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AVALIAÇÃO DOS FATORES COMPORTAMENTAIS E NEUROQUIMICOS ASSOCIADOS AO EFEITO DA HESPERIDINA EM MODELOS DA DOENÇA DE PARKINSON E DE DEPRESSÃO EM CAMUNDONGOS

TESE DE DOUTORADO

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Itaqui, RS, Brasil.
2017

AVALIAÇÃO DOS FATORES COMPORTAMENTAIS E NEUROQUIMICOS ASSOCIADOS AO EFEITO DA HESPERIDINA EM MODELOS DA DOENÇA DE PARKINSON E DE DEPRESSÃO EM CAMUNDONGOS

por

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

Orientador: Prof. Dr. Cristiano Ricardo Jesse

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PARTE I

RESUMO

A doença de Parkinson (DP) é a segunda doença neurodegenerativa mais prevalente na população idosa, tendo como principal característica neuropatológica a depleção de dopamina (DA) estriatal, condição esta, que conduz ao aparecimento dos sintomas motores da doença. Além de sintomas motores, os pacientes acometidos com essa doença apresentam ainda sintomas não motores e neuropsiquiátricos onde destaca-se a depressão. Atualmente, nenhuma das drogas utilizadas é totalmente eficaz para a DP, nesse contexto, terapias promissoras ainda precisam ser exploradas. Alguns estudos demonstraram que o consumo de flavonoides podem diminuir a incidência e os sintomas de doenças neurodegenerativas e de transtornos depressivos. Nosso estudo anterior já constatou que o flavonoide hesperidina (4'-methoxy-7-O-rutinosyl-3',5-dihydroxyflavanone), intervém na DP e no comportamento tipo depressivo via estresse oxidativo, em um modelo induzido por 6-hidroxidopamina (6-OHDA) em camundongos. Baseando-se nas evidências, se torna interessante investigar o efeito da hesperidina em outros parâmetros da DP no modelo da 6-OHDA, além de seu efeito antidepressivo no modelo da bulbectomia olfatória (BO). Para avaliar a DP, a hesperidina ou o veículo (50 mg/kg) foram administrados via oral (gavagem) durante 28 dias. Após este período, os camundongos foram submetidos a testes comportamentais. Para avaliar a depressão no modelo OB, a hesperidina ou o veículo (50 mg/kg) foram administrados via oral (gavagem) durante 13 dias. Após este período, os camundongos foram submetidos aos testes comportamentais e neuroquímicos. No modelo induzido pela 6-OHDA, o tratamento com a hesperidina protegeu contra a inibição das enzimas mitocondriais dos complexos I, II, IV e V, da enzima Na^+/K^+ ATPase, diminuição do potencial de membrana mitocondrial, e aumento das atividades das caspases 3 e 9 e níveis de marcadores de inflamação. Além disso, a administração do flavonoide culminou na restauração dos níveis de fatores neurotroficos, assim como dos neurônios positivos para tirosina hidroxilase (TH), e níveis de DA e seus metabolitos no estriado. O tratamento com hesperidina também atenuou as alterações comportamentais de rotação, ansiedade, anedonia, memória e nos danos olfatórios induzidos pela 6-OHDA. No modelo de depressão induzido pela OB, o tratamento oral com hesperidina foi efetivo em reverter o declínio dos fatores neurotróficos, o aumento das citocinas pro-inflamatórias e da atividade da enzima acetilcolinesterase (AchE) no hipocampo, bem como o aumento da atividade locomotora, os prejuízos na memória, o comportamento tipo depressivo e a anedonia decorrentes da remoção dos bulbos. No modelo de DP induzido pela 6-OHDA pode-se propor que a hesperidina atua na restauração dos neurônios dopaminérgicos através do restabelecimento dos fatores neurotróficos, controle das citocinas, e modulação da atividade mitocondrial e apoptótica no estriado, o que reflete na recuperação dos prejuízos comportamentais. No modelo da depressão, indica-se que a reparação hipocampal sobre os fatores neurotróficos e marcadores inflamatórios são responsáveis pelo reestabelecimento comportamental no tratamento com hesperidina. Estes resultados certificam o efeito potencial da hesperidina no tratamento da DP e dos distúrbios depressivos, indicando que esse flavonoide pode atuar como terapia multialvo, associando DP e os transtornos mentais. Isso se deve à

regulação de fatores neurotróficos, inflamatórios e apoptóticos, atividades enzimáticas (AchE e Na^+/K^+ ATPase), disfunção mitocondrial e recuperação neuronal dopaminérgica.

Palavras chave: Neurodegeneração; Depressão; 6-hidroxidopamina; Bulbectomia olfatória; Hesperidina; Inflamação; Plasticidade; Dopamina.

ABSTRACT

Parkinson's disease (PD) is the second most prevalent neurodegenerative disease in the elderly population. Its main neuropathological characteristic is the striatal dopamine (DA) depletion, which leads to the appearance of the motor symptoms of the disease. In addition to motor symptoms, the patients with this disease also present non-motor and neuropsychiatric symptoms where depression stands out. Currently, none of the drugs used is fully effective for PD, in this context, promising therapies still need to be explored. Some studies have shown that consumption of flavonoids may decrease the incidence and symptoms of neurodegenerative diseases and depressive disorders. Our previous study has found that the flavonoid hesperidin (4'-methoxy-7-O-rutinosyl-3 ', 5-dihydroxyflavanone) plays a role in PD and in the depressive behavior via oxidative stress in mice induced by 6-hydroxydopamine (6 -OHDA). Based on the evidence, is interesting to investigate the effect of hesperidin in other parameters of PD in the 6-OHDA model, besides the its antidepressant effect in olfactory bulbulometric (BO) model. To evaluate the PD, hesperidin or vehicle (50 mg / kg) were administered orally (gavage) for 28 days. After this period, the mice were submitted to behavioral tests. To assess depression in the OB model, hesperidin or vehicle (50 mg / kg) were administered orally (gavage) for 13 days. After this period, the mice were submitted to behavioral and neurochemical tests. In the 6-OHDA-induced model, treatment with hesperidin protected against inhibition of mitochondrial enzymes of complexes I, II, IV and V, Na + / K + ATPase enzyme, against the decrease in mitochondrial membrane potential, and against the increase of caspases 3 and 9 activities and inflammatory markers levels. In addition, flavonoid administration culminated in restoration of neurotrophic factors levels, as well as tyrosine hydroxylase (TH) positive neurons, DA and its metabolites levels in the striatum. The treatment with hesperidin also attenuated the behavioral changes of rotation, anxiety, anhedonia, memory and olfactory damage induced by 6-OHDA. In addition, in depression model OB-induced, oral hesperidin treatment was effective in reversing the decline of neurotrophic factors, increase of proinflammatory cytokines levels and acetylcholinesterase (AchE) activity in the hippocampus, as well as increase of locomotor activity, memory impairment, depressive behavior and anhedonia due to removal of bulbs. In DP model induced by 6-OHDA it can be proposed that hesperidin acts in the restoration of dopaminergic neurons through the reestablishment of neurotrophic factors, cytokine control, and modulation of mitochondrial and apoptotic activity in striatum, which reflects in the recovery of damages behavioral. In depression model, it is indicated that hippocampal repair on neurotrophic factors and inflammatory markers are responsible for behavioral reestablishment by hesperidin treatment. These results confirm the potential effect of hesperidin in the treatment of PD and depressive disorders, indicating that this flavonoid can act as a multi-target therapy, associating PD and mental disorders. This is due to the regulation of neurotrophic, inflammatory and apoptotic factors, enzymatic activities (AchE and Na^+/K^+ ATPase), mitochondrial dysfunction and dopaminergic neuronal recovery.

Keywords: Neurodegeneration; Depression; 6-hydroxydopamine; Olfactory bulbectomy; Hesperidin; Inflammation; Plasticity; Dopamine.

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LISTA DE ABREVIATURAS

ACh = Acetilcolina

AchE = Acetilcolinesterase

ATP =Trifosfato de adenosina

BO = Bulbectomia olfatória

BDNF = Fator neurotrófico derivado do cérebro (do inglês, *brain-derived neurotrophic fator)*

DA = Dopamina

DAT = Transportador de dopamina

DOPAC = 3,4=di=hidroxifenilacético (do inglês, *3,4=dihydroxyphe=nylacetic acid*)

DP = Doença de Parkinson

EPMT = Teste do labirinto em cruz elevado (do inglês, *elevated plus-maze test*)

FST = Teste do nado forçado (do inglês: *forced swimming test*)

GDNF = fator neurotrófico derivado de células gliais (do inglês, *glial cell line-derived neurotrophic fator)*

HVA = Ácido homovanílico (do inglês, *homovanillic acid*)

IFN-γ = Interferon-gama

IL-1β = Interleucina 1-beta

IL-6 = Interleucina 6

IL-10 = Interleucina 10

IL-12 = Interleucina 12

IL-18 = Interleucina 18

MWMT = Teste do labirinto aquatic de Morris (do inglês: *Morris water maze task*)

NGF = Fator de crescimento neuronal (do inglês: *nerve Growth Factor*)

NTs = Neurotrofinas

NT-3 = Neurotrofina-3

OFT = Teste de campo aberto (do inglês, *open=field test*)

ORT = Teste de reconhecimento de objetos (do inglês, *object recognition test*)

ROS = Espécies reativas de oxigênio (do inglês, *reactive oxygen species)*

SNC = Sistema nervoso central

SNpc = Substância negra pars compacta

TH = Tirosina hidroxilase

TNF-α = Fator de necrose tumoral-alfa (do inglês, *tumor necrosis factor-alpha*)

TST = Testes de suspensão da cauda (do inglês, *tail suspension test*)

6-OHDA = 6-hidroxidopamina

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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 dois manuscritos e um artigo publicado em periódico científico, os quais se encontram nos itens **MANUSCRITO I e MANUSCRITO II e ARTIGO,** na **parte II** deste trabalho. As seções materiais e métodos, resultados, discussão dos resultados e referências bibliográficas, encontram-se nos próprios manuscritos e artigo 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** refere-se somente às citações que aparecem nos itens introdução e discussão desta tese. No item **PERSPECTIVAS**, estão expostos os possíveis estudos para dar continuidade a este trabalho.

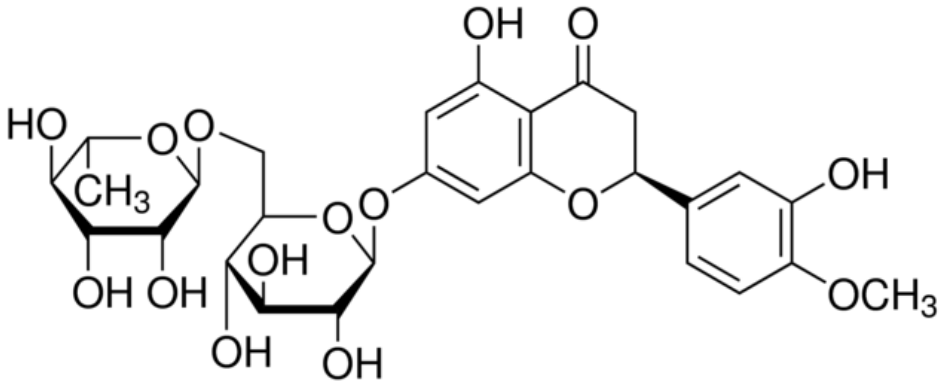
PARTE I

INTRODUÇÃO

1. Hesperidina

A hesperidina (3',5,7-tri-hidroxi-4'-metoxi-flavanona-7-ramnoglucosídeo (**Figura 1**), um flavonoide da subclasse flavanona, é encontrada principalmente em frutos cítricos sendo assim classificada como um bioflavonóide cítrico, está principalmente contida na casca, foi isolada pela primeira vez por Lebreton em 1827. A aglicona de hesperidina é hesperetina. A hesperetina acoplada com o dissacarídeo rutinose é o composto hesperidina em si (GARG et al, 2001). É bem estabelecido que esse polifenol exerce uma variedade de efeitos farmacológicos, entre as principais atividades exercidas pela hesperidina estão: atividades anti-aterogênico, anti-alérgica, antioxidante e anti-inflamatória (AMEER et al, 1996; ANTUNES et al., 2016; BORRADAILE et al, 1999; DONATO et al., 2014; GALATI et al, 1994; WILCOX et al, 2001).

Figura 1 - Estruturas químicas de hesperidina e hesperetina.



Fonte: Iranshahi et al., 2015.

Antes de ser absorvida, a hesperidina é hidrolisada pela microflora intestinal e absorvida no cólon em sua forma aglicona (hesperitina) (MANACH et al., 2003) e as principais enzimas envolvidas na hidrólise são as hidrolases (florisina hidrolase lactase (LPH) e glicosidases (g-glicosidades, glicosidade citosólica (CBG) (ERLUND, 2004). Posteriormente, na parede intestinal, a hesperitina sofre reações de conjugação, tais como glicuronidação e metilação (MANACH et al., 1998; SPENCER

et al., 1999), forma na qual é conduzida pelo sistema-porta dos enterócitos ao fígado, onde sofrerá outras reações, como metilação, sulfatação e glicuronidação, formando uma variedade de metabólitos (SCALBERT et al., 2002, MATSUMOTO et al., 2004). É importante ressaltar que uma parte da hesperidina absorvida é convertida em hesperitina, entretanto a outra parte da hesperidina que não sofre deglicosilação é parcialmente conjugada, e imediatamente liberada da circulação e excretada sob a forma de metabólitos na urina sem que haja reabsorção renal tubular (MATSUMOTO et al., 2004; YAMADA et al., 2006,). Finalmente, os metabólitos (hesperitina-glicuronídeos e sulfoglicoronídeos) podem ser excretados pela bile ou conduzidos aos tecidos periféricos pelo sistema sanguíneo, onde poderá desempenhar as atividades funcionais no organismo, amplamente descritas na literatura (HEIM et al., 2002). Após o administração de hesperidina, a detecção da hesperitina se dá após 6h, com seu pico máximo entre 9 e 12h, a cerca de aproximadamente 1,3 μM. É a quantidade de substancia livre no plasma que caracteriza sua ação farmacológica. Além disso, o autor relata que a hesperidina não é toxica, mesmo em tratamentos com altas doses e tempos de administração prolongados (YAMADA, 2006).

2. Hesperidina e sistema nervoso

A capacidade de hesperitina e hesperidina para atravessar a barreira hematoencefálica já é estabelecida e os torna candidatos naturais ideais no tratamento de diferentes distúrbios do SNC (DIMPFEL, 2006). Portanto, os efeitos de ambos os compostos sobre o sistema nervoso central têm sido objeto estudos. Nosso estudo anterior propõe que o flavonoide hesperidina exerce proteção na DP através da redução da produção de espécies reativas, cuja concentração é susceptível de desempenhar um papel patológico crucial no cérebro envelhecido, reduzindo o estresse oxidativo, revertendo os baixos níveis de DA, comportamento tipo depressivo e memória, em modelo induzido por 6-OHDA em camundongos (ANTUNES et al., 2013). Além disso, estudos de nosso grupo já demostraram algumas vias de sinalização envolvidas nos efeitos tipo antidepressivo do flavonoide reportado, em testes comportamentais, através da modulação da vias l-arginina-NO-cGMP e receptores Kappa-opióides e serotononérgicos (DONATO et al., 2012, DONATO et al., 2014; FILHO et al., 2012; SOUZA et al., 2013). Considerando as variadas aplicações biológicas da hesperidina, se torna também considerável investigar novos

mecanismos desse flavonoide na DP além de seus efeitos antidepressivos em modelos experimentais de depressão.

3. Doença de Parkinson

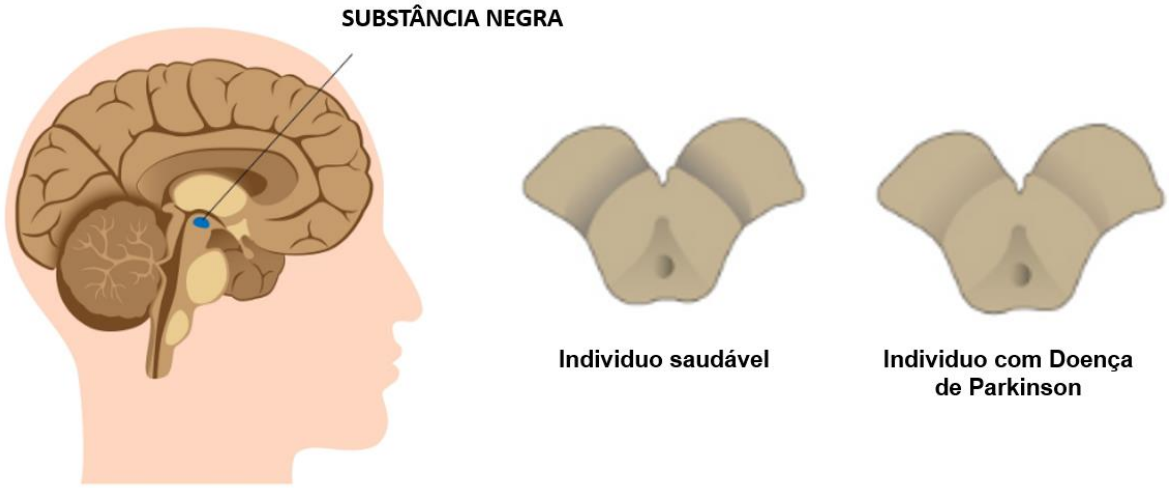
O aumento da população idosa tem conduzido a uma crescente incidência de doenças neurodegenerativas em todo o mundo, com isso, o interesse por estudos que visem a prevenção e recuperação destas enfermidades são considerados de grande valia. A Doença de Parkinson (DP) é a segunda doença neurodegenerativa mais comum em humanos (OMS, 2008), sendo precedida apenas pela doença de Alzheimer. A média de idade para ocorrência da DP é de 55 anos, sendo que o seu risco de desenvolvimento aumenta em cinco vezes por volta dos 70 anos de idade (HALD & LOTHARIUS, 2005). Em 2006, Barbosa e colaboradores demostraram em estudo epidemiológico que 3,3% da população idosa brasileira é acometida por essa doença, como a idade é um dos principais fatores de risco para a DP, é possível que sua prevalência venha a crescer, devido ao aumento da expectativa de vida (BARBOSA et al., 2006). Dados recentes do IBGE, estimam que em 2060 o Brasil terá 5 milhões de idosos com mais 90 anos. O que corresponde a uma população dez vezes maior que a atual, sendo que a expectativa de vida média em 2060 chegará a 81,2 anos (IBGE, 2013).

Os sinais clínicos, dessa doença, foram inicialmente descritos em 1817 por James Parkinson (médico inglês e membro do colégio real de 15 cirurgiões) na sua clássica publicação: “Ensaio sobre a paralisia agitante”. Porém, apenas em 1920, Jean-Martin Charcot reconheceu o pioneirismo de James Parkinson na descrição da paralisia agitante denominando-a com o nome de seu descobridor (TOULOUSE & SULLIVAN, 2008).

Clinicamente, a DP é caracterizada por anormalidades motoras que consistem em tremores em repouso, bradicinesia, acinesia, rigidez muscular, perda de equilíbrio e instabilidade postural, as quais são atribuídas à perda de neurônios dopaminérgicos de uma região em particular, localizada no mesencéfalo, denominada substância negra pars compacta (SNpc). A redução na densidade neuronal dessa população celular gera uma diminuição na projeção de fibras dopaminérgicas que aferentam o estriado dorsal causando por sua vez, uma consequente redução dos níveis de dopamina (DA) nessa estrutura dos núcleos da base, como demostrado na **Figura 2** (QUINN 1995; SHULMAN et al., 2011). A DA é o neurotransmissor essencial para as

atividades do sistema modulador da motricidade voluntária, seu detrimento promove também uma diminuição dos níveis de seus metabólitos ácido homovanílico (HVA) e 3,4- dihidroxifenilacético (DOPAC), além da redução da atividade da enzima tirosina hidroxilase (TH), limitante na síntese dos neurotransmissores de catecolaminas (CHO et al., 2013; LANG & LOZANO, 1998; MIZUNO, 1999).

Figura 2 - Corpo estriado de paciente normal e em seguida de paciente com DP, mostrando a depleção da DA na doença.



Fonte: Antunes 2017.

Enquanto a DP está associada com uma patofisiologia complexa que pode potencialmente afetar a maior parte do cérebro, seus sintomas motores são, em grande parte, devido a degeneração dos neurônios dopaminérgicos da SNpc (ALBIN et al., 1989). Estes neurônios dopaminérgicos projetam os seus axônios para o corpo estriado, formando assim a via dopaminérgica nigro-estriatal, que em conjunto, formam uma espécie de equilíbrio, crucial no controle dos movimentos. Estima-se que os sintomas motores da DP aparecem quando a perda de neurônios dopaminérgicos atinge cerca de 50% a 60%, o que corresponde a uma diminuição de 70% a 80% nos níveis de DA no estriado, se manifestando depois que a patologia atingiu um nível avançado (NANDHAGOPAL et al., 2009). Estudos de neuroimagem confirmam que a perda neuronal nessa região inicia de 4-6 anos antes do aparecimento dos sintomas (PONSEN et al. 2010). A origem dessa degeneração neuronal é ainda desconhecida e, provavelmente envolve diversos eventos celulares e moleculares (DAUER &

PRZEDBORSKI, 2003). Estudos apontam que a etiopatogenia da doença envolve mecanismos como estresse oxidativo, fatores gliais e inflamatórios, neurotoxinas ambientais e fatores genéticos (SHAPIRA & OLANOW, 2004). Uma vez iniciado o processo neurodegenerativo por esses fatores causais, uma cascata de eventos secundários deletérios provocaria as alterações neuroquímicas observadas nos pacientes com DP (HIRSCH et al., 2013). Além desses sintomas motores, os pacientes acometidos com a DP apresentam prejuízos não motores que incluem: distúrbios olfativos e de sono, constipação, incontinência urinária, hipotensão ortostática, disfunção erétil, insuficiência autonômica, e vários sintomas neuropsiquiátricos como depressão, alucinações e demência, que podem aparecer tanto antes quanto durante os sintomas motores (CHAUDHURI et al., 2006; FARRER, 2006; SHULMAN et al., 2011).

Após quase dois séculos da descrição inicial, a DP ainda é uma doença de patogênese pouco compreendida, sendo seu tratamento basicamente sintomático, representando uma alternativa meramente paliativa à doença, visto que atuam restabelecendo temporariamente a função dopaminérgica no sistema nervoso central (SNC), sem exercer ação neuroprotetora comprovada (LEV et al., 2003; SHIMOHAMA, 2003). Contudo, os esforços que visam melhorar as limitações dos fármacos utilizados atualmente no tratamento dessa patogênese vêm crescendo, há uma grande procura por tratamentos alternativos ou adjuvantes que visem atenuar os sintomas motores e não-motores, que provoquem menos efeitos colaterais e, principalmente, que sejam capazes de inibir ou retardar o avanço do processo neurodegenerativo.

4. Disfunção mitocondrial e apoptose na morte neuronal da DP

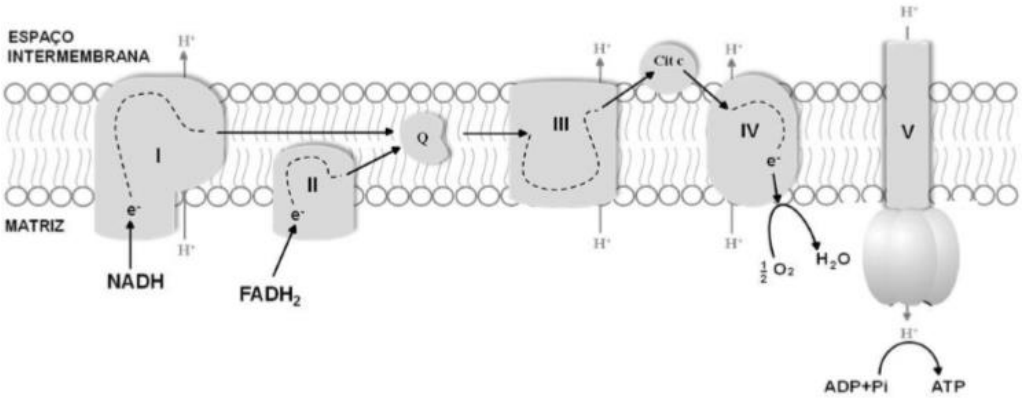
Crescentes evidencias tem destacado o papel da disfunção mitocondrial e da bioenergética na DP. A mitocôndria é uma organela central no metabolismo energético, descoberta em 1857 por Rudolph Albert Von Kolliker, tem em seu interior a ocorrência reações de extrema importância para a célula, como reações do ciclo de Krebs, β-oxidação e fosforilação oxidativa (MITCHELL & MOYLE, 1967). Anos mais tarde, mais um papel da mitocôndria foi descoberto, em 1972 os pesquisadores Kerr e seus colaboradores, demostraram o envolvimento dessa organela na regulação da

homeostasia celular, evidenciando seu papel direto na ativação de vias pró-apoptóticas. Dentre as funções metabólicas relatadas para ocorrer na matriz mitocondrial, destaca-se a síntese de trifosfato de adenosina (ATP) por meio do acoplamento da fosforilação oxidativa com a cadeia de transporte de elétrons. São os danos nas enzimas mitocondriais da cadeia transportadora de elétrons e membranas mitocondriais que interferem na formação do ATP e podem, dessa forma, induzir a apoptose celular.

A mitocôndria é constituída por duas membranas, uma membrana externa e outra membrana interna, um estreito espaço intermembranar e uma ampla matriz (TEDESCHI, 1950). A membrana externa dessa organela é lisa, esférica e permeável a pequenas moléculas e íons, que se movem livremente através dos canais transmembranares. Já a membrana interna é impermeável à maior parte de moléculas de dimensões reduzidas e íons, é nessa membrana que se encontram organizados grandes complexos enzimáticos, estruturados em um sistema nomeado de Cadeia Respiratória Mitocondrial, onde ocorre o processo de fosforilação oxidativa (GRAZINA, 2004).

A fosforilação oxidativa envolve a redução de O_2 a H_2O pelos elétrons cedidos pelos equivalentes redutores – NADH e $FADH_2$, por intermédio de uma série de complexos proteicos (complexo I, II, III, IV – citocromo C oxidase e V – ATP sintase) da cadeia respiratória, permitindo que a energia libertada por estas reações seja armazenada nas ligações do ATP, como esquematizado na **Figura 3**. No entanto, a energia libertada pela oxidação do NADH e $FADH_2$ não é utilizada diretamente para a produção de ATP, o gradiente eletroquímico de prótons gerado durante o transporte de elétrons é utilizado para impulsionar a conversão de ADP (adenosina difosfato) e Pi (fosfato inorgânico) em ATP, por meio do complexo ATP sintase (MITCHELL & MOYLE 1967).

Figura 3: Representação esquemática da fosforilação oxidativa



Fonte: Campos 2012.

De forma simplificada, os elétrons são doados para carreadores específicos, NAD^+ E FAD, formando NADH e $FADH_2$. Os elétrons provenientes de NADH são transferidos para o complexo I que em seguida é oxidado promovendo a redução da coenzima Q. Os elétrons provenientes do $FADH_2$ são transferidos via complexo II diretamente para coenzima Q reduzida. Da coenzima Q os elétrons são transferidos para o complexo III, que reduz o citocromo C. Posteriormente, o citocromo C reduz o complexo IV que irá transferir os elétrons para o O_2, aceptor final da cadeia respiratória. A passagem de elétrons por esses carreadores acompanha uma liberação de prótons para o espaço intermembranas, o que gera um gradiente eletroquímico que favorece a reentrada de prótons por meio da ATP sintase, que finalmente utiliza a energia próton-motriz para fosforilar ADP em ATP (MITCHELL & MOYLE, 1967).

Porém, nem sempre todos os elétrons transportados pela cadeia respiratória chegam a seu aceptor final. Postula-se que aumentos no consumo de O_2 ou no fluxo de elétrons na cadeia, assim como o potencial da membrana interna mitocondrial são determinantes para o extravasamento de elétrons e, consequente, a formação de espécies reativas de oxigênio. Em que os complexos I e III, ao invés de doar os elétrons para o próximo componente da cadeia, os doam ao O_2, formando assim o ânion superóxido ($O^{\bullet}{}_2^-$), um radical livre capaz de causar dano oxidativo em diversos componentes mitocondriais e celulares. Em acordo, Friguet e colaboradores 2008, demostraram que a produção exacerbada de espécies reativas de oxigênio podem inativar diretamente proteínas mitocondriais ou contribuir para oxidação de lipídios e carboidratos gerando uma disfunção mitocondrial a qual é potencialmente perigosa para a célula. Evidências sugerem a hipótese que o complexo mitocondrial I tem

importante papel na etiologia da DP. A atividade do complexo I está reduzida em 35–40% em homogenatos de substância negra de pacientes com DP em estudos *post mortem* (SCHAPIRA et al.,1989; SCHAPIRA et al.,1990)

Em linha ao exposto acima, a apoptose dos neurônios dopaminérgicos é um evento envolvido no processo de neurodegeneração na DP (BATTISTI et al., 2008). Os processos de apoptose acontecem através das enzimas caspases, que pertencem a uma família de proteases. A indução de apoptose intracelular ocorre com a participação da mitocôndria, essas organelas são induzidas a liberar a proteína carreadora de elétrons, citocromo C para o citosol. No citosol, o citocromo C se liga a uma proteína adaptadora chamada Apaf-1, que ativada liga-se a moléculas de procaspases-9 gerando um agregado. Logo em seguida as procaspases-9 são clivadas e ativadas, e seguem ativando outras procaspases, ocasionando uma cascata de morte celular. O envolvimento da apoptose na neurodegeneração dos neurônios dopaminérgicos já foi evidenciado em achados clínicos e estudos com modelos experimentais de DP (ADAMS et al., 2012; SINGH & DIKSHIT, 2007). Ainda na apoptose, a enzima Na^+/K^+ ATPase desempenha um papel fundamental na função celular, regulando a troca de Na^+ e K^+ entre a célula e o espaço intercelular. A diminuição de sua atividade pode motivar a apoptose, devido a alterações na homeostase de K^+, exercendo também um papel central na manutenção neuronal (WANG et al., 2003). A enzima Na^+/K^+ ATPase é, além disso, associada com o processo de envelhecimento, a diminuição da sua atividade pode afetar a via de transdução de sinal, contratilidade, excitabilidade e as funções celulares, em modelos animais, como demostrado por estudos de Kaur e colaboradores 2001, preconizando a diminuição na atividade dessa enzima como fator contribuinte no desenvolvimento de distúrbios neurológicos. Nesse sentido, novos estudos são conduzidos a fim de prover novas estratégias de intervenção visando a modulação dos componentes da cadeia respiratória e da apoptose na fisiopatologia da DP.

5. Depressão associada à DP

A depressão é um distúrbio afetivo grave, caracterizado por um estado de baixo ânimo prevalente e persistente, que é acompanhado por culpa inapropriada, baixa auto-estima, desesperança e pensamentos de morte ou suicídio, o que promove um impacto negativo significativo na qualidade de vida, bem-estar social e profissional,

acarretando um pesado fardo para os pacientes, suas famílias e sociedade (BRENES, 2007; RAPAPORT et al., 2005).

Esses sintomas depressivos acometem os pacientes com DP com uma prevalência de 40-70%, indicando que este transtorno de humor está profundamente relacionado com a referida neurodegeneração (BARNUM & TANSEY 2012; WOLTERS 2008; FRISINA et al., 2009). Estudos tem reportado que a taxa de prevalência de depressão é quase duas vezes maior em pacientes com a DP do que na população geral, sendo mais comum nesta patologia do que em outras doenças crônicas (ROJO et al., 2003). É também relatado que pacientes com depressão associada a DP apresentam maior ansiedade e impulsos suicidas do que pacientes com transtornos depressivos não relacionados a DP (WICHOWICZ et al., 2006; DISSANAYAKA et al., 2011). Outro sintoma comum entre essas duas patologias é a anedonia, acometendo 45% dos indivíduos com DP e, 79% dos pacientes com depressão vinculada a DP (LEMKE et al., 2006). Adicionalmente, as pessoas com DP e depressão são mais propensas a sentir-se desamparadas e apresentam maior deterioração da função cognitiva e dos sintomas motores do que indivíduos com DP sem depressão (BAE et al., 2013).

Entre os pacientes acometidas com a DP, a depressão é reconhecida como uma das principais causas de incapacidade e declínio da qualidade de vida, especialmente nos estágios mais avançados (KANO et al., 2011). A depressão pode enfrentar flutuações, da mesma forma que as manifestações motoras, podendo não progredir de forma linear com a gravidade dos sintomas motores, ocorrendo nos estágios precoces ou tardios da DP, ou até mesmo antes de qualquer comprometimento motor (FINKEL et al., 1996; WELLS et al., 1988). Segundo dados do The Global Parkinson's Disease Survey Steering Comitee, apenas 1% dos pacientes com DP chegam a revelar seus sintomas depressivos a seus médicos. Consequentemente, tais manifestações não são diagnosticados em 65% dos casos e, quando são corretamente diagnosticados, apenas 35% recebem tratamento adequado (SHULMAN 2003; WEINTRAUB et al., 2003). Um problema adicional, é o fato de que pode ocorrer superposição dos sintomas depressivos da DP com outras co-morbidades dessa doença neurogenerativa, visto que, a lentidão psicomotora, diminuição de iniciativa e afeto restrito são sintomas depressivos que podem ser confundidos com a bradicinesia, postura inclinada e com a hipomimia da DP,

desempenhando um papel significativo na redução da qualidade de vida dos pacientes (DOWDING et al., 2006; MARSH et al., 2006; SCHRAG, 2006).

A epidemiologia sugere que relatos de depressão, e ainda, de ansiedade podem preceder o diagnóstico da DP (SHIBA et al., 2000; RICHARD, 2005; TANBERG et al, 1997). No Brasil, dois estudos relatam a ocorrência da depressão em 24% a 38.33% dos pacientes com DP (PRADO et al.,2005, TUMAS et al., 2008). A razão para esta alta prevalência ainda não está totalmente esclarecida, no entanto, observações de características patológicas do SNpc de pacientes com DP deprimidos, sugerem que o circuito nigrostriatal, onde a homeostase de DA é conhecida por ser prejudicada, está envolvido na depressão (FRISINA et al., 2009).

Essa relação entre depressão e PD já vem sendo explorada, recentemente Gustafsson e colaboradores, 2015, investigaram o risco de DP após depressão utilizando o banco de dados de um grande grupo sueco. No estudo, mais de 140.000 indivíduos com depressão foram acompanhados por um período médio de 6,8 anos. Os autores relataram que o risco de ser diagnosticado com PD é acentuadamente maior nos primeiros 3 meses após o diagnóstico de transtorno depressivo maior, e esse risco vai diminuindo gradualmente. Nessa linha, várias hipóteses neurobiológicas sugerem que a depressão é um sintoma prodrômico precoce ou um fator de risco para DP (ARABIA et al., 2007; FANG et al., 2010; WIRDEFELDT et al., 2011). Assim, achados de Gustafsson e colaboradores, 2015, o maior na ligação entre depressão e PD até a data, corrobora com outros resultados, relatando que os sintomas depressivos podem estar presente muitos anos antes da manifestação dos sintomas motores da DP (KANO et al., 2011). Nesse sentido, tal sintoma requer uma atenção especial da comunidade científica a fim de propiciar uma ampliação nas buscas de novas moléculas farmacologicamente ativas, contribuindo assim, para o futuro desenvolvimento de novas alternativas terapêuticas.

6. O papel da neuroinflamação na DP e nos transtornos depressivos

A inflamação é a resposta dos tecidos a agressões locais, depende da imunidade inata e envolve leucócitos e mediadores inflamatórios. A inflamação é a primeira linha de defesa do organismo contra lesões teciduais ou infecções, todavia, uma resposta inflamatória excessiva pode se tornar fonte de uma lesão tecidual ainda maior do que a provocada pelo estímulo inicial (GAO et al., 2003). O termo

neuroinflamação vem sendo utilizado para designar uma inflamação específica no SNC, que não reproduz as características da inflamação periférica, como inchaço, calor e dor. Infecções, traumas, toxinas e outros estímulos são capazes de ativar o sistema imune inato no SNC, impulsionando assim, a microglia. As células microgliais funcionam como uma defesa imune especializada e fazem a mediação da resposta imune inata à invasão de patógenos secretando uma gama de fatores, que incluem: citocinas, quimiocinas, prostaglandinas, espécies reativas de oxigênio e nitrogênio e fatores de crescimento. Alguns desses fatores exercem funções neuroprotetoras e neurotróficas, ajudando no processo de reparação do cérebro, enquanto outros aumentam o estresse oxidativo e desencadeiam a apoptose (TANSEY & GOLDBERG, 2010). As citocinas são pequenas proteínas secretoras que regulam as respostas do nosso corpo a infecções, reações imunes, inflamações e lesões. Incluem citocinas pró-inflamatórias tais como: interleucina-1 beta (IL-1β), interleucina-6 (IL-6), interleucina-12 (IL-12), interleucina-18 (IL-18), fator de necrose tumoral alfa (TNF-α) e interferon gama (IFN-γ) e a citocina anti-inflamatória como a interleucina-10 (IL-10) (CAVAILLON, 2001, HSU et al., 2014). No que se refere a DP e a depressão, essa proteínas, exercem influencias através de suas ações iminentes, bem como a partir de seu impacto sobre o sistema neurotransmissor.

A hipótese da neuroinflamação é uma via em comum entre depressão e DP, nos últimos anos, o pressuposto de que a inflamação contribui ativamente na fisiopatologia da depressão na DP vêm sendo fortemente apoiada (TEISMANN et al., 2003). Uma vez que os neurônios, apresentam pouca ou nenhuma habilidade em se dividir e uma reduzida capacidade de recuperação frente a lesões, tornam-se, portanto extremamente vulneráveis a processos autoimunes e inflamatórios (GAO et al., 2003). Sabe-se que dentro do microambiente cerebral, as células gliais desempenham um papel fundamental nos mecanismos homeostáticos proporcionando a sobrevivência neuronal. Se essa ativação microglial protege ou exacerba a perda neuronal ainda é assunto de debate, embora a maioria das evidências sugiram efeitos tóxicos para os neurônios (HIRSCH et al., 2005).

Na degeneração parkinsoniana, o papel da inflamação na têm estado sob intensa investigação, os fatores neuroinflamatórios têm sido abordados como possíveis promotores da neurodegeneração nigral dopaminérgica, tendo uma função importante nessa patogênese. Em 1988, McGeer e colaboradores evidenciaram pela

primeira vez, um possível envolvimento da neuroinflamação na DP, demostrando ativação da microglia no SNpc em analises *post-mortem* de tecido cerebral humano (MCGEEr et al., 1988). Corroborando, estudos mais recentes de Brodacki, et al., 2008, demonstram níveis elevados de IL-1β, IL-6, TNF-α, no estriado de indivíduos com DP quando comparados a pacientes não parkinsonianos, indicando presença de processo neuroinflamatório. De acordo com resultados de Collins et al. 2012, quando as células da glia são ativadas na DP, elas passam a secretar altos níveis de mediadores pró-inflamatórios, que induzem a morte dos neurônios dopaminérgicos e aumentam ainda mais a ativação destas células, o que resulta em um ciclo de propagação de inflamação e neurodegeneração. Estes dados sugerem que ativação da microglia e neuroinflamação na DP é um alvo anti-parkinsoniano digno de estudo, assim, a modulação da neuroinflamação pode ser uma estratégia potencial de evitar ou reverter a degeneração dopaminérgica (QIAN et al., 2010).

No que se refere a depressão, essa patofisiologia também caracteriza-se pela ativação do sistema de resposta inflamatória com o aumento da produção de citocinas pró-inflamatórias (DANTZER et al., 2008). Os primeiros trabalhos publicados acerca da depressão na década de 80, já descreveram essa patologia pelo envolvimento da inflamação e da ativação glial, desde então, essa hipótese tem papel chave nos transtornos depressivos (MAES et al., 1990). Nos dias de hoje, a hipótese da inflamação ainda vem sendo explorada, um crescente corpo de evidências tem demonstrado que a depressão está associada à hiperativação microglial. Estudos de Tyring, 2006 e Zunszain, 2013, revelam concentrações aumentadas de citocinas pró-inflamatórias no soro e líquido cefalorraquidiano de pacientes com depressão, contrastando com baixas concentrações de citocinas anti-inflamatórias, e ainda correlacionam essa característica com a gravidade da doença. Suplementando essas evidências, resultados de Salazar et al., 2012, e de Wang et al., 2011, indicam que mediadores pró-inflamatórios induzem comportamentos depressivos em modelos experimentais, os quais são inibidos pelo bloqueio da ativação microglial (ARAKAWA et al., 2012; MOLINA-HERNÁNDEZ et al., 2008). Estando de acordo com investigações clínicas que demonstram que a inibição de mediadores pró-inflamatórios exibem efeitos antidepressivos também em pacientes humanos (KÖHLER et al., 2014).

Além de todas essas consequências relatadas, a ativação microglial é apontada para convergir em diminuição do suporte neurotrófico e da neurogênese, através da baixa regulação de marcadores de plasticidade, potencializando assim, seus efeitos negativos a nível de SNC (BARRIENTOS et al., 2006; BEN MENACHEM-ZIDON et al., 2008; WU et al., 2007). Nesse contexto, torna-se conveniente buscar terapias imunomodulatórias, capazes de proteger o cérebro contra insultos inflamatórios e suas consequências.

7. Neurotrofinas e SNC

As neurotrofinas (NTs) são um grupo de proteínas diméricas de baixo peso molecular (aproximadamente 13KDa), que se relacionam profundamente com o desenvolvimento do sistema nervoso em todos os vertebrados. São ligantes extracelulares que influenciam na diferenciação, manutenção e sobrevivência neuronal, sendo reguladores críticos da formação e plasticidade das redes neuronais (HUANG et al., 2001). Desde a sua descoberta, receberam um interesse significativo da comunidade científica devido suas capacidades. Na década de 50, Rita Levi-Montalcini e seus colaboradores revelaram, em um experimento envolvendo glândulas salivares de cobaias, a presença de uma substância capaz de promover o crescimento de neurônios do gânglio sensitivo e do sistema nervoso simpático, a qual denominaram de fator de crescimento nervoso (NGF). Seus estudos demonstraram ainda que, apesar do grande número de neurônios em desenvolvimento presentes em gânglios embrionários, apenas aqueles que estabelecem conexões bem sucedidas com outros neurônios ou fibras musculares conseguem se manter vivos, revelando o papel essencial das interações celulares no desenvolvimento (HAMBURGER, LEVI-MONTALCINI, 1949; LEVI-MONTALCINI et al., 1951). A partir desses resultados, Riccio e colaboradores, 1997, reforçam e estabelecem a teoria neurotrófica, através da qual o equilíbrio entre a sobrevivência e a morte dos neurônios é regulada pelas NTs, uma vez que a ausência desses fatores neutróficos podem motivar a morte neuronal (RICCIO et al., 1997).

Os fatores neurotróficos compõem duas principais famílias: a das NTs que incluem o NGF, o fator neurotrófico derivado do cérebro (BDNF), a neurotrofina-3 (NT-3), e a família do fator neurotrófico derivado da glia (GDNF). Estes fatores são responsáveis por influenciar na sobrevida, no crescimento e na maturação neuronal

embrionária, tendo seus efeitos sobre a função neuronal persistentes na vida adulta, convergindo assim como potenciais neuroprotetores e restauradores funcionais em distúrbios neurológicos (AUTRY & MONTEGGIA, 2012).

Entre os promotores da plasticidade cerebral, como referido anteriormente o NGF foi o primeiro a ser descrito (LEVI-MONTALCHINI, 1951), e atualmente é o melhor caracterizado. É produzido principalmente no córtex, hipocampo, glândula pituitária e medula espinhal. Essa neurotrofina tem característica de promover a sobrevivência de neurônios sensoriais primários, assim como de neurônios simpáticos e colinérgicos do gânglio basal, além de proteger contra neurodegeneração (SHOVAL & WEIZMAN, 2005). Durante a fase de desenvolvimento embrionário, essa neurotrofina aumenta a sobrevida de neurônios do gânglio dorsal e da grande maioria dos neurônios do sistema simpático e dos neurônios sensitivos, mas não dos neurônios do sistema parassimpático (LEVI-MONTALCINI 1987; YIN et al., 1998). Seu papel em patologias como depressão e doenças neurodegenerativas vem sendo amplamente abordado (FURUKAWA 2015; MOOSAVI et al., 2015).

Décadas mais tarde, isolou-se outra proteína desta família, o BDNF. Esse segundo membro das NTs purificado e identificado em 1982, por Barde e colaboradores, é uma proteína formada por 120 aminoácidos e sua estrutura é aproximadamente 50% semelhante ao NGF (GONUL et al., 2005). O BDNF é sintetizado e liberado por células de Schwann e por células do músculo esquelético (HADJICONSTANTINOU et al., 2001). Estudos *in vitro* e *in vivo* demonstraram que esse fator neurotrófico possui importante efeito na sobrevivência e na diferenciação de neurônios sensitivos e de neurônios motores (HADJICONSTANTINOU et al., 2001; HAMILTON et al., 1960). Seus efeitos podem variar conforme a fase do desenvolvimento, no início da fase fetal essa neurotrofina é importante para a formação e maturação dos neurônios em geral, já durante a fase adulta, tem papel fundamental no processo de consolidação da memória (POST, 2007). Estudos prévios de Katoh-Semba, 2007, indicam que essa neurotrofina tem produção aumentada no ser humano até por volta dos 40 anos, apresentando queda após essa idade. Frente a isso, seu papel em doenças relacionadas ao envelhecimento, como na DP vem sendo alvo de interesse da comunidade científica (MOOSAVI et al., 2015). Além disso, suas atribuições em transtornos psiquiátricos também vem sendo evidenciadas (FURUKAWA 2015).

A NT-3 foi o terceiro membro das NTs a ser identificado. Essa proteína é formada por 119 aminoácidos e sua estrutura é 55% semelhante ao NGF, é encontrada tanto no SNC (bulbo olfatório, cerebelo e hipocampo) como também no sistema nervoso periférico (timo, músculo, fígado, rins e baço) (FUKUMOTO et al., 2001; GONUL et al., 2005). Atua promovendo sobrevivência e diferenciação de neurônios sensitivos e do sistema parassimpático, possui pouca influência em neurônios do sistema simpático e promove a sobrevivência de neurônios motores *in vivo* (GONUL et al., 2005; HAMILTON et al., 1960). A diminuição dessa neurotrofina é observada em modelos animais de neurodegeneração e possui papel bastante apreciado na fisiopatologia da DP (MENDOZA et al., 2014; NGEMA & MABANDLA 2017).

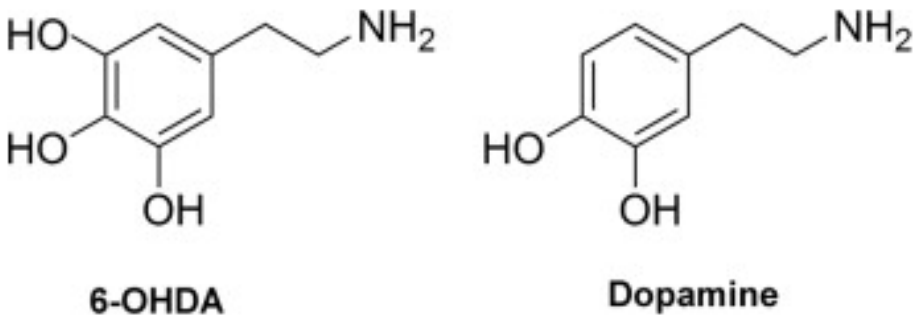
O GDNF é largamente encontrado nas regiões cerebrais, principalmente em regiões do estriado e áreas prosencefálicas (AIRAKSINEN & SAARMA, 2002). É inicialmente produzido na forma de um precursor e sua ativação ocorre, provavelmente por clivagem proteolítica. Exerce papel fundamental no desenvolvimento e manutenção de células gliais, serotoninérgicas e dopaminérgicas (DUCRAY et al, 2006). Evidencias de Airaksinen & Saarma, 2002 demonstram que esse fator neurotrófico apresenta como característica apreciável, a capacidade de exercer efeito neuroprotetor na população de neurônios centrais e periféricos (AIRAKSINEN & SAARMA, 2002). Em linha, estudos de Ngema e Mabandla, 2017, apontam que a reversão da DP induzida experimentalmente por 6-OHDA em ratos, se dá pela modulação de fatores neurotróficos como GDNF e NT-3 no estriado (NGEMA & MABANDLA 2017). Embasando-se nas evidencias acima, postula-se a hipótese da plasticidade para constituir um importante instrumento de avaliação no âmbito da neurobiologia da neurodegeneração.

8. 6-hidroxidopamina (6-OHDA) como modelo de DP

O uso de toxinas indutoras em modelos animais tem sido crucial para a elucidação da fisiopatologia da DP e para o desenvolvimento de estratégias terapêuticas voltadas para o tratamento de seus sintomas. Além disso, esses modelos são empregados para lançar descobertas sobre mecanismos patogênicos envolvidos nessa doença com o objetivo final de desenvolver compostos neuroprotetores (BOVÉ & PERIER, 2012).

6-OHDA é um análogo hidroxilado do neurotransmissor DA (BLUM et al., 2001) **(Figura 4)**. Foi inicialmente isolado através de Senoh e colaboradores em 1959. Seus efeitos biológicos foram demonstrados pela primeira vez em 1963 por Porter e colaboradores, que constataram que 6-OHDA é capaz de induzir depleção de noradrenalina em nervos simpáticos para o coração (PORTER et al., 1963). A 6-OHDA é uma das neurotoxinas mais frequentemente utilizadas em modelos experimentais de degeneração da substancia negra, tanto *in vitro* como *in vivo* (SCHOBER, 2004). A caracterização desse análogo hidroxilado de DA, como uma toxina de indução de degeneração dos neurônios dopaminérgicos no trato nigro-estriatal o tornou uma ferramenta amplamente utilizada para induzir parkinsonismo em roedores, porem, 6-OHDA não é eficiente em atravessar a barreira sangue-cérebro, dessa forma, requer injeção direta no cérebro. Esse foi o primeiro modelo conhecido de indução de DP, proposto por Ungerstedt, 1968. Embora raro, 6-OHDA também tem sido utilizado em gatos, cobaias, cães, e macacos (BÉZARD et al 1998; BÉZARD & PRZEDBORSKI 2011).

Figura 4 - Comparação entre a estrutura química da toxina 6-OHDA e do neurotransmissor DA.

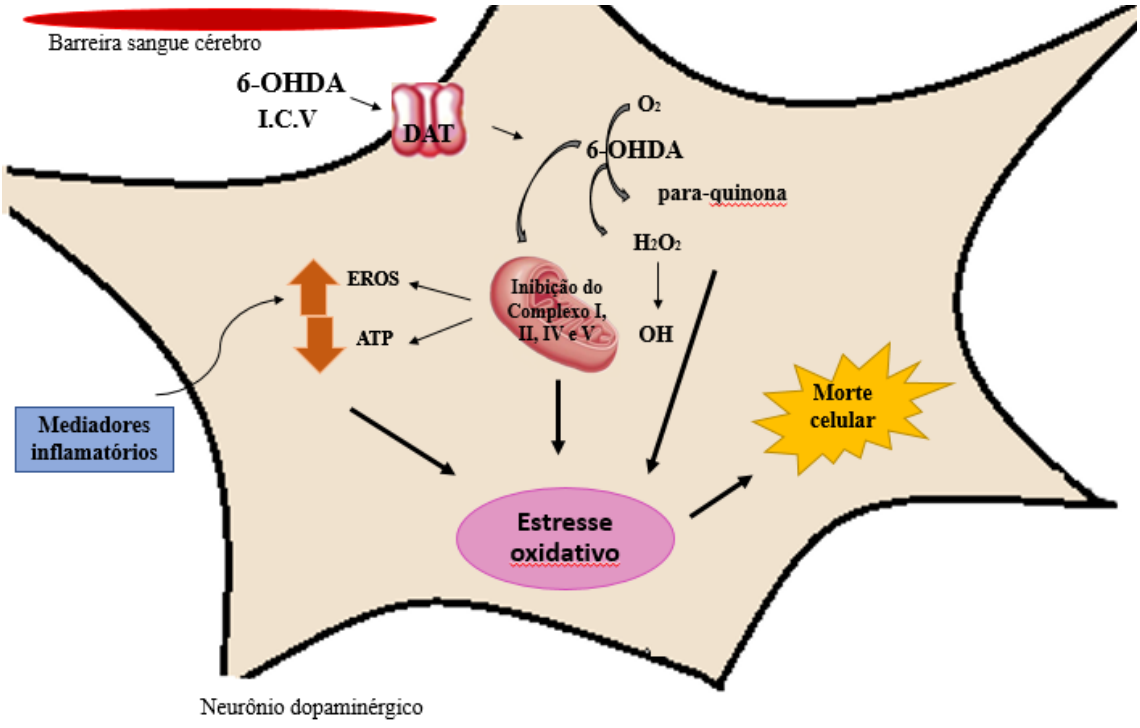


Fonte: Antunes, 2017.

Após sua injeção no estriado, essa neurotoxina é levada aos neurônios dopaminérgicos através do transportador de DA, DAT, que têm elevada afinidade para esta molécula devido à sua semelhança química com a DA (LUTHMAN et al., 1989). Uma vez transportada a 6-OHDA degenera seletivamente a via dopaminérgica nigroestriatal induzindo o estresse oxidativo, a inflamação e finalmente a morte celular,

como esquematizado na **Figura 5**. A injeção, unilateral intraestriatal de 6-OHDA induz acentuadas assimetrias de comportamento, déficits neuroquímicos e alterações histológicas semelhantes à DP, esse modelo reproduz muitas das características, incluindo redução dos níveis de DA no corpo estriado e da TH, também atua na ativação da microglia no estriado como demostrado por Cicchetti et al., 2002 e Mogi et al., 2000 atuando na elevação de marcadores inflamatórios estriatais, replicando, ainda mais, o que é visto no cérebro *post-mortem* de pacientes com a DP (BOVÉ & PERIER, 2012; MOGI et al., 1994). Ainda, uma vez dentro dos neurônios, a 6-OHDA se acumula e sofre auto-oxidação enzimática promovendo a formação de espécies reativas de oxigênio, redução dos níveis de proteínas anti-oxidantes, assim como interação direta com os complexos I e IV da cadeia respiratória mitocondrial, conduzindo ao estresse oxidativo (GLINKA et al., 1997; KUNIKOWSKA & DUTY, 2001; MAZZIO et al., 2004). Muitos desses eventos parecem ocorrer no cérebro durante o desenvolvimento da DP, o que fornece suporte para a validade do modelo com 6- OHDA.

Figura 5 - Neurotoxicidade induzida pela 6-OHDA. DAT: transportador de DA. EROs: espécies reativas de oxigênio.



Fonte: Antunes, 2017.

A 6-OHDA é uma candidata como possível toxina endógena para o início do processo neurodegenerativo da DP, uma vez que é um produto do metabolismo da DA e é o resultado de ataque de radicais hidroxilas (GRAHAM, et al., 1974). Vale a pena enfatizar que 6-OHDA foi encontrada no núcleo caudado humano e na urina de pacientes tratados com L-dopa (ANDREW et al., 1993; CURTIUS et al., 1974).

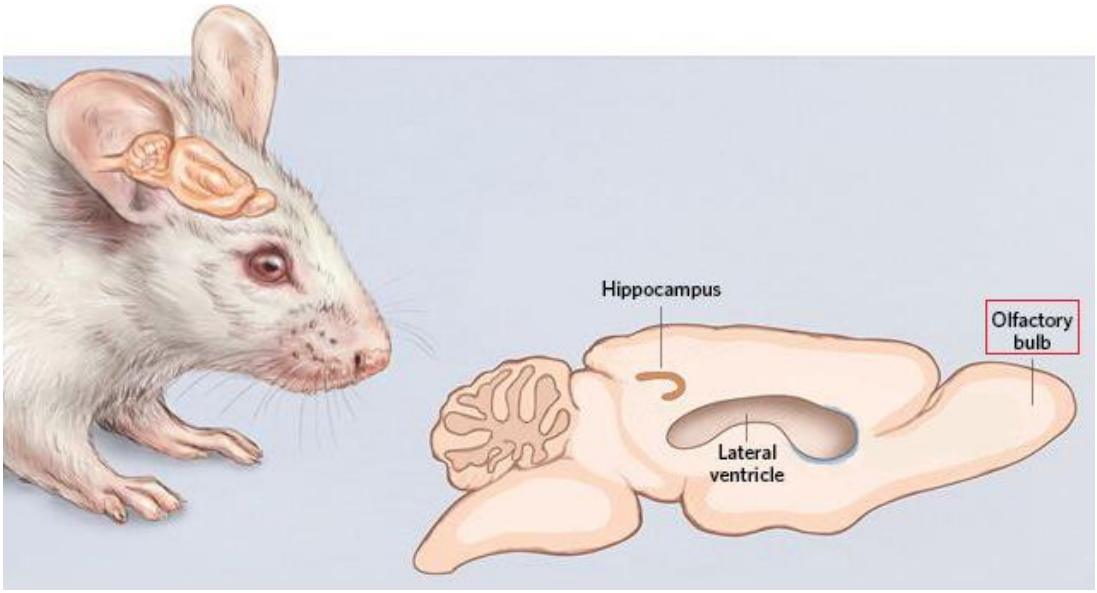
O modelo de 6-OHDA compartilha uma falha comum com muitos outros modelos animais de PD, uma vez que não conduz à formação de uma característica patológica da DP, os corpos de Lewy. Os corpos de Lewy são inclusões eosinófilas que contêm proteínas ubiquitinadas tais como α-sinucleína acumuladas no cérebro de pacientes com DP (BABA et al., 1998; BRAAK et al., 2001; SPILLANTINI et al., 1997). Um relatório recente da formação de agregados contendo parkin em ratos lesionados com 6-OHDA é, portanto, um avanço importante, mas requer confirmação (UM et al., 2010). Em geral, o modelo unilateral de 6-OHDA é extensivamente usado como um modelo pré-clínico a fim de avaliar os efeitos antiparkinsonianos e neuroprotetores de novas terapias farmacológicas (CHAN et al., 2010; ILIJIC et al., 2011; JIANG et al., 1993).

9. Bulbectomia olfatória (BO) como modelo de depressão

Baseando-se nas evidencias de nossos estudos anteriores, onde hesperidina atuou revertendo o comportamento tipo depressivo no teste de suspensão de cauda (TST) no modelo de 6-OHDA, e em dados de nosso grupo demonstrando o papel desse flavonoide em algumas vias de sinalização na depressão em testes comportamentais, se torna interessante investigar o efeito antidepressivo de hesperidina utilizando o modelo da BO (ANTUNES et al., 2014; DONATO et al., 2012, DONATO et al., 2014). A remoção cirúrgica dos bulbos olfatórios em ratos ou camundongos é um modelo farmacológico frequentemente utilizado para prever eficácia antidepressiva, esse modelo interrompe as conexões eferentes e produz efeitos extensos e variados em diferentes regiões do cérebro, promovendo alterações complexas, acarretando uma série de modificações comportamentais, cognitivas e neuroquímicas muitas das quais são compatíveis com aquelas encontradas em indivíduos deprimidos, se tornando uma ferramenta valiosa para avaliar os efeitos de

novos medicamentos antidepressivos **(Figura 6)** (DEUSSING, 2006; HENDRIKSEN et al, 2012; VARELA-NALLA 2013).

Figura 6 - Esquema de bulbo olfatório de camundongos.



Fonte: Varela-Nalla, 2013.

Dentre as modificações neuroquímicas influenciadas pela remoção dos bulbos no hipocampo, estudos enfatizam uma maior ativação de citocinas pró inflamatórias, juntamente com um declínio na neuroplasticidade, dada pela redução dos fatores neurotróficos nessa estrutura (RINWA, et al., 2013). Além disso, ratos bulbectomizados apresentam uma diminuição da liberação de serotonina, noradrenalina e dopamina no cérebro (SATO et al., 2010b; ZUEGER et al., 2005; SONG; LEONARD, 2005). Considerando as alterações comportamentais, o sistema dopaminérgico permanece fortemente envolvido em comportamentos relacionados à emoção em roedores no modelo da BO, estando associado à memória, ao prazer e à motivação (Mele et al., 2004; Setlow e McGaugh, 1998). O modelo experimental da BO tem sido proposto como de alta validade para indução de depressão, uma vez que, uma vasta gama de medicamentos antidepressivos conhecidos ou presumíveis foram testados quanto à sua capacidade para normalizar a típica hiperatividade resultante da remoção dos bulbos (LEONARD, 1984; SONG & LEONARD, 2005). Nessa perspectiva, o modelo da BO pode ser empregado para fornecer evidencias

quanto aos efeitos biológico do flavonoide hesperidina em um modelo especifico de depressão.

OBJETIVOS

Objetivo geral

Investigar a eficácia do flavonoide hesperidina nas alterações comportamentais e neuroquímicas no modelo de DP induzida por 6-OHDA, e no modelo de depressão induzida por OB em camundongos.

Objetivos específicos

1. Avaliar o potencial neuroprotetor da hesperidina, sobre o comportamento motor, atividade rotatória, ansiedade, anedonia, memória e disfunção olfatória nos animais submetidos a microinjeção intra-estriatal de 6-OHDA;

2. Avaliar o potencial neuroprotetor da hesperidina sobre o dano neuronal causado pela lesão com 6-OHDA, analisando:

- Imunorreatividade da TH, um marcador dopaminérgico, no estriado;

- Concentração de DA e dos metabólitos DOPAC e HVA no estriado;

3. Determinar os efeitos de hesperidina sobre os níveis de BDNF, NGF, NT-3 e GDNF no estriado de camundongos infundidos com 6-OHDA;

4. Verificar o efeito protetor de hesperidina sobre as alterações neuroinflamatórias induzida por 6-OHDA no estriado, verificando:

- níveis das citocinas pró inflamatórias: IFN-γ, TNF-α, IL-1β, IL-II e IL-6;

- níveis da citocina antinflamatórias: IL-10;

5. Investigar o efeito protetor de hesperidina frente as alterações mitocondriais associadas à neurotoxicidade induzida por 6-OHDA no estriado, verificando:

- Atividade dos complexos mitocondriais I, II, IV e V no estriado;

- O potencial da membrana mitocondrial no estriado;

6. Avaliar os efeitos da hesperidina na atividade da enzima Na^+/K^+ ATPase no estriado de camundongos após a administração de 6-OHDA;

7. Determinar o efeito antiapoptótico de hesperidina frente a neurotoxicidade de 6-OHDA no estriado através das caspases 3 e 9.

8. Examinar um possível efeito antidepressivo de flavonoide hesperidina na depressão induzida pela BO em camundongos analisando:

- Comportamento motor, memória, comportamento tipo-depressivo e anedonia nos camundongos que tiveram os bulbos olfatórios removidos.

9. Analisar os níveis de BDNF e NGF, no hipocampo de camundongos após tratamento com hesperidina no modelo da BO;

10. Verificar o efeito do tratamento com hesperidina na neuroinflamação através dos níveis de IL-1β e IL-6 no hipocampo de camundongos após a BO;

11. Determinar a atividade da enzima AchE no hipocampo de camundongos no modelo da BO.

PARTE II

RESULTADOS

MANUSCRITO I

Submetido à Parkinsonism & Related Disorders e apresentado na tese conforme normas da revista

Manuscript Number:

Title: Neuroprotective effects of hesperidin on 6-OHDA-induced Parkinson's model through apoptosis, mitochondrial dysfunction and rescue of dopaminergic neurodegeneration

Article Type: Full Length Article

Keywords: Neurodegenerative disease; flavonoid; treatments; dopaminergic neurons.

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Abstract: Introduction
Parkinson's disease (PD) is the second most common neurodegenerative disease. The potential causes of this pathophysiology remain uncertain, but recent studies suggest a significant role of mitochondrial dysfunction and apoptosis, with several pathways being suggested as playing a role in dopaminergic degeneration. Conversely, none of the drug is fully effective, acting only to decrease symptoms, promising therapies for this disease still need to be explored.
Methods
Our previous study demonstrated hesperidin to exhibit strong neuroprotective effect through anti-oxidative. In the present study, we investigated the potential contribution of hesperidin (50mg/kg, per oral, during 28 days) in apoptosis, mitochondrial dysfunction, dopaminergic neurons restoration and behavioral changes (locomotor and rotational activities, olfactory and memory tests), in 6-hydroxydopamine (6-OHDA) model of PD in mice.
Results
Hesperidin treatment restored the mitochondrial enzymes activities of the complexes I, II, IV and V, mitochondrial membrane potential and Na+/K+ ATPase. The treatment also reversed the increase of caspase 3 and 9 activities and the depletion of tyrosine hydroxylase (TH) positive neurons, dopamine (DA) and its metabolites in striatum, besides recovering the behavior changes.
Conclusions
Taken together, our results indicate that hesperidin reversed the dopaminergic neurodegeneration through of mitochondrial dysfunction and apoptosis pathways modulations in 6-OHDA model, thus providing a potential therapeutic strategy for PD.

Neuroprotective effects of hesperidin on 6-OHDA-induced Parkinson's model through apoptosis, mitochondrial dysfunction and rescue of dopaminergic neurodegeneration

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Highlights

- 6-OHDA microinjection caused dopamine depletion and behavioral déficits
- Hesperidin reversed deficits observed in the Parkinson’s disease model
- Hesperidin protected against mitochondrial dysfunction and apoptosis
- Depletion of tyrosine hydroxylase positive neurons are protected

Abstract

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease. The potential causes of this pathophysiology remain uncertain, but recent studies suggest a significant role of mitochondrial dysfunction and apoptosis, with several pathways being suggested as playing a role in dopaminergic degeneration. Conversely, none of the drug is fully effective, acting only to decrease symptoms, promising therapies for this disease still need to be explored.

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Our previous study demonstrated hesperidin to exhibit strong neuroprotective effect through anti-oxidative. In the present study, we investigated the potential contribution of hesperidin (50mg/kg, *per oral*, during 28 days) in apoptosis, mitochondrial dysfunction, dopaminergic neurons restoration and behavioral changes (locomotor and rotational activities, olfactory and memory tests), in 6-hydroxydopamine (6-OHDA) model of PD in mice.

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Keywords

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1. Introduction

Parkinson's disease (PD) is a neurodegenerative movement disorder that is characterized by the progressive degeneration of dopamine (DA) neurons in the substantia nigra (SN) that massively projects into the striatum [1]. The PD is the second most important neurodegenerative disorder in the elderly population, after Alzheimer's disease, affecting 1.7% of the aged population [2]. This disorder is characterized by a wide range of motor and non-motor symptoms, however, its first motor symptoms are clinically undetectable until 50% of the neurons in the SN are damaged or more than 70% of the terminals in the striatum are lost [3].

The PD cause severe cognitive, psychosocial and social consequences. In line, clinical and basic neuroscientists have recently started to concentrate on non-motor symptoms such as depression, anxiety, memory deficits, apathy, insomnia, sleep disturbances, olfactory dysfunction, which are closely related to the daily quality of life of PD patients, since those deficits are frequently observed during all stages of disease [4, 5]. The pathogenesis of PD is complex, current therapeutic strategies available only provide symptomatic improvements. These complexities are accompanied by clinical challenges, its etiology and treatment is serious challenge to modern medicine. In this regard, novel compounds devoid of psychomotor, biochemical, neuropsychiatric and autonomic complications are under experimental scrutiny in preclinical studies and clinical trials [6]. The causes of PD are still unclear, recent evidences show the involvement of mitochondria dysfunction, oxidative stress and apoptosis [7, 8, 9].

Neurodegeneration models have been developed to understand the physiopathological mechanisms underlying PD [10, 11]. In this study, we used 6-hydroxydopamine (6-OHDA) exposure to induce unilateral lesion, a well-known experimental model of dopaminergic toxicity. Among these neurochemical effects are attribute to PD like lower levels of striatal monoamines [12]. It is also known that this model induces a mitochondrial dysfunction, this organelle is a major source of reactive oxygen species (ROS), being related to disease progression [13, 14]. As our previous data has already demonstrated the antioxidant potential of hesperidin (4′-methoxy-7-O-rutinosyl-3′,5-dihydroxyflavanone) in striatum, we will extend our study to the role of mitochondrial dysfunction, as well in mitochondrial membrane potential (MMP) and Na^+/K^+ ATPase activities in PD. The apoptosis is another pathogenic factor with a key

role under exposure of cells to neurotoxins like 6-OHDA [15], thus, we also will evaluate caspase 3 and 9 activities in striatum.

In recent years, natural products, especially plant polyphenols, have attracted progressively more attention as supplemental interventions to modulate neuronal function and control central nervous system (CNS) diseases, including PD [16, 17]. The polyphenols effect appears to be underpinned by the processes that they interact with important neuronal signaling cascades in the brain [18]. In this context, hesperidin is a specific flavonoid glycoside that is frequently found in citrus fruits [19], it has been reported to possess significant antioxidant, anti-inflammatory and antidepressant-like properties in mice [20, 21, 22]. Our previous results indicated that hesperidin exerts therapeutic effects of PD through antioxidant perspective and provides substantial evidence for preclinical application [16].

Thus, the present study tested the hypothesis of hesperidin on apoptosis, mitochondrial dysfunction, dopaminergic neurons restoration and behavior changes in a model of PD induced by 6-OHDA in mice.

2. Materials and methods

2.1 Animals

The experiments were conducted using male C57BL/6 mice (25-35 g, 3-6 months old). Animals were maintained at constant room temperature (21 ± 1 °C) with free access to water and food, under a 12:12 h light:dark cycle (lights on at 07:00 h), manipulations were carried out during the light portion of the cycle. 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 #001/2013) of the Federal University of Pampa, Brazil. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2 Reagents

All reagents, used in this study, were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.3 Experimental design

All animals were submitted to exteriotaxic surgery, to 6-OHDA infusion (6-OHDA group) or only surgery (SHAM). The animals remained 7 days of recovery, then, they were exposed to treatment with hesperidin or saline (50ml/kg of body weight).

Hesperidin was dissolved in distilled water, given by gavage (*per oral*, p.o.). Mice were treated with vehicle or hesperidin, once a day for twenty eight days. The solutions were freshly prepared each day. Controls received an identical volume of saline (vehicle). Mice were assigned to one of the following groups: (I) SHAM-operated/saline (SHAM/vehicle) as the control group; (II) SHAM-operated/hesperidin treated mice (SHAM/hesperidin); (III) 6-OHDA-mice treated with saline (6-OHDA/vehicle); (IV) 6-OHDA-mice treated with hesperidin. After finish of treatments, the mice were submitted to behavior tests and neurochemical analysis **(Fig. 1)**.

2.4 Stereotaxic surgical injection of 6-OHDA

Surgery was performed under anesthesia with 10 mL/kg of 1% ketamine and 0.2% xylazine. 6-OHDA (5 µg in 2 µL of 0.9% NaCl with 0.2 µg/mL 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) [23]. After the injection, the syringe was maintained in the brain for an additional 3 min before it was slowly retracted. Controls were injected with vehicle

2.5 Behavioral assessment

2.5.1 Open field test (OFT)

To verify the effects of hesperidin and 6-OHDA administration on locomotor activity, the animals were submitted individually to a 5 min OFT (Insight model EP 154C) 24 h after the last treatment. The apparatus was cleaned with an ethanol solution (10% v/v) and dried with paper towels after each trial in order to prevent odors from carrying-over between trials. The parameter observed included the distance (unit: mm) [24].

2.5.2 Olfactory Discrimination Task (ODT)

The olfactory discrimination ability was assessed using a protocol previously described by Prediger et al. [25]. This task is based on the fact that rodents usually disclose preference for places impregnated with their own odor (familiar compartments) than places with other non-familiar odors. In short, each mice was placed for 5 min in a cage divided in two equal areas separated by an open door, where it could choose between one compartment with fresh sawdust (non-familiar compartment) and another with unchanged sawdust (familiar compartment) that the same mice had occupied for 3 days before the test. Each animal was initially placed in the center of the non-familiar compartment, and the time spent by the mice in both

compartments (familiar vs. non-familiar) was recorded. Generally, mature male mice are able to discriminate between the familiar and the non-familiar compartments, spending much more time in the familiar compartment since they significantly prefer their own odor. The results were expressed as the percentage of time the mice remained in the familiar compartment.

2.5.3 Barnes Maze

Spatial learning and working memory were evaluated using the Barnes maze [26]. The maze consists of a circular platform (92-cm diameter) surrounded by 20 holes (5-cm diameter, equidistant), one providing an escape route. The mice was placed in a dark chamber in the center of the maze and aversive stimuli subsequently triggered (bright light and noise). The animal was then given the opportunity to leave the maze by crawling through the hole allowing the escape. In the spatial acquisition phase, animals were subjected to four trials per day for four consecutive days, with a constant inter-trial interval time of 20 min. The number of holes explored and the time required for the mice to locate the escape hole were quantified. For the reference memory phase or probe trials, animals were tested on the 5th day (short-term retention) and 12th day (long-term retention) following the first trials, omitting training sessions between test trials. During the probe trials, the escape route was removed and replaced with a standard hole through which the animal could not enter. The mice was tested during a 90-s period. The latency to reach the virtual target hole was recorded, the outcome measure corresponding to the spatial memory capacity of the animal.

2.5.4 Rotational behavior

Twenty-eight days after surgery, animals were tested for rotational behavior after receiving an intraperitoneal bolus injection of apomorphine hydrochloride (0.6 mg/kg; intraperitoneal, i.p.; from Sigma-Aldrich). Rotational testing was conducted following the methodology initially described by Ungerstedt [27]. Briefly, animals were placed inside a cylindrical container (33 cm diameter and 35 cm height) and contralateral rotations (the number of 360° contralateral turns) were counted for 60 min in a quiet isolated room.

2.6 Tissue preparation

After behavioral tests, mice were sacrificed with a barbiturate overdose (pentobarbital sodium 150 mg/kg, intraperitoneal, i.p.). The striatum was removed and rapidly homogenized in 50 mM Tris-Cl, pH 7.4. The homogenate was centrifuged at

2400*g* for 15 min at 4°C, and a low-speed supernatant fraction (S1) was used for assays.

2.7 Neurochemical analysis

2.7.1 Immunohistochemistry of Tyrosine hydroxylase (TH)

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 and Paxinos, [28] and Oorchot, [29] 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 [30, 31, 32]. 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-positive 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.

2.7.2 Determination of monoamine 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 [33]. Tissue homogenate (10% w/v) was sonicated in 0.1 M $HClO_4$ for 30 s, centrifuged at 4 °C for 15 min at 15,000 rpm, and the supernatant was filtered (0.2 µm, Millipore). A 20-µL sample was then injected into the C-18 HPLC column. The mobile phase was 0.163 M citric acid (pH 3.0), containing 0.02 mM NaCl with 0.69 mM sodium octanesulfonic acid as the ion pairing reagent, 4% v/v acetonitrile and 1.7% v/v tetrahydrofuran. DA, DOPAC and HVA were electrochemically detected, using an amperometric detector (Shimadzu, Japan). The amount of monoamines was determined by comparison with freshly prepared standards, and their concentrations were expressed as ng/mg of tissue.

2.7.3 Mitochondria Isolation

Mitochondria from mice brain striatum were isolated by following standard protocol [34]. The mitochondrial protein content was estimated using the method of Lowry et al. [35].

2.7.4 Estimation of NADH dehydrogenase (Complex-I) activity

NADH dehydrogenase activity was measured by catalytic oxidation of NADH with potassium ferricyanide as an artificial electron acceptor at excitation (350 nm) and emission (470 nm) wavelengths for NADH [36]. The reaction mixture consisted of 200 µl of 10 mM potassium ferricyanide, 60 µl of 1 mM NADH in 2 mM potassium phosphate

buffer, and 2.64 ml of 0.12 M potassium phosphate buffer. Activity of NADH dehydrogenase was expressed as nmol NADH oxidized/min/mg protein.

2.7.5 Estimation of succinate dehydrogenase (Complex-II) activity

The mitochondrial succinate dehydrogenase (SDH) was determined by the progressive reduction of nitro blue tetrazolium (NBT) to an insoluble colored compound, diformazan (dfz), which was measured at 570 nm [37]. Briefly, the reaction mixture consisted of 1.5 ml of phosphate buffer (0.2 M, pH 7.8), 0.2 ml of succinic acid (0.6 M, pH 7.8), 0.3 ml of BSA (1%, w/v), and 0.1 ml of 0.03 M potassium ferricyanide and the reaction was started by addition of mitochondrial preparation. The SDH activity was expressed as micromole formazan produced/min/mg protein.

2.7.6 Estimation of cytochrome-C oxidase (Complex-IV) activity

Complex-IV activity was measured in mitochondrial preparation according by Storrie and Madden [38]. Briefly, first the cytochrome c was reduced by the addition of a few crystals of sodium borohydride and then neutralized to pH 7.0 by 0.1 M HCl. The reduced cytochrome c (0.3 mM) was added to 0.075 M phosphate buffer (pH 7.4) and the reaction was initiated by addition of appropriate amount of mitochondrial suspension. The decrease in absorbance was measured at 550 nm for 3 min. Results were expressed as nmol cytochrome-c oxidized/min/mg protein ($\varepsilon 550 = 19.6\ mmol^{-1}\ cm^{-1}$).

2.7.7 Estimation of F1F0 ATP synthase (Complex-V) activity

Mitochondrial F1F0 synthase was measured according to Griffiths and Houghton [39]. Briefly, mitochondrial suspension was incubated in 500 ml of ATPase buffer (50 mM Tris HCl and 5 mM $MgCl_2$, pH 7.5) at 37 °C with 5 mM ATP for 10 min. The reaction was stopped by adding 500 ml of 10% (w/v) trichloroacetic acid. The contents were centrifuged at 3000 g for 20 min and then 500 ml of supernatant was mixed with 500 ml of water. After, the inorganic phosphate concentration was measured by the method of Fiske and Subbarao [40]. Results were expressed as nmol ATP hydrolyzed/min/mg protein.

2.7.8 MMP

MMP is monitored with the help of fluorescent cationic dye tetra-methyl rhodamine methyl ester. Briefly, 100 µl of mitochondrial suspension was added to 890 µl of assay buffer (80 mM NaCl, 75 mM KCl, 25 mM d-glucose, and 25 mM HEPES, pH 7.4) and then 10 µl of 15 µM of TMRM solution was added to the above mixture

and incubated for 15 min at 37 °C. After incubation 400 µl of PBS was added and centrifuged at 10,000 rpm. The pellet was collected in PBS and the rhodamine dye taken up by mitochondria was measured with a spectrofluorometer (Hitachi, F-2500, Japan) [41]. The florescence emission was read at an excitation λ of 535 ± 10 nm and emission λ of 580 ± 10 nm using slit no. 10. The peak fluorescence intensity recorded was around 570 ± 5 nm. The results were expressed as fluorescence intensity value/mg protein.

2.7.9 Measurement of Na^+/K^+ ATPase activity

Activity of Na^+/K^+ ATPase was measured in striatum according to Silva et al. [42]. Briefly, the reaction medium consisted of 30 mM Tris–HCl buffer (pH 7.4), 0.1 mM EDTA, 50 mM NaCl, 5 mM KCl, 6 mM $MgCl_2$, and 50 µg protein in the presence or absence of ouabain (1 mM) to 350 µL final volume. The reaction was started by adding adenosine triphosphate (ATP) to 5 mM final concentration. After 30 min at 37 °C, reaction was stopped by adding 70 µL trichloroacetic acid (50 %). Appropriate controls were included in the assays for ATP non-enzymatic hydrolysis. The amount of inorganic phosphate released was quantified by the colorimetric method described by Fiske and Subbarow [40], and the $Na^{+/}K^+$ ATPase activity was calculated by subtracting the ouabain-insensitive activity from the overall activity (in the absence of ouabain).

2.7.10 Estimation of caspase 3 and 9 activities

The striatal tissue homogenates were tested for caspase-3 and 9 activities by the addition of a caspase-specific peptide that is conjugated to the colour reporter molecule p-nitroanaline (pNA). The cleavage of the peptide by the caspase releases the chromophore pNA, which was assessed spectrophotometrically at a wavelength of 405 nm. The enzymatic reaction for caspases activities were carried out using caspase3 and caspase-9 colorimetric kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

2.8 Statistical analysis

The results are presented as the means ± standard error of the mean (SEM). Comparisons between the experimental and control groups were performed using two-way analysis of variance (ANOVA), followed by Newman–Keuls post hoc tests when appropriate. A value of $p < 0.05$ was considered significant. All tests were performed using GraphPad Prism software 5.0 (San Diego, CA, USA).

3. Results

3.1. Behavioral assessment

3.1.1 OFT

The **Fig 2A**, shows the effect of hesperidin (50 mg/kg) on 6-OHDA-induced changes in the distance traveled in OFT. Two-way ANOVA revealed that the total distance in OFT was not changed significantly by interaction factor (6-OHDA X hesperidin) $F(1,16) = 0.58$, $p<0.456$, hesperidin $F(1,16) = 0.15$, $p<0.703$ or 6-OHDA $F(1,16) = 0.01$, $p<0.984$. Using this test, it is possible to observe that neither 6-OHDA nor hesperidin treatments caused sedative or excitatory effects on the animals.

3.1.2 Rotation behavior

As shows the **Fig 2B**, two-way ANOVA reveals a significant effect for interaction factor (6-OHDA X hesperidin) $F(1,16) = 35.65$, $p<0.001$, hesperidin $F(1,16) = 35.65$, $p<0.001$ and 6-OHDA $F(1,16) = 94.72$, $p<0.001$ in contralateral rotations. *Post hoc* comparisons revealed that 6-OHDA group significantly increased the number of apomorphine-induced contralateral rotations and the hesperidin treatment reversed this effect when compared to 6-OHDA group.

3.1.2 ODT

Two-way ANOVA revealed a significant effect for interaction factor (6-OHDA X hesperidin) $F(1,12) = 19.10$, $p<0.005$, hesperidin $F(1,12) = 23.59$, $p<0.002$ and 6-OHDA $F(1,12) = 14.04$, $p<0.001$ on olfactory discrimination ability in mice **(Fig 3A)**. *Post hoc* test indicated that 6-OHDA-lesioned mice presented an early disruption in olfactory discrimination ability, spending less of time in familiar compartment when compared with control group. On the other hand, this behavior was reversed by hesperidin treatment, whose olfactory discrimination abilities were similar to control group.

Two-way ANOVA revealed a significant effect for interaction factor (6-OHDA X hesperidin) $F(1,12) = 18.64$, $p<0.005$, hesperidin $F(1,12) = 28.26$, $p<0.001$ and 6-OHDA $F(1,12) = 50.42$, $p<0.001$ in olfactory discrimination ratio in mice **(Fig 3B)**. *Post hoc* test indicated that 6-OHDA microinjection reflected impaired recognition ability, and hesperidin treatment revealed a protective activity when compared to the neurotoxic 6-OHDA group.

3.1.3 Barnes maze

As shows the **Fig 4A**, two-way ANOVA yield significant effect for interaction factor (6-OHDA X hesperidin) $F(1,16) = 12.77$, $p<0.002$, hesperidin $F(1,16) = 7.88$, $p<0.012$ and 6-OHDA $F(1,16) = 13.70$, $p<0.001$ in 5th day. The **Fig 4B**, two-way ANOVA showed significant effect for interaction factor (6-OHDA X hesperidin), $F(1,16) = 7.66$, $p<0.0137$, hesperidin $F(1,16) = 9.57$, $p<0.007$ and 6-OHDA $F(1,16) = 37.63$, $p<0.001$ in 12th day. *Post hoc* comparisons demonstrated that hesperidin treatment reversed the increase of time promoted by 6-OHDA microinjection in 5th and 12th days on Barnes maze test.

In addition, two-way ANOVA demonstrated significant effect for interaction factor (6-OHDA X hesperidin) $F(1,16) = 5.81$, $p<0.028$, hesperidin $F(1,16) = 7.21$, $p<0.016$ and 6-OHDA $F(1,16) = 6.59$, $p<0.02$ in number of errors in 5th day (**Fig 4C**). In **Fig 4D**, two-way ANOVA showed significant effect for interaction factor (6-OHDA X hesperidin) $F(1,16) = 6.59$, $p<0.020$ and a main effects of hesperidin $F(1,16) = 13.31$, $p<0.002$, but not for 6-OHDA $F(1,16) = 2.07$, $p<0.169$ in number of errors in 12th day. *Post hoc* comparisons revealed that hesperidin treatment reversed memory impairment promoted by 6-OHDA injection in Barnes maze in mice.

3.2 Neurochemical determinations

3.2.1 Determinations of TH-positive neurons, DA, DOPAC and HVA levels

Two-way ANOVA showed significant effect for interaction factor (6-OHDA X hesperidin) $F(1,16) = 8.63$, $p<0.009$, hesperidin $F(1,16) = 4.80$, $p<0.043$ and 6-OHDA $F(1,16) = 49.38$, $p<0.001$ in TH positive striatal content **(Fig 5A-G)**. *Post hoc* comparisons indicated that 6-OHDA group significantly decreased the TH-positive neurons and the hesperidin treatment (hesperidin 50 mg/kg, *p.o*. during 28 days) promotes attenuation of this parameter.

Two-way ANOVA yield significant main effect of 6-OHDA $F(1,16) = 8.53$, $p<0.01$ in DA levels in striatum of mice. Results of *post hoc* indicated that 6-OHDA microinjection displayed a significant decrease in the DA levels in striatum of mice compared to control animals. Hesperidin (50 mg/kg, p.o.) treatment promotes attenuation of this damage **(Fig 6A)**.

Two-way ANOVA revealed significant main effect of 6-OHDA $F(1,16) = 14,79$ $p<0.0024$ in DOPAC levels in striatum. P*ost hoc* comparisons indicated that after 6-

OHDA microinjection displayed a significant decrease in the DOPAC levels compared to control animals and hesperidin treatment promotes attenuation of this loss **(Fig 6B)**.

Two-way ANOVA demonstrated significant main effect of 6-OHDA $F(1,16) = 15.53$, $p<0.002$ in HVA in striatum. Results of *post hoc* showed that 6-OHDA exposure displayed a significant decrease in the HVA levels compared to control animals. Hesperidin treatment promotes protection of this damage, but did not restore to control levels **(Fig 6C)**.

3.2.2 Estimation of mitochondrial complex-I, II, IV and V activity

Two-way ANOVA of complex-I activity in striatum of mice revealed a significant effect for interaction factor (6-OHDA X hesperidin) $F(1,16) = 0.835$ $p<0.0107$, hesperidin $F(1,16) = 35.81$, $p<0.001$ and 6-OHDA $F(1,16) = 60.28$, $p<0.001$. Results of *post hoc* indicated that after 6-OHDA administration in striatum of mice displayed a significant decrease of complex-I activity compared to control group. Hesperidin treatment protected against the inhibition of the enzyme induced by 6-OHDA (**Fig 7A**).

Two-way ANOVA of complex-II activity in striatum of mice yield a significant main effect of 6-OHDA $F(1,16) = 7.82$, $p<0.012$. *Post hoc* comparisons indicated that 6-OHDA exposure displayed a significant decrease in complex-II activity compared to control group (**Fig 7B).**

Two-way ANOVA of complex-IV activity showed a significant effect for interaction factor (6-OHDA X hesperidin) $F(1,16) = 5.60$, $p<0.031$, hesperidin $F(1,16) = 4.79$, $p<0.043$ and 6-OHDA $F(1,16) = 29.43$, $p<0.001$. Results of *post hoc* indicated that after 6-OHDA displayed a significant decrease the complex-VI activity of striatum compared to control group and the treatment with hesperidin promotes recovery of this parameter (**Fig 7C**).

Two-way ANOVA of complex-V activity demonstrated a significant effect for interaction factor (6-OHDA X hesperidin) $F(1,16) = 9.61$, $p<0.006$, hesperidin $F(1,16) = 15.72$, $p<0.001$ and 6-OHDA $F(1,16) = 16.09$, $p<0.001$. *Post hoc* results revealed that 6-OHDA significantly inhibited the complex-V compared to control group. Hesperidin treatment protected against the inhibition of the enzyme induced by 6-0HDA in striatum of mice (**Fig 7D**).

3.2.3 Evaluation of MMP

Two-way ANOVA of MMP in striatum demonstrated a significant effect for interaction factor (6-OHDA X hesperidin) $F(1,16) = 13.37$, $p<0.002$, hesperidin $F(1,16)$

= 3.66, p<0.073 and 6-OHDA F(1,16) = 14.73, p<0.001. *Post hoc* comparisons revealed that 6-OHDA group significantly decreased the MMP and hesperidin treatment (50 mg/kg, p.o.) reversed this inhibition (**Fig. 8A**).

3.2.4 Measurement of Na^+/K^+ ATPase activity

Two way ANOVA of Na^+/K^+ ATPase activity yield a significant main effect of 6-OHDA F(1,16) = 11.76, p<0.001 **(Fig 8B)**. *Post hoc* comparisons revealed that 6-OHDA injection significantly decreased Na^+/K^+ ATPase activity in striatum and the hesperidin treatment reversed this inhibition when compared to 6-OHDA group.

3.2.5 Estimation of caspase 3 and 9 activities

Two-way ANOVA of caspase-3 activity in striatum of mice yield a significant effect for interaction factor (6-OHDA X hesperidin) F(1,16) = 14.84, p<0.001, hesperidin F(1,16) = 15.63, p<0.001 and 6-OHDA F(1,16) = 36.06, p<0.001. Results of *post hoc* showed that hesperidin treatment significantly protected the increase of caspase-3 activity in striatum after 6-OHDA exposure **(Fig 9A)**.

Two-way ANOVA of caspase-9 activity demonstrated a significant effect for interaction factor (6-OHDA X hesperidin) F(1,16) = 19.19, p<0.005, hesperidin F(1,16) = 14.85, p<0.001 and 6-OHDA F(1,16) = 47.46, p<0.001. *Post hoc* results indicated that hesperidin treatment (50 mg/kg during 28 days) significantly reversed the increase of caspase-9 activity in striatum of mice caused by 6-OHDA injection **(Fig 9B).**

4. Discussion

PD is a daunting public health problem, the mystery of its molecular pathology remains elusive. Current medications act just on a single target and alleviate symptoms via enhancing DA levels directly or indirectly, thus, preventing/reversing the dopaminergic neurodegenerative process through neuronal protection or restoration remains a big challenge [43]. Naturally occurring flavonoids are widely present in vegetables, fruits and seeds, are potential candidates for therapy of neurodegenerative diseases [44]. Among the salient findings of the present study, is that hesperidin at a daily dosage of 50 mg/kg during four weeks, promoted the neuroprotetive effect in behavioral parameters evaluated (locomotor activity, rotation, olfactory dysfunction and memory tests), as well as, in the striatal modifications (TH-positive neurons, DA and its metabolites levels, mitochondrial dysfunction and apoptosis) in PD model induced by 6-OHDA.

This model of PD seems to produce cognitive deficits, particularly in procedural memory. The striatum has been the main area implicated in procedural learning dysfunction [45, 46]. We observed a clear deficit in the ability of mice 6-OHDA group in 5th day (short-term retention) and 12th day (long-term retention) in spatial learning and working memory in Barnes maze. These results corroborated with our previous study, where 6-OHDA caused memory impairment in Morris water maze test, and treatment with hesperidin was able to reverse these changes in mice, reinforcing its potential cognitive effect in this model, which is strongly correlated through modulation of striatal DA [16]. Corroborating, early pharmacological studies of DA modulation of spatial navigation in rodents lend support for the dissociation of aspects of spatial learning and their modulation via the dopaminergic system. Striatal injection of DA receptor agonists (e.g., amphetamine, D1 and D2 receptor agents) facilitated performance in the win-stay radial maze and in the water maze task, which mainly involved the formation of stimulus-response associations between cues and locations [47; 48; 49]. Thus, the dopaminergic neuronal restoration achieved by hesperidin treatment may to mediate the memory improvement in this test.

Olfactory dysfunction is also a common non-motor symptom among patients with PD, about 75%–90% of patients present with hyposmia [50, 51]. The present study associates these results with the marked reduction in TH-positive neurons, DA and its metabolites levels by injection of 6-OHDA. We propose that recovery of olfactory deficits may be related to dopaminergic system, shown that dopaminergic denervation is directly related to deficit olfactory after 6-OHDA, corroborating with study by Valle-Leija [52]. In the same way, Winner, [53] have shown that the recovery of olfactory deficits is related to an dopaminergic neogenesis growth factors. In addition, our group previously showed that hesperidin acts in the neurogenesis mediated by BDNF, therefore this mechanism can act on the olfactory defects [21]. In this context, hesperidin administration (50mg/kg) for 28 days may reverse olfactory dysfunction 6-OHDA-caused in mice.

The specific neurotoxin 6-OHDA has high validity for induction of experimental PD in rodents, some of its observed effects are the same that develop in brain of parkinsonian patients [12]. Among these various effects are observed lower levels of DA, its metabolites and TH positive neurons in striatum [54, 55]. We observed decrease of TH-positive neurons in striatum, which it reversed by reported flavonoid.

Results of DA, DOPAC and HVA levels, observed in this study, confirm our previous results, which showed decrease of DA and its metabolites levels in striatum after 6-OHDA in mice are reversed by hesperidin treatment. In line, these modifications in monoaminergic system, lead to prominent motor asymmetry, as observed in this study by contralateral rotations in 6-OHDA-lesioned mice. Oral administration of hesperidin attenuated the 6-OHDA-induced catecholamine neurotoxicity. Thus, we attributed the decrease of contralateral rotations (cardinal behavioral) with the potential of hesperedin in rescue of dopaminergic neurons. In this line, the treatment with hesperidin acts recovering the compromised cells in striatum, acting in direct control of the viability of dopaminergic neurons, which are the histological hallmark of PD, clearly suggesting its neuroprotective effect.

The present study also revealed that 6-OHDA cause mitochondrial dysfunction (reduction in activity of complex I, II, IV and V) in striatum of mice. These data corroborated with results of Kupsch [13], which showed that the respiratory control ratio, the main parameter of mitochondrial function, is distinctly impaired in 6-OHDA model of PD. Likewise, 6-OHDA microinjection decreased the MMP in striatum suggesting loss of mitochondrial integrity and compromised mitochondrial bioenergetics in this structure. It is suggested that 6-OHDA causes mitochondrial dysfunction, decreased the ATP production and subsequently causes DA depletion due to energy insufficiency. The consequence of mitochondrial dysfunction is reactive species generation and subsequent accumulative oxidative damage is the ultimate demise of the dopaminergic neurons [56]. The protective effect of hesperidin was evidenced by reversal of mitochondrial dysfunction and preservation of mitochondrial integrity, suggesting that this flavonoid prevented the reactive species production, which is closely related to the death of dopaminergic neurons. In support of this idea, previous study of our group already showed the potential antioxidant effect of this flavonoid [16, 22]. These results indicated that hesperidin maintained the striatal mitochondrial bioenergetics and integrity in dopaminergic neurotoxicity induced by 6-OHDA, attenuated the 6-OHDA-induced decrease in ATP synthase activity, indicating increased energy available for DA biosynthesis. Hence, we suggest that mitochondrial function performed by hesperidin may be an important target to treat or delay dementia in PD patients.

The enzyme Na^+/K^+ ATPase plays a key role in cell function, regulating the exchange of Na^+ and K^+ between the cell and the intercellular space. This change may activate primarily a neuronal apoptosis by altering a K^+ homeostasis [57]. In this study, we suggest that the reduction of Na+/K+ ATPase activity striatal , due to the administration of 6-OHDA, can lead to apoptosis, since, we have already demonstrated that this neurotoxin acts in the mitochondria, decreasing the synthesis of ATP, a molecule essential for the proper functioning of enzyme. Contributing, we demonstrated that hesperidin treatment attenuated the inhibition of Na^+/K^+ ATPase activity in the striatum of mice, which may contribute to interrupt apoptosis due to lack of energy in this structure. Once, in nerve tissue, the activity of this enzyme represents about 50% of the total energy consumption [58]. The Na^+/K^+ ATPase pump is central to neuronal survival, thus, hesperidin is able to act as a neuroprotective by reducing the mitochondrial dysfunction, apoptosis and preventing dopaminergic neuronal death.

The mitochondrial membrane potential is reduced due to 6-OHDA microinjection, leading to increased mitochondrial permeability and results in the enhanced release of cytochrome C from the mitochondria, which can trigger activation of caspase-3 and 9 culminating with cell death [56]. In this way, we have previously shown that this neurotoxin also acts increasing the oxidative stress in the striatum [16], which may contribute to the activation of these caspases. As described above, the release of cytochrome C after mitochondrial dysfunction may be involved in caspase-3 activation, which confirms studies of Li et al., 1997 [59]. Finally, hesperidin administration was able to attenuate the activation of caspase-3 and 9 revealed an anti-apoptotic effect. These evidences confirmed hesperidin intervene on mitochondrion mediated apoptotic pathway, playing a key role in striatal monoamines recovery in PD. Finally, the histopathological reports that hesperidin has effect in repair of DA neurons, through mitochondrial restoration and apoptosis, which is correlated with recovery in behavioral tests.

5. Conclusion

Evidences collected in this study with behavioral and neurochemical combination, suggested that hesperidin offered neuroprotection against 6-OHDA induced damage of dopaminergic cells in striatum of mice. The protection may be associated with reversal of the mitochondrial dysfunction and of the mitochondrion

mediated apoptotic pathways. Observations in this study constituted a relevant approach for developing therapeutic strategy for neuroprotection in PD.

Conflict of interest

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

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References

[1] O. Hornykiewicz, Ageing and neurotoxins as causative factors in idiopathic Parkinson's disease–a critical analysis of the neurochemical evidence, Prog. Neuropsychopharmacol. Biol. Psychiatry, 13 (1989) 319–328.

[2] Z. Zhang, L.Houa, X. Lib, C. Juc, J. Zhanga, X. et al., Neuroprotection of inositol hexaphosphate and changes of mitochondrion mediated apoptotic pathway and α-synuclein aggregation in 6-OHDA induced parkinson′s disease cell model, Brain Res. 15 (2016) 87-95.

[3] H. Bernheimer, W. Birkmayer, O. Hornykiewicz, K. Jellinger, F. Seitelberger, Brain dopamine and the syndromes of Parkinson and Huntington. Clinical, morphological and neurochemical correlations, J. Neurol. Sci. 20 (1973) 415–455.

[4] Y. Wang, W. Liu, J. Yang, F. Wang, Y. Sima, et al., Parkinson's disease-like motor and non-motor symptoms in rotenone-treated zebrafish, Neurotoxicology. 58 (2017) 103-109.

[5] K.R. Chaudhuri, A.H. Schapira, Non-motor symptoms of Parkinson's disease: dopaminergic pathophysiology and treatment, Lancet Neurol. 8 (2009) 464–474.

[6] R. Cacabelos, Parkinson’s disease: Old concepts and new challenges, Sci. Pages Alzheimers Dis. Dement. (2016) 1-001.

[7] C. Perier, J. Bové, D.C. Wu, B. Dehay, D.K. Choi, et al., Two molecular pathways initiate mitochondria-dependent dopaminergic neurodegeneration in experimental Parkinson’s disease, Proc Natl Acad Sci USA. 104 (2007) 8161–8166.

[8] S. Nikam, P. Nikam, S.K. Ahaley, A.V. Sontakke, Oxidative stress in Parkinson’s disease. Indian J Clin Biochem 24 (2003) 98–101.

[9] J.D. Adams, Parkinson’s disease-apoptosis and dopamine oxidation, Open J Apoptosis. 1 (2012):1–8.
[10] W. Dauer, S. Przedborski, Parkinson's disease: mechanisms and models, Neuron. 39 (2003) 889–909.
[11] G.E. Meredith, U.J. Kang, Behavioral models of Parkinson's disease in rodents: a new look at an old problem, Mov. Disord. 21 (2006), 1595–1606.
[12] U. Ungerstedt, 6-Hydroxy-dopamine induced degeneration of central monoamine neurons, Eur. J. Pharmacol. 5 (1968) 107–110.
[13] A. Kupsch, W. Schmidt, Z. Gizatullina, G. Debska-Vielhaber, J. Voges, et al., 6-Hydroxydopamine impairs mitochondrial function in the rat model of Parkinson's disease: respirometric, histological, and behavioral analyses, J. Neural. Transm. (Vienna). 121 (2014) 1245-57.
[14] B. J. Ryan, S. Hoek, E.A, Fon, R. Wade-Martins, Mitochondrial dysfunction and mitophagy in Parkinson’s: from familial to sporadic disease, Trends in biochemical sciences. 40, 200–210.

[15] C.K. Hwang, H.S. Chun, Isoliquiritigenin isolated from licorice Glycyrrhiza uralensis prevents 6-hydroxydopamine-induced apoptosis in dopaminergic neurons. Biosci. Biotechnol. Biochem. 76 (2012) 536–543.

[16] M.S. Antunes, A.T. Goes, S.P, Boeira, M. Prigol, C.R. Jesse, Protective effect of hesperidin in a model of Parkinson's disease induced by 6-hydroxydopamine in aged mice, Nutrition. 30 (2014) 1415-22.

[17] A.I. Abushouk, A. Negida, H. Ahmed, M.M. Abdel-Daim, Neuroprotective mechanisms of plant extracts against MPTP induced neurotoxicity: Future applications in Parkinson's disease, Biomed Pharmacother. 85 (2017) 635-645.

[18] D. Vauzour, K. Vafeiadou, A. Rodriguez-Mateos, C. Rendeiro, J.P. Spencer, The neuroprotective potential of flavonoids: a multiplicity of effects, Genes Nutr. 3 (2008) 115–126.
[19] A. Garg, S. Garg, L.J. Zaneveld, A.D. Singla, Chemistry and pharmacology of the citrus bioflavonoid Hesperidin, Phytother. Res. 15 (2001) 655–669.

[20] V. Gaur, A. Kumar, Hesperidin pre-treatment attenuates NO-mediated cerebral ischemic reperfusion injury and memory dysfunction, Pharmacol. Rep. 62 (2010) 635–648.

[21] M.S. Antunes, C.R. Jesse, J.R. Ruff, D. de Oliveira Espinosa, N.S. Gomes, et al., Hesperidin reverses cognitive and depressive disturbances induced by olfactory bulbectomy in mice by modulating hippocampal neurotrophins and cytokine levels and acetylcholinesterase activity, Eur J Pharmacol. 15 (2016) 789:411.

[22] F. Donato, M.G. de Gomes, A.T. Goes, C.B. Filho, L. Del Fabbro, et al., Hesperidin exerts antidepressant-like effects in acute and chronic treatments in mice: possible role of l-arginine-NO-cGMP pathway and BDNF levels, Brain Res Bull. 104 (2014) 19-26.

[23] S.R. Kim, T. Kareva, O. Yarygina, N. Kholodilov, R.E. Burke, AAV transduction of dopamine neurons with constitutively active Rheb protects from neurodegeneration and mediates axon regrowth, Mol. Ther. 20 (2012) 275–286.

[24] L. Prut, C. Belzung, The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review, Eur. J. Pharmacol. 463 (2003) 3–33

[25] R.D. Prediger, A.S. Aguiar Jr, A.E. Rojas-Mayorquin, C.P. Figueiredo, F.C. Matheus, et al., Single intranasal administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in C57BL/6 mice models early preclinical phase of Parkinson's disease, Neurotox. Res. 17 (2010) 114–29.

[26] J.M. Walker, S.W. Fowler, D.K. Miller, A.Y. Sun, G.A. Weisman, et al., Spatial learning and memory impairment and increased locomotion in a transgenic amyloid precursor protein mouse model of Alzheimer's disease, Behav. Brain Res. 12 (2011) 169-75.

[27] U. Ungerstedt, Postsynaptic supersensitivity after 6-hydroxy-dopamine induced degeneration of the nigro-striatal dopamine system, Acta Physiol. Scand. 367 (1971) 69–93.

[28] K. Franklin, G. Paxinos, The mouse brain in stereotaxic coordinates, New York: Academic. Press. (1997).

[29] D.E. Oorchot, Total number of neurons in the neostriatal, pallidal, subthalamic, and substantia nigral nuclei of the rat basal ganglia: A stereological study using the cavalieri and optical disector methods Journal of Comparative, Neurology. 366 (1996) 580–599.

[30] H.J.G. Gundersen, P. Bagger, T.F. Bendtsen, The new stereoloical tools: disector, fractionator, nucleator and point sampled intercepts and their use in pathological research and diagnosis. Acta Pathol. Microbiol. Immunol. Scand. 96 (1988) 857–881.
[31] H.J.G. Gundersen, The smooth fractionator, J. Microsc. 207 (2002) 191–210.
[32] C. Schmitz, P.R. Hof, Design-based stereology in neuroscience. Neuroscience. 130 (2005) 813–831.
[33] W.H. Habig, W.B. Jakoby, Glutathione S-transferases (rat and humam), Methods Enzymol. 77 (1981) 218–231.
[34] P.L. Pedersen, J.W. Greenawalt, B. Reynafarje, J. Hullihen, G.L. Decker, et al., Preparation and characterization of mitochondria and sub-mitochondrial particles of rat liver-derived tissues, Methods Cell Biol. 20 (1978) 411–481.
[35] O.H. Lowry, N.J. Rosenborough, A.L. Farr, R.J. Randall, Protein measurement with folin phenol reagente, J. Biol. Chem. 193 (1951) 265–275.
[36] B.L. Shapiro, R.J. Feigal, F.H. Lam, Mitochondrial NADH dehydrogenase in cystic fibrosis, Proc. Natl. Acad. Sci. U S A. 76 (1979) 2979–2983.
[37] L.O. Sally, A.J. Margaret, Methods of microphotometric assay of succinate dehydrogenase and cytochrome-C oxidase activities for use on human skeletal muscle, Histochem. J. 21 (1989) 545–555.
[38] B. Storrie, E.A. Madden, Isolation of subcellular organelles, Methods Enzymol. 182 (1990) 203–225.
[39] D.E. Griffiths, R.L. Houghton, Studies on energy linked reactions: modified mitochondrial ATPase of oligomycin-resistant mutants of Saccharomyces cerevisiae, Eur. J. Biochem. 46 (1974) 157–167.
[40] C.H. Fiske, Y. Subbarao, The colorimetric determination of phosphorus, J. Biol. Chem. 66 (1925) 375–400.
[41] S.G. Huang, Development of a high throughput screening assay for mitochondrial membrane potential in living cells, J. Biomol. Screen. 7 (2002) 383–389.
[42] L.F. Silva, M.S. Hoffmann, R. Gerbatin, F. Fiorin, F. Dobrachinski, et al., Treadmill exercise protects against pentylenetetrazol-induced seizures and oxidative stress after traumatic brain injury. J Neurotrauma. 14 (2013) 1278-87.
[42] A. Xie, J. Gao, L. Xu, D. Meng, Shared mechanisms of neurodegeneration in Alzheimer’s disease and Parkinson's disease. Biomed Res Int 2014 (2014) 648740.

[44] I. Solanki, P. Parihar, M.S. Parihar, Neurodegenerative diseases: From available treatments to prospective herbal therapy, Neurochem Int. 95 (2016) 100-8.
[45] J.A. Saint-Cyr, A.E. Taylor, A.E. Lang, Procedural learning and neostriatal dysfunction in man, Brain. 111 (1988) 941–959.
[46] M.T. Tadaiesky, P.A. Dombrowski, C. Da Cunha, R.N. Takahashi, Effects of SR141716 A on cognitive and depression-related behavior in an animal model of premotor Parkinson's disease, Parkinsons Dis. 238491 (2010) 26–32.
[47] M.G. Packard, S.F. Vecchioli, J.P. Schroeder, A. Gasbarri, Task-dependent role for striatum metabotropic glutamate receptors in memory, Learn Mem. 8 (2001) 96–103.
[48] F. Thurm, N.W. Schuck, M. Fauser, C.F. Doeller, Y. Stankevich, R. Evens, O. Riedel, A. Storch, U. Lueken, S.C. Li, Dopamine modulation of spatial navigation memory in Parkinson's disease, Neurobiol Aging. 38 (2016) 93-103.
[49] Sato T, Tanaka Ki, Ohnishi Y, Teramoto T, Irifune M, Nishikawa T, Effects of steroid hormones on (Na+, K+)-ATPase activity inhibition-induced amnesia on the step-through passive avoidance task in gonadectomized mice. Pharmacol Res. 2004 Feb; 49(2):151-9.
[50] G.W. Ross, H. Petrovitch, R.D. Abbott, C.M. Tanner, J. Popper, et al., Association of olfactory dysfunction with risk for future Parkinson’s disease, Ann. Neurol. 63 (2008) 167–173.
[51] M.E. Domellöf, K.F. Lundin, M. Edström, L. Forsgre, Olfactory dysfunction and dementia in newly diagnosed patients with Parkinson's disease, Parkinsonism Relat. Disord. 21 (2017) 30067-6.
[52] P. Valle-Leija, R. Drucker-Colín, Unilateral olfactory deficit in a hemiparkinson's disease mouse model, Neuroreport. 12 (2014) 948-53.
[53] B. Winner, S. Couillard-Despres, M. Geyer, R. Aigner, U. Bogdahn, et al., Dopaminergic lesion enhances growth factor-induced striatal neuroblast migration, J. Neuropathol. Exp. Neurol. 67 (2008) 105-16.
[54] E.A. Kozina, G.R. Khakimova, V.G. Khaindrava, V.G. Kucheryanu, N.E. Vorobyeva, et al., Tyrosine hydroxylase expression and activity in nigrostriatal dopaminergic neurons of MPTP-treated mice at the presymptomatic and symptomatic stages of parkinsonism, J. Neurol. Sci. 340 (2014) 198–207.

[55] R. Soto-Otero, E. Méndez-Alvarez, A. Hermida-Ameijeiras, A.M. Muñoz-Patiño, J.L. Labandeira-Garcia, Autoxidation and neurotoxicity of 6-hydroxydopamine in the presence of some antioxidants: potential implication in relation to the pathogenesis of Parkinson's disease, J. Neurochem. 74 (2000) 1605–1617.
[56] M.T. Lin, M.F. Beal, Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases, Nature. 443 (2006) 787–795.
[57] X.Q. Wang, A.Y. Xiao, C. Sheline, K. Hyrc, A. Yang, et al., Apoptotic insults impair Na+, K+-ATPase activity as a mechanism of neuronal death mediated by concurrent ATP deficiency and oxidant stress, J. Cell Sci. 116 (2003) 2099–2110.
[58] W.L. Stahl. The Na,K-ATPase of nervous tissue. Neurochem Int. 8 (1986) 449-76.
[59] P. Li, D. Nijhawan, I. Budihardjo, S.M. Srinivasula, M. Ahmad, et al., Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell. 91 (1997) 479–489.

Figure Legends

Fig. 1. Schematic representation of the experimental protocol describing the treatment periods with hesperidin (50 mg/kg, *p.o*), behavior tests and neurochemistry analyzes.

Fig. 2. (A) Effect of hesperidin treatment in the OFT – total travelled distance. **(B)** Effect of hesperidin treatment in contralateral rotations induced by apomorphine. Values are mean ± S.E.M. (n=6 per group). *: $p<0.05$, ***: $p<0.001$ when compared 6-OHDA/vehicle with SHAM/vehicle; #: $p<0.05$ when compared 6-OHDA/hesperidin with 6-OHDA/vehicle (two-way ANOVA and Newman–Keuls multiple comparison test).

Fig. 3. (A) Effects of hesperidin treatment during twenty-eight days after 6-OHDA in ODT. **(B)** Social recognition: the test evaluated the working memory of mice using the ratio of investigation duration (RDI)—ratio between the time of the second exposure and the first exposure to the same juvenile. Values are mean ± S.E.M. (n=6 per group). ***: $p<0.001$, when compared 6-OHDA/vehicle with SHAM/vehicle; #: $p<0.05$ when compared 6-OHDA/hesperidin with 6-OHDA/vehicle (two-way ANOVA and Newman–Keuls multiple comparison test).

Fig. 4. Effects of hesperidin treatment during twenty-eight days after 6-OHDA microinjection in Barnes Maze – time day 5 **(A)**, time day 12 **(B),** erros day 5 **(C)** and – erros day 12 **(D)**. Values are mean ± S.E.M. (n=6 per group). **: $p<0.01$, ***: $p<0.001$ when compared 6-OHDA/vehicle with SHAM/vehicle; #: $p<0.05$ when compared 6-OHDA/hesperidin with 6-OHDA/vehicle (two-way ANOVA and Newman–Keuls multiple comparison test).

Fig. 5. Photomicrographs showing the optical disector probe application, where could be observed TH-positive neurons, from substantia nigra pars compacta in different focal planes: Control (A, E and I), 6-OHDA (B, F and J), Hesperidin (C, G and K), 6-OHDA + hesperidin (D, H and L) 5X, 20X and 63X respectively. The figure **(M)** show effects of hesperidin treatment during two weeks in quantification of TH-positive cells in striatum after 6-OHDA. Values are mean ± S.E.M. (n=7 per group). **: $p<0.01$; ***: $p<0.001$ when compared 6-OHDA/vehicle with SHAM/vehicle; #: $p<0.05$ when compared 6-OHDA/hesperidin with 6-OHDA/vehicle (two-way ANOVA and Newman–Keuls multiple comparis.

Fig. 6. Effects of hesperidin 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). *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$ when compared 6-

OHDA/vehicle with SHAM/vehicle; #: p<0.05 when compared 6-OHDA/hesperidin with 6-OHDA/vehicle (two-way ANOVA and Newman–Keuls multiple comparison test).

Fig. 7. Effects of hesperidin treatment during twenty-eight days in complex I **(A)**, complex II **(B)**, complex IV **(C)** and complex V **(D)** activities in striatum after 6-OHDA. Values are mean ± S.E.M. (n=6 per group). *: p<0.05, ***: p<0.001 when compared 6-OHDA/vehicle with SHAM/vehicle; #: p<0.05 when compared 6-OHDA/hesperidin with 6-OHDA/vehicle (two-way ANOVA and Newman–Keuls multiple comparison test).

Fig. 8. Effects of hesperidin treatment during twenty-eight days in function of mitochondrial integrity – MMP **(A)** and Na^+/K^+ ATPase activity **(B)** in striatum after 6-OHDA. Values are mean ± S.E.M. (n=10 per group). *: p<0.05, **: p<0.01, ***: p<0.001 when compared 6-OHDA/vehicle with SHAM/vehicle; #: p<0.05 when compared 6-OHDA/hesperidin with 6-OHDA/vehicle (two-way ANOVA and Newman–Keuls multiple comparison test).

Fig. 9. Effect of hesperidin treatment in caspase-3 **(A)** and caspase-9 **(B)** activities in striatum after microinjection of 6-OHDA neurotoxine. Values are mean ± S.E.M. (n=6 per group). **: p<0.01, ***: p<0.001 when compared 6-OHDA/vehicle with SHAM/vehicle; #: p<0.05 when compared 6-OHDA/hesperidin with 6-OHDA/vehicle (two-way ANOVA and Newman–Keuls multiple comparison test).

Graphical abstract

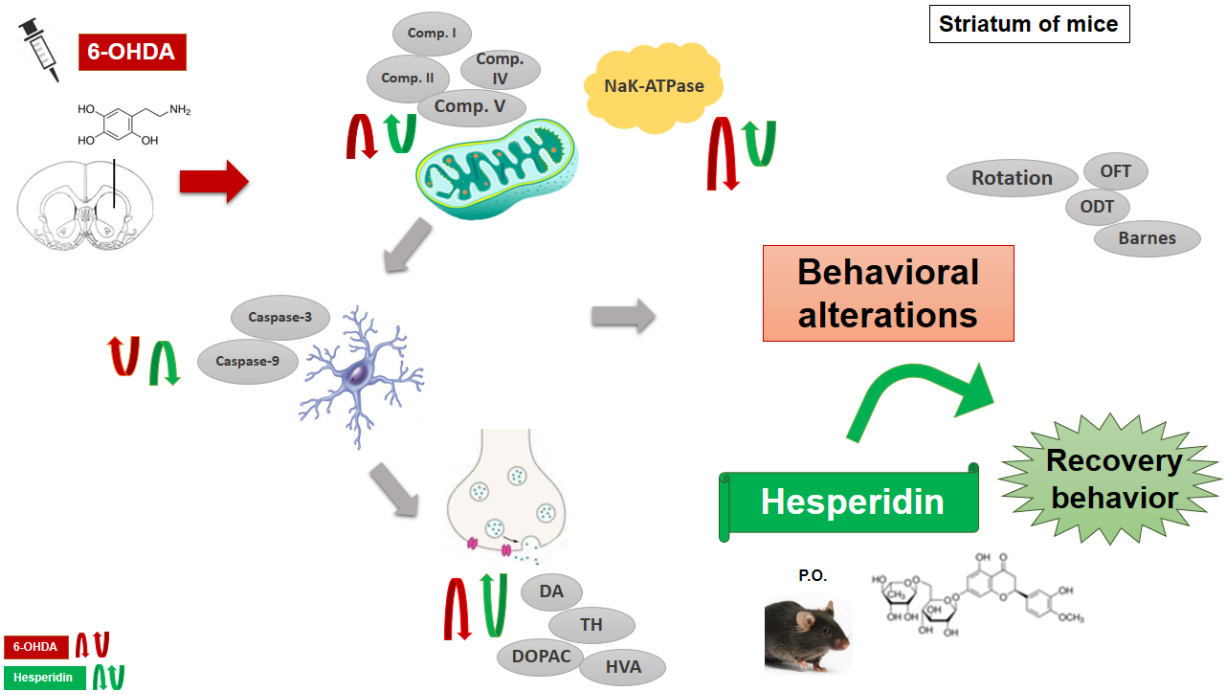


FIG. 1

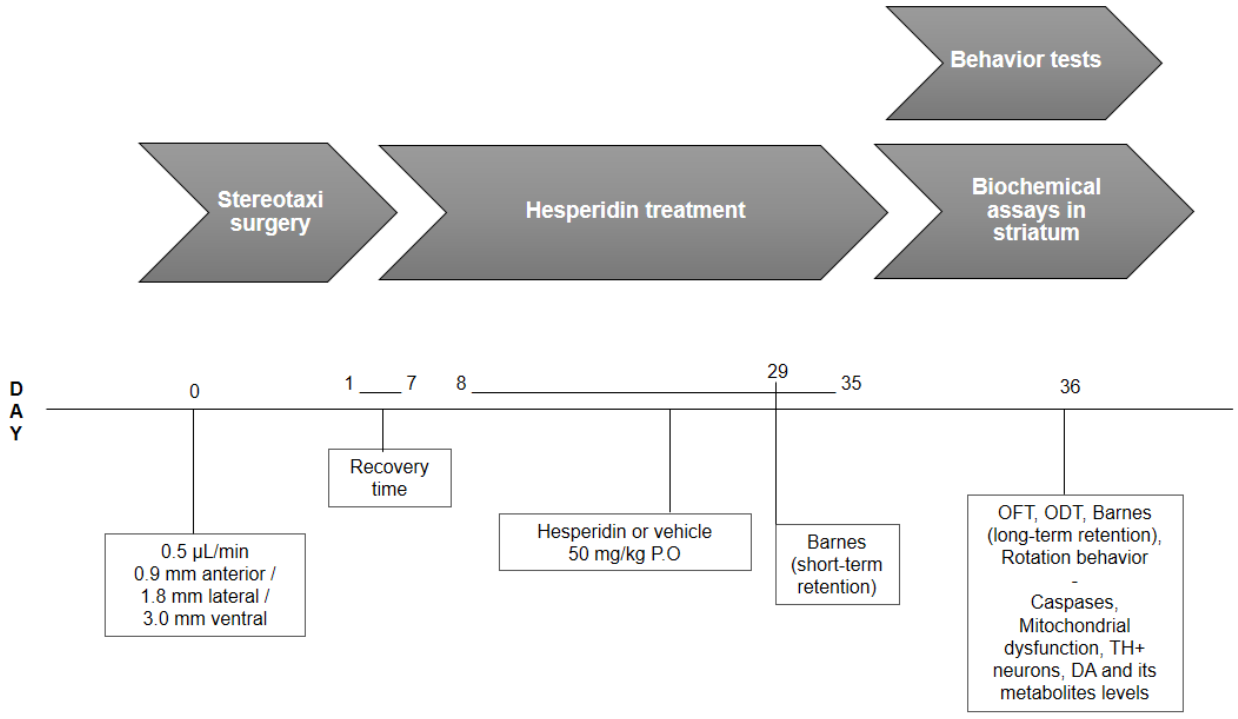
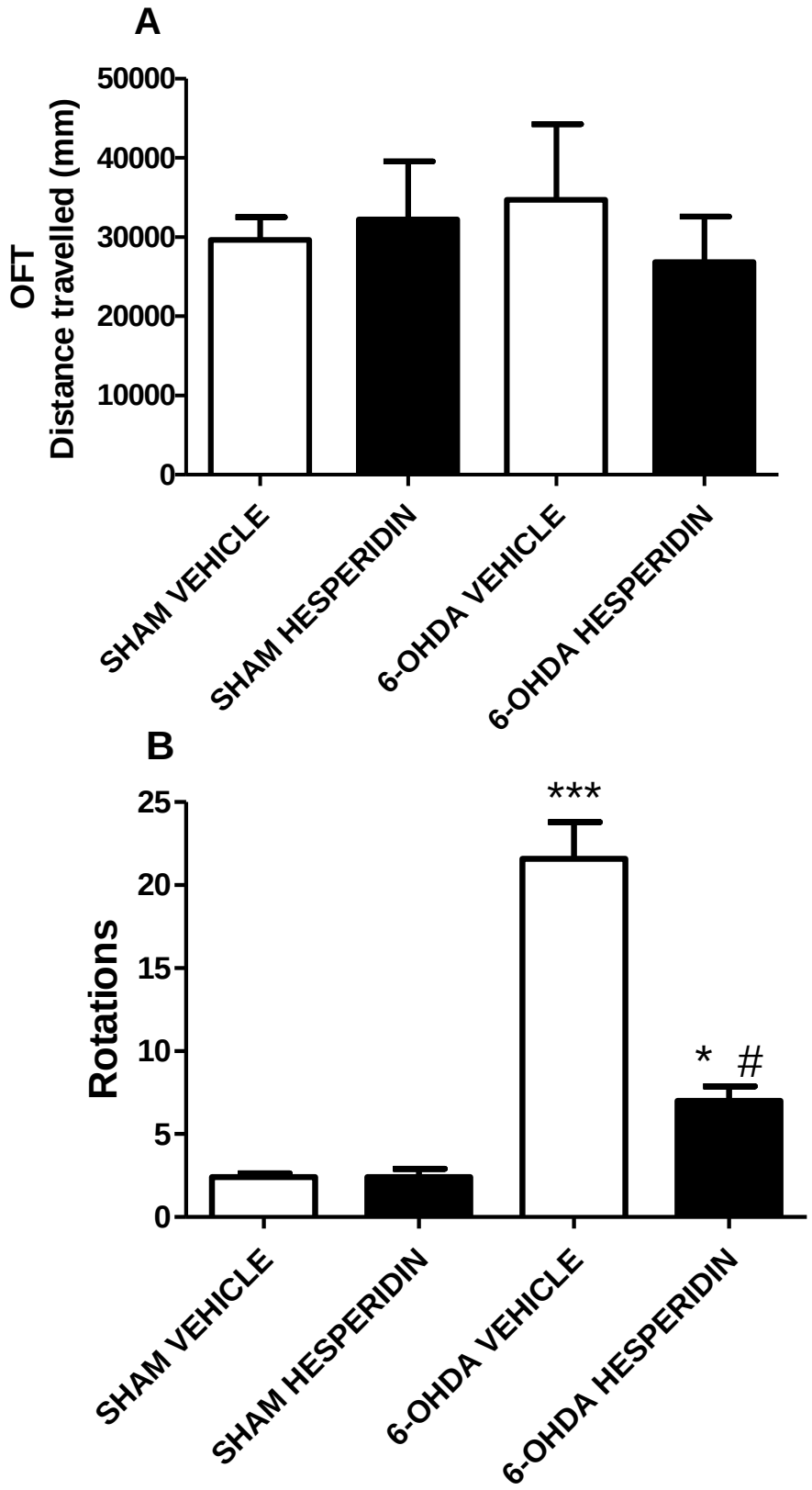


FIG. 2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Distance travelled (mm)
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SHAM HESPERIDIN
6-OHDA VEHICLE
6-OHDA HESPERIDIN
B
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20
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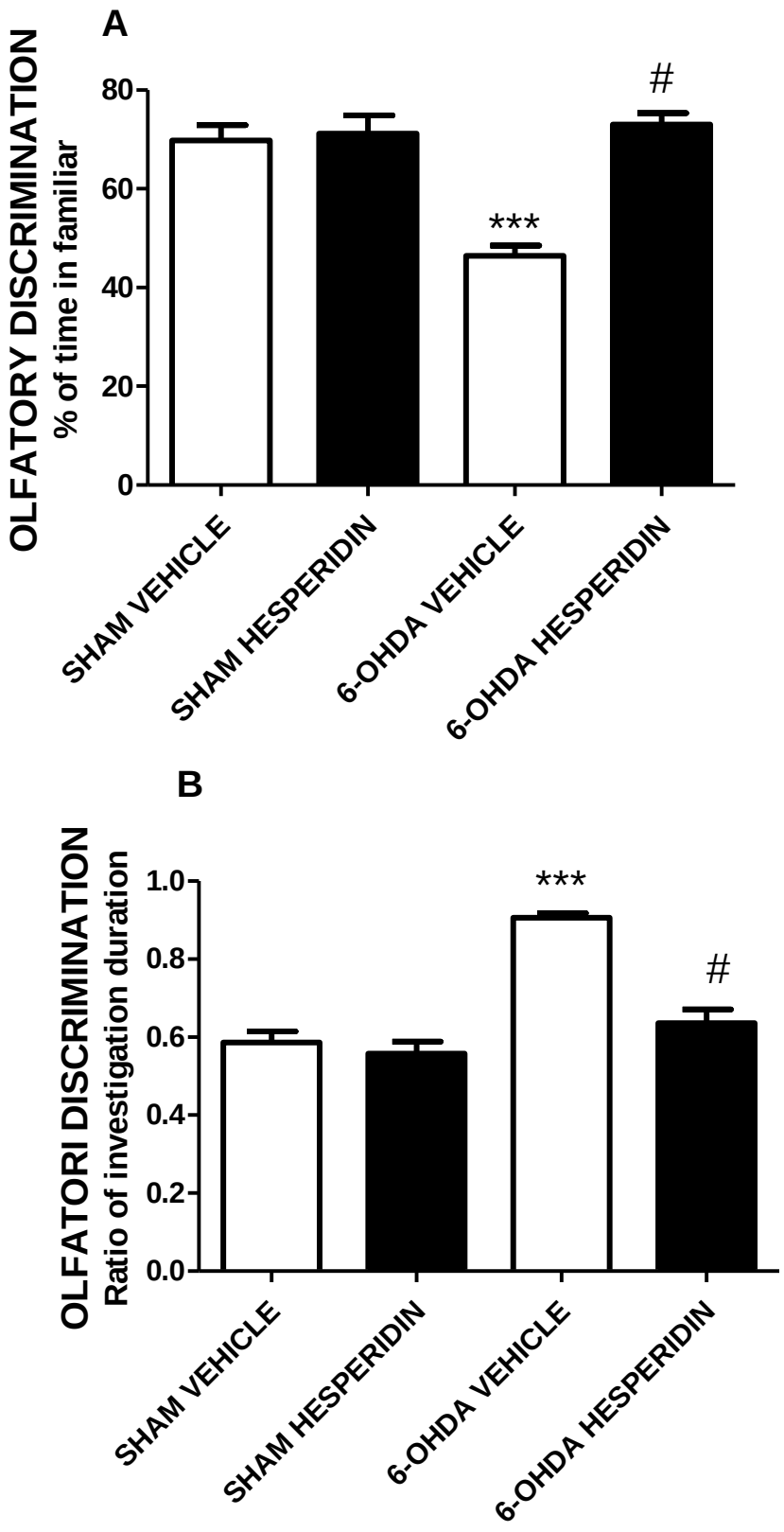
SHAM HESPERIDIN
6-OHDA VEHICLE
6-OHDA HESPERIDIN

FIG. 3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SHAM HESPERIDIN
6-OHDA VEHICLE
6-OHDA HESPERIDIN
B
OLFATORI DISCRIMINATION
Ratio of investigation duration
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0.6
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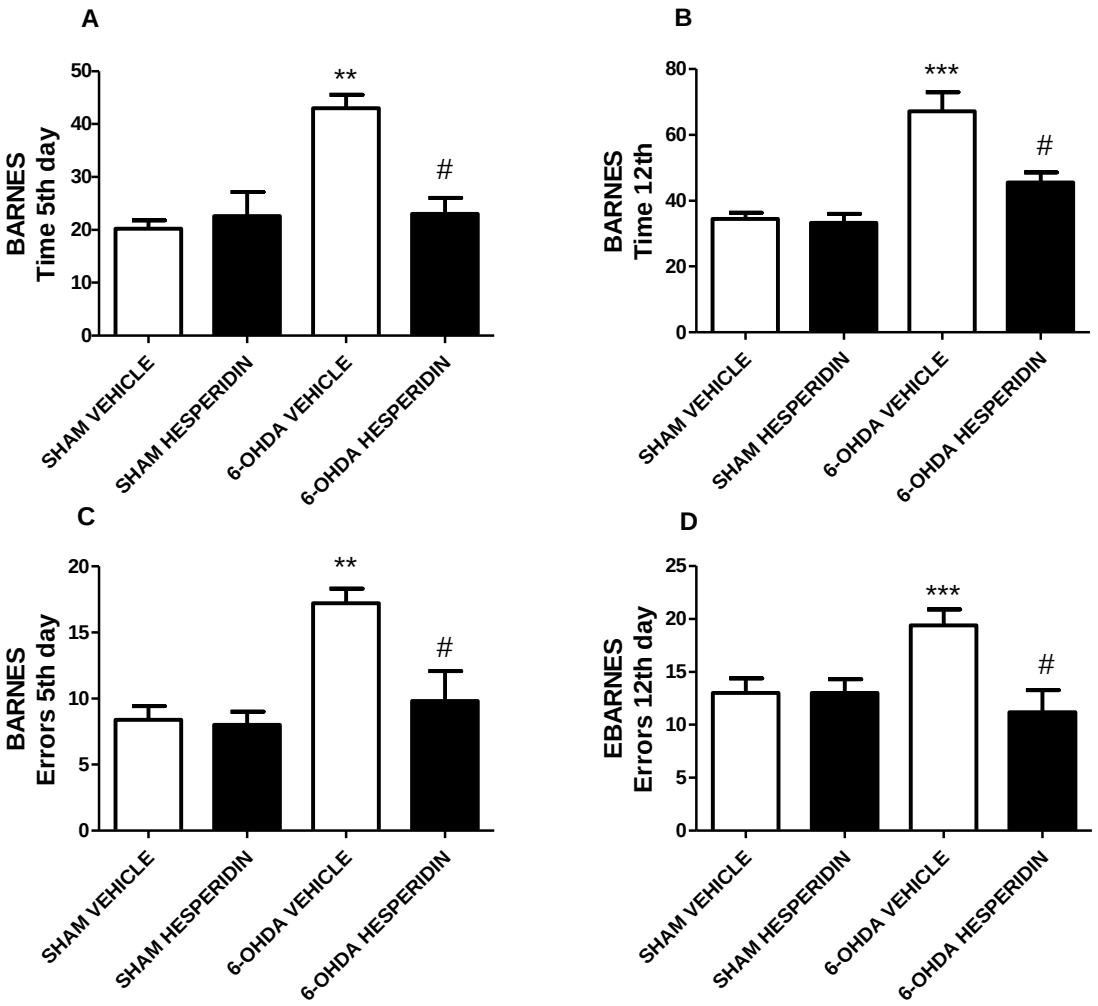
SHAM HESPERIDIN
6-OHDA VEHICLE
6-OHDA HESPERIDIN

FIG. 4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Time 12th
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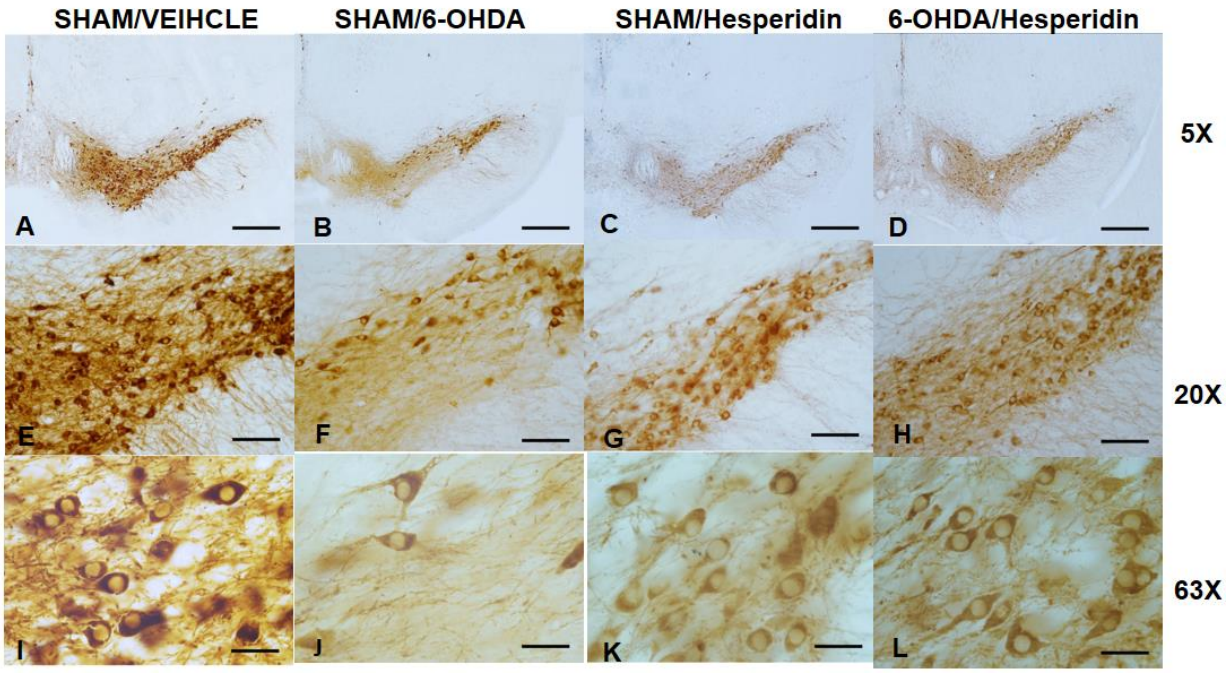
FIG. 5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SHAM/6-OHDA
SHAM/Hesperidin
6-OHDA/Hesperidin
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20X
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B
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D
E
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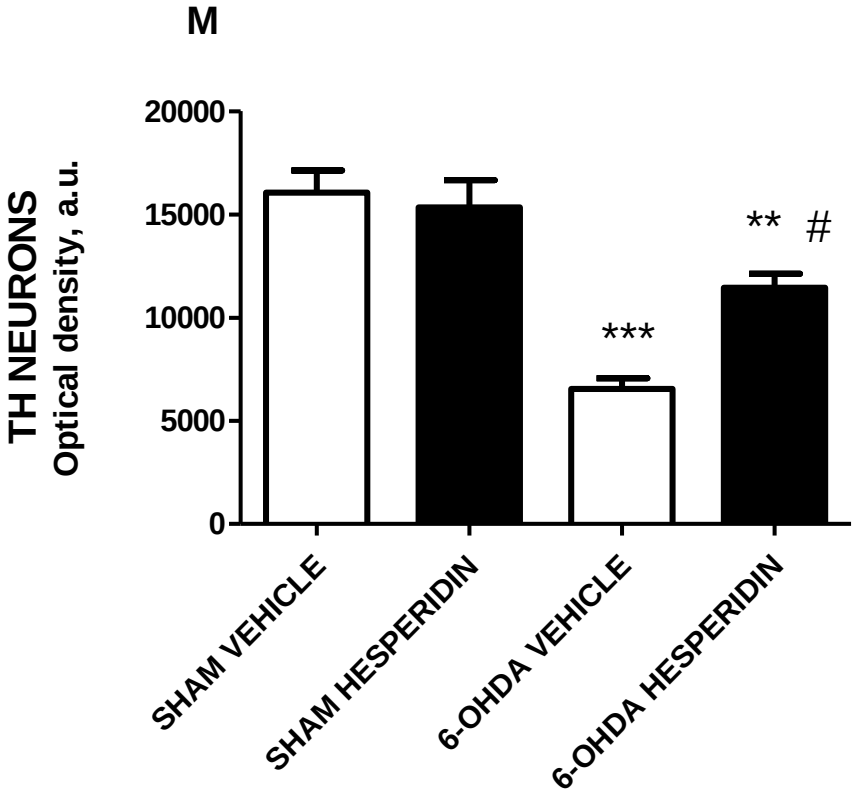
I
J
K
L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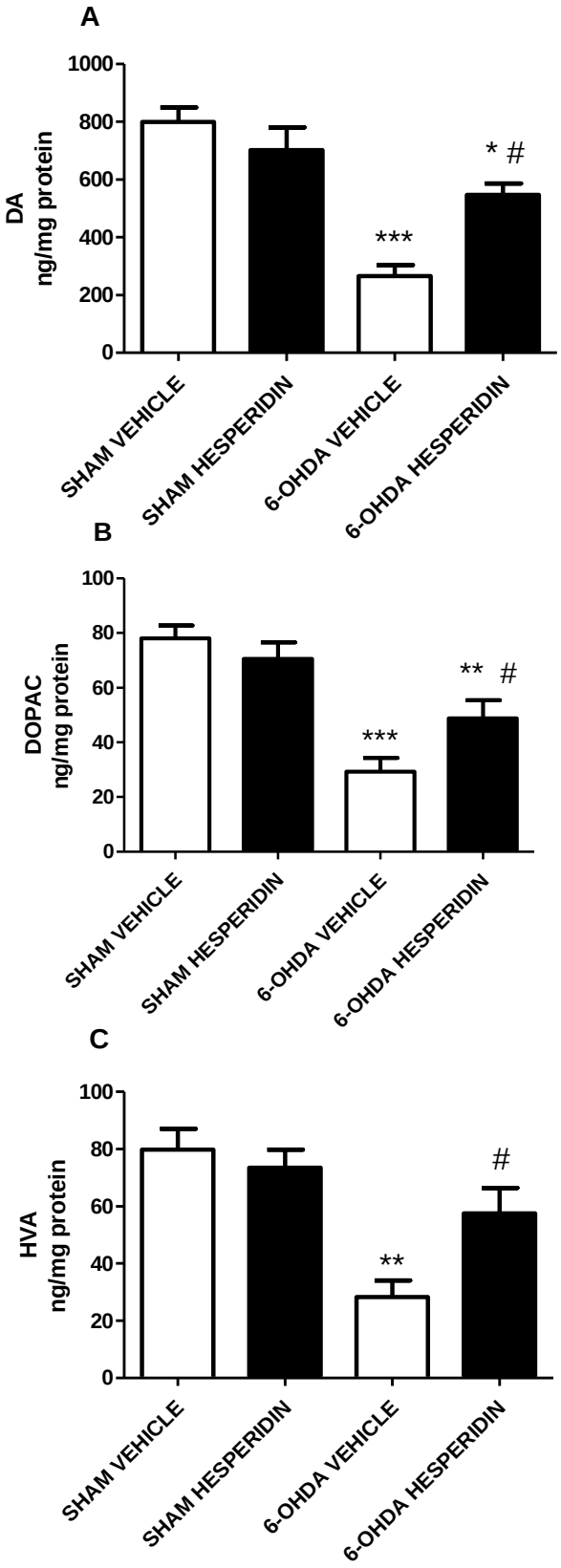
** #
SHAM VEHICLE
SHAM HESPERIDIN
6-OHDA VEHICLE
6-OHDA HESPERIDIN

FIG. 6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ng/mg protein
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400
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* #
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SHAM HESPERIDIN
6-OHDA VEHICLE
6-OHDA HESPERIDIN
B
DOPAC
ng/mg protein
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** #
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SHAM HESPERIDIN
6-OHDA VEHICLE
6-OHDA HESPERIDIN
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HVA
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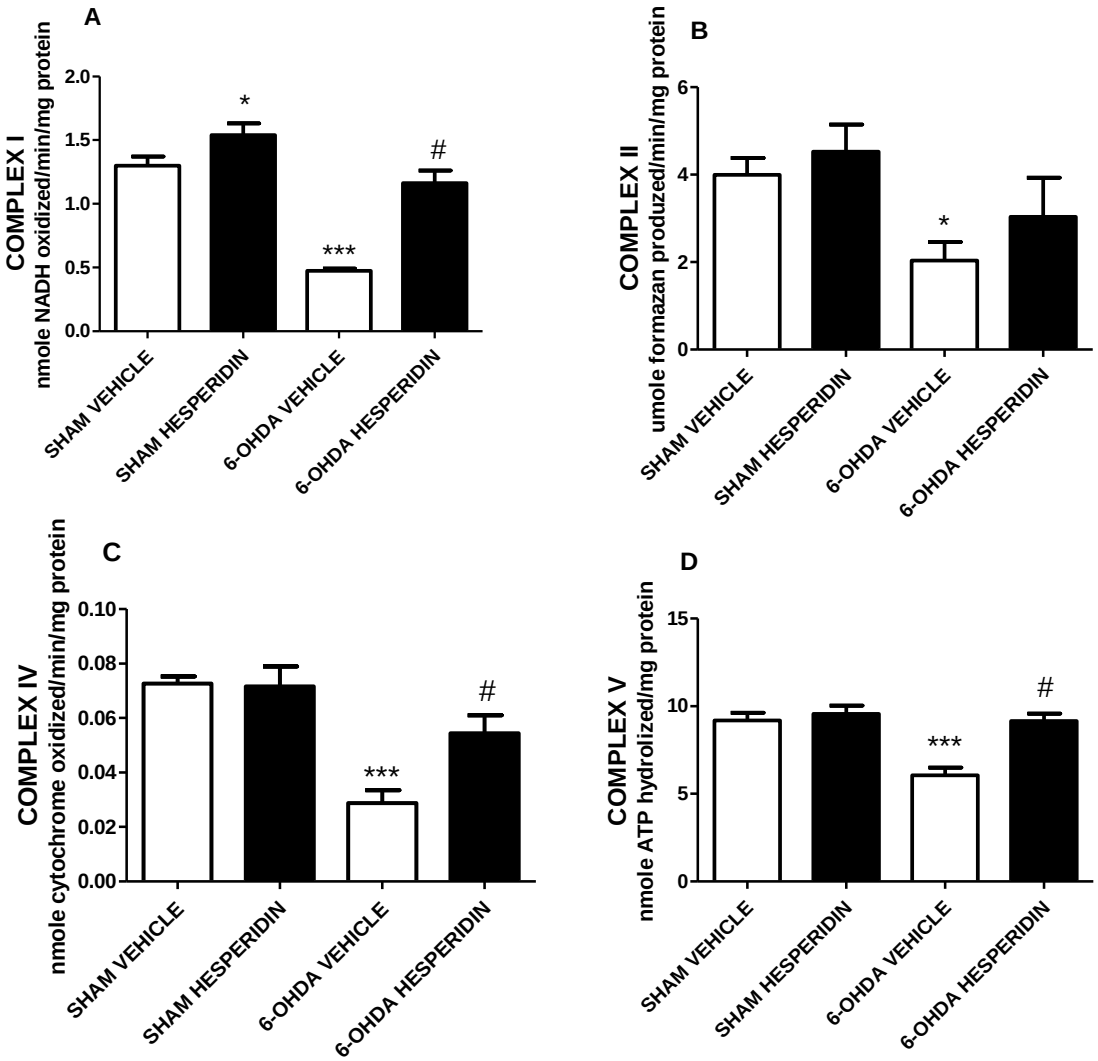
SHAM HESPERIDIN
6-OHDA VEHICLE
6-OHDA HESPERIDIN

FIG. 7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COMPLEX I
nmole NADH oxidized/min/mg protein
2.0
1.5
1.0
0.5
0.0
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SHAM HESPERIDIN
6-OHDA VEHICLE
6-OHDA HESPERIDIN
B
COMPLEX II
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4
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*
SHAM VEHICLE
SHAM HESPERIDIN
6-OHDA VEHICLE
6-OHDA HESPERIDIN
C
COMPLEX IV
nmole cytochrome oxidized/min/mg protein
0.10
0.08
0.06
0.04
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0.00
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6-OHDA HESPERIDIN
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COMPLEX V
nmole ATP hydrolized/mg protein
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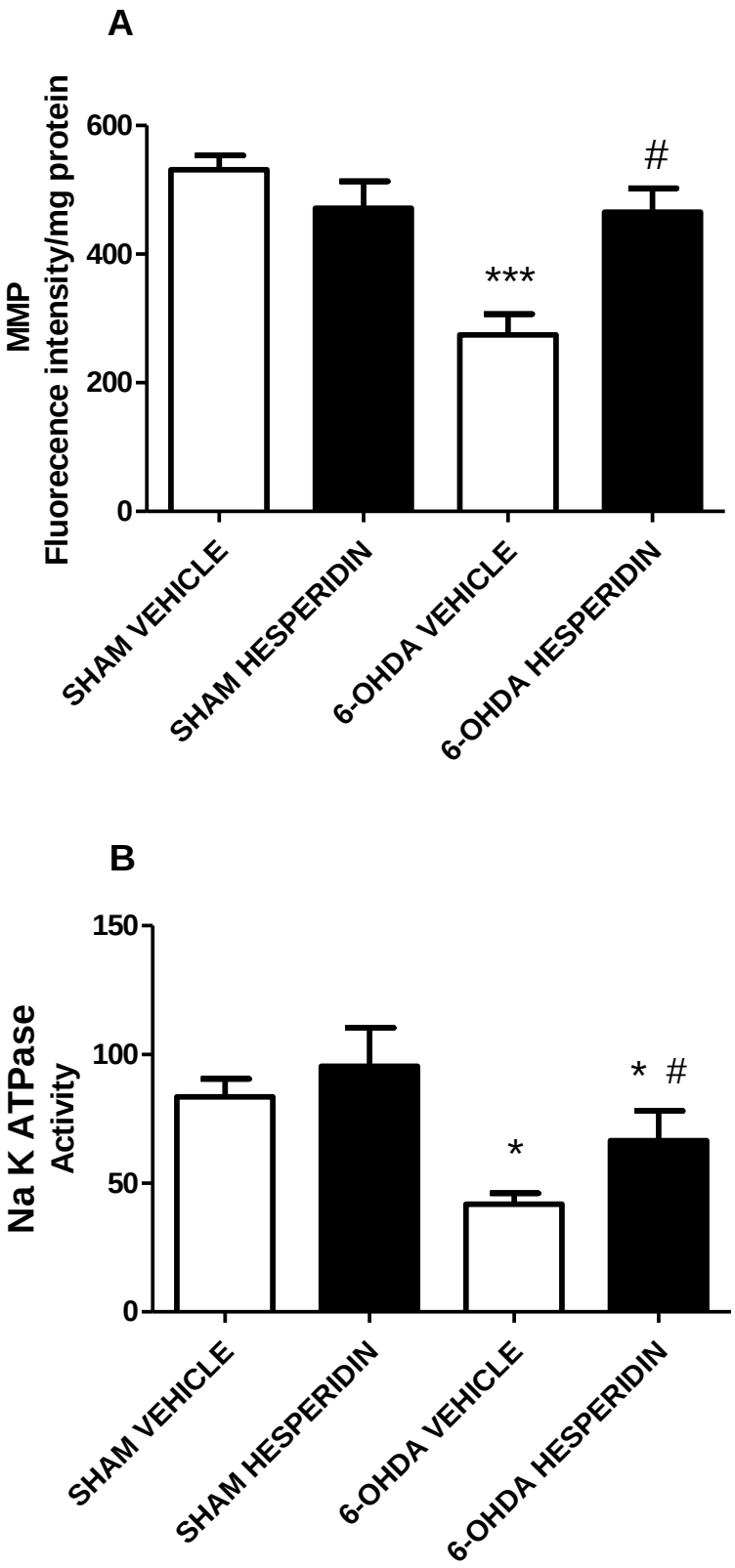
6-OHDA VEHICLE
6-OHDA HESPERIDIN

FIG. 8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Fluorecence intensity/mg protein
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6-OHDA HESPERIDIN
B
Na K ATPase
Activity
150
100
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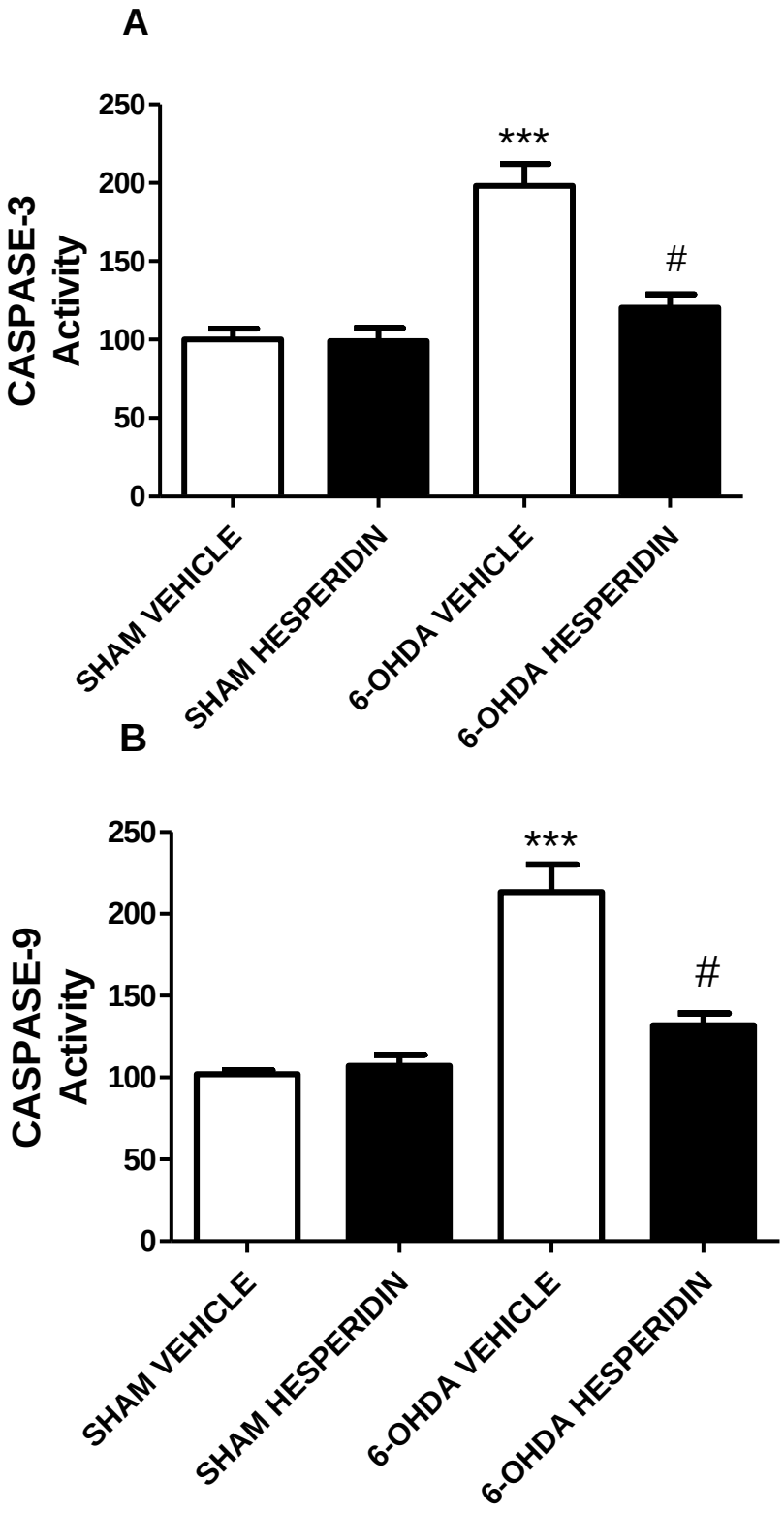
SHAM HESPERIDIN
6-OHDA VEHICLE
6-OHDA HESPERIDIN

FIG. 9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SHAM HESPERIDIN
6-OHDA VEHICLE
6-OHDA HESPERIDIN
B
CASPASE-9 Activity
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SHAM VEHICLE
SHAM HESPERIDIN
6-OHDA VEHICLE
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MANUSCRITO II

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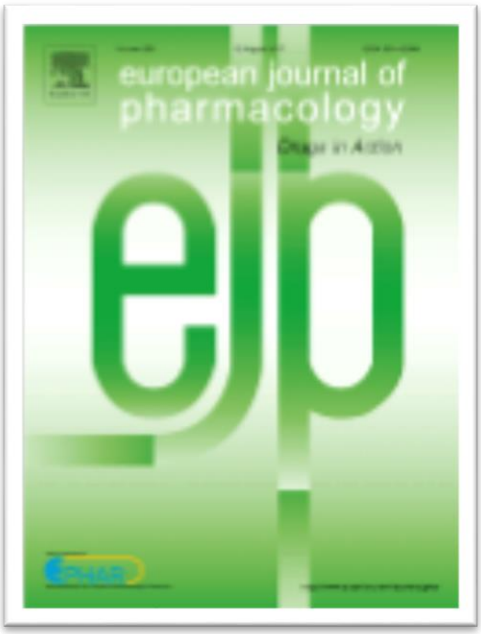

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Abstract: Hesperidin is a specific flavonoid glycoside that is frequently found in citrus fruits and our previous results indicated that this flavonoid exerts therapeutic effects for PD through antioxidant. Neuroinflammation, plasticity and neuronal recovery factors associated with the neuroprotective effect of this flavonoid require further investigations. Thus, now we investigated the possible involvement of pro-inflammatory cytokines, markers of neuroplasticity, neuronal recovery and neurotrophic factors in the effect of hesperidin in 6-hydroxidopamine (6-OHDA), a well-established model of Parkinson's disease, in mice. The 6-OHDA microinjection induced an anxiety-like (characterized by decrease in central time in the open field test, in time and entries in the elevated plus-maze test) and depressive-like behavior (characterized by decrease in the time of grooming in the splash test). 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, reduced the neurotrophin 3 (NT-3), brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF) and glial cell line-derived neurotrophic factor (GDNF) levels. The injection of 6-OHDA also induced an decrease of dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanylic acid (HVA) levels and tyrosine hydroxylase (TH) positive neurons in this structure. Oral treatment with hesperidin (50 mg/kg during 28 days) culminated in the prevention of these alterations occasioned by 6-OHDA. These results corroborated with the neuroprotective effect of hesperidin in the treatment of Parkinson's disease and indicated the mechanism involved throught the pro-inflammatory cytokines, neurotrophic factors and recovery of dopaminergic neurons in striatum.

Hesperidin improves depression and anxiety behavior, neurotrophins damage and neuroinflammation following 6-hydroxydopamine-induced Parkinson's disease model in mice

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Abstract

Hesperidin is a specific flavonoid glycoside that is frequently found in citrus fruits and our previous results indicated that this flavonoid exerts therapeutic effects for PD through antioxidant. Neuroinflammation, plasticity and neuronal recovery factors associated with the neuroprotective effect of this flavonoid require further investigations. Thus, now we investigated the possible involvement of pro-inflammatory cytokines, markers of neuroplasticity, neuronal recovery and neurotrophic factors in the effect of hesperidin in 6-hydroxidopamine (6-OHDA), a well-established model of Parkinson's disease, in mice. The 6-OHDA microinjection induced an anxiety-like (characterized by decrease in central time in the open field test, in time and entries in the elevated plus-maze test) and depressive-like behavior (characterized by decrease in the time of grooming in the splash test). 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, reduced the neurotrophin 3 (NT-3), brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF) and glial cell line-derived neurotrophic factor (GDNF) levels. The injection of 6-OHDA also induced an decrease of dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanylic acid (HVA) levels and tyrosine hydroxylase (TH) positive neurons in this structure. Oral treatment with hesperidin (50 mg/kg during 28 days) culminated in the prevention of these alterations occasioned by 6-OHDA. These results corroborated with the neuroprotective effect of hesperidin in the treatment of Parkinson's disease and indicated the mechanism involved throught the pro-inflammatory cytokines, neurotrophic factors and recovery of dopaminergic neurons in striatum.

Keywords

Neurotrophic factor; Flavonoid; Inflammation; Dopamine; Plasticity

1. Introduction

Parkinson's disease (PD) is the second most prevalent neurodegenerative disease, after Alzheimer's disease. This disorder is characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc), decrease of dopamine (DA) levels, DA biosynthesis capacity and surviving neurons in the striatum of the basal ganglia (Lees, et al., 2009). 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 (Shuman et al., 2011). It is also reported that patients with this disease have non-motor symptoms such as anxiety and depressive disorders (Wichowicz et al., 2006; Dissanayaka et al., 2011).

The processes of PD are still unclear, 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 (Moosavi et al., 2015; Pramanik et al., 2016). Furthermore, inflammation has also been a well-investigated feature in PD pathology (Bassani et al., 2015). There is growing recognition that neuroinflammatory mechanisms might contribute to the cascade of events leading to neuronal degeneration in this disease, and some data have suggested it could be the primary pathological cause for SN neuronal cell death. Accumulated evidences suggest that microglial activation and insufficient support of neurotrophic factors, may be crucial for the initiation and progression of this pathology (Kim, et al., 2016; Vivekanantham et al., 2015).

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 destroys the dopaminergic nigrostriatal neurons (Blandini and Armentero 2012). In this context, the mechanism of 6-OHDA neurotoxicity has often been involved in the production of the reactive species which can lead to the induction of inflammation and ultimately cell death and behavioral changes (Lev et al., 2013; Ungerstedt 1968).

The current pharmacological treatments for PD are primarily DA replacement therapies, which can only alleviate certain symptoms and may cause side effects, thus neuroprotective therapies have been focused in recent decades (Olanow and

Schapira, 2013). In line, there has been considerable interest in the development of neuroprotective drugs from natural origins as a therapeutic strategy for PD, the bioflavonoid hesperidin (4′-methoxy-7-O-rutinosyl-3′,5-dihydroxyflavanone) is a specific flavonoid glycoside that is frequently found in citrus fruits (Garg et al., 2001). It has been reported to possess significant antioxidant, anti-inflammatory, anticancer, and antidepressant-like properties in mice (Antunes, et al., 2016; Donato et al, 2014; Gaur and Kumar 2010). Our previous results indicated that hesperidin exerts therapeutic effects for PD through antioxidant perspective and provides substantial evidence for preclinical application (Antunes et al., 2013). In this study, we hope modulation of inflammation, neurotrophic fators and tyrosine hydrolysase, as well as, the reversal of behavioral alterations in PD model induced by 6-OHDA, in order to lead to a new PD therapy.

2. Materials and methods

2.1. Animals

Experiments were performed using male C57 BL/6 mice (25–35 g, 3-6 months old). Animals were maintained at 22°C to 25°C with free access to water and food, under a 12-h light–dark cycle, with lights on at 0700. All manipulations were carried out during the light phase of the day. 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 # 001/2013) of the Federal University of Pampa, Brazil. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2 Experimental design

The mice were subjected to stereotaxic surgical infusion i.c.v. of 6-OHDA or vehicle. Seven d after the microinjections, treatment with oral hesperidin (50 mg/kg) was initiated for 28 days. Mice were randomly assigned to four groups: (I) SHAM-operated/saline (SHAM/vehicle) as the control group; (II) SHAM-operated/hesperidin treated mice (SHAM/hesperidin); (III) 6-OHDA-mice treated with saline (6-OHDA/vehicle); (IV) 6-OHDA-mice treated with hesperidin. After finish of treatments, the mice were submitted to behavior and biochemistry tests **(Fig. 1)**. All analyses were performed in a blinded fashion.

2.3 Stereotaxic surgical intrastriatal microinjection of 6-OHDA

Surgery was performed under anesthesia with 10 mL/kg of 1% ketamine and 0.2% xylazine. 6-OHDA (5 µg in 2 µL of 0.9% NaCl with 0.2 µg/mL 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 infusion i.c.v., the syringe was maintained in the brain for an additional 3 min before it was slowly retracted. Controls were injected with the vehicle. All reagents were purchased from Sigma Chemical.

2.4 Behavioral assessment

2.4.1 Open field test (OFT)

To verify the effects of hesperidin and 6-OHDA administrations on locomotor activity, the animals were submitted individually to a 5 min OFT (Insight model EP 154C) 24 h after the last treatment. The apparatus was cleaned with an ethanol solution (10% v/v) and dried with paper towels after each trial in order to prevent odors from carrying-over between trials. The parameter observed included the distance and time spent in central area (unit: mm) (Prut and Belzung, 2003).

2.4.2 Elevated plus-maze test (EPMT)

After OFT, the EPMT was used to evaluate anxiety-like behavior in mice, according with the method of Pellow et al., (1985). The experimental apparatus (Insight Ltd., Ribeirao Preto, SP, Brazil) was shaped as a plus sign and consisted of two open arms (30 × 5 cm) and two equal-sized closed arms (30 × 5 × 15 cm) extending from a common central platform (5 × 5 cm). The maze was made of opaque grey PVC and was kept elevated at a height of 50 cm above the floor. The experiments were conducted in a sound-attenuated room under low intensity light (12 lx). The animals were individually placed in the central area of the maze facing an enclosed arm and were observed for 5 min. The apparatus was cleaned with an ethanol solution (10% v/v) and dried with paper towels after each trial. During a 5 min test period, the number of entries either the open or enclosed arms, plus the time spent in the open arms we recorded. An entry was defined as placing all four paws within the boundaries of the arm. The following measures were obtained from the test: (a) the time spent in the open arms compared to the total time spent in the plus-maze (300 s); (b) the number of entries into the open arms; (c) the number of entries into the closed arms. In this test, the anxiolytic effect of a drug is defined by a significant increase in the entries/or time spent in the open arms (Clénet et al., 2006).

2.4.3 Splash test (SPT)

The splash test was adapted from Yalcin et al. (2005). This test evaluates grooming behavior, defined as cleaning of the fur by licking or scratching, after vaporization of 10% sucrose solution onto the mouse's dorsal coat. The solution's viscosity prompts mice to initiate grooming behavior, with depressive symptoms characterized by an increased latency (idle time between spray and initiation of grooming) and decreased time spent grooming (d'Audiffret et al., 2010). Latency and time spent grooming were recorded for 5 min.

2.5 Tissue preparation

After behavioral tests, mice were sacrificed with a barbiturate overdose (intraperitoneal 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 2400g for 15 min at 4°C, and a low-speed supernatant fraction (S1) was used for assays.

2.6 Biochemical determinations

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 (1976), 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 Neurotrophic factors levels

Protein levels of neurotrophin 3 (NT-3), brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF) and glial cell line-derived neurotrophic factor (GDNF) 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 NT-3, BDNF, NGF and GDNF levels were expressed as pg/mg wet weight of tissue.

2.6.3 DA, 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanylic acid (HVA) levels

Striatal tissues were homogenized with 300 µL of 200 mM ice-cold perchloric acid containing 10 mM disodium EDTA. After centrifugation (10 000g for 10 min at 4°C), the supernatant was filtered and then injected directly into a high-performance liquid chromatography system (Shimadzu; Kyoto, Japan) with an electrochemical detector (ECD; Eicom, Kyoto, Japan). The appendant potential of the ECD (carbon electrode versus Ag/AgCl reference electrode) was set at 700 mV. The analytical 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) (Habig and Jakoby 1981).

2.6.4. Immunohistochemistry for tyrosine hydroxylase (TH)

Design-based stereology was performed for cell counts and volume measurements of TH-positive 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 (SNpc) were delineated according to Franklin and Paxinos, (1997) and Oorchot, (1996) by anatomical landmarks (Bregma−4.70 to −6.30 mm) with a 2,5× magnification on live microscopic video images displayed on a monitor, as we have previously described (Filichia et al., 2015).

The SNpc TH-positive neurons were counted by the optical fractionator design (Gundersen et.al., 1988; Oorchot, 1996; Gundersen et.al., 2002; Schmitz and Hof, 2005). 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-positive 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.

2.7 Statistical analysis

The results are presented as the means ± standard error of the mean (SEM). Comparisons between the experimental and control groups were performed using two-way analysis of variance (ANOVA), followed by Newman–Keuls post hoc tests when appropriate. A value of $p < 0.05$ was considered significant. All tests were performed using GraphPad Prism software 5.0 (San Diego, CA, USA).

3. Results

3.1. Behavioral assessment

3.1.1. OFT

The **Fig.2.A** shows the effect of hesperidin (50 mg/kg) on 6-OHDA-induced changes in time spent in central area in OFT. Two way ANOVA revealed main effects of hesperidin $F(1,16) = 5.59$, $p<0.03$ and of 6-OHDA $F(1,16) = 11.30$, $p<0.004$. *Post hoc* comparisons revealed that thirty-five days after the 6-OHDA infusion, mice showed decreased of time spent in central area on the OFT when compared with the SHAM group.

In OFT total travel time, two way ANOVA did not revealed significant effect for interaction factor (6-OHDA X hesperidin) $F(1,16) = 0.53$, $p<0.47$, nor by main effects of hesperidin $F(1,16) = 0.31$, $p<0.58$ or 6-OHDA $F(1,16) = 0.02$, $p<0.86$ **(Fig.2.B)**. This test indicated that neither 6-OHDA nor hesperidin treatments caused sedative or excitatory effects on the animals.

3.1.2. EPMT

Two-way ANOVA showed a significant main effect of hesperidin $F(1,16) = 24,80$. $p<0.001$ and 6-OHDA $F(1,16) = 36.11$, $p<0.001$ in open arms total time **(Fig.3 A).** In **Fig.3 B,** two-way ANOVA showed a significant main effect of hesperidin $F(1,16) = 36.87$, $p<0.001$ and 6-OHDA $F(1,16) = 29.17$, $p<0.001$ in open arms entries. *Post hoc* analyses reveled that anxiety-like behavior was evident after 6-OHDA microinjection and hesperidin treatment reversed this alteration in EPTM, acting as anxiolytic compound as anxiolytic.

3.1.3. SPT

The **fig. 3 C** shows the effect of hesperidin treatment on the performance in SPT**.** Two-way ANOVA revealed a significant effect for interaction factor (6-OHDA X hesperidin) $F(1,16) = 6.69$, $p<0.01$, hesperidin $F(1,16) = 18.91$, $p<0.005$ and 6-OHDA $F(1,16) = 4.06$, $p<0.05$. *Post hoc* comparisons revealed that after 6-OHDA infusion presented a significant decrease on sucrose preference when compared to the control group (SHAM/vehicle). Hesperidin administration (50 mg/kg during 28 days) prevented the reduction of time grooming, suggesting a role in anhedonia.

3. 2 Biomarkers of neurochemical alterations

3.2.1. Effect of hesperidin on Cytokines levels in striatum

As illustrated in **Fig. 4 A,** two-way ANOVA of TNF-α levels revealed a significant effect for interaction factor (6-OHDA X hesperidin) $F(1,16) = 44.37$, $p<0.001$, hesperidin $F(1,16) = 40.03$, $p<0.001$ and 6-OHDA $F(1,16) = 93.82$, $p<0.001$. Moreover, two-way ANOVA of IFN-γ levels revealed a significant effect for interaction factor (6-OHDA X hesperidin) $F(1,16) = 27.36$, $p<0.002$, hesperidin $F(1,16) = 23.88$, $p<0.004$ and 6-OHDA $F(1,16) = 59.26$, $p<0.001$. **(Fig. 4 B)**. *Post hoc* comparisons showed that after 6-OHDA administration induced a significant increase of IFN-γ and TNF-α levels in striatum. Hesperidin treatment protected against the increase of these inflammatory markers induced by 6-OHDA in striatum of mice.

In **Fig. 5 A,** two-way ANOVA of IL-1β levels demonstrated a significant effect for interaction factor (6-OHDA X hesperidin) $F(1,16) = 21.42$, $p<0.006$, hesperidin $F(1,16) = 31.10$, $p<0.001$ and 6-OHDA $F(1,16) = 94.13$, $p<0.001$. As illustrated in **Fig. 5 B**, two-way ANOVA of IL-2 levels revealed a main effect of 6-OHDA $F(1,16) = 42.97$, $p<0.001$. In **Fig. 5 C,** two-way ANOVA of IL-6 levels showed a significant effect for interaction factor (6-OHDA X hesperidin) $F(1,16) = 22.04$, $p<0.005$, hesperidin $F(1,16) = 15.49$, $p<0.002$ and 6OHDA $F(1,16) = 70.04$, $p<0.001$. Two-way ANOVA of IL-10 yield a main effect of 6-OHDA $F(1,16) = 20.96$, $p<0.006$ (**Fig. 5 D)**. *Post hoc* comparisons revealed that 6-OHDA injection significantly increased the pro-inflammatory mediators (IL-1β, IL-2 and IL-6 levels) and it decreased the anti-inflammatory cytokine (IL-10 levels) in striatum. Treatment with hesperidin prevented the increase of IL-1β and IL-6 levels in striatum in the PD model induced by 6-OHDA striatal.

3.2.2. Effect of hesperidin on neurotrophic factors

In **Fig. 6 A,** two-way ANOVA of NGF levels revealed a significant effect for interaction factor (6-OHDA X hesperidin) $F(1,16) = 4.57$, $p<0.05$, hesperidin $F(1,16) = 4.41$, $p<0.05$ and of 6-OHDA $F(1,12) = 4.88$, $p<0.04$ in striatum. Two-way ANOVA of GDNF levels yield a main effect of 6-OHDA $F(1,16) = 32.45$, $p<0.001$ (**Fig. 6 B**). Analysis statistical of BDNF levels showed a main effect of hesperidin $F(1,16) = 9.07$, $p<0.01$ and 6-OHDA $F(1,16) = 14.61$, $p<0.002$ (**Fig. 6 C)**. In addition, two-way ANOVA of NT-3 levels revealed a significant effect for interaction factor (6-OHDA X hesperidin) $F(1,16) = 8.78$, $p<0.01$ and hesperidin $F(1,16) = 6.18$, $p<0.02$ **(Fig. 6 D)**. *Post hoc*

comparisons revealed that after 6-OHDA exposure decreased the neurotrophic factors and the treatment with hesperidin acts reversing this damage (NGF, BDNF and NT-3) in striatum of mice.

3.2.3. Effect of hesperidin in DA, DOPAC and HVA levels and TH positive neurons

Two way ANOVA of DA levels revealed a significant effect for interaction factor (6-OHDA X hesperidin) $F(1,16) = 9.08$, $p<0.01$ and 6-OHDA $F(1,16) = 17.95$, $p<0.001$ **(Fig. 7 A)**. As illustrated in **Fig. 7 B**, two way ANOVA of DOPAC levels exhibited a significant effect for interaction factor (6-OHDA X hesperidin) $F(1,16) = 6.31$, $p<0.02$, hesperidin $F(1,16) = 4.07$, $p<0.05$ and 6-OHDA $F(1,16) = 16.54$, $p<0.001$. In **Fig. 7 C**, two way ANOVA of HVA levels showed a main effect of 6-OHDA $F(1,16) = 25,03$ $p<0.0003$. Two way ANOVA of TH content evidenced a significant effect for interaction factor (6-OHDA X hesperidin) $F(1,16) = 4.90$, $p<0.04$, hesperidin $F(1,16) = 9.35$, $p<0.007$ and 6-OHDA $F(1,16) = 48.37$, $p<0.001$ (**Fig. 8 A–I)**. *Post hoc* comparisons demonstrated that 6-OHDA exposure decreased DA, DOPAC and HVA levels and TH^+ neurons in striatum of mice and treatment with hesperidin (50 mg/kg/day, p.o.) attenuated these neurochemical damages.

4. Discussion

In the present study, 6-OHDA exposure caused anxiety-like behavioral in the OFT and EPTM, and anhedonia-like behavior in splash test. 6-OHDA infusion also decreased TH positive neurons, DA, DOPAC, HVA and neurotrophins levels in the striatum, besides, caused massive increased of pro-inflammatory cytokines levels in this structure. The above neurological, behavior and biochemical changes were restored by oral hesperidin treatment (50 mg/kg daily). These results, demonstrated that this flavonoid overcomes the 6-OHDA-induced deficits in emotional behaviors, reversed the neuroinflammation and reduction of neurotrophins and monoamine neurotransmitters in striatum, suggesting a potential role for hesperidin in this model of PD.

Motor dysfunction and cognitive impairments are seen in a high percentage of patients with PD. Mood disorders such as anhedonia, depression and anxiety are insidious non-motor symptoms of this disease and very detrimental to the quality of life in patients (Akhmadeeva et al., 2017; Martinez-Martin et al., 2011). The current study demonstated that infusion i.c.v. of 6-OHDA caused increase of anxiety and anhedonia

in behavior tests, corroborating with results of Matheus et al. (2016) and O'Connor et al. (2016). Together with the literature, our data supports and extends the notion that an inflammatory response, reflected by a cytokine dysregulation and DA and neurotrophins alterations can be an important neurobiological mechanism of 6-OHDA-induced behavior impairment in mice. Clinical studies of Chaudhuri and Schapira (2009), has been suggested that anxiety and depression might be related to the degeneration of DA cells. In support of this idea, is conceivable to propose that the neuroprotective effect of hesperidin, evidenced in this study by dopaminergic neurotransmitters restoration in striatum, is correlated with the reversion of anxiety and anhedonic-like behavior in mice. These data are in agreement with our previous results, where hesperidin reversed the behavioral impairments through modulation of DA in striatum (Antunes et al., 2013). Still, anti-inflammatory agents can protect DA neurons against neurotoxin molecules in animal models of PD (Kim et al., 2010), which suggests that the control of microglial activation, achieved by hesperidin, may be important for the DA neurons protection. Our study suggested that microglia-mediated inflammatory responses and decrease of plasticity contribute to the pathophysiology of PD through the neurodegeneration of SNpc dopaminergic neurons, and, consequently anxiety-like behavior and anhedonia in this model.

Activation of glial cell is a common feature in patients with PD and animal models, performing critical roles in the neuroinflammatory processes associated with PD (Wang, et al., 2015). 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 infusion. According, studies have shown that excessive activation of microglia leads to the elevation of pro-inflammatory cytokines, which could rapidly induce the degeneration of SNpc dopaminergic neurons, which is the hallmark of PD (Cagnin, et al., 2006; Norden, et al., 2015). In this line, the inhibition of microglia cascade reactions could prevent the degradation of neurons (Burguillos et al., 2011). Our results demonstrated that hesperidin treatment during 28 days, reversed alterations in pro-inflammatory cytokines in striatum. In line, other study of group, there are demonstrated that this flavonoid reversed the increase of these pro-inflammatory cytokines also in hippocampus in olfactory bulbectomy model (Antunes et al, 2016). Accordingly, the present study supports and expands the anti-inflammatory role of hesperidin in the

brain, evidencing its involvement in this model of PD. Thus, we suggested that these anti-inflammatory properties of hesperidin contributed to the neuroprotective effect in dopaminergic neurons in PD.

This increase of pro-inflammatory cytokines levels are correlated with decrease of plasticity in brain, as showed earlier by Anusha et al., (2017) and Johnson and Sharma, (2003), where microglial activation is linked with decrease in neurotrophins expression in mice. In our study, a reduction in BDNF, GDNF, NGF and NT-3 levels in striatum after 6-OHDA indicated an alteration of synaptic plasticity. It is known that these substances are already known be vital for neuronal survival (Moosavi et al., 2015; Pramanik et al., 2016). In support of this idea, hesperidin treatment acted in recovery of BDNF, NGF and NT-3 after 6-OHDA in striatum, like this, act in PD by increasing survival of DA neurons, since, decrease neuronal is the main characteristic of this disease (Espejo, et al., 2000). These findings suggest that neurotrophins deficit becomes more critical to dopaminergic dynamics and related behavioral activities, evidenced in this study by anhedonia and anxiety analysis. Being in accordance with results of Heinz et al. 1994, the anhedonia, a common depressive disorder, is supposed to be a consequence of altered dopaminergic reward mechanisms. Moreover, the reduction in DA release in striatum is also correlated with anxiety (Shannonhouse et al., 2016). In line, hesperidin appears to act via plasticity mechanisms that rescue the compromised cells in striatum in 6-OHDA model, may be useful as therapy to protect or restore function or to promote neuronal survival.

PD is associated with depletion of the neurotransmitter DA in the striatum due to the degeneration of the nigrostriatal dopaminergic neurons (Hirsch, 1994) and the decreased DA biosynthesis capacity in the surviving neurons (Haavik and Toska, 1998). The activities of dopaminergic neurons can be inferred by determining the DA, DOPAC, and HVA levels as well as TH – positive neurons, the rate-limiting enzyme of DA synthesis in striatum (Kozina et al. 2014; Lee et al. 2013; Soto-Otero,et al., 2000). In this context, we found that TH, DA, DOPAC, and HVA levels were significantly decreased following 6-OHDA exposure, which is similar our previous report, showing that 6-OHDA is toxic to this cells (Antunes et al., 2014). It is possible, from this study, affirm that oral administration of hesperidin (50 mg/kg) daily for 4 week attenuated the 6-OHDA-induced catecholamine neurotoxicity, thereby maintaining the concentration of TH, DA and its levels of metabolites at normal levels. We suggest that this reversion

is linked to the stabilization of inflammation and neurotrophic factors in striatum after treatment, agreeing with Nagatsu et al., (2000) and Tansey and Goldberg, (2010), which correlated the degeneration of nigrostriatal dopaminergic neurons in PD with increased of cytokines and/or decreased of neurotrophins levels in brain. Hesperidin treatment opperated reversing striatal and behavioral changes 6-OHDA-caused, its effect may be related to antioxidant and anti-inflammatory potential (Antunes et al., 2013;, 2016; Donato et al., 2014; Filho, et al., 2013; Souza et al., 2013)., besides to its antidepressant effect, emphasizing that may represent a new therapeutic tool for the treatment of PD.

5. Conclusion

Our data show that 6-OHDA microinjection induced behavior alterations (anxiety and anhedonia) in mice and that the depletions of monoamines, neurotrophic factors and increase of neuroinflammation in striatum contributing to these behaviorals disorders. Finally, we provide evidence that flavonoid hesperidin administered for 28 days opperated reversing the alterations 6-OHDA caused in mice, through its antioxidant, anti-inflammatory effect, modulation in neurotrophins and dopaminergic neuronal restoration in striatum, indicating that may represent a new therapeutic tool for PD.

6. Conflict of interest

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

7. Acknowledgement

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References

Akhmadeeva, G.N., Magzhanov, R.V., Tayupova, G.N., Bajtimerov, A.R., Hidijatova, I.M., 2017. Anxiety and depressive disorders in Parkinson's disease. Zh Nevrol Psikhiatr Im S S Korsakova. 117, 54-58.

Antunes, M.S., Goes, A.T., Boeira, S.P., Prigol, M., Jesse, C.R., 2014. Protective effect of hesperidin in a model of Parkinson's disease induced by 6-hydroxydopamine in aged mice. Nutrition. 30, 1415–1422.

Antunes, M.S., Jesse, C.R., Ruff, J.R., de Oliveira Espinosa, D., Gomes, N.S., Altvater, E.E., Donato, F., Giacomeli, R., Boeira, S.P., 2016. Hesperidin reverses cognitive and depressive disturbances induced by olfactory bulbectomy in mice by modulating hippocampal neurotrophins and cytokine levels and acetylcholinesterase activity. Eur. J. Pharmacol. 789, 411-20.

Anusha, C., Sumathi, T., Joseph, L.D., 2017. Protective role of apigenin on rotenone induced rat model of Parkinson's disease: Suppression of neuroinflammation and oxidative stress mediated apoptosis. Chem Biol Interact. 269, 67-79.

Burguillos, M. A., Deierborg, T., Kavanagh, E., Persson, A., Hajji, N., Garcia-Quintanilla, A., Cano, J., Brundin, P., Englund, E., Venero, J.L., Joseph, B., 2011. Caspase signalling controls microglia activation and neurotoxicity. *Nature.* 472, 319–324.

Blandini, F., Armentero, M.T., 2012. Animal models of Parkinson's disease. FEBS J. 279, 1156-66.

Blomster, L.V., Vukovic, J., Hendrickx, D.A.E., Jung, S., Harvey, A.R., Filgueira, L., Ruitenberg, M.J., 2011. CX(3)CR1 deficiency exacerbates neuronal loss and impairs early regenerative responses in the target-ablated olfactory epithelium. Mol. Cell. Neurosci. 48, 236-45

Bassani, T.B., Vital, M.A., Rauh, L.K., 2015. Neuroinflammation in the pathophysiology of Parkinson's disease and therapeutic evidence of anti-inflammatory drugs.Arq Neuropsiquiatr. 73, 616-23.

Brochard, V., Combadière, B., Prigent, A., Laouar, Y., Perrin, A., Beray-Berthat, V., Bonduelle, O., Alvarez-Fischer, D., Callebert, J., Launay, J.M., Duyckaerts, C., Flavell, R.A., Hirsch, E.C., Hunot, S., 2009. Infiltration of CD4 + lymphocytes into the brain contributes to neurodegeneration in a mouse model of Parkinson disease. J. Clin. Invest., 119, 182–192.

Cagnin, A., Kassiou, M., Meikle, S.R., Banati, R.B., 2006. In vivo evidence for microglial activation in neurodegenerative dementia. Acta Neurol. Scand. Suppl. 185, 107–14.

Chaudhuri, K.R., Schapira, A.H. 2009. Non-motor symptoms of Parkinson's disease: dopaminergic pathophysiology and treatment. Lancet Neurol. 8, 464–474.

Clénet, F., Bouyon, E., Hascoet, M., Bourin, M., 2006. Light/dark cycle manipulation influences mice behavior in the elevated plus maze. Behav. Brain Res. 166, 140–149.

d'Audiffret, A.C., Frisbee, S.J., Stapleton, P.A., Goodwill, A.G., Isingrini, E, Frisbee, J.C., 2010. Depressive behavior and vascular dysfunction: a link between clinical depression and vascular disease? J. Appl. Physiol. 108, 1041–1051.

Dissanayaka, N.N., Sellbach, A., Silburn, P.A., O'Sullivan, J.D., Marsh, R., Mellick, G.D. 2011. Factors associated with depression in Parkinson's disease. J Affect Disord. 132, 82-8.

Donato, F., de Gomes, M.G., Goes, A.T., Filho, C.B., Del Fabbro, L., Antunes. M.S., Souza, L.C., Boeira, S.P., Jesse, C.R. 2014. Hesperidin exerts antidepressant-like effects in acute and chronic treatments in mice: possible role of l-arginine-NO-cGMP pathway and BDNF levels. Brain Res. Bull. 104, 19-26.

Espejo, M., Cutillas, B., Arenas, T.E., Ambrosio, S., 2000. Increased survival of dopaminergic neurons in striatal grafts of fetal ventral mesencephalic cells exposed to neurotrophin-3 or glial cell line-derived neurotrophic fator. Cell Transplant. 9, 45–53.

Filichia, E., Shen, H., Zhou, X., Qi, X., Jin, K., Greig, N., Hoffer, B., Luo, Y., 2015. Forebrain neuronal specific ablation of p53 gene provides protection in a cortical ischemic stroke model. Neuroscience, 295, 1–10.

Filho, C.B., Del Fabbro, L., de Gomes, M.G., Goes, A.T., Souza, L.C., Boeira, S.P., Jesse, C.R., 2013. Kappa-opioid receptors mediate the antidepressant-like activity of hesperidin in the mouse forced swimming test. Eur J Pharmacol. 698, 286-91.

Franklin, K., Paxinos, G., 1997. The mouse brain in stereotaxic coordinates, New York: Academic. Press.

Garg, A., Garg, S., Zaneveld, L.J., Singla, A.D., 2001. Chemistry and pharmacology of the citrus bioflavonoid Hesperidin. Phytother. Res. 15, 655–669.

Gaur, V., Kumar, A., 2010. Hesperidin pre-treatment attenuates NO-mediated cerebral ischemic reperfusion injury and memory dysfunction. Pharmacol. Rep. 62, 635–648

Gundersen, H.J.G., Bagger, P., Bendtsen, T.F., 1988. The new stereoloical tools: disector, fractionator, nucleator and point sampled intercepts and their use in pathological research and diagnosis. Acta Pathol. Microbiol. Immunol. Scand. 96, 857–881.

Gundersen, H.J.G., 2002. The smooth fractionator.J. Microsc. 207, 191–210.

Habig, W.H., Jakoby, W.B., 1981. Glutathione S-transferases (rat and humam). Methods Enzymol. 77, 218–231.

Heinz, A., Schmidt, L.G., Reischies, F.M., 1994. Anhedonia in schizophrenic, depressed, or alcohol-dependent patients–neurobiological correlates. Pharmacopsychiatry. 27, 7–10.

Hirsch, E.C., 1994. Biochemistry of Parkinson's disease with special reference to the dopaminergic systems. Mol. Neurobiol. 9, 135–142.

Johnson, V.J., Sharma, R.P., 2003. Aluminum disrupts the pro-inflammatory cytokine/neurotrophin balance in primary brain rotation-mediated aggregate cultures: possible role in neurodegeneration. Neurotoxicology. 24, 261–268.

Kim, H.D., Jeong, K.H., Jung, U.J., Kim, S.R., 2016. Naringin treatment induces neuroprotective effects in a mouse model of Parkinson's disease in vivo, but not enough to restore the lesioned dopaminergic system. J. Nutr. Biochem. 28,140-6.

Kozina, E.A., Khakimova, G.R., Khaindrava, V.G., Kucheryanu, V.G., Vorobyeva, N.E., Krasnov, A.N., Georgieva, S.G., Kerkerian-Le Goff, L., Ugrumov, M.V., 2014. Tyrosine hydroxylase expression and activity in nigrostriatal dopaminergic neurons of MPTP-treated mice at the presymptomatic and symptomatic stages of parkinsonism. J. Neurol. Sci. 340,198–207.

Lee, K.W., Im, J.Y., Woo, J.M., Grosso, H., Kim, Y.S., Cristovao, A.C., Sonsalla, P.K., Schuster, D.S., Jalbut, M.M., Fernandez, J.R., Voronkov, M., Junn, E., Braithwaite, S.P., Stock, J.B., Mouradian, M.M., 2013. Neuroprotective and anti-inflammatory properties of a coffee component in the MPTP model of Parkinson’s disease. Neurotherapeutics. 10,143–153.

Lees, A.J.J., Hardy, Revesz T., 2009. Parkinson's disease. Lancet. 373, 2055–2066.

Lev, N., Barhum, Y., Ben-Zur, T., Melamed, E., Steiner, I., Offen, D., 2013. Knocking out DJ-1 attenuates astrocytes neuroprotection against 6-hydroxydopamine toxicity. J. Mol. Neurosci. 50, 542–550

Martinez-Martin, P., Rodriguez-Blazquez, C., Kurtis, M.M., Chaudhuri, K.R., 2011. The impact of non-motor symptoms on health-related quality of life of patients with Parkinson's disease. Mov. Disord. 3, 399–406.

Matheus, F.C., Rial, D., Real, J.I., Lemos, C., Takahashi, R.N., Bertoglio, L.J., Cunha, R.A., Prediger, R.D., 2016. Temporal Dissociation of Striatum and Prefrontal Cortex Uncouples Anhedonia and Defense Behaviors Relevant to Depression in 6-OHDA-Lesioned Rats. Mol Neurobiol. 53, 3891-9.

Moosavi, F., Hosseini, R., Saso, L., Firuzi, O., 2015. Modulation of neurotrophic signaling pathways by polyphenols.Drug Des Devel Ther. 21, 10:23-42.

Nagatsu, T., Mogi, M., Ichinose, H., Togari, A., 2000. Cytokines in Parkinson's disease. J. Neural. Transm. Suppl. 58, 143-51.

Norden, D.M., Muccigrosso, M.M., Godbout, J.P. 2015. Microglial priming and enhanced reactivity to secondary insult in aging, and traumatic CNS injury, and neurodegenerative disease. Neuropharmacology. 96, 29–41.

Olanow, C.W., Schapira, A.H., 2013. Therapeutic prospects for Parkinson disease. Ann. Neurol. 74, 337–347.

O'Connor, K.A., Feustel, P.J., Ramirez-Zamora, A., Molho, E., Pilitsis, J.G., 2016. Investigation of diazepam efficacy on anxiety-like behavior in hemiparkinsonian rats.Shin DS. Behav Brain Res. 301, 226-37.

Oorchot, D.E., 1996. Total number of neurons in the neostriatal, pallidal, subthalamic, and substantia nigral nuclei of the rat basal ganglia: A stereological study using the cavalieri and optical disector methods Journal of Comparative Neurology. 366, 580–599.

Owen, A.M., Sahakian, B.J., Hodges, J.R., Summers, B.A., Polkey, C.E., Robbins, T.W., 1995. Dopamine-dependent frontostriatal planning deficits in early Parkinson's disease. Neuropsychology. 9, 126–140.

Pellow, S., Chopin, P., File, S.E., Briley, M., 1985. Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. J. Neurosci. Methods. 14, 149–167.

Pramanik, S., Sulistio, Y.A., Heese, K., 2016. Neurotrophin Signaling and Stem Cells-Implications for Neurodegenerative Diseases and Stem Cell Therapy.Mol Neurobiol. Nov 5. [Epub ahead of print]

Prut, L., Belzung, C., 2003. The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review. Eur. J. Pharmacol. 463, 3–33.

Schmitz, C., Hof, P.R. 2005. Design-based stereology in neuroscience. Neuroscience. 130, 813–831.

Shannonhouse, J.L., DuBois, D.W., Fincher, A.S., Vela, A.M., Henry, M.M., Wellman, P.J., Frye, G.D., Morgan, C., 2016. Fluoxetine disrupts motivation and GABAergic signaling in adolescent female hamsters. Prog Neuropsychopharmacol Biol Psychiatry. 1, 69:19-30.

Shuman, J.M., De Jager, P.L., Feany, M.B., 2011. Parkinson's disease: genetics and pathogenesis. Annu Rev. Pathol. 6, 193–222.

soto-Otero, R., Méndez-Alvarez, E., Hermida-Ameijeiras, A., Muñoz-Patiño, A.M., Labandeira-Garcia, J.L., 2000. Autoxidation and neurotoxicity of 6-hydroxydopamine in the presence of some antioxidants: potential implication in relation to the pathogenesis of Parkinson's disease. J Neurochem. 74, 1605–1617.

Souza, L.C., de Gomes, M.G., Goes, A.T., Del Fabbro, L., Filho, C.B., Boeira, S.P., Jesse, C.R., 2013. Evidence for the involvement of the serotonergic 5-HT(1A) receptors in the antidepressant-like effect caused by hesperidin in mice.Prog Neuropsychopharmacol Biol Psychiatry. 40, 103-9.

Tansey, M.G., Goldberg, M.S., 2010. Neuroinflammation in Parkinson's disease: its role in neuronal death and implications for therapeutic intervention. Neurobiol. Dis. 37,510-8.

Ungerstedt, U., 1968. 6-Hydroxydopamine induced degeneration of central monoamine neurons. Eur. J. Pharmacol. 5, 107–117.

Vivekanantham, S., Shah, S., Dewji, R., Dewji, A., Khatri, C., Ologunde, R., 2015. Neuroinflammation in Parkinson's disease: role in neurodegeneration and tissue repair. Int. J. Neurosci. 125, 717–725.

Wang, Q., Liu, Y., Zhou, J., 2015. Neuroinflammation in Parkinson’s disease and its potential as therapeutic target. Transl. Neurodegener. 12, 4-19.

Wichowicz, H.M., Sławek, J., Derejko, M., Cubała, W.J., 2006. Factors associated with depression in Parkinson's disease: a cross-sectional study in a Polish population. Eur. Psychiatry. 21, 516-20.

Yalcin, I., Aksu, F., Belzung, C., 2005. Effects of desipramine and tramadol in a chronic mild stress model in mice are altered by yohimbine but not by pindolol. Eur. J. Pharmacol. 514, 165–174.

Figure Legends

Fig. 1. Schematic representation of the experimental protocol describing the treatment periods with hesperidin (50 mg/kg, *p.o*), behavior tests and neurochemistry analyzes.

Fig. 2. (A) Effect of hesperidin treatment in the OFT – central time. **(B)** Effect of hesperidin treatment in OFT – total travelled distance. Values are mean ± S.E.M. (n=7 per group). *: $p<0.05$ when compared 6-OHDA/vehicle with SHAM/vehicle (two-way ANOVA and Newman–Keuls multiple comparison test).

Fig. 3. (A) Effects of hesperidin treatment during twenty-eight days after 6-OHDA in EPTM - total. **(B)** Effects of hesperidin treatment during twenty-eight days after 6-OHDA in EPTM - entries. **(C)** Effects of hesperidin treatment during twenty-eight days after 6-OHDA infusion in splash test. Values are mean ± S.E.M. (n=7 per group). **: $p<0.01$, ***: $p<0.001$ when compared 6-OHDA/vehicle with SHAM/vehicle; #: $p<0.05$ when compared 6-OHDA/hesperidin with 6-OHDA/vehicle (two-way ANOVA and Newman–Keuls multiple comparison test).

Fig. 4. Effects of hesperidin treatment during twenty-eight days after 6-OHDA microinjection in TNF-α (**A**), IFN-γ (**B**) levels in the striatum. Values are mean ± S.E.M. (n=7 per group). ***: $p<0.001$ when compared 6-OHDA/vehicle with SHAM/vehicle; #: $p<0.05$ when compared 6-OHDA/hesperidin with 6-OHDA/vehicle (two-way ANOVA and Newman–Keuls multiple comparison test).

Fig. 5. Effects of hesperidin treatment during twenty-eight days after 6-OHDA infusion in IL-1β **(A)**, IL-2 **(B)**, in IL-6 **(C)** and IL-10 **(D)** levels in striatum of mice. Values are mean ± S.E.M. (n=7 per group). *: $p<0.05$; **: $p<0.01$ ***: $p<0.001$ when compared 6-OHDA/vehicle with SHAM/vehicle; #: $p<0.05$ when compared 6-OHDA/hesperidin with 6-OHDA/vehicle (two-way ANOVA and Newman–Keuls multiple comparison test).

Fig. 6. Effects of hesperidin treatment during twenty-eight days after 6-OHDA microinjection in NGF **(A)**, GNDF **(B)**, BDNF **(C)** and NT-3 **(D)** levels in the striatum. Values are mean ± S.E.M. (n=7 per group). *: $p<0.05$; **: $p<0.01$ when compared 6-OHDA/vehicle with SHAM/vehicle; #: $p<0.05$ when compared 6-OHDA/hesperidin with 6-OHDA/vehicle (two-way ANOVA and Newman–Keuls multiple comparison test).

Fig. 7. Effects of hesperidin treatment during twenty-eight days after 6-OHDA infusion in DA **(A)**, DOPAC **(B)** and HVA **(C)** levels in the striatum. Values are mean ± S.E.M. (n=7 per group). **: $p<0.01$ when compared 6-OHDA/vehicle with SHAM/vehicle; #:

p<0.05 when compared 6-OHDA/hesperidin with 6-OHDA/vehicle (two-way ANOVA and Newman–Keuls multiple comparison test).

Fig. 8. Photomicrographs showing the optical disector probe application, where could be observed TH-positive neurons, from substantia nigra pars compacta in different focal planes: Control (A and E), 6-OHDA (B and F), Hesperidin (C and G), 6-OHDA + hesperidin (D and H) 5X and 20X respectively. The figure **(I)** show effects of hesperidin treatment during two weeks in quantification of TH-positive cells in striatum after 6-OHDA. Values are mean ± S.E.M. (n=7 per group). *: p<0.05; ***: p<0.001 when compared 6-OHDA/vehicle with SHAM/vehicle; #: p<0.05 when compared 6-OHDA/hesperidin with 6-OHDA/vehicle (two-way ANOVA and Newman–Keuls multiple comparis.

Grapical abstract

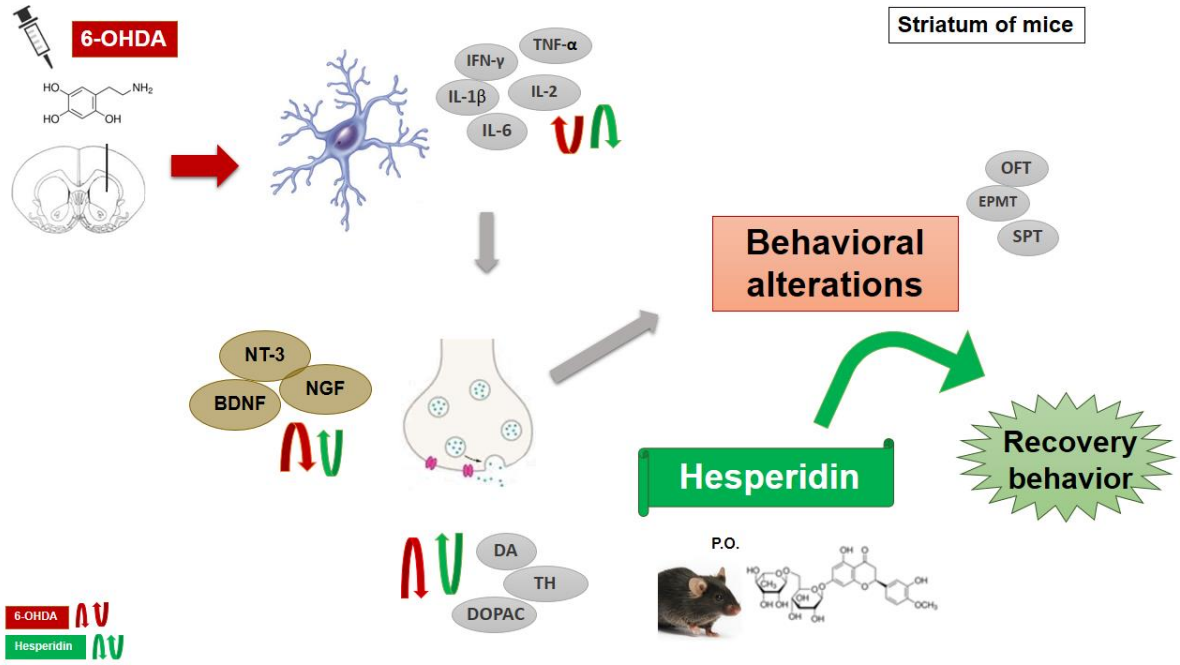


Fig. 1

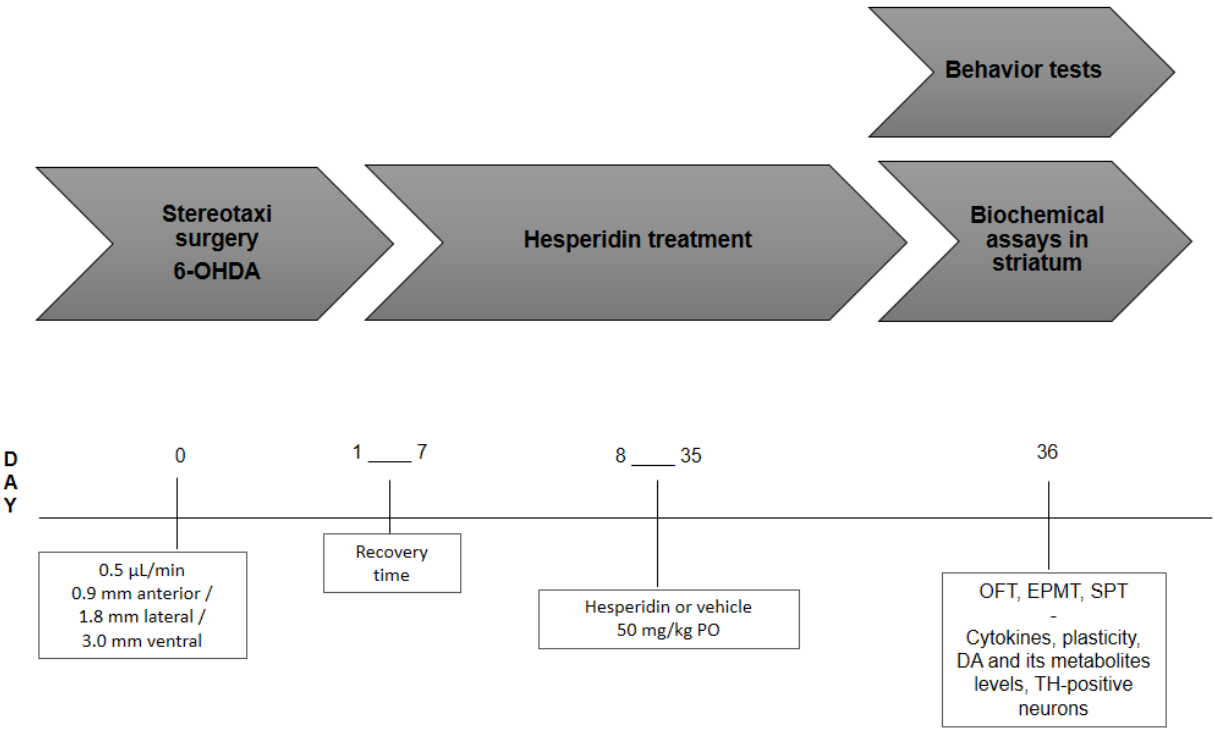
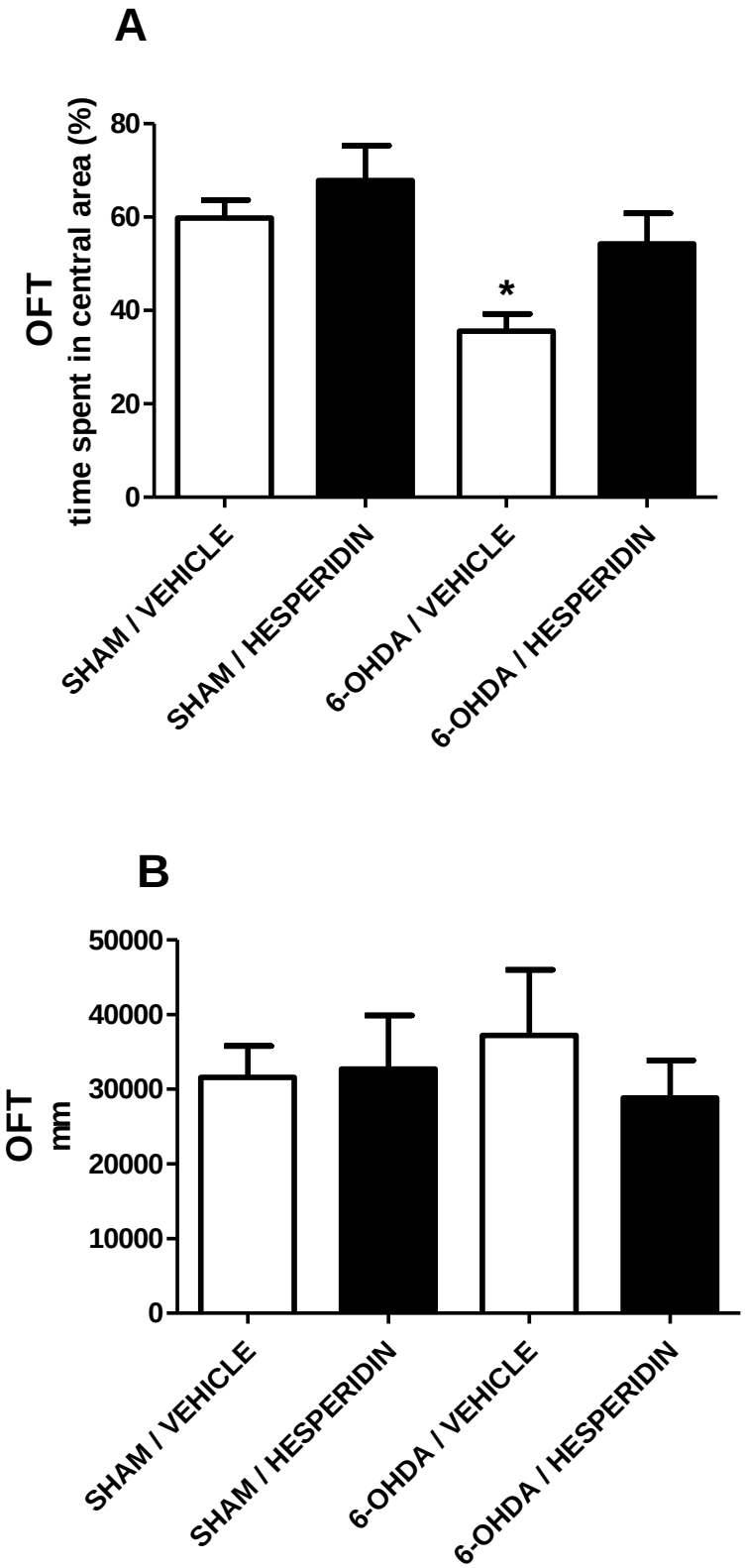


Fig. 2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*
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SHAM / HESPERIDIN
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6-OHDA / HESPERIDIN
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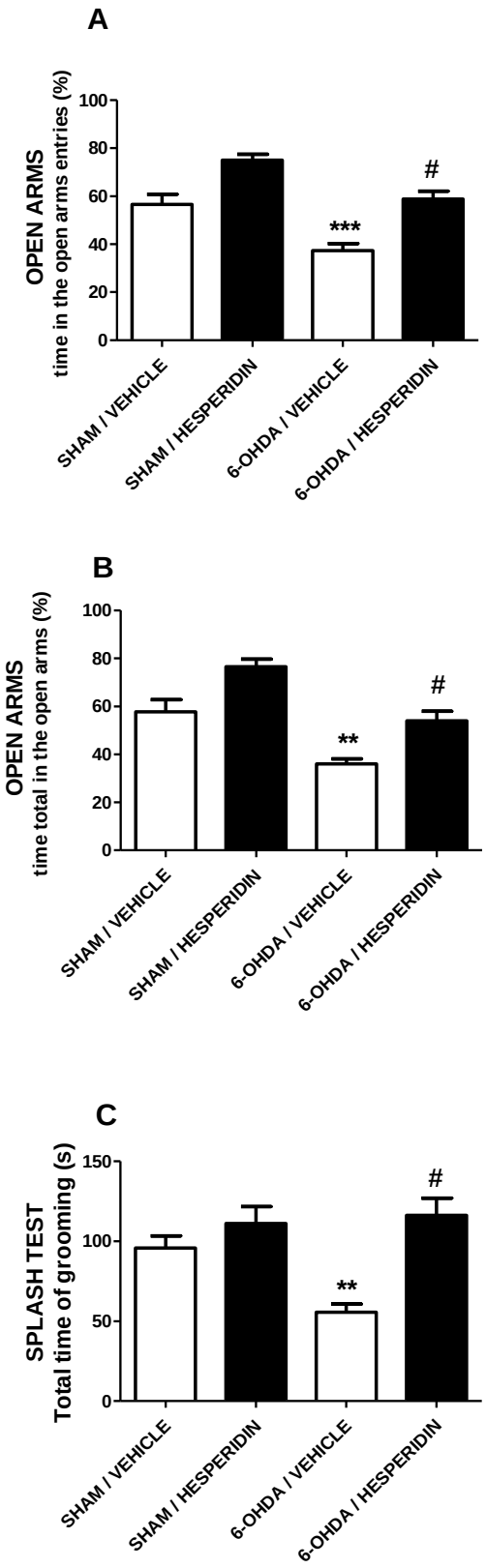
Fig. 3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6-OHDA / HESPERIDIN
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time total in the open arms (%)
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6-OHDA / HESPERIDIN
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Fig. 4

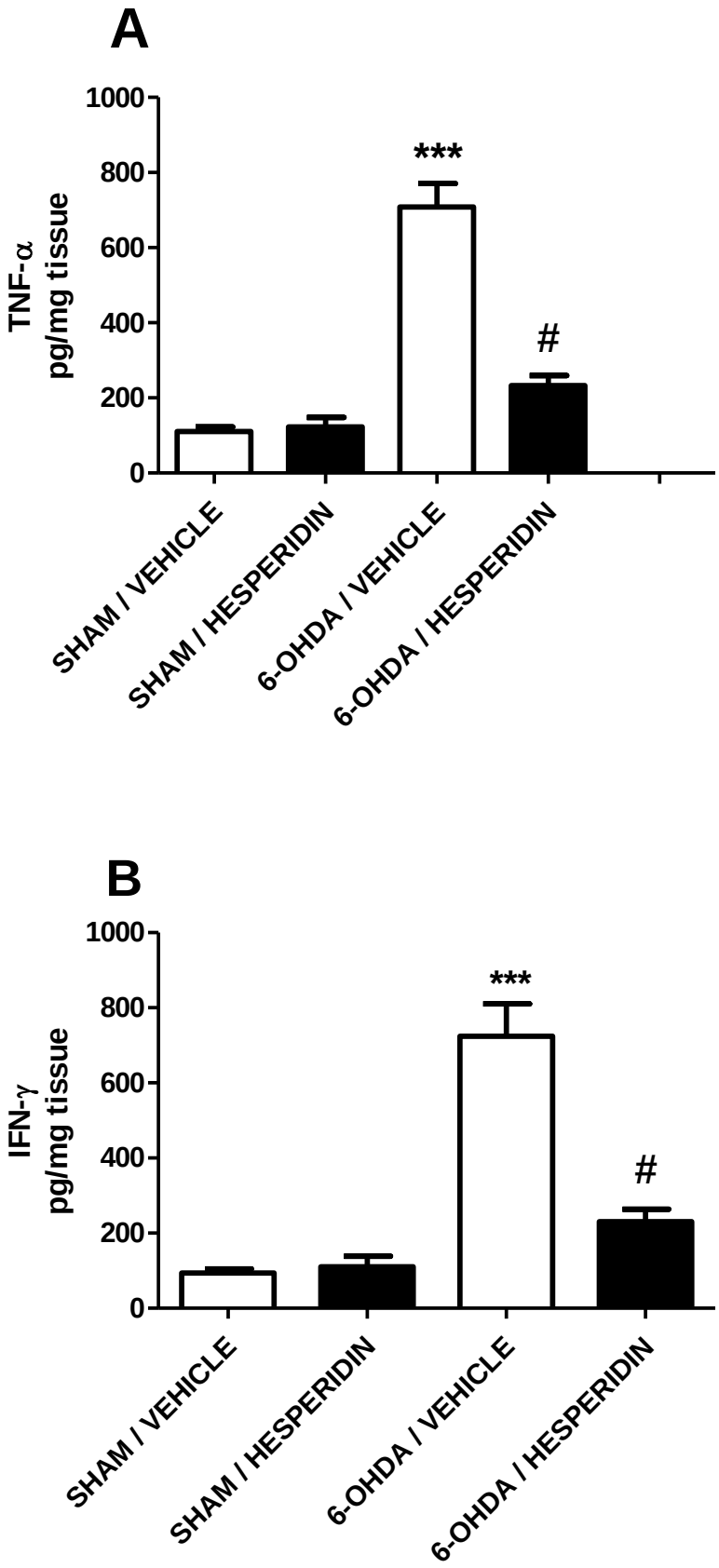
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Fig. 5

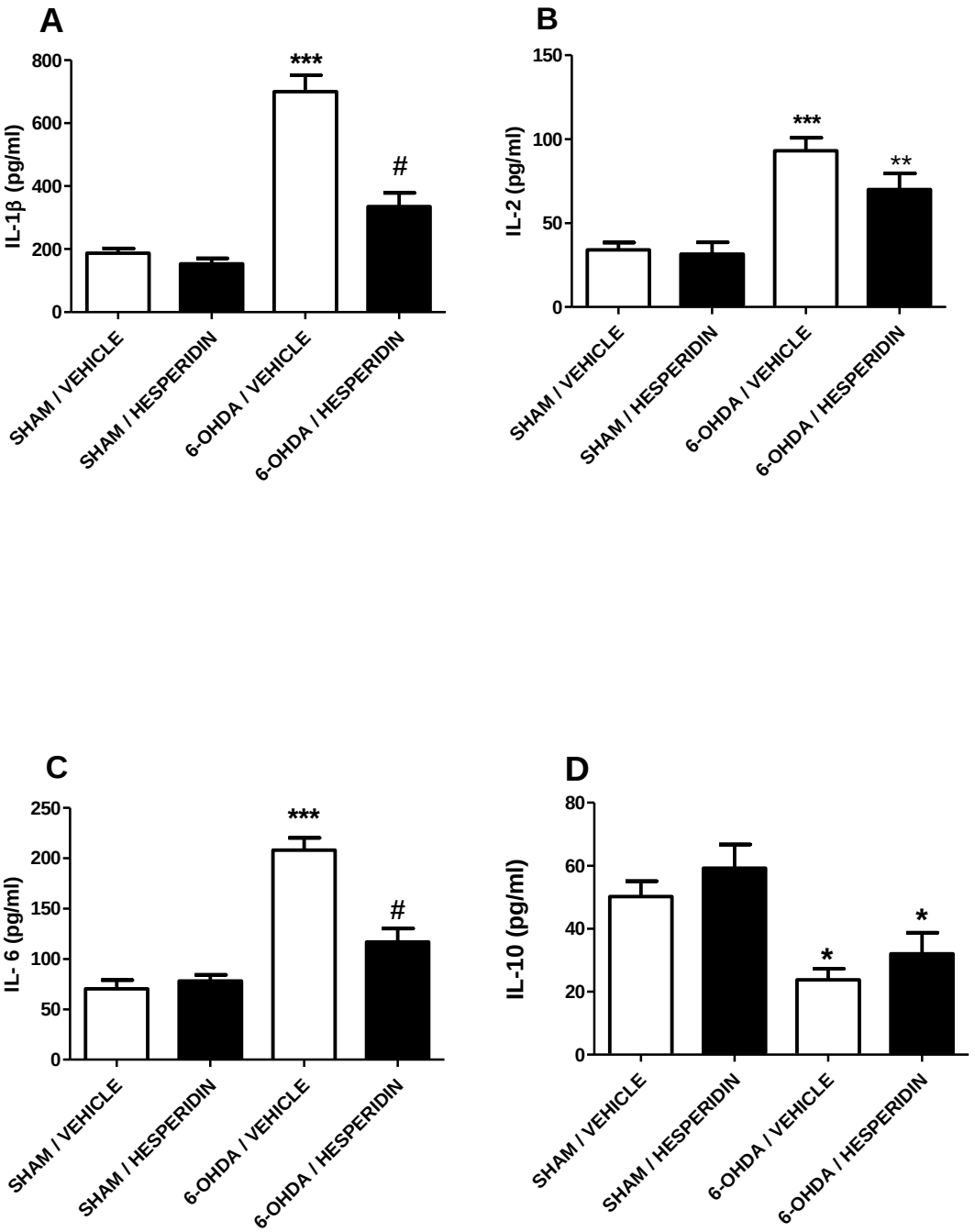
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Fig. 6

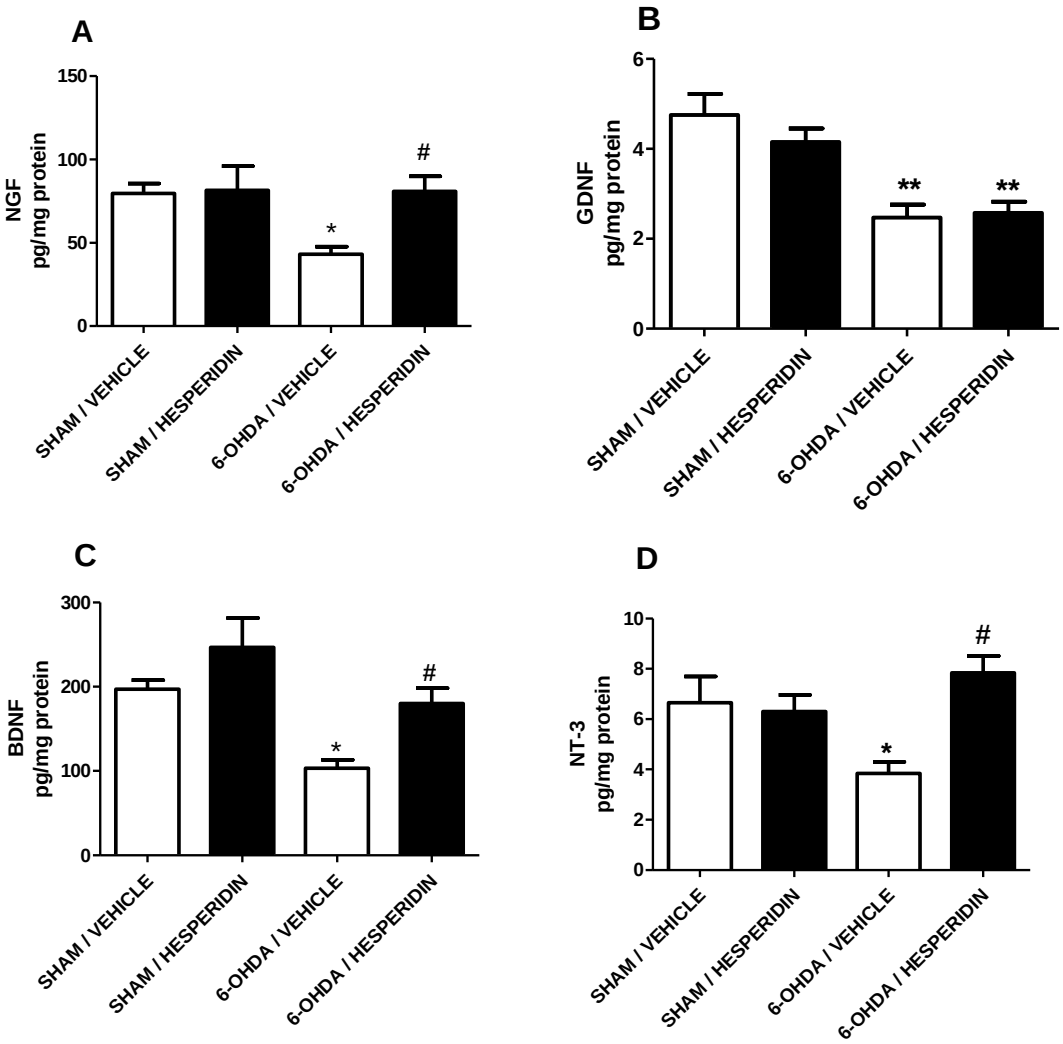
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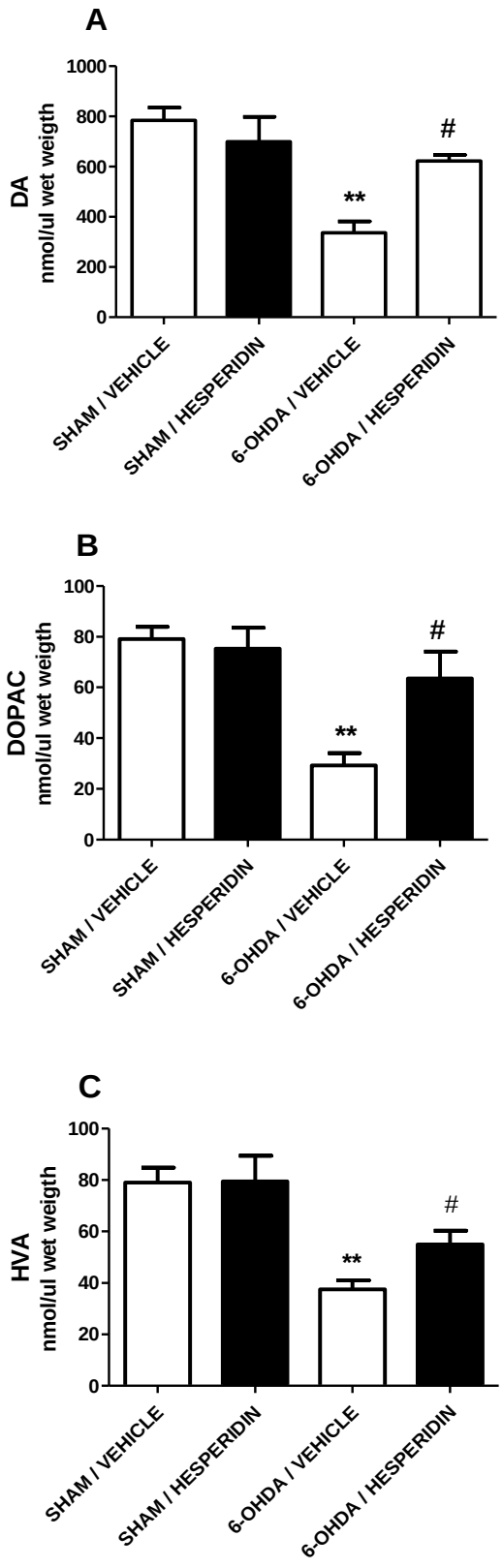
Fig. 7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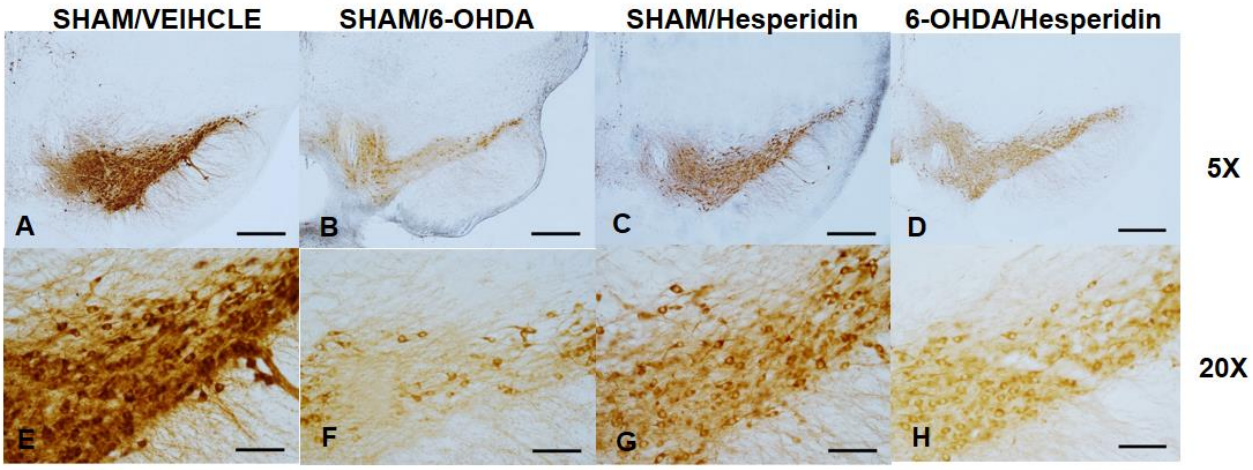
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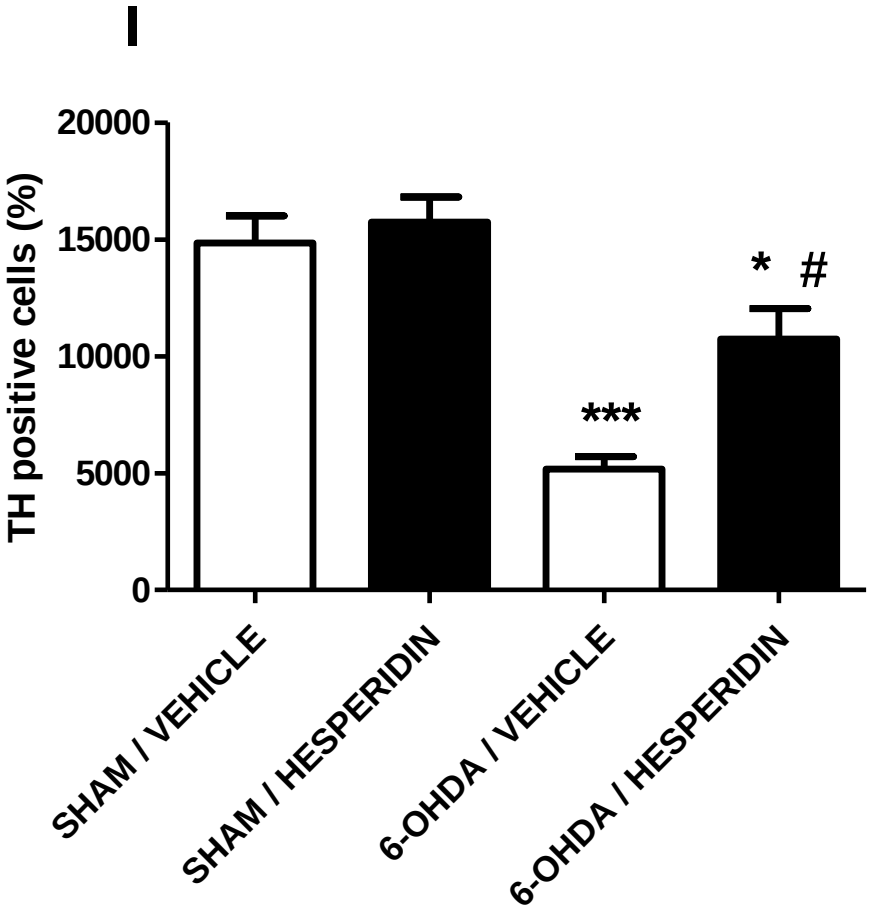
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Behavioural pharmacology

Hesperidin reverses cognitive and depressive disturbances induced by olfactory bulbectomy in mice by modulating hippocampal neurotrophins and cytokine levels and acetylcholinesterase activity

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Abstract

Depression is a serious mental disorder that is becoming more common. To better treat patients suffering from this illness, elucidation of the underlying psychopathological and neurobiological mechanisms of depression is needed. Based on the evidence, we sought to investigate the effects of hesperidin in a model of depression induced by olfactory bulbectomy (OB). C57BL/6 mice were treated with hesperidin (50 mg/kg) and imipramine (10 mg/kg, positive control) after OB induction. The brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), interleukin 1β (IL-1β) and interleukin 6 (IL-6) levels and acetylcholinesterase activity were analyzed in the hippocampus of the mice. The behavioral parameters were also verified in the model of depression induced by OB. This study demonstrated that OB increased the pro-inflammatory cytokines levels and acetylcholinesterase activity in the hippocampus, exploratory activity in the open field test and immobility in the forced swimming test in mice. In addition, OB decreased the BDNF and NGF levels in the hippocampus, grooming time in the splash test and memory consolidation in the Morris water maze task. Treatment with hesperidin, similar to imipramine, was effective in preventing these behavioral and neurochemical alterations. We suggest that the main targets of hesperidin are pro-inflammatory cytokine modulation, helping to maintain brain plasticity and acetylcholinesterase activity regulation, which are closely linked with antidepressant-like action, as shown by behavior tests. This study demonstrated that there is a pharmacological effect of hesperidin in alterations induced by OB in mice, indicating that hesperidin could be useful as a treatment for depression.

Keywords: Hesperidin; Olfactory bulbectomy; Neurotrophins; Cytokines

1. Introduction

Depression is a serious mental disorder that is becoming more common. In 2005, 17% of the population around the globe was affected by this condition (Kessler et al., 2005), and it is the fourth major cause of morbidity worldwide at present and will become the second by 2020 according to the World Health Organization (Kessler et al., 2003, 2011). Depressive disorders are clinically characterized by a prevalent and persistent low mood, accompanied by inappropriate guilt, low self-esteem, hopelessness and thoughts of death or suicide (De Bodinat et al., 2010). Despite the increasing number of available antidepressants drugs, over 30% of patients do not respond to pharmacotherapy, and full remission was only able to be achieved in only half of patients (Mihaljević-Peleš et al., 2011); there is still a clear need for drugs that have improved efficacy and fewer side effects. Based on the evidence, we examined the antidepressant-like activity of the bioflavonoid hesperidin, a specific flavonoid glycoside that is predominant in citrus fruits (Yang et al., 2012). Hesperidin has been reported to possess significant anti-inflammatory, antiviral, anticancer (Gaur and Kumar, 2010), and antidepressant-like properties in mice (Antunes et al., 2014; Donato et al., 2014).

In the current study, olfactory bulbectomy (OB) has been adopted as animal model to evaluate the effects of hesperidin in mice. OB causes numerous behavioral changes, such as hyperactivity after being exposed to a novel environment, signs of anhedonia, and cognitive deficits (Harkin et al., 2003; Hendriksen et al., 2015). Moreover, OB could cause an increase of inflammatory reactions in several brain regions (Myint et al., 2007; Rinwa and Kumar, 2013). These changes are similar to the clinical symptoms of human depression and could be improved by repeated antidepressant treatment; therefore, this model is often used to study the pathophysiology of depression and to screen antidepressants (Eisenstein et al., 2010; Oral et al., 2013). Our present study investigated the effects of hesperidin on the brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), interleukin 1β (IL-1β) and interleukin 6 (IL-6) levels and acetylcholinesterase (AChE) activity in the hippocampus of mice. Moreover, we analyzed behavior in the open field test (OFT), forced swimming test (FST), splash test, Morris water maze task (MWMT) and object recognition test (ORT) in the model of depression induced by OB in mice.

2. Materials and methods

2.1. Animals

The experiments were conducted using male C57BL/6 mice (25–35 g, 4–6 months old). The animals were maintained at constant room temperature (21±1 °C) with free access to water and food under a 12:12 h light: dark cycle (lights on at 07:00 h). The manipulations were carried out during the light portion of the cycle. 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 #001/2013) of the Federal University of Pampa, Brazil.

2.2. Reagents

All reagents used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA) with a degree of purity of 99%.

2.3. Experimental design

After undergoing an OFT, all animals were submitted to surgery to remove their olfactory bulbs or only to surgery (SHAM). The animals had 14 days of recovery and were then again subjected to an OFT after exposure to treatments. The 48 animals were divided in 6 groups. Imipramine (10 mg/kg of body weight, positive control) or hesperidin (50 mg/kg of body weight) were used as treatments. Imipramine was administered intraperitoneally (i.p.), and hesperidin was dissolved in distilled water and given by gavage (per oral, p.o.). Mice were treated with vehicle, imipramine or hesperidin once a day for two weeks. Solutions were freshly prepared each day. The controls received an identical volume of distilled water (vehicle). After finishing the treatments, the mice were subjected to behavior (we used different animal groups for each behavioral test) and biochemistry tests (Fig. 1).

2.4. Bilateral olfactory bulbectomy (OB) surgical procedure

After a 2-week acclimatization period, OB was performed according to the procedure described by Leonard and Tuite (1981). Briefly, mice were anesthetized with xylazin (20 mg/kg) in combination with ketamine (100 mg/kg) diluted in saline (0.9% NaCl) administered intraperitoneally (i.p., 10 ml/kg body weight). The skull covering the olfactory bulbs was exposed by skin incision, and two burr holes were drilled using

a dentist drill. The olfactory bulbs were bilaterally aspirated using a blunt hypodermic needle (1.0–1.2 cm long and with a rounded tip of 0.80–1.2 mm in diameter) attached to a 10-ml syringe, taking care not to cause damage to the frontal cortex. Finally, the burr hole was filled with acrylic resin to avoid bleeding and contamination of the surgical site. SHAM-operations were performed in the same way, but the olfactory bulbs were left intact. After surgery, all animals were allowed to recover in a post-operative cage (maintained at 24 °C) for 3 h. After this time period, the mice were returned to their home cage. The technique was adapted (Leonard and Tuite, 1981; Van Riezen and Leonard, 1990). After behavioral testing, all animals were killed and the presence of lesions was verified. The bulbectomized animals that showed incomplete removal of olfactory bulbs or damage to other brain areas were excluded from the subsequent analysis following the criteria previously described (Jarosik et al., 2007 and Kelly et al., 1997).

2.5. Behavioral assessment

2.5.1. Open field test (OFT)

To verify the effects of imipramine and hesperidin administration on locomotor activity, animals were submitted individually to 5 min in the OFT (Insight model EP 154C) 24 h after the last treatment in the morning. The parameters observed included the distance traveled (unit: mm) (Prut and Belzung, 2003).

2.5.2. Forced swimming test (FST)

The test conducted using the method described by Porsolt et al. (1977). Briefly, the mice were individually forced to swim in open cylinders (25-cm height×10-cm diameter) containing 19 cm of water at 25±1 °C. The duration of immobility was scored during the 6 min test period as described previously (Rodrigues et al., 2002). The test occurred in the morning. Each mouse was recorded as immobile when floating motionless or making only those movements necessary to keep its head above the water.

2.5.3. Splash test

The splash test was adapted from Yalcin et al. (2005). This test evaluates grooming behavior, defined as cleaning of the fur by licking or scratching, after vaporization of a 10% sucrose solution onto the mouse’s dorsal coat. The solution’s viscosity prompts mice to initiate grooming behavior, with depressive symptoms

characterized by an increased latency (idle time between spray and the initiation of grooming) and decreased time spent grooming (d'Audiffret et al., 2010). Latency and time spent grooming were recorded for 5 min.

2.5.4. Morris water maze task (MWMT)

The Morris water maze task (MWMT) was performed in a circular swimming pool similar to that described by Morris et al. (1982). The pool consisted of black painted fiberglass, 97 cm in diameter and 60 cm in height. For the tests, the tank was filled with water maintained at 23+2 °C. The target platform (10×10 cm2) was made of transparent Plexiglas and was submerged 1–1.5 cm beneath the surface of the water. The starting points for the animals were marked on the outside of the pool as north (N), south (S), east (E) and west (W). Four distant visual cues (55×55 cm2) were placed on the walls of the water maze room. The cues were positioned such that their the lower edges were situated 30 cm above the upper edge of the water tank, and in the standard setting, the position of each symbol marked the midpoint of the perimeter of a quadrant (circle¼NE quadrant, square¼SE quadrant, cross¼SW quadrant, and diamond¼NW quadrant). The apparatus was located in a room with indirect incandescent illumination. The mice were submitted to a spatial reference memory version of the water maze using a previously described protocol by Prediger et al. (2007). The training session consisted of ten consecutive trials during which the animals were left in the tank facing the wall and then allowed to swim freely to the submerged platform. The platform was located in a constant position (middle of the southwest quadrant), equidistant from the center and the wall of the pool. If the animal did not find the platform during a period of 60 s, it was gently guided to the platform. The animal was allowed to remain on the platform for 10 s after escaping to it and was then removed from the tank for 20 s before being placed at the next starting point in the tank. This procedure was repeated ten times, with the starting points (the axis of one imaginary quadrant) varying in a pseudo-randomized manner. The test session was carried out 24 h later and consisted of a single probe trial wherein the platform was removed from the pool and each mouse was allowed to swim for 60 s in the maze. The time spent in the correct quadrant (i.e., the quadrant in which the platform was located on the training session) was recorded, and the percentage of the total time was analyzed.

2.5.5. Object recognition test (ORT)

In this test, mice were placed in an open box to evaluate the preference for a novel object; short-term memory and long-term memory could be assayed. The ORT was performed as described by Ennaceur and Delacour (1988) with some modifications. The behavioral task was performed in a 45×45 cm open field surrounded by 30-cm high walls. Four objects were used: A1, A2, B and C. The “A” objects were two identical triangles, the “B” object was a ball and the “C” object was a rectangle. All objects were made of plastic material, with 10 cm×10 cm (length×height). Each object had following pattern of color: blue, red and yellow. Twenty-four hours after habituation, training was conducted by placing each individual rat for 5 min into the field, in which two identical objects (objects A1 and A2) were positioned in two adjacent corners, 10 cm from the walls. In a short-term memory (STM) test given 1.5 h after training, the mice explored the open field for 5 min in the presence of one familiar (A) and one novel (B) object. All objects presented similar textures, colors, and sizes, but distinctive shapes. Between trials, the objects were washed with a 10% ethanol solution. In a long-term memory (LTM) test given 24 h after training, the same rat explored the field for 5 min in the presence of a familiar object A and a novel object C. Recognition memory was evaluated as for the STM test. Exploration was defined as sniffing or touching the object with the nose and/or forepaws. The data are expressed as the mean±S.E.M. percentage time exploring any of the objects (training) or the novel objects. Exploratory preference in:Training =(A2/(A1+A2))×100; STM =(B /(A1+B))×100; LTM =(C /(A1+C))×100.

The positions of the objects in the test and the objects used as novel or familiar were counterbalanced between the animals. Exploratory behavior was defined as sniffing or touching the object with the nose and/or forepaws. Any other behavior, such as sitting on or turning around the object, was not considered to be exploration. The time each animal spent actively investigating the objects was manually measured, and the discrimination index was calculated as the time exploring the novel or familiar object divided by the total time exploring both objects.

2.6. Tissue preparation for neurochemical determinations

After the behavioral tests, the mice were killed with a barbiturate overdose (pentobarbital sodium 150 mg/kg; i.p. route) and transcardially perfused with 10 ml of ice-cold saline via the aorta. Brain dissection was performed according to the method of Spijker (2011), a method to dissect multiple brain regions from a single brain based on existing atlases (Williams, 1999). The hippocampus was bilaterally removed and rapidly homogenized in 50 mM Tris–Cl, pH 7.4. The homogenate was centrifuged at 2400g for 15 min at 4 °C, and a low-speed supernatant fraction (S1) was used for the assays.

2.7. Hippocampal biomarkers of neurochemical alterations

2.7.1. Brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) levels

The concentrations of the BDNF and NGF proteins were assessed using a two-site enzyme immunoassay kit (Promega, Madison, WI, USA). In brief, 96-well immunoplates (NUNC) were coated with 50 µl/well with the corresponding capture antibody that binds the neurotrophin of interest and were stored overnight at 4 °C. The next day, serial dilutions of known amounts of BDNF and NGF ranging from 0 to 500 pg/ml were performed in duplicate to generate a standard curve. Then, the plates were washed three times with wash buffer, and the standard curves and supernatants of hippocampus tissue homogenates were incubated in the coated wells (100 µl each) for 2 h at room temperature (RT) with shaking. After additional washes, the antigen was incubated with a second specific antibody for 2 h at RT (BDNF) or overnight at 4 °C (NGF), as specified in the protocol. The plates were washed again with wash buffer and then incubated with an anti-IgY HRP for 1 h at RT. After another wash, the plates were incubated with a TMB/Peroxidase substrate solution for 15 min and phosphoric acid 1 M (100 µl/well) was added to the wells. The colorimetric reaction product was measured at 450 nm using a microplate reader (Dynatech MR 5000, Germany). Neurotrophin concentrations were determined from the regression line for the neurotrophin standard (ranging from 7.8 to 500 pg/ml-purified mouse BDNF or NGF) incubated under similar conditions in each assay. The neurotrophin concentration was expressed as the pg/g wet weight, and all assays were performed in triplicate.

2.7.2. Measurement of cytokines interleukin-1β (IL-1β) and interleukin-6 (IL-6) levels

The levels of IL-1β and IL-6 in the hippocampus were quantified using commercial mouse enzyme-linked immunosorbent assay kits (eBioscience, San Diego, CA, USA), according to the manufacturer's instructions (DuoSet Kits, R&D Systems; Minneapolis, USA). The results are shown as pg/mg tissue.

2.7.3. Determination of acetylcholinesterase (AChE) activity

The AChE activity was measured by the method described by Ellman et al. (1961), using acetylthiocholine iodide as a substrate in homogenates of the hippocampus. Each sample was assayed in triplicate. The rate of hydrolysis of acetylthiocholine iodide was measured at 412 nm through the release of thiol compounds, which reacted with 5,5′-dithiobis-(2-nitrobenzoic acid (DTNB), producing the colored product thionitrobenzoic acid.

2.8. Data analysis

The data were analyzed using Prism 5 (GraphPad) software. Comparisons between the experimental and control groups were performed by one-way analysis of variance (ANOVA), followed by the Tukey post hoc test or two-way ANOVA (OB×treatments – hesperidin or imipramine) and by Bonferroni post hoc tests, when appropriate. Descriptive statistics data were expressed as the mean(s)±S.E.M. Probability values less than 0.05 ($P < 0.05$) were considered statically significant.

3. Results

3.1. Behavioral assessment

3.1.1. Open field test (OFT)

Two-way ANOVA showed a significant main effect for OB [$F(1,30)=50.85$, $P<0.001$], treatments (hesperidin or imipramine) [$F(2,30)=3.80$, $P<0.03$] and OB×treatments interaction [$F(1,30)=6.36$, $P<0.006$] in the crossing numbers. One-way ANOVA revealed a hyperactivity induced by OB in the crossing numbers in the OFT ($P<0.001$) (Fig. 2A). In the rearing episodes, two-way ANOVA demonstrated a significant main effect for OB [$F(1,30)=13.01$, $P<0.001$], treatments (hesperidin or imipramine) [$F(2,30)=10.63$, $P<0.005$] and OB×treatments interaction [$F(2,30)=7.75$, $P<0.002$]. One-way ANOVA revealed the significant effect of OB in the number of rearings ($P<0.001$) (Fig. 2B). Post hoc comparisons demonstrated that treatment with

hesperidin or imipramine reversed the increase in crossing numbers and rearing episodes induced by OB in mice (P<0.01) (Fig. 2A and B).

3.1.2. Forced swimming test (FST)

Two-way ANOVA of depressive-like behavior in the FST revealed a significant main effect for OB [F (1,30)=39.51, P<0.001], treatments (hesperidin or imipramine) [F (2,30)=20.66, P<0.001] and OB×treatments interaction [F (2,30)=7.36, P<0.005]. One-way ANOVA demonstrates that immobility time produced by OB was significantly increased (P<0.05) when compared to the control group. The results also indicated that imipramine (P<0.05) and hesperidin (P<0.01) produced an effect per se in the FST. Post hoc comparisons revealed hesperidin (P<0.01) or imipramine (P<0.01) treatments attenuated the depressive-like behavior, promoting an antidepressant-like effect (Fig. 3A).

3.1.3. Splash test

Two-way ANOVA showed a significant main effect for OB [F (1,30)=32.52, P<0.001], treatments (hesperidin or imipramine) [F (2,30)=4.38, P<0.05] and OB×treatments interaction [F (2,30)=5.09, P<0.01] in the splash test. One-way ANOVA indicates that OB increased the total time of grooming, an indicative of loss of self-car and motivational behavior, when compared to control group (Sham/saline) (P<0.001) (Fig. 3B). Post hoc comparisons showed that chronic treatment with hesperidin (P<0.001) or imipramine (P<0.01) significantly protected against the change in the behaviors (latency to grooming and time spent grooming) in the mice (Fig. 3B).

3.1.4. Morris water maze task (MWMT)

One-way ANOVA measures revealed that the OB and treatments did not interfere with the spatial learning and memory of the animals, since no alterations in the escape latency (training session) were observed (Fig. 4A). The animals from all of the experimental groups were able to learn the task (Fig. 4A).

Two-way ANOVA revealed a significant main effect for OB [F (1,30)=33.84, P<0.001], treatments (hesperidin or imipramine) [F (2,30)=10.91, P<0.004] and OB×treatments interaction [F (2,30)=6.58, P<0.005] in the escape latency. A one-way ANOVA revealed significant effect of OB surgery in the latency to target plataform location during the test session when compared to control group (P<0.001) (Fig. 4B). Post hoc comparisons revealed that imipramine (P<0.01) or hesperidin (P<0.001) treatments

reversed this memory deficit induced by OB in the mice (Fig. 4B). Two-way ANOVA revealed a significant main effect for OB [F (1,30)=10.89, P<0.003], treatments (hesperidin or imipramine) [F (2,30)=9.97, P<0.001] and OB×treatment interaction [F (2,30)=7.33, P<0.003] in the crossing numbers. In addition, a one-way ANOVA revealed significant effect of OB in number of crossing in the plataform in test session when compared to control group (P<0.001) (Fig. 4C). Post hoc comparisons revealed that administration of imipramine or hesperidin (P<0.001) protected against memory impairments induced by OB in the MWMT in the mice (Fig. 4C).

3.1.5. Object recognition test (ORT)

Two-way ANOVA of cognitive performance in the ORT revealed a significant main effect for OB [F (1,30)=4.67, P<0.04], treatments (hesperidin or imipramine) [F (2,30)=3.89, P<0.05] and OB×treatments interaction [F (2,30)=7.79, P<0.002]. One-way ANOVA demonstrates that recognition time by OB was significantly increased when compared to the control group (P<0.001). Post hoc comparisons demonstrated that hesperidin and imipramine (P<0.05) improved the recognition index in memory impairment promotion by bulbectomy (Fig. 5A). Two-way ANOVA of the total time exploration in STM did not reveal an interaction in the groups in ORT (Fig. 5B). One-way ANOVA measures revealed that the OB and treatments did not interfere with cognitive performance in the ORT, since no difference was observed in the total time of exploration.

3.2. Hippocampal biomarkers of neurochemical alterations

3.2.1. Determination of the Brain-derived neurotrophic factor (BDNF) level

Two-way ANOVA of the BNDF level in the hippocampus of mice demonstrated a significant main effect for OB [F (1,30)=29.72, P<0.001], treatments (hesperidin or imipramine) [F (2,30)=22.11, P<0.001] and OB×treatments interaction [F (2,30)=3.88, P<0.05]. One-way ANOVA measures revealed that treatment with hesperidin (P <0.001) caused a significant increase (effect per se) in the BNDF levels in the hippocampus when compared to control group (Sham saline). Post hoc comparisons revealed that OB (P<0.01) significantly decreased the BNDF levels in the hippocampus and that the hesperidin (P<0.01) or imipramine (P<0.01) administrations prevented the reduction of this neurotrophin (Fig. 6A).

3.2.2. Determination of nerve growth factor (NGF) level

Two-way ANOVA of the NGF level demonstrated a significant main effect for treatments (hesperidin or imipramine) [F (2,30)=9.92, P<0.001] and OB×treatments interaction [F (2,30)=5.13, P<0.01], but not for the OB factor [F (1,30)=0.63, P<0.43]. One-way ANOVA demonstrated that OB (P<0.001) caused a significant decrease in the NGF levels in the hippocampus when compared to control group. Post hoc comparisons revealed that treatments (hesperidin (P<0.01) or imipramine (P<0.001)) prevented against the decrease of the neurotrophin level in the hippocampus of the mice (Fig. 6B).

3.2.3. Measurement of cytokines interleukin-1β and interleukin-6 levels

Two-way ANOVA of the IL-1β levels in the hippocampus of the mice demonstrated a significant main effect for OB [F (1,30)=55.90, P<0.001], treatments (hesperidin or imipramine) [F (2,30)=15.44, P<0.001] and OB×treatments interaction [F (2,30)=12.14, P<0.05] (Fig. 7A). One-way ANOVA demonstrated that OB (P<0.001) caused a significant increase in the IL-1β levels in the hippocampus when compared to Sham/saline group. Post hoc comparisons revealed that hesperidin (P<0.001) and imipramine (P<0.001) partially prevented the increase in these interleukin levels caused by OB in the hippocampus of mice (Fig. 7A). There was also a significant main effect for OB [F (1,30)=15.47, P<0.001], treatments (hesperidin or imipramine) [F (2,30)=20.30, P<0.001] and OB×treatments interaction [F (2,30)=13.66, P<0.002] on the IL-6 levels (Fig. 7B). One-way ANOVA demonstrated that OB (P<0.001) caused a significant increase in the IL-6 levels in the hippocampus when compared to sham/saline group. Post hoc comparisons revealed that hesperidin (P<0.001) and imipramine (P<0.001) partially prevented the increase in the IL-6 levels caused by OB in the hippocampus of mice (Fig. 7B).

3.2.4. Acetylcholinesterase activity

Two-way ANOVA revealed a main effect for OB [F (1,30)=30.34, P<0.001], treatments (hesperidin or imipramine) [F (2,30)=6.54, P<0.005] and OB×treatments interaction [F (2,30)=7.51, P<0.002]. One-way ANOVA demonstrated that OB (P<0.001) caused a significant increase in the Acetylcholinesterase activity in the hippocampus when compared to sham/saline group. Post hoc comparisons revealed that hesperidin (P<0.05) and imipramine (P<0.05) treatments decreased the activity in the hippocampus of bulbectomized mice (Fig. 8).

4. Discussion

The current study demonstrated the pharmacological effects of hesperidin on behavior and neurochemistry changes induced by OB in mice. Bulbectomized mice exhibited various behavioral alterations, such as increases in the OFT, anhedonia in the splash test, immobility time in the FST and impairment of memory in the MWMT and ORT. Moreover, mice exhibited decreases of BNDF and NGF levels and increases of acetylcholinesterase activity and IL-1β and IL-6 levels in the hippocampus. Hesperidin administration reversed these changes, as did the antidepressant imipramine (positive control). We suggest that this flavonoid acts to regulate neurotrophin and cytokine levels and acetylcholinesterase activity in the hippocampus to reverse the alterations in behavioral tests.

The bilateral removal of the olfactory bulbs in rodents has been extensively accepted as an effective animal model of depression (Morales-Medina et al., 2013; Song and Leonard, 2005). OB remarkably increased spontaneous activity in the OFT and immobility time in the FST, corroborating the results of Bhatt et al. (2013) and Morales-Medina et al. (2013), showing that behavioral symptoms are associated with this model. Furthermore, studies by Bhatt et al. (2013), indicated that chronic treatment with antidepressants could reverse these changes. The present study investigated the ability of repeated treatment with hesperidin to reverse induced OB-depressive behavior in mice (hyperactivity in the open field test, immobility in the forced swimming and anhedonic behavior in the splash test). The ability of hesperidin to block the symptoms of agitated depression reinforces the antidepressant-like property of this compound and corroborates the findings of several studies that have shown this pharmacological property in mice (Donato et al., 2014; Filho et al., 2013; Souza et al., 2013).

In addition to the cognitive deficits, OB causes different signs of anhedonia. Anhedonia, or hyposensitivity to pleasure, is a key symptom of depression diagnosis (Song and Leonard, 2005). In the present study, this behavior was inferred by a decrease in grooming time in the splash test in bulbectomized mice compared with control mice, similar to Romeas et al. (2009) and Sato et al. (2010). It is known that compounds with antidepressant properties, such as the classical antidepressant imipramine, recover this damage (Detanico et al., 2009); this damage was also abolished by hesperidin treatment. Our results are in line with the thought that

hesperidin acts by counteracting the loss in motivational and self-care behavior induced by OB in mice. All of these behavioral tests respond selectively to chronic hesperidin treatment and demonstrated the usefulness of hesperidin for the management of depression associated with anhedonia.

Hippocampus-dependent spatial memory in the MWMT and performance in the ORT were also impaired in our study. Bulbectomized mice demonstrated an increase in the latency time to the platform in the MWMT and low performance in the ORT compared with the control group. Treatment with hesperidin improved performance on both tests, demonstrating that hesperidin could contribute to learning and memory; our previous study revealed that chronic administration of hesperidin over 28 days was effective in reversing these changes in memory (Antunes et al., 2014). We demonstrated that hesperidin administration contributes to learning and memory in different models in mice. Therefore, our data corroborate clinical observations and provide additional evidence that cognitive dysfunction accompanies depression and psychobehavioral signs. We suggest that the neurotrophic deficiency and neuroinflammation in the hippocampus that occurs in response to OB is related to the impaired recognition memory and depressive-like behaviors observed in our study.

In addition to behavioral alterations, our study showed that OB causes neurochemistry changes in mice. We observed a significant reduction in the BDNF and NGF levels in the hippocampus, indicating altered synaptic plasticity, after OB. Neurotrophic factors are proteins that play a vital role in regulating all of the aspects of neural circuit development and function in the mammalian brain, including neuronal survival, synaptic plasticity, neurogenesis and depression; the role of these factors in the mechanism of action of antidepressant drugs is well appreciated (Castren and Rantamaki, 2010; Li et al., 2013; Middeldorp et al., 2010). Hesperidin treatment potentiated plasticity, increasing the BDNF and NGF levels in the hippocampus after OB. Downregulation of the BDNF and NGF levels in the hippocampus resulted in the reduction of neurogenesis and the development of depression and cognitive impairment (Dwivedi et al., 2005; Kimpton, 2012). Therefore, we suggest that behavioral disturbances (depression and memory) elicited by OB are driven by the BDNF and NGF pathways in the hippocampus of mice. It could be suggested that the increase in the BDNF and NGF levels might result from a compensatory up-regulation of these neurotrophins following OB that was prevented by hesperidin treatment.

Therefore, the BDNF and NGF pathways are important to the action of hesperidin in this depression model in mice.

Together with neurotrophins changes, our study showed that OB increases pro-inflammatory cytokines levels, such as those of IL-1β and IL-6, in the hippocampus of mice compared with the control group. These data were in accordance with the data from Rinwa et al. (2013) and Poretti et al. (2015), showing that depressive disorder is associated with an increase of these pro-inflammatory cytokines. Moreover, this increase could be correlated with a decrease of neuroplasticity, as shown previously by Blomster et al. (2011) and Johnson and Sharma (2003), wherein an increase of pro-inflammatory cytokines was linked with a decrease of NGF and BDNF expression in mice. Together with the literature results, the current study supports and extends the thought that an inflammatory response, reflected by cytokine dysregulation in the brain regions implicated with memory and depressive alterations, could be an important neurobiological mechanism of OB-induced cognitive impairment and emotional alterations in mice. Based on these results, we have shown that hesperidin treatment attenuated the changes in cytokine levels in mice, and its anti-inflammatory properties could contribute, in part, to the neuroprotective effect of this flavonoid.

It has been previously implied that the cholinergic system plays a role in the pathophysiology of depression. Acetylcholinesterase catalyzes acetylcholine (ACh) hydrolysis in the synaptic cleft and neuromuscular junctions (Soreq, 2001). In this study, we observed that acetylcholinesterase activity increased in the hippocampus of mice after OB, and hesperidin treatment decreased this hyperactivity. This result is similar to that of Müller et al. (2002), who showed that treatment with antidepressants decreased A acetylcholinesterase activity in human serum. This reversion in acetylcholinesterase activity could also be linked with cognitive processes because the enzyme breaks down ACh. Furthermore, studies by Song et al. (2008) reported that the administration of IL-1β for 7 days reduced ACh and NGF expression in the dentate gyrus of mice, and this reduction was associated with memory loss. Hesperidin treatment modulated changes in depression and memory tests through its anti-inflammatory properties, as well as BDNF and NGF upregulation and regulation of acetylcholinesterase activity, in the hippocampus of mice in the model induced by OB.

5. Conclusion

The findings of this study demonstrate that hesperidin reversed the depressive-like behavior and decreases of memory caused by OB; this response was accompanied by the modulation of pro-inflammatory cytokines, maintenance of brain plasticity, and regulation of acetylcholinesterase activity. The present results support the claim that hesperidin could be an attractive tool the treatment of depression-related responses. Moreover, hesperidin it is a flavonoid that is found naturally in plants and might have few side effects. Future studies will investigate the effects of hesperidin in other models of depression and its possible side effects. Finally, the data presented here might be of therapeutic relevance; these results indicate that this flavonoid could constitute an attractive tool for the treatment of depressive disorders.

Acknowledgments

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References

Antunes, M.S., Goes, A.T., Boeira, S.P., Prigol, M., Jesse, C.R., 20014. Protective effect of hesperidin in a model of Parkinson's disease induced by 6-hydroxydopamine in aged mice. Nutrition 30, 1415-22.

Bhatt, S., Mahesh, R., Devadoss, T., Jindal, A., 2013. Anti-depressant like activity of N-n-butyl-3-methoxyquinoxaline-2-carboxamide (6o) a 5-HT3 receptor antagonista. Indian J Exp Biol. 51,435–443.

De Bodinat, C., Guardiola-Lemaitre, B., Mocaër, E,, Renard, P., Muñoz, C., Millan, M.J., 2010. Agomelatine, the first melatonergic antidepressant: discovery, characterization and development. Nat. Rev. Drug. Discov. 9, 628–642.

Blomster, L.V., Vukovic, J., Hendrickx, D.A.E., Jung, S., Harvey, A.R., Filgueira, L., et al., 2011. CX(3)CR1 deficiency exacerbates neuronal loss and impairs early regenerative responses in the target-ablated olfactory epithelium. Mol. Cell. Neurosci. 48,236–245.

Castren, E., Rantamaki, T, 2010. The role of BDNF and its receptors in depression and antidepressant drug action: reactivation of developmental plasticity. Dev. Neurobiol. 70, 289–297.

d'Audiffret, A.C., Frisbee, S.J., Stapleton, P.A., Goodwill, A.G., Isingrini, E, Frisbee, J.C., 2010. Depressive behavior and vascular dysfunction: a link between clinical depression and vascular disease? J. Appl. Physiol. 108, 1041–1051.

Detanico, B.C., Piato, A.L, Freitas, J.J, Lhullier, F.L., Hidalgo, M.P., Caumo, W., et al. 2009. Antidepressant-like effects of melatonin in the mouse chronic mild stress model. Eur. J. Pharmacol. 607, 121–125.

Donato, F., de Gomes, M.G., Goes, A.T., Filho, C.B., Del Fabbro, L., Antunes, M.S., Souza, L.C., Boeira, S.P., Jesse, C.R., 2014. Hesperidin exerts antidepressant-like effects in acute and chronic treatments in mice: possible role of l-arginine-NO-cGMP pathway and BDNF levels. Brain Res. Bull. 104, 19-26.

Dowlati, Y., Herrmann, N., Swardfager, W., Liu, H., Sham, L., Reim, E.K., et al., 2010. A meta-analysis of cytokines in major depression. Biol. Psychiatry 67, 446–457.

Dwivedi, Y., Mondal, A.C., Rizavi, H.S., Conley, R.R., 2005. Suicide brain is associated with decreased expression of neurotrophins. Biol. Psychiatry 58, 315–324.

Eisenstein, S.A., Clapper, J.R., Holmes, P.V., Piomelli, D., Hohmann, A.G., 2010. A role for 2-arachidonoylglycerol and endocannabinoid signaling in the locomotor response to novelty induced by olfactory bulbectomy. Pharmacol. Res. 61, 419–429.

Ennaceur, A., Delacour, J., 1988. A new one-trial test for neurobiological studies of memory in rats. 1: Behavioral data. Behav. Brain Res. 31, 47-59.

Filho, C.B., Del Fabbro, L., de Gomes, M.G., Goes, A.T., Souza, L.C., Boeira, S.P., Jesse, C.R., 2013. Kappa-opioid receptors mediate the antidepressant-like activity of hesperidin in the mouse forced swimming test. Eur. J. Pharmacol. 698, 286-91.

Gaur, V., Kumar, A., 2010. Hesperidin pre-treatment attenuates NO-mediated cerebral ischemic reperfusion injury and memory dysfunction. Pharmacol. Rep. 62, 635–648.

Harkin, A., Shanahan, E., Kelly, J.P., et al. 2003. Methylenendioxyamphetamine produces serotonin nerve terminal loss and diminished behavioural and neurochemical responses to the antidepressant fluoxetine. Eur. J. Neurosci. 18, 1021-7.

Howren, M. B., Lamkin, D.M., Suls, J. 2009. Associations of depression with C-reactive protein, IL-1, and IL-6: a meta-analysis. Psychosom. Med. 71, 171–186.

Janowsky, D.S., El-Yousef, M.K., Davis, J.M. 1974. Acetylcholine and depression. Psychosom. Med. 36, 248–257.

Jarosik, J., Legutko, B., Unsicker, K., et al. 2007. Antidepressant-mediated reversal of abnormal behavior and neurodegeneration in mice following olfactory bulbectomy. Exp. Neurol. 204, 20-8.

Johnson, V.J., Sharma, R.P. 2003. Aluminum disrupts the pro-inflammatory cytokine/neurotrophin balance in primary brain rotation-mediated aggregate cultures: possible role in neurodegeneration. Neurotoxicology 24, 261–268.

Kelly, J.P., Wrynn, A.S., Leonard, B.E., 1997. The olfactory bulbectomized rat as a model of depression: an update. Pharmacol. Ther. 74, 299–316.

Kessler, R.C., Berglund, P., Demler, O., Jin, R., Koretz, D., Merikangas, K.R., et al., 2003. The epidemiology of major depressive disorder: results from the National Comorbidity Survey Replication (NCS-R). JAMA 289, 3095–3105.

Kessler, R.C., Berglund, P., Demler, O., Jin, R., Merikangas, K.R., Walters, E.E., 2005. Lifetime prevalence and age-of-onset distributions of DSM-IV disorders in the national comorbidity survey replication. Arch Gen Psychiatry 62, 593–602.

Kessler, R.C., Ormel, J., Petukhova, M., McLaughlin, K.A., Green, J.G., Russo, L.J., et al., 2011. Development of lifetime comorbidity in the World Health Organization world mental health surveys. Arch. Gen. Psychiatry 68, 90–100.

Kimpton, J., 2012. The brain derived neurotrophic factor and influences of stress in depression. Psychiatr. Danub. 24, S169–S171.

Knoppman, D., 2001. Pharmacothephy for Alzheimer's disease. Curr. Neurol. Neurosci. Rep. 1, 428–432.

Leonard, B.E., Tuite, M., 1981. Anatomical, physiological, and behavioral aspects of olfactory bulbectomy in the rat. Int. Rev. Neurobiol. 22:251-86.

Li, J., Geng, D., Xu, J.L., Weng, J., Liu, Q., Yi, L.T., 2013. Antidepressant-like effect of macranthol isolated from Illicium dunnianum tutch in mice. Eur. J. Pharmacol. 707, 112–119.

Middeldorp, C.M., Slof-Op't Landt, M.C., Medland, S.E., van Beijsterveldtm, C.E., Bartels, M., Willemsen, G., et al. 2010. Anxiety and depression in children and adults: influence of serotonergic and neurotrophic genes? Genes Brain Behav. 9, 808–816.

Mihaljević-Peleš, A., Sagud, M., Janović, M.B., Mikulić, S.K., Jevtović, S., 2011. Do we need new therapeutic strategies for depression? Psychiatr. Danub. 23, 300–301.

Morales-Medina. J.C., Juarez, I., Venancio-Garcia, E., Cabrera, S.N., Menard, C., Yu, W., et al., 2013. Impaired structural hippocampal plasticity is associated with emotional and memory deficits in the olfactory bulbectomized rat. Neuroscience 236, 233–243.

Morris, R.G.M., Garrud, P., Rawlins, J.N.P., O'Keefe, J., 1982. Place navigation impaired in rats with hippocampal lesions. Nature 297, 681–683.

Myint, A.M., Steinbusch, H.W., Goeghegan, L., Luchtman, D., Kim, Y.K., Leonard, B.E., 2007. Effect of the COX-2 inhibitor celecoxib on behavioural and

immune changes in an olfactory bulbectomised rat model of depression. Neuroimmunomodulation 14, 65–71.

Oral, E., Aydin, M.D., Aydin, N., Ozcan, H., A., 2013. Hacimuftuoglu, S. Sipal, et al. How olfaction disorders can cause depression? The role of habenular degeneration. Neuroscience 240, 63–69.

Porsolt, R.D., Le Pichon, M., Jalfre, M., 1977. Depression: a new animal model sensitive to antidepressant treatments. Nature 21, 730-2.

Prediger, R.D., Franco, J.L., Pandolfo, P., Medeiros, R., Duarte, F.S., Di Giunta, G., et al., 2007. Differential susceptibility following beta-amyloid peptide-(1-40) administration in C57 BL/6 and Swiss albino mice: evidence for a dissociation between cognitive deficits and the glutathione system response. Behav. Brain Res. 177, 205–218.

Prut, L., Belzung, C., 2003. The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review. Eur. J. Pharmacol. 463, 3-33.

Rinwa, P., Kumar, A., 2013. Quercetin suppress microglial neuroinflammatory response and induce antidepressant-like effect in olfactory bulbectomized rats. Neuroscience 255, 86–98.

Rodrigues, A.L.S., Da Silva, G.L., Mateussi, A.S., Fernandes, E.S., Miguel, O.G., Yunes, R.A., et al., 2002. Involvement of monoaminergic system in the antidepressant-like effect of the hydroalcoholic extract of Siphocampylus verticillatus Life Sci. 70, 1347–1358.

Romeas, T., Morissette, M.C., Mnie-Filali, O., Piñeyro, G., Boye, S.M., 2009. Simultaneous anhedonia and exaggerated locomotor activation in an animal model of depression. Psychopharmacology (Berl) 205, 293-303.

Sato, A., Nakagawasai, O., Tan-No, K., Onogi, H., Niijima, F., Tadano, T., 2010. Effect of non-selective dopaminergic receptor agonist on disrupted maternal behavior in olfactory bulbectomized mice. Behav. Brain Res. 210, 251-6.

Song, C., Leonard, B.E., 2005. The olfactory bulbectomised rat as a model of depression. Neurosci. Biobehav. Rev. 29, 627–647.

Song, C., Taepavarapruk, P., Zhang, Y., 2008. Acute and chronic IL-1β administrations differentially modulate learning and memory, acetylcholine efflux and

neurotrophin expressions in the hippocampus: possible mechanisms involved in neuroprotection and neurodegeneration. Brain Behav. Immun. 22, S41–S42.

Soreq, H., Seidman, S., 2001. Acetylcholinesterase--new roles for an old actor. Nat. Ver. Neurosci. 2, 294-302.

Souza, L.C., de Gomes, M.G., Goes, A.T., Del Fabbro, L., Filho, C.B., Boeira, S.P., Jesse, C.R., 2013. Evidence for the involvement of the serotonergic 5-HT(1A) receptors in the antidepressant-like effect caused by hesperidin in mice. Prog. Neuropsychopharmacol. Biol. Psychiatry 40, 103-9.

Van Riezen, H., Leonard, B.E., 1990. Effects of psychotropic drugs on the behavior and neurochemistry of olfactory bulbectomized rats. Pharmacol. Ther. 47, 21–34.

Yalcin, I., Aksu, F., Belzung, C., 2005. Effects of desipramine and tramadol in a chronic mild stress model in mice are altered by yohimbine but not by pindolol. Eur. J. Pharmacol. 514, 165–174.

Yang, H.L., Chen, S.C., Kumar, K.J.S., Yu, K.N., Chao, D.L., Tsai, S.Y., Hou, Y.C., Hse, Y.C., 2012. Antioxidant and anti-inflammatory potential of hesperetin metabolites obtained from Hesperitin-administered rat serum: Na ex vivo approach. J. Agric. Food Chem. 60, 522–532.

Figure Legends

Fig. 1. Schematic representation of the experimental protocol describing the surgery day, treatment periods with imipramine, (10 mg/kg, i.p.) or hesperidin (50 mg/kg, *p.o*), behavior tests and neurochemistry analyzes.

Fig. 2. (A) Effect of imipramine or hesperidin treatment in Crossing total in the OFT in bulbectomized mice. **(B)** Effects of imipramine or hesperidin treatment in Rearing total in the OFT in bulbectomized mice. Values are mean ± S.E.M. (n=10 per group). ***: p<0.001 when comparing Sham/saline. #: p<0.01 when compared to OB/vehicle.

Fig. 3. (A) Effects of hesperidin or imipramine treatment during thirteen days after OB surgery in FST. **(B)** Effect of hesperedin or imipramine treatment in the splash test after surgery. Values are mean ± S.E.M. (n=10 per group). * .p<0.05, ** .p<0.01 and ***: p<0.001 when comparing Sham/saline #: p<0.01 when compared to OB/vehicle.

Fig. 4. (**A**) Effects of hesperidin or imipramine treatment on escape latency training trials, **(B)** during thirteen days after OB surgery in MWMT and (**C**) crossing in MWMT. Values are mean ± S.E.M. (n=10 per group). ***: p<0.001 when comparing SHAM/vehicle. #: p<0.01 when compared to OB/vehicle.

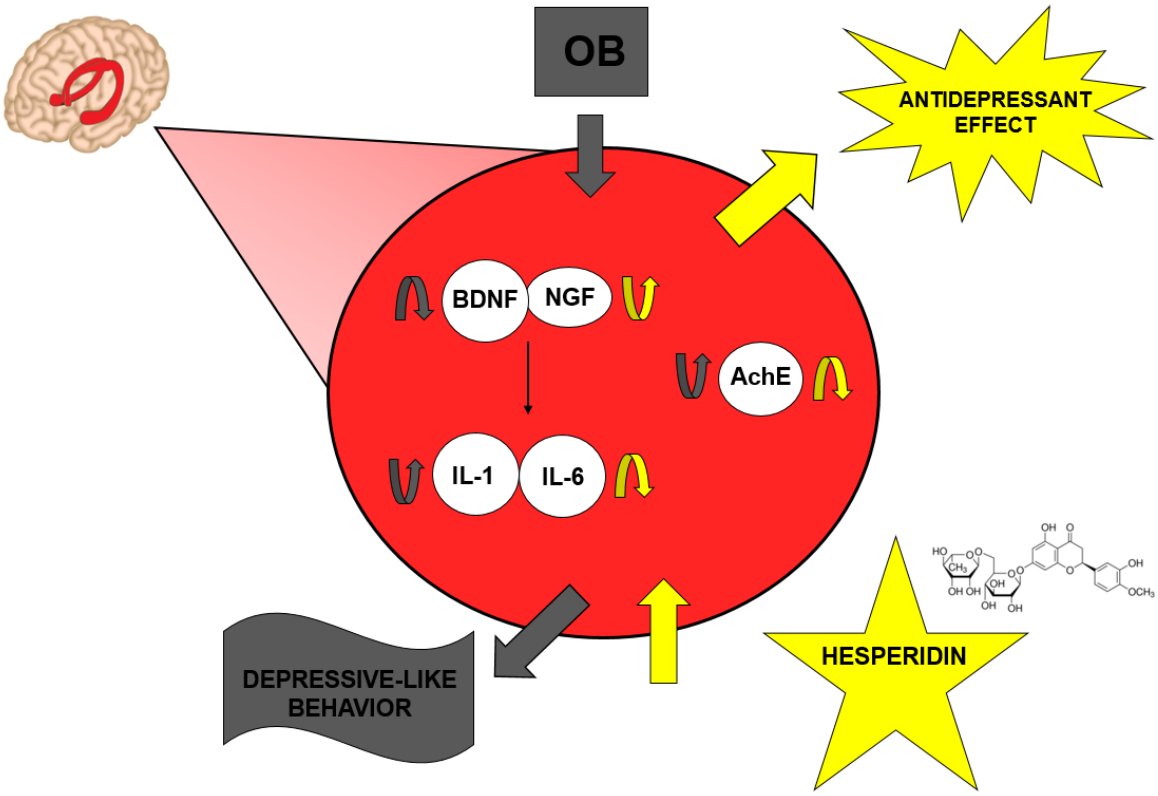
Fig. 5. (A) Effect of 2-weeks of hesperidin or imipramine in bulbectomized mice in STM - ORT. **(B)** Effect of 2-weeks of hesperidin or imipramine in bulbectomized mice in memory consolidation in LTM - ORT. Values are mean ± S.E.M. (n=10 per group). ***: p<0.001 when comparing SHAM/vehicle. #: p<0.01 when compared to OB/vehicle.

Fig. 6. (A) Effects of hesperidin or imipramine treatment during thirteen days in BDNF level in hippocampus after OB. **(B)** Effects of hesperidin or imipramine treatment during thirteen days in NGF levels in hippocampus after OB. Values are mean ± S.E.M. (n=10 per group). **.p<0.01 and ***: p<0.001 when comparing SHAM/vehicle. #: p<0.01 when compared to OB/vehicle.

Fig. 7. (A) Effects of hesperidin or imipramine treatment during thirteen days in IL-1β levels in hippocampus after OB. **(B)** Effects of hesperidin or imipramine treatment during thirteen days in IL-6 levels in hippocampus after OB. Values are mean ± S.E.M. (n=10 per group). *.p<0.05 ***: p<0.001 when comparing SHAM/vehicle. #: p<0.01 when compared to OB/vehicle.

Fig. 8. Effect of imipramine or hesperidin treatment on AchE activity in hippocampus of bulbectomized mice. Values are mean ± S.E.M. (n=10 per group). ***: $p<0.001$ when comparing Sham/saline. #: $p<0.01$ when compared to OB/vehicle.

Graphical abstract



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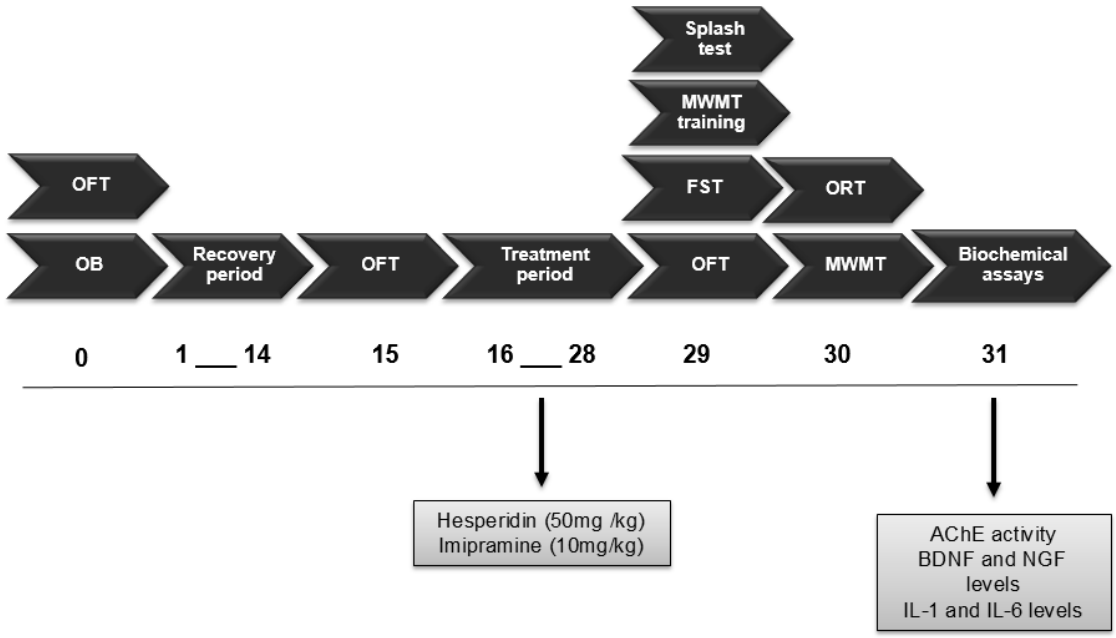

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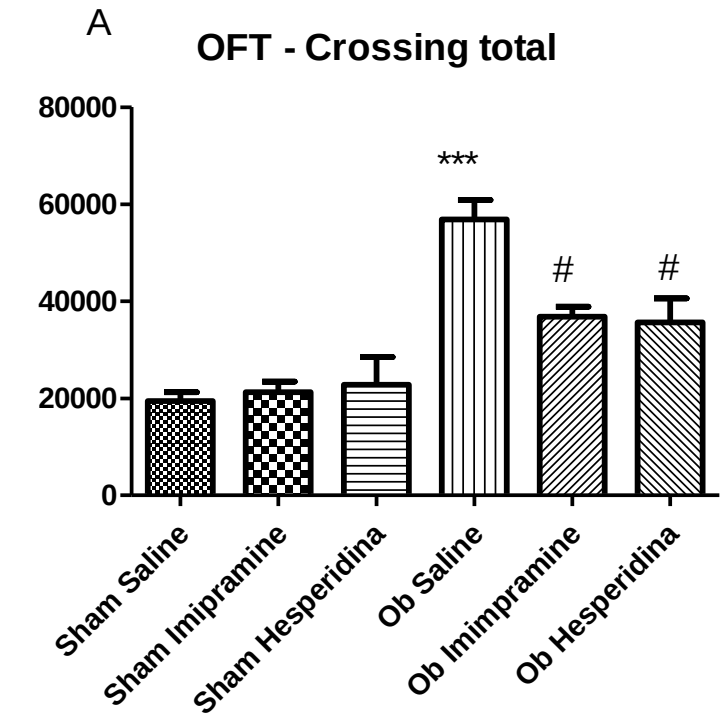
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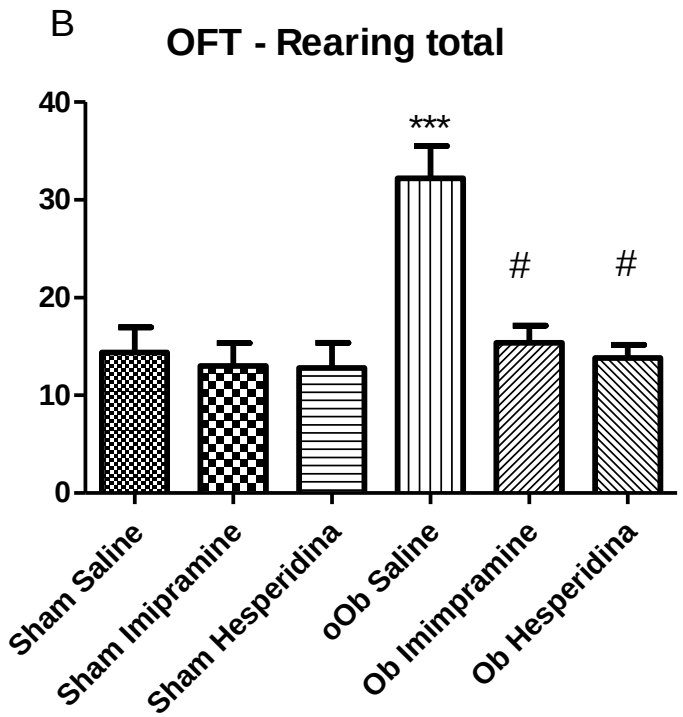
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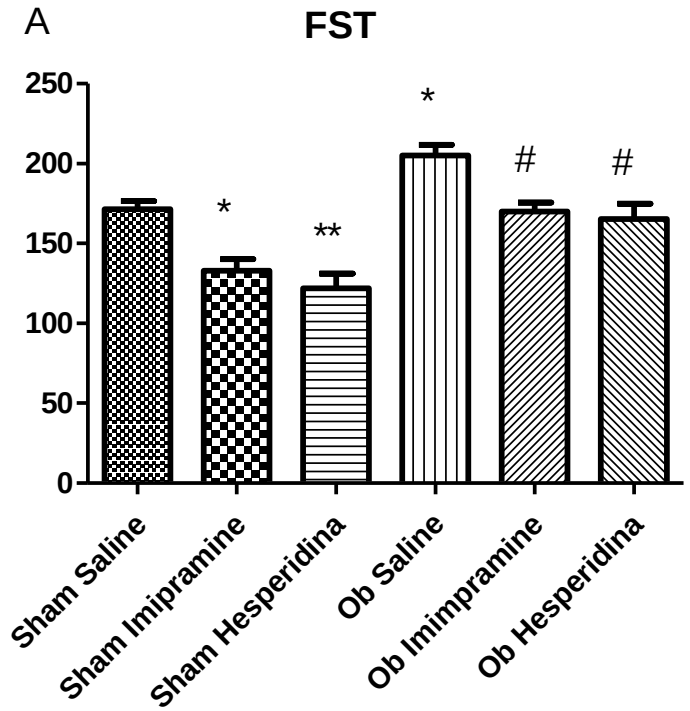
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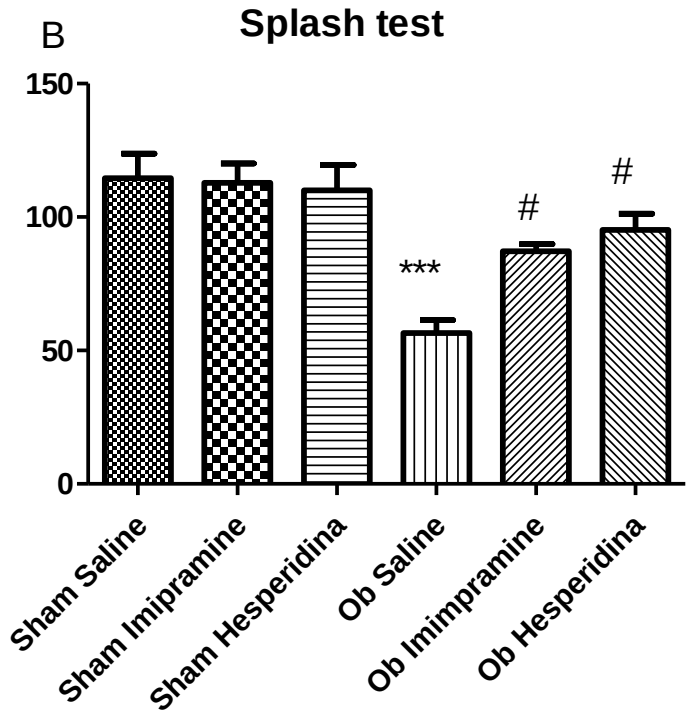
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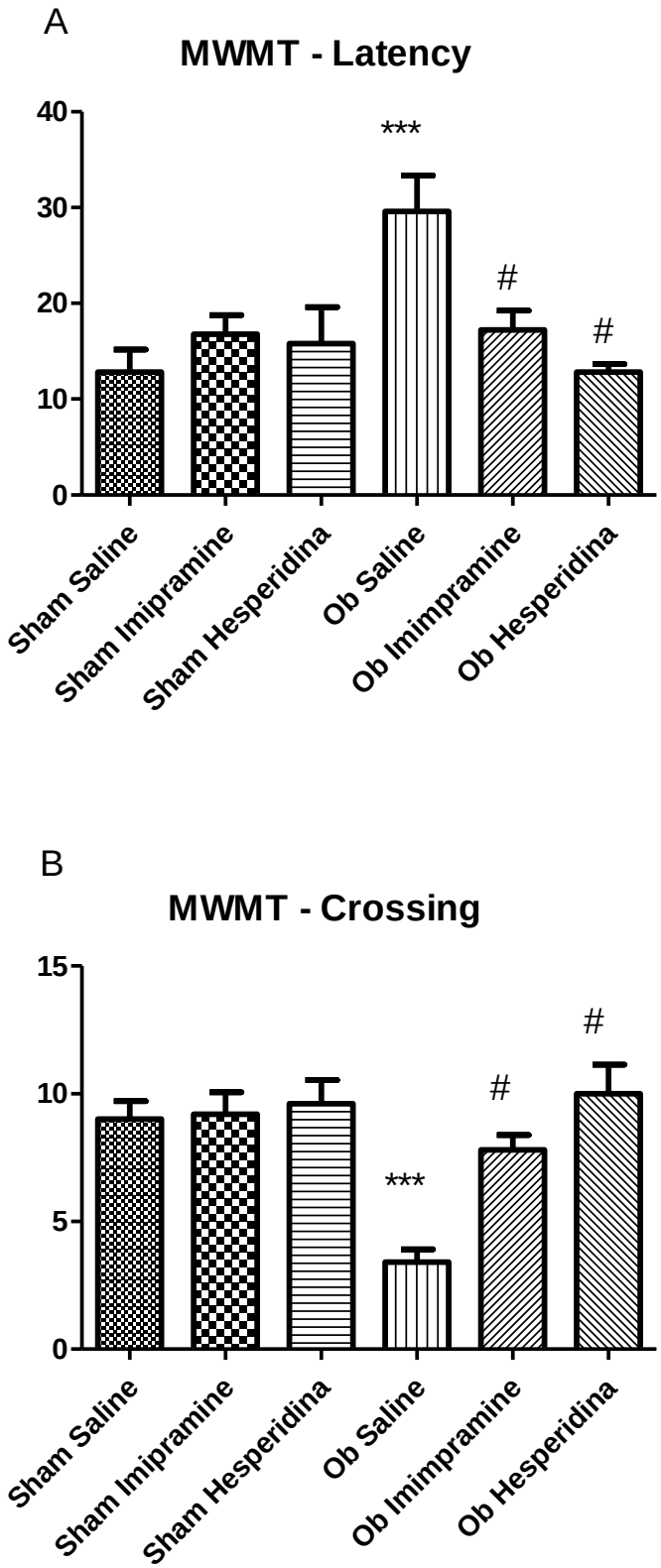
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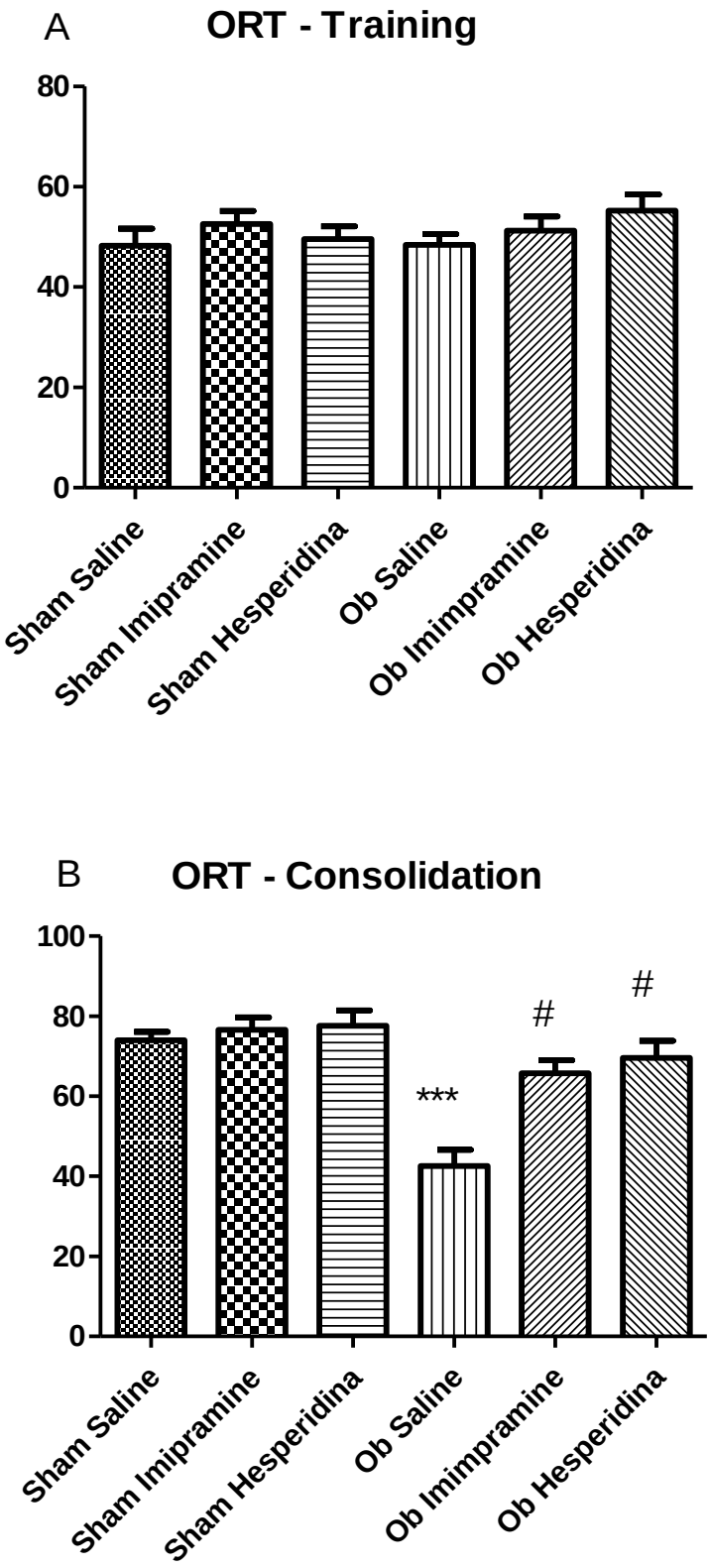
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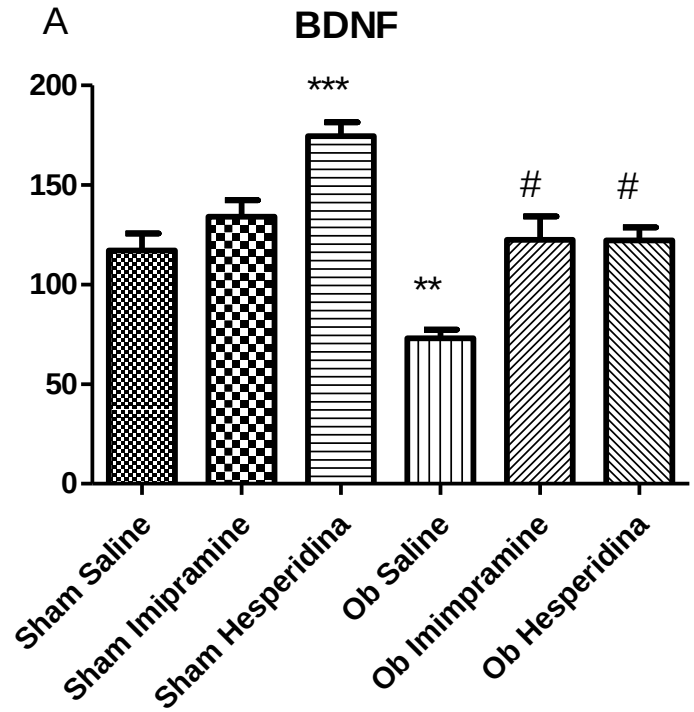
Figura 6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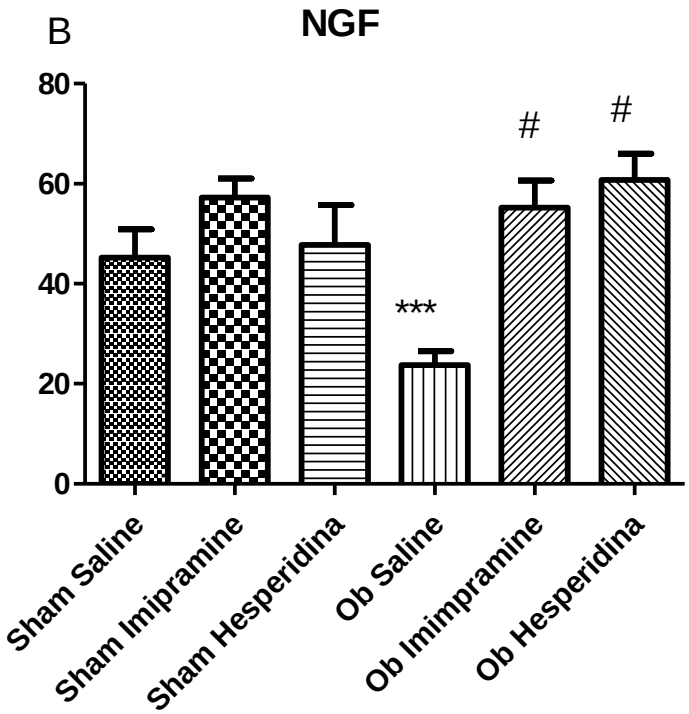
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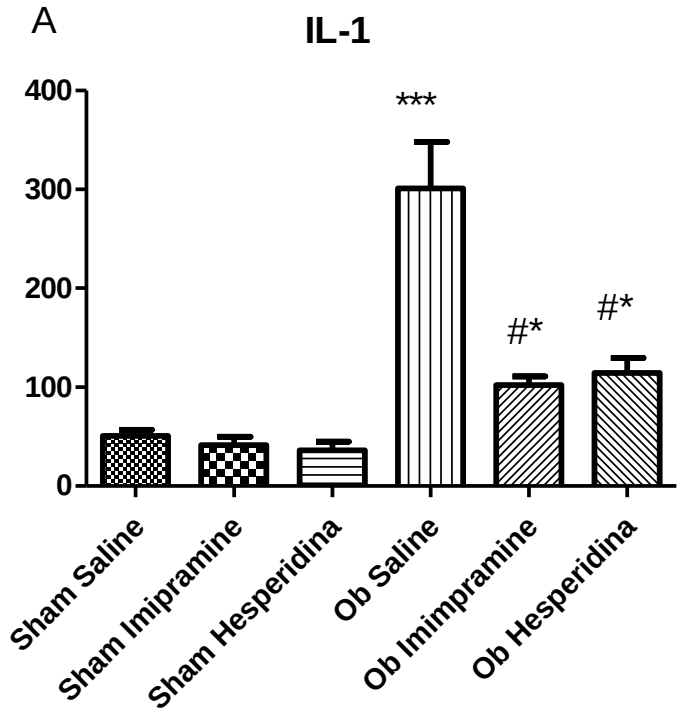
Figura 7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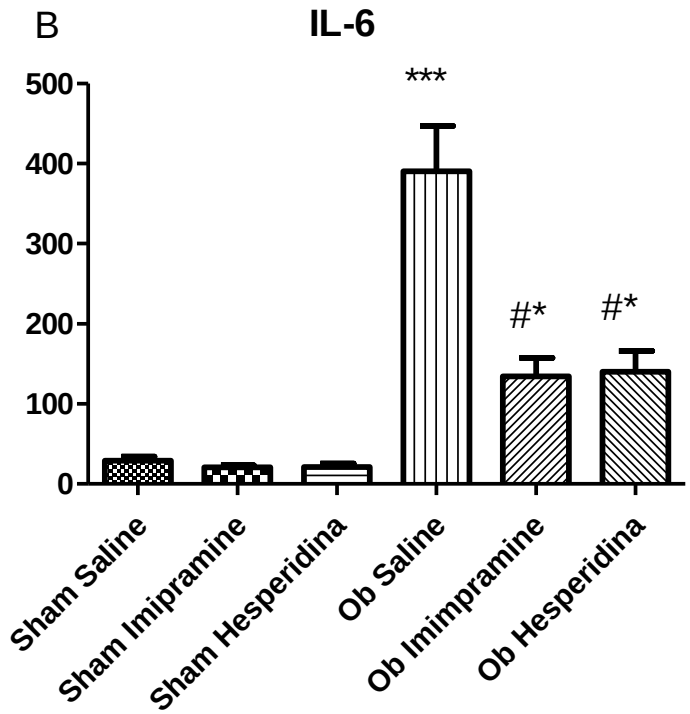
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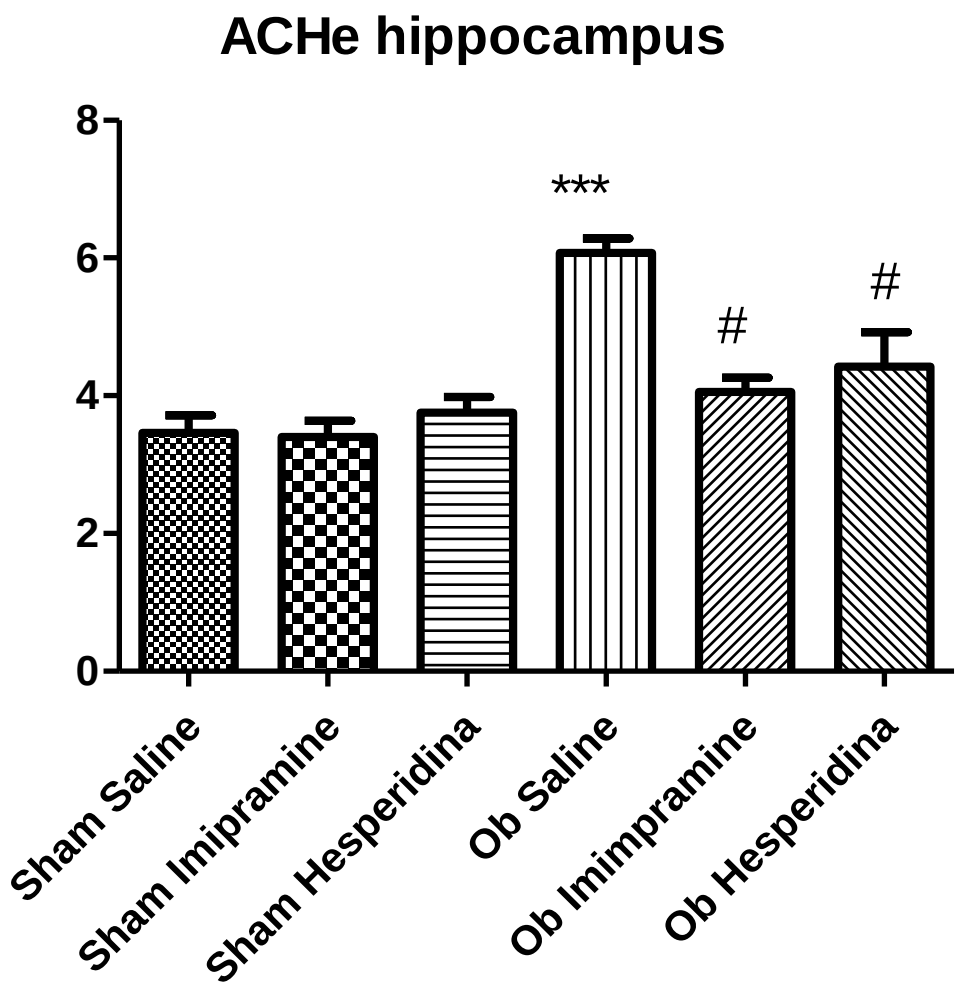
Figura 8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PARTE III

DISCUSSÃO GERAL

Os flavonoides são pigmentos polifenólicos naturalmente presentes em plantas como metabólitos secundários, esses compostos podem concentrar diversos efeitos biológicos, atuando como mensageiros químicos e reguladores fisiológicos (QUIDEAU et al., 2011). Alguns estudos prévios publicados por nosso grupo, já demonstraram uma variedade dos efeitos farmacológicos vinculados ao flavonoide hesperidina, desses, destacamos seu potencial antioxidante, neuroprotetor e antidepressivo (ANTUNES et al., 2014; 2016; DONATO et al., 2014). Com base nessas constatações, os trabalhos que fazem parte desta pesquisa têm por objetivo aprofundar a investigação quanto aos mecanismos pelos quais a hesperidina exerce seus efeitos neuroprotetores e antidepressivos. Esse estudo mensura o potencial terapêutico do referido polifenol, a fim de minimizar os sintomas da DP e da depressão, utilizando dois modelos experimentais já bem descritos na literatura como, 6-OHDA e BO, respectivamente.

Dentre os principais achados da primeira parte do estudo, destaca-se o potencial da hesperidina (50 mg/kg diariamente, durante quatro semanas), em reverter as modificações neuroquímicas e comportamentais decorrentes da microinjeção de 6-OHDA no estriado de camundongos. Essa neurotoxina específica, 6-OHDA, tem alta validade para a indução experimental de PD em roedores, alguns dos seus efeitos observados em modelos animais são os mesmos que se desenvolvem no cérebro de pacientes parkinsonianos. Entre estes vários efeitos, pode-se destacar uma acentuada diminuição nos níveis de DA, seus metabólicos e do conteúdo de TH no estriado (KOZINA et al., 2014; SOTO-OTERO et al., 2000; UNGERSTEDT, 1968). O presente resultado dos níveis de DA, DOPAC e HVA, observados neste estudo, confirmam nossos dados anteriores, revelando diminuição estriatal dessas monoaminas em camundongos 38 dias após administração de 6-OHDA, evidenciando assim, o efeito tóxico de 6-OHDA para estas células nesse intervalo de tempo (ANTUNES et al., 2014). Além disso, agora estendemos nossas observações para

avaliar o conteúdo da enzima TH no estriado que, da mesma forma, teve uma expressiva redução de sua atividade ocasionada pela neurotoxina. Estas modificaçoes neuroquímicas estriatais foram revertidas pelo tratamento diário com hesperidina. Assim, é possível propor que a administração oral de hesperidina (50 mg/kg) durante 4 semanas é capaz de atenuar a neurotoxicidade catecolaminérgica induzida por 6-OHDA, preservando assim e os níveis de DA, seus metabolitos DOPAC e HVA e os neurônios TH-positivos no estriado.

Essas alterações no sistema monoaminérgico são apontadas para conduzir a assimetrias motoras, como observada pelas proeminentes rotações contralaterais apresentadas pelos camundongos lesionados por 6-OHDA. O que está em linha com resultados de Ungerstedt & Arbuthnott, 1970, que indicam que essa lesão decorrente da administração de 6-OHDA no estriado é responsável por motivar o comportamento rotatório contralateral à lesão, quando os animais são tratados com agonistas dopaminérgicos como a apomorfina. Tal comportamento ocorre porque os receptores pós-sinápticos dopaminérgicos estriatais estão hipersensibilizados no lado desnervado, ocasionando uma regulação positiva dos receptores de DA (MEREDITH & KANG, 2006). Nesse contexto, nosso estudo também demonstra que os animais submetidos à lesão com 6-OHDA apresentam um aumento significativo do número de rotações contralaterais, estando de acordo ainda com outros estudos (KHAN et al., 2010; RIZELIO et al., 2010; TEIXEIRA et al., 2013). Conseguinte, verificou-se que o tratamento com o flavonoide hesperidina apresentou efeito significativo na reversão desse comportamento rotatório. Assim, atribuímos tal resultado ao potencial de hesperidina em atuar no reestabelecimento neuronal, como evidenciado pela manutenção dos níveis de DA, seus metabólicos e dos neurônios TH-positivos no estriado. Dessa forma, intervindo no controle direto da viabilidade dos neurônios dopaminérgicos, marca histológica da DP, o que sugere claramente seu efeito neuroprotetor.

Além da evidente morte neuronal, a DP está associada com uma diminuição da capacidade de biossíntese dos neurônios dopaminérgicos sobreviventes (HAAVIK & TOSKA, 1998). Nesse sentido, observou-se uma redução significativa nos níveis de fatores neurotróficos (BDNF, NGF, NT-3 e GDNF), no estriado após administração de 6-OHDA, indicando ressaltada alteração na plasticidade estriatal. Estas substâncias são conhecidas por serem vitais para a sobrevivência neuronal. Em apoio a esta ideia,

a restauração dos níveis de BDNF, NGF e NT-3 no estriado alcançada pelo tratamento com hesperidina, poderia intervir no aumento da sobrevida dos neurônios dopaminérgicos, intensificando assim seu efeito neuroprotetor, uma vez que a diminuição neuronal é a principal característica da doença estudada (BUJ-BELLO et al., ESPEJO et al., 2000; 1995, LINDSAY, 1994). É presumível indicar que um dos mecanismos, pelo qual esse flavonoide atua para o reestabelecimento das células estriatais comprometidas nesse modelo, é através da recuperação desses fatores neurotroficos, podendo, diante disso, ser útil como terapia para promover e restaurar a função neuronal.

A ativação glial é outra característica relevante da DP, desempenhando papel crítico nos processos neuroinflamatórios associados a patologia (WANG, et al., 2015). Levando em conta sua importância, nosso estudo investigou e constatou um aumento dos níveis de citocinas pró-inflamatórias, incluindo TNF-α, IFN-γ, IL-1β, IL-2 e IL-6 no estriado, além de uma diminuição dos níveis da citocina anti-inflamatória IL-10 nessa estrutura após a administração 6-OHDA, o que pode ter contribuído para a degeneração dos neurônios dopaminérgicos. Em linha a essas evidências, estudos demonstram que a ativação excessiva da microglia leva à elevação dessas citocinas pró-inflamatórias, o que pode induzir rapidamente a degeneração neuronal na SNpc. Ainda, a inibição das reações em cascata da microglia pode prevenir a degradação desses neurônios através da modulação neuinflamatória (BURGUILLOS et al., 2011; CAGNIN, et al., 2006). Em acordo, observou-se, nesse estudo, que o tratamento com hesperidina durante 28 dias, também reverteu significativamente as alterações nos níveis de TNF-α, IFN-γ, IL-1β e IL-6 no estriado. Corroborando com dados do grupo que demonstram que esse flavonoide atua na regulação de citocinas pró-inflamatórias também no hipocampo, no modelo da BO em camundongos (ANTUNES et al, 2016). Nesse sentido, estudos de Kim et al., 2010 demostram que agentes anti-inflamatórios podem proteger os neurônios dopaminérgicos contra danos causados por 6-OHDA em modelos animais de DP, sustentando que este controle da ativação da microglia pode ser importante para a proteção neuronal (KIM et al., 2010). Nesse contexto, sugere-se que o tratamento com hesperidina atenua as alterações nos níveis de citocinas em camundongos após a microinjeção de 6-OHDA no estriado devido suas propriedades anti-inflamatórias, o que pode ser útil para retardar a morte dos neurônios dopaminérgicos contribuindo, para o efeito deste flavonoide na

fisiopatologia da DP. Em linha, as propriedades anti-inflamatórias desse flavanoide podem atuar na depressão, um dos sintomas não motores que mais incapacitam os pacientes com DP, uma vez que as alterações nos mediadores pró-inflamatórios também são observadas em pacientes com desordens depressivas (BARNUM & TANSEY 2012; KÖHLER et al., 2014)

O presente estudo revelou que a infusão i.c.v. de 6-OHDA também promoveu disfunção mitocondrial no estriado, o que corrobora com os resultados de Kupsch 2014, que demostra que a razão do controle respiratório, principal parâmetro da função mitocondrial, é claramente prejudicada nesse modelo da 6-OHDA. Nessa perspectiva, verificou-se em nosso estudo uma diminuição da atividade das enzimas mitocondriais dos complexos I, II, IV e V após a administração de 6-OHDA. Da mesma forma, essa neurotoxina atuou diminuindo o potencial de membrana mitocondrial no estriado, indicando perda de integridade mitocondrial e comprometimento bioenergético nessa estrutura. Juntamente com nossos dados preliminares, sugere-se que a neurotoxina 6-OHDA atua promovendo disfunção mitocondrial, geração de espécies reativas e consequente diminuição na produção de ATP induzindo a morte dos neurônios dopaminérgicos devido à insuficiência energética (ANTUNES, et al., 2014; LIN & BEAL, 2006). Nesses parâmetros, o flavonoide hesperidina também conferiu efeitos favoráveis, evidenciados pela reversão da disfunção mitocondrial e pela preservação da integridade mitocondrial decorrentes do seu potencial efeito antioxidante, já reportado anteriormente por nosso grupo (ANTUNES et al., 2012; DONATO et al., 2014).

Ainda nesse contexto energético, a Na^+/K^+ ATPase é uma complexa proteína transmembranar cuja atividade enzimática utiliza ATP para o transporte íons de Na e K em células eucariotas. Essa enzima é conhecida por desempenhar um papel chave na função celular, regulando as trocas iônicas entre a célula e o espaço intercelular e, as alterações em sua atividade pode estimular a apoptose neuronal devido a alteração na homeostase de K (WANG et al., 2003). Nesse estudo sugerimos que a diminuição da atividade estriatal dessa enzima, decorrente da administração de 6-OHDA, pode culminar na apoptose, uma vez que já constatamos que essa neurotoxina atua na mitocôndria diminuindo a síntese de ATP, molécula essencial para o funcionamento adequado da enzima. Demonstramos que o flavonoide por nós avaliado, atenuou a inibição da enzima Na^+/K^+ ATPase no estriado dos camundongos, o que pode

colaborar para interromper a apoptose por insuficiência energética nessa estrutura. Uma vez que, no tecido nervoso, a atividade dessa enzima representa cerca de 50% do consumo total de energia (Stahl, 1986).

Em linha a apoptose, devido à redução do potencial de membrana mitocondrial, ocorre uma maior permeabilidade, o que permite uma liberação aumentada de citocromo C que consequentemente pode também desencadear a ativação de cascata de apoptose e culminar em morte celular (Kozina et al., 2014; LI et al., 1997). De fato, demostramos o envolvimento dos demais complexos mitocondriais na neurotoxicidade induzida por 6-OHDA, assim sugerimos que possa ter ocorrido também a liberação do citocromo C, e essa possível liberação pode estar envolvida na ativação das Caspases 3 e 9 decorrente da injeção dessa neurotoxina no estriado. Nossos dados demostram que o tratamento com hesperidina possui efetividade em inibir a atividade exacerbada das caspases 3 e 9, revelando um potencial efeito anti-apoptótico. O que está de acordo com resultados de Shaban et al., 2017 que já demostram a capacidade desse flavonoide em reverter a diminuição da expressão de proteínas anti-apoptóticas no testículo de ratos após exposição à radiação gama. Assim, nossas evidências confirmaram a implicação da hesperidina também na via apoptótica mediada por mitocôndrias no estriado, o que pode ser relacionado com seu potencial na recuperação das monoaminas estriatais observadas em nosso estudo. Incrementando, esse flavonoide também atua mantendo a integridade e a bioenergética mitocondrial no estriado através da reversão da inibição das atividades das enzimas mitocondriais incluindo a ATP-sintase, compelindo assim em um aumento da energia disponível para a biossíntese DA. Neste contexto, a hesperidina é capaz de atuar reduzindo a disfunção mitocondrial e a apoptose, podendo, dessa forma, preservar a DA estriatal. Os resultados do presente estudo indicam que as propriedades antiparkinsonianas de hesperidina podem se relacionar, pelo menos em parte, com a inibição da inflamação, apoptose, disfunção mitocondrial, recuperação da atividade da enzima Na^+/K^+ ATPase e dos fatores neurotróficos, resultando assim, na recuperação neuronal dopaminérgica.

Além das alterações estriatais, as disfunções não motoras e deficiências cognitivas possuem alta prevalência nos pacientes com DP. Distúrbios de humor, como a ansiedade, anedonia e depressão, são sintomas não-motores insidiosos desta doença e são muito prejudiciais para a qualidade de vida dos pacientes (MARTINEZ-

MARTIN et al., 2011). Entre os achados do nosso estudo, demonstra-se que a administração i.c.v. de 6-OHDA promove uma diminuição do tempo gasto na área central no OFT, e no tempo e entradas nos braços abertos no teste do labirinto em cruz elevado (EPMT), evidenciando o comportamento tipo-ansioso provocado pela neurotoxina. Além disso, a 6-OHDA provoca redução do tempo de lambida no teste de splash, confirmando comportamento tipo anedônico já revelado anteriormente por nosso grupo (ANTUNES et al., 2014). O tratamento oral com hesperidina durante os 28 dias foi capaz de reverter as referidas alterações comportamentais. Esse efeito ansiolítico, por nos observados, colabora com estudos de Viswanatha e colaboradores 2012, que também relatam melhoria na ansiedade após administração oral do flavonoide, ademais, o seu efeito positivo na andeonia, confirma nossos resultados anteriores, sustentando seus efeitos nesse modelo (ANTUNES, et al., 2014). É concebível que esses resultados tenham relação com as reparações neuroquímicas e histopatológicas estriatais aqui elucidadas. Corroborando, estudo de Timmer e colaboradores, 2017, já associaram o estriado com déficits relacionados com a depressão na DP. Nesse sentido, o presente estudo apoia e amplia que o tratamento com hesperidina reestabelece os níveis de diversos marcadores, apoiando a suposição de que ansiedade e DP podem compartilhar alguns mecanismos biológicos subjacentes, portanto, a ansiedade e comportamento tipo depressivo são fortemente dependentes da depleção DA estriatal.

Entre os prejuízos não motores, este modelo de DP parece conduzir também a déficits cognitivos, particularmente na memória procedural, como constado pelo claro déficit na capacidade de aprendizagem espacial e na memória no 5º dia (retenção de curta duração) e no 12º dia (retenção de longo prazo) dos camundongos do grupo 6-OHDA avaliados pelo teste de Barnes. Estes novos resultados corroboram com o nosso estudo anterior, em que 6-OHDA demostra motivar comprometimento da memória em camundongos no teste do labirinto aquático de Morris (MWMT) (ANTUNES et al., 2014). Assim como publicado anteriormente, o tratamento com hesperidina também se revelou efetivo na recuperação da memória nesse novo teste, reforçando seu potencial efeito cognitivo. Correlacionamos nossos achados com a modulação da DA estriatal resultante do tratamento com hesperidina, visto que esse neurotransmissor desempenha um papel chave na regulação de certos estágios da aprendizagem, memória e plasticidade sináptica (ANTUNES et al., 2014; CALABRESI

et al., 2007; JAY, 2003). Em acordo, observações de Saint-Cyr et al., 1988 e Tadaiesky et al., 2010, já implicaram o estriado como principal área envolvida na disfunção de aprendizagem. Além disso, a inibição da atividade da enzima Na^+/K^+ ATPase causada por 6-OHDA é apontada por produzir edema e morte celular a nível de SNC o que também se relaciona com prejuízos no aprendizado e na memória (SATO et al., 2004). Assim, o efeito da hesperidina sobre esta enzima, pode ser um dos mecanismos envolvidos na restauração cognitiva alcançada por esse flavonoide. É importante enfatizar que a hesperidina não causa efeito sedativo ou excitatório nos animais, como demonstrado no teste do campo aberto (OFT).

Ainda entre os prejuízos não motores ocasionados pela microinjeção de 6-OHDA, observou-se um déficit olfatório nos camundongos infundidos com a neurotoxina. Esse é um sintoma não-motor comum da DP, cerca de 75% a 90% dos pacientes apresentam hiposmia (ROSS et al., 2008; DOMELLOF et al., 2017). Sugere-se que a recuperação dos déficits olfatórios, alcançada pelo tratamento com hesperidina, se relaciona com a restauração do sistema dopaminérgico, o que também está em linha com os estudos de Valle-Leija, que demonstram que a denervação dopaminérgica está diretamente associada com o déficit olfatório causado por 6-OHDA. Ainda, sustentando nossos resultados, estudo realizado por Winner et al., 2008, revela que a recuperação dos déficits olfatórios se correlaciona com a neogênese dopaminérgica, a qual também revelamos ser modulada pelo tratamento com hesperidina nesse estudo pela regulação de fatores neurotróficos como BDNF, NGF e NT-3. Finalmente, os resultados discutidos até o momento apontam para a hipótese de que o efeito neuroprotetor do flavonoide hesperidina frente a DP pode estar relacionado com seu potencial antioxidante (ANTUNES et al., 2013), anti-inflamatório, antiapoptótico e sua influência na neuroplasticidade, disfunção mitocondrial e atividade da enzima Na^+/K^+ ATPase no estriado. Com base nessa evidências, supõe-se que através desses mecanismos, o flavonoide hesperidina atua na reversão da perda neuronal e que estas restaurações neuroquímicas estão correlacionadas com os efeitos comportamentais alcançados. Dessa forma, o flavonoide reportado por nosso estudo pode ser um composto promissor para a pesquisa translacional da DP, unindo assim descobertas básicas com a investigação clínica e produzir resultados benefícios para a comunidade.

Adicionalmente, levando em consideração a importância e a carga social da depressão nos pacientes com DP bem como na sociedade em geral, sendo um dos transtornos mentais mais prevalentes e uma das principais causas de incapacidade em todo o mundo e, que o flavonoide estudado, comtempla efeitos positivos diante desses sintomas, se torna de grande valia determinar os efeitos da hesperidina frente a um modelo de depressão. Assim sendo, o próximo passo do presente estudo foi investigar a ação do flavonoide hesperidina no modelo de depressão conferido pela BO.

A remoção bilateral dos bulbos olfatórios em roedores tem sido amplamente aceita como um modelo animal efetivo de depressão (MORALES-MEDINA et al., 2013). Utilizando-o em nosso estudo, foi possível constatar notáveis modificações neuroquímicas, entre estas certificamos uma significativa redução nos níveis de BDNF e NGF no hipocampo. Esses fatores neurotróficos são proteínas que desempenham um papel vital na regulação do desenvolvimento e da função do circuito neural no cérebro de mamíferos, incluindo sobrevivência neuronal, plasticidade sináptica e neurogênese, o seu papel no mecanismo de ação dos fármacos antidepressivos tem sido bastante apreciado (CASTREN & RANTAMAKI, 2010; PARK & POO, 2013). Verificou-se, em nossa pesquisa, que o tratamento com hesperidina possui habilidade em restaurar os níveis dessas proteínas no hipocampo, diminuídas em decorrência da BO. Nossos dados estão em linha com resultados de Dwivedi et al., 2005 e Kimpton, 2012, que demostram que a regulação negativa dos níveis de BDNF e NGF no hipocampo está relacionada com a redução da neurogênese, desenvolvimento de depressão e deficiências cognitivas, os quais também foram evidenciados em nosso estudo pelo detrimento da memória, motivação e autocuidado (DWIVEDI et al., 2005 e KIMPTON, 2012). Nesse contexto, propõem-se que os distúrbios comportamentais induzidos pela BO são impulsionados, em parte, pelas vias do BDNF e NGF, assim a regulação destas NTs no hipocampo, proporcionadas pelo tratamento com a hesperidina pode representar uma importante aplicação para o campo de estudos dos transtornos depressivos. Corroborando, esse flavonoide já demonstrou atuação nesses paramentros de neuroplasticidade também no estriado, em modelo de DP indizido por 6-OHDA.

Juntamente com as alterações neurotróficas, demonstrou-se que a remoção dos bulbos olfatórios opera ainda, elevando os níveis de citocinas pró-inflamatórias

como IL-1β e IL-6 no hipocampo, o que está de acordo com estudos de Rinwa et al., 2013; Poretti et al., 2015, que reportaram aumento destas citocinas em distúrbios depressivos decorrentes da BO (RINWA et al., 2013; PORETTI et al., 2015). Em consonância com os dados da literatura, o presente estudo apoia a ideia de que uma resposta inflamatória, refletida pela desregulação de citocinas no hipocampo é implicada com as alterações comportamentais aqui observadas, podendo ser um importante mecanismo neurobiológico induzido pela BO em camundongos. Baseando-se nesses resultados, demonstra-se que as propriedades anti-inflamatórias de hesperidina, podem contribuir, em parte, para o efeito neuroprotetor deste flavonoide contra insultos inflamatórios, como o gerado pela remoção dos bulbos olfatórios.

Adicionalmente, o sistema colinérgico também tem sido implicado para desempenhar um papel importante na patofisiologia da depressão (MACHADO et al., 2012). Já é conhecido que inibidores da enzima acetilcolinesterase (AchE) podem atuar nos sintomas de depressão e melhorar a eficácia de antidepressivos através da diminuição da atividade dessa enzima no soro humano (MÜLLER et al. 2002). De fato, observou-se que a hiperatividade da AchE no hipocampo de camundongos após a BO foi revertida pelo tratamento com hesperidina durante 13 dias, fornecendo suporte para a hipótese de que a sinalização colinérgica pode mediar os efeitos antidepressivos desse flavonoide. Ainda, o déficit colinérgico refletido no hipocampo pela BO, pode refletir em importantes papéis na cognição, estudos de Song et al. 2008, relataram que a administração de IL-1β por 7 dias reduz a expressão de acetilcolina (ACh) e de NGF no giro dentado de ratos, e que esta redução relaciona-se com os prejuízos na memória.

Nesse contexto, a remoção dos bulbos olfatórios também promoveu comprometimento na memória espacial dependente do hipocampo, verificada pelo aumento no tempo de latência para a plataforma no MWMT e pelo baixo desempenho na retenção da memória de curto e de longo prazo no ORT dos camundongos bulbectomizados, o que está em conformidade com dados de Borre e colaboradores 2012, que revelaram que prejuízos cognitivos são frequentemente observados em ratos bulbectomizados. O tratamento com hesperidina intervém melhorando o desempenho em ambos os testes, reafirmando nosso estudo anterior, que já revelou

que a administração sub aguda de hesperidina é eficaz na reversão dessa alteração cognitiva (ANTUNES et al., 2014).

Em continuidade, observamos em nosso estudo um aumento da atividade locomotora espontânea no OFT e da imobilidade no teste do nado forçado (FST) após a remoção dos bulbos, o que está em consonância com os resultados de Bhatt et al., 2013 e Morales-Medina et al., 2013, que também demostram essas alterações em modelos de depressão e recomendam ainda, que o tratamento crônico com antidepressivos atua na reversão de tais mudanças. Em acordo, certificamos que o tratamento com hesperidina durante 28 dias foi efetivo em reverter a hiperatividade e o comportamento tipo depressivo induzidos pela BO, reforçando as propriedades farmacológicas deste composto, já demonstrada anteriormente por nosso laboratório (ANTUNES et al., 2014; DONATO et al., 2014; FILHO et al., 2013; SOUZA et al., 2013). Ainda entre as alterações comportamentais, a BO causou sinais de anedonia ou hipossensibilidade ao prazer, condição esta que é fundamental para o diagnóstico da depressão (SONG & LEONARD, 2005). Esse comportamento foi inferido por uma diminuição no tempo de lambidas observado nos camundongos bulbectomizados no teste de splash, o que se assemelha com resultados obtidos por Romeas et al., 2009; Sato et al., 2010 nesse modelo (ROMEAS et al., 2009; SATO et al., 2010a). Essa alteração pode ser reestabelecida por compostos que possuem propriedades antidepressivas, tais como o antidepressivo clássico imipramina (DETANICO et al., 2009). Nesse contexto, o tratamento com hesperidina também reverte esta modificação comportamental, sugerindo sua atuação no reestabelecimento do comportamento motivacional e autocuidado, demostrando a utilidade desse flavonoide no controle da anedonia associada a depressão. Por fim, esses dados corroboram com observações clínicas e fornecem evidências adicionais de que a disfunção cognitiva acompanha a depressão e seus sinais psico-comportamentais. A partir desses resultados, sugerimos que os prejuízos comportamentais aqui observados estão associados com o comprometimento da neuroplasticidade e com a neuroinflamação ocasionada pela BO no hipocampo, estando em linha com dados de Han et al. 2008 e Song et al. 2009, que evidenciam que deficiência neurotrófica e inflamação hipocampal resultam nessas irregularidades comportamentais após a remoção dos bulbos (HAN et al. 2008 e SONG et al. 2009).

Os achados deste estudo revelam que o tratamento com hesperidina possui efetividade em reverter os prejuízos na memória, hiperatividade, comportamento tipo depressivo e anedonia provocados pela BO. Esta resposta foi acompanhada pela modulação de citocinas pró-inflamatórias, manutenção da plasticidade cerebral e regulação da atividade da AchE no hipocampo de camundongos. Os presentes resultados suportam o argumento de que esse flavonoide, pode ser uma ferramenta atrativa para o tratamento de perturbações depressivas.

CONCLUSÕES

Considerando os resultados obtidos, podemos afirmar que o flavonoide hesperidina, exerce efeito neuroprotetor em um modelo de parkinsonismo experimental induzido pela microinjeção de 6-OHDA no estriado. Hesperidina atua através de mecanismos que modulam fatores neurotróficos (BDNF, NGF e NT-3), inflamação (TNF-α, IFN-γ, IL-1β e IL-6), componentes mitocondriais (complexos I, II, IV e V), e apoptose (caspases 3 e 9), para manter a viabilidade neuronal dopaminérgica nessa estrutura cerebral. Em adição, seu papel na DP é fortalecido pela recuperação dos déficits comportamentais nos testes de atividade rotatória, ansiedade, anedonia, memória e disfunção olfatória, nesse modelo. Adicionalmente, baseando-se em nossos resultados anteriores, esse estudo amplia o efeito da hesperidina na depressão. Por intermédio do modelo da BO, constata-se que o flavonoide reportado também exibe um pronunciado efeito tipo antidepressivo em um modelo exclusivamente de depressão, que se dá, pelo menos em parte, por seu potencial efeito na modulação da inflamação (IL-1β e IL-6), e de fatores neurotróficos (BDNF e NGF), no hipocampo. Por fim, com base nesses resultados, nós sugerimos que hesperidina é um importante alvo terapêutico para o desenvolvimento de drogas promissoras para o tratamento da DP e depressão.

REFERENCIAS

AIRAKSINEN, M; SAARMA, M. **The GDNF family:** signaling, biological functions and therapeutic value. Nat Rev Neurosci, 3:383-94, 2002.

AUTRY, AE; MONTEGGIA, LM. **Brain-derived neurotrophic factor and neuropsychiatric disorders**. Pharmacol Ver, 64:38-58, 2012.

ADAMS, JD; CHANG, ML; KLAIDMAN, L. **Parkinson's disease--redox mechanisms.** Curr Med Chem, 8:809-14. 2001.

ALBERTS, et al. Molecular Biology of The Cell. Garland Science, 5th Edition, 2008.

ALBIN, RL; YOUNG, AB; PENNEY, JB. **The functional anatomy of basal ganglia disorders**. Trends Neurosci, 12:366–375. 1989.

AMEER, B; WEINTRAUB, RA; JOHNSON, JV; YOST, RA; ROUSEFF, RL. **Flavanone absorption after naringin, hesperidin, and citrus administration**. Clin Pharmacol Ther, 60:34-40, 1996.

ANDREW, R; WATSON, DG; BEST, SA; MIDGLE, JM; WENLONG, H; PETTY, RK. **The determination of hydroxydopamines and other trace amines in the urine of parkinsonian patients and normal controls**. Neurochem Res, 18:1175–1177, 1993.

ANTUNES, MS; GOES, AT; BOEIRA, SP; PRIGOL, M; JESSE, CR. **Protective effect of hesperidin in a model of Parkinson's disease induced by 6-hydroxydopamine in aged mice.** Nutrition, 30:1415-22, 2014.

ANTUNES, MS; JESSE, CR; RUFF, JR; de OLIVEIRA ESPINOSA, D; GOMES, NS; ALTVATER, EE; DONATO, F; GIACOMELI, R; BOEIRA, SP. **Hesperidin reverses cognitive and depressive disturbances induced by olfactory bulbectomy in mice by modulating hippocampal neurotrophins and cytokine levels and acetylcholinesterase activity.** Eur J Pharmacol, 789, 411-20, 2016.

ARABIA, G; GROSSARDT, BR; GEDA, YE; CARLIN, JM; BOWER, JH; AHLSKOG, JE; MARAGANORE, DM; ROCCA, WA. **Increased risk of depressive and anxiety disorders in relatives of patients with Parkinson disease**. Arch Gen Psychiatry, 64: 1385–1392, 2007.

ARAKAWA, S; SHIRAYAMA, Y; FUJITA, Y; ISHIMA ,T; HORIO, M; MUNEOKA, K; IYO, M; HASHIMOTO, K. **Minocycline produced antidepressantlike effects on the learned helplessness rats with alterations in levels of monoamine in the amygdala and no changes in BDNF levels in the hippocampus at baseline**. Pharmacol Biochem Behav, 100:601–606, 2012.

BAE, SK; HEO, CH; CHOI, DJ; SEN, D; JOE, EH; CHO, BR; KIM, HM. **A ratiometric two-photon fluorescent probe reveals reduction in mitochondrial H2S production in Parkinson's disease gene knockout astrocytes.** J Am Chem Soc, 135:9915-23, 2013.

BABA, M*;* NAKAJO, S*;* TU, PH*;* TOMITA, T*;* NAKAYA, K*;* LEE, VM. TROJANOWSKI, JQ; IWATSUBO, *T.* **Aggregation of α-synuclein in Lewy bodies of sporadic Parkinson's disease and dementia with Lewy bodies***.* Am J Pathol, 152*:*879–884, 1998.

BARBOSA, MT. CARAMELLI,P; MAIA, DP; CUNNINGHAM, MC, GUERRA, HL; LIMA-COSTA, MF; CARDOSO, F. **Parkinsonism and Parkinson's disease in the elderly: a community-based survey in Brazil (the Bambui study).** Mov Disord, 21:800-808, 2006.

BARDE, YA; EDGAR, D; THOENEM, H. **Purification of a new neurotrophic factor from mammalian brain.** EMBO J, 1:549-5531982.

BARNUM, CJ; TANSEY, MG. **Neuroinflammation and non-motor symptoms: the dark passenger of Parkinson's disease.** Curr Neurol Neurosci Rep, 12:350–358, 2012.

BARRIENTOS, RM; SPRUNGER, DB; CAMPEAU, S; HIGGINS, EA; WATKINS, LR; RUDY, JW; Maier SF. **Brain-derived neurotrophic factor mRNA downregulation produced by social isolation is blocked by intrahippocampal interleukin-1 receptor antagonist.** Neuroscience, 121:847–853, 2003.

BHATT, S; MAHESH, R; DEVADOSS, T; JINDAL, A. **Anti-depressant like activity of N-n-butyl-3-methoxyquinoxaline-2-carboxamide (6o) a 5-HT3 receptor antagonista**. Indian J Exp Biol, 51:435–443, 2013.

BATTISTI, C; FORMICHI, P; RADI, E; FEDERICO, A. **Oxidative-stress-induced apoptosis in PBLs of two patients with Parkinson disease secondary to alpha-synuclein mutation**. J Neurol Sci, 267:120-4, 2007.

BEN MENACHEM-ZIDON, IO; GOSHEN, T; KREISEL, Y; BEN MENAHEM, E; REINHARTZ, T; BEN HUR, R. **Yirmiya. Intrahippocampal transplantation of transgenic neural precursor cells overexpressing interleukin-1 receptor antagonist blocks chronic isolation-induced impairment in memory and neurogenesis.** Neuropsychopharmacology, 33:2251–2262, 2008.

BÉZARD, E. DOVERO, S; PRUNIER, C; RAVENSCROFT, P; CHALON, S; GUILLOTEAU, D; CROSSMAN, AR; BIOULAC, B; BROTCHIE, JM; GROSS, CE. **Relationship between the appearance of symptoms and the level of nigrostriatal degeneration in a progressive 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine lesioned macaque model of Parkinson's disease**. J Neurosci, 21:6853-6861, 2001.

BEZARD, E; IMBERT, C; GROSS, CE. **Experimental models of Parkinson's disease: From the static to the dynamic**. Rev Neurosci, 9:71–90, 1998.

BORRE, Y; SIR, V; DE KIVIT, S; WESTPHAL, KG; OLIVIER, B; Oosting, RS. Minocycline restores spatial but not fear memory in olfactory bulbectomized rats. Eur J Pharmacol, 697:59–64, 2012.

BORRADAILE, NM; CARROLL, KK; KUROWSKA, EM. **Regulation of HepG2 cell apolipoprotein B metabolism by the citrus flavanones hesperedin and naringenin.** Lipids, 34:591-598, 1999.

BOVÉ, J; PERIER, C. **Neurotoxin-based models of Parkinson's disease**. Neuroscience, 211:51-76, 2012.

BUJ-BELLO, A; BUCHMAN, VL; HORTON, A; ROSENTHAL, A; DAVIS, AM. **GDNF is an age-specific survival factor for sensory and autonomic neurons**. Neuron, 15:821–828, 1995.

BURGUILLOS, MA; DEIERBORG, T; KAVANAGH, E; PERSSON, A; HAJJI, N; GARCIA-QUINTANILLA, A; CANO, J; BRUNDIN, P; ENGLUND, E; VENERO, JL; Joseph, B. **Caspase signalling controls microglia activation and neurotoxicity**. Nature, 472:319–324, 2011.

BLUM, D; TORCH, S; LAMBENG, N; NISSOU, M; BENABID, AL; SADOUL, VERNA, JM. **Molecular pathways involved in the neurotoxicity of 6-OHDA, dopamine and MPTP: contribution to the apoptotic theory in Parkinson's disease**. Prog Neurobiol, 65:135–172, 2001.

BRAAK, E.*;* SANDMANN-KEIL, D.*;* RUB, U.*;* GAI, WP.; DE VOS, RA.*;* STEUR, EN.; *et al.* **α-synuclein immunopositive Parkinson's disease-related inclusion bodies in lower brain stem nuclei***.* Acta Neuropathol 101*:*195–201*,* 2001*.*

BRENES, GA; **Anxiety, depression, and quality of life in primary care patients. Prim. Care Companion.** J Clin Psychiatry, 9:437–443, 2007.

BRODACKI, B; STASZEWSKI, J; TOCZYŁOWSKA, B; KOZŁOWSKA, E; DRELA, N; CHALIMONIUK, M. STEPIEN, A. **Serum interleukin (IL-2, IL-10, IL-6, IL-4),**

TNFalpha, and INFgamma concentrations are elevated in patients with atypical and idiopathic parkinsonism. Neuroscience letters, 441:158–62, 2008.

CALABRESI, P; GALLETTI, F; SAGGESE, E; GHIGLIERI, V; PICCONI, B; **Neuronal networks and synaptic plasticity in Parkinson's disease: beyond motor deficits.** Parkinsonism Relat Disord, 13:259-62, 2007.

CAVAILLON, JM. **Pro- versus anti-inflammatory cytokines: myth or reality**. Cell Mol Biol, 47:695–702, 2001.

CAGNIN, A; KASSIOU, M; MEIKLE, SR; BANATI, RB. **In vivo evidence for microglial activation in neurodegenerative dementia.** Acta Neurol Scand Suppl 185:107–14, 2006.

CICCHETTI, F*;* BROWNELL, AL*;* WILLIAMS, K*;* CHEN, YI*;* LIVNI, E*;* ISACSON, O. **Neuroinflammation of the nigrostriatal pathway during progressive 6-OHDA dopamine degeneration in rats monitored by immunohistochemistry and PET imaging***.* Eur J Neurosci*,* 15*:*991–998*,* 2002*.*

COLLINS, LM; TOULOUSE, A; CONNOR, TJ; NOLAN, YM. **Contributions of central and systemic inflammation to the pathophysiology of Parkinson's disease**. Neuropharmacol, 62: 2154-68, 2012.

CAMPOS, JC. **Caracterização da função e da dinamica mitochondrial em modelo animal de disfunção cardíaca associada ao infarto do miocardio: efeitos do treinamento fisico aeróbico.** Dissertação de mestrado, USP, 2012.

CURTIUS, HC; WOLFENSBERGER, M; STEINMANN, B; REDWEIK, U; SIEGFRIED, J. **Mass fragmentography of dopamine and 6-hydroxydopamineApplication to the determination of dopamine in human brain biopsies from the caudate nucleus.** J Chromatogr, 99:529–540, 1974.

CHAUDHURI, KR; HEALY, DG; SCHAPIRA, AH. **Non-motor symptoms of Parkinson's disease: diagnosis and management.** Lancet Neurol, 5:235–245, 2006.

CHAUDHURI, KR; SCHAPIRA, AH. **Non-motor symptoms of Parkinson's disease: dopaminergic pathophysiology and treatment.** Lancet Neurol, 8:464–474, 2009.

CHAN, H; PAUR, H*;* VERNON, AC*;* ZABARSKY, V*;* DATLA, KP*;* CROUCHER, MJ*;* DEXTER, DT*.* **Neuroprotection and functional recovery associated with decreased microglial activation following selective activation of mGluR2/3**

receptors in a rodent model of Parkinson's disease. *Parkinsons Dis,* 2010. doi: 10.4061/2010/190450.

CHO, HS; SHIN, MS; SONG, W; JUN, TW; LIM, BV; KIM, YP; KIM, CJ. **Treadmill exercise lleviates short-term memory impairment in 6-hydroxydopamine-induced Parkinson's rats.** J Exerc Rehabil, 9:354–361, 2013.

DAUER, W; PRZEDBORSKI, S. **Parkinson's Disease: Mechanisms and Models**. Neuron, 39:889-909, 2003.

DANTZER, R; O'CONNOR, JC; FREUND, GG; JOHNSON, RW; KELLEY, KW. **From inflammation to sickness and depression: when the immune system subjugates the brain.** Nat Rev Neurosci, 9:46-56, 2008.

DE RIJK, MC; BRETELER, MMB; DEN BREEIJEN, JH; LAUNER, LJ; GROBBEE, DE; VAN DER MECHÉ, FG; HOFMAN, A. **Dietary antioxidants and Parkinson disease: the Rotterdam Study.** Arch Neurol, 54:762–765, 1997.

DETANICO, BC; PIATO, AL; FREITAS, JJ; LHULLIER, FL; HIDALGO, MP; CAUMO, W; ELISABETSKY, E. **Antidepressant-like effects of melatonin in the mouse chronic mild stress model**. Eur J Pharmacol, 607:121–125, 2009.

DEUSSING, JM. **Animal models of depression**. Drug Discov Today, 3:375–383, 2007.

DIMPFEL, W. **Different anticonvulsive effects of hesperidin and its aglycone hesperetin on electrical activity in the rat hippocampus in-vitro**. J Pharm Pharmacol, 58:375–379, 2006.

DISSANAYAKA, NN; SELLBACH, A; SILBURN, PA; O'SULLIVAN, JD; MARSH, R; MELLICK, GD. **Factors associated with depression in Parkinson's disease.** J Affect Disord, 132:82-8, 2011.

DOMELLÖF, E; LUNDIN, KF; EDSTRÖM, M; FORSGRE, L. **Olfactory dysfunction and dementia in newly diagnosed patients with Parkinson's disease.** Parkinsonism Relat Disord, 21:30067-6, 2017.

DONATO, F; DE GOMES, MG; GOES, AT; FILHO, CB; DEL FABBRO, L; ANTUNES, MS; SOUZA, LC; BOEIRA, SP; JESSE; CR. **Hesperidin exerts antidepressant-like effects in acute and chronic treatments in mice: possible role of l-arginine-NO-cGMP pathway and BDNF levels**. Brain Res Bull, 104:19-26, 2014.

DWIVEDI, Y; MONDAL, AC; RIZAVI, HS; CONLEY, RR. **Suicide brain is associated with decreased expression of neurotrophins.** Biol Psychiatry, 58:315–324, 2005.

DOWDING, CH; SHENTON, CL; SALEK, SS. **A review of the health-related quality of life and economic impact of Parkinson's disease**. Drugs & Aging, 23:693–721, 2006.

DUCRAY, A; KREBS, SH; SCHALLER, B; SEILER, RW; MEYER, M; WIDMER, HR. **GDNF-family ligands display distinct action profiles on cultured GABAergic and serotonergic neurons of rat ventral mesencephalon.** Brain Res, 1069:104-112, 2006.

DUTY, S; JENNER, P. **Animal models of Parkinson's disease: a source of novel treatments and clues to the cause of the disease.** Br J Pharmacol, 164:1357-1391, 2011.

ERLUND, I.; MERIRINNE, E.; ALFTHAN, G.; ARO, A. **Plasma kinetics and urinary excretion of the flavanonas naringenina and hesperetin in humans after ingestion of orange juice and grapefruit juice.** J Nutr, 131: 235-241, 2001.

ESPEJO, M; CUTILLAS, B; ARENAS, TE; AMBROSIO, S. **Increased survival of dopaminergic neurons in striatal grafts of fetal ventral mesencephalic cells exposed to neurotrophin-3 or glial cell line-derived neurotrophic fator**. Cell Transplant, 9:45–53, 2000.

FANG, F; XU, Q; PARK, Y; HUANG, X; HOLLENBECK, A; BLAIR, A; SCHATZKIN, A; KAMEL, F; CHEN, H. **Depression and the subsequent risk of Parkinson's disease in the NIH-AARP Diet and Health Study**. Mov Disord, 25:1157–1162, 2010.

FARRER, MJ. **Genetics of Parkinson disease: paradigm shifts and future prospects**. Nat Rev Genet, 7: 306–318, 2006.

FRISINA, PG; HAROUTUNIAN, V; LIBOW, LS. **The neuropathological basis for depression in Parkinson's disease**. Parkinsonism Relat Disord, 15:144–148, 2009.

FILHO, CB; DEL FABBRO, L; DE GOMES, MG; GOES, AT; SOUZA, LC; BOEIRA, SP; JESSE, CR. **Kappa-opioid receptors mediate the antidepressant-like activity of hesperidin in the mouse forced swimming test.** Eur J Pharmacol, 698:286-91, 2013.

FINKEL, SI; COSTA E SILVA, J; COHEN, G; MILLER, S; SARTORIUS, N. **Behavioral and psychological signs and symptoms of dementia: a consensus statement on current knowledge and implications for research and treatment**. Int Psychogeriatr, 3:497-500, 1996.

FUKUMOTO, T; MORINOBU, S; OKAMOTO, Y; KAGAYA, A; YAMAWAKI, S. **Chronic lithium treatment increases the expression of brain-derived neurotrophic factor in the rat brain.** Psychopharmacology (Berl), 158:100-6, 2001.

FURUKAWA, S. **Basic Research on Neurotrophic Factors and Its Application to Medical Uses.** Yakugaku Zasshi, 135:1213-26, 2015.

FRISINA, PG; HAROUTUNIAN, V; LIBOW, LS. **The neuropathological basis for depression in Parkinson's disease.** Parkinsonism Relat Disord, 15:144–148, 2009.

FRIGUET, B; BULTEAU, AL; PETROPOULOS, I. **Mitochondrial protein quality control: implications in ageing.** Biotechnol JJun, 3:757-64, 2008.

GAO, HM; LIU, B; ZHANG, W; HONG, JS. **Novel anti-inflammatory therapy for Parkinson's disease.** Trends Pharmacol Sci, 24:395-401, 2003.

GALATI, EM; MONFORTE, MT; KIRJAVAINEN, S; FORESTIERI, AM; TROVATO, A; TRIPODO, MM. **Biological effects of hesperidin, a Citrus flavonoid (Note I), anti-inflammatory and analgesic activity**. Farmaco, 40:709-12, 1994.

GARG, A; GARG, S; ZANEVELD, LJ; SINGLA, AK. **Chemistry and pharmacology of the Citrus bioflavonoid hesperidin.** Phytother Res, 15:655-69, 2001.

GONUL, AS; AKDENIZ, F; TANELI, F; DONAT, O; EKER, C; VAHIP, S. **Effect of treatment on serum brain-derived neurotrophic factor levels in depressed patients**. Eur Arch Psychiatry Clin Neurosci, 255:381-6, 2005.

GUSTAFSSON, H; NORDSTROM, A; NORDSTROM, P. **Depression and subsequent risk of Parkinson's disease; a nationwide cohort study.** Neurology, 84:2422–2429, 2015.

GUZMÁN-GUTIÉRREZ, SL; NAVARRETE, A. **Pharmacological exploration of the sedative mechanism of hesperidin identified as the active principle of Citrus sinensis flowers.** Planta Med, 75:295–301, 2009.

GLINKA, Y; GASSEN, M; YOUDIM, MB. **Mechanism of 6-hydroxydopamine neurotoxicity.** J Neural Transm Suppl, 50:55-66, 1997.

GLOBAL PARKINSON'S DISEASE SURVEY STEERING COMMITTEE. **Factors impacting on quality of life in Parkinson's disease: results from an international survey.** Mov Disord, 17:60-7, 2002.

GRAHAM, JD; LEWIS, MJ; WILLIAMS, J. **Proceedings: The effect of delta-1-tetrahydrocannabinol on the noradrenaline and dopamine content of the brain and heart of the rat.** Br J Pharmacol, 52:446P, 1974.

GRAZINA, M. **Genoma Mitocondrial e Défice Energético no diagnóstico das Doenças da Cadeia Respiratória Mitocondrial**. Coimbra: Faculdade de Medicina, Universidade de Coimbra. Tese de Doutorado, 2004.

HAAVIK, J; TOSKA, K. **Tyrosine hydroxylase and Parkinson's disease**. Mol Neurobiol, 16:285–309, 1998.

HADJICONSTANTINOU, M; MCGUIRE, L; DUCHEMIN, AM; LASKOWSKI, B; KIECOLT-GLASER, J; GLASER, R. **Changes in plasma nerve growth factor levels in older adults associated with chronic stress.** J Neuroimmunol, 116:102-6, 2001.

HALD, A; LOTHARIUS, J. **Oxidative stress and inflammation in Parkinson's** disease: Is there a casual link? Experim Neurol, 184:561-564, 2005.

HAMILTON, M. **A rating scale for depression.** J Neurol Neurosurg Psychiatry, 23:56-62, 1960

HAMBURGER, V; LEVI-MONTALCINI, R. **Proliferation, differentiation and degeneration in the spinal ganglia of the chick embryo under normal and experimental conditions**. J Exp Zool, 111:457–501, 1949.

HAN, F; SHIODA, N; MORIGUCHI, S; QIN, ZH; FUKUNAGA, K. **The vanadium (IV) compound rescues septo-hippocampal cholinergic neurons from neurodegeneration in olfactory bulbectomized mice.** Neuroscience, 151:671–679, 2008.

HEIM, K.E.; TAGLIAFERRO, A.R.; BOBILVA, D.J. **Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships.** J Nutr Biochem, 13: 572- 584, 2002.

HENDRIKSEN, H; MEULENDIJKS, D; DOUMA, TN; BINK, DI; BREUER, ME; WESTPHAL, KG; OLIVIER, B; OOSTING, RS. **Oosting. Environmental enrichment has antidepressant-like action without improving learning and memory deficits in olfactory bulbectomized rats.** Neuropharmacology, 62:270–277, 2012.

HIRSCH, EC; HUNOT, S; HARTMANN, A. **Neuroinflammatory processes in Parkinson's disease**. Parkinsonism Relat Disord, 11:9-15, 2005.

HIRSCH, EC; JENNER, P; PHARM, S; PRZEDBORSKI, S. **Pathogenesis of Parkinson's disease**. Mov Disorders, 28:24-30, 2013.

HUANG, EJ; REICHARDT, LF. **Neurotrophins: roles in neuronal development and function.** Annu Rev Neurosci, 24:677–736, 2001.

HSU, JW; LIRNG, JF; WANG, SJ; LIN, CL; YANG, KC; LIAO, MH; CHOU, YH. **Association of thalamic serotonin transporter and interleukin-10 in bipolar I disorder: a SPECT study.** Bipolar Disord, 16:241–248, 2014.

IBGE. **Idosos no Brasil.** 2013. Disponível em http://www.ibge.gov.br/home/estatistica/populacao/perfilidoso/default.shtm.

ILIJIC, E; GUZMAN, JN; SURMEIER, DJ. **The L-type channel antagonist isradipine is neuroprotective in a mouse model of Parkinson's disease**. Neurobiol Dis, 2011.

IRANSHAHI, M; REZAEE, R; PARHIZ, H; ROOHBAKHSH, A; SOLTANI, F. **Protective effects of flavonoids against microbes and toxins: The cases of hesperidin and hesperetin.** Life Sci 137:125-32, 2015.

JAY, T. **Dopamine: a potential substrate for synaptic plasticity and memory mechanisms**. Prog Neurobiol, 69:375-90, 2003.

JIANG, H; JACKSON-LEWIS, V; MUTHANE, U; DOLLISON, A; FERREIRA, M; ESPINOSA, A; PARSONS, B; PRZEDBORSKI, S. **Adenosine receptor antagonists potentiate dopamine receptor agonist-induced rotational behavior in 6-hydroxydopamine-lesioned rats.** Brain Res 613:347–351, 1993.

KAUR, J; SHARMA, D; SINGH, R. **Acetyl-l-carnitine enhances Na(+) K(+)-ATPase glutathione-S-transferase and multiple unit activity and reduces lipid peroxidation and lipofuscin concentration in aged rat brain regions**. Neurosci Lett, 301:1–4, 2001.

KANO, O; IKEDA, K; CRIDEBRING, D; TAKAZAWA, T; YOSHII, Y; IWASAKI, Y. **Neurobiology of depression and anxiety in Parkinson's disease**. Parkinsons Dis, 2011:143–547, 2011.

KATOH-SEMBA, R; WAKAKO, R; KOMORI, T; SHIMEGI, H; MIYASAKI, N; ITO, H; KUMAGAI, T; TSUZUKI, M; SHIMEGI, K; YOSHIDA, F; NAKAYAMA. A. **Age-related changes in BDNF protein levels in human serum: differences between autism cases and normal controls**. Int J Dev Neurosci, 25:367-72, 2007.

KERR, JF; WYLLIE, AH; CURRIE, AR. **Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics**. Br J Cancer, 26:239-57, 1972.

KIMPTON, J; **The brain derived neurotrophic factor and influences of stress in depression**. Psychiatr Danub, 24: 169–171, 2012.

KÖHLER, O; BENROS, ME; NORDENTOFT, M; FARKOUH, ME; IYENGAR, RL; MORS, O; KROGH J. **Effect of anti-inflammatory treatment on depression, depressive symptoms, and adverse effects: a systematic review and meta-analysis of randomized clinical trials**. JAMA Psychiatry, 71:1381–1391, 2014.

KOZINA, EA; KHAKIMOVA, GR; KHAINDRAVA, VG; KUCHERYANU, VG; VOROBYEVA, NE; KRASNOV, AN; GEORGIEVA, SG; KERKERIAN-LE GOFF, L; UGRUMOV, MV. **Tyrosine hydroxylase expression and activity in nigrostriatal dopaminergic neurons of MPTP-treated mice at the presymptomatic and symptomatic stages of parkinsonism.** J Neurol Sci 340:198–207, 2014.

KUNIKOWSKA, G; JENNER, P. **6-Hydroxydopamine-lesioning of the nigrostriatal pathway in rats alters basal ganglia mRNA for copper, zinc- and manganese-superoxide dismutase, but not glutathione peroxidase**. Brain Res, 922:51–64, 2001.

KUPSCH, A; SCHMIDT, W; GIZATULLINA, Z; DEBSKA-VIELHABER, G; VOGES, J; et al. **6-Hydroxydopamine impairs mitochondrial function in the rat model of Parkinson's disease: respirometric, histological, and behavioral analyses.** J Neural Transm (Vienna), 121:1245-57, 2014.

KHAN, MM; AHMAD, A; ISHRAT, T; KHAN, MB; HODA, MN; KHUWAJA, G; RAZA, SS; KHAN, A; JAVED, H; VAIBHAV, K; ISLAM, F. **Resveratrol attenuates 6-hydroxydopamine-induced oxidative damage and dopamine depletion in rat model of Parkinson's disease.** Brain Res, 1328:139-51, 2010.

LANG, AE; LOZANO, AM. **Parkinson's disease. Second of two parts**. N Engl J Med, 339:1130-43, 1998.

LEONARD, BE; TUITE, M. **Anatomical, physiological, and behavioral aspects of olfactory bulbectomy in the rat**. Int Rev Neurobiol, 22:251-86, 1981.

LEV, N; MELAMED, E; OFFEN, D. **Apoptosis and Parkinson’s disease**. Prog Neuropsychopharmacol, 27:245-250, 2003.

LEVI-MONTALCINI, R. **The development to the acoustico-vestibular centers in the chick embryo in the absence of the afferent root fibers and of descending fiber tracts.** J Comp Neurol, 91:209–241, 1949.

LEVI-MONTALCINI, R; HAMBURGER, V. **Selective growth stimulating effects of mouse sarcoma on the sensory and sympathetic nervous system of the chick embryo**. J Exp Zool, 116:321-361, 1951.

LEVI-MONTALCINI, R. **The nerve growth factor 35 years later.** Science, 237:1154-62, 1987.

LEMKE, MR; BRECHT, HM; KOESTER, J; REICHMANN, H. **Effects of the dopamine agonist pramipexole on depression, anhedonia and motor functioning in Parkinson's disease.** J Neurol Sci, 248:266-270, 2006.

LI, S; PU, X. **Neuroprotective Effect of Kaempferol against a 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine-Induced Mouse Model of Parkinson's Disease**. Biol Pharm Bull, 34:1291-1297, 2011.

LI, P; NIJHAWAN, D; BUDIHARDJO, I; SRINIVASULA, SM; AHMAD, M; ALNEMRI, ES; WANG, X. **Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade**. Cell, 91:479–489, 1997.

LIN, MT; BEAL, MF; **Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases**. Nature, 443:787–795, 2006.

LINDSAY, RM. **Trophic protection of motor neurons: clinical potential in motor neuron diseases**. J Neurol, 242:8–11, 1994.

LUTHMAN, J; FREDRIKSSON, A; SUNDSTROM, E; JONSSON, G; ARCHER, T. **Selective lesion of central dopamine or noradrenaline neuron systems in the neonatal rat: Motor behavior and monoamine alterations at adult stage**. Behav Brain Res, 33:267–277, 1989.

MACHADO, DG; CUNHA, MP; NEIS, VB; BALEN, GO; COLLA, A; GRANDO, J; BROCARDO, PS; BETTIO, LE; CAPRA, JC; RODRIGUES, AL. **Fluoxetine reverses depressive-like behaviors and increases hippocampal acetylcholinesterase activity induced by olfactory bulbectomy**. Pharmacology Biochem Behav, 103:220–229, 2012.

MAES, M; BOSMANS, E; SUY, E; VANDERVORST, C; DE JONCKHEERE, C; RAUS, J. **Immune disturbances during major depression: upregulated expression of interleukin-2 receptors**. Neuropsychobiology, 24:115-20, 1990.

MANACH, C.; MORAND, C.; GIL-IZQUIERDO, A. et al. **Bioavailability in humans of the flavanones hesperidin and narirutin after the ingestion of two doses of orange juice.** Eur J Clin Nutr, 57: 235-242, 2003.

MARCHETTI, B; ABBRACCHIO, MP. **To be or not to be (inflamed)--is that the question in anti-inflammatory drug therapy of neurodegenerative disorders?** Trends Pharmacol Sci, 26:517-25, 2005.

MARSH, L; MCDONALD, WM; CUMMINGS, J; RAVINA, B. **Provisional diagnostic criteria for depression in Parkinson's disease: report of an NINDS/NIMH Work Group**. Mov Disord, 21:148-58, 2006.

MARTINEZ-MARTIN, P; RODRIGUEZ-BLAZQUEZ, C; KURTIS, MM; CHAUDHURI, KR. **The impact of non-motor symptoms on health-related quality of life of patients with Parkinson's disease**. Mov Disord, 3:399–406. 2011.

MATSUMOTO, H.; IKOMA, Y.; SUGIURA, M.; MASAMICHI Y.; HASEGAXA, Y. **Identification and quantification of the conjugated metabolites derived from orally administered hesperidin in rat plasma.** Agric Food Chem, 52: 6653-6659, 2004.

MAZZIO, EA; REAMS; RR; SOLIMAN, KF. **The role of oxidative stress, impaired glycolysis and mitochondrial respiratory redox failure in the cytotoxic effects of 6 hydroxydopamine in vitro.** Brain Res, 1004:29–44, 2004.

MELE, A; AVENA, M; ROULLET, P; DE LEONIBUS, E; MANDILLO. S; SARGOLINI, F; COCCURELLO, R; OLIVERIO, A. **Nucleus accumbens dopamine receptors in the consolidation of spatial memory.** Behav Pharmacol, 15:423–31, 2004.

MENDOZA, E; MIRANDA-BARRIENTOS, JA; VÁZQUEZ-ROQUE, RA; MORALES-HERRERA, E; RUELAS, A; DE LA ROSA, G; FLORES, G; HERNÁNDEZ-ECHEAGARAY, E. **In vivo mitochondrial inhibition alters corticostriatal synaptic function and the modulatory effects of neurotrophins.** Neuroscience, 280:156-70, 2014.

MENZE, ET; TADROS, MG; ABDEL-TAWAB, AM; KHALIFA, AE. **Potential neuroprotective effects of hesperidin on 3-nitropropionic acid-induced neurotoxicity in rats.** NeuroToxicology, 33:1265–1275, 2012.

MEREDITH, GE; KANG, UJ. **Behavioral models of Parkinson's disease in rodents: a new look at an old problem.** Mov Disord, 21:1595–1606, 2006.

MIZUNO, Y. **Genetics of Parkinson´s disease.** Biomed Pharmacother, 53:109- 116, 1999.

MITCHELL, P; MOYLE, J. **Chemiosmotic hypothesis of oxidative phosphorylation**. Nature, 213:137-9, 1967.

MOOSAVI, F; HOSSEINI, R; SASO, L; FIRUZI, O. **Modulation of neurotrophic signaling pathways by polyphenols.** Drug Des Devel Ther, 10:23-42, 2015.

MOGI, M; TOGARI, A; KONDO, T; MIZUNO, Y; KOMURE, O; KUNO, S; ICHINOSE, H; NAGATSU, T. **Caspase activities and tumor necrosis factor receptor R1 level are elevated in the substantia nigra in Parkinson's disease**. J Neural Transm, 107:355-341, 2000.

MOLINA-HERNÁNDEZ, M; TELLEZ-ALCÁNTARA, NP; PÉREZ-GARCÍA, J; OLIVERA-LOPEZ, JI; JARAMILLO-JAIMES, MT. **Antidepressant-like actions of minocycline combined with several glutamate antagonists**. Prog Neuropsychopharmacol Biol Psychiatry, 32:380–386, 2008.

MORALES-MEDINA, JC; JUAREZ, I; VENANCIO-GARCIA, E; CABRERA, SN; MENARD, C; YU, W; FLORES, G; MECHAWAR, N; QUIRION, R. **Impaired structural hippocampal plasticity is associated with emotional and memory deficits in the olfactory bulbectomized rat**. Neuroscience, 236:233–243, 2013.

MÜLLER, TC1; ROCHA, JB; MORSCH, VM; NEIS, RT; SCHETINGER, MR; **Antidepressants inhibit human acetylcholinesterase and butyrylcholinesterase activity**. Biochim Biophys Acta, 1587:92-8, 2002.

MCGEER, PL; ITAGAKI, S; BOYES, BE; MCGEER, EG. **Reactive microglia are positive for HLA-DR in the substantia nigra of Parkinson's and Alzheimer's disease brains.** Neurology, 38:1285–91, 1988.

NABAVI, SM; DAGLIA, M; BRAIDY, N; NABAVI, SF. **Natural products, micronutrients, and nutraceuticals for the treatment of** depression**: A short review**. Nutr Neurosci, 2015.

NANDHAGOPAL, R; KURAMOTO, L; SCHULZER, M; MAK, E; CRAGG, J; LEE, CS; MCKENZIE, J; MCCORMICK, S; SAMII, A; TROIANO, A; RUTH, TJ; SOSSI, V; DE LA FUENTE-FERNANDEZ, R; CALNE, DB; STOESSL, AJ. **Longitudinal progression of sporadic Parkinson's disease: a multi-tracer positron emission tomography study**. Brain, 132:2970–2979, 2009.

Ngema, PN; Mabandla, MV. **Post 6-OHDA lesion exposure to stress affects neurotrophic factor expression and aggravates motor impairment.** Metab Brain Dis, 2017. doi: 10.1007/s11011-017-9988-1.

NISHI, A; FISONE, G; SNYDER, GL; DULUBOVA, I; APERIA, A; NAIRN, AC; GREENGARD, P. **Regulation of Na,K-ATPase isoforms in rat neostriatum by dopamine and protein kinase C.** J Neurochem, 73:1492–1501, 1999.

OMS. **Global burden disease.** 2008. Retrieved from http://www.who.int/research/en/. 2008.

PRADO, RC; BARBOSA, ER. **Depression in Parkinson's disease: study of 60 cases**. Arq Neuropsiquiatr, 63:766-71, 2005.

PONSEN, MM; STOFFERS, D; WOLTERS, EC; BOOIJ, J; BERENDSE, HW. **Olfactory testing combined with dopamine transporter imaging as a method to detect prodromal Parkinson's disease.** J Neurol Neurosurg Psychiatry, 81:396–9, 2010.

PORETTI, MB; RASK-ANDERSEN, M; KUMAR, P; RUBIALES DE BARIOGLIO, S; FIOL DE CUNEO, M; SCHIÖTH, HB; CARLINI VP. **Ghrelin effects expression of several genes associated with depression-like behavior.** Prog Neuropsychopharmacol Biol Psychiatry, 56:227-34, 2015.

PORTER, CC; TOTARO, JA; STONE, CA. **Effect of 6-hydroxydopamine and some other compounds on the concentration of norepinephrine in the hearts of mice.** J Pharmacol Exp Ther, 140:308–316, 1963.

POST, RM. **Role of BDNF in bipolar and unipolar disorder: clinical and theoretical implications.** J Psychiatr Res, 41:979-90, 2007.

QIAN, L; FLOOD, PM; HONG, JS. **Neuroinflammation is a key player in Parkinson's disease and a prime target for therapy**. J Neural Transm (Vienna), 117:971-9, 2010.

QUIDEAU, S; DEFFIEUX, D; DOUAT-CASASSUS, C; POUYSÉGU, L. **Plant polyphenols: chemical properties, biological activities, and synthesis**. Angew Chem Int Ed Engl, 50:586-621, 2011.

QUINN, N. **Parkinsonism--recognition and differential diagnosis.** BMJ, 310:447-52, 1995.

RAPAPORT, MH; CLARY, C; FAYYAD, R; ENDICOTT, J. **Quality-of-life impairment in depressive and anxiety disorders**. Am J Psychiatry, 162:1171–1178, 2005.

RICCIO, A; PIERCHALA, BA; CIARALLO, CL; GINTY, DD. **An NGF-TrkA-mediated retrograde signal to transcription factor CREB in sympathetic neurons**. Science, 277:1097–1100, 1997.

RICHARD, IH. **Anxiety disorders in Parkinson's disease**. Adv Neurol, 96:42-55, 2005.

RINWA, P; KUMAR, A. **Quercetin suppress microglial neuroinflammatory response and induce antidepressent-like effect in olfactory bulbectomized rats.** Neuroscience, 255:86-98, 2013.

RINWA, P; KUMAR, A; GARG, S. **Suppression of neuroinflammatory and apoptotic signaling cascade by curcumin alone and in combination with piperine in rat model of** olfactory bulbectomy **induced depression**. PLoS One, 17:8-12. 2013.

RIZELIO, V; SZAWKA, RE; XAVIER, LL; ACHAVAL, M; RIGON, P; SAUR, L; MATHEUSSI, F; DELATTRE, AM; ANSELMO-FRANCI, JA; MENESES, M; FERRAZ, AC. **Lesion of the subthalamic nucleus reverses motor deficits but not death of nigrostriatal dopaminergic neurons in a rat 6-hydroxydopamine-lesion model of Parkinson's disease.** Braz J Med Biol Res, 43:85-95, 2010.

ROJO, A; AGUILAR, M; GAROLERA, MT; CUBO, E; NAVAS, I; QUINTANA, S. **Depression in Parkinson´s disease: clinical correlates and outcome**. Parkinsonism and Related Disorders, 10:23-28, 2003.

ROMEAS, T; MORISSETTE, MC; MNIE-FILALI, O; PIÑEYRO, G; BOYE, SM. **Simultaneous anhedonia and exaggerated locomotor activation in an animal model of depression.** Psychopharmacology (Berl), 205:293-303, 2009.

ROSS, GW; PETROVITCH, H; ABBOTT, RD; TANNER, CM; POPPER, J; MASAKI, K; LAUNER, L; WHITE, LR. **Association of olfactory dysfunction with risk for future Parkinson's disease.** Ann Neurol, 63:167–173, 2008.

SAINT-CYR, JA; TAYLOR, AE; LANG, AE. **Procedural learning and neostriatal dysfunction in man.** Brain, 111:941–959, 1988.

SALAZAR, A; GONZALEZ-RIVERA, BL; REDUS, L; PARROTT, JM; O'CONNOR, JC. **Indoleamine 2,3-dioxygenase mediates anhedonia and anxiety-like behaviors caused by peripheral lipopolysaccharide immune challenge.** Horm Behav, 62:202–209, 2012.

SATO, T; TANAKA, KI; OHNISH, IY; TERAMOTO, T; IRIFUNE, M; NISHIKAWA, T. **Effects of steroid hormones on (Na+, K+)-ATPase activity inhibition-induced amnesia on the step-through passive avoidance task in gonadectomized mice.** Pharmacol Res, 49:151-9, 2004.

SATO, A; NAKAGAWASAI, O; TAN-NO, K; ONOGI, H; NIIJIMA, F; TADANO, T. **Effect of non-selective dopaminergic receptor agonist on disrupted maternal behavior in olfactory bulbectomized mice**. Behav Brain Res, 210: 251-6, 2010b.

SATO, A; NAKAGAWASAI, O; TAN-NO, K; ONOGI, H; NIIJIMA, F; TADANO, T. **Influence of olfactory bulbectomy on maternal behavior and dopaminergic function in nucleus accumbens in mice.** Behav Brain Res, 215:141-5, 2010a.

SETLOW, B; MCGAUGH, J.L. **Sulpiride infused into the nucleus accumbens posttraining impairs memory of spatial water maze training.** Behav Neurosci, 112:603–10, 1998.

SINGH, S; DIKSHIT, M. **Apoptotic neuronal death in Parkinson's disease: involvement of nitric oxide.** Brain Res Rev, 54:233-50, 2007.

SONG, C; LEONARD, BE. **The olfactory bulbectomised rat as a model of depression**. Neurosci Biobehav Rev, 29:627–647, 2005.

SOUZA, LC; DE GOMES, MG; GOES, AT; DEL FABBRO, L; FILHO, CB; BOEIRA, SP; JESSE, CR. Evidence **for the** involvement **of the** serotonergic 5-HT**(**1A**)** receptors **in the** antidepressant-like effect caused **by** hesperidin **in** mice**. Prog. Neuropsychopharmacol.** Biol Psychiatry, 40:103-9, 2013.

SONG, C; TAEPAVARAPRUK, P; ZHANG, Y. **Acute and chronic IL-1β administrations differentially modulate learning and memory, acetylcholine efflux and neurotrophin expressions in the hippocampus: possible mechanisms involved in neuroprotection and neurodegeneration.** Brain Behav Immun, 22:41–42, 2008.

SONG, C; ZHANG, XY; MANKU, M. **Increased phospholipase A2 activity and inflammatory response but decreased nerve growth factor expression in the olfactory bulbectomized rat model of depression: effect of chronic ethyl-eicosapentaenoate treatment.** Neurobiol Dis, 7:14–22, 2009.

SCHAPIRA, AH; COOPER, JM; DEXTER, D; JENNER, P; CLARK, JB; MARSDEN, CD. **Mitochondrial complex I deficiency in Parkinson's disease**. J Neurochem, 54:823-7, 1990.

SCALBERT A.; WILLIAMSON, G. **Dietary intake and bioavailability of polyphenols.** J Nutr, 130: 2073S-2085S, 2000.

SCHAPIRA, AH; OLANOW, CW. **Neuroprotection in Parkinson disease: mysteries, myths, and misconceptions.** Jama, 291:358-64, 2004.

SCHRAG, A. **Quality of life and depression in Parkinson's disease**. J Neurol Sci, 248:151-7, 2006.

SCHOBER, A. **Classic toxin-induced animal models of Parkinson's disease. 6-OHDA and MPTP**. Cell Tissue Res, 318:215–224, 2004.

SHIBA, M; BOWER, JH; MARAGANORE, DM; MCDONNELL, SK; PETERSON, BJ; AHLSKOG, JE; SCHAID, DJ; ROCCA, WA. **Anxiety disorders and depressive disorders preceding Parkinson's disease: a case-control study**. Mov Disord, 15:669-77, 2000.

SHIMOHAMA, S; SAWADA, H; KITAMURA, Y; TANIGUCHI, T. **Disease model: Parkinson's disease.** Trends Mol Med, 9:360-365, 2003.

SHOVAL, G; WEIZMAN, A. **The possible role of neurotrophins in the pathogenesis and therapy of schizophrenia.** Eur Neuropsychopharmacol, 15:319-29, 2005.

SHULMAN, RB. **Maintenance ECT in the treatment of PD. Therapy improves psychotic symptoms, physical function.** Geriatrics, 58:43-5, 2003.

SHULMAN, JM; DE JAGER, PL; FEANY, MB. **Parkinson's disease: genetics and pathogenesis.** Annu Rev Pathol, 6:193–222, 2011.

SPENCER, J.P.E.; CHOWRIMOOTOO, G.; CHOUDHURY, R. et al. **The small intestine can both absorb and glucuronidate luminal flavonoids.** FEBS Lett., 458: 224- 230, 1999.

SPILLANTINI, MG*;* CROWTHER, RA*;* JAKES, R*;* HASEGAWA, M*;* GOEDERT, M. **alpha-Synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with lewy bodies***.* Proc Natl Acad Sci USA, 95:6469–6473, 1998.

TADAIESKY, MT; DOMBROWSKI, PA; DA CUNHA, C; TAKAHASHI, RN. **Effects of SR141716 A on cognitive and depression-related behavior in an animal model of premotor Parkinson's disease.** Parkinsons Dis 238491 26–32, 2010.

TANSEY, MG; GOLDBERG, MS. **Neuroinflammation in Parkinson's disease: its role in neuronal death and implications for therapeutic intervention**. Neurobiol Dis, 37:510-8, 2010.

TANDBERG, E; LARSEN, JP; AARSLAND, D; LAAKE, K; CUMMINGS, JL. **Risk factors for depression in Parkinson disease.** Arch Neurol, 54:625-30, 1997.

TEDESCHI, H. **The structure of the mitochondrial membrane: inferences from permeability properties.** J Biophys Biochem Cytol, 6:241-52, 1959.

TEISMANN, P; SCHULZ, JB. **Cellular pathology of Parkinson's disease: astrocytes, microglia and inflammation**. Cell. Tissue Res, 318:149-161, 2004.

TEIXEIRA, MD; SOUZA, CM; MENEZES, AP; CARMO, MR; FONTELES, AA; GURGEL, JP; LIMA, FA; VIANA, GS; ANDRADE, GM. **Catechin attenuates behavioral neurotoxicity induced by 6-OHDA in rats**. Pharmacol Biochem Behav, 110:1-7, 2013.

TELES-GRILO RUIVO, LM; MELLOR, JR. **Cholinergic modulation of hippocampal network function.** Front Synaptic Neurosci, 2013. doi: 10.3389/fnsyn.2013.00002.

TIMMER, MH; SESCOUSSE, G; VAN DER SCHAAF, ME; ESSELINK, RA; COOLS, R. **Reward learning deficits in Parkinson's disease depend on depression.** Psychol Med, 4:1-10, 2017.

TOULOUSE, A; SULLIVAN, AM. **Progress in Parkinson's disease-Where do we stand?** Prog Neurobiol, In Press, 2008.

TUMAS, V; RODRIGUES, GG; FARIAS, TL; CRIPPA, JA. **The accuracy of diagnosis of major depression in patients with Parkinson's disease: a comparative study among the UPDRS, the geriatric depression scale and the Beck depression inventory.** Arq Neuropsiquiatr, 66:152-6, 2008.

TYRING, S; GOTTLIEB, A; PAPP, K; GORDON, K; LEONARDI, C; WANG, A. **Etanercept and clinical outcomes, fatigue, and depression in psoriasis: double-blind placebo-controlled randomised phase III trial**. Lancet, 367:29–35, 2006.

UM, JW; PARK, HJ; SONG, J; JEON, I; LEE, G; LEE, PH; CHUNG, KC. **Formation of parkin aggregates and enhanced PINK1 accumulation during the pathogenesis of Parkinson's disease.** Biochem Biophys Res Commun, 393:824-8, 2010.

UNGERSTEDT, U. **6-Hydroxy-dopamine induced degeneration of central monoamine neurons.** Eur J Pharmcol, 5:107-117, 1968.

UNGERSTEDT, U; ARBUTHNOTT, GW. **Quantitative recording of rotational behavior in rats after 6-hydroxy-dopamine lesions of the nigrostriatal dopamine system.** Brain Res, **24:485-93,** 1970.

VALLE-LEIJA, P; DRUCKER-COLÍN, R. **Unilateral olfactory deficit in a hemiparkinson's disease mouse model**. Neuroreport 12:948-53, 2014.

VARELA-NALLAR, L; NIBALDO, C; INESTROS, A. **Wnt signaling in the regulation of adult hippocampal neurogenesis.** Front Cell Neurosci, 2013. doi: 10.3389/fncel.2013.00100

VISWANATHA, GL; SHYLAJA, H; SANDEEP RAO; KS; SANTHOSH KUMAR, VR; JAGADEESH. M. **Hesperidin ameliorates immobilization-stress-induced behavioral and biochemical alterations and mitochondrial dysfunction in mice by modulating nitrergic pathway**. ISRN Pharmacol, 2012. doi: 10.5402/2012/479570.

WANG, XQ; XIAO, AY; SHELINE, C; HYRC, K; YANG, A; Goldberg, MP; CHOI, DW; YU, SP. **Apoptotic insults impair Na+, K+-ATPase activity as a mechanism of neuronal death mediated by concurrent ATP deficiency and oxidant stress.** J Cell Sci, 116:2099–2110, 2003.

WANG, XQ; XIAO, AY; SHELINE, C; HYRC, K; YANG, A; ET AL. Apoptotic insults impair Na+, K+-ATPase activity as a mechanism of neuronal death mediated by concurrent ATP deficiency and oxidant stress. J Cell Sci, 116, 2099–2110, 2003.

WANG, Y; CUI, XL; LIU, YF; GAO, F; WEI, D; LI, XW; Wang, HN; TAN, QR; JIANG, W. **LPS inhibits the effects of fluoxetine on depression-like behavior and hippocampal neurogenesis in rats.** Prog Neuropsychopharmacol Biol Psychiatry, 35:1831–1835, 2011.

WANG, Q; LIU, Y; ZHOU, J. **Neuroinflammation in Parkinson's disease and its potential as therapeutic target.** Transl Neurodegener, 12:4-19, 2015.

WELLS, K B; GOLDING, JM; BURNAM, MA. **Psychiatric disorder in a sample of the general population with and without chronic medical conditions**. Am J Psychiatry, 145:976-981, 1988.

WEINTRAUB, D; MOBERG, PJ; DUDA, JE; KATZ, IR; STERN, MB. **Recognition and treatment of depression in Parkinson's disease.** J Geriatr Psychiatry Neurol, 16:178-83, 2003.

WICHOWICZ, HM; SŁAWEK, J; DEREJKO, M; CUBAŁA, WJ. **Factors associated with depression in Parkinson's disease: a cross-sectional study in a Polish population.** Eur Psychiatry, 21:516-20, 2006.

WINNER, B; COUILLARD-DESPRES, S; GEYER, M; AIGNER, R; BOGDAHN, U; AIGNER, L; KUHN, HG; WINKLER, J. **Dopaminergic lesion enhances growth factor-induced striatal neuroblast migration.** J Neuropathol Exp Neurol, 67:105-16, 2008.

WILCOX, L; BORRADAILE, NM; DE DREU, LE; HUFF, MW. **Secretion of hepatocyte apoB is inhibited by the flavonoids, naringenin and hesperedin, via reduced activity and expression of ACAT2 and MTP.** J Lipid Res, 42:725-759, 2001.

WIRDEFELDT, K; ADAMI, HO; COLE, P; TRICHOPOULOS, D; MANDEL, J. **Epidemiology and etiology of Parkinson's disease: a review of the evidence**. Eur J Epidemiol, 26:1–58, 2011.

WOLTERS, EC. **Variability in the clinical expression of Parkinson's disease**. J Neurol Sci, 266:197–203, 2008.

WU, CW; CHEN, YC; YU, L; CHEN, HI; JEN, CJ; HUANG, AM; TSAI, HJ; CHANG, YT; KUO, YM. **Treadmill exercise counteracts the suppressive effects of peripheral lipopolysaccharide on hippocampal neurogenesis and learning and memory.** J. Neurochem, 103:2471–2481, 2007.

YAMADA, M.; TANABE, F.; ARAI, N. et al. **Bioavailability of glucosyl hesperidin in rats. Biosci.** Biotechnol Biochem, 70: 1386-1394, 2006.

YIN, Q; KEMP, GJ; FROSTICK, SP. **Neurotrophins, neurones and peripheral nerve regeneration.** J Hand Surg Br, 23:433-7, 1998.

ZUNSZAIN, PA; HEPGUL, N; PARIANTE. CM. **Inflammation and depression**. Curr Top Behav Neurosci, 14:135–51, 2013.

PERSPECTIVAS

Como perspectiva, destacamos que outros parâmetros precisam ser investigados no intuito de melhor compreender a atuação da hesperidina no SNC. Adicionalemte, pretende-se investigar um possível dano hepático e renal que o tratamento com hesperidina possa vir a causar, através da verificação da atividade de aspartato aminotransferase (AST), alanina aminotransferase (ALT), fosfatase alcalina (FAL), gama glutamil transferase (ɣGT), lactato desidrogenase (LDH), e os níveis de uréia e creatinina no plasma sanguíneo, além de seus efeitos em outros modelos de DP e de depressão.

ANEXO I

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

Pró-Reitoria de Pesquisa

COMISSÂO DE ÉTICA NO USO DE ANIMAIS - CEUA

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

Data: 22 de Março de 2013

PROTOCOLO N° 001/2013

Pesquisador: CRISTIANO RICARDO JESSE

Campus: ITAQUI

Telefone: (55)- 3433-1669

Título: EFEITO DO FLAVONOIDE HESPERIDINA NO TRATAMENTO DE DOENÇA DE PARKINSON INDUZIDA POR 6-HIDROXIDOPAMINA EM CAMUNDONGOS

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

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Luiz E. Henkes
Professor Adjunto
Coordenador do CEUA/Unipampa