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Tese de Doutorado

Efeito da α-(fenilselenil) acetofenona na díade dor e depressão em camundongos

Fernanda Severo Sabedra Sousa

Pelotas, 2018

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Apresentação de tese de doutorado ao Programa de Pós-Graduação em Bioquímica e Bioprospecção da Universidade Federal de Pelotas

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Kömmling

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“Determinação, coragem e autoconfiança são fatores decisivos para o sucesso. Se estamos possuídos por uma inabalável determinação, conseguiremos superá-los. Independentemente das circunstâncias, devemos ser sempre humildes, recatados e despidos de orgulho.”

Dalai Lama

Resumo

Efeito da α-(fenilselenil) acetofenona na díade dor e depressão em camundongos

Episódios recorrentes de dor muitas vezes podem estar em comorbidade com outras patologias, como é o caso da depressão. Sabe-se que 65% dos pacientes com dor sofrem de depressão em todo o mundo. Os indivíduos que são acometidos por estas condições são menos propensos a aderir aos tratamentos e apresentam uma maior probabilidade de recidivas após os mesmos. Assim sendo, diversos grupos de pesquisa impulsionam seus estudos na busca de novas terapias para o tratamento da díade dor e depressão, as quais possam melhorar a qualidade de vida dos pacientes. Tendo isto em vista o mencionado acima, a molécula α- (fenilselenil) acetofenona (PSAP) tem sido alvo do nosso estudo, pois apresenta efeitos farmacológicos como atividade antinociceptiva, tipo-antidepressiva e antioxidante já elucidados na literatura. Neste sentido, o objetivo deste trabalho foi avaliar, o efeito da PSAP na comorbidade entre dor e depressão em camundongos. O **artigo 1** demonstra que o tratamento agudo com a PSAP (10 mg/Kg administrado pela via intragástrica [i.g.]) reduz a comorbidade dor e depressão induzida por reserpina (0,5 mg/Kg/dia, durante 3 dias, intraperitoneal [i.p.]) em camundongos swiss machos. O efeito do tipo-antidepressivo e anti-hiperalgésico da PSAP envolve a modulação do estresse oxidativo e a sua afinidade pela enzima monoamino oxidase A. Neste mesmo sentido, o **artigo 2** mostra que a PSAP (10 mg/Kg, i.g.) reverteu o comportamento do tipo depressivo, tipo ansiogênico e de dor induzidos pelo estresse de restrição agudo em camundongos. Sendo que esse efeito envolve a redução do estresse oxidativo assim como os níveis de corticosterona plasmáticos. Os resultados do **manuscrito 1** elucidam que a PSAP (0,001-10 mg/Kg, i.g.) foi capaz de reverter a comorbidade dor, depressão e ansiedade induzidas por lipopolissacarídeo (100 µg/Kg, i.p.) (LPS). Além disso, a administração aguda da PSAP (10 mg/Kg, i.g.) apresentou efeito anti-inflamatório ao reverter a ativação da proteína quinase ativada por mitógeno p38 (p38 MAPK), diminuindo os níveis de fator nuclear-κB (p-p65NF-κB) e ciclooxigenase 2 (COX-2) e aumentando os níveis do fator neurotrófico derivado do cérebro (mBDNF) induzidos pelo LPS em córtex. O **manuscrito 2** relata que a PSAP (1-50 mg/Kg, i.g.) apresenta efeito antinociceptivo e do tipo antidepressivo em um modelo de comorbidade entre dor e depressão induzido pela ligação parcial do nervo ciático (PSNL) em camundongos, associado a um efeito anti-inflamatório. A PSNL causou o aumento dos níveis de citocinas pro-inflamatórias no córtex cerebral e hipocampo, de camundongos, bem como a ativação do ácido ribonucleico mensageiro (RNAm) de indoleamina 2,3-dioxigenase (IDO), o aumento dos níveis de NF-κB e do fator neurotrófico derivado do cérebro (TNF-α) e a diminuição dos níveis de ácido ribonucleico mensageiro RNAm de BDNF. O tratamento agudo com a PSAP (50 mg/kg, i.g.) reverte estes parâmetros. Para finalizar a ideia dessa tese o **manuscrito 3** apresenta que o tratamento agudo com a PSAP (10 mg/Kg, i.g.) reverte os efeitos do tipo-depressivo, hiperalgésicos e o estresse oxidativo induzidos pela injeção de TNF-α intracerebroventricular em camundongos. Assim sendo, a PSAP apresenta atividade frente a indução do estado tipo-depressivo e da hiperalgesia induzida por diferentes modelos em camundongos, como: a depleção das monoaminas através da reserpina, ativação do eixo HPA por EAR,

neuroinflamação através da injeção de LPS, dor crônica através da PSNL e aumento das citocinas pró inflamatórias através da injeção de TNF-α. Tendo em vista que a díade dor-depressão é uma condição multipatogênica e a inflamação pode ter um papel central nessa comorbidade, a PSAP poderia ser considerada uma interessante alternativa terapêutica para tratar a dor crônica associada à depressão.

Palavras-chaves: Antidepressivo; Antinociceptivo; Ansiolítico; Selênio.

Abstract

Effect of α- (phenylselenyl) acetophenone on dyad pain and depression in mice

Recurrent episodes of pain can often be in comorbidity with other pathologies, such as depression. It is known that 65% of patients with pain suffer from depression worldwide. Individuals who are affected by these conditions are less likely to adhere to treatments and are more likely to relapse after treatment. Thus, several research groups are promoting their studies in the search for new therapies for the treatment of dyad pain and depression, which can improve patients' quality of life. In view of the above, the α- (phenylselenyl) acetophenone (PSAP) molecule has been the subject of our study, since it has pharmacological effects such as antinociceptive, antidepressant-like and antioxidant activity already elucidated in the literature. In this sense, the objective of this work was to evaluate the effect of PSAP on the comorbidity between pain and depression in mice. **Article 1** demonstrates that acute treatment with PSAP (10 mg/kg administered intragastric [i.g.]) reduces pain and depression comorbidity induced by reserpine (0.5 mg/kg/day for 3 days, intraperitoneal [i.p.]) in male swiss mice. The anti-depressant and antihyperalgesic effect of PSAP involves the modulation of oxidative stress and its affinity for the enzyme monoamine oxidase A. In the same sense, **article 2** shows that PSAP (10 mg/kg, ig) reversed the behavior of the depressive type, anxiogenic type and pain induced by the stress of acute restriction (ARS) in mice. This effect involves the reduction of oxidative stress as well as plasma corticosterone levels. The results of the **manuscript 1** elucidated that PSAP (0.001-10 mg/kg, i.g.) was able to reverse comorbid pain, depression and anxiety induced by lipopolysaccharide (100 µg/kg, i.p.) (LPS). In addition, acute administration of PSAP (10 mg/kg, i.g.) showed anti-inflammatory effect by reversing the activation of p38 mitogen-activated protein kinase (p38 MAPK), decreasing nuclear factor-κB levels (p-p65NF- κB) and cyclooxygenase-2 (COX-2) and increasing levels of brain-derived neurotrophic factor (mBDNF) induced by LPS in cortex. **Manuscript 2** reports that PSAP (1-50 mg/kg, i.g.) shows an antinociceptive and antidepressant-like effect in a model of comorbidity between pain and depression induced by partial sciatic nerve attachment (PSNL) in mice, associated with an effect anti-inflammatory. PSNL caused increased levels of proinflammatory cytokines in the cerebral cortex and hippocampus of mice, as well as the activation of indoleamine 2,3-dioxygenase (IDO) messenger ribonucleic acid (mRNA), increased levels of NF-κB and brain-derived neurotrophic factor (TNF-α) and decreased levels of ribonucleic acid messenger BDNF mRNA. Acute treatment with PSAP (50 mg/kg, i.g.) reverses these parameters. To conclude the idea of this thesis, **manuscript 3** shows that acute treatment with PSAP (10 mg/kg, i.g.) reverses the effects of the depressive-like, hyperalgesic and oxidative stress induced by the injection of intracerebroventricular TNF-α in mice. Therefore, the PSAP presents activity against the induction of the depressive-like behaviour and the hyperalgesia induced by different models in mice, such as: monoamine depletion through reserpine, activation of the HPA axis by ARS, neuroinflammation through LPS injection, pain chronic inflammation through PSNL and increased pro-inflammatory cytokines through TNF-α injection. Since pain-depression dyad is a multipathogenic condition and inflammation may play a central role in this

comorbidity, PSAP could be considered an interesting alternative therapy for treating chronic pain associated with depression.

Keywords: Antidepressant; Antinociceptive; Anxiolytic; Selenium.

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LISTA DE ABREVIATURAS

µL – Microlitros

µg – Microgramas

δ-ALA-D - Delta-aminolevulinato desidratase

5-HT - 5-hidroxitriptamina

ABTS - 2,2-azinobis (3-etilbenzotiazolina-6-ácido sulfônico)

ACTH - Hormônio adrenocorticotrófico

AINES - Fármacos anti-inflamatórios não esteroidais

ALT - Alanina aminotransferase

AMPA - α-amino-3-hidroxi-5-metil-4-isoxazol propiônico

AST - Aspartato aminotransferase

ATP - Adenosina trifosfato

AP- 1 - Proteína ativadora -1

BDNF - Fator neurotrófico derivado do cérebro

BH_2 - Dihidrobiopterina

BH_4 – Tetrahidrobiopterina

CAT - Catalase

CNC - Constrição do nervo ciático

COX 1 - Ciclooxigenase-1

COX 2 - Ciclooxigenase-2

CRF - Fator liberador de corticotropina

DA- Dopamina

DAT - Transportador de dopamina

DNA - Ácido desoxirribonucleico

DPPH - 2,2-difenil-1-picril-hidrazila

EAR - Estresse agudo de restrição

EAAT - Transportadores de aminoácidos excitatórios

EAAT2 - Transportadores de aminoácidos excitatórios tipo 2

EO - Estresse oxidativo

ERK - Proteína quinase regulada por sinal extracelular

ER- Espécies reativas

ERN – Espécies reativas de nitrogênio

ERO - Espécies reativas de oxigênio

FRAP – Potencial redutor do íon férrico

Glu - Glutamato

GPCRs - Receptores acoplados à proteína G

GPx - Glutationa peroxidase

HPA - Eixo hipotalâmico-pituitário-adrenal

IASP - Associação Internacional para o Estudo da Dor

ICV - Intracerebroventricular

IDO - Indoleamina 2,3- dioxigenase

i.g - Intragástrico

IKK - Quinase IKK

IL-1- interleucina-1β

IL-4 - interleucina-4

IL-6 - interleucina-6

IL-10 - interleucina-10

INF-γ - interferon gama

iNOS - Óxido nítrico síntase induzível

i.pl - Intraplantar

ISRS - Inibidores seletivos da recaptação de serotonina

ISRSN - Inibidores da recaptação de serotonina e noradrenalina

IκB: Proteína Inibidora de κB

JNK - Quinase Jun N-terminal

LPS - Lipopolissacarídeo

MAO - Monoamino oxidade

MAPKKK - Proteína quinase quinase quinase ativada por mitógenos

MAPK-p38 - Proteína quinase ativada por mitógeno

MDA - Malondialdeído

NA – Noradrenalina

NFκB - Fator nuclear kappa B

NET - Transportador de noradrenalina

NMDA - N-metil D-Aspartato

NO - Óxido nítrico

NOS - Óxido nítrico sintase

NPS - Nitroprussiato de sódio

NPSH - Tiol não proteico

PAMPs - Padrões moleculares associados a patógenos

PG - Prostaglandina

PGE_2- Prostaglandina E_2

PSNL – Constrição parcial do nervo ciático

P_2X_4 - Purinoceptores

PVN - Núcleo paraventricular

QUIN - ácido quinolínico

RNA - Ácido ribonucleico

RRP - Receptores de reconhecimento de padrões

SelP - Selenoproteína P

SERT - Transportador de serotonina

SNC - Sistema nervoso central

SNP - sistema nervoso Periférico

SOD - Superóxido dismutase

TBARS - Espécies reativas ao ácido tiobarbitúrico

TH - Tirosina hidroxilase

TPH - Triptofano Hidroxilase

TNF- α - Fator de necrose tumoral- alfa

TRx - Tioredoxina redutase

TSC - Teste de suspensão da cauda

$VMAT_2$ - Transportadores vesiculares das monoaminas

SUMÁRIO

1. INTRODUÇÃO

A depressão é um transtorno neuropsiquiátrico que afeta aproximadamente 20% da população, apresentando elevada taxa de morbidade e mortalidade. De acordo com a Organização Mundial de Saúde (OMS, 2017), estima-se que 322 milhões de pessoas sofrem de depressão em todo o mundo. Segundo a Classificação Internacional de Doenças da OMS (2017) e o Manual Diagnóstico e Estatístico de Transtornos Mentais - DSM-V-TR (2013), os principais sintomas da depressão são: perda de interesse ou prazer por quase todas as atividades, humor deprimido na maior parte do tempo, insônia, agitação psicomotora, aumento ou perda de peso, ideação suicida e sentimento de desvalorização. Esses sintomas devem estar presentes praticamente todos os dias, por um período de mais de duas semanas para ser considerado um episódio depressivo.

A dor, por outro lado, é um fenômeno multidimensional que pode ser evidenciada como uma experiência sensorial e emocional desagradável, associada a uma lesão potencial ou real dos tecidos (SCHOLZ; WOOLF, 2002). Assim sendo, a dor serve como alerta para despertar a atenção de que há algo errado com o corpo, ela é o principal sintoma clínico que leva à busca por serviços de saúde, representando um grande problema clínico, social e econômico. A prevalência estimada de várias condições de dor varia de 8% até 60% (CURRIE; WANG, 2004; MILLER; RAISON, 2015).

Vale ressaltar que 65% das pessoas no mundo que são acometidas por dor crônica sofrem também de depressão (MONROE; HARKNESS, 2011; YIEND *et al.*, 2009). As observações clínicas reconhecem há muito tempo a comorbidade da dor e da depressão. Essas observações levaram alguns autores a rotular está comorbidade como a síndrome da dor-depressão ou a díade dor-depressão. Ambas as condições geralmente coexistem, respondem a tratamentos semelhantes, exacerbam-se e compartilham mecanismos patogênicos (BURKE; FINN; ROCHE, 2015; RICHARDS *et al.*, 2016). A natureza comórbida da depressão e da dor foi amplamente revisada (BAIR *et al.*, 2003; CAMPBELL; CLAUW; KEEFE, 2003; HAN, C.; PAE, 2015; NICOLSON *et al.*, 2009), no

entanto, os mecanismos neurobiológicos exatos permanecem obscuros, e os estudos pré-clínicos baseados nestes mecanismo são raros (LIU, M.; CHEN, J., 2014).

Baseando-se nisso, indivíduos que sofrem de ambas as condições são menos propensos a aderir a tratamentos e possuem uma maior probabilidade de recidivas após o tratamento. Além disso, apresentam uma incapacidade prolongada e têm uma qualidade de vida mais baixa em comparação com aquelas pessoas que possuem apenas dor ou depressão crônica (READ *et al.*, 2017).

Neste sentido, diversos grupos de pesquisa aumentaram seus estudos na busca de novas terapias para o tratamento da díade dor e depressão, as quais possam melhorar a qualidade de vida dos pacientes e estes tenham melhor aderência ao tratamento (CALVÓ-PERXAS *et al.*, 2016). Assim, baseado em estudos prévios, nosso grupo de pesquisa tem buscado compostos orgânicos de selênio que apresentem atividades que melhorem os sinais e sintomas da comorbidade dor e depressão. Com isso, a molécula α- (fenilselenil) acetofenona (PSAP) tem sido alvo de nosso estudo.

Sabe-se que a PSAP apresenta atividade tipo-antidepressiva, antioxidante, antinociceptiva já bem elucidada na literatura, e para somar com estes efeitos, não apresenta toxicidade aguda e nem crônica nos testes realizados. Também, foi realizado um estudo de *docking* molecular. Tendo em vista que os fármacos disponíveis no mercado apresentam efeitos adversos e uma eficácia nem sempre satisfatória, estes motivos nos levaram a avaliar os efeitos da PSAP na díade dor e depressão induzida por diferentes mecanismos em camundongos (CASARIL *et al.*, 2015a; GERZSON *et al.*, 2012; SOUSA *et al.*, 2017a).

2. OBJETIVOS

Objetivo Geral

O objetivo deste estudo foi avaliar o efeito da PSAP em modelos experimentais da díade dor e depressão em camundongos Swiss machos.

Objetivos Específicos

- ✓ Avaliar a atividade do tipo-antidepressiva e antinociceptiva da PSAP em animais induzidos com reserpina;
- ✓ Observar a ação da PSAP frente a atividade das enzimas antioxidantes (catalase, superóxido dismutase) e seu efeito frente ao estresse oxidativo em camundongos induzidos com reserpina;
- ✓ Verificar a interação da PSAP com as enzimas monoamino oxidase (MAO- A e Total) em camundongos que receberam reserpina;
- ✓ Explorar a interação da PSAP com as enzimas MAO-A e MAO-B, por estudos de *docking* molecular;
- ✓ Observar o efeito do tipo-antidepressivo, tipo-ansiolítico e anti-hiperalgésico da PSAP frente ao estresse agudo de restrição (EAR);
- ✓ Avaliar a atividade antioxidante da PSAP frente à peroxidação lipídica, níveis de espécies reativas e de óxido nítrico em camundongos submetidos ao EAR;
- ✓ Examinar os efeitos da PSAP nos níveis plasmáticos de corticosterona de camundongos submetidos ao EAR;
- ✓ Verificar o efeito da atividade da PSAP frente a indução de dor, ansiedade e depressão por lipopolissacarídeo (LPS);
- ✓ Observar quais são os mecanismos de ação do efeito da PSAP através da análise do fator neurotrófico derivado do cérebro (mBDNF), Proteína kinase ativada por mitógeno (MAPK), fator nuclear-κB (NFκB) e Ciclooxigenage-2 em córtex cerebral, frente a indução com LPS;

- ✓ Verificar a atividade da PSAP em reduzir a dor neuropática em comorbidade com a depressão em animais que sofreram a constrição parcial do nervo ciático (CPNC);
- ✓ Quantificar os níveis de RNAm dos genes NFκB, Fator de necrose tumoral α (TNF-α), Indoleamina 2,3- dioxigenase (IDO) e BDNF em córtex cerebral e hipocampo de camundongos que sofreram CPNC;
- ✓ Observar o efeito antioxidante da PSAP em córtex cerebral e hipocampo de camundongos que sofreram CPNC;
- ✓ Induzir um efeito do tipo-depressivo e hiperalgésico com o fator de necrose tumoral alfa (TNF-α) e avaliar os efeitos da PSAP;
- ✓ Avaliar os níveis plasmáticos de corticosterona nos animais que receberam a injeção de TNF-α e foram tratados com a PSAP.

3. REVISÃO BIBLIOGRÁFICA

Depressão

A depressão é um transtorno neuropsiquiátrico cujo os principais sintomas são: fadiga, perda de energia, insônia ou sono excessivo, alterações psicomotoras (agitação ou retardação), sentimento de culpa, problemas de concentração, baixa autoestima, tendências suicidas, bem como distúrbios autonômicos e gastrintestinais (OMS, 2017). Os critérios DSM-5 (APA, 2013) para depressão são atendidos quando um indivíduo exibe pelo menos cinco sintomas psiquiátricos durante o mesmo período de 2 semanas, com pelo menos um sintoma de humor deprimido ou perda de interesse.

Diante da variedade de sintomas, a depressão é considerada uma doença heterogênea e cada um destes sintomas tem fatores de risco e gravidade específicos (FRIED *et al.*, 2014). Esta heterogeneidade gera desafios importantes para o diagnóstico e tratamento da depressão (PERLIS, 2014).

Os transtornos de humor estão entre as formas mais prevalentes de doenças psiquiátricas em todo o mundo. De acordo com a organização mundial da saúde (OMS, 2017), 322 milhões da população mundial é atingida pela depressão em pelo menos um momento da vida. Acredita-se que em 2030 o número de casos irá aumentar em torno de 9,8%. Conforme o que foi relatado, existe uma necessidade urgente de novas estruturas conceituais para a compreensão do desenvolvimento da depressão para, assim, desenvolver novos tratamentos e terapias para melhorar os sintomas (MILLER; RAISON, 2015).

A depressão foi considerada sobretudo como um distúrbio centrado em torno da disfunção das vias monoaminérgicas (serotonina [5-HT], dopamina [DA] e noradrenalina [NA]) (ELHWUEGI, 2004). No entanto, foi evidenciado nos últimos anos o envolvimento do sistema imune-inflamatório (MAES *et al.*, 2012; MILLER *et al.*, 2011), neuroendócrino e estresse oxidativo (EO) no estabelecimento da doença (MOYLAN *et al.*, 2014). Nesse contexto, postulou-se que a interação destes sistemas conduz à alterações estruturais e funcionais

do cérebro, num processo referido como neuroprogressão associado à depressão (VARGHESE; BROWN, 2001).

Hipótese monoaminérgica

A hipótese das monoaminas tem sido reconhecida como um conceito central na patogênese da depressão. Ela foi proposta por SCHILDKRAUT, (1965) e postula que a depressão é causada pela redução das monoaminas cerebrais (NA, DA e 5-HT) na fenda sináptica. Tal proposição é reforçada pelo conhecimento do mecanismo de ação dos medicamentos antidepressivos, que se baseia, principalmente, no aumento da disponibilidade desses neurotransmissores na fenda sináptica.

A diminuição da concentração monoaminérgica está associada com a disfunção da atividade de proteínas transportadoras (RECEPTOR *et al.*, 1994), auto receptores e de enzimas envolvidas na síntese e degradação de monoaminas (LEONARD; MAES, 2012; MILLER; RAISON, 2015). Devido a isso, os antidepressivos disponíveis atuam modulando esse sistema, através da regulação dessas vias (XU *et al.*, 2013).

O aumento da concentração dos neurotransmissores na fenda sináptica pode ocorrer através do bloqueio da recaptação e/ou pela inibição da enzima responsável pela degradação destas monoaminas, a monoamina oxidase (MAO). De acordo com esta hipótese, a depleção de NA, 5-HT e DA na fenda sináptica desencadeia o desenvolvimento de sintomas depressivos (Figura 1).

Por outro lado, esta hipótese não é totalmente aceita, pois existem fármacos como as anfetaminas, que aumentam a disponibilidade das monoaminas na fenda sináptica, porém, não são consideradas fármacos antidepressivos. A partir dessa observação é sugerido que não só as monoaminas, mas também outros fatores podem estar envolvidos no quadro depressivo (KRISHNAN E NESTLER, 2012).

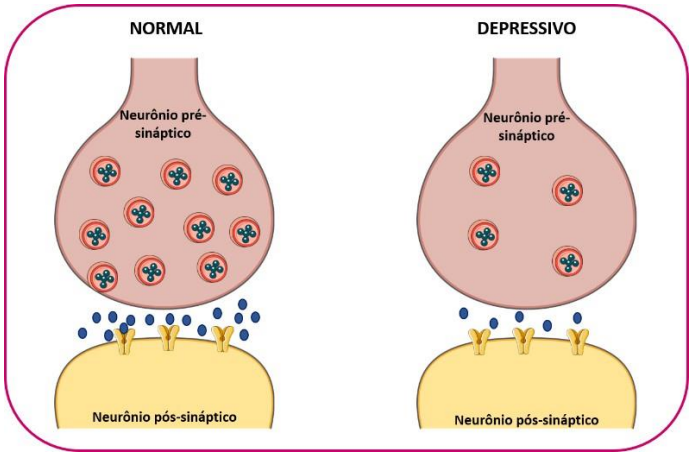

Figura 1. Hipótese monoaminérgica da depressão, elucidando a diminuição das monoaminas na fenda sináptica quando há o estado depressivo (Fonte própria).

Hipótese do estresse oxidativo

Além de ser bem estabelecido que a neuroinflamação e o EO têm papéis importantes em doenças neurodegenerativas e no envelhecimento, evidências crescentes sugerem seu envolvimento na patogênese do transtorno depressivo (HERKEN *et al.*, 2007). O cérebro representa mais de 20% do consumo total de oxigênio e apesar do oxigênio ser essencial para os neurônios, alguns de seus produtos podem ser neurotóxicos (GANDHI; ABRAMOV, 2012). As espécies reativas de oxigênio (ERO) são moléculas altamente reativas derivadas do oxigênio, os quais facilmente oxidam e modificam as funções de RNA (ácido ribonucleico), DNA (ácido desoxirribonucleico), proteínas e lipídios, levando a danos inevitáveis aos neurônios (TURRENS, 2003). Em condições normais, os níveis de ERO são equilibrados por um sistema de defesa antioxidante, mas quando ocorre um desequilíbrio entre moléculas oxidantes e antioxidantes, um estado de EO é alcançado.

As células do cérebro, como as células da glia e os neurônios, são especialmente vulneráveis aos efeitos prejudiciais do EO devido à sua elevada taxa metabólica, a abundância de substratos altamente peroxidáveis e os níveis modestos de antioxidantes presentes (BAKUNINA; CARMINE; ZUNSZAIN, 2015). Os níveis elevados de biomarcadores de estresse oxidativo, tais como o

malondialdeído (MDA), um subproduto de peroxidação de ácidos graxos poli-insaturados e ácido araquidônico, são um indicativo de dano oxidativo os quais são uma característica da depressão (DANTZER *et al.*, 2011).

Em um estado depressivo, pode ocorrer a ativação do sistema imune, gerando um aumento na produção de citocinas pró-inflamatórias. Isto ocorre devido ao desequilíbrio entre as espécies reativas e as defesas antioxidantes, gerando a ativação de fatores de transcrição [fator nuclear kappa B (NFκB), proteína quinase regulada por sinal extracelular (ERK) e proteína quinase ativada por mitógeno (MAPK-p38)] e a liberação de citocinas pró-inflamatórias. Estas citocinas podem ser induzidas por espécies reativas de oxigênio e nitrogênio (ERO e ERN, respectivamente) que aumentam a lesão tecidual e a peroxidação lipídica (DANTZER *et al.*, 2011).

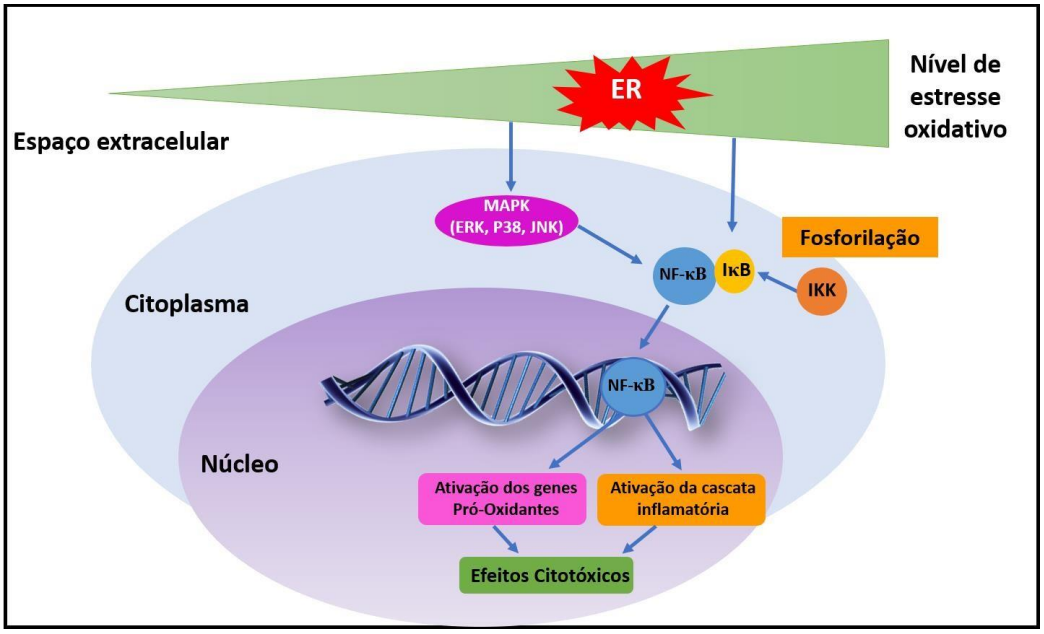

Figura 2. Esquema da Hipótese do estresse oxidativo. Abreviações: ER: Espécies reativas; MAPK: Proteína quinase ativada por mitógeno; NFκB: Fator nuclear κB; ERK: Quinase reguladora de sinal extracelular; JNK: Quinase Jun N-terminal; IKK: Quinase IKK; IκB: Proteína inibidora de κB (adaptado de BAKUNINA; CARMINE; ZUNSZAIN, 2015).

Hipótese da Neuroinflamação

A patogênese da depressão também está associada com a ativação do sistema imunológico, uma vez que, pacientes com depressão possuem altos níveis de citocinas pró-inflamatórias no plasma sanguíneo e no fluido cérebro-espinhal (JEON, KIM, 2016; MAES *et al.*, 2011). As principais citocinas pró-inflamatórias são interleucina 1 - alfa e beta (IL-1 α e β), interferon gama (IFN-γ) e fator de necrose tumoral- alfa (TNF-α). Em contrapartida, as citocinas anti-inflamatórias são a IL-4, IL-10 e IL-14, que geralmente apresentam efeitos opostos aos das pró-inflamatórias (ANDERSON *et al.*, 2014).

As citocinas IL-1, IL- 6, TNF-α e IFN-γ, produzidas no SNC sob condições normais, apesar de seus efeitos deletérios, contribuem com o desenvolvimento neuronal, plasticidade, sinaptogênese e reparo tecidual (BEATTIE, 2002; MUÑOZ-FERNÁNDEZ; FRESNO, 1998). Em contraste, a expressão desses mediadores pelas células gliais, pode ser disparado por algum estimulo antigênico local, periférico e até mesmo por algum dano cerebral (CSERR; KNOPF, 1992).

A correlação da depressão com a neuroinflamação é bem estabelecida, uma vez que a administração de citocinas pró-inflamatórias em animais induz sintomas tipo-depressivos. Somando-se a isso, as citocinas também ativam o eixo hipotalâmico-pituitário-adrenal (HPA), sistema que está intimamente associado com a patofisiologia da depressão, devido a capacidade desses mediadores de estimular o hormônio liberador de corticotropina e o hormônio adrenocorticotrófico (JEON; KIM, 2016).

Em geral, a depressão está associada com a interligação de todos sistemas. As citocinas pró-inflamatórias periféricas apresentam a capacidade de alcançar o sistema nervoso central (SNC) aumentando a produção de mediadores inflamatórios locais. Assim, ativam a ciclooxigenase-2 (COX-2), a enzima óxido nítrico síntase induzível (iNOS) e o NF-κB, aumentando a produção de prostaglandina, óxido nítrico (ON), ER e citocinas, tanto pelas células

endoteliais, como pelos macrófagos perivasculares e micróglia (FELGER; LOTRICH, 2013; WON JEON *et al.*, 2016).

Através da geração das ER, as citocinas contribuem para a redução da tetrahidrobiopterina (BH_4), cofator necessário para a síntese das monoaminas, que é altamente sensível ao EO (MILLER; RAISON, 2015). Além disso, as citocinas pró-inflamatórias IL-1β e TNF-α, através da via das MAPK pela indução de p38, podem diminuir a expressão dos transportadores vesiculares das monoaminas (o $VMAT_2$) e aumentar a expressão de transportadores de membrana de 5-HT e DA, o 5-HTT e DAT. Esses fatores corroboram com redução da concentração das monoaminas na fenda sináptica e assim, acredita-se que ocorram os sintomas depressivos (FELGER; LOTRICH, 2013; MILLER; RAISON, 2015; WON JEON *et al.*, 2016). Além disso, as citocinas pró-inflamatórias diminuem a expressão do fator neurotrófico derivado do cérebro (BDNF), prejudicando a neurogênese e a plasticidade neuronal.

Através das vias de sinalização da MAPK e NF-κB, as citocinas pró-inflamatórias ativam a enzima indoleamina 2,3- dioxigenase (IDO). Essa ativação contribui com o metabolismo do triptofano (precursor do 5-HT) à quinurenina, reduzindo os níveis de 5-HT na fenda sináptica. A quinurenina é novamente metabolizada formando um composto neurotóxico, o ácido quinolínico. O ácido quinolínico é um agonista dos receptores de glutamato N-metil D-aspartato (NMDA) e ácido α-amino-3-hidroxi-5-metil-4-isoxazol propiônico (AMPA). Considerando-se que o glutamato é um neurotransmissor excitatório, essa ação agonista aumenta a excitotoxicidade neuronal (Figura 3). É importante salientar que o excesso de citocinas, espécies reativas e quinurenina também reduz a expressão dos transportadores de glutamato (EAAT) nos astrócitos e, consequentemente isso diminui a recaptação desse neurotransmissor aumentando a sua disponibilidade na fenda sináptica (FELGER; LOTRICH, 2013; MILLER; RAISON, 2015; WON JEON *et al.*, 2016). Além de aumentar a excitotoxicidade, a ligação do glutamato aos receptores NMDA também diminui a produção de BDNF, reduzindo a sobrevida neuronal (Figura 3) (FELGER; LOTRICH, 2013; MILLER; RAISON, 2015).

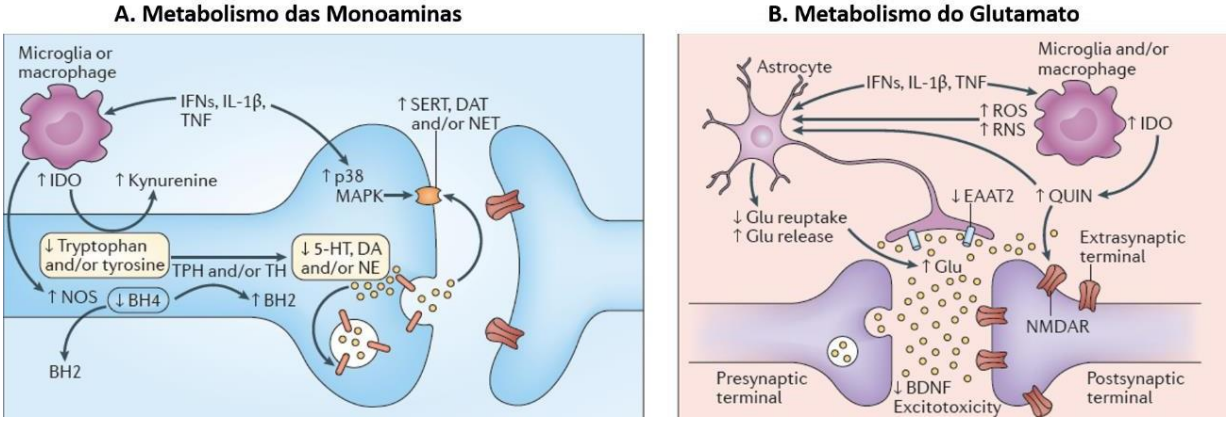

Figura 3. Esquema da hipótese neuroinflamatória. 3A: A nível molecular, as citocinas pró-inflamatórias, incluindo os INF I e II, a IL-1β e o TNF podem reduzir a disponibilidade de monoaminas - 5-HT, DA e NA - aumentando a função e a expressão das proteínas que realizam a recaptação pré-sináptica (transportadores) de 5-HT, DA e NA através da ativação da MAPK e pela redução da síntese de monoaminas através do cofator enzimático BH_4, a qual é altamente sensível ao estresse oxidativo induzido por citocinas e está envolvida na produção de ON através da NOS. Muitas citocinas, incluindo IFNγ, IL-1β e TNF, também podem diminuir os precursores de monoaminas, ativando a enzima IDO, a qual degrada o triptofano, o precursor primário da 5-HT, a quinurenina. Por outro lado, a micróglia ativada pode converter a quinurenina em ácido quinolínico, que se liga ao receptor de NMDA (um receptor de glutamato), aumentando a ligação glutamato/receptor, aumentando a excitotoxicidade. O aumento do ácido quinolínico acarreta na ativação dos receptores glutamatérgicos, liberação de glutamato e bloqueio da reabsorção deste neurotransmissor pelos astrócitos. Os efeitos do ácido quinolínico sobre o glutamato convergem com os efeitos diretos das citocinas pró-inflamatórias sobre o metabolismo deste neurotransmissor, que incluem a diminuição da recaptação de glutamato pelos astrócitos e estimulam a liberação deste também nos astrócitos. Essas ações contribuem com o excesso de glutamato dentro e fora da sinapse aumentando a ligação desse neurotransmissor com os receptores do tipo NMDA. Com isso, há um aumento da excitotoxicidade e a diminuição da produção do BDNF. O BDNF promove a neurogênese, que é um pré-requisito importante para uma resposta antidepressiva, e demonstrou ser reduzido pela IL-1β e TNF e as suas vias de sinalização, incluindo NF-κB em modelos de depressão induzidos pelo estresse. *Abreviações: BH_2: dihidrobiopterina; DAT: transportador de dopamina; EAAT2: transportador de aminoácidos excitatórios tipo 2; NET: transportador de noradrenalina; NF-κB: fator nuclear-κB; SERT: transportador de serotonina; TH: tirosina hidroxilase; TPH: triptofano hidroxilase; BH_4: tetrahidrobiopterina; Glu: Glutamato; QUIN: ácido quinolínico; IDO: indoleamina 2,3-dioxigenase; ROS: Espécies reativas de oxigênio; RSN: Espécies reativas de nitrogênio; NMDA, receptor de N-metil-D-aspartato; NOS: óxido nítrico sintase.* Fonte: (MILLER; RAISON, 2015).

Hipótese Neuroendócrina

O estresse pode não ser suficiente para induzir depressão, mas é um fator importante na patogênese desta doença. Assim, o eixo HPA, é um fator importante na resistência ao estresse e tem atraído considerável atenção científica. O eixo HPA é ativado por uma ampla variedade de estímulos estressantes os quais resultam no aumento de glicocorticoides na corrente sanguínea, assim, aumentando a gliconeogênese e lipólise (WON JEON *et al.*, 2016).

O fator liberador de corticotropina (CRF), que é secretado a partir do núcleo paraventricular (PVN) do hipotálamo, aumenta a secreção de adrenocorticotropina (ACTH) na hipófise. Como resultado, o glicocorticoide (cortisol humano) é segregado do córtex da adrenal e assim afeta funções neurocomportamentais em muitos domínios do cérebro. O eixo HPA forma um *loop de feedback* com domínios cerebrais, como o hipocampo e a amígdala (BELUJON; GRACE, A. A., 2011) (Figura 4). O dano nos neurônios piramidais do hipocampo ocorre quando o estresse severo continua por um longo tempo e uma concentração sanguínea elevada de glicocorticoides é sustentada (ANACKER *et al.*, 2013; BREMNER *et al.*, 2000). A hipercortisolemia aumenta a excitotoxicidade dos neurônios piramidais no hipocampo e, eventualmente, provoca atrofia dendrítica e, até mesmo, morte celular nervosa em casos extremos. Além disso, a nova formação de células nervosas na camada granular é inibida. Esses problemas funcionais no hipocampo causados por estresse prolongado podem deteriorar o tom inibitório que o hipocampo dá ao eixo HPA (KRISHNAN E NESTLER, 2012).

Consequentemente, o eixo HPA pode ser excessivamente ativado. Esta atividade excessiva é observada em aproximadamente 50% dos pacientes deprimidos, e a administração contínua de antidepressivos é conhecida por aliviar esses fenômenos (MARIC; ADZIC, 2013). Além disso, o tratamento contínuo com antidepressivos pode aumentar uma nova formação neuronal e

reverter as funções anormais no hipocampo acima mencionadas (JEON; KIM, 2016).

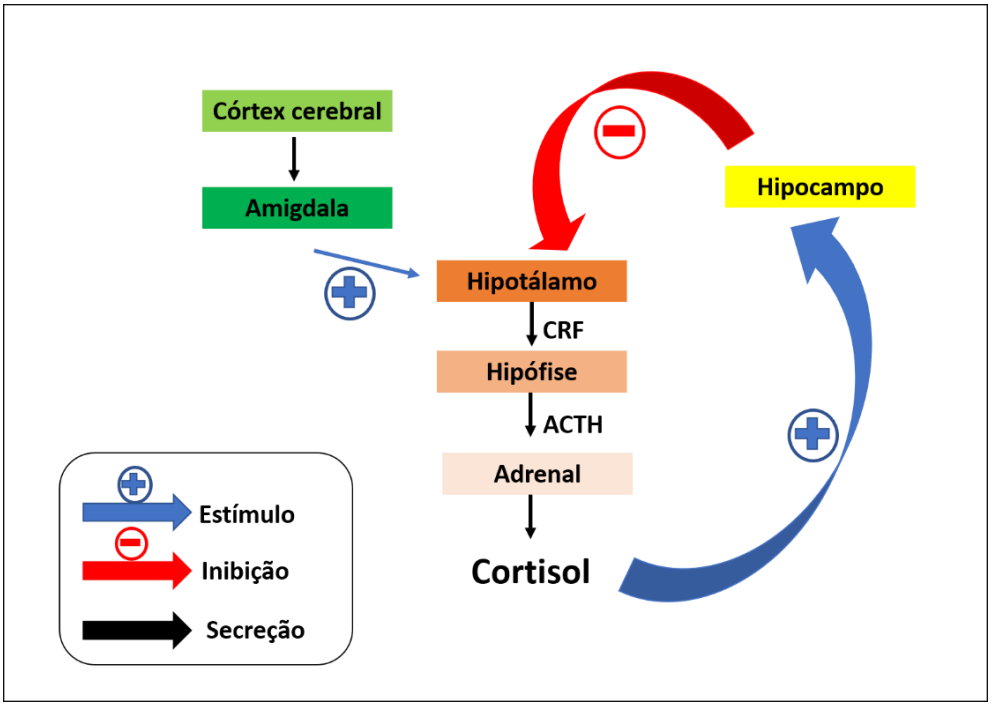

Figura 4. Circuito neural do eixo hipotálamo-hipófise-adrenal (HPA) como um *loop* de *feedback*. Adaptado de WON JEON *et al.*, 2016.

Dor e Nocicepção

A palavra dor é derivada da palavra em latim *dolore,* que significa desgosto, aflição, tristeza e sofrimento tanto físico quanto moral. Atualmente, a definição que melhor explica o que é a dor foi elaborada pela Associação Internacional para o Estudo da Dor (IASP), a qual classifica a dor como uma "experiência sensitiva e emocional desagradável associada a uma real ou potencial lesão tecidual" (LOESER; TREEDE, R., 2008). A dor é um processo de extrema importância para o ser vivo, visto que ela faz com que tenhamos consciência de que a integridade corporal ou mental está sendo ameaçada.

A percepção de estímulos nocivos pelo SNC é realizada pela dor, mais especificamente, por nociceptores, que são os próprios sistemas neurais, ou

seja, o sistema nociceptivo. A dor parece ser influenciada tanto por fatores sensoriais quanto psicológicos, e a nocicepção refere-se apenas a parte fisiológica da dor, sem levar em consideração os aspectos psicológicos, os quais também influenciam na percepção final da dor. A nocicepção está relacionada com os sinais dolorosos reconhecidos pelo SNC e é mediada pelas terminações nervosas axonais livres, nas quais seus canais iônicos são sensíveis a inúmeros estímulos térmicos, químicos e mecânicos que formulam informações relacionadas à lesão. Por meio da detecção dos receptores dos neurônios sensoriais periféricos, a nocicepção é capaz de detectar estímulos capazes de afetar a integridade física do organismo. Por outro lado, a percepção é de cunho psicológico, motivacional e emocional, porque cada pessoa reage de modo diferente a estímulos de dor (APKARIAN; BALIKI; GEHA, 2009).

Classificação da dor

De acordo com a sua duração, a dor pode ser classificada em: dor transitória, aguda e crônica. Na dor transitória, os nociceptores da pele e outros tecidos são ativados sem que haja qualquer lesão, desempenhando um papel essencialmente protetor sem necessidade de atenção clínica. Por sua vez, a dor aguda caracteriza-se por lesão tecidual provocada frequentemente por trauma, intervenção cirúrgica ou doença e pode durar de poucos dias a poucas semanas, desaparecendo com a resolução da lesão. Normalmente a intervenção clínica é útil no sentido de bloquear ou reduzir a dor ou acelerar o processo de restabelecimento do tecido lesado. Já a dor crônica permanece mesmo após a resolução da lesão e pode durar de meses a anos, causando muito sofrimento e incapacidade para o indivíduo. Na dor crônica a lesão tecidual excede a capacidade de reparação do organismo, devido à perda de parte do tecido, a extensão do trauma ou a lesão do próprio sistema nervoso. As terapias clínicas disponíveis normalmente não são efetivas e fornecem apenas alívio transitório sendo que fatores ambientais e afetivos, bem como o estresse podem contribuir significativamente para a intensidade e persistência da dor. Além disso, tanto a

dor aguda quanto a crônica estão frequentemente associadas a processos inflamatórios, como resultado da lesão tecidual, reatividade imune anormal ou lesão nervosa (LOESER, 1992; STEIN; SCHÄFER; MACHELSKA, 2003).

A dor crônica resultante de lesão a um nervo do sistema nervoso periférico (SNP) ou SNC, devido a uma doença, lesão ou inflamação é chamada de dor neuropática. Segundo a IASP 7–8 % dos casos de dor crônica na população em geral apresentam característica neuropática. Esse tipo de dor geralmente é muito mais severa, debilitante e de difícil tratamento, principalmente porque os mecanismos patofisiológicos são pouco conhecidos (HARDEN; COHEN, M., 2003). Os sintomas da dor neuropática podem incluir alodínia (dor resultante de um estímulo que normalmente não é nocivo), hiperalgesia (uma resposta excessiva a um estímulo nocivo) e dor espontânea (WOOLF; MANNION, 1999).

3.3. Relação entre dor e depressão

A dor é ainda mais prevalente em pacientes com alguma comorbidade psiquiátrica, particularmente distúrbios de humor. A sobreposição entre dor e depressão varia de 30% a 60% (BAIR *et al.*, 2003; MAGNI *et al.*, 1993). A dor é um forte preditor tanto do início quanto da persistência da depressão (OHAYON; SCHATZBERG, 2003), e a depressão também é um poderoso preditor de dor, particularmente da dor persistente (BREIVIK, 2002; GUREJE *et al.*, 1998). Reciprocamente, a prevalência de depressão em pacientes com dor crônica pode chegar a 85% (BAIR *et al.*, 2004; WILLIAMS *et al.*, 2004). A dor e a depressão simultâneas têm um impacto muito maior do que qualquer desordem por si só em múltiplos domínios de estado funcional, bem como na utilização de cuidados de saúde (BAIR *et al.*, 2003).

Estudos têm apontado que o desenvolvimento da díade dor-depressão pode estar intimamente relacionado com eventos inflamatórios, e a neuroinflamação seria o mecanismo comum desta comorbidade (WALKER *et al.*, 2013; ZHOU *et al.*, 2015; ZUCOLOTO *et al.*, 2017). Vale ressaltar que existe uma comunicação entre o sistema imune e o SNC e as condições inflamatórias

podem afetar o ambiente neuronal e induzir sintomas depressivos (DANTZER *et al.*, 2008), mas ao mesmo tempo também podem levar ao desenvolvimento e manutenção da dor crônica (VALLEJO *et al.*, 2010).

Interessantemente, embora múltiplas condições possam gerar a dor neuropática, a presença de inflamação no local do nervo lesionado, é um mecanismo muito comum, causando hipersensibilidade e alodínia transitórias. No entanto, em alguns casos, essa condição torna-se crônica. As citocinas pró-inflamatórias contribuem para este processo através da hipersensibilização das fibras aferentes transmissoras do estímulo doloroso e também sensibilizando nervos vicinais que não sofreram danos (MCMAHON; CAFFERTY; MARCHAND, 2005; SOMMER; KRESS, 2004).

A lesão a um nervo periférico também leva à ativação da micróglia com liberação de citocinas pró-inflamatórias, indução da cicloxigenase-2 (COX-2) e de óxido nítrico sintase induzível (iNOS) no SNC (DELEO; YEZIERSKI, 2001; WATKINS; MILLIGAN; MAIER, 2001). A ativação da micróglia pode ocorrer devido à fosforilação da MAPK p38. O mecanismo pelo qual a MAPK p38 é ativada no SNC após a lesão a um nervo periférico ainda não foi completamente elucidado. Sabe-se, porém, que a regulação da ativação da MAPK p38 ocorre em resposta aos níveis intracelulares de Ca^{2+}. A lesão a um nervo periférico leva a ativação de canais expressos na micróglia como, por exemplo, purinoceptores (P_2X_4 ou P_2X_7), receptores acoplados a proteína G (GPCRs, por exemplo, purinoceptores CCR2, CB2 ou P2Y). A ativação desses receptores pelos seus ligantes pode levar a um aumento das concentrações intracelulares de Ca^{2+} e à ativação da MAPK p38. Estes sinais intracelulares podem levar à liberação de fatores difusíveis bioativos, como citocinas, quimiocinas e fatores neurotróficos. Somando-se a isso, a lesão a um nervo periférico induz a liberação de neurotransmissores como glutamato e adenosina trifosfato (ATP) a partir das fibras nervosas aferentes (JI *et al.*, 2003). O glutamato liberado ativa os receptores NMDA da micróglia que, por sua vez despolarizam a membrana celular causando a abertura dos canais de Ca^{2+} dependentes de voltagem. O grande influxo de íons Ca^{2+} ativa a MAPK p38 e sua via de sinalização com

consequente ativação da micróglia (CHIANG; HUANG; TSAI, 2013; KIM; KO, 1998; LOHR; KUCZENSKI; NICULESCU, 2003), a qual desempenha um importante papel no desenvolvimento e potenciação da dor neuropática (Figura 5) (MCMAHON; CAFFERTY; MARCHAND, 2005; VALLEJO *et al.*, 2010; WATKINS; MILLIGAN; MAIER, 2001). De fato, inúmeros estudos com modelos animais têm demonstrado a ocorrência tanto da dor neuropática como depressão após a constrição de um nervo periférico, como o nervo ciático (CHIANG; HUANG; TSAI, 2013; GAI *et al.*, 2014). Os sinais inflamatórios podem ter diversos efeitos sobre a função neuronal e alterar diferentes vias de neurotransmissores, como descrito anteriormente. A inflamação crônica pode levar a permanente reestruturação dessas vias e à transição de mal-estar inicial à depressão e da dor aguda à dor crônica, mesmo que a resposta inflamatória inicial tenha sido dissipada (WALKER *et al.*, 2013).

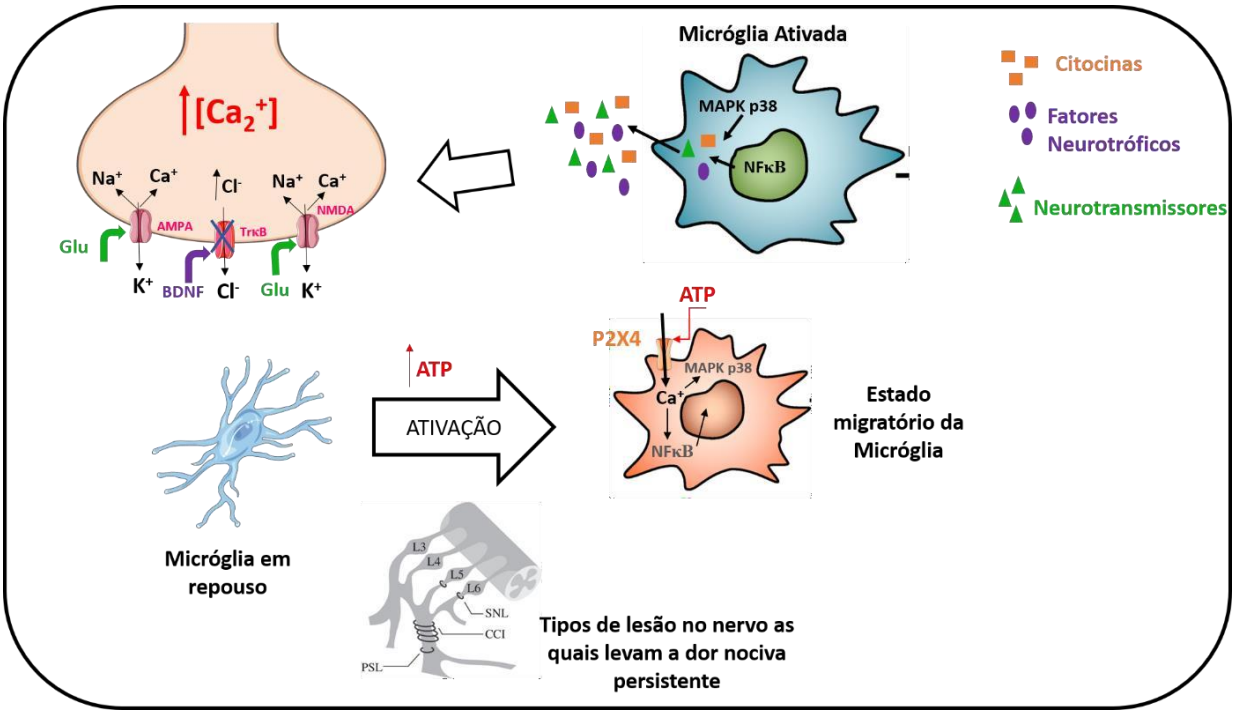

Figura 5. Esquema da dor neuropática causada por lesão do nervo periférico. A lesão do nervo periférico causa uma ativação de células microgliais, as quais estavam em repouso, estas por sua vez, migram para a fonte de ATP. O ATP pode se ligar aos receptores P_2X_4 na superfície da micróglia, o que resulta em um aumento no Ca^{2+} intracelular. O influxo de Ca^{2+} resulta na translocação de NFκB para o núcleo e indução da via MAPK p38. A forma nuclear do NFκB e a

indução da via MAPK p38 iniciam transcrição de vários agentes neuroinflamatórios, incluindo citocinas, fatores neurotróficos e neurotransmissores. A liberação destes agentes neuroinflamatórios na fenda sináptica e subsequente ligação a vários receptores resulta num aumento nos íons intracelulares dentro do neurônio, como Ca^{2+}e Cl^{-}, que despolariza a célula e, desse modo, provoca sensibilização. Dois receptores proeminentes que estão envolvidos com o influxo de Ca^{2+} no neurônio são os receptores AMPA e NMDA. O BDNF liga-se ao receptor TrκB e inibe o efluxo de Cl^{-} do neurônio. *Abreviações: ATP: adenosina trifosfato; BDNF: fator neurotrófico derivado do cérebro; MAPKp38: proteína quinase ativada por mitogênio p38; Ca^{2+}: Cálcio; Na^{+}: Sódio; Cl^{-}: Cloro; Glu: Glutamato.* Adaptado de (VALLEJO *et al.*, 2010).

Alterações no metabolismo da 5-HT, no sistema glutamatérgico e no eixo HPA, pela ação das citocinas pró-inflamatórias, além de induzirem o estado depressivo, também podem ter estreita relação com o desenvolvimento da dor neuropática. De fato, em um modelo animal de artrite inflamatória, a indução de alodínia mecânica e térmica, bem como o comportamento do tipo depressivo, foi associada à ativação da IDO no hipocampo, culminando no aumento na taxa quinurenina/triptofano e, por consequência, na diminuição dos níveis de 5-HT (KIM et al., 2012). Conforme discutido previamente, baixos níveis de 5-HT estão diretamente relacionados aos sintomas depressivos e diminuem a inibição da transmissão do impulso nociceptivo pela via descendente.

O sistema glutamatérgico também desempenha um papel importante na díade dor-depressão. Além do glutamato ser o principal neurotransmissor das fibras aferentes nociceptivas primárias, ele também contribui para a sensibilização central e desenvolvimento da dor crônica (COULL *et al.*, 2003; INQUIMBERT *et al.*, 2012; JI *et al.*, 2003). Neste sentido, o antagonista dos receptores NMDA, a cetamina possui tanto efeito antidepressivo como analgésico (SIGTERMANS *et al.*, 2009), e de fato bloqueia o comportamento do tipo depressivo associado à dor neuropática (WANG *et al.*, 2011). Não menos importante, a ativação do eixo HPA parece contribuir significativamente para a comorbidade entre dor e depressão (BLACKBURN-MUNRO, 2001). A dor crônica pode ser considerada uma forma crônica de estresse, sendo que muitos estímulos nociceptivos podem ativar o eixo HPA e vários componentes deste eixo estão envolvidos na resposta à dor (TAYLOR *et al.*, 1998). Em conformidade

com o descrito anteriormente, em diferentes condições de dor crônica como fibromialgia e artrite reumatoide, onde eventos inflamatórios também estão presentes, ocorre aumento da liberação de hormônios do estresse *(HARBUZ et al.,* 1999). Inicialmente altos níveis de glicocorticoides resultam em aumento dos disparos dos neurônios serotoninérgicos do núcleo da rafe, mas à medida que o estresse se torna crônico pode ocorrer a depleção de 5-HT, o que leva ao desenvolvimento da depressão e facilitação da via nociceptiva ascendente (MASON, 1999). Os altos níveis de glicocorticoides e as citocinas pró-inflamatórias induzem também a resistência dos receptores glicocorticoides e mineralocorticoides, especialmente no hipocampo, onde há uma maior concentração desses receptores (BLACKBURN-MUNRO, 2001). Isso prejudica o mecanismo de retroalimentação negativa do eixo HPA e também induz atrofia nos dendritos apicais hipocampais (MAGARIÑOS; DESLANDES; MCEWEN, 1999; MALETIC, 2009). O hipocampo é considerado uma região chave na comorbidade entre dor e depressão, uma vez que é uma região comum à diferentes vias e neurotransmissores que regulam tanto a dor crônica quanto a depressão (FASICK *et al.*, 2015).

O tratamento da dor crônica apresenta um difícil desafio, uma vez que pode exigir uma abordagem multidisciplinar, incluindo a farmacoterapia, terapia cognitiva, psicoterapia e neurocirurgia. Dadas as diversas origens da dor crônica, a controvérsia circunda a relação que ela tem com a depressão com a qual muitas vezes é coexpressada (BLACKBURN-MUNRO, 2001).

A comorbidade entre estas duas patologias diminui a probabilidade de uma resposta favorável de qualquer das condições ao tratamento e também a satisfação do paciente com o tratamento (BAIR *et al.*, 2003; KROENKE *et al.*, 2008). Assim, avaliar a presença e gravidade da dor em pacientes com depressão, particularmente aqueles que não respondem ao tratamento inicial, bem como estratégias eficazes e eficientes para os cuidados com a depressão baseados em evidências no tratamento de pacientes com dor crônica, é extremamente necessário (KROENKE *et al.*, 2011).

De fato, desde 1960, os antidepressivos foram usados para tratar dor, especialmente a comorbidade entre dor e depressão, embora estudos clínicos posteriores demonstrem que os efeitos analgésicos dos antidepressivos podem ser separáveis de suas atividades antidepressivas (LI, 2015). Em adição à complexidade, os pacientes com ambas as condições são altamente heterogêneos. As chances do desenvolvimento de depressão podem ser diferentes para as diversas condições de dor (MEDAWAR, 2012). Para a depressão, o espectro de sintomas não é desenvolvido de forma uniforme em diferentes condições de dor. A este respeito, estudos com animais pré-clínicos estão posicionados de forma única para abordar esta questão, porque as condições experimentais podem ser cuidadosamente controladas e as variáveis relacionadas à dor e à depressão podem ser medidas com precisão (GERRITS *et al.*, 2014; GILRON; JENSEN, 2013).

Baseando-se nisso, o tratamento para a comorbidade dor e depressão é de difícil aceitação pelos pacientes os quais nem sempre o realizam de forma correta o que faz com que ocorra uma maior probabilidade de recidivas, com isso, estes pacientes apresentam uma qualidade de vida mais baixa em comparação com aquelas pessoas que possuem apenas dor ou depressão crônica. Em conformidade a isto, o tratamento para esta díade apresenta vários efeitos adversos e uma eficácia nem sempre satisfatória (READ *et al.*, 2017). Por esta razão, diversos grupos de pesquisa têm aumentando seus estudos com compostos orgânicos que sejam capazes de melhorar os sintomas e aumentar a qualidade de vida de pacientes que sofrem desta comorbidade.

Terapia Farmacológica

Tratamento farmacológico com antidepressivos para a dor crônica

O tratamento para depressão é feito por medicamentos que possuem em comum a capacidade de aumentar a disponibilidade sináptica de um ou mais neurotransmissores, através da ação em receptores, transportadores e enzimas específicas. Estes fármacos estão sendo muito utilizados para tratar não só a

depressão, mas também a dor crônica. Assim sendo, segundo a IASP os fármacos antidepressivos de primeira-linha utilizados para a dor crônica são os medicamentos da classe dos tricíclicos (ex: amitriptilina e nortriptilina), inibidores da recaptação de serotonina e noradrenalina (ISRSN) (ex: duloxetina e venlafaxina) e os inibidores seletivos da recaptação de serotonina (ISRS) (ex: paroxetina e citalopram). Entretanto, esses fármacos apresentam eficácia somente em 60-70% dos pacientes com depressão, o início do efeito é tardio, e fornecem pouca proteção à recaída após o término do tratamento (MILLAN, 2004, 2006).

Fármacos tricíclicos

Os fármacos tricíclicos além de serem utilizados para o tratamento da depressão, também são bem aceitos para o tratamento da dor crônica. O mecanismo de ação destes fármacos é o bloqueio das proteínas transportadoras que fazem a recaptação das monoaminas (5-HT, NA e DA) na fenda sináptica. As aminas terciárias inibem preferencialmente a recaptura de 5-HT enquanto que as secundárias a de NA. Embora o mecanismo de ação exato não tenha sido totalmente elucidado, sabe-se que os antidepressivos tricíclicos promovem o aumento na disponibilidade de 5-HT, NA e DA na fenda sináptica.

Segundo SINDRUP *et al.*, 2005, os fármacos tricíclicos possuem uma ação analgésica, por isso são muito utilizados para tratar a comorbidade de dor e depressão. O medicamento mais utilizado desta classe para tratar a comorbidade de dor e depressão é a amitriptilina. Isto não significa que os outros antidepressivos tricíclicos sejam menos eficazes, mas a maior parte dos estudos clínicos disponíveis são em relação à amitriptilina (MICO, 2012). Além disso, alguns estudos relatam que há uma ligação dos antidepressivos tricíclicos aos receptores opioides, porém, alguns autores revelam que está afinidade é muito baixa nas doses terapêuticas utilizadas (VERDU, DECOSTERD, BUCLIN, 2008).

Outra ação elucidada dos fármacos tricíclicos é o antagonismo de receptores do tipo NMDA, o qual é acoplado à canais de sódio voltagem

dependente (SINDRUP *et al.*, 2005). Este mecanismo pode estar relacionado ao seu papel na analgesia da dor crônica (VERDU, DECOSTERD, BUCLIN, 2008), já que a inibição destes canais pode diminuir impulsos nervosos, levar a hiperpolarização dos neurônios envolvidos na dor, inibir a exocitose de substâncias estimulantes da resposta à dor (ex. glutamato e substância P), dentre outras ações indiretas a este bloqueio.

Estes fármacos apresentam menos efeitos adversos quando utilizados para o tratamento da dor, pois as doses utilizadas são menores, assim, seus efeitos indesejáveis são menos comuns e severos do que quando os mesmos são usados no tratamento da depressão (MICO, 2012). Porém, seus efeitos adversos representam a principal desvantagem do uso, fazendo com que muitos pacientes interrompam o tratamento. Além disso, seu tempo de ação pode ser demorado, em torno de três semanas (PARK; MOON, 2010).

Inibidores seletivos da recaptação de serotonina (ISRS)

Os antidepressivos ISRS surgiram como alternativa para os pacientes que não podem utilizar os tricíclicos devido à presença de alterações clínicas ou efeitos adversos. Estes fármacos apresentam poucos problemas de tolerabilidade e segurança, quando comparado com as demais classes. Os ISRS inibem de forma potente e seletiva as proteínas que fazem a recaptação de serotonina, resultando em potencialização da neurotransmissão serotonérgica.

Aserotonina é conhecido por modular os mecanismos de sinalização da dor. Juntamente com a bradicinina, histamina e prostaglandina, a serotonina faz parte da “sopa inflamatória”, a qual contribui com a dor gerada por injúria ou inflamação. A serotonina é liberada pelas plaquetas e interage com vários receptores presentes em diversos tecidos. Resultados descritos por AIRA *et al.*, 2010, demonstraram que a administração de agonistas dos receptores 5-HT_{1a}, 5-HT_{1b}, 5-HT_{2c} ou 5-HT_3 foi capaz de reduzir os potenciais de ação das fibras nociceptivas C na medula espinhal de ratos submetidos à dor neuropática. Porém, além do uso de agonistas estar envolvido com certa condição de dor,

sabe-se também, que o uso de antagonistas 5-HT apresenta potencial analgésico, visto que leva ao bloqueio da nocicepção ao bloquear os receptores serotoninérgicos (VIGUIER *et al.*, 2013). Têm-se bem claro na literatura, que a sensação de dor pode ser modificada por vias descendentes inibitórias no SNC, e que o principal neurotransmissor envolvido com essa via descendente é a serotonina (BRÜNING *et al.*, 2014). A ativação dessas vias descendentes a partir do núcleo da raphe até o corno dorsal, mediadas pela serotonina, são capazes de inibir a transmissão da dor (MILLAN, 2002). Tendo em vista essa informação, compostos antidepressivos que modulam o sistema serotoninérgico são amplamente utilizados no tratamento da dor, visto que a analgesia desencadeada por tais compostos pode ser obtida através de uma única dose (FISHBAIN, 2000).

Os principais fármacos desta classe utilizados para o tratamento da dor crônica são paroxetina e o citalopram que mostram certa eficácia no manuseio da dor neuropática, sendo considerados fármacos de terceira linha para o tratamento da dor (HENNEMANN-KRAUSE; SREDNI, 2016).

Inibidores da recaptação de serotonina e noradrenalina (IRSN)

Em paralelo com o sistema serotoninérgico e dopaminérgico, o envolvimento do sistema noradrenérgico também está envolvido na fisiopatologia da nocicepção. Na medula espinal, a noradrenalina é liberada a partir de vias descendentes e suprime a dor pela sua ação inibitória sobre os receptores α_2-adrenérgicos nos terminais centrais dos nociceptores aferentes primários (inibição pré-sináptica) ou por ativação dos interneurônios inibitórios α_1-adrenérgicos. O sistema noradrenérgico modulador da dor interage com outros sistemas de neurotransmissores no nível da medula espinhal, incluindo os sistemas opioide, gabaérgico, serotoninérgico e adenosinérgico (PERTOVAARA, 2006)

A sinergia potencial entre esses agentes indica vínculos importantes entre as áreas do tronco encefálico (particularmente as drogas que afetam a liberação

de noradrenalina e serotonina) e a eficácia do tratamento (BANNISTER; BEE; DICKENSON, 2009; TRACEY; MANTYH, 2007). De fato, os dados pré-clínicos mostram que os fármacos que atuam nos sistemas das monoaminas interagem dentro da medula espinhal, os quais incluem centros cerebrais importantes em respostas emocionais e aversivas à dor. Esses centros cerebrais serão ativados não só pela dor, que desloca o equilíbrio da inibição noradrenérgica para a facilitação serotoninérgica, mas também pelo medo, depressão, ansiedade e outros eventos da vida que começam a dominar nos estados de dor crônica. Essas vias, em seguida, descem para facilitar os mecanismos espinhais da dor, mostrando a interação entre os eventos sensoriais e psicológicos no processamento da dor (OSSIPOV, 2010).

Mudanças nestes sistemas explicam a eficácia da gabapentina ou da pregabalina ao lado das alterações nos canais de Ca^{2+} com tensão medular. Mas também podem ser modulados pelos fármacos opioides. Assim, a ação dos opioides não só é confinada à inibição pré-sináptica e pós-sináptica da medula espinhal, mas também pode alternar controles descendentes no tronco cerebral para inibição por interação com os neurônios na origem dos controles descendentes (BEE; ANTHONY, 2008).

Os IRSN ou antidepressivos duais, duloxetina e venlafaxina, em doses mais baixas agem predominantemente como os ISRS, em doses mais altas inibem também a recaptação da noradrenalina. Estes são fármacos de primeira linha de tratamento da dor crônica (HENNEMANN-KRAUSE; SREDNI, 2016). Os efeitos adversos mais frequentes relatados com o uso da venlafaxina são: náuseas, tonturas, sonolência, hipertensão, sudorese abundante, tremores dentre outros (HORST; PRESKORN, 1998).

Farmacoterapia combinada para o tratamento da dor crônica

A combinação de uma terapia ajustada para o tratamento da dor crônica, tem sido amplamente utilizada. Neste sentido tem-se utilizado não só

antidepressivos, mas também anticonvulsivantes e fármacos anti-inflamatórios não esteroidais (AINES).

A dor crônica geralmente é dividida em três classes principais: inflamatória (por exemplo, artrite), neuropática (por exemplo, neuralgia pós herpética) e idiopática (por exemplo, fibromialgia). Devido a estas divisões a farmacoterapia para o tratamento da dor crônica teve que se diversificar (GILRON; TROELS; JENSEN, 2013).

Assim sendo, a dor crônica geralmente é caracterizada por maior sensibilidade e resposta aumentada a estímulos nocivos (hiperalgesia) e dor produzida por estímulos normalmente não-nocivos (alodínia) (JENSEN; BARON, 2003). Esta hiperexcitabilidade sensorial pode muitas vezes ser suprimida por analgésicos, incluindo anticonvulsivantes, antidepressivos e opioides. Os fármacos anti-hiperalgésicos exercem seus efeitos nos canais de cálcio (Ca^{2+}), sódio (Na^{+}), nos mecanismos de captação e recaptação de monoaminas e nos receptores de membrana acoplados a proteína G expressos nos neurônios que estão espalhados por todo o sistema nervoso e nas estruturas periféricas, espinhais, tronco, limbo e cortical. Como tal, os depressores do SNC também podem causar sedação, tonturas e problemas de memória, enquanto combinações de fármacos com efeitos analgésicos aditivos, mas não efeitos cumulativos adversos, representam a possibilidade de diminuir a dose de agentes individuais e assim reduzir os efeitos colaterais (FINNERUP; JENSEN, 2006). Estes efeitos podem ser observados na Tabela 1.

Por outro lado, os fármacos opioides podem exercer algumas das suas ações na medula espinhal, onde a combinação de inibição pré-sináptica e pós-sináptica reduz a liberação de neurotransmissores e assim inibindo a atividade neuronal. Após a lesão do nervo, por exemplo, os receptores pré-sinápticos produzidos no gânglio da raiz dorsal podem ser vulneráveis à lesão ou doença do nervo e, portanto, a dosagem de opioides pode ser maior em comparação com a dor inflamatória (MELLO; DICKENSON, 2008).

Tabela 1. Tratamentos farmacológicos sistêmicos recomendados para o tratamento de dor crônica adaptado de GILRON; TROELS; JENSEN, 2013.

	RECOMENDAÇÕES DE PRIMEIRA E SEGUNDA LINHA	**RECOMENDAÇÕES DE TERCEIRA E QUARTA LINHA**	**NÃO RECOMENDADOS**
Dor neuropática	**Primeira linha:** antidepressivos tricíclicos, antidepressivos IRSN, anticonvulsivantes (gabapentina ou pregabalina); **Segunda linha:** tramadol, opioides	Bupropiona, citalopram, paroxetina, carbamazepina, lamotrigina, oxcarbazepina, topiramato, ácido valproico, dextrometorfano, memantina e mexiletina	----------
Fibromialgia	Relaxantes musculares, antidepressivos tricíclicos, antidepressivos ISRS e IRSN, tramadol, anticonvulsivantes (gabapentina ou pregabalina)	----------	Opioides
Osteoartrite do quadril ou joelho	Paracetamol, AINEs, tramadol	----------	Glucosamina, opioides, duloxetina

Em teoria, o aumento da atividade em sistemas inibitórios, ao mesmo tempo que diminui a transmissão excitatória, pode ser preferível. Combinar drogas com locais de ação periféricos e centrais pode bloquear a transmissão no local da neuropatia e modular seus resultados centrais. Assim, a lidocaína administrada perifericamente para uma neuropatia focal pode ser combinada com um agente de atuação central (por exemplo, opioide, anticonvulsivante ou antidepressivo). No entanto, o momento da administração deve explicar as variáveis farmacocinéticas dos agentes combinados, de modo que a eficiência máxima de ambos os agentes se sobreponha temporariamente (MATTHEWS *et al.*, 2002; YAMAMOTO, T., 1992).

Apesar das possíveis alternativas terapêuticas listadas acima, as opções farmacológicas para o tratamento da comorbidade entre dor e depressão ainda são bastante limitadas e apresentam baixa eficácia. Considerando a origem multifatorial e o complexo perfil clínico desta comorbidade, de fato é improvável que todos os sintomas sejam controlados por um fármaco possuindo um único mecanismo de ação e desta forma o conceito de um tratamento multimodal tem atraído a atenção. No que se refere à farmacoterapia, duas estratégias centrais podem ser aplicadas. Primeiro, o uso de fármacos que tenham dois ou mais mecanismos complementares e, segundo a coadministração de dois ou mais fármacos diferentes. Ambas as estratégias poderiam aumentar a eficácia, acelerar a ação e tratar simultaneamente os diversos sintomas desta complexa díade (MILLAN, 2014). No entanto, a administração de diversos fármacos pode ser bastante inconveniente, diminuir a adesão ao tratamento e provocar diversas reações adversas (PRUDENT *et al.*, 2008). Dessa forma, o desenvolvimento de novas moléculas que possam agir em diferentes alvos, aumentando a janela terapêutica e reduzindo os efeitos adversos torna-se bastante interessante.

Modelos animais de indução de dor e depressão

Os modelos animais são importantes ferramentas para investigar a etiologia da dor e depressão, bem como o progresso no desenvolvimento de alvos terapêuticos eficazes para o seu tratamento. Um modelo animal da comorbidade dor e depressão deve ser semelhante à sintomatologia clínica, induzir mudanças comportamentais que podem ser medidas objetivamente e revertidas pelos tratamentos que são efetivos em humanos e também ser reprodutível entre pesquisadores (DEDIC; WALSER; DEUSSING, [s.d.]). É importante destacar que para utilização de modelos animais de experimentação, é necessário a aprovação do comitê de ética em pesquisa animal. Este órgão é responsável pela ética e orientação da experimentação animal.

3.3.1 Desafio com lipopolissacarídeo (LPS)

O lipopolissacarídeo (LPS) é uma endotoxina e o principal componente da membrana externa de bactérias Gram negativas (GUHA; MACKMAN, 2001). Com a finalidade de compreender os mecanismos de ação de novas moléculas e descobrir novos antidepressivos com efeitos analgésicos, o LPS vem sendo utilizado como modelo de indução tanto de dor quanto de depressão (LI, 2015). A infecção por bactérias gram-negativas provoca dor, inflamação, sepse, choque séptico e até mesmo a morte, e estes fatores reduzem a qualidade de vida dos pacientes (HO; TAMBYAH; PATERSON, 2010; RÔÇAS *et al.*, 2002; WADACHI; HARGREAVES, 2006). Essa endotoxina é constituída por três subunidades principais: a porção polissacarídica, o antígeno O (que varia de acordo o sorotipo de bactérias); núcleo, que liga o a porção lipídica ao antígeno O e o lipídeo A que confere a sua imunogenicidade (GUHA; MACKMAN, 2001).

A resposta imune inata é ativada através da ligação de padrões moleculares associados a patógenos (PAMPs) aos receptores de reconhecimento de padrões (RRP) presentes na membrana de células imunocompetentes (BRYANT *et al.*, 2010). O LPS, um PAMP, induz a resposta imune inata pela sua ligação aos receptores tipo-toll 4 presente na superfícies de macrófagos, monócitos, micróglia, astrócitos e neurônios (BRYANT *et al.*, 2010; KUBERA *et al.*, 2013).

No SNC, o tratamento dos astrócitos e da microglia com baixas concentrações de LPS pode produzir prostaglandina E_2 (PGE_2) através de sistemas dependentes de receptores Toll-4 (JOHANN *et al.*, 2008). O PGE_2 pode ativar diretamente os neurônios sensíveis à dor para induzir nocicepção (CAO *et al.,* 2009; QUIRION, 2008). Ao mesmo tempo, os receptores de PGE_2 que estão localizados em tecidos periféricos podem se espalhar até o final das terminações nervosas nociceptivas, sensibilizando assim o SNC para a existência de estimulação nociceptiva (SOUTHALL; VASKO, 2001). As COXs são enzimas que catalisam a síntese de PGs. Existem três isoformas distintas: COX-1, COX-2 e COX-3 (BERENBAUM, 2004).

A COX-1 é constitutivamente expressa para regular as condições fisiológicas

normais, enquanto a COX-2 é iniciada em resposta a sinais inflamatórios, como citocinas e LPS (ZHAO *et al.*, 2014). Além disso, nas condições de dor inflamatória, a própria COX-2 pode atuar como um estimulador nociceptivo para causar diretamente dor. A COX-2 é regulada pelo fator nuclear NF -kB, que é um fator de transcrição bem conhecido que está envolvido em inflamação ou lesão. Estudos revelam que o NF-kB também está implicado na hiperalgesia, que é regulada por uma série de adaptadores (HSU *et al.*, 2013).

Em condições normais, o NF-kB está inativo e é ligado ao inibidor kB (IkB) através das suas subunidades, P65 e P50, no citoplasma. A ativação das células por LPS promove a secreção de um amplo espectro de mediadores endógenos, tais como, as citocinas pró-inflamatórias IL-1β, IL- 6, TNF-α, PGE_2, tromboxano, leucotrienos e ER (HU *et al.*, 2016; ZHU *et al.*, 2015). Após a ativação da IkB quinase (IKK), o IkB é fosforilado, resultando em sua ubiquitinação e subsequente degradação pela 26S proteosoma. NF-kB então transloca para o núcleo para regular a transcrição de genes que codificam citocinas inflamatórias e substâncias nociceptivas (HADDAD; ABDEL-KARIM, 2011; NIEDERBERGER; GEISSLINGER, 2008). A transdução de sinal mediada pela endotoxina leva a ativação de vários fatores de transcrição, como por exemplo, NF-kB (p50) e proteína ativadora -1 (AP-1) (c-Fos/c-Jun). Esses fatores, regulam a indução de genes que codificam inúmeros mediadores inflamatórios (Figura 6) (ALEXANDER; RIETSCHEL, 2001; GUHA; MACKMAN, 2001).

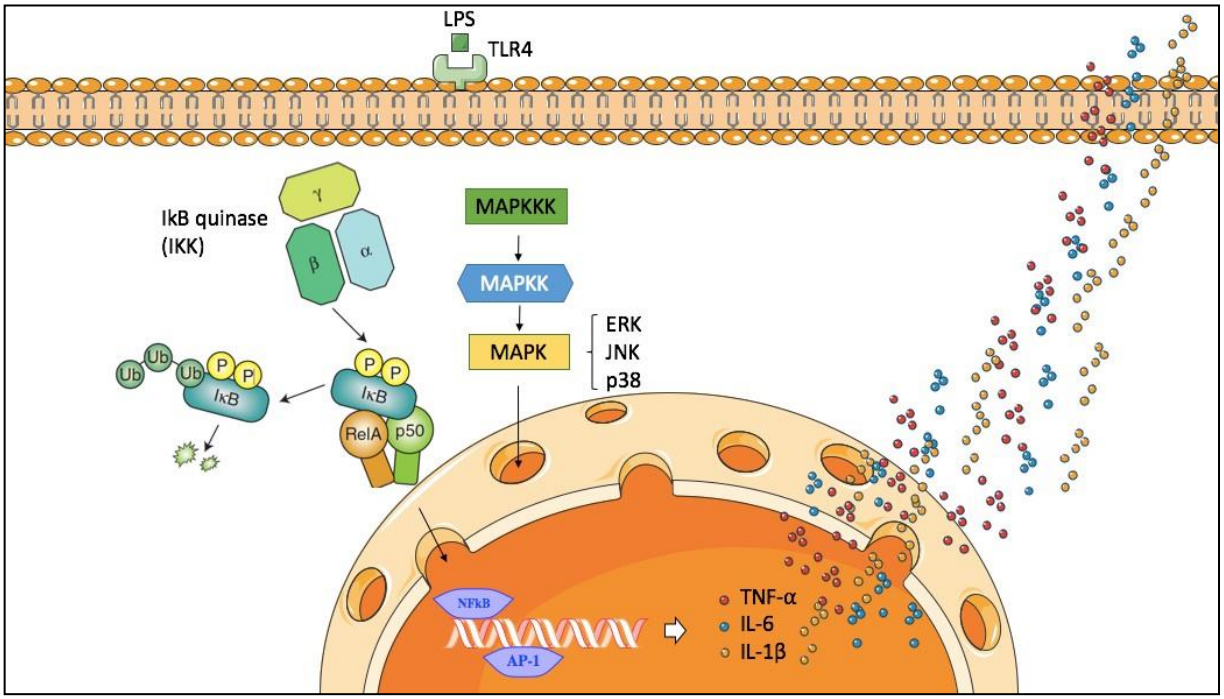

Figura 6. Transdução de sinal mediada por LPS. A endotoxina se liga ao receptor tipo-Toll ativando a cascata de sinalização do NF-κB e MAPK. Quando o LPS se liga aos receptores TLR4, a enzima IκB quinase (γβα) é ativada e fosforila a porção IκB do complexo IκB/RelA/p50. A subunidade IκB fosforilada sofrerá ubiquitinação e então degradação proteossomal, já a porção RelA/p50 (NF-κB) livre será translocada para o núcleo aonde se ligará na sequencia promotora especifica conduzindo a transcrição de citocinas pró-inflamatórias. A ativação do TLR4 pelo LPS, também ativa a via da MAPK. A MAPKKK fosforila resíduos de serina-treonina da MAPKK que por sua vez se tornará ativa. A MAPKK fosforila a MAPK ativando-a. Essa última é translocada para o núcleo celular ativando fatores de transcrição como por exemplo a proteína ativadora-1 (AP-1) que leva o aumento da produção de citocinas pró-inflamatórias. Proteína quinase ativada por mitógenos (MAPK); proteína quinase quinase ativada por mitógenos (MAPKK); proteína quinase quinase quinase ativada por mitógenos (MAPKKK); LPS (lipopolissacarídeo) (ALEXANDER; RIETSCHEL, 2001; GUHA; MACKMAN, 2001) Domingues et al., 2015 [TCC]).

Constrição do nervo ciático (CNC)

Conforme já descrito no item 3.3, este método consiste em uma operação em camundongos, na qual o nervo ciático é constringido. Esta constrição pode ser total, parcial ou espinhal. Neste ensaio, o animal terá uma dor similar a dor crônica do nervo ciático em humanos. A dor crônica pode ocasionar um estado tipo-depressivo nos animais dependendo do tempo de duração da constrição do

nervo. A CNC resulta em liberação de múltiplos mediadores inflamatórios e nociceptivos, causando um aumento na duração e intensidade do potencial de ação em fibras sensoriais primárias. Esta atividade leva a alterações no fenótipo de células neuronais, neurogliais e neuroimunes no SNC, gerando sintomas depressivos (HU, X. *et al.*, 2016; MA, W. *et al.*, 2010).

Citocinas Pró-inflamatórias

Está bem elucidado que a administração de agentes imunomoduladores como citocinas pró-inflamatórias aumentam os riscos do desenvolvimento de depressão. As citocinas são polipeptídios ou glicoproteínas extracelulares, hidrossolúveis, variando entre 8 e 30 kDa. Elas são produzidas por diversos tipos de células no local da lesão e por células do sistema imunológico através da ativação de proteína quinases ativadas por mitógenos (OLIVEIRA *et al.*, 2011).

Estas citocinas possuem a capacidade de regular a proliferação e o crescimento das células da glia, além de modular a atividade dos peptídeos opióides endógenos e ativar o eixo HPA. Além disso, as citocinas podem afetar o metabolismo dos sistemas noradrenérgico, serotoninérgico e dopaminérgico. Por exemplo, a IL-1 pode induzir a síntese de 5-HT, DA e NA; e a IL-2 pode diminuir a transmissão de NA e a transmissão de DA (DUNN; SWIERGIEL; BEAUREPAIRE, 2005).

Neste estudo, será utilizado o TNF-α para induzir o estado tipo-depressivo dos camundongos. Assim sendo, o TNF-α foi descrito em 1975 como um fator solúvel no soro capaz de provocar a necrose de células tumorais. O TNF-α é uma citocina pró-inflamatória a qual possui importantes funções, tais como a indução de mediadores lipídicos da inflamação, de proliferação, também apresenta a capacidade de induzir outras citocinas, de fazer diferenciação celular e apoptose. O TNF-α é induzido por vários estímulos, incluindo microrganismos, mediadores lipídicos, imune complexos, células tumorais e citocinas. PAMPs microbianos, atuando no receptor do tipo-Toll, induzem a produção de TNF-α, que é produzida rapidamente e que induz uma cascata de

mediadores inflamatórios (BEMELMANS *et al.*, 1996; TOGBE *et al.*, 2007; TRACEY, D. *et al.*, 2008).

As atividades biológicas do TNF-α são mediadas pela sua interação com seus receptores específicos, os quais fazem parte de uma superfamília de proteínas. O TNF-α pode exercer seus efeitos pela interação com dois subtipos de receptores (TNFR), que são o TNFR1 (p55 ou CD120a), constitutivamente expresso em todas as células, com exceção dos eritrócitos, e o TNFR2 (p75 ou CD120b), que é geralmente induzido e preferencialmente expresso em células do sistema imune. Esses receptores são proteínas transmembrânicas tipo I, com um a cinco motivos ricos em cisteína no seu domínio extracelular e um domínio de morte intracitoplasmático, porém o TNFR2 não possui o domínio de morte. A interação do TNF-α com o TNFR pode levar, alternativamente, à ativação de NFκB, que controla a expressão de genes de mediadores inflamatórios, ou à ativação de uma via de caspases, causando apoptose. Assim, a sinalização via TNFR1 pode levar à ativação celular ou à apoptose, enquanto a sinalização via TNFR2 não leva diretamente à apoptose, mas pode cooperar com o TNFR1 para induzi-la (GUPTA, 2002; TRACEY *et al.*, 2008; WARZOCHA *et al.*, 2000). Os TNFR ligados à membrana podem ser proteoliticamente clivada da membrana celular pela ação proteolítica de uma metaloproteinase chamada enzima conversora de TNF-α (TACE). Portanto, os os receptores de citocinas, como o TNFR2 (p75), controlem a atividade da citocina *in vivo*, inibindo a capacidade das citocinas de se ligarem a seus receptores de membrana e, assim, inibindo uma resposta biológica. Os níveis plasmáticos elevados de TNFR2 indicam um efeito inflamatório (Figura 7) (BERTHOLD-LOSLEBEN; HIMMERICH, 2008).

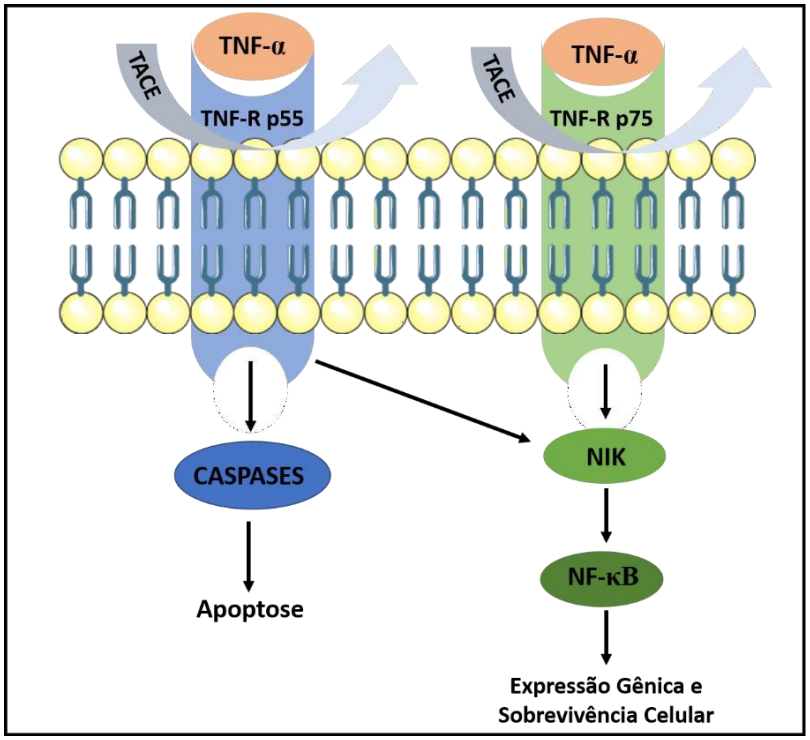

Figura 7. Esquema simplificado de sinalização do TNF-α. A ação do TNF-α à nível celular é mediada pelos receptores de superfície celular TNFR p55 e TNFR p75. O TNF-α induz a diferenciação celular ou a apoptose dependendo da via de sinalização ativada dentro da célula. A via de sobrevivência celular e expressão gênica induzida pelo TNF-α parece ser mediada pelo fator de transcrição NF-κB. Nos casos em que o TNF-α induz a apoptose, o evento crucial de sinalização intracelular é a ativação sequencial das caspases. Os TNFR ligados a membrana podem ser clivados proteoliticamente da membrana celular pela ação de uma metaloproteinase de desintegrina denominada enzima conversora de TNF-α. Abreviaturas: fator alfa de necrose tumoral (TNF-α), receptor de TNF-α (TNFR), enzima conversora de TNF-α (TACE), fator nuclear κB (NFκB), proteína quinase indutora de NFκB (NIK). Adaptado de (BERTHOLD-LOSLEBEN; HIMMERICH, 2008).

Tem sido sugerido que o TNF-α na forma solúvel interage preferencialmente com TNFR1, enquanto que a forma associada à membrana interage preferencialmente com o TNFR2 (MACEWAN, 2002). Além da ativação da via NFκB, o TNF/TNFR1 ativa a via das MAPK, JNK e p38, gerando o fator de transcrição AP-1. Também ativa a via da PI3K, que ativa a quinase Akt, que juntamente com a MAPK ERK, está geralmente associada à sobrevivência e proliferação celular. Portanto, os TNFR ativam genes inflamatórios, controlam a proliferação e morte celular, podendo ser postulado, de maneira geral, que o TNFR1 está associado com funções citotóxicas e pró-inflamatórias, causando

injúria tecidual, enquanto o TNFR2 promove a ativação, migração e proliferação celular, atuando no reparo tecidual e angiogênese (AGGARWAL, 2003).

Estudos relatam que a depressão pode estar associada a um aumento nos níveis séricos de TNF-α (MIKOVA *et al.*, 2001), bem como IL, especificamente com o aumento dos níveis de IL-1 no SNC e IL-6 no plasma. Em experiências pré-clínicas, os animais apresentaram comportamento depressivo após a injeção intracerebroventricular (i.c.v.) de TNF-α (REYNOLDS, Jessica L. *et al.*, 2004). Em conjunto, parece que as citocinas e o TNF-α em particular, são fundamentais na patogênese tanto da depressão como da dor crônica. O TNF-α (CLARK; ALLEVA; VISSEL, 2010; REN, W. J. *et al.*, 2011) e interleucina-1 (IL-1) (REY, DEL *et al.*, 2011) também podem estar envolvidos na diminuição da neurogênese evidenciada nos modelos de dor e depressão.

Reserpina

A reserpina é um alcaloide proveniente do arbusto *Rauwolfia serpentina*, da família das *Apocynaceae*, nativo da Índia. Foi o primeiro fármaco a atuar no sistema nervoso simpático dos humanos. Contudo, na experimentação animal, tem sido utilizada como um modelo de comorbidade entre dor e depressão. Este modelo é caracterizado pela capacidade de induzir a depleção de monoaminas na fenda sináptica (ARORA *et al.*, 2011). Esta substância atua bloqueando a capacidade de captação e armazenamento de aminas biogênicas das vesículas transmissoras aminérgicas, levando à depleção de NA, 5-HT e DA em neurônios tanto centrais quanto periféricos (Figura 8) (KHADRAWY *et al.*, 2017).

Assim sendo, a reserpina leva a uma depleção de monoaminas, fazendo um bloqueio no transportador das monoaminas vesiculares para transmissão ou armazenamento neuronal, promovendo a autooxidação da dopamina e o catabolismo oxidativo pela MAO (LOHR; KUCZENSKI; NICULESCU, 2003). Este mecanismo leva à formação de dopamina-quinase e peróxido de hidrogênio, relacionadas ao processo de estresse oxidativo (BILSKA; DUBIEL, 2007). Muitas regiões do cérebro, especialmente aquelas como hipocampo e córtex cerebral,

devido à sua vulnerabilidade a insultos metabólicos, atuam em conjunto para mediar os sintomas de depressão acompanhados de dor (KUMAR, A. *et al.*, 2009). Ambos os pacientes que sofrem de depressão e dor já demonstraram níveis elevados de biomarcadores oxidativos, como níveis séricos mais elevados de MDA, juntamente com a redução sérica de superóxido dismutase (SOD) (BAGIS *et al.,* 2005).

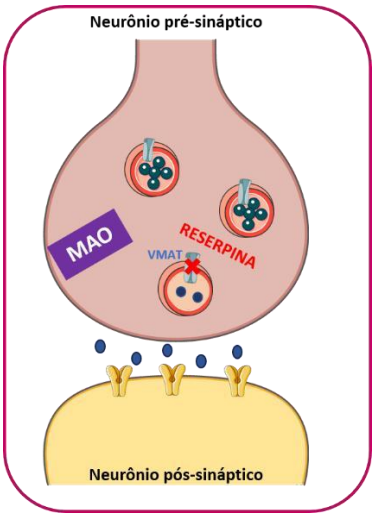

Figura 8. Mecanismo de ação da reserpina no neurônio pré-sináptico (Fonte própria).

Estresse agudo de restrição (EAR)

Vários estudos têm demonstrado que experiências de vida estressantes são consideradas como os principais requisitos responsáveis pelo desenvolvimento de doenças neuropsiquiátricas (CHU *et al.*, 2016; MUSAZZI *et al.*, 2010; OYADEYI *et al.*, 2005; ROPER *et al.*, 2010), incluindo a depressão. Diversos achados documentados demonstraram a relação entre a vida estressante e subsequentes sintomas depressivos (MAZURE, 1998). Além disso, as consequências da produção de ERO devido ao EO e ao sistema antioxidante endógeno alterado levam a comportamentos depressivos semelhantes (MICHEL *et al.*, 2007).

A situação estressante pode ser responsável pela ativação do eixo HPA, que posteriormente resulta no desenvolvimento de ansiedade e depressão. O estímulo para a ativação do eixo HPA é o fator de liberação de CRF que resulta em produção de hormônio ACTH na glândula pituitária e posterior liberação de cortisol e corticosterona no córtex adrenal (BHUTADA *et al.*, 2010; KAWABATA; KAWAI; TERAO, 2010).

Numerosos estudos documentados revelam que os roedores submetidos ao EAR induziram sintomas depressivos significativos (BUDNI *et al.*, 2013; FREITAS *et al.*, 2014). Para realização da indução do EAR, os camundongos são imobilizados por um período de 4 h usando um local de retenção de roedor individual feito com um tubo plástico que possui várias fenestrações, este recipiente impede todo e qualquer movimento físico do animal, sem causar dor. Vale ressaltar que os animais são privados de comida e água durante todo o período de exposição ao estresse (FREITAS *et al.*, 2014; KUMAR; GOYAL, 2008).

Selênio e compostos orgânicos de selênio

O selênio é um calcogênio que faz parte do grupo 16 da tabela periódica e que pode ser encontrado em quatro formas de oxidação: seleneto (Se^{-2}), selênio elementar (Se^{0}), selenito (Se^{+4}) e selenato (Se^{+6}). Diversos estudos trazem a importância do Se para o nosso organismo e como este elemento químico deveria ser introduzido na dieta da população (NOGUEIRA *et al.,* 2004). Sendo assim, o selênio pode ser caracterizado como sendo um micronutriente essencial para a saúde humana (SCHWARZ, K.; FOLTZ, 1957). Porém, uma ingestão exagerada de selênio pode causar toxicidade.

Grande parte da influência benéfica do selênio sobre a saúde humana é atribuída à sua presença em pelo menos 25 proteínas (KRYUKOV *et al.*, 2003). Dentre essas proteínas, vale destacar a glutationa peroxidase (GPx), a tioredoxina redutase (TRx) e a selenoproteína P (SelP), as quais têm sido amplamente estudadas devido suas funções na regulação do sistema redox da

célula (FERGUSON *et al.*, 2012; KUDVA; SHAY; PRABHU, 2015; SANMARTÍN *et al.*, 2011). Ao contrário de outros elementos metálicos que interagem com as proteínas como cofatores, o selênio está incorporado na cadeia polipeptídica como parte do aminoácido selenocisteína (KRYUKOV *et al.*, 2003).

A biossíntese de selenoproteínas, ocorre de maneira bastante especial e depende da disponibilidade de selênio. Os baixos níveis desse elemento, inviabilizam a síntese dessas proteínas pelas células, sendo este o efeito adverso em relação abaixa ingestão de selênio (KUDVA; SHAY; PRABHU, 2015).

Apesar da sua importância para o funcionamento de enzimas antioxidantes, as quantidades totais de selênio no cérebro são relativamente baixas em relação a outros órgãos. No entanto, em condições de deficiência desse elemento, o cérebro o armazena melhor do que os demais tecidos, sugerindo a grande importância do selênio no sistema nervoso central. Em uma análise da distribuição geral de selênio no cérebro, foi visto que cerca de 20% do total desse elemento é incorporado na enzima GPx. Além disso, foi evidenciado que nas regiões com maior concentração de massa cinzenta, os níveis de selênio são mais elevados (PROHASKA; GANTHER, 1976).

A deficiência de selênio tem sido associada a várias condições, tais como aumento do risco de infecção, infertilidade masculina, diminuição da função imune e da tireoide, e várias condições neurológicas, incluindo a doença de Alzheimer e de Parkinson (FAIRWEATHER-TAIT *et al.*, 2011). O aumento no consumo de selênio diminui o estado depressivo e outros sintomas negativos do humor como ansiedade, confusão e hostilidade (BENTON; COOK, 1991; BRUNING *et al.*, 2009; SARI *et al.*, 2018; OLIVEIRA *et al.*, 2016a; BROD *et al.*, 2016; SOUSA *et al.*, 2017b).

Tendo em vista os benefícios que podem ser obtidos através da suplementação alimentar com selênio, esse elemento já foi reconhecido como sendo indispensável para a dieta e é recomendada por associações nutricionais uma ingestão diária de cerca de 55 µg deste calcogênio diariamente. Deve-se observar que não é recomendado ingerir mais que 400 µg de selênio

diariamente, pois este elemento é tóxico em altas concentrações e pode levar o indivíduo a ter selenoses (DUMONT; VANHAECKE; CORNELIS, 2006). Os seguintes alimentos ricos em selênio são recomendados: castanha-do-pará, salmão, carne vermelha, peixes, brócolis, oleaginosas e cereais.

Com o decorrer do tempo, o selênio passou a ser empregado em estruturas químicas, formando assim os compostos orgânicos de selênio, que por sua vez, tornaram-se alvos atrativos para farmacoterapia sintética (MORO *et al.*, 2005) e, somando-se a isso, estudos realizados por nosso grupo de pesquisa mostraram que o efeito de vários compostos orgânicos de selênio. Como exemplo, BIRMANN, *et al.*, 2018 relatou que o 3- (4-Clorofenilselanil) -1-metil-1H-indole (CMI) (Figure 9) apresenta efeito antinociceptivo mediado pela modulação dos sistemas monaminérgicos, opioidérgicos e adenosinérgicos e pode ser uma molécula promissora capaz de modular diferentes vias para o tratamento da dor e da inflamação. Neste mesmo sentido, o pré-tratamento com CMI levou a melhora da neuroinflamação induzida por LPS, reduzindo os níveis de interleucina (IL) -1β, IL-4 e IL-6 no hipocampo e no córtex pré-frontal, bem como marcadores de dano oxidativo. Adicionalmente, foram investigados os efeitos toxicológicos do CMI (200 mg/Kg, i.g.) no fígado, rim e cérebro através da determinação da atividade da aspartato aminotransferase (AST), alanina aminotransferase (ALT), δ-aminolevulinato desidratase (δ-ALA -D) e níveis de creatinina. Esses biomarcadores não foram modificados, indicando a possível ausência de efeitos neuro, hepato e nefrotóxicos. Este estudo revelou que o CMI pode ser uma abordagem terapêutica para o tratamento da depressão e outros transtornos neuropsiquiátricos associados à inflamação e ao estresse oxidativo (CASARIL *et al.*, 2017).

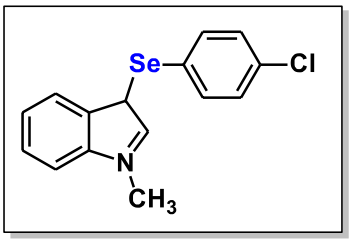

Figura 9. Estrutura química do 3- (4-Clorofenilselanil) -1-metil-1H-indole (CMI).

Os compostos orgânicos de selênio sintéticos têm recebido bastante atenção ultimamente devido a diversas propriedades farmacológicas que os mesmos têm apresentado (CHAGAS *et al.*, 2014; ROSA, Suzan G. *et al.*, 2015). Interessantemente, alguns desses novos compostos têm demonstrado tanto efeito antinociceptivo como antidepressivo, como é o caso do disseleneto de difenila $(PhSe)_2$ (SAVEGNAGO *et al.*, 2007, 2008), 3-(4-fluorofenilselenil) -2,5-difenilselenofeno (F-DPS) (GAI *et al.*, 2014), disseleneto de *m*-trifluormetil-fenila $(m\text{-}CF3\text{-}PhSe)_2$ (BRÜNING *et al.*, 2014; BRUNING, *et al.*, 2009), p,p'- metoxil disseleneto de difenila $(OMePhSe)_2$ (OLIVEIRA e colab., 2016a) e a α-(fenilselenil) acetofenona (PSAP) (SOUSA *et al.*, 2017a) (Figura 10).

O efeito tipo-antidepressivo da PSAP foi demonstrado em um estudo realizado por GERZSON e colab., 2012 no qual o composto teve a capacidade de reduzir o tempo de imobilidade no teste do nado forçado (TNF). Somando-se a isso, a PSAP também apresentou a capacidade de diminuir o tempo de imobilidade no teste de suspensão da cauda (TSC), e não foi observado diferença significativa entre os grupos testados no teste do campo aberto, o qual foi utilizado para descartar resultados falsos positivos na atividade locomotora dos camundongos. Somando-se a isso, foi demostrado que a PSAP apresenta envolvimento do sistema serotoninérgico em sua atividade tipo-antidepressiva. Agregando com estes resultados, foi observado que a PSAP apresenta atividade antioxidante *in vitro* nos ensaios da capacidade de sequestrar os radicais livres sintéticos 2,2-difenil-1-picril-hidrazila (DPPH) e 2,2-azinobis (3-etilbenzotiazolina-6-ácido sulfônico) (ABTS), além de demonstrar que têm a capacidade de reduzir o íon férrico a íon ferroso no teste do FRAP e reduzir a peroxidação lipídica induzida por nitroprussiato de sódio (NPS).

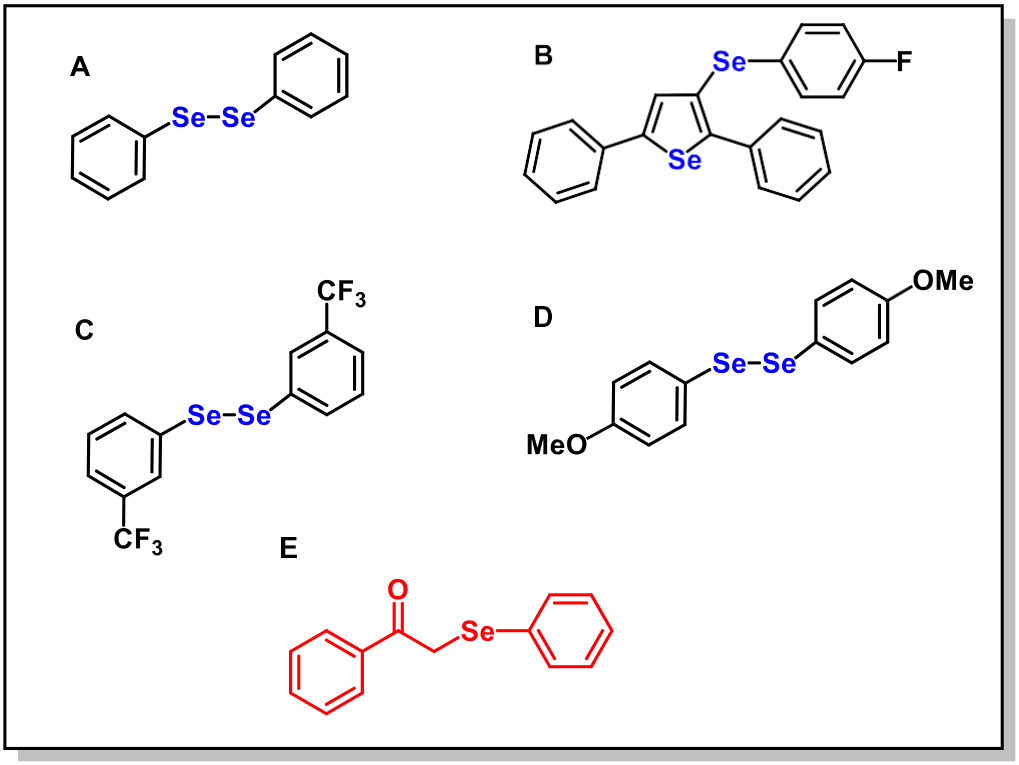

Figura 10. Estrutura química de compostos orgânicos de selênio. Disseleneto de difenila (PhSe)2 (A), 3- (4-fluorofenilselenil) -2,5-difenilselenofeno (F-DPS) (B), disseleneto de *m*-trifluormetil-fenila (*m*– CF3–PhSe)2 (C), p,p'-metoxil disseleneto de difenila (OMePhSe)2 (D) e α- fenilseleno acetofenona (PSAP) (E).

Assim sendo, em um estudo realizado para avaliar a toxicidade da PSAP demonstra que o composto administrado em altas doses (400 mg/Kg, por via intragástrica [i.g.]) não alterou os níveis de TBARS (espécies reativas ao ácido tiobarbitúrico), a atividade das enzimas delta-aminolevulinato desidratase (δ-ALA-D) e catalase em camundongos (GERZSON *et al.*, 2012). Um outro estudo também realizado por nosso grupo de pesquisa demonstrou que tanto o tratamento agudo quanto crônico com PSAP não causou genotoxicidade no ensaio com leucócitos de camundongos. O composto apresentou efeito citotóxico no ensaio *in vitro* com células de ovários de Hamster chinês apenas em uma concentração testada (CASARIL *et al.*, 2015b). Neste estudo também avaliou-se os testes bioquímicos toxicológicos de alanina aminotransferase (ALT), aspartato aminotransferase (AST) e creatinina, confirmando que este

composto não apresenta toxicidade nas doses avaliadas em camundongos (CASARIL *et al.*, 2015b).

Em um estudo realizado por nosso grupo de pesquisa para avaliar a atividade antinociceptiva da PSAP, pode-se observar que o composto causou inibição na fase neurogênica (primeira fase) e reduziu o edema da pata causado pela injeção intraplantar (i.pl.) de formalina. Adicionando-se a estes resultados, a PSAP também diminuiu a resposta nociceptiva na fase inflamatória (segunda fase) do teste de formalina e no comportamento de lamber a pata desencadeado por glutamato nas diferentes doses testadas. Neste mesmo estudo também verificou-se que a atividade antinociceptiva da PSAP foi através do sistema noradrenérgico e dopaminérgico (ousa et al., 2017b).

Tendo em vista (i) os múltiplos aspectos patofisiológicos da díade entre dor e depressão discutidos anteriormente, (ii) a necessidade do desenvolvimento de terapias mais efetivas que tratem simultaneamente os diferentes sintomas dessa díade, (iii) os efeitos antinociceptivo e antidepressivo da PSAP já demonstrados, (iv) o efeito não tóxico da PSAP e (v) as evidências de que este composto possa agir em diferentes sistemas associadas tanto à dor quanto à depressão, torna-se interessante a investigação do possível efeito da PSAP na comorbidade entre dor e depressão.

4 Resultados

Artigo 1

Os resultados que fazem parte desta tese de doutorado estão apresentados sob a forma de artigo, o qual se encontra assim organizado. Os itens Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas encontram-se no próprio artigo. O artigo foi publicado na revista **Brain Research Bulletin.**

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Research report

α- (phenylselanyl) acetophenone mitigates reserpine-induced pain–depression dyad: Behavioral, biochemical and molecular docking evidences

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ABSTRACT

Chronic pain and depressive disorders have been estimated to co-occur in up to 80% of patients and traditional antidepressants and analgesics have shown limited clinical efficacy. α- (phenylselanyl) acetophenone (PSAP) is an organic selenium compound which has already demonstrated antioxidant, antidepressant and antinociceptive activities in animal models, without showing acute toxicity. In view of develop more effective treatments to comorbid pain and depression, the purpose of this study was to evaluate the behavioral and biochemical effects of PSAP on reserpine induced pain-depression dyad model in mice as well to analyze the interaction of PSAP with specific targets by molecular docking analysis. Reserpine (0.5 mg/kg daily, for 3 days, i.p.) decreased the latency for the first episode of immobility and the swimming time, as well as increased the immobility time of mice in the modified forced swimming test (mFST). Reserpine also led to a significant decrease in nociceptive threshold in thermal hyperalgesia in the hot plate test. PSAP or imipramine (10 mg/kg daily, for 2 days, i.g.) reversed these alterations in both mFST and hot plate test. Additionaly, PSAP reduced nitrite and malondialdehyde (MDA) levels and catalase (CAT) activity in the cerebral cortex and hippocampus of reserpinised mice. PSAP also normalized monoamine oxidase (MAO-A and MAO-T) activity increased in reserpinised mice. According to the molecular docking analysis, PSAP has affinity to MAO-A, suggesting an inhibition of this enzyme. The data presented here show that PSAP had reversed effects in the pain-depression dyad induced by reserpine, possibly by its antioxidant property and MAO-A inhibition.

1. Introduction

The arylselenyl acetophenones are a class of organoselenium compounds with a range of interesting biological activities. Studies have shown that α- (phenylselanyl) acetophenone (PSAP) has antidepressant-like properties in mice, dependent of an interaction with the serotonergic system, and it may be of interest as a therapeutic agent for the treatment of depressive disorders (Gerzson et al., 2012). Our group of research also showed the antinociceptive and anti-edematogenic activities of PSAP in glutamate and formalin tests in mice. The antinociceptive effect of PSAP was dependent of dopaminergic and adrenergic modulation (Sousa et al., 2017). In addition, PSAP exhibits glutathione peroxidase-like activity, antioxidant effect and capacity to inhibit tumor promoter-induced down-regulation in intercellular communication between liver epithelial cells (Gerzson et al., 2012; Sousa et al., 2017; Wang et al., 2013), and it does not present acute and chronic toxicity in rodents (Casaril et al., 2015; Gerzson et al., 2012).

Chronic pain and depressive disorders have been estimated to co-occur in up to 80% of patients, and this comorbidity is more disabling and more expensive to both patients and society than the disorders alone. Moreover, the coexistence of depression and chronic pain is associated with increased severity and duration of depressive and

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physical symptoms, poor treatment response, and it is a significant risk factor for relapse and also diminishes patient satisfaction with treatment (Burger et al., 2005; Fuentes et al., 2007; Victoria et al., 2009).

Taking into consideration the pharmacological properties of PSAP, together with the poor efficacy of commonly used antidepressants and analgesics to treat both conditions, it is important to develop more effective treatments against pain-depression dyad. An interesting model to induce comorbidity between pain and depression is the reserpine model. Reserpine presents high affinity for the vesicular monoamine transporter type 2 (VMAT-2) and blocks monoamine binding to its site, inhibiting its vesicular storage and preventing the release of monoamine into the synaptic cleft (Burger et al., 2005; Colpaert, 1987; Fuentes et al., 2007).

Thus, the aim of this study was to investigate the behavioral, biochemical and neurochemical effects of PSAP on pain-depression dyad induced by reserpine in mice. Additionally, we also explored the PSAP interaction with the enzymes MAO-A and MAO-B, responsible by monoamines degradation, by molecular docking studies.

2. Materials and methods

2.1. Animals

The experiments were conducted using male adult Swiss mice (25–35 g) from our own breeding colony. The animals were kept in a separate animal room, on a 12 h light/dark cycle with lights on at 7:00 a.m., at room temperature (22 ± 1 °C) with free access to water and food. All experimental procedures were conducted in accordance with the guidelines of the Committee on the Care and Use of Experimental Animal Resources of Federal University of Pelotas, Brazil (number CEEA 6408-2016).

2.2. Drugs

PSAP (Fig. 1) was prepared and characterized in the Laboratory of Clean Organic Synthesis (LASOL) according to the method previously described (Victoria et al., 2009). PSAP was dissolved in canola oil and administered to mice intragastrically (i.g.) at dose of 10 mg/kg in a volume of 10 mL/kg. Reserpine (Sigma St. Louis, MO, USA) was dissolved in glacial acetic acid, diluted to a final concentration of 0.5% acetic acid with distilled water. The pH was adjusted with 5 M NaOH, giving a solution with pH 5.0 (Arora et al., 2011; Blasco-Serra et al., 2015; Dhingra and Sharma, 2006), and reserpine was administered to mice at dose of 0.5 mg/kg by intraperitoneally (i.p.) route, in a volume of 1 mL/kg. Reserpine and PSAP dilutions were prepared every day before the experiments. Appropriate vehicle treated groups were also simultaneously assessed. The standard drug used was imipramine (IMI) at the dose of 10 mg/kg (i.g.), in a volume of 10 mL/kg, and it was purchased from a commercial pharmacy. All other chemicals were obtained at the highest available commercial grade.

2.3. Experimental design

The mice were randomly assigned into six groups:

(1) Saline + canola oil (control group);

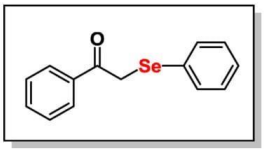


Fig. 1. Chemical structure of α-(phenylselanyl) acetophenone (PSAP).

(2) Saline + PSAP (10 mg/kg, i.g.);
(3) Saline + IMI (10 mg/kg, i.g);
(4) Reserpine (0.5 mg/kg, i.p.) + canola oil (reserpine treatment group);
(5) Reserpine (0.5 mg/kg, i.p.) + PSAP (10 mg/kg, i.g.);
(6) Reserpine (0.5 mg/kg, i.p.) + IMI (10 mg/kg, i.g.).

The current choice of PSAP dosage was based on our previous findings (Gerzson et al., 2012; Sousa et al., 2017). The pain-depression symptoms were induced by administration of reserpine (0.5 mg/kg daily, i.p.) for 3 consecutive days (Nagakura et al., 2009), and on the next 2 days, the animals were treated with PSAP (10 mg/kg, once a day), IMI (10 mg/kg, once a day) or their respective vehicles. On the fifth day, after the PSAP or IMI treatment, the animals from all groups were evaluated in the open field test (OFT) and then subjected to the hot plate test and the modified forced swimming test (mFST). Biochemical and oxidative stress tests were assessed in different group of animals (Fig. 2).

2.4. Behavioral tests

2.4.1. Modified forced swimming test (mFST)

The mFST was carried out as described by Detke et al., 1995 with some modifications. This test permits to study the involvement of monoamines in the antidepressant-like effect of a compound. Briefly, the mice were individually forced to swim in an open cylindrical container (diameter 10 cm, height 25 cm), containing 19 cm of water at 25 ± 1 °C. In the test, the time of climbing, swimming and immobility were measured during a 6-min period. The first two minutes are for adaptation of the animal to the water, and it is recorded the latency time for the first episode of immobility, so the immobility and swimming time are observed in the next 4 min. Climbing behavior consisted of upward directed movements of the forepaws along the side of the swim chamber. Swimming behavior was defined as movement (usually horizontal) throughout the swim chamber, which also included crossing into another quadrant. Immobility was assigned when no additional activity was observed other than that required to keep the mice head above the water. A decrease in the duration of immobility is indicative of an antidepressant-like effect.

2.4.2. Hot plate test

The hot plate test was carried out according to the method previously described (Derrien et al., 1993). In this test, the animals were placed in a glass cylinder on a heated metal plate maintained at 52 ± 1 °C. The latency of nociceptive responses, such as licking or shaking one of the paws or jumping, was recorded. To avoid damage to the paws of the animals, the time standing on the plate was limited to 45 s.

2.4.3. Open field test (OFT)

The spontaneous locomotor activity of mice was accessed in the OFT (Walsh and Cummins, 1976). The open-field was made of plywood and surrounded by walls 30 cm in height. The floor of the open-field, 40 cm in length and 40 cm in width, was divided by masking tape markers into 9 squares (3 rows of 3). Each animal was placed individually in the center of the arena, and the number of segments crossed (four-paw criterion) and rearings were recorded in a 5 min session.

2.5. Ex vivo assays

2.5.1. Oxidative stress

To investigate the effect of PSAP on oxidative stress, malondialdehyde (MDA), reactive species (RS), non-protein thiols (NPSH) and nitric oxide (NO) levels, as well catalase (CAT) activity were determined in total cerebral cortex and hippocamppus of mice. The cortices and hippocampus of different animals were removed, weighed and

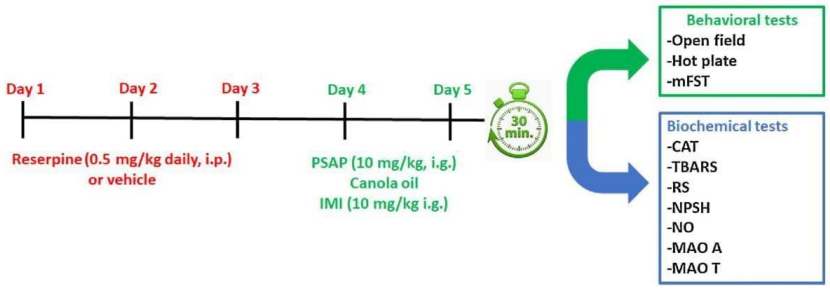
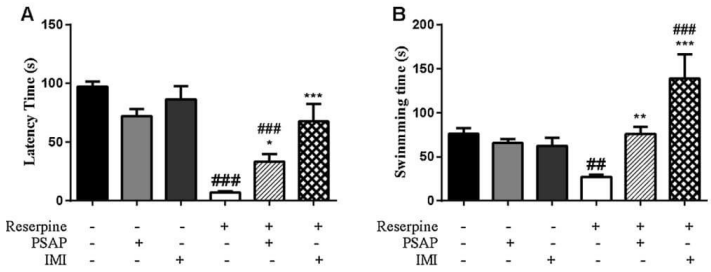


Fig. 2. Schematic protocol of the induction of pain and depression dyad through reserpine admnistration. *Abbreviations:* PSAP: α-(phenylselanyl) acetophenone; IMI: imipramine; i.p.: intraperitoneal; i.g.: intragastric; mFST: modified forced swim test; CAT: catalase; TBARS: thiobarbituric acid reactive species; ER: reactive species; NPSH: non-protein thiols; NO: nitric oxide; MAO: monoamine oxidase.



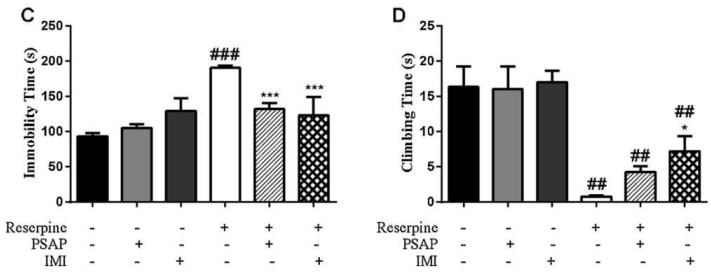


Fig. 3. Effects of PSAP (10 mg/kg, i.g.) on active behaviors in the modified forced swimming test in reserpinised mice. (A) Latency time; (B) swimming time; (C) immobility time and (D) climbing time; Each column represents the mean ± SEM of 6–8 animals for group. Statistical analysis was performed by one-way ANOVA followed by the Newman–Keuls test when appropriate. $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ as compared with the control group, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ as compared with the reserpinised group. *Abbreviations:* PSAP: α-(phenylselanyl) acetophenone; IMI: imipramine.

homogenized in 50 mM Tris–HCl, pH 7.4 (1/4, weight/volume), and centrifuged at 2.400 g at 4 °C for 15 min. The low-speed supernatant fraction (S_1) was collected and used for oxidative stress analyses.

2.5.1.1. Malondialdehyde (MDA) levels. MDA is an end product of the lipid peroxidation. MDA reacts with thiobarbituric acid (TBA) to generate a colored product that can be optically measured at 532 nm (Ohkawa et al., 1979). An aliquot of the S_1 (10 μL) was incubated with 8.1% sodium dodecyl sulfate (SDS), 0.8% TBA and acetic acid/HCl (pH 3.4) at 95 °C during 60 min. The absorbance of the samples was measured at 532 nm, and the results were expressed as nmol MDA/g tissue.

2.5.1.2. Reactive species (RS) levels. Quantification of RS levels of cerebral cortex hemisphere and hippocampus of mice was performed according to Loetchutinat et al. (2005). Briefly, an aliquot of S_1 (10 μL) was 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 RS. The DCF fluorescence intensity emission was recorded at 520 nm (with 480 nm excitation) and RS levels were expressed as arbitrary units (AU) of fluorescence.

2.5.1.3. Non-protein thiols (NPSH) levels. NPSH levels, were determined by the method of Ellman (Ellman, 1959). Cerebral cortex and hippocampus (10 μL) were mixed (1:1) with 10% trichloroacetic acid (TCA). After the centrifugation (3.000 × g for 5 min), the protein pellet was discarded and free –SH groups were determined in the clear supernatant. An aliquot of supernatant was added in 1 M potassium phosphate buffer pH 7.4 and 10 mM 5,5'-dithiobis-(2-nitrobenzoic

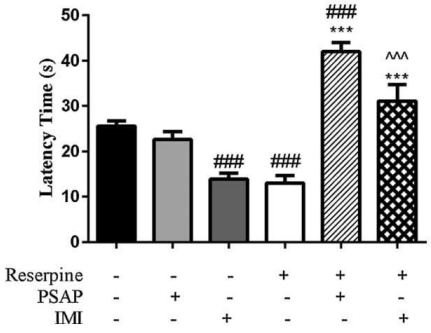


Fig. 4. Effects of PSAP (10 mg/kg, i.g.) on the response latency to thermal stimuli of reserpinised mice in the hot plate test. Each column represents the mean ± SEM of 7–10 animals for group. Statistical analysis was performed by one-way ANOVA followed by the Newman–Keuls test when appropriate. $^{###}p < 0.001$ as compared with the control group, $^{***}p < 0.001$ as compared with the reserpinised group, $^{\wedge\wedge\wedge}p < 0.001$ as compared with the reserpinised + PSAP group. *Abbreviations:* PSAP: α-(phenylselanyl) acetophenone; IMI: imipramine.

Table 1
Effect of administration of PSAP and IMI on behavior parameter in the OFT in mice.

Experimental groups	Number of crossings	Number of rearings
Vehicle (Saline + Canola oil)	106.5 ± 3.58	31.5 ± 4.28
PSAP (10 mg/kg)	96.5 ± 6.98	29.0 ± 4.40
IMI (10 mg/kg)	127.0 ± 14.19	40.0 ± 7.88
Reserpine	7.0 ± 1. 85 ###	3.50 ± 0.89 ###
Reserpine + PSAP 10 mg/kg	26.0 ± 2.29 ###*	2.0 ± 1.50 ###
Reserpine + IMI 10 mg/kg	37.0 ± 6.00 ###*	1.0 ± 0.27###

acid). The color reaction was measured at 412 nm. NPSH levels were expressed as μmol NPSH/g tissue.

2.5.1.4. Catalase (CAT) activity. CAT is an enzymatic antioxidant defense that is involved in protecting against the injurious effects of reactive species. CAT activity was assayed spectrophotometrically by the method of Beers and Sizer (1952), which involves monitoring the consumption of H_2O_2 in the cerebral cortex and hippocampus (S_1) presence at 240 nm. Enzymatic reaction was initiated by adding an aliquot of 20 μl of S_1 and the substrate (H_2O_2) to a concentration of 0.3 mM in a medium containing 50 mM phosphate buffer, pH 7.0. Thfere enzymatic activity was expressed in international units (IU) per milligram of protein (1 IU decomposes1 μmol of H_2O_2 per min at pH 7 at 25 °C).

2.5.1.5. Nitric oxide (NO) levels. NO is an unstable compound being rapidly oxidized to nitrate and nitrite after its production. The cerebral cortex and hippocampus were removed, rinsed in isotonic saline and weighed. A 10% (w/v) tissue homogenate was prepared with 0.1 M phosphate buffer (pH 7.4). A different group of animals was used because the homogenization buffer used was different from the other assays. The post nuclear fraction was obtained by centrifugation of the homogenate at 12,000 × g for 20 min at 4 °C. The metabolites were determined using nitrogen oxides (NOx = nitrite plus nitrate) analysis (adapted from (Cryan et al., 2002). The nitrate was converted into nitrite by nitrate reductase and measured using the colorimetric Griess reaction in a microplate reader at a wavelength of 462 nm. The values obtained from this assay represent the amount of nitrite and nitrate derived from NO contained in the cerebral cortex homogenates. A standard curve was taken with sodium nitrate (0–100 μM).

2.5.2. Monoamino oxidase (MAO) activity

2.5.2.1. Mitochondria preparation. The mitochondrial preparation of cerebral cortex and hippocampus was used for the MAO assay as previously described by Soto-otero and Méndez-álvarez (Soto-otero and Méndez-álvarez, 2001). The cerebral cortex and hippocampus were immediately removed and washed in ice-cold isolation medium (pH 7.4, Na_2PO_4/KH_2PO_4 isotonized with sucrose). Mitochondria from cerebral cortex were then obtained by differential centrifugation. Briefly, after removing blood vessels and membranes, cerebral cortices were manually homogenized with four volumes (w/v) of the isolation medium. Then, the homogenate was centrifuged at 900 g at 4 °C for 5 min. The supernatant was centrifuged at 12.500 g for 15 min. The mitochondria pellet was then washed once with isolation medium and re-centrifuged under the same conditions. Finally, the mitochondrial pellet was reconstituted in a buffer solution (Na_2PO_4/KH_2PO_4 isotonized with KCl, pH 7.4) and stored in aliquots.

2.5.2.2. Enzyme assay. The MAO activity was determined as described by Krajl (1965) with some modifications (Matsumoto et al., 1984). Aliquots of samples were incubated at 37 °C for 5 min in a medium containing buffer solution (Na_2PO_4/KH_2PO_4 isotonized with KCl, pH 7.4) and specific inhibitor, pargiline (selectively inhibits type MAO-B, an enzyme that catalyzes the oxidative deamination and inactivation of certain catecholamines, such as norepinephrine and dopamine, within the presynaptic nerve terminals, 250 nM), at a final volume of 700 μl. Then kynuramine dihydrobromide (final concentration 90 mM to MAO-A assay) was added to the reaction mixture as substrate. Samples were then incubated at 37 °C for 30 min. After incubation, the reaction was terminated by adding of 10% of TCA. After cooling and centrifugation at 16,000 g for 5 min, an aliquot of supernatant was added to 1 M NaOH. The fluorescence intensity was detected spectrofluorimetrically with excitation at 315 nm and emission at 380 nm. The concentration of

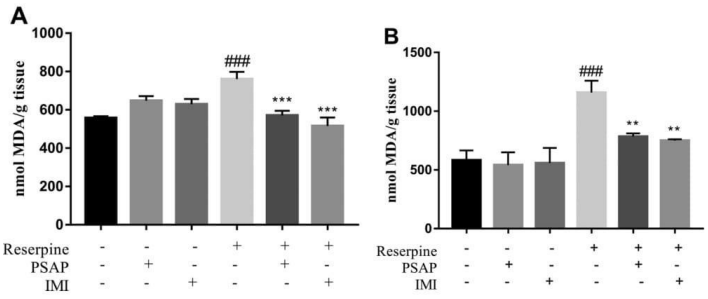


Fig. 5. Effects of PSAP (10 mg/kg, i.g.) on lipid peroxidation levels in the cerebral cortex (A) and hippocampus (B) of reserpinised mice. Each column represents the mean ± SEM of 5–6 animals for group. Statistical analysis was performed by one-way ANOVA followed by the Newman–Keuls test when appropriate. $^{###}p < 0.001$ as compared with the control group, $^{**}p < 0.01$ and $^{***}p < 0.001$ as compared with the reserpinised group. *Abbreviations:* PSAP: α-(phenylselanyl) acetophenone; IMI: imipramine; MDA: malondialdehyde.

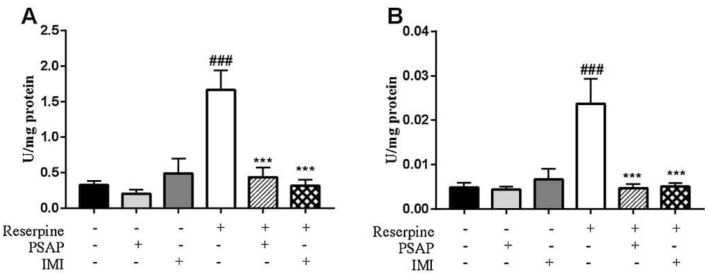


Fig. 6. Effects of PSAP (10 mg/kg, i.g.) on CAT activity in the cerebral cortex (A) and hippocampus (B) of reserpinised mice. The CAT activity is expressed as U/mg protein. Each column represents the mean ± SEM of 5–6 animals for group. Statistical analysis was performed by one-way ANOVA followed by the Newman–Keuls test when appropriate. $^{###}p < 0.001$ as compared with the control group, $^{***}p < 0.001$ as compared with the reserpinised group. *Abbreviations:* PSAP: α-(phenylselanyl) acetophenone; IMI: imipramine; CAT: catalase.

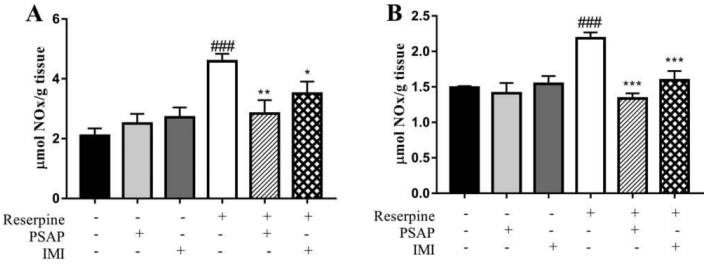


Fig. 7. Effects of PSAP (10 mg/kg, i.g.) on the nitrite and nitrate levels in the cerebral cortex (A) and hippocampus (B) of mice administered with reserpine. The results are expressed in µmol NOx/g of tissue. Each column represents the mean ± SEM of 6 animals for group. Statistical analysis was performed by one-way ANOVA followed by the Newman–Keuls test when appropriate. $^{###}p < 0.001$ as compared with the control group, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ as compared with the reserpinised group. *Abbreviations:* PSAP: α-(phenylselanyl) acetophenone; IMI: imipramine.

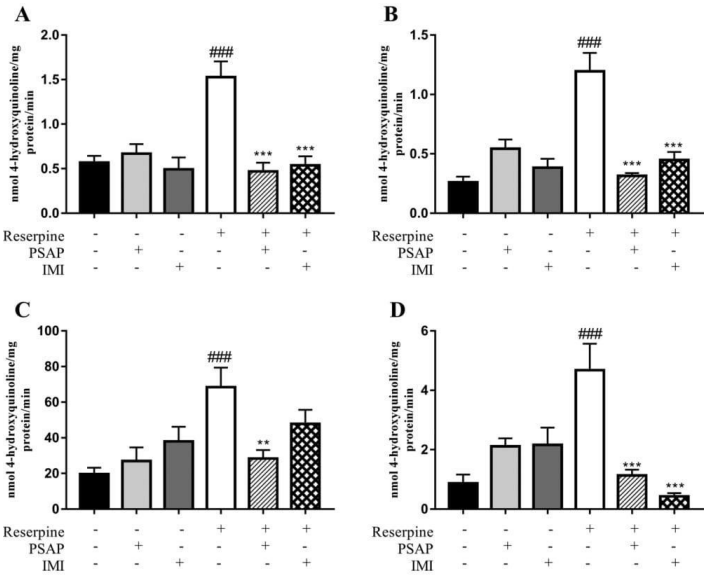


Fig. 8. Effects of PSAP (10 mg/kg, i.g.) on the MAO-A activity in the cerebral cortex (A) and hippocampus (B) and MAO-T activity in the cerebral cortex (C) and hippocampus (D) of reserpinised mice. The activity of MAO is expressed as ηmol 4-hydroxyquinoline/mg protein/min. Each column represents the mean ± SEM of 6–8 animals for group. Statistical analysis was performed by one-way ANOVA followed by the Newman–Keuls test when appropriate. $^{###}p < 0.001$ as compared with the control group, $^{**}p < 0.01$ and $^{***}p < 0.001$ as compared with the reserpinised group. *Abbreviations:* PSAP: α-(phenylselanyl) acetophenone; IMI: imipramine; MAO: monoamine oxidase.

4-hydroxyquinoline was estimated from a corresponding standard fluorescence curve of 4-hydroxyquinoline. MAO-A and MAO-T activities were expressed as ηmol of 4-hydroxyquinoline formed/mg protein/min.

2.5.3. Protein determination

The protein concentration was measured by the method by Bradford, 1976 using bovine serum albumin as standard.

2.6. Molecular docking

In view of extending our knowledge about PSAP mechanism of action, molecular docking tool was utilized. To reach this goal, PSAP was drew using ChemDraw and their 3D geometry optimized using the

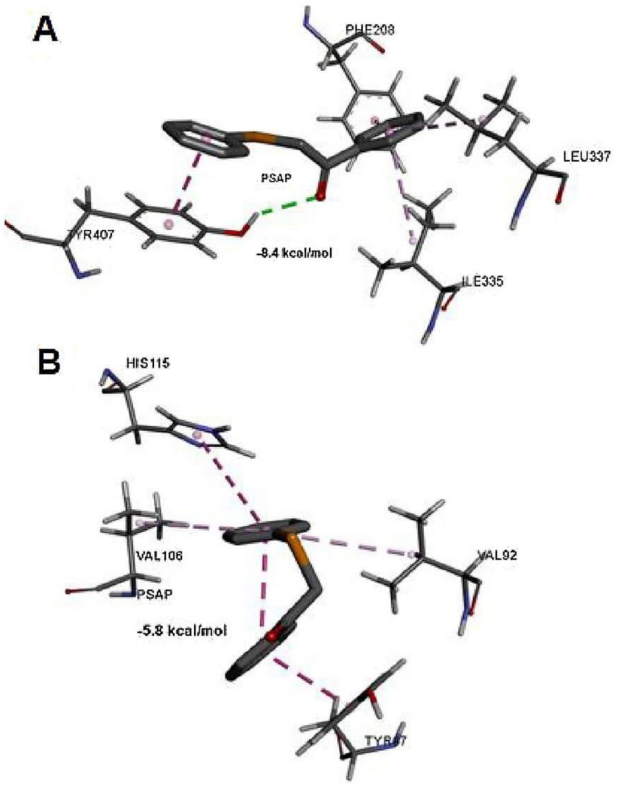


Fig. 9. Binding modes of PSAP in MAO-A (A) and with MAO-B (B).

software Avogadro 0.9.4 following the MMFF94 method (Hanwell et al., 2012). The molecular docking simulation was performed using the software Autodock Vina (Trott and Olson, 2009). We used crystallographic structures of different MAO isoforms from Protein Data Bank (PDB) (http://www.pdb.org/): MAO-A: 2BXR and MAO-B: 2BYB. The CHIMERA 1.5.3 software was used to remove ligands, ions, and water and to minimize the structure of proteins, using the Gasteiger charges with 500 steps of minimization in all molecular targets (Pettersen et al., 2004). The interactions between the tested molecule and protein were determined using Discovery Studio Visualizer.

After obtaining the ligands and enzymes, their structures were improved, using the Auto Dock Tools 1.5.4 program, in which all the rotatable bonds of ligands were allowed to rotate freely, and the receptors were considered rigid (Morris et al., 2009). For docking studies, we used the Auto Dock Vina 1.1.1. utilizing a grid box centered on the active site of MAO A and B with high resolution, allowing the program to search for additional places of probable interactions.

2.7. Statistical analysis

All experimental results are given as a mean ± standard error of the mean (SEM). Behavioural and neurochemical comparisons between experimental and control groups were performed by one-way analysis of variance (ANOVA). When ANOVA revealed a significant, the Newman-Keuls 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 Graph Pad Prism version 7.0 for Windows, Graph Pad Software (San Diego, CA, USA).

3. Results

3.1. The effects of PSAP on pain and depression-like behavior induced by reserpine in mice

3.1.1. Effects of PSAP on reserpine-induced depression-like behavior

In order to investigate if PSAP has effect in the depressive-like behavior induced by reserpine in mice, the animals were tested in the mFST. The latency time to the first episode of immobility was drastically reduced by reserpine and both PSAP and IMI treatments partially prevented this effect [$F_(5,34_) = 15.41$ ($p < 0.001$)] (Fig. 3A). In the same way, PSAP and IMI also prevented the reduction of swimming time induced by reserpine [$F_(5,39_) = 10.4$ ($p < 0.001$)] (Fig. 3B). In relation to immobility time of mice, reserpine induced an increase in this parameter and both PSAP and IMI blocked this effect [$F_(5,45_) = 12.2$ ($p < 0.001$)] (Fig. 3C). Additionally, reserpine induced a reduction in the climbing time and only IMI partially prevented this effect (Fig. 4D).

3.1.2. Effects of PSAP on reserpine-induced hyperalgesia

With the purpose of evaluating the PSAP effects on thermal hyperalgesia in mice, the hot plate test was carried out. The animals that received reserpine had a decrease in the latency time to antinociceptive response and both PSAP or IMI prevented this effect of reserpine [$F_{(5,40)} = 27.5$ ($p < 0.001$)] (Fig. 4). In addition, the reserpinised animals that received both IMI or PSAP presented a higher latency time to antinociceptive response when compared with control group.

3.1.3. Effects of PSAP on locomotor activity in reserpine-treated mice

Reserpine induced a decrease in the number of crossings and rearings of mice in the open-field test, as observed in Table 1. The treatment of mice with PSAP or IMI partially prevented the reduction of crossings [$F_{(5, 50)} = 52,92$ ($p < 0.001$)] induced by reserpine, but did not prevent the reduction in the number of rearings [$F_{(5, 50)} = 25$ ($p < 0.001$)].

The effect of treatment with PSAP and IMI on behavior of mice in the open-field test. Data presented are mean values ± S.E.M of 10–12 animals for group. Results are expressed as mean ± SEM. ($^{###}$) $p < 0.001$ when compared with the control group. (*) $p < 0.05$ when compared with the reserpine group. *Abreviations*: PSAP: α-(phenylalanyl) acetophenone; IMI: imipramine.

3.2. Effect of PSAP on oxidative stress parameters

MDA levels were significantly increased in the cerebral cortex and hippocampus of reserpinised mice as compared to control group (Fig. 5). Treatment with PSAP or IMI blocked this effect of reserpine in both cerebral cortex [$F_{(}5,27_{)} = 8.8$ ($p < 0.001$)] (Fig. 5A) and hippocampus [$F_{(}5,32_{)} = 7.04$ ($p < 0.001$)] (Fig. 5B).

The activity of CAT was significantly increased by reserpine in the cortex and hippocampus of mice as compared with control group (Fig. 6A and 6B). PSAP or IMI treatments prevented the increase of CAT activity in both cerebral cortex [$F_{(}5,31_{)} = 10.7$ ($p < 0.001$)] and hippocampus [$F_{(}5,49_{)} = 9.26$ ($p < 0.001$)].

The levels of NO were also increased by reserpine in both cerebral cortex [$F_{(}5,30_{)} = 6.8$ ($p < 0.001$)] (Fig. 7A) and hippocampus [$F_{(}5,30_{)} = 9.07$ ($p < 0.001$)] (Fig. 7B) when compared with the control group. PSAP or IMI treatments prevented this effect of reserpine. The one-way ANOVA for NPSH and RS levels (data not shown) revealed no significant differences among groups.

3.3. Effects of PSAP in monoamine oxidase (MAO) activity

As observed in Fig. 8, reserpine induced a great increase in the activities of MAO-A and MAO-T, in both cerebral cortex and hippocampus. The treatment of mice with PSAP or IMI prevented the increase of MAO-A activity induced by reserpine in cerebral cortex [$F_{(}5,40_{)} = 11.6$ ($p < 0.001$)] (Fig. 8A) and hippocampus [$F_{(}5,34_{)} = 15.2$ ($p < 0.001$)] (Fig. 8B). In addition, PSAP also prevented the increase of MAO-T activity in cerebral cortex [$F_{(}5,31_{)} = 6.53$ ($p < 0.001$)] and hippocampus [$F_{(}5,38_{)} = 11.2$ ($p < 0.001$)]. Imipramine only prevented the increase of MAO-T activity in the hippocampus.

3.4. Molecular docking

The molecular docking studies revealed the affinity of PSAP in binding MAO-A, which is described by the high docking score of -8.4 kcal/mol. As demonstrated in Fig. 9, PSAP is able to interact with some important active site residues for MAO-A inhibition: Tyr407, Phe208, Leu 337, Ile335. In other way, when the MAO-B was tested, docking studies showed a lower docking PSAP score of -5.8 kcal/mol, which is not too representative. Besides, PSAP made protein interactions mainly with Val106, Val92, His115 and Tyr 97. Although these residues were not found to be crucial for MAO-B inhibition in literature, suggesting PSAP is a MAO-A specific inhibitor.

4. Discussion

Reserpine is known to produce symptoms of major depression in both humans and rodents (Akiskal and Mckinney, 2016). In this study, animals that received reserpine demonstrated an increase in immobility time, as well as a decrease in swimming time and latency time to the first episode of immobility in mFST, features of a depressive-like behavior. In addition, the reserpinised mice remained for less time in the hot plate before a nociceptive response, indicating a hyperalgesic effect of reserpine. Interestingly, the treatment of mice with PSAP prevented both the depressive-like behavior and hyperalgesia induced by reserpine, similarly to the positive control imipramine. In addition, reserpinised mice had an increase in oxidative parameters in cerebral cortex and hippocampus, such as lipid peroxidation, NO levels and CAT activity and PSAP prevented all these alterations. PSAP also prevented the increase of MAO-T and MAO-A activities induced by reserpine and analysis of molecular docking indicates that PSAP could be an inhibitor of MAO-A. For this study, IMI was used as a positive control because it is a tricyclic antidepressant used to treat neurogenic pain, rheumatologic pain, nocturnal enuresis and depression.

The FST is a test broadly employed as a behavioral instrument for screening of antidepressant drugs (Cryan et al., 2002). Nevertheless, in the current study we opted for a modified version of FST (mFST), because it allows differentiating the mechanism of action elicited by antidepressant-like drugs. The serotonin (5-HT)-related compounds such as SSRIs, decrease the immobility and increase the swimming behavior, but do not modify the climbing behavior of mice, that is a characteristic of noradrenergic agents, which decrease the immobility and in parallel increase the climbing behavior (Slattery and Cryan, 2012). Our results revealed that PSAP reduced the immobility time and increased the swimming time without altering the climbing behavior in reserpinised mice, which indicate that PSAP has antidepressant-like effect and this effect is probably dependent of a modulation of serotoninergic system.

The effect of PSAP in the depressive-like behavior induced by reserpine was also observed in the open-field test, which evaluates the spontaneous locomotor activity of mice. Reserpinised mice showed a reduction in the number of crossings and rearings and the treatment with PSAP partially prevented the reduction of crossings, but had no effect in number of rearings. Similar effects were found after administration of imipramine. Accordingly, some studies have associated the decreasing in the number of spontaneous crossings and rearings in the open-field test with a depressive phenotype, after reserpine administration (Gao et al., 2016; Khadrawy et al., 2017).

(Oliveira et al., 2016), (Arora et al., 2011) and (Arora and Chopra, 2013) have been reporting the relationship between reserpine injection and increased pain sensitivity, which in our study led the mice stay less on the hot plate before a nociceptive response when compared to the control animals, characterizing an hyperalgesic effect. Administration of PSAP was performed for two days after treatment with reserpine and this compound was effective in decreasing thermal hyperalgesia in the hot plate test. This effect suggests central actions of PSAP, once the hot plate test produces a non-inflammatory nociception response and it is a good model to investigate the central effect of analgesic drugs (Oliveira et al., 2009). The present results further reinforce the previously demonstrated antinociceptive effect of PSAP, attributed to a modulation of noradrenergic and dopaminergic systems (Sousa et al., 2017).

Reserpine is a monoamine depletory substance, that exerts a blockade on the vesicular monoamine transporter for neuronal transmission or storage, promoting dopamine-autoxidation and oxidative catabolism by MAO (Lohr et al., 2003). This mechanism leads to the formation of dopamine-quinones and hydrogen peroxide, related to the oxidative stress process (Bilska and Dubiel, 2007). Many brain regions, especially hippocampus and cerebral cortex, due to their vulnerability to metabolic insults, act in concert to mediate the symptoms of

depression accompanied with pain (Kumar et al., 2009). Both patients suffering from depression and pain already demonstrated elevated levels of oxidative biomarkers such as higher serum levels of MDA, together with serum superoxide dismutase (SOD) reduction (Bagis et al., 2005). NO is an important signaling molecule for neurotransmission, but over-stimulated by nitrosative stress, NO can react with RS to form highly toxic reactive nitrogen species (Szabó et al., 2007) and the first line antioxidant defense system includes enzymes, such as CAT, which are involved in superoxide detoxification in neuronal metabolism. Taking this into account, we investigated the antioxidant activity of PSAP in the model of reserpine-induced pain-depression dyad. The results showed that the injection of reserpine increased the levels of MDA, a reliable marker of oxidative stress, NO and also CAT activity, although RS and NPSH were not altered. The administration of PSAP was effective in restoring all these parameters to the baseline levels in both cerebral cortex and hippocampus further confirming the already reported antioxidant property of PSAP (Gerzson et al., 2012). Considering these facts, it can be suggested that the antioxidant effect of PSAP has a role in its antidepressant-like and antinociceptive action.

We also observed in this study that reserpine induced a great increase in the MAO-T and MAO-A activities and this effect was prevented by PSAP. The inhibition of MAO activity could prevent the breakdown of monoamine neurotransmitters and contribute to their increase in the synaptic cleft (Medvedev et al., 1996; Tsugeno et al., 1995; Velasquez et al., 2017). Although the results demonstrated that PSAP prevented the increase in MAO-T and MAO-A activities, we cannot affirm that this compound increases extracellular 5-HT concentration.

MAO inhibitors are effective antidepressants in human populations and rodent models, sometimes relieving depression when other treatments have failed (Pesarico et al., 2015). To have an indication if PSAP is an inhibitor of MAO activity or only prevented its increase by indirect mechanisms we performed molecular docking analysis. Our results demonstrated that PSAP could be a MAO-A specific inhibitor. This effect can be attributed to interaction with the residue Ile335 which is determinant for the substrate and consequently inhibitor specificities as related by Colibus et al., 2005; Geha et al., 2001. Furthermore, the additional hydrogen bond with the residue Tyr407 is responsible to set the PSAP conformation between the “aromatic cage” of active site, an important feature for the enzyme functionality. In addition, the residues located in the inner portion of MAO-A as Leu337 and Phe208 in the favorable binding mode of PSAP may suggest noncompetitive mechanism of action (Coelho Cerqueira et al., 2011).

Considering PSAP as a reversible inhibitor of MAO-A, the fact that this compound *per se* did not inhibited the MAO-A activity could be attributed to the time-consuming process to mitochondrial preparation of cerebral cortex and hippocampus. The multiple washes and centrifugations could wash out PSAP and its inhibitory effect on MAO-A. However more studies are needed to evaluate the effect of PSAP on MAO activity.

Therefore, the findings of the present study support PSAP as an interesting approach in pain-depression comorbidity treatment, especially due the poor availability of alternative treatments for this syndrome as well as the puzzled mechanisms implicated in this condition. The results of the present study raised the possibility that PSAP was effective against pain-depression dyad induced by reserpine due its antioxidant property and inhibitory effect on MAO-A activity. As previously described, this compound already showed well-elicited antinociceptive activity and antidepressant-like effect due a modulation of monoaminergic system, together with the absence of toxicity. In addition, other studies are essential to better explain the mechanisms implicated in the antinociceptive and antidepressant effects demonstrated by PSAP to contribute in the development of more effective treatments to the pain-depression comorbidity.

Conflict of interest

All authors declare that they have no conflicts of interest.

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Artigo 2

Os resultados que fazem parte desta tese de doutorado estão apresentados sob a forma de artigo, o qual se encontra assim organizado. Os itens Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas encontram-se no próprio artigo. O artigo foi publicado na revista **Neurochemistry International**

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α-(phenylselanyl) acetophenone abolishes acute restraint stress induced-comorbid pain, depression and anxiety-related behaviors in mice

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ABSTRACT

α-(phenylselanyl) acetophenone (PSAP) is an organoselenium compound that presents antidepressive-like and antinociceptive effect in animal models. In this study, we evaluate the potential pharmacological effects of PSAP on acute stress restriction (ARS)-induced depressive and anxiogenic-like behavior associated with hyperalgesia and mechanical allodynia in male adult Swiss mice (25–35 g). The ARS is an unavoidable stress situation that was applied for a period of 4 h using an individual rodent restraint. Ten min after ARS, the animals were treated with the PSAP (10 mg/kg, intragastrically [IG]) or imipramine (IMI, 10 mg/kg, IG) and after thirty min they were submitted to the behavioral observations in the forced swimming test (FST), sucrose preference test, hot plate, Von-Frey Hair filaments (VHF), marble burying test and elevated plus maze (EPM). It was also evaluated the levels of plasma corticosterone and some parameters of oxidative stress in cortex and hippocampus. The ARS caused a decrease in the latency to the first episode of immobility in the FST, the sucrose preference, latency time in the hot plate test, frequency of paw withdrawal in the VFH and time spent in the open arms of the EPM. ARS also increased the immobility time of mice in the FST, the number of marbles buried and enclosed entries number in the EPM. PSAP or IMI reversed all these parameters. ARS increased the levels of lipid peroxidation, reactive species (RS) and nitrite and nitrate (NO_x) levels in cortex and hippocampus and the treatment with PSAP or IMI also reduced these parameters, demonstrating its effect on the reduction of oxidative stress. In addition, ARS also caused an increase in plasma corticosterone levels and PSAP treatment reversed this effect. Hence, PSAP exhibited antidepressant-like, anxiolytic-like, anti-hyperalgesic and anti-allodynic effect in the ARS model and could be a promising molecule to treat these comorbidities.

1. Introduction

Depression is a neuropsychiatric disorder that affects 20% of the population, presenting a high rate of morbidity and mortality. According to the World Health Organization (WHO, 2017), it is estimated that 300 million people suffer from depression worldwide. According to the WHO International Classification of Diseases (2013) and the Diagnostic and Statistical Manual of Mental Disorders - DSM-V-TR (2013), the main symptoms of depression are loss of interest or pleasure for almost all activities and depressed mood most of the time. These symptoms should be present almost every day for a period of more than two weeks.

The relationship between onset of a major depressive episode, pain, anxiety and prior stressful life events has been extensively reported (Hammen et al., 2009; Mazure, 1998). It is believed that depression could be related to the neurotoxic effects of the deregulation of the negative feedback of glucocorticoids induced by stress and this effect could be partly explained by the oxidative and inflammatory hypothesis of depression, which postulates that oxidative and nitrosative stresses contribute to neurodegenerative processes in depression (Anisman et al., 2002; Lang and Borgwardt, 2013; Maes et al., 2011, 2009). Abnormalities of the hypothalamic-pituitary-adrenal (HPA) axis have been

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shown to play a critical role in development of depressive symptoms, persistence of symptoms, and recurrence of depression (Morris et al., 2012; Plotsky et al., 1998).

In this same sense, hyperalgesia (increased sensitivity to painful stimuli) or allodynia (pain triggered by innocuous stimuli) are also correlated with stressful life (Bardin et al., 2009). In pain conditions, there is an increase in neural activity due to neuronal excitability, with more utilization of metabolic substrates and increased production of reactive oxygen and nitrogen species (RS) (Scheid et al., 2013).

The prevalence of pain among people with depression can be as high as 65% (Bair et al., 2003), and the concomitant presence of symptoms of depression and pain is associated with worse health status for patients compared to the presence of one of these conditions alone (Bair et al., 2008; Moussavi and Chatterji, 2007). Our understanding of the neurobiological mechanisms of depression is still poor, and the therapeutic effects of antidepressants are limited. Most of drugs available to depression treatment have in common the ability to increase the synaptic availability of one or more neurotransmitters through action on specific receptors, transporters, and enzymes (Jeon and Kim, 2016). These drugs are being widely used to treat not only depression but also chronic pain and anxiety. The disadvantage of such drugs is that they take a long time to obtain treatment effects, and the overall remission rate is low. In this way, synthetic organic selenium compounds have received considerable attention due to several pharmacological properties that they have presented, such as antidepressant-like and antinociceptive actions (Casaril et al., 2017; Da Rocha et al., 2013; Jesse et al., 2009; Marcondes Sari et al., 2016; Nogueira et al., 2004; Pinto Brod et al., 2016).

Our research group has already demonstrated that α-(phenylselanyl) acetophenone (PSAP) has antidepressant-like properties in mice dependent of an interaction with the serotonergic system, and it may be of interest as a therapeutic agent for the treatment of depressive disorders (Gerzson et al., 2012). It also has been shown the antinociceptive and anti-edematogenic activities of PSAP (Sousa et al., 2017a). In addition, PSAP not present acute or chronic toxicity (Casaril et al., 2015; Gerzson et al., 2012). As we already know that PSAP has an antidepressant-like and anti-inflammatory effect, it is worth correlating these findings with stress. As stated earlier, stress is closely correlated with episodes of pain, depression, and anxiety. Therefore, the objective of this study was to evaluate the possible effects of PSAP on depressive phenotype correlated with pain and anxiety, as well as brain oxidative parameters, in mice submitted to acute restraint stress.

2. Materials and methods

2.1. Animals

The experiments were conducted using male adult Swiss mice (25–35 g) from our own breeding colony. The animals were kept in a separate animal room, on a 12 h light/dark cycle with lights on at 7:00 a.m., at room temperature (22 ± 1 °C) with free access to water and food. All experimental procedures were conducted in accordance with the guidelines of the Committee of Ethics in Research (number 8328–2017). All efforts were made to minimize animal suffering and the number of animals used in the experiments.

2.2. Drugs and treatment

The α- (phenylselanyl) acetophenone (PSAP) (Fig. 1) was prepared and characterized in the Laboratory of Clean Organic Synthesis (LASOL) according to the method previously described (Victoria et al., 2009). PSAP was dissolved in canola oil and administered to mice intragastrically (IG) at dose of 10 mg/kg in a volume of 10 ml/kg. This dose of PSAP was chosen according to previous studies conducted by our research group (Gerzson et al., 2012; Sousa et al., 2017b). The standard drug used was imipramine (IMI) at the dose of 10 mg/kg (IG), in a volume of 10 ml/kg, and it was purchased from a commercial pharmacy. IMI is a tricyclic antidepressant used to treat neurogenic pain, rheumatologic pain, nocturnal enuresis and depression. All other chemicals were obtained at the highest available commercial grade.

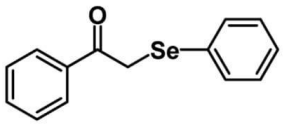

Fig. 1. Chemical structure of α-(phenylselanyl) acetophenone (PSAP).

All the behavioral tests were assessed in independent groups of mice 30 min after an acute administration of PSAP or IMI. In the acute restraint stress (ARS) protocol, PSAP (10 mg/kg, IG) was administered 10 min after the ARS. The animals were assigned to the following groups: (a) unstressed/vehicle, (b) unstressed/PSAP, (c) unstressed/IMI, (d) ARS/vehicle, (e) ARS/PSAP and (f) ARS/IMI. A diagram of all experimental schedules is given in Fig. 2.

2.3. Acute restraint stress procedure (ARS)

ARS procedure was performed by a method described previously (Kumar et al., 2009; Zafir et al., 2009). The immobilization was applied for a period of 4 h using an individual rodent restraint device made of Plexiglas fenestrate. This restrained all physical movements without causing pain. The animals were deprived of food and water during the entire period of exposure to stress. The choice of this method was based on the literature, considering that several studies use the acute stress of restriction (4 h) to reproduce depressive-like, anxiogenic-like and pain behavior (Colín-González et al., 2015; Pesarico et al., 2015; Spiers et al., 2016). The unstressed-groups were treated with vehicle or PSAP or IMI and were kept without food and water during the entire period of exposure to stress. Ten min after ARS, the animals were treated with the PSAP or IMI and after thirty min, submitted to the behavioral observations, and then to the biochemistry analysis (with a different group of animals).

2.4. Behavioral tests

2.4.1. Forced swimming test (FST)

Mice were individually forced to swimming in an open cylindrical container (diameter 10 cm, height 25 cm), containing 19 cm of water (depth) at 25 ± 1 °C; the animals were observed for 6 min, the first two minutes were evaluated the latency time for the first episode of immobility (in seconds [s]), and the other 4 min the time of immobility (s) was evaluated by the animals (Brocardo et al., 2008; Freitas et al., 2010). 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 latency and increase of immobility time is indicative of a depressant-like behavior (Porsolt et al., 1977). The animals are divided in number of 7 animals per group.

2.4.2. Sucrose preference test

Anhedonia was measured, with a different group of animals (number of 7 animals per group), by preference for a sucrose solution over water, using a two-bottle free choice method as previously described (Shi et al., 2010; Yue et al., 2017). Briefly, each mouse was presented simultaneously with two bottles (40 ml), one with 2% sucrose solution and the other containing tap water. Mice were then given a free choice between either tap water or 2% sucrose in tap water solution for 24 h. Twenty-four hours later, were measured through a beaker, the amount of water or sucrose consumed in the mice. Sucrose preference was calculated as sucrose consumption/(sucrose

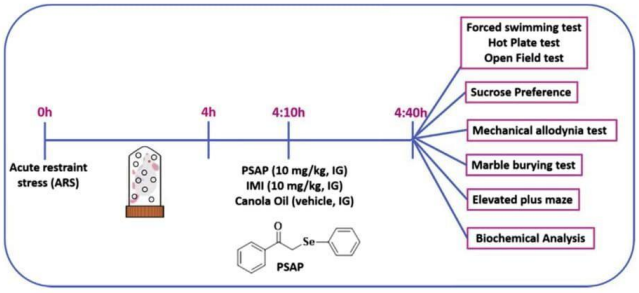

Fig. 2. Schematic protocol of the induction of the acute restraint stress. Abbreviations: PSAP: α-(phenylselanyl) acetophenone; IG: Intragastrically.

consumption + water consumption) × 100%.

2.4.3. Marble burying test

The marble burying test was adapted from Kedia and Chattarji (2014). A cage was filled approximately 5 cm deep with husk bedding material that was evenly distributed into a flat surface across the whole cage. Eight glass marbles (1.4 cm in diameter, plain dark glass) were then spaced evenly in a 4 × 5 grid on the surface of the bedding. During the testing phase each mouse was placed in the cage and allowed to explore it for 30 min. At the end of the test, mice were removed from the cage and the number of marbles buried was counted. The more marbles are buried, greater the anxiogenic behavior of mice. This test was performed with a different group of animals (number of 5 animals per group).

2.4.4. Elevated plus maze test (EPM)

The elevated plus maze test (EPM) was used to evaluate anxiety-like behavior and performed in a black Plexiglas apparatus with four arms, two open and two closed, set in cross from a neutral central square elevated 40 cm above the floor. Five-minute test sessions were performed, the time spent in the open arms and closed arms entries was determined, as previously reported (Busquets-Garcia et al., 2011). Number of 5 animals per group.

2.4.5. Hot plate test (thermal hyperalgesia)

For the evaluation of the development of thermal hyperalgesia, the animals were submitted to the hot plate test. The mice (number of 7 animals per group) were placed in an acrylic cylinder (20 cm in diameter) on the hot plate apparatus, maintained at 52 ± 1 °C. The time (s) between the placement on the heated surface and licking of their hindpaws or jumping was recorded as the response latency. A 45 s cut-off was used to prevent tissue damage (Brüning et al., 2015; Savegnago et al., 2007).

2.4.6. Mechanical allodynia test

The mechanical allodynia was measured as described before (Bortalanza et al., 2002; De Sousa et al., 2014). The response frequency was measured after ten applications (duration of 1–2 s each) of 1.0 g Von-Frey Hair (VFH, Stoelting, Chicago, IL). To this end, mice (number of 8 animals per group) were further habituated in individual clear Plexiglas boxes (9 × 7 × 11 cm) on an elevated wire mesh platform to allow access to the ventral surface of the hind paws. To this end, immediately after ARS, the animals received either PSAP or IMI, and were then placed in the Von Frey box for 30 min, then, the number of withdrawals with the right paw was considered.

2.4.7. Locomotor activity

The open field test (OFT) was made of plywood and surrounded by walls 30 cm in height. The floor of the open field, 45 cm in length and 45 cm in width, was divided by masking tape markers into 9 squares (3 rows of 3). Each animal (number of 7 animals per group) was placed individually at the center of the apparatus and observed for 5 min to record the number of segments crossed with the four paws and the number of time rearing on the hindlimbs (Walsh and Cummins, 1976).

2.5. Biochemical analysis

To investigate the effect of PSAP on oxidative stress, malondialdehyde (MDA), reactive species (RS) and nitric oxide (NO) levels were determined in total cerebral cortex and hippocampus of mice. The cortices and hippocampus of different animals were removed, weighed and homogenized in 50 mM Tris–HCl, pH 7.4 (1/4, weight/volume), and centrifuged at 2.400 g at 4 °C for 15 min. The low-speed supernatant fraction (S_1) was collected and used for oxidative stress analyses.

2.5.1. Malondialdehyde (MDA) levels

MDA is an end product of the lipid peroxidation. MDA reacts with thiobarbituric acid (TBA) to generate a colored product that can be optically measured at 532 nm (Ohkawa et al., 1979). An aliquot of the cerebral cortex and hippocampus (S_1) was incubated with 8.1% SDS, 0.8% TBA and acetic acid/HCl (pH 3.4) at 95 °C during 60 min. The absorbance of the samples was measured at 532 nm, and the results were expressed as nmol TBARS/g of tissue.

2.5.2. Reactive species (RS) levels

Quantification of RS levels of cerebral cortex and hippocampus of mice was performed according to (Loetchutinat et al., 2005). Briefly, an aliquot of S_1 was 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 RS. The DCF fluorescence intensity emission was recorded at 520 nm (with 480 nm excitation) and RS levels were expressed as arbitrary units (AU) of fluorescence.

2.5.3. Nitrite and nitrate (NO_x) levels

NO_x is an unstable compound being rapidly oxidized to nitrate and nitrite after its production. The different group of animals, the cerebral cortex and hippocampus were removed, rinsed in isotonic saline and weighed. A 10% (w/v) tissue homogenate was prepared with 0.1 M phosphate buffer (pH 7.4). A different group of animals was used because the homogenization buffer used was different from the other assays. The post nuclear fraction was obtained by centrifugation of the homogenate at 12000 × g for 20 min at 4 °C. The metabolites were determined using nitrogen oxides (NOx = nitrite plus nitrate) analysis (adapted from (Slattery and Cryan, 2012). The nitrate was converted into nitrite by nitrate reductase and measured using the colorimetric Griess reaction in a microplate reader at a wavelength of 462 nm. The values obtained from this assay represent the amount of nitrite and

nitrate derived from NO contained in the cerebral cortex homogenates. A standard curve was taken with sodium nitrate (0–100 μM).

2.5.4. Corticosterone level in plasma

Determination of plasma corticosterone levels was performed according to Zenker and Bernestein (1957) with a different group of animals. For the collection of blood plasma, heparin was used as anticoagulant, the mice were euthanized by isoflurane inhalation and soon after cardiac puncture was performed. For plasma separation, the blood was centrifuged at 4.000 rotation per minute (RPM) for 10 min at 4 °C. 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 2500 rpm) and incubated at room temperature for 2 h. Fluorescence intensity emission, corresponding to plasma corticosterone levels, was recorded at Ex: 247, EM: 540 and corticosterone levels were expressed as ng/ml.

2.6. Statistical analysis

All experimental results are given as a mean ± standard error of the mean (SEM). Comparisons between experimental and control groups were performed by one-way analysis of variance (ANOVA). When ANOVA revealed a significant effect, the Newman-Keuls 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 Graph Pad Prism version 7.0 for Windows, Graph Pad Software (San Diego, CA, USA).

3. Results

3.1. Effect of acute treatment with PSAP on the immobility time of mice in the FST

Fig. 3 shows the results of FST performed shortly after 4 h of ARS and 30 min of treatment with PSAP or IMI. Observing Fig. 3A, it is possible to verify that the mice submitted to ARS had a decrease in the latency to the first episode of immobility when compared with the non-stressed control group. Treatment with PSAP reverted this parameter, increasing the immobility time of animals that suffered ARS [$F_{(5,42)} = 16.7$, $p < 0.001$]. The same was observed with the standard drug, IMI. In addition, PSAP *per se* increased the latency to the first episode of immobility, when compared to the non-stressed control group. The results depicted in Fig. 3B show that ARS increased the immobility time of mice and the treatment with PSAP or IMI reversed this effect [$F_{(5,42)} = 7.77$, $p < 0.001$].

3.2. Sucrose preference and marble burying tests

Through the sucrose preference test, we can see in Fig. 4A that animals that suffered 4 h of restriction stress had a decrease in preference for the bottle containing 2% of sucrose when compared to the control group. The treatment with PSAP or IMI had the capacity to revert these parameters [$F_{(5,41)} = 33.4$, $p < 0.001$], increasing the consumption of sucrose of the animals suffering from ARS.

After 4 h of immobilization stress and 30 min of treatment with PSAP or IMI (Fig. 4B), control and stressed mice were tested in the marble burying apparatus. At the end of the 30-min test period, the total number of marbles buried by the ARS mice was significantly higher than that buried by the non-stressed control mice. The animals treated with PSAP buried less marbles when compared to the ARS group [$F_{(5,30)} = 8.41$, $p < 0.001$]. Animals that received IMI also reduced the number of buried marbles when compared with the ARS group, being similar to the non-stressed control group ($p < 0.001$).

3.3. Elevated plus maze test (EPM)

The animals that suffered the ARS reduced the time spent in the open arms, as well as increased the number of entries in the closed arms in the EPM (Fig. 5). Treatment with the PSAP or IMI (positive control) reversed these effects, leading to an increase in the time spent in the open arms [$F_{(5,30)} = 11.9$, $p < 0.001$] and a decrease in the number of entries in the closed arms [$F_{(5,30)} = 7.74$, $p < 0.001$] in the EPM.

3.4. Hot plate test (thermal hyperalgesia) and mechanical allodynia test

In the hot plate test, ARS significantly reduced the latency time to nociceptive response when compared with unstressed animals. PSAP [$F_{(5,42)} = 5.56$, $p < 0.01$] blocked the effect of ARS (Fig. 6A). Similar results were observed with IMI.

Response frequency of VFH stimulation was significantly increased by ARS in right paw when compared with the control group (unstressed group). In addition, PSAP acute treatment decrease the response frequency of VFH stimulation (Fig. 6B), when compared with ARS group [$F_{(5,47)} = 26.2$, $p < 0.001$]. Administration of IMI at a dose of 10 mg/kg, *per se*, 30 min before mechanical allodynia test, was effective in reducing pain sensitivity in the right paw. In addition, IMI administration reduced response frequency of VFH stimulation significantly different from the ARS and ARS + PSAP groups.

3.5. Locomotor activity

To avoid the possibility that the increase of latency and immobility time of mice in the FST could be due to locomotor impairment, the number of crossings and rearings was assessed in the OFT. As depicted in Table 1, the treatments did not produce significant differences in rearing [$F_{(5,42)} = 1.95$, $p = 0.11$] and crossings [$F_{(5,42)} = 2.08$, $p = 0.09$].

3.6. Biochemical analysis

3.6.1. Malondialdehyde (MDA) levels

The results depicted in Fig. 7 illustrate that ARS significantly

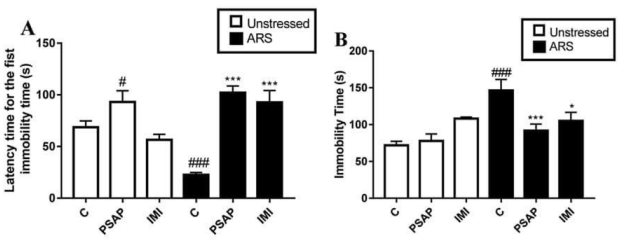

Fig. 3. Effects of PSAP or IMI (10 mg/kg, IG) on active behaviors in forced swimming test in ARS mice. (A) Latency time (s); (B) Immobility time (s). Each column represents the mean ± SEM. of 7 animals for group. Statistical analysis was performed by one-way ANOVA followed by the Newman–Keuls test when appropriate. #$p < 0.05$, ###$p < 0.001$ as compared with the control group, **$p < 0.01$ and ***$p < 0.001$ as compared with the ARS group. Abbreviations: PSAP: α-(phenylselanyl) acetophenone; IMI: imipramine.

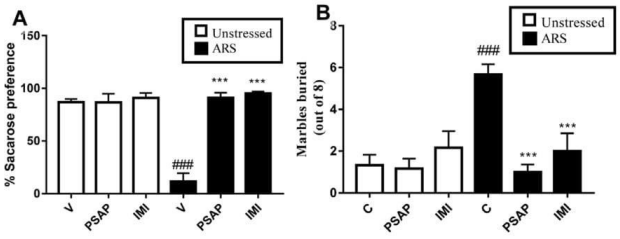

Fig. 4. Effects of PSAP or IMI (10 mg/kg, IG) on the sucrose preference test and marble burying behavior in ARS mice. Each column represents the mean ± SEM of 5–7 animals for group. Statistical analysis was performed by one-way ANOVA followed by the Newman–Keuls test when appropriate. ###p < 0.001 as compared with the control group and ***p < 0.001 as compared with the ARS group. Abbreviations: V: vehicle; PSAP: α-(phenylselanyl) acetophenone; IMI: imipramine.

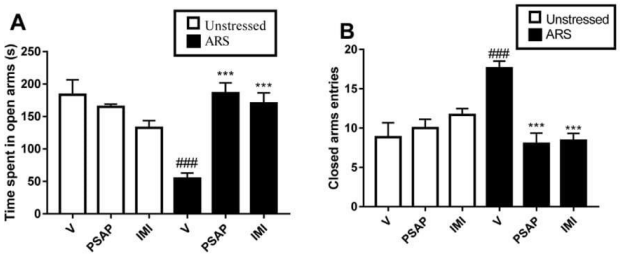

Fig. 5. Evaluation of the PSAP (10 mg/kg, IG) or IMI (10 mg/kg, IG) anxiogenic activity against the induction of the ARS induced anxiolytic-like in elevated plus maze test (EPM). Time spent in open arms (s) (A); closed arms entries (B). Each column represents the mean ± SEM of 5 animals for group. Statistical analysis was performed by one-way ANOVA followed by the Newman–Keuls test when appropriate. ###p < 0.001 as compared with the control group, ***p < 0.001 as compared with the ARS group. Abbreviations: V: vehicle; PSAP: α-(phenylselanyl) acetophenone; IMI: imipramine.

increased the levels of TBARS (an indicative of lipid peroxidation) in both cortex (Fig. 7A) and hippocampus (Fig. 7B) and this effect was significantly blocked by PSAP treatment in hippocampus, and in the cortex. No changes were observed in the unstressed animals. The one-way ANOVA revealed significant differences in cortex [$F_{(5,22)} = 26.1$, $p < 0.001$] and hippocampus [$F_{(5,22)} = 8.52$, $p < 0.001$]. The same was observed with the positive control IMI, which also blocked the increase of TBARS levels induced by ARS just in hippocampus.

3.6.2. Reactive species (RS) levels

Fig. 8 shows that in cortex and hippocampus, after 4 h of ARS challenge and 30 min of treatment with PSAP, there was a significant enhancement in RS production in ARS group when compared with control animals. The treatment with PSAP reversed alterations in RS production in the cortex [$F_{(5,22)} = 15.8$, $p < 0.001$] (Fig. 8A) and hippocampus [$F_{(5,22)} = 5.54$, $p < 0.001$] (Fig. 8B) when compared with the ARS group. The treatment of mice with PSAP alone did not change RS levels. In addition, IMI also reduced RS levels increased by ARS in the hippocampus, but not in the cortex.

3.6.3. Nitrite and nitrate (NO_x) levels

The levels of NO_x were also increased by restraint stress in cortex [$F_{(5,30)} = 55.6$ ($p < 0.001$)] (Fig. 9A) and hippocampus [$F_{(5,30)} = 12.4$ ($p < 0.001$)] (Fig. 9B) when compared with non-stressed mice. The treatment with PSAP and IMI restored NO_x levels in cortex and hippocampus when compared with the ARS mice. In addition, it is possible to verify that in cortex both PSAP and IMI reduced NO_x levels in ARS animals over the control levels.

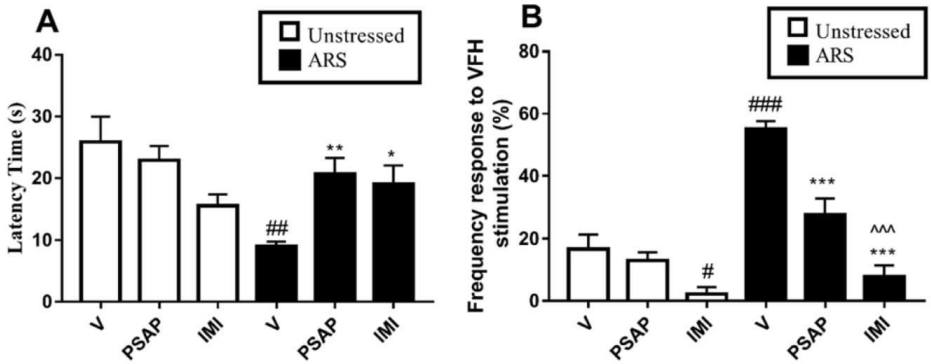

Fig. 6. Effects of PSAP or IMI (10 mg/kg, IG) on the response latency to thermal stimuli on the hot plate test and response frequency to VFH stimulation in right paw of ARS in mice. Each column represents the mean ± SEM of 7–8 animals for group. Statistical analysis was performed by one-way ANOVA followed by the Newman–Keuls test when appropriate. #p < 0.05, ##p < 0.01 and ###p < 0.001 as compared with the control group; *p < 0.05, **p < 0.01 and ***p < 0.001 as compared with the ARS group; ^^^ p < 0.001 compared with ARS + PSAP group. Abbreviations: V: vehicle; PSAP: α-(phenylselanyl) acetophenone; IMI: imipramine; VFH: Von-Frey Hair.

Table 1
Effect of administration of PSAP and IMI on behavior parameter in the open field test in mice.

Experimental groups	Number of crossings	Number of rearings
Vehicle (Saline + Canola oil)	86.0 ± 2.17	25.5 ± 2.74
PSAP (10 mg/kg)	91.5 ± 5.07	25.0 ± 1.38
IMI (10 mg/kg)	102.0 ± 3.77	33.0 ± 1.96
ARS	63.0 ± 8.51	25.0 ± 2.80
ARS + PSAP 10 mg/kg	76.0 ± 5.60	26.0 ± 2.63
ARS + IMI 10 mg/kg	86.0 ± 4.73	18.0 ± 2.05

The effect of treatment with PSAP and IMI on behavior of mice in the open-field test. Data presented are mean values ± SEM. Results are expressed as mean ± SEM. $^{###}p < 0.001$ when compared to the control group. ***p < 0.001 as compared with the ARS group. *Abbreviations*: PSAP: α-(phenylalanyl) acetophenone; IMI: imipramine; ARS: acute restraint stress.

3.6.4. Corticosterone levels in plasma

Plasma corticosterone levels were significantly increased in ARS mice when compared with non-stressed controls (Fig. 9C). Both PSAP and IMI administered after ARS reversed this increase [$F_{(5,30)} = 20.7$, $p < 0.001$].

4. Discussion

This study demonstrated that acute administration of PSAP exerts antidepressant-like, anxiolytic-like, anti-hyperalgesic and anti-allodynia effects in the model o ARS in mice. PSAP also reduced lipid peroxidation, RS and NOx levels increased by ARS in both cortex and hippocampus. This model is validated because we can obtain an increase in plasma corticosterone levels, thus indicating that the ARS model can activate the HPA axis and this factor induces depressive, anxiogenic and pain symptoms.

The ARS has been proposed as a model of depression induced by stress, which combines both emotional and physical components in addition to affect the brain's intra-cellular redox status (Buynitsky and Mostofsky, 2009; Glavin et al., 1994). Various experimental findings demonstrated that, animals exposed to restraint stress, in different duration of stressful conditions showed increase immobility time in FST model (Budni et al., 2013; Freitas et al., 2014).

Depressive-like and anhedonia (loss of ability to feel pleasure), in the ARS model, agreement that the increase in corticosterone levels lead to these findings. As expected, restraint stress disturbed the HPA axis, as shown by the increase in plasma corticosterone levels compared with the nonstressed group. Corticosterone is the hormone characteristic of the stress response (Lee et al., 2012). Corticosterone is produced by the adrenal cortex in response to stress, which triggers sequential activation of the hypothalamus (release of corticotropin-releasing hormone) and the anterior pituitary gland (release of adrenocorticotropic hormone) (Lupien et al., 2009). In the long term, a high concentration of corticosterone contributes to neuronal atrophy, especially in the hippocampus, cortex, and amygdala, and to the pathogenesis of neurodegenerative diseases (Patel et al., 2002). Accordingly, abnormal increases in corticosterone concentrations might augment the impairment of stress-induced oxidative damage in brain tissue. As described, ARS induces an excessive activation in HPA, which increases corticosterone levels in the blood plasma of stressed mice. The treatment with PSAP and IMI were able to decrease plasma corticosterone levels altered by ARS in mice, contributing to its pharmacological effects. These findings corroborate further to predict that PSAP is a promising molecule for the treatment of stress-related disorders.

The marble burying test is a good example of behavioral method for studying repetitive and compulsive-like behaviors in mice (Albelda and Joel, 2012; Deacon, 2006). The value of this test derives from the fact that it is a validated model of human psychiatric disorders such as anxiety model. The marble burying test is a sensitive assay for characterizing the increase in anxiety-like behavior triggered by a single episode of 4-h of acute immobilization stress in mice. Importantly, the anxiogenic effect of this acute stress is detected by the marbles burying test even 10 days later (Angoa-Pérez et al., 2013). Through the results found in this model, we can see that the PSAP and IMI presents anticompulsive activity in the ARS model.

The EPM is a type of highly aversive environment that has been used to study the neurobiology of fear-induced antinociception (Mendes-Gomes et al., 2011) and defensive behaviors (Sorregotti et al., 2013). Exposure of mice to the EPM enhances plasma corticosterone titers and elicits defensive behaviors (Sorregotti et al., 2018). Thus, we can suggest that PSAP and IMI reversed the fear and the anxious-like composition provoked by EPM through its decrease in plasma corticosterone levels in addition to its antioxidant activity in the ARS model.

The hot plate test produces a non-inflammatory nociception and that is a good model to investigate the central effect of analgesic drugs with supraspinally integrated responses (Oliveira et al., 2008). Also, the hot plate test produces, at constant temperature, two kinds of behavioral response, which are paw licking and jumping. Both of these are considered to be supraspinally integrated responses (Chapman et al., 1985). In line with these results, PSAP and IMI reduced the nociceptive behavior from the thermal stimulus in the hot-plate test, suggesting central actions of these compounds in the ARS model.

Von Frey filaments is a method used to evaluate the tissue sensitivity to the mechanical stimulus, being widely used clinically. This method was also used for laboratory experiments in order to evaluate the influence of drugs on nociceptive sensitivity in animals (Silva et al., 2013). Through the results, we can verify that the ARS model induces a mechanical allodynia, leading to a greater sensibility in the right side of the mice. According to the findings, PSAP or IMI have an anti-allodynic effect.

Hence, the effects of PSAP or IMI were tested for possible locomotor activity by OFT. Pretreatment with PSAP or IMI produced no significant alterations on number of crossings and rearings by mice in both non-stressed as well as ARS, suggesting the antidepressant activity observed due to PSAP or IMI in ARS is independent of any locomotor effect.

During oxidative stress occur lipid peroxidation and is considered a critical mechanism of injury occurring in cells (Niki, 2012). Lipid peroxidation may be increased during emotional stress, which

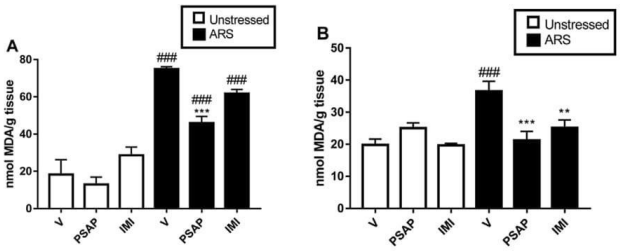

Fig. 7. Effects of PSAP or IMI (10 mg/kg, IG) on lipid peroxidation levels in the cerebral cortex (A) and hippocampus (B) of ARS mice. Each column represents the mean ± SEM. of 4–5 animals for group. Statistical analysis was performed by one-way ANOVA followed by the Newman–Keuls test when appropriate. ##p < 0.01 and ###p < 0.001 as compared with the control group, *p < 0.05, **p < 0.01 and ***p < 0.001 as compared with the ARS group. Abbreviations: V: vehicle; PSAP: α-(phenylselanyl) acetophenone; IMI: imipramine; MDA: malondialdehyde.

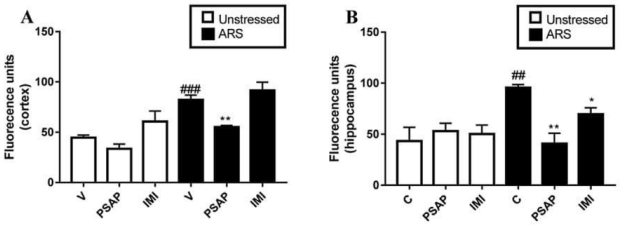

Fig. 8. Effects of PSAP or IMI (10 mg/kg, IG) on formation of reactive species levels in the cerebral cortex (A) and hippocampus (B) of ARS mice. Each column represents the mean ± SEM. of 4–5 animals for group. Statistical analysis was performed by one-way ANOVA followed by the Newman–Keuls test when appropriate. ##p < 0.01, ###p < 0.001 as compared with the control group, *p < 0.05 and **p < 0.01 as compared with the ARS group. Abbreviations: V: vehicle; PSAP: α-(phenylselanyl) acetophenone; IMI: imipramine.

accompanies severe depression, and clinical studies have directly demonstrated higher levels of MDA in patients with affective disorders. (Lang and Borgwardt, 2013). In addition, several studies have demonstrated that the restraint stress significantly elevated lipid peroxidation level in the cortices and hippocampus of mice (Budni et al., 2013; Moretti et al., 2013). In line with this, our results show that the ARS procedure caused a significant lipid peroxidation, as evidenced by increased amount of TBARS and RS levels in ARS-mice, which was abolished by PSAP treatment, in both cortex and hippocampal structures. Through these results we can say that the PSAP has the capacity to reversed lipid peroxidation of the mice submitted to the ARS, suggesting thus to have antioxidant effect against the symptoms of pain, depression and anxiety.

NO also has the capability of inducing peripheral and central sensitization by reducing receptor thresholds (Goupille et al., 1998) and is able to reduce the inhibitory activity of the central nervous system leading to central sensitization of dorsal horn neurons. NO is an important signaling molecule for neurotransmission, but over-stimulated by nitrosative stress, NO can react with RS to form highly toxic reactive nitrogen species. In this experiment we can verify that the animals that suffered ARS had an increase of nitrite and nitrate levels (NO_x), an indirect measure of NO levels, in both cortex and hippocampus when compared with the animals that did not suffer stress (Szabó and Harry Ischiropoulos, 2007). The treatment with PSAP or IMI reduced this increase of NO_x in both structures analyzed. With the results obtained in this study, we can suggest that the PSAP presents central actions to reduce hyperalgesia and allodynia.

5. Conclusion

Altogether, our data demonstrated that intragastric administration of PSAP reversed behavioral alterations induced by ARS in the FST, sucrose preference, hot plate, mechanical allodynia and marble burying test. PSAP also reversed the increase in TBARS, RS and NO_x levels in the cerebral cortex and hippocampus as well as the increase in plasma corticosterone levels of animals subjected to the ARS. With these results, we can suggest that PSAP presents antidepressant-like, anxiolytic-like, anti-hyperalgesic, anti-allodynic and antioxidant activity in the ARS model. In this way, PSAP can be a promising molecule for the treatment of comorbid pain, depression and anxiety.

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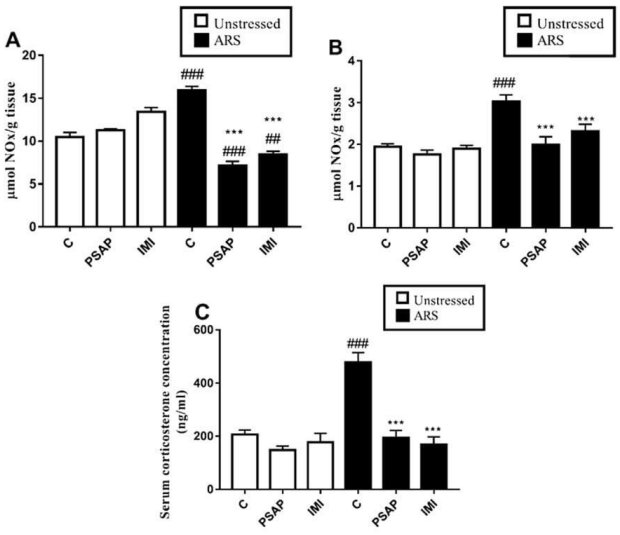

Fig. 9. Effects of PSAP or IMI (10 mg/kg, IG) on the nitrite and nitrate levels in the cerebral cortex (A) and hippocampus (B) and corticosterone level in plasma (C) of ARS mice. The results are expressed in µmol NOx/g of tissue. Each column represents the mean ± SEM of 5–6 animals for group. Statistical analysis was performed by one-way ANOVA followed by the Newman–Keuls test when appropriate. ###p < 0.001 as compared with the control group, ***p < 0.001 as compared with the ARS group. Abbreviations: V: vehicle; PSAP: α-(phenylselanyl) acetophenone; IMI: imipramine.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.neuint.2018.08.006.

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Manuscrito 1

Os resultados que fazem parte desta tese de doutorado estão apresentados sob a forma de manuscrito, o qual se encontra assim organizado. Os itens Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas encontram-se no próprio manuscrito. O manuscrito foi submetido na revista **Neuropharmacology.**

Lipopolysaccharide-induced depressive-like, anxiogenic-like and hyperalgesic behavior is attenuated by acute administration of α-(phenylselanyl) acetophenone in mice

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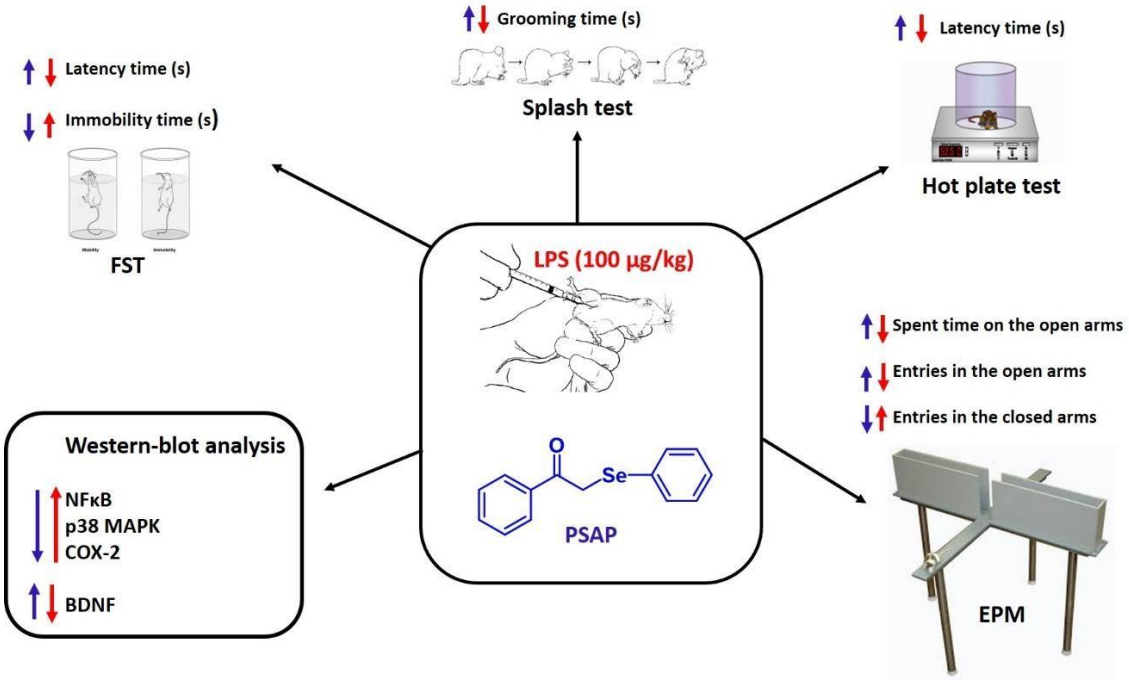
Abstract

The lipopolysaccharide (LPS) is an endotoxin derived from gram-negative bacteria, which induces inflammation. The interest in organoselenium biochemistry and pharmacology has increased due to a variety of compounds that show biological activity. The aims of the present study were to evaluate the possible α-(phenylselanyl) acetophenone (PSAP) activity in reducing comorbid hyperalgesia, depressive-like and anxiogenic-like symptoms induced by LPS in mice. The LPS (100 µg/kg, intraperitoneally) or saline were administered and after 4h the treatment with PSAP (0.001 - 10 mg/kg, intragastric route [i.g.]) or FLX (10 mg/kg, i.g.) was performed, and after 30 min, the behavioral tests were carried out. LPS reduced the latency time for the first episode of immobility and increased the immobility time in the FST as well as decreased the grooming time in the splash test. PSAP reversed these alterations demonstrating an antidepressive-like effect. LPS also enhances the anxiogenic behavior in the elevated plus maze test (EPM). PSAP reversed these parameters, showing anxiolytic-like effect. LPS also decreased the latency time (s) on the hot plate and the treatment with PSAP at all doses significantly reversed the hyperalgesic effect of LPS. LPS increased the activation of p38MAPK and p-p65NF-κB pathways as well as the COX-2 levels in the cerebral cortex, which are indicative of an inflammatory response. Besides, it also reduced the levels of mBDNF, involved in neuroplasticity. Treatment with PSAP restored all these neurochemical alterations induced by LPS. The results demonstrated that PSAP presents antidepressive-like, anxiolytic-like and anti-hyperalgesic effects related to reduction in neuroinflammation.

Keywords: Selenium, depression, anxiety, pain, synaptic signaling

Graphical Abstract



1. Introduction

People suffering from chronic pain are more likely to experience symptoms of depression and anxiety (BAGLIONI *et al.*, 2011; BAIR *et al.*, 2003, 2008). The prevalence of pain among people with depression can be as high as 65%, and the concomitant presence of symptoms of depression, anxiety, and pain is associated with worse health status for patients compared to the presence of one of these conditions alone (MELLO, M. T. DE *et al.*, 2013). Additionally, the co-occurrence of symptoms of depression and pain results in higher healthcare utilization costs. Despite the impact that comorbid depression, anxiety and pain brings for patients and society, the mechanisms underpinning this relationship remain largely unclear (PINHEIRO, M. B. *et al.*, 2018).

Thus, several studies have used lipopolysaccharide (LPS), an endotoxin derived from gram-negative bacteria, which induces inflammation and causes anxiety, pain and depression (HO; TAMBYAH; PATERSON, 2010). The LPS is a prototypical ligand of membrane-bound pattern recognition receptor (PRR) toll-like receptor 4 (TLR4), covering up to 75% of its outer surface. In the central nervous system (CNS), treatment of astrocytes and microglia with low concentrations of LPS can produce prostaglandin-2 (PGE_2) via toll-like receptor 4-dependent manner (HO; TAMBYAH; PATERSON, 2010; NIKAIDO, 2003). PGE_2 can directly trigger pain-sensitive neurons to induce nociception (GINSBURG; KOREN, 2008).

The peripheral administration of LPS in rodents is a well-established animal model to investigate the link between immune activation, oxidative stress, pain and depressive symptoms. Binding of LPS to TLR4 activates a downstream signaling cascade leading to activation and nuclear translocation of nuclear factor kappa B (NF-κB) followed by production of pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6, tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ) (CHO, Y. H. *et al.*, 2016; PINHO-RIBEIRO *et al.*, 2016). These mediators largely contribute to the amplification of the inflammatory response with activation of cyclooxygenase-2 (COX-2), increases the production of reactive oxygen species (ROS) through mitochondrial dysfunction and decreased

neurotrophins such as brain-derived neurotrophic factor (BDNF) (REN, J. D. *et al.*, 2016). These factors lead to leading to behavioral and neurochemical alterations resembling to those found in subjects with major depression (DANTZER, Robert *et al.*, 2011).

In this context, many researches comprise the search for new synthetic drugs with antidepressive-like, anxiolytic-like and pain activity. In the last two decades, the interest in organoselenium biochemistry and pharmacology has increased due to a variety of compounds that show biological activity (Casaril et al., 2017; Gai et al., 2014; Oliveira et al., 2016; Brod et al., 2016; Sousa et al., 2017). With this in mind, arylselanyl acetophenones are a class of organoselenium compounds that have a number of interesting biological activities. Studies have shown that α-(phenylselanyl) acetophenone (PSAP) exhibits glutathione peroxidase-like activity (Cotgreave et al., 1992; Engman et al., 1994; Nikolic, 2007), has the capacity to inhibit tumor promoter-induced downregulation of intercellular communication between liver epithelial cells via gap junction. In addition, the PSAP have antioxidant, antidepressant-like (GERZSON *et al.*, 2012) and antinociceptive activities (Sousa et al., 2017), without showing acute or chronic toxicity (CASARIL *et al.*, 2015b).

Taking into account the previously described biological activities of PSAP and LPS-induced dysfunctions, the aims of the present study were to: (1) Evaluate the possible PSAP activity in reducing hyperalgesia induced by LPS; (2) Reduced depressive symptoms caused by systemic administration of LPS; (3) Observed the anxiolytic action of PSAP against LPS-induced anxiogenic phenotype; (4) Check possible mechanisms involved in the PSAP effects.

2. Materials and Methods

Animals

The experiments were conducted using male adult Swiss mice (25-35g) from our own breeding colony. The animals were kept in a separate animal room, on a 12 h light/dark cycle with lights on at 7:00 a.m., at room temperature (22 ± 1 °C) with free access to water and food. All experimental procedures were conducted

in accordance with the guidelines of the Committee of Ethics in Research (number 6408-2016). All efforts were made to minimize animal suffering and the number of animals used in the experiments.

Drugs

The lipopolysaccharide (LPS) from *Escherichia coli* (L-3129, serotype 0127:B8) was purchased from Sigma-Aldrich., St Louis, USA. LPS was dissolved in saline and administered to mice intraperitoneally route (i.p.) at dose of 100 µg/kg. The α- (phenyselanyl) acetophenone (PSAP) (Fig. 1) was prepared and characterized in the Laboratory of Clean Organic Synthesis (LASOL) according to the method previously described (VICTORIA, Francine Novack *et al.*, 2009). PSAP was dissolved in canola oil and administered to mice intragastrically (i.g.) at dose range of 0.001 to 10 mg/kg in a volume of 10 ml/kg. The standard drug used was fluoxetine (FLX) at the dose of 10 mg/kg (i.g.), in a volume of 10 ml/kg, and it was purchased from a commercial pharmacy. All other chemicals were obtained at the highest available commercial grade.

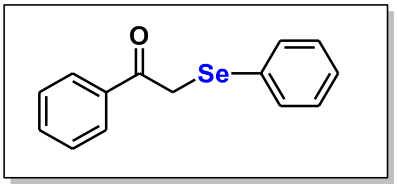


Figure 1. Chemical structure of α-(phenylselanyl) acetophenone (PSAP).

Experimental design

Animals were randomly divided in fourteen experimental groups (n= 7 per group) to assess the effect of PSAP in mice with depressive-like, hyperalgesia and anxiogenic-like behavioral. After 4h of LPS administration, the mice received PSAP (0.001 - 10 mg/kg) or FLX (10 mg/kg) and after 30 min the forced swimming test (FST), splash test, hot plat test, elevated plus maze test (EPM) or open field test (OFT) were performed (Figure 2).

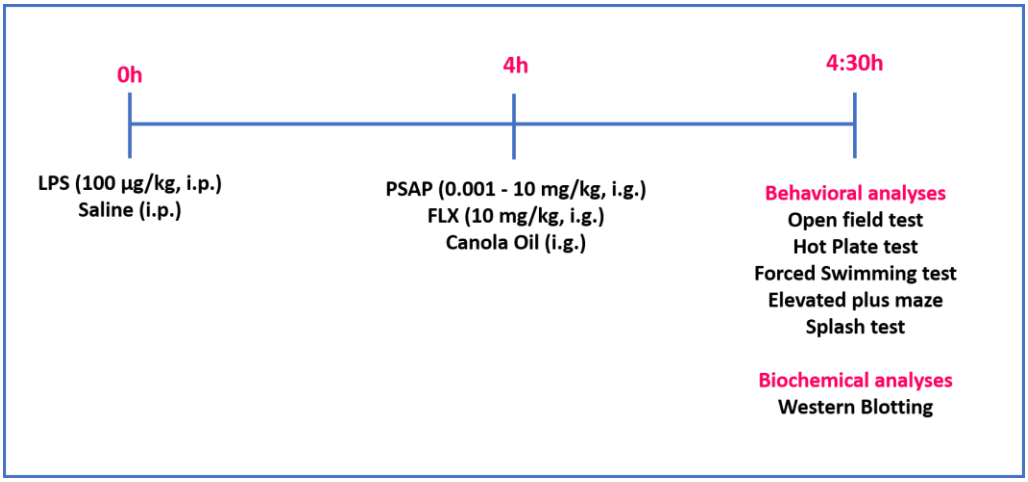


Figure 2. Schematic protocol of the induction of depressive-like, hyperalgesia and anxiogenic-like behavior with lipopolysaccharide (LPS) injection. *Abbreviations:* PSAP: α-(phenylselanyl) acetophenone; i.g.: Intragastrically; i.p. Intraperitoneally, FLX: Fluoxetine, FST: forced swimming test, EPM: Elevated plus maze, OFT: Open field test.

All observations were performed by an observer blinded to the study plan. Biochemical parameters were evaluated by taking different animals (following the same experimental design) to avoid interferences of behavioral assessment on neurochemical parameters. The animals used for neurochemical determinations were killed by cervical dislocation, followed by brain removal and isolation of total cortex for analysis.

Behavioral determinations

Forced Swimming test (FST)

The FST, as originally described by Porsolt et al. (1977), is the most widely used pharmacological assay for assessing antidepressant-like activity. Mice were individually placed in a cylinder (diameter 10 cm, height 25 cm), containing 19 cm of water maintained at 25 ± 1°C. The water in the cylinder was changed after each swim session. After 2 min of adaptation (latency time for first episode of immobility), the immobility time (in seconds) was observed for 4 min. Mice were considered immobile when they ceased to undertake any movement such as swimming, diving and climbing, making only movements necessary to keep its

head or nose above the water. A decrease in the duration of immobility is indicative of an antidepressant-like effect.

Splash Test

The splash test consists of squirting a 10% sucrose solution on the dorsal coat of a mouse in its home cage. Because of its viscosity, the sucrose solution dirties the mice fur and animals initiate grooming behavior. After applying sucrose solution, the time spent grooming was recorded for a period of 5 min as an index of self-care and motivational behavior according to a protocol used elsewhere (ISINGRINI *et al.*, 2010).

Elevated plus maze test (EPM)

The elevated plus maze test (EPM) was used to evaluate anxiety-like behavior and performed in a black Plexiglas apparatus with four arms, two open and two closed, set in cross from a neutral central square elevated 40 cm above the floor. Five-minute test sessions were performed, and the percentage of entries in the open arms, time spent in the open arms and percentage of entries in the closed arms was determined, as previously reported (BUSQUETS-GARCIA *et al.*, 2011).

Hot Plate test

For the evaluation of the development of thermal hyperalgesia, the animals were subjected to the hot plate test. The mice were placed in an acrylic cylinder (20 cm in diameter) on the hot plate apparatus, maintained at 55 ± 1 °C. The time (s) between the placement on the heated surface and licking of their hindpaws or jumping was recorded as the response latency. A 45 s cut-off was used to prevent tissue damage (BRÜNING *et al.*, 2015; SAVEGNAGO *et al.*, 2007).

Open field test (OFT)

The OFT was carried out before the FST and hot plate test (WALSH; CUMMINS, 1976) to assess the possible effect of the treatments on locomotor

activity. The mice were placed individually in the centre of a box (30 x 30 x 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 activity (expressed by the number of times the mice stood on rear limbs).

Western-blot analysis

With another group of animals (n = 5 animals per group) were perform the western-blot analysis, the 10 mg/kg dose of the PSAP was chosen. The parameter of choice for this dose is since this was the most effective dose in all behavioural tests The samples of cortex were homogenized in RIPA buffer (Sigma-Aldrich Co., St. Louis, Missouri, USA) containing protease inhibitor cocktail (Sigma-Aldrich Co., St. Louis, Missouri, USA) as well as phosphatase inhibitors cocktail (sodium orthovanadate 50 mM; sodium fluoride 25 mM and β-glycerophosphate 250 mM) and they were centrifuged at 14000 ×g at 4 °C for 10 min. The supernatant was collected, and the pellet was discarded. The samples were diluted to a final protein concentration of 2 µg/µl in 3x laemmli sample buffer (essentially 150 mM Tris-HCl (pH 6.8), 6% SDS, 0.3% bromophenol blue, and 30% glycerol as well as β-mercaptoethanol as reducing agent). The samples (20 µg of protein) and pre-stained molecular weight standards (Sigma-Aldrich Co., St. Louis, Missouri, USA) were separated by 12% SDS-PAGE electrophoresis and transferred to nitrocellulose membrane (0.45 µm, Bio-rad) using TransferBlot® Turbo™ Transfer System (1.0 A; 50 min for proteins above 25 kDa or 10 min for proteins below 25 kDa), and equal protein loading was confirmed by Ponceau S staining. After blocking with 3% bovine serum albumin or 3% skimmed milk solution, the blots were incubated overnight or for 2 days at 4 °C with rabbit anti-phospho-p38 MAPK Thr180/Tyr182 (1:1000; Cell Signaling) and rabbit anti-p38 MAPK (1:1000; Cell Signaling), rabbit anti-phospho-NF-κB p65 (Ser536) (1:1000; Cell Signaling) and rabbit anti-NF-κB p65 (1:1000; Cell Signaling), rabbit anti-COX-2 (1:1000; Cell Signaling), and rabbit anti-mBDNF (1:1000; Abcam). Mouse anti-β-actin (1:5000; Cell Signaling) was stained as

additional control of the protein loading. After primary antibody incubation, membranes were washed and incubated with their respective secondary antibodies (1:5000) conjugated with horseradish peroxidase (Bio-Rad Laboratories, Hercules, CA, USA) for 1 h at room temperature. For protein detection, we used chemiluminescence kit (Amersham, São Paulo/Brazil) and the signals were captured with Amersham Imager 600 (GE healthcare life sciences). Optical density (O.D.) of the western blotting bands was quantified using Image J (NIH, Bethesda, MD, USA) software for Windows. Each value was derived from the ratio between arbitrary units obtained by the protein band and the respective β-actin band. For these analyzes, the most effective dose of PSAP (10 mg/kg) administered by i.g. In addition to this, we chose the structure of the cortex to perform the Western-blot analysis.

Statistical analysis

All experimental results are given as a mean ± standard error of the mean (SEM). Behavioral and neurochemical comparisons between experimental and control groups were performed by one-way analysis of variance (ANOVA). When ANOVA revealed a significant, the Newman-Keuls 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 Graph Pad Prism version 7.0 for Windows, Graph Pad Software (San Diego, CA, USA).

3. Results

Effects of PSAP on behavioral evaluations

PSAP has antidepressant-like in the Forced Swimming Test

To evaluate the influence of treatment of mice with PSAP (0.001-10 mg/kg, i.g.) or fluoxetine (10 mg/kg, i.g.) on the depressive-like behavior elicited by intraperitoneal injection of LPS, the FST was performed (Figure 3). Figure 3A shows the latency time to the first episode of immobility of mice in the FST. It can

be observed that the animals that received the injection of LPS had a decrease in the latency time to the first episode of immobility when compared to the control group. The treatment with PSAP at the highest dose (10 mg/kg) [$F_{(13,95)}$ = 5.67, P < 0.001] and with the standard drug (FLX) reversed this effect of LPS.

The LPS administration (Figure 3B) induced remarkable increase of immobility time of mice in the FST when compared to the control group (P < 0.01). The treatment with PSAP (0.01-10 mg/kg) [$F_{(13,95)}$ = 13.7, P < 0.001] significantly reversed this parameter, reducing the time of immobility of mice in the FST. The same effect was observed when the animals received FLX.

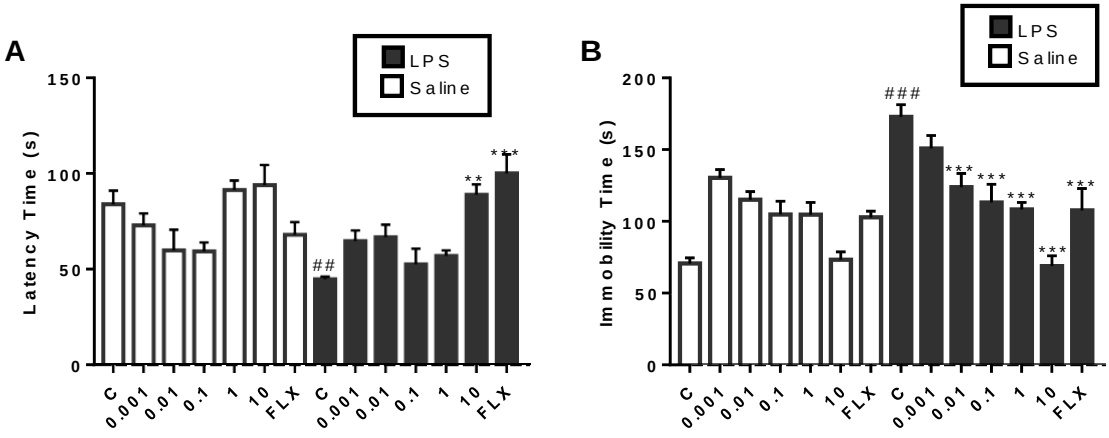


Figure 3. Effects of PSAP (0.001-10 mg/kg, i.g.) or FLX (10 mg/kg, i.g.) on active behaviors in the forced swimming test. (A) Latency time (s); (B) Immobility time (s). Each column represents the mean ± SEM. of 7 animals for group. Statistical analysis was performed by one-way ANOVA followed by the Newman–Keuls test when appropriate. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ as compared with the control (vehicle) group, $^{**}P < 0.01$ and $^{***}P < 0.001$ as compared with the LPS group. *Abbreviations:* V: vehicle; PSAP: α-(phenylselanyl) acetophenone; FLX: Fluoxetine.

PSAP has antidepressant-like effect in the splash test

Figure 4 shows that LPS injection 4 hours prior to PSAP treatment caused a reduction in grooming time when compared to control group and treatment with PSAP [$F_{(9,70)}$ = 9.16, P < 0.001] at doses of 1 and 10 mg/kg as well as FLX reversed this effect, demonstrating the antidepressant-like activity of PSAP.

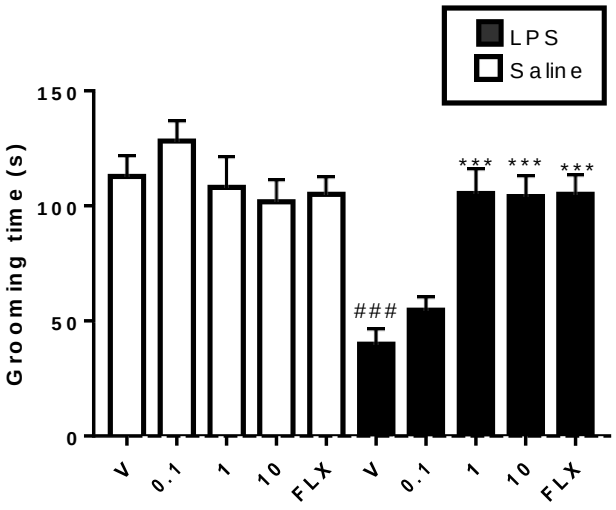


Figure 4. Effects of PSAP (0.1-10 mg/kg, i.g.) or FLX (10 mg/kg, i.g.) on the total grooming time in the splash test as an index of motivational and self-care behavior. Each column represents the mean ± SEM of 7 animals for group. Statistical analysis was performed by one-way ANOVA followed by the Newman–Keuls test when appropriate. $^{###}P < 0.001$ as compared with the control (vehicle) group, $^{***}P < 0.001$ as compared with the LPS group. *Abbreviations:* V: vehicle; PSAP: α-(phenylselanyl) acetophenone; FLX: Fluoxetine.

3.1.4 PSAP has anxiolytic-like action in the elevated plus maze

Figure 5A demonstrates the time spent by mice in the open arms of the EPM. LPS decreased the time spent in the open arms when compared to control group. The treatment with PSAP (10 mg/kg) or FLX (10 mg/kg) [$F_{(7,49)} = 8.62$, $P < 0.001$] reversed this effect of LPS. Figure 5B shows the number of the entries of the mice in the open arms. Animals that received LPS presented a reduction in the number of open arms entries compared to the control group and the treatment with PSAP (1 and 10 mg/kg) or FLX (10 mg/kg) [$F_{(7,52)} = 4.74$, $P < 0.001$] reversed this effect of LPS. Figure 5C shows the number of entries of mice in the closed arms of the EPM. The animals that received the LPS injection had an increase in the number of entries in the closed arms when compared to the control group. The treatment with PSAP (1 and 10 mg/kg) as well as the FLX reversed this alteration induced by LPS [$F_{(7,52)} = 11.2$, $P < 0.001$].

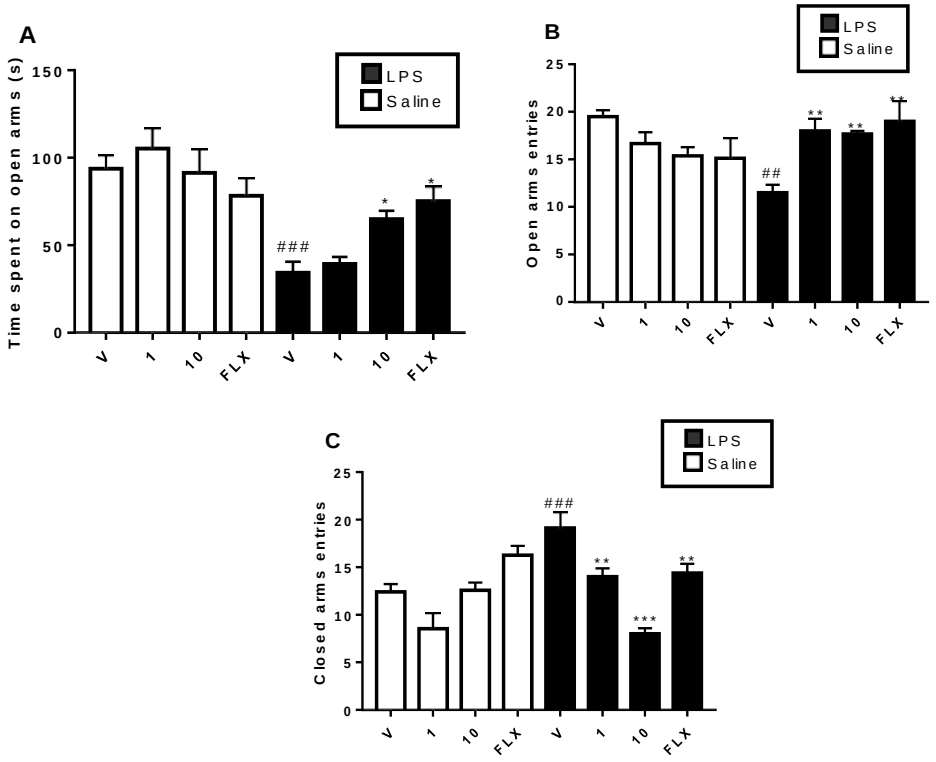


Figure 5. Effects of PSAP (1-10 mg/kg, i.g.) or FLX (10 mg/kg, i.g.) on LPS-induced anxiolytic-like activity in the elevated plus maze test (EPM). Time spent in open arms (s) (A); Open arms entries (B) and closed arms entries (C). Each column represents the mean ± SEM of 7 animals for group. Statistical analysis was performed by one-way ANOVA followed by the Newman–Keuls test when appropriate. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ as compared with the control (vehicle) group, $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ as compared with the LPS group. *Abbreviations:* V: vehicle; PSAP: α-(phenylselanyl) acetophenone; FLX: Fluoxetine.

PSAP has anti-hyperalgesic effect in the hot plate test

The injection of LPS significantly decreased the latency time (s) to nociceptive response on the hot plate, when compared with the control animals (Figure 6). The treatment with PSAP at all doses [$F_{(13,95)} = 9.84$, $P < 0.001$] significantly reversed the hyperalgesic effect of LPS. However, FLX did not reverse this effect. In addition, animals that received saline and PSAP at doses of 0.1 and 1 mg/kg also had a significant reduction in latency time to nociceptive

response when compared to control. The same was observed in the animals that received FLX.

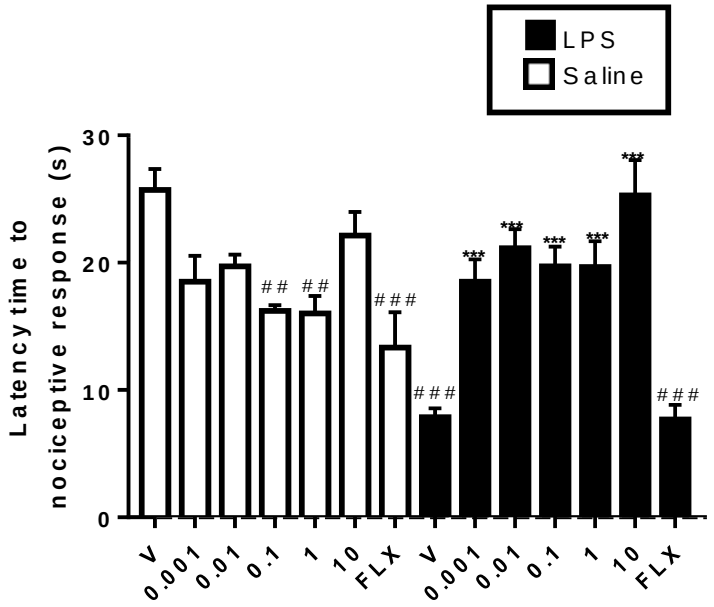


Figure 6. Effects of PSAP (0.001-10 mg/kg, i.g.) or FLX (10 mg/kg, i.g.) on the response latency to LPS induced hyperalgesia of mice in the hot plate test. Each column represents the mean ± SEM of 7 animals for group. Statistical analysis was performed by one-way ANOVA followed by the Newman–Keuls test when appropriate. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ as compared with the control (vehicle) group, $^{***}P < 0.001$ as compared with the LPS group. *Abbreviations:* V: vehicle; PSAP: α-(phenylselanyl) acetophenone; FLX: Fluoxetine.

Locomotor and exploratory activity of PSAP on the open field test

In order to investigate whether the decrease in decrease in the immobility time in the FST and EPM is due to locomotor impairment, the number of crossings and rearings was assessed in the OFT. As depicted in Table 1, the treatments did not produce significant differences in crossings and rearings among groups.

Table 1. Effect of administration of PSAP or FLX on behavior parameter in the open field test in mice.

Experimental groups	Number of crossings	Number of rearings
Vehicle (Saline + Canola oil)	87.0 ± 8.21	44.0 ± 2.34
PSAP (0.001 mg/kg)	106.0 ± 6.00	47.5 ± 4.98
PSAP (0.01 mg/kg)	101.0 ± 9.11	55.0 ± 3.09
PSAP (0.1 mg/kg)	89.5 ± 8.25	57.0 ± 3.91
PSAP (1 mg/kg)	102.0 ± 7.50	46.0 ± 3.54
PSAP (10 mg/kg)	102.0 ± 6.14	30.0 ± 2.91
FLX (10 mg/kg)	104.0 ± 9.80	32.0 ± 7.14
LPS+ Canola oil	85.5 ± 10.9	42.0 ± 2.02
LPS+ PSAP 0.001 mg/kg	105.0 ± 6.93	34.0 ± 5.70
LPS+ PSAP 0.01 mg/kg	94.0 ± 3.08	49.0 ± 3.57
LPS+ PSAP 0.1 mg/kg	111.0 ± 6.90	42.0 ± 3.75
LPS+ PSAP 1 mg/kg	83.0 ± 5.83	44.0 ± 3.07
LPS+ PSAP 10 mg/kg	94.0 ± 3.88	42.5 ± 3.12
LPS+ FLX 10 mg/kg	96.5 ± 3.97	40.5 ± 4.70

The effect of treatment with PSAP or FLX on behavior of mice in the open-field test. Data presented are mean values ± S.E.M. *Abbreviations*: PSAP: α-(phenylalanyl) acetophenone; FLX: Fluoxetine; LPS: Lipopolysaccharide.

PSAP reversed activation of p38 MAPK and p65 NF-κB pathways and alterations in COX-2 and mBDNF levels induced by LPS

The treatment with PSAP reversed the increase in the protein content ratio of p-p38/p38MAPK ($F_{(3,16)}$ = 9.45, P < 0.001) (Fig. 7A) and p-p65NFκB/p65NFκB ($F_{(3,16)}$ = 7.94, P < 0.001) (Fig. 7B) in the cerebral cortex of mice treated with LPS.

LPS also induced an increase in COX-2 levels and a decrease in mBDNF levels in the cerebral cortex and the treatment with PSAP normalized the levels of both COX-2 and mBDNF ($F_{(3,16)}$= 7.71, P < 0.001 and $F_{(3,16)}$ = 11.7, P < 0.001, respectively) (Fig. 7C and D).

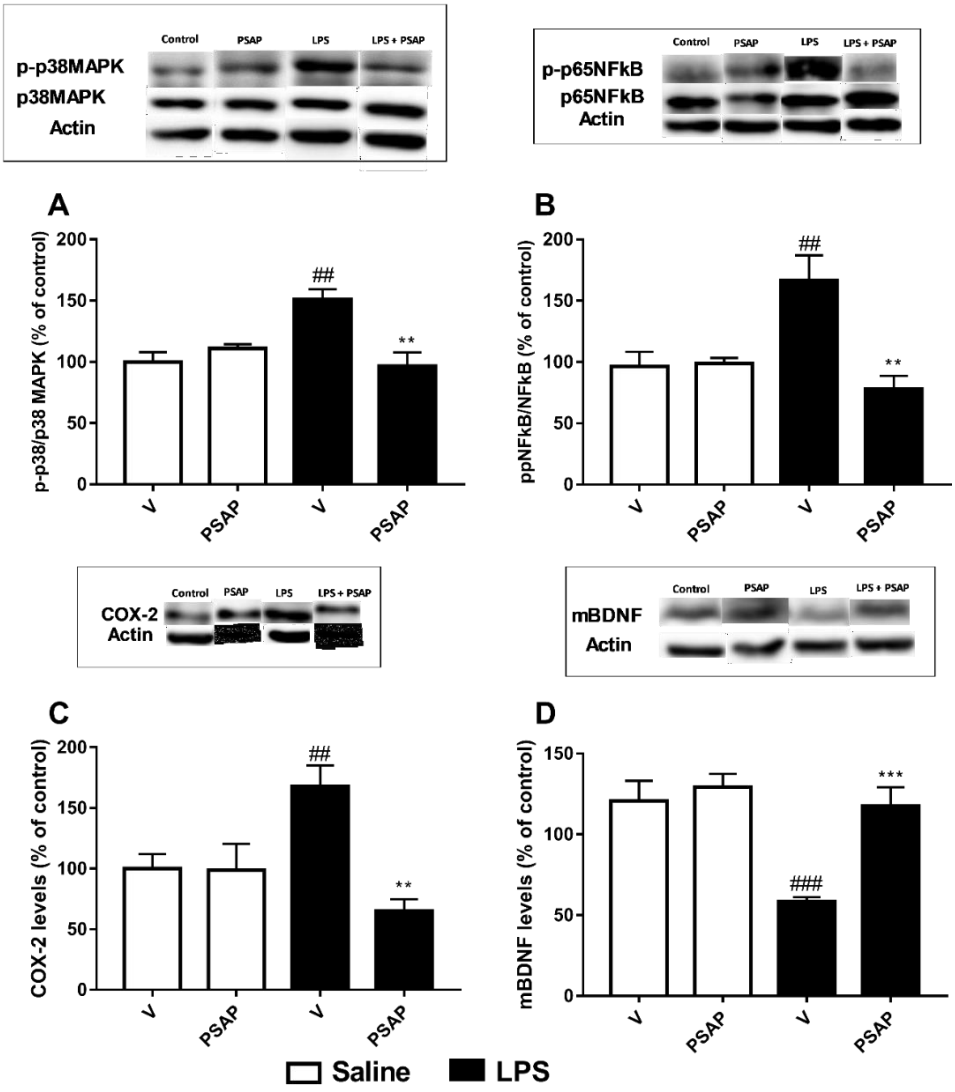


Figure 7. Effects of PSAP (10 mg/kg, i.g.) on (A) p-p38/p38MAPK ratio, (B) p p65NFκB/ p65NFκB ratio, (C) COX-2 levels and (D) mBDNF levels in the cerebral cortex. Each column represents the mean ± SEM of 5 animals for group. Statistical analysis was performed by one-way ANOVA followed by the Newman–Keuls test when appropriate. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ as compared with the control (vehicle) group, $^{**}P < 0.01$ and $^{***}P < 0.001$ as compared with the LPS group.

4. Discussion

In the present study, we evaluated of the PSAP, a selenium-containing compound, to reversed LPS-induced depressive-like, anxiogenic-like and hyperalgesic behavior. This effect was accompanied by the reversion of LPS-induced alterations in brain inflammatory mediators, through the reduction of

p38MAPK and p65NF-κB activation, in addition to modulate restoring COX-2 and mBDNF levels.

LPS, which is a component of the cell wall of gram-negative bacteria, is known to activate a number of cellular signals in various cell types and tissues during inflammation and infection. Based on the role of cytokines in depression, the systemic administration of LPS is acknowledged as a validated animal model to elicit depressive-like behavior in rodents (DANTZER, Robert *et al.*, 2008; OHGI *et al.*, 2013). The behavioral and neurochemical alterations caused by LPS administration are time-dependent. Sickness behavior is noticeable 2 h after LPS administration, and is outlined by cytokine peak release, hypolocomotion, anhedonia, among others. After 4 h of LPS administration, it is possible to detect depressive-like and hyperalgesic behavior in mice (Savignac et al., 2016; Zhang et al., 2016; Zucoloto et al., 2017).

The development of depressive disorders is accompanied by activation of immune-inflammatory pathways consisting of innate immune system and inflammatory process (Xiang et al., 2011). These findings suggest that inflammation played a vital role in depressive symptoms, and inhibition of inflammatory process might relieve these symptoms. In our study, the results showed that LPS induced depressive-like behavior in mice by increasing the latency time for the first episode of immobility and reducing the immobility time in the FST. PSAP reverse these alterations. The same is observed with the treatment with positive control, FLX, which can increase the latency time and reduce the time of immobility induced by LPS in the FST. In the same sense, the animals that received LPS had a reduction in the time spent with grooming in the splash test. The treatment with PSAP and FLX reverted this parameter, improving the depressive-like symptoms induced by the injection of LPS. In agreement with the findings of Gerzson et al., 2012, PSAP presents an antidepressive-like effect per se in low doses. Thus, a promising molecule for the treatment of depression.

Therefore, it was also evaluated whether PSAP had an effect on anxiety.

The EPM has been described as a simple method for assessing anxiety responses of rodents. The EPM relies upon rodents' proclivity toward dark,

enclosed spaces (approach) and an unconditioned fear of heights/open spaces. The results of this study demonstrate that the LPS leads to an anxiogenic type state in the mice, demonstrated by less time spent in the open arms, more time spent in the closed arms and lower number of entries into the open arms of the EPM. PSAP demonstrated an anxiolytic-like effect, by reversing these effects of LPS. Our study also shows that FLX has an anxiolytic effect at the dose tested.

The hyperalgesic effect of LPS was verified by the shortened latency time to nociceptive response using the hot-plate test. The hot plate is widely used to assess responses to heat stimuli. This test has been used for over half a century because it is well standardized and considered reference for testing analgesic drugs. The hot-plate test was used focuses on the pathophysiological process central level (BALLOU *et al.*, 2000). Using this model, we were able to investigate the peripheral and central antinociceptive effects of PSAP. The results showed that the treatment with PSAP from the lowest dose was able to increase the latency time to nociceptive response in hot plate. Thus, this compound shows activity in reversing the hyperalgesia caused by LPS injection. On the other hand, the FLX is not able to increase the latency time in the hot plate test. FLX is a serotonin selective reuptake inhibitors (SSRIs) used for the treatment of depression or anxiety disorders. SSRIs decrease the symptoms of depression by blocking serotonin from being reuptake to nerve synapses (KORNHUBER *et al.*, 2009), resulting in an increase of the synaptic concentration level, hence facilitating serotonergic neurotransmission.

The cerebral cortex was used to evaluate possible mechanisms involved in the effects of PSAP. Studies have reported that there is a decrease in the volume of the cortex in depressed patients and this factor is closely related to the severity of depression (KANG *et al.*, 2012). In addition, individuals with depression in post-mortem studies also observed a significantly reduced number of synapses of cerebral cortex, which decreases synaptic functions (GOLD, Phillip W.; MACHADO-VIEIRA; PAVLATOU, 2015). Impairment of emotion regulation is thought to be because of functional and anatomical defects of emotion-associated cortical and limbic brain regions including the cerebral cortex

and amygdala (OCHSNER *et al.*, 2004; QUIRK, G. J.; BEER, 2006; URRY, 2006). In particular, the inhibitory influence of the cortex on amygdalar neurons is a major regulator of emotion. This regulation system from the cortex to the amygdala also plays an essential role in the regulation of anxiety and fear behaviors and extinction process of fear memory (LIKHTIK *et al.*, 2008; MILAD; QUIRK, G. J. J., 2002; QUIRK, G. J.; BEER, 2006).The cortex is also involved in episodes of pain, and probably influences pain via several mechanisms (XIE, Y.; HUO; TANG, 2009). It has been proposed that the cortex may reduce pain by interrupting the transmission of noxious information from the spinal cord level by activating

Our results show that LPS activated the p38MAPK pathway in the cerebral cortices of mice, and PSAP treatment reversed this activation, demonstrated by a reduction in p-p38/p38MAPK ratio. Studies have suggested that exposure of mice to LPS can lead to release of proinflammatory cytokines, which in turn activate a second level of inflammatory cascades including cytokines, lipid mediators and adhesion molecules such as nitric oxide (NO), PGE_2, TNF-α, lL-1β, ROS, inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2) (Cohen, 2002; Zhang et al., 2016; Zong et al., 2012). The mitogen-activated protein kinase (MAPK) signaling pathway in macrophages is one of the most extensively investigated intracellular signaling cascades involved in LPS-induced proinflammatory responses (EHLTING *et al.*, 2011; HSIEH *et al.*, 2011). Activation of TLR4 by LPS activates the MAPK pathway. MAPK kinase (MAPKK) phosphorylates serine-threonine residues from MAPK which in turn will become active. The latter is translocated to the cell nucleus and activates transcription factors such as the activator-1 (AP-1) protein that leads to increased production of pro-inflammatory cytokines (ALEXANDER; RIETSCHEL, 2001; GUHA; MACKMAN, 2001). In addition, the peripheral activation of the innate immune system with LPS leads to a rapid stimulation of CNS 5-HT transporter (SERT) activity, accompanied by an acceleration of 5-HT clearance rate and alterations in SERT-dependent behaviors. Thus, the activation of MAPK through the peripheral injection of LPS leads to an induction of the increase of inflammatory

cytokines and a reduction of the availability of serotonin in the synaptic cleft, thus generating symptoms of depression pain and anxiety. According to the described above, the inhibition of p38MAPK pathway by PSAP could inhibit the activation of SERT by LPS and ultimately lead to an increase in 5-HT availability in the synaptic cleft, which could contribute to the antidepressive-like, anxiolytic-like and anti-hyperalgesic effects of PSAP.

The mechanisms underlying the effects of PSAP could be also attributed to inhibition of NF-κB activation and cytokine production. LPS triggers NF-κB activation in a toll-like receptor 4 manner leading to the release of IL-1β, IL-6, TNF-α, IL-1β and secondary mediators, such as leukotrienes and prostaglandins (PGs) which in turn contributes to hyperalgesia and depression (CALIL *et al.*, 2014; ELIOPOULOS *et al.*, 2002). PSAP was shown to inhibit IL-1β and TNF-α-induced NF-κB translocation to the nucleus in endothelial cells. Pro-inflammatory cytokines may in turn induce the translocation of more NF-κB and create a vicious circle (CHANG, W. C. *et al.*, 2010; CHEN, J.-W., 2003; ZUCOLOTO *et al.*, 2017). Thus, we believe that PSAP reduced the neuroinflammation caused by LPS by inhibiting the activation of the NF-κB pathway.

To further elucidate the mechanism of action of PSAP, this study also evaluated inflammatory parameters such as COX-2. There are two types of COX: COX-1 is constitutively expressed in most cell types and plays a role in gastrointestinal and reproductive function and the COX-2 is expressed at very low levels and is strongly induced by growth factors and pro-inflammatory stimuli, including LPS. The significance of COX-2 in prostaglandin synthesis and inflammation is highlighted by the observation that COX-2 inhibitors block the synthesis of PGE_2 and, as a result, they inhibit inflammation and confer analgesia (CAIVANO; COHEN, P., 2000; ELIOPOULOS *et al.*, 2002). As described, our results show that LPS injection increases COX-2 levels in cerebral cortex of mice and PSAP treatment was able to decrease COX-2 levels, thus confirming its anti-inflammatory activity against LPS.

The BDNF is one of the major protein regulators of diverse biological functions in the nervous system. It is synthesized as a precursor protein

(proBDNF) that undergoes proteolytic cleavage in order to become a mature molecule (mBDNF). Decreased levels of BDNF in brain have been related to pathophysiology of depression and also to neuropathic pain concurrent with mood disorders. It is reported that the administration of the cytokine-inducer LPS causes a significant reduction of BDNF gene expression Fukuhara et al., 2012). These findings support the possibility that inflammation contributes to the development of depression by compromising neuroplasticity via reduction of BDNF (CALABRESE *et al.*, 2014). In agreement with these findings, in the present study i.p. injection of LPS reduced mBDNF levels in the cerebral cortex of mice, and PSAP treatment had the ability to increase mBDNF levels in the cerebral cortex, indicating that the PSAP could help restore neuroplasticity via increase of mBDNF.

In conclusion, PSAP, a new organic compound of selenium, reversed all these alterations demonstrating antidepressive-like, anti-hyperalgesic and anxiolytic-like activities in LPS model in mice. In this sense, the effect of PSAP on these pathologies is due to the increased p-p38MAPK/p38MAPK and p-p65NF-κB/p65NF-κB ratio, increased COX-2 levels and reduced mBDNF levels.In this way, PSAP could be a promising molecule to treat mood disorders associated with pain.

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4.4 Manuscrito 2

Os resultados que fazem parte desta tese de doutorado estão apresentados sob a forma de manuscrito, o qual se encontra assim organizado. Os itens Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas encontram-se no próprio manuscrito. O manuscrito foi submetido na revista **Progress in Neuro-Psychopharmacology & Biological Psychiatry.**

α-(phenylselanyl) acetophenone reverses comorbid depressive-like behavior and mechanical allodynia induced by partial sciatic nerve ligation in mice

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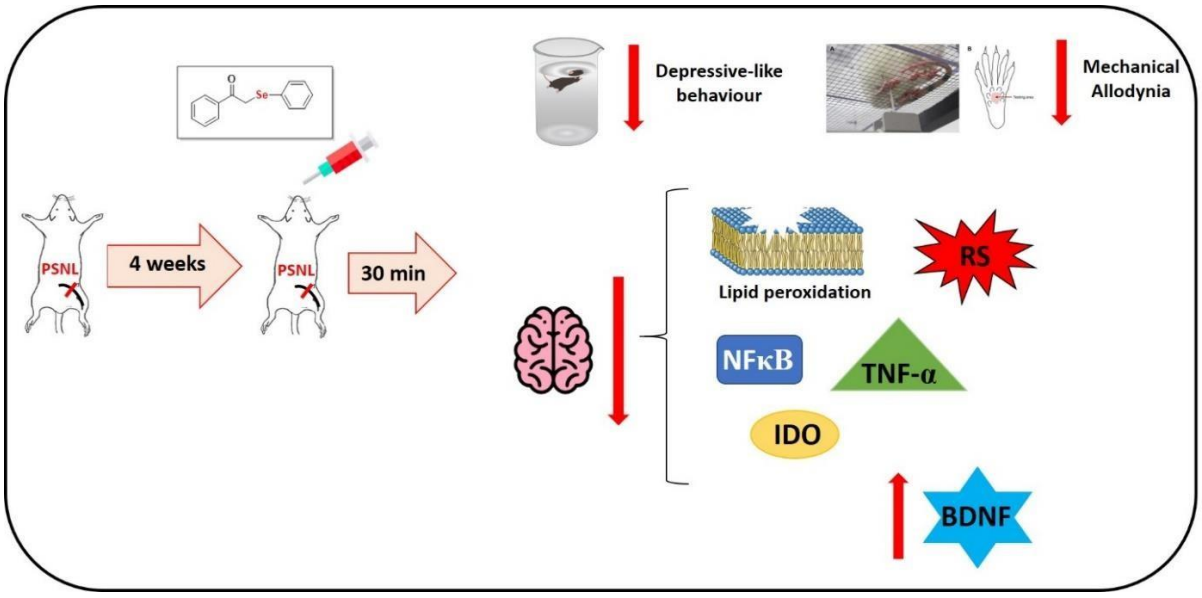
Abstract

Patients suffering from chronic pain usually also have mood disorders such as depression and traditional antidepressants and analgesics have shown limited clinical efficacy. Based on this, this study evaluated the effect of α-(phenylselanyl) acetophenone (PSAP), in the comorbidity of chronic pain and depression induced by partial sciatic nerve ligation (PSNL) in an inflammatory approach. Mice were submitted to PSNL for 4 weeks and treated with PSAP (1 - 50 mg/kg, intragastrically [i.g.]) or imipramine (50 mg/kg, [i.g.]) 30 min before the behavioral tests. Both treatments reversed PSNL-increased pain sensitivity and depressive-like behavior observed in Von-Frey hair and forced swimming tests, respectively. These effects could be mainly associated with an anti-inflammatory action of PSAP which reduced the mRNA levels of pro-inflammatory cytokines, nuclear factor kappa (NF-κB), tumor necrosis factor-α (TNF-α) and indoleamine-2,3-dioxygenase (IDO) in total cortex and hippocampus that were increased by PSNL. PSAP also increased the brain derived neurotrophic factor (BDNF) mRNA levels in hippocampus altered by PSNL. In addition, PSAP reversed the lipid peroxidation and reactive species levels that were increased in cortex and hippocampus in PSNL mice. Considering the potential common mechanisms involved in the comorbidity of inflammation-induced depression and chronic pain. The results found in this study indicate that PSAP could become an interesting molecule to treat dyad pain and depression.

Keywords: antidepressant; analgesic; organoselenium; chronic pain.

Graphical Abstract



1. Introduction

α-(phenylselanyl) acetophenone (PSAP), an organoselenium compound, has been reported to have antinociceptive, antioxidant and antidepressant-like effects in animal models (GERZSON *et al.*, 2012; SOUSA, Fernanda S.S. *et al.*, 2017b). In addition Molecular docking indicated that PSAP could act through selective inhibition of monoamine oxidase A (MAO-A) activity (SOUSA, Fernanda Severo Sabedra; BIRMANN, Paloma Taborda; BALDINOTTI; *et al.*, 2018). More recently, our research group found that PSAP reduces the levels of corticosterone, the major stress hormone, in the plasma of mice undergoing acute restraint stress (ARS). The main mechanism of PSAP in this model is through its antioxidant activity, which may decrease/inhibit the activation of the hypothalamic-pituitary-adrenal axis (SOUSA, Fernanda Severo Sabedra; BIRMANN, Paloma Taborda; BALAGUEZ; *et al.*, 2018). Also, PSAP not present acute and chronic toxicity (CASARIL *et al.*, 2015b; GERZSON *et al.*, 2012). Although PSAP has been reported to be a multi-target compound, detailed characterization of its effects remains largely unknown.

In this sense, it is of great interest to evaluate the mechanisms involved in the effects of PSAP on the dyad of chronic pain and depression. Considering the depression and pain comorbidity in the context of inflammation and the fact that the efficacy of traditional antidepressants and analgesics yields success rates lower than 50%. In this sense it is important the development of novel effective pharmaceutical interventions to treat simultaneously both conditions (KROENKE, Kurt; KREBS; BAIR, 2009). In additional, it is known that 65% of patients with chronic pain are accompanied by depressive disorders (ARNOW *et al.*, 2006; MONROE; HARKNESS, 2011). In fact, previous studies have shown depression-related behavior in rodents subjected to sciatic nerve injury, a well-recognized model for neuropathic pain (FUKUHARA *et al.*, 2012; JESSE, Cristiano R.; WILHELM, Ethel A.; NOGUEIRA, Cristina W., 2010).

The partial sciatic nerve ligation (PSNL) is a rodent model of neuropathic pain that incorporates a less peripheral inflammatory component compared with other peripheral nerve injury models. Thus, is a robust mechanical hypersensitivity and

high responsiveness to analgesic drugs and novel therapies for chronic pain (Crisp et al., 2003; Dowdall et al., 2005; Narita et al., 2005; Gay et al., 2014). After PSNL, the first cytokines formed are interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), which act directly on specific receptors of the sensory neurons and lead to the cascade synthesis of other effectors (GRACE, P. M. *et al.*, 2014). In this sense, the increase of proinflammatory cytokines after PSNL leads to the activation of the enzyme indoleamine 2,3-dioxygenase (IDO) leading to tryptophan (a precursor of serotonin synthesis) depletion (CALABRESE *et al.*, 2014).The depression-like behavior caused by some pro-inflammatory cytokines might also be underlain by their capacity to down regulate hippocampal brain-derivate neurotrophic factor (BDNF), consequently impairing neurogenesis (POSTAL *et al.*, 2016).

Therefore, in the light of reported antioxidant, anti-inflammatory, antinociceptive, antidepressant-like and anxiolytic activities of PSAP, the present study was designed to investigate the possible beneficial effect of PSAP in PSNL-induced neuropathic pain in mice by assessing behavioral, biochemical and gene expression parameters.

2. Materials and methods

Animals

The experiments were conducted using male adult Swiss mice (25-35g) from our own breeding colony. The animals were kept in a separate animal room, on a 12 h light/dark cycle with lights on at 7:00 a.m., at room temperature (22 ± 1 °C) with free access to water and food. All experimental procedures were conducted in accordance with the guidelines of the Committee of Ethics in Research (number 6408-2016). All efforts were made to minimize animal suffering and the number of animals used in the experiments.

Drugs and treatment

α- (phenylselanyl) acetophenone (PSAP) (Fig. 1) was prepared and characterized in the Laboratory of Clean Organic Synthesis (LASOL) according

to the method previously described (VICTORIA, Francine Novack *et al.*, 2009). PSAP was dissolved in canola oil and administered by intragastrically (i.g.) route at dose range of 1-50 mg/kg in a volume of 10 ml/kg. The standard drug used was imipramine (IMI) at the dose of 50 mg/kg (i.g.), in a volume of 10 ml/kg, and it was purchased from a commercial pharmacy. IMI is a tricyclic antidepressant used to treat neurogenic pain. All other chemicals were obtained at the highest available commercial grade.

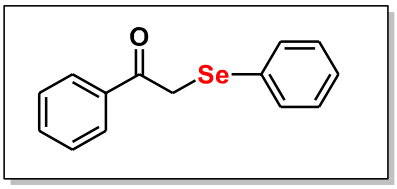


Figure 1. Chemical structure of α-(phenylselanyl) acetophenone (PSAP).

Surgical procedure

PSNL has been shown to produce neuropathic pain and increased depressive-like behavior in rodents (JESSE, Cristiano R.; WILHELM, Ethel A.; NOGUEIRA, Cristina W., 2010; YALCIN *et al.*, 2011). PSNL was performed based on the original description (NARITA, Minoru *et al.*, 2005) under intraperitoneal ketamine/xylazine (150 and 10 mg/kg, respectively) anesthesia. Briefly, the right sciatic nerve was exposed after the incision of skin and blunt separation of the muscle. The sciatic nerve was freed of the adhering tissue gently for about 7 mm, and one ligature (8/0 Ethicon GmbH, Norderstedt, Germany) was made around approximately one-third to one-half of the diameter of the sciatic nerve. In sequence, the incisions were closed with surgical clips and covered with iodine solution. Immediately after surgery, animals were kept in a warm room until full recovery from the anesthesia. In sham procedures, the same steps were performed; the nerve was similarly exposed and freed of the adherent tissue and muscle, but no ligatures were placed. After 4 weeks, the sciatic nerve ligates mice had depressive-like behavior and mechanical hypernociception (Malmberg and Basbaum, 1998), and the well-characterized changes in neuronal and biochemical processing at the central nervous system (OSSIPOV, 2010).

Experimental procedure

At the end of the 4th week after surgery, PSNL mice were treated with vehicle (canola oil) (10 ml/kg), PSAP (1- 50 mg/kg, i.g.) or IMI (50 mg/kg, i.g.). Thirty minutes after treatment, mice were then tested in the open field test, forced swimming test (FST) and with another group of animals, were evaluated mechanical allodynia test. The PSAP pretreatment time was based on a previous study from our research group, which established 30 min as the acute PSAP antidepressant-like and antinociceptive effect (GERZSON *et al.*, 2012; SOUSA, Fernanda S.S. *et al.*, 2017a). For the purpose the effect of PSAP on the neuropathic pain induced by PSNL, a separate group of animals received vehicle (10 ml/kg), PSAP or IMI and was evaluated in the mechanical allodynia test with the Von-Frey filaments (VHF) 30 min after treatment (Fig. 2).

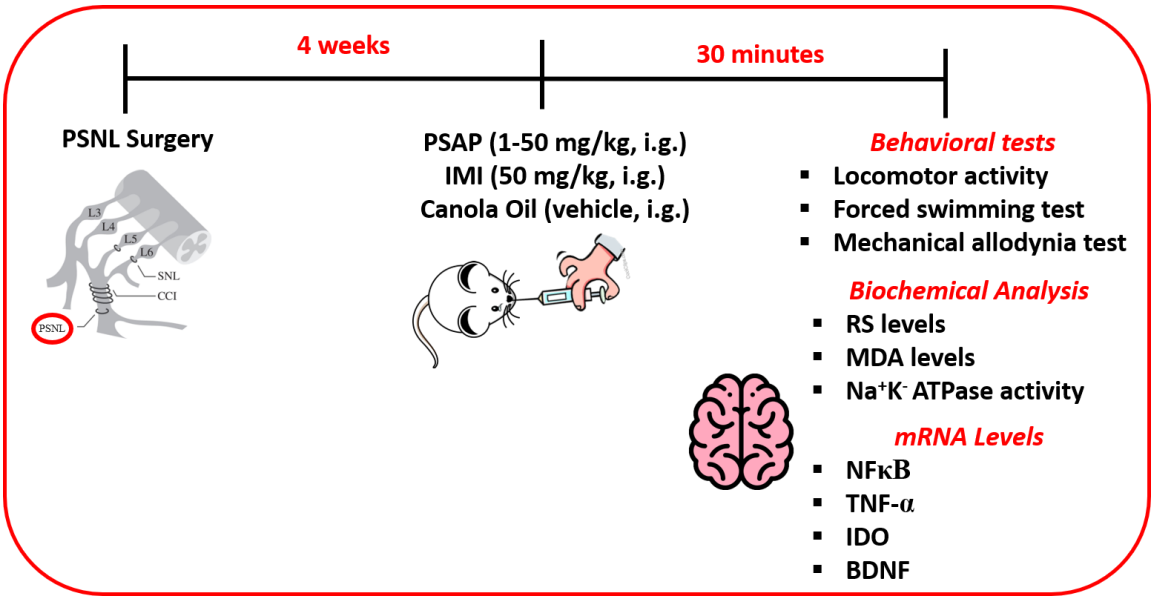


Figure 2. Schematic protocol of the induction of partial sciatic nerve ligation (PSNL). *Abbreviations:* PSAP: α-(phenylselanyl) acetophenone; IMI: Imipramine; i.g.: Intragastrically; PSNL: Partial sciatic nerve ligation; RS: Reactive Species; MDA: malondialdehyde; qRT-PCR: Quantitative real-time polymerase chain reaction.

Behavioral tests

2.5.1. Forced swimming test (FST)

Mice were individually forced to swimming in an open cylindrical container (diameter 10 cm, height 25 cm), containing 19 cm of water (depth) at 25 ± 1 °C; the total amount of time each animal remained immobile during a 4-min session was recorded (in seconds) as immobility time, as described previously (BROCARDO *et al.*, 2008; FREITAS *et al.*, 2010). 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. In addition, the latency time (observed during the first two minutes), which corresponds to the first episode of immobility, was also observed. The increased of latency time and decrease of immobility time is indicative of an antidepressant-like behavior (Porsolt et al., 1977).

Mechanical allodynia test

The mechanical allodynia was measured as described before (BORTALANZA *et al.*, 2002; SOUSA, M. V. P. DE *et al.*, 2014) with a different group of animals. The response frequency of nociception was measured after ten applications (duration of 2 s each) of 1.0 g Von-Frey Hair (VFH, Stoelting, Chicago, IL). Mice were habituated in individual clear Plexiglas boxes (9 x 7 x 11 cm) on an elevated wire mesh platform to allow access to the ventral surface of the hind paws) for 4h. After this time, the animals received PSAP or IMI and after 30 min the animals were submitted to the VFH assay.

Both the ipsilateral (right hind paw) and the contralateral hind paws, were tested in order to evaluate the occurrence, of mirror-image pain. The contralateral allodynia (bilateral allodynia or mirror-image pain) to an injury has been described both in humans and various models of neuropathic and inflammatory pain in animals (HUANG, D.; YU, B., 2010; JAGGI; SINGH, 2011).

Locomotor activity

The open field test (OFT) was made of plywood and surrounded by walls 30 cm in height. The floor of the open field, 45 cm in length and 45 cm in width,

was divided by masking tape markers into 9 squares (3 rows of 3). Each animal was placed individually at the center of the apparatus and observed for 5 min to record the number of segments crossed with the four paws and the number of time rearing on the hindlimbs (WALSH; CUMMINS, 1976).

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

The Total mRNA was extracted in hippocampus and cerebral cortex with different group of mice were performed by using TRIzol (Invitrogen™, Carlsbad, USA) followed by DNase treatment with DNA-free® kit (Ambion™, USA) and mRNA quantification. The cDNA synthesis was performed using 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. 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 $2^{\Delta\Delta CT}$ (Delta-Delta Comparative Threshold) method was used to normalize the fold change in gene expressions. The following genes were analyzed: nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB; fwd 5'-GCT TTC GCA GGA GCA TTA AC-3', rev 5'-CCG AAG CAG GAG CTA TCA AC-3'), tumor necrosis factor-α (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'),'), 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').

Biochemical analysis

Malondialdehyde (MDA) and reactive species (RS), were determined in cerebral cortex and hippocampus with a different group of mice. The cerebral

cortices and hippocampus of different animals were removed, weighed and homogenized in 50 mM Tris–HCl, pH 7.4 (1/4, weight/volume) and centrifuged at 2.400g at 4 °C for 15 min. The low-speed supernatant fraction (S_1) was collected and used for biochemical analyses. For biochemical analyses, we chose the highest effective dose of PSAP at a dose of 50 mg/kg.

Thiobarbituric acid (TBARS) levels

MDA is an end product of the lipid peroxidation. MDA reacts with thiobarbituric acid (TBA) to generate a colored product that can be optically measured at 532 nm (OHKAWA; OHISHI; YAGI, 1979). An aliquot of the S_1 was incubated with 8.1% SDS, 0.8% TBA and acetic acid/HCl (pH 3.4) at 95ºC during 60 min. The absorbance of the samples was measured at 532 nm, and the results were expressed as nmol TBARS/g of tissue.

Reactive species (RS) levels

Quantification of RS levels of cerebral cortex and hippocampus of mice was performed according to Loetchutinat et al., 2005. Briefly, an aliquot of S_1 was 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 RS. The DCF fluorescence intensity emission was recorded at 520 nm (with 480 nm excitation) and RS levels were expressed as arbitrary units (AU) of fluorescence.

Statistical analysis

All experimental results are given as a mean ± standard error of the mean (SEM). Comparisons between experimental and control groups were performed by one-way analysis of variance (ANOVA). When ANOVA revealed a significant effect, the Newman-Keuls 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 Graph Pad Prism version 7.0 for Windows, Graph Pad Software (San Diego, CA, USA).

3. Results

PSAP antidepressant-like effect in PSNL model in mice

A one-way ANOVA revealed a significant effect of PSAP and IMI treatments on latency [$F_{(9,57)}$= 8.27, $p < 0.001$] and immobility time [$F_{(9.57)}$ = 4.2, $p < 0.001$] of PSNL mice in the FST (Fig. 3). PSAP (1 - 50 mg/kg, i.g.) and IMI (50 mg/kg, i.g.) increased the latency for the first immobility episode ($p < 0.001$, Fig. 3A). In addition, total immobility time of PSNL mice was decreased by PSAP (10 and 50 mg/kg) and IMI (50 mg/kg) administration ($p < 0.01$; Fig. 3B).

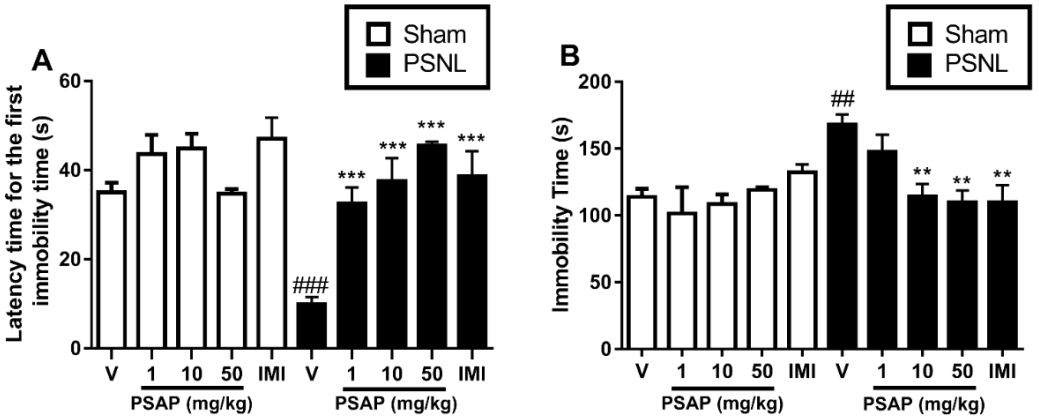


Figure 3. Effects of PSAP (1-50 mg/kg, i.g.) or IMI (50 mg/kg, i.g.) in the forced swimming test in PSNL mice. (A) Latency time to the first immobility episode (s); (B) Immobility time (s). Each column represents the mean ± SEM of 6 animals for group. Statistical analysis was performed by one-way ANOVA followed by the Newman–Keuls test when appropriate. $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ as compared with the sham group, $^{**}p < 0.01$ and $^{***}p < 0.001$ as compared with the PSNL group. *Abbreviations:* V: vehicle; PSAP: α-(phenylselanyl) acetophenone; IMI: imipramine; PSNL: partial sciatic nerve ligation.

PSAP has anti-allodynic effect in PSNL model in mice

Response frequency of VFH stimulation was significantly increased by PSNL in ipsilateral paw when compared to the sham group. PSAP (1-50 mg/kg) reduced the frequency of VFH stimuli when compared to the PSNL [$F_{(9,72)}$= 12.8, $p < 0.001$] group (Fig. 4A). The same was observed with the animals that received

IMI. In addition, the response frequency of contralateral paw was also increased by PSNL, demonstrating the presence of “mirror pain” (Fig. 4B). PSAP or IMI (50 mg/kg) [$F_{(9,72)}$= 4.83, $p < 0.001$] reduced the response frequency to VFH when compared to the PSNL group.

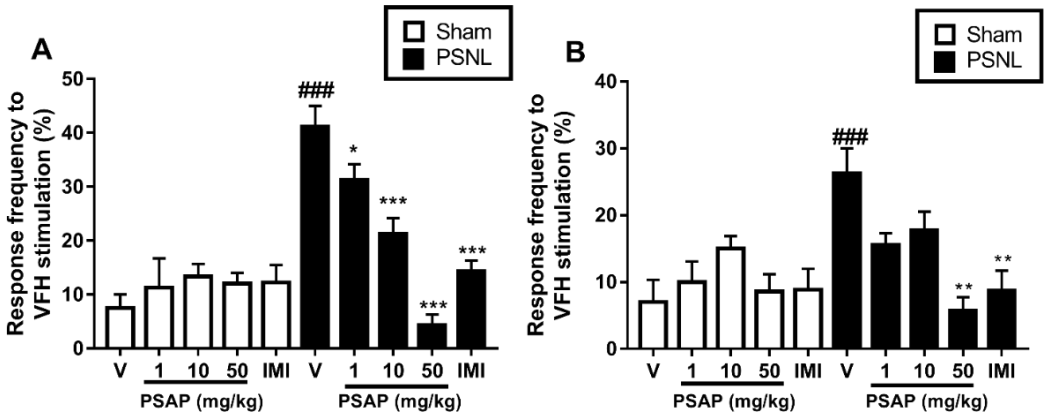


Figure 4. Effect of with PSAP (1-50 mg/kg, i.g.) or IMI (50 mg/kg, i.g.) on the response frequency to VFH stimulation in ipsilateral paw in PSNL mice. Each column represents the mean ± SEM of 8 animals for group. Statistical analysis was performed by one-way ANOVA followed by the Newman–Keuls test when appropriate. $^{###}p < 0.001$ as compared with the sham group, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ as compared with the PSNL group. *Abbreviations:* V: vehicle; PSAP: α-(phenylselanyl) acetophenone; IMI: imipramine; VFH: Von-Frey Hair; PSNL: partial sciatic nerve ligation.

Effect of PSAP in locomotor activity

To avoid the possibility that the increase of latency to immobility, immobility time of mice in the FST and VHF could be due to locomotor impairment, the numbers of crossings and rearings were assessed in the OFT. As depicted in Table 1, the treatments did not produce significant differences in crossings [$F_{(9,57)}$ = 2.11, p = 0.04] and rearing [$F_{(9,57)}$ = 1.07, p = 0.40].

Table1. Effect of administration of PSAP and IMI on behavior parameters in the open field test in mice.

Experimental groups	Number of crossings	Number of rearings
Sham	105.0 ± 8.16	26.0 ± 2.16
Sham + PSAP 1 mg/kg	98.5 ± 7.40	32.5 ± 2.77
Sham + PSAP 10 mg/kg	90.5 ± 9.30	28.0 ± 3.28
Sham + PSAP 50 mg/kg	87.0 ± 4.29	22.5 ± 3.62
Sham + IMI 50 mg/kg	98.0 ± 12.60	26.0 ± 2.66
PSNL	92.0 ± 4.17	25.0 ± 2.01
PSNL + PSAP 1 mg/kg	81.5 ± 9.81	19.0 ± 4.47
PSNL + PSAP 10 mg/kg	71.5 ± 8.24	19.0 ± 4.27
PSNL + PSAP 50 mg/kg	88.0 ± 4.35	22.5 ± 3.38
PSNL + IMI 50 mg/kg	81.5 ± 7.20	22.0 ± 4.98

The effect of treatment with PSAP and IMI in mice behavior in the open-field test. Data presented are mean values ± SEM. *Abbreviations*: PSAP: α-(phenylalanyl) acetophenone; IMI: imipramine; PSNL: partial sciatic nerve ligation.

3.4.4 NFκB, TNF-α, IDO and BDNF levels

PSNL led to an increase in NFκB mRNA levels in the hippocampus (Fig. 5B) when compared with sham group and the treatment with PSAP [$F_{(3,16)} = 6.58$, $p = 0.004$] reversed this effect. On the other hand, PSNL showed no significant difference in cortex [$F_{(3,16)} = 2.06$, $p = 0.15$] (Fig. 5A).

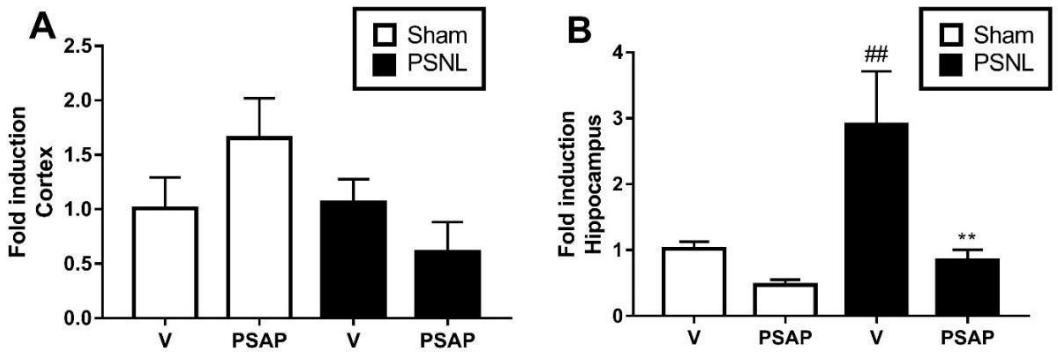


Figure 5. The influence of PSAP (50 mg/kg, i.g.) on NFκB mRNA levels in the cerebral cortex (A) and hippocampus (B) of PSNL mice. Each column represents the mean ± SEM of 4 animals for group. Statistical analysis was performed by one-way ANOVA followed by the Newman–Keuls test when appropriate. $^{\#\#}p < 0.01$ as compared with the sham group and $^{**}p < 0.01$ as compared with the PSNL

group. *Abbreviations:* PSAP: α-(phenylselanyl) acetophenone; PSNL: partial sciatic nerve ligation.

According to Fig. 6, animals that suffered PSNL had an increase in TNF-α mRNA levels in total cortex and hippocampus when compared to the sham group. Treatment with PSAP reduced TNF-α mRNA levels in hippocampus [$F_{(3,16)}$ = 5.14,*p* = 0.01] and in total cortex [$F_{(3,16)}$ = 8.02, *p* = 0.002] when compared to the PSNL group.

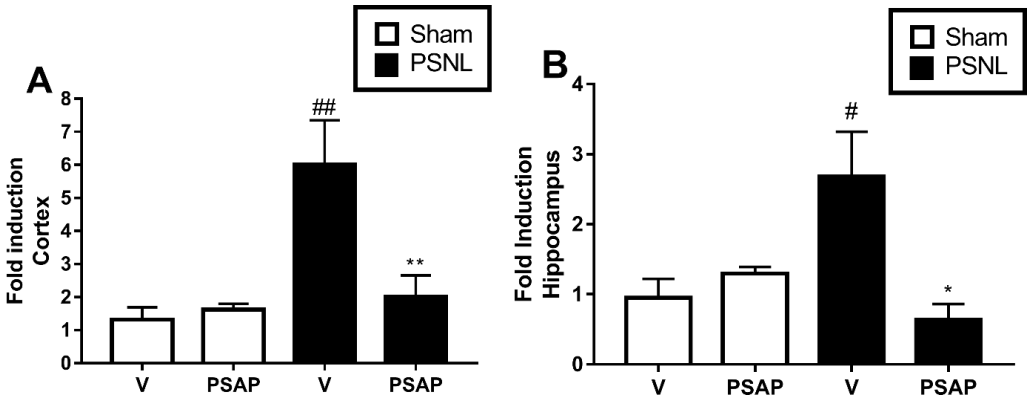


Figure 6. The influence of PSAP (50 mg/kg, i.g.) on TNF-α mRNA levels in the cerebral cortex (A) and hippocampus (B) of PSNL mice. Each column represents the mean ± SEM of 4 animals for group. Statistical analysis was performed by one-way ANOVA followed by the Newman–Keuls test when appropriate. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ as compared with the sham group and $^{*}p < 0.05$ and $^{**}p < 0.01$ as compared with the PSNL group. *Abbreviations:* PSAP: α-(phenylselanyl) acetophenone; PSNL: partial sciatic nerve ligation.

The animals that underwent PSNL increased levels of the IDO mRNA levels in the total cortex and hippocampus (Fig. 7A and B, respectively) when compared with sham group. PSAP treatment reversed these effect in both cortex and hippocampus ([$F_{(3,16)}$ = 10.6, *p* = 0.003] and [$F_{(3,16)}$ = 11.2, *p* = 0.001], respectively).

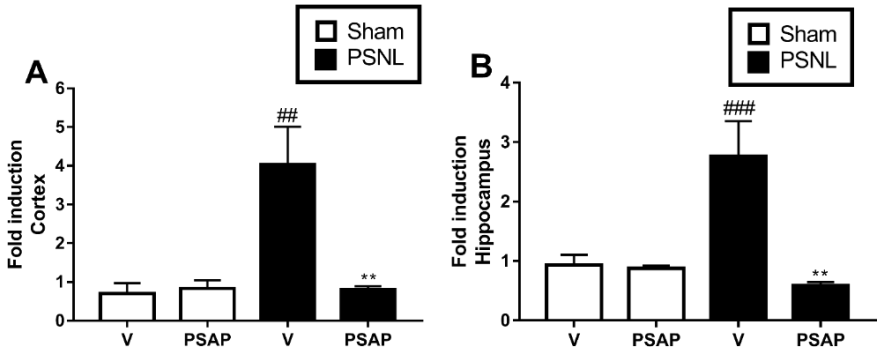


Figure 7. The influence of PSAP (50 mg/kg, i.g.) on IDO mRNA levels in the cerebral cortex (A) and hippocampus (B) of PSNL mice. Each column represents the mean ± SEM of 4 animals for group. Statistical analysis was performed by one-way ANOVA followed by the Newman–Keuls test when appropriate. $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ as compared with the sham group and $^{**}p < 0.01$ as compared with the PSNL group. *Abbreviations:* PSAP: α-(phenylselanyl) acetophenone; PSNL: partial sciatic nerve ligation.

Animals that suffered PSNL had a decrease in BDNF mRNA levels in hippocampus of mice. The acute treatment with PSAP led to an increase in BDNF mRNA levels in hippocampus [$F_{(3,16)} = 5.66$, $p = 0.008$]. No significant changes were observed in BDNF mRNA levels between the groups tested in total cortex [$F_{(3,16)} = 3.23$, $p = 0.05$] (Fig. 8).

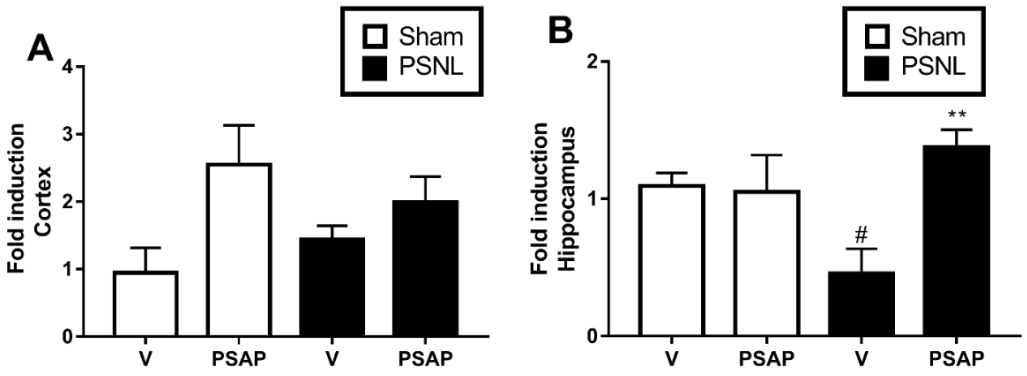


Figure 8. The influence of PSAP (50 mg/kg, i.g.) on BDNF mRNA levels in the cerebral cortex (A) and hippocampus (B) of PSNL mice. Each column represents the mean ± SEM of 4 animals for group. Statistical analysis was performed by one-way ANOVA followed by the Newman–Keuls test when appropriate. $^{\#}p < 0.05$ as compared with the sham group and $^{**}p < 0.01$ as compared with the PSNL

group. *Abbreviations:* PSAP: α-(phenylselanyl) acetophenone; PSNL: partial sciatic nerve ligation.

Biochemical analysis

Thiobarbituric acid (TBARS) levels

The results depicted in Fig. 9 illustrate that PSNL significantly increased the levels of TBARS (an indicative of lipid peroxidation) in both cortex ($p < 0.001$; Fig. 9A) and hippocampus ($p < 0.05$; Fig. 9B). The PSAP and IMI reduced TBARS levels in cortex compared to the PSNL group [$F_{(5,25)} = 9,8$, $p < 0.001$]. However, PSAP did not present the ability to reduced TBARS levels in the hippocampus when compared to the PSNL group [$F_{(5,25)} = 4.08$, $p = 0.009$]. On the other hand, treatment with IMI reduced levels of TBARS in the hippocampus.

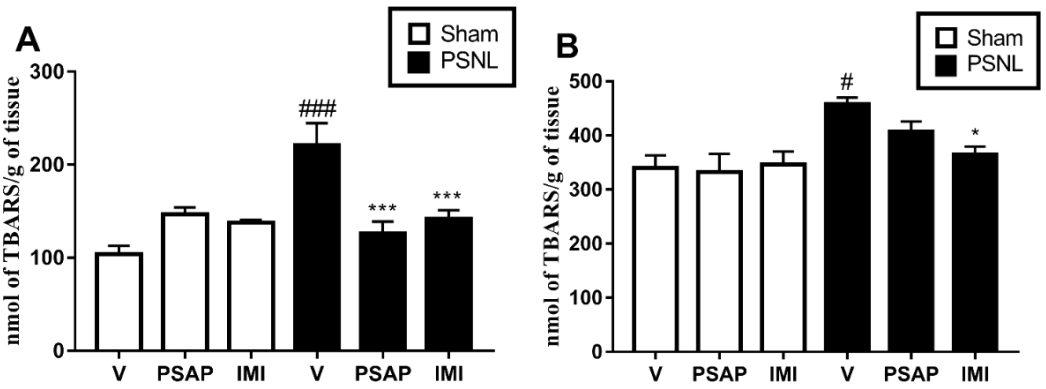


Figure 9. Effects of PSAP (50 mg/kg, i.g.) or IMI (50 mg/kg, i.g.) on TBARS levels in the cerebral cortex (A) and hippocampus (B) of PSNL mice. Each column represents the mean ± SEM of 5 animals for group. Statistical analysis was performed by one-way ANOVA followed by the Newman–Keuls test when appropriate. $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ as compared with the sham group, $^{*}p < 0.05$ and $^{***}p < 0.001$ as compared with the PSNL group. *Abbreviations:* V: vehicle; PSAP: α-(phenylselanyl) acetophenone; IMI: imipramine; MDA: malondialdehyde; PSNL: partial sciatic nerve ligation.

Reactive species (RS) levels

Fig. 10 shows that in cortex ($p < 0.05$) and hippocampus ($p < 0.001$) of mice, after 4 weeks of PSNL challenge, there was a significant enhancement in RS production when compared to sham group. The treatment with PSAP or IMI

reduced alterations in RS production in the cortex [$F_{(5,25)} = 4.22$, $p = 0.007$] (Fig. 10A) and hippocampus [$F_{(5,25)} = 27.3$, $p < 0.001$] (Fig. 10B) when compared with the PSNL group. The treatment of mice with PSAP or IMI alone decreased RS levels in hippocampus, but not in cortex.

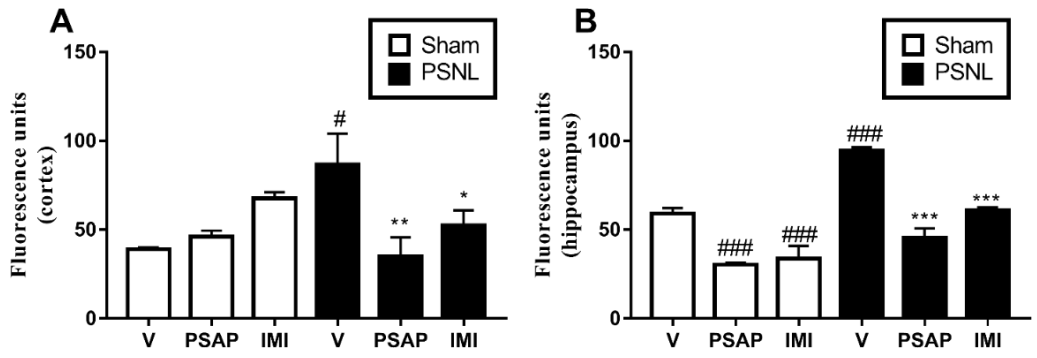


Figure 10. Effects of PSAP (50 mg/kg, i.g.) or IMI (50 mg/kg, i.g.) on formation of reactive species levels in the cerebral cortex (A) and hippocampus (B) of PSNL mice. Each column represents the mean ± SEM of 5 animals for group. Statistical analysis was performed by one-way ANOVA followed by the Newman–Keuls test when appropriate. $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ as compared with the sham group, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ as compared with the PSNL group. *Abbreviations:* V: vehicle; PSAP: α-(phenylselanyl) acetophenone; IMI: imipramine; PSNL: partial sciatic nerve ligation.

4. *Discussion*

The aim of the present study was to evaluate the effect of treatment with PSAP on behavior consequences induced by neuropathic pain in the mouse PSNL model. The main pharmacological finding of this study was that acute PSAP treatments blocked depression-like behavior and mechanical allodynia in PSNL mice. In addition, PSAP did not cause locomotor changes in the open field test. The activity of PSAP appears to be involved with its antioxidant effect and inhibition of the NFκB pathway and consequently decrease of pro-inflammatory cytokines such as TNF-α in hippocampus. PSAP also reduced the IDO mRNA levels in total cortex and hippocampus and increased BDNF mRNA levels in hippocampus. Although we have not determined protein levels, an increase in mRNA levels for NFκB, TNF-α, IDO and BDNF strongly indicates an increase in the expression of these molecules. These findings permit further understanding

the pharmacological properties of PSAP as well as the mechanisms behind its antidepressant-like and anti-allodynic action.

Considering that PSAP has already demonstrated antidepressive-like and antinociceptive effects (GERZSON *et al.*, 2012; SOUSA, Fernanda S.S. *et al.*, 2017a; SOUSA, Fernanda Severo Sabedra; BIRMANN, Paloma Taborda; BALAGUEZ; *et al.*, 2018; SOUSA, Fernanda Severo Sabedra; BIRMANN, Paloma Taborda; BALDINOTTI; *et al.*, 2018) and the constant search for new compounds that influence the treatment of pain and depression, the antidepressive and antiallodynic effects of PSAP in the comorbid pain-depression were evaluated in this study. The depressive-like behavior of mice after PSNL was evaluated through the FST, where the immobility time of mice in an adverse situation is indicative of depression. PSNL increased the immobility time and the latency to immobility and PSAP reversed this effect. In the other hand, the allodynic effect of PSNL was evaluated trough the VFH. VFH stimulation is widely used in pain research (FRUHSTORFER; GROSS, W.; SELBMANN, 2001). The principle of VFH stimulation is to apply a calibrated and graduated force to a sensory field, the rated force being that which is reached at the point when the VFH buckles (LAMBERT; MALLOS; ZAGAMI, 2009). The results of the present study indicated that PSNL in mice produced a significant increase in the response frequency of VFH stimulation of the ipsilateral and contralateral hind paws and the administration of PSAP reversed this effect. These results imply that a single administration of PSAP decreases comorbid depressive-like behavior and mechanical allodynia induced by PSNL in mice.

Neuropathic pain, defined as “pain caused by a lesion or disease of the somatosensory nervous system” (JENSEN *et al.*, 2011), is a specific type of chronic pain that results from disease of the neurons or injury to the peripheral or central nervous system (CNS), and does not primarily signal noxious tissue stimulation, and is often accompanied by depressive symptoms. In the PSNL model, animals developed depression-like behavior 4 weeks after the surgery as evidenced by increased immobility in both FST and tail suspension test (TST) (GAI; BORTOLATTO, Cristiani Folharini; BRÜNING; *et al.*, 2014), suggesting that

the depressive symptoms are manifested only once pain persists along time (PORTA, LA *et al.*, 2016). Damage to the peripheral nerve tissue contributes to activation of neurons and glial cells and releases various pro-inflammatory mediators like interleukins and TNF-α (ASWAR, M. *et al.*, 2014). When activated, microglial cells release neuroinflammatory agents into the synaptic cleft and local astrocytic surface receptors bind to various agents and results in an influx of Ca^{2+}. Microglial cells can cause expression of nitric oxide synthase within the postsynaptic neuron, which freely diffuses through cell membranes and can also induce astrocyte activation and result in Ca^{2+} influx. High levels of intracellular Ca^{2+} result in translocation of NFκB from the cytoplasm to the nucleus of astrocytes, induction of the p38 mitogen-activated protein kinase (p38MAPK) pathway, as well as a dose-dependent release of glutamate. Upon activation, the astrocyte undergoes hypertrophy and increased production of neuroinflammatory agents that are secreted into the synaptic cleft. Astrocyte activation in conjunction with microglial activation significantly depolarizes the neuron increasing its sensitivity and potentiating the neuropathic pain state (MCMAHON; CAFFERTY; MARCHAND, 2005; VALLEJO *et al.*, 2010; WATKINS; MILLIGAN; MAIER, 2001). According to our findings, the reduction of neuropathic pain and consequently depressive-like symptoms by PSAP seems to involve the reduction of TNF-α and NFκB mRNA levels increased by PSNL.

NFkB is a transcription factor required to induce the expression of several inflammatory and immune responses, which can be activated by a variety of pathogenic stimuli, including cytokines, such as IL-1β, IL-6 and TNF-α, growth factors and oxidative stress (MAKAROV, 2001). Accordingly, it has been shown that NF-kB activation plays a critical role in the procession of neuropathic pain and targeting of the NFκB pathway is considered a potential novel approach in treatment of chronic pain mainly because of the stimulus-evoked pro-inflammatory role of NFkB in immune cells (NIEDERBERGER; GEISSLINGER, 2008). In this way, a decrease of NFkB activation could contribute to the pharmacological effects of PSAP after peripheral nerve injury.

PSNL increased the TNF-α levels in hippocampus. The TNF-α is a cytokine that produces different stimuli in various physiological and pathological conditions (HIMMERICH, H. *et al.*, 2008). TNF-α exerts its biological effects mainly by binding to tumour necrosis factor receptor 1 (TNFR1) and receptor 2 (TNFR2), causing activation of complex signalling cascades that mediate different intracellular effects. In the brain, TNFR1 seems to show a constitutive pattern of expression whereas TNFR2 is mainly expressed under stimulatory conditions (KARIN, 2001). TNF-α may underlie the mechanism of depression by an activation of the hypothalamus-pituitary-adrenocortical (HPA) axis, an activation of neuronal serotonin transporters and the stimulation of the IDO (KRISHNADAS; CAVANAGH, 2012).

The activation of IDO by TNF-α leads to tryptophan depletion, the precursor of serotonin synthesis (POSTAL *et al.*, 2016). These findings are in line with we have found in the PSNL model in mice in this study, i.e., an increase in both TNF-a and IDO mRNA levels in total cortex and hippocampus. The depletion of tryptophan due to IDO activation may justify the reduced availability of serotonin and links TNF-α to depression and pain, once serotonin is an important neurotransmitter not only to emotional networks, but also in the nociceptive control through the descending inhibition pathway (BERTHOLD-LOSLEBEN; HIMMERICH, Hubertus, 2008; HIMMERICH, H. *et al.*, 2008). In addition, TNF-α activates the neuronal p38 MAPK, which in turn could activate serotonin transporter (SERT) (BERTHOLD-LOSLEBEN; HIMMERICH, Hubertus, 2008; ZHU, C. Bin; BLAKELY; HEWLETT, 2006). The availability of serotonin in the synaptic cleft is reduced, a feature of depressive patients. Drugs like selective serotonin reuptake inhibitors (SSRI) are used in the therapy of depression because SSRIs lead to recovery from depression via inhibition of SERT (BERTHOLD-LOSLEBEN; HIMMERICH, Hubertus, 2008). Considering this, PSAP reduction of depressive-like and allodynic symptoms induced by PSNL could be due a restorative action in the serotonergic system, through the decrease of TNF-α and IDO mRNA levels in both cortex and hippocampus. However, it is also possible that PSAP causes a direct activation of serotonergic

system, not related to reduction of NFκB, TNF-α and IDO levels. In a study of molecular docking, PSAP showed potential selective inhibition of monoamine oxidase A (MAO-A) activity, an enzyme responsible by serotonin degradation, which in turn could increase serotonin availability (SOUSA, Fernanda Severo Sabedra; BIRMANN, Paloma Taborda; BALDINOTTI; *et al.*, 2018). In addition, Gerzson et al., (2012) showed that the antidepressant-like effect of PSAP was related to the serotonergic receptor 5-HT_{1A}, once WAY 100635, a 5-HT_{1A} receptor antagonist, blocked its effect (Gerzson et al., 2012). Interestingly, in various models of inflammatory or neuropathic pain, numerous studies showed that systemic administration of a highly selective 5-HT_{1A} receptor agonist, befiradol (F-13640), abolished pain behavior (VIGUIER *et al.*, 2013).

PSNL also reduced BDNF mRNA levels in hippocampus. A large consensus has led to the conclusion that depression is associated with BDNF down regulation in brain areas such as the hippocampus and total cortex (CAI; HUANG, S.; HAO, W., 2015), and it has been recently proposed that depression-like behavior caused by some pro-inflammatory cytokines might be underlain by their capacity to down regulate hippocampal BDNF (CALABRESE *et al.*, 2014). BDNF is distributed widely in the sensory and limbic systems and is essential for maintenance of the related functions of pain, memory, and depression/anxiety. BDNF plays an important role in development, neurostructural and synaptic plasticity, including axonal sprouting, and enhancement of neurotransmission (KAFITZ *et al.*, 1999). Deletion of BDNF in adult mice has been reported to produce hyperalgesia and/or depression (HELDT *et al.*, 2007). Thus, there is a relationship between the lack of BDNF and chronic pain associated with depression-like behavior. In this study, PSAP reversed the decrease of BDNF mRNA levels induced by PSNL and this effect may corroborate to antidepressive-like and antillodynic action of PSAP.

The activation of IDO by TNF-α additionally leads to the production of glutamatergic agonists. The role of increased glutamatergic neurotransmission in the pathogenesis of depression remains inconclusive, but it is believed that an activation of the microglia and an increase of the pro-inflammatory cytokines,

increase the release of glutamate and decrease its reuptake by its transporters. Glutamatergic up-regulation can lead to an increase in excitotoxicity and is strongly correlated with the symptoms of dyad pain and depression (MÜLLER; SCHWARZ, M. J., 2007; WICHERS, M. C.; MAES, Michael, 2004; WICHERS, M.; MAES, Michael, 2002). The excitotoxicity in turn could lead to oxidative stress in brain (ATIF; YOUSUF; AGRAWAL, 2008). In accordance, oxidative stress as well as production of free radicals has long been hypothesized to be involved in the pathogenesis of neuropathic pain and depression (NAIK *et al.*, 2006). In this study, PSAP decreased TBARS and RS levels in cerebral cortex and hippocampus, an effect that may be the contributing factor for its role in alleviation neuropathic pain along with depressive-like disorder.

PSAP attenuates the neuropathic pain and depressive-like behaviour induced by PSNL in mice. This effect may be attributed to anti-inflammatory and antioxidant effects of this compound. Considering that neuropathic pain and depression are multi-pathogenic disorders, these results suggest that PSAP might be an attractive therapeutic tool in the development of novel therapies for pain-emotion diseases.

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4.5 Manuscrito 3

Os resultados que fazem parte desta tese de doutorado estão apresentados sob a forma de manuscrito, o qual se encontra assim organizado. Os itens Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas encontram-se no próprio manuscrito. O manuscrito foi submetido na revista **Journal of Psychiatric Research.**

Antidepressant-like and anti-hyperalgesic effects of α-(phenylselanyl) acetophenone after intracerebroventricular tumor necrosis factor-α injection in mice.

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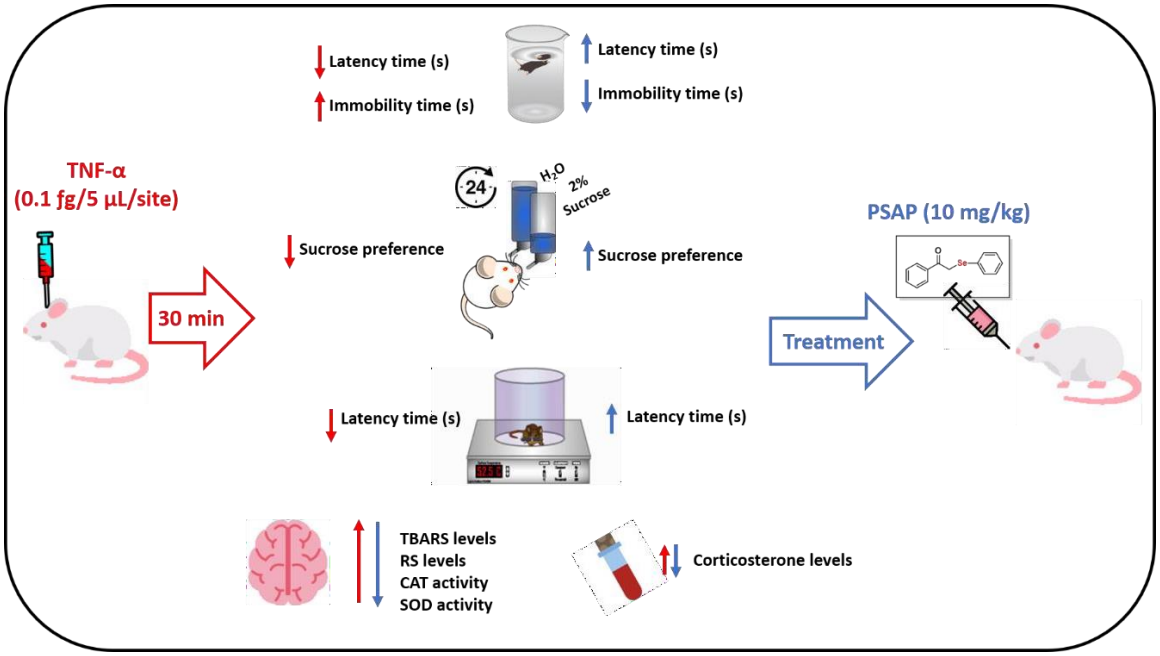
ABSTRACT

The relationship between pain and depression is closely related to the increase of proinflammatory cytokines. The increased of tumor necrosis factor-α (TNF-α),a proinflammatory cytokine, can lead to a depressive-like state and hyperalgesia.In this sense, the objective of this study was to evaluate the antidepressant-like and analgesic, as well as antioxidant effects of acute administration of PSAP after intracerebroventricular (i.c.v.) injection of TNF-α (0.1 ƒg/5 µL/site) in mice. The experiments were conducted using male adult Swiss mice. TNF-α was injected 1 h before the behavioral tests and acute treatment with PSAP (10 mg/kg, intragastrically [i.g.]) was performed 30 min after TNF-α injection. The TNF-α injection reduced the latency time to the first episode of immobility (s) and increased the immobility time in the forced swimming test (FST). Also, TNF-α injection reduced sucrose preference. PSAP reversed the latency time to immobility and decreased the time of immobility in FST and increased the sucrose preference induced by TNF-α. TNF-α also induced a decrease in the latency time to nociceptive response in the hot plate test and PSAP reversed this effect. TNF-α and acute PSAP treatment did not change any locomotor parameter. TNF-α injection increased TBARS, RS levels and CAT, SOD activity in cerebral cortex and hippocampus of mice. The acute treatment with PSAP reversed these parameters. In additional, plasma corticosterone levels were significantly increased in TNF-α mice and PSAP reversed this increase. Considering these results, PSAP could be a promising molecule for the treatment of pain and depression comorbidity.

Keywords: Depression; inflammation; organoselenium; pain; antioxidant.

Graphical Abstract



1. Introduction

It is well known in the primary care setting that both depression and chronic pain result in decreased quality of life, inability to interact with others, and an overall patient disability, even to the point of not being able to function in everyday life (Bair et al., 2004). Often patients who have injuries which result in pain lasting longer than six months (chronic) will go on to develop depressive symptoms. On the other hand, patients with a long history of depressive episodes will often have vague, unclassified pain syndromes (Bair et al., 2003). These two disorders when seen together often exacerbate each other, leading to a vicious cycle that is difficult to treat medically (M'Dahoma et al., 2015; Williams et al., 2004).

It is known that the relationship between pain and depression is closely related to the increase of proinflammatory cytokines, with ligation between the peripheral immune system and the central nervous system (CNS). This communication is mediated largely by cytokines produced by immune cells found both centrally and peripherally. In fact, patients with depression and pain have increased serum levels of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) (Fasick et al., 2015; Himmerich et al., 2008). Both central and peripheral administration of recombinant proinflammatory cytokines in rodents can induce a spectrum of symptoms collectively known as "sickness behavior" that has been linked to symptoms of major depressive disorder (Dantzer, 2004; Kaster et al., 2012).

TNF-α was originally identified as a factor that leads to rapid necrosis of transplantable tumors in mice and now it is considered a proinflammatory cytokine involved in the innate immune response (Clark et al., 2010). In the CNS TNF-α exerts both homeostatic and pathophysiological roles (Montgomery and Bowers, 2012). Studies show that increased of TNF-α levels may lead to a depressive-like state and hyperalgesia (Fasick et al., 2015; Leung and Cahill, 2010). An increase in TNF-α levels may lead to the imbalance between reactive species (RS) and antioxidant defenses, generating the activation of transcription factors and the release of more proinflammatory cytokines. These cytokines may

induce the formation of RS that increase tissue injury and lipid peroxidation (Dantzer et al., 2011). In addition, this increase in proinflammatory cytokines and RS leads to the activation of the hypothalamic-pituitary-adrenal (HPA) axis, which results in increased glucocorticoids in the bloodstream, leading to depressive and pain symptoms (Krishnan e Nestler, 2012). Neuroinflammation and excitotoxicity have key roles as triggers and sustainers of the neurodegenerative process and thus, elevated levels of TNF-α have been found in pain and depression (Pickering et al., 2005).

Antidepressant medications are commonly used to manage both chronic pain and depression, but unfortunately many cases of depression and/or chronic pain are refractory to medical management (Sairanen, 2005; Santarelli et al., 2003). In this sense, synthetic organic selenium compounds have received a lot of attention lately due to several pharmacological properties that they have presented (Birmann et al., 2018; Casaril et al., 2017; Nogueira et al., 2004; Nogueira and Rocha, 2011; Pinto Brod et al., 2016; Sousa et al., 2017).

Selenium is a trace element widely distributed throughout the human body, forming part of the chemical composition of selenoproteins such as glutathione peroxidase, thioredoxin reductase and selenoprotein P, which are known for their important role in protecting the body against lipid peroxidation and oxidative cellular damage (Steinbrenner and Sies, 2013). α-(Phenylselanyl) acetophenone (PSAP) is an organoselenium compound that has antioxidant, antidepressive-like, antinociceptive and anxiolytic-like effect in animal models (Casaril et al., 2015a; Gerzson et al., 2012; Nikolic, 2007; Sousa et al., 2017; Sousa et al., 2018). Also, PSAP have effect in reserpine induced-depressive-like and hyperalgesic behavior. In this sense, PSAP shows interaction with monoamine oxidase A (MAO A) enzyme, evidenced by molecular docking (Sousa et al., 2018). In addition, PSAP not present acute or chronic toxicity (Casaril et al., 2015b; Gerzson et al., 2012).

In view of the described pharmacological properties of PSAP and the need for the search for compounds that have fewer adverse effects and a better efficacy for the treatment of pain and depression associated with neuroinflammation, the

objective of this study was to evaluate the antidepressive-like, anti-anhedonic, analgesic and antioxidant effects of acute administration of PSAP after intracerebroventricular injection of TNF-α in mice.

2. Materials and methods

Chemicals

PSAP (Fig. 1) was prepared and characterized in the Laboratory of Clean Organic Synthesis (LASOL) according to the method previously described (Victoria et al., 2009). PSAP was dissolved in canola oil and administered to mice intragastrically (i.g.) at dose of 10 mg/kg in a volume of 10 ml/kg. TNF-α was dissolved in sterile saline and administered by the intracerebroventricular (i.c.v.) route. The i.c.v. injection of TNF-α
was performed using a “free hand” method, without stereotaxic setup, under light isoflurane anesthesia (just the necessary for the loss of the postural reflex) as originally described by Haley and Mccormick, 1957 and modified by Laursen and Belknap, 1986 with the bregma fissure as a reference. The asepsis of the injection site was carried out using gauze embedded in 70% ethanol. Bregma was found by lightly rubbing the point of the needle over the skull until the suture was felt through the skin. Each animal was gently restrained by hand at the neck with the thumb and forefinger and a 0.4 mm external diameter hypodermic needle, which was linked to a 10 mL Hamilton syringe, was inserted unilaterally 1 mm to bregma and perpendicular to the plane of the skull and no more than 2 mm into the brain (a retainer was attached to the needle). The volume of 5 µL of sterile saline containing TNF-α was injected gradually into the ventricle over 1 min and the needle remained in place for more 30 s in order to avoid the reflux of the TNF-α. All other chemicals were obtained at the highest available commercial grade.

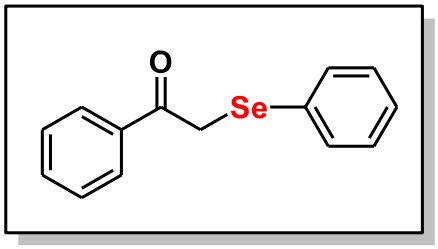


Figure 1. Chemical structure of α-(phenylselanyl) acetophenone (PSAP).

Animals

The experiments were conducted using male adult Swiss mice (25-35g) from our own breeding colony. The animals were kept in a separate animal room, on a 12 h light/dark cycle with lights on at 7:00 a.m., at room temperature (22 ± 1 °C) with free access to water and food. All experimental procedures were conducted in accordance with the guidelines of the Committee on the Care and Use of Experimental Animal Resources of Federal University of Pelotas, Brazil (number CEEA 8328-2017).

Experimental design

The animals were divided in number of 5-7 per group. The mice were randomly assigned into four groups:

(1) Saline (i.c.v.) + canola oil (control group);

(2) Saline (i.c.v.) + PSAP (10 mg/kg, i.g.);

(3) TNF-α (0.1 ƒg/5 µL/site, i.c.v.) + canola oil;

(4) TNF-α (0.1 ƒg/5 µL/site, i.c.v.) + PSAP (10 mg/kg, i.g.);

TNF-α was injected 1 h before the behavioral tests and acute treatment with PSAP was performed 30 min after TNF-α injection. The effective concentration of TNF-α to induce mouse depressive-like and pain behaviors in the tests was 0.1 ƒg/5 µL/site (Kaster et al., 2012). The current choice of PSAP dosage was based on our previous findings (Gerzson et al., 2012; Sousa et al., 2017). The pain-depression effect of TNF-α was evaluated in the open field test (OFT) (locomotor activity), forced swimming test (FST) (depressive-like test),

splash test (depressive-like test), sucrose preference test (anhedonia test) and the hot plate test (hyperalgesic test). Biochemical and oxidative stress tests were assessed in different group of animals (Fig. 2).

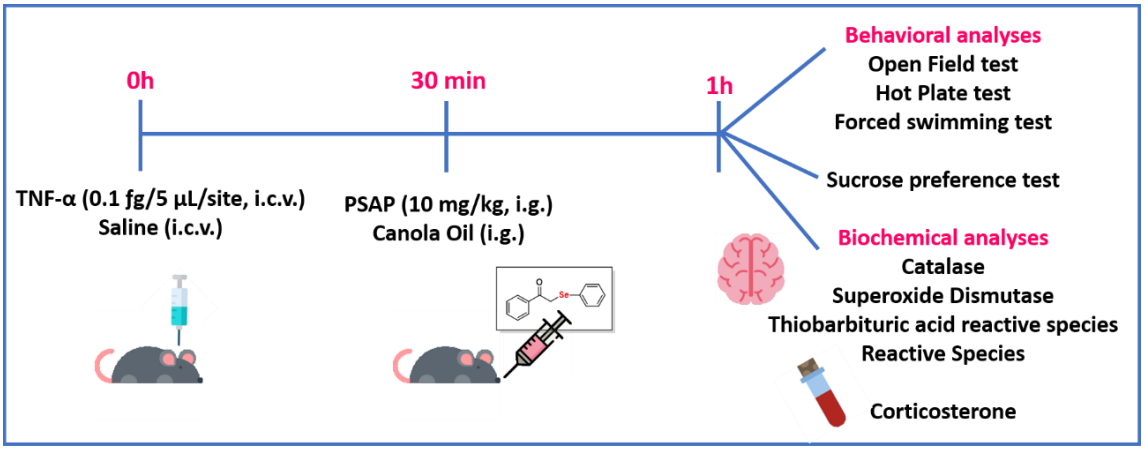


Figure 2. Schematic protocol of the induction of pain and depression dyad through TNF-α administration. *Abbreviations:* PSAP: α-(phenylselanyl) acetophenone; i.c.v: intracerebroventricular; i.g.: intragastric route; FST: forced swim test; CAT: catalase; SOD: superoxide dismutase; TBARS: thiobarbituric acid reactive species; RS: reactive species; TNF-α: tumor necrosis factor-α.

Behavioral tests

Forced swimming test (FST)

Mice were individually forced to swimming in an open cylindrical container (diameter 10 cm, height 25 cm), containing 19 cm of water (depth) at 25 ± 1 °C. The animals were observed for 6 min. In the first two minutes were evaluated the latency time to the first episode of immobility (in seconds [s]), and the other 4 min the time of immobility (s) was evaluated (Brocardo et al., 2008; Freitas et al., 2010). 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 latency and increase of immobility time is indicative of a depressant-like behavior (Porsolt et al., 1977).

Sucrose preference test

Anhedonia was measured, with a different group of animals, by preference for a sucrose solution over water, using a two-bottle free choice method as previously described (Shi et al., 2010; Yue et al., 2017). Briefly, each mouse was presented simultaneously with two bottles (40 ml), one with 2% sucrose solution and the other containing tap water. Mice were then given a free choice between either tap water or 2% sucrose in tap water solution for 24 h. Twenty-four hours later, the amount of water or sucrose consumed by mice were measured through a beaker. Sucrose preference was calculated as sucrose consumption/(sucrose consumption+ water consumption)×100%.

Hot plate test

The hot plate test was carried out according to the method previously described (Derrien et al., 1993)**.** In this test, the animals were placed in a glass cylinder on a heated metal plate maintained at 55 ± 1 °C. The latency of nociceptive responses, such as licking or shaking one of the paws or jumping, was recorded. To avoid damage to the paws of the animals, the time standing on the plate was limited to 45 s.

Open field test (OFT)

The spontaneous locomotor activity of mice was accessed in the OFT (Walsh and Cummins, 1976). The open-field was made of plywood and surrounded by walls 30 cm in height. The floor of the open-field, 40 cm in length and 40 cm in width, was divided by masking tape markers into 9 squares (3 rows of 3). Each animal was placed individually in the center of the arena, and the number of segments crossed (four-paw criterion) and rearings were recorded in a 5 min session.

Oxidative stress analysis

To investigate the effect of PSAP on oxidative stress, thiobarbituric acid reactive species (TBARS) and reactive species (RS) levels, as well as catalase (CAT) and superoxide dismutase (SOD) activities were determined in total

cerebral cortex and hippocampus of mice. The cortices and hippocampus of different animals were removed, weighed and homogenized in 50 mM Tris–HCl, pH 7.4 (1/4, weight/volume), and centrifuged at 2.400g at 4 °C for 15 min. The low-speed supernatant fraction (S_1) was collected and used for oxidative stress analyses.

Thiobarbituric acid reactive species (TBARS) levels

MDA is an end product of the lipid peroxidation. MDA reacts with thiobarbituric acid (TBA) to generate a colored product that can be optically measured at 532 nm (Ohkawa et al., 1979). An aliquot of the S_1 (10 µL) was incubated with 8.1% sodium dodecyl sulfate (SDS), 0.8% TBA and acetic acid/HCl (pH 3.4) at 95ºC during 60 min. The absorbance of the samples was measured at 532 nm, and the results were expressed as nmol TBARS/g tissue.

Reactive species (RS) levels

Quantification of RS levels of cerebral cortex and hippocampus of mice was performed according to Loetchutinat et al., 2005. Briefly, an aliquot of S_1 (10 µL) was 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 RS. The DCF fluorescence intensity emission was recorded at 520 nm (with 480 nm excitation) and RS levels were expressed as arbitrary units (AU) of fluorescence.

Catalase (CAT) activity

CAT is an enzymatic antioxidant defense that is involved in protecting against the injurious effects of reactive species. CAT activity was assayed spectrophotometrically by the method of Beers and Sizer, 1951, which involves monitoring the consumption of H_2O_2 in the cerebral cortex and hippocampus (S_1) presence at 240 nm. Enzymatic reaction was initiated by adding an aliquot of 20 µl of S_1 and the substrate (H_2O_2) to a concentration of 0.3 mM in a medium containing 50 mM phosphate buffer, pH 7.0. The enzymatic activity was

expressed in international units (IU) per milligram of protein (1 IU decomposes1 µmol of H_2O_2 per min at pH 7 at 25 °C).

Superoxide Dismutase (SOD) activity

SOD is an antioxidant enzyme that is involved in protecting against the injurious effects of oxidative stress. SOD activity was assayed spectrophotometrically as described by Misra and Fridovich, 1972. This method is based on the capacity of SOD in inhibiting autoxidation of epinephrine to epinechrome. The color reaction was measured at 480 nm. Aliquots of S1 were added in a 50 mM Na_2CO_3 buffer pH 10.3 and the enzymatic reaction was initiated by adding epinephrine. One unit of SOD was defined as the amount of enzyme required to inhibit the rate of epinephrine auto oxidation by 50% at 26 °C. The enzymatic activity was expressed as U/mg protein.

Corticosterone level in plasma

Determination of plasma corticosterone levels was performed according to Zenker, 1957 with a different group of animals. For the collection of blood plasma, heparin was used as anticoagulant, the mice were euthanized by isoflurane inhalation and soon after cardiac puncture was performed. For plasma separation, the blood was centrifuged at 4.000 rotation per minute (RPM) for 10 min at 4°C. 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 2500 rpm) and incubated at room temperature for 2 h. Fluorescence intensity emission, corresponding to plasma corticosterone levels, was recorded at Ex: 247, EM: 540 and corticosterone levels were expressed as ng/ml.

Statistical analysis

All experimental results are given as a mean ± standard error of the mean (SEM). Behavioural and neurochemical comparisons between experimental and control groups were performed by one-way analysis of variance (ANOVA). When

ANOVA revealed a significant, the Newman-Keuls 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 Graph Pad Prism version 7.0 for Windows (San Diego, CA, USA).

3. Results

The effect of PSAP on depression-like behavior induced by TNF-α in mice

Fig. 3 shows the results of FST performed shortly after 1h of TNF-α injection and 30 minutes of treatment with PSAP. Observing Fig. 3A, it is possible to verify that the mice submitted to TNF-α injection had a decrease in the latency to the first episode of immobility when compared with the control group. Treatment with PSAP reverted this parameter, increasing the immobility time of animals that suffered TNF-α injection [$F_{(3,24)} = 29.6$ ($P < 0.001$)] . The results depicted in Fig. 3B show that TNF-α injection increased the immobility time of mice and the treatment with PSAP reversed this effect [$F_{(3,24)} = 23.3$ ($P < 0.001$)].

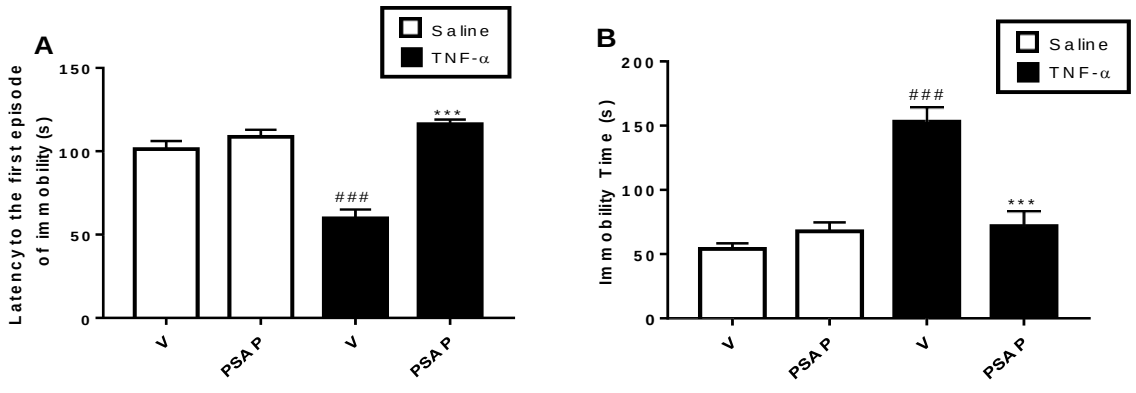


Figure 3. Effects of PSAP (10 mg/kg, i.g.) on active behaviors in the forced swimming test in TNF-α mice. (A) Latency time to the first episode of immobility (s); (B) immobility time (s). Each column represents the mean ± SEM. Statistical analysis was performed by one-way ANOVA followed by the Newman–Keuls test when appropriate. $^{###}P < 0.001$ as compared with the control group, $^{***}P < 0.001$

as compared with the TNF-α group. *Abbreviations:* PSAP: α-(phenylselanyl) acetophenone; V: vehicle; TNF-α: tumor necrosis factor-α.

Fig. 4 shows that TNF-α injection reduced sucrose preference of mice when compared with control group, and the treatment with PSAP increased sucrose consumption [$F_{(3,24)}$ = 46.0 (P < 0.001)] in the sucrose preference test.

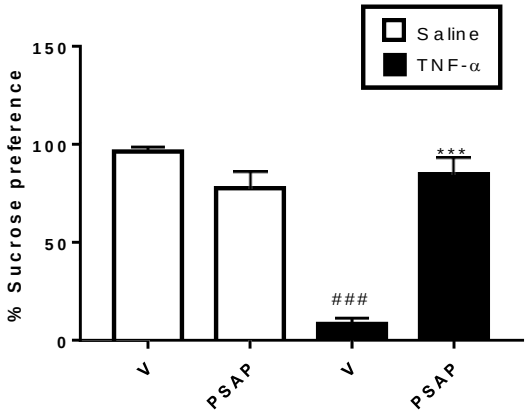


Figure 4. Effects of PSAP (10 mg/kg, i.g.) on the sucrose preference test in TNF-α mice. Each column represents the mean ± SEM. Statistical analysis was performed by one-way ANOVA followed by the Newman–Keuls test when appropriate. ###P < 0.001 as compared with the control group and ***P < 0.001 as compared with the TNF-α group. *Abbreviations:* V: vehicle; PSAP: α-(phenylselanyl) acetophenone; TNF-α: tumor necrosis factor-α.

Effects of PSAP on TNF-α induced hyperalgesia in mice

With the purpose of evaluating the PSAP effects on thermal hyperalgesia, the hot plate test was carried out (Fig. 5). The animals that received TNF-α had a decrease in the latency time to nociceptive response when compared with the control group and PSAP reversed the nociceptive effect of TNF-α [$F_{(3,24)}$ = 37.6 (P < 0.001)] (Fig. 5).

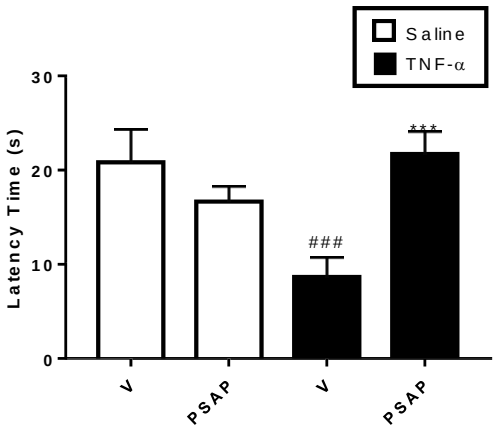


Figure 5. Effects of PSAP (10 mg/kg, i.g.) on the response latency to thermal stimuli in TNF-α mice on the hot plate test. Each column represents the mean ± SEM. Statistical analysis was performed by one-way ANOVA followed by the Newman–Keuls test when appropriate. $^{###}P < 0.001$ as compared with the control group and $^{***}P < 0.001$ as compared with the TNF-α group. *Abbreviations:* V: vehicle; PSAP: α-(phenylselanyl) acetophenone; TNF-α: tumor necrosis factor-α.

Effects of PSAP on TNF-α induced locomotor activity in mice

The injection of TNF-α and acute PSAP treatment did not change any locomotor parameter evaluated in the OFT (Table 1).

Table 1. Effects of administration of PSAP and TNF-α on locomotor and exploratory behavior parameters in the OFT in mice.

Experimental groups	Number of crossings	Number of rearings
Vehicle (Saline + Canola oil)	105.0 ± 8.46	46.0 ± 3.93
PSAP (10 mg/kg)	91.0 ± 12.10	28.0 ± 4.02
TNF-α	125.0 ± 8.48	49.0 ± 4.25
TNF-α + PSAP (10 mg/kg)	86.5 ± 10.30	28.0 ± 3.37

The effect of treatment with PSAP on behavior of mice in the open-field test. Data presented are mean values ± SEM. Results are expressed as mean ± SEM. The 5-6 animals per group. *Abbreviations*: PSAP: α-(phenylalanyl) acetophenone; TNF-α: tumor necrosis factor-α.

Effect of PSAP on oxidative stress parameters

TBARS levels were significantly increased in the cerebral cortex and hippocampus of TNF-α mice as compared with the control group (Fig. 6). Treatment with PSAP reversed the effect of TNF-α in both cerebral cortex [$F_{(3,19)}$ = 13.8 (P < 0.001)] (Fig. 6A) and hippocampus [$F_{(3,19)}$ = 10.2 (P < 0.001)] (Fig. 6B).

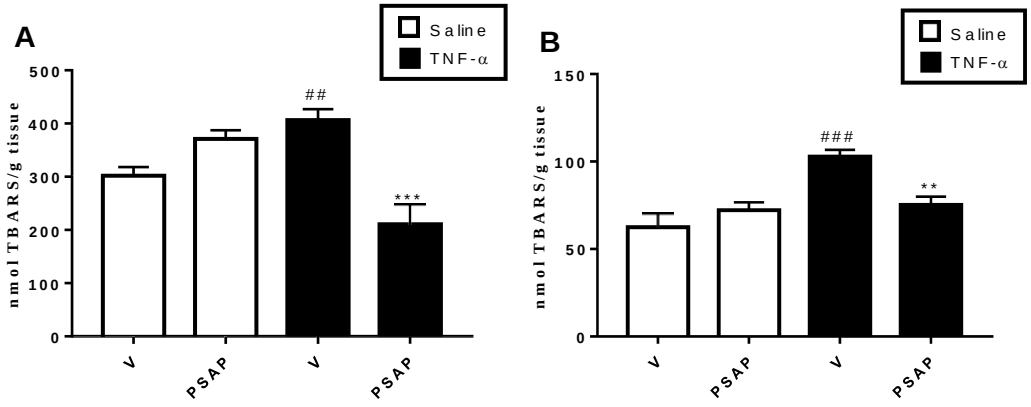


Figure 6. Effects of PSAP (10 mg/kg, i.g.) on lipid peroxidation levels in the cerebral cortex (A) and hippocampus (B) of TNF-α mice. Each column represents the mean ± SEM. of 5-6 animals for group. Statistical analysis was performed by one-way ANOVA followed by the Newman–Keuls test when appropriate. $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ as compared with the control group, $^{**}P < 0.01$ and $^{***}P < 0.001$ as compared with the TNF-α group. *Abbreviations:* V: vehicle; PSAP: α-(phenylselanyl) acetophenone; MDA: malondialdehyde; TNF-α: tumor necrosis factor-α.

Fig. 7 shows that in cortex and hippocampus, after 1h of TNF-α challenge there was a significant enhancement in RS production when compared to control animals. The treatment with PSAP reversed alterations in RS production in the cerebral cortex [$F_{(3,19)}$ = 14.2, P < 0.001] (Fig. 7A) and hippocampus [$F_{(3,19)}$ = 21.4, P < 0.001] (Fig. 7B) induced by TNF-α group.

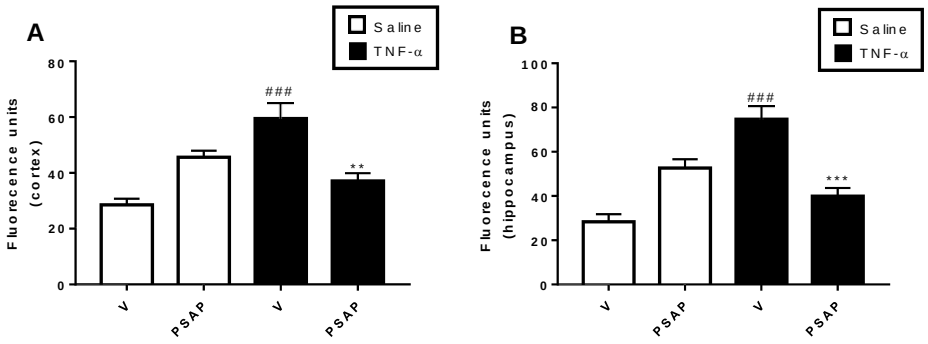


Figure 7. Effects of PSAP (10 mg/kg, i.g.) on formation of reactive species levels in the cerebral cortex (A) and hippocampus (B) of TNF-α mice. Each column represents the mean ± SEM. of 5-6 animals for group. Statistical analysis was performed by one-way ANOVA followed by the Newman–Keuls test when appropriate. $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ as compared with the control group, $^{**}P < 0.01$ and $^{***}P < 0.001$ as compared with the TNF-α group. *Abbreviations:* V: vehicle; PSAP: α-(phenylselanyl) acetophenone; MDA: malondialdehyde; TNF-α: tumor necrosis factor-α.

The CAT activity was significantly increased by TNF-α in the cerebral cortex and hippocampus of mice as compared with control group (Fig 8A and 8B). PSAP treatment reversed the increase of CAT activity in both cerebral cortex [$F_{(3,19)} = 19.9$ ($P < 0.001$)] and hippocampus [$F_{(3.19)} = 16.4$ ($P < 0.001$)].

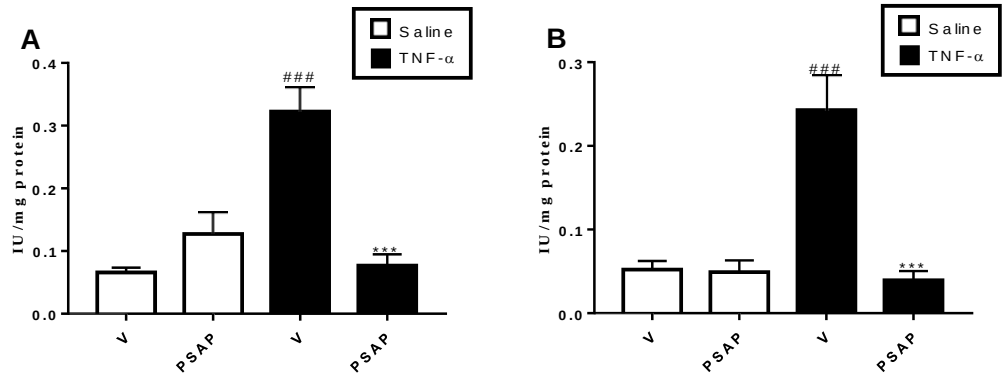


Figure 8. Effects of PSAP (10 mg/kg, i.g.) on CAT activity in the cerebral cortex (A) and hippocampus (B) of TNF-α mice. The CAT activity is expressed as IU/mg protein. Each column represents the mean ± SEM of 5-6 animals for group. Statistical analysis was performed by one-way ANOVA followed by the Newman–Keuls test when appropriate. $^{\#\#\#}P < 0.001$ as compared with the control group, $^{***}P < 0.001$ as compared with the TNF-α group. *Abbreviations:* V: vehicle;

PSAP: α-(phenylselanyl) acetophenone; TNF-α: tumor necrosis factor-α; CAT:catalase.

Through the results shown in Fig. 9 we can verify that the injection of TNF-α increases the activity of the SOD enzyme in cerebral cortex and hippocampus when compared with the control animals. PSAP treatment reduces SOD enzyme activity in cerebral cortex $F_{(3,19)} = 74.7$ ($P < 0.001$)] and hippocampus $F_{(3,19)} = 40.6$ ($P < 0.001$)] when compared with the animals receiving TNF-α.

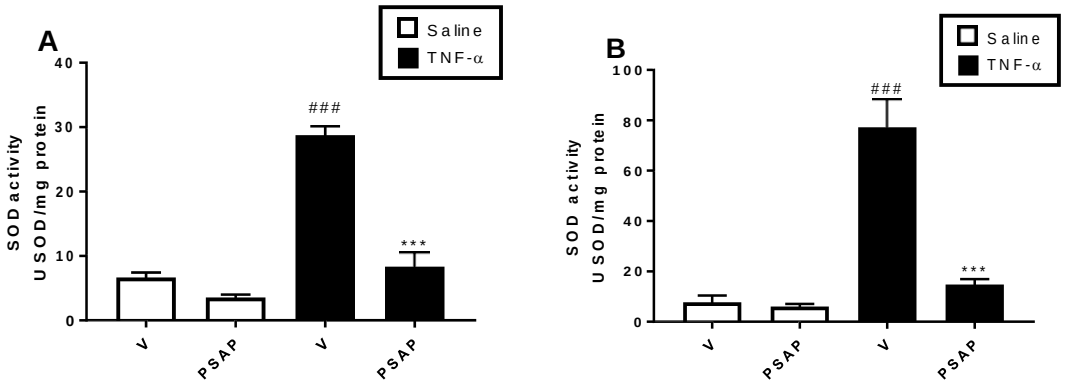


Figure 9. Effects of PSAP (10 mg/kg, i.g.) on SOD activity in the cerebral cortex (A) and hippocampus (B) of TNF-α mice. The SOD activity is expressed as U SOD/mg protein. Each column represents the mean ± SEM of 5-6 animals for group. Statistical analysis was performed by one-way ANOVA followed by the Newman–Keuls test when appropriate. ###$P < 0.001$ as compared with the control group, ***$P < 0.001$ as compared with the TNF-α group. *Abbreviations:* V: vehicle; PSAP: α-(phenylselanyl) acetophenone; TNF-α: tumor necrosis factor-α; SOD: superoxide dismutase.

3.4 Corticosterone levels in plasma

Plasma corticosterone levels were significantly increased in TNF-α mice when compared with control group (Fig. 10). PSAP administered after TNF-α reversed this increase [$F_{(5,19)} = 7.88$, $P = 0.002$].

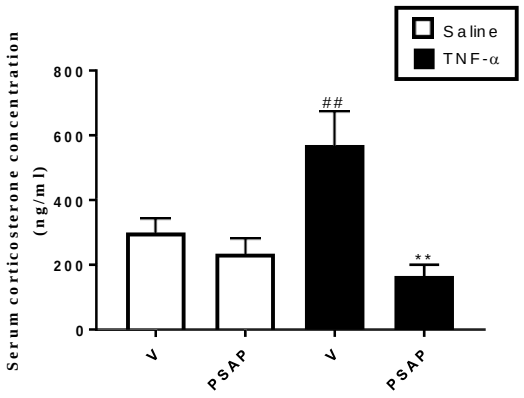


Figure 10. Effects of PSAP (10 mg/kg, i.g.) on the corticosterone levels in plasma. Each column represents the mean ± SEM of 5-6 animals for group. Statistical analysis was performed by one-way ANOVA followed by the Newman–Keuls test when appropriate. $^{\#\#}P < 0.01$ as compared with the control group, $^{**}P < 0.01$ as compared with the TNF-α group. *Abbreviations:* V: vehicle; PSAP: α-(phenylselanyl) acetophenone; TNF-α: tumor necrosis factor-α.

4. Discussion

This study demonstrated that acute administration of PSAP exerts antidepressant-like and anti-hyperalgesic effects in the TNF-α model in mice. PSAP also reduced lipid peroxidation, RS levels and CAT and SOD activities in cerebral cortex and hippocampus, as well as plasma corticosterone levels after TNF-α injection.

The antidepressant-like effect of PSAP was observed in FST, is a test broadly employed as a behavioral instrument for screening of antidepressant drugs (Cryan et al., 2002). In this assay, the TNF-α decreased the latency time for the first immobility time and increased the immobility time. The treatment with PSAP reversed these parameters in the FST in mice. In addition, the TNF-α induced anhedonia in mice, characterized by decreased sweet solution preference. Anhedonia is a state of depressive patients that includes reduced motivation or ability to experience pleasure (Pollak and Yirmiya, 2002; Yirmiya, 1996). PSAP administration reversed the anhedonia effect in TNF-α model in mice.

In the same sense, the hot plate test produces a non-inflammatory nociception and that is a good model to investigate the central effect of analgesic drugs with

supraspinally integrated responses (Oliveira et al., 2008). Also, the hot plate test produces, at constant temperature, two kinds of behavioral response, which are paw licking and jumping. Both of these are considered to be supraspinally integrated responses (Chapman et al., 1985). In line with these results, PSAP reduced the nociceptive behavior from the thermal stimulus in the hot-plate test, suggesting central actions of these compounds in the TNF-α model.

The pain and depression comorbid model were induced through i.c.v. injection of TNF-α in mice. The TNF-α is one of the proinflammatory cytokines that playsa significant role in mood regulation (Reynolds et al., 2004). Pro-inflammatory cytokines, including TNF-α, can activate the adrenal pituitary axis (HPA) (Bernardini et al., 2015), increase the activation of neuronal serotonin transporters (Malynn et al., 2013) and stimulate the enzyme indoleamine 2,3-dioxygenase (IDO), which in turn attenuates the brain conversion of tryptophan, a precursor of serotonin synthesis, to into kynurenine pathway (Maes, 1995). The decrease in the availability of tryptophan for the production of serotonin causes it to reduce the levels of this neurotransmitter in the synaptic cleft leading to episodes of pain and depression (Müller and Schwarz, 2007; Wichers and Maes, 2002, 2004). Through the results demonstrated in this study, we confirmed that the i.c.v. injection of TNF-αled to an increase in the depressive-like and the hyperalgesia behavior in mice.

The TNF-α injection i.c.v activates the immune/inflammatory system. This cytokine is active at the level of the hypothalamus and hippocampus and activate afferent autonomic nerve pathways involved in transducing cytokine signals to the central nerve system (CNS) (Blackburn-Munro, 2001; Miller et al., 2009). Increased TNF-α levels in the hippocampus decreases the hippocampal norepinephrine (NE) levels and decreases brain derivate neurotrophic factor (BDNF), effects involved in the development of chronic pain and depression (Reynolds et al., 2005). TNF-α injection reduces both the number and the function of glucocorticoid receptors and plays a role in altering the negative feedback from the hippocampus. Loss of negative feedback control of glucocorticoid production is responsible for the continual increase in cytokine activity and in adrenal

medulla production of epinephrine and NE, which may increase the production of pro-inflammatory cytokine by macrophages (Blackburn-Munro, 2001; Miller et al., 2009). Depression/pain-induced sympathetic outflow similarly influences cytokine production from immune/inflammatory cells. Taken together, dysregulation of the HPA axis and an unrestrained immune response are related to chronic pain and depression (Fasick et al., 2015; Maletic, 2009). As described, TNF-α induces an excessive activation in HPA, which increases corticosterone levels in the blood plasma of mice (Bernardini et al., 2015). The same was observed in the present study and the treatment with PSAP decreased plasma corticosterone levels altered by TNF-α injection in mice, which could contribute to its pharmacological effects.

TNF-α is reported to stimulate RS production by several mechanisms, including its direct toxic phenomena and its effects on mitochondrial function (Fernández-Checa et al., 1997; Schulze-Osthoff et al., 1992). RS, sometimes referred to as free radicals, describes a number of molecules, including chemical species with one unpaired electron, derived from the metabolism of molecular oxygen. RS are formed as natural byproducts of the normal metabolism of oxygen, nitrogen and are involved in a variety of cellular processes from cell proliferation to cell adaptation to hypoxia, from apoptosis to carcinogenesis, to maintain or reestablish redox homeostasis, acting as intracellular second messengers or modulating signal transduction pathways (Chandel and Schumacker, 2000; Dröge, 2002). Under physiological conditions, the deleterious effects of RS are minimized by antioxidant defense mechanisms that prevent their formation in excess, act as scavengers or repair the resultant damage. Such defense mechanisms involve a strong antioxidant system including SOD, glutathione peroxidase, CAT and a variety of DNA-repairing enzymes (Kowaltowski et al., 2009). Uncontrolled RS production and/or decreased antioxidant activity, however, results in a deleterious state called oxidative stress.

In accord with this, in the present study, the TNF-α model increased the lipid peroxidation, RS levels and CAT and SOD activities in cerebral cortex and hippocampus of mice and these are probably some of the factors that are

involved in the pathophysiology of TNF-α induced pain and depression in the present study. PSAP reversed these alterations. The same was observed in a study carried out by Gerzson et al., 2012, where PSAP showed in vitro antioxidant properties in four test systems (DPPH, ABTS, FRAP and inhibition of lipid peroxidation). According with this, PSAP has a potent antioxidant activity and protects against lipid peroxidation and these effects could be related to its pharmacological action in the comorbid pain and depression induced by TNF-α.

Therefore, the results set of the present study demonstrated that PSAP significantly attenuates TNF-α-induced depressive-like behavior and hyperalgesia in mice. PSAP reduced plasmatic corticosterone levels and reduced parameters of oxidative stress. Both mechanisms are closely correlated with dyad pain and depression. Considering these results, this organoselenium compound could be a new interesting approach in the pharmacological intervention of pain-depression comorbidity.

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5. Discussão

A PSAP pertence a uma classe de compostos orgânicos de selênio com diversas propriedades farmacológicas (NOGUEIRA e ROCHA, 2004), dentre elas efeito antinociceptivo e atividade do tipo antidepressiva demonstrados em estudos previamente publicados (GERZSON *et al.*, 2012; SOUSA *et al.*, 2017a). O efeito antinociceptivo foi demonstrado em modelos químicos de nocicepção em camundongos, como o teste da injeção de formalina e glutamato na pata. No teste de indução da nocicepção induzido com glutamato a pré-administração de antagonistas monoaminérgicos. Neste sentido, esses dados demonstraram o envolvimento dos receptores dopaminérgicos e noradrenérgicos no efeito antinociceptivo da PSAP (SOUSA *et al.*, 2017a).

Por outro lado, o efeito do tipo antidepressivo da PSAP foi demonstrado no TNF e no TSC em camundongos. A pré-administração do antagonista de receptor serotoninérgico 5-HT_{1A}, bloqueou a redução do tempo de imobilidade dos animais induzida pelo PSAP no TSC, demonstrando assim que o sistema serotoninérgico está envolvido no efeito do tipo antidepressivo da PSAP (GERZSON *et al.*, 2012).

Outro estudo realizado por nosso grupo de pesquisa demonstrou que tanto o tratamento agudo quanto crônico com PSAP não causou genotoxicidade no ensaio com leucócitos de camundongos. Neste estudo também avaliou-se as enzimas marcadores de danos toxicológicos como: ALT, AST e creatinina, confirmando que este composto não apresenta toxicidade nas doses avaliadas em camundongos (CASARIL *et al.*, 2015b). Devido aos resultados obtidos nestes trabalhos, a PSAP foi escolhida para continuar os estudos e elucidar melhor quais são seus mecanismos de ação envolvidos em seu efeito do tipo-antidepressivo e antinociceptivo em camundongos.

Neste sentido, os resultados do **artigo 1** demonstraram que a PSAP foi eficaz em reverter o comportamento do tipo depressivo e a hiperalgesia induzidas pela reserpina, um depletor de monoaminas. Acredita-se que este composto restaureos níveis das monoaminas na fenda sináptica devido a sua propriedade antioxidante e possível efeito inibitório seletivo sobre a atividade da enzima

MAO-A. Como já descrito anteriormente, a PSAP apresenta uma ação antinociceptiva bem elucidada e o efeito do tipo-antidepressivo envolve a modulação do sistema monoaminérgico e parece não causar toxicidade quando usado em doses terapêuticas (GERZSON *et al.*, 2012).

Neste mesmo sentido, para avaliar um modelo da comorbidade dor, ansiedade e depressão na ausência de um indutor químico, foi utilizado o modelo de EAR conforme descrito no **artigo 2**. Estudos apontam que o eixo HPA é ativado por uma ampla variedade de estímulos estressantes, como o EAR, os quais resultam no aumento de glicocorticoides na corrente sanguínea (FREITAS *et al.*, 2014). Assim sendo, foi possível verificar que a PSAP reduz o aumento da corticosterona no plasma sanguíneo de camundongos submetidos ao EAR. Os resultados demonstraram também, que o EAR aumentou os níveis de ER, ON e de MDA, e o tratamento com a PSAP diminuiu estes níveis em córtex cerebral e hipocampo de camundongos. Desta forma, podemos concluir que a PSAP tem atividade do tipo-antidepressiva, tipo-ansiolítica, anti-hiperalgésica com o envolvimento do sistema antioxidante e do eixo HPA.

O **manuscrito 1,** também foi desenvolvido com o intuito de induzir a comorbidade dor, depressão e ansiedade através da administração de uma endotoxina, o LPS. Sabe-se que o desenvolvimento de transtornos depressivos e suas comorbidades são acompanhados pela ativação de vias imuno-inflamatórias que consistem no sistema imune inato e no processo inflamatório (ALEXANDER; RIETSCHEL, 2001; ZHU, L. *et al.*, 2015). Esses achados sugerem que a inflamação desempenha um papel vital nos sintomas depressivos, de dor e ansiedade e a inibição do processo inflamatório pode aliviar esses sintomas (XIANG *et al.*, 2011). Estudos sugeriram que a exposição de camundongos ao LPS pode levar à liberação de citocinas pró-inflamatórias, que por sua vez ativam cascatas inflamatórias incluindo citocinas e moléculas de adesão como ON, PGE2, TNF-α, IL -1β, ER, óxido nítrico sintase indutível (iNOS)e COX-2 (CRUZ-MACHADO, 2010; MENDES *et al.*, 2016; ZHANG, X. *et al.*, 2016).

A injeção i.p. de LPS induziu um comportamento do tipo-depressivo, tipo-ansiogênico e hiperalgésico em camundongos. O tratamento agudo com a PSAP nas diferentes doses reverteu estes comportamentos induzidos por LPS em camundongos. Em adição a estes achados, a administração de LPS causou um aumento dos níveis de NF-κB e ativação da MAPK p38 no córtex cerebral e no hipocampo dos camundongos, duas importantes regiões envolvidas na regulação do humor. Essas duas proteínas estão associadas à cascata de sinalização dos receptores tipo-toll 4, e tanto o NF-κB quando a p38 MAPK estão envolvidos na indução de diversos fatores inflamatórios (FRAZIER *et al.*, 2012). Além disso, a MAPK p38 pode ter efeitos diretos no sistema serotoninérgico, aumentando a recaptação de 5-HT e diminuindo a disponibilidade deste neurotransmissor na fenda sináptica (RABELO *et al.*, 2016). Notavelmente, a PSAP, reverteu o aumento dos níveis de NF-κB e a ativação da MAPK p38 induzidas pelo LPS. Em acréscimo ao que foi exposto, a administração de LPS também levou ao aumento da COX-2 e uma diminuição do BDNF. A PSAP reverteu estes parâmetros, demonstrando que este composto também apresenta efeito anti-inflamatório, o que poderia contribuir para seu efeito do tipo-antidepressivo, anti-hiperalgésico e do tipo-ansiolítico.

Tendo em vista as propriedades farmacológicas da PSAP observadas e o fato da comorbidade entre dor e depressão ser uma condição multipatogênica podendo ter a neuroinflamação como mecanismo central (WALKER, A. K. *et al.*, 2013), procurou-se avaliar se este composto orgânico de selênio poderia ser efetivo na reversão desta comorbidade. Os resultados apresentados no **manuscrito 2** demonstraram que a PSNL em camundongos induziu alodínia mecânica, após 4 semanas, observada no teste dos filamentos de Von-frey, e o comportamento do tipo-depressivo no TNF, sem alterações da atividade locomotora. O tratamento dos animais com baixas doses da PSAP reverteu tanto a alodínia quanto o comportamento do tipo-depressivo.

De um modo geral, a PSNL induziu um significativo aumento dos níveis de RNAm (TNF-α, IDO e NFκB) e uma redução do RNAm BDNF, tanto em córtex cerebral quanto em hipocampo e o tratamento com a PSAP normalizou essas

alterações. A PSNL também induziu alterações oxidativas observado pelo aumento dos níveis de RS e TBARS em córtex cerebral e hipocampo, sendo que o tratamento agudo com a PSAP reverteu esses efeitos. Como amplamente discutido, todas essas alterações induzidas pela PSNL podem estar envolvidas na indução e manutenção da dor crônica e do estado do tipo-depressivo e as citocinas pró-inflamatórias podem ser o fator chave no desencadeamento das mesmas. De acordo com os resultados expostos, a PSAP ao reverter grande parte dessas alterações, demonstrou amplo espectro de ação, podendo vir a se tornar um fármaco com amplo espectro de ação.

Inúmeras evidências têm apontado que o sistema imune pode induzir diversos efeitos no SNC e eventos inflamatórios podem ter um papel importante na patogênese da dor e depressão (DANTZER *et al.*, 2011; WALKER *et al.*, 2013). Por muito tempo o cérebro foi considerado um órgão imunologicamente privilegiado, porém sabe-se que citocinas pró-inflamatórias circulantes podem atravessar a barreira hematoencefálica e/ou ativar a micróglia a produzirem mais citocinas via ativação de nervos aferentes, e dessa forma iniciar um processo de neuroinflamação (BANKS, 2009; GRACE, P. M. *et al.*, 2014; VITKOVIC *et al.*, 2000). Além disso, LOUVEAU *et al.*, 2015 identificaram, pela primeira vez, a presença de um sistema linfático no SNC em camundongos, o que permitiria a comunicação direta entre o sistema imune e o cérebro. Para acrescentar estes achados, foi realizado o **manuscrito 3,** o qual o estado do tipo-depressivo e a hiperalgesia foram induzidos através da administração i.c.v da citocina pró-inflamatória TNF-α em camundongos. Neste estudo a PSAP foi eficaz em reverter o comportamento do tipo-depressivo, anedônico e hiperalgésico provocados pelo TNF-α em camundongos no TNF, teste de preferência por sacarose e teste da placa quente, respectivamente. É importante salientar também que nenhum dos tratamentos alterou a atividade locomotora dos animais observada no teste do campo aberto, o que descarta que o comportamento dos camundongos no TNF possa ter sido influenciado por alterações na atividade locomotora. A injeção de TNF-α levou a um aumento das enzimas antioxidantes como a SOD e CAT, assim como aumentou os níveis de

TBARS e ER. Acredita-se que o efeito da PSAP está relacionado com a normalização da atividade das enzimas SOD e CAT, dos níveis de TBARS e ER, correlacionando assim a atividade da PSAP na comorbidade dor e depressão no modelo de TNF-α com seu efeito antioxidante.

A propriedade multialvo de uma molécula tem sido considerada a melhor alternativa terapêutica de doenças multipatogênicas e complexas, como é caso da depressão, da dor crônica e da díade dor-depressão (MILLAN, 2014). O principal desafio no desenvolvimento de fármacos multialvo é a integração de dois ou mais farmacóforos em pequenas estruturas com peso molecular idealmente não ultrapassando 500, a fim de conciliar os efeitos farmacológicos com adequadas propriedades farmacocinéticas, como acesso ao SNC e bioestabilidade (KROENKE, Kurt *et al.*, 2008; MILLAN, 2006). Os resultados do presente estudo e de anteriores, resumidos na Figura 11, demonstram que a PSAP é uma molécula multialvo concentrando propriedades do tipo antidepressiva, anti-hiperalgésica e ansiolítica.

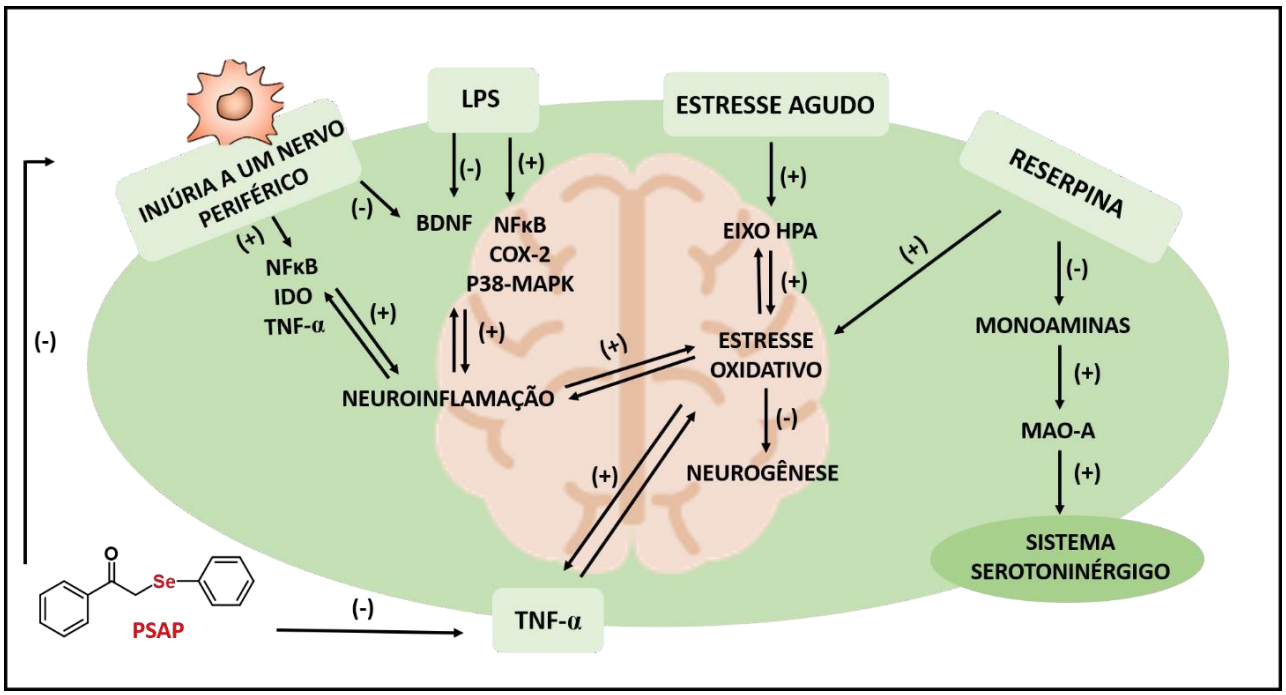

Figura 11. Esquema geral dos mecanismos envolvidos nos efeitos farmacológicos da PSAP. (+) representa modulação positiva enquanto (-) representa modulação negativa. PSAP: molécula α- (fenilselenil) acetofenona (Fonte própria).

6. Conclusão

Os resultados apresentados nesse estudo indicam que a PSAP apresentou efeito do tipo antidepressivo, anti-hiperalgésico e ansiolítico em modelos experimentais de comorbidade dor e depressão através da (I) afinidade pela enzima MAO-A; (II) reversão do estresse oxidativo; (III) diminuição da peroxidação lipídica; (IV) minimização dos níveis de corticosterona plasmáticas; (V) normalização do aumento das citocinas pró-inflamatórias e da diminuição das citocinas anti-inflamatórias ;(VI) redução da ativação da MAPK p38 e IDO (VII) reversão do aumento dos níveis de NF-κB (VIII) normalização dos níveis de BDNF.

A díade dor-depressão apresenta múltiplos mecanismos patofisiológicos e moléculas multialvo seriam possivelmente a melhor maneira de tratar essa comorbidade. Considerando os efeitos da PSAP demonstrados no presente estudo bem como suas propriedades já conhecidas, este composto orgânico de selênio poderia ser uma interessante alternativa terapêutica para o tratamento da dor crônica associada à depressão.

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Anexos