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EFEITO DA CRISINA SOBRE AS ALTERAÇÕES COMPORTAMENTAIS E NEUROQUÍMICOS EM UM MODELO DA DOENÇA DE PARKINSON CAUSADA PELA 6-HIDROXIDOPAMINA EM CAMUNDONGOS

DEFESA DE TESE

André Tiago Rossito Goes

Uruguaiana, RS, Brasil.
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por

André Tiago Rossito Goes

Tese apresentada como requisito parcial para obtenção do
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Co-orientador: Prof. Dr. Leandro Cattelan Souza

Uruguaiana, RS, Brasil.

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

COMISSÃO EXAMINADORA:

Prof. Dr. Leandro Cattelan Souza (presidente, coorientador)

Prof. Dr. Carlos Borges Filho (UNIPAMPA)

Prof. Dr. Leonardo Magno Rambo (UNIPAMPA)

Prof. Dr. Gustavo Petri Guerra (UNIPAMPA)

Prof. Dr. Mauro Schneider Oliveira (UFSM)

Uruguaiana, RS, Brasil.

2018

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por toda a inspiração e luz
no caminho percorrido até o momento.
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Tese de Doutorado

Programa de Pós-Graduação em Bioquímica

Universidade Federal do Pampa, RS, Brasil

EFEITO DA CRISINA SOBRE AS ALTERAÇÕES COMPORTAMENTAIS E NEUROQUÍMICOS EM UM MODELO DA DOENÇA DE PARKINSON CAUSADA PELA 6-HIDROXIDOPAMINA EM CAMUNDONGOS

Autor: André Tiago Rossito Goes

Orientador: Cristiano Ricardo Jesse / Leandro Cattelan Souza

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A doença de Parkinson (DP) é caracterizada por uma degeneração progressiva dos neurônios dopaminérgicos do sistema nigroestriatal e depleção de dopamina (DA) no corpo estriado. A crisina tem demonstrado ser uma abordagem não farmacológica promissora para reduzir o risco de doenças neurodegenerativas. Este estudo investigou o potencial efeito neuroprotetor da crisina em um modelo animal de DP induzida por 6-hidroxidopamina (6-OHDA) em camundongos. O presente estudo demonstrou que uma dose de crisina (10mg/kg/dia p.o.) durate 28 dias foi eficaz em modular as seguintes alterações resultantes da exposição à 6-OHDA: atenuar à alteração no comportamento tipo-depressivo (teste de suspensão de cauda) e disfunções locomotoras (teste de rota-rod e teste do cilindro), proteger contra o estresse oxidativo (GPx, GSH, GST, GH), reduzir os níveis de citocinas pró-inflamatórias (TNF-α, IFN-γ, IL-1β, IL-2 e IL-6), bem como atenuou a redução dos níveis de DA, DOPAC e HVA. Além disso, preveniu a redução de neurônios TH^+ causados pela exposição a 6-OHDA, além de elevar os níveis BDNF, GDNF e NGF no estriado de camundongos. Estes resultados reforçam que um dos efeitos neuroproterores induzidos pela crisina na DP, são decorrentes dos efeitos antioxidante, anti-inflamatório e modulador de neurotrofinas. Portanto, o presente estudosugere que a crisina atenua os declínios cognitivos e motores, depressão, estresse oxidativo e neuroinflamação induzidas pela

6-OHDA suportando a hipótese de que a crisina pode ser usado como um tratamento coadjuvante ao tratamente farmacológico atual para reduzir os sinais da DP.

Palavras-chave: Doença de Parkinson, crisina, 6-hidroxidopamina, deficit cognitivo, estresse oxidativo, neuroinflamação.

ABSTRACT

Doctoral Thesis

Program of Post-Graduation in Biochemistry

Federal University of Pampa

EFFECT OF CRISINE ON BEHAVIORAL AND NEUROCHEMICAL INFECTIONS IN A MODEL OF PARKINSON DISEASE CAUSED BY 6-HYDROXIDOPAMINE IN MICE

Author: André Tiago Rossito Goes

Advisor: Cristiano Ricardo Jesse / Leandro Cattelan Souza

Site and Date of Defence: Itaqui, February 24, 2018.

Parkinson's disease (PD) is characterized by a progressive degeneration of dopaminergic neurons of the nigrostriatal system and dopamine depletion (DA) in the striatum. Chrysin has been shown to be a promising non-pharmacological approach to reducing the risk of neurodegenerative diseases. This study investigated the potential neuroprotective effect of chrysin in an animal model of PD induced by 6-hydroxydopamine (6-OHDA) in mice. The present study demonstrated that a dose of chrysin (10 mg / kg / day po) for 28 days was effective in modulating the following changes resulting from exposure to 6-OHDA: attenuating the change in type-depressive behavior (tail suspension test) (GPx, GSH, GST, GH), reduce levels of proinflammatory cytokines (TNF-α, IFN-γ, IL-1β) , IL-2 and IL-6), as well as attenuated the reduction of AD, DOPAC and HVA levels. In addition, it prevented the reduction of TH + neurons caused by exposure to 6-OHDA, in addition to raising the BDNF, GDNF and NGF levels in the striatum of mice. These results reinforce that one of the neuroproteror effects induced by chrysin in PD is due to the antioxidant, anti-inflammatory and modulating effects of neurotrophins. Therefore, the present study suggests that chrysin attenuates the 6-OHDA-induced cognitive and motor declines, depression, oxidative stress and neuroinflammation by supporting the hypothesis that chrysin may be used

as a adjuvant treatment to the current pharmacological treatment to reduce the signs of PD

Key words: Parkinson´s disease, chrisin, 6-hydroxydopamine, cognitive impairment, oxidative stress, neuroinflammation.

LISTA DE ABREVIATURAS

DP – doença de Parkinson

DA – dopamina

SNc – substancia negra parte compacta

SNC – sistema nervoso central

EROs – espécies reativas de oxigênio

ERNs – espécies reativas ao nitrogênio

SOD – superóxido dismutase

CAT – catalase

GPx – glutationa peroxidase

TNF-α – *tumor necrosis fator-alpha* = fator de necrose tumoral-alfa

IL-1ß – interleucina 1-beta

BDNF – *Brain-derived neurotrophic fator* = fator de crescimento derivado do cérebro

6-OHDA – 6-hidroxidopamina

MPTP – 1-metil 4-fenil-1,2,3,6 tetrahidropiridina

MAO – monoamina oxidase

H_2O_2 – peróxido de hidrogênio

LPS – lipopolissacarídeo

COX-2 – cicloxigenase-2

NA – noradrenalina

5-HT – 5-hidroxitriptofano (serotonina)

Ach – acetilcolina

TST – *tail suspension test* = teste de suspensão de cauda

TRO – teste de reconhecimento de objetos

MLP – memória de longo prazo

ER – espécies reativas

HVA – ácido homovanílico

DOPAC – 3,4-di-hidroxifenilacético

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PARTE I

APRESENTAÇÃO

A presente tese foi dividida em três partes principais. Na **parte I** encontram-se a Introdução e objetivos. Os resultados que fazem parte desta tese estão apresentados sob a forma de um artigo publicado em periódico científico e um manuscrito, os quais se encontram nos itens artigo e manuscrito na **parte II** deste trabalho. As seções materiais e métodos, resultados, discussão dos resultados e referências bibliográficas, encontram-se nos próprios artigo e manuscrito e representam a íntegra deste estudo. Os itens Discussão e Conclusão, encontrados na **parte III** desta tese, apresentam interpretações e comentários gerais sobre os resultados apresentados na parte II deste trabalho. O item Referências Adicionais refere-se somente às citações que aparecem nos itens introdução, discussão e conclusão desta tese. No ítem Perspectivas, estão expostos os possíveis estudos para dar continuidade a este trabalho.

INTRODUÇÃO

1.1 Doença de Parkinson

Em 1817, o médico inglês James Parkinson descreveu a doença de Parkinson (DP) no “Ensaio da paralisia agitante” vindo a receber seu nome posteriormente (Meneses e Teive, 2003; Lim, 2005). Lewy, em 1912, observou contenções citoplasmáticas hialinas em neurônios dopaminérgicos de pacientes com DP. Os corpos de Lewy, como são conhecidas até os dias de hoje, estão intimamente ligas a DP (Takahashi e Wakabayashi, 2005). Em 1930, Hassler descreveu a perda de neurônios nos núcleos basais de Meynert (Mori, 2005) e ainda em 1938, observou que a substância negra e *locus coeruleus* encontravam-se lesados em pacientes da DP (Takahashi e Wakabayashi, 2005). Conforme Lee Mosley et al. (2006) a doença manifesta-se em média por volta dos 55 anos de idade e a sua incidência tende a aumentar com o envelhecimento e ainda, é aproximadamente 1.5 vezes maior no homem que nas mulheres em todas as idades (Elbaz et al., 2007). Uma possível explicação para o fato que a DP tenha maior incidência no sexo masculino seria o papel neuroprotetor dos esteroides sexuais femininos, em especial a progesterona (hormônio esteroide feminino produzido nas células do corpo lúteo do ovário) que possui receptores por todo o cérebro e que poderia atuar como neuroprotetor, além de reforçar as capacidades cognitivas e neurogenese (Casas et al., 2011).

A DP é um transtorno neurodegenerativo, sendo relacionada mais comumente à idade, tendo a secunda maior prevalência em humanos (atrás apenas da doença de Alzheimer) (Tadaiesky, 2010). Aproximadamente 1% da população mundial acima dos 55 anos é portadora da DP (Hayes et al., 2010). Com o aumento da população idosa, acredita-se que o número de pessoas diagnosticadas com DP deve aumentar rapidamente nos próximos anos.

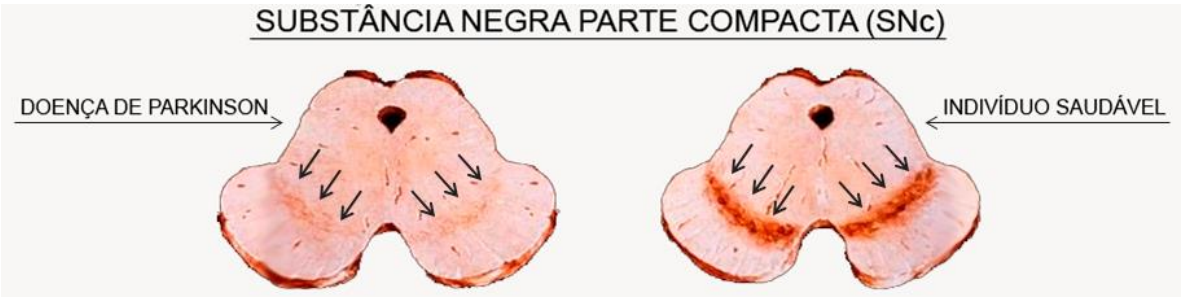
Clinicamente, a DP é caracterizada pelos sinais motores clássicos (**Tabela 1**) relacionados à patologia: tremor em repouso, diminuição dos movimentos voluntários, bradicinesia, rigidez, postura curvada e instabilidade postural.

Tabela 1. Características dos sinais motores clássicos

Sinais motores clássicos	Consequências
Tremor em repouso	Sintoma inicial em cerca de 60-70% dos pacientes, não sendo necessariamente incapacitante por ser atenuado pelo movimento voluntário (Yanagisawa, 2006)
Diminuição dos movimentos voluntários	Dificuldade de programação e execução dos movimentos (Klockgether, 2004)
Rigidez muscular	Aumento da resistência da articulação durante um movimento passivo (Dauer e Przedborski, 2003) e capacidade reduzida de relaxar os músculos dos membros (Klockgether, 2004)
Instabilidade postural	Compromete a capacidade do paciente de manter o equilíbrio durante as tarefas diárias, tais como levantar, andar e curvar-se, aumentando o risco de quedas do paciente (Morris, 2000)

A principal característica neuropatológica, lesão dos neurônios dopaminérgicos (**Figura 1**) localizados na substância negra parte compacta (SNc), com o papel de enviar projeções para os gânglios da base, ocasiona à redução dos níveis de dopamina (DA) no corpo estriado, além de alterar suas conexões com o córtex pré-frontal (Blandini et al., 2000; Araki et al., 2001). Contudo, a neuropatologia da DP não está restrita exclusivamente à via nigroestriatal, de forma que são notadas anomalias histológicas também em outros grupos celulares dopaminérgicos e não dopaminérgicos, tais como os sistemas colinérgicos, noradrenérgicos e serotonérgicos, bem como córtex e sistema nervoso autônomo (Dauer e Przedborski, 2003).

Figura 1. Degeneração da substância negra parte compacta (SNc).
Degeneração dos neurônios dopaminérgicos na SNc em indivíduo saudável e DP.



Fonte: Aguiar Junior, 2011

Apesar de vários estudos epidemiológicos apontarem que alguns fatores ambientais podem estar envolvidos no desenvolvimento da doença, tais como a exposição a alguns tipos de herbicidas e pesticidas (Liou et al., 1997; Gorell et al., 1998; Fall et al., 1999; Vanacore et al., 2002), a causa primária da DP não está totalmente esclarecida. Além disso, o fato dos neurônios dopaminérgicos serem sensíveis ao estresse oxidativo, por fatores como o metabolismo da DA, auto-oxidação, níveis aumentados de ferro e reduzidos de glutationa total em comparação a outras regiões cerebrais (Jenner, 2003; Berg et al., 2004), sugerem um importante papel do estresse oxidativo na patologia. O envelhecimento cerebral, a predisposição genética, anomalias mitocondriais e toxinas ambientais (Olanow et al., 1998; Alexi et al., 2000) são os possíveis desencadeadores da DP.

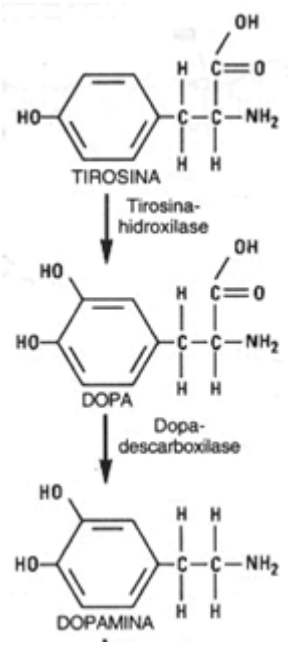
O aparecimento dos sintomas se dá quando de 60-80% dos níveis de DA no estriado já foram depletados quando comparado a níveis normais (Rang et al., 2007). Deste modo, a hipótese é de que a cooperação pós-sináptica entre acetilcolina (Ach) (excitatória) e a DA (inibitória) no estriado contribui para os sintomas da DP (Barbeau, 1962; Rang et al., 2007; Threlfell e Cragg, 2011), ainda mais que anticolinérgicos eram os primeiros medicamentos utilizados para o tratamento da DP (White e Westerbeke, 1961).

Além dos sintomas motores, existem sintomas pré-motores, como distúrbios emocionais, cognitivos e psicossociais frequentemente observados antes dos sintomas motores portadores da DP (Tadaiesky et al., 2008; Savica et al., 2010; Hayes et al., 2010). Desde modo, tratar-se de uma doença multidimensional, associando a sintomatologia da perda dos movimentos há

diversos sintomas que resultam na diminuição da qualidade de vida do paciente (Gupta e Bathia, 2000). Dentre os sintomas pré-motores, pode-se destacar: depressão (Santamaria et al., 1986; Cummings, 1992; Oertel et al., 2001; Hayes et al., 2010; Casas et al., 2011), ansiedade (Maricle et al., 1995; Witjas et al., 2002; Richard et al., 2004) e prejuízos cognitivos (Owen et al., 1995; Pillon et al., 1997; Goldman et al., 1998). Dentre elas, a depressão possui a maior incidência (76% dos casos), sendo que a severidade aumenta conforme a evolução da DP (Leentjens et al., 2003), seguindo pela ansiedade (aproximadamente 40%) (Richard, 2005), sendo ambas podem ocorrer simultaneamente (Menza et al., 1993; Marinus et al., 2002), e as disfunções cognitivas aparecem em todos os estágios da doença (prevalência entre 11 e 36% dos pacientes) (Giladi et al., 2000). Essas alterações se desenvolverem antes do aparecimento dos sinais motores e do diagnóstico da DP, sugerindo assim a ocorrência de alterações cerebrais na fase inicial da doença, fato esse que levaria a uma maior predisposição ao desenvolvimento de sintomas psiquiátricos e alterações cognitivas (Tolosa et al., 2009). Tais sintomas não são exclusivos da DP, por isso, geralmente, não são considerados para diagnóstico da doença em seus estágios iniciais.

No cérebro a distribuição de DA é mais abundante no corpo estriado, parte do sistema motor extrapiramidal envolvido na coordenação motora, e elevadas concentrações também ocorrem em certas partes do sistema límbico e do hipotálamo. Sua síntese segue a mesma rota da noradrenalina (NA; **Figura 2**), especificamente a conversão de tirosina para dopa (passo limitante da velocidade da reação), seguida pela descarboxilação para formar DA (Rang et al., 2007).

Figura 2. Rota da síntese de DA.



Fonte: <http://www.hu.uel.br/index.php?pagina=129&pai=5>

Como a DP ainda não possui cura, o tratamento farmacológico se dá pela neuroproteção e pelo controle dos sintomas motores e cognitivos. O fármaco mais utilizado no tratamento é a levodopa, precursor metabólico da DA (Hauser et al., 2009; Katezenschlager e Lees, 2002), desenvolvido na década de 60 (Cotzias et al., 1969). Deste modo, aumenta os níveis de DA cerebral. Para aumentar a sua biodisponibilidade, geralmente se administra sinergicamente inibidores periféricos da dopa-descarboxilase (Rinaldi, 2011). Contudo, o uso prolongado deste medicamento pode acarretar em efeitos colaterais indesejados, tais como, aparição dos sintomas antes da próxima dose e discinesias (Katezenschlager e Lees, 2002). Recentemente, a levodopa tem sido considerada a terapia padrão para os pacientes com DP e apresenta maior eficácia com o menor grau de efeitos colaterais indesejados quando comparada com os agonistas dopaminérgicos (Abbruzzese, 2008; Murata, 2009). Outros medicamentos (**Quadro 1**) também são utilizados na DP: anticolinérgicos, liberadores de dopamina, inibidores periféricos da dopa-descarboxilase, agonistas dopaminérgicos, inibidores da monoamina oxidase (MAO), principalmente a MAO-B, e da COMT (Jankovic e Stacy, 2007; Archibald e Burn, 2008).

Quadro 1. Classificação, princípio ativo e efeitos colaterais indesejados dos principais fármacos utilizados no tratamento da DP. Adaptado de Rinaldi, (2011).

Classificação dos Fármacos	Princípio Ativo	Efeitos Colaterais Indesejados
Anticolinérgicos	Antagonistas da ação de fibras nervosas parassimpáticas que liberam acetilcolina. Ou seja, que inibem a produção da acetilcolina.	Confusão, alucinações
Liberadores de Dopamina	Estimula a liberação de dopamina no cérebro além de impedir a retirada deste neurotransmissor da fenda sináptica	Disfunção cognitiva, alucinações
Precursor dopaminérgico	Sofre ação da dopa-descarboxilase, dando origem à DA	Náusea, vômito, alucinações visuais, sonolência, discinesia
Inibidores periféricos da dopa-descarboxilase	Impedem o metabolismo da levodopa antes de atingir o SNC	Náusea, vômito, alucinações visuais, sonolência, discinesia
Agonistas dopaminérgicos	Não necessitam de transformação enzimática para serem ativas. Agem nos receptores de DA na SNc	Náusea, hipotensão, alucinações, dores de cabeça, problemas no sono, fibrose pulmonar
Inibidores da MAO-B	Agem no SNC impedindo a remoção da DA após utilizada pelo receptor	Perda de peso, vômito, problemas no equilíbrio, hipotensão
Inibidores da COMT	Agem tanto no SNC quanto fora dele, junto com a MAO-B. Esta enzima também inibe a transformação da levodopa em 3-Ometildopa, substância sem efeito terapêutico	Diarréia, discinesia, toxicidade no fígado

1.2 6-OHDA

A neurotoxina 6-hidroxidopamina (6-OHDA) injetada localmente no estriado é o modelo (**Tabela 2**) mais comumente utilizado em roedores para a DP, gerando uma lesão nigroestriatal (Tadaiesky, 2010). A injeção bilateral de 6-OHDA na SNc é capaz de causar degeneração anterógrada do sistema dopaminérgico nigroestriatal, causando acinesia e alta taxa de mortalidade,

sendo assim gerado o primeiro modelo de DP (Ungerstedt, 1968). Devido à sua baixa complexidade e custo do procedimento, além de ser altamente reprodutível, em contraste com modelos mais recentes, a 6-OHDA ainda é a mais utilizada para mimetizar a degeneração dos neurônios dopaminérgicos (Tadaiesky, 2010; Blesa et al., 2012). Além disso, ao contrário de outras toxinas utilizadas para a indução de DP, tal como o 1-metil 4-fenil-1,2,3,6 tetrahidropiridina (MPTP), a 6-OHDA apresenta baixo risco de toxicidade relacionada à sua manipulação (Tadaiesky, 2010; Blesa et al., 2012).

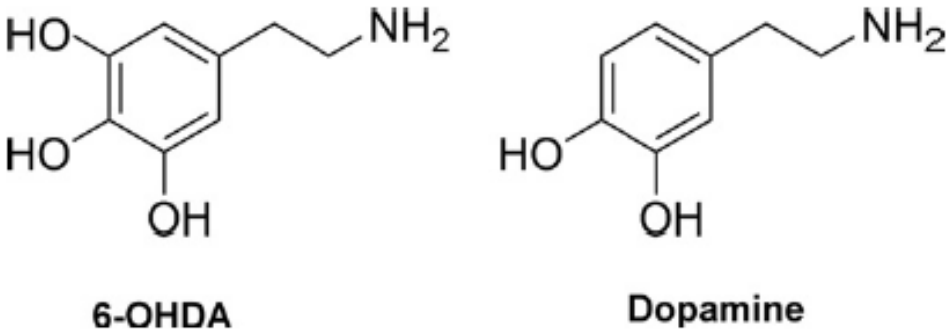
Tabela 2. Diferentes modelos utilizados para DP em animais. Adaptado de Blesa et al., 2012

Modelo	Sintomas comportamentais	Dano nigroestriatal	Agregados de sinucleína/ corpo de Lewy	Usos do modelo	Desvantagens
6-OHDA	Comportamento rotatório após a injeção unilateral	Perda de inervação no local da injeção (estriado)	Sem inclusões	Terapias para melhora dos sintomas e mecanismos de morte celular	Requer a injeção diretamente no estriado
MPTP	Deficiência motora primária e aguda em roedores	Perda de neurônios dopaminérgicos dose-dependente (95% em doses elevadas). Diminuição dos níveis de DA no estriado	Inclusões não prevalentes	Terapias para melhora dos sintomas e mecanismos de morte celular	Morte das células não é progressivo (efeito agudo)
Rotenone	Diminuição da atividade motora em roedores	Perda de inervação e de neurônios dopaminérgicos no estriado	Agregados de sinucleína nos neurônios dopaminérgicos	Testar compostos neuroprotetores	Morbidade e mortalidade substancial. Trabalho e tempo intensivo
Paraquat	Sem danos motores claros	Diminuição da imunorreatividade da tirosina hidroxilase no estriado	Sem inclusões mas com aumento da imunorreatividade sinucleína em neurônios dopaminérgicos	Testar estratégias neuroprotetoras	Não testado extensivamente. Efeitos em outros sistemas de neurotransmissores

A 6-OHDA é tóxica tanto a nível periférico quanto central, Porter et al. (1963) demonstrou que a 6-OHDA (análogo estrutural da DA e NA; **Figura 3**) era capaz de causar depleção de NA nos nervos simpáticos do coração, entretanto, por não possuir a capacidade de ultrapassar a barreira hematoencefálica, ela deve ser injetada diretamente no cérebro através de cirurgia estereotáxica para que possa gerar toxicidade no SNC. Os efeitos neurotóxicos da 6-OHDA ocorrem

através do acúmulo da toxina nos neurônios catecolaminérgicos, seguido por alterações na homeostase celular e dano neuronal (Tadaiesky, 2010). O armazenamento intracelular de 6-OHDA é mediado pelos transportadores de membrana de DA e NA, que reconhecem e captam a 6-OHDA devido à sua similaridade estrutural com as catecolaminas endógenas (Tadaiesky, 2010; Bové e Perier, 2012).

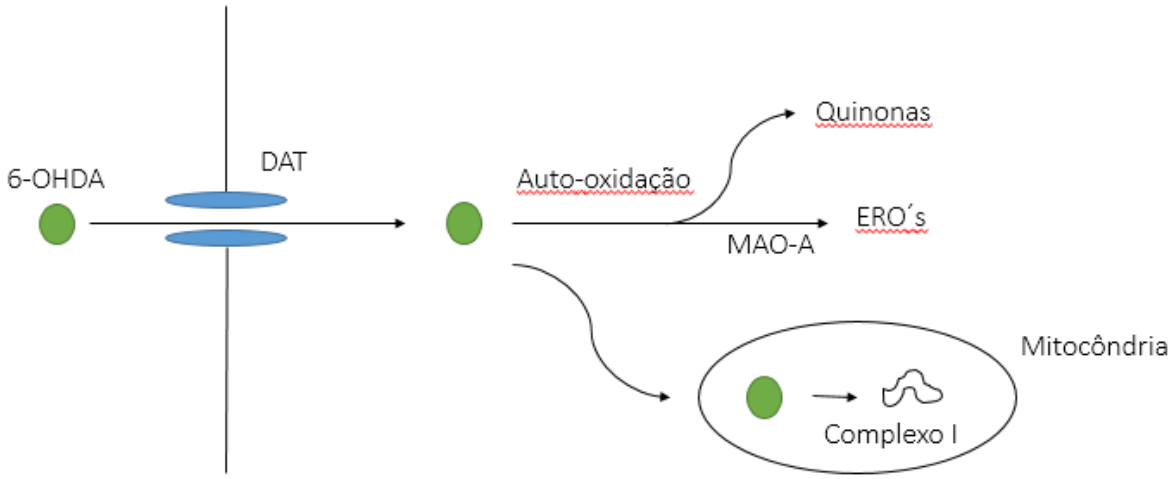
Figura 3. Comparação entre as estruturas químicas da 6-OHDA e da DA.



Fonte: Bové e Perier, 2012

Uma vez infundida diretamente no estriado, a 6-OHDA produz espécies citotóxicas através de mecanismos enzimáticos e não-enzimáticos (Choi et al., 1999): a oxidação de 6-OHDA pela MAO gera peróxido de hidrogênio (H_2O_2), o qual, além de ser citotóxico, induz a produção de outros radicais de oxigênio (**Figura 4**). Além disso, a 6-OHDA sofre um processo de auto-oxidação, gerando H_2O_2, EROs e quinonas (Kabuto & Yamanushi, 2011). Os aumentos nos níveis de EROs e outras espécies reativas resultam na rápida depleção das enzimas antioxidantes, amplificando a neurotoxicidade no metabolismo e estrutura celular resultando, assim, em dano neuronal (Blum et al., 2001). A 6-OHDA pode, além de acentuar o estresse oxidativo, induzir a neurotoxicidade alterando a função mitocondrial, sendo demonstrado que esta toxina prejudica a atividade do complexo I em mitocôndrias isoladas (Glinka e Youdim, 1995).

Figura 4. Neurotoxicidade induzida pela 6-OHDA. DAT: transportador de DA. MOA-A; monoamina oxidase A. ERO's: espécies reativas de oxigênio.



Fonte: Tadaiesky, 2010

Sendo dose-dependente, a lesão causada pela 6-OHDA depende da concentração injetada. A variância da neurodegeneração pode variar do massivo ao moderado, uma vez que o grau da degeneração depende da dose injetada (Fearnley e Lees, 1991; Lee et al., 1996; Tadaiesky, 2010; Bové e Perier, 2012). A injeção de 6-OHDA na SNc ou no trato nigroestriatal leva a uma destruição imediata e potencialmente completa dos neurônios dopaminérgicos da SNc e, em menor grau, da área tegmentar ventral, resultando em depleção de 80-90% de DA estriatal, produzindo assim, um modelo de DP severo (Kirik et al., 1998; Tadaiesky, 2010).

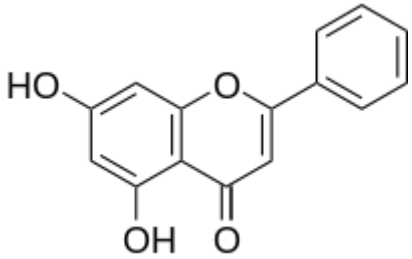
A variação do procedimento original, na qual a 6-OHDA é injetada no corpo estriado, foi proposta na década de 1990, sendo a localização dos terminais dos neurônios da SNc (Sauer e Oertel, 1994). Uma vez injetada no corpo estriado, a 6-OHDA é capaz de produzir degeneração retrógrada lenta do sistema nigroestriatal (Lee et al., 1996), fato esse que reproduz de maneira mais próxima a progressão da DP, viabilizando assim a investigação de tratamentos neuroprotetores (Georgievska et al., 2002).

1.3 Crisina

Pertencente à classe das flavonas, o flavonoide crisina (**Figura 5**) é encontrada naturalmente no mel, própolis e diversas espécies de plantas (Pichichero et al., 2010; Medic-Saric, 2011). Encontrada principalmente na *Passiflora coerulea* (mais comumente conhecida como maracujá do mato, muito

abundante no bioma Pampa), a crisina foi isolada primeiramente em 1990 e utilizada como anticonvulsivante (Medina et al., 1990). Posteriormente começou a ser utilizada principalmente por fisiculturistas para inibir a amoratase, evitando assim a biotransformação da testosterona em estradiol (Kao et al., 1998; Darwish et al., 2014).

Figura 5. Estrutura química do flavonoide crisina.



Devido principalmente a presença do grupamento hidroxila no carbono 5, a crisina possui características antioxidante (Pushpavalli et al., 2010). Além disso, a crisina possui também propriedades anticonvulsivante (Medina et al., 1990), anti-hipertensivo (Vilar et al., 2002), anti-inflamatório (Bae et al., 2011), antineoplásico (Pichichero et al., 2011), anti-hiperlipidêmico (Zarzecki et al., 2014) e antidepressivo (Borges Filho et al., 2015).

Estudos vêm demonstrando que a crisina modula os níveis do fator de crescimento derivado do cérebro (BDNF), que é uma molécula neurotrófica reguladora chave do desenvolvimento e função do sistema neural. Esta neurotrofina medeia muitos processos no cérebro de mamíferos, incluindo diferenciação e crescimento neuronal, formação de sinapses e plasticidade, bem como modula a sobrevivência neuronal, a liberação de neurotransmissão e a fixação da memória (Souza et al., 2015; Park and Poo, 2013). Sendo assim, a crisina vem demonstrando eficiência na regulação dos níveis de BDNF (Souza et al., 2015). Tal ação pode ser um dos principais mecanismos de neuroproteção apresentado pela crisina.

1.4 Estresse oxidativo e neuroinflamação

Acredita-se que a produção de EROs bem como a de espécies reativas ao nitrogênio (ERNs) no cérebro seja importante na defesa inespecífica deste órgão. Contudo, a hiperativação desse processo pode levar à perda de neurônios

devido à alta susceptibilidade destes ao dano oxidativo (Ischiopoulos e Beckman, 2003).

As EROs e ERNs são produzidos normalmente pelo metabolismo celular, sugerindo o duplo papel desempenhado no organismo. Em baixas e moderadas concentrações, essas espécies auxiliariam na defesa do organismo contra agentes infecciosos e envolvidas em sistemas de sinalização celular, já em níveis elevados, elas produzem dano celular conhecido como estresse oxidativo (Valko et al., 2007).

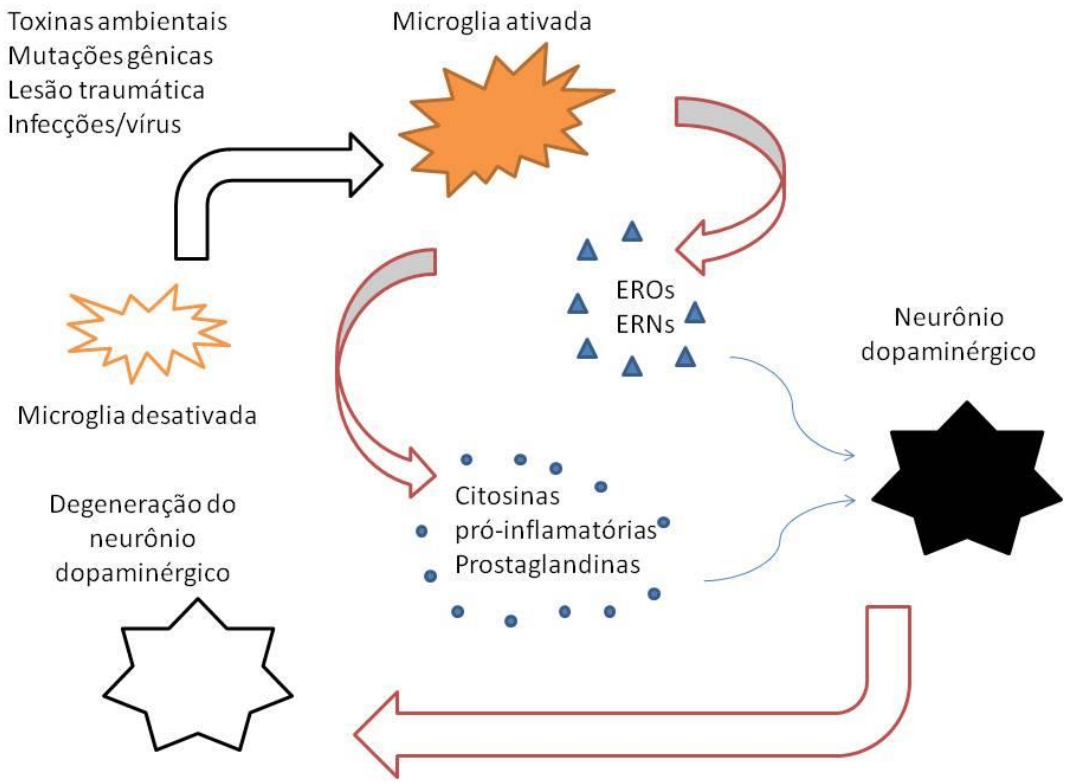
Com o aumento desacerbado na produção de espécies reativas, a capacidade de remoção pelos antioxidantes endógenos é alterada e diminuída, gerando danos oxidaivos nos componentes biológicos como DNA, lipídeos, proteínas e outras moléculas, resultando na alteração da célula e podendo, assim, gerar sua morte (Ischiopoulos e Beckman, 2003).

Schapira et al. (1989) relatou pela primeira vez que pacientes com DP apresentam a atividade do complexo I da cadeia transportadora de elétrons mitocondrial reduzida na SNc. O possível envolvimento desse complexo na patogênese foi intensificado com a comprovação de que a neurotoxina MPTP, assim como a 6-OHDA (Glinka e Youdim, 1995; Blum et al., 2001), é capaz de produzir sinais característicos da doença em modelos animais.

A resposta imune no cérebro não envolve o sistema imune periférico, sendo assim, não há a participação de anticorpos e células. Ao invés disso, depende da síntese de componente inflamatório pelos neurônios, glia e micróglia (McGeer e McGeer, 2004).

Segundo Fahn e Sulzer (2004) células da glia e micróglia, quando há um insulto tóxico, podem produzir substâncias nocivas à célula, tais como citocinas pró-inflamatórias, prostaglandinas, EROs e ERNs (**Figura 6**).

Figura 6. Potencias fatores de ativação da microglia no SNC na contribuição para a degeneração de neurônios dopaminérgicos na DP.



Fonte: Adaptado de Bové e Perier, 2012

Seguindo nessa linha, para McGeer e McGeer (2004) a ativação microglial pode resultar no aumento da produção de ânions superóxidos e outras neurotoxinas e tais produtos podem contribuir para processos neurotóxicos, incluindo dano a neurônios dopaminérgicos (Hirsch et al., 2012). Nesse viés, foram relatadas na DP aumento no número de células microgliais ativas (Hirsch et al., 2012).

Devido a alguns achados, o envolvimento de processos inflamatórios na DP foi mais bem elucidado, como a descoberta de macrófagos derivado de micróglia na SNc de pacientes com DP e que a atenuação, ou até mesmo a inibição, da resposta imune microglial aumentava a sobrevivência neuronal em modelos animais da 6-OHDA e MPTP (Croisier et al., 2005).

Neste sentido, o presente estudo tem o intuído de estudar uma nova alternativa para o tratamento da DP, uma vez que os tratamentos conhecidos possuem diversos efeitos colaterais indesejados.

2 OBJETIVOS

2.1 Objetivo geral

- Avaliar o efeito neuroprotetor do flavonoide crisina em um modelo da DP induzido pela 6-OHDA.

2.2 Objetivos específicos

- Analisar parâmetros comportamentais (Comportamento circular induzido por apomorfina, teste de suspensão de cauda e teste do cilindro) e de locomoção (Rota rod, teste de campo aberto) nos camundongos após a administração da 6-OHDA.
- Analisar os parâmetros de estresse oxidativo (espécies reativas) e atividade de enzimas antioxidantes (GPx, GSH, GST, GH, CAT) no estriado dos camundongos após a administração da 6-OHDA.
- Analisar os níveis de citocinas (TNF-α, IFN-γ, IL-1β, IL-2, IL-6, IL-10 e S110B) no estriado de camundongos após a administração da 6-OHDA.
- Analisar os níveis de neurotrofinas (BDNF, GDNF e NGF) no estriado de camundongos após a administração da 6-OHDA.
- Caracterizar as alterações neuroquímicas (TH^+) associadas à neurotoxicidade induzida pela 6-OHDA, no estriado de camundongos por imunohistoquímica.
- Analisar os níveis da dopamina e seus metabólitos (HVA e DOPAC) no estriado de camundongos após a administração da 6-OHDA.

Parte II

Artigo

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Protective role of chrysin on 6-hydroxydopamine-induced neurodegeneration a mouse model of Parkinson's disease: Involvement of neuroinflammation and neurotrophins

André T.R. Goes[a], Cristiano R. Jesse[a,*], Michelle S. Antunes[a], Fernando V. Lobo Ladd[b], Aliny A.B. Lobo Ladd[c], Cristiane Luchese[d], Natalia Paroul[e], Silvana P. Boeira[a]

[a] Laboratory of Pharmacological and Toxicological Evaluations Applied to Bioactive Molecules, Federal University of Pampa, Itaqui, CEP 97650-000, RS, Brazil
[b] Department of Morphology, Laboratory of Neuroanatom, Biosciences Center, Federal University of Rio Grande do Norte, Natal, RN, Brazil
[c] Laboratory of Stochastic Stereology and Chemical Anatomy, Department of Surgery, College of Veterinary Medicine and Animal Science, University of São Paulo, Brazil
[d] Centro de Ciências Químicas, Farmacêuticas e de Alimentos, Universidade Federal de Pelotas, Campus Universitário, s/n, 96160-000, Capão do Leão, RS, Brazil
[e] Universidade Regional Integrada, Campus Erechim, CEP 99700-000, RS, Brazil

Protective role of chrysin on 6-hydroxydopamine-induced neurodegeneration a mouse model of Parkinson's disease: Involvement of neuroinflammation and neurotrophins

André T. R. Goes[1], Cristiano R. Jesse[1]*, Michelle S. Antunes[1], Fernando Vagner Lobo Ladd[2], Aliny Antunes Barbosa Lobo Ladd[3], Silvana Peterini Boeira[1]

[1]Laboratory of Pharmacological and Toxicological Evaluations Applied to Bioactive Molecules, Federal University of Pampa, Itaqui, CEP 97650-000, RS, Brazil.

[2]Department of Morphology/Laboratory of Neuroanatom, Biosciences Center, Federal University of Rio Grande do Norte, Natal-RN, Brazil.

[3]Laboratory of Stochastic Stereology and Chemical Anatomy, Department of Surgery, College of Veterinary Medicine and Animal Science, University of São Paulo, Brazil.

*Correspondence should be sent to:

Cristiano R. Jesse

Phone and FAX number: +55-55-34321853

E-mail: cristianoricardojesse@yahoo.com.br

Abstract

Chrysin is a natural flavonoid which is found in bee propolis, honey and various plants, and neuroprotective effect of chrysin in mice was previously demonstrated by our group. Neuroinflammation, neurotrophic factors and neuronal recovery factors associated with the neuroprotective effect of this flavonoid require further investigations. Thus, now we investigated the possible involvement of inflammatory cytokines, neurotrophic factors and neuronal recovery in the effect of chrysin in 6-hydroxidopamine (6-OHDA), a well-established model of Parkinson's disease, in striatum of mice. The 6-OHDA microinjection induced behavioral alterations on the rotarod test and apomorphine-induced circling behavior in mice. 6-OHDA administration elevated levels of tumor necrosis factor-α, interferon-gamma, interleukin-1β, interleukin-2, interleukin-6 and decreased the interleukin-10 levels in striatum, as well as, modified the calcium-binding protein B (S100B), brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF) and glial cell line-derived neurotrophic factor (GDNF) levels. The intrastriatal injection of 6-OHDA also induced an decrease of dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanylic acid (HVA) levels and tyrosine hydroxylase (TH) content. Oral treatment with chrysin (10 mg/kg, 28 days), culminated with the prevention of these alterations occasioned by 6-OHDA. These results corroborated with the neuroprotective effect of chrysin in

the treatment of Parkinson's disease and, indicated the mechanism involved throught the inflammatory cytokines, neurotrophic factors and recovery of dopaminergic neurons in striatum.

Keywords

Neurotrophic factor; Flavonoid; Inflammation; Dopamine; neurodegenerative disease.

Abreviations

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP); 3,4-dihydroxyphenylacetic acid (DOPAC); 6-hydroxydopamine (6-OHDA); advanced glycation end products (RAGE); brain-derived neurotrophic factor (BDNF); Calcium-binding protein B (S100B); dopamine (DA); enzyme-linked immunosorbent assay (ELISA); ethical Committee for Animal Use (CEUA); glial cell line-derived neurotrophic factor (GDNF); homovanillic acid (HVA); interferon-gamma (IFN-γ); interleukin 1-beta (IL-1β); interleukin 2 (IL-2); interleukin 6 (IL-6); interleukin-10 (IL-10); nerve growth factor (NGF); Parkinson's disease (PD); per oral (p.o.); reactive oxygen species (ROS); *substantia nigra pars compacta* (SNpc); supernatant fraction (S_1); tumour necrosis factor-alpha (TNF-α); tyrosine hydroxylase (TH).

1. Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder of unknown aetiology, which is characterized by selective, abnormal loss of nigrostriatal dopaminergic neurons of the *substantia nigra pars compacta* (SNpc), resulting in the loss of nerve terminals, accompanied by dopamine (DA) deficiency in the striatum [1]. The motor symptoms of PD include resting tremor, postural instability, bradykinesia, rigidity, and gait impairment, these features are attributable to dopaminergic cell loss and the resultant dysfunction of the basal ganglia, a cluster of deep nuclei that participate in the initiation and execution of movements [2]. The mechanisms responsible for neurodegeneration in PD are still unknown, but oxidative stress, excitotoxicity and neuroinflammation are believed to play a key role in neuron death [3,4]. There has been considerable interest in studying the involvement of neurotrophic factors, that are substances known to be vital for survival of specific neurochemical-phenotype classes of neuron [5,6]. Accumulated evidences suggest that microglial activation and insufficient support of neurotrophic factors may be crucial for the initiation and progression of this pathology [5,6]. A well-established model of PD is the catecholamine neurotoxin 6-hydroxydopamine (6-OHDA), hydroxylated analogue of DA, administered into the striatum of mice selectively destroyed the dopaminergic nigrostriatal neurons [7].

A limited number of drugs are available for the management of PD and, thus, new therapeutic and alternative agents acting on multiple levels of the pathology are needed. Recent findings suggest that phytochemical compounds with neuroprotective features may be important resources in the discovery of candidate drug against PD [8]. Compounds, such as curcumin, catechins and

resveratrol, have markable neuroprotective properties [9]. Chrysin (5,7-dihydroxyflavone) is a flavonoid which is found in propolis, honey, mushroom and flowers, such as the blue passion flower (*Passiflora caerulea)* and the *Indian* trumpet flower [10]. Research has shown that chrysin have a broad of biological and pharmacological properties, such as antioxidant, anti-inflammatory and antineoplastic actions [11-13]. Chrysin has also been found to have neuroprotective effects, preventing neurons from oxidative insults and apoptosis [13]. It can also improved cognitive deficits and brain damage induced by chronic cerebral hypoperfusion in rats [14] and age-related cognitive decline associated to brain oxidative stress in aged mice [15]. Conversely, no studies were found regarding the protective effect of chrysin in a rodent model of PD.

Thus, we sought to investigate the effect of chrysin treatment (10 mg/kg, orally*, per day*) over 28 days in a mouse model of PD induced by injection of 6-OHDA. For this purpose, we verified whether chrysin treatment can protect against behavioral impairment elicited by 6-OHDA. In addition, we investigated the protective effect of chrysin on inflammation markers, neurotrophins levels and dopaminergic neurons restoration in striatum of mice exposed to 6-OHDA.

2. Materials and methods

2.1. Animals

Experiments were performed using male C57B/6J mice (20-30g, 90 days old). Animals were maintained at 22-25ºC with free access to water and food, under a 12:12h light/dark cycle, with lights on at 7:00 a.m. All manipulations were carried out during light phase on the day. All efforts were made to minimize animal

suffering and to reduce the number of animals used. The procedures of this study were conducted according to the guidelines of the Committee on Care and Use of Experimental Animals Resources and with the approval of Ethical Committee for Animal Use (CEUA protocol # 038/2012).

2.2. Experimental design

Mice were randomly assigned to four groups (n=6 per group): (1) Vehicle/ Vehicle; (2) Vehicle/Chrysin; (3) 6-OHDA/Vehicle and (4) 6-OHDA/ Chrysin. The mice were subjected to stereotaxic surgical injections of 6-OHDA or vehicle. Seven days after the injections, treatment with chrysin (10 mg/kg, *per oral*, p.o.) was initiated for 28 days, and the after behavioral testing, mice were euthanized and the striatum was removed for neurochemical assays (**Figure 1**).

2.3. Stereotaxic surgical injection of 6-OHDA

Surgery was performed under anesthesia with 10 mL/kg of 1% ketamine (Bela-Pharm, Vechta, Germany) and 0.2% xylazine (Bayer HealthCare, Leverkusen, Germany). 6-OHDA (Sigma; 5 µg in 2 µL of 0.9% NaCl with 0.2 µg/lL ascorbic acid) was injected slowly (0.5 µL/min) into the right striatum (0.9 mm anterior and 1.8 mm lateral from bregma, 3.0 mm ventral from the dura). After the injection, the syringe was kept for additional 3 min in the brain, before it was slowly retracted. Controls were vehicle-injected [16].

2.4 Behavioral assessment

2.4.1. Rota rod (muscular coordination)

For motor-coordination ability, 36 days after lesioning, the mice were evaluated on rotarod apparatus [17]. The rotarod unit (Omni Rotor, Omnitech Electronics, Inc., Columbus, OH, USA) consist of a rotating rod, 55 mm diameter, on which mice were allowed to retain. After twice daily training for 2 successive days (speed 7 rpm on the first day and 10 rpm on second day) the rotational speed of the test was increased to 15 rpm on the third day in a test session. The time for each mice to remain on the rotating bar was recorded. The maximum time was 120 sec per trial. The apparatus automatically records the time in 0.1 s and stop the counting when the mice fall of the rotating shaft. The speed was set at 15 r.p.m. and cut off time was 120 sec. The animals were trained on the rod, so that they could stay on it at least for the cut-off time. Data were presented as retention time on the rotating bar over the three test trials.

2.4.2. Apomorphine-induced circling behavior

After 36 days of lesioning the mice were tested for drug-induced rotational behavior. Contralateral rotations of animals were recorded after giving 0.5 mg/kg apomorphine (in ascorbic acid-saline) subcutaneously and their rotational scores were collected over a period of 30 min intervals. The animals tested for 5 min over a period of 30 min (6 sessions) for apomorphine rotations. The results were expressed in number of rotations [18].

2.5.Tissue preparation

After behavioral tests, mice were euthanized with a barbiturate overdose (pentobarbital sodium 150 mg/kg). The striatum was removed and rapidly

homogenized in 50 mM Tris-Cl, pH 7.4. The homogenate was centrifuged at 2,400×g for 15 min at 4 °C, and a low-speed supernatant fraction (S_1) was used for assays.

2.6. Neurochemical assays

2.6.1. Cytokine levels

Brains were removed immediately and the striatum was dissected and homogenized with PBS buffer containing 0.05% Tween 20, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzethonium chloride, 10 mM EDTA and 20 IU aprotinin A. The homogenates were centrifuged at 3000×g for 10 min and the supernatants stored at −80 °C until assays for the determination of levels of interferon-gamma (IFN-γ), tumour necrosis factor-alpha (TNF-α), interleukin 1-beta (IL-1β), interleukin 2 (IL-2), interleukin 6 (IL-6) and interleukin-10 (IL-10) were carried out. The amount of protein in each sample was measured using the method of Bradford [19], using bovine serum albumin as a standard. The levels of each cytokine were evaluated using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's recommendations (R&D systems, Minneapolis, MN, USA) and the results are expressed in pg/mg of protein in each sample.

2.6.2. Trophic factors measurement

Calcium-binding protein B (S100B), brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF) and nerve growth factor (NGF) were measured using a commercially available sandwich enzyme-linked immune sorbent assay (ELISA) kit (Chemicon International, Temecula, CA, USA) according to the manufacturer's instructions. These plasticity markers levels were evaluated in striatum. The S100B, BDNF, GDNF and NGF levels were expressed as pg/mg wet weight of tissue.

2.6.3. DA, DOPAC and HVA levels

The measurement of DA, 3,4-dihydroxyphenylacetic acid (DOPAC) and Homovanillic acid (HVA) in striatal tissue was carried out by HPLC, as previously described [20]. Striatal tissues were homogenized with 300 µL of 200 mM ice-cold perchloric acid containing 10 mM disodium EDTA. After centrifugation (10,000 g for 10 min at 4 ºC), the supernatant was filtered and then injected directly into an HPLC system (Shimadzu; Kyoto, Japan) with an electrochemical detector (ECD; Eicom, Kyoto, Japan). The appendant potential of the ECD (carbon electrode vs. Ag/AgCl reference electrode) was set at 700 mV. The analytic column was a TSKgel Super-ODS (4.6 mm I.D. 9 100 mm; Tosoh, Tokyo, Japan), and the mobile phase consisted of 0.1 M citrate-sodium acetate buffer (pH 3.9) containing methanol (18%, v/v), disodium EDTA (4 mg/l), and sodium octanesulfonate (0.8 mM). The amount of monoamines was determined by comparison with freshly prepared standards, and their concentrations were expressed as ng/mg of tissue.

2.7. Stereological procedures

Design-based stereology was performed for cell counts and volume measurements of TH^+ neurons in SNpc, using a Leicamicroscopy (DMR 6000) coupled with stereological system newCast (Visiopharm version 4.5.6.857), and a video-camera Olympus DP72. The regions of interest (e.g. SNpc) were delineated according to Franklin e Paxinos [21] and Oorchot [22] by anatomical landmarks (Bregma−4.70 to −6.30 mm) with a 2,5× magnification on live microscopic video images displayed on a monitor.

The SNpc TH^+ neurons were counted by the optical fractionator design [23,24]. Briefly, the prefixed brains were embedded in 8% agar solution and cut exhaustively in coronal sections by a vibrotome (Leica VT1000S) in 40-µm-thick sections, cover all region of interest, along the frontal-caudal axis. Every fifth serial section containing SNpc was selected, and then we obtained the section sampling fraction (ssf=1/5). In this sample sections were applied free-floating immunohistochemical methods to identify the dopaminergic neurons in SNpc. So, the sections were washed with PBS, incubated with a 0.3% Triton X-100 solution, exposed to 0.3% hydrogen peroxide in distilled water to block endogenous peroxidases, placed in a 10% non-immune normal goat serum (Jackson Immuno Research Labs), incubated with a primary antibody (rabbit anti-Tirosinehidroxilase, 1:1000, Abcam) and with a secondary antibody (anti-rabbit IgG peroxidase conjugate, 1:200, KPL) in PBS. Immunoreactivity was visualized with 3,3- diaminobenzidine in PBS containing 0.01% hydrogen peroxide, and then the sections were mounted in a glass slide and cover with a coverslip.

In each sampled section some unbiased counting frames were created by the software and randomly placed over the region of interest. The counting frames were replaced systematically by stepwise movements in x- and y-directions.The area of the unbiased counting frame (a (frame) = 6400 µm²) relative to the area associated with the x and y steps (step length = 70000 µm) gives the area sampling fraction (asf=1/11). The optical disector height, along the z-axis, was determined by excluding a up guard region and a bottom guard region. The height of the optical disector relative to the actual thickness of the section results in the height sampling fraction (hsf=20). Only counting frames for which at least a part of the frame fell within the delineated contour were used for counting. Cells were marked if they were positive and in focuswithin the counting area. Furthermore, the counting frames have forbidden lines to avoid edge effects.

The estimated total number of positive cells (N) was calculated from the number of TH^+ neurons counted according to the formula:

NTH-neurons= 1/ssf-1. 1/asf-1. 1/hsf-1. Q-

Where Q- is the TH^+ neurons selected by the optical disector, ssf is the section sampling fraction; asf is the area sampling faction (a (frame) / step length) and hsf is the high sampling fraction.

2.8. Protein determination

Protein content was measured colorimetrically according to the method of Bradford [19], and bovine serum albumin (1 mg/ml) was used as the standard.

2.9. Statistical analysis

Results were presented as the means ± S.E.M. First, we evaluated the normality of data using the D'Agostino and Pearson omnibus normality test. Comparisons between experimental and control groups were performed by two-way analysis of variance (ANOVA) followed by Newman–Keuls test for post hoc comparison when appropriate. Main effects of first-order interactions are presented only when interaction was not significant. Comparisons between experimental and control groups were performed by one-way (chrysin or 6-OHDA = independent variable) or two-way ANOVAs (chrysin X 6-OHDA = independent variables). A value of $p < 0.05$ was considered to be significant. All tests and plotting graphics were executed using the GraphPad Prism 6 software (San Diego, CA, U.S.A.).

3. Results

3.1. Effects caused by chrysin on the and rotarod test and apomorphine-induced circling behavior

Statistical analysis of the latency for the first fall yielded significant effect of 6-OHDA ($F_{1, 24} = 30.09$, $p < 0.001$), chrysin ($F_{1, 24} = 7.57$, $p < 0.01$) and 6-OHDA × chrysin interaction ($F_{1, 24} = 12.35$, $p < 0.002$). Post hoc comparisons showed that injection of 6-OHDA significantly decreased the latency for the first fall of mice when compared to control group ($p < 0.001$). Chrysin treatment protected against the reduction of the latency for the first fall induced by 6-OHDA ($p < 0.05$) (**Fig. 2A**).

Two-way ANOVA of the number of rotations demonstrated a significant effect of 6-OHDA ($F_{1, 24} = 47.85$, $p < 0.001$), chrysin ($F_{1, 24} = 21.99$, $p < 0.001$) and 6-OHDA × chrysin interaction ($F_{1, 24} = 20.91$, $p < 0.001$). Post hoc comparisons revealed that injection of 6-OHDA significantly increased the number of rotations of mice compared to control group ($p < 0.001$). Chrysin treatment attenuated the increase of number of rotations induced by 6-OHDA ($p < 0.05$) (**Fig. 2B**).

3.2. Effect of chrysin and 6-OHDA injection on cytokine levels

Two-way ANOVA of IL-1β levels in the striatum demonstrated a significant effect of 6-OHDA ($F_{1, 24} = 66.80$, $p < 0.001$), chrysin ($F_{1, 24} = 27.47$, $p < 0.001$) and 6-OHDA × chrysin interaction ($F_{1, 24} = 33.44$, $p < 0.001$). Post hoc comparisons revealed that chrysin treatment attenuated the increase of IL-1β levels caused by 6-OHDA ($p < 0.05$; **Fig. 3A**).

Statistical analysis of TNFα levels in the striatum yielded a significant effect of 6-OHDA ($F_{1, 24} = 50.31$, $p < 0.001$), chrysin ($F_{1, 24} = 30.72$, $p < 0.001$) and 6-OHDA × chrysin interaction ($F_{1, 24} = 20.93$, $p < 0.001$). Post hoc comparisons showed that the increased TNFα levels observed in the striatum of 6-OHDA-treated mice was markedly abrogated by chrysin treatment, but not at control levels ($p < 0.05$; **Fig. 3B**).

Two-way ANOVA of IL-6 levels in the striatum demonstrated a significant effect of 6-OHDA ($F_{1, 24} = 40.50$, $p < 0.001$), chrysin ($F_{1, 24} = 16.23$, $p < 0.001$) and 6-OHDA × chrysin interaction ($F_{1, 24} = 7.54$, $p < 0.01$). Post hoc comparisons revealed that the increase of IL-6 levels induced by 6-OHDA was significantly attenuated by administration of chrysin ($p < 0.001$; **Fig. 3C**).

Statistical analysis of IL-2 levels in the striatum demonstrated a significant effect of 6-OHDA ($F_{1, 24} = 19.52$, $p < 0.001$), nor main effect of chrysin ($F_{1, 24} = 0.05$, $p < 0.85$) and 6-OHDA × chrysin interaction ($F_{1, 24} = 0.03$, $p < 0.97$). Post hoc comparisons revealed that 6-OHDA significantly increased IL-2 levels in the striatum of mice compared to the control group. Chrysin administration did not alter the increase of IL-2 in the striatum of mice caused by 6-OHDA (**Fig. 3D**).

Two-way ANOVA of IFNγ levels in the striatum demonstrated a significant effect of 6-OHDA ($F_{1, 24} = 30.52$, $p < 0.001$), chrysin ($F_{1, 24} = 11.25$, $p < 0.005$) and 6-OHDA × chrysin interaction ($F_{1, 24} = 11.66$, $p < 0.005$). Post hoc comparisons revealed that 6-OHDA significantly increased IFNγ levels in the striatum of mice compared to the control group. Chrysin treatment significantly normalized the IFNγ levels, but not at control levels ($p < 0.05$; **Fig. 3E**).

Statistical analysis of IL-10 levels in the striatum demonstrated a significant effect of 6-OHDA ($F_{1, 24} = 4.65$, $p < 0.001$), nor main effect of chrysin ($F_{1, 24} = 2.83$, $p < 0.11$) and 6-OHDA × chrysin interaction ($F_{1, 24} = 0.86$, $p < 0.37$). Post hoc comparisons revealed that the increase of IL-10 levels induced by 6-OHDA was significantly prevented by administration of chrysin ($p < 0.05$; **Fig. 3F**).

3.3. Effect of chrysin and 6-OHDA injection on neurotrophic levels

Two-way ANOVA of S100B levels in the striatum demonstrated a significant effect of 6-OHDA ($F_{1, 24} = 4.83$, $p < 0.04$), but not a main effect of chrysin ($F_{1, 24} = 3.79$, $p < 0.07$) and 6-OHDA × chrysin interaction ($F_{1, 24} = 0.89$, $p < 0.36$). Post hoc comparisons revealed that chrysin treatment attenuated the increase of S100B levels caused by 6-OHDA in the striatum of mice ($p < 0.05$; **Fig. 4A**).

Statistical analysis of BDNF levels in the striatum demonstrated a significant effect of 6-OHDA ($F_{1, 24} = 21.11$, $p < 0.001$), chrysin ($F_{1, 24} = 7.37$, $p < 0.01$) and 6-OHDA × chrysin interaction ($F_{1, 24} = 4.69$, $p < 0.04$). Post hoc comparisons revealed that the 6-OHDA-induced decrease of BDNF levels compared to control group ($p < 0.01$). In addition, BDNF levels in sham/chrysin mice were significantly increased compared to that of control group. Post hoc comparisons showed that chrysin administration attenuated the loss of BDNF levels induced by 6-OHDA ($p < 0.05$; **Fig. 4B**)

Two-way ANOVA of GDNF levels in the striatum demonstrated a significant effect of 6-OHDA ($F_{1, 24} = 23.24$, $p < 0.001$), chrysin ($F_{1, 24} = 4.55$, $p < 0.04$), but not 6-OHDA × chrysin interaction ($F_{1, 24} = 4.69$, $p < 0.04$). Post hoc comparisons revealed that 6-OHDA significantly decreased GDNF levels in the striatum of mice compared to the control group. Chrysin treatment partially restored the decrease of GDNF levels caused by 6-OHDA ($p < 0.05$; **Fig. 4C**).

Statistical analysis of NGF levels in striatum demonstrated a significant effect of 6-OHDA ($F_{1, 24} = 8.14$, $p < 0.01$), chrysin ($F_{1, 24} = 10.13$, $p < 0.007$) and 6-OHDA × chrysin interaction ($F_{1, 24} = 15.38$, $p < 0.002$). Post hoc comparisons showed that the decreased NGF levels observed in the striatum of 6-OHDA-treated mice was also normalized by chrysin treatment ($p < 0.001$; **Fig. 4D**).

3.4. Effect of chrysin and 6-OHDA injection on DA, DOPAC and HVA levels

Two-way ANOVA of DA levels in the striatum demonstrated a significant effect of 6-OHDA ($F_{1, 24} = 44.45$, $p < 0.001$), chrysin ($F_{1, 24} = 4.10$, $p < 0.05$) and 6-OHDA × chrysin interaction ($F_{1, 24} = 4.87$, $p < 0.04$).

Statistical analysis of DOPAC levels in the striatum revealed a significant effect of 6-OHDA ($F_{1, 24} = 31.36$, $p < 0.001$), and 6-OHDA × chrysin interaction ($F_{1, 24} = 4.12$, $p < 0.05$), nor main effect of chrysin ($F_{1, 24} = 2.18$, $p < 0.16$).

Two-way ANOVA of HVA levels in the striatum demonstrated a significant effect of 6-OHDA ($F_{1, 24} = 27.13$, $p < 0.001$), but not a main effect of chrysin ($F_{1, 24} = 3.87$, $p < 0.07$) and 6-OHDA × chrysin interaction ($F_{1, 24} = 3.45$, $p < 0.08$).

Post hoc comparisons revealed that 6-OHDA significantly decreased DA, DOPAC and HVA levels in the striatum of mice compared to the control group. Chrysin treatment attenuated the decrease of DA, DOPAC and HVA levels caused by 6-OHDA, but not at control levels ($p < 0.05$; **Fig. 5A-C**).

3.5. Effect of chrysin and 6-OHDA injection on TH$^+$ neurons

Statistical analysis of TH$^+$ neurons in the striatum demonstrated a significant effect of 6-OHDA ($F_{1, 24} = 23.70$, $p < 0.01$), chrysin ($F_{1, 24} = 4.26$, $p < 0.04$) and 6-OHDA × chrysin interaction ($F_{1, 24} = 8.92$, $p < 0.008$). Post hoc comparisons revealed that 6-OHDA significantly decreased TH$^+$ neurons levels in the striatum of mice compared to the control group. Chrysin treatment partially restored the decrease of TH$^+$ neurons caused by 6-OHDA (**Fig. 6 and 7**).

4. Discussion

The purpose of this study was to investigate the chrysin treatment (10 mg/kg, orally*, per day*) on a chronic experimental model of PD with a moderate level of neurodegeneration and to explore possible mechanisms of

neuroprotection. We found that chrysin treatment protected the morphological integrity of the nigrostriatal neurons and the amount of striatal TH^+ neurons, DA, DOPAC and HVA levels were significantly less impaired than those found in the control group. The neuroprotective activity of chrysin was confirmed by behavioral improvement with performing better in the rotarod and dyskinesia tests. Treatment with chrysin showed to raise the levels of endogenous S100B, BDNF, GDNF and NGF in the striatum of mice. We also found that chrysin reduced the level of striatal interleukins possibly due to 6-OHDA-induced neuroinflammation. We concluded that flavonoid chrysin protect against behavioral alterations with anti-inflammatory and neurotrophic activities in the nigrostriatal neurons in the PD mouse model induced by 6-OHDA. These findings supported the notion that chrysin could be a promising candidate for antiparkinsonian action.

Locomotor dysfunction is a kind of clinical symptom of PD [25]. In the behavioural analysis, animals treated with chrysin exhibited better muscular coordination in the rotarod test. We also demonstrated that chrysin treatment attenuated apomorphine-induced rotations (dyskinesia) in mice. In this way, behavioral impairments observed in PD correlate with nigrostriatal neurodegeneration [26,27] and the benefical effect of chrysin on TH^+ neurons in the striatum corroborated with the achieved results on motor test. Together with the literature, the current study supported and extended the notion that neurotrophic and inflammatory responses [11,12,15] can be an important

neurobiological mechanism of chrysin on 6-OHDA-induced behavioral impairment.

Inflammation in the brain has increasingly been recognized to play an important role in the pathogenesis of several neurodegenerative disorders, including PD [28,29]. Cytokines are small proteins that correlated with inflammatory processes and the regulation of the immune system in PD [30,31]. Several reports show that oxidative stress activates microglial cells upregulating the production of pro-inflammatory cytokines (TNF-α, IFN-γ, IL-1β, IL-2 and IL-6), leading to degeneration of dopaminergic neurons [32]. Glial cells released deleterious compounds such as proinflammatory cytokines (TNF-α, IFN-γ, IL-1β) with deleterious effect on dopaminergic neurons by activating receptors that contain intracytoplasmic death domains involved in apoptosis [33[. Studies indicated that serum concentrations of TNF-α, IL-2 and IL-6 were elevated in PD patients [34]. Another study reported that the concentrations of IFN-γ were elevated in PD patients blood plasma, and IFN-γ-deficient mice displayed attenuated dopaminergic cell loss in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced PD mouse model [35]. Meanwhile, anti-inflammatory cytokines, such as IL-10, have also been reported in the central nervous system or serum of PD patients, which is believed to be from activated microglia as well [36,37]. Chrysin has been shown to have anti-inflammatory activity and inhibited chronic stress-induced inflammatory response [11,12]. Our

study showed increase of pro-inflammatory cytokines levels, including TNF-α, IFN-γ, IL-1β, IL-2 and IL-6 and decrease of anti-inflammatory cytokine such as IL-10 levels in striatum after 6-OHDA microinjection. In addition, our results demonstrated that chrysin treatment during 28 days reversed alterations in cytokines in striatum. Accordingly, the present study expanded the anti-inflammatory role of chrysin in the brain, evidencing its involvement in this model of neurodegeneration. Thus, we suggested that chrysin-induced modulation of immune response is an important neurobiological mechanism against 6-OHDA-induced behavioral impairment in PD model.

Neurotrophic factors have been recognized as a key regulator of synaptic development and plasticity, and have therefore attracted keen interest in relation to neurodegenerative disorders such as PD [38,39]. Several neurotrophic factors in the brain have been recognized as supporting the survival of adult neurons and as playing a role in neuroprotection in PD models [40]. BDNF, GDNF a NGF prevents neuronal degeneration of the lesioned nigroestriatal system *in vivo* [41,42] and improves motor deficits [43]. S100B may be a biomarker neurodegenerative disease, incluinding PD [44]. Moreover, S100B via receptor for advanced glycation end products (RAGE) activates overproduction of reactive oxygen species (ROS) and inflammatory cytokines, triggering damaging and neurodegeneration [45]. These changes occurred after treatment with 6-OHDA, suggesting that neurotrophins imbalance are the trigger for activation of other

downstream molecules involved in the pathogenesis of PD. In support of this idea, chrysin treatment acted in recovery of S100B, BDNF, GDNF and NGF after 6-OHDA in striatum, like this, could act in PD by increasing survival of DA neurons, since, decrease neuronal is the main characteristic of this disease [46]. These findings suggested that neurotrophins deficit becomes more critical to dopaminergic dynamics and related behavioral activities, evidenced in this study with the neuroprotective effect of chrysin.

PD is characterized by the loss of dopaminergic neurons with depletion of DA and metabolites in the striatum [8]. To assess the potential protective effect of chrysin on 6-OHDA-inducing alteration in nigrostriatal vulnerability, we analyzed TH immunoreactivity and DA levels. TH is the rate-limiting enzyme responsible for conversion of L-DOPA to DA [47]. The measurement of TH-immunoreactivity is thus a measure of the functionality of dopaminergic neurons and fibres present in the striatum [47]. Of the particular importance, chrysin treatment prevented the reduction of TH-stained cells and the loss DA and metabolites in the striatum. It is believed that the main mechanism of dopaminergic neuronal loss in these animals is oxidative stress and inflammation generated in response to 6-OHDA exposure [48]. Chrysin treatment protected the dopaminergic neurons in the striatum of mice after 6-OHDA injection and this protection may be correlated to antioxidant and anti-inflammatory activities of this compound [11,12]. Moreover, the neuroprotective action of chrysin in PD model

could be due to the neuronal development and survival and requires neurotrophic support, mainly BDNF and NGF [15,49]. Our findings suggested that chrysin have anti-inflammatory and neurotrophic potential, demonstrated in dopaminergic cell survival.

In summary, chrysin have neuroprotective activity against the 6-OHDA-induced parkinsonian mouse model, indicating that may represent a new therapeutic tool for PD. This neuroprotective effect are attributed to their strong antioxidant potential and inhibitory role on different key events involved in neuroinflammation and reserving neurotrophic factor levels, as well as regulating TH^+ neurons in striatum, which consequently retained neurotransmitters such as DA, DOPAC and HVA and behavioral changes.

Conflict of interest

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

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Legends

Fig. 1. Schematic representation of the experimental protocol describing the treatment periods with chrysin (10 mg/kg, *p.o*), behavior tests and neurochemistry analyzes.

Fig. 2. (A) Effect of chrysin treatment on the rotarod test and **(B)** on the number rotations induced by apomorphine. Values are mean ± S.E.M. (n=6 per group). *: $p<0.05$, ***: $p<0.001$ when compared with SHAM/control; #: $p<0.05$ when compared with 6-OHDA/control (Two-way ANOVA and Newman–Keuls multiple comparison test).

Fig. 3. Effects of chrysin treatment during twenty-eight days after 6-OHDA microinjection in IL-1β **(A)**, TNFα **(B)**, IL-6 **(C),** IL-2 **(D),** lNFγ **(E)** and IL-10 **(F)** levels in striatum of mice. Values are mean ± S.E.M. (n=6 per group). *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$ when compared with SHAM/control; #: $p<0.05$ when

compared with 6-OHDA/control (Two-way ANOVA and Newman–Keuls multiple comparison test).

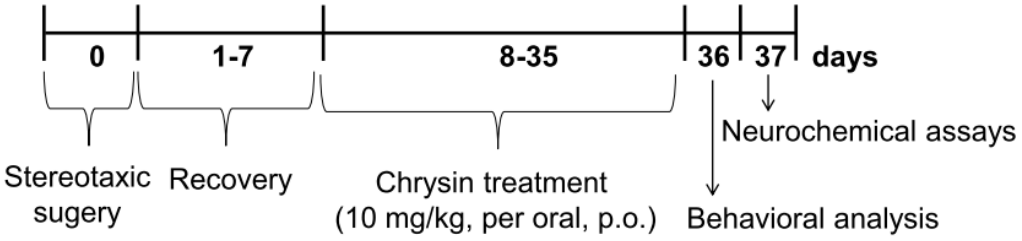
Fig. 4. Effects of crhsyin treatment during twenty-eight days after 6-OHDA microinjection in S100B **(A)**, BDNF **(B)**, GDNF **(C)** and NGF **(D)** levels in the striatum. Values are mean ± S.E.M. (n=6 per group). *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$ when compared with SHAM/control; #: $p<0.05$ when compared 6- with 6-OHDA/control (Two-way ANOVA and Newman–Keuls multiple comparison test).

Fig. 5. Effects of crhsyin treatment during twenty-eight days after 6-OHDA microinjection in DA **(A)**, DOPAC **(B)** and HVA **(C)** levels in the striatum. Values are mean ± S.E.M. (n=6 per group). *: $p<0.05$, ***: $p<0.001$ when compared with SHAM/control; #: $p<0.05$ when compared with 6-OHDA/control (Two-way ANOVA and Newman–Keuls multiple comparison test).

Fig. 6. Photomicrographs showing the optical disector probe application, where could be observed TH^+ neurons, from substantia nigra pars compacta about effect of chrysin treatment (10 mg/kg, p.o.) and surgery injection of 6-OHDA in different scale bar: 5x=100µm, 20x=25 µm and 40x=5 µm.

Fig. 7. Effects of chrysin treatment during four weeks in quantification of TH^+ neurons in striatum after 6-OHDA injection. *: $p<0.05$, **: $p<0.01$ when compared with SHAM/control; #: $p<0.05$ when compared with 6-OHDA/control (Two-way ANOVA and Newman–Keuls multiple comparison test).

Figure 1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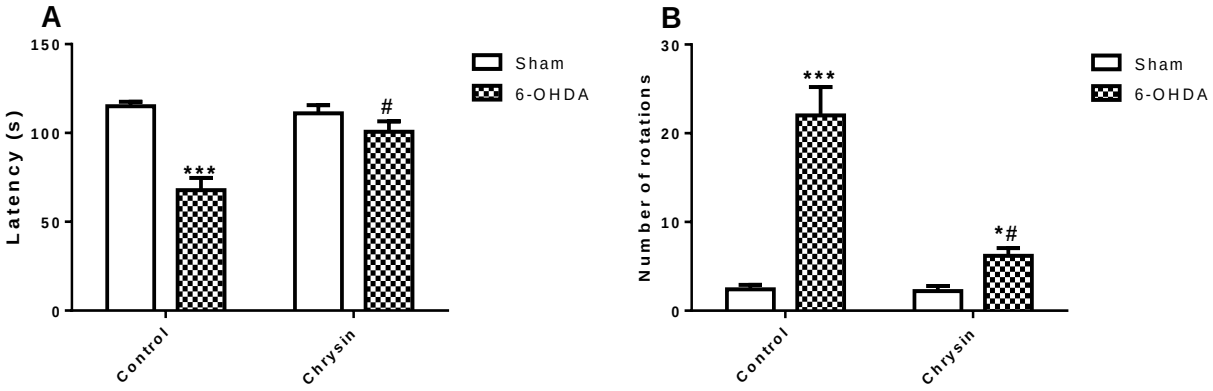
1-7
8-35
36
37
days
Neurochemical assays
Stereotaxic sugery
Recovery
Chrysin treatment (10 mg/kg, per oral, p.o.)
Behavioral analysis

Figure 2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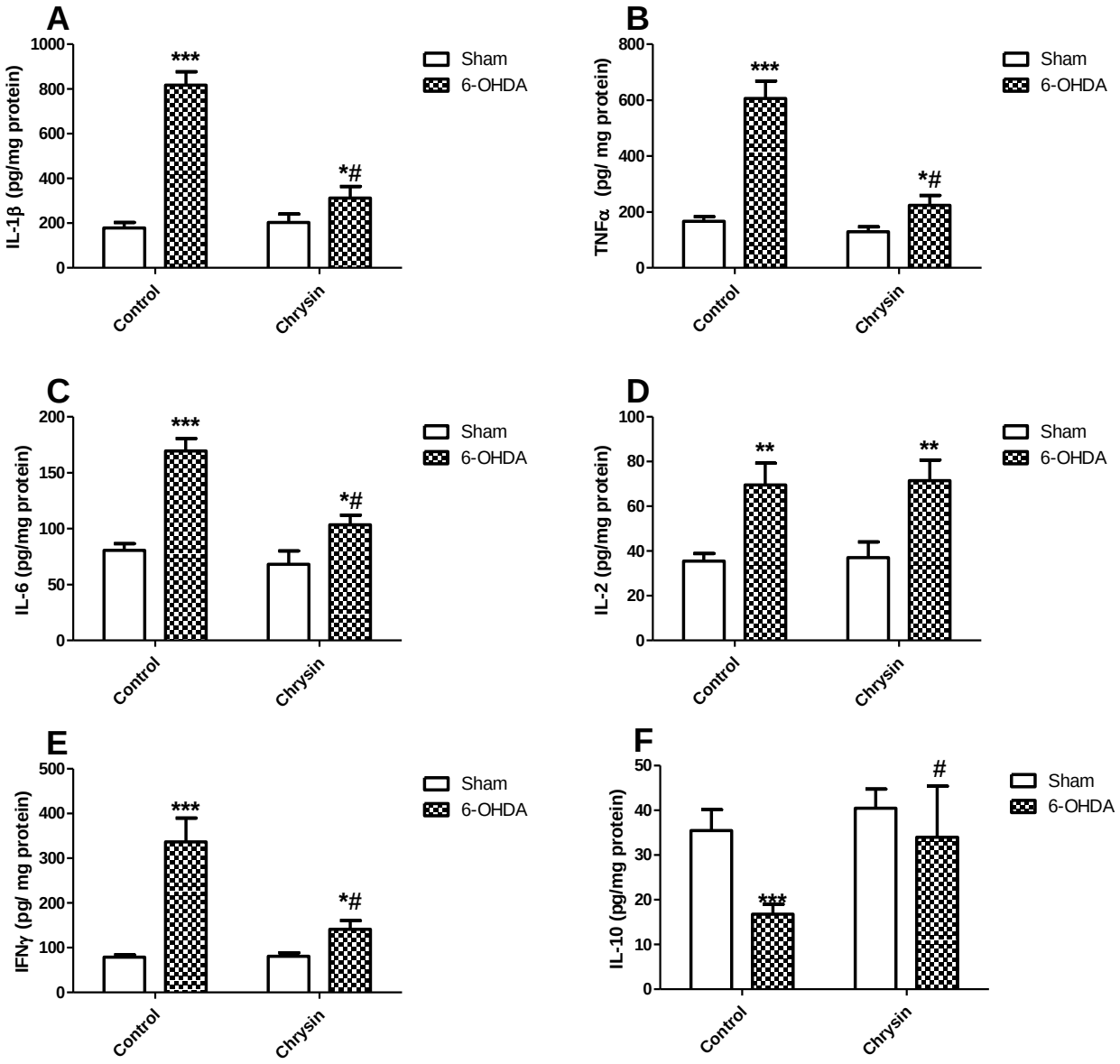
100
50
0
***
#
Control
Chrysin
Sham
6-OHDA
B
Number of rotations
30
20
10
0
***
*#
Control
Chrysin
Sham
6-OHDA

Figure 3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200
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***
*#
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Chrysin
Sham
6-OHDA
B
TNFα (pg/ mg protein)
800
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400
200
0
***
*#
Control
Chrysin
Sham
6-OHDA
C
IL-6 (pg/mg protein)
200
150
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6-OHDA
D
IL-2 (pg/mg protein)
100
80
60
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Control
Chrysin
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E
IFNγ (pg/ mg protein)
500
400
300
200
100
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*#
Control
Chrysin
Sham
6-OHDA
F
IL-10 (pg/mg protein)
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40
30
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10
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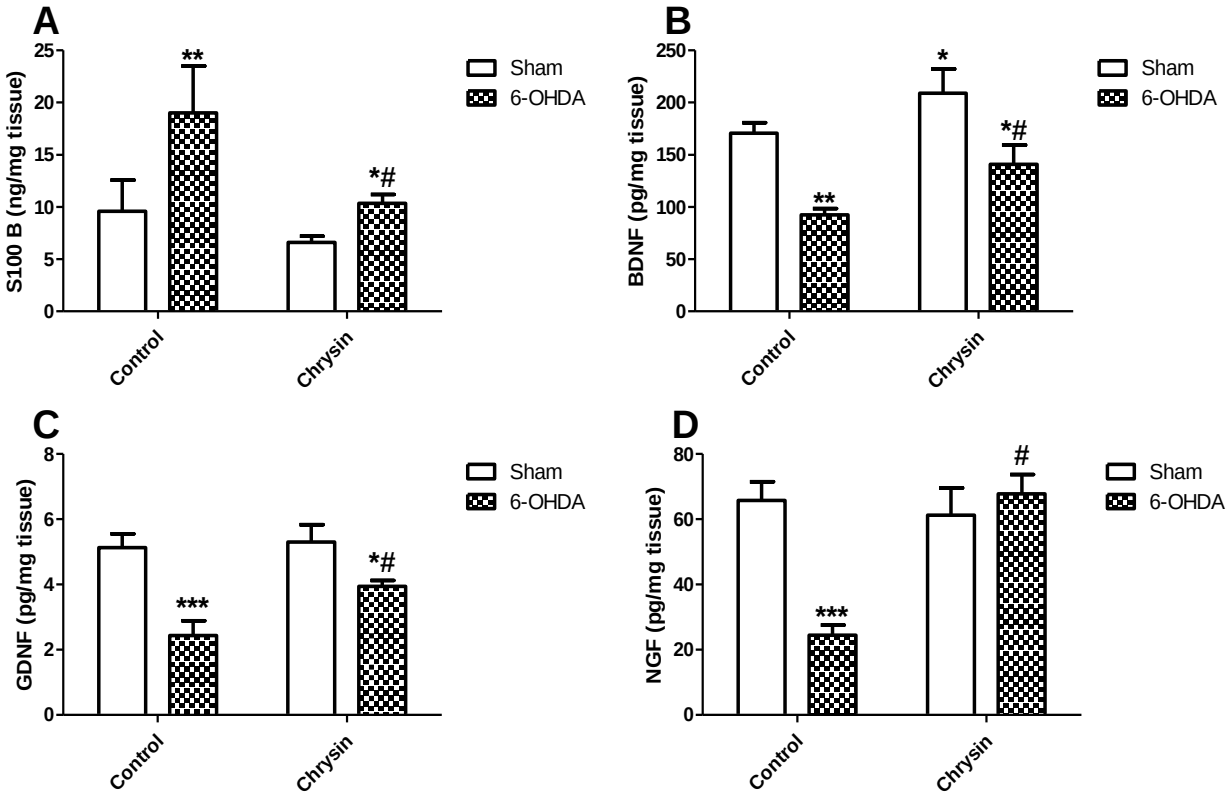
***
#
Control
Chrysin
Sham
6-OHDA

Figure 4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S100 B (ng/mg tissue)
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20
15
10
5
0
**
*#
Control
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Sham
6-OHDA
B
BDNF (pg/mg tissue)
250
200
150
100
50
0
*
**
*#
Control
Chrysin
Sham
6-OHDA
C
GDNF (pg/mg tissue)
8
6
4
2
0
***
*#
Control
Chrysin
Sham
6-OHDA
D
NGF (pg/mg tissue)
80
60
40
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#
Control
Chrysin
Sham
6-OHDA

Figure 5

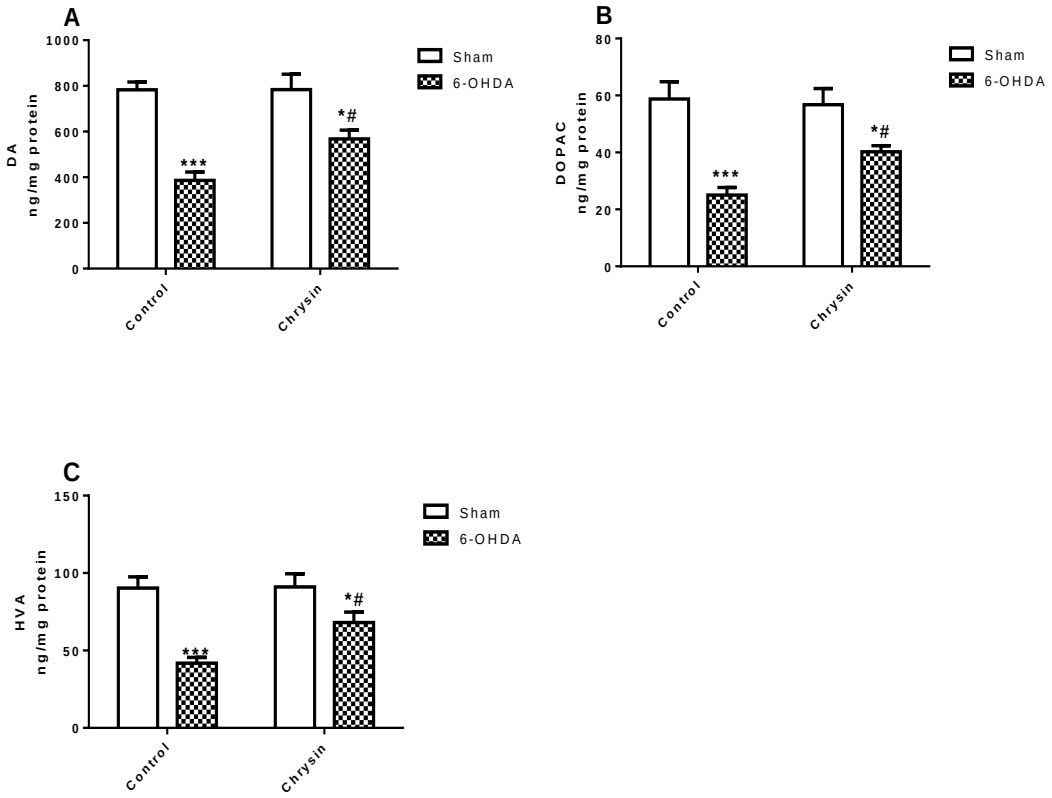

A
DA
ng/mg protein
1000
800
600
400
200
0
***
*#
Control
Chrysin
Sham
6-OHDA
B
DOPAC
ng/mg protein
80
60
40
20
0
***
*#
Control
Chrysin
Sham
6-OHDA
C
HVA
ng/mg protein
150
100
50
0
***
*#
Control
Chrysin
Sham
6-OHDA

Figure 6

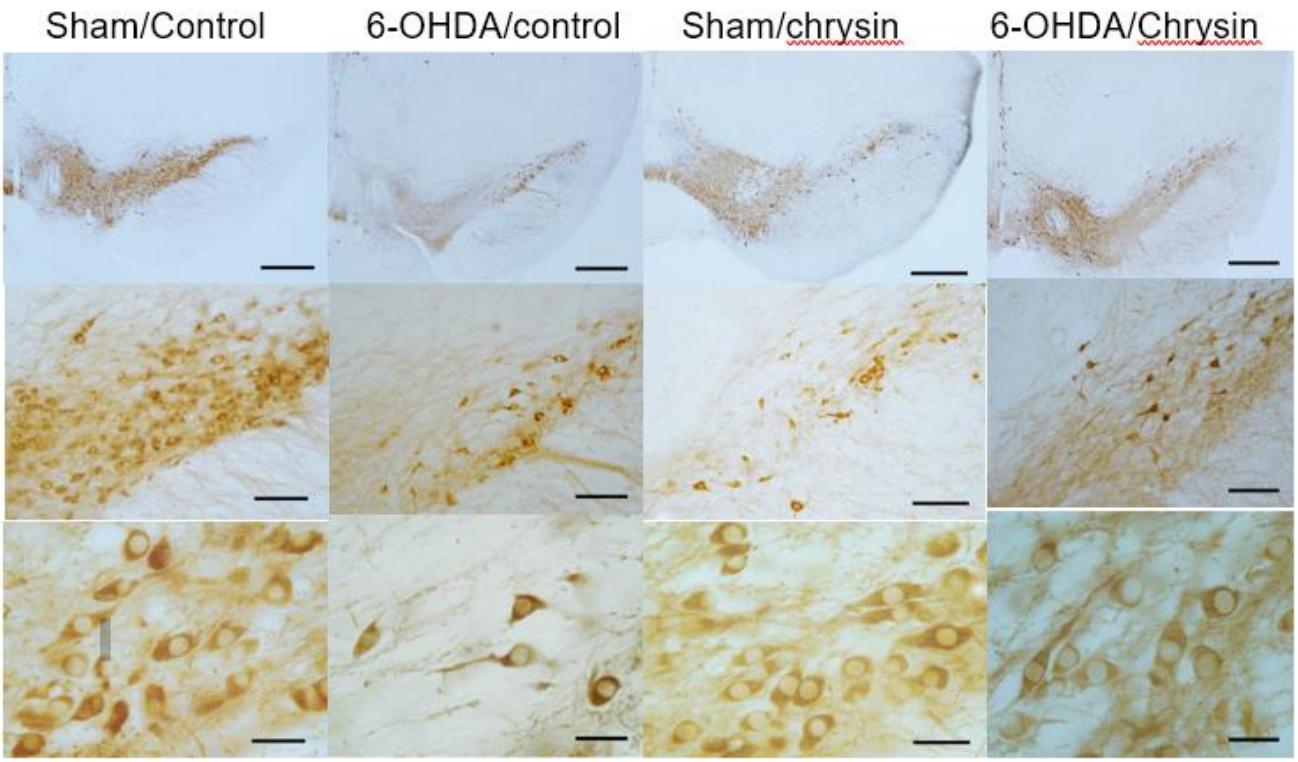
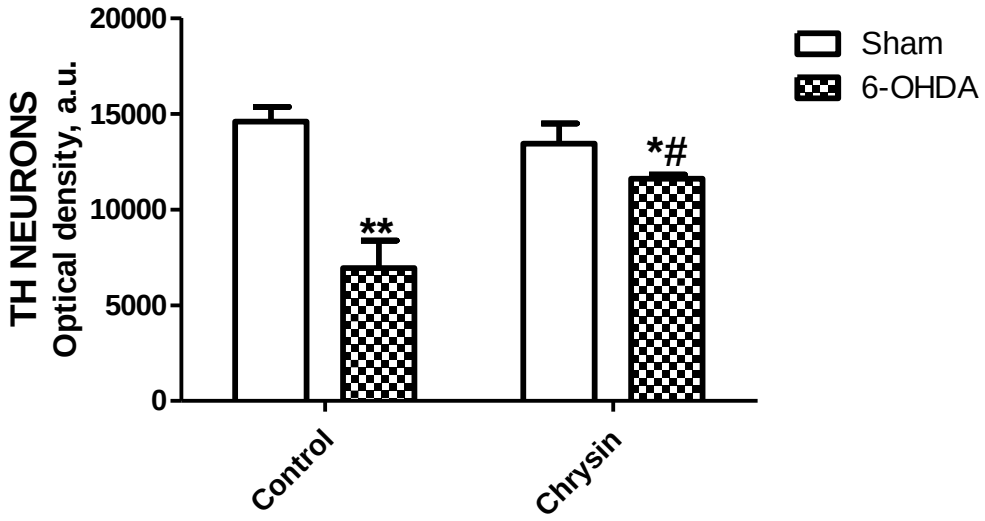


Figure 7


TH NEURONS
Optical density, a.u.
20000
15000
10000
5000
0
**
*#
Control
Chrysin
Sham
6-OHDA

MANUSCRITO

Neuroprotective effects of chrysin on oxidative stress parameters and depressive-like behavior in 6-hydroxydopamine-induced a mouse model of Parkinson's disease

André T. Rossito Goes, Cristiano R. Jesse, Leandro Cattelan Souza, Michelle S. Antunes, Fernando Vagner Lobo Ladd, Aliny Antunes Barbosa Lobo Ladd, Silvana Peterini Boeira

Submetido à Nutritional Neuroscience

Neuroprotective effects of chrysin on oxidative stress parameters and depressive-like behavior in 6-hydroxydopamine-induced a mouse model of Parkinson's disease

André T. Rossito Goes[1], Cristiano R. Jesse[1]*, Leandro Cattelan Souza[1], Michelle S. Antunes[1], Fernando Vagner Lobo Ladd[2], Aliny Antunes Barbosa Lobo Ladd[3], Silvana Peterini Boeira[1]

[1]Laboratory of Pharmacological and Toxicological Evaluations Applied to Bioactive Molecules, Federal University of Pampa, Itaqui, CEP 97650-000, RS, Brazil.

[2]Department of Morphology/Laboratory of Neuroanatom, Biosciences Center, Federal University of Rio Grande do Norte, Natal-RN, Brazil.

[3]Laboratory of Stochastic Stereology and Chemical Anatomy, Department of Surgery, College of Veterinary Medicine and Animal Science, University of São Paulo, Brazil.

*Correspondence should be sent to:

Cristiano R. Jesse

Phone and FAX number: +55-55-34321853

E-mail: cristianoricardojesse@yahoo.com.br

Abstract

Objetives: Parkinson's disease (PD) may be due to the interaction of a number of factors, including genetic influence, toxins, oxidative stress, mitochondrial abnormalities and aging. Studies have shown that consumption of a diet rich in antioxidants may reduce the incidence of neurodegenerative diseases. This study was designed to investigate the potential neuroprotective effect of chrysin in a mouse model of PD induced by 6-hidroxidopamine (6-OHDA).

Methods: The enzymatic activities of catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPx), and glutathione S-transferase (GST), the levels of glutathione (GSH), reactive species (RS), dopamine (DA) and its levels of metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), was analyzed in the striatum of mice. The behavioral parameters (depressive-like, locomotor and the degree of forepaw asymmetry) were also measured.

Results: The present study demonstrated that treatment with chrysin (10 mg/kg, per oral during 28 days) was effective in attenuating the following impairments resulting from 6-OHDA exposure: (1) depressive-like behavior in the tail suspension test; (2) increase of the RS levels; (3) decrease of the GSH, DA, DOPAC and HVA levels; (4) inhibition of GPx activity, (5) rises in GST and GR activities and (7) decreased tyrosine hydroxylase positive (TH^+) neurons from substantia nigra pars compacta. *Discussion:* We suggest that treatment with chrysin attenuates oxidative stress in striatum of mice and the depressive-like behavior induced by 6-OHDA injection, supporting the hypothesis that chrysin can be used as a new pharmacological tool to reduce the symptoms of PD.

Key words: chrysin, depression, neurogenerative disease, flavonoid, dopamine

Introduction

Parkinson's disease (PD) is characterized by progressive degeneration of dopaminergic neurons in the nigrostriatal system and dopamine (DA) depletion in the striatum. While the pathogenesis of PD is not clear, damage of dopaminergic neurons by oxygen-derived free radicals is considered to be an important contributing mechanism[1]. PD is most often considered a disorder of movement. Although most of the typical motor impairments are due to the loss of nigrostriatal dopaminergic neurons, PD affects multiple neuronal systems both centrally and peripherally, leading to a constellation of non-motor symptoms including olfactory deficits, affective disorders such as depression, as well as autonomic and digestive dysfunction[2,3].

The administration of 6-hydroxydopamine (6-OHDA) into the striatum of mice produces a well-established model of PD. The toxicity of 6-OHDA is thought to be related to its ability to produce free radicals and to cause oxidative stress and cell death[4,5]. 6-OHDA is susceptible to autooxidation, resulting in the formation of 6-OHDA quinine and hydrogen peroxide (H_2O_2), superoxide radical ($\cdot O^{2-}$), and hydroxyl radical ($\cdot OH$)[1]. These active oxygen forms are neurotoxic because of their strong oxidizing potential[1]. The unilateral, intrastriatal injection of 6-OHDA induces pronounced behavioural alterations as well as biochemical and neurochemical deficits similar to PD[4,5,6,7].

The glutathione (GSH) system, which is responsible for removing free radicals and maintaining protein thiols in their appropriate redox state, is an important protective mechanism for minimizing oxidative stress[8]. Moreover, antioxidant enzymes, such as glutathione reductase (GR), glutathione-

peroxidase (GPx), glutathione-s-transferse (GST) and catalase (CAT), are also important mediators in the reduction of oxidative stress[9].

A variety of antioxidant compounds, such as flavonoids derived from natural products, have demonstrated neuroprotective activity in either *in vitro* or *in vivo* models of PD[10,11,12]. These polyphenols are found in fruit, vegetables and plant-derived beverages and may have important roles as dietary components via cytoprotective actions in many organs[13]. The flavonoid chrysin (5,7-dihydroxylflavone) is a natural product extracted from plants, honey, and propolis[14]. Researchers have shown that chrysin has a wide range of biological and pharmacological properties, such as anti-inflammation, anti-oxidation and protective effects[14]. Thus antioxidants may provide protection against the oxidative damage occurring in PD, and the use of anti-oxidant therapies in the management of PD is limited[13].

According to the evidence presented, we sought to investigate the effects of chrysin treatment (10 mg/kg, *per oral*, p.o.) over 28 days in an *in vivo* mouse model of PD induced by the injection of 6-OHDA. We investigated the protective effect of chrysin on behavior alterations (depressive-like, locomotor and the degree of forepaw asymmetry), modifications in antioxidant enzymes (CAT, GST, GR and GPx), as well as levels of reactive species (RS) and GSH, in the striatum of mice. In addition, we evaluated the protective effects of chrysin against neurochemical alterations of DA and its levels of metabolites (3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA)) and of tyrosine hydroxylase positive (TH^+) neurons induced by the striatal injection of 6-OHDA in the mice.

Materials and Methods

Animals

Experiments were performed using male C57B/6J mice (20-30g, 90 days old). Animals were maintained at 22-25ºC with free access to water and food, under a 12:12h light/dark cycle, with lights on at 7:00 a.m. All manipulations were carried out during light phase on the day. All efforts were made to minimize animal suffering and to reduce the number of animals used. The procedures of this study were conducted according to the guidelines of the Committee on Care and Use of Experimental Animals Resources and with the approval of Ethical Committee for Animal Use (CEUA protocol # 038/2012).

Experimental design

Mice were randomly assigned to four groups (n=10 per group): (1) Vehicle/ Vehicle; (2) Vehicle/Chrysin; (3) 6-OHDA/Vehicle and (4) 6-OHDA/ Chrysin. The mice were subjected to stereotaxic surgical injections of 6-OHDA or vehicle. Seven days after the injections, treatment with chrysin (10 mg/kg, per oral, p.o.) was initiated for 28 days, and the after behavioral testing, mice were euthanized and the striatum was removed for biochemical assays (**Figure 1**).

Stereotaxic surgical injection of 6-OHDA

Surgery was performed under anesthesia with 10 mL/kg of 1% ketamine (Bela-Pharm, Vechta, Germany) and 0.2% xylazine (Bayer HealthCare, Leverkusen, Germany). 6-OHDA (Sigma; 5 µg in 2 µL of 0.9% NaCl with 0.2 µg/lL ascorbic acid) was injected slowly (0.5 µL/min) into the right striatum (0.9 mm anterior and 1.8 mm lateral from bregma, 3.0 mm ventral from the dura). After the

injection, the syringe was kept for additional 3 min in the brain, before it was slowly retracted. Controls were vehicle-injected (sham-OP)[15].

Behavioral assessment

Open field test (OFT)

To verify the effects of treatments on the locomotor activity, the animals were submitted individually to a 5 min OFT (Insight model EP 154C) 24 hr after pretreatment. The parameters observed included the distance (unit: mm)[16].

Tail suspension test (TST)

Antidepressant-like effects were measured using the tail suspension test[17], with minor alterations[18]. Each mouse was suspended by its tail using adhesive tape, placed approximately 1 cm from the tip of the tail, and hung approximately 30 cm above a table. The animals were suspended for a period of 6 min, and the duration of immobility was scored manually during the last 4 min interval of the test (activity in the first 2 min was discarded because animals predominantly try to escape during this period). Mice were considered immobile only when they hung passively.

Cylinder test

We performed the cylinder test for behavioral evaluation at 1, 2, 3 and 4 weeks after 6-OHDA injections. The cylinder test was used to assess the degree of forepaw asymmetry. Mice were placed in a transparent cylinder (diameter: 20 cm, height: 30 cm) for 3 min, with the number of forepaw contacts to the cylinder wall counted. The score of cylinder test in this study was calculated as a contralateral bias: [(the number of contacts with the contralateral limb)−(the

number of contacts with the ipsilateral limb)/ (the number of total contacts)×100]

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Tissue preparation

After behavioral tests, mice were euthanized with a barbiturate overdose (pentobarbital sodium 150 mg/kg; i.p.). The striatum was removed and rapidly homogenized in 50 mM Tris-Cl, pH 7.4. The homogenate was centrifuged at 2,400×g for 15 min at 4 °C, and a low-speed supernatant fraction (S_1) was used for assays.

Biochemical Determinations

GSH levels

GSH Content was determined fluorometrically using ortho-phthalaldehyde (OPA) as the fluorophore[20]. S^1 (100 µl) was incubated with 100 µl of OPA (0.1 % in methanol) and 1.8 ml of 0.1 M phosphate buffer (pH 8.0) for 15 min at room temperature in the dark. Fluorescence was measured with a fluorescence spectrophotometer at the excitation wavelength of 350 nm and at the emission wavelength of 420 nm. GSH levels were expressed as nmol/g of tissue.

RS levels

To determine RS levels, S_1 (fresh preparation) was diluted (1:10) in 50 mM Tris-Hcl (pH 7.4) and incubated with 10 µl of 2′,7′-dichlorofluorescein diacetate (DCHF-DA; 1 mM) at 37ºC for 30 min. The RS levels were determined by a spectrofluorimetric method using the DCHF-DA assay, as described by Loetchutinat et al.[21]. The DCHF-DA is enzymatically hydrolyzed by intracellular esterases to form nonfluorescent DCFH, which is then rapidly oxidized to form

highly fluorescent 2´,7´-dichlorofluorescein (DCF) in the presence of RS. DCF fluorescence intensity is proportional to the amount of RS that is formed. The DCF fluorescence intensity emission was recorded at 520 nm (with 480 nm excitation) 30 min after the addition of DCHF-DA to the medium. The RS levels were expressed in arbitrary units (AU).

CAT activity

CAT activity in S_1 was assayed spectrophotometrically by the method proposed by Aebi[22], which involves monitoring the disappearance of H_2O_2 in the presence of S_1 at 240 nm. Enzymatic reaction was initiated by adding S_1 and the substrate H_2O_2 (0.3 mM) in a medium containing 50 mM potassium phosphate buffer (pH 7.0). One unit of enzyme was defined as the amount of enzyme required for monitoring the disappearance of H_2O_2. The enzymatic activity was expressed as Units (U)/mg protein (1U decomposes 1 µmol H_2O_2/min at pH 7 at 25 °C).

GR activity

GR activity was determined spectrophotometrically as described by Calberg and Mannervick[23]. In this assay, GSSG is reduced by GR at the expense of NADPH consumption, which was followed at 340 nm. GR activity is proportional to NADPH decay. An aliquot of S_1 was added in the system containing 0.15 M potassium phosphate buffer (pH 7.0), 1.5 mM EDTA, 0.15 Mm NADPH. After the basal reading, the substrate (GSSG 20 mM) was added. The enzymatic activity was expressed as nmol NADPH/min/mg protein.

GPx activity

GPx activity in S_1 was assayed spectrophotometrically by the method described by Wendel[24] through the GSH/NADPH/glutathione reductase system, by the dismutation of H_2O_2 at 340 nm. S_1 was added to the medium containing the GSH/NADPH/glutathione reductase system and the enzymatic reaction was initiated by adding H_2O_2 (4 mM). In this assay, the enzyme activity was indirectly measured by means of NADPH decay. H_2O_2 is reduced and generates GSSG from GSH. GSSG is regenerated back to GSH by the glutathione reductase that is present in the assay media, at the expense of NADPH. The enzymatic activity was expressed as nmol NADPH/min/mg protein.

GST activity

GST activity was assayed through the conjugation of GSH with CDNB at 340 nm, as described by Habig and Jakoby[25]. An aliquot of S_1 was added to a medium containing 0.1 M potassium phosphate buffer (pH 7.4). Then, 100 mM CDNB and GSH were added to the medium. CDNB was used as a substrate. The enzymatic activity was expressed as nmol CDNB conjugated/min/mg protein.

DA, DOPAC and HVA levels

Striatal tissues were homogenized with 300 µL of 200 mM ice-cold perchloric acid containing 10 mM disodium EDTA. After centrifugation (10,000 g for 10 min at 4 ºC), the supernatant was filtered and then injected directly into an HPLC system (Shimadzu; Kyoto, Japan) with an electrochemical detector (ECD; Eicom, Kyoto, Japan). The appendant potential of the ECD (carbon electrode vs. Ag/AgCl reference electrode) was set at 700 mV. The analytic column was a TSKgel Super-ODS (4.6 mm I.D. 9 100 mm; Tosoh, Tokyo, Japan), and the mobile phase consisted of 0.1 M citrate-sodium acetate buffer (pH 3.9) containing

methanol (18%, v/v), disodium EDTA (4 mg/l), and sodium octanesulfonate (0.8 mM)[26].

Stereological procedures

Design-based stereology was performed for cell counts and volume measurements of TH-positive neurons in substantia nigra pars compacta (SNpc), using a Leicamicroscopy (DMR 6000) coupled with stereological system newCast (Visiopharm version 4.5.6.857), and a video-camera Olympus DP72. The regions of interest (e.g. Substantia nigra pars compacta SNpc) were delineated according to Franklin e Paxinos, [27] and Oorchot, [28] by anatomical landmarks (Bregma−4.70 to −6.30 mm) with a 2,5× magnification on live microscopic video images displayed on a monitor.

The SNpc TH-positive neurons were counted by the optical fractionator design[29,28,30,31]. Briefly, the prefixed brains were embedded in 8% agar solution and cut exhaustively in coronal sections by a vibrotome (Leica VT1000S) in 40-µm-thick sections, cover all region of interest, along the frontal-caudal axis. Every fifth serial section containing SNpc was selected, and then we obtained the section sampling fraction (ssf=1/5). In this sample sections were applied free-floating immunohistochemical methods to identify the dopaminergic neurons in SNpc. So, the sections were washed with PBS, incubated with a 0.3% Triton X-100 solution, exposed to 0.3% hydrogen peroxide in distilled water to block endogenous peroxidases, placed in a 10% non-immune normal goat serum (Jackson Immuno Research Labs), incubated with a primary antibody (rabbit anti-Tirosinehidroxilase, 1:1000, Abcam) and with a secondary antibody (anti-rabbit IgG peroxidase conjugate, 1:200, KPL) in PBS. Immunoreactivity was visualized

with 3,3- diaminobenzidine in PBS containing 0.01% hydrogen peroxide, and then the sections were mounted in a glass slide and cover with a coverslip.

In each sampled section some unbiased counting frames were created by the software and randomly placed over the region of interest. The counting frames were replaced systematically by stepwise movements in x- and y-directions.The area of the unbiased counting frame (a (frame) = 6400 μm^2) relative to the area associated with the x and y steps (step length = 70000 µm) gives the area sampling fraction (asf=1/11). The optical disector height, along the z-axis, was determined by excluding a up guard region and a bottom guard region. The height of the optical disector relative to the actual thickness of the section results in the height sampling fraction (hsf=20). Only counting frames for which at least a part of the frame fell within the delineated contour were used for counting. Cells were marked if they were positive and in focuswithin the counting area. Furthermore, the counting frames have forbidden lines to avoid edge effects.

The estimated total number of positive cells (N) was calculated from the number of TH-neurons counted according to the formula:

NTH-neurons= 1/ssf-1. 1/asf-1. 1/hsf-1. Q-

Where Q- is the TH-positive neurons selected by the optical disector (**Fig. 3**), ssf is the section sampling fraction; asf is the area sampling faction (a (frame) / step length) and hsf is the high sampling fraction.

Protein determination

Protein content was measured colorimetrically according to the method of Bradford[32], and bovine serum albumin (1 mg/ml) was used as the standard.

Statistical analysis

Results were presented as the means ± SD. First, we evaluated the normality of data using the D'Agostino and Pearson omnibus normality test. Comparisons between experimental and control groups were performed by two-way analysis of variance (ANOVA) followed by Newman–Keuls test for post hoc comparison when appropriate. Main effects of first-order interactions are presented only when interaction was not significant. Comparisons between experimental and control groups were performed by one-way (chrysin or 6-OHDA = independent variable) or two-way ANOVAs (chrysin X 6-OHDA = independent variables). A value of $p < 0.05$ was considered to be significant. All tests and plotting graphics were executed using the GraphPad Prism 6 software (San Diego, CA, U.S.A.).

Results

Behavioral assessment

Locomotor activity in the OFT

Two-way ANOVA revealed that total distance in OFT was not changed significantly by 6-OHDA × chrysin interaction ($F_{1.28} = 0.48$; $p = 0.4944$), chrysin ($F_{1.28} = 0.59$; $p = 0.4504$) and 6-OHDA ($F_{1.28} = 0.4$; $p = 0.8493$) (**Fig. 2A**).

Depressive-like behavior in the TST

A two-way ANOVA of depressive-like behavior in the TST revealed a significant 6-OHDA × chrysin interaction ($F_{1.28} = 35.19$; $p = 0.0001$), as well as main effects of chrysin ($F_{1.28} = 56.22$; $p = 0.0001$) and 6-OHDA ($F_{1.28} = 14.06$; $p = 0.0008$). Post hoc comparisons demonstrated that animals in the 6-

OHDA/vehicle group showed an increase in immobility time when compared to that of animals in the vehicle/vehicle group, exhibiting 6-OHDA-induced depressive-like behavior in the TST. Chrysin treatment (10 mg/kg per day, p.o.) protected against the increase in the time spent immobile in the TST caused by 6-OHDA (**Figure 2B**).

Cylinder test

The scores of the cylinder test in the chrysin group were ameliorated over time at 2, 3, and 4 weeks after 6-OHDA injections (contralateral bias: 33±6%, 28±3%, and 17±4%), compared to those in the control group (contralateral bias: 74±7%, 63±5%, and 60±3%) (data not shown).

Biomarkers of oxidative stress and neurochemical alterations

GSH levels

A two-way ANOVA revealed that striatal GSH levels were changed significantly by chrysin ($F_{1.16} = 11.12$; $p = 0.0042$) and 6-OHDA ($F_{1.16} = 24.47$; $p = 0.0001$). Post hoc comparisons demonstrated that 6-OHDA significantly decreased the striatal GSH levels. Oral administration of chrysin (10 mg/kg per day) prevented the inhibition of GSH levels caused by 6-OHDA in striatum of mice (**Table 1**).

RS levels

A two-way ANOVA of striatal RS levels demonstrated a significant 6-OHDA × chrysin interaction ($F_{1.16} = 10.39$; $p = 0.0053$), including main effects of chrysin ($F_{1.16} = 16.24$; $p = 0.0010$) and 6-OHDA ($F_{1.16} = 37.86$; $p = 0.0001$). Post hoc comparisons revealed that 6-OHDA significantly increased striatal RS levels.

Chrysin treatment (10 mg/kg per day) protected against the elevated striatal RS levels induced by injections of 6-OHDA **(Table 1)**.

CAT activity

Two-way ANOVA revealed that CAT activity in striatum of mice was not changed significantly by 6-OHDA × chrysin interaction ($F_{1.32}$ = 0.01; p = 0.9361), chrysin ($F_{1.32}$ = 0.73; p = 0.3992) and 6-OHDA ($F_{1.32}$ = 0.25; p = 0.6175) (**Table 2**).

GST activity

Two-way ANOVA of GST activity in striatum demonstrated a significant 6-OHDA × chrysin interaction ($F_{1.27}$ = 25.98; p = 0.0001) and a main effect of chrysin ($F_{1.27}$ = 40.86; p = 0.0001) and 6-OHDA ($F_{1.27}$ = 31.73; p = 0.0001). Post hoc comparisons revealed that 6-OHDA significantly increased GST activity in striatum of vehicle mice. Chrysin treatment (10 mg/kg per day, p.o.) protected against the increase of GST activity caused by 6-OHDA in striatum of mice (**Table 2**).

GR activity

Two-way ANOVA of GR activity in striatum demonstrated a significant 6-OHDA × chrysin interaction ($F_{1.16}$ = 16.77; p = 0.0008) and a main effect of 6-OHDA ($F_{1.16}$ = 25.56; p = 0.0001). Post hoc comparisons revealed that 6-OHDA significantly increased GR activity in striatum of vehicle mice. Oral treatment with chrysin prevented the increase of GR activity caused by 6-OHDA in striatum of mice (**Table 2**).

GPx activity

Statistical analysis of GPx activity in striatum revealed a significant a main effect chrysin ($F_{1.16}$ = 12.64; p = 0.0026) and of 6-OHDA ($F_{1.16}$ = 11.68; p = 0.0035). Post hoc comparisons demonstrated that 6-OHDA significantly inhibited GPx activity in striatum of vehicle mice. Chrysin treatment (10 mg/kg per day, p.o.) protected against the inhibition of GPx activity caused by 6-OHDA in striatum of mice (**Table 2).**

DA, DOPAC and HVA levels and TH^+ neurons

Statistical analysis of DA levels in striatum revealed a significant 6-OHDA × chrysin interaction ($F_{1.16}$ = 5.98; p = 0.0264) and a main effect of 6-OHDA ($F_{1.16}$ = 41.39; p = 0.0001). Post hoc comparisons demonstrated that 6-OHDA significantly decreased DA levels in striatum of vehicle mice. Chrysin treatment protected against the decrease of DA levels caused by 6-OHDA in striatum of mice (**Table 3**).

A two-way ANOVA of DOPAC levels in the striatum demonstrated a significant 6-OHDA × chrysin interaction ($F_{1.16}$ = 4.68; p = 0.0460) and yielded a main effect of chrysin ($F_{1.16}$ = 6.18; p = 0.0244) and 6-OHDA ($F_{1.16}$ = 19.31; p = 0.0005) (**Table 3**). Post hoc comparisons demonstrated that 6-OHDA significantly decreased striatal DOPAC levels, and the administration of chrysin (10 mg/kg per day) protected against reductions in DOPAC levels caused by 6-OHDA.

A significant 6-OHDA × chrysin interaction ($F_{1.16}$ = 24.30; p = 0.0002) and a main effect of chrysin ($F_{1.16}$ = 11.56; p = 0.0037) and 6-OHDA ($F_{1.16}$ = 67.46; p = 0.0001) in striatum HVA levels in striatum of mice was observed (**Table 3**). Post hoc comparisons demonstrated that 6-OHDA significantly decreased HVA levels

in striatum of vehicle mice. Chrysin treatment significantly restored HVA levels in striatum of 6-OHDA/chrysin mice.

Two-way ANOVA showed a significant effect for interaction 6-OHDA × chrysin interaction ($F_{1.16}$ = 31.29; p = 0.001) in TH^+ striatal neurons, as well as main effects of chrysin ($F_{1.16}$ = 31.29; p = 0.001) and 6-OHDA ($F_{1.16}$ = 31.29; p = 0.001) **(Table 3)**. Post hoc comparisons indicated that 6-OHDA group has significantly decrease of TH^+ neurons when compared with control group. Chrysin treatment at the dose of 10 mg/kg partially restored the TH^+ neurons in striatum of mice (**Figure 3**).

DISCUSSION

This study demonstrated the potential protective effects of chrysin against nigrostriatal dopaminergic neuronal toxicity induced by 6-OHDA injection in mice. Chrysin treatment (10 mg/kg per day during 28 days) attenuated behavioral alterations observed in the depressive-like activity and protected the striatum of mice against oxidative stress, as well as against reductions of DA, DOPAC and HVA levels and TH^+ neurons caused by 6-OHDA exposure, probably through its demonstrated modulation of antioxidant status.

Regarding PD symptoms, an increasing number of studies have demonstrated that PD seems to be a multidimensional disease, and in addition to motor deficits, it is associated with depressive disturbances that result in a loss of quality of life of the afflicted individuals[33]. Depression is among the most common psychiatric conditions accompanying PD. Indeed, depending on the criteria measured, depression can affect 10–45% of PD patients[3]. Furthermore,

it has been shown that depression may largely precede the onset of motor symptoms of PD[3] It was found in the TST, one of the most commonly used tests to evaluate depression-like behavior in laboratory rodents[34], are in line with the clinical data[35]. Furthermore, such an alteration in TST has been found 5 weeks after the lesion, when no alteration is yet present, according to locomotor activity analysis. Although very subtle motor impairments not revealed by the latter analysis cannot be ruled out, these results suggest that the appearance of depressive-like behavior precedes that of motor symptoms. In addition, alterations in the dopaminergic system suggest, therefore, that neurotransmitter systems play an important role in depressive-like behaviors in the current model tested, thereby further supporting the involvement of these neurotransmitter systems in PD-related depression[7]. Our results demonstrated that the 6-OHDA/Chrysin group demonstrated less immobility time than the 6-OHDA/Vehicle group, showing that flavonoid had antidepressant like effect in this model of 6-OHDA-induced PD in mice. These results represent the first evidence that chrysin may prevent depressive-like behavior in an experimental model of PD. In a recent study, we demonstrated that chrysin treatment is also linked to the modulation of depression in mice[36]. The antidepressant-like effects induced by 6-OHDA caused by chrysin, seems to be uncorrelated with motor effects when assessed with the OFT.

In this study, 6-OHDA infusion caused an overproduction of free radicals which, in turn, caused oxidative damages to membrane lipids and protein levels, and ultimately led to a modification in the activity of antioxidant enzymes. This oxidative neuronal damage in 6-OHDA-treated rodents is consistent with previous reports[4,5]. 6-OHDA is a selective catecholamine neurotoxin and could easily undergo autoxidation to yield hydrogen peroxide and superoxide radicals

which take part in a secondary metal-catalyzed Haber-Weiss reaction producing hydroxyl free radicals[37]. As a result, the vehicle group of mice exposed to 6-OHDA exhibited inhibition of GPx activity and increases in GST and GR. In addition, our data confirms that the tested toxin initiated an oxidative cascade of events in the striatum, through the formation of RS, and decreased levels of GSH. We demonstrated that oral administration of chrysin (10 mg/kg per day) for 4 weeks completely reversed the inhibition of GSH level and GPx activity. In contrast, our study showed that the activity of GR and GST are elevated in the striatum of mice infused with 6-OHDA, compared to the control. Furthermore, increased activity of GST and GR suggests an adaptive mechanism to compensate for increased RS levels, as well as decreased levels of GSH. This work is in accordance with results obtained by Jhoo et al[38] and Prediger et al[39] in other neurodegenerative diseases. Our protocol treatment with chrysin for 4 weeks was efficient at preventing increases of enzymes GST and GR activity and decrease of GPx activity in the striatum prior to 6-OHDA injection. Indeed, across different biological systems, chrysin has been demonstrated to act as an antioxidant in other neurodegenerative diseases, such as in Alzheimer's and Huntington's diseases, that stabilizes biomembranes and thus prevents cell membrane damage[40,41]. Thus, these results show that chrysin may prevent oxidative stress and maintain the integrity of the striatum of mice that received 6-OHDA in this PD model.

The measurement of monoamine neurotransmitters in the basal ganglia serves as an important method to determine whether or not a particular drug has a therapeutic effect on dopaminergic neurons and nigrostriatal vulnerability in the TH immunoreactivity[42]. The activities of dopaminergic neurons can thus be inferred by determining the levels of DA, DOPAC and HVA and the TH^+ neurons

in the brain[42]. To this end, we performed HPLC ECD to detect monoamine neurotransmitters in the striatum of mice, and found that levels of DA, DOPAC and HVA were significantly decreased following 6-OHDA exposure, which is similar to previous reports[5]. In addition, 6-OHDA induced a significant reduction in the number of TH^+ neurons in the striatum, indicating degeneration of the dopaminergic nigrostriatal pathway. We can infer from our study that chrysin may inhibit the 6-OHDA-induced catecholamine neurotoxicity and maintain the concentration of DA and its metabolites at normality or close to normality. In the same way, chrysin treatment prevented the reduction of TH-stained cells and loss of striatal dopaminergic fibers. Therefore, chrysin appears to act, in this PD model induced by 6-OHDA, via antioxidant and DA- enhancing mechanisms that rescue the compromised cells in striatum of mice and the behavior impairments.

The present study demonstrated that treatment with chrysin (10 mg/kg per day, for 28 days) was effective in attenuating the following impairments resulting from 6-OHDA exposure in mice: (1) depressive-like behavior in the TST; (2) increased RS levels; (3) decreased GSH levels; (4) inhibition of GPx activity; (5) rises in GST and GR activity; (6) decreased DA, DOPAC and HVA levels and (7) decreased TH^+ neurons from substantia nigra pars compacta. In view of our results, we have provided the first preclinical data indicating that chrysin acts as a protective agent through the analysis of behavioral, neurochemical and biochemical parameters in mice submitted to an experimental model of PD induced by 6-OHDA. The mechanisms involved in this study include the modulation of GPx, GST and GR activity, as well as GSH and RS levels, in a 6-OHDA-induced PD model, thereby protecting against reductions in striatal DA, DOPAC and HVA levels and TH^+ cells.

CONCLUSION

These results provide new insights into experimental models of PD, indicating that chrysin may represent a new therapeutic tool for the treatment of PD. Conversely, further research is needed to clarify the precise molecular mechanisms involved in the protective effects induced by chrysin in the 6-OHDA model of PD.

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Legends of figures

Figure 1. Experimental design.

Figure 2. Effect of chrysin treatment (10 mg/kg, p.o.) and surgery injection of 6-OHDA on OFT (A) and TST (B). The values were analyzed by two-way ANOVA and Newman-Keuls multiple comparison test. Each value is expressed as the mean ± SD. [a] $P<0.05$ when compared 6-OHDA/Vehicle with Vehicle/Vehicle. [b] $P<0.05$ when compared 6-OHDA/Chrysin with 6-OHDA/ Vehicle.

Figure 3. Photomicrographs showing the optical disector probe application, where could be observed TH^+ neurons from substantia nigra pars compacta

about effect of chrysin treatment (10 mg/kg, p.o.) and surgery injection of 6-OHDA in different scale bar: 5x=100µm and 20x=25 µm.

Figure 1

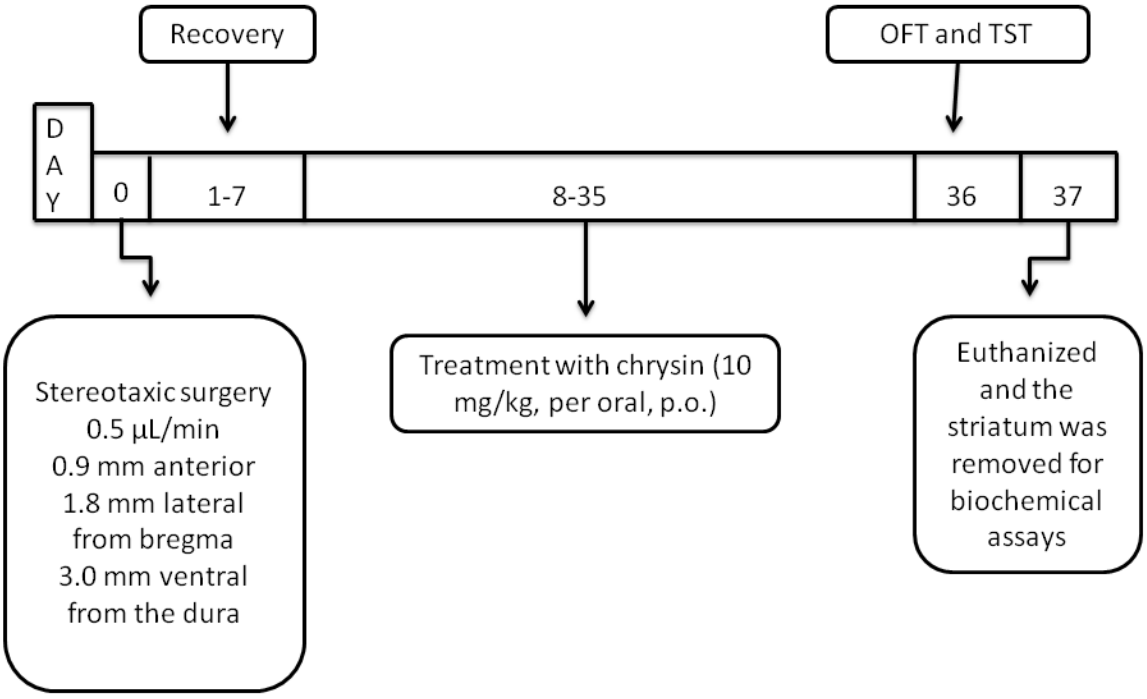
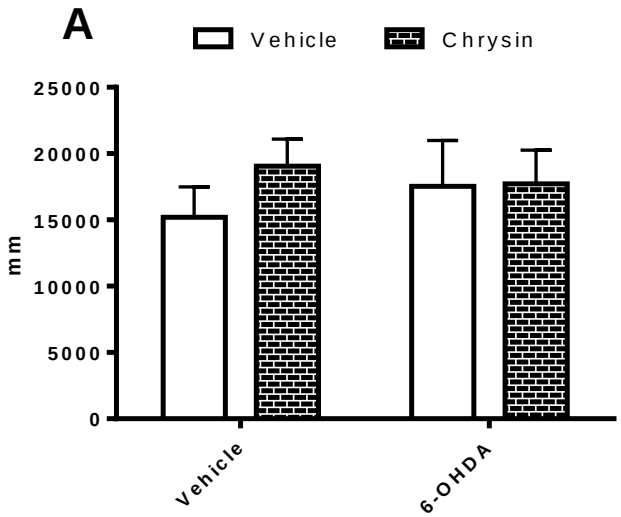

Recovery
OFT and TST
DAY
0
1-7
8-35
36
37
Stereotaxic surgery
0.5 µL/min
0.9 mm anterior
1.8 mm lateral
from bregma
3.0 mm ventral
from the dura
Treatment with chrysin (10 mg/kg, per oral, p.o.)
Euthanized and the striatum was removed for biochemical assays

Figure 2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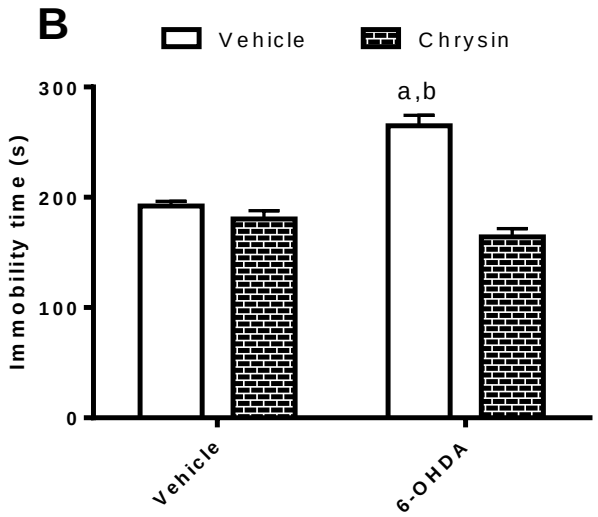
6-OHDA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6-OHDA

Figure 3

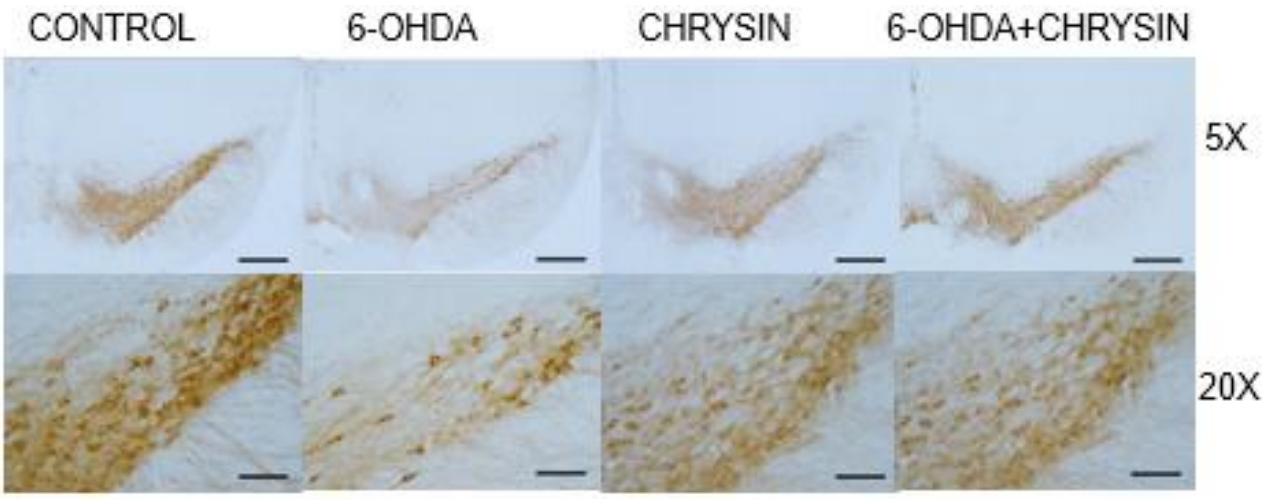

Table 1. Effect of chrysin treatment (10 mg/kg, p.o.) and surgery injection 6-OHDA on RS and GSH levels in striatum of mice

Groups	RS	GSH
Vehicle/Vehicle	13.13±3.13	12.65±2.18
Vehicle/Chrysin	11.66±1.55	13.97±2.65
6-OHDA/Vehicle	30.15±4.22[a,b]	6.53±1.08[a,b]
6-OHDA/Chrysin	16.98±5.99	11.21±1.79

The values were analyzed by two-way ANOVA and Newman-Keuls multiple comparison test, each value is expressed as the mean ± SD. [a] $P<0.05$ when compared 6-OHDA/Vehicle with Vehicle/Vehicle. [b] $P<0.05$ when compared 6-OHDA/Chrysin with 6-OHDA/ Vehicle. The RS levels were expressed as arbitrary unit (AU) and GSH levels were expressed as mol/g of tissue.

Table 2. Effect of chrysin treatment (10 mg/kg, p.o.) and surgery injection 6-OHDA on CAT, GST, GPx and GR activities in striatum of mice

Groups	CAT	GST	GPx	GR
Vehicle/Vehicle	0.91 ± 0.15	29.5 ± 2.88	50.2 ± 1.98	29.7 ± 1.58
Vehicle/Chrysin	0.88 ± 0.13	27.5 ± 3.51	51.4 ± 3.21	34.41 ± 2.84
6-OHDA/Vehicle	0.71 ± 0.13	82.5 ± 4.02[a]	24.6 ± 1.89[a]	48.84 ± 2.41[a]
6-OHDA/Chrysin	0.75 ± 0.17	34.5 ± 4.21[b]	47.5 ± 2.12[b]	32.41 ± 2.98[b]

The values were analyzed by two-way ANOVA and Newman-Keuls multiple comparison test, each value is expressed as the mean ± SD. [a] $P<0.05$ when compared 6-OHDA/Vehicle with Vehicle/Vehicle. [b] $P<0.05$ when compared 6-OHDA/Chrysin with 6-OHDA/ Vehicle. CAT activity are expressed as (U)/mg protein; GR activiy are expressed as nmol NADPH/min/mg protein; GPx activity are expressed as nmol NADPH/min/mg protein and GST activity are expressed as nmol CDNB conjugated/min/mg protein.

Table 3. Effect of chrysin treatment (10 mg/kg, p.o.) and surgery injection 6-OHDA on DA, DOPAC, and HVA levels and TH^+ neurons in striatum of mice

Groups	DA	DOPAC	HVA^3	TH^+ neurons
Vehicle/Vehicle	745.4±63.49	27.72± 2.88	68.92± 6.13	14567.2± 854.2
Vehicle/Chrysin	730.6±74.35	25.06± 5.33	53.32± 5.01	15124.3± 658.9
6-OHDA/Vehicle	444.4±60.65[a,b]	18.35± 4.22[a,b]	24.78± 6.33[a,b]	6145.8± 598.3[a,b]
6-OHDA/Chrysin	595.4±98.72[c]	34.04± 4.49	55.88± 6.65[c]	10359.6± 719.6[c]

The values were analyzed by two-way ANOVA and Newman-Keuls multiple comparison test, each value is expressed as the mean ± SD. [a] $P<0.05$ when compared 6-OHDA/Vehicle with Vehicle/Vehicle. [b] $P<0.05$ when compared 6-OHDA/Chrysin with 6-OHDA/ Vehicle. [c] $P<0.05$ when compared 6-OHDA/Chrysin with Vehicle/Vehicle. DA, DOPAC and HVA levels are expressed as ng/g wet weight of striatum.

DISCUSSÃO

O objetivo deste estudo foi investigar o tratamento com crisina (10 mg / kg, por via oral), em um modelo experimental crônico da DP com um nível moderado de neurodegeneração e explorar possíveis mecanismos de neuroproteção. Foi demonstrado os potenciais efeitos terapêuticos da crisina contra a toxicidade neuronal dopaminérgica nigrostriatal induzida pela injeção de 6-OHDA em camundongos. O tratamento com crisina foi capaz de atenuar as alterações comportamentais e locomotoras, proteger contra aumento do estresse oxidativo, reduzir os níveis de citocinas pró-inflamatórias, bem como preveniu a redução dos níveis de DA, DOPAC e HVA. Além disso, preveniu a redução de neurônios TH^+ causados pela exposição a 6-OHDA, além de elevar os níveis BDNF, GDNF e NGF no estriado de camundongos. Esses achados apoiam a noção de que a crisina pode ser um candidato promissor para ação antiparkinsoniana.

Quanto aos sintomas de DP, um número crescente de estudos demonstrou que a DP é uma doença multidimensional, e, além dos déficits motores, está associada a distúrbios depressivos que resultam na diminuição da qualidade de vida dos pacientes (Matheus et al., 2012; Noyce et al., 2012; Tadaiesky, 2010; Tadaiesky et al., 2008). A depressão está entre as condições psiquiátricas mais comuns que acompanham a DP. Na verdade, dependendo dos critérios medidos, a depressão pode afetar 45% dos pacientes com DP (Noyce et al., 2012). Além disso, foi demonstrado que a depressão pode preceder, em grande parte, o início dos sintomas motores da DP (Noyce et al., 2012). Foi encontrado no TST, um dos testes mais comumente usados para avaliar comportamentos semelhantes a depressão em roedores (Cryan et al., 2005), resultados semelhantes aos que estão na literatura (Filho et al., 2016; Pollak et al., 2010). Além disso, tal alteração no comportamento tipo- depressivo, foi encontrado 5 semanas após a lesão, quando ainda não existem alterações locomotoras, de acordo com a análise da atividade locomotora. Embora deficiências motoras muito sutis não tenham sido reveladas pela última análise, não é possível descartar que esses resultados sugerem que o comportamento depressivo precede os sintomas motores. Sendo assim, alterações no sistema dopaminérgico sugerem que os sistemas de neurotransmissores desempenham um papel importante no comportamento depressivo neste modelo da DP,

sustentando ainda mais o envolvimento desses sistemas de neurotransmissores nos sintomas depressivos relacionados a DP (Santiago et al., 2010)7. Nossos resultados demonstraram que o grupo 6-OHDA / Crisina obteve menor tempo no TST quando comparado com ao grupo 6-OHDA /Veículo, mostrando que o flavonoide possui um efeito tipo-antidepressivo neste modelo de DP induzida por 6-OHDA em camundongos (Singh et al., 2016). Estes resultados representam a primeira evidência de que a crisina pode prevenir o comportamento tipo-depressivo em um modelo experimental de DP.

A disfunção locomotora é um tipo de sintoma clínico de DP (Jankovic, 2008). Na análise comportamental, os animais tratados com crisina apresentaram melhor coordenação muscular no teste rotarod. Também demonstramos que o tratamento com crisina atenuou as rotações induzidas por apomorfina (discinesia) em camundongos. Desta forma, deficiências comportamentais observadas na DP correlacionam-se com a neurodegeneração nigrostriatal (Gordon et al., 2016; Jayaraj et al., 2014) e o efeito benéfico da crisina nos neurônios TH^+ no estriado corroborado com os resultados alcançados no teste motor. Juntamente com a literatura, o presente estudo apoiou e ampliou a noção de que as respostas neurotróficas e inflamatórias (Filho et al., 2016a; Filho et al., 2016b; Souza et al., 2015) podem ser um importante mecanismo neurobiológico da crisina no comprometimento induzido por 6-OHDA.

Neste estudo, a infusão de 6-OHDA causou uma superprodução de radicais livres no estriado de camundongos que, por sua vez, causaram danos oxidativos aos níveis em lipídios e proteínas e, que resultou uma modificação na atividade de enzimas antioxidantes. Este dano neuronal oxidativo, causado pela 6-OHDA, é consistente com os relatórios anteriores (Ahmad et al., 2012; Shobana et al., 2012; Tadaiesky et al., 2008). A 6- OHDA é uma neurotoxina de catecolamina seletiva e pode ser facilmente submetida a autoxidação, resultando na formação de 6-OHDA quinase, de peróxido de hidrogênio e superóxido que participam de uma reação de Sier-Weiss catalisada por metal secundário produzindo radicais livres, além do radical hidroxila (Opacka-Juffry et al., 1998). No presente estudo, o grupo 6-OHDA /Veículo demonstrou uma inibição da atividade de GPx e aumento de GST e GR. Além disso, nossos dados confirmam que a toxina testada iniciou uma cascata oxidativa de eventos no estriado com diminuição dos níveis de GSH. Demonstramos que a administração oral de crisina (10 mg / kg por dia) durante 4 semanas reverteu completamente a inibição

dos níveis de GSH e GPx. Em contraste, nosso estudo mostrou que a atividade de GR e GST são elevadas no estriado de camundongos infundidos com 6-OHDA, em comparação com o controle. Além do mais, o aumento da atividade de GST e GR, em resposta a toxina 6-OHDA, sugere um mecanismo adaptativo para compensar o aumento dos níveis de ER, bem como a diminuição dos níveis de GSH, sendo assim, estando de acordo com os resultados obtidos por Jhoo et al., (2004) e Prediger et al., (2007). Nosso protocolo de tratamento com crisina foi eficiente na prevenção do aumento das atividades das enzimas GST e GR e diminuição da atividade de GPx no estriado, em resposta à injeção de 6-OHDA. De fato, foi demonstrado que a crisina atua como antioxidante em outros modelos induzidos por neurotoxinas de doenças neurodegenerativas, como as doenças de Alzheimer e Huntington, esbalizizando as biomembranas e, portanto, evitando o dano da membrana celular (Hung et al., 2012; Menze et al., 2001). Portanto, os resultados apresentados mostram que a crisina pode prevenir o estresse oxidativo e manter a integridade do estriado de camundongos que receberam 6-OHDA.

A neuroinflamação é bem reconhecida como um importante processo na patogênese de diversas doenças neurodegenerativas, incluindo DP (Naughton et al., 2016; Tentillier et al., 2016). Tem sido amplamente demonstrada que as citocinas são pequenas proteínas que atuam em processos inflamatórios e na regulação do sistema imunológico na DP (Dursun et al., 2015; Lindqvist et al., 2013). Neste sentido, foi reportado que o estresse oxidativo ativa as células microgliais para aumentar a produção de citocinas pró-inflamatórios (TNF-α, IFN-γ, IL-1β, IL-2 e IL-6), levando a degeneração dos neurônios dopaminérgicos, uma vez que tais são muito sensíveis a essas alterações (Pellegrini et al., 2017). As células gliais liberam citocinas pró-inflamatórias (TNF-a, IFN-γ, IL-1β) com efeito deletério sobre os neurónios dopaminérgicos, ativando receptores envolvidos na apoptose (Avagliano et al., 2016). Um estudo prévio indicou que as concentrações séricas de TNF-α, IL-2 e IL-6 estão elevadas em pacientes com DP (Quin et al., 2016). Outro estudo relatou que os níveis de IFN-γ estavam elevados no plasma sanguíneo de pacientes com DP e camundongos transgênicos com deficiência de IFN-γ atenuaram a perda de células dopaminérgicas através da indução de 1-metil-4-fenil-1,2,3,6-tetra-hidropiridina (MPTP) em um modelo da DP, apontando que a delação gênica desta citocina protege contra a perda de tais células, reforçando assim o envolvimento da IFN-

γ na perda neural relacionada a DP (Barcia et al., 2012). Enquanto isso, em resposta a ativação da micróglia, menores níveis de citocinas anti-inflamatórias, como IL-10, também foram relatados no sistema nervoso central ou soro de pacientes com DP (Collins et al., 2012; Brodacki et al., 2008) Estudos prévios do nosso laboratório demonstraram que a crisina tem atividade anti-inflamatória e inibição da resposta inflamatória induzida pelo estresse crônico (Filho et al., 2016a; Filho et al., 2016b). Nosso estudo mostrou um aumento dos níveis de citocinas pró-inflamatórias, incluindo TNF-α, IFN-γ, IL-1β, IL-2 e IL-6 e uma diminuição da citocina anti-inflamatória como a IL-10 no estriado após a injeção de 6-OHDA. Por outro lado, o tratamento com crisina atenuou o aumento de citocinas pró-inflamatórias e a diminuição de anti-inflamatórias no estriado. Consequentemente, o presente estudo ampliou as evidencias sobre o papel anti-inflamatório da crisina no cérebro, sustentando o seu efeito neste modelo de neurodegeneração. Assim, nós sugerimos que a modulação da resposta induzida pela crisina é um importante mecanismo neurobiológico contra as auterações comportamentais induzida pela 6-OHDA.

Os fatores neurotróficos são reconhecidos como reguladores chave do desenvolvimento sináptico e da plasticidade e, portanto, estão sendo implicados em relação a distúrbios neurodegenerativos, como a DP (Anusha et al., 2017; Li et al., 2017). Vários fatores neurotróficos no cérebro foram reconhecidos como moduladores da sobrevivência de neurônios, e como desempenhando um papel na neuroproteção em modelos da DP (Garea-Rodriguez et al., 2016). Estudos demonstram que o BDNF, GDNF e NGF previnem a degeneração neuronal do sistema nigroestriatal lesionado *in vivo* (Sampaio et al., 2017; Sarkar et al., 2016) e diminuem os déficits motores (Drinkut et al., 2016). A S100B, em níveis elevados (na ordem de micromols), pode ser um biomarcador de doenças neurodegenerativas, incluindo a DP (Carvalho et al., 2015), sendo que a S100B via ativação de receptor, para produtos finais de glicação avançada (AGEs), ativa a superprodução de espécies reativas de oxigênio (EROs) e citocinas inflamatórias, provocando danos e neurodegeneração (Niranjan, 2014). No presente estudo, o aumento de EROs e citocinas inflamatórias ocorreram após o tratamento com 6-OHDA, sugerindo que o desequilíbrio das neurotrofinas, juntamente com o aumento dos níveis de EROs é o gatilho para a ativação de outras moléculas envolvidas na patogênese da DP. Em contraste, o tratamento com crisina atuou na recuperação dos níveis de S100B, BDNF, GDNF e NGF

após a injeção de 6-OHDA no estriado, aumentando a sobrevivência dos neurônios dopaminérgicos, uma vez que a diminuição da quantidade desses neurônios é a principal característica da DP (Espejo et al., 2000). Esses achados sugerem que o déficit de neurotrofinas se torna mais crítico para sobrevivência dos neurônios dopaminérgicos e atividades comportamentais relacionadas, evidenciadas neste estudo com o efeito neuroprotetor da crisina.

A mensuração dos níveis dos neurotransmissores de monoamanias nos gânglios basais é um método importante para determinar se um medicamento específico possui efeito terapêutico sobre neurônios dopaminérgicos (Moore et al., 2005). As atividades dos neurônios dopaminérgicos podem assim ser inferidas determinando os níveis de DA, DOPAC e HVA e os neurônios TH^+ no cérebro (Moore et al., 2005). Para este fim, realizamos HPLC ECD para detecção de neurotransmissores de monoamina no estriado de camundongos e descobrimos os níveis de DA, DOPAC e HVA diminuíram significativamente após a exposição à 6-OHDA, corroborando com achados anteriores (Shobana et al., 2012). Além disso, a 6-OHDA induziu uma redução significativa no número de neurônios TH^+ no estriado, quantificados através de imunohistoquimica, indicando uma degeneração da via nigrostriatal dopaminérgica. Nosso estudo sustenta que a crisina tem uma ação inibitória da neurotoxicidade da catecolamina 6-OHDA e, mantendo a concentração de DA e seus metabólitos em normalidade ou próximo da normalidade. Do mesmo modo, o tratamento com crisina evitou a redução das células com TH^+ e a perda de fibras dopaminérgicas no estriado. Portanto, no modelo utilizado nesse estudo a ação da crisina se dá através de mecanismos antioxidantes e da redução dos níveis DA no estriado de camundongos, atenuando também as deficiências de comportamento. A TH é a enzima limitante de taxa responsável pela conversão de L-DOPA em DA (Daubner et al., 2011). A medida da imunorreatividade TH é, portanto, uma medida da funcionalidade dos neurônios dopaminérgicos e fibras presentes no estriado (Daubner et al., 2011). Da importância particular, o tratamento com crisina evitou a redução das células TH e a perda DA e seus metabolitos no estriado. Acredita-se que o principal mecanismo de perda neuronal dopaminérgica nesses animais é o estresse oxidativo e a inflamação gerada em resposta à exposição a 6-OHDA (Hernandez-Baltazar et al., 2015). O tratamento com crisina atenuou os danos causados aos neurônios dopaminérgicos no estriado de camundongos após a injeção de 6-OHDA e esta atenuação pode

estar correlacionada com atividades antioxidantes e anti-inflamatórias deste composto (Filho et al., 2016a; Filho et al., 2016b). Além disso, a ação neuroprotetora da crisina no modelo de DP pode ser devida ao desenvolvimento neuronal e à sobrevivência e requer suporte neurotrófico, principalmente BDNF e NGF (Souza et al., 2015; Filho et al., 2015). Nossas descobertas sugeriram que a crisina possui potencial anti-inflamatório e neurotrófico, demonstrada na sobrevivência das células dopaminérgicas, principalmente dada pela modulação do BDNF.

Em resumo, a crisina demonstriu atividade neuroprotetora no modelo de DP induzido pela 6-OHDA, indicando que pode representar uma nova opção terapêutica para DP. Este efeito neuroprotetor é atribuído ao seu forte potencial antioxidante e papel inibitório em diferentes eventos-chave envolvidos na neuroinflamação e preservando os níveis de fatores neurotróficos. Desta maneira, a crisina também causa uma regulação de neurônios TH^+ no estriado, que consequentemente mantem níveis homeostáticos, ou próximos a homeostase, de neurotransmissores como DA, DOPAC e HVA e alterações comportamentais características da doença.

CONCLUSÕES

- A crisina foi capaz de atenuar o dano locomotor no teste de rota rod, bem como no teste de comportamento rotatório. Além disso a crisina foi capaz de reverter o comportamente tipo depressivo no teste suspensação de causa causada pela 6-OHDA.
- Nos parâmetros de estresse oxidativo, houve diminuição nos níveis de espécies reativas, bem como o aumento dos níveis de GSH. Além disso, as enzimas CAT, GST, GPx e GR tiverão suas atividades restauradas há níveis homeostáticos.
- A crisina foi capaz de atenuar o dano causado pela 6-OHDA no estriado, diminuindo a degeneração dos neurônios TH^+.
- Os níveis de DA, HVA e DOPAC foram aumentados com o tratamento de 28 dias com crisina quando comparado ao grupo 6-OHDA/Veículo.

PERSPECTIVAS

Afim de elucidar melhor o papel da crisina como possível medida no auxílio da prevenção e ao tratamento farmacológico da DP, pretendemos utilizar outros protocolos, desde o modelo (pretende-se utilizar o MPTP ao invés da 6-OHDA) até o acréscimo de um protocolo de exercício físico (sendo que nesse caso, pretende-se averiguar o papel do exercício juntamente com a crisina no modelo induzido a DP). Além disso, pretende-se averiguar os mecanismos pela qual a crisina age (através de marcação de carbono) e, analisar a atividade e expressão de enzimas antioxidantes.

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ANEXO

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 4721, E-mail: ceua@unipampa.edu.br

PROTOCOLO Nº 038/2012
DATA: 19/11/12

Título: EFEITO TERAPÊUTICO DO EXERCÍCIO AERÓBIO E DA CRISINA EM UM MODELO INDUZIDO POR 6-HIDROXIDOPAMINA EM CAMUNDONGOS

Pesquisador: Cristiano Ricardo Jesse / André Tiago Rossito Goes

Campus: Itaqui

Telefone: 055 3433-1669

E-mail: cristianoricardojesse@yahoo.com.br / andrerossitogoes@gmail.com

Após a análise detalhada do projeto de pesquisa a relatoria da CEUA-Unipampa emite parecer **FAVORÁVEL** para o cadastro do protocolo e execução do referido projeto.

Att.

Luiz E. Henkes
Professor Adjunto
Coordenador do CEUA/Unipampa