UNIVERSIDADE FEDERAL DO PAMPA

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOQUÍMICA

CARLOS BORGES FILHO

ESTUDO DOS FATORES NEUROQUÍMICOS ASSOCIADOS AO EFEITO TIPO-ANTIDEPRESSIVO DO FLAVONOIDE CRISINA EM CAMUNDONGOS

TESE DE DOUTORADO

Uruguaiana, RS, Brasil.

2016

ESTUDO DOS FATORES NEUROQUÍMICOS ASSOCIADOS AO EFEITO TIPO-ANTIDEPRESSIVO DO FLAVONOIDE CRISINA EM CAMUNDONGOS

por

Carlos Borges Filho

Tese de doutorado apresentada ao Programa de Pós-Graduação em Bioquímica, da Universidade Federal do Pampa (UNIPAMPA), como requisito parcial para obtenção do grau de **Doutor em Bioquímica**

Área de concentração: Bioprospecção Molecular

Orientador: Prof. Dr. Cristiano Ricardo Jesse

Coorientadora: Profª Drª Marina Prigol

Uruguaiana, RS, Brasil.

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A comissão examinadora, abaixo nominada, aprova a Tese de Doutorado

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Elaborada por

Carlos Borges Filho

Como requisito parcial para a obtenção do grau de **Doutor em Bioquímica**

COMISSÃO EXAMINADORA:

_____

Dr. Cristiano Ricardo Jesse (Unipampa)

(Presidente, orientador)

_____

Drª Camila Simonetti Pase (Unipampa)

_____

Dr. Daniel Henrique Roos (Unipampa)

_____

Drª Ethel Antunes Wilhelm (UFPel)

_____

Dr. Leonardo Magno Rambo (Unipampa)

Uruguaiana, RS, Brasil.

2016

DEDICATÓRIA

Humildemente, dedico este trabalho a Deus, meu Criador, Salvador, Guia, Mantenedor, Consolador, Companheiro, Fiel Amigo, e Pai.

...DEDICATÓRIA

À minha amada esposa, Marianne. Que desde o princípio desta caminhada tem estado ao meu lado em amor, auxiliando-me em tudo e complementando-me física, emocional e espiritualmente.

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"...Pois desde a criação do mundo os atributos invisíveis de Deus, seu eterno poder e sua natureza divina, têm sido vistos claramente, sendo compreendidos por meio das coisas criadas, de forma que tais homens são indesculpáveis; porque, tendo conhecido a Deus, não o glorificaram como Deus, nem lhe renderam graças, mas os seus pensamentos tornaram-se fúteis e o coração insensato deles obscureceu-se. Dizendo-se sábios, tornaram-se loucos..."

(*Carta de Paulo aos Romanos,1:20-22)*

"Um pouco de ciência nos afasta de Deus. Muito, nos aproxima".

(*Louis Pasteur,1822-1895)*

RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Bioquímica
Universidade Federal do Pampa

ESTUDO DOS FATORES NEUROQUÍMICOS ASSOCIADOS AO EFEITO TIPO-ANTIDEPRESSIVO DO FLAVONOIDE CRISINA EM CAMUNDONGOS

Autor: Carlos Borges Filho
Orientador: Cristiano Ricardo Jesse
Coorientadora: Marina Prigol

Local e Data da defesa: Uruguaiana, 30 de setembro de 2016.

A depressão é uma doença altamente incapacitante e que tem acometido um percentual crescente da população mundial. Ainda que vários antidepressivos estejam comercialmente disponíveis há décadas, os efeitos colaterais destas drogas, aliados ao fato de que nem todos os pacientes respondem satisfatoriamente ao tratamento, levam a uma busca contínua por novas alternativas para o tratamento ou complementação do tratamento da depressão. Assim, expande-se cada vez mais o número de estudos que avaliam compostos candidatos a antidepressivos. Neste contexto é que o efeito tipo-antidepressivo da crisina, um flavonóide natural abundante no maracujá do mato (*Passiflora coerulea*), em camundongos submetidos ao estresse crônico imprevisível (UCS) foi demonstrado anteriormente por nosso grupo. No entanto, os fatores neuroquímicos associados a este efeito carecem de maiores investigações. Deste modo, o objetivo deste estudo foi avaliar os fatores neuroquímicos associados ao efeito tipo-antidepressivo do flavonoide crisina em dois modelos animais de depressão, o modelo do UCS e o modelo da bulbectomia olfatória (OB), ambos em camundongos. No modelo do UCS foram avaliados o córtex pré-frontal (PFC) e o hipocampo (HP), enquanto no modelo da OB foi avaliado o HP. O UCS e a OB induziram um comportamento tipo-depressivo, caracterizado pela diminuição no tempo de lambida no teste de borrifagem de sacarose e pelo aumento no tempo de imobilidade no teste de suspensão de cauda ou no teste de nado forçado. Ainda, a OB ocasionou alterações no teste de campo aberto, decorrentes da hiperatividade caracteristicamente induzida por este modelo. O tratamento oral com crisina (5 ou 20 mg/kg, durante 28 dias no modelo do UCS, e por 14 dias no modelo da OB), de forma semelhante à fluoxetina (10 mg/kg, controle positivo), culminou na prevenção destas alterações, confirmando a ação tipo-antidepressiva da crisina nos parâmetros comportamentais avaliados. O UCS ocasionou o aumento nos níveis plasmáticos do hormônio liberador de corticotrofina, do hormônio adrenocorticotrófico, e a atividade das caspases 3 e 9 nas estruturas cerebrais avaliadas, enquanto a OB ocasionou a redução dos níveis hipocampais do fator neurotrófico derivado do encéfalo. O UCS e a OB resultaram no aumento dos níveis de citocinas pró-inflamatórias nas estruturas cerebrais avaliadas, como fator de necrose tumoral-α, interferon-γ, interleucina-1β, interleucina-6, além do aumento dos níveis de quinurenina. O UCS e a OB também induziram a diminuição dos níveis de 5-hidroxitriptamina (5-HT) e o aumento da

atividade da enzima indoleamina-2,3-dioxigenase. O tratamento com crisina, de forma semelhante à fluoxetina, promoveu a atenuação de todas estas alterações ocasionadas pelo UCS ou pela OB. Em suma, os resultados deste estudo vêm a corroborar com a hipótese de que o flavonoide crisina é um alvo potencial no estudo de novas alternativas para o tratamento ou para a complementação do tratamento da depressão. Adicionalmente, este trabalho indica a associação das citocinas pró-inflamatórias, da via da quinurenina, do metabolismo da 5-HT, das neurotrofinas e da atividade das caspases na ação tipo-antidepressiva exercida pela crisina em camundongos expostos ao UCS ou à OB. Finalmente, o presente trabalho expõe o maracujá do mato como um importante alvo para o estudo dos produtos naturais no combate à depressão, mostrando a fundamentalidade da investigação da funcionalidade e constituição bioativa desta e outras plantas do bioma pampa.

Palavras-chave: Estresse crônico, bulbectomia olfatória, inflamação, IDO, BDNF.

ABSTRACT

Doctoral Thesis
Program of Post-Graduation in Biochemistry
Federal University of Pampa

STUDY OF NEUROCHEMICAL FACTORS ASSOCIATED WITH THE ANTIDEPRESSANT-LIKE EFFECT OF FLAVONOID CHRYSIN IN MICE

Author: Carlos Borges Filho
Advisor: Cristiano Ricardo Jesse
Co-advisor: Marina Prigol

Site and Date of defence: Uruguaiana, September 30, 2016.

Depression is a highly incapacitating disease that has affected a crescent percentage of the world population. Although various antidepressants have been commercially available for decades, the side effects of these drugs, together with the fact that not all patients respond satisfactorily to treatment, lead to continuous search for new alternatives for the treatment or supplementary treatment of depression. Thus, the number of studies evaluating compounds candidate to antidepressants expands increasingly. In this context, the antidepressant-like effect of chrysin, a natural flavonoid abundant in passion fruit bush (*Passiflora coerulea*), in mice subjected to unpredictable chronic stress (UCS) has been previously demonstrated by our group. However, neurochemical factors associated with this effect require further investigations. Thus, the objective of this study was to evaluate the neurochemical factors associated with the antidepressant-like effect of chrysin in two animal models of depression, the model of UCS and the model of olfactory bulbectomy (OB), both in mice. In the UCS model the prefrontal cortex (PFC) and the hippocampus (HP) were evaluated, in the OB model the HP was evaluated. The UCS and OB induced a depressive-like behavior, characterized by the decrease in the total time of grooming in the splash test and by increase on immobility time in the tail suspension test or forced swimming test. Still, OB induced changes in open field test, resulting from the hyperactivity characteristically induced by this model. The oral treatment with chrysin (5 or 20 mg/kg for 28 days in the UCS model and for 14 days in OB model), similarly to fluoxetine (10 mg/kg, positive control) resulted in the prevention of these changes, confirming the antidepressant-like action of chrysin in the behavioral parameters evaluated. The UCS led to an increase in plasma levels of corticotropin-releasing hormone, adrenocorticotropic hormone and activity of caspases 3 and 9 in the brain structures evaluated, while the OB caused a reduction of hippocampal levels of brain-derived neurotrophic factor. The UCS and OB resulted in increase of proinflammatory cytokines levels in the brain structures evaluated, such as tumor necrosis factor-α, interferon-γ, interleukin-1β, interleukin-6, and increase kynurenine levels. UCS and OB also induced the decrease in 5-hydroxytryptamine (5-HT) levels and the increase of the activity of indoleamine-2,3-dioxygenase enzyme. Treatment with chrysin, similarly to fluoxetine,

promoted the attenuation of all these changes caused by UCS or OB. In summary, results of this study come to corroborate the hyphotesis that the flavonoid chrysin is a potential target in the study of new alternatives for the treatment or complement treatment of depression. Additionally, this study indicates the association of pro-inflammatory cytokines, of kynurenine pathway, of 5-HT metabolism, of neurotrophins and of caspases activities in the antidepressant-like action exerted by chrysin in mice exposed to UCS or OB. Finally, this paper exposes the passion bush as an important target for the study of natural products to combat depression, showing the importance of research of functionality and bioactive constitution of this and other plants of the pampa biome.

Key-words: Chronic stress, olfactory bulbectomy, inflammation, IDO, BDNF.

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LISTA DE ABREVIATURAS E SIGLAS

5-HIAA, Ácido 5-hidroxindolacético (do inglês *5-Hydroxyindoleacetic acid*)**;**

5-HT, Serotonina;

ACTH, Hormônio adrenocorticotrófico (do inglês *Adrenocorticotropic hormone);*

BDNF, Fator neurotrófico derivado do encéfalo *(do inglês Brain-derived neurotrophic fator)*

CMS, Estresse crônico moderado (do inglês *Chronic mild stress);*

CRH, Hormônio liberador de corticotrofina (do inglês *Corticotropin-releasing hormone);*

UCS, Estresse crônico imprevisível (do inglês *Unpredictable chronic stress*);

DA, Dopamina;

DSM, Manual Diagnóstico e Estatístico de Transtornos Mentais (do inglês *Diagnostic and Statistical Manual of Mental Disorders*);

FST, Teste de nado forçado (do inglês *Forced swimming test*);

HP, Hipocampo;

HPA, Hipotálamo-pituitária-adrenal;

IDO, Indoleamina-2,3-dioxigenase;

IFN-γ, Interferon-γ;

IL-1β, Interleucina 1β;

IL-6, Interleucina 6;

KYN, Quinurenina (do inglês *Kynurenine*);

NE, Norepinefrina;

OB, Bulbectmomia olfatória (do inglês *Olfactory bulbectomy*);

OFT, Teste de campo aberto (do inglês *Open field test*);

PFC, Córtex pré-frontal (do inglês *Prefrontal cortex*);

SNC, Sistema nervoso central;

ST, Teste de borrigafem de sacarose (do inglês *Splash test*);

TDO, Triptofano dioxigenase;

TNF-α, Fator de necrose tumoral α (do inglês *Tumor necrosis factor α*);

TRP, Triptofano;

TST, Teste de suspensão de cauda (do inglês *Tail suspension test*);

WHO, Organização mundial da saúde (do inglês *World Health Organization*).

SUMÁRIO

1 INTRODUÇÃO

A depressão é uma doença altamente debilitante, comumente ocorrente, com uma prevalência mundial de cerca de 16% (Liu et al., 2013). A depressão é o principal tipo de transtorno afetivo e está associada a fatores biológicos, psicológicos, sociais e outros, a síndrome é caracterizada por baixa significativa e duradoura no humor (Lu et al., 2015).

Embora sua fisiopatologia ainda não esteja totalmente esclarecida, sabe-se que a depressão está associada a transtornos no sistema monoaminérgico, sobretudo na disponibilidade da serotonina (5-hidroxitriptamina, 5-HT) e à estimulação excessiva do eixo hipotálamo-pituitária-adrenal (HPA), caracterizada pela hipersecreção do hormônio liberador de corticotrofina (CRH – do inglês *Corticotropin-releasing hormone*), do hormônio adrenocorticotrópico (ACTH – do inglês *Adrenocorticotropic hormone*) e glicocorticoides, principalmente o cortisol (corticosterona em roedores) (Lee et al., 2010).

A estimulação excessiva do HPA tem sido associada com a produção acentuada de citocinas pró-inflamatórias, incluindo fator de necrose tumoral-α (TNF-α - do inglês *Tumor necrosis factor-α*), interferon-γ (IFN-γ), interleucina-1β (IL-1β) e interleucina-6 (IL-6), que têm sido relacionadas com a redução da síntese cerebral de 5-HT devido à interferência destas citocinas na via da quinurenina (KP – do inglês *Kynuranine pathway*) por meio da ativação da enzima indoleamina-2,3-dioxigenase (IDO), envolvida com a metabolização do triptofano (TRP), precursor da 5-HT (Maes et al., 2011).

Também tem sido mostrado que a ativação excessiva do eixo HPA produz efeitos neurotóxicos em várias regiões do cérebro relacionadas com a depressão, tais como o córtex pré-frontal (PFC – do inglês *Prefrontal cortex*) e o hipocampo (HP) (Anacker et al., 2011, Liu et al., 2014b), e que em animais estressados pode ser observado o aumento da atividade de caspases em estruturas cerebrais (Bachis et al., 2008), sugerindo a ocorrência da intensificação da apoptose em animais que apresentam a estimulação excessiva de eixo HPA.

Pesquisas têm também postulado a hipótese do envolvimento das neurotrofinas na depressão, que sugere a associação da deficiência do fator neurotrófico derivado do encéfalo (BDNF - do inglês *Brain-derived neurotrophic fator*) com a ocorrência da depressão, e que antidepressivos atuam via restauração dos níveis de BDNF (Liu et al., 2014a; Mao et al., 2014). A hipótese da relação entre a depressão e as neurotrofinas é sustentada basicamente pelos seguintes fatores: I. A depressão está associada com a redução das concentrações sanguíneas e cerebrais de neurotrofinas; II. Tratamentos com antidepressivos aumentam a

expressão de neurotrofinas; III. Glicocorticoides podem suprimir a síntese de neurotrofinas (Vaidya e Duman, 2001; Shiet al., 2010; Liu et al., 2014a; Mao et al., 2014).

Como visto, o estresse, sobretudo o estresse crônico, tem sido consideravelmente relacionado ao crescente índice de ocorrência da depressão em humanos. A partir disso, para simular o desenvolvimento e o progresso da depressão clínica em seres humanos, foi desenvolvido o modelo animal de estresse crônico imprevisível (UCS – do inglês *Unpredictable chronic stress*). Alguns trabalhos mostram que o UCS pode induzir mudanças comportamentais e fisiológicas que se assemelham a sintomas da depressão clínica (Liu et al, 2014a, Willner et al, 2005).

Outro modelo animal que tem sido utilizado no estudo da depressão é o de bulbectomia olfatória (OB – do inglês *Olfactory Bulbectomy*). A OB vem sendo utilizada como modelo experimental para depressão, onde a destruição bilateral dos bulbos olfatórios causa alterações complexas em diferentes parâmetros comportamentais e bioquímicos, muitos dos quais são compatíveis com aqueles encontrados em indivíduos deprimidos (Hendriksen et al., 2012).

Dante disto, os modelos de animais de UCS e OB são utilizados em estudos visando a elucidação da fisiopatologia da depressão e também para avaliar a eficácia de compostos candidatos a antidepressivos através de testes comportamentais e de avaliações bioquímicas (Mao et al, 2009; Tõnissaar et al, 2008; Willner et al, 2005; Zhou et al, 2007).

Apesar de vários antidepressivos esterem disponíveis há décadas, a maioria deles não é completamente eficaz, além destes medicamentos estarem associados a muitos efeitos adversos (ex: alteração do sono e apetite, alterações gastrintestinais (diarréia ou obstipação intestinal), retenção urinária, alergias de pele, sudorese, diminuição da libido ou retardo da ejaculação, aumento ou diminuição de peso, náusea, tontura, tremores) (Perović et al., 2010). Disto decorre que pesquisas recentes estejam concentrando-se na possibilidade do uso de produtos naturais, especialmente flavonoides, para o desenvolvimento de medicamentos antidepressivos, ou como uma alternativa complementar para o tratamento da depressão (Borges Filho et al., 2013; Borges Filho et al., 2015; Liu et al., 2014a; Mao et al., 2014).

A Crisina (5,7-Dihidroxiflavona, Fig.1) faz parte da classe flavona de flavonoides e pode ser encontrada naturalmente no mel, própolis e várias espécies de plantas, incluindo o localmente denominado maracujá do mato (*Passiflora coerulea*) (Pichichero et al., 2011 e Medic-Saric, 2011). A bioatividade da crisina tem sido constatada e já foi demonstrado o seu efeito antioxidante (Pushpavalli et al., 2010), anti-inflamatório, (Bae et al., 2011), antineoplásico (Pichichero et al., 2011) e anti-hiperlipidêmico (Zarzecki et al., 2014).

Além dos efeitos supracitados, um estudo prévio de nosso grupo demonstrou o efeito tipo-antidepressivo do tratamento por 28 dias com crisina em camundongos submetidos ao UCS (Borges Filho et al., 2015). Neste estudo prévio, além do efeito nos testes comportamentais, foi observado o papel da crisina na regulação dos níveis plásmáticos de corticosterona, dos níveis de espécies reativas de oxigênio e tióis não protéicos, da atividade de enzimas antioxidantes e da Na^+,K^+-ATPase, e dos níveis de neurotrofinas no PFC e no HP (Borges Filho et al., 2015). Entretanto, os fatores neuroquímicos possivelmente associados ao efeito tipo-antidepressivo da crisina no modelo do UCS carecem de maiores elucidações. Adicionalmente, a avaliação do efeito da crisina em outros modelos animais de depressão também faz-se necessária.

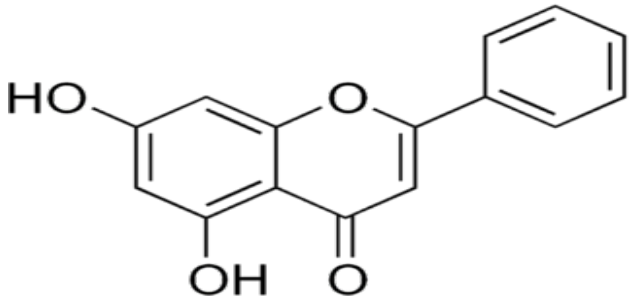

Figura 1. Estrutura química do flavonoide crisina.

2 REVISÃO BIBLIOGRÁFICA

2.1 Depressão

De acordo com a Organização Mundial de Saúde (OMS) a depressão deve se tornar o maior transtorno incapacitante nos próximos 20 anos. Além disso, ela será a doença que mais gerará custos econômicos e sociais para os governos, devido aos gastos com tratamento e às perdas de produção (Alencastro, 2013). O transtorno depressivo é também fator de risco para suicídio e para a ocorrência de doença cardíaca isquêmica (Ferrari et al., 2013; Lichtman et al., 2014), duas das mais importantes causas de mortalidade, sendo sua identificação e tratamento uma prioridade mundial. A depressão também pode apresentar comorbidade com outras doenças psiquiátricas e neurológicas, como ansiedade e doença de Parkinson, entre outros diversos distúrbios somáticos, os quais limitam atividades normais (Gotlib e Joormann, 2010).

A prevalência mundial estimada para o transtorno depressivo é de cerca 4,4% (Ferrari et al., 2013), com uma prevalência ao longo da vida de até 16.2% (Kessler et al., 2003) em estudos americanos. Estudos recentes calculam que o transtorno depressivo foi responsável por cerca de 2,5% da incapacidade ajustada aos anos de vida em 2010, sendo a 2ª causa mundial de anos vividos com incapacidade (Vos et al., 2012).

Embora acometa pessoas de todas as idades, etnias e classes socioeconômicas (Brhlikova et al., 2011), comumente, as mulheres apresentam maior prevalência de depressão do que os homens (taxa de risco durante a vida para transtorno depressivo de 10-25% entre as mulheres e 5-10% entre os homens). Isto pode ser resultado de características hormonais e psicossociais singulares entre homens e mulheres, que podem explicar as altas taxas de depressão (Crema, 2011).

Para que um paciente seja diagnosticado como depressivo, há de ser feita a observação clínica dos sintomas enumerados na Tabela 1, que são altamente variáveis e muitas vezes contrastantes. Para o indivíduo preencher os critérios para o diagnóstico de depressão maior, deve apresentar pelo menos um entre os dois primeiros sintomas e mais o número necessário para perfazer um total de cinco entre os sintomas três a nove, com duração mínima de duas semanas (American Psychiatric Association, 1994).

Tabela 1. Critérios diagnósticos da depressão de acordo com o Manual Diagnóstico e Estatístico de Transtornos Mentais, quarta edição (DSM-IV).

1. Humor deprimido
2. Anedonia
3. Falta de esperança, desespero, sentimento de culpa ou desvalia
4. Perda de peso e apetite/ ganho de peso ou apetite
5. Agitação psicomotora/ letargia
6. Fadiga ou falta de energia
7. Pensamentos recorrentes de morte ou suicídio
8. Dificuldade de concentração
9. Insônia/hipersônia

A partir da natureza dos sintomas, podem ser diagnosticados subtipos de depressão: não-melancólica e melancólica. A depressão não-melancólica é o tipo mais comum de depressão, também referida como "depressão maior" e caracterizada pelos seguintes sintomas: baixa auto-estima, elevada auto-crítica, humor deprimido, alterações no apetite e distúrbios do sono (Alencastro, 2013). A depressão melancólica se caracteriza pela presença de distúrbio psicomotor marcante, expresso como agitação espontânea ou retardo psicomotor (Parker e Brotchie, 1992). Além das alterações no movimento, a literatura tem documentado déficits cognitivos na depressão melancólica, com prejuízos na formação de conceitos, flexibilidade mental e aquisição de memórias (Alencastro, 2013).

Como dito anteriormente, as opções para a terapia antidepressiva disponíveis atualmente estão frequentemente relacionadas a vários efeitos colaterais indesejáveis, e a sua eficácia só alcança uma parcela da população (Perović et al., 2010). Diante do exposto, percebe-se que a heterogeneidade da resposta clínica aos antidepressivos e a susceptibilidade aos efeitos adversos são significativos problemas clínicos da terapia antidepressiva, e disto surge a importância da investigação de novos agentes terapêuticos para tratar a depressão e/ou para complementar o tratamento.

2.2 Fisiopatologia da depressão

Apesar da etiologia da depressão ainda não ser bem esclarecida, alguns fatores fisiopatológicos têm sido elencados como possíveis responsáveis pela ocorrência da depressão. Neste texto, faremos menção de alguns destes inúmeros fatores, dando maior ênfase aos fatores explorados neste estudo.

2.2.1 A teoria monoaminérgica

É conhecido que o transtorno depressivo decorre, ao menos em parte, de transtornos na atividade monoaminérgica no cérebro (Elhwuegi, 2004). A teoria ou hipótese monoaminérgica surgiu em 1965 e postula que o principal processo neuroquímico envolvido na depressão é a disfunção na neurotransmissão monoaminérgica e concomitante diminuição das monoaminas (norepinefrina (NE) e/ou 5-HT) na fenda sináptica, e também pode ser estendida para dopamina (DA). Deste modo os níveis de monoaminas nas fendas podem ser alterados por disfunções na síntese, armazenamento ou liberação, ou estes podem manter-se inalterados, mas as atividades dos receptores e/ou mensageiros intracelulares podem estar alteradas.

Esta teoria é sustentada principalmente pelo fato de a maioria dos antidepressivos utilizados na clínica aumentarem os níveis de monoaminas no cérebro através da inibição da recaptação de 5-HT, NE ou DA e/ou ainda pela inibição da enzima monoamina oxidase, enzima que biotransforma as monoaminas (Nemeroff, 2007). Estudos neurobiológicos também corroboram com esta teoria demonstrando que a normalização das funções dos sistemas serotoninérgico e dopaminérgico tem papel direto no sucesso da terapia antidepressiva (Elhwuegi, 2004).

Entretanto, em decorrência do fato de que a normalização dos níveis de monoaminas em estruturas cerebrais nem sempre está associado a uma melhora significativa no quadro depressivo, outras modificações neuroquímicas têm sido estudadas e correlacionadas com a ocorrência da depressão. Dentre estas modificações, a seguir serão brevemente expostas aquelas que foram alvo deste estudo.

2.2.2 O papel da neuroinflamação

Vários estudos têm sugerido o envolvimento da desregulação imunológica na fisiopatologia da depressão (Dantzer e Kelley, 2007; Kim et al, 2007; Leonard e Maes, 2012). A hipótese apresentada por Smith (1991) indica que as citocinas pró-inflamatórias, as quais são produzidas por macrófagos ativados, contribuem para muitos dos sintomas da depressão.

O papel das citocinas na depressão é suportado pelo alto índice de depressão clínica visto durante o tratamento com citocinas (ex: interferon gama, usado no combate a infecções e alguns tipos de câncer) que conduz ao modelo de depressão induzida por citocinas (Raison et

al, 2005). Dahl et al. (2014) verificaram o aumento nos níveis de uma gama de citocinas (ex: IL-1β, IL-6, TNF-α) em homens e mulheres depressivas, sendo restaurados aos níveis do controle após 12 semanas de terapia antidepressiva. Estudos evidenciam que os transtornos depressivos são mais prevalentes em pacientes afetados com condições que levam à inflamação crônica (como doenças cardiovasculares, diabetes tipo 2 e artrite reumatóide), do que na população em geral (Yirmiya et al., 1999; Dantzer et al., 2008; Loftis et al., 2010). Além disso, regiões cerebrais com as maiores concentrações de receptores de citocinas pró-inflamatórias, especificamente receptores para IL-1β, IL-6, e TNF-α, incluem o PFC e o HP (Parnet et al., 1994; Khairova et al., 2009), que são regiões críticas na resposta antidepressiva (Loftis et al, 2010).

Existem diversos mecanismos pelos quais as citocinas pró-inflamatórias podem induzir a depressão. Um desses mecanismos pode estar relacionado com o fato de que a depressão frequentemente está associada com a desregulação do eixo HPA (Vreeburg et al., 2009) e as citocinas pró-inflamatórias são potentes ativadoras desse eixo (Sapolsky et al., 1987; Kenis e Maes, 2002; O'Brien et al., 2004). Um segundo possível mecanismo pode estar associado com as citocinas pró-inflamatórias modulando a neurogênese hipocampal. Neste contexto, as citocinas pró-inflamatórias podem inibir a neurogênese no hipocampo (Wichers e Maes, 2002; Ekdahl et al., 2003; Santarelli et al., 2003), o que pode levar à redução do volume hipocampal (Marsland et al., 2008) que também é observada na depressão (Campbell et al., 2004). Adicionalmente, as citocinas pró-inflamatórias afetam o metabolismo da 5-HT, pela ativação da IDO (Fujigaki et al., 2006).

2.2.3 A neuroinflamação e a ativação da IDO

Como já mencionado, as citocinas pró-inflamatórias afetam o metabolismo da 5-HT, pela estimulação da IDO (Fujigaki et al., 2006). Isto se dá porque a IDO catalisa a conversão de TRP, precursor da 5-HT, em quinurenina (KYN), reduzindo indiretamente a intensidade da síntese e, consequentemente, a disponibilidade de 5-HT (Mellor e Munn, 1999; Dantzer et al., 2008) (Figura 2).

Além do efeito na disponibilidade de 5-HT, a ativação da IDO também influencia na excitotoxicidade (Turner et al., 2006). Tem sido sugerido que a ativação da IDO ocasiona o aumento da produção de certos metabólitos da KP, como a 3-hidroxiquinurenina e o ácido quinolínico, que podem alterar a neurotransmissão ao longo das vias glutamatérgicas

elevando o risco de excitotoxicidade (Wichers e Maes, 2004; Wichers et al., 2005; McNally et al., 2008; Myint et al., 2012). Tanto a 3-hidroxiquinurenina quanto o ácido quinolínico são substâncias neurotóxicas que estão implicadas em diversas doenças neurodegenerativas (Schiepers et al., 2005). Então, o fato das citocinas estarem mediando esse processo indica que elas podem influenciar a biossíntese serotonérgica e a neurotransmissão encefálica resultando em consequências neurobiológicas significantes (Loftis et al, 2010).

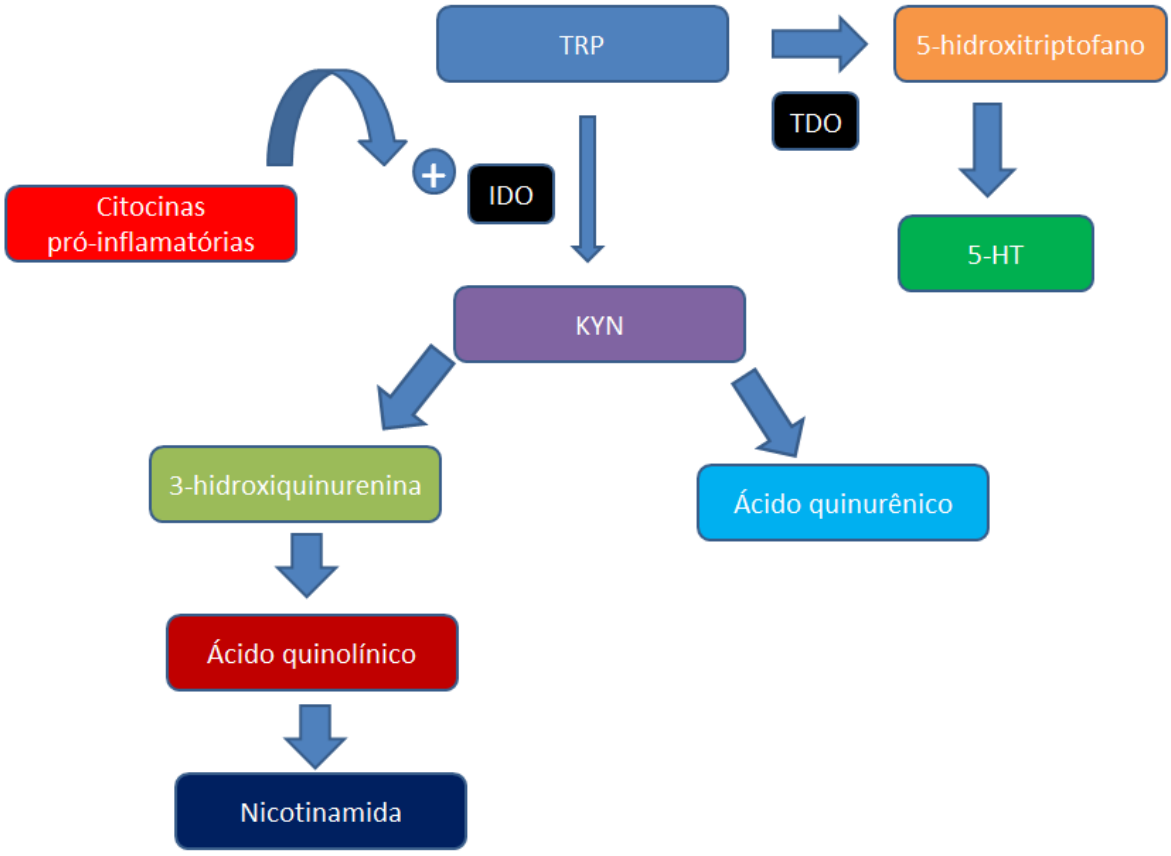

Figura 2: Indução da enzima IDO por citocinas pró-inflamatórias. A oxidação do TRP é catalisada pela triptofano dioxigenase (TDO). A oxidação do TRP, no entanto, pode ocorrer também de maneira extra-hepática pela enzima IDO. Embora a degradação do TRP pela IDO seja normalmente insignificante, a IDO é altamente induzida por citocinas pró-inflamatórias (Adaptado de Neis, 2013).

2.2.4 O papel da apoptose

A exposição ao estresse crônico, é conhecida por induzir, sobretudo em decorrência da hipersecreção de glicocorticoides, a atrofia e a morte neuronal, que causam inúmeros prejuízos comportamentais (Nacher et al., 2001; Stockmeier et al., 2004; Vyas et al., 2002). A atrofia hipocampal é considerada um dos achados neuropatológicos mais confiáveis no estudo da depressão (McEwen et al., 1999).

Um dos principais mecanismos responsáveis pela atrofia neuronal parece ser a intensificação da ocorrência da apoptose, isto é, a morte celular programada (Kim et al., 2013; Zhu et al., 2006).

Com relação à apoptose em estruturas cerebrais, sobretudo no HP, estudos têm demonstrado que diferentes fatores estressantes intensificam a ocorrência da apoptose (Kim et al., 2013; Lehner et al., 2015; Zhu et al., 2006). Diante disso, as enzimas caspases têm sido utilizadas como marcadores da intensidade de ocorrência dos processos apoptóticos, pela mensuração da atividade, expressão proteica ou pelo número de células imunorreativas destas enzimas (Lehner et al., 2015).

2.2.5 O papel das neurotrofinas

Neurotrofinas ou fatores neurotróficos são conhecidos por serem potentes reguladores da plasticidade e sobrevivência de células neurais e gliais adultas. Assim, a hipótese neurotrófica sugere que a diminuição dos níveis de fatores neurotróficos contribui para o déficit na função hipocampal durante o desenvolvimento da síndrome depressiva, sendo esta condição, revertida pelo tratamento antidepressivo (Vaidya e Duman, 2001). Além disso, a normalização dos níveis de neurotrofinas se dá por volta de 15 dias após o início da terapia com um antidepressivo, coincidindo com o tempo necessário para a ação terapêutica do fármaco (Vaidya e Duman, 2001; Shi et al., 2010).

Esta hipótese tem dado maior enfoque ao BDNF, um dos principais fatores neurotróficos no encéfalo (Nestler et al., 2002), o qual apresenta níveis de concentração diminuída no soro e no hipocampo de pacientes que apresentaram estados depressivos, como anedonia. Adicionalmente, foi demonstrada a diminuição dos níveis de BDNF no cérebro de roedores submetidos ao UCS (Liu et al., 2014a; Mao et al., 2014), além de já ter sido sugerido que animais submetidos à OB também apresentam reduções nos níveis de BDNF em estruturas cerebrais (Hellweg et al., 2007; Yang et al., 2014).

2.3 Modelos animais utilizados no estudo da depressão

Embora existam inúmeros modelos animais de depressão e que são amplamente utilizados atualmente, como depressão induzida por estresse, por bulbectomia olfatória, por ovariectomia, por costicosterona, por citocinas, entre outros, serão mencionados neste texto apenas os modelos animais utilizados neste estudo.

2.3.1 Estresse crônico imprevisível (UCS)

Embora existam outros modelos animais de depressão induzida por estresse, como o modelo do estresse agudo e o modelo de estresse crônico repetido, será descrito neste texto o modelo do UCS, já que foi o modelo utilizado neste estudo.

Em 1987, Willner e colaboradores desenvolveram o paradigma do estresse crônico moderado, o qual incluía uma variedade de estressores moderados aplicados por um longo período (Willner et al., 1987). A apresentação de diferentes tipos de estressores é essencial para o modelo, ao invés de aplicar um único estressor de forma repetida que frequentemente induziria uma habituação comportamental (Muscat e Willner, 1992). Este modelo parece simular melhor a condição do ambiente humano, principalmente pela exposição do indivíduo a estressores diários moderados e variados do que a eventos traumáticos. Algumas das anormalidades vistas no CMS (do inglês *Chronic Mild Stress*), designado neste trabalho como UCS, coincidem com muitos sintomas do tipo depressivos observados em humanos.

Os roedores, como a maioria dos humanos, normalmente preferem consumir soluções doces (Willner et., 1987) em relação à água normal. Willner e colaboradores demonstraram que o UCS pode induzir significante redução na preferência pelo consumo de sacarose, comportamento designado como anedonia, um dos principais sintomas do transtorno depressivo (Willner, 1987).

Além disso, o modelo citado diminui comportamentos de agressividade, sexuais e de atividade locomotora em ratos durante a fase ativa (Yan et al., 2010). Alterações circadianas e do ritmo diurno (Gorka et al., 1996), distúrbios do sono, como mudanças no padrão de sono (Cheeta et al.,1997) foram também observados, além de perda de peso (Willner e Jones, 1996), distúrbios na regulação simpática das funções cardíacas (Grippo et al., 2003), aumento dos níveis séricos de citocinas, como o fator de necrose tumoral α (TNF- α) (Kumar et al., 2011) e aumento da atividade do eixo HPA (Muscat e Willner, 1992). Assim, o UCS é um

modelo com grande aplicabilidade e valia no estudo da fisiopatologia da depressão e na avaliação de compostos candidatos a antidepressivos.

2.3.2 Bulbectomia olfatória (OB)

A OB vem sendo utilizada como modelo experimental para depressão, onde a remoção bilateral dos bulbos olfatórios causa alterações complexas em diferentes variáveis comportamentais e bioquímicas, muitas das quais são compatíveis com aquelas encontradas em indivíduos deprimidos (Hendriksen et al., 2012). Como o bulbo olfatório possui extensas conexões eferentes com regiões mesocorticais e subcorticais, é previsível que a bulbectomia olfatória gere um grande impacto sobre as projeções para o PFC, HP, amígdala, locus coeruleus e os núcleos da rafe, promovendo prejuízos sobre as funções reguladas por essas estruturas (Song e Leonard, 2005).

Como consequência da remoção do bulbo olfatório, observam-se majoritariamente disfunções nos sistemas noradrenergico, dopaminérgico e serotoninérgico, ocasionando mudanças comportamentais, identificadas tipicamente duas semanas após a realização do procedimento, como hiperatividade, déficit no aprendizado, função cognitiva e hiper-responsividade ao estresse (Sato et al., 2010).

Uma marcante consequência comportamental da OB é a hiperatividade, que segundo estudos pode ser revertida pelo tratamento crônico com antidepressivos, imitando o início lento da ação antidepressiva em estudos clínicos (Machado et al., 2012). Além disso, a OB em roedores tem sido associada a inúmeras alterações químicas em estruturas cerebrais, principalmente no HP, incluindo a produção acentuada de citocinas pró-inflamatórias (Yang et al., 2014), a desregulação do sistema 5-HT (Hellweg et al., 2007), e a síntese diminuída de neurotrofinas, sobretudo do BDNF (Hellweg et al, 2007; Yang et al, 2014).

2.4 Testes comportamentais utilizados no estudo da depressão em modelos animais

Muitos testes comportamentais visando o esclarecimento da etiologia da depressão e o estudo de candidatos a antidepressivos foram desenvolvidos. Como regra, estes testes devem representar diversos aspectos da depressão nas espécies pesquisadas, comumente roedores (ratos e camundongos) (Willner, 1997). Como princípio destes testes, existe uma hipótese de que algumas espécies de animais podem, através dos testes, exibir alterações de comportamento do tipo-depressivo (do inglês *depressive-like*), ou seja, parecido com alguns

comportamentos apresentados pelos humanos. Dentre as múltiplas possibilidades de escolha nos testes existentes, trabalhamos neste estudo com o teste de suspensão de cauda (TST – do inglês *Tail suspension test*), com o teste de nado forçado (FST – do inglês *Forced swimming test*), com o teste de campo aberto (OFT – do inglês *Open field test*), com o teste do Rota rod, e com o teste de borrifagem de sacarose (ST – do inglês *Splash test*), os quais serão descritos a seguir.

2.4.1 Teste de Suspensão de Cauda (TST)

O TST foi apresentado em 1985 por Steru e colaboradores. O TST é um dos modelos mais tradicionais para o estudo da depressão em animais de laboratório, por apresentar alto valor preditivo devido à resposta aos medicamentos antidepressivos existentes. Neste teste, os roedores são pendurados pela cauda e após um período de movimentos de tentativas de fuga, estes desenvolvem uma postura de imobilidade, o que é resultado de uma situação estressante e inescapável. Esta imobilidade é observada durante um tempo total de teste de 6 minutos.

2.4.2 Teste de Nado forçado (FST)

O FST apresenta um princípio semelhante ao do TST. Esse teste possui alto valor preditivo para o efeito tipo antidepressivo e também constitui um teste comum para o estudo de novas drogas. Proposto por Porsolt e colaboradores, em 1977, neste teste os roedores são expostos a uma situação aversiva, nadar em um tanque cilíndrico com água, onde eles não podem tocar o fundo do cilindro ou fugir (escape). Assim como no TST, o FST geralmente tem duração de 6 minutos onde se observa o tempo total de imobilidade apresentado pelos animais. Tanto ratos como camundongos podem ser usados para o estudo do efeito do tipo antidepressivo de drogas através do FST.

A hipótese que justifica o comportamento animal no TST e no FST é baseada na ideia de que o animal “perde a esperança de escapar” de tal situação, em outras palavras a falta de persistência em escapar é percebida como uma desistência e refletida em tempo de imobilidade descrito como um estado depressivo (Thierry et al., 1984). As substâncias antidepressivas revertem esse quadro diminuindo assim o tempo de imobilidade fazendo com que o animal não desista de escapar das situações impostas a ele, dessa maneira os antidepressivos clássicos empregados na clínica, como a imipramina e a fluoxetina são utilizados como controles positivos nestes testes.

2.4.3 Teste de Campo Aberto (OFT)

Embora não seja um teste utilizado unicamente no estudo da depressão, o OFT é utilizado para verificar os efeitos de substâncias ou determinados procedimentos sobre o sistema motor dos animais, a fim de excluir a possibilidade de que a diminuição do tempo de imobilidade exibida nos testes preditivos de efeito tipo antidepressivo, como no TST ou no FST, seja devido a uma estimulação motora.

Substâncias estimulantes do sistema nervoso central (SNC) tendem a aumentar os parâmetros comportamentais registrados no modelo enquanto que substâncias depressoras tendem a diminuí-los. Este teste, em geral, é realizado em uma caixa medindo 45 x 45 x 30 cm, com o chão dividido em 9 quadrantes iguais. Durante a sessão de teste, o número de quadrantes cruzados com todas as patas e elevações em um período de 5 minutos são utilizados como parâmetro para a avaliação da atividade locomotora e exploratória dos animais (Walsh e Cummins, 1976).

2.4.4 Teste do Rota rod

Embora também não seja um teste utilizado unicamente no estudo da depressão, o teste do Rota rod é útil em experimentos relacionados à depressão porque avalia o efeito do relaxamento muscular ou incoordenação motora produzidos por drogas ou dados procedimentos nos animais (Carlini e Burgos, 1979). Para este teste, os camundongos são colocados com as quatro patas sobre uma barra de 2,5 cm de diâmetro, elevada a cerca de 25 cm do piso, em uma rotação média de 12 rpm, por um período de 3 minutos. São registrados o tempo de permanência na barra giratória, em segundos (s), e o número de quedas, com três reconduções, no máximo (Dunham e Miya, 1957).

2.4.5 Teste de Borrifagem de Sacarose (ST)

O ST é utilizado para avaliar o comportamento de autolimpeza dos animais, após a borrifagem com solução de sacarose a 10%. O tempo em que o animal permanece neste comportamento é cronometrado durante 5 minutos (Ducottet e Belzung, 2004). O ST é um válido marcador comportamental, uma vez que animais submetidos a modelos comportamentais de depressão apresentam um menor tempo de autolimpeza quando comparados aos animais controle (Kalueff et al., 2002; 2004; Moretti et al., 2012). Em

modelos animais de depressão, a administração crônica de antidepressivos clássicos aumenta o tempo despendido neste comportamento (Yalcin et al., 2005).

2.5 Compostos fenólicos naturais na terapia da depressão

Os compostos fenólicos derivados de plantas são divididos em diversas categorias, como fenóis simples, ácidos fenólicos, cumarinas, flavonóides, taninos condensados e hidrolisáveis, lignanas e ligninas (Naczk e Shahidi, 2004). Os flavonoides são compostos polifenólicos presentes em alimentos e bebidas de origem vegetal e que têm despertado interesse em decorrência da bioatividade destes compostos (Sequeto et al., 2012). Nesse contexto, flavonoides isolados de plantas, como luteolina, hespiridina, apigenina, rutina, quercetina e crisina têm demonstrado efeitos protetores em doenças cardíacas, renais, hepáticas, cerebrais, e neoplásicas (Pietta, 2000; Sequeto et al., 2012).

Um ensaio clínico revelou que a administração crônica e subcrônica de compostos fenólicos leva a uma menor prevalência de sintomas depressivos em indivíduos idosos japoneses (Niu et al., 2009). Zhu et al. (2012) observaram a menor incidência das alterações comuns a um quadro de depressão sob parâmetros comportamentais e bioquímicos em camundongos que foram administrados com polifenóis do chá verde. A atividade biológica dos compostos fenólicos em doenças neurodegenerativas, inflamação, câncer e doenças cardiovasculares envolve a regulação do crescimento celular e a proliferação, a atividade enzimática, e a modulação da sinalização celular (Pandey et al., 2009; Darvesh et al., 2010).

Embora muitos antidepressivos estejam disponíveis há décadas, a maioria deles não é completamente eficaz, além destes medicamentos estarem associados a muitos efeitos adversos (ex: alteração do sono e apetite, alterações gastrintestinais (diarréia ou obstipação intestinal), retenção urinária, alergias de pele, sudorese, diminuição da libido ou retardo da ejaculação, aumento ou diminuição de peso, náusea, tontura, tremores) (Perović et al., 2010). Assim, pesquisas recentes concentram-se na possibilidade do uso de produtos naturais, especialmente flavonoides, para o desenvolvimento de medicamentos antidepressivos, ou como uma alternativa complementar para o tratamento da depressão (Kumar et al., 2011; Borges Filho et al., 2013; Liu et al., 2014a, Mao et al., 2014).

2.5.1 Crisina

A Crisina pertence à classe flavona de flavonoides. É encontrada naturalmente em mel, própolis, e várias espécies de plantas, incluindo espécies do gênero *Pelargonium, Passiflora* e da família *Pinaceae* (Pichichero et al., 2010; Medic-Saric , 2011). Uma das principais fontes naturais da crisina é a planta *Passiflora coerulea* (regionalmente conhecida como maracujá do mato), da qual foi isolada em 1990 por Medina e colaboradores e apresentada como um composto com propriedades anticonvulsivantes (Medina et al., 1990).

2.5.1.1 Biodistribuição da crisina

A característica fundamental dos flavonoides quanto à farmaco ou toxicocinética são os grupos hidroxila livres, que são rapidamente e eficientemente metabolizados via glucuronidação e sulfatação (Griffiths e Barrow, 1972; Bokkenheuser et al., 1987).

Os flavonóides, em geral, apresentam-se em duas formas, a forma glicosilada (conjugados com açúcares) e a forma agliconada (livre). De um modo geral, assume-se que os flavonóides glicosilados são absorvidos na forma agliconada após passar por hidrólise no trato digestivo. Na forma agliconada, nos enterócitos, as principais transformações que ocorrem com os flavonoides incluem reações de glucuronidação e sulfatação, que podem ser seguidas de efluxo, culminando na eliminação via fezes, urina e dióxido de carbono (mediada por bactérias do trato intestinal), ou absorção e distribuição para os tecidos via sistema circulatório.

A crisina é um flavonoide agliconado que, como a maioria dos flavonoides, é considerada um composto de relativamente baixa taxa de absorção oral (Walle, 2004). Tsuji et al. (2006) avaliaram a acumulação da crisina em peixes após 8h de exposição, verificando, em ordem decrescente, maiores concentrações em fígado, intestino, pele e cérebro. Quanto ao metabolismo, o mesmo grupo de pesquisadores observou que a crisina sofre glucuronidação após a absorção, presumivelmente nos carbonos 5 e 7 e é secretada na bile. Walle et al. (2001) monitoraram os níveis de crisina e dos metabólitos crisina sulfatada e crisina glucuronada em sangue, urina e fezes de humanos saudáveis durante 48h após a administração de 400 mg de crisina via oral. Nas análises sanguíneas, foram verificadas baixas concentrações de crisina livre, com picos de em média 6 ng/ml após 6 horas. Já para a crisina conjugada com sulfato, o pico foi de 200 ng/ml (cerca de 30 vezes maior em relação à crisina livre) e se deu aproximadamente 6h após a ingestão. Para a crisina glucuronada, não foram encontrados

níveis plasmáticos suficientes para uma detecção precisa. No que se refere à eliminação via urina, Walle et al. (2001) demonstraram que a excreção de crisina livre na urina se deu entre 0,2 e 3,1mg. Já para a crisina glucuronada foram encontradas concentrações entre 2 e 26 mg na urina. Para a crisina sulfatada foram verificados apenas traços não detectáveis de forma precisa. Ainda, observou-se que a taxa de eliminação de crisina e seus metabólitos via urina é baixa, entre 1 e 7%. Quanto à eliminação via fezes, verificou-se que a taxa de eliminação de crisina e metabólitos chegou a até cerca de 98% da crisina administrada, com valores que variaram entre 40 e 390 mg. A partir disso, com base nas análises plasmáticas, observa-se que as possíveis formas nas quais a crisina exerce a sua constatada bioatividade seriam a forma livre e, talvez principalmente, a forma sulfatada. Já a forma glucuronada é a forma predominante de eliminação.

2.5.1.2 Bioatividade da Crisina

A crisina é um flavonóide antioxidante com propriedades complexantes, devido principalmente à presença de um grupo hidroxila no carbono 5 (Pushpavalli e al., 2010). Estudos sobre a atividade antioxidante de flavonóides sugerem a relação da presença de hidroxilas na bioatividade destes compostos (Pushpavalli e al., 2010). Mais especificamente, a capacidade antioxidante de um flavonóide geralmente está relacionada principalmente com a hidoxilação do anel B. No entanto, as hidroxilas presentes nos carbonos 5 e 7 do anel A da crisina desempenham uma função significativa na atividade antioxidante deste composto (Torel et al., 1986).

Além da função antioxidante (Pushpavalli et al., 2010), a crisina já tem sido apresentada como tendo efeitos anticonvulsivantes (Medina et al., 1990), anti-hipertensivos (Vilar et al., 2002), anti-inflamatórios (Bae et al., 2011), antineoplásicos (Pichichero et al., 2011) e anti-hiperlipidêmicos (Zarzecki et al., 2014). Também há relatos que sugerem que a crisina aumenta os níveis de testosterona pela inibição da enzima aromatase (Kao et al., 1998), que converte a testosterona em estradiol, e em decorrência disto, a crisina já está disponível no mercado como um suplemento dietético (500 mg por cápsula).

Como já mencionado neste escrito, o tratamento por 28 dias com o flavonóide crisina mostrou satisfatório efeito tipo-antidepressivo em animais submetidos ao UCS (Borges Filho et al., 2015). Neste estudo prévio, além do efeito nos testes comportamentais, foi observado o papel da crisina na regulação dos níveis plásmáticos de corticosterona, dos níveis de espécies reativas de oxigênio e tióis não protéicos, da atividade de enzimas antioxidantes e da Na^+,K^+-

ATPase, e dos níveis de neurotrofinas no PFC e no HP (Borges Filho et al., 2015). Entretanto, os fatores neuroquímicos possivelmente associados ao efeito tipo-antidepressivo da crisina no modelo do UCS carecem de maiores elucidações.

Diante do exposto, visando o aprofundamento dos resultados já obtidos, percebe-se que alguns fatores neuroquímicos fortemente relacionados à depressão e possivelmente associados ao efeito da crisina em animais submetidos ao UCS ainda carecem de ser estudados. Além disso, a avaliação do efeito da crisina em outros modelos animais de depressão, como o modelo de OB, também faz-se necessária e relevante.

3 OBJETIVOS

3.1 Objetivo Geral

Avaliar os fatores neuroquímicos associados ao efeito tipo-antidepressivo da administração do flavonoide crisina em camundongos.

3.2 Objetivos Específicos

- Analisar, sob aspectos comportamentais, o efeito do tratamento com o flavonoide crisina em camundongos submetidos ao UCS ou à OB;

- Avaliar os fatores neuroquímicos associados ao efeito do flavonoide crisina em camundongos submetidos ao UCS ou à OB.

4 PRODUÇÃO CIENTÍFICA

Os resultados que fazem parte desta tese de doutorado estão apresentados sob a forma de 1 artigo aceito para publicação e 1 manuscrito. Os itens Introdução, Materiais e Métodos, Resultados, Discussão e Referências encontram-se nos próprios documentos e representam a íntegra deste estudo. Os documentos estão dispostos da mesma forma que foram submetidos para as respectivas revistas. O artigo foi aceito para publicação na revista "European Journal of Pharmacology". O manuscrito foi submetido à revista "Chemico-Biological Interactions".

4.1 ARTIGO

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Neurochemical factors associated with the antidepressant-like effect of flavonoid chrysin in chronically stressed mice

Carlos Borges Filho, Cristiano Ricardo Jesse, Franciele Donato, Lucian Del Fabbro, Marcelo Gomes de Gomes, André Tiago Rossito Goes, Leandro Cattelan Souza, Renata Giacomeli, Michelle Antunes, Cristiane Luchese, Silvane Souza Roman, Silvana Peterini Boeira

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Neurochemical factors associated with the antidepressant-like effect of flavonoid chrysin in chronically stressed female mice

Carlos Borges Filho[1], Cristiano Ricardo Jesse[1*], Franciele Donato[1], Lucian Del Fabbro[1], Marcelo Gomes de Gomes[1], André Tiago Rossito Goes[1], Leandro Cattelan Souza[1], Renata Giacomeli[1], Michelle Antunes[1], Cristiane Luchese[2], Silvane Souza Roman[3], Silvana Peterini Boeira[1]

[1] Laboratório de Avaliações Farmacológicas e Toxicológicas Aplicadas às Moléculas Bioativas - LaftamBio Pampa - Universidade Federal do Pampa, CEP 97650-000, Itaqui, RS, Brazil.

[2] Centro de Ciências Químicas, Farmacêuticas e de Alimentos, Universidade Federal de Pelotas, Campus Universitário, s/n, 96160-000 Capão do Leão, RS, Brasil.

[3] Universidade Regional Integrada, Campus Erechim, CEP 99700-000, RS, Brazil

Cristiano R. Jesse E-mail: cristianoricardojesse@yahoo.com.br

Laboratório de Avaliações Farmacológicas e Toxicológicas Aplicadas às Moléculas Bioativas - *LaftamBio* Pampa - Universidade Federal do Pampa, CEP 97650-000, Itaqui, RS, Brazil.

Phone: +55-55-34331669

FAX: +55-55-34331669

Abstract

Chrysin is a flavonoid which is found in bee propolis, honey and various plants. Antidepressant-like effect of chrysin in chronically stressed mice was previously demonstrated by our group. Conversely, neurochemical factors associated with this effect require further investigations. Thus, we investigated the possible involvement of pro-inflammatory cytokines, kynurenine pathway (KP), 5-hydroxytryptamine (5-HT) metabolism and caspases activities in the effect of chrysin in mice exposed to unpredictable chronic stress (UCS). UCS applied for 28 days induced a depressive-like behavior, characterized by decrease in the time of grooming in the splash test and by increase in the immobility time in the tail suspension test. Oral treatment with chrysin (5 or 20 mg/kg, 28 days), similarly to fluoxetine (10 mg/kg, positive control), culminated in the prevention of these alterations. UCS elevated plasma levels of corticotropin-releasing hormone and adrenocorticotropic hormone, as well the tumor necrosis factor-α, interleukin-1β, interleukin-6 and kynurenine levels in the prefrontal cortex (PFC) and hippocampus (HP). UCS induced the decrease in the 5-HT levels in the HP and the increase in the indoleamine-2,3-dioxygenase, caspase 3 and 9 activities in the PFC and HP. Treatment with chrysin, similarly to fluoxetine, promoted the attenuation of these alterations occasioned by UCS. These results corroborated with the antidepressant potential of chrysin in the treatment of psychiatric diseases. Furthermore, this work indicated the association of pro-inflammatory cytokines synthesis, KP, 5-HT metabolism and caspases activities with the action exercised by chrysin in mice exposed to UCS.

Keywords: Flavonoid, depression, chronic stress, antidepressant-like.

1 Introduction

Initially developed as a model to screen antidepressant drugs, unpredictable chronic stress (UCS) is increasingly used as a means to investigate behavioral, endocrine and neurochemical changes underlying depression (Willner et al., 1987, 1997). Depression is a debilitating, commonly occurring, and life-threatening psychiatric disorder, with a worldwide prevalence of approximately 17% (Liu et al., 2013). Depression is the main type of affective disorders which are due to the biological, psychological, social, and other factors. In this way, the syndrome is characterized by significant and lasting low mood (Lu et al., 2015). Depression is a major cause of disability, and imposes a substantial health threat to the modern society.

Although the pathophysiology of depression is not yet fully clarified, it is known that depression is generally associated with reductions in the central monoamines levels (mainly 5-hydroxytryptamine, 5-HT), although not all depressed subjects having serotonin reductions. Furthermore, also is observed excessive stimulation of hypothalamic-pituitary-adrenal (HPA) axis, characterized by hypersecretion of corticotropin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH) and glucocorticoids, mainly cortisol (corticosterone in rodents) (Lee et al., 2010). Excessive stimulation of HPA has been associated with the production of pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1 β) and interleukin-6 (IL-6), that have been found to reduce the production of 5-HT by interference of cytokines in the kynurenine (KYN) pathway (KP), by activation of the tryptophan (TRP)-metabolizing enzyme indoleamine-2,3-dioxygenase (IDO) (Maes et al., 2011). In addition, it has been shown that the excessive activation of HPA axis produces neurotoxic effects in several brain regions related to depression, such as prefrontal cortex (PFC) and hippocampus (HP) (Anacker et al., 2011; Liu et al., 2014), and that stressed animals exhibit the increase of caspases activities in cerebral structures (Bachis et al., 2008), suggesting the occurrence of apoptosis in animals that present excessive stimulation of HPA axis.

Chrysin (5,7-dihydroxyflavone, Fig. 1) is a flavonoid which is found in bee propolis, honey and various plants (Barbaric et al., 2011). As a result of its effect on inhibition of the aromatase enzyme, which converts testosterone to estradiol, chrysin is commercially available as a dietary supplement (500 mg per capsule), aiming the elevation of testosterone levels. Research has shown that chrysin has multiple other biological activities, such as anti-

inflammatory, antineoplastic, hipolipdemic and antioxidant (Borges Filho et al., 2013; Cho et al., 2004; Lapidot et al., 2002; Zarzecki et al., 2014). A recent study of our group demonstrated the antidepressant potential of crhysin when administered for 28 days in chronically stressed mice (Borges Filho et al., 2015). However, this study needs to be expanded for the evaluation of other neurochemical parameters strongly associated with depression.

Thus, our study investigated the possible involvement of the pro-inflammatory cytokines and 5-HT levels, KP and caspases activities in the antidepressant-like effect of chrysin treatment in female mice exposed to UCS. Our working hypothesis is that pro-inflammatory cytokines and 5-HT levels, KP, and caspases activities may be associated with the antidepressant-like effect of chrysin in mice subjected to UCS.

2 Materials and methods

2.1 Animals

Experiments were realized with 42 female C57B/6J mice (20-25g, 90 days old). Animals were maintained at 22-25 °C, with free access to water and food, under a 12:12-h light/dark cycle (except when the stressful activity involved continuous light during 24h), with lights on at 7:00 a.m. The procedures of this study were conducted according to the guidelines of the Committee on the Care and Use of Experimental Animal Resources and with the approval of the Ethics Commission for Animal Use (CEUA protocol # 035/2013). It is important to clarify that this work was realized in female mice because women are more susceptible to development of depressive disorder than men (Mazure et al., 2003; Parker et al., 2010; Posmontier et al., 2008).

2.2 Drug solutions and administrations

Chrysin and fluoxetine were purchased from Sigma (St. Louis, MO, USA). All other chemicals used were obtained from standard commercial suppliers.

Chrysin was dissolved in a distilled water/propyleneglycol solution (80:20). Fluoxetine was dissolved in distilled water. Both drugs were administered per oral (p.o.) in the volume of 10ml/kg. Mice were treated with chrysin at doses of 5 or 20 mg/kg, corresponding to a low dose and a high dose, respectively [13, 15], or fluoxetine at the dose of

10mg/kg (Kumar et al., 2011). Both drugs were daily administered for 28 days, 30min before the stressful activity (Borges Filho et al., 2015).

2.3 Experimental design and Unpredictable Chronic Stress (UCS)

The mice were divided into eight groups (*n =5-7*): [1] Control (No stress + vehicle) {V},[2] Fluoxetine 10mg/kg (No stress + fluoxetine 10mg/kg) {F10}, [3] Chrysin 5mg/kg (No stress + chrysin 5 mg/kg) {C5}, [4] Chrysin 20mg/kg (No stress + chrysin 20mg/kg) {C20}, [5] Stress (Stress + vehicle) {V}, [6] Stress + fluoxetine 10mg/kg {F10}, [7] Stress + Chrysin 5mg/kg {C5} and [8] Stress + Chrysin 20mg/kg {C20}. The UCS regimen was based on the procedures described by other researchers, with minor modifications (Borges Filho et al., 2015; Chen et al., 2012; Grippo et al., 2008; Liu et al., 2013; Liu et al., 2014; Peng et al., 2012; Zhang et al., 2012). The mice were housed in separate cages. Briefly, UCS-exposed mice were subjected to various stressors in a chronic and unpredictable way according to a random schedule for 28 days. The stressors were: damp bedding for 12 h; 45° cage tilting for 12 to 18h; continuous light during 24h; water and food deprivation for 12 to 18h; strong level shaking for 5 min; electric shock foot (2 min; 0,5mA, 3s duration, average 1 shock/min) in an electrified grid; 2min in the electrified grid, but without shock foot; 45°C oven for 5 min; physically restraint for 2h. Aleatory stressors were applied at random times in order to be completely unpredictable. All mice in the stress group were exposed to the same single stressor simultaneously in 1 day. None of stressful procedures was applied on two consecutive days. During the whole process of stress, each stressor was applied two to four times. On the 28th day, in the end of UCS, the animals were subjected to splash test (ST) and after 24h were exposed to rota rod test and subsequently to tail suspension test (TST). After 24h the animals were anesthetized and blood was collected by cardiac puncture and the mice were euthanized by decapitation and PFC and HP were dissected (Fig. 2).

2.4 Behavioral assessment

2.4.1 Splash Test (ST)

This test was performed on the 28th day of stressful protocol, 24h after the last stressing procedure, and consisted of squirting 200 µl of a 10% sucrose solution on the mouse's snout. Because of its viscosity, the sucrose solution dirties the mouse fur and animals

initiate grooming behavior. After applying sucrose solution, the time spent for grooming was recorded in s for a period of 5 min. A decrease in grooming, which is a particular feature of mice submitted to stress was used as an index of self-care and motivational behavior (Petit et al., 2014; Surget et al., 2008).

2.4.2 Rota rod test

The evaluation of the motor coordination was performed 24 h after the ST and was carried out using a Rota-Rod setup (Rota-Rod Treadmill, ENV-575M, Neuro-science Co., Ltd., Tokyo, Japan). For this test, each mouse was placed with all four feet onto a bar of 2.5 cm diameter, 25 cm high from the floor, in a rotation of 17 rpm for a period of 6 min. The duration of permanence in the swivel bar (s), and the number of falls, with three renewals at maximum were recorded (Fortes et al., 2013; Nunes et al., 2015).

2.4.3 Tail suspension test (TST)

The TST was realized based on the method described previously (Steru et al., 1985), was carried out immediately after to the rota rod test. Mice both acoustically and visually isolated were suspended 50 cm above the floor by means of an adhesive tape, placed approximately 1 cm from the tip of the tail. The total duration of immobility (s) was quantified during a test period of 6 min. Mice were considered immobile only when they hung passively and completely motionless.

2.5 Plasma and tissue preparation

24h after the TST, blood was collected by cardiac puncture into tubes containing heparin (1 UI/µl). Plasma was obtained by centrifuging of the blood at 2,400 x g for 10 min and used for hormonal determinations. After the blood collection, mice were euthanized for decapitation and HP and PFC were dissected and homogenized (1:5) in Tris-HCl 50mM, pH 7.5. The homogenate was centrifuged at 2,400 x g for 5 min and supernatant fraction (S1) was used for neurochemical determinations.

2.6 Hormonal determinations

2.6.1 CRH and ACTH levels

The plasma levels of CRH and ACTH were determined using an AssayMax ELISA kit (Assaypro LLC, St. Charles, MO) according to the manufacturer's instructions. CRH and ACTH plasma levels were expressed as ng/l.

2.7 Neurochemical determinations

2.7.1 TNF-α, IL-1β and IL-6 levels

Levels of TNF-α, IL-1β and IL-6 in the PFC and HP were determined using commercially available ELISA assays, following the instructions supplied by the manufacturer (DuoSet Kits, R&D Systems; Minneapolis). Results are shown as pg/mg tissue.

2.7.2 IDO activity, KYN and TRP levels and KYN/TRP ratio

IDO activity in the PFC and HP was determined as previously described (Sono, 1989), with minor modifications: the tissue used in the original study was rabbit small intestine and in our study it was prefrontal cortex (PFC) and hippocampus (HP) of mice; the amount of the enzyme as expressed in terms of its heme content based on the absorbance at 406 nm and in our method, the protein concentration was measured by the method of Bradford (1976), using bovine serum albumin as the stardard; the temperature was 25ºC and in our study was 37ºC; the original method did not describe the block with trichloroacetic acid and the rotations of the centrifugation. The S1 (0.2 ml) were added to 0.8 ml of the reaction mixture containing 400 µM L-tryptophan, 20 mM ascorbate, 10 µM methylene blue, and 100 µg catalase in 50 mM potassium phosphate buffer pH 6.5. The reaction was carried out at 37ºC under agitation for 60 min. Then, it was blocked by adding 0.2 ml of 30% trichloroacetic acid and further incubated at 50ºC for 30 min to convert the N-formylkynurenine to L-kynurenine. Samples were centrifuged at 13,000g for 10 min at 4ºC. The supernatants were filtered through microspin ultrafiltrates with a cut-off of 10,000M_r before being taken for measurement of IDO. The amount of L-kynurenine formed from TRP was determined by reversed phase high pressure liquid chromatography (HPLC). One hundred µl of the reaction product was injected

onto a Merck LiChrospher column (150mm long, 4.6mm diameter, packed with 5 lm silica beads holding 18C long carbon chains). A cartridge guard column containing the same material as the analytical column was used. The mobile phase consisted of 0.1M ammonium acetate buffer (pH 4.65) with 5% acetonitrile. Flow rate was 1 ml/min. KYN was detected using a spectrometer measuring absorbency at a wavelength of 365nm and was quantified using known amounts of L-kynurenine. The retention time of KYN was around 5.35 min. All determinations were performed in duplicate. One unit of the activity was defined as 1 nmol KYN/h/mg protein at 37ºC.

KYN and TRP levels were measured in the PFC and HP samples using HPLC. The mobile phase contained 50 nM glacial acetic acid, 100 mM zinc acetate and 3% acetonitrile dissolved in double-distilled NANO pure water HPLC grade H_2O. The pH was adjusted to 4.9 using 5 M NaOH. S1 of PFC and HP were sonicated in 1 ml of mobile phase containing 7% perchloric acid spiked with 50 ng/20 µl of N-methyl 5-HT as an internal standard. The resultant solution was centrifuged at 20,000 rpm for 20 min and the supernatants were placed into new Eppendorf tubes using a syringe fitted with a 0.45-µm filter (Phenomenex). Approximately 20 µl of the filtered supernatant was injected using a Waters auto sampler and a Reverse Phase analytical column (Kinetex™ Core Shell Technology column with specific area of 4.6 mm and particle size of 2.6 µl, Phenomenex) was used for the separation of metabolites. A PDA-UV detector (Shimadzu SPD-M10A VP), calibrated to integrate at 230 and 250 nm, as well as a fluorescent detector (Shimadzu RF- 20A XS prominence fluorescence detector), set to excitation wavelength 254 nm and emission wavelength 404 nm, were used to detect the metabolites. Chromatographs were generated by CLASS-VP software (Shimadzu). The results are expressed as ng/g tissue. KYN/TRP ratio was calculated by ratio between KYN and TRP concentrations.

2.7.3 5-HT and 5-HIAA levels and 5-HIAA/5-HT ratio

The levels of 5-HT and its metabolite 5-Hydroxyindoleacetic acid (5-HIAA) in the PFC and HP were analyzed by HPLC with electrochemical detection, as previously described (Harkin et al., 2003). The mobile phase, used at a flow rate of 0.8 ml/min, consisted of 0.02 M phosphate/citrate buffer and 90/10 methanol (v/v), 0.12 mMNa2 EDTA, and 0.0556% heptane sulphonic acid as ion pair. The pH was adjusted to 2.64 with H_3PO_4 at 22 °C. A 5-µm (220 × 4.6) Spheri-5 RP-18 column from Brownlee Laboratory was used. Electrochemical detection was performed with a Shimadzu L-ECD-6A electrochemical detector with a potential of 0.75

V. The peak area of the internal standard (DHBA) was used to quantify the sample peaks. The tissue levels were expressed in ng/g tissue. 5-HIAA/5-HT ratio was calculated by ratio between 5-HIAA and 5-HT concentrations.

2.7.4 Caspase 3 and 9 activities

Caspase 3 and 9 activities in the PFC and HP were performed by using caspase colorimetric assay kits (Sigma, St. Louis, MO, USA). The results were expressed as units/μg of protein.

2.7.5 Protein quantification

Protein concentration in the PFC and HP was measured by the method of Bradford (1976), using bovine serum albumin as the standard.

2.8 Statistical analysis

Results are expressed as the mean ± standard error of the mean (S.E.M.). Statistical analysis was performed using a two-way analysis of variance (ANOVA), analyzing the effect of stress, treatments (chrysin or fluoxetine), and stress x treatments interaction. Two-way ANOVA was followed by Newman-Keuls test when appropriate. Person's correlation test also was realized to verify the possible statistic relation between the evaluated parameters. The main results of Person's correlation test were mentioned in the results and in the discussion. The level of significance was set at $P<0.05$. The statistical analysis was performed using the software GraphPad Prism version 5 (GraphPad Software, Inc., La Jolla, CA, USA).

3 Results

3.1 Splash test

Two-way ANOVA showed significant main effect for stress [$F(1.29)=9.12$, $P<0.01$], treatments [$F(3.29)=6.05$, $P<0.01$] and stress X treatments interaction [$F(3.29)=8.53$, $P<0.001$] in the total time of grooming in the ST. Results of *post hoc* indicated that after the UCS exposition the animals displayed significant decrease in the total time of grooming

compared to control animals (No stress + vehicle). The administration of chrysin (5 or 20 mg/kg) or fluoxetine (10 mg/kg) prevented the decrease in the total time of grooming compared to stress group (Stress + vehicle) (Fig. 3A). Pearson's correlation tests demonstrated significant correlation between time of grooming in the ST and CRH, ACTH, TNF-α, IL-1β, IL-6, KYN, TRP, 5-HT, 5-HIAA levels; IDO, caspase 3, caspase 9 activity; KYN/TRP, 5-HIAA/5-HT ratio (Table 1).

3.2 Rota rod test

Two-way ANOVA revealed no significant main effect for stress [$F(1.33)=0.34$, $P=0.56$], treatments (fluoxetine and chrysin) [$F(3.33)=0.81$, $P=0.50$] and stress X treatments interaction [$F(3.33)=1.20$, $P=0.32$] in the total performance time in the rota rod test (Fig. 3B). Similarly, two-way ANOVA showed no significant main effect for stress [$F(1.29)=0.93$, $P=0.76$], treatments (fluoxetine and chrysin) [$F(3.29)=0.18$, $P= 0.90$] and stress X treatments interaction [$F(3.29)=0.44$, $P=0.72$] on number of falls in the rota rod test (Fig. 3C).

3.3 Tail suspension test

Two-way ANOVA showed significant main effect for stress [$F(1.28)=16,68$, $P<0.001$], treatments [$F(3.28)=30.63$, $P<0.0001$] and stress X treatments interaction [$F(3.28)= 5.47$, $P<0.01$] in the total immobility time in the TST. Results demonstrated that chrysin administration (5 or 20 mg/kg) or fluoxetine (10 mg/kg) occasioned a decrease in the total immobility time in the TST when compared to control group (No stress + vehicle). Results showed that after the UCS exposition the animals displayed a significant increase in immobility time in TST when compared to control group (No stress + vehicle). Administration of chrysin prevented the increase in immobility time in mice exposed to UCS compared to stress group (Stress + vehicle). Moreover, chrysin administration occasioned a decrease in immobility time in mice exposed to UCS when compared to control group (No stress + vehicle) (Fig. 3D). Pearson's correlation tests demonstrated significant correlation between immobility time in the TST and CRH, ACTH, TNF-α, IL-1β, IL-6, KYN, TRP, 5-HT, 5-HIAA levels; IDO, caspase 3, caspase 9 activity; KYN/TRP, 5-HIAA/5-HT ratio (Table 1).

3.4 CRH and ACTH levels

Two-way ANOVA showed significant main effect for stress [F(1.20)= 53.62, P<0.0001], treatments [F(3.20)= 4.401, P= 0.0156] and stress X treatments interaction [F(3.20)= 7.713, P= 0.0013] in the plasma levels of CRH. Similarly, two-way ANOVA showed significant main effect for stress [F(1.20)= 40.23, P<0.0001], treatments [F(3.20)= 10.34, P=0.0003] and stress X treatments interaction [F(3.20)= 8.564, P= 0.0007] in the plasma levels of ACTH. Results showed that after UCS protocol the animals displayed a significant increase in the CRH and ACTH levels compared to control animals (No stress + vehicle). The treatment with chrysin or fluoxetine promoted the attenuation of the increase in the CRH and ACTH levels compared to stress group (Stress + vehicle) (Fig. 4). Pearson's correlation tests demonstrated significant positive correlation (ACTH X TNF-α, ACTH X IL-1ß, ACTH X IL-6, ACTH X caspase 3 and ACTH X caspase 9 in the PFC and in the HP) (Table 2).

3.5 TNF-α, IL-1β and IL-6 levels

Two-way ANOVA showed significant main effect for stress [F(1.24)=75.46, P<0.0001], treatments [F(3.24)=13.51, P<0,0001] and stress X treatments interaction [F(3.24)=10.85, P=0.0001] in the TNF-α levels in the PFC. Results of *post hoc* indicated that after the UCS exposition the animals displayed a significant increase in the TNF-α levels in the PFC compared to control animals (No stress + vehicle). The administration of chrysin or fluoxetine attenuated the increase in the TNF-α levels in the PFC compared to stress group (Stress + vehicle) (Fig. 5A). Pearson's correlation tests demonstrated a significant positive correlation (TNF-α levels in the PFC X IDO activity in the PFC) (Table 2).

Two-way ANOVA showed significant main effect for stress [F(1.24)= 244.7, P<0.001], treatments [F(3.24)=54.75, P<0.001] and stress X treatments interaction [F(3.24)= 52.23, P<0.001] in the IL-1β levels in the PFC. Results of *post hoc* indicated that after the UCS exposition the animals displayed a significant increase in the IL-1β levels in the PFC compared to control animals (No stress + vehicle). The administration of chrysin or fluoxetine attenuated the increase in the IL-1β levels in the PFC compared to stress group (Stress + vehicle) (Fig. 5B). Pearson's correlation tests demonstrated a significant positive correlation (IL-1β levels in the PFC X IDO activity in the PFC) (Table 2).

Two-way ANOVA showed significant main effect for stress [F(1.24)=162.1, P<0.0001], treatments [F(3.24)=35.00, P<0.0001] and stress X treatments interaction [F(3.24)=31.68, P<0.0001] in the IL-6 levels in the PFC. Results of *post hoc* indicated that after the UCS exposition the animals displayed a significant increase in the IL-6 levels in the PFC compared to control animals (No stress + vehicle). The administration of chrysin or fluoxetine attenuated the increase in the IL-6 levels in the PFC compared to stress group (Stress + vehicle) (Fig. 5C). Pearson's correlation tests demonstrated a significant positive correlation (IL-6 levels in the PFC X IDO activity in the PFC) (Table 2).

Two-way ANOVA showed significant main effect for stress [F(1.24)=104.4, P<0.0001], treatments [F(3.24)=11.00, P<0.0001] and stress X treatments interaction [F(3.24)=13.67, P<0.0001] in the TNF-α levels in the HP. Results of *post hoc* indicated that after the UCS exposition the animals displayed a significant increase in the TNF-α levels in the HP compared to control animals (No stress + vehicle). The administration of chrysin or fluoxetine attenuated the increase in the TNF-α levels in the HP compared to stress group (Stress + vehicle) (Fig. 5D). Pearson's correlation tests demonstrated a significant positive correlation (TNF-α levels in the HP X IDO activity in the HP) (Table 2).

Two-way ANOVA showed significant main effect for stress [F(1.24)=110.1, P<0.0001], treatments [F(3.24)=9.813, P=0.0002] and stress X treatments interaction [F(3.24)=9.514, P=0.0003] in the IL-1β levels in the HP. Results of *post hoc* indicated that after the UCS exposition the animals displayed a significant increase in the IL-1β levels in the HP compared to control animals (No stress + vehicle). The administration of chrysin or fluoxetine attenuated the increase in the IL-1β levels in the HP compared to stress group (Stress + vehicle) (Fig. 5E). Pearson's correlation tests demonstrated a significant positive correlation (IL-1β levels in the HP X IDO activity in the HP) (Table 2).

Two-way ANOVA showed significant main effect for stress [F(1.24)=263.8, P<0.0001], treatments [F(3.24)=50.39, P<0.0001] and stress X treatments interaction [F(3.24)= 32.92, P<0.0001] in the IL-6 levels in the HP. Results of *post hoc* indicated that after the UCS exposition the animals displayed a significant increase in the IL-6 levels in the HP compared to control animals (No stress + vehicle). The administration of chrysin or fluoxetine attenuated the increase in the IL-6 levels in the HP compared to stress group (Stress + vehicle) (Fig. 5F). Pearson's correlation tests demonstrated a significant positive correlation (IL-6 levels in the HP X IDO activity in the HP) (Table 2).

3.6 IDO activity, KYN and TRP levels and KYN/TRP ratio

Two-way ANOVA showed significant main effect for stress [F(1.24)=96.66, P<0.001], treatments [F(3.24)=15.03, P<0.001] and stress X treatments interaction [F(3.24)= 7.75, P<0.001] in the IDO activity in the PFC. Results showed that UCS occasioned a significant increase in the IDO activity in the PFC compared to control group (No stress + vehicle). The treatment with chrysin (5 or 20 mg/kg) or fluoxetine (10 mg/kg) attenuated the increase in the IDO activity in the PFC resulting from UCS compared to stress group (Stress + vehicle) (Fig. 6A). Pearson's correlation tests demonstrated a significant negative correlation (IDO activity in the PFC X 5-HT levels in the PFC) (Table 2).

Two-way ANOVA revealed significant main effect for stress [F(1.24)=18.49, P=0.0002], and no significant for treatments [F(3.24)=0.30, P=0.82] and stress X treatments interaction [F(3.24)=058, P=0.63] in the TRP levels in the PFC (Fig. 6B). Pearson's correlation tests showed significant negative correlation (TRP levels in the PFC X ST), and no significant correlation (TRP levels in the PFC X TST) (Table 1).

Two-way ANOVA demonstrated significant main effect for stress [F(1.24)=60.20, P<0.0001], treatments [F(3.24)=9.49, P=0.0003] and stress X treatments interaction [F(3.24)=7.39, P=0.0011] in the KYN levels in the PFC. Results revaluated that UCS protocol induced a significant elevation in the KYN levels in the PFC compared to control group (No stress + vehicle). The treatment with chrysin (5 or 20 mg/kg) or fluoxetine (10 mg/kg) promoted the prevention in the increase in the KYN levels in the PFC occasioned by UCS when compared to stress group (Stress + vehicle) (Fig. 6C). Pearson's correlation tests showed a significant negative correlation (KYN levels in the PCF X ST), and a significant positive correlation (KYN levels in the PCF X TST) (Table 1).

Two-way ANOVA showed significant main effect for stress [F(1.24)=31.38, P<0.0001], treatments [F(3.24)=6.77, P=0.0018] and stress X treatments interaction [F(3.24)=6.15, P=0.0030] in the KYN/TRP ratio in the PFC. *Post hoc* test demonstrated that UCS occasioned a significant increase in the KYN/TRP ratio in the PFC compared to control group (No stress + vehicle). The treatment with chrysin (5 or 20 mg/kg) or fluoxetine (10 mg/kg) prevented the increase in the KYN/TRP ratio in the PFC resulting from UCS compared to stress group (Stress + vehicle) (Fig. 6D).

Two-way ANOVA showed significant main effect for stress [F(1.24)=98.31, P<0.0001], treatments [F(3.24)=9.780, P=0.0002] and stress X treatments interaction [F(3.24)=6.264, P=0.0027] in the IDO activity in the HP. Results showed that UCS

occasioned a significant increase in the IDO activity in the HP compared to control group (No stress + vehicle). The treatment with chrysin or fluoxetine attenuated the increase in the IDO activity in the HP resulting from UCS compared to stress group (Stress + vehicle) (Fig. 7A). Pearson's correlation tests demonstrated a significant negative correlation (IDO activity in the HP X 5-HT levels in the HP) (Table 2).

Two-way ANOVA revealed significant main effect for stress [$F(1.24)=13.33$, $P=0.0013$], and no significant for treatments [$F(3.24)=0.58$, $P=0.64$] and stress X treatments interaction [$F(3.24)=0.47$, $P=0.70$] in the TRP levels in the HP (Fig. 7B).

Two-way ANOVA demonstrated significant main effect for stress [$F(1.24)=90.39$, $P<0.0001$], treatments [$F(3.24)=20.83$, $P<0.0001$] and stress X treatments interaction [$F(3.24)=16.77$, $P<0.0001$] in the KYN levels in the HP. Results revaluated that UCS protocol induced a significant elevation in the KYN levels in the HP compared to control group (No stress + vehicle). The treatment with chrysin (5 or 20 mg/kg) or fluoxetine (10 mg/kg) promoted the prevention in the increase in the KYN levels in the HP occasioned by UCS when compared to stress group (Stress + vehicle) (Fig. 7C).

Two-way ANOVA also showed significant main effect for stress [$F(1.24)=61.70$, $P<0.0001$], treatments [$F(3.24)= 18.24$, $P<0.0001$] and stress X treatments interaction [$F(3.24)=13.42$, $P<0.0001$] in the KYN/TRP ratio in the HP. *Post hoc* test demonstrated that UCS occasioned a significant increase in the KYN/TRP ratio in the HP compared to control group (No stress + vehicle). The treatment with chrysin (5 or 20 mg/kg) or fluoxetine (10 mg/kg) prevented the increase in the KYN/TRP ratio in the HP resulting from UCS compared to stress group (Stress + vehicle) (Fig. 7D).

3.7 5-HT and 5-HIAA levels and 5-HIAA/5-HT ratio

Two-way ANOVA showed significant main effect for stress [$F(1.24)=5.88$, $P=0.02$], and treatments [$F(3.24)=8.03$, $P=0.0007$] and no significant main effect for stress X treatments interaction [$F(3.24)=1.43$, $P=0.27$] in the 5-HT levels in the PFC. (Fig. 8A).

Two-way ANOVA revealed significant main effect for stress [$F(1.24)=5.67$, $P=0.0255$], and no significant for treatments [$F(3.24)=0.35$, $P=0.79$] and stress X treatments interaction [$F(3.24)=1.17$, $P=0.34$] in the 5-HIAA levels in the PFC (Fig. 8B).

Two-way ANOVA demonstrated significant main effect for stress [$F(1.24)=13.80$, $P=0.0011$] and treatments [$F(3.24)=4.32$, $P=0.01$], and no significant main effect stress X treatments interaction [$F(3.24)=1.003$, $P=0.41$] in the 5-HIAA/5-HT ratio in the PFC. Results

showed that after UCS exposition the animals displayed a significant increase in the 5-HIAA/5-HT ratio in the PFC compared to control animals (No stress + vehicle). Only the treatment with fluoxetine (10 mg/kg) attenuated the decrease in the 5-HIAA/5-HT ratio in the PFC compared to stress group (Stress + vehicle) (Fig. 8C).

Two-way ANOVA showed significant main effect for stress [F(1.24)=7.704, P=0.0105] and treatments [F(3.24)=10.34, P=0.0001], and no significant main effect stress X treatments interaction [F(3.24)=1.696, P=0.1945] in the 5-HT levels in the HP. Results of *post hoc* indicated that after the UCS exposition the animals displayed a significant decrease in the 5-HT levels in the HP compared to control animals (No stress + vehicle). The administration of chrysin (5 or 20 mg/kg) or fluoxetine (10 mg/kg) attenuated the decrease in the 5-HT levels in the HP compared to stress group (Stress + vehicle) (Fig. 8D).

Two-way ANOVA revealed significant main effect for stress [F(1.24)= 10.42, P=0.0036], and no significant main effect for treatments [F(3.24)=0.845, P=0.4824] and stress X treatments interaction [F(3.24)=1.377, P=0.2737] in the 5-HIAA levels in the HP (Fig. 8E).

Two-way ANOVA revaluated significant main effect for stress [F(1.24)=19.47, P=0.0002], treatments [F(3.24)=16.10, P<0.0001] and stress X treatments interaction [F(3.24)=10.03, P=0.0002] in the 5-HIAA/5-HT ratio in the HP. Results showed that after UCS exposition the animals displayed a significant increase in the 5-HIAA/5-HT ratio in the HP compared to control animals (No stress + vehicle). The treatment with chrysin (5 or 20 mg/kg) or fluoxetine (10 mg/kg) attenuated the increase in the 5-HIAA/5-HT ratio in the HP compared to stress group (Stress + vehicle) (Fig. 8F).

3.8 Caspase 3 and 9 activities

Two-way ANOVA showed significant main effect for stress [F(1.24)=93.79, P<0.0001], treatments [F(3.24)=22.55, P<0.0001] and stress X treatments interaction [F(3.24)= 8.575, P=0.0005] in the caspase 3 activity in the PFC. Results showed that after UCS protocol the animals displayed a significant increase in the caspase 3 activity in the PFC compared to control animals (No stress + vehicle). The treatment with chrysin (5 or 20 mg/kg) or fluoxetine (10 mg/kg) promoted the attenuation of the increase in the caspase 3 activity in the PFC compared to stress group (Stress + vehicle) (Fig. 9A).

Two-way ANOVA showed significant main effect for stress [F(1.24)=87.97, P<0.0001], treatments [F(3.24)=15.43, P<0.0001] and stress X treatments interaction [F(3.24)=14.66, P<0.0001] in the caspase 9 activity in the PFC. Results demonstrated that

after UCS mice displayed a significant increase in the caspase 9 activity in the PFC compared to control animals (No stress + vehicle). The treatment with crhysin (5 or 20 mg/kg) or fluoxetine (10 mg/kg) attenuated of the increase in the caspase 9 activity in the PFC compared to stress group (Stress + vehicle) (Fig. 9B).

Two-way ANOVA demonstrated significant main effect for stress [$F(1.24)=142.4$, $P<0.0001$], treatments [$F(3.24)=9.164$, $P=0.0003$] and stress X treatments interaction [$F(3.24)=4.970$, $P=0.0080$] in the caspase 3 activity in the HP. Results showed that after UCS the animals presented a significant increase in the caspase 3 activity in the HP compared to control animals (No stress + vehicle). The treatment with chrysin (5 or 20 mg/kg) or fluoxetine (10 mg/kg) promoted the attenuation of the increase in the caspase 3 activity in the HP compared to stress group (Stress + vehicle) (Fig. 9C).

Two-way ANOVA demonstrated significant main effect for stress [$F(1.24)=83.01$, $P<0.0001$], treatments [$F(3.24)=13.12$, $P<0.0001$] and stress X treatments interaction [$F(3.24)=11.81$, $P<0.0001$] in the caspase 9 activity in the HP. Results showed that after UCS the animals presented a significant increase in the caspase 9 activity in the HP compared to control animals (No stress + vehicle). The treatment with chrysin (5 or 20 mg/kg) or fluoxetine (10 mg/kg) promoted the attenuation of the increase in the caspase 9 activity in the HP compared to stress group (Stress + vehicle) (Fig. 9D).

4 Discussion

This work showed the antidepressant-like effect of chronic treatment with chrysin in mice exposed to UCS, similarly to fluoxetine. The antidepressant-like effect of chrysin on behavioral, hormonal, oxidative and neurotrophic parameters had been previously demonstrated by our group (Borges Filho et al., 2015). Meanwhile, in this work we extended the evaluations of neurochemical factors, demonstrating with this the association of pro-inflammatory cytokines, KP, 5-HT and caspases with antidepressant-like effect of chrysin in female mice exposed to UCS.

Treatment with chrysin or fluoxetine presented an antidepressant-like action in the TST in non-stressed mice. This result indicated the efficacy of chrysin or fluoxetine treatment even when there is not a chronic stress situation. UCS occasioned a depressive-like behavior in ST and TST in stressed mice. Depressive behavior showed in ST and TST demonstrating the lack of self-care and the conformity with adverse situations, respectively (Petit et al., 2014; Steru et al., 1985; Surget et al., 2008). These results demonstrated the efficiency of

UCS to induce a depressive-like status in rodents, such as showed previously (Borges Filho et al., 2015; Kumar et al., 2011; Mao et al., 2014). Chrysin or fluoxetine administrations promoted the total prevention of depressive behavior in ST and TST in stressed mice. Additionally, the fact that no significant difference in rota rod test was observed among any of the experimental groups indicates that behavioral changes in the ST and TST are not related to psychomotor-stimulant action. In another study of our group, the antidepressant-like action of chrysin in the forced swimming test and sucrose preference test in stressed mice was demonstrated (Borges Filho et al., 2015). Results observed in ST and TST comes to confirm the antidepressant potential of treatment with the flavonoid chrysin in female mice, similarly to fluoxetine.

In a previous work, our group showed the elevation of corticosterone levels in animals subjected to UCS, and the effect of chrysin treatment in the regulation of the levels of this hormone (Borges Filho et al., 2015). In this work, we demonstrated the fact that UCS also occasioned the increase in the plasma levels of CRH and ACTH in stressed mice, confirming the efficacy of UCS as a stress model by induction of the HPA axis dysfunction. Our results showed that the groups treated with chrysin or fluoxetine presented normal levels of CRH and ACTH and that plasma levels of these hormones presented a correlation with behavioral tests, which reinforces the concept that the antidepressant-like effect of chrysin is associated which the regulation of the HPA axis in stressed mice, as suggested in our previous work.

Correlated with deregulation on of the HPA axis, our results demonstrated the elevation of TNF-α, IL-1β and IL-6 levels in the PFC and HP resulting from UCS exposition in mice and the action of chrysin in the attenuation of these alterations. Change in pro-inflammatory cytokines levels has been strongly associated with depression occurrence in humans (Dahl et al., 2014; Dowlati et al., 2010). Dinkel et al. (2003) suggested that glucocorticoids, abundant in stressful situations, increase the number of inflammatory cells such as macrophages and microglia. Positive correlation observed between pro-inflammatory cytokines levels and ACTH levels consistently suggests the association of pro-inflammatory cytokines synthesis with the HPA axis dysfunction. In addition, correlation verified between TNF-α, IL- 1β and IL-6 levels and behavioral parameters indicate the role of pro-inflammatory cytokines in the depressive-like behavior observed in mice exposed to UCS. The role of chrysin in the attenuation of inflammation has been reported (Ahad et al., 2014; Feng et al., 2014; Xiao et al., 2014). Our work confirms the effect of chrysin in the suppression of inflammatory status, showing the effect of chronic treatment with chrysin in the attenuation of increase of TNF-α, IL-1β and IL-6 levels in the PFC and HP occasioned by

UCS. This result suggests the probable role of anti-inflammatory action in the antidepressant-like effect of chrysin treatment in mice exposed to UCS.

Our work showed that UCS occasioned the dysfunction of KP in mice, characterized by increase in the IDO activity, KYN levels and KYN/TRP ratio in PFC and HP. Treatment with chrysin or fluoxetine promoted the attenuation of this changes in stressed mice. The KP has been hypothesized to play a key role in processes linking peripheral inflammation and central nervous system alterations by i) reducing tryptophan availability, and ii) production of highly potent neurotoxins (Hochstrasser et al., 2011). Thus, TRP degradation and its role in the availability of 5-HT have brought attention to the KP as a potential target for future research into alternative treatments for depression. About 99% of TRP is metabolized by tryptophan 2,3-dioxygenase (TDO) into KYN in the liver. However, during active inflammation, IDO is activated in extrahepatic tissues to convert TRP to KYN (Leklem et al., 1971). The final products of the TRP degradation pathway have also been shown to directly alter activity of certain receptors in the brain. The correlations observed between IDO activity, KYN levels, KYN/TRP ratio and behavioral parameters indicate the role of KP in the depressive-like behavior occasioned by UCS. The positive correlation observed between pro-inflammatory cytokines and IDO activity suggests the strong association of inflammatory status with IDO activity. KP has been assumed to represent a link between pro-inflammatory cytokines and the neurochemical or neuroendocrine alterations that may be responsible for depression (Castanon et al., 2002; Schiepers et al., 2005). Furthermore, microglia is known to produce inflammatory mediators, so it is possible that these pro-inflammatory cytokines may induce the KP in the brain (Myint et al., 2012). Thus, is probably that, due to role of pro-inflammatory cytokines production in the activation of KP, the anti-inflammatory action of chrysin is associated with KP regulation and, consequently, with antidepressant-like behavior observed in animals treated with chrysin.

This work also demonstrated the effect of UCS in the 5-HT metabolism, characterized by increase in the 5-HIAA/5-HT ratio in the PFC and HP, and decrease in the 5-HT levels in the HP of mice. Treatment with chrysin or fluoxetine promoted the attenuation of this alterations. As stated previously, IDO can be induced by an increased production of pro-inflammatory cytokines. IDO activation may convert more TRP into neurotoxic tryptophan catabolites (TRYCATs) rather than to 5-HT (Neumeister et al., 2003), which decreases the bioavailability of TRP for the synthesis of 5-HT. In our work, the correlations observed between 5-HT, 5-HIAA levels, 5-HIAA/5-HT ratio and behavioral tests reinforce the serotonergic hypothesis of depression developed by Van Praag and Korf (1971), and the role

of 5-HT system in the depressive-like behavior induced by UCS. In addition, the negative correlation between 5-HT levels and IDO activity indicate strongly the role of activation of IDO in the decrease of 5-HT levels. Thus, our data suggest that decreased 5-HT level in HP was, at least in part, caused by elevated IDO activity although other factors which cause depletion of 5-HT could not be excluded in our study. Furthermore, the antidepressant-like effect of chrysin treatment in mice exposed to UCS is probably associated with regulation of 5-HT system occasioned by effect of chrysin in the normalization of pro-inflammatory cytokines production and IDO activity in brain structures.

Our results showed the increase in the caspase 3 and caspase 9 activities in the PFC and HP in stressed mice. Treatment with chrysin or fluoxetine promoted the attenuation of this damages. The increase in the caspase 3 and caspase 9 activities observed in mice exposed to UCS indicate the role of stress in the increase of apoptosis, programmed cell death. In addition, the correlation observed between caspase 3, caspase 9 activities and behavioral tests indicate the role of the apoptosis in the depressive status triggered by UCS. Stress is thought to induce apoptosis in many ways, including an increase in plasma corticosterone (Magarinos et al., 1985; Zhu et al., 2008). Some studies show the effect of chrysin treatment in the amelioration of apoptosis (Darwish et al., 2014; Khan et al., 2012). In both studies, chrysin treatment occasioned the regulation of caspase 3 expression or caspase 3 activity, suggesting the key role played by caspases in the effect of chrysin in the attenuation of apoptosis. As stated previously, some works indicate the role of hypersecretion of glucocorticoids in the increase of apoptosis. Thus, it is possible that the effect of chrysin in the regulation of HPA axis is associated with the action of chrysin in the amelioration of apoptosis. This possibility is enhanced by the correlation observed between caspase 3, caspase 9 activities and ACTH levels.

5 Conclusion

In conclusion, our study demonstrated the association existing between pro-inflammatory cytokines synthesis, KP, 5-HT levels, caspases activities and the antidepressant-like effect of chrysin treatment in mice exposed to UCS. These results clarify some of the mechanisms related to the action of chrysin in stressed mice. In addition, the fact of results presented by chrysin treatment are similar to presented by fluoxetine, a drug extensively used in antidepressant therapy, support that the flavonoid chrysin should be considered as a drug

with potential to depression treatment, corroborating the importance of further studies about the possible use of chrysin intake in the treatment or complementary treatment of depression.

Conflict of interest

The authors declare that there are no conflicts of interest in the present work.

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Legends

Fig. 1: Chemical structure of chrysin.

Fig. 2: Experimental design.

Fig. 3: Effect of chrysin and fluoxetine administration on splash test (ST) (A), rota rod test (B and C) and tail suspension test (TST) (D) in mice exposed to UCS. ST was realized in the 28th day of UCS. Rota rod test was realized 24h after to ST. TST was realized immediately after to rota rod test. *Indicates $P<0.05$ compared to control group (No stress + vehicle). $^{\#}$Indicates $P<0.05$ compared to stress group (Stress + vehicle).

Fig. 4: Effect of chrysin or fluoxetine treatment in the plasma levels of CRH (A) and ACTH (B) of mice exposed to UCS. The blood collection was realized 24h after TST. ^{a}Indicates $P<0.05$ compared to control group (No stress + vehicle). *Indicates $P<0.05$ compared to control group (No stress + vehicle). $^{\#}$Indicates $P<0.05$ compared to stress group (Stress + vehicle).

Fig. 5: Effect of chrysin or fluoxetine treatment in the TNF-α levels in the PFC (A) and HP (D); IL-1ß in the PFC (B) and HP (E); and IL-6 in the PFC (C) and HP (F). The brain structures were dissected immediately after blood collection. *Indicates $P<0.05$ compared to control group (No stress + vehicle). $^{\#}$Indicates $P<0.05$ compared to stress group (Stress + vehicle).

Fig. 6: Results of chrysin or fluoxetine treatment in the IDO activity (A), TRP levels (B), KYN levels (C) and KYN/TRP ratio (D) in the PFC of mice exposed to UCS. The brain structures were dissected immediately after blood collection. *Indicates $P<0.05$ compared to control group (No stress + vehicle). $^{\#}$Indicates $P<0.05$ compared to stress group (Stress + vehicle).

Fig. 7: Results of chrysin or fluoxetine treatment in the IDO activity (A), TRP levels (B), KYN levels (C) and KYN/TRP ratio (D) in the HP of mice exposed to UCS. The brain structures were dissected immediately after blood collection. *Indicates $P<0.05$ compared to

control group (No stress + vehicle). #Indicates P<0.05 compared to stress group (Stress + vehicle).

Fig. 8: Effect of chrysin or fluoxetine treatment in the 5-HT levels in the PFC (A) and HP (D); 5-HIAA levels in the PFC (B) and HP (E); and 5-HIAA/5-HT ratio in the PFC (C) and HP (F). The brain structures were dissected immediately after blood collection. *Indicates P<0.05 compared to control group (No stress + vehicle). #Indicates P<0.05 compared to stress group (Stress + vehicle).

Fig. 9: Effect of chrysin or fluoxetine treatment in the caspase 3 activity in the PFC (A) and HP (C); and caspase 9 activity in the PFC (B) and HP (D) of mice exposed to UCS. The brain structures were dissected immediately after blood collection. *Indicates P<0.05 compared to control group (No stress + vehicle). #Indicates P<0.05 compared to stress group (Stress + vehicle).

Figures

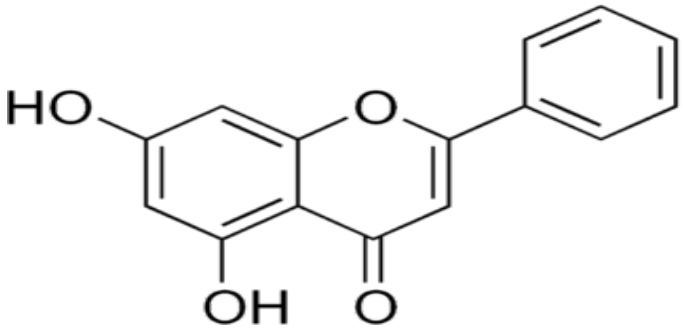

Fig. 1

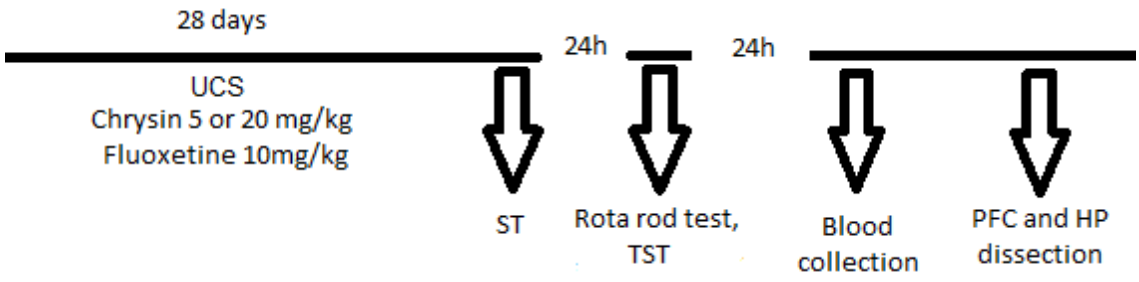
28 days
UCS
Chrysin 5 or 20 mg/kg
Fluoxetine 10mg/kg
24h
24h
ST
Rota rod test,
TST
Blood
collection
PFC and HP
dissection

Fig. 2

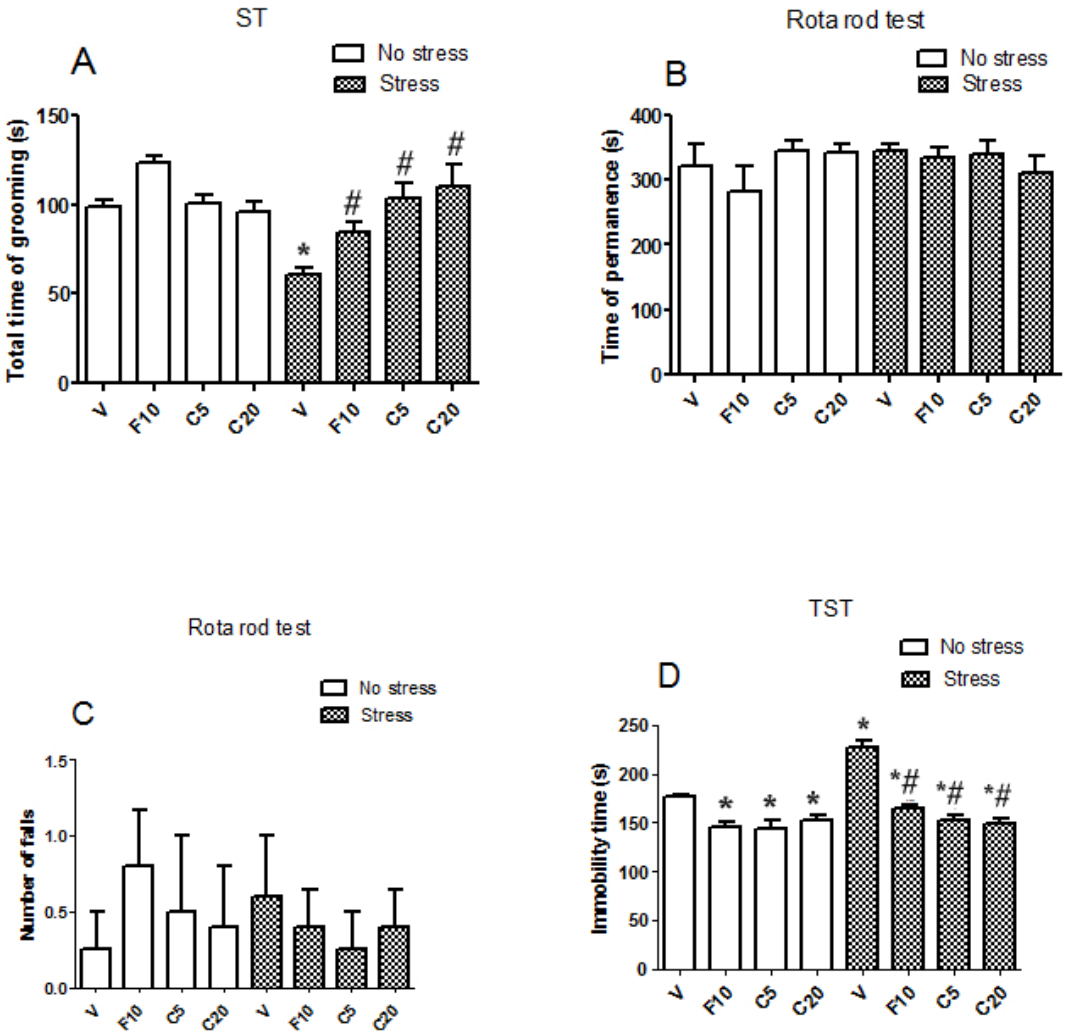
ST
A
No stress
Stress
Total time of grooming (s)
150
100
50
0
V
F10
C5
C20
*
#
Rota rod test
B
Time of permanence (s)
400
300
200
100
0
Rota rod test
C
Number of falls
1.5
1.0
0.5
0.0
TST
D
Immobility time (s)
250
200
150
100
50
0
*
*#

Fig. 3

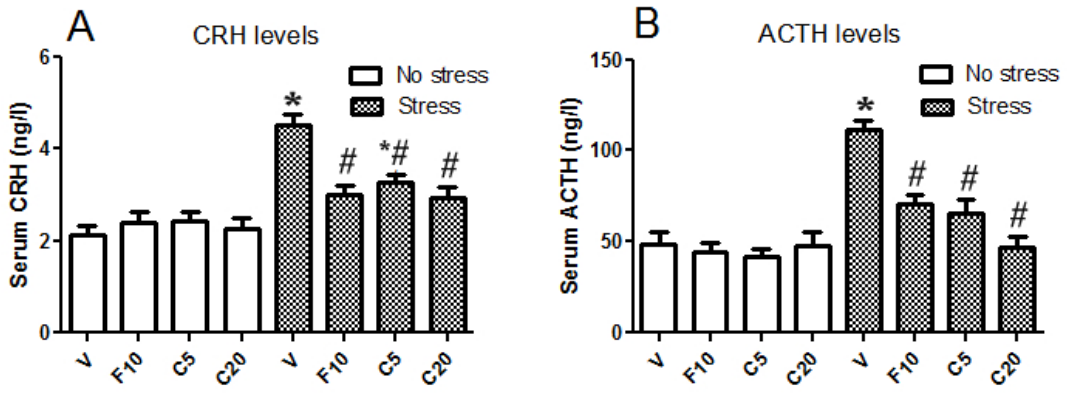
A
CRH levels
No stress
Stress
Serum CRH (ng/l)
6
4
2
0
*
#
*#
#
V
F10
C5
C20
V
F10
C5
C20
B
ACTH levels
No stress
Stress
Serum ACTH (ng/l)
150
100
50
0
*
#
#
#
V
F10
C5
C20
V
F10
C5
C20

Fig. 4

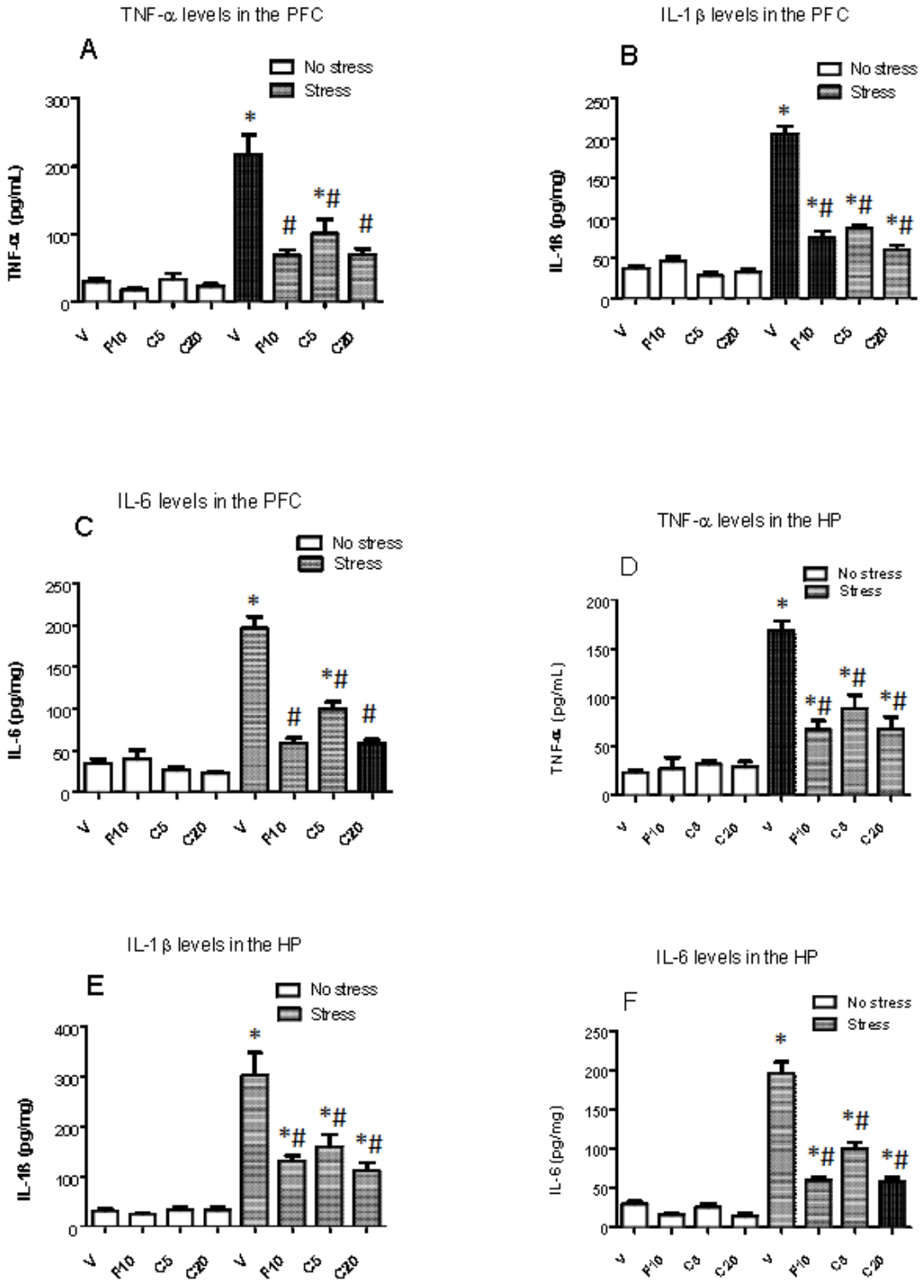
TNF-α levels in the PFC
A
No stress
Stress
TNF-α (pg/mL)
V
P10
C5
C20
IL-1 β levels in the PFC
B
IL-1ß (pg/mg)
IL-6 levels in the PFC
C
IL-6 (pg/mg)
TNF-α levels in the HP
D
IL-1 β levels in the HP
E
IL-1ß (pg/mg)
IL-6 levels in the HP
F
IL-6 (pg/mg)

Fig. 5

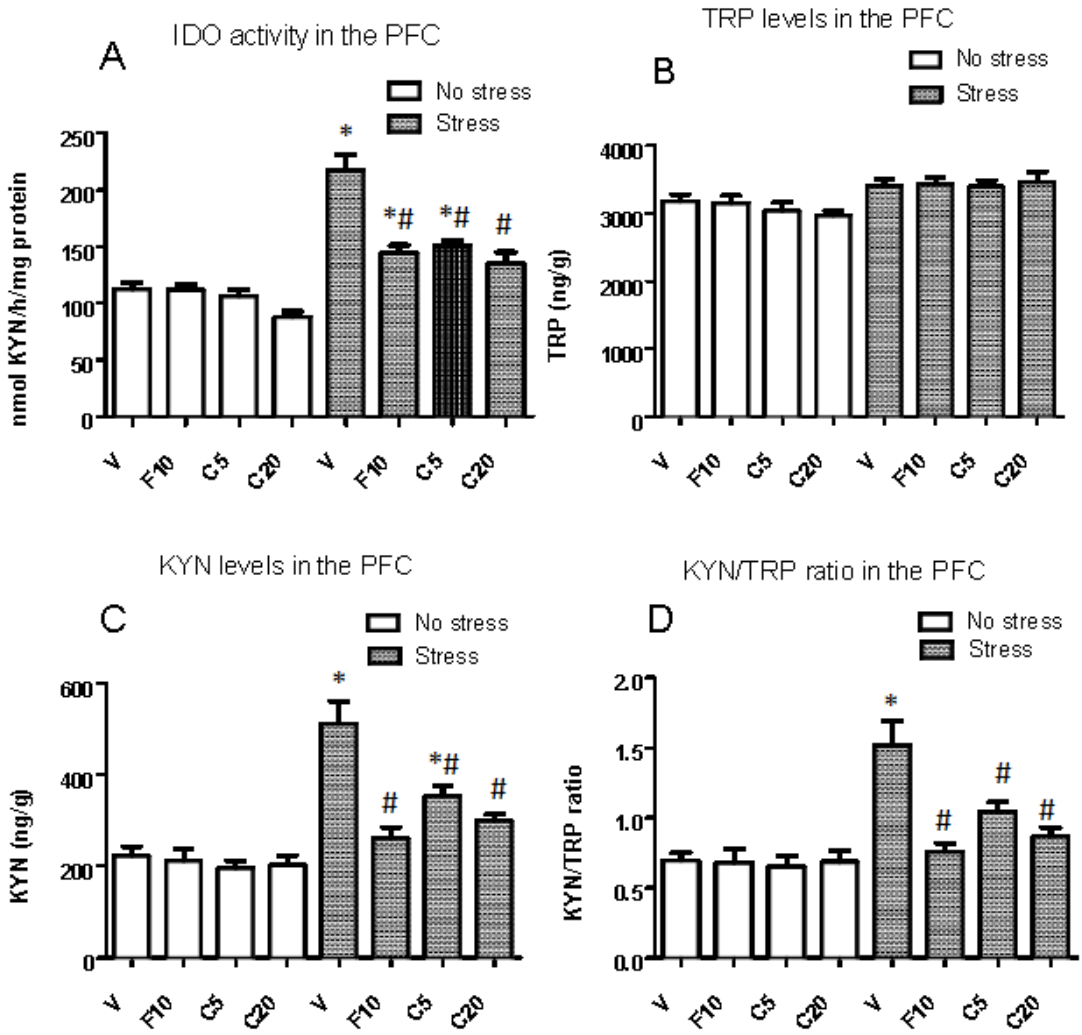
A
IDO activity in the PFC
No stress
Stress
nmol KYN/h/mg protein
250
200
150
100
50
0
V
F10
C5
C20
B
TRP levels in the PFC
TRP (ng/g)
4000
3000
2000
1000
C
KYN levels in the PFC
KYN (ng/g)
600
400
200
D
KYN/TRP ratio in the PFC
KYN/TRP ratio
2.0
1.5
1.0
0.5
0.0

Fig. 6

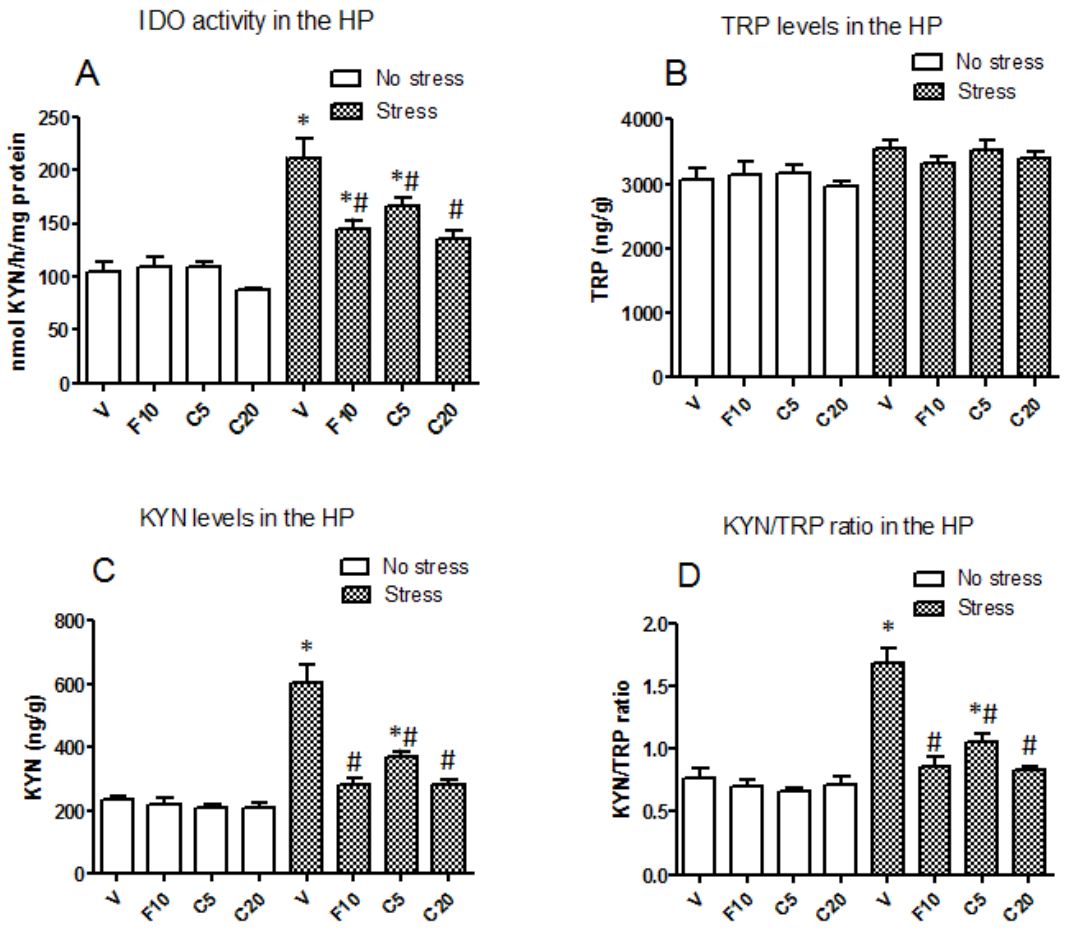
IDO activity in the HP
A
No stress
Stress
nmol KYN/h/mg protein
250
200
150
100
50
0
*
*#
*#
#
V
F10
C5
C20
V
F10
C5
C20
TRP levels in the HP
B
No stress
Stress
TRP (ng/g)
4000
3000
2000
1000
0
KYN levels in the HP
C
No stress
Stress
KYN (ng/g)
800
600
400
200
0
*
#
*#
#
KYN/TRP ratio in the HP
D
No stress
Stress
KYN/TRP ratio
2.0
1.5
1.0
0.5
0.0
*
#
*#
#

Fig. 7

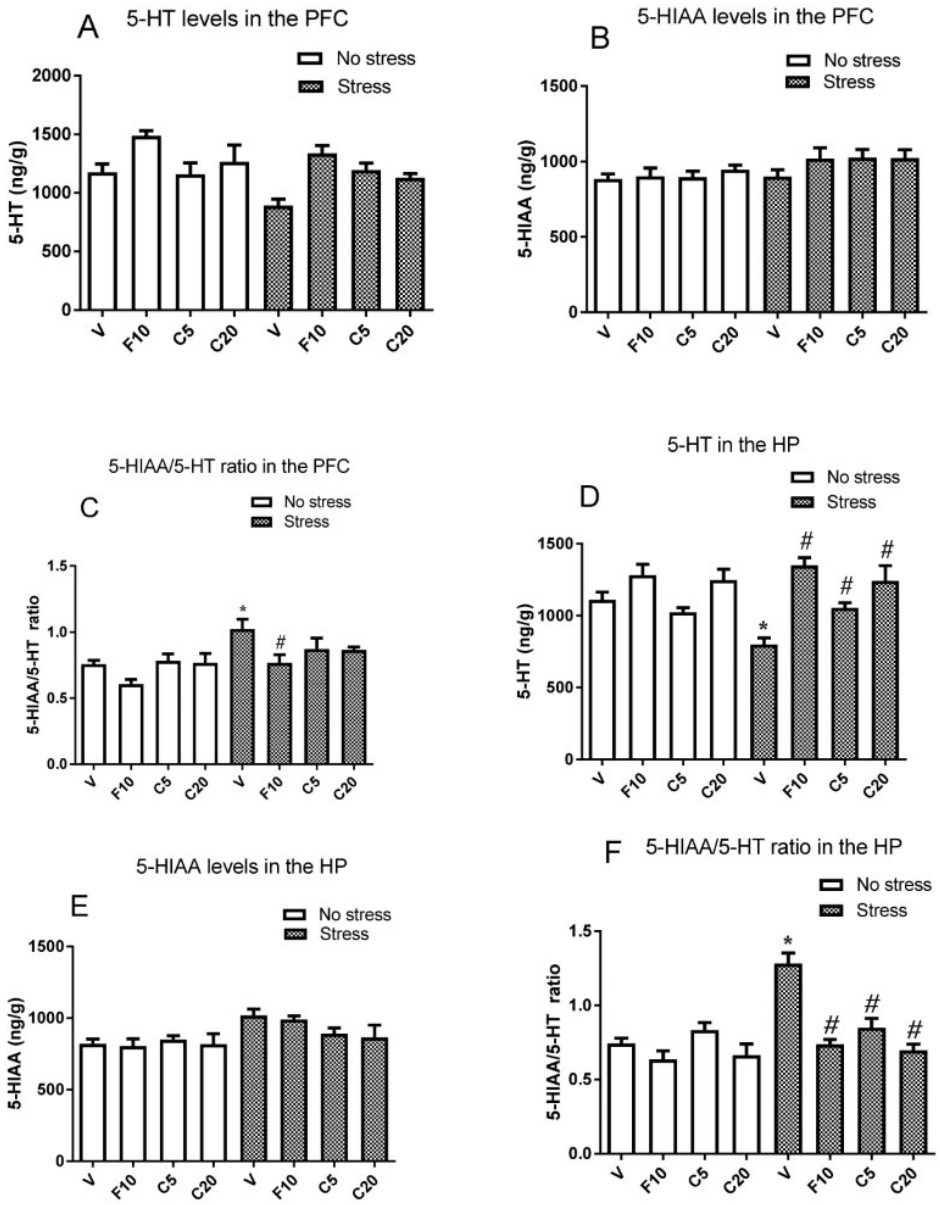
A
5-HT levels in the PFC
No stress
Stress
5-HT (ng/g)
2000
1500
1000
500
0
V
F10
C5
C20
B
5-HIAA levels in the PFC
5-HIAA (ng/g)
C
5-HIAA/5-HT ratio in the PFC
5-HIAA/5-HT ratio
1.5
1.0
0.5
0.0
D
5-HT in the HP
E
5-HIAA levels in the HP
F
5-HIAA/5-HT ratio in the HP

Fig. 8

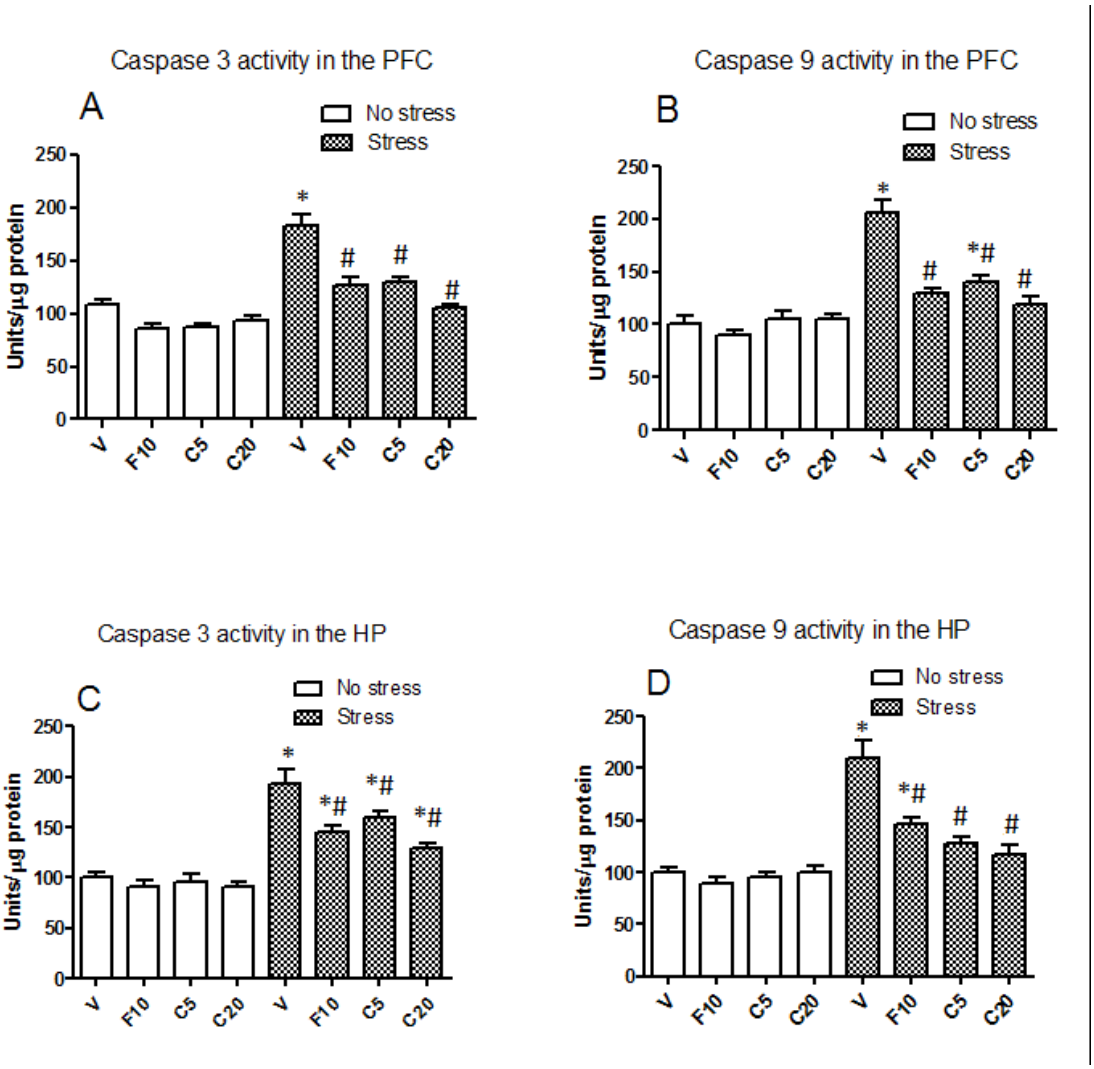
Caspase 3 activity in the PFC
A
No stress
Stress
Units/µg protein
250
200
150
100
50
0
V
F10
C5
C20
Caspase 9 activity in the PFC
B
Caspase 3 activity in the HP
C
Caspase 9 activity in the HP
D

Fig. 9

Tables

Table 1. r values resulting from Pearson's correlation test for endocrine and neurochemical factors X behavioral parameters. [a]denoted P<0.05.

	ST	TST
CRH levels	-0.41[a]	0.62[a]
ACTH levels	-0.66[a]	0.76[a]
TNF-α levels	PFC=-0.48[a] HP=-0.43[a]	PFC=0.66[a] HP= 0.68[a]
IL-1β levels	PFC=-0.43[a] HP=-0.54[a]	PFC=0.74[a] HP=0.65[a]
IL-6 levels	PFC=-0.41[a] HP=-0.49[a]	PFC=0.70[a] HP=0.70[a]
IDO activity	PFC=-0.45[a] HP=-0.54[a]	PFC=0.70[a] HP=0.60[a]
KYN levels	PFC=-0.39[a] HP=-0.34	PFC=0.62[a] HP=0.70[a]
TRP levels	PFC=-0.46[a] HP=0.19	PFC=0.25 HP=0.27
KYN/TRP ratio	PFC=-0.31[a] HP=-0.39[a]	PFC=0.61[a] HP=0.72[a]
5-HT levels	PFC=0.37[a] HP=0.10	PFC=-0.40[a] HP=-0.50[a]
5-HIAA levels	PFC=-0.18 HP=-0.58[a]	PFC=0.068 HP=0.41[a]
5-HIAA/5-HT ratio	PFC=-0.43[a] HP=-0.42[*]	PFC=0.48[a] HP=0.65[a]
Caspase 3 activity	PFC=-0.55[a] HP=-0.54[*]	PFC=0.77[a] HP= 0.60[a]
Caspase 9 activity	PFC=-0.50[a] HP=-0.60[a]	PFC=0.69[a] HP=0.70[a]

Table 2. r values resulting from Pearson's correlation test for hormonal and neurochemical parameters. [a]denoted P<0.05.

	ACTH levels	TNF-α levels (PFC or HP)	IL-1β levels (PFC or HP)	IL-6 levels (PFC or HP)	5-HT levels (PFC or HP)
ACTH levels	-	PFC=0.81[a] HP= 0.90[a]	PFC=0.85[a] HP= 0.84[a]	PFC=0.87[a] HP= 0.87[a]	PFC=-037 HP=-0.54[a]
IDO activity PFC	0.82[a]	0.92[a]	0.88[a]	0.92[a]	-0.47[a]
IDO activity HP	0.78[a]	0.82[a]	0.83[a]	0.91[a]	-0.43[a]
5-HT levels PFC	-	-0.59[a]	-0.50[a]	-0.54[a]	-
5-HT levels HP	-	-0.52[a]	-0.52[a]	-0.58[a]	-
Caspase 3 activity PFC	0.87[a]	0.85[a]	0.88[a]	0.88[a]	-0.53[a]
Caspase 3 activity HP	0.74[a]	0.83[a]	0.82[a]	0.88[a]	-0.33[a]
Caspase 9 activity PFC	0.81[a]	0.91[a]	0.90[a]	0.90[a]	-0.53[a]
Caspase 9 activity HP	0.83[a]	0.80[a]	0.82[a]	0.88[a]	-0.47[a]

4.2 MANUSCRITO

Submetido à revista “Chemico-Biological Interactions”.

Chrysin promotes attenuation of depressive-like behavior and hippocampal dysfunction resulting from olfactory bulbectomy in mice

Carlos Borges Filho[1], Cristiano Ricardo Jesse[1*], Franciele Donato[1], Lucian Del Fabbro[1], Marcelo Gomes de Gomes[1], André Tiago Rossito Goes[1], Leandro Cattelan Souza[1], Silvana Peterini Boeira[1]

[1] Laboratório de Avaliações Farmacológicas e Toxicológicas Aplicadas às Moléculas Bioativas - LaftamBio Pampa - Universidade Federal do Pampa, CEP 97650-000, Itaqui, RS, Brazil.

Cristiano R. Jesse E-mail: cristianoricardojesse@yahoo.com.br

Laboratório de Avaliações Farmacológicas e Toxicológicas Aplicadas às Moléculas Bioativas - *LaftamBio* Pampa - Universidade Federal do Pampa, CEP 97650-000, Itaqui, RS, Brazil.

Phone: +55-55-34331669

FAX: +55-55-34331669

Abstract

Chrysin is a flavonoid which is found in bee propolis, honey and various plants, and antidepressant-like effect of chrysin in chronically stressed mice was previously demonstrated by our group. In this work, we investigate the action of chrysin treatment (5 or 20 mg/kg) for 14 days in the depressant-like behavior and in the hippocampal dysfunction induced by olfactory bulbectomy (OB), a model of agitated depression. Results demonstrated that OB occasioned a depressant-like behavior in the splash test, open field test and forced swimming test. Chrysin administration, similarly to fluoxetine (positive control), promoted the attenuation of these behavioral modifications. OB also caused the elevation of tumor necrosis factor-α, interferon-γ, interleukin-1β, interleukin-6, kynurenine (KYN) levels and indoleamine-2,3-dioxygenase activity, as well as occasioned the decrease of 5-hydroxytryptamine (5-HT) and brain-derived neurotrophic factor (BDNF) levels and increase KYN/tryptophan and 5-hydroxyindoleacetic acid/5-HT ratio in the hippocampus. Chrysin therapy prevented against all these alterations in the hippocampus. In addition, chrysin treatment (20 mg/kg) resulted in the up-regulation of BDNF levels in the control animals, reinforcing our hypothesis that up-regulation of BDNF synthesis play a key role in the antidepressant action of chrysin. In conclusion, this study showed that chrysin, similarly to fluoxetine, is capable of promoting the attenuation of depressant-like behavior and hippocampal dysfunction resulting from OB in mice. These results reinforce the potential of chrysin for the treatment or supplementary treatment of depression, as well showed that chrysin also is effective with 14 days of therapy in a model of agitated depression.

Keywords: Flavonoid, depression, olfactory bulbectomy, antidepressant-like effect.

Abreviations: 5-HIAA, 5-Hydroxyindoleacetic acid; 5-HT, 5-hydroxytryptamine; ANOVA, analysis of variance; BDNF, brain-derived neurotrophic factor; CRH, corticotropin-releasing hormone; ELISA, enzyme-linked immune sorbent assay; FST, forced swimming test; HP, hippocampus; HPLC, high pressure liquid chromatography; IDO, indoleamine-2,3-dioxygenase; IFN-γ, interferon-γ; IL-1β, interleukin-1β; IL-6, interleukin-6; KP, kynureninepathway; KYN, kynurenine; KYNA, kynurenic acid; QUIN, quinolinic acid; S1, supernatant fraction; ST, splash test; TNF-α, tumor necrosis factor-α; TRP, tryptophan; TRYCATs, tryptophan catabolites.

1 Introduction

Etiology and treatment of depression is one of the most serious challenges to modern medicine. The number of patients suffering from depression is growing. Unfortunately, in many cases of depression, the effectiveness of currently used antidepressants is not sufficient [1].

The bilateral olfactory bulbectomy (OB) creates a chronically altered brain state with complex changes of behavioral and neurochemical parameters, many of which are comparable to those seen in patients with major depression [2]. Thus, OB in rodents has been proposed to represent an animal model that appears to fulfill many of the necessary criteria for a depression model, especially agitated depression [3,4].

A hyperactivity response, the major behavioral change in OB, can be reversed by chronic treatment with antidepressants, mimicking the slow onset of antidepressant action reported in clinical studies [4]. Studies have analyzed self-care, motivational and/or anhedonic behavior associated with hyperactivity in bulbectomized rodents [4,5]. Moreover, OB in rodents has been associated with chemical alterations in the hippocampus (HP), including pro-inflammatory cytokines production [6], serotonin (5-HT) system [2], and brain-derived neurotrophic factor (BDNF) levels [2,6].

Chrysin (5,7-dihydroxyflavone, Fig. 1) is a flavonoid which is found in bee propolis, honey and various plants [7]. Research has shown that chrysin has multiple biological activities, such as anti-inflammatory, antineoplastic, hipolipidemic and antioxidant [8-11]. In addition, a recent study of our group demonstrated the antidepressant potential of chrysin treatment in mice exposed to chronic unpredictable mild stress (CUMS) [11]. However, we understand that the antidepressant-like effect of chrysin still requires investigations in other animal models of depression. Thus, the aim of this work was to investigate the effect of treatment with chrysin in an animal model of depression induced by OB, comparing it to the effect of fluoxetine, a positive control [4]. Our working hypothesis is that chrysin may promote the attenuation of depressive-like behavior and hippocampal damage induced by OB in mice.

2 Materials and methods

2.1 Animals

Experiments were realized with male C57B/6J mice (20-25g, 90 days old). Animals were maintained at 22-25 °C, with free access to water and food, under a 12:12-h light/dark cycle, with lights on at 7:00 a.m. The procedures of this study were conducted according to the guidelines of the Committee on the Care and Use of Experimental Animal Resources.

2.2 Drug solutions and administrations

Chrysin and fluoxetine were purchased from Sigma (St. Louis, MO, USA). All other chemicals used were obtained from standard commercial suppliers.

Chrysin was dissolved in a distilled water/propyleneglycol solution (80:20). Fluoxetine was dissolved in distilled water. Both drugs were administered per oral (p.o.) in the volume of 10ml/kg.

2.3 Bilateral olfactory bulbectomy (OB) surgical procedure

After a 2-week acclimatization period, OB was performed according to the procedure described by Leonard and Tuit [12]. Briefly, mice were anesthetized with xylazin (20 mg/kg) in combination with ketamine (100 mg/kg) diluted in saline (0.9% NaCl) administered intraperitoneally (i.p., 10 ml/kg body weight). The skull covering the olfactory bulbs was exposed by skin incision, and two burr holes were drilled using a dentist drill. The olfactory bulbs were bilaterally aspirated using a blunt hypodermic needle (1.0 to 1.2 cm long and with a rounded tip of 0.80 to 1.2 mm in diameter) attached to a 10-ml syringe, taking care not to cause damage to the frontal cortex. Finally, the burr hole was filled with acrylic resin to avoid bleeding and contamination of the surgical site. SHAM-operations were performed in the same way, but the olfactory bulbs were left intact. After surgery, all animals were allowed to recover in a post-operative cage (maintained at 24°C) for 3h. After this time period, the mice were returned to their home cage. After behavioral testing, all animals were sacrificed and the presence of lesions was verified. The bulbectomized animals that showed incomplete removal of olfactory bulbs or damage to other brain areas were excluded from the subsequent analysis following the criteria previously described [13,14].

2.4 Drug treatments and experimental design

After undergoing an OFT, all animals were submitted to surgery to remove their olfactory bulbs or only to surgery (SHAM). The animals had 14 days of recovery and were then again subjected to an OFT after exposure to treatments. The mice were divided into eight groups (n = 4-6): [1] Control (SHAM + vehicle) {V},[2] Fluoxetine 10mg/kg (SHAM + fluoxetine 10mg/kg) {F10}, [3] Chrysin 5mg/kg (SHAM + chrysin 5 mg/kg) {C5}, [4] Chrysin 20mg/kg (SHAM + chrysin 20mg/kg){C20}, [5] OB (OB + vehicle) {V}, [6] OB + fluoxetine 10mg/kg {F10}, [7] OB + Chrysin 5mg/kg {C5} and [8] OB + Chrysin 20mg/kg {C20}. Mice were treated with chrysin at doses of 5 or 20 mg/kg, corresponding to a low dose and a high dose, respectively [11], or fluoxetine at the dose of 10mg/kg [15,16], daily for 14 days.

After the treatments, the animals were subjected to splash test (ST) and after 24h were exposed to open field test (OFT) and subsequently to forced swimming test (FST). After 24h the animals were euthanized by decapitation and HP was dissected for hippocampal determinations (Fig. 2).

2.5 Behavioral assessment

2.5.1 Splash test (ST)

This test was performed after the treatment period and consisted of squirting 200 µl of a 10% sucrose solution on the mouse's snout. Because of its viscosity, the sucrose solution dirties the mouse fur and animals initiate grooming behavior. After applying sucrose solution, the time spent for grooming was recorded in seconds for a period of 5 min. A decrease in grooming, which is a particular feature of mice submitted to stress was used as an index of self-care and motivational behavior [17,18].

2.5.2 Open field test (OFT)

OFT was performed 24 hours after the ST. In brief, the locomotor activity of mice was tested by a digital actophotometer. Each mouse was placed in the center of the actophotometer apparatus, and the locomotor activity was assessed. Total distance traveled (mm) and number of rearing were evaluated in the 5-min test period to evaluate locomotor activity [19].

2.5.3 Forced swimming test (FST)

FST was performed immediately after the OFT. FST consist of cylindrical jar with dimension of 10 cm diameter, 25 cm height and filled with19 cm of water at 24 ± 1 °C as defined previously [20]. Each mouse was placed gently and was allowed to swim freely for 6 min. The mouse was assumed as immobile when it showed disparity and became motionless in the water. During the period of immobility they only made those movements which were necessary to keep their head outside the water. The immobility time was recorded in seconds.

2.6 Tissue preparation

24h after the FST, mice were euthanized for decapitation and HP was dissected and homogenized (1:5) in Tris-HCl 50mM, pH 7.5. The homogenate was centrifuged at 2,400 x g for 5 min and supernatant fraction (S1) was used for hippocampal determinations.

2.7 Hippocampal determinations

2.7.1 Tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), interleukin-1β (IL-1β) and interleukin-6 (IL-6) levels

Levels of TNF-α, IFN-γ, IL-1β and IL-6 in the HP were determined using commercially available ELISA assays, following the instructions supplied by the manufacturer (DuoSet Kits, R&D Systems; Minneapolis). Results are shown as pg/mg tissue.

2.7.2 Indoleamine-2,3-dioxygenase (IDO) activity

IDO activity in the HP was determined as previously described [21], with minor modifications. The S1 (0.2 ml) were added to 0.8 ml of the reaction mixture containing 400 µM L-tryptophan, 20 mM ascorbate, 10 µM methylene blue, and 100 µg catalase in 50mM potassium phosphate buffer pH 6.5. The reaction was carried out at 37ºC under agitation for 60 min. Then, it was blocked by adding 0.2 ml of 30% trichloroacetic acid and further incubated at 50ºC for 30 min to convert the N-formylkynurenine to L-kynurenine. Samples were centrifuged at 13,000g for 10 min at 4ºC. The supernatants were filtered through microspin ultrafiltrates with a cut-off of 10,000M_r before being taken for measurement of

IDO. The amount of L-kynurenine formed from TRP was determined by reversed phase high pressure liquid chromatography (HPLC). One hundred µl of the reaction product was injected onto a Merck LiChrospher column (150mm long, 4.6mm diameter, packed with 5 lm silica beads holding 18C long carbon chains). A cartridge guard column containing the same material as the analytical column was used. The mobile phase consisted of 0.1M ammonium acetate buffer (pH 4.65) with 5% acetonitrile. Flow rate was 1 ml/min. KYN was detected using a spectrometer measuring absorbency at a wavelength of 365nm and was quantified using known amounts of L-kynurenine. The retention time of KYN was around 5.35 min. All determinations were performed in duplicate. One unit of the activity was defined as 1 nmol KYN/h/mg protein at 37ºC.

2.7.3 Tryptophan (TRP) and kynurenine (KYN) levels and KYN/TRP ratio

TRP and KYN levels were measured in the HP samples using HPLC. The mobile phase contained 50 nM glacial acetic acid, 100 mM zinc acetate and 3% acetonitrile dissolved in double-distilled NANO pure water HPLC grade H_2O. The pH was adjusted to 4.9 using 5 M NaOH. S1 of PFC and HP were sonicated in 1 ml of mobile phase containing 7% perchloric acid spiked with 50 ng/20 µl of N-methyl 5-HT as an internal standard. The resultant solution was centrifuged at 20,000 rpm for 20 min and the supernatants were placed into new Eppendorf tubes using a syringe fitted with a 0.45-µm filter (Phenomenex). Approximately 20 µl of the filtered supernatant was injected using a Waters auto sampler and a Reverse Phase analytical column (Kinetex™ Core Shell Technology column with specific area of 4.6 mm and particle size of 2.6 µl, Phenomenex) was used for the separation of metabolites. A PDA-UV detector (Shimadzu SPD-M10A VP), calibrated to integrate at 230 and 250 nm, as well as a fluorescent detector (Shimadzu RF- 20A XS prominence fluorescence detector), set to excitation wavelength 254 nm and emission wavelength 404 nm, were used to detect the metabolites. Chromatographs were generated by CLASS-VP software (Shimadzu). The results were expressed as ng/g tissue. KYN/TRP ratio was calculated by ratio between KYN and TRP concentrations.

2.7.4 5-HT and 5-Hydroxyindoleacetic acid (5-HIAA) levels and 5-HIAA/5-HT ratio

The levels of 5-HT and its metabolite, 5-HIAA in the HP were analyzed by HPLC with electrochemical detection, as previously described [22]. The mobile phase, used at a flow rate of 0.8 ml/min, consisted of 0.02 M phosphate/citrate buffer and 90/10 methanol (v/v), 0.12 mMNa2 EDTA, and 0.0556% heptane sulphonic acid as ion pair. The pH was adjusted to 2.64 with H_3PO_4 at 22 °C. A 5-µm (220 × 4.6) Spheri-5 RP-18 column from Brownlee Laboratory was used. Electrochemical detection was performed with a Shimadzu L-ECD-6A electrochemical detector with a potential of 0.75 V. The peak area of the internal standard (DHBA) was used to quantify the sample peaks. The tissue levels were expressed in ng/g tissue. 5-HIAA/5-HT ratio was calculated by ratio between 5-HIAA and 5-HT concentrations.

2.7.5 BDNF levels

BDNF levels in the HP were determined using commercially available ELISA assays, following the instructions supplied by the manufacturer (DuoSet Kits, R&D Systems; Minneapolis). Results were shown as ng/g tissue.

2.7.6 Protein quantification

Protein concentration in the PFC and HP was measured by the method of Bradford (1976), using bovine serum albumin as the standard.

2.8 Statistical analysis

Results were expressed as the mean ± standard error of the mean (SEM). Statistical analysis was performed using a two-way analysis of variance (ANOVA), analyzing the effect of OB, treatments (chrysin or fluoxetine), and OB x treatments interaction. Two-way ANOVA was followed by Newman-Keuls test when appropriate. Person's correlation test was also realized to verify the possible statistic relation between the evaluated parameters. The main results of Person's correlation test were mentioned in the results and in the discussion. The level of significance was set at $p<0.05$. The statistical analysis was performed using the software GraphPad Prism version 5 (GraphPad Software, Inc., La Jolla, CA, USA).

3 Results

3.1 ST

Two-way ANOVA showed significant main effect for OB [$F(1.32)=9.62$, $p<0.01$] and treatments [$F(3.32)=3.05$, $p<0.05$] and no significant OB X treatments interaction [$F(3.32)=2.89$, $p=0.0505$] in the total time of grooming in the ST. Results of post hoc indicated that after OB the animals displayed significant decrease in the total time of grooming compared to control animals (SHAM + vehicle). The administration of chrysin (20 mg/kg) or fluoxetine (10 mg/kg) prevented the decrease in the total time of grooming compared to OB group (OB + vehicle) (Fig. 3A).

3.2 OFT

Two-way ANOVA revealed significant main effect for OB [$F(1.32)=59.86$, $p<0.001$], treatments (fluoxetine and chrysin) [$F(3.32)=9.83$, $p<0.0001$] and OB X treatments interaction [$F(3.32)=9.38$, $p<0.0001$] in the travelled distance in the OFT. Results of post hoc indicated that after OB the animals displayed significant increase in the travelled distance compared to control group (SHAM + vehicle). The administration of chrysin (5 or 20 mg/kg) or fluoxetine (10 mg/kg) prevented the increase in the travelled distance compared to OB group (OB + vehicle) (Fig. 3B).

In addition, two-way ANOVA revealed significant main effect for OB [$F(1.32)=4.88$, $p<0.05$] and OB X treatments interaction [$F(3.32)=5.06$, $p<0.01$], and no significant main effect for treatments (fluoxetine and chrysin) [$F(3.32)=2.74$, $p=0.0593$] in the number of rearing in the OFT. Post hoc test demonstrated that after OB the animals displayed significant increase in the number of rearing compared to control group (SHAM + vehicle). The administration of chrysin (5 or 20 mg/kg) or fluoxetine (10 mg/kg) prevented the increase in the number of rearing compared to OB group (OB + vehicle) (Fig. 3C).

3.3 FST

Two-way ANOVA showed significant main effect for OB [$F(1.32)=27.68$, $p<0.0001$], treatments [$F(3.32)=32.63$, $p<0.0001$] and OB X treatments interaction [$F(3.32)= 3.09$, $p<0.05$] in the total immobility time in the FST. Results demonstrated that chrysin

administration (5 or 20 mg/kg) or fluoxetine (10 mg/kg) occasioned a decrease in the total immobility time in the FST when compared to control group (SHAM + vehicle). Results showed that after the OB the animals displayed a significant increase in immobility time in FST when compared to control group (SHAM + vehicle). Administration of chrysin (5 or 20 mg/kg) or fluoxetine (10mg/kg) prevented the increase in immobility time in mice exposed to OB compared to OB group (OB + vehicle). Moreover, chrysin (20 mg/kg) administration occasioned a decrease in immobility time in mice exposed to OB when compared to control group (SHAM + vehicle) (Fig. 3D).

3.4 TNF-α, IFN-γ, IL-1β and IL-6 levels

Two-way ANOVA showed significant main effect for OB [$F(1.24)=123.4$, $p<0.0001$], treatments [$F(3.24)=16.76$, $p<0.0001$] and OB X treatments interaction [$F(3.24)=16.02$, $p<0.0001$] in the TNF-α levels in the HP. Results of post hoc indicated that after the OB the animals displayed a significant increase in the TNF-α levels in the HP compared to control animals (SHAM + vehicle). The administration of chrysin or fluoxetine attenuated the increase in the TNF-α levels in the HP compared to OB group (OB + vehicle) (Fig. 4A). Pearson's correlation tests demonstrated a significant negative correlation (TNF-α levels in the HP X ST), a significant positive correlation (TNF-α levels in the HP X FST and TNF-α levels in the HP X OFT) (Table 1), a significant positive correlation (TNF-α levels in the HP X IDO activity in the HP), and a significant negative correlation (TNF-α levels in the HP X BDNF levels in the HP) (Table 2).

Two-way ANOVA showed significant main effect for OB [$F(1.24)= 77.92$, $p<0.0001$], treatments [$F(3.24)=19.62$, $p<0.0001$] and OB X treatments interaction [$F(3.24)= 20.86$, $p<0.0001$] in the IFN-γ levels in the HP. Results of post hoc indicated that after the OB the animals displayed a significant increase in the IFN-γ levels in the HP compared to control animals (SHAM + vehicle). The administration of chrysin or fluoxetine attenuated the increase in the IFN-γ levels in the HP compared to OB group (OB + vehicle) (Fig. 4B). Pearson's correlation tests demonstrated a significant negative correlation (IFN-γ levels in the HP X ST), a significant positive correlation (IFN-γ levels in the HP X FST and IFN-γ levels in the HP X OFT) (Table 1), a significant positive correlation (IFN-γ levels in the HP X IDO activity in the HP), and a significant negative correlation (IFN-γ levels in the HP X BDNF levels in the HP) (Table 2).

Two-way ANOVA showed significant main effect for OB [$F(1.24)= 78.25$, $p<0.0001$], treatments [$F(3.24)=19.24$, $p<0.0001$] and OB X treatments interaction [$F(3.24)= 17.05$, $p<0.0001$] in the IL-1β levels in the HP. Results of post hoc indicated that after the OB the animals displayed a significant increase in the IL-1β levels in the OB compared to control animals (SHAM + vehicle). The administration of chrysin or fluoxetine attenuated the increase in the IL-1β levels in the HP compared to OB group (OB + vehicle) (Fig. 4C). Pearson's correlation tests demonstrated a significant negative correlation (IL-1β levels in the HP X ST), a significant positive correlation (IL-1β levels in the HP X FST and IL-1β levels in the HP X OFT) (Table 1), and a significant positive correlation (IL-1β levels in the HP X IDO activity in the HP), and a significant negative correlation (IL-1β levels in the HP X BDNF levels in the HP) (Table 2).

Two-way ANOVA showed significant main effect for OB [$F(1.24)=54.12$, $p<0.0001$], treatments [$F(3.24)=6.17$, $p<0.01$] and OB X treatments interaction [$F(3.24)=6.16$, $p<0.01$] in the IL-6 levels in the HP. Results of post hoc indicated that after the OB the animals displayed a significant increase in the IL-6 levels in the HP compared to control animals (SHAM + vehicle). The administration of chrysin or fluoxetine attenuated the increase in the IL-6 levels in the HP compared to OB group (OB + vehicle) (Fig. 4D). Pearson's correlation tests demonstrated a significant negative correlation (IL-6 levels in the HP X ST), a significant positive correlation (IL-6 levels in the HP X FST and IL-6 levels in the HP X OFT) (Table 1), a significant positive correlation (IL-6 levels in the HP X IDO activity in the HP), and a significant negative correlation (IL-6 levels in the HP X BDNF levels in the HP) (Table 2).

3.5 IDO activity

Two-way ANOVA showed significant main effect for OB [$F(1.24)=56.36$, $p<0.0001$], treatments [$F(3.24)=8.02$, $p<0.001$] and OB X treatments interaction [$F(3.24)= 11.19$, $p<0.0001$] in the IDO activity in the HP. Results showed that CUMS occasioned a significant increase in the IDO activity in the HP compared to control group (SHAM + vehicle). The treatment with chrysin or fluoxetine attenuated the increase in the IDO activity in the HP resulting from OB compared to OB group (OB + vehicle) (Fig. 5A). Pearson's correlation tests demonstrated a significant negative correlation (IDO activity in the HP X ST), a significant positive correlation (IDO activity in the HP X FST and IDO activity in the HP X OFT) (Table 1), and a significant negative correlation (IDO activity in the HP X 5-HT levels in the HP) (Table 2).

3.6 TRP and KYN levels and KYN/TRP ratio

Two-way ANOVA demonstrated no significant main effect for OB [$F(1.24)=0.0024$, $p=0.9612$], treatments [$F(3.24)=0.57$, $p=0.6385$] and OB X treatments interaction [$F(3.24)=0.37$, $p=0.7771$] in the TRP levels in the HP (Fig. 5B).

Two-way ANOVA revealed significant main effect for OB [$F(1.24)=48.86$, $p<0.0001$], treatments [$F(3.24)=19.74$, $p<0.0001$] and OB X treatments interaction [$F(3.24)=19.09$, $p<0.0001$] in the KYN levels in the HP. Results revaluated that OB induced a significant elevation in the KYN levels in the HP compared to control group (SHAM + vehicle). The treatment with chrysin or fluoxetine promoted the prevention of the increase in the KYN levels in the HP occasioned by OB when compared to OB group (OB + vehicle) (Fig. 5C). Pearson's correlation tests showed a significant negative correlation (KYN levels in the HP X ST), and a significant positive correlation (KYN levels in the HP X FST and KYN levels in the HP X OFT) (Table 1).

Two-way ANOVA showed significant main effect for stress [$F(1.24)=34.78$, $p<0.0001$], treatments [$F(3.24)=11.97$, $p<0.0001$] and stress X treatments interaction [$F(3.24)=13.50$, $p<0.0001$] of the KYN/TRP ratio in the HP. Post hoc test demonstrated that OB occasioned a significant increase of the KYN/TRP ratio in the HP compared to control group (SHAM + vehicle). The treatment with chrysin or fluoxetine prevented the increase of the KYN/TRP ratio in the HP resulting from OB compared to OB group (OB + vehicle) (Fig. 5D). Pearson's correlation tests showed significant negative correlation (KYN/TRP ratio in the HP X ST) and significant positive correlation (KYN/TRP ratio in the HP X FST and KYN/TRP ratio in the HP X OFT) (Table 1).

3.7 5-HT and 5-HIAA levels and 5-HIAA/5-HT ratio

Two-way ANOVA showed no significant main effect for OB [$F(1.24)=3.20$, $p=0.0861$], and for OB X treatments interaction [$F(3.24)=1.90$, $p=0.1558$], and a significant main effect for treatments [$F(3.24)=6.57$, $p<0.01$] in the 5-HT levels in the HP. Post hoc test demonstrated that OB occasioned a significant decrease in the 5-HT levels in the HP compared to control group (SHAM + vehicle). The treatment with chrysin or fluoxetine prevented the decrease in the 5-HT levels in the HP resulting from OB compared to OB group (OB + vehicle) (Fig. 6A). Pearson's correlation tests demonstrated a significant negative

correlation (5-HT levels in the HP X FST and 5-HT levels in the HP X OFT) (Table 1), and a significant positive correlation (5-HT levels in the HP X BDNF levels in the HP) (Table 2).

Two-way ANOVA revealed significant main effect for OB [$F(1.24)=6.73$, $p<0.05$] and for treatments [$F(3.24)=3.07$, $p<0.05$], and no significant main effect for OB X treatments interaction [$F(3.24)=2.61$, $p=0.0747$] in the 5-HIAA levels in the HP. Post hoc test demonstrated that OB occasioned a significant increase in the 5-HIAA levels in the HP compared to control group (SHAM + vehicle). The treatment with chrysin or fluoxetine prevented the increase in the 5-HIAA levels in the HP resulting from OB compared to OB group (OB + vehicle) (Fig. 6B). Pearson's correlation tests showed significant negative correlation (5-HIAA levels in the HP X ST), and significant positive correlation (5-HIAA levels in the HP X FST and 5-HIAA levels in the HP X OFT) (Table 1).

Two-way ANOVA demonstrated significant main effect for OB [$F(1.24)=15.31$, $p<0.001$], treatments [$F(3.24)=14.03$, $p<0.0001$], and OB X treatments interaction [$F(3.24)=9.91$, $p<0.001$] in the 5-HIAA/5-HT ratio in the HP. Post hoc test demonstrated that OB occasioned a significant increase of the 5-HIAA/5-HT ratio in the HP compared to control group (SHAM + vehicle). The treatment with chrysin or fluoxetine prevented the increase of the 5-HIAA/5-HT ratio in the HP resulting from OB compared to OB group (OB + vehicle) (Fig. 6C). Pearson's correlation tests showed significant negative correlation (5-HIAA/5-HT ratio in the HP X ST), and significant positive correlation (5-HIAA/5-HT ratio in the HP X FST and 5-HIAA/5-HT ratio in the HP X OFT) (Table 1).

3.8 BDNF levels

Two-way ANOVA revealed significant main effect for OB [$F(1.24)=88.77$, $p<0.0001$] and treatments [$F(3.24)=14.38$, $p<0.0001$] and no significant main effect for OB X treatments interaction [$F(3.24)=0.99$, $p=0.4154$] in the BDNF levels in the HP. Results demonstrated that chrysin (20 mg/kg) and fluoxetine (10 mg/kg) induced a significant elevation in the BDNF levels in the HP of control animals compared to control group (SHAM + vehicle). In addition, results showed that OB induced a significant decrease in the BDNF levels in the HP compared to control group (SHAM + vehicle). The treatment with chrysin or fluoxetine promoted the prevention in the decrease in the BDNF levels in the HP occasioned by OB when compared to OB group (OB + vehicle) (Fig. 7). Pearson's correlation tests showed a significant positive correlation (BDNF levels in the HP X ST), and a significant negative correlation (BDNF levels in the HP X FST and BDNF levels in the HP X OFT) (Table 1).

4 Discussion

In this work, we investigated the effect of chrysin treatment in depressive-like behavior and hippocampal alterations occasioned by OB in male mice. Our study demonstrated the antidepressant-like effect of chrysin treatment in all behavioral parameters evaluated (ST, OFT, FST), as well in the hippocampal modifications (TNF-α, IFN-γ, IL-1β, IL-6, TRP, KYN, 5-HT, 5-HIAA, BDNF levels and IDO activity).

Results of ST, OFT and FST, observed in this study, confirm that OB induces behavioral alterations in mice, occasioning loss of self-care, hyperactivity and loss of motivation, respectively [4]. These modifications in behavior are common in depressive individuals, especially patients with agitated depression [3,4]. Treatment with chrysin, as well with fluoxetine, prevented the behavioral modifications occasioned by OB. Our group demonstrated previously the antidepressant-like of chrysin treatment in other model of depression, assessing OFT, FST and sucrose preference test [11]. Thus, the results of this work confirm the potential of chrysin for antidepressant therapy. In addition, in an unprecedented manner, this study showed the behavioral action of chrysin in a model of agitated depression, preventing the hyperactivity commonly resulting from OB.

Strongly correlated with behavioral modifications, mice exposed to OB presented elevated levels of TNF-α, IFN-γ, IL-1β and IL-6 in the HP. The overproduction of pro-inflammatory cytokines is a normal characteristic of depressive patients [24], and also is recurrent in brain structures of rodents subjected to OB [6,25]. These changes are not only similar to clinical symptoms of human depression, but can also be improved by repeated long-term antidepressant treatment [26]. In this context, we observed that chrysin flavonoid promoted the attenuation of inflammatory status induced by OB in mice, confirming the anti-inflammatory potential of chrysin previously demonstrated by other studies [27-29], and suggesting the role of anti-inflammatory action of chrysin in the antidepressant effect observed in this work. Reinforcing this hypothesis, our results demonstrated the strong correlation between behavioral tests and pro-inflammatory cytokines levels in the model of depression induced by OB.

Our study also showed that the overproduction of pro-inflammatory cytokines is strongly correlated with dysregulation of kynurenine pathway (KP), resulting of exacerbated activation of IDO. Though studies linking KP with depressive-like behavior induced by OB in rodents, the role of hyperactivation of KP is recurrently associated with depression occurrence in other animal models of depression, as depression occasioned by ovariectomy [30], and

depression resulting from stress [31]. The exacerbated activation of IDO in the HP, observed in our work, that results in the overproduction of KYN and in the increase of KYN/TRP ratio is occasioned due to the elevated synthesis of pro-inflammatory cytokines. In this way, it has been showed that the production of cytokines lead to activation of IDO [32], which is corroborated in this study by the positive correlation observed between IDO activity and pro-inflammatory cytokines levels. Thus, IDO activation appears to play a key role between inflammatory status and 5-HT depletion in brain, because IDO competes for the same substrate used in the 5-HT synthesis, TRP. This information is confirmed by negative correlation observed between IDO activity and 5-HT levels. In addition, IDO activity and KYN levels also presented correlation with behavioral tests, suggesting the role of KP in the depressive-like status induced by OB. Treatment with chrysin occasioned the attenuation of increased activation of KP. As noted above, the anti-inflammatory action of chrysin is fairly reported in previous studies. Thus, we suggest that chrysin probably acts in the regulation of KP through attenuation of inflammatory status.

Also associated with KP hyperactivation, we demonstrated that OB occasioned the decrease of 5-HT levels in the HP. In addition, we verified the increase of 5-HIAA levels and, consequently, the elevation of 5-HIAA/5-HT ratio. Treatment with chrysin or fluoxetine promoted the attenuation of this alterations. As stated previously, IDO can be induced by an increased production of pro-inflammatory cytokines. IDO activation may convert more TRP into neurotoxic tryptophan catabolites (TRYCATs) rather than to 5-HT [33], which decreases the bioavailability of TRP for the synthesis of 5-HT. Besides IDO, disruption in 5-HT signaling in depression might also be caused by other factors, such as anti-5- HT antibody which could be generated by cell mediated immunity [34], and inflammatory cytokines which could cause 5-HT turnover [35]. In our work, the correlations observed between 5-HT, 5-HIAA levels, 5-HIAA/5-HT ratio and behavioral tests reinforce the serotonergic hypothesis of depression developed by Van Praag and Korf [36], and the role of 5-HT system in the depressive-like behavior induced by OB. In addition, the negative correlation between 5-HT levels and IDO activity indicate the role of activation of IDO in the decrease of 5-HT levels. Thus, our data suggest that decreased 5-HT level in HP was, at least in part, caused by elevated IDO activity although other factors which cause depletion of 5-HT could not be excluded in our study. Furthermore, the antidepressant-like effect of chrysin treatment in mice exposed to OB it's probably associated with regulation of 5-HT system occasioned by the effect of chrysin in the normalization of pro-inflammatory cytokines production and IDO activity in the HP.

Finally, our study showed the effect of OB in the decrease the BDNF levels in the HP, as well as the effect of chrysin in the prevention of this alteration. Furthermore, we demonstrated the action of chrysin (20 mg/kg) and fluoxetine (10 mg/kg) treatment in the up-regulation of BDNF levels in the SHAM animals. In a previous work, we reported that oral chrysin (20 mg/kg) treatment for 28 days occasioned up-regulation of BDNF levels in the HP of female mice [11]. In the same study, we suggested that antidepressant effect of chrysin could, at least in part, be associated with the up-regulation of this neurotrophin in the brain structures [11]. This hypothesis is supported basically for these factors: I. depression is associated with a decrease in blood or brain concentrations of neurotrophins; II. antidepressant treatments increase the expression of neurotrophins; III. Administration of neurotrophins in animals promotes the attenuation of depressive status [37,38]. Thus, results of the present study reinforced the role of up-regulation of neurotrophins levels in the HP in the antidepressant effect of chrysin. In addition, these results showed that chrysin treatment is effective in the increase of BDNF levels with 14 days of therapy, and not just with 28 days, as showed in the previous study. The role of BDNF in the depressive behavior resulting from OB was reported previously [2,6]. This role was also demonstrated by our results and confirmed by significant correlations observed between BDNF levels in the HP and behavioral tests. Finally, the role of BDNF in the antidepressant action of chrysin was also reinforced.

5 Conclusion

In summary, our work showed that chrysin treatment promoted the attenuation of behavioral (ST, OFT, FST) and hippocampal (TNF-α, IFN-γ, IL-1β, IL-6, TRP, KYN, 5-HT, 5-HIAA, BDNF levels and IDO activity) modifications occasioned by OB in mice. These results reinforce the potential of chrysin for the treatment or supplementary treatment of depression and showed that flavonoid is also effective with 14 days of therapy in a model of agitated depression. In addition, this work corroborates with the role of BDNF up-regulation in the antidepressant-like action of chrysin.

Conflict of interest

The authors declare that there are no conflicts of interest in the present work.

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Legends

Figure 1: Chemical structure of chrysin.

Figure 2: Experimental design.

Figure 3: Effect of chrysin and fluoxetine administration on ST (A), OFT (B and C) and FST (D) in mice exposed to OB. ST was realized in the 30th day of protocol. OFT was realized 24h after to ST. FST was realized immediately after to OFT. [a]Indicates $p<0.05$ compared to control group (SHAM + vehicle). [b]Indicates $p<0.05$ compared to OB group (OB + vehicle).

Figure 4: Effect of chrysin or fluoxetine treatment in the TNF-α (A), IFN-γ (B), IL-1ß (C) and IL-6 (D) levels in the HP of mice exposed to OB. HP was dissected immediately after FST. [a]Indicates $p<0.05$ compared to control group (SHAM + vehicle). [b]Indicates $p<0.05$ compared to OB group (OB + vehicle).

Figure 5: Results of chrysin or fluoxetine treatment in the IDO activity (A), TRP levels (B), KYN levels (C) and KYN/TRP ratio (D) in the HP of mice exposed to OB. HP was dissected immediately after FST. [a]Indicates $p<0.05$ compared to control group (SHAM + vehicle). [b]Indicates $p<0.05$ compared to OB group (OB + vehicle).

Figure 6: Effect of chrysin or fluoxetine treatment in the 5-HT levels (A), 5-HIAA levels (B), and 5-HIAA/5-HT ratio (C) in the HP of mice exposed to OB. HP was dissected immediately after FST. [a]Indicates $p<0.05$ compared to control group (SHAM + vehicle). [b]Indicates $p<0.05$ compared to OB group (OB + vehicle).

Figure 7: Effect of chrysin or fluoxetine treatment in the BDNF levels in the HP of mice exposed to OB. HP was dissected immediately after FST. [a]Indicates $p<0.05$ compared to control group (SHAM + vehicle). [b]Indicates $p<0.05$ compared to OB group (OB + vehicle).

Figures

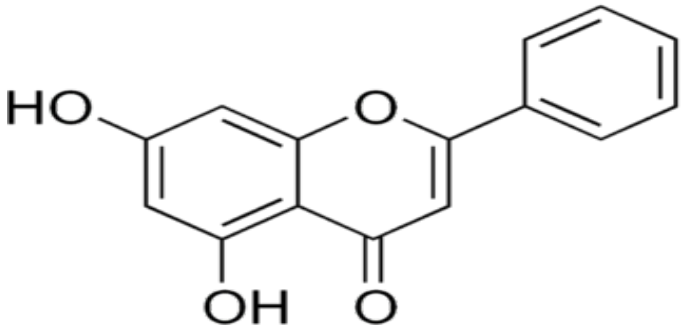

Figure 1

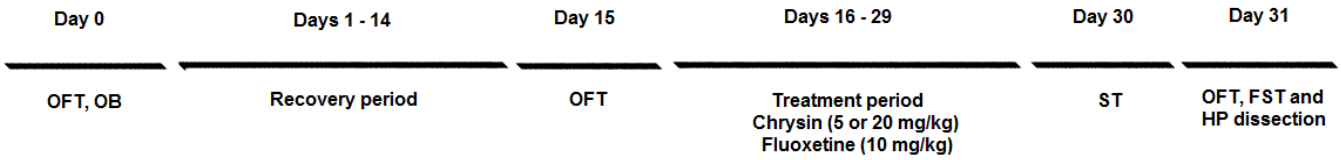
Day 0
Days 1 - 14
Day 15
Days 16 - 29
Day 30
Day 31
OFT, OB
Recovery period
OFT
Treatment period
Chrysin (5 or 20 mg/kg)
Fluoxetine (10 mg/kg)
ST
OFT, FST and
HP dissection

Figure 2

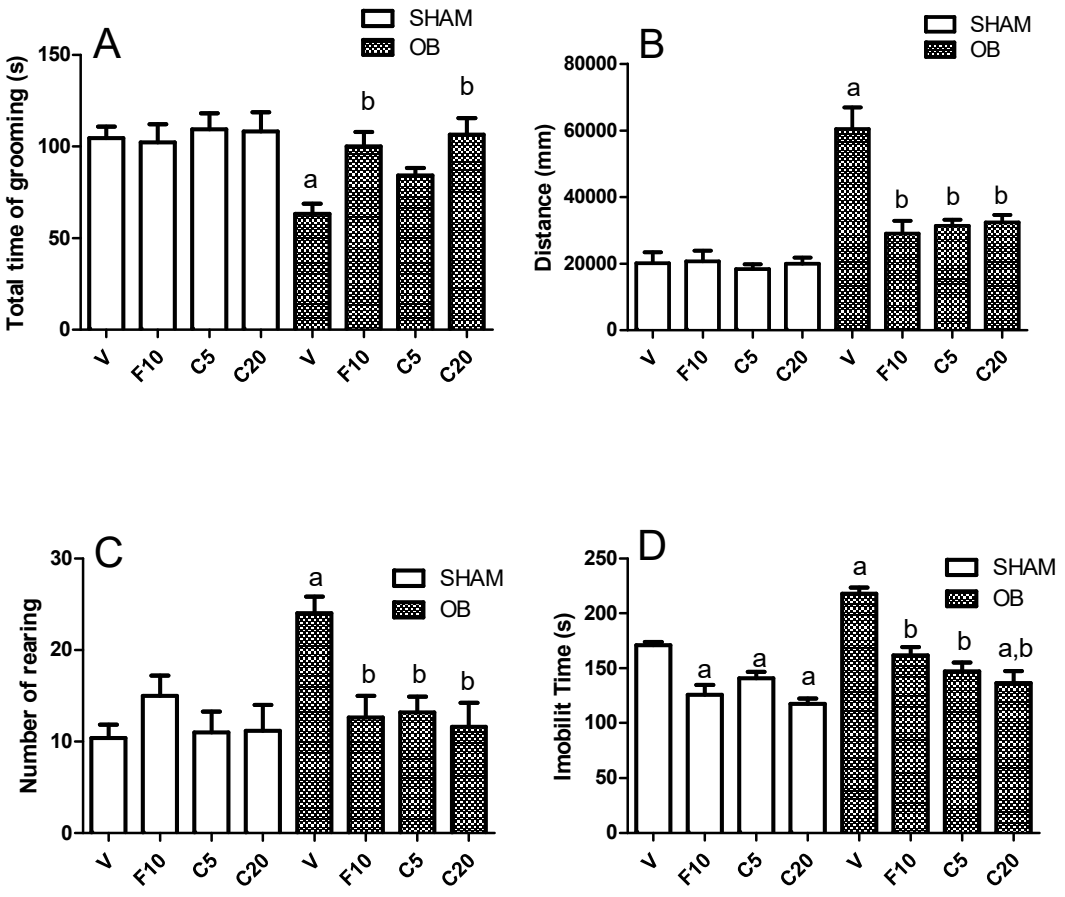
A
SHAM
OB
Total time of grooming (s)
150
100
50
0
a
b
b
V
F10
C5
C20
B
Distance (mm)
80000
60000
40000
20000
C
Number of rearing
30
20
10
D
Imobilit Time (s)
250
200
150
100
a,b

Figure 3

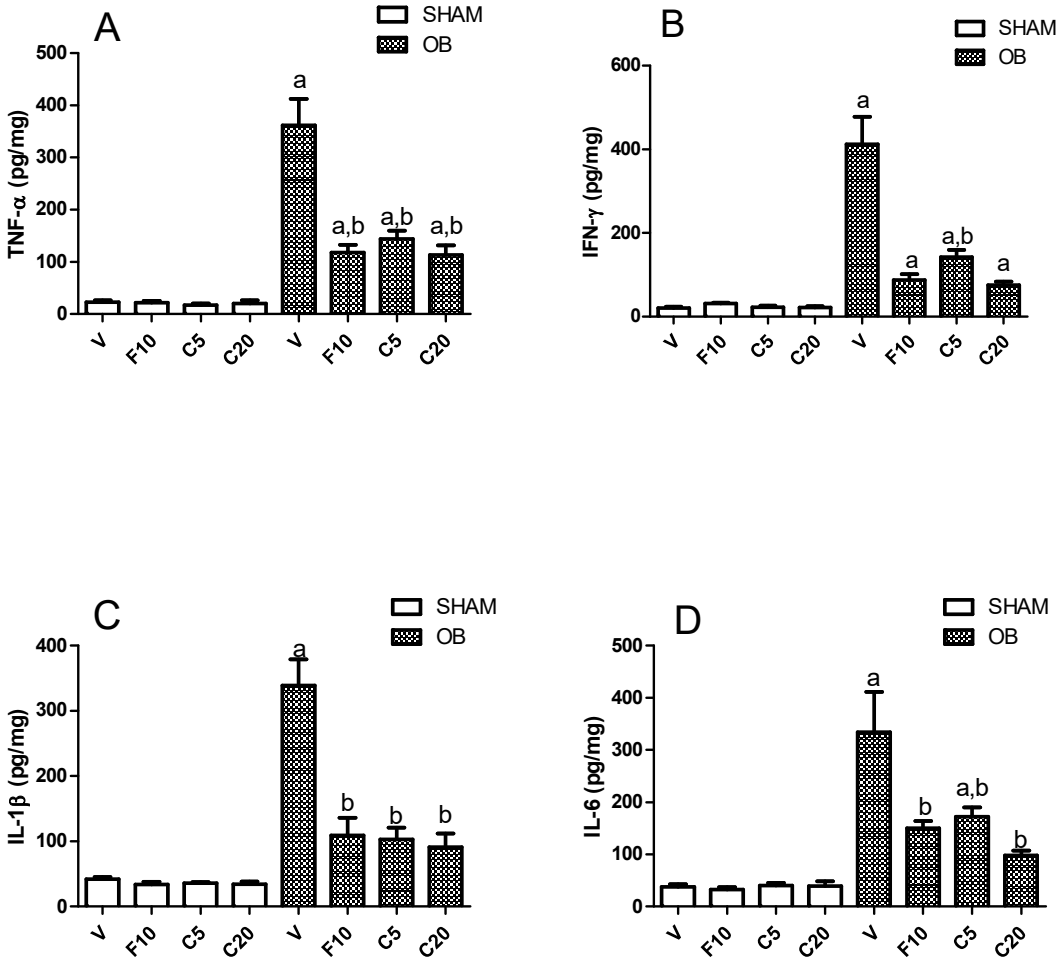
A
SHAM
OB
TNF-α (pg/mg)
500
400
300
200
100
0
a
a,b
a,b
a,b
V
F10
C5
C20
B
SHAM
OB
IFN-γ (pg/mg)
600
400
200
0
a
a
a,b
a
C
SHAM
OB
IL-1β (pg/mg)
400
300
200
100
0
a
b
b
b
D
SHAM
OB
IL-6 (pg/mg)
500
400
300
200
100
0
a
b
a,b
b

Figure 4

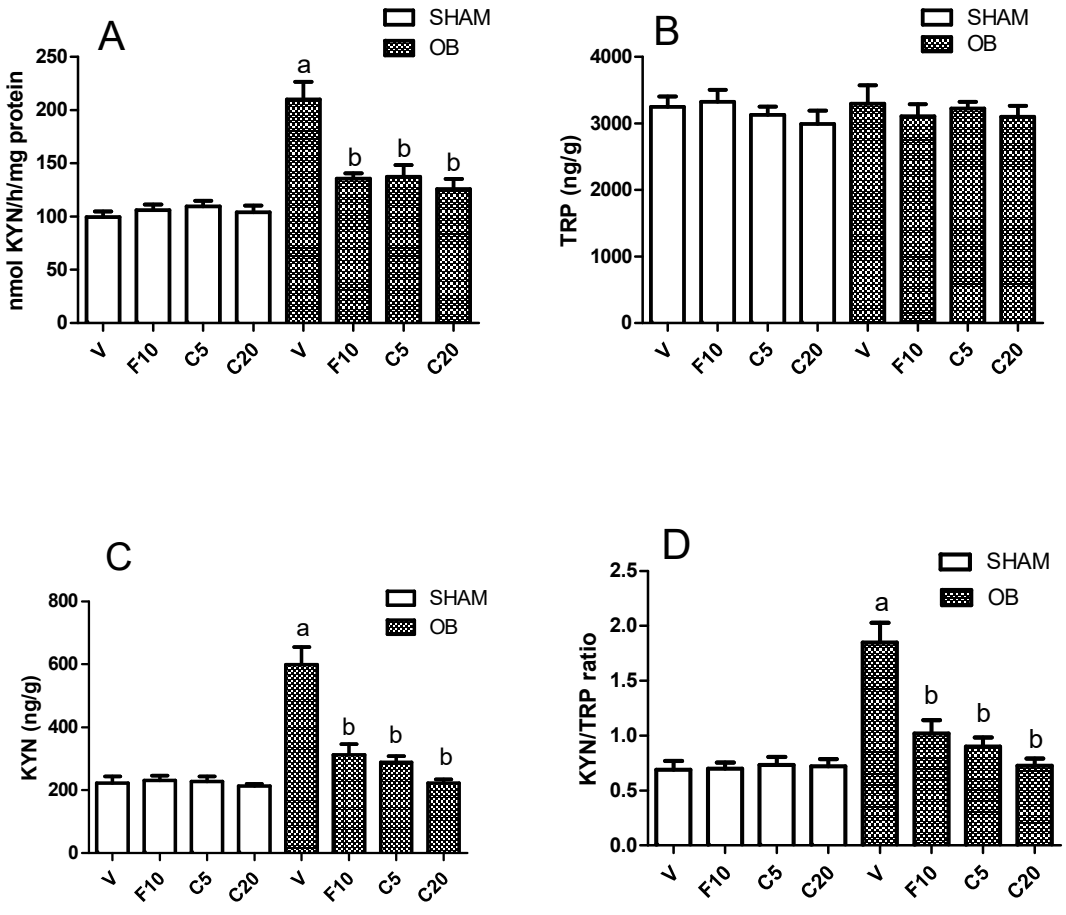
A
SHAM
OB
nmol KYN/h/mg protein
250
200
150
100
50
0
a
b
b
b
V
F10
C5
C20
V
F10
C5
C20
B
SHAM
OB
TRP (ng/g)
4000
3000
2000
1000
0
V
F10
C5
C20
V
F10
C5
C20
C
SHAM
OB
KYN (ng/g)
800
600
400
200
0
a
b
b
b
V
F10
C5
C20
V
F10
C5
C20
D
SHAM
OB
KYN/TRP ratio
2.5
2.0
1.5
1.0
0.5
0.0
a
b
b
b
V
F10
C5
C20
V
F10
C5
C20

Figure 5

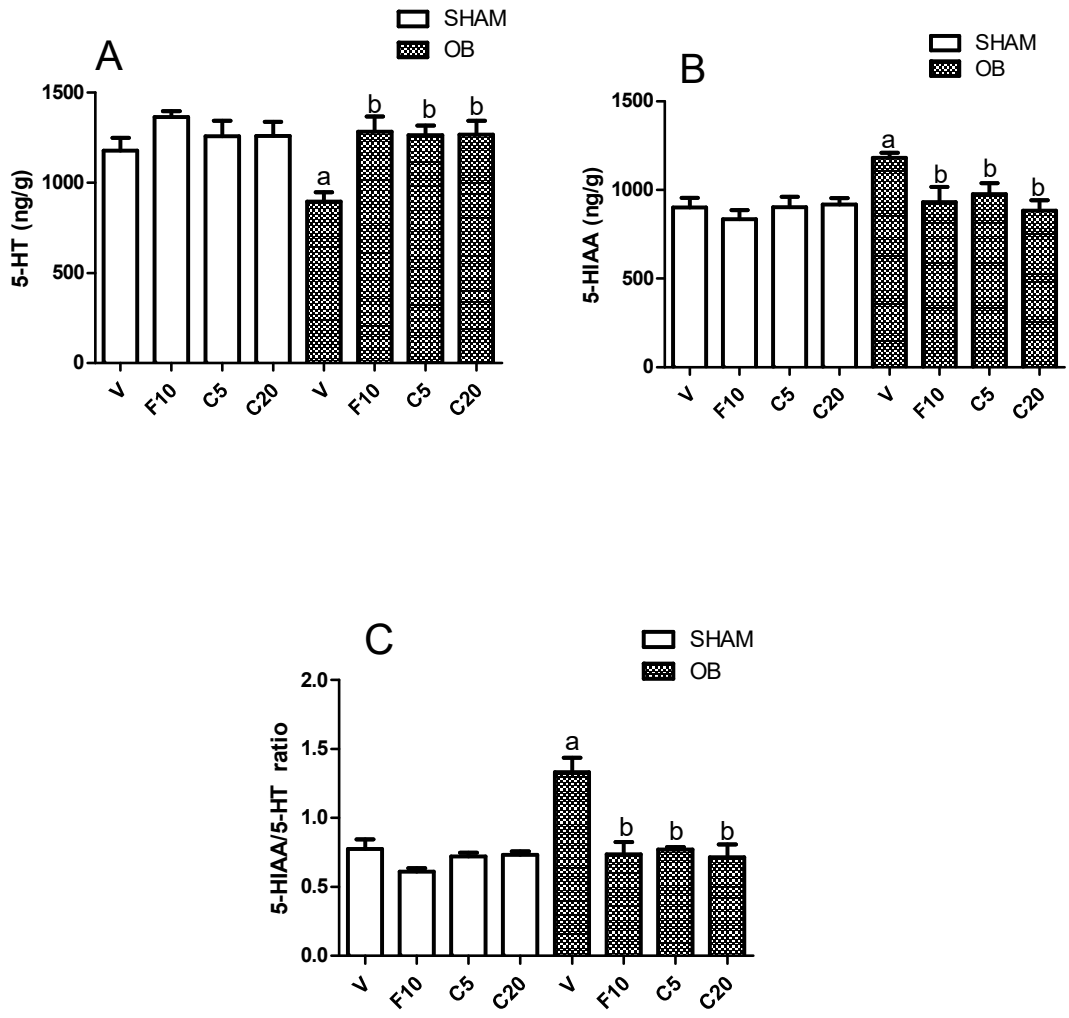
A
SHAM
OB
5-HT (ng/g)
1500
1000
500
0
a
b
b
b
V
F10
C5
C20
B
5-HIAA (ng/g)
C
5-HIAA/5-HT ratio
2.0
1.5
1.0
0.5
0.0

Figure 6

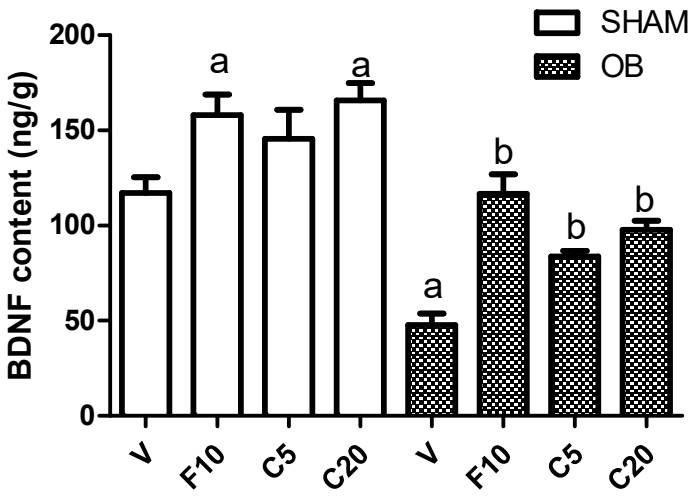
SHAM
OB
BDNF content (ng/g)
200
150
100
50
0
a
a
a
b
b
b
V
F10
C5
C20
V
F10
C5
C20

Figure 7

Tables

Table 1. r values resulting from Pearson's correlation test for neurochemical factors X behavioral parameters. *denoted $p<0.05$.

	ST	OFT (Crossing)	OFT (Rearing)	FST
TNF-α levels	-0.58*	0.82*	0.53*	0.78*
IFN-γ levels	-0.64*	0.84*	0.59*	0.73*
IL-1 levels	-0.53*	0.81*	0.65*	0.74*
IL-6 levels	-0.43*	0.73*	0.56*	0.65*
IDO activity	-0.54*	0.88*	0.58*	0.67*
TRP levels	-0.17	0.05	0.12	0.15
KYN levels	-0.57*	0.76*	0.54*	0.77*
KYN/TRP ratio	-0.54*	0.77*	0.54*	0.74*
5-HT levels	0.29	-0.64*	-0.56*	-0.63*
5-HIAA levels	-0.39*	0.61*	0.35*	0.49*
5-HIAA/5-HT ratio	-0.42*	0.79*	0.61*	0.69*
BDNF levels	0.40*	-0.66*	-0.41*	-0.74*

Table 2. r values resulting from Pearson's correlation test for neurochemical parameters. *denoted $p<0.05$.

	TNF-α levels	**IFN-γ levels**	**IL-1β levels**	**IL-6 levels**	**5-HT levels**
IDO activity	0.86*	0.86*	0.78*	0.76*	-0.59*
BDNF levels	-0.76*	-0.71*	-0.74*	-0.74*	0.41*

5 DISCUSSÃO

Este estudo mostrou o efeito tipo-antidepressivo do tratamento com crisina em camundongos expostos ao UCS e à OB. O efeito tipo-antidepressivo da crisina em parâmetros comportamentais, hormonais, oxidativos e neurotróficos havia sido demonstrado anteriormente pelo nosso grupo (Borges Filho et al., 2015). Entretanto, neste trabalho nós aprofundamos as avaliações dos fatores neuroquímicos associados ao efeito da crisina em animais estressados e avaliamos o efeito da crisina em animais submetidos à OB. Neste sentido, demonstramos o papel das citocinas pró-inflamatórias, da KP, do metabolismo da 5-HT e da atividade das caspases no efeito tipo-antidepressivo da crisina em camundongos expostos ao UCS. Além disso, mostramos o efeito da crisina em animais submetidos à OB, demonstrando o papel das citocinas pró-inflamatórias, da KP, do metabolismo da 5-HT e do BDNF neste efeito.

O tratamento com crisina, semelhantemente à fluoxetina, apresentou ação tipo-antidepressiva nos testes comportamentais realizados nos animais controle (não estressados ou SHAM) dos dois modelos avaliados. Estes resultados indicam a eficácia do tratamento com crisina mesmo quando não há uma situação de estresse crônico ou de remoção do bulbo olfatório, ou seja, mesmo em situações fisiológicas. O UCS ocasionou um comportamento tipo-depressivo nos animais, demostrado pelos testes de ST e TST. Já a OB mostrou efeito tipo-depressivo através dos testes comportamentais de ST, OFT e FST. Os testes de ST e TST ou FST sugerem a falta de auto-cuidado e a conformidade com as situações adversas, respectivamente (Petit et al, 2014;.. Steru et al, 1985;. Surget et al, 2008). Já o teste de OFT, que mostrou diferença apenas no modelo da OB, indica a hiperatividade resultante da OB. Estes resultados demonstraram a eficiência do UCS e da OB para induzir um quadro tipo-depressivo em animais, corroborando com estudos anteriores (Borges Filho et al, 2015; Kumar et al, 2011;. Mao et al, 2014). A administração com crisina ou fluoxetina preveniu todas as alterações comportamentais visualizadas nos modelos utilizados. Além disso, o fato de que não houve diferença significativa no teste de rota rod entre qualquer um dos grupos do experimento usando o modelo do UCS indica que as alterações comportamentais observadas neste experimento não estão relacionados a qualquer alteração psicomotora. Em outro estudo do nosso grupo, a ação antidepressiva da crisina no teste de FST e no teste de preferência por sacarose em camundongos estressados foi demonstrado (Borges Filho et al., 2015). Neste sentido, os resultados observados nos testes comportamentais realizados no

presente estudo vêm a confirmar o potencial antidepressivo do tratamento com o flavonoide crisina em camundongos.

Em um trabalho anterior, o nosso grupo mostrou a elevação dos níveis de corticosterona nos animais submetidos ao UCS, e o efeito do tratamento com crisina na regulação dos níveis deste hormônio (Borges Filho et al., 2015). No presente estudo, nós demonstramos que o UCS também ocasionou o aumento nos níveis plasmáticos de CRH e ACTH em camundongos estressados, o que confirma a eficácia do UCS como um modelo de estresse induzindo a disfunção do eixo HPA. Nossos resultados mostraram que os grupos tratados com crisina ou fluoxetina apresentaram níveis normais de CRH e ACTH e que os níveis plasmáticos destes hormônios apresentaram correlação com os testes comportamentais, o que reforça o conceito de que o efeito antidepressivo da crisina está associado com a regulação do eixo HPA em animais estressados, como sugerido no nosso trabalho anterior.

Correlacionado com a desregulação no do eixo HPA, os nossos dados demonstraram a elevação dos níveis de TNF-α, IL-1β e IL-6 no PFC e HP resultante do UCS. Também demonstramos que os níveis de TNF-α, IFN-γ, IL-1β e IL-6 foram aumentados no HP dos animais submetidos à OB. A alteração nos níveis de citocinas pró-inflamatórias tem sido fortemente associada à ocorrência de depressão em seres humanos (Dahl et al, 2014; Dowlati et al, 2010). Dinkel et al. (2003) sugeriram que os glicocorticoides, abundantes em situações de estresse, aumentam o número de células inflamatórias, tais como macrófagos e microglias. A correlação positiva entre os níveis de citocinas pró-inflamatórias e os níveis de ACTH no experimento do UCS sugere de forma consistente a associação entre a síntese de citocinas pró-inflamatórias com a disfunção do eixo HPA. Além disso, a correlação verificada entre os níveis das citocinas avaliadas e os parâmetros comportamentais estudados tanto no modelo do UCS quanto no modelo da OB indicam o papel das citocinas pró-inflamatórias no comportamento tipo-depressivo induzido pelo UCS e pela OB. O papel da crisina na atenuação da inflamação tem sido relatado previamente (Ahad et ai, 2014; Feng et ai, 2014; Xiao et ai, 2014). Em nosso trabalho, os resultados corroboram o efeito anti-inflamatório da crisina, já que demonstramos a ação da crisina na atenuação do aumento de dos níveis de TNF-α, IFN-γ, IL-1β e IL-6 decorrentes do UCS ou da OB nas estruturas cerebrais estudadas. Estes resultados sugerem o provável papel da ação anti-inflamatória da crisina no efeito tipo-antidepressivo demonstrado por este flavonoide nos modelos experimentais estudados.

Este trabalho demonstrou que UCS e a OB ocasionaram a disfunção da KP nas estruturas cerebrais avaliadas, desencadeando o aumento na atividade da IDO, dos níveis de KYN e da razão KYN/TRP. O tratamento com fluoxetina ou crisina promoveu a atenuação destas alterações em ambos os experimentos. Tem sido documentado que a KP desempenha papel chave na ligação entre os processos inflamatórios e a ocorrência da depressão por basicamente duas razões. I) redução da disponibilidade de TRP, e II) produção de neurotoxinas altamente ativas (Hochstrasser et al., 2011). Assim, a degradação do TRP e o seu papel na disponibilidade de 5-HT tem chamado a atenção para a KP como um alvo potencial para pesquisas futuras de tratamentos alternativos para a depressão. Cerca de 99% do TRP é metabolizado pela triptofano 2,3-dioxigenase (TDO) em KYN no fígado. No entanto, durante a inflamação acentuada, a IDO é ativada em tecidos extra-hepáticos para converter TRP em KYN (Leklem et al., 1971). As correlações observadas entre a atividade IDO, os níveis KYN, a razão KYN/TRP e os parâmetros comportamentais indicam o papel da KP no comportamento tipo-depressivo ocasionado pelo UCS e pela OB. A correlação positiva entre os níveis de citocinas pró-inflamatórias e a atividade IDO sugere a forte associação do estado inflamatório com a atividade IDO. Como dito, a KP hoje é aceita como sendo o elo de ligação entre as citocinas pró-inflamatórias e as alterações neuroquímicas ou neuroendócrinas que podem ser responsáveis pela depressão (Castanon et ai, 2002;. Schiepers et al., 2005). Assim, é provável que, devido ao papel da produção de citocinas pró-inflamatórias na ativação da KP, a ação anti-inflamatória da crisina esteja associada com a regulação da KP e, consequentemente, com o comportamento tipo-antidepressivo observado nos animais tratados com crisina.

Este estudo também demonstrou o efeito do UCS e da OB no metabolismo da 5-HT, caracterizado pelo aumento da razão 5-HIAA/5-HT e diminuição dos níveis de 5-HT nas estruturas cerebrais estudadas. O tratamento com fluoxetina ou crisina promoveu a atenuação dessas alterações. Como afirmado anteriormente, a IDO pode ser induzida por um aumento da produção de citocinas pró-inflamatórias. A ativação da IDO ocasiona a maior conversão de TRP em catabolitos neurotóxicos do triptofano (TRYCATs) (Neumeister et al., 2003), o que diminui a biodisponibilidade do TRP para a síntese de 5-HT. No nosso trabalho, as correlações observadas entre os níveis de 5-HT e 5-HIAA, a razão 5-HIAA/5-HT e os testes comportamentais reforçam a hipótese serotonérgica da depressão desenvolvida por Van Praga e Korf (1971), e o papel do sistema 5-HT no comportamento tipo-depressivo induzido pelo UCS e pela OB. Além disso, a correlação negativa entre os níveis de 5-HT e atividade da IDO indicam o papel de ativação da IDO na diminuição dos níveis de 5-HT nos modelos

estudados. Assim, nossos dados sugerem que a diminuição dos níveis de 5-HT nas estruturas cerebais avaliadas foi, ao menos em parte, causada pela acentuada atividade da IDO, embora outros fatores que causam o esgotamento dos níveis de 5-HT não possam ser excluídos. Além disso, o efeito tipo-antidepressivo do tratamento com crisina parece estar relacionado com a regulação do sistema de 5-HT ocasionado pelo efeito da crisina na regulação da produção de citocinas pró-inflamatórias e consequente normalização da atividade da IDO nas estruturas cerebrais estudadas.

Nossos resultados mostraram o aumento da atividade das caspase 3 e 9 no PFC e HP dos camundongos submetidos ao UCS. Nos animais submetidos à OB, optamos por não avaliar estes parâmetros, por entendermos que não são indicadores comumente avaliados neste modelo e por este se tratar ainda de um estudo prévio do modelo da OB. O tratamento com crisina ou fluoxetina promoveu a atenuação das alterações decorrentes do UCS. A elevação da atividade das caspases 3 e 9 indica o papel do estresse no aumento da apoptose, a morte celular programada. Além disso, a correlação observada entre a atividade das caspases e os testes comportamentais sugerem o papel da apoptose no estado tipo-depressivo desencadeado pelo UCS. É conhecido que o estresse atua acentuando a ocorrência da apoptose de muitas formas, mas a principal parece ser o aumento da concentração plasmática de corticosterona (Magarinos et al, 1985;. Zhu et al., 2008). Alguns estudos mostram o efeito do tratamento crisina na prevenção da apoptose (Darwish et al, 2014; Khan et al, 2012). Em ambos os estudos, o tratamento com crisina ocasionou a regulação da atividade ou expressão das caspases, sugerindo o papel fundamental desempenhado pelas caspases no efeito da crisina na atenuação da apoptose. Como foi mencionado anteriormente, alguns trabalhos indicam o papel de hipersecreção de glicocorticoides no aumento da apoptose. Assim, é possível que o efeito de crisina na regulação do eixo HPA esteja associada com a ação de crisina na prevenção da apoptose. Esta possibilidade é reforçada pela correlação observada entre a atividade das caspases 3 e 9 e os níveis plasmáticos de ACTH.

Finalmente, o presente estudo mostrou o efeito da OB na diminuição dos níveis de BDNF no HP, bem como o efeito de crisina na prevenção desta alteração. Além disso, demonstramos a ação da crisina (20 mg/kg) e da fluoxetina na elevação dos níveis de BDNF acima dos níveis do controle nos animais SHAM. No experimento com o modelo do UCS não foi avaliado este parâmetro em virtude de o mesmo já ter sido estudado em um trabalho anterior (Borges Filho et al., 2015). Neste trabalho anterior, mostramos que o tratamento com crisina (20 mg/kg) também ocasionou o aumento dos níveis de BDNF em níveis acima do grupo controle (Borges Filho et al., 2015). No mesmo estudo, foi sugerido que o efeito tipo-

antidepressivo de crisina poderia, ao menos em parte, estar associado com a superprodução de BDNF nas estruturas cerebrais (Borges Filho et al., 2015). Esta hipótese é sustentada basicamente pelos seguintes fatores: I. a depressão está associada com uma diminuição das concentrações sanguíneas e cerebrais de neurotrofinas; II. tratamentos com antidepressivos aumentam a expressão de neurotrofinas; III. a administração de neurotrofinas em animais promove a atenuação do estado depressivo (Vaidya e Duman, 2001; Shi et al., 2010). Assim, os resultados obtidos no experimento da OB reforçam o papel da superprodução de neurotrofinas no efeito tipo-antidepressivo de crisina. Além disso, os resultados do presente estudo mostraram que o tratamento com crisina é eficaz no aumento dos níveis de BDNF com 14 dias de terapia, e não apenas com 28 dias, como sugerido no estudo anterior. No presente trabalho, também mostrou-se que a OB ocasionou a diminuição dos níveis de BDNF no HP e que o tratamento com crisina preveniu estas modificações. O papel do BDNF no comportamento tipo-depressivo resultante de OB foi relatado anteriormente (Hellweg et ai, 2007; Yang et al, 2014). Este papel também foi demonstrado pelos nossos resultados e confirmado por correlações significativas observadas entre os níveis de BDNF no HP e os testes comportamentais realizados. Além disso, o papel do BDNF na ação tipo-antidepressiva da crisina foi também reforçado. No entanto, o mecanismo através do qual a crisina atua na regulação dos níveis de BDNF é ainda desconhecido, e permanece como um alvo para estudos futuros.

6 CONCLUSÕES

- Corroborando com nossos estudos anteriores, os testes comportamentais deste estudo demonstraram o efeito tipo-antidepressivo da crisina em camundongos submetidos ao UCS ou à OB;

- Foi demonstrado o envolvimento de alguns fatores neuroquímicos no efeito da crisina nos animais submetidos ao UCS e à OB. Dentre estes fatores estão a produção de citocinas pró-inflamatórias e neurotrofinas, a KP, a atividade de caspases, e o metabolismo da 5-HT;

- Também foi ratificado o efeito da crisina na regulação do eixo HPA em animais estressados, eixo comumente desregulado em situações de estresse e em quadros de depressão;

- Corroborando com resultados anteriores, demonstramos o efeito do tratamento com crisina na regulação ou elevação dos níveis de BDNF em estruturas cerebrais. Mostramos também, no modelo da OB, que este efeito pode ser observado já com 14 dias de tratamento, e não apenas com 28 dias, como sugerido no modelo do UCS;

- A partir destes dados, embora mais estudos sejam necessários, sugere-se que um dos posssíveis, talvez o principal mecanismo envolvido na atividade tipo-antidepressiva da crisina é o papel deste flavonoide na atenuação dos processos inflamatórios, o que tem relação direta com a regulação da KP e com a normalização dos níveis de 5-HT nas estruturas cerebrais, sobretudo no HP;

- Sugere-se também que outro possível mecanismo envolvido seja o papel da crisina na elevação dos níveis de BDNF a níveis acima do grupo controle nas estruturas cerebrais avaliadas;

- Ainda, este trabalho expõe o maracujá do mato como um importante alvo para o estudo dos produtos naturais no combate à depressão, mostrando a fundamentalidade da investigação da funcionalidade e constituição bioativa desta e outras plantas do bioma Pampa.

7 PERSPECTIVAS

- Estender o presente estudo avaliando o efeito da crisina em outros modelos animais de depressão, como depressão induzida por corticosterona, depressão induzida por ovariectomia e depressão induzida por citocinas;

- Avaliar alguns mecanismos envolvidos no efeito da crisina na atenuação dos processos inflamatórios;

- Esclarecer alguns mecanismos envolvidos no papel da crisina no aumento dos níveis de BDNF;

- Por meio da análise da crisina e seus metabólitos em estruturas cerebrais, tentar elucidar a taxa de absorção cerebral e as concentrações cerebrais necessárias para a observação do efeito antidepressivo, bem como tentar mostrar qual a forma (crisina ou metabólito) mais efetivamente envolvida no efeito farmacológico demonstrado neste estudo.

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ANEXO A –Protocolo de aprovação do projeto pela Comissão de ética no uso de animais (CEUA-UNIPAMPA)

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

Pró-Reitoria de Pesquisa

COMISSÃO DE ÉTICA NO USO DE ANIMAIS - CEUA

Fone: (55) 3413 4321, E-mail: ceua@unipampa.edu.br

CERTIFICADO DE APROVAÇÃO DE PROTOCOLO PARA USO DE ANIMAIS EM PESQUISA

Número de protocolo da CEUA: **035/2013**

Título: **AVALIAÇÃO DO EFEITO DO FLAVONÓIDE CRISINA EM CAMUNDONGOS SUBMETIDOS AO ESTRESSE CRÔNICO IMPREVISÍVEL**

Data da aprovação: **20/12/2013**

Período de vigência do projeto: De: **12/2013** Até: **12/2016**

Pesquisador: **CRISTIANO RICARDO JESSE**

Campus: **ITAQUI**

Telefone: **(55) 3433-1669**

E-mail: cristianoricardojesse@yahoo.com.br

Alessandra S. K. Tamajusuku Neis
Professor Adjunto
Coordenadora da CEUA/UNIPAMPA