.

The body weight of the mice was measured on day 3 (Fig. 2C) and on day 21 (Fig. 2D). With the exception of the reduced body weight

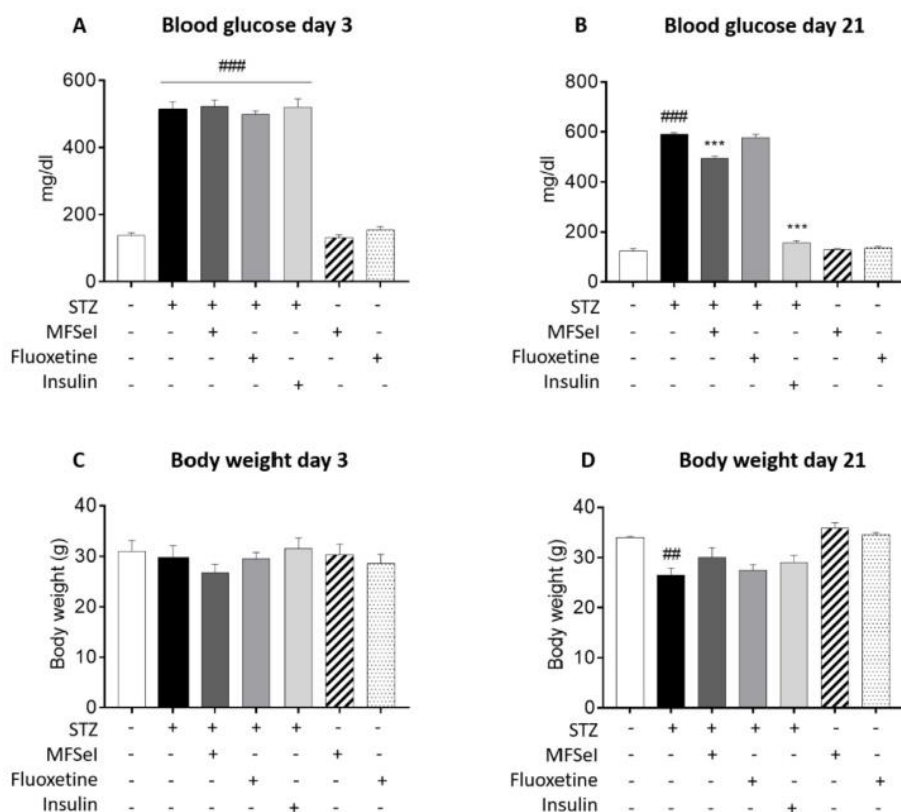


Fig. 2. MFSeI treatment decreased blood glucose levels without altering the body weight of STZ-treated mice. Fasting blood glucose levels on (A) day 3 and (B) day 21, and body weight on (C) day 3 and (D) day 21. $^{##}p < 0.01$ and $^{###}p < 0.001$ when compared to control group (white bar). $^{***}p < 0.001$ when compared to the STZ-treated group (black bar). $n = 6$ mice/group.

found in the STZ-treated mice on day 21 ($p < 0.01$), no alteration was found for any of the treatments ($F_{6,35} = 8.62$; $p < 0.001$). Because of the altered body weight of STZ-treated mice, we performed a Pearson's correlation analysis to verify if the body weight (on day 21) was influencing the behavior of mice. No correlation was found between the body weight of mice and the immobility time in the TST ($r = 0.152$, $p = 0.34$) and FST ($r = 0.042$, $p = 0.79$) (data not shown). The absence of the correlation indicated to us that the body weight was not influencing the behavior of mice and therefore, it was not addressed as a confounding variable.

Chronic hyperglycemia can lead to the dysregulation of other biochemical parameters and complications in the liver and kidneys. As depicted in Tables 2 and 3, the STZ injection increased the plasma levels of triglycerides ($F_{6,35} = 10.70$; $p < 0.001$), urea ($F_{6,35} = 5.30$; $p < 0.001$) and creatinine ($F_{6,35} = 46.80$; $p < 0.001$). In STZ-treated mice, the triglyceride levels were reduced by treatment with MFSeI ($p < 0.001$) and fluoxetine ($p < 0.05$). The increased levels of creatinine induced by STZ were decreased by the treatment with MFSeI, fluoxetine, and insulin ($p < 0.001$). Total cholesterol, AST and ALT

were not altered by any of the treatments. Administration of MFSeI and fluoxetine without the induction of diabetes mellitus did not alter these parameters.

3.2. MFSeI treatment presented antidepressant-like effect in STZ-induced diabetes mellitus and depression-like behavior

In order to exclude the possibility of the drugs having exercised a psychostimulant effect, the OFT was performed. The number of crossings ($F_{6,35} = 0.74$; $p = 0.61$) and rearings ($F_{6,35} = 0.26$; $p = 0.95$) were not altered by any of the treatments (data not shown).

As expected, STZ-induced diabetic mice also presented depression-like behavior when compared to the control group, as observed in the TST and FST. Interestingly, we found that MFSeI reversed the increased immobility time in the TST ($F_{6,35} = 15.80$; $p < 0.001$, Fig. 3A) and FST ($F_{6,35} = 12.00$; $p < 0.001$, Fig. 3B). Moreover, the positive controls fluoxetine and insulin also presented antidepressant-like effect in the TST and FST. Administration of MFSeI and fluoxetine without the induction of diabetes mellitus did not alter the behavior of mice.

Of note, correlation analysis showed that the increase in immobility time in TST and FST was positively correlated with the increase in the blood glucose levels ($r = 0.551$; $p = 0.02$, Fig. 3C and $r = 0.550$; $p = 0.02$, Fig. 3D, respectively).

3.3. MFSeI treatment reduced the levels of RS in STZ-induced diabetic mice

In the present study, we verified that diabetic mice with depression-like behaviour presented increased levels of RS in the prefrontal cortex ($F_{6,35} = 8.20$; $p < 0.001$, Fig. 4A), hippocampus ($F_{6,35} = 16.30$; $p < 0.001$, Fig. 4B), liver ($F_{6,35} = 6.37$; $p < 0.001$, Fig. 4C), and kidneys ($F_{6,35} = 6.49$; $p < 0.001$, Fig. 4D) in comparison to the control group. Treatment with MFSeI and insulin after the STZ injection significantly decreased the RS levels in all tissues. Fluoxetine had similar effects in the prefrontal cortex, hippocampus, and kidneys. Administration of MFSeI and fluoxetine without the induction of diabetes

Table 2

Effect of MFSeI treatment on triglycerides and total cholesterol levels in mice with and without STZ injection.

	Triglycerides (mg/dl)	Total cholesterol (mg/dl)
Control	198.0 ± 18.3	83.5 ± 3.8
STZ	262 ± 18.3 [#]	92.2 ± 5.9
STZ + MFSeI	115.0 ± 19.6 ^{***}	91.5 ± 6.6
STZ + Flx	187.0 ± 13.3 [*]	79.4 ± 4.4
STZ + insulin	218.0 ± 11.8	73.6 ± 5.66
MFSeI	228.0 ± 5.34	82.6 ± 4.8
Flx	244.0 ± 14.8	85.2 ± 4.3

STZ: streptozotocin; MFSeI: 1-methyl-3-(phenylselanyl)-1H-indole; Flx: fluoxetine. [#] $p < 0.05$ when compared to the control group; ^{*} $p < 0.05$ and ^{***} $p < 0.001$ when compared to the STZ group.

Table 3

Effect of MFSeI treatment on markers of hepatic and renal toxicity in mice with and without STZ injection.

	AST (U/L)	ALT (U/L)	Urea (mg/dl)	Creatinine (mg/dl)
Control	83.1 ± 5.08	41.4 ± 2.06	59.5 ± 3.8	0.235 ± 0.042
STZ	92.3 ± 11.1	53.0 ± 3.72	80.1 ± 4.8 [#]	1.810 ± 0.194 ^{###}
STZ + MFSeI	107.0 ± 9.63	45.2 ± 2.82	77.2 ± 4.4	0.229 ± 0.027 ^{***}
STZ + Flx	108.0 ± 4.62	54.0 ± 4.9	69.5 ± 6.7	0.193 ± 0.084 ^{***}
STZ + insulin	113.0 ± 5.87	52.1 ± 3.61	75.7 ± 5.66	0.387 ± 0.046 ^{***}
MFSeI	81.5 ± 12.2	41.9 ± 5.35	54.6 ± 4.35	0.307 ± 0.049
Flx	82.5 ± 3.34	46.8 ± 3.86	58.4 ± 2.1	0.177 ± 0.022

STZ: streptozotocin; MFSeI: 1-methyl-3-(phenylselenanyl)-1H-indole; Flx: fluoxetine; AST: aspartate aminotransferase; ALT: alanine aminotransferase. [#] $p < 0.05$ and ^{###} $p < 0.001$ when compared to the control group. ^{***} $p < 0.001$ when compared to the STZ group.

mellitus by STZ did not alter the levels of RS in any of the tissues analyzed.

3.4. MFSeI treatment reduced the lipid peroxidation levels in STZ-induced diabetic mice

The levels of lipid peroxidation in the prefrontal cortices, hippocampus, liver, and kidney of mice are shown in Fig. 5. The statistical analysis showed that TBARS levels were increased in STZ-induced diabetic mice when compared to the control group in all tested tissues. Treatment with MFSeI and the positive controls fluoxetine and insulin abolished the increase of TBARS levels in the prefrontal cortices ($F_{6,35} = 11.8$; $p < 0.001$, Fig. 5A) and hippocampus ($F_{6,35} = 5.11$; $p < 0.001$, Fig. 5B), while only MFSeI and insulin decreased the TBARS levels in the liver ($F_{6,35} = 7.57$; $p < 0.001$, Fig. 5C) and kidney ($F_{6,35} = 5.64$; $p < 0.001$, Fig. 5D) when compared to STZ group. Administration of MFSeI and fluoxetine without the induction of diabetes mellitus did not alter the levels of lipid peroxidation in any of the tissues analyzed.

3.5. MFSeI treatment beneficially affects the gene expression in STZ-induced diabetic mice

The statistical analyses demonstrated that the STZ decreased the expression of IRS-1 in the prefrontal cortex ($F_{6,35} = 11.00$; $p < 0.001$, Fig. 6A) and hippocampus ($F_{6,35} = 7.11$; $p < 0.001$, Fig. 6B) of mice. The treatments with MFSeI ($p < 0.05$) and insulin ($p < 0.05$) increased the IRS-1 mRNA expression in the prefrontal cortex and only MFSeI ($p < 0.05$) increased the expression of IRS-1 in the hippocampus.

The GLUT-4 mRNA expression was decreased in the prefrontal cortices of mice exposed to STZ. The treatment with MFSeI ($p < 0.001$), but not insulin and fluoxetine, abolished the decrease of GLUT-4 mRNA expression ($F_{6,35} = 9.47$; $p < 0.001$, Fig. 6C). The GLUT-4 mRNA expression in the hippocampus was not altered by any of the treatments ($F_{6,35} = 1.16$; $p = 0.35$, Fig. 6D).

The statistical analysis showed that the mRNA expression of TNF- α was increased in the prefrontal cortices ($F_{6,35} = 29.90$; $p < 0.001$, Fig. 7A) and hippocampus ($F_{6,35} = 36.20$; $p < 0.001$, Fig. 7B) of mice exposed to the STZ when compared to the control group. In the prefrontal cortex, all treatments (MFSeI, insulin, and fluoxetine) abolished the increase of TNF- α mRNA expression. On the other hand, only the treatment with MFSeI decreased the TNF- α mRNA expression in the

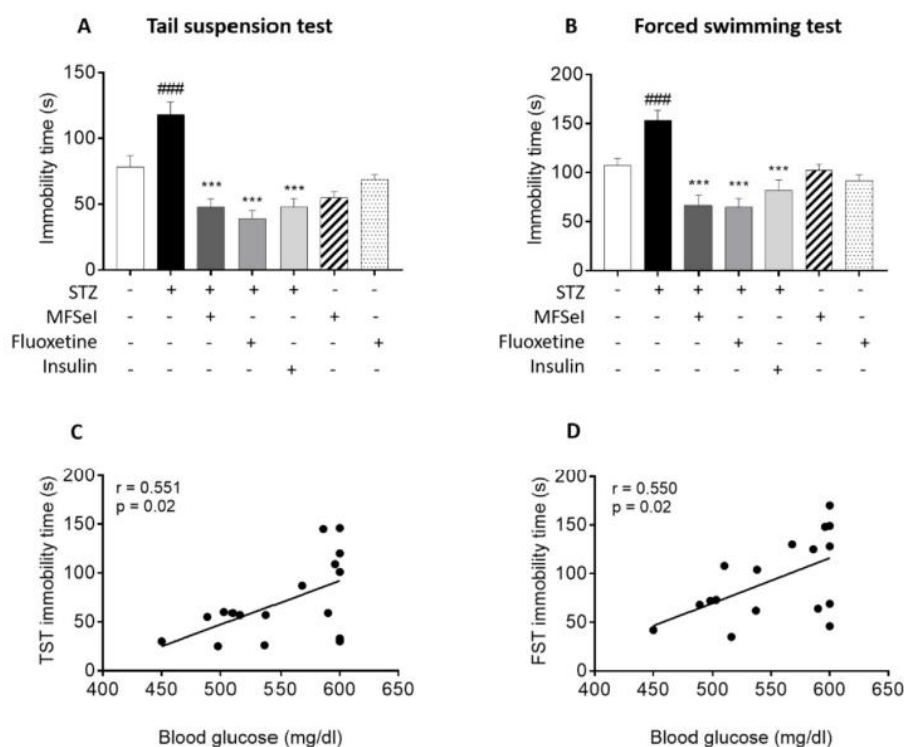


Fig. 3. MFSeI treatment presented antidepressant-like effect in STZ-induced diabetic mice. Effects of MFSeI on the (A) TST and (B) FST in mice with and without STZ injection. Correlation between the increase in immobility time in the (C) TST and (D) FST with increase in blood glucose levels (Pearson's correlation coefficient). ^{###} $p < 0.001$ when compared to control group (white bar). ^{***} $p < 0.001$ when compared to the STZ-treated mice (black bar). $n = 6$ mice/group.

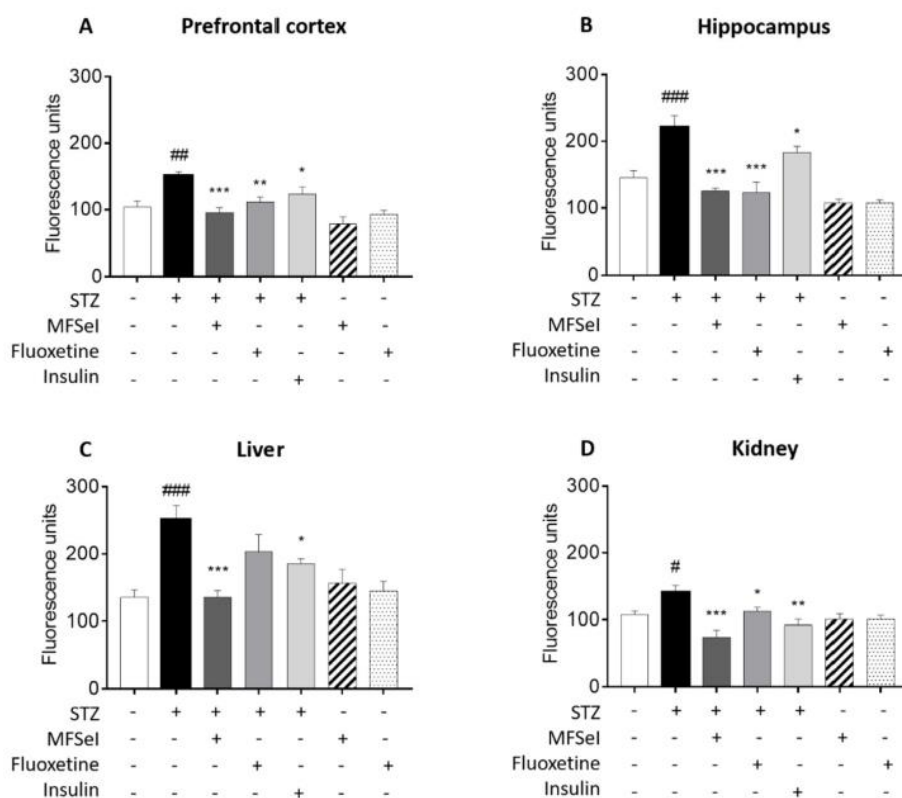


Fig. 4. MFSeI treatment decreased the levels of RS induced by STZ in mice. RS levels in the (A) prefrontal cortex, (B) hippocampus, (C) liver, and (D) kidney of mice with or without STZ injection. [#] $p < 0.05$, ^{##} $p < 0.01$, and ^{###} $p < 0.001$ when compared to the control group (white bar). ^{*} $p < 0.05$, ^{**} $p < 0.01$, and ^{***} $p < 0.001$ when compared to STZ-treated mice. $n = 6$ mice/group.

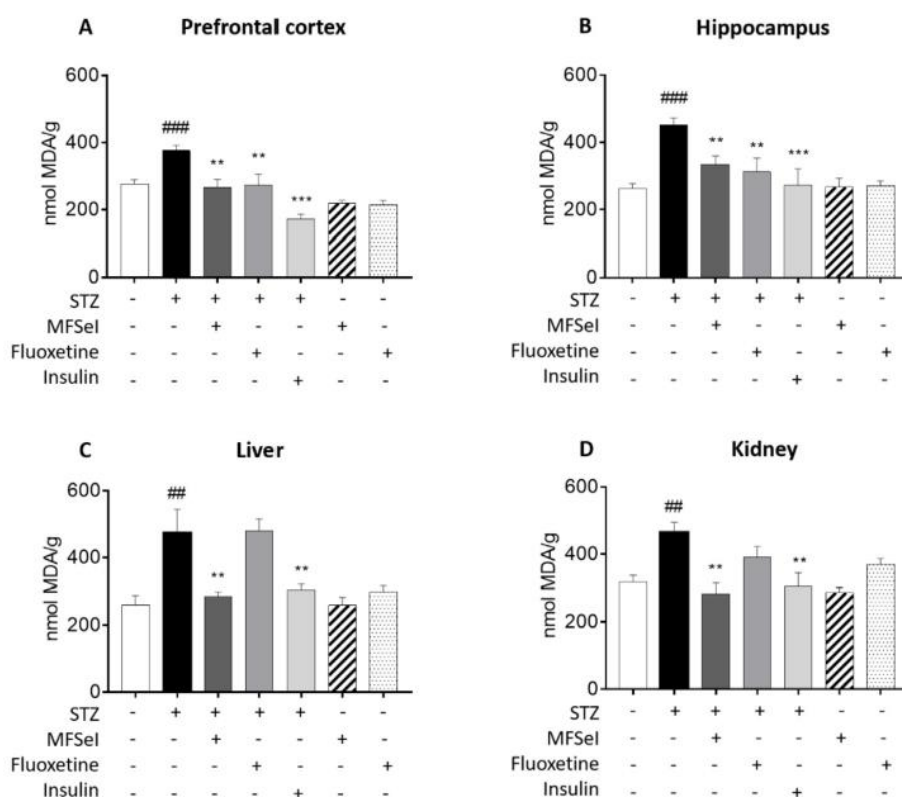


Fig. 5. MFSeI treatment reduced the lipid peroxidation induced by STZ in mice. TBARS levels in the (A) prefrontal cortex, (B) hippocampus, (C) liver, and (D) kidney of mice with and without STZ injection. ^{##} $p < 0.01$ and ^{###} $p < 0.001$ when compared to the control group (white bar). ^{**} $p < 0.01$ and ^{***} $p < 0.001$ when compared to STZ-treated mice. $n = 6$ mice/group.

hippocampus of diabetic mice ($p < 0.001$).

The STZ injection induced a statistically significant decrease in the mRNA expression of IL-10 in the prefrontal cortex ($F_{6,35} = 5.40$; $p < 0.001$, Fig. 7C) and hippocampus ($F_{6,28} = 7.88$; $p < 0.001$, Fig. 7D) of mice when compared to the control group. The treatments

with MFSeI ($p < 0.001$) and fluoxetine ($p < 0.01$) reversed the decreased expression of IL-10 in the prefrontal cortices, but only MFSeI ($p < 0.001$) increased the expression of IL-10 in the hippocampus of diabetic mice.

The expression of iNOS was increased in the prefrontal cortex of

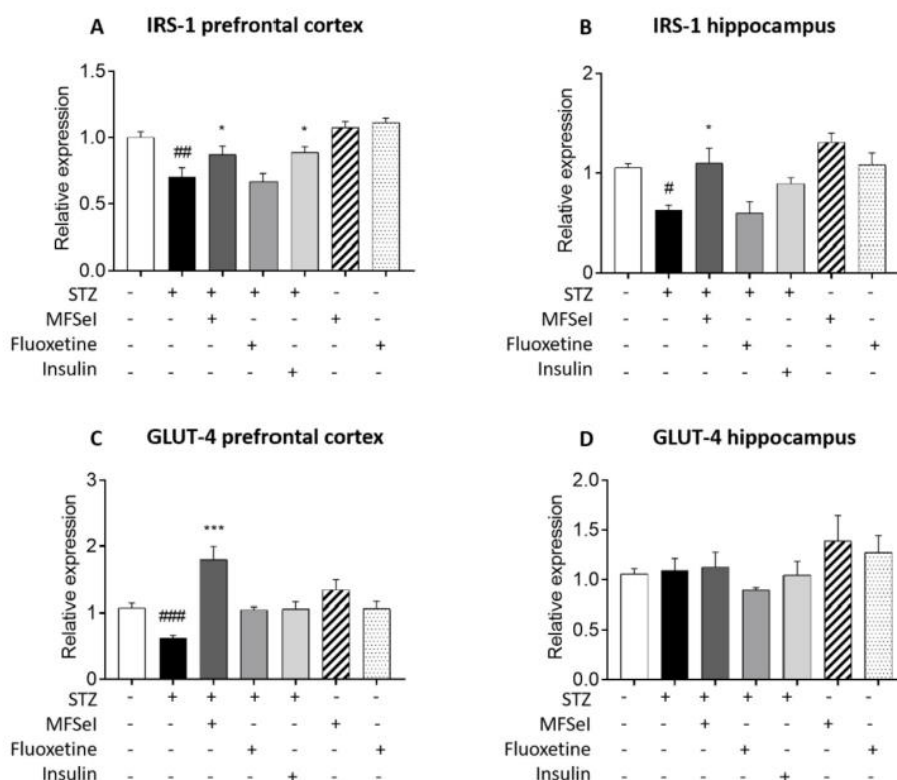


Fig. 6. MFSeI treatment restores the expression of genes related to the glucose metabolism in the brain of mice. Relative expression of IRS-1 and GLUT-4 in the (A and C) prefrontal cortex and (B and D) hippocampus of mice with and without STZ injection. # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$ when compared to the control group (white bar). * $p < 0.05$ and *** $p < 0.001$ when compared to STZ-treated mice. $n = 5-6$ mice/group.

diabetic mice ($F_{6,35} = 26.30$; $p < 0.001$, Fig. 7E), and treatment with MFSeI ($p < 0.01$) and fluoxetine ($p < 0.01$) were able to reduce it. The mRNA expression iNOS in the hippocampus of mice was not altered by any of the treatments (Fig. 7F).

The decrease in the availability of tryptophan was assessed by IDO mRNA expression in the prefrontal cortex (Fig. 7G) and hippocampus (Fig. 7H). Diabetic mice presented increased mRNA expression in the prefrontal cortex ($F_{6,35} = 6.35$; $p < 0.001$) and hippocampus ($F_{6,35} = 5.63$; $p < 0.001$). Treatment with MFSeI decreased the IDO expression in the prefrontal cortex ($p < 0.05$) and hippocampus ($p < 0.001$), while fluoxetine ($p < 0.01$) and insulin ($p < 0.001$) decreased the expression of IDO only in the hippocampus of diabetic mice.

The treatment with MFSeI and fluoxetine without the induction of diabetes mellitus by STZ did not alter the expression of any of the genes in the prefrontal cortex and hippocampus of mice.

4. Discussion

It has been acknowledged that diabetes mellitus and MDD frequently co-occur (Anderson et al., 2001; Knol et al., 2006), and the functional and physiological consequences of their co-occurrence are measurable and important. In the present study, we investigated the putative relationship between diabetes mellitus and depression-like behavior, and we tested the hypothesis that treatment with MFSeI would be able to counteract behavioral and biochemical alterations in STZ-induced diabetic mice (Fig. 8). The results showed for the first time that the depressive symptoms of diabetic mice can be effectively minimized by MFSeI treatment. At the same time, MFSeI treatment reduced the high blood glucose levels and plasma levels of triglycerides, urea, and creatinine induced by STZ. Moreover, MFSeI treatment reversed the STZ-induced increased production of RS and the levels of lipid peroxidation in the brain, liver, and kidneys of mice. Treatment with MFSeI also increased the expression of IRS-1 and GLUT-4, decreased the expression of TNF- α and increased the expression of IL-10 in the prefrontal cortex and hippocampus of mice. Additionally, MFSeI

decreased the expression of enzymes related to the production of nitric oxide (iNOS) and of deviation from serotonin production (IDO) in brain structures of mice treated with STZ.

Even though several neural pathways are linked to the development of depression, the hippocampus and prefrontal cortex have been repeatedly implicated in the pathophysiology and progression of this disorder (Belleau et al., 2019). Patients with major depression have shown structural changes in both brain regions (McKinnon et al., 2009; Treadway et al., 2015), increased resting-state functional connectivity between hippocampal and frontal regions (Goveas et al., 2011), and abnormal hippocampal activation during the processing of negative-valence stimuli (Sheline et al., 2009). Despite the fact that insulin receptors are ubiquitous in the brain, insulin receptor density is highest in the olfactory bulb, hypothalamus, hippocampus, cerebral cortex, striatum, and cerebellum (Arnold et al., 2018). Particularly, it has been shown that diabetic patients may suffer from weaknesses in their prefrontal cortex functions (Sun et al., 2017) and reduced hippocampal functional connectivity with the bilateral frontal cortex (Zhou et al., 2010; Sun et al., 2018). In this sense, we decided to focus our efforts on elucidating the possible effects of MFSeI on these two brain regions.

It seems that there is a double directional association between depression and diabetes, an important and complex relation that might share biological mechanisms. This is evident when considering that depression may increase the risk of developing type 2 diabetes by 60% (Rubin et al., 2008) and depression occurrence is two to three times higher in people with diabetes mellitus (Roy and Lloyd, 2012). Therefore, our present study could provide better treatment and improve the outcomes for these pathologies.

STZ is a widely used diabetes mellitus type 1 inducer in mice since it has preferential toxicity toward pancreatic β cells (Goyal et al., 2016). In the current study, STZ resulted in hyperglycemia three days after its administration and this status remained detectable until the end of the experiment. STZ-induced diabetic mice also exhibited weight loss on the last day of the experiment, which confirmed the establishment of the diabetic condition. The 14 days of treatment with MFSeI partially reversed hyperglycemia caused by STZ, which was not achieved with

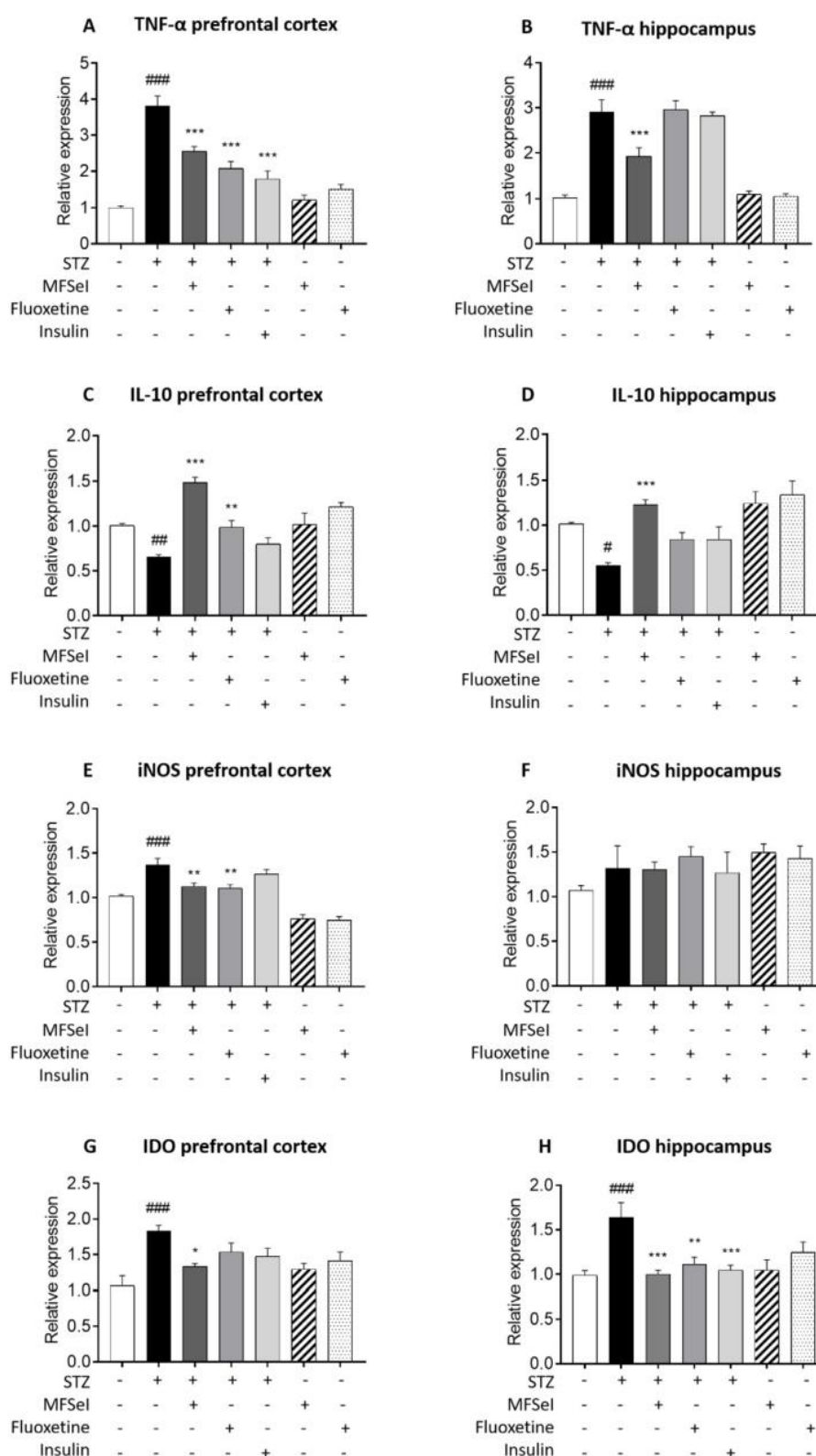


Fig. 7. MFSel treatment restored the expression of genes related to neuroinflammation in mice. Relative expression of TNF- α (A: prefrontal cortex; B: hippocampus), IL-10 (C: prefrontal cortex; D: hippocampus), iNOS (E: prefrontal cortex; F: hippocampus), and IDO (G: prefrontal cortex; H: hippocampus) in mice with and without STZ injection. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ when compared to the control group (white bar). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ when compared to STZ-treated mice. $n = 5-6$ mice/group.

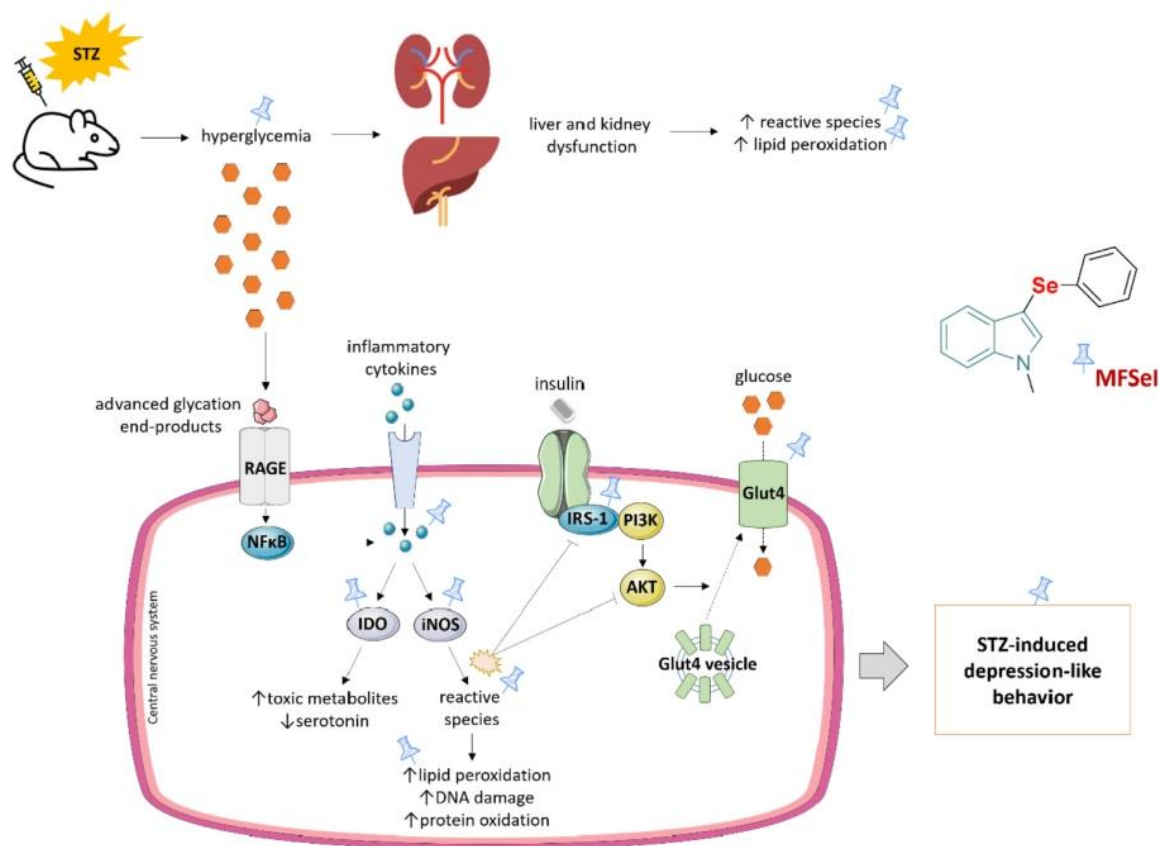


Fig. 8. Overview of the effects of STZ and the modulatory effects of MFSeI. Blue pins indicate the parameters modulated by MFSeI in the present study. STZ: streptozotocin; RAGE: receptor for advanced glycation end products; NF- κ B: nuclear factor kappa-light-chain-enhancer of activated B cells; IDO: indoleamine 2,3-dioxygenase; iNOS: inducible nitric oxide synthase; IRS-1: insulin receptor substrate-1; PI3K: phosphoinositide 3-kinases; AKT: protein kinase B, also known as AKT; Glut-4: glucose transporter-4.

the antidepressant fluoxetine. As expected, the treatment with insulin decreased the hyperglycemia induced by STZ, leading to glucose levels similar to the control group. By using an antidepressant and the classic treatment for diabetes, our objective here was to validate our experimental model and investigate if the antidepressant-like effect of MFSeI was also dependent on the modulation of the glucose metabolism in STZ-induced diabetic mice.

Previous studies reported that STZ-induced diabetic mice exhibit depression-like behavior 14 days after treatment (Hilakivi-Clarke et al., 1990; Castillo-Gomez et al., 2015). Treatment with MFSeI elicited antidepressant-like effect in STZ-induced diabetic mice by reducing the immobility time in TST and FST, an effect that was also observed after fluoxetine and insulin treatment, reference drugs used to treat patients with major depression and type 1 diabetes mellitus (Ho et al., 2013). Mice had no locomotor and exploratory alterations in the OFT, excluding the possibility that the increased immobility time in the TST and FST was a consequence of psychostimulant behavior. It is important to note that by the time that MFSeI presented antidepressant-like effect, it also reduced the blood glucose levels. Both MFSeI and insulin treatment presented antidepressant-like effect while reducing blood glucose levels. However, insulin-related hypoglycemia is responsible for more than 95,000 visits to emergency departments in the USA each year, raising some concerns about its chronic use (Moheet and Seaquist, 2014). In this sense, the milder effect of MFSeI in reducing blood glucose levels when compared to insulin may be seen as an advantage, as both drugs still present similar effects in the TST and FST.

Diabetes mellitus-related complications in multiple vital organs are a major concern of this chronic disorder. Diabetes mellitus is associated with lipid disorders (Vergès, 2009), liver diseases (Harrison, 2006) and

kidney failure (Alicic et al., 2017). Here we reported that treatment with MFSeI was able to decrease the plasma levels of triglycerides and creatinine, but not urea, in STZ-treated mice. On the other hand, the STZ injection did not alter the levels of total cholesterol, AST, and ALT. However, the STZ injection increased the levels of RS and lipid peroxidation in the liver and kidney of mice, which were reversed by treatment with MFSeI and insulin. Fluoxetine only reversed the increased levels of RS in the kidneys of STZ-treated mice. Altogether, these results show the ability of MFSeI, not only in reducing the blood glucose levels but also in reversing the complications induced by diabetes mellitus in the liver and kidney.

The oxidative stress present in the periphery of diabetic mice was also found in the prefrontal cortex and hippocampus. In addition to increased RS and TBARS levels, diabetic mice also presented increased expression of TNF- α , iNOS, and IDO, and decreased expression of IL-10 in the prefrontal cortex and hippocampus. Mechanistically, STZ-induced hyperglycemia increases the production of advanced glycation end-products (AGEs) (Kesavan et al., 2013), which can bind to protein and enzymes and induce the production of RS while inactivating antioxidant defenses, leading to oxidative stress. Also, AGEs can bind to their receptor (RAGE) and increase the production of reactive species and induce the expression of inflammatory cytokines. Here we showed that STZ-induced oxidative stress and neuroinflammation were reversed by treatment with MFSeI in the prefrontal cortex and hippocampus of mice. It is interesting to note that, while fluoxetine and insulin were able to improve oxidative stress in both brain regions, MFSeI was the only treatment able to restore the levels of TNF- α and IL-10 in the hippocampus of diabetic mice. This is in agreement with previous data showing that another selenium-containing compound belonging to

the same class as MFSeI modulated the neuroinflammation in mice models of depression-like behavior (Casaril et al., 2017a; Casaril et al., 2019a,b). In addition, MFSeI reversed the upregulation of IDO induced by STZ. It has been shown that IDO mediates inflammation-induced depression-like behavior (O'Connor et al., 2009) and accumulation of toxic metabolites of tryptophan degradation (Le Floch et al., 2011), therefore, decreasing its expression may account for the improvement of depression-like behavior evidence in the TST and FST after MFSeI administration.

In animals, the rise of blood glucose stimulates the secretion of the hormone insulin. The insulin action initiates when it docks into the insulin receptor and results in its phosphorylation. This subsequently activates the IRS-1, phosphatidylinositol-3-kinases (PI3K), and protein kinase B (PKB or Akt), resulting in the translocation of GLUT-4 to promote the glucose uptake. Impaired insulin signaling in the brain increases the complication of diabetes and increases the risk of Alzheimer's disease (Hölscher, 2019). Since STZ-induced diabetic mice produce less insulin, the glucose uptake through tends to be impaired, as reflected by the decreased expression of IRS-1 and GLUT-4 in the brain. Interestingly, treatment with MFSeI increased the expression of IRS-1 in the prefrontal and hippocampus and increased the expression of GLUT-4 in the prefrontal cortex. These effects suggest that MFSeI helps in glucose disposal by promoting insulin-mediated glucose uptake. As insulin signaling also regulates neuronal growth, repair, and functions, the neuroprotective effects of MFSeI should also be investigated. In addition, insulin signaling also activates the nuclear factor erythroid 2-related factor 2 (Nrf2) to increase the cellular response against oxidative stress (Song et al., 2018). The activation of this pathway would help to explain the ability of MFSeI to decrease the oxidative stress status (i.e. RS and TBARS levels) in the brain of diabetic mice. Interestingly, treatment with fluoxetine did not increase the expression of IRS-1 and GLUT-4 in diabetic mice, while insulin only increased the expression of IRS-1 in the prefrontal cortex, but not in the hippocampus, probably because the amount of insulin passing the blood-brain barrier varies drastically among species (Woods et al., 2003). Although the association between peripheral insulin resistance and brain insulin resistance has been reported, the causative relationship between these events has not been established yet. Therefore, even though we believe that in our model of STZ-induced diabetes and depression-like behavior, the altered expression of IRS-1 and GLUT4 in the brain (and possibly insulin resistance) may be a consequence of peripheral insulin resistance, we would have to perform a timeline study to investigate the order of events. We believe that brain insulin resistance may be a consequence of diabetes since studies have suggested that peripheral insulin resistance can metastasize to the brain leading to insulin resistance (Kamal Sachdeva et al., 2019; Sripetchwandee et al., 2018; Filippi et al., 2017; Pathan et al., 2008).

Our study was focused on the characterization of MFSeI impact on various aspects of diabetic complications, such as peripheral, neurochemical, and behavioral alterations. However, we are aware that the metabolic profile of diabetes and STZ-induced diabetes is more complex than what we studied here. An additional limitation of our study is the use of a single animal model. Nonetheless, the injection with STZ mimicked several alterations found in diabetic patients, and the use of two positive controls validated the diabetic and depressive nature of mice injected with STZ, allowing the characterization of MFSeI. Moreover, we tested the effects of MFSeI in male mice, knowing that depression is more prevalent in women than in men. We chose to initiate the characterization of MFSeI in male mice to avoid any confounding factors with the hormonal changes that take place in female mice. However, this limitation needs to be addressed if we want to make a translational approach to humans. In that front, we already reported that MFSeI has no toxic effect in mice (Bampi et al., 2019), which would encourage us to keep investigating this molecule until we have a strong pre-clinical characterization of MFSeI that could be used to prompt the clinical research. The characterization of the pharmacokinetics and

pharmacodynamics of MFSeI, a stronger toxicological evaluation, and the use of other animal species need to be conducted to fully characterize the pharmacological profile and the safety of MFSeI before we can translate its effects to humans.

It is also important to consider that the studies analyzing the anti-diabetic effects of selenium supplementation only considered selenium in its inorganic form (especially sodium selenate). Due to the role of selenium in the active site of antioxidant enzymes, it is suggested that its ability to protect cells against oxidative stress may be implicated in its antidiabetic effect (Steinbrenner and Sies, 2009; Mueller et al., 2009a,b). Additionally, it has been shown that insulin-like properties of inorganic selenium may be a consequence of its ability to activate kinases involved in the insulin signaling, such as the kinases Akt and the p70 S6 (Steinbrenner et al., 2011). Other studies have shown that selenium may improve insulin signaling via stimulating glucose uptake through translocation of glucose transporters to the plasma membrane (Hei et al., 1998), influencing calcium and zinc homeostasis, decreased the expression of IL-1 β and TNF- α (Ayaz and Turan, 2006), normalizing the Na⁺/K⁺ ATPase activity (Aydemir-Koksoy and Turan, 2008), improving insulin sensitivity in type 2 diabetic db/db mice (Mueller and Pallauf, 2006), and reversing the abnormal expression and activity of glycolytic and gluconeogenic liver enzymes (Becker et al., 1996).

In summary, the present study supports the notion that treatment with MFSeI ameliorates the peripheral, neurochemical, and behavioral alterations induced by STZ in mice (Fig. 8). Specifically, MFSeI reversed the depression-like behavior induced by STZ, and this effect may be dependent on its ability to modulate the plasma glucose levels, liver, and kidney oxidative stress, and brain oxidative stress, neuroinflammation, and insulin pathway. Further studies to characterize the molecular mechanisms of MFSeI – via the modulation of the IRS1/PI3K/Akt/mTOR/GSK-3 β pathway – will be carried out.

Declaration of competing interest

All authors declare that they have no conflicts of interest.

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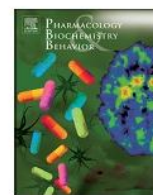
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Repeated administration of a selenium-containing indolyl compound attenuates behavioural alterations by streptozotocin through modulation of oxidative stress in mice

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ABSTRACT

Although the pathophysiology of major depression disorder (MDD) is still poorly understood, mounting evidence suggests that the brains of depressed patients are under oxidative stress, leading to depressive symptoms that may include anxiety and cognitive impairment. This study aimed to investigate if the seleno-organic compound 1-methyl-3-(phenylselanyl)-1H-indole (MFSeI) reverses the depression- and anxiogenic-like behaviour, cognitive impairment and oxidative stress induced by the intra-cerebroventricular injection of streptozotocin (STZ; 0.2 mg/4 µl/per mouse) in Swiss male mice. Twenty-four hours after the STZ injection, mice were treated with MFSeI (10 mg/kg, intra-gastrically), or vehicle solution, once daily for seven days. The behavioural tests were performed 30 min after the final MFSeI administration, followed by euthanasia and collection of the cerebral cortex and hippocampus. Administration of MFSeI reversed the depression- and anxiogenic-like behaviour and cognitive impairment induced by STZ, in mice. Neurochemical analyses demonstrated that MFSeI reversed the STZ-increased levels of reactive species, nitrite, lipid peroxidation and acetylcholinesterase activity in the cerebral cortex and hippocampus of mice. Moreover, a single administration of MFSeI (300 mg/kg, intra-gastrically) did not cause acute toxicity in Swiss male mice. Altogether, our data suggest that MFSeI exhibits antidepressant- and anxiolytic-like effects and improves the cognition of STZ-treated mice, without any toxicity.

1. Introduction

Selenium is an essential micronutrient that is most active as a constituent of seleno-proteins that act as oxidoreductases (Brigelius-Flohe and Flohe, 2017). Selenium deficiency has been associated with mood disorders, such as depression and anxiety (Pasco et al., 2012; Wang et al., 2018). Indeed, psychiatric disorders are characterised by increased oxidative stress (Salim, 2017), and, therefore, it is no surprise that several research groups are studying the behavioural and biochemical effects of selenium-containing organic compounds. For example, seleno-organic compounds have been shown to possess antioxidant (Vieira et al., 2017; Vogt et al., 2018), antidepressant-like and anxiolytic-like effects (Domingues et al., 2019), as well as anti-inflammatory and anti-nociceptive actions (Birmann et al., 2018; Rosa et al., 2018), in preclinical studies. Similarly, the indole group has also received extensive attention in preclinical investigations, since it is one

of the most widely occurring heterocycles in nature. For example, it is present in the amino acid tryptophan, in the hormone melatonin, and in the neurotransmitter serotonin. In addition, indole derivatives possess antioxidant (Casaril et al., 2017), antidepressant-like (Casaril et al., 2019), antitumour (Rosales et al., 2019) and neuroprotective (Chen et al., 2018) effects.

The intra-cerebroventricular (ICV) administration of streptozotocin (STZ) has been shown to cause neuroinflammation, neuronal loss and oxidative stress in the brain of mouse models, leading to depression- and anxiogenic-like behaviour and symptoms of cognitive impairment (Ishrat et al., 2009; Souza et al., 2013; Zhang et al., 2016). The first line of treatment for depression, i.e., selective serotonin reuptake inhibitors (SSRIs), show considerable adverse effects (such as nausea, insomnia, sexual dysfunction and sleep disturbances) and modest efficacy. It is, therefore, important to find better treatments for depression and to use validated preclinical models to investigate novel antidepressant

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molecules (Lader, 2007; Shelton, 2019).

The involvement of oxidative stress in depression has been investigated for many years; studies have shown that depressed patients have either increased reactive species levels or decreased antioxidant defences (Bajpai et al., 2014; Michel et al., 2012). Of note, it has been shown that 1-methyl-3-(phenylselenanyl)-1*H*-indole (MFSeI), an indole- and selenium-containing compound, displays antioxidant activity *in vitro* (Vieira et al., 2017). Considering the promising biological effects of seleno-organic and indole compounds, and the urgent need for better molecules to treat depression, the aim of this study was to investigate if MFSeI can reverse the depression- and anxiogenic-like behaviour, cognitive impairment and oxidative stress induced by STZ, in mice.

2. Materials and methods

2.1. Animals

The behavioural experiments were conducted using adult male Swiss mice (25–35 g). All animals were maintained on a 12-h light/12-h dark cycle (lights turned on at 7:00 a.m.). The environment temperature (22–25 °C) was kept constant. Mice were housed in cages with free access to food and water. The animals were maintained in regular cages, with 5 animals per cage. The experiments were performed according to a randomized schedule and each group of animals was used in determined tests and a separate group was used to euthanasia. All manipulations were carried out between 08.00 a.m. and 04.00 p.m. The present experimental study was approved by the Institutional Ethics Committee on Care and Use of Experimental Animal Resources from the Federal University of Pelotas, Brazil (6305-2017). All efforts were made to minimize animals suffering and to reduce the number of animals used in the experiments.

2.2. Drugs

The MFSeI (Fig. 1A) was prepared and characterised in the Laboratory of Clean Organic Synthesis at the Federal University of

Pelotas, Brazil. The MFSeI was dissolved in canola oil and administered intragastrically (i.g.) at a dose of 10 mg/kg. The dose of based in the previous studies not published yet. Fluoxetine hydrochloride (Pfizer, Brazil) was diluted in saline and administered at dose 10 mg/kg (i.g.) (Shafia et al., 2017). All drugs were administered at a constant volume of 10 ml/kg.

I.g. procedure is commonly used by our research group for administration of organic compounds and oil-soluble drugs, in this method compounds are administered by using a gastroesophageal probe that releases them directly into the stomach. The i.g. is route was chosen because is more common used in the laboratories (Casaril et al., 2019; Domingues et al., 2018; Fronza et al., 2017) to administration of compounds and this route mimics the oral route used by human and it is more close to use in the clinical study futures.

STZ was purchased from Sigma-Aldrich. (St Louis USA), dissolved in saline 0.9% and administered by ICV injection (0.2 mg/4 µl/per mouse). All other chemicals were of analytical grade and obtained from standard commercial suppliers. STZ could be easily disassembled, but a STZ solution pH 3 is impossible administer ICV in mice; it could cause a severe toxicity. Furthermore, with purpose to reduce disassemble we take care about to maintain in low temperatures and in the dark during the experiment. Dose and the administration time of STZ were selected based on our pilot studies and previously published data (Amiri et al., 2017; Pinton et al., 2011; Souza et al., 2017a).

2.3. Experimental protocol

The experimental design is illustrated in Fig. 1B. Three different sets of mice underwent the same treatment regimen (n = 6 mice/group): First, mice received STZ or saline (vehicle) through ICV injection. After 24 h, the animals received MFSeI (10 mg/kg), fluoxetine (10 mg/kg) or vehicle (canola oil) once daily for seven consecutive days. Thirty minutes after the last treatment, the behavioural tests were performed. The first set of mice was tested in the open-field test, elevated plus maze and forced swimming test, and the second set of mice was tested in the social interaction test, Y-maze and splash test. The third set of mice was

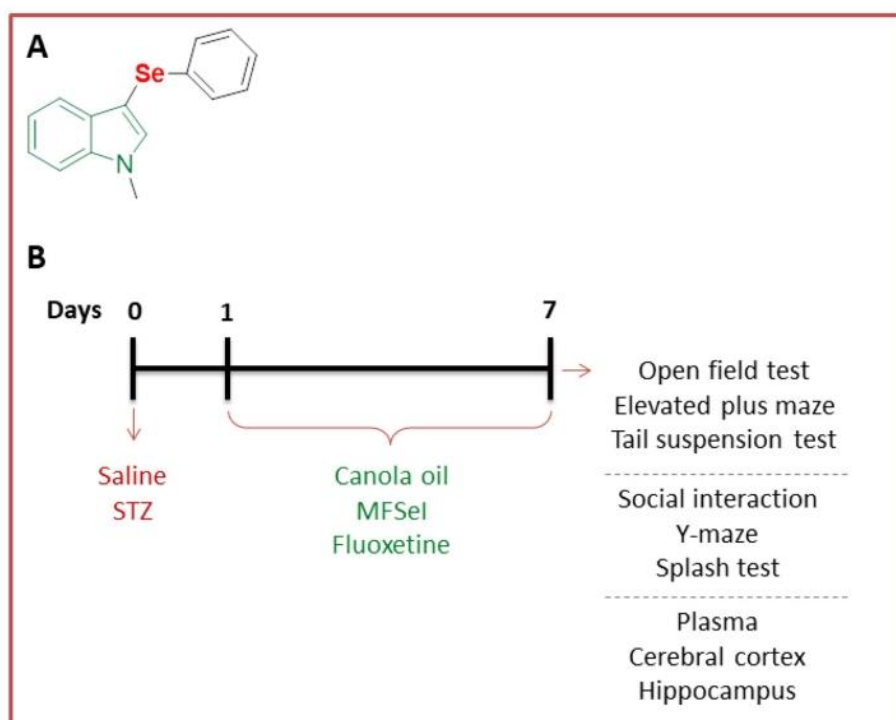


Fig. 1. Overview of the present study. (A) Chemical structure of 1-methyl-3-(phenylselenanyl)-1*H*-indole (MFSeI). (B) Schematic representation of the experimental employed. STZ: streptozotocin.

euthanized for collection of plasma and removal of cerebral cortex and hippocampus.

In this current study we choose use separate groups of animals for different behavioural tests and for euthanasia. We have some explanation for this choice. First, we used six behavioural tests, and to reduce the stress of animals and the to remove the possible interaction the animals between tests were separate in two sets, one set to open field test, elevated plus maze and tail suspension test and the another set to social interaction, Y-maze and splash test. The third set of animals was used to *ex vivo* analyses.

It is important highlight that the first and second sets of animals were euthanized after the end of behavioural tests that occurs exactly 30 min after the last treatment with MFSeI and fluoxetine. The third set used to *ex vivo* analysis was euthanized 30 min after the last treatment too. Immediately after euthanasia the samples of prefrontal cortex, hippocampus and plasma were excised and stored at -80°C for *ex vivo* analyses.

2.4. ICV injection

For the ICV injection, mice were anaesthetized by isoflurane (Iso Fo Abbott Laboratories) inhalation. The ICV injection was performed through “free-hand” method, as previously described by [Haley and McCormick \(1957\)](#) and modified by [Laursen and Belknap \(1986\)](#), using the bregma fissure as a reference point. For this method, a microsyringe (25 μL , Hamilton) was inserted perpendicularly at 0.8 mm posterior to bregma, 1.0 mm lateral to sagittal suture, and 3.0 mm beneath the surface of the brain. The needle was inserted unilaterally 1 mm into the midline point equidistant from each eye at an equal distance between the eyes and the ears and perpendicular to the plane of the skull. The animals were immobilized and a gauze soaked in 70% ethanol was utilized for the asepsis of the injection site. The injection was given over 30 s, and the needle was kept in place for a further 30 s to avoid reflux of the injected solution.

2.5. Behavioural tests

2.5.1. Open field test (OFT)

With the purpose of excluding sedative or motor abnormality, the mice were tested in the open field test. The mice were placed in the center of a wooden box (30 $\times$ 30 $\times$ 15 cm) divided into nine squares of equal areas. During a 5 min session, the number of groomings (as an indication of anxiety) and the locomotor (through the number of crossed squares) and exploratory (number of elevations) activity were evaluated ([Walsh and Cummins, 1976](#)). The apparatus was cleaned with a solution of 70% ethanol between tests in order to hide animal clues.

2.5.2. Tail suspension test

The total duration of immobility induced by tail suspension was measured according to the method described by [Steru et al. \(1985\)](#). Mice (acoustically and visually isolated) were suspended 50 cm above the floor by adhesive tape placed approximately 1 cm from the tip of the tail during 6 min. Mice were considered immobile only when they hung passively and completely motionless. Immobility time was manually recorded during the last 4-min period.

2.5.3. Splash test

The splash test was carried out as described by [Detanico et al. \(2009\)](#) and [Pesarico et al. \(2016\)](#). The grooming behaviour was observed as a measurement of motivational and self-care difficulties. The test consisted of squirting a 10% sucrose solution on the dorsal coat of a mouse placed individually in a cage. The total grooming activity (including nose/face grooming, head washing and body grooming) was recorder for 5 min.

2.5.4. Social interaction test

The social interaction test was realized in the same apparatus that was used for the OFT and it was performed based on previous data showing that mice with depressive-like behaviour spend less time interacting with an unknown mouse ([Gupta et al., 2016](#)). The intruder mice were the same age, sex, weight and unknown to the test mouse. For the test, the test mouse and the intruder mouse were placed on different sides of the open field. The social interaction behaviour was recorded during a 5 min session and consisted of grooming, mounting and crawling under the intruder mouse, including the passive interaction (number of crossing to the stimulus mouse). Manifestation of an aggressive behaviour from either mice resulted in interruption of the test.

2.5.5. Y-maze test

Y-maze is used to assess memory function as spatial short-term memory based on the willingness of mice in exploring new environments ([Sarter et al., 1988](#)). The Y-maze is a three-arm horizontal maze (40 cm long and 3 cm wide with walls 12 cm high) in which the three arms are symmetrically separated at 120° . Mice were initially placed in one arm (A), and the arm entry sequence (e.g. ABCACB, where letters indicate arm codes) and the number of arm entries was recorded manually for each mouse over a 5 min period. The entry was defined as placing all four paws within the boundaries of arm. The alternation was defined as entries into all three arms consecutively (i.e. ABC, BAC or CBA but not BAB) without repetition. The following formula determined percentage alternation: $\% \text{ Alternation} = [(\text{Number of alternations} \times 3) / (\text{Total arm entries} - 2)] \times 100$.

2.5.6. Elevated plus maze test

Anxiolytic-like behaviour was accomplished using the elevated plus maze task as previously described ([Frussa-Filho et al., 1999](#); [Rubin et al., 2000](#)). The apparatus consists of a wooden structure raised to 50 cm from the floor. This apparatus is composed of four arms of the same size, with two closed-arms (walls 40 cm) and two open-arms connected by a central platform. Initially, the animals were placed on the central platform of the maze in front an open arm. An entry was defined as placing all four paws within the boundaries of the arm. The mice had 5 min to explore the apparatus, and the time spent and the number of entries in open and closed-arms were recorded.

2.6. Tissue preparation

The separate group of animals was euthanized and immediately the samples of prefrontal cortex, hippocampus and plasma were excised and stored at -80°C for *ex vivo* analyses. The cerebral cortex and hippocampus were separated in two hemispheres in order to submit each sample to all analyses. The brain hemispheres were homogenized in 50 mM Tris-HCl, pH 7.4 (1:4, w/v) for the determination of the levels of reactive species (RS), nitrite metabolites and thiobarbituric acid reactive species (TBARS). To evaluate the acetylcholinesterase (AChE) activity prefrontal cortex and hippocampus were homogenized in 25 mM sucrose buffer (1:10, w/v).

2.6.1. Reactive species quantification

The quantification of RS was performed according [Loetchutinat et al. \(2005\)](#). In this assay, dichlorodihydro-fluorescein diacetate (DCHF-DA) oxidation in to fluorescent dichlorofluorescein (DCF) is evaluated for intracellular RS detection. The fluorescence intensity was recorded in spectrofluorophotometer in 520 nm emission and 480 nm excitation and results were expressed as arbitrary units of fluorescence.

2.6.2. Nitrite production

The accumulation of nitrite, an indicator of the production of nitric oxide (NO), was determined by a colorimetric assay using Griess reagent (0.1% N-[1-naphthyl] ethylene diamine dihydro chloride, 1%

sulfanilamide and 2.5% phosphoric acid) (Beda and Nedospasov, 2005). Absorbance was determined at 550 nm and the concentration of nitrite was expressed as $\mu\text{mol}/\text{mg}$ protein.

2.6.3. Thiobarbituric acid reactive species assay

TBARS levels were estimated by the amounts of malondialdehyde (MDA) formed using the thiobarbituric acid (TBA) reagent (Ohkawa et al., 1979). Submission of samples to high temperatures favours the binding of TBA to MDA, forming a pink chromophore that was measured spectrophotometrically at 532 nm. The results were expressed as nmol MDA/g tissue.

2.6.4. Acetylcholinesterase activity

Assessment of AChE activity was carried out using dithiobis-2-nitrobenzoate (DTNB) (Ellman reagent) as described by Ellman et al. (1961) using acetylthiocholine as substrate. In this assay, thiocholine (8 mM, acetyl choline analog) complexed to DTNB 10 mM reduced DTNB to 5-thio-2-nitro-benzoic acid (TNB). TNB has a yellowish color proportional to AChE activity. The change in absorbance was measured spectrophotometrically immediately at zero and 1 min at 412 nm. The AChE activity in the supernatant was expressed as nmol/min/mg protein.

2.7. Plasma glucose levels

Glucose levels were measured in the plasma of mice by an enzymatic colorimetric method using a commercial kit (Labtest, Diagnostica S.A., Minas Gerais, Brazil). Glucose levels were expressed as mg/dL.

2.8. Acute toxicity and biochemical parameters

The determination of acute toxicity biomarkers were performed using two groups of adult male Swiss mice ($n = 6$ mice/group): canola oil (vehicle, i.g.) and MFSeI (300 mg/kg, i.g.).

Observations were carried out daily during the first 72 h after the treatment to look for any clinical signs of toxicity, like changes in fur, skin, eyes and nasal secretions (mucus and bleeding), incidence of secretions and excretions, lacrimation, piloerection and unusual respiratory pattern. After 72 h, mice were anaesthetized (inhalation of isoflurane) for blood collection by cardiac puncture followed by euthanasia by cervical dislocation for the removal of brain, liver and kidney.

Blood was collected and centrifuged at $2000 \times g$ for 10 min to obtain the plasma, which was used for the determination of aspartate aminotransferase (AST), alanine aminotransferase (ALT) activity, glucose levels, lactate dehydrogenase (LDH), urea and creatinine. The total brain, liver and kidney were homogenized in 50 mM Tris-HCl, pH 7.4 (1:10, w/v), and the homogenate was centrifuged at $2500 \times g$ for 10 min at 4°C and was used for the measurement of RS, TBARS, δ -aminolevulinic dehydratase (δ -ALA-D) activity. RS and TBARS levels were assessed as previously described.

2.8.1. δ -aminolevulinic dehydratase activity

δ -ALA-D is a sulfhydryl-containing and Zn^{2+} -containing enzyme and highly sensitive to prooxidants and heavy metals. The δ -ALA-D activity was assayed according to the method of Sassa (1982) by measuring the rate of porphobilinogen (PBG) formation. An aliquot of S1 was incubated for 0.5 h (liver), 1 h (kidney) and 3 h (brain) at 37°C . The reaction was stopped by addition of trichloroacetic acid (TCA). The reaction product was determined using modified Ehrlich's reagent at 555 nm. δ -ALA-D activity was expressed as nmol PBG/mg protein/h.

2.8.2. Biochemical parameters

Plasmatic AST, ALT, glycemia, LDH, urea and creatinine were quantified using commercial kits (LABTEST, Diagnostica SA, Brazil), the results were expressed as conventional units (mg/dL).

2.9. Protein quantification

Protein quantification was performed using assay of Bradford (1976) measuring the formation of a blue color at a wavelength of 595 nm. Bovine serum albumin (BSA) was used as the control.

2.10. Statistical analysis

Data from behavioural and neurochemical experiments of mice challenged with STZ and treated with MFSeI and fluoxetine were analyzed by one-way analysis of variance (ANOVA), followed by the Newman-Keuls *post hoc* test. Data from toxicological evaluation were analyzed by Student *t*-test. All data are represented as mean $\pm$ standard error of the mean (S.E.M.) and were analyzed by GraphPad Prism 7.0. Values < 0.05 ($p \leq 0.05$) were considered statistically significant.

3. Results

3.1. Plasma glucose levels

The blood glucose levels were the same in all experimental groups ($F_{(5,30)} = 0.569$; $p = 0.72$) (data not shown).

3.2. MFSeI treatment abolishes the depressive-like behaviour induced by STZ

Fig. 2A–D shows the immobility time of mice in the TST, grooming time in the splash test, duration of social interaction and number of social interactions in the social interaction test, respectively.

STZ increased the immobility time in TST (Fig. 2A), compared to the control group; MFSeI and fluoxetine were effective against this increase ($F_{(5,30)} = 12.4$; $p < 0.001$). In the splash test, STZ decreased the grooming time (Fig. 2B), compared to the control group; treatment with MFSeI and fluoxetine increased the grooming time induced by STZ ($F_{(5,30)} = 13.7$; $p < 0.001$).

Both treatments, MFSeI and fluoxetine, reversed the depressive-like behaviour induced by STZ, for duration of social interaction ($F_{(5,30)} = 6.7$; $p < 0.001$) (Fig. 2C) and number of social interactions ($F_{(5,30)} = 3.81$; $p = 0.009$) (Fig. 2D).

3.3. MFSeI treatment improves the anxiogenic-like behaviour and deficit cognition induced by STZ

Mice subjected to STZ spent more time at grooming behaviour (Fig. 3A), compared to the control group. The time spent grooming decreased in mice treated with MFSeI, but not fluoxetine ($F_{(5,29)} = 5.14$; $p = 0.002$). The grooming behaviour observed in the open-field box represented anxious behaviour.

STZ decreased the time spent on open arms (Fig. 3B) and reduced the number of open arm entries (Fig. 3C), compared to the control group; both MFSeI and fluoxetine treatment reversed this alteration in the EPM test ($F_{(5,30)} = 12.4$; $p < 0.001$).

Treatment by MFSeI and fluoxetine reversed the decrease in the number of alternations in the Y-maze test, induced by STZ administration ($F_{(5,29)} = 7.24$; $p < 0.001$) (Fig. 3D).

3.4. MFSeI effects on locomotor activity of mice exposed to STZ

Fig. 4 shows the effects of treatment with MFSeI and fluoxetine on locomotor activity of mice exposed to the STZ. Neither STZ nor treatment by MFSeI or fluoxetine changed the numbers of crossings (Fig. 4A) ($F_{(5,30)} = 0.737$; $p = 0.60$) and rearings (Fig. 4B) ($F_{(5,30)} = 0.396$; $p = 0.85$) by mice.

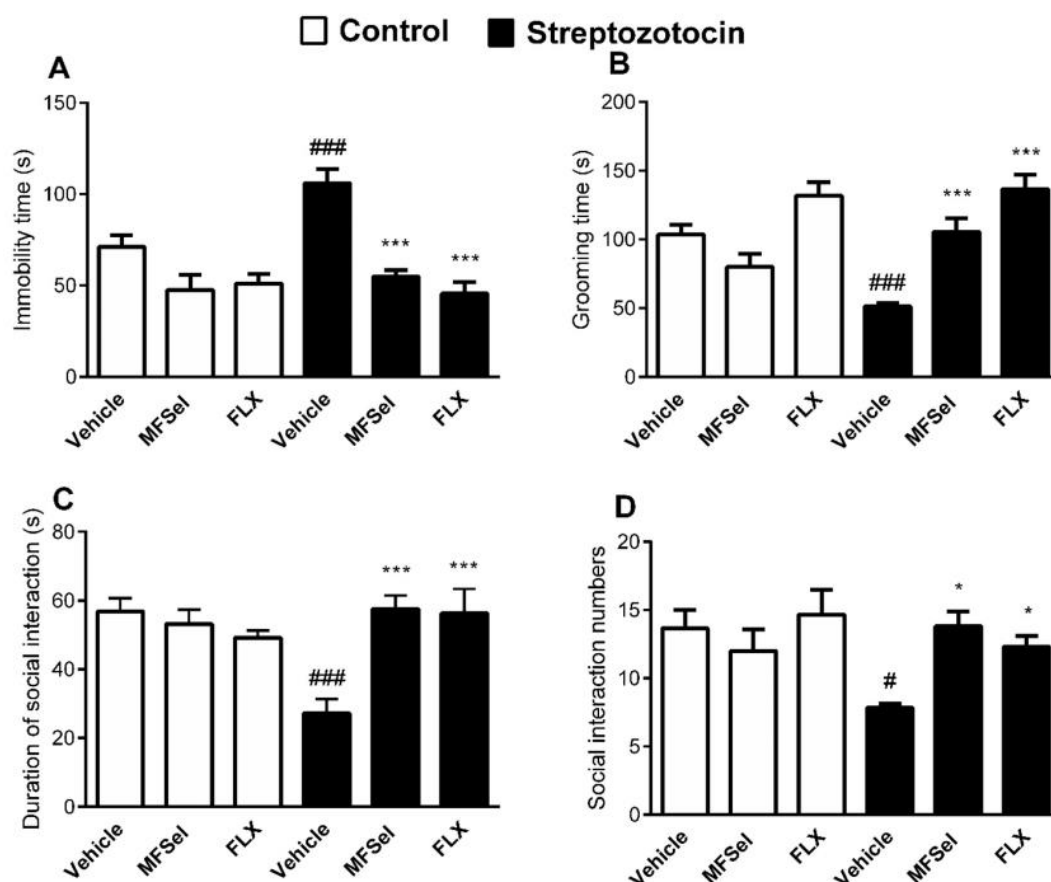


Fig. 2. The MFSel and fluoxetine effect on immobility time in the tail suspension test (A), grooming time in the splash test (B), duration of interaction social (C) and number of interaction social (D) in the interaction social test in a mouse model induced by STZ. Data are shown as means $\pm$ S.E.M. of 6 animals per group. # $p < 0.05$ and ### $p < 0.001$ compared to the control group; * $p < 0.05$ and *** $p < 0.001$ compared to the STZ group (one-way ANOVA followed by the Newman-Keuls). MFSel: 1-methyl-3-(phenylselenanyl)-1H-indole; FLX: fluoxetine;

3.5. Involvement of oxidative stress

Fig. 5A and B show that administration of STZ increased lipid peroxidation levels in the prefrontal cortex ($F_{(5,30)} = 4.78$; $p = 0.002$) and hippocampus ($F_{(5,30)} = 5.66$; $p < 0.001$) of mice, compared to the control group. MFSel and fluoxetine reversed the lipid peroxidation levels induced by STZ, in both structures.

Fig. 5C demonstrates that STZ increased RS levels in the prefrontal cortices of mice; both MFSel and fluoxetine treatment abolished this increase ($F_{(5,30)} = 6.35$; $p < 0.001$). The same results occurred in the hippocampus (Fig. 5D), and both treatments were effective in reversing the increase induced by STZ ($F_{(5,30)} = 5.04$; $p = 0.002$).

Nitrite production is an indirect measurement of nitric oxide. The results show an increase in the production of nitrite induced by STZ, compared to the control group, and this effect was reversed by MFSel and fluoxetine treatment in the prefrontal cortex (Fig. 5E) ($F_{(5,30)} = 8.74$; $p < 0.001$) and hippocampus (Fig. 5F) ($F_{(5,30)} = 7.05$; $p < 0.001$).

3.6. Involvement of the cholinergic system

As shown in Fig. 6A and B, STZ increased AChE activity, compared to the control group, in the prefrontal cortex ($F_{(5,20)} = 4.85$; $p = 0.005$) and in the hippocampus ($F_{(5,22)} = 4.48$; $p = 0.006$), respectively. Furthermore, MFSel and fluoxetine attenuated STZ-induced AChE activity.

3.7. MFSel has no toxic effect on blood and biochemical parameters

After administration of MFSel (300 mg/kg) no clinical signs of toxicity and no deaths were observed. Tables 1 and 2 show that there was no alteration of toxicity biomarkers in the brain, kidney, liver and heart of mice.

4. Discussion

The results of the present study clearly show that MFSel treatment exhibits a significant antidepressant-like and anxiolytic-like effect and reverses cognitive impairment induced by STZ. In terms of a theoretical mechanism of action for MFSel, this study shows that this seleno-organic compound reverses the STZ-induced increase in RS, nitrite, and TBARS levels, and AChE activity, in the cerebral cortex and hippocampus of mice. Importantly, MFSel treatment did not affect the locomotor and exploratory behaviours and did not produce any signs of acute toxicity, in the mice, suggesting the treatment is safe to administer.

Previous studies have demonstrated that ICV injection of STZ causes depression- and anxiogenic-like behaviours, and memory impairment, in rodents (Amiri et al., 2017; Grieb, 2016; Souza et al., 2013), making it useful for research into novel antidepressant molecules. Accordingly, we replicated these findings, as we found that a single injection of STZ induced behavioural and biochemical alterations in mice, which allowed us to investigate the potential pharmacological effects of a novel synthetic organo-selenium compound. The antidepressant-like effect of MFSel was observed in its ability to decrease immobility time in the

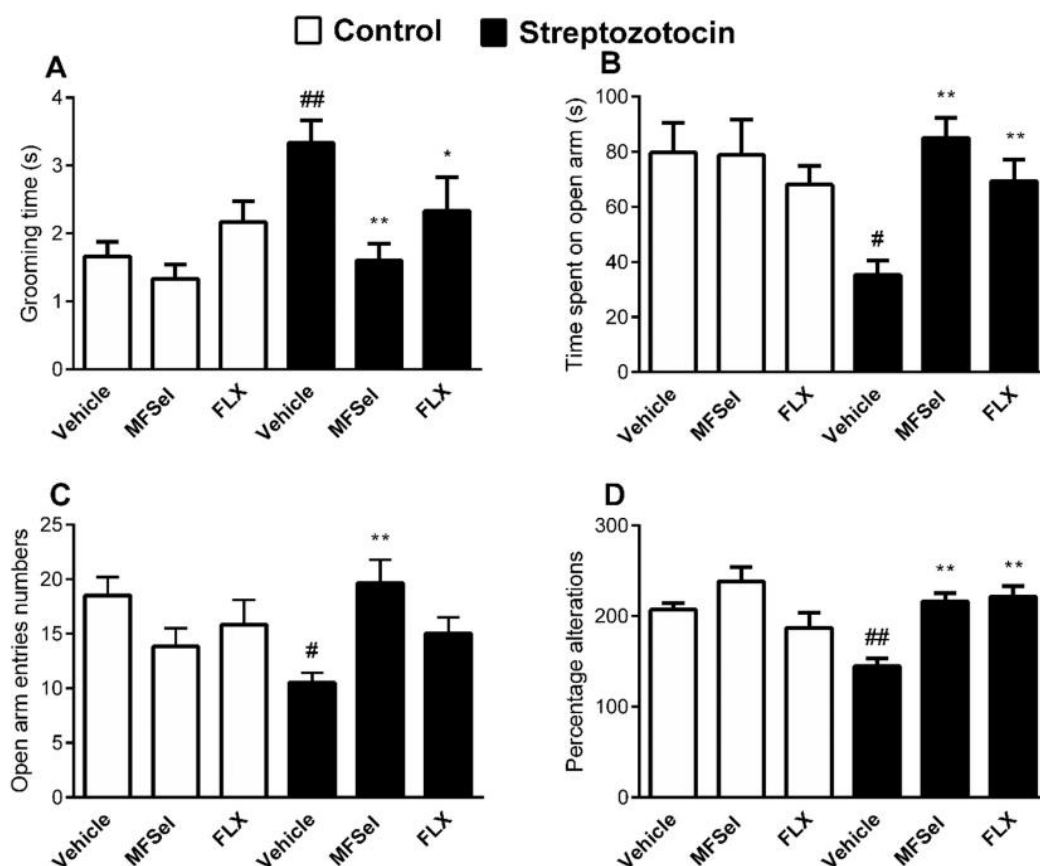


Fig. 3. The MFSeI and fluoxetine effect on grooming time in the open-field box (A), time spent on open arm (B) and number of open arm entries (C) in the EPM test and percentage alterations in Y-maze test (D) in a mouse model induced by STZ. Data are shown as means $\pm$ S.E.M. of 6 animals per group. [#] $p < 0.05$ and ^{##} $p < 0.01$ compared to the control group; ^{*} $p < 0.05$ and ^{**} $p < 0.01$ compared to the STZ group (one-way ANOVA followed by the Newman-Keuls). MFSeI: 1-methyl-3-(phenylselenanyl)-1H-indole; FLX: fluoxetine;

TST, increase grooming time in the splash test and increase interaction time in the social interaction test, in STZ-treated mice. Furthermore, the ability of MFSeI to reverse the decreased number of entries and the decreased time spent in the open arms in the EPM, induced by STZ, suggests that MFSeI possesses anxiolytic-like effects. MFSeI administration reversed the decrease in the number of spontaneous alterations induced by STZ in the Y-maze, indicating that this seleno-organic compound can also improve cognition.

In addition to the behavioural effects, MFSeI treatment also restored neurochemical alterations induced by STZ. Previously, studies have suggested that an increase in oxidative stress in the brain may play a role in the pathogenesis of depression and that antidepressant drugs modulate this imbalance (Eren et al., 2007; Moylan et al., 2014). In agreement with this, ICV injection of STZ alters the proinflammatory cytokine pathways and brain-derived neurotrophic factor (BDNF) kynurenine and tryptophan levels (Souza et al., 2017a; Souza et al.,

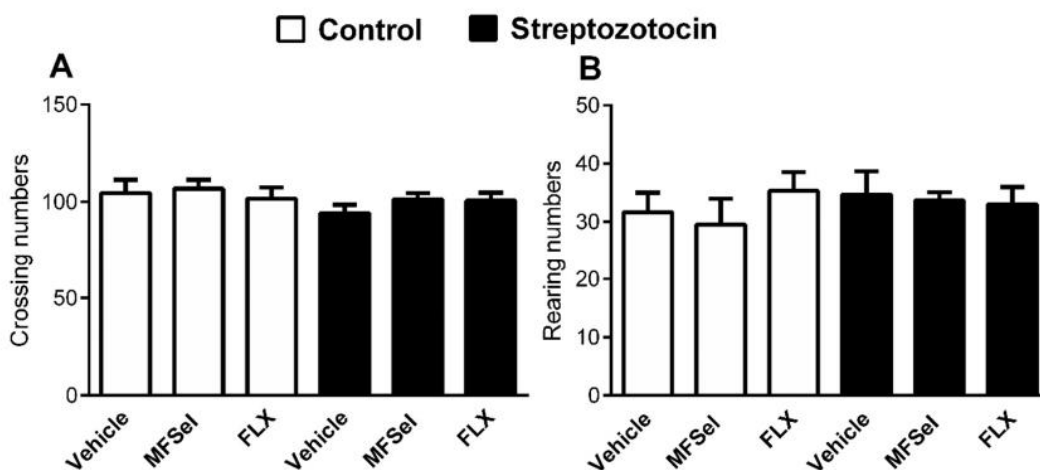


Fig. 4. The MFSeI and fluoxetine effect on crossing numbers (A) and rearing numbers (B) in the open-field test in a mouse model induced by STZ. Data are shown as means $\pm$ S.E.M. of 6 animals per group. (one-way ANOVA followed by the Newman-Keuls). MFSeI: 1-methyl-3-(phenylselenanyl)-1H-indole; FLX: fluoxetine;

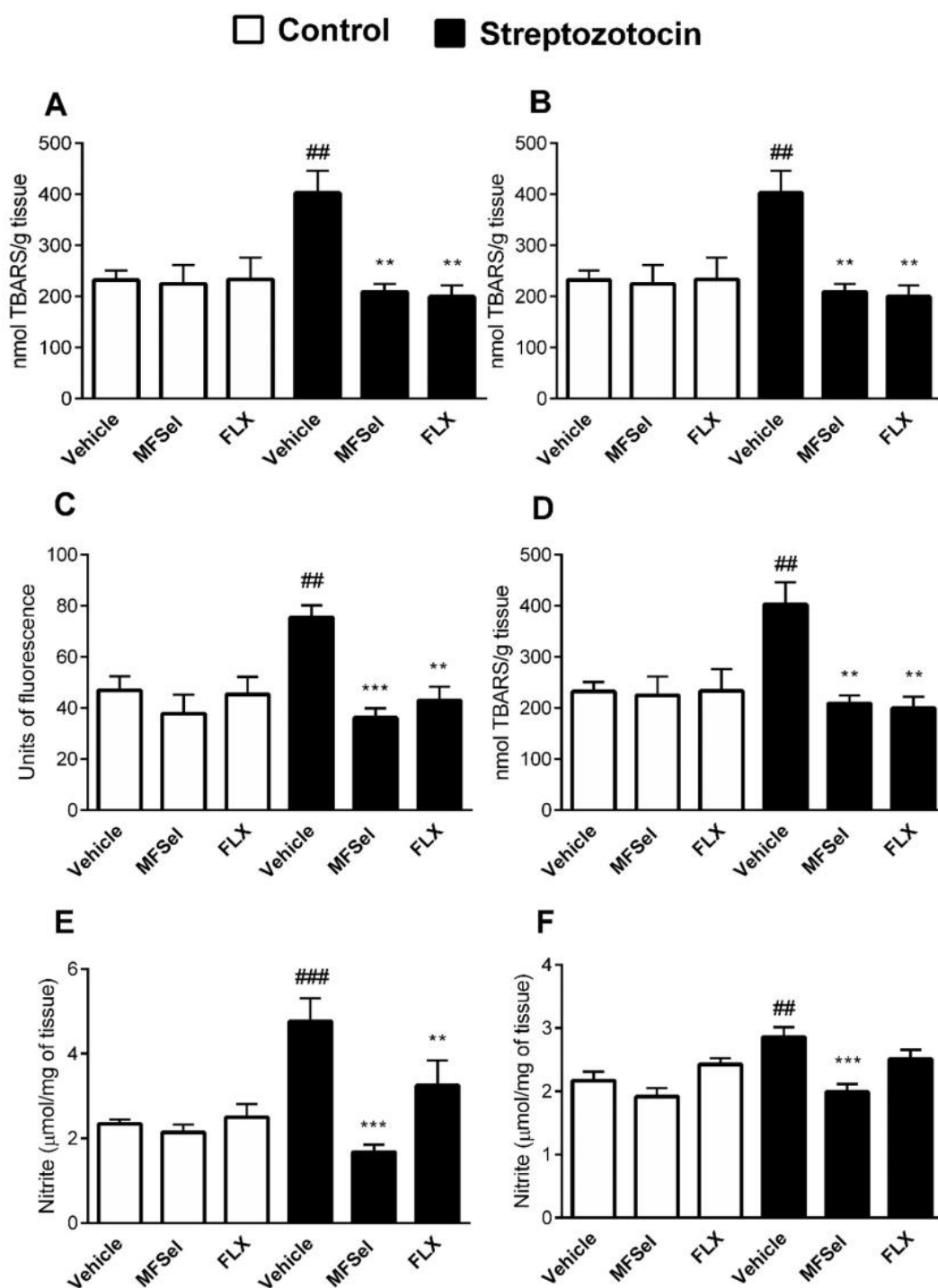


Fig. 5. The MFSeI and fluoxetine effect on the TBARS levels of prefrontal cortex (A) and hippocampus (B), on the RS levels of prefrontal cortex (C) and hippocampus (D) and on the nitrite levels of prefrontal cortex (E) and hippocampus (F) in a mouse model induced by STZ. Data are shown as means $\pm$ S.E.M. of 6 animals per group. ## p < 0.01 and ### p < 0.01 compared to the control group; ** p < 0.01 and *** p < 0.001 compared to the STZ group (one-way ANOVA followed by the Newman-Keuls). MFSeI: 1-methyl-3-(phenylselanyl)-1H-indole; FLX: fluoxetine;

2017b), leading to oxidative damage (Thome et al., 2018). Analyses of the oxidative stress parameters demonstrated that MFSeI reversed the increased levels of RS, nitrite and TBARS in the cerebral cortex and hippocampus of mice treated with STZ. In this way, STZ administration caused lipid peroxidation that was abolished by treatment with MFSeI, indicating the involvement of antioxidant effects in the decrease of oxidative stress caused by the STZ protocol. Accordingly, MFSeI has been reported to have antioxidant properties *in vitro* (Vieira et al.,

2017). According to our results, the MFSeI antidepressant- and anxiolytic-like action and cognition improvements are related to its effectiveness in maintaining anti-oxidative homeostasis in the prefrontal cortices and hippocampus of mice.

In addition, some studies have demonstrated that RS might be generated during nitrite metabolism, as a breakdown product of nitric oxide (Abdel-Wahab and Moussa, 2019; May et al., 2004). In this study, we found that nitrite levels were increased by STZ administration, and

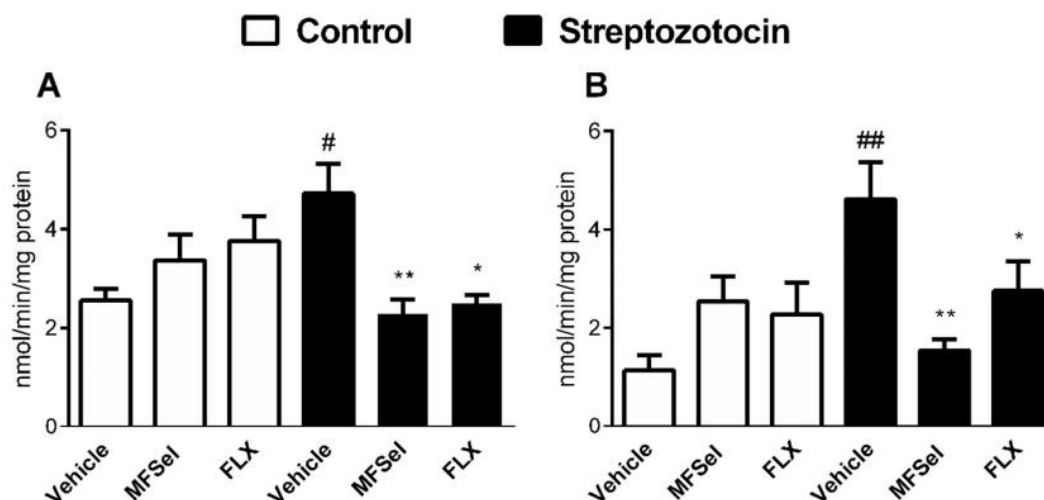


Fig. 6. The MFSeI and fluoxetine effect on the AChE activity of prefrontal cortex (A) and hippocampus (B) in a mouse model induced by STZ. Data are shown as means $\pm$ S.E.M. of 6 animals per group. [#] $p < 0.05$ and ^{##} $p < 0.01$ compared to the control group; ^{*} $p < 0.05$ and ^{**} $p < 0.001$ compared to the STZ group (one-way ANOVA followed by the Newman-Keuls). MFSeI: 1-methyl-3-(phenylselenanyl)-1H-indole; FLX: fluoxetine;

Table 1

Effect of acute administration of MFSeI (300 mg/kg; i.g.) in toxicology parameters in the plasma of mice.

Analysis	Control	MFSeI	p	t
AST	80.20 $\pm$ 2.97	69.10 $\pm$ 9.75	0.29	1.10
ALT	133.00 $\pm$ 4.31	146.00 $\pm$ 19.00	0.51	0.67
Glycemia	136.00 $\pm$ 8.87	141.00 $\pm$ 12.10	0.75	0.32
LDH	14.80 $\pm$ 3.41	14.20 $\pm$ 2.51	0.87	0.15
Urea	51.60 $\pm$ 1.51	45.30 $\pm$ 3.14	0.10	1.80
Creatinine	0.10 $\pm$ 0.07	0.09 $\pm$ 0.06	0.74	0.32

AST and ALT activities were expressed in U/L. Glycemia levels was expressed in mg/dL. LDH was expressed in U/L. Urea and creatinine levels were expressed in mg/dL. Results are expressed as mean $\pm$ SEM. ALT: alanine aminotransferase; AST: aspartate aminotransferase; LDH: Lactic dehydrogenase; MFSeI: 1-methyl-3-(phenylselenanyl)-1H-indole.

Table 2

Effect of acute administration of MFSeI (300 mg/kg; i.g.) in toxicological parameters in brain, liver and kidney of mice.

Analysis	Control	MFSeI	p	t
RS				
Brain	72.80 $\pm$ 3.42	75.20 $\pm$ 7.60	0.77	0.29
Liver	304.00 $\pm$ 11.90	295.00 $\pm$ 6.46	0.98	0.01
Kidney	245.00 $\pm$ 12.00	245.00 $\pm$ 12.00	0.53	0.64
TBARS				
Brain	34.10 $\pm$ 2.61	36.40 $\pm$ 4.01	0.64	0.46
Liver	37.80 $\pm$ 5.08	30.40 $\pm$ 6.72	0.39	0.88
Kidney	42.00 $\pm$ 9.03	43.30 $\pm$ 4.80	0.90	0.12
δ -ALA-D				
Brain	3.20 $\pm$ 0.52	2.82 $\pm$ 0.28	0.57	0.88
Liver	10.10 $\pm$ 0.92	7.68 $\pm$ 1.85	0.24	1.23
Kidney	3.80 $\pm$ 0.75	4.72 $\pm$ 0.45	0.31	1.05

RS quantification was expressed in units of fluorescence. TBARS levels were expressed in nmol MDA/g tissue. δ -ALA-D activity was expressed in nmol PBG/mg/protein/h. Results are expressed as mean $\pm$ S.E.M. RS: reactive species; TBARS: thiobarbituric acid reactive species; MDA: malondialdehyde; δ -ALA-D: δ -aminolevulinatase dehydratase.

MFSeI and fluoxetine treatment decreased these levels, corroborating the results of oxidative stress; this suggests that the increase in RS levels is due to an increase in nitrite levels.

In the present study, the repeated administration of MFSeI and fluoxetine reversed the STZ- increased activity of AChE in the cerebral

cortex and hippocampus of mice. AChE inhibitors (such as donepezil, galanthamine and rivastigmine), which prevent the hydrolysis of acetylcholine, reduced the cognitive decline in neurodegenerative conditions. Deficit in the cholinergic system is one of the major neuropathological characteristics associated with memory loss (Grossberg, 2003; Hasselmo, 2006). Therefore, the reduced cognitive impairment associated with MFSeI administration, observed in the Y-maze test, may be a consequence of its ability to modulate the cholinergic system.

Because of the promising effects of MFSeI as a therapeutic agent, we investigated the possibility of toxicological effects following administration. A dose 10 times higher than the effective dose on behavioural and neurochemical parameters (Casaril et al., 2017) was chosen to rule out the possibility of toxicity for MFSeI. The absence of increased circulating biomarkers of renal, hepatic and cardiac toxicity, together with the absence of altered RS and TBARS levels and δ -ALA-D activity in the brain, liver and kidney of mice, suggests that MFSeI does not induce toxicity in mice, following administration. These results have encouraged us to continue investigating the pharmacological effects of this organo-selenium compound.

It is possible that the activity of MFSeI is closely related to its chemical structure. The indole nucleus is one of the most commonly occurring heterocycles, and is present in the structures of tryptophan, melatonin and serotonin. Additionally, the redox potential of selenoproteins in the brain highlight the importance of controlling RS generation and neutralization. In this sense, molecular hybridisation involving the indole nucleus and the selenium atom is a potential approach in the development of synthetic molecules with promising biological effects under various conditions.

The current study provides preliminary results on the effectiveness of repeated MFSeI treatment on depression, anxiety and cognitive deficit, but further investigations are needed into the applicability of MFSeI, compared to SSRIs. Presently, we are not able to recommend MFSeI over SSRI, but the fact that low doses of MFSeI were effective could reduce the risk of any undesirable side-effects.

We acknowledge some limitations of this study: (1) the lack of further investigations into the involvement of the cholinergic nervous system, e.g., antagonists of acetylcholine receptors; (2) the fact that only oxidative stress was evaluated, and there were no experiments investigating the neuroprotective effect involved in depressive-like behaviour. To address these limitations, we will conduct further studies to show the involvement of other pathways in the mechanism of MFSeI.

5. Conclusion

The present study revealed that MFSeI abolishes the depressive-like, anxiogenic-like behaviour and cognitive impairment induced by STZ injection in mice. The behavioural effects of this seleno-organic compound appear to involve the reduction of oxidative stress (evidenced by decreased ROS, NO_x and TBARS levels) and AChE activity in the PFC and HC of mice treated with STZ. Importantly, toxicity biomarkers were not altered after MFSeI treatment, demonstrating the safety of administering this compound. Overall, the results highlight the potential biological effects of a novel selenium-containing compound endowed with antioxidant activity, which could be useful in the treatment of disorders characterised by oxidative stress, such as depression and anxiety. Further studies are being carried out to investigate the effects of MFSeI in different animal models.

Declaration of Competing Interest

All authors declare that they have no conflicts of interest.

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Selanylimidazopyridine Prevents Lipopolysaccharide-Induced Depressive-Like Behavior in Mice by Targeting Neurotrophins and Inflammatory/Oxidative Mediators

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Inasmuch, as the major depressive disorder (MDD) has been characterized as a heterogeneous disease as the inflammatory processes, neurotrophic factors' dysfunction and oxidative/nitrosative stress are believed to play a vital role in its establishment. Organoselenium compounds stand out due to their antioxidant, anti-inflammatory, neuroprotective, and antidepressant effects. In this sense, the present study investigated the effect of 3-((4-methoxyphenyl)selanyl)-2-phenylimidazo[1,2-a]pyridine (MPI; 20 and 50 mg/kg, intragastrically) pretreatment [30 min prior lipopolysaccharide (LPS) challenge (0.83 mg/kg)] on acute LPS induced depressive-like behavior, neuroinflammation, and oxidative stress. MPI was able to prevent the increased immobility time induced by LPS on the forced swimming test (FST), the increase in pro-inflammatory cytokines' expression in the hippocampus (HC) of mice after LPS challenge via NFκB downregulation, and the increase of the reactive oxygen species generation and lipid peroxidation in the prefrontal cortex and HC of mice. It was observed that at the doses tested, MPI protected against reducing levels of BDNF in the cortex and HC of mice challenged with LPS. These observations suggest that the antidepressant-like effect of MPI depends on its capacity to modulate the inflammatory, antioxidant, and neurotrophic systems.

Keywords: major depressive disorder, lipopolysaccharide, imidazopyridines, selenium, depression, NFκB, mouse models of depressive disorder, neuroinflammation

INTRODUCTION

Major depressive disorder (MDD) is a serious illness with great loss in quality of life, increased morbidity and mortality, and a high rate of recurrence and chronicity (Réus et al., 2016). For many years, the pathophysiology of MDD has been focused on the monoaminergic theory, proposing that depression is caused by a decreased monoaminergic function in the brain (Maes et al., 2011).

Although the monoaminergic systems are clearly involved in the etiology of depression, it is now accepted that inflammatory processes, neurotrophic factors' dysfunction, and oxidative/nitrosative stress might also be involved in the establishment of this disorder (Maes et al., 2013). Based on these observations, rodent models of inflammation-associated depression have been developed, such as administration of bacterial lipopolysaccharide (LPS), which is a potent activator of the immune system (Dantzer et al., 2008).

Classic antidepressants, which are effective in around 30–40% of the patients (Moreno et al., 1999), are often associated with numerous side effects, which compromise their acceptance and use by patients (Wang et al., 2016). Hence, in recent years, there has been a growing search for new molecules which display antidepressant activity with multitarget characteristics. In this context, the selenium-containing compounds have received attention, since these molecules demonstrate an amplitude of biological properties (da Rocha et al., 2012). Among the biological functions, they have been widely explored in the literature as anti-inflammatory, antioxidant, and antidepressant (Tiegs et al., 1998; Bortolatto et al., 2013; Pinto Brod et al., 2016). Donato et al. (2015) demonstrated the antidepressant-like effect of an organoselenium molecule in the tail suspension test (TST) through modulation of the noradrenergic and dopaminergic systems. Besides, Casaril et al. (2017) showed the anti-neuroinflammatory effect of a selenium-containing indolyl compound. In this sense, normalization of activated inflammation pathways may be a novel target for the discovery of new antidepressant molecules (Stefânia et al., 2013). In addition to the organoselenium compounds, the imidazopyridines have been found to possess interesting biological properties (Casaril et al., 2017). These molecules have been used to treat insomnia, and have shown promising antioxidant, anti-inflammatory, and neurotrophic effects (Parekh et al., 2013; Dymńska, 2015; Qian et al., 2016). Considering the pharmacological proprieties of organoselenium compounds and imidazopyridines, the combination of both molecules may be a relevant strategy for the development of more efficient drugs with multitarget profile for the treatment of depression.

We reported in this study that 3-((4-methoxyphenyl)selenyl)-2-phenylimidazo[1,2-a]pyridine (MPI) mitigates the LPS-induced depressive-like behavior and investigated the mechanisms of this molecule in the biomarkers of oxidative stress, neuroinflammation, and neurotrophic factor.

MATERIALS AND METHODS

Animals

The experiments were conducted in male Swiss mice (25–30 g). Six animals per box were kept under standard environmental conditions ($24 \pm 1^\circ\text{C}$ and light/dark cycles of 12 h) with free access to water and food. All procedures were performed according to the guidelines of the Ethics Committee on Animal Experimentation of the Federal University of Pelotas (CEEa/UFPel-1870-2016). All efforts were made to minimize

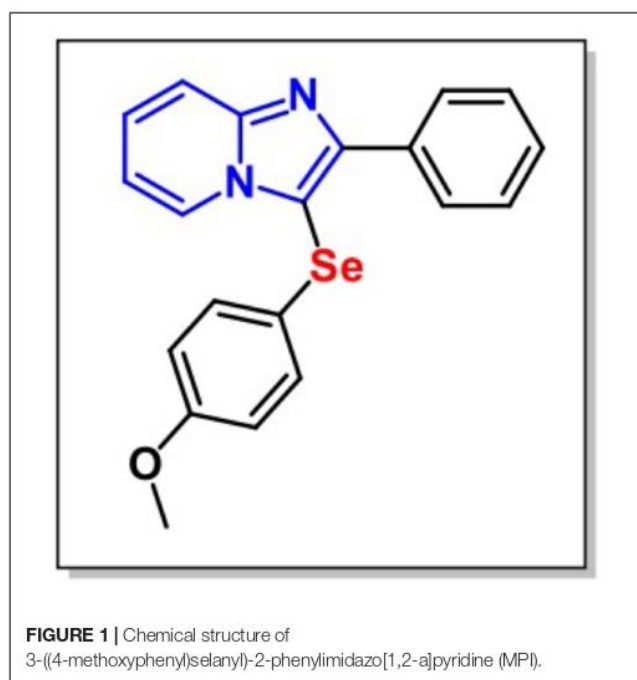
animals suffering and to reduce the number of animals used in the experiments.

Drugs

3-((4-Methoxyphenyl)selenyl)-2-phenylimidazo[1,2-a]pyridine (Figure 1) was synthesized by the Laboratory of Clean Organic Synthesis (LASOL-UFPel). *Escherichia coli* LPS (L-3129, serotype 0127:B8) was purchased from Sigma-Aldrich Co. (St. Louis, MO, United States). MPI was diluted in canola oil (a non-polar and inert substance) and administered intragastrically (i.g.) at a constant volume of 10 ml/kg body weight. LPS was diluted in saline at a constant dose of 0.83 mg/kg and administered intraperitoneally (i.p.). Drug solutions were prepared freshly in the morning. All other chemicals used in the present study were of analytical grade. Appropriate vehicle-treated groups were simultaneously assessed.

Experimental Procedure

Animals were randomly divided into six experimental groups, each group consisting of six mice ($n = 6$). The groups were used as follows: Group I: canola oil was administered 30 min prior to saline injection. This group served as a normal control group; Group II: Canola oil was administered 30 min prior to LPS injection. This group served as a disease control group; Group III: MPI (20 mg/kg, i.g.) was administered 30 min prior to saline injection; Group IV: MPI (50 mg/kg, i.g.) was administered 30 min prior to saline injection; Group V: MPI (20 mg/kg, i.g.) was administered 30 min prior to LPS injection; and Group VI: MPI (50 mg/kg, i.g.) was administered 30 min prior to LPS injection. The different doses of MPI (20 and 50 mg/kg) were selected from previous literature evaluating organoselenium



compounds' antidepressant-like effects in mice (Gerzson et al., 2012; Casaril et al., 2015; Donato et al., 2015).

The LPS dose was chosen based on previous studies (O'Connor et al., 2009). After 24 h of LPS or saline administration, depressive-like behavior was evaluated. Behavior tests were carried out by a trained observer blind to the treatments. The behavior analyses, open field test (OFT), and forced swimming test (FST) were assessed after 24 h of the LPS or saline injection (Zhang et al., 2016). All the behavioral tests were made in all the animals. The sequence of the protocol was OFT–FST (Martinez et al., 2014).

The cytokines and neurochemical determinations were evaluated by taking different animals (following the same experimental design) in order to avoid interferences from behavioral assessment on neurochemical parameters (Casaril et al., 2017). It is important to notice that the mice were anesthetized by isoflurane before the sacrifice. They were then killed by cervical dislocation, followed by brain removal and isolation of prefrontal cortex (PFC) and hippocampus (HC) for analysis. The brain tissues were removed and the right hemisphere was used to measure cytokines (TNF- α , IL-1 β), NF- κ B (using a primer sequence corresponding to subunit p65), and BDNF expression, while the left hemisphere was used to determine reactive oxygen species (ROS) formation and lipid peroxidation.

Open Field Test (OFT)

Open field test was the first behavioral test performed. Before all the treatments, we determined the baseline between all the groups. After this first phase, we submitted the animals to the respective treatments and the OFT was performed 24 h after LPS or saline administration. This procedure evaluates the possible effects of the compound, whether it interferes in the locomotor and exploratory activity of the animals. Briefly, the mice were placed in the center of a wooden box (30 cm $\times$ 30 cm $\times$ 15 cm) divided into nine squares of equal areas, and during 5 min, the locomotor (through the number of crossed squares) and exploratory (number of elevations) activities were evaluated.

Forced Swimming Test (FST)

Forced swimming test is used to evaluate the immobility time as the absence of escape-oriented behavior, which is an important symptom of depression (Porsolt et al., 1977). In this test, mice were individually placed into a cylinder (25 cm height, 10 cm diameter) containing 8 cm of water maintained at 25°C. After a habituation period (2 min), the immobility time (s) of the animals was rated for a further 4 min time. This test was performed by two experienced raters, who were blind to the treatment group.

Neurochemical Determinations

The animals were euthanized 24 h after administration of saline and LPS. The brains were dissected for the removal of HC and PFC. The left hemispheres were homogenized in Tris-HCl (50 mM, pH 7.4; 1:10, w/v). The homogenate was centrifuged at 2500 $\times$ g for 10 min at 4°C, and a low-speed supernatant fraction (S₁) was used for the measuring of the thiobarbituric acid reactive species (TBARS) levels and

determination of ROS. The right hemispheres were immersed in Trizol, stored at -80°C , and used for the qRT-PCR assay.

Evaluation of Lipid Peroxidation

The lipid peroxidation in the cerebral structures (PFC and HC) was evaluated by measuring the thiobarbituric acid (TBA) reacting substances as an index of RS (Ohkawa et al., 1979). Briefly, S₁ was incubated with 8.1% sodium dodecyl sulfate (SDS), 0.8% TBA and acetic acid/HCl (pH 3.4) at 95°C during a period of 2 h. Lipid peroxidation was detected by the absorbance at 532 nm in a spectrophotometer. The results were expressed as nmol MDA/g tissue.

Reactive Oxygen Species (ROS) Quantification

The ROS levels formed in PFC and HC of the left hemisphere were determined by conventional spectrofluorimetry using the dichloro-dihydro-fluorescein diacetate (DCHF-DA) reagent (Loetchutinat et al., 2005). Briefly, DA-DCHF (1 mM) was incubated together with S₁ and Tris-HCl buffer (10 mM, pH 7.4). The oxidation of DCHF to fluorescent dichlorofluorescein (DCF) was measured for intracellular RS detection. The fluorescence intensity is measured with emission at 520 nm and excitation at 488 nm in spectrofluorimeter and the results are expressed in units of fluorescence.

RNA Extraction and Gene Expression Evaluation by qRT-PCR

Total RNA was purified from right hemispheres PFC and HC using Trizol reagent (Invitrogen™, Carlsbad, CA, United States). cDNA synthesis was accomplished with 1 μ g RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems™, United Kingdom) according to the manufacturer's protocol. SYBR Green PCR Master Mix (Applied Biosystems™, United Kingdom) was used for performing Real-time qRT-PCR, according to the manufacturer's protocol. Relative values of gene expression were normalized using GAPDH and the conditions for the reaction included 95°C for 15 s, 60°C for 60 s, and 72°C for 30 s. The $2^{-\Delta\Delta CT}$ (Delta-Delta Comparative Threshold) method was used to normalize the fold change in gene expressions. Primer sequences and full name of the genes are provided in **Table 1**. It is importante to notice that p65 NF κ B analyzed was obtained in the whole tissue, which is a limitation in this study.

Statistical Analysis

Data from behavior and neurochemical analysis were expressed as mean $\pm$ SEM (standard errors of the mean). The results were analyzed by one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test for *post hoc* comparison. Results were considered significant ($P < 0.05$). The statistical analysis was accomplished by using Graph Pad Prism version 7.0 for mac, Graph pad Software (San Diego, CA, United States).

TABLE 1 | Primers' sequences.

Gene	Sequência 5'–3'
GAPDH	S-AGGTCGGTGTGAACGGATTTC A-TGTAGACCATGTAGTTGAGGTCA
BDNF	S-OCATAAGGACGCGGACTGTAC A-AGACATGTTTGCGGCATCCAGG
TNF- α	S-CATCTTCTCAAAATTCGAGTGACAA A-TGGGAGTAGACAAGGTACAACCC
IL-1 β	S-CTGTGCTTTCCCGTGGACC A-CAGCTCATATGGGTCGACA
NF κ B p65	S-GCT TTC GCA GGA GCA TTA AC A-OCT TTC GCA GGA GCA TCA AC

RESULTS

Effects of Pretreatment With MPI on the Psychocomotor Activity

In order to exclude possible inhibitory or excitatory effects of LPS and MPI, the number of crossings and rearings was measured in the OFT before FST. It is importante to notice that the baseline was analyzed before the treatments and no significant differences were observed in the groups analyzed in the number of crossing [$F(5,30) = 0.9765$, $p = 0.4480$] and number of rearing [$F(5,30) = 1.492$, $p = 0.222$] (data not shown). As shown in **Figures 2A,B**, the treatments did not produce significant differences in the number of rearing [$F(5,30) = 0.7789$, $p = 0.57$] and crossing [$F(5,30) = 0.5698$, $p = 0.72$]. These results indicate that LPS and MPI did not cause alterations in the psychomotor and exploratory activity of the animals.

Pretreatment With MPI Prevented the Increase in the Immobility Time Induced by LPS

Effect of MPI on the immobility time determined in FST is depicted in **Figure 3**. The systemic administration of LPS caused a significant increase in the duration of the immobility time when compared to saline-treated mice (control group; $P < 0.001$). However, pretreatment with MPI (20–50 mg/kg)

prevented the increase in the immobility time induced by LPS ($P < 0.001$), suggesting an antidepressant-like effect of the compound. Pretreatment with MPI (20 and 50 mg/kg) followed by administration of saline indicated no change when compared to saline-treated mice.

Pretreatment With MPI Prevented Lipid Peroxidation Induced by LPS in PFC and HC of Mice

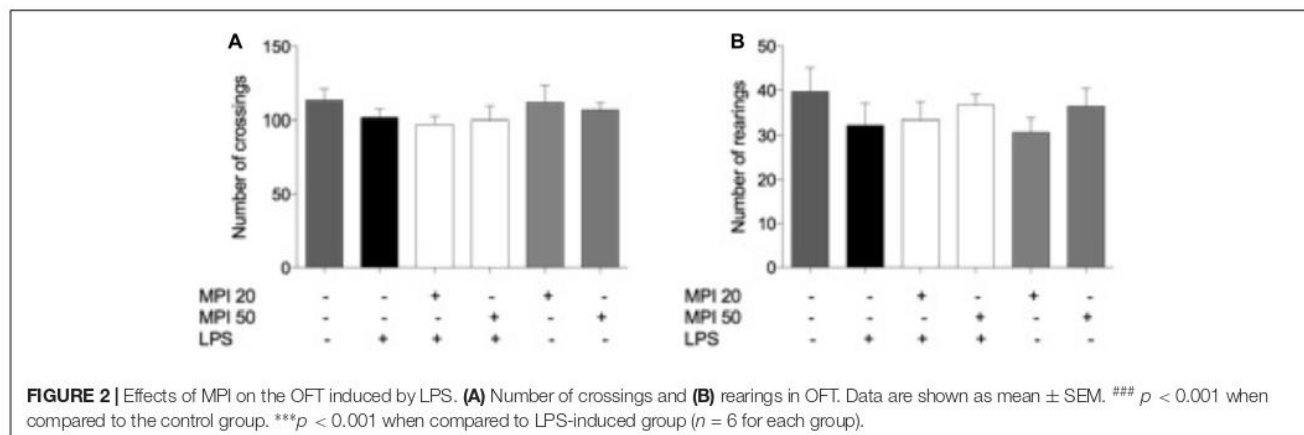
Lipopolysaccharide challenge induced significant increase in levels of lipid peroxidation in the PFC and HC ($P < 0.001$; **Figures 4A,B**). Pretreatment with MPI (20 and 50 mg/kg) prevented lipid peroxidation induced by LPS in the PFC ($P < 0.0001$) and HC ($P < 0.001$). However, administration of the compound alone did not influence lipid oxidation, maintaining similar values to the control group.

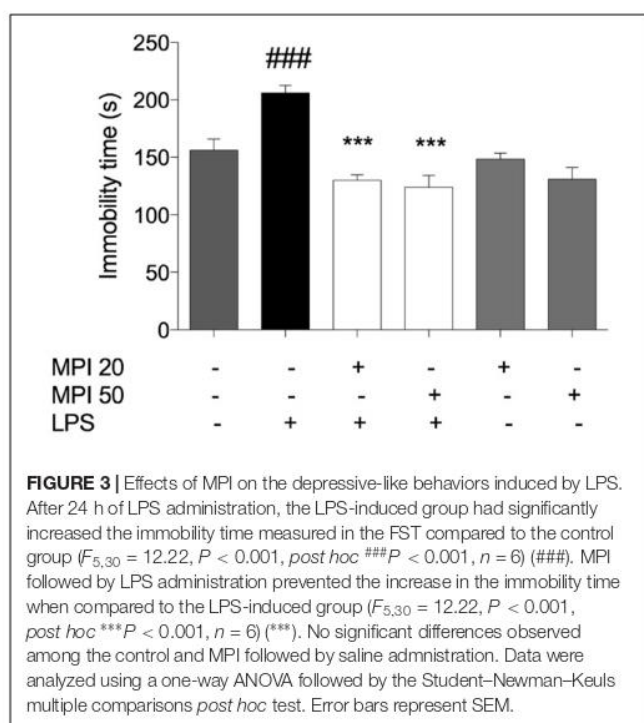
Pretreatment With MPI Prevented the RS Formation Induced by LPS in PFC and HC of Mice

The level of RS formed in the PFC ($P < 0.001$) and HC ($P < 0.001$) of mice is expressed in **Figures 5A,B**. LPS challenged-mice showed significant increase in the RS production in PFC and HC, when compared to the control group. Noteworthy, the pretreatment with MPI (20 and 50 mg/kg) prevented the RS formation induced by endotoxin in the PFC ($P < 0.001$) and HC ($P < 0.001$). In addition, the pretreatment by MPI, followed by saline administration, maintained similar values to the control (saline).

MPI Prevented the Pro-inflammatory Cytokine Production Induced by LPS in the PFC and HC of Mice

In order to investigate the mechanism underlying the behavioral changes induced by LPS, the levels of pro-inflammatory cytokines IL-1 β and TNF- α were evaluated (**Figure 6**). LPS challenge caused a significant increase of cytokines levels in the PFC and HC, when compared to the control ($P < 0.001$). The increase of TNF- α (**Figure 4B**) was prevented by pretreatment with MPI at the





highest dose in the PFC ($P < 0.05$) and in both doses in the HC ($P < 0.001$). MPI in both doses was capable of preventing IL-1 β expression in the HC ($P < 0.001$; **Figure 6D**) and in PFC (**Figure 6C**) at the highest dose (50 mg/kg; $P < 0.05$; **Figure 6C**).

MPI Prevented the Increase of NF κ B Levels in the PFC and HC of Mice

Lipopolysaccharide significantly increased NF κ B levels compared to control in PFC ($P < 0.001$) and HC ($P < 0.001$). MPI (20 and 50 mg/kg) significantly prevented the increase of this transcription factor, both in the PFC and in the HC ($P < 0.001$ and $P < 0.001$; **Figures 7A,B**). Administration of MPI alone did not cause any change in baseline neurochemical parameters.

MPI Prevented the Decrease in BDNF Levels in the PFC and HC of Mice Due to LPS Administration

Lipopolysaccharide significantly reduced BDNF levels compared to control in PFC, ($P < 0.001$) and HC ($P < 0.001$). MPI (20 and 50 mg/kg) significantly prevented the reduction of this neurotrophic factor, in the PFC ($P < 0.01$ and $P < 0.001$; **Figure 8A**) and in the HC ($P < 0.05$ and $P < 0.001$; **Figure 8B**). Administration of MPI alone did not cause any change in baseline BDNF levels.

DISCUSSION

In this study, we have demonstrated the antidepressant-like effect of MPI, a novel multitarget compound, in the LPS-induced depressive-like behavior. This effect was accompanied

by the prevention of LPS-induced NF κ B activation and pro-inflammatory cytokines (TNF- α and IL-1 β) expression in the brain. Besides, our study has also demonstrated that the antidepressant-like effect of MPI might be related with its antioxidant properties, since it prevented the LPS-induced oxidative damage by reducing the ROS formation and lipid peroxidation in PFC and HC. Another major finding was that MPI was able to prevent the LPS-induced BDNF downregulation, by increasing its expression in PFC and HC of mice.

The observed effects of MPI may be related to its chemical structure, which combines selenium and imidazopyridine. Organoselenium compounds can exert a variety of biological actions, including anti-inflammatory, neuroprotective, antioxidant, and antidepressant activities (da Rocha et al., 2012; Pinto Brod et al., 2016; Casaril et al., 2017). Similarly, imidazopyridines also present several pharmacological properties, such as anti-inflammatory (Qian et al., 2016), antiviral, antioxidant, and neuroprotective (Dymińska, 2015) activities. These multimodal pharmacological effects provided a rationale for the study of their multitarget action to treat MDD (Jiang et al., 2016). Considering that MDD is accompanied by activation of immuno-inflammatory pathways, oxidative stress, and BDNF reduction, the combination of both pharmacophore groups in MPI can be a promising alternative to treat this disorder (Maes et al., 2012; Moylan et al., 2013; Slyepchenko et al., 2016).

Lipopolysaccharide administration is a widely accepted model to investigate the link between depressive-like behavior and related neurochemical alterations (Ferreira Mello et al., 2013). This occurs because this endotoxin is recognized by the Toll-like receptor 4 (TLR-4), thereby inducing neurochemical changes in rodent brains, such as increase of oxidative stress, pro-inflammatory cytokines expression, and the decrease in BDNF levels, through the NF κ B pathway activation. The LPS administration increases peripheral cytokines and leads to sickness behaviors, including a decrease in food and water intake and lack of movement. Around 24 h postinjection, mice begin eating and moving identically to control animals, as well as their sick behaviors, have resolved. Pro-inflammatory cytokines' increased levels lead to an elevation of indoleamine 2,3 dioxygenase (IDO) 24 h after LPS injection. During the same time that IDO is elevated, animals will present depressive-like behavioral changes including anhedonia and spend more time immobile in the FST (O'Connor et al., 2009). Regarding depressive-like behavior induced by LPS 24 h after its administration, antidepressant and anti-inflammatory treatments can reverse or prevent this condition (Yirmiya, 1996; Ohgi et al., 2013).

The results of our study remained consistent with previous ones since the increase of immobility time in FST was prevented by pretreatment with MPI at doses of 20 and 50 mg/kg, indicating the antidepressant-like effect of this compound. It is worth noting that administration of MPI alone did not cause a significant change when compared to the control group, being effective only in the disease condition. In addition, MPI has not influenced the locomotor and exploratory activity in mice and this result is in accordance with previous works which have demonstrated that

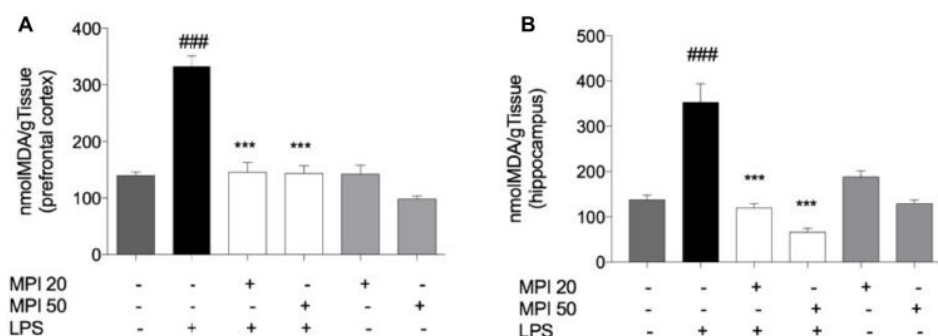


FIGURE 4 | Effect of MPI on lipid peroxidation in LPS-challenged mice. After 24 h of LPS administration, the LPS-induced group had significantly increased the TBARS levels in PFC (A) and HC (B) $^{###}P < 0.001$ when compared to the control group. MPI followed by LPS administration prevented the increase of TBARS levels in PFC ($F_{5,30} = 35.47$, $P < 0.001$, *post hoc* $^{***}P < 0.001$, $n = 6$) and HC ($F_{5,30} = 26.18$, $P < 0.001$, *post hoc* $^{***}P < 0.001$, $n = 6$) (***) when compared to the LPS-induced group. No significant differences observed among the control and MPI followed by saline administration. Data were analyzed using a one-way ANOVA followed by the Student–Newman–Keuls multiple comparisons *post hoc* test. Error bars represent SEM.

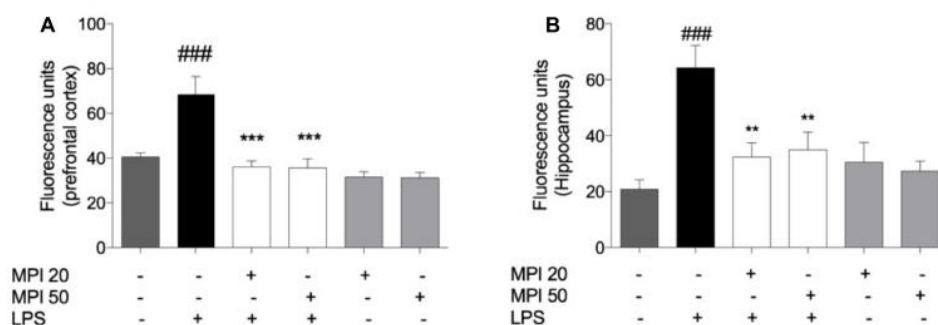


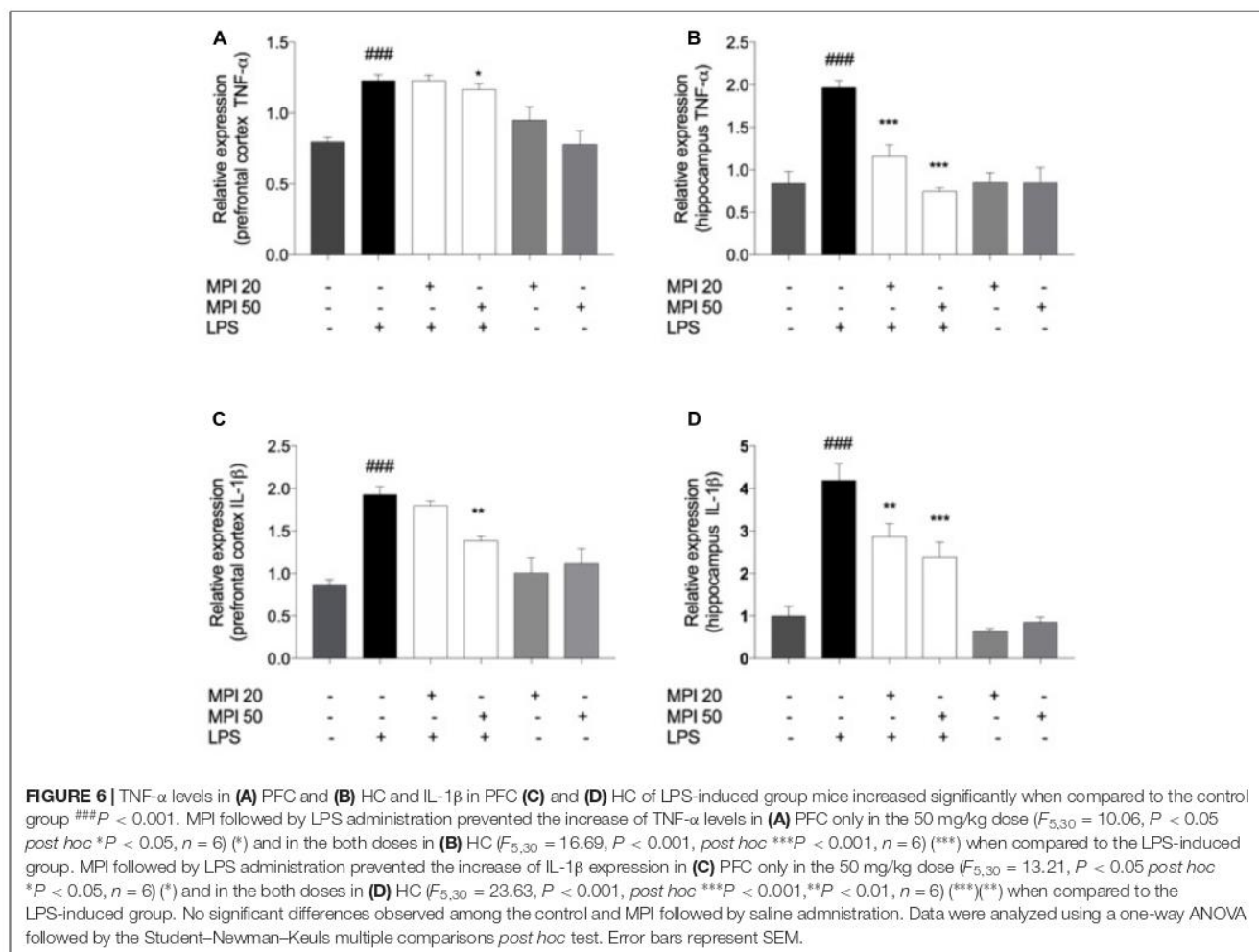
FIGURE 5 | Effect of MPI on RS formation in LPS-challenged mice. After 24 h of LPS administration, the LPS-induced group had significantly increased the RS formation in PFC (A) and HC (B) $^{###}P < 0.001$ when compared to the control group. MPI followed by LPS administration prevented the increase of RS formation in PFC ($F_{5,30} = 4.472$, $P < 0.001$ *post hoc* $^{***}P < 0.001$, $n = 6$) (***) and HC ($F_{5,30} = 6.317$, $p < 0.01$, *post hoc* $^{**}P < 0.01$, $n = 6$) (**) when compared to the LPS-induced group. No significant differences observed among the control and MPI followed by saline administration. Data were analyzed using a one-way ANOVA followed by the Student–Newman–Keuls multiple comparisons *post hoc* test. Error bars represent SEM.

24 h after injection of LPS the motor activity is back to normal when compared to the other groups. These results exclude the possibility that the FST results were affected by alterations in locomotor activity.

MPI antidepressant-like effect was accompanied by the restoration of LPS-induced alterations of inflammatory mediators (i.e., TNF- α and IL-1 β), neurotrophic factors through the NF κ B downregulation. Moreover, MPI decreased lipid peroxidation and ROS formation in PFC and HC. Slyepchenko et al. (2016) demonstrated that increased oxidative stress could induce neuroinflammation and contribute to the development of depression. Maes et al. (2011) also demonstrated that peripherally administered cytokines could reach the brain and lead the immune activation and ROS production via NF κ B pathway. Once in the brain, these mediators can modulate the release of monoamines, by activation of the tryptophan-kynurenine pathway-cytokine induced, enhancing the re-uptake of monoamines and decreasing the concentration of the cofactor tetrahydrobiopterin (BH4), thereby reducing the synthesis of monoamines. Besides we

analyzed the levels of p65 NF κ B, it was obtained in the whole tissue in both structure of mice, which is a limitation in this study.

In this study, the antidepressant-like effects of MPI were accompanied by decreasing TNF- α and IL-1 β levels in brain areas related to mood regulation. There is significant evidence that both cytokines play important roles in modulating mood behavior (Drevets et al., 2008). Interestingly, IL-1 β plays a pivotal role of microglial activation induced by repeated stress, since the genetic deletion of IL-1 β mitigates microglial activation induced by social defeat stress (Wohleb et al., 2011). Zhang et al. (2016) showed that IL-1 β impairs the differentiation of hippocampal neural progenitor cells into serotonergic neurons indicating the antidepressant effect of IL-1 β antagonists. The pro-inflammatory cytokine TNF- α also contributes to the pathogenesis of depression by an activation of neuronal serotonin transporters, stimulation of indoleamine 2,3-dioxygenase, and activation of hypothalamic-pituitary-adrenocortical (HPA) axis (Bossù et al., 2012; Zhang et al., 2016). Additionally, classically activated macrophages (M1) pro-inflammatory cells are activated by LPS,



while alternatively activated macrophages (M2) exert an anti-inflammatory effect, and act by reducing the neuroinflammation (Slyepchenko et al., 2016). Accordingly, a recent study has found evidence that selenium supplementation increases the polarization of macrophages from M1 to M2-like phenotype, decreasing neuroinflammation (Kudva et al., 2015), suggesting that the antidepressant effect of MPI associated to its anti-inflammatory activity can be related to the presence of selenium in its structure.

Lipopolysaccharide alone decreased the levels of BDNF by increasing the levels of pro-inflammatory cytokines. Several reports demonstrated that LPS-induced cytokines expression decreased BDNF and others neurotrophins expression. Previous studies have shown that LPS has the potential to cause cognitive dysfunction by this mechanism.

Besides the above-mentioned effects of cytokines in the brain, it is known that these pro-inflammatory mediators can reduce the expression of BDNF. The signaling pathway of this neurotrophic factor plays an important role in brain neuroplasticity and depression pathogenesis (Lee and Kim, 2010). Peripheral administration of LPS reduces the amount of BDNF in the brain (Guan and Fang, 2006), which was prevented in

MPI-treated mice. Accumulated evidences point to a reduction in the HC size in depressive patients (Eyre and Baune, 2012; Slyepchenko et al., 2016). It is known that BDNF levels are much higher in HC when compared to other brain structures, due to the great biological importance of the HC in memory maintenance and involvement with emotions (Eyre and Baune, 2012). Studies have shown that neuroinflammation interferes in the generation of new neurons, since the systemic administration of LPS, IL-1 β , and TNF- α contributes to the reduction of BDNF expression (Kohman and Rhodes, 2014). Our results showed that MPI prevented the changes in BDNF levels induced by LPS administration, when compared to the LPS group. We assumed that MPI prevented the BDNF reduction since MPI was also able to prevent the increase of pro-inflammatory cytokines expression in the rodent brains. Of note, the expression of the pro-inflammatory cytokines may be dependent on the NF κ B expression, as also demonstrated in the present study. These results show us a new perspective about the effect of MPI, since BDNF is involved with cognition control and depressive patients present potential cognitive dysfunction, due to BDNF levels decrease. Interestingly, the HC is the brain region that has the highest recruitment of cells of the immune system

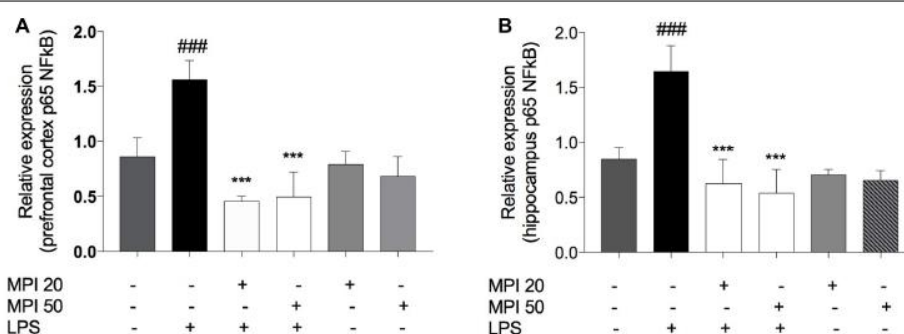


FIGURE 7 | p65 NFκB expression in (A) PFC and (B) HC of LPS-induced group mice increased significantly when compared to the control group $^{###}P < 0.001$. MPI (20–50 mg/kg) followed by LPS administration prevented the increase of NFκB expression in (A) PFC ($F_{5,30} = 30.45$, $P < 0.001$ *post hoc* $^{***}P < 0.001$, $n = 6$) and in (B) HC ($F_{5,30} = 31.82$, $P < 0.001$, *post hoc* $^{***}P < 0.001$, $n = 6$) (***) when compared to the LPS-induced group. No significant differences observed among the control and MPI followed by saline administration. Data were analyzed using a one-way ANOVA followed by the Student-Newman-Keuls multiple comparisons *post hoc* test. Error bars represent SEM.

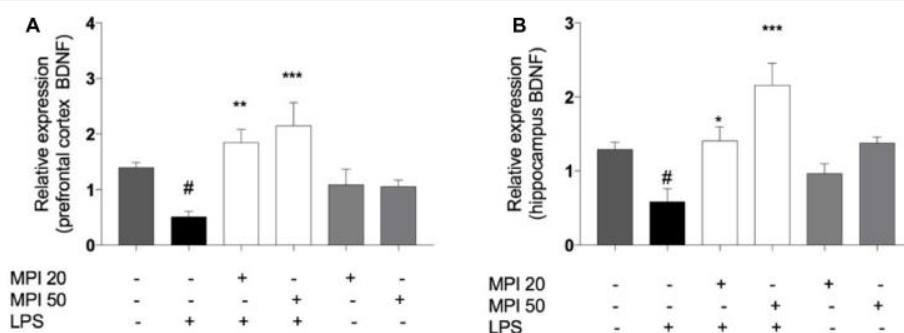


FIGURE 8 | BDNF expression in (A) PFC and (B) HC of LPS-induced group mice decreased significantly when compared to the control group $^{###}P < 0.001$. MPI (20–50 mg/kg) followed by LPS administration prevented the decrease of BDNF expression in (A) PFC ($F_{5,30} = 8.751$, $P < 0.001$; *post hoc* $^{**}P < 0.01$, $^{***}P < 0.001$, $n = 6$) and in (B) HC ($F_{5,30} = 6.775$, $P < 0.001$, *post hoc* $^{*}P < 0.05$, $^{***}P < 0.001$, $n = 6$) (***)(**) when compared to the LPS-induced group. $^{#}P < 0.05$ when compared to the control group. No significant differences observed among the control and MPI followed by saline administration. Data were analyzed using a one-way ANOVA followed by the Student-Newman-Keuls multiple comparisons *post hoc* test. Error bars represent SEM.

after some kind of stress, setting up a specific response to the stressor, reducing the levels of these pro-inflammatory cytokines (Brachman et al., 2015). However, the compound did not show effect in TNF- α levels in PFC, indicating its anti-inflammatory effect mainly in HC, main brain area associated to MDD. Thus, it may be suggested that the compound acts as an immunomodulator, increasing the recruitment of M2 cells to HC, in order to diminish the damage caused by LPS administration, by preventing the increase of IL-1 β and TNF- α levels in this brain area through reduction of NFκB expression. Moreover, BDNF and its interaction with ROS may play a role in several symptoms of neuropsychiatric abnormalities. The downregulation of BDNF increases the vulnerability to oxidative damage under stressful circumstances (Hacioglu et al., 2016). The oxidative stress plays an important role in the neurobiology of MDD (Tomaz et al., 2014; Liu et al., 2015; Casaril et al., 2017). High metabolic rate and an abundance of peroxide substrates make the brain cells more vulnerable to oxidative damage. Various studies have showed that depressed patients present higher levels of oxidative stress biomarkers such as malondialdehyde (Painsipp et al., 2011;

Tomaz et al., 2014; Casaril et al., 2017). It is known that high levels of this biomarker lead to mitochondrial dysfunction causing cell death, biomolecules damage, and more ROS production. Interestingly, lipid peroxidation products decrease CREB-dependent BDNF promoter activity in rat hippocampal neurons. Thus, products of lipid peroxidation are associated to BDNF and related cognitive function (Pugazhenthil et al., 2006). In addition, excess of ROS also influences the depletion of monoamines in the synaptic cleft, through the oxidation of BH4 (Miller and Raison, 2016). Peripheral immune activation by LPS causes change in the brain's redox system, by increasing the ROS directly by activating microglia (Maes et al., 2012; Liu et al., 2015; Slyepchenko et al., 2016). Exposure to high doses of LPS triggering prolonged production of cytokines which lead to increase of free radicals production might cause deleterious condition termed oxidative stress (O'Connor et al., 2009). ROS are involved in the mechanism of LPS toxicity, in particular in NFκB activation. In general, there is a balance between ROS generation and antioxidant defenses in cells. When ROS generation overcomes the cellular antioxidants, oxidative

stress is generated and its beneficial functions might harm the host leading to neuroprogression of MDD. Cytokines increased levels induced by LPS administration lead to NF κ B activation which contributes to upregulation of NADPH oxidase (NOx) and inducible nitric oxide synthase (iNOS) and consequently culminate in the free radicals raise.

In the present study, MPI significantly prevented the increase of ROS production in brain areas, demonstrated by the quantification of ROS by the use of DCFH-DA (Loetchutin et al., 2005) indicating the antioxidant profile of the compound. Considering that LPS increases lipid peroxidation as a consequence of oxidative stress, in the present study, MDA levels, measured by TBARS (Ohkawa et al., 1979), were prevented by MPI in HC and PFC. This suggests that this molecule also prevents lipid peroxidation caused by the oxidative condition. In this view, it can be stated that the compound has antioxidant activity, suggesting that the antidepressant-like effect may also be due to this property. Considering that the compound presented protective effect against NF- κ B upregulation induced by LPS, the neuroprotector effect of the compound may be associated with its anti-inflammatory effect.

Current treatments are estimated to only reduce about one-third of the disease burden of depressive disorders, in this sense the preventive alternatives may be a strategy to treat this progressive disease since it causes several brain changes. Hence, pretreatment with MPI is a promising strategy to further reduce the disease burden of depression, due to its several proprieties.

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CONCLUSION

To sum up, the present study demonstrated that the antidepressant effect of MPI at doses of 20 and 50 mg/kg might be related, in part, to its ability to modulate the oxidative, immune-inflammatory pathways and restore BDNF levels, altered by LPS induction. These multitarget proprieties make MPI a promising treatment to MDD. However, more studies are needed to accurately understand the molecular basis of the antidepressant activity of this molecule, and then progress to clinical studies.

AUTHOR CONTRIBUTIONS

MD and AC designed the study and the protocol, managed literature searches and manuscript writing, and collected, assembled, analyzed, and interpreted data with LS, PB, DL, KB, FS, and TC. BV and EL synthesized and characterized the organoselenium compound. All authors revised the manuscript and approved the final manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer GA and handling Editor declared their shared affiliation.

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**Anexo B – Demais artigos publicados durante o período de doutoramento
cujos temas não se relacionam a presente tese**

Paschoal, J.D.F., Lopes, I.A., Borges, M., Feijó, A.L., Simões, L.D., Segatto, N.V., Campos, V.F., Seixas, F.K., **Casaril, A.M.**, Savegnago, L., Lenardão, E.J., Collares, T., 2020. Toxicological evaluation of 3-(4-Chlorophenylselenanyl)-1-methyl-1H-indole through the bovine oocyte in vitro maturation model. *Toxicol. In vitro.* 62, 104678. <https://doi.org/10.1016/j.tiv.2019.104678>

Abstract

The development of new bioactive molecules based on the molecular hybridization has been widely explored. In line with this, reliable tests should be employed to give information about the toxicology of these new molecules. In this sense, the use of in vitro tests is a valuable tool, especially the in vitro maturation of oocytes (IVM), which is an efficient resource to discover the potential toxicity of synthetic molecules. Thus, the aim of the present study was to evaluate the toxicological effects of the selenium-containing indolyl compound 3-(4-Chlorophenylselenanyl)-1-methyl-1H-indole (CMI), on different quality parameters of bovine oocytes through the IVM. Different concentrations of the CMI compound (0, 25, 50, 100, 200 μ M) were supplemented during the in vitro maturation process. After, the oocyte maturation rate, glutathione (GSH) levels, reactive oxygen species (ROS) levels, membrane, and mitochondrial integrity were evaluated. The results showed that the lowest concentration of CMI induced the highest GSH production ($P < 0.05$), an important marker of cytoplasmic quality and maturation. All treatments increased ROS production in relation to non-supplementation ($P < 0.05$). In addition, oocyte maturation was reduced only with the highest concentration of CMI ($P < 0.05$). Supplementation with CMI did not impact mitochondrial activity, integrity and cell membrane. To our knowledge, this is the first study that evaluates CMI on the oocyte in vitro maturation process. Importantly, our results did not find any toxic effect of CMI on bovine oocytes. CMI was efficient for cytoplasmic maturation by promoting an increase in the intracellular levels of glutathione.

Alves, M.D.S., Neves, R.N., Sena-Lopes, A., Domingues, M., **Casaril, A.M.**, Segatto, N.V., Nogueira, T.C.M., Souza, M.V.N., Savegnago, L., Seixas, F.K., Collares, T.V., Borsuk, S., 2020. Antiparasitic activity of furanyl N-acylhydrazone derivatives against *Trichomonas vaginalis*: *in vitro* and *in silico* analyses. *Parasite Vector*, 13:59. <https://doi.org/10.1186/s13071-020-3923-8>

Abstract

Background: *Trichomonas vaginalis* is the causative agent of trichomoniasis, which is one of the most common sexually transmitted diseases worldwide. Trichomoniasis has a high incidence and prevalence and is associated with serious complications such as HIV transmission and acquisition, pelvic inflammatory disease and preterm birth. Although trichomoniasis is treated with oral metronidazole (MTZ), the number of strains resistant to this drug is increasing (2.5–9.6%), leading to treatment failure. Therefore, there is an urgent need to find alternative drugs to combat this disease. Methods: Herein, we report the *in vitro* and *in silico* analysis of 12 furanyl N-acylhydrazone derivatives (PFUR 4, a-k) against *Trichomonas vaginalis*. *Trichomonas vaginalis* ATCC 30236 isolate was treated with seven concentrations of these compounds to determine the minimum inhibitory concentration (MIC) and 50% inhibitory concentration (IC₅₀). In addition, compounds that displayed anti-*T. vaginalis* activity were analyzed using thiobarbituric acid reactive substances (TBARS) assay and molecular docking. Cytotoxicity analysis was also performed in CHO-K1 cells. Results: The compounds PFUR 4a and 4b, at 6.25 μ M, induced complete parasite death after 24 h of exposure with IC₅₀ of 1.69 μ M and 1.98 μ M, respectively. The results showed that lipid peroxidation is not involved in parasite death. Molecular docking studies predicted strong interactions of PFUR 4a and 4b with *T. vaginalis* enzymes, purine nucleoside phosphorylase, and lactate dehydrogenase, while only PFUR 4b interacted *in silico* with thioredoxin reductase and methionine gamma-lyase. PFUR 4a and 4b led to a growth inhibition (7.4). Conclusions: Our results showed that PFUR 4a and 4b are promising molecules that can be used for the development of new trichomonacidal agents for *T. vaginalis*.

Sena-Lopes, A., Neves, R.N., Dié, M.A.S., Perin, G., Alves, D., **Casaril, A.M.**,
Savegnago, L., Begnini, K.R., Seixas, F.K., Collares, T., Borsuk, S., 2019. Quinolines-
1,2,3-triazolylcarboxamides exhibit antiparasitic activity in *Trichomonas vaginalis*.
Biotech. Res. Innov. 3, 265-274. <https://doi.org/10.1016/j.biori.2019.06.003>

Abstract

Increased prevalence of metronidazole-resistant infections has resulted in a search for
alternative drugs for the treatment of trichomoniasis. In the present study, we report
the evaluation of *in vitro* activity of three quinolines-1,2,3-triazolylcarboxamides
(QTCA-1, QTCA-2 and QTCA-3) against *Trichomonas vaginalis*, evaluation of
cytotoxicity in CHO cells and expression of genes related to hydrogenosome by real
time PCR. Nine concentrations of these compounds were analyzed for *in vitro* activity
against ATCC 30236 isolate of *T. vaginalis*. QTCA-2 reported a cytotoxic effect against
100% of *T. vaginalis* trophozoites at a final concentration of 80 μ M with an IC₅₀ of
50 μ M. The kinetic growth curve of trophozoites indicated that QTCA-2 reduced the
growth by 70% at a concentration of 80 μ M after an exposure of 12 h, and induced
complete parasite death at 24 h. QTCA-2 induced less than 30% of cytotoxicity in
CHO-K1 cells at 80 μ M and data showed this concentration and lower ones had no
significant cytotoxic effect when compared to the control. There was no significant
difference in gene expression (pyruvate-ferredoxin oxidoreductase A and B; Malic
enzyme D; Hydrogenase; β -tubulin) when compared to control and MTZ. Further *in*
silico analysis showed that QTCA-2 had significant binding free energy with *T.*
vaginalis lactate dehydrogenase (−9.3 kcal/mol), purine nucleoside phosphorylase
(−9.1 kcal/mol) and triosephosphate isomerase (−7.3 kcal/mol). The present study
offers new perspectives for exploring the potential of this class of molecules as an
additional option for the treatment of trichomoniasis.

Das Neves, R.N., Sena-Lopes, A., Dié, M.A.S., Fonseca, B.R., Silva, C.C., **Casaril, A.M.**, Savegnago, L., Pereira, C.M.P., Ramos, D.F., Borsuk, S., 2019. 2-Hydroxychalcones as an alternative treatment for trichomoniasis in association with metronidazole. *Parasitol. Res.* 1-12. <https://doi.org/10.1007/s00436-019-06568-4>

Abstract

The treatment for trichomoniasis, based on 5'-nitroimidazol agents, has been presenting failures related to allergic reactions, side effects, and the emergence of resistant isolates. There are no alternative drugs approved for the treatment of these cases; thus, the search for new active molecules is necessary. In this scenario, chalcones have been extensively studied for their promising biological activities. Here, we presented the synthesis of three hydroxychalcones (3a, b, and c), in vitro and in silico analyses against *Trichomonas vaginalis*. The in vitro biological evaluation showed that hydroxychalcone 3c presented anti-*T. vaginalis* activity, with complete death in 12 h of incubation at minimum inhibitory concentration (MIC) of 100 μ M. 3c showed a dose-dependent cytotoxicity against mammalian VERO cell line, but the association of 3c at 12.5 μ M and metronidazole (MTZ) at 40 μ M showed 95.31% activity against *T. vaginalis* trophozoites after 24 h of exposure and did not affect the VERO cell growth, appearing to be a good alternative. In silico analysis by molecular docking showed that 3c could inhibit the activity of TvMGL (methionine gamma-lyase), TvLDH (lactate dehydrogenase), and TvPNP (purine nucleoside phosphorylase) affecting the *T. vaginalis* survival and also suggesting a different mechanism of action from MTZ. Therefore, these results propose that hydroxychalcones are promising anti-*T. vaginalis* agents and must be considered for further investigations regarding trichomoniasis treatment.

Anexo C – Patentes depositadas durante o período de doutoramento

Savegnago, L., Bampi, S., **Casari, A.M.**, Domingues, M., Lourenço, D.A.,
Vieira, B., Padilha, N.B., Lenardao, E.J., Alves, D. Número do registro:
BR1020180045377. Título: "Molécula 1-metil-3-(fenilselenil)-1H-indol
antidiabetogênica e antidepressiva". Patente: Privilegio de Inovação. Instituição de
registro: INPI - Instituto Nacional da Propriedade Industrial. Depósito: 07/03/2018



**Pedido nacional de Invenção, Modelo de Utilidade, Certificado de
Adição de Invenção e entrada na fase nacional do PCT**

Número do Processo: BR 10 2018 004537 7

Casari, A.M., Savegnago, L., Domingues, M., Lenardao, E., Vieira, B.M.,
Padilha, N.B., Perin, G., Alves, D., Jacob, R.G., Lourenço, D.A., Número do registro:
BR102018000526. Título: "Composição farmacêutica contendo 2-fenil-3-
(fenilselenil)imidazo[1,2-a]piridina como um antidepressivo". Patente: Privilegio de
Inovação. Instituição de registro: INPI - Instituto Nacional da Propriedade Industrial.
Depósito: 10/01/2018



**Pedido nacional de Invenção, Modelo de Utilidade, Certificado de
Adição de Invenção e entrada na fase nacional do PCT**

Número do Processo: BR 10 2018 000526 0

Savegnago, L., Domingues, M., **Casari, A.M.**, Bampi, S., Lourenço, D.A.,
Lenardao, E.J., Vieira, B., Perin, G., Alves, D., Jacob, R. Número do registro:
BR1020180004565. Título: "Composição farmacêutica contendo 3-((4-
metoxifenil)selenil)-2-fenilimidazo[1,2-a]piridina como molécula antidepressiva
moduladora do sistema neuroinflamatório". Patente: Privilegio de Inovação. Instituição
de registro: INPI - Instituto Nacional da Propriedade Industrial. Depósito: 09/01/2018



**Pedido nacional de Invenção, Modelo de Utilidade, Certificado de
Adição de Invenção e entrada na fase nacional do PCT**

Número do Processo: BR 10 2018 000456 5

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4744 Domingues, M., **Casari, A.M.**, Savegnago, L., Lenardao, E.J., Perin, G., Vieira,
4745 B.M., Alves, D., Jacob, R.G. Número do registro: BR1020160262062. Título: "Uso da
4746 3-((4-metoxifenil)selenil)-2-fenilimidazo[1,2-a]piridina como um antidepressivo".
4747 Patente: Privilegio de Inovação. Instituição de registro: INPI - Instituto Nacional da
4748 Propriedade Industrial. Depósito: 09/11/2016

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**Pedido nacional de Invenção, Modelo de Utilidade, Certificado de
Adição de Invenção e entrada na fase nacional do PCT**

Número do Processo: BR 10 2016 026206 2

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4752 **Casari, A.M.**, Savegnago, L., Domingues, M., Fronza, M.G., Vieira, B.M.,
4753 Lenardao, E.J., Begnini, K., Seixas, F.K., Collares, T., Perin, G., Alves, D., Jacob, R.G.
4754 Número do registro: BR1020160267510. Título: "Utilização do 3-(4-clorofenilselenil)-
4755 1-metil-1H-indol como uma molécula antidepressiva". Patente: Privilegio de Inovação.
4756 Instituição de registro: INPI - Instituto Nacional da Propriedade Industrial. Depósito:
4757 16/11/2016

4758



**Pedido nacional de Invenção, Modelo de Utilidade, Certificado de
Adição de Invenção e entrada na fase nacional do PCT**

Número do Processo: BR 10 2016 026751 0

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Anexo D – Aprovações da Comissão de Ética em Experimentação Animal

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Pelotas, 18 de novembro de 2015

De: M.V. Dra. Anelize de Oliveira Campello Felix
Presidente da Comissão de Ética em Experimentação Animal (CEEA)

Para: Profa. Lucielli Savegnago
Biotecnologia – Centro de Desenvolvimento Tecnológico

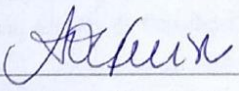
Senhora Professora:

A CEEA analisou o projeto intitulado: **“Atividade de selenoindóis no comportamento tipo-depressivo em camundongos após a administração de lipopolissacarídeo”**, processo nº23110.007034/2015-78, que envolve a utilização de animais pertencentes ao filo Chordata, Subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino, sendo de parecer **FAVORÁVEL** a sua execução, pois está de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA).

Solicitamos, após tomar ciência do parecer, reenviar o processo à CEEA.

Salientamos também a necessidade deste projeto ser cadastrado junto ao COBALTO para posterior registro no COCEPE (código para cadastro nº CEEA 7034-2015).

Vigência do Projeto: 01/12/2015 a 01/12/2017
Espécie/Linhagem: *Mus musculus*/ Swiss
Nº de animais: 312
Idade: 60 dias
Sexo: Machos
Origem: Biotério Central – UFPeI


M.V. Dra. Anelize de Oliveira Campello Felix
Presidente da CEEA

Assinatura do Professor Responsável:  Ciente em: ____/____/2015



Pelotas, 08 de agosto de 2017

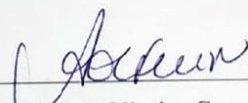
Certificado

Certificamos que a proposta intitulada **“Estudo da atividade de compostos heterocíclicos selenonitrogenados no comportamento tipo-depressivo induzido por estresse de restrição de camundongos”**, registrada com o nº 23110.004034/2017-88, sob a responsabilidade de **Lucieli Savegnago** - que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) – encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e recebeu parecer **FAVORÁVEL** a sua execução pela Comissão de Ética em Experimentação Animal, em reunião de 10/07/2017.

Finalidade	(X) Pesquisa () Ensino
Vigência da autorização	Início: 08/2017 Término: 10/2018
Espécie/linhagem/raça	Mus musculus / swiss
Nº de animais	660
Idade	-----
Sexo	Masculino
Origem	Biotério Central da UFPEL

Solicitamos, após tomar ciência do parecer, reenviar o processo à CEEA.

Salientamos também a necessidade deste projeto ser cadastrado junto ao **COBALTO** para posterior registro no **COCEPE** (código para cadastro nº **CEEA 4034-2017**).


M.V. Dra. Anelize de Oliveira Campello Felix
Presidente da CEEA

Assinatura do Professor Responsável:  Ciente em: 14 / 08 / 2017



Pelotas, 03 de outubro de 2017

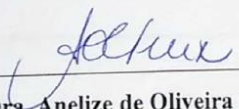
Certificado

Certificamos que a proposta intitulada “**Estudo da capacidade do 3-((4-clorofenil)selenil)-1-metil-1H-indol em reverter o comportamento tipo-depressivo, tipo-ansiosgênico e o déficit cognitivo induzido por lipopolissacarídeo em camundongos**”, registrada com o nº **23110.008331/2017-01**, sob a responsabilidade de **Lucielli Savegnago** - que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) – encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e recebeu parecer **FAVORÁVEL** a sua execução pela Comissão de Ética em Experimentação Animal, em reunião de 02/10/2017.

Finalidade	(X) Pesquisa () Ensino
Vigência da autorização	Início: 11/2017 Término: 11/2018
Espécie/linhagem/raça	Mus musculus / Swiss
Nº de animais	112
Idade	RD
Sexo	Masculino
Origem	Biotério Central da Universidade Federal de Pelotas

Solicitamos, após tomar ciência do parecer, reenviar o processo à CEEA.

Salientamos também a necessidade deste projeto ser cadastrado junto ao **COBALTO** para posterior registro no **COCEPE** (código para cadastro nº **CEE A 8331-2017**).


M.V. Dra. Anelize de Oliveira Campello Felix
Presidente da CEEA

Assinatura do Professor Responsável:  Ciente em: 19 / 09 / 2017



UNIVERSIDADE FEDERAL DE PELOTAS

PARECER N°

28/2018/CEEA/REITORIA

PROCESSO N°

23110.002208/2018-59

INTERESSADO:

LUCIELLI SAVEGNAGO

Pelotas, 12 de abril de 2018

Certificado

Certificamos que a proposta intitulada **“Estudo da capacidade do 3-((4-clorofenil)selenil)-1-metil-1H-indol em reverter o comportamento tipo-depressivo, tipo-ansiosgênico e déficit cognitivo induzido pela administração de corticosterona em camundongos”**, processo n° 23110.002208/2018-59, sob a responsabilidade de **Lucieli Savegnago** - que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) – encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e recebeu parecer **FAVORÁVEL** a sua execução pela Comissão de Ética em Experimentação Animal, em reunião de 09/04/2018.

Finalidade	(X) Pesquisa () Ensino
Vigência da autorização	01/05/2018 a 01/10/2019
Espécie/linhagem/raça	<i>Mus musculus</i> /Swiss
N° de animais	352
Idade	RD
Sexo	Machos
Origem	Biotério Central - UFPel



Pelotas, 06 de outubro de 2017

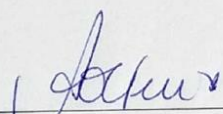
Certificado

Certificamos que a proposta intitulada “**Estudo da capacidade do 3-((4-clorofenil)selenil)-1-metil-1H-indol em reverter alterações comportamentais e bioquímicas em um modelo de depressão induzida por câncer**” processo número 23110.008373/2017-33, de responsabilidade de **Lucielli Savegnago** - que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) – encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e recebeu parecer **FAVORÁVEL** a sua complementação pela Comissão de Ética em Experimentação Animal, em reunião de 02/10/2017.

Finalidade	(X) Pesquisa	() Ensino
Vigência da autorização	01/12/2017 a 01/11/2018	
Espécie/linhagem/raça	<i>Mus musculus</i> /Balb/c	
Nº de animais	306	
Idade	5-6 semanas	
Sexo	Fêmeas	
Origem	Biotério Central - UFPEL	

Solicitamos, após tomar ciência do parecer, reenviar o processo à CEEA.

Salientamos também a necessidade deste projeto ser cadastrado junto ao *COBALTO* para posterior registro no *COCEPE* (código para cadastro nº CEEA 8373-2017).


M.V. Dra. Anelize de Oliveira Campello Felix
Presidente da CEEA

Assinatura do Professor Responsável:  Ciente em: 23 / 12 /2017

4766

4767

Anexo E – Outras informações relevantes

- 2019:** Agraciada com uma bolsa para participação e apresentação de trabalho em congresso internacional. *Travel award: Michael Irwin Equity and Diversity*. Congresso: PsychoNeuroImmunology Research Society (PNIRS), Berlin, Alemanha.
- 2019:** Primeiro lugar na apresentação “Tese em 3 minutos (3MT)” organizada pela Universidade Federal de Pelotas. Título: “Em busca da felicidade...”
- 2019:** Destaque na apresentação oral de trabalho no XXI Encontro de Pós-Graduação, da 5ª Semana Integrada de Inovação, Ensino, Pesquisa e Extensão, UFPel.
- 2018:** Internacionalização (Ciência sem Fronteiras, CNPq, 1 ano). The University of Texas MD Anderson Cancer Center. Supervisor: Robert Dantzer.
- 2018:** Destaque na área de Saúde Humana na II Mostra Acadêmica do VI Simpósio de Biotecnologia: Campo, Bancada e Indústria (co-autoria), Universidade Federal de Pelotas.
- 2018:** Menção Honrosa pela apresentação de trabalho (co-autoria), XII Reunião Regional da FeSBE.
- 2018:** Destaque na apresentação de poster no II Workshop on Biochemistry and Bioprospecting (co-autoria), UFPel.
- 2017:** Destaque na apresentação de poster na I Mostra Científica do V Simpósio de Biotecnologia, Universidade Federal de Pelotas.
- 2017:** Destaque na apresentação oral de trabalho no 26 Congresso de Iniciação Científica (co-autoria), Universidade Federal do Rio Grande.
- 2016:** Primeiro lugar na apresentação oral de trabalho no XVIII Encontro de Pós-Graduação, Universidade Federal de Pelotas.
- 2016:** Internacionalização (estágio, 4 meses). University of Copenhagen. Supervisor: Michael J. Davies.

4796 **Anexo F – Autorizações para o reuso dos artigos científicos**

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Selenium-containing indolyl compounds: Kinetics of reaction with inflammation-associated oxidants and protective effect against oxidation of extracellular matrix proteins

Author:

Angela M. Casaril, Marta T. Ignasiak, Christine Y. Chuang, Beatriz Vieira, Nathalia B. Padilha, Luke Carroll, Eder J. Lenardão, Lucielli Savegnago, Michael J. Davies

Publication: Free Radical Biology and Medicine

Publisher: Elsevier

Date: December 2017

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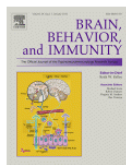
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The antioxidant and immunomodulatory compound 3-[(4-chlorophenyl)selanyl]-1-methyl-1H-indole attenuates depression-like behavior and cognitive impairment developed in a mouse model of breast tumor

Author:

Angela Maria Casaril, Micaela Domingues, Suely Ribeiro Bampi, Darling de Andrade Lourenço, Thiago Ângelo Smaniotto, Natália Segatto, Beatriz Vieira, Fabiana K. Seixas, Tiago Collares, Eder João Lenardão, Lucielli Savegnago

Publication: Brain, Behavior, and Immunity

Publisher: Elsevier

Date: February 2020

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**Repeated administration of a selenium-containing indolyl compound attenuates behavioural alterations by streptozotocin through modulation of oxidative stress in mice****Author:**

Suely Ribeiro Bampi, Angela Maria Casaril, Fernanda S. Sabedra Sousa, Ana Paula Pesarico, Beatriz Vieira, Eder João Lenardão, Lucielli Savegnago

Publication: Pharmacology Biochemistry and Behavior**Publisher:** Elsevier**Date:** August 2019

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