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RICIELLE LOPES AUGUSTO

ÁCIDOS ANACÁRDICOS E EFEITOS NEUROPROTETORES NA DOENÇA DE PARKINSON EXPERIMENTAL: ANÁLISES DE ASPECTOS ANTIOXIDANTES E ANTI-INFLAMATÓRIOS

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Tese apresentada ao Programa de Pós-Graduação em Bioquímica e Fisiologia da Universidade Federal de Pernambuco, como requisito parcial para obtenção do título de Doutora em Bioquímica e Fisiologia.

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Orientadora: Profª Drª Belmira Lara da Silveira Andrade da Costa

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ÁCIDOS ANACÁRDICOS E POSSÍVEIS EFEITOS NEUROPROTETORES NA DOENÇA DE PARKINSON EXPERIMENTAL: ANÁLISES DE ASPECTOS ANTIOXIDANTES E ANTI-INFLAMATÓRIOS

Tese de Doutorado apresentada como um dos requisitos para o cumprimento parcial das exigências para obtenção do título de Doutora em Bioquímica e Fisiologia pela Universidade Federal de Pernambuco.

Aprovada em:_____/_____/_____.

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RESUMO

O aumento da população idosa leva a uma crescente incidência de doenças relativas à fase senil, como as degenerativas. A doença de Parkinson (DP) se destaca por ser a segunda doença neurodegenerativa mais prevalente no mundo. É um distúrbio do movimento de caráter progressivo que ainda não tem cura. Entre os fatores de risco para o desenvolvimento da DP estão as neurotoxinas ambientais, como por exemplo os pesticidas. Muitos estudos que utilizam pesticidas para indução da DP experimental, mostram que sua patogênese é multifatorial e envolve o aumento de substancias oxidantes e inflamatórias. Neste sentido, cresce a busca por agentes terapêuticos que possam atuar no maior número de alvos moleculares possíveis e desta maneira sejam mais eficazes em retardar ou inibir a neurodegeneração. Os ácidos anacárdicos (AAs) compostos fenólicos obtidos principalmente do *Anacardium occidentale*, possuem propriedades benéficas tais como: antimicrobiana, gastroprotetora, anticarcinogênica e anti-inflamatória. Evidências *in vitro* e *in vivo* mostraram que os AAs possuem baixa toxicidade e efeitos antioxidantes, sugerindo seu potencial uso como agente nutraceutico. Neste estudo hipotetizamos que AAs podem exercer neuroproteção no sistema nigroestriatal contra o estresse oxidativo e inflamação induzidos pela rotenona. Camundongos Swiss adultos foram divididos em 4 grupos e tratados por via oral com AAs 50 mg/kg ou veículo, com rotenona apenas (2,5 mg/kg) por via subcutânea, ou com AAs 50mg/kg 1 hora antes da administração de rotenona durante 7 dias. Os sinais de neurodegeneração na substância negra e estriado foram verificados através das análises de neurônios dopaminérgicos e astrócitos por quantificação dos níveis proteicos de tirosina hidroxilase (TH) e proteína ácida fibrilar glial (GFAP). O status oxidante foi verificado pela lipoperoxidação, óxido nítrico e níveis de glutationa reduzida (GSH) e oxidada (GSSG). Também foram avaliados marcadores que traçam um perfil inflamatório como factor nuclear kappa beta ativado (NF-kβ-p65), metaloproteinase 9 (MMP-9), seu inibidor TIMP-1, além de pró-interleucina 1 beta (IL-1β), sua forma ativa de 17 KDa. A administração de rotenona aumentou significativamente os níveis de lipoperoxidação e óxido nítrico e reduziu a relação GSH/GSSG na substância negra e estriado. Também aumentou significativamente os níveis de pró e ativa IL-1b, MMP-9, GFAP, NF-kβ -p65, e reduziu a expressão da proteína TH e TIMP-1 em comparação aos grupos controle e AAs. Os AAs, por si só, foram capazes de reduzir os níveis de pró-IL-1b no estriado e de MMP-9 tanto na substância negra quanto no estriado. O tratamento concomitante de AAs com rotenona reverteu os níveis aumentados de lipoperoxidação e NO e restaurou o balanço redox dado pela razão GSH/GSSG em ambos os

núcleos. Também aumentou os níveis de TH e TIMP-1, e reduziu pró-IL-1b e MMP-9 em ambas as regiões, NF-kB p65 na substância negra e GFAP no estriado. Em conjunto, os dados mostraram que a ação protetora de AAs em modelo experimental de DP induzido por rotenona envolve múltiplos alvos que podem ser atribuídos a propriedades antioxidantes e antiinflamatórias potentes.

Palavras-chaves: Rotenona. Estresse oxidativo. Ácidos Anacárdicos. NF-kβ. Metaloproteinase 9.

ABSTRACT

Parkinson´s Disease (PD) induced by environmental toxins begins to be clarified as due to a multifactorial cascade of harmful factors, which has motivated the search for therapeutic neuroprotector agents able to act on the greatest number of molecular targets. The present study evaluated the efficacy of 50mg/Kg anacardic acids (AAs) isolated from cashew nut (Anacardium occidentale L.) shell liquid on multiple steps of oxidative stress and inflammation induced by 2.5 mg/Kg/day rotenone for 7 days in the substantia nigra and striatum . Adult Swiss mice were divided in 4 groups: Control (C), rotenone-treated, AAs +rotenone treated and AAs-treated. Biochemical levels of lipoperoxidation, GSH/ GSSG ratio, nitric oxide were evaluated as oxidative markers in pooled tissue of substantia nigra and striatum. Western blot or ELISA was adopted for quantifying NFkB-p65, pro-IL-1b, cleaved IL-1b, Metalloproteinase 9 (MMP-9), Tyrosine hydroxilase and Glial fibrilary acidic protein (GFAP) levels. All the experiments were carried out in triplicates in at least three independent moments. Rotenone administration significantly increased lipoperoxidation and nitric oxide levels and reduced GSH/GSSG ratio in both substantia nigra and striatum. It also significantly enhanced the levels of NFkB-p65, pro and cleaved IL-1b, MMP-9, and GFAP and reduced TH and TIMP-1 protein expression compared to control and AAs-treated groups. AAs per se were able to reduce pro-IL-1b in the striatum and MMP-9 levels in both substantia nigra and striatum. The concomitant treatment of AAs with rotenone reversed the increased lipoperoxidation and NO levels and restored the redox balance given by GSH/GSSG ratio in both nuclei. It also increased TH and TIMP-1 and reduced pro-IL-1b and MMP-9 levels in both regions, NF-kB p65 in the substantia nigra and GFAP protein expression in the striatum. Taken together, the data showed that protective action of AAs in a rotenone-induced-PD involve multiple targets which can be attributed to its potent antioxidative and anti-inflammatory properties.

Keywords: Rotenone. Oxidative stress. Anacardic acids. NFK-b. Metalloproteinase 9.

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIAÇÕES E SIGLAS

AAs	Ácidos anacárdicos
ATP	Trifosfato de adenosina
BSA	Albumina de soro bovino, do inglês *bovine serum albumin*
CLs	Corpos de Lewy
DMSO	Sulfóxido de dimetilo, do inglês *dimethyl sulfoxide*
DP	Doença de Parkinson
EDS	Sonolência diurna excessiva, do inglês *excessive daytime sleepiness*
GFAP	Proteína ácida fibrilar glial, do inglês *glial fibrilary acidic protein*
GSH	Glutationa reduzida, do inglês *reduced glutathione*
GSSG	Glutationa oxidada, do inglês *oxidized glutathione*
IkBα	Do inglês, *inhibitory subunit of factor nuclear kappa B alpha*
IL-1β	Interleucina 1 beta
LCCC	Líquido da casca da castanha de caju
MCI	Comprometimento cognitivo leve, do inglês *mild cognitive impairment*
MDA	Malondialdeído
MMPs	Metaloproteinases da matrix
NF-kβ	Fator nuclear kappa beta, do inglês *factor nuclear kappa beta*
NO	Oxido nitrico, do inglês nitric oxide
PMSF	Fluoreto de fenilmetilsulfonil, do inglês *phenylmethylsulfonyl fluoride*
RBD	Distúrbio comportamental do sono, do inglês *rest behavioral disorder*
REM	Movimento rápido dos olhos, do inglês *rapid eyes movement*
SOD	Superóxido dismutase
SN	Substância negra
SNC	Sistema nervoso central
SNpc	Substância negra pars compacta
TBA	Ácido tiobarbitúrico, do inglês *thiobarbituric acid*
TBARS	Substâncias reativas ao ácido tiobarbitúrico
TH	Tirosina hidroxilase
TNF-α	Fator de necrose tumoral alfa, do inglês *tumor necrosis fator alpha*

SUMÁRIO

1 INTRODUÇÃO

Muitas mudanças, notadamente as socioeconômicas, contribuíram para elevação da expectativa de vida mundial. O aumento da população idosa tem, consequentemente, levado a uma crescente incidência de doenças relativas à fase senil, tais como as neurodegenerativas. Dentre estas, a doença de Parkinson (DP) continua sendo umas das principais causas de deficiência neurológica, dominando o segundo lugar no ranque mundial, atrás apenas da doença de Alzheimer, sendo, portanto, considerada um grave problema de saúde pública (DEXTER & JENNER, 2013). Sua prevalência acomete todos os grupos raciais e étnicos, independente de gênero, o que impulsiona muitos esforços destinados a sua prevenção e melhoraria do seu tratamento (ARMANDO et al, 2016). Entretanto, até o momento não há uma terapêutica definitiva que cure ou impeça, de forma efetiva, a progressão da doença, sendo as drogas utilizadas atualmente no tratamento da DP apenas sintomáticas, ou seja, minimizam os sintomas, mas nenhuma delas faz desaparecer a degeneração neuronal. Por isso, estudos que visem à prevenção e cura desta enfermidade são de grande interesse.

Várias evidências têm demonstrado que a DP pode ser deflagrada por fatores genéticos e/ou ambientais e diversos mecanismos têm sido propostos para explicar os eventos que culminam com a morte neuronal (GAO & HONG 2018; SUBRAMANIAM & CHESSELETA 2013). Modelos experimentais desta doença mostram que sua patogênese é multifatorial e está relacionada com o aumento do estresse oxidativo e de substâncias pro-inflamatórias. Dentre estes modelos os que usam pesticidas como agente indutor da doença tem uma grande importância epidemiológica devido ao fato dessas neurotoxinas estarem associadas a etiologia da doença por fatores ambientais, diretamente relacionados com a atividade humana, principalmente na agricultura. A maioria dos pesticidas podem induzir efeitos epigenéticos, inflamatórios e oxidantes, havendo também evidencias de que alguns, como por exemplo, a rotenona, afetam a expressão da enzima tirosina hidroxilase, necessária à síntese de dopamina. Todos estes fatores contribuem com a neurodegeneração observada na DP. Assim, cresce a busca por agentes terapêuticos que possam atuar no maior número de alvos moleculares possíveis e desta maneira sejam mais eficazes em retardar ou inibir a neurodegeneração. Neste sentido, os constituintes naturais derivados de plantas medicinais ganham destaque, uma vez que esses compostos apresentam uma gama de atividades biológicas, dentre elas, atividade antioxidante e/ou anti-inflamatória com potencialidades terapêuticas.

Os ácidos anacárdicos (AAs) extraídos de plantas nativas do nordeste brasileiro têm atraído interesse por serem substâncias bioativas naturais promissoras a serem usadas como agentes nutracêuticos. São caracterizados quimicamente como compostos fenólicos e consistem de uma mistura de lípidos não isoprenóides, que apresentam um núcleo de ácido salicílico. Estes compostos fenólicos podem apresentar cerca de 4 configurações, sendo uma saturada, e as demais contendo de 2 a 6 insaturações na molécula. Estão presentes em todos os componentes do caju, inclusive na casca da castanha, bem como em folhas de Ginkgo biloba (GELLERMAN et al, 1976) e no exo e mesocarpo de Pistachio (ERŞAN et al, 2016). Estudos *in vitro* e *in vivo*, demonstraram atividades antimicrobiana, anticarcinogênica, anti-inflamatória e antitumoral, sendo que estas atividades podem ser diferenciadas entre as 4 configurações (TREVISAN et al. 2006; CASTILLO-JUÁREZ et al. 2007; SUNG et al. 2008; TAN et al. 2012; RADDE et al. 2016; MUZAFFAR et al. 2016), de modo que a mistura de AAs pode exercer uma maior variedade de efeitos quando utilizada em diversos ensaios.

Parte das atividades benéficas associadas aos AAs são devidas a uma gama de ações biológicas que incluem inibição da síntese de lipídios (KIM et al. 2018) e da atividade de diversas enzimas como, lipoxigenase, tirosinase, xantina oxidase, sintase endoperoxidase de prostaglandinas, histona acetiltransferase e algumas metaloproteinases de matriz (MMPs) como a MMP-2 e MMP-9. Adicionalmente, estudos in vitro utilizando células tumorais também evidenciaram ações de AAs sobre a ativação do fator nuclear -κB (NFk-B) bem como ativação da Aurora quinase (GRAZZINI et al. 1991; MASUOKA & KUBO 2004; BALASUBRAMANYAM et al. 2003; SUNG et al. 2008; KISHORE et al. 2008; OMANAKUTTAN et al. 2012; HUNDT et al. 2015; NAMBIAR et al. 2016). Há também evidencias de que AAs induzem apoptose em câncer de próstata via mecanismos de autofagia (TAN et al. 2017).

Além destas características, os AAs podem ser obtidos de fontes naturais renováveis cujos resíduos não são poluentes, com baixo custo de produção, o que pode estimular a economia local. Todos estes fatores abrem a perspectiva para o desenvolvimento de um novo fármaco em condições que estimulam medidas de sustentabilidade, como por exemplo, a da utilização da casca da castanha, geralmente colocada no lixo por não ser comestível.

Assim, considerando a vasta gama de ações descritas para os AAs e a patogênese multifatorial da DP o presente estudo visa testar a hipótese de que os AAs, administrados por via oral podem exercer atividade neuroprotetora na substância negra, estriado e córtex cerebral contra o estresse oxidativo e a neuroinflamação induzidos pelo pesticida rotenona,

através da combinação de múltiplos mecanismos moleculares que em conjunto permitirão combater a neurodegeneração já na fase inicial da DP.

1.1 OBJETIVOS

1.1.1 Objetivo Geral

Investigar mecanismos moleculares envolvidos com a ação neuroprotetora da administração sistêmica de AAs em um modelo experimental de DP induzido pela rotenona em ratos e camundongos.

1.1.2 Objetivos Específicos

Investigar na substância negra, estriado e córtex cerebral de ratos e na substância negra e estriado de camundongos adultos ação do tratamento com AAs *per se* ou na presença de rotenona, sobre a função mitocondrial, níveis de estresse oxidativo e marcadores de inflamação que incluem:

- Para parâmetros mitocondriais:

Quantificar atividade sobre o complexo mitocondrial 1 imediatamente após tratamento com rotenona

- Para parâmetros oxidativos:

Estimar níveis de peroxidação lipídica, glutationa reduzida e oxidada, e a produção de óxido nítrico.

Avaliar se AAS per se alteram a expressão gênica da SOD mitocondrial e citoplasmática bem como atividade da SOD total.

- Para parâmetros inflamatórios:

Quantificar por Western blot:

a) níveis de NF-kβ fosforilada e de seu modulador IKB-alpha;

b) Níveis de MMP-9 e de seu regulador inibitório TIMP-1;

c) Níveis de citocinas pró inflamatórias, como, fator de necrose tumoral alfa (TNF-alfa), interleucina 1 beta e interleucina 6;

- Nível da citocina anti-inflamatória interleucina 10.

- Para parâmetros celulares:

Quantificar por Western blot níveis da enzima tirosina hidroxilase, como marcador dos neurônios dopaminérgicos e da proteína fibrilar ácida glial, específica de astrócitos.

2 REVISÃO DE LITERATURA

2.1 DOENÇA DE PARKINSON

A DP é uma doença degenerativa do sistema nervoso central (SNC), de caráter crônico e progressivo. Leva a uma acentuada diminuição da produção dopamina, um importante neurotransmissor que está relacionado com diversas funções no cérebro. Com a dopamina é possível realizar movimentos voluntários do corpo de forma automática, sem que seja necessário pensar em cada movimento que os músculos realizam. Com a diminuição acentuada desse neurotransmissor, particularmente numa pequena região encefálica chamada substância negra, o controle motor do indivíduo é perdido, ocasionando sinais e sintomas característicos, que veremos adiante.

Esse conjunto de sinais e sintomas neurológicos foi denominada de síndrome parkinsoniana ou parkinsonismo. Diferentes doenças e causas muito diversas podem produzir essa síndrome parkinsoniana. Entretanto, a principal causa dessa síndrome é a própria DP. Os demais casos relacionam-se a enfermidades ou condições clínicas nas quais os sintomas são semelhantes, porém outras características estão presentes e a história clínica e a evolução vão ajudar no diagnóstico diferencial.

2.1.1 Relevância Epidemiológica

O aumento da expectativa de vida leva há um crescimento exponencial da população idosa no mundo, o que converge com maiores ocorrências das doenças neurodegenerativas (TYSNES & STORSTEIN 2017). Com essa tendência há uma preocupação em estabelecer políticas públicas que desenvolvam estratégias para atender às necessidades de saúde desses pacientes. Surge, então, uma atenção especial, por parte da sociedade cientifica e dos profissionais de saúde, com as doenças que têm como fator de risco a idade (BOVOLENTA & FELÍCIO 2017). Dentre as doenças que acometem o SNC a DP apresenta grande importância, pois é a segunda doença neurodegenerativa progressiva mais comum, atrás apenas da doença de Alzheimer (COELHO & FERREIRA 2012; LEE & GILBERT 2016). Estudos epidemiológicos sobre sua incidência são fundamentais para compreender melhor os fatores de risco e de proteção para DP. Ela é predominantemente em indivíduos adultos e acomete mais pessoas do sexo masculino, o que dentre as possíveis razões são discutidos

fatores hormonais e estilo de vida (ASCHERIO & SCHWARZSCHILD 2016; PICILLO et al. 2017). Além disso, nas mulheres a DP apresenta um fenótipo mais brando (MARTINEZ-MARTIN et al. 2012; PICILLO et al. 2017). A prevalência mundial é aceita como variando de 100 a 200 por 100.000 pessoas e a incidência anual é estimada em torno de 17 por 100.000 pessoas (HIRSCH et al. 2016; TYSNES & STORSTEIN 2017). Há um predomínio na população idosa, pois o início da doença geralmente ocorre entre 65 a 70 anos. O diagnóstico da DP é baixo antes dos 40 anos de idade, menos de 5%, mas aumenta velozmente com a idade, atingindo o pico em média aos 75 anos e estabilizando a partir dos 80 anos (HIRSCH et al. 2016; TYSNES & STORSTEIN 2017). No Brasil não há dados oficiais que contenham a relação do número de pacientes portadores da DP. Isto pode ser pelo fato da divulgação da DP não ser compulsória e também pela falta de métodos adequados em busca desses números o que leva a subnotificação dos casos de DP no país (BOVOLENTA & FELÍCIO 2017).

Neste sentido, muitos trabalhos, clínicos e experimentais, têm mostrado a existência de fatores protetores contra o risco de desenvolvimento da DP. No topo da lista de intervenções benéficas para o não desenvolvimento da DP está a atividade física, seguida de uma alimentação saudável (ASCHERIO & SCHWARZSCHILD 2016)). Atividade física moderada ao longo da vida, bem como o consumo moderado de cafeína, chá verde e preto e aumento da ingestão de flavonoides podem ser vistos não apenas por gerar um perfil favorável a prevenção da DP, como também podem ser propostas alternativas ao tratamento desta doença (LEE & GILBERT, 2016; ASCHERIO & SCHWARZSCHILD ET AL 2016).

2.1.2 Sintomas

A DP foi descrita pela primeira vez em 1817 por James Parkinson, em “Um ensaio sobre a paralisia agitante”, após observar a sintomatologia em seis pacientes. Neste trabalho foram relatados sinais como tremor de repouso, festinações e postura flexionada os quais o autor chamou de Paralisia agitante (GOETZ 2011). A estes sintomas foram somados bradicinesia e rigidez, pelo médico neurologista Charcot que acrescentou detalhes extensivos às observações de James Parkinson. Após os achados de Charcot e amplos estudos sobre outras condições de tremores a Paralisia agitante passou a ser chamada de mal de Parkinson ou doença de Parkinson em 1888, em homenagem a James Parkinson(Goetz 2011).

Atualmente, os sintomas da DP podem ser divididos em dois grupos: sintomas motores e não motores. Os sintomas motores podem ser vistos no Quadro. 1 e são inicialmente unilaterais, aparecendo quando a doença já está num estágio mais avançado.

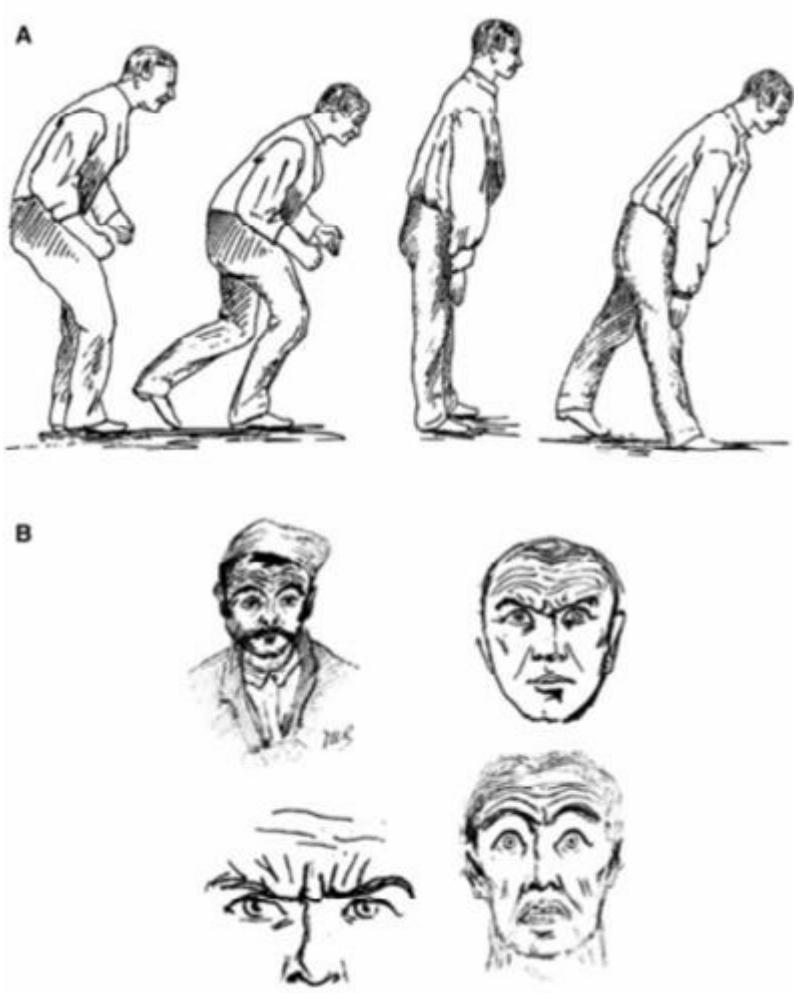
Quadro 1: Principais sintomas motores da doença de Parkinson.

SINTOMAS	CARACTERÍSTICAS
Tremores	Surgem tanto no repouso quanto nas tentativas de movimento voluntário. Geralmente começam nas mãos ou braços e depois estendem-se para as pernas.
Alterações de marcha	Impossibilidade de conseguir elevar as pernas na altura inicialmente planejada, comprimento do passo reduzido e festinação.
Postura flexionada	Atualmente chamada de camptocormia que é a propensão a se inclinar para a frente (Figura 1).
Bradicinesia	Caminhar lento. Também pode haver perda da capacidade de iniciar e se manter em movimento. Pode existir também a acinesia, ou seja, perda total dos movimentos do corpo.
Rigidez	Hipertonia plástica, acometendo a musculatura flexora, determinando alterações típicas de postura. Os músculos se tornam tensos e contraídos, e algumas pessoas podem sentir dor.
Expressões faciais	Dificuldade de controle dos músculos faciais (Figura. 1).

Fonte: Elaboração própria

Dentre esses sintomas motores clássicos 3 são fundamentais para apoiar o diagnóstico clínico: presença marcante de bradicinesia, tremor de repouso e rigidez. Os distúrbios do reflexo postural geralmente ocorrem mais tarde, no decorrer da evolução da DP e não são mais considerados características diagnósticas essenciais (OBESO et al. 2017).

Figura 1. Comprometimento de natureza motora involuntária.

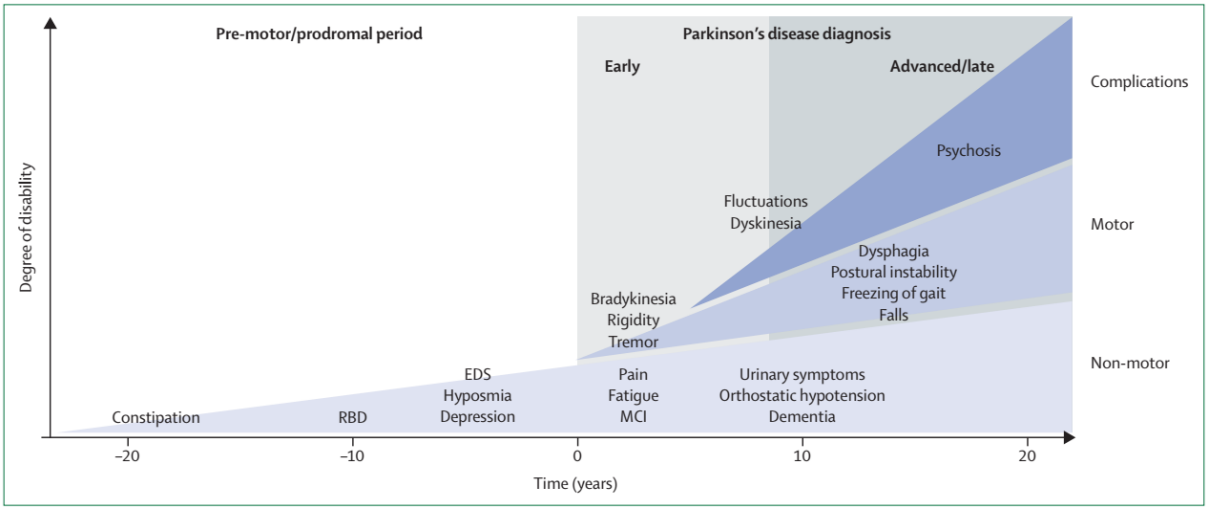


FONTE: GOETZ, 2011. Dificuldades tanto na marcha e postura (A) como no controle de expressões faciais (B).

Apesar de muito bem definidos os sintomas motores, hoje a sintomatologia da DP é reconhecida como heterogênea, com características não motoras clinicamente significativas. Os sinais motores são precedidos por manifestações não motoras muitos anos antes, as quais são consideradas as primeiras incursões da DP. São sintomas sutis e de progressão lenta podendo ser facilmente confundidos com outros distúrbios do SNC, esta é a chamada fase prodrômica (figura. 2) da DP (KALIA & LANG 2016; OBESO et al, 2017). E por esta razão retardam o diagnóstico precoce da doença que seria de fundamental importância para que se desse início ao tratamento nas fases iniciais evitando assim o aparecimento dos sintomas motores e melhorando a qualidade de vida dos pacientes. Essa fase onde só há percepção de sintomas não motores também pode ser chamada de período pré-sintomático substancial que é quando a doença está camuflada possivelmente devido à existência de mecanismos

compensatórios (NAVNTOFT & DREYER 2016). A constipação intestinal pode ser vista como um dos sintomas mais remotos da DP (figura 2) e por esta razão, muitos trabalhos sugerem a hipótese de que a DP pode começar no intestino, antes de chegar ao SNC (JANKOVIC 2017). Esta hipótese vem sendo corroborada pela detecção de corpos de Lewy (CLs) no sistema nervoso entérico em análises post-mortem de pacientes com DP (BOGER et al. 2010; REICHMANN 2011) e por análises em indivíduos assintomáticos. CLs são inclusões presentes no corpo celular dos neurônios que contem diferentes tipos de proteínas celulares, sendo a-sinucleína a mais abundante (BENSKEY ET AL. 2016).

Figura 2: Sintomas clínicos e evolução temporal da progressão da DP.



KALIA & LANG 2016. RBD = REM - distúrbio comportamental do sono com movimento rápido dos olhos. EDS - sonolência diurna excessiva. MCI - comprometimento cognitivo leve. Fonte:

Além da constipação intestinal uma extensa variedade de sintomas como, disfunção olfativa (em aproximadamente 90% dos casos), depressão, ansiedade e distúrbio comportamental do sono podem também ocorrer como manifestações mais precoces da DP (LANG 2011; PONT-SUNYER et al. 2015). Estes sintomas evidenciam o envolvimento de neurônios noradrenérgicos e colinérgicos na DP (ZIEMSSEN & REICHMANN 2007).

Tudo isso leva à hipótese dual-hit, que teoriza que antes do acometimento da substância negra (SN) e de outros núcleos cerebrais pela agregação de alfa-sinucleína e formação de CLs, esses fenômenos ocorrem primordialmente, em uma etapa pré-clínica, no bulbo olfatório e no sistema nervoso entérico (HAWKES et al. 2007; BOGER et al. 2010; REICHMANN 2011; LEMA TOMÉ et al. 2013; SAITO et al. 2016).

Com a progressão da DP o quadro clínico fica composto por complicações motoras relacionadas à dopamina, características motoras não-dopaminérgicas como problemas de fala

e deglutição, congelamento de marcha e quedas e características não motoras cada vez mais incapacitantes, como distúrbios psiquiátricos e demência (OBESO et al, 2017).

Vale ressaltar que as manifestações clínicas podem ser distintas entre os pacientes, além de apresentarem diferentes taxas de progressão e responsividade ao tratamento, sugerindo a presença de subtipos biologicamente distintos da DP (OBESO et al, 2017;MARRAS & LANG 2013).

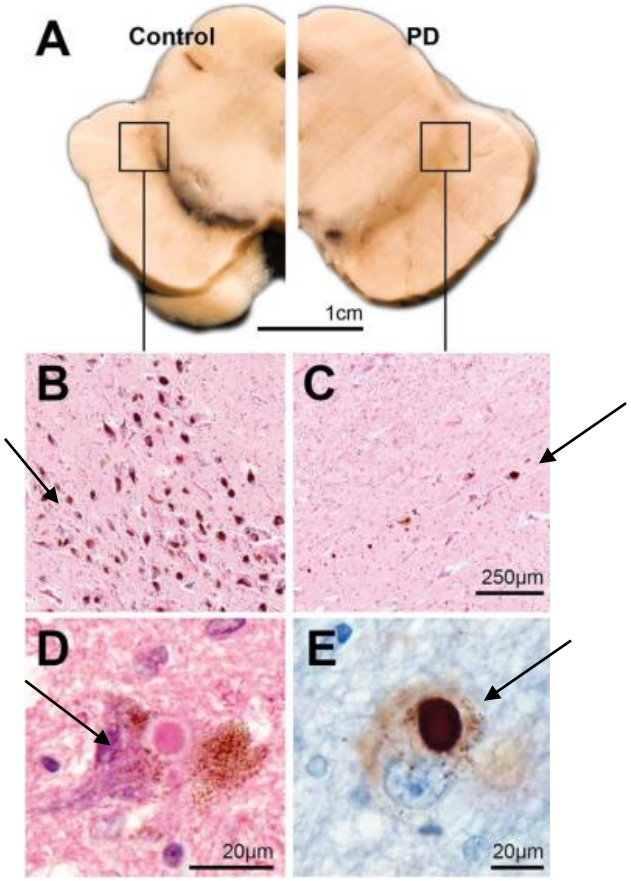
Além de todos esses sintomas, existe ainda uma confirmação da DP post-mortem que pode ser feita pela presença dos CLs no SNC (OLANOW & BRUNDIN 2013). Também é possível encontrar essa forma aberrante de alfa sinucleína dentro do axônio, formando os chamados neuritos de Lewy (DICKSON et al. 2018).

2.1.3 Características Neuropatológicas

Apesar de ao longo dos últimos anos os sintomas não motores da DP receberem uma atenção considerável, são os sintomas motores que a classificam como um distúrbio do movimento e são as características mais importantes para diagnóstico de DP, sendo marca da doença mesmo hoje com exames de imagem ou laboratoriais modernos para ajudar no desafio do diagnóstico (TYSNES et al, 2017). E o que por trás da DP estaria causando esse cortejo de sintomas? A neurodegeneração (Figura. 3). A degeneração inicialmente é seletiva e acontece em neurônios dopaminérgicos da SN, notadamente na região da substância negra pars compacta (SNpc), os quais fazem projeções para o estriado formando a via nigrostriatal (OBESO et al. 2017). A deficiência de dopamina no estriado leva aos distúrbios do movimento porque este neurotransmissor está relacionado principalmente com a função de coordenação dos circuitos intrínsecos dos núcleos da base, envolvendo movimentos, funções executivas, límbicas e cognitivas (NANDIPATI & LITVAN 2016). Apesar desse ser o padrão neuropatológico mais consistente encontrado em todos os pacientes com diagnóstico clínico de DP, ele não é uma condição específica da DP, pois a neurodegeneração também é observada em outros distúrbios classificados como síndromes parkinsonianos, que podem ter algumas ou todas as características clínicas da DP (DICKSON 2018). No entanto, o tipo degeneração dopaminérgica na substância negra (SN) é distintiva para DP, com perda mais severa das células localizadas na região ventrolateral, por serem as mais vulneráveis ao estresse oxidativo. Neurônios dopaminérgicos presentes na SN rostro-dorsomedial são

lesionados nas fases mais tardias da doença e os da área tegmentar ventral são geralmente poupados. (HALL et al. 2014).

Figura 3 - Principais achados neuropatológicos em pacientes com DP.



Fonte: OBESO et al., 2017. (A) Hemisecção transversa do mesencéfalo do paciente controle à esquerda e do paciente com DP à direita mostrando a redução acentuada dos pigmentos preto na região SN. (B-C) Hematoxilina e eosina da secção da região ventrolateral identificando neurônios pigmentados da SN do paciente controle (B) e do paciente com PD (C). (D-E) Intracitoplasmático CL no neurônio pigmentado remanescente da SN do paciente com DP mostrando o núcleo eosinofílico e o halo mais pálido na coloração com hematoxilina e eosina (D) e a agregação escura de a-sinucleína usando imunoperoxidase (E).

Prejuízos nos processos celulares envolvidos na regulação da homeostase protéica parecem estar também envolvidos na patogênese da DP. Estes incluem anormalidades na agregação das proteínas intracelulares, no tráfico através da membrana e na eliminação de proteínas pelos sistemas ubiquitina-proteassoma e lisossoma-autofagia (KALIA E LANG 2016). As anormalidades que modificam a solubilidade e afinidade de ligação da proteínas causam sua precipitação intracelular e levam a formação dos CLs (Figura. 3) que como já referido anteriormente tem a alfa sinucleína como componente principal, mas mais de 90 outras moléculas são reconhecidas nessas estruturas celulares anormais (OBESO et al. 2017;

(BENSKEY et al. 2016). Sabe-se que esses agregados de alfa sinucleína com progressão da doença podem se espalhar por todo SNC. Propagação dessas formas anormais de alfa sinucleína é umas das hipóteses mais atuais para explicar o progressivo envolvimento de diversos sistemas neuronais vistos na DP (BRAAK et al. 2004). Hawkes e colaboradores chamaram a atenção para a possibilidade dos CLs serem formados inicialmente no sistema nervoso periférico e posteriormente obter acesso ao SNC por via nasal ou gástrica, e assim, se espalhar de uma região cerebral suscetível para a próxima através das sinapses (HAWKES et al, 2007; BRAAK et al. 2004). Outros estudos corroboram essa hipótese ao mostrarem que em quatro casos distintos de pacientes com DP que receberam transplante de neurônios mesencefálicos embrionários em seu putâmen, apresentaram inclusões parecidas com CLs mostrando que essas inclusões podem se desenvolver dentro dos neurônios enxertados (KORDOWER et al. 2008a; KORDOWER et al. 2008b; LI et al. 2008). Trabalhos com animais também evidenciam essa hipótese ao mostrarem que ao colocarem uma injeção de fibra sintética de alfa sinucleína em várias regiões do cérebro de camundongos transgênicos superexpressando alfa sinucleína ou de camundongos do tipo selvagem houve formação de inclusões parecidas com CLs em locais próximos e distantes dos locais de injeção (LUK et al. 2012; MASUDA-SUZUKAKE et al. 2013; SACINO et al. 2014). Esta inclusões também podem aparecer em células gliais e parecem estar aumentadas na DP provocada por mutações no gene da alfa sinucleína (FUJISHIRO et al. 2013).

É importante ressaltar que essas inclusões citoplasmáticas também podem ser observadas em outras patologias neurológicas, como é o caso do mal de Alzheimer, onde cerca de 60% dos pacientes apresentam CLs em seus cérebros (HALLIDAY et al. 2011).

2.1.4 Patogênese

Cada vez mais estudos mostram que tanto DP têm diversas vias envolvidas na patogênese, tais como, a disfunção mitocondrial, o estresse oxidativo, comprometimento tanto do sistema ubiquitina-proteosoma como do autofagolisossomos e neuroinflamação induzida por ativação da micróglia, (CHUNG et al. 2001; WANG et al. 2005; ABOU-SLEIMAN et al. 2006; JENNER & OLANOW 2006; SUN et al. 2007; TANSEY et al. 2007; YANG & TIFFANY-CASTIGLIONI 2007; CHENG et al. 2009) que são componentes chaves no desenvolvimento e progressão da neurodegeneração (OBESO et al. 2017; TIWARI & PAL 2017; PUSPITA et al. 2017; ROCHA et al. 2018).

Há décadas numerosos estudos pós-morte, de imagiologia cerebral, epidemiológicos e em animais mostram o envolvimento da imunidade inata e adaptativa na DP (GELDERS et al. 2018). A relação do sistema imune com a DP abre a possibilidade de que alterações na autoimunidade seja um fator de risco para o desenvolvimento do Parkinson, com isso a neuroinflamação se tornou forco de muitos estudos envolvendo a DP (ARMANDO et al. 2016).

McGeer et al. 2008 relataram em estudo pós-morte a presença de microglia reativa ativada na SNpc de pacientes com DP, além de infiltrado de células T CD8 + e CD4 +, acumulação astrócitos, e alterações na morfologia e função das células gliais. A mircoglia ativada pode ser prejudicial, pois ela aumenta os níveis de citocinas, quimiocinas e outros mediadores inflamatórios no local da lesão/dano podendo levar a uma exacerbação da resposta imune. A micróglia inativa pode se tornar reativa na presença de mediadores inflamatórios, levando a uma amplificação da resposta inflamatória (GELDERS et al. 2018). Quais estímulos são responsáveis por essa ativação microglial e consequente resposta inflamatória ainda estão sendo elucidadas, mas proteínas anormalmente desdobradas como α-sinucleína extracelular têm sido propostas como um dos principais candidatos (GELDERS et al 2018). Os neurônios dopaminérgicos expressam uma ampla gama de receptores de citocinas e quimiocinas, com isso acredita-se que eles são responsivos a esses mediadores inflamatórios derivados da microglia.

Muitos trabalhos evidenciam diversos sinais inflamatórios na via nigroestriatal de pacientes com DP. Níveis elevados das interleucinas (ILs) proinflamatórias IL1β, IL2, IL6 e fator de necrose tumoral α (TNFα), bem como o fator de crescimento transformador anti-inflamatório β1 (TGFβ1) foram detectados no estriado e concentrações de TNFα, IL1β, interferon-γ (IFNγ), óxido nítrico sintase (NOS) e espécies reativas de oxigênio (EROS) foram verificadas aumentadas na SN de amostras pós-morte. Estas citocinas inflamatórias também foram detectadas no líquido cefalorraquidiano de pacientes com DP (BRODACKI et al. 2008; KATSAROU et al 2007; PERREIRA et al 2016; NICOLETTI et al, 2011).).

Além dos fatores como, ILs, TNF-a, óxido nítrico (NO), prostaglandina E2 (PGE2) a micróglia também é capaz de produzir metaloproteinases da matriz (MMPs), que também são consideradas fatores pró-inflamatórios que são tóxicos para os neurônios (ROSENBERG et al. 2009). A MMPs atuam na degradação da matriz extracelular e estão envolvidas em diversos processos neuropatológicos, bem como neuroinflamação, lesão da barreira cerebral (BHE) e morte celular neuronal, sendo consideradas importantes na patogênese das doenças neurodegenerativas, incluindo a DP (CHEN et al. 2016). Indutores da expressão e atividade

de MMPs, como as citocinas, o NO e as EROS estão implicadas na fisiopatologia da DP. Dentre a família das MMPs, as gelatinases MMP-9 e MMP-2 mostraram estar relacionadas com a DP (CHEN et al 2016).

Além da microglia, astrócitos também participam da neuropatologia da DP (VENKATESHAPPA et al. 2012). Os astrócitos são distribuídos heterogeneamente no mesencéfalo, com baixa densidade astrocitária na SN (GELDERS et al 2018). Mas na DP uma elevação no número de astrócitos na SN, bem como alterações patológicas nos astrócitos seguindo um padrão de distribuição específico, foi relatada post-mortem (BRAAK et al 2007). Além disso, a regulação positiva da proteína de ligação ao cálcio S100b, que é expressa principalmente pelos astrócitos e atua como uma citocina, foi demonstrada no SN de pacientes com DP pós-morte (BRAAK et al 2007). A S100b pode aumentar a expressão da sintase induzida do óxido nítrico (iNOS) que, por sua vez, pode resultar na ativação da enzima pró-inflamatória ciclooxigenase-2 (COX-2) na microglia, bem como aumento da produção de óxido nítrico (NO) e radicais superóxido (GOMES et al 2008; BIANCHI et al 2007). Esses eventos podem causar, direta ou indiretamente, morte celular neuronal.

2.1.5 Etiologia

Embora as causas da DP permaneçam ainda elusivas, exposição a substâncias químicas ambientais além de mutações genéticas são indicadas como fatores envolvidos na etiologia (OBESO et al., 2017). Por isso, atualmente, o desenvolvimento da DP tem sido visto como resultado de uma combinação de fatores genéticos e/ou ambientais (KALIA & LANG, 2016). A maioria dos casos de DP é esporádica com apenas 10% identificados por causas genéticas, encontrados na forma familiar da doença (ARMANDO et al. 2016).

Nas últimas duas décadas a hipótese ambiental da DP tem despertado bastante interesse da comunidade científica (NISTICÒ et al. 2011). Tanto estudos epidemiológicos como de análise histopatológica post-mortem em cérebros humanos têm mostrado que a exposição a pesticidas está associada a um maior risco para o desenvolvimento desta doença (KANTHASAMY et al. 2005; DINIS-OLIVEIRA et al. 2006). Estudos toxicológicos experimentais reforçam esta hipótese, demonstrando que a exposição de animais a pesticidas causa neurodegeneração e sintomatologia comportamental similar a da DP. Neste sentido, modelos experimentais utilizando administração sistêmica ou local do herbicida paraquat e do pesticida rotenona vêm sendo utilizados no sentido de melhor entender potenciais

mecanismos envolvidos com os danos neurais que levam ao aparecimento e progressão da DP. Alterações ao nível de expressão gênica também são observadas com o uso de toxinas ambientais. Evidências experimentais utilizando linhagem de células neuronais indicam que modificações epigenéticas na acetilação e desacetilação de histonas podem estar também envolvidas na neurotoxicidade induzida por pesticidas como o paraquat e dieldrin (KANTHASAMY et al., 2005). Na linhagem de células dopaminérgicas mesencefálicas N27, 100 μM dieldrin provocou um aumento dependente de tempo na acetilação das histonas H3 e H4 (SONG et al., 2010) enquanto que o paraquat aumentou a acetilação apenas da histona H3 (SONG et al., 2011).

Além de induzirem os potenciais mecanismos patogênicos acima citados, as toxinas ambientais também são capazes de aumentar a atividade de algumas MMPs. Esse aumento pode estar também envolvido na neurodegeneração de células dopaminérgicas induzida pelo pesticida rotenona (SHING et al., 2012). A MMPs atuam na degradação da matriz extracelular e são consideradas importantes na patogênese das doenças neurodegenerativas, incluindo a DP (CHEN et al. 2016).

Assim, a patogênese da DP induzida por toxinas ambientais começa a ser esclarecida como uma cascata multifatorial de fatores deletérios, o que tem motivado a busca por alternativas terapêuticas capazes de alcançar diferentes alvos moleculares.

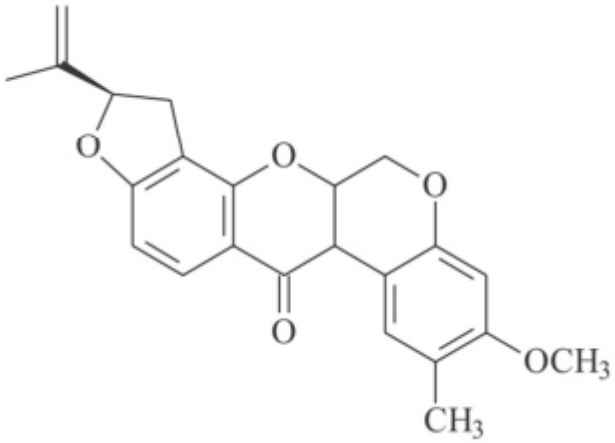
2.2 MODELO EXPERIMENTAL DA DOENÇA DE PARKINSON INDUZIDO POR ROTENONA

A exposição humana aos pesticidas ocorre de maneira bastante usual através da ingestão em resíduos de alimentos, água potável e, de forma mais significativa, em uso ocupacional, nos trabalhadores agrícolas e trabalhadores da indústria de pesticidas (NANDIPATI & LITVAN 2016).

A rotenona (Figura 4) é um pesticida natural de origem vegetal, e sua ação piscicida e inseticida é conhecida há séculos, sendo utilizada até os dias atuais em práticas ecológicas no controle populacional de espécies daninhas de peixes e insetos (RAYNER & CREESE 2006). Atua inibindo o complexo I da cadeia transportadora de elétrons, por inibição da enzima nicotinamida adenina dinucleotídeo desidrogenase que resulta numa considerável queda na produção de trifosfato de adenosina e aumento de espécies reativas que leva ao dano oxidativo, inflamação, reação microglial e apoptose (BERNDT et al. 2013; LEE et al. 2014). Estudos epidemiológicos em humanos mostram que a DP foi mais frequente em pessoas que

fizeram uso de rotenona em comparação com não usuários, independentemente do uso de luvas de proteção (NANDIPATI & LITVAN 2016).

Figura 4. Estrutura química da rotenona



Fonte: SANTOS et al. 2007. Sua fórmula química é $C_{23}H_{22}O_6$.

Betarbet e colaboradores (2000) foram os primeiros a demonstrar que a rotenona é capaz de reproduzir as características da DP após sua administração sistêmica e foi este trabalho que chamou a atenção dos estudiosos da doença para este agente. O modelo da DP induzida pela rotenona foi sugerido dois anos mais tarde por Alam & Schmidt (2002). Apesar de seu uso relativamente recente em pesquisas relacionadas a DP, a rotenona vem se mostrando um excelente modelo toxicológico tanto em roedores como em células (JOHNSON & BOBROVSKAYA 2015; OJHA et al. 2015; ZHANG et al. 2016). E uma clara vantagem da rotenona, em comparação a vários dos agentes utilizados para induzir efeitos parkinsonianos em animais, diz respeito à sua permeabilidade à barreira hematoencefálica devido a sua natureza lipofílica (RAVENSTIJN et al. 2008).

Em camundongos e ratos déficits motores característicos da doença como bradicinesia, instabilidade postural e rigidez, induzidos pela rotenona foram observados em testes comportamentais, tais como, teste do cilindro, teste de campo aberto, teste de iniciação de movimento, entre outros (KANDIL et al. 2016). Ao mesmo tempo, ela é capaz de promover nesses animais um aumento na apoptose dos neurônios da SN, além de decréscimo da viabilidade celular (TONG et al. 2016). Dependendo da dose utilizada e do período de tratamento com a rotenona, estudos com ratos post-mortem relataram uma progressiva perda de neurônios dopaminérgicos na SN e estriado. Também há vários estudos com este pesticida mostrando a diminuição na expressão da enzima tirosina hidroxilase o que leva a uma menor

concentração de dopamina estriatal (RAVENSTIJN et al. 2008; DU et al. 2014; TONG et al. 2016). Alguns estudos em animais utilizando rotenona também mostraram que o declínio motor nem sempre está associado à perda de células dopaminérgicas, o que sugere que a rotenona pode causar disfunção mitocondrial difusa em células não-dopaminérgicas dentro e fora do SNC (FLEMING et al. 2004; RICHTER et al. 2007).

A rotenona também é capaz de promover agregados de alfa-sinucleína e poliubiquitina em neurônios dopaminérgicos da SN, similar aos CLs encontrados na DP (SINDHU et al. 2005; MILUSHEVA et al. 2005; CANNON et al. 2009) e no sistema nervoso entérico (DROLET et al. 2009; PAN-MONTOJO et al. 2010). Este pesticida pode induzir a fosforilação e agregação de outras proteínas, como a tau e a beta amiloide (CHAVES et al. 2010; HONGO et al. 2012).

A rotenona promove sintomas extra-nigrais o que sustenta a hipótese dual-hit da DP, como lesão gastrointestinal e no bulbo olfatório, verificados pela agregação de alfa-sinucleína entérica e hiposmia (verificada pelas mudanças oxidativas da proteína DJ-1 no bulbo olfatório), respectivamente, embora tenha falhado em favorecer esses agregados no bulbo olfatório. Associado a tudo isso, o distúrbios do sono e a alteração do ritmo circadiano, a diminuição da testosterona sérica dos ratos machos, comportamentos de ansiedade e depressão crescentes atestam a validade da rotenona como modelo da DP (JOHNSON & BOBROVSKAYA, 2015). Desta forma, o foco recente das pesquisas utilizando rotenona, está na busca por novos tratamentos, visto que ela representa um modelo animal clinicamente relevante para o triagem de novos agentes (JOHNSON & BOBROVSKAYA 2014; OJHA et al, 2016).

2.3. BUSCA POR TRATAMENTOS NUTRACÊUTICOS PARA A DOENÇA DE PARKINSON

Com o aumento da expectativa de vida humana, haverá cada vez mais indivíduos procurando por tratamentos para doenças neurodegenerativas relacionadas à idade, tal como a DP. Como visto anteriormente, a base do tratamento da DP é sintomática com drogas que aumentam as concentrações de dopamina ou estimulam diretamente os receptores de dopamina (KALIA & LANG, 2016). Medicamentos que reduzam ou interrompam a neurodegeneração são a maior necessidade terapêutica ainda não preenchida na DP. No entanto, a compreensão da patogênese da doença está se expandindo e, assim, ajudando a identificar alvos potenciais para inibir a progressão da doença (KALIA & LANG, 2016).

Tendo em vista que a elevada morbidade, os custos socioeconômicos e a falta de tratamentos específicos são fatores chaves que definem a relevância das patologias cerebrais degenerativas, um número crescente de estudos vem sendo realizados com substâncias naturais como novos agentes preventivos e neuroprotetores. Neste sentido, substâncias bioativas naturais vêm sendo propostas como alternativas nutracêuticas em doenças do sistema nervoso.

Dentre estas substâncias, destacam-se algumas com atividade antioxidante, como por exemplo, polifenóis incluindo os flavonoides, por causa da variedade de efeitos benéficos relatados para os mesmos em vários estudos epidemiológicos (KUMAR & PANDEY 2013; de FARIAS et al. 2016; JUNG & KIM 2018). No sistema nervoso, ressaltam-se ações neuroprotetoras, especialmente pela capacidade de alguns flavonoides em modular a sinalização intracelular envolvida na sobrevivência de neurônios e glia (DAJAS et al. 2003). Em modelos experimentais de DP, polifenóis como a quercetina e outras moléculas como a troxiorutin, hesperitin, curcumin, quando administrados localmente foram capazes de proteger neurônios dopaminérgicos contra o insulto oxidativo induzido pela 6-hidroxi-dopamina (DAJAS et al. 2003; KIASALARI et al. 2016; BALUCHNEJADMOJARAD et al. 2017 WANG et al., 2017; SHARMA & NEHRU 2018). Flavonoides derivados do chá verde, como a epigalocatequina vêm sendo apontados como uma alternativa promissora para a prevenção e tratamento individualizado de doenças degenerativas embora a dose ótima para estes efeitos ainda seja matéria de debate (MÄHLER et al. 2013). Em uma revisão recente, vem sendo relatados efeitos benéficos de plantas iridoides como alternativas bioativas capazes de atuar em vários mecanismos de neuroproteção tanto em modelos experimentais de DP como de Mal de Alzheimer (DINDA et al. 2019). A suplementação dietética com ácidos graxos essenciais da família Omega 3, derivados de plantas ou do óleo de peixe parece ser também uma estratégia eficaz em amenizar os danos celulares característicos da DP por reduzir eventos inflamatórios e oxidativos (MORI et al. 2018). Ácido ferrulico abundante em folhas e sementes de várias plantas, especialmente em cereais como arroz integral, aveia e trigo integral, bem como o ácido glicirrínico apresentaram também efeitos neuroprotetores contra administração de rotenona por 4 semanas (OJHA et al. 2015; OJHA et al. 2016). Compostos fenólicos como, por exemplo, o ácido helágico foram testados recentemente em modelo de DP induzida por 6-hidroxi-dopamina, apresentando efeitos neuroprotetores via redução da apoptose e do estresse oxidativo, supressão das enzimas monoamino oxidases A e B sendo estes efeitos parcialmente mediados por várias vias de sinalização celular (BALUCHNEJADMOJARAD et al. 2017). Quimicamente, o ácido anacárdico é uma mistura

de vários compostos orgânicos intimamente relacionados. Cada um consiste de um ácido salicílico substituído por uma cadeia alquílica que possui 15 ou 17 átomos de carbono; O ácido anacárdico é uma mistura de moléculas saturadas e insaturadas. A mistura exata depende da espécie da planta e o componente principal é C5: 3 all-Z.

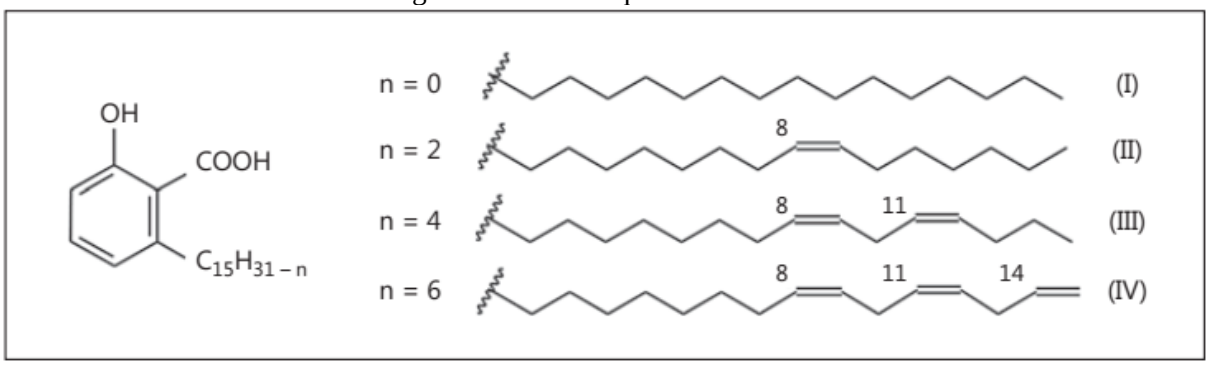
2.3.1 Ácidos Anacárdicos como Alternativas Nutracêuticas

Os AAs são compostos fenólicos (ácido 2-hidroxi-6-pentadecil benzoico) presentes principalmente em plantas da família Anacardiaceae como o caju. O líquido da casca da castanha de caju (LCCC) é um subproduto obtido durante o processamento das castanhas a partir das quais são extraídos AAs, cardanol, cardol entre outros, sendo os AAs os principais compostos ativos do LCCC (HEMSHEKHAR et al. 2012; HAMAD & MUBOFU 2015). Os AAs consistem de uma mistura de lípidos não isoprenóides que apresentam um núcleo de ácido salicílico (Figura. 5). Por ser uma mistura lipossolúvel tem facilidade em atravessar a membrana plasmática. Sua biodisponibilidade e capacidade de atingir os tecidos rapidamente também foi demonstrada, mesmo quando administrado 30 min antes de outros tratamentos para análise do sistema nervoso (Gomes-Junior et AL., 20018). Trata-se de um agente fitoquímico de grande interesse devido a sua ampla bioatividade, tais como, inseticida, moluscicida (KUBO ET AL. 2003; FERREIRA DE CARVALHO et al. 2019; DE CARVALHO et al. 2019), parasiticida (CUI et al., 2008; MATUTINO BASTOS et al. 2019), bactericida (SAJEEVAN et al. 2018; ASHRAF & RATHINASAMY 2018), antimutagênica (MASUOKA & KUBO 2004), anti-inflamatória (TAN et al. 2012; CARVALHO et al. 2013; DE SOUZA et al. 2018), anti-viral (HUNDT et al. 2015) e gastroprotetora (MORAIS et al. 2010). Também foi demonstrado que os AAs foram capazes de induzir apoptose independente de caspase em linhagem de células tumorais de pulmão, fígado e pâncreas comprovando sua atividade anticancerígena (SUNG et al. 2008; PARK et al. 2018). Além disso, estudos *in vitro* demonstraram que os AAs também apresentam um potente efeito antioxidante, o que vem sendo atribuído à sua cadeia lateral e anel fenólico (TREVISAN et al., 2006). Discute-se que esta capacidade antioxidante foi provavelmente, responsável por exercer um papel protetor na pele (KUBO et al., 2006) bem como proteger contra o espessamento epidérmico induzido pelos raios ultravioletas (Kim et al. 2013 & 2017), proteger o estômago contra danos induzido pelo etanol (MORAIS et al., 2010).

Um estudo recente demonstrou atividade anticonvulsivante de AAs administrado sistemicamente (i.p.) em camundongos Swiss nas doses de 25, 50 e 100 mg/Kg 30 minutos

antes de drogas convulsivantes em 3 diferentes modelos experimentais de epilepsia (GOMES JÚNIOR et al. 2018). Além disto, foi também observada atividade ansiolítica após administração aguda de AAs nas doses de 25 e 50 mg/Kg, associada a um aumento na atividade da enzima catalase e nos níveis de glutationa reduzida tanto no córtex pré-frontal como no hipocampo (GOMES-JUNIOR et al., 2018b). Os dados destes 2 recentes trabalhos sugerem atividade de AAs em receptores GABAérgicos do tipo GABA-A.

Figura 5. Estrutura química dos AAs.



Fonte: SILVEIRA et al, 2014. AA: saturado (I), radical monoeno (II), radical dieno (III) e radical trieno (IV). n indica o número de átomos de H removido.

Vale ressaltar que os AAs apresentam um perfil de segurança, não sendo tóxicos na administração oral em dose única de 2000 mg / kg, bem como, sendo seguros em diferentes doses administradas sucessivamente durante 30 dias (CARVALHO et al. 2011). Análise de toxicidade realizado com o teste Cometa também não demonstrou dano ao DNA do hipocampo e córtex pré-frontal em doses agudas de 10, 25 e 50 mg/Kg de AAs administrados por via intraperitonial em camundongos (GOMES-JUNIOR et al., 2018). Levando em conta a busca por alvos terapêuticos para a DP, hipotetizamos que os AAs, administrados por via oral têm potencialidade como agente nutracêutico neuroprotetor. Tal hipótese baseia-se não apenas pelas capacidades antioxidante e anti-inflamatória destes compostos, previamente demonstradas, mas também por atuarem em múltiplos alvos moleculares e por atender a critérios de adequada biodisponibilidade e inexistência de toxicidade sistêmica.

3 RESULTADOS

3.1 ARTIGO CIENTÍFICO 2 (em forma de manuscrito)

Anacardic acids from cashew nuts are potential nutraceutical agents with multiple anti-inflammatory effects in rodent experimental model of Parkinson`s disease

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Abstract

Parkinson´s Disease (PD) induced by environmental toxins begins to be clarified as due to a multifactorial cascade of harmful factors, which has motivated the search for therapeutic agents able to act on the greatest number of molecular targets. The present study evaluated the efficacy of 50mg/Kg anacardic acids (AAs) isolated from cashew (Anacardium occidentale L.) nut shell liquid on multiple steps of oxidative stress and inflammation induced by 2.5 mg/Kg/day rotenone for 7 days in the substantia nigra (SN) and striatum . Adult Swiss mice were divided in 4 groups : Control (C), rotenone-treated, AAs +rotenone treated and AAs-treated. Biochemical levels of lipoperoxidation, GSH/ GSSG ratio, nitric oxide were evaluated as oxidative markers in pooled tissue of SN and striatum. Western blot or ELISA was adopted for quantifying NFkB-p65, pro-IL-1β, cleaved IL-1 β, metalloproteinase 9, Tissue Inhibitory Factor-1 (TIMP-1), tyrosine hydroxilase and glial fibrilary acidic protein (GFAP) levels. All the experiments were carried out in triplicates in at least three independent moments. Rotenone administration significantly increased lipoperoxidation and nitric oxide levels and reduced GSH/GSSG ratio in both SN and striatum. It also significantly enhanced the levels of NFkB-p65, pro and cleaved IL-1β, MMP-9 and GFAP and TH protein expression compared to control and AAs-treated groups. AAs per se were able to reduce pro-IL-1β in the striatum and MMP-9 levels in both SN and striatum while augmented TIMP1 amount in both nuclei. The concomitant treatment of AAs with rotenone reversed the increased lipoperoxidation and NO levels and restored the redox balance given by GSH/GSSG ratio. It also increased TH and attenuated pro-IL-1β and MMP-9 levels in both regions, NF-kB p65 in the SN and GFAP protein expression in the striatum. Taken together, the data suggest that AAs have promising neuroprotective effect against degenerative changes in PD acting in multiple targets involved in the oxidative and inflammatory condition induced by rotenone.

Key words: Anacardic acids, neuroprotection, Parkinson´s disease, rotenone, inflammation, oxidative stress.

Introduction

Parkinson`s disease (PD) is one prevalent neurodegenerative disorder, characterized by an expressive dopaminergic cell loss in the substantia nigra, and impairment of many other neurotransmitter systems of the brainstem and basal ganglia, as well in the peripheral autonomic nervous system (Obeso et al. 2017). The multiple neuronal loss induces deleterious consequences to the motor, cognitive, executive and emotional functions which compromise the quality of life, inducing also elevated socioeconomic costs (Kalia and Lang 2016). Although the causes remain elusive, epidemiological studies and post-mortem histopathological analysis in human brains have shown that exposure to pesticides is associated with a higher risk for the development of this disease (Kanthasamy et al. 2005; Dinis-Oliveira et al. 2006; Nisticò et al. 2011). Multiple pathways and mechanisms involving increased production of reactive oxygen species due to mitochondrial dysfunction, changes in calcium homeostasis, imbalanced α-synuclein proteostasis, epigenetic changes as well as glial cells activation promotes oxidative stress and concomitant inflammation that contributes to progressive neurogeneration in PD (Tansey et al. 2007; Sanchez-Guajardo et al. 2013; Sarkar et al. 2016) . Therefore, the pathogenesis of PD provoked by environmental toxins begins to be clarified as a multifactorial cascade of these harmful factors, which has motivated the search for therapeutic agents able to act on the greatest number of molecular targets.

In this sense, natural constituents derived from medicinal plants such as polyphenols and phenolic compounds are highlighted, since these compounds present a wide range of biological activities, among them, antioxidant and / or anti-inflammatory actions. Anacardic acids (AAs) extracted from cashew nuts have attracted interest as promising bioactive substances to be used as nutraceutical agents. Chemically, AAs consist of a mixture of non-isoprenoid lipids, characterized as phenolic compounds which contain a salicylic acid core bearing a C15 side chain attached at the 6-position. The alkyl long chain occurs as saturated (I) or unsaturated – monoene (II), diene (III) and triene (IV) – in which the double bonds are respectively located at the 8-, the 8-, 11- and the 8-, 11-, 14-positions, all of which have the Z-configuration (dos Santos and de Magalhães, 1999; Trevisan et al. 2006).

In vitro and *in vivo* studies have demonstrated antimicrobial, anticarcinogenic, anti-inflammatory and antitumoral activities of AAs (Castillo-Juárez et al. 2007; Sung et al. 2008; Tan et al. 2012; Carvalho et al. 2013; Radde et al. 2016; Muzaffar et al. 2016; de Souza et al. 2018; Park et al. 2018). In addition, *in vitro* studies have also shown that AAs exert a potent antioxidant activity (Trevisan et al., 2006). It has been discussed that this antioxidant capacity

is probably responsible for its protective effect in the skin against the ultraviolet-induced epidermal thickening (Kubo et al. 2006; Kim et al. 2013, 2017) and against stomach damage induced by ethanol (Morais et al. 2010). Some of the beneficial effects associated with AAs are due to a range of biological actions including inhibition of the activity of various enzymes such as lipoxygenase, tyrosinase, xanthine oxidase, prostaglandin endoperoxidase synthase, histone. acetyltransferase and some matrix metalloproteinases (MMPs) such as MMP-2 and MMP-9, as well as inhibition of lipid synthesis (KIM et al. 2018). Additionally, in vitro studies using tumor cells also showed AAs actions on nuclear factor -κB (NFk-B) and aurora kinase activation (GRAZZINI et al. 1991; MASUOKA & KUBO 2004; BALASUBRAMANYAM et al. 2003; SUNG et al. 2008; KISHORE et al. 2008; Omanakuttan et al. 2012; HUNDT et al. 2015; NAMBIAR et al. 2016). There is also evidence that AAs induce apoptosis in prostate cancer via autophagy mechanisms (TAN et al. 2017).

In the central nervous system, a recent study demonstrated anti- epileptic activity of AAs systemically administered in Swiss mice at doses of 25, 50 and 100 mg/kg, 30 minutes prior to convulsant drugs in different experimental models of epilepsy (Gomes Júnior et al. 2018a). Anxiolytic activity was also observed after acute administration of AAs at doses of 25 and 50 mg/kg, associated with increasing levels of reduced glutathione (GSH) and catalase enzymatic activity in both prefrontal cortex and hippocampus of mice (Gomes- Junior et al., 2018b). These two later studies indicated action of AAs on GABA-A receptors.

A previous evidence from our group showed that oral administration of AAs for 5 days, prevented rotenone-induced behavioral changes in rats and reduced lipoperoxidation in the cerebral cortex, substantia nigra and striatum, in part due to a direct action on the mitochondrial complex 1. Moreover, AAs per se (at 100mg/kg/day) increased the gene expression of cytoplasmic and mitochondrial superoxide dismutase (SOD) enzimes as well the total-SOD activity in the same brain regions (Medeiros-Linard et al. 2018).

In the present study, considering the multifactorial pathogenesis of PD and the wide range of actions previously described for AAs, we hypothesized that these phenolic compounds can promote protective activity against oxidative stress and inflammation in the substantia nigra and striatum through the combination of multiple molecular mechanisms. Thus, these nutraceutical agents, may represent a potential alternative for early intervention approach able to prevent or reduce the progressive neurodegeneration in PD.

MATERIAL AND METHODS

Animals

In the present study, the experiments were conducted with adult male Swiss mice weighing 40 to 50 g (70 days of age), from the Animal Production Unit, Federal University of Paraíba, Brazil (UFPB). The animals were maintained under standard 30 environmental conditions (12h light/dark cycle; light on 6 am) and temperature (22 ± 4 °C) and fed with a balanced commercial chow (Presence®, Purina, Brazil) and water ad libitum. Experimental protocols were approved by the Ethics Committee on Animal Use of the Federal University of Pernambuco, Brazil, under license no. 23076.005404/2015- 04, according to recommendations of the Brazilian College for Animal Experimentation guidelines, which follow the "Principles of Laboratory Animal Care" (NIH, Bethesda, USA).

Drugs and Solvents

Rotenone was purchased from Sigma (St. Louis, MO, USA) and dissolved (2.5 mg/mL) in dimethyl sulfoxide (DMSO 10% w/v) and in sunflower oil (90% w/v), dissolved 1 hour before use in animals.

Extraction, Isolation and Hydrogenation of AA

The AAs were isolated from cashew nut shell liquid, extracted from fresh cashew nuts (*Anacardium occidentale*) supplied by Iracema Cashew Industry Ltda., Fortaleza, Brazil. The plant extract preparation and the chemical characterization of these compounds followed the protocol previously described by Silveira et al. (2014).

Treatments

The animals were randomly divided into four experimental groups and treated over seven consecutive days (n = 6-10 animals/group). Each experimental condition was reproduced in three independent moments, generating triplicates of each group. The daily treatment with the drugs was carried out in the afternoon, between 1 and 2 pm. Mice from group 1 received sunflower oil as a vehicle (w/v) by gavage and 1 h after, received sunflower oil 90% + DMSO

10% (w/v) s.c.. Group 2 received sunflower oil as a vehicle (w/v) by gavage and 1 h after, rotenone (2.5 mg/kg/day s.c.). Group 3 was treated with AAs 50 mg/kg/day by gavage and 1 h after, rotenone (2.5 mg/kg/day, s.c.). Group 4 was treated with AAs 50 mg/kg/day by gavage and 1 h after received sunflower oil 90% + DMSO 10% as a vehicle (w/v) s.c.

Tissue Processing for Biochemical analysis

Twenty-four hours after the last day of pharmacological treatment, the animal groups were sacrificed by decapitation under isoflurane anesthesia. For biochemical analysis, pooled tissue was obtained from three independent control and experimental animal groups. The striatum and substantia nigra (SN) were dissected and weighed. After weighing, the pooled tissue of each region was homogenized (1:5 w/v) in 50 mM Tris buffer (pH 7.4) containing 0.5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM orthovanadate, 1% Nonidet and 1% protease inhibitor cocktail (Sigma-Aldrich, USA) at 4 °C, centrifuged for 10 min at 1000 g at 4 °C and aliquots of supernatant were separated for biochemical analysis and stored at −80 °C. Each individual experiment was carried out in triplicate and total protein concentration was estimated using the Bradford method (Bradford 1976).

Biochemical Studies

Measurement of Lipid Peroxidation

Lipoperoxidation (LP) was measured by estimating malondialdehyde (MDA) levels using a thiobarbituric acid (TBA) reaction (TBARS method) according to Ohkawa et al. (1979). In the TBARS test reaction, MDA- or MDA-like substances and TBA react to produce a pigment with maximum absorption at 532 nm. The reaction was developed by the sequential addition of 80 μL of 8.1 % sodium dodecyl sulfate, 600 μL of 20 % acetic acid pH 3.5, and 600 μL of 0.8 % TBA solutions boiled in water for 60 min to 200 μL of striatum and SN samples. After tap water cooling, 600 μL of n-butanol was added to the sample, centrifuged at 2500 g for 10 min, and the organic phase was read using a plate reader (Thermo Scientific, Varioskan flash spectral scanning multimode reader). Experiments were carried out in triplicate. The results were expressed as nmol per mg of protein using a standard curve generated using different concentrations of a 1,1,3,3-tetramethoxy propane solution.

Estimation of Nitric Oxide Production

Nitrite levels were estimated using the Griess reagent (Sigma-Aldrich) which serves as an indicator of nitric oxide (NO) production as described by Green et al. (1982). Equal volumes (100 μL) of samples and the Griess reagent were placed in 96-well plates and reacted for 10 min at room temperature (~22 °C). The absorbance of the diazonium compound was measured at a wavelength of 540 nm. The results were expressed as nmol per mg of protein with reference to a standard curve built with known sodium nitrite concentrations.

Reduced Glutathione Levels

Glutathione (GSH) levels were analyzed according to Hissin and Hilf (1976) method: 450 μL of phosphate buffer 100 mM with EDTA (5 mM) pH 8.0 were added to 50 μL of the samples. 50 μL of this mixture plus 140 μL of phosphate buffer 100 mM plus 10 μL orthophtaldehyde solutions (OPT) were placed in 96- well plates and incubated for 15 min at room temperature, protected from light. Fluorescence was recorded in a spectrofluorimeter using a wavelength of 350 nm. The results were expressed as μmol per mg of protein with reference to a standard curve built with known GSH(Sigma-Aldrich) concentrations.

Oxidized Glutathione Levels

GSH levels were analyzed according to Hissin and Hilf (1975) method. First of all, 50 μL of the samples were incubated at room temperature with 20 μL of N-ethylmaleimide (NEM) 0.04 M for 30 min to interact with GSH present in the tissue. To this mixture, 430 μL of sodium hydroxide (NaOH) 0.1 M was added. 50 μL of this mixture plus 140 μL of NaOH 0.1 M and 10 μL OPT were placed in the wells of a 96-well plate. This mixture was incubated for 15 min at room temperature and protected from light. The reading was made in a spectrofluorimeter using a wavelength of 350 nm. The results were expressed as μmol per mg of protein with reference to a standard curve built with known oxidized glutathione (GSSG; Sigma-Aldrich) concentrations.

Western blotting

Striatum and SN samples homogenates were diluted in sample buffer (62.5 mM tris/HCl, pH 7.4, containing 4% SDS, 10% glycerol, 1% β-mercaptoethanol and 0.002% bromophenol blue) and boiled for 5 min at 90 ºC. Fifty or thirty micrograms of protein per lane were electrophoretically separated in 10% sodium dodecyl sulphate–polyacrylamide gel at 60 mA. After separation, the proteins were transferred onto Hybond-nitrocelullose membrane (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) for 1.5 hrs at 350 mA. Membranes were blocked for 1 hr in Tris-buffered saline-Tween 20 (TBS-T) containing 5% of skimmed milk. Membranes were incubated with different antibodies: rabbit anti-TH (1:1000, in TTBS; Cell Signaling), mouse anti-GFAP (1:1000 in TTBS; Sigma-Aldrich), rabbit anti-IL-1beta (1:500 in TTBS; Immuny), mouse anti-MMP-9 (1:1000 in TTBS; Santa Cruz Biotechnology), rabbit anti-NFKB p65 (1:1000 in TTBS; Sigma) and anti-B actina (1:10,000 in TTBS; Santa Cruz Biotechnology) overnight at 4 °C. Membranes were rinsed in TTBS and incubated with the anti-rabbit or anti-mouse peroxidase–conjugated secondary antibody (1:50,000 in TTBS; Jackson ImmunoResearch) for 1 h at room temperature. Following three washes in TTBS (10 min each), labeling was detected with LUMINATA reagent (Millipore) via chemiluminescence using a ChemiDoc imaging system (Bio-Rad, USA). Band intensities were analyzed using Quantity One 4–6 software (BioRad Laboratories, Inc.).

Immunohistochemical procedure

Mice (six animals per group) were anesthetized with isoflurane) and perfused transcardially with saline (0.9% NaCl; 50 ml) followed by 4% paraformaldeyde in 0.1 M phosphate buffer (PB), pH 7.4 (200 ml). Perfusion was always performed between 12:00 and 18:00, with a continuous infusion pump (Harvard equipment) at a rate of 7.64 ml/min. After perfusion, the brains were dissected starting from the prefrontal cortex back to the inferior limit of the brainstem (the olfactory bulb and cochleas were excluded). They were then postfixed overnight in the same fixative, rinsed in PB and weighed (wet weight). Afterward, the brains were cryoprotected in solutions of 10%, 20% and 30% sucrose in PB. Brain blocks were serially cut on a criostate (Leica, Germany) into 40 mm-thick sections in the parasagittal plane. All sections were collected serially in PB and arranged in six series. The atlas of Paxinos and Watson (1986) was used to delimit the cytoarchitectonic regions of SN. The series of sections used for immunohistochemistry was first treated with 0.01 M citrate buffer, pH 6.0, at 90 ºC for 1 min in the microwave. Thereafter, free-floating sections were rinsed in

PB several times and incubated with rabbit anti-TH polyclonal antibody (Millipore, USA) diluted 1:500 in PB containing 0.3% Triton X- 100 (PBX) and 1% bovine serum albumin for 24 h at 4 ºC. Sections were then incubated for 1.5 h in secondary antiserum (biotinylated goat anti-rabbit IgG; Vector Labs, Burlingame, CA) diluted 1:200 in PBX, and processed for immunoperoxidase staining using the avidin biotin peroxidase complex (Standard ABC kit, Vector Labs). The binding of antibodies was revealed by the addition of diaminobenzidine tetrahydrochloride 0.05% (Sigma) and 0.01% H_2O_2 in PB, for 10 min. Subsequently, the free-floating sections were rinsed in PB and mounted on gelatin-coated slides. These procedures were carried out simultaneously in brain sections from both control and experimental animals. For the control of the staining specificity, some sections were processed omitting the primary antibody.

Statistical Analysis

All data sets are expressed as means ± standard deviation (SD). All the groups were tested for normality using the Kolmogorov–Smirnov test. In all the groups, the parameters showed a normal distribution. Accordingly, statistical significance was evaluated with one-way or two-way analysis of variance (ANOVA) followed by post hoc Tukey's or Sidak's multiple comparisons test, using GraphPad Prism software version 7.0 for Windows (San Diego, CA, USA). Differences were considered to be statistically significant when $p \leq 0.05$.

MATERIAL AND METHODS

Animals

In the present study, the experiments were conducted with adult male Swiss mice weighing 40 to 50 g (70 days of age), from the Animal Production Unit, Federal University of Paraíba, Brazil (UFPB). The animals were maintained under standard environmental conditions (12h light/dark cycle; light on 6 am) and temperature (22 ± 4 °C) and fed with a balanced commercial chow (Presence®, Purina, Brazil) and water ad libitum. Experimental protocols were approved by the Ethics Committee on Animal Use of the Federal University of Pernambuco, Brazil, under license no. 23076.005404/2015- 04, according to recommendations of the Brazilian College for Animal Experimentation guidelines, which follow the “Principles of Laboratory Animal Care” (NIH, Bethesda, USA).

Drugs and Solvents

Rotenone

Rotenone was purchased from Sigma (St. Louis, MO, USA) and dissolved (2.5 mg/mL) in dimethyl sulfoxide (DMSO 10% w/v) and in sunflower oil (90% w/v), dissolved 1 hour before use in animals.

Extraction, Isolation and Hydrogenation of AA

The AAs were isolated from cashew nut shell liquid, extracted from fresh cashew nuts (*Anacardium occidentale*) supplied by Iracema Cashew Industry Ltda., Fortaleza, Brazil. The plant extract preparation and the chemical characterization of these compounds followed the protocol previously described by Silveira et al. (2014).

Treatments

The animals were randomly divided into four experimental groups and treated over seven consecutive days (n = 6-10 animals/group). Each experimental condition was reproduced in three independent moments, generating triplicates of each group. The daily treatment with the drugs was carried out in the afternoon, between 1 and 2 pm. Mice from group 1 received sunflower oil as a vehicle (w/v) by gavage and 1 h after, received sunflower oil 90% + DMSO 10% (w/v) s.c.. Group 2 received sunflower oil as a vehicle (w/v) by gavage and 1 h after, rotenone (2.5 mg/kg/day s.c.). Group 3 was treated with AAs 50 mg/kg/day by gavage and 1 h after, rotenone (2.5 mg/kg/day, s.c.). Group 4 was treated with AAs 50 mg/kg/day by gavage and 1 h after received sunflower oil 90% + DMSO 10% as a vehicle (w/v) s.c.

Homogenate preparation

Twenty-four hours after the last day of pharmacological treatment, the animal groups were sacrificed by decapitation under isoflurane anesthesia. For biochemical analysis, pooled tissue was obtained from three independent control and experimental animal groups. The striatum and substantia nigra were dissected and weighed. After weighing, the pooled tissue of each region was homogenized (1:5 w/v) in 50 mM Tris buffer (pH 7.4) containing 0.5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM orthovanadate, 1% Nonidet and

1% protease inhibitor cocktail (Sigma-Aldrich, USA) at 4 °C, centrifuged for 10 min at 1000 g at 4 °C and aliquots of supernatant were separated for biochemical analysis and stored at −80 °C. Each individual experiment was carried out in triplicate and total protein concentration was estimated using the Bradford method (Bradford 1976).

Tissue preparation for tyrosine hydroxylase immunohistochemistry

The animals were anesthetized with isoflurane and perfused transcardially first with saline (0.9 % NaCl) followed by 20 0mL of 4 % paraformaldehyde in 0.1 M phosphate buffer (PB) pH 7.4. Perfusion was always carried out between 13:00 and 17:00 h, with a continuous infusion pump (Harvard equipment). After perfusion, the brains were post fixed for 2 h in the same fixative and rinsed in PB. Subsequently, the brains were cryoprotected in sequential solutions of 10, 20 and 30 % sucrose in PB. Brain blocks were serially cut on a cryostat microtome (Leica) into 40-μm-thick sections across the parasagittal plane of each hemibrain. All sections were collected serially in PB and arranged in six series. The Paxinos and Watson atlas (PAXINOS 1986) was used to delimit the cytoarchitectonic regions of the lateral cerebellum between the stereotaxic coordinates corresponding to lateral 3.9 and 2.9 mm. The series of sections used for tyrosine hydroxylase (TH) immunohistochemistry were treated with a 0.01 M citrate buffer (pH 6.0) at 60 °C for 1 h. Thereafter, free-floating sections were rinsed in PB, treated with 3 % H2O2 in 20 % methanol for 20 min, and incubated with a rabbit anti-TH monoclonal antibody (Cell Signaling; USA; 1:500) diluted in PB containing 0.3 % Triton X-100 (PBX) and 1 % bovine serum albumin for overnight at 4 °C. Sections were then rinsed in PB and incubated for 1 h in secondary antiserum (biotinylated goat anti-rabbit Jackson Laboratories, USA) diluted 1:1000 in PBX, and processed for immunoperoxidase staining using the streptavidin–peroxidase complex (Standard ABC kit, Vector Labs, USA). Antibody binding was revealed with 3-3′-diaminobenzidine tetrahydrochloride 0.05 % (Sigma-Aldrich, USA). Subsequently, the free-floating sections were rinsed in PB and mounted on gelatin-coated glass slides. These procedures were carried out simultaneously in brain sections from both the control and rotenone animals.

Biochemical Studies

Measurement of Lipid Peroxidation

Lipoperoxidation was measured by estimating malondialdehyde (MDA) levels using a thiobarbituric acid (TBA) reaction (TBARS method) according to Ohkawa et al. (1979). In the TBARS test reaction, MDA- or MDA-like substances and TBA react to produce a pigment with maximum absorption at 532 nm. The reaction was developed by the sequential addition of 80 μL of 8.1 % sodium dodecyl sulfate, 600 μL of 20 % acetic acid pH 3.5, and 600 μL of 0.8 % TBA solutions boiled in water for 60 min to 200 μL of striatum and substantia nigra samples. After tap water cooling, 600 μL of n-butanol was added to the sample, centrifuged at 2500 g for 10 min, and the organic phase was read using a plate reader (Thermo Scientific, Varioskan flash spectral scanning multimode reader). Experiments were carried out in triplicate. The results were expressed as nmol per mg of protein using a standard curve generated using different concentrations of a 1,1,3,3-tetramethoxy propane solution.

Estimation of Nitric Oxide Production

Nitrite levels were estimated using the Griess reagent (Sigma-Aldrich) which serves as an indicator of nitric oxide (NO) production as described by Green et al. (1982). Equal volumes (100 μL) of samples and the Griess reagent were placed in 96-well plates and reacted for 10 min at room temperature (~22 °C). The absorbance of the diazonium compound was measured at a wavelength of 540 nm. The results were expressed as nmol per mg of protein with reference to a standard curve built with known sodium nitrite concentrations.

Reduced Glutathione Levels

Reduced Glutathione (GSH) levels were analyzed according to Hissin and Hilf (1976) method: 450 μL of phosphate buffer 100 mM with EDTA (5 mM) pH 8.0 were added to 50 μL of the samples. 50 μL of this mixture plus 140 μL of phosphate buffer 100 mM plus 10 μL orthophtaldehyde solutions were placed in 96- well plates and incubated for 15 min at room temperature, protected from light. Fluorescence was recorded in a spectrofluorimeter using a wavelength of 350 nm. The results were expressed as μmol per mg of protein with reference to a standard curve built with known GSH (Sigma-Aldrich) concentrations.

Oxidized Glutathione Levels

Oxidized Glutathione (GSSG) levels were analyzed according to Hissin and Hilf (1975) method. First of all, 50 μL of the samples were incubated at room temperature with 20 μL of N-ethylmaleimide 0.04 M for 30 min to interact with GSH present in the tissue. To this mixture, 430 μL of sodium hydroxide (NaOH) 0.1 M was added. 50 μL of this mixture plus 140 μL of NaOH 0.1 M and 10 μL OPT were placed in the wells of a 96-well plate. This mixture was incubated for 15 min at room temperature and protected from light. The reading was made in a spectrofluorimeter using a wavelength of 350 nm. The results were expressed as μmol per mg of protein with reference to a standard curve built with known oxidized glutathione (GSSG; Sigma-Aldrich) concentrations.

Western blotting

Striatum and substantia nigra samples homogenates were diluted in sample buffer (62.5 mM tris/HCl, pH 7.4, containing 4% SDS, 10% glycerol, 1% β-mercaptoethanol and 0.002% bromophenol blue) and boiled for 5 min at 90 ºC. Fifty or thirty micrograms of protein per lane were electrophoretically separated in 10% sodium dodecyl sulphate–polyacrylamide gel at 60 mA. After separation, the proteins were transferred onto Hybond-nitrocelullose membrane (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) for 1:30 hrs at 350 mA. Membranes were blocked for 1 hr in Tris-buffered saline-Tween 20 (TBS-T) containing 5% of skimmed milk. Membranes were incubated with different antibodies: rabbit anti-TH (1:1000, in TTBS; Cell Signaling), mouse anti-GFAP (1:1000 in TTBS; Sigma-Aldrich), rabbit anti-IL-1beta (1:500 in TTBS; Immuny), mouse anti-MMP-9 (1:1000 in TTBS; Santa Cruz Biotechnology), rabbit anti-NFKB p65 (1:1000 in TTBS; Sigma) and anti-B actina (1:10,000 in TTBS; Santa Cruz Biotechnology) overnight at 4 °C. Membranes were rinsed in TTBS and incubated with the anti-rabbit or anti-mouse peroxidase–conjugated secondary antibody (1:50,000 in TTBS; Jackson ImmunoResearch) for 1 h at room temperature. Following three washes in TTBS (10 min each), labeling was detected with LUMINATA reagent (Millipore) via chemiluminescence using a ChemiDoc imaging system (Bio-Rad, USA). Band intensities were analyzed using Quantity One 4–6 software (BioRad Laboratories, Inc.).

Immunohistochemical procedure

Mice (six animals per group) were anesthetized with isoflurane) and perfused transcardially with saline (0.9% NaCl; 50 ml) followed by 4% paraformaldeyde in 0.1 M phosphate buffer (PB), pH 7.4 (200 ml). Perfusion was always performed between 12:00 and 18:00, with a continuous infusion pump (Harvard equipment) at a rate of 7.64 ml/min. After perfusion, the brains were dissected starting from the prefrontal cortex back to the inferior limit of the brainstem (the olfactory bulb and cochleas were excluded). They were then postfixed overnight in the same fixative, rinsed in PB and weighed (wet weight). Afterward, the brains were cryoprotected in solutions of 10%, 20% and 30% sucrose in PB. Brain blocks were serially cut on a criostate (Leica, Germany) into 40 mm-thick sections in the parasagittal plane. All sections were collected serially in PB and arranged in six series. The atlas of Paxinos and Watson (1986) was used to delimit the cytoarchitectonic regions of SN. The series of sections used for immunohistochemistry was first treated with 0.01 M citrate buffer, pH 6.0, at 90 ºC for 1 min in the microwave. Thereafter, free-floating sections were rinsed in PB several times and incubated with rabbit anti-TH polyclonal antibody (Millipore, USA) diluted 1:500 in PB containing 0.3% Triton X- 100 (PBX) and 1% bovine serum albumin for 24 h at 4 ºC. Sections were then incubated for 1.5 h in secondary antiserum (biotinylated goat anti-rabbit IgG; Vector Labs, Burlingame, CA) diluted 1:200 in PBX, and processed for immunoperoxidase staining using the avidin biotin peroxidase complex (Standard ABC kit, Vector Labs). The binding of antibodies was revealed by the addition of diaminobenzidine tetrahydrochloride 0.05% (Sigma) and 0.01% H_2O_2 in PB, for 10 min. Subsequently, the free-floating sections were rinsed in PB and mounted on gelatin-coated slides. These procedures were carried out simultaneously in brain sections from both control and experimental animals. For the control of the staining specificity, some sections were processed omitting the primary antibody.

Statistical Analysis

All data sets are expressed as means ± standard deviation (SD). All the groups were tested for normality using the Kolmogorov–Smirnov test. In all the groups, the parameters showed a normal distribution. Accordingly, statistical significance was evaluated with one-way or two-way analysis of variance (ANOVA) followed by post hoc Tukey's or Sidak's multiple comparisons test, using GraphPad Prism software version 7.0 for Windows (San Diego, CA, USA). Differences were considered to be statistically significant when $p \leq 0.05$.

RESULTS

3.1. Body weights of animal groups

Table 1 shows the body weights of control, AAs 50 mg/Kg, rotenone 2.5mg/Kg and rotenone + AAs groups at the first and last day of the treament. No significant difference was found in the body weight of all animals in the 8th day of the pharmacological treatment or when compared to the control group, indicating that neither rotenone or AAs per se were able to modify this somatic parameter.

TABLE 1. Body weights of animal groups before and after treatments

Experimental groups	**Body weight (g) 1st day (Mean ± SD)**	**Body weight (g) 8th day (Mean ± SD)**	***p* values**
Control	47.3±4.508	47.15±2.852	>0.9999
AAs	46.16±3.705	43.44±3.203	0.1837
ROT	46.38±4.437	45.78±2.255	0.9348
ROT + AAs	43.79±4.829	42.55±5.241	0.9638

3.2. *TH-immunoreactivity pattern in the* substantia nigra *of Control and Rotenone groups*

The distribution of dopaminergic cells TH^+ was analyzed in 6 parasagittal sections throughout the latero-medial extension of substantia nigra of the groups Control and Rotenone 2.5mg/Kg. **Figure 2** ilustrates the TH-immunoreactivity pattern in one representative animal of both groups. Partial analysis of these data indicates a moderate loss of dopaminergic cells especially those located in the ventro-lateral region of substantia nigra in the rotenone group when compared to the control one. This data corroborates the higher sensitivity of this dopaminergic cell population to oxidative damage induced by this pesticide.

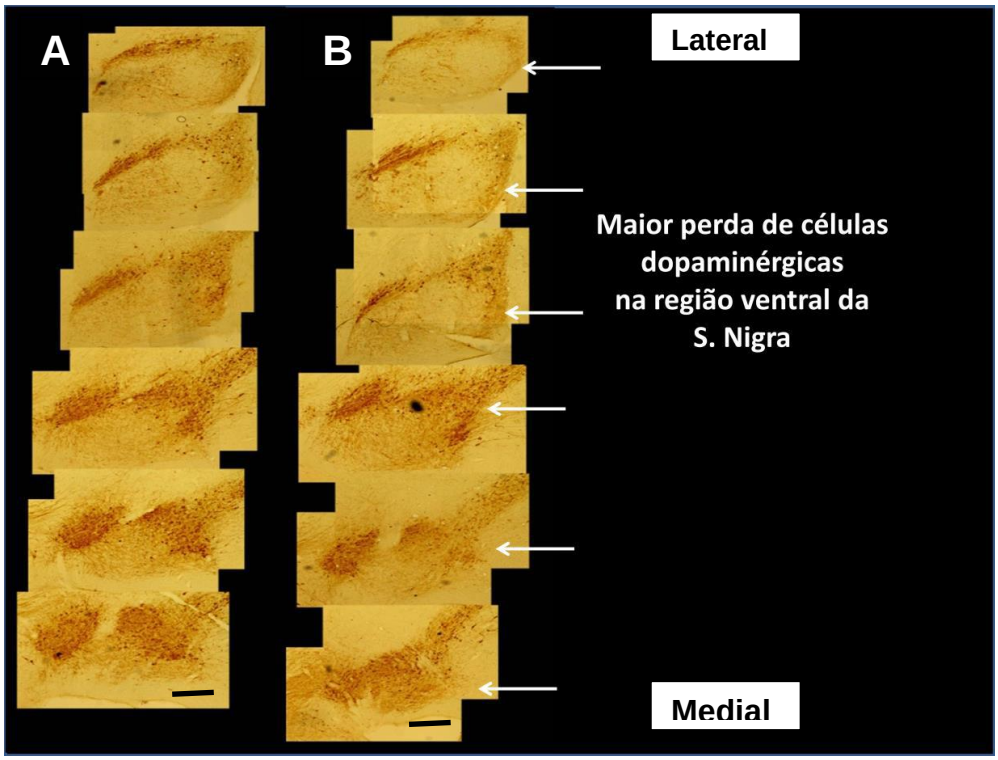

Figure 1*:* Representative photomicrographs of TH-immunoreactive parasagittal sections at the latero-medial extension of the substantia nigra from mice of control (A) or rotenone (B) groups . White arrows indicate the location of caudo-ventro-lateral region of substantia nigra where we detected reduced number of TH-immunorreactive cells in 2 animals of these groups. The analysis of this data is in progress at this moment. Scale bar = 250 µm.

3.3. Tyrosine hydroxilase and GFAP protein levels in the striatum and substantia nigra

Quantitative analysis of TH protein levels in homogenates of substantia nigra or striatum is depicted in the figure 2A and 2C. A pronounced decline in the protein expression of this enzyme was observed in the rotenone group when compared to the control ($P < 0.0001$ in both nuclei*).* AAs 50 mg/Kg/ day *per se* did not modify these levels (1,802 ± 0,2446) compared to the control (1,808 ± 0,3715) but was able to partially reverse the effect of rotenone in both nuclei (~35 % in the striatum; $P < 0.01$ and 52 % in the SN; $P = 0.0001$).

Astroglial reactivity was assessed analyzing GFAP protein expression. As shown in the figure 2B and 2D, rotenone increased ~2 fold GFAP levels in both nuclei compared to control ($P < 0.0001$ in the substantia nigra and striatum) and AAs partially reversed this effect in the striatum ($P = 0.0001$) but not in the substantia nigra. AAs 50 mg/Kg/ day per se did not modify the glial reactivity when compared to control condition.

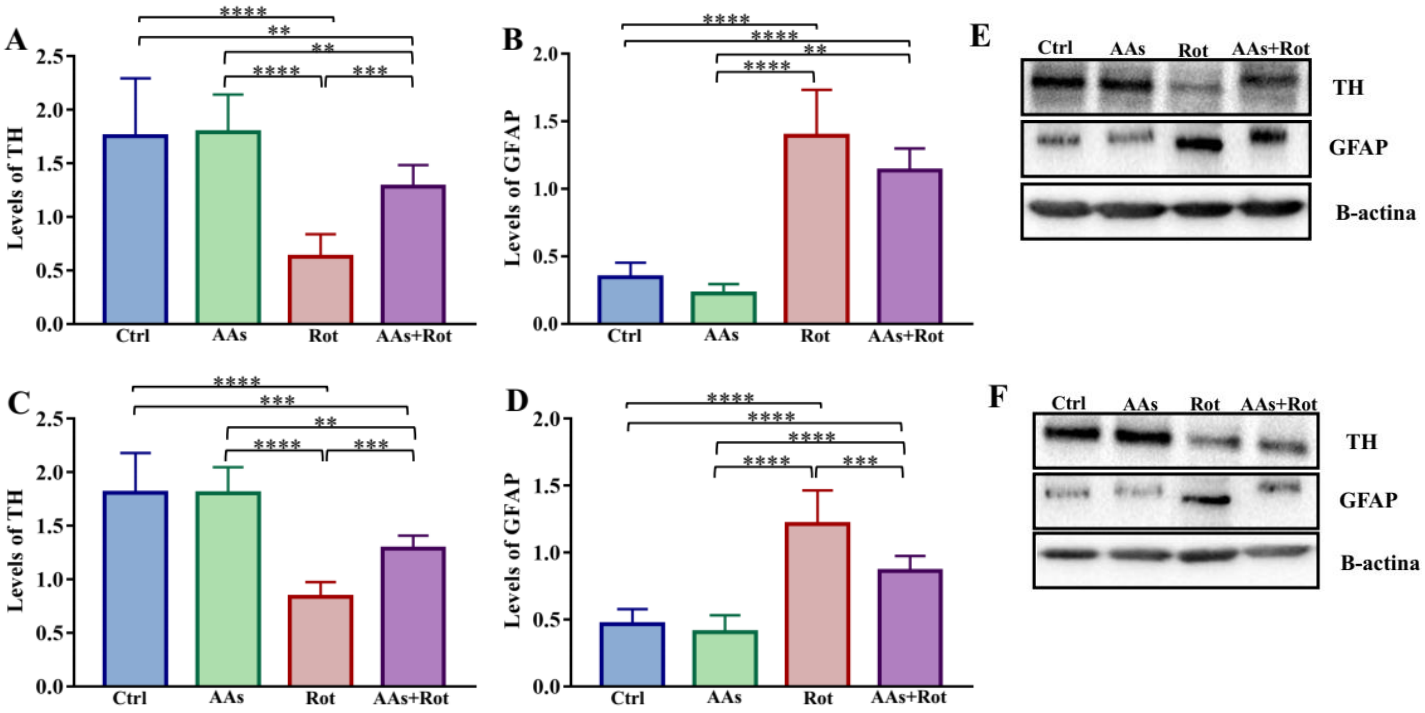

Figure 2. TH protein levels (A and C) and GFAP protein levels (B and D) in the pools of substantia nigra and striatum. Western blot analysis for TH, GFAP and B-actina in the pools of substantia nigra (E) and striatum (F). Data are expressed as mean ± standard deviation. ** indicates $P < 0.01$, *** indicates $P = 0.0001$, **** $P < 0.0001$ (One way ANOVA and post hoc Tukey's multiple comparisons test).

3.4. Effects of AAs on biochemical parameters indicative of oxidative stress

3.4.1 Lipoperoxidation and Nitric oxide levels

In the substantia nigra, rotenone treatment increased ~2 fold lipoperoxidation levels compared to the control and AAs groups ($P < 0.0001$). This deleterious effect was completely reversed by the concomitant treatment with AAs 50 mg/Kg/day (Figure 3A). No significant difference was detected between AAs alone and the control group. In the striatum of rotenone-treated animals, rotenone increased ~3fold lipoperoxidation levels as compared to control and AAs groups ($p<0.001$). AAs 50 mg/Kg/day alone did not induce lipid damage and was able to completely reverse the effect of rotenone in both regions.

Nitrite levels were elevated by rotenone ~3 fold in the substantia nigra (p <) and 20 % in the striatum (p <). AAs alone did not modify their levels when compared to the control condition but completely reversed the effect of rotenone in both regions

3.4.2. Glutathione levels

Figure 3C shows also GSH, GSSG levels and GSH/GSSG ration in the substantia nigra and striatum of the control and experimental groups (C and F). Co-treatment of AAs with rotenone completely recovered the redox balance when compared to control condition (p= 53 in the substantia nigra and p=0.288 in the striatum). AAs alone did not modify the GSH/GSSG ratio as compared to the control (p= 0.45). On the other hand, rotenone exposure significantly decreased GSH/GSSG redox ratio by 26 % in the striatum when compared to control (p=0.002) and AAs (p=0.0001) groups (figure 3D).

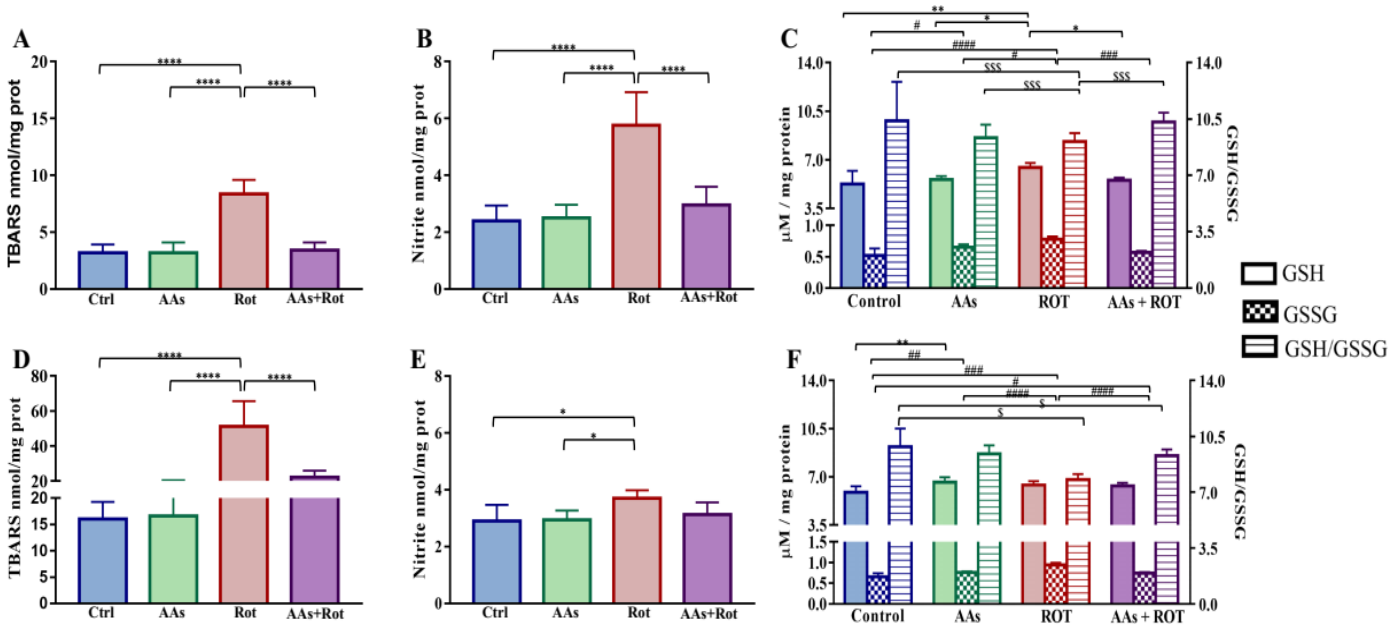

Figure 3. TBARS levels (A and D), nitrate concentration as an indicator of NO production (B and E) and GSH, GSSH levels and GSH/GSSG ratio (C and F) in the pools of substantia nigra and striatum. Data are expressed as mean ± standard deviation. * indicates $P < 0.05$, **** $P < 0.0001$ (one way ANOVA and post hoc Tukey's multiple comparisons test).
* indicates GSH, # indicates GSSG and $ indicates GSH/GSSG.

3.5. Effects of AAs on inflammatory markers

Targeting NF-κB has been proposed as an approach to the treatment of acute and chronic inflammatory conditions, and the use of inhibitors specific for this gene transcription factor has been found to inhibit neurodegeneration of TH+ neurons in murine and primate

models of Parkinson's disease (Flood et al. 2011). Therefore, we analyzed the effects of AAs on protein levels of the active form NF-κB-p65, which is able to be translocated to the nucleus and induce the transcription of several genes with inflammatory actions (Kumar et al. 2004).

In the SN of rotenone-treated animals an expressive increase of ~3fold was detected in the of NF-κB-p65 protein levels, compared to control and AAs groups (*P = 0.0001*). AAs per se did not modify these levels (p < 0.0001 vs control) but it was able to reduce ~50% the deleterious effect of rotenone (*P = 0.0001;* Figure 4A).

Similarly in the striatum of rotenone- treated animals (Figure 5A), increase NF-κB-p65 protein levels were observed when compared to control and AAs (*P = 0.0001*) groups.). AAs per se did not modify these levels (p < 0.0001 vs control) but it was able to reduce ~50% the deleterious effect of rotenone (*P = 0.0001;* Figure 4D).

The levels of the pro-inflammatory interleukin IL-1β (34 KDa) was also increased by the subchronic treatment with rotenone either in the substantia nigra (~3 fold) or in the striatum (~ 2 fold) as compared to the control condition. AAs *per se* reduced the levels of the pro-IL-1 β in the striatum (*P < 0.05*), but not in the substantia nigra (p= 0.9097), when compared to the respective controls. AAs also completely reversed the effect of rotenone in the striatum (p<0,0001). In the substantia nigra, the co-treatment of AA + rotenone, reduced ~60 % the levels of pro-IL-1β. Analysis of the active form of IL-1β was carried out using ELISA immunoassay. Preliminar data of 1 pool of 6 animals per group showed a significant increase in the levels of the 17 KDa IL-1b isoform and apparently, the co-treatment with AAs was able to partially or completely reverse this effect in the striatum and substantia nigra respectively.

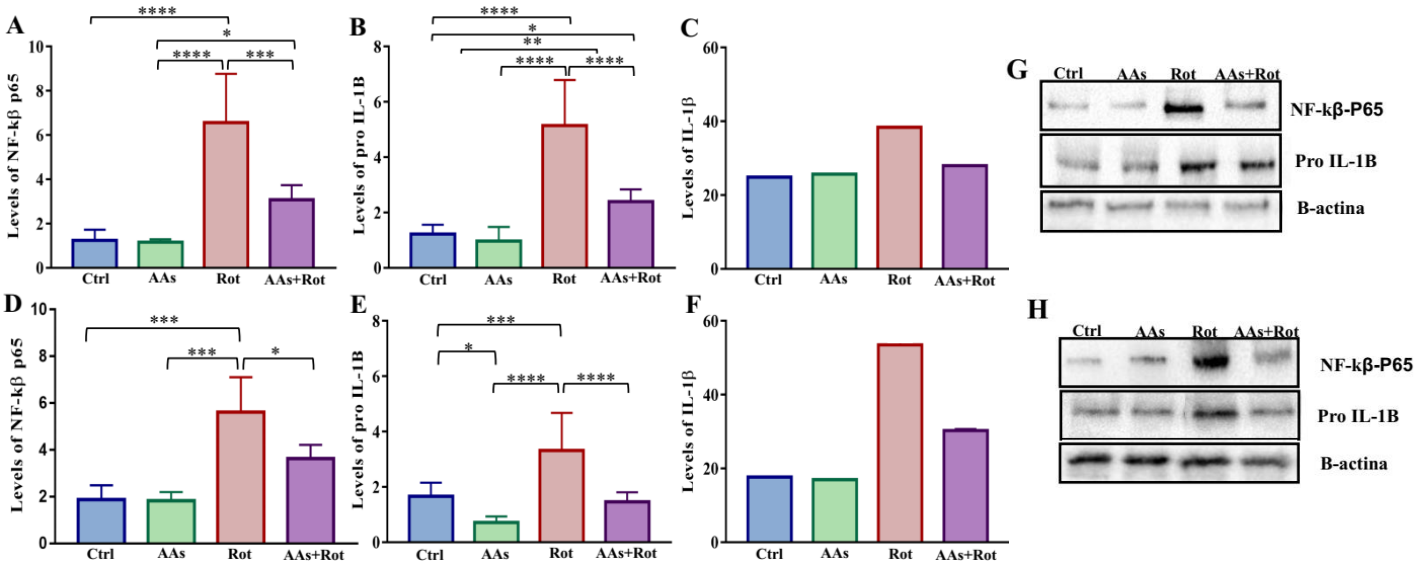

Figure 4. NF-kβ p65 (A and D), pro IL-1*β* (B and E) and IL-1*β* (C and F) levels and in the pool of substantia nigra and striatum. The mean ± standard deviation is presented. Western blot analysis for NF-kβ p65, pro IL--1*β* and B-actina in the pools of substantia nigra (G) and striatum (H). *indicates $P < 0.05$, ** indicates $P < 0.01$, *** indicates $P = 0.0001$, **** $P < 0.0001$ (one way ANOVA and post hoc Tukey's multiple comparisons test).

3.5. Effects of AAs on MMP-9 and TIMP-1

Rotenone exposure increased about 3 fold MMP-9 protein levels either in the substantia nigra or striatum (*P = 0.0001* for both regions). AAs per se were able to reduce the expression of this protein, when compared to control condition in both nuclei (*P < 0.01* in the substantia nigra) and (*P < 0.05* in the striatum). The co-treatment of AA with rotenone attenuated ~60 % the effect induced by rotenone alone in both nuclei.

Rotenone did not modify TIMP-1 protein levels in both Substantia nigra and striatum when compared to the control condition. Nevertheless, AAs alone increased ~2 fold (in the substantia nigra) and ~1.7 fold (in the striatum) the amount of this protein. The concomitant treatment of rotenone + AAs also augmented TIMP-1 levels in the substantia nigra when compared to the control (p <), but this effect was not observed in the striatum.

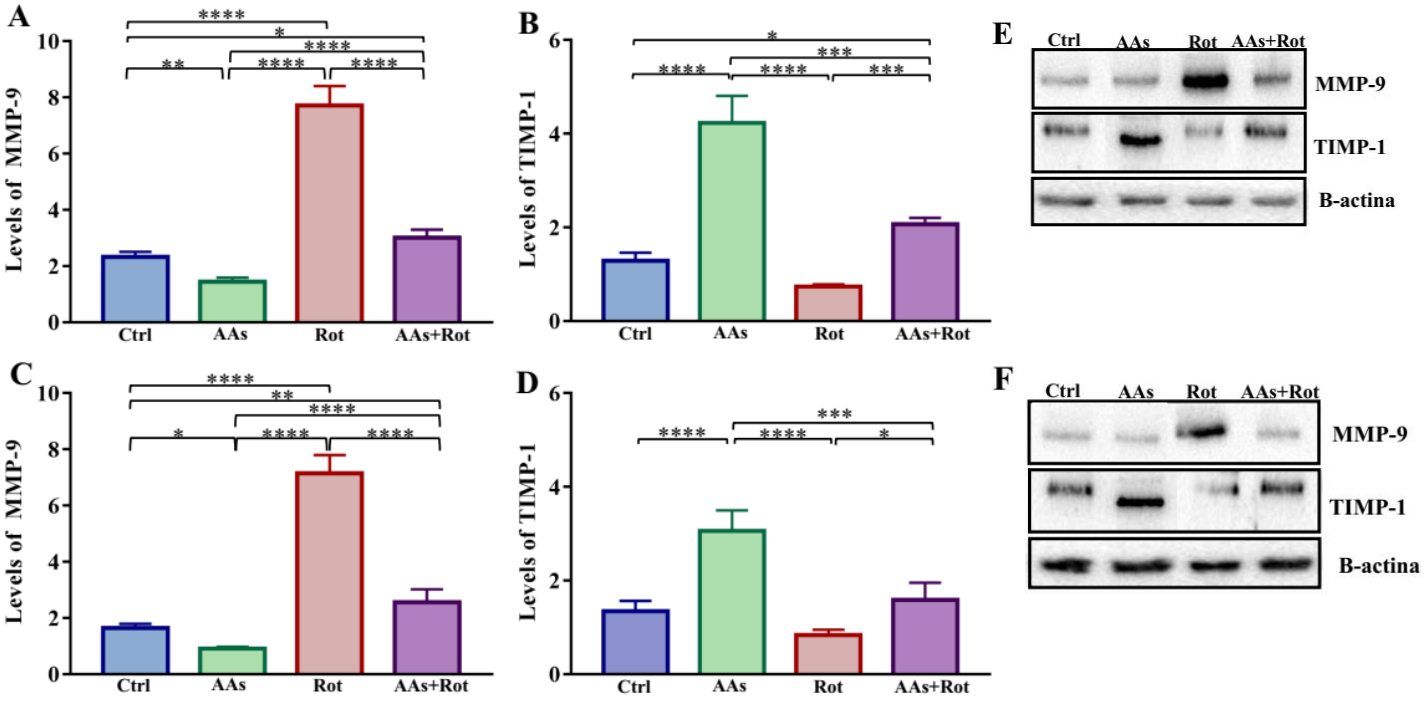

Figure 5. MMP-9 (A and C) and TIMP-1 (B and D) in the pool of substantia nigra and striatum. The mean ± standard deviation is presented. Western blot analysis for MMP-9, TIMP-1 and B-actina in the pools of substantia nigra (E) and striatum (F). *indicates *P* <

0.05, ** indicates $P < 0.01$, *** indicates $P = 0.0001$, **** $P < 0.0001$ (one way ANOVA and post hoc Tukey's multiple comparisons test).

Discussion

In the present study, we investigated the effect of AAs against rotenone-induced model of PD considering the well established evidence that exposure to this pesticide mimics many of the key pathological features of human PD including inflammation, oxidative stress, glial activation and selective cell loss (Niranjan R, 2014; Litteljohn et al., 2011; Johnson ME, Bobrovskaya L 2014). It was used 2.5 mg/kg body weight dose of rotenone for 7 days to provoke moderate dopaminergic neuronal death in the SN which is typical of early stages of PD. The main motivation was to test the hypothesis that AAs may represent an alternative nutraceutical agent for prevention and early intervention approach considering their ability to act in several molecular targets involved in the progression of the neurodegenerative process.

The subchronic rotenone treatment here adopted was able to reduce ~20% the number of dopaminergic cells, especially in the ventro-caudo-lateral region of the SN pars compacta. This result is in agreement with previous evidence that this region is more vulnerable to oxidative stress (Poulin et al., 2014) because the dopamine cell population in this SN region display a distinct neurochemical profile that favors their demise first than those located in the rostro-dorso-medial region. Part of this difference is due to an increased expression of genes encoding pro-inflammatory cytokines and decreased expression of several glutathione-related genes among others involved in the redox balance (Duke et al., 2007; Poulin et al., 2014). Analysis post mortem in humans also demonstrated that dopaminergic neurons present in the ventral tier are the subpopulation that first die during the progression of PD (Damier et al., 1999; Duke et al., 2007).

Another biomarker of dopaminergic damage herein detected was the reduced level of TH protein expression induced by rotenone (~40% of the control condition in both SN and striatum). TH catalyses the formation of L-dihydroxyphenylalanine (L-DOPA), the rate-limiting step in the biosynthesis of dopamine. It has been shown that redox state changes provoked by an oxidative injury, can directly or indirectly affect the TH activity, metabolism and expression (Giovanni et al 2012). Pesticides such as Deltamethrin, for example, also inhibits the activity and mRNA/protein expression of TH in striatum [97]; thereby implicating TH as a molecular target of pesticides within the nigrostriatal pathway. AAs significantly

attenuated the deleterious effect of rotenone on TH protein expression, and this effect was more expressive in the SN (~52% reduction) compared to that was observed in the striatum (~35%). This finding support its anti-oxidative activity in the brain, previously demonstrated in rat model of PD (Linard-Medeiros et al., 2018) and here in mice, considering its ability to completely reverse the rotenone-induced lipoperoxidation, increased NO levels and the reduced redox balance given by GSH/GSSG ratio in both SN and striatum. In addition, AAs per se were able to increase GSH levels compared to the control condition in both regions. This stimulant effect on GSH levels was also found in mice prefrontal cortex and hipoccampus after acute intraperitoneal administration of 25 e 50 mg/Kg AAs (GOMES-JUNIOR et al., 2018b) suggesting that it can occur independent of brain region, dose and route of drug administration. Altogether, this evidence increases the number of molecular targets where AAs can act to prevent or reduce oxidative damage. Reactive oxygen species production and mitochondrial damage are two mechanisms of neuronal degeneration proposed to have a role in Parkinson's disease and an important link between these two mechanisms can be the scavenging activity of glutathione and superoxide dismutase against accumulation of oxygen radicals. In our previous study it was shown that 50 and 100 mg/Kg AAs per se increased total SOD enzymatic activity compared to control condition and at 100 mg/Kg AAs were also able to intensify mitochondrial and cytoplasmic SOD gene expression (Linard-Medeiros et al., 2018). Moreover, AAs treatment (0.4 or 1.5 µM) 1 min after rotenone application (1.8 µM) on isolated mitochondria completely reversed the inhibition of complex I of the electron transport chain, restoring the oxygen consumption (Linard-Medeiros et al., 2018). Therefore, considering that mitochondrial damage represents the initial insult induced by rotenone followed by oxidative stress, most of the beneficial anti-oxidative effects induced by AAs treatment reveal the ability of these phenolic compounds in acting as a multi-modal drug in the early and subsequent steps of the progressive neurodegeneration.

Experimental and clinical evidence have indicated that cellular damage resultant of oxidative stress triggers the inflammatory process via activation of the transcription factor NF-kB, inducing the synthesis and secretion of several cytokines in neurodegenerative diseases, including PD (Kumar et al., 2004; Popa-Wagner et al., 2013). A conspicuous increase in NF-κB activation within the midbrain of animals submitted to MPTP-induced PD, as well as in the SNpars compacta of PD patients (Hunot at al., 1977; Ghosh et al., 2007) has been demonstrated. According to these studies, this activation occurs in TH positive neurons, astrocytes and microglia. In addition, compounds such as PPARγ agonists and curcumin for example, are able to reduce dopamine neurodegeneration, by inhibiting NF-κB activation and

its translocation to the nucleus.(Yang et al., 2008; Ghosh et al., 2007; Flood et al., 2011). Therefore, NF-κB has been considered a target for therapy in Parkinson's Disease (Flood et al., 2011). In the present study, we have shown that AAs per se did not modify the activated NF-κB p65 protein levels but significantly reduced the activation of this transcription factor provoked by rotenone (~50% in the SN and ~30% in the striatum).

The inhibition of NF-κB p65 activation provoked by AAs in our experimental animals was accompanied by a concomitant reduction in the protein levels of pro-IL-1β of 34 KDa and its activated form of 17 KDa whose levels were increased by rotenone. AAs per se were capable to reduce protein levels of Pro-IL-1β in the striatum. Neuroinflammation mediated by IL-1β increases susceptibility of dopamine neurons to degeneration in experimental models of Parkinson's disease (Koprich et AL., 2008; Leal et al., 2013; Stojakovic et AL., 2017). This pro-inflammatory cytokine has also been found in the cerebrospinal fluid and post-mortem striata of PD patients (Nagatsu et al., 2000). It is mainly produced by microglia (Herx et al., 2000) followed by astrocytes (Rappold and Tieu, 2010) and some neurons (Silverman et al., 2005) under pathological conditions.

In the present study, subchronic treatment with 2,5 mg/Kg rotenone increased ~2 fold GFAP protein levels, when compared to the control or AAs alone conditions, which is in agreement with previous evidence that astrocyte activation in the SN and striatum is a key step in rotenone induced toxicity (Swarnkar et al., 2013). AAs alone did not modify GFAP protein expression but they were able to reduce rotenone effect in the striatum (~40%) but not in the SN, although a tendency for decrease ($p = 0.07$) was observed in this region. A growing body of evidence indicates that astrocytes can exert a dual role in neuronal survival and function in the context of PD. While these cells are involved in production and release of GSH, neurotrophic factors, clearance of α-synuclein, under overactivation it can be also involved in the inflammatory process (Rappold and Tieu, 2010). In this aspect, astrocytes have a reciprocal interaction with IL-1β. Besides to directly release IL-1β and other pro-inflammatory cytokines, astrocytes can also be stimulated by TNF-α and IL-1β from microglia, leading to production of reactive oxygen and nitrogen species (Rappold and Tieu, 2010). Using a co-culture model of glia and neurons, Saijo et al., (2009) reported that astrocytes can enhance microglial responses through a NF-κB-dependent mechanism, leading to more dopaminergic toxicity under condition of inflammation induced by LPS.

MMP-9 and its inhibitor TIMP1 were analyzed in the present investigation considering the recognized inhibitory action of AAs on the activity of metaloproteinases

(Omanakuttan et al., 2012) and the recent report in tumor cell lineages demonstrating that AA inhibits both the gene expression as well as the activity of MMP-2 and MMP-9 from fibrosarcoma cells, HT1080 (Nambiar et al., 2016). Associations of MMP-9 and TIMP1 polymorphisms have been also associated with PD in human beings (HE et al., 2013; Chen et al., 2016). MMP-9 contributes to glial activation and neurodegeneration in both the monkey and mouse MPTP- or rotenone-induced PD (Annese et al. (2015; SINGH et al., 2015). One of the mechanisms underlying rotenone toxicity which promotes neuronal death involves microglial phagocytosis (Mansour et al., 2018). Previous reports state that apoptosis of dopaminergic neurons releases MMP-3 which can provoke microglial activation, elevated nitric oxide activating MMP-9, which results in the disruption of the extracellular matrix, and leads to cell detachment [Shin et AL., 2012)

In our experimental animals, AAs per se were able to reduce MMP-9 and increased TIMP1 protein levels when compared to the control condition in both SN and striatum, suggesting that in addition to a direct effect on MMP activity, this phenolic compounds can also negatively modulate the gene expression of this inflammatory marker in brain regions, as was described in tumor cell lineages (Nambiar et al., 2016). The expressive increase (~4 fold) of MMP-9 amount induced by rotenone was also reduced by AAs about 85% in both regions. On the other hand, the concomitant treatment of AAs + rotenone increased TIMP1 levels above the basal levels detected in the control condition. Thus, considering previous evidence that pharmacological inhibition of MMP-9 protected dopaminergic neuronal loss, and diminished dopamine depletion in MPTP-PD (Lorenzl et al. (2003), the present findings using rotenone show that AAs are multimodal drugs that in fact act in multiple targets to reduce extracellular matrix degradation and inflammatory status in the context of PD.

Altogether, all the neuroprotective actions of AAs herein detected reinforce the initial hypothesis that these nutraceutic agents have the potential to be further developed as a therapeutic candidate for preventive treatments that can lower the risk of developing PD or reduce the progression of this neurological disorder. The main results obtained are illustrated in the schematic drawing showed in the Figure 6.

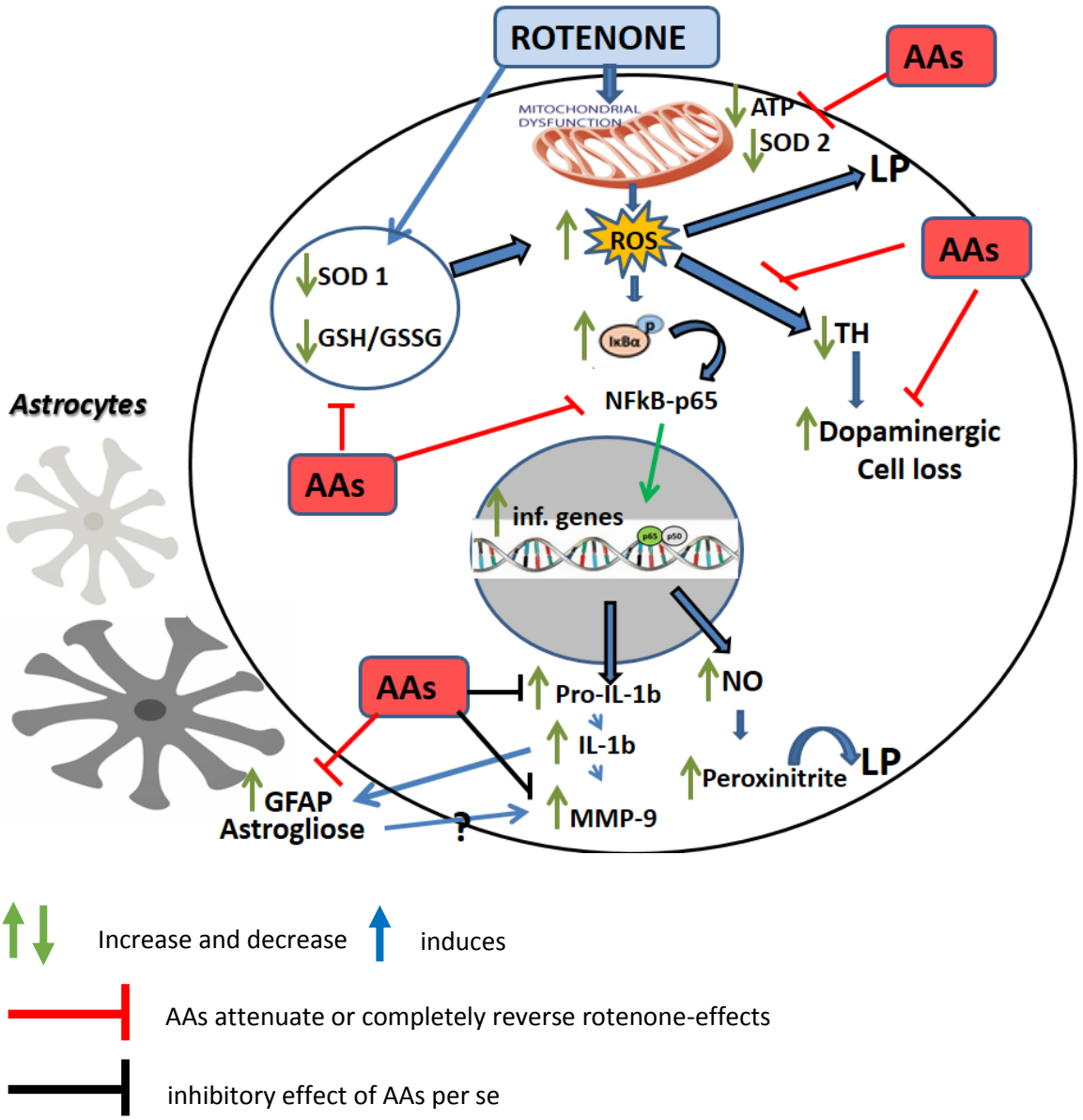

Figure 6: Schematic drawing illustrating multiple effects of AAs involved in its anti-oxidative and anti-inflammatory capacity against rotenone-induced damage observed in this study. SOD 1= cytoplasmatic superoxide dismutase; SOD 2= mitochondrial superoxide dismutase; ROS= reactive oxygen species; LP= lipoperoxidation; GSH= gluthatione reduced; GSSG= gluthatione oxidized; pIkBα = phosphorylated nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; NFkB-p65 = activated nuclear factor kappa-light-chain-enhancer of activated B cells; inf.genes = inflammatory genes; Pro-IL-1b = pro-interleukin 1b; IL-1b; active form of pro-interleukin 1b; MMP-9= metalloproteinase 9; NO= nitric oxide; GFAP= glial fibrilary acidic protein; TH= tyrosine hydroxylase.

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4 CONCLUSÕES

Os dados obtidos em camundongos corroboram a nossa hipótese inicial de que os AAs exercem atividade neuroprotetora na SN e estriado contra os danos induzidos pelo pesticida rotenona. Tal atividade deve-se em parte à sua capacidade antioxidativa desde a sua ação direta sobre a mitocôndria bem como sobre o estímulo para produção de substancia antioxidantes. Também apresenta ação anti-inflamatória no sistema nervoso, atuando de forma modulatória em múltiplos mecanismos celulares e moleculares que são capazes de induzir neurodegeneração e são alvos terapêuticos para a prevenção e tratamento da Doença de Parkinson.

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APÊNDICE A - ARTIGO PUBLICADO

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ORIGINAL ARTICLE

Anacardic Acids from Cashew Nuts Prevent Behavioral Changes and Oxidative Stress Induced by Rotenone in a Rat Model of Parkinson's Disease

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Abstract
Anacardic acids (AAs) are alkyl phenols mainly presenting in cashew nuts. The antioxidant effects of these compounds have been an area of interest in recent research, with findings suggesting potential therapeutic use for certain diseases. Nevertheless, none of these studies were performed in order to test the hypothesis of whether anacardic acids are capable of preventing behavioral changes and oxidative stress induced by the pesticide rotenone in experimental model of Parkinson's disease. In our research, adult male rats were treated orally with AAs (1, 3, 10, 25, 50, or 100 mg/kg/day) 1 h before rotenone (3 mg/kg; s.c.) for five consecutive days. The behavioral testing strategies, including tests for general locomotor activity (open field), motor coordination (rotarod), and spatial memory performance (elevated T-maze), were carried out. Lipoperoxidation levels and total superoxide dismutase (t-SOD) activity, as well as cytoplasmic and mitochondrial SOD gene expression, were assessed in the substantia nigra (SN), striatum, and cerebral cortex. The results showed that AAs dose-dependently prevented the rotenone-induced learning and motor impairment from 10 mg/kg/day. AAs also precluded rotenone-induced lipoperoxidation in all doses, acting directly on the mitochondria, and improved the t-SOD activity in the doses 25–100 mg/kg/day. AAs per se (100 mg/kg/day) increased SOD gene expression and t-SOD activity. Our findings indicate that the oral administration of AAs prevents rotenone-induced behavioral changes and oxidative stress, in part due to a modulatory action on the mitochondria and SOD gene expression. These data suggest that AAs have promising neuroprotective action against degenerative changes in Parkinson's disease.

Keywords Rotenone · Lipoperoxidation · Superoxide dismutase · Substantia nigra · Motor behavior

Introduction

Parkinson's disease (PD) is a prevalent neurodegenerative disorder characterized by motor, limbic and cognitive impairment that negatively affects the quality of life during aging (Jankovic 2008; de Farias et al. 2016). Population-based studies have indicated that the number of individuals looking for treatment for PD is likely to increase significantly over the

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next decades (Lees et al. 2009). Therefore, preventive therapeutic intervention at early stages of this disorder has been proposed for minimizing its long-term development. It is noteworthy that a wide range of environmental toxins may be risk factors for the etiology and pathogenesis of PD, inducing both degeneration of monoaminergic systems and increased levels of oxidative stress (OS) in the brainstem (de Farias et al. 2016; Liu et al. 2016). In fact, OS has been found in PD patients who have decreased glutathione (GSH) levels, increased levels of iron, neuromelanin-associated redox-active iron, lipoperoxidation, protein oxidation, and DNA damage in the substantia nigra (SN) (Dexter et al. 1989; Faucheux et al. 2003; Wang et al. 2016).

Epidemiological studies have found that exposure to pesticides is increasingly recognized as a factor involved in the pathogenesis of PD (Greenamyre et al. 2010; Kanthasamy et al. 2012), with multiple effects on motor behavior. In the present study, we used the rotenone, a potent lipophilic pesticide, to imitate the clinical and pathological features of PD. Rotenone induces neurotoxicity, mediated by its ability to inhibit mitochondrial complex I activity, leading to generation of reactive oxygen species (Duty and Jenner 2011). The present study adopted a short-term administration of the rotenone (3 mg/kg/day, s.c.). This dosage induces OS motor as well as cognitive and motor behavior in a similar manner as previously described for early stages of a toxicological PD (Sherer et al. 2003; Morais et al. 2010; Johnson and Bobrovskaya 2015; de Farias et al. 2016). In addition, the well-known systemic rotenone model of PD is dopamine-dependent, given that the motor deficiency observed in rats is reversed by apomorphine (a dopamine receptor agonist), consistent with a lesion of the nigrostriatal dopamine system (Cannon et al. 2009).

Although PD cannot be cured completely at this present point in time, a number of therapies may markedly improve its symptoms. Antioxidant therapy has been suggested to ameliorate the negative effects of this disease (Lau et al. 2005; de Farias et al. 2016). A growing number of studies have been carried out in the form of drug screening for nutraceutical antioxidants as novel preventive and neuroprotective agents, especially those obtained from natural compounds which have low toxicity and can be used via oral administration or taken in the diet (Lau et al. 2005; Giardi et al. 2011; Melo et al. 2011; Kumar and Pandey 2013; de Farias et al. 2016).

Anacardium occidentale, popularly known as the cashew tree, is a tropical tree native to northeastern Brazil. Peduncles and nuts can be eaten raw or converted into various nutritional products (juice, tea, jam, and beverages). The cashew nut shell liquid (CNSL) is a by-product obtained during the processing of cashew nuts from which anacardic acids (AAs), cardanol, cardol, and other phenols are extracted, with AAs as the main active compounds of CNSL (Hemshekhar et al. 2011; Hamad and Mubofu 2015). In vitro assays suggest that the side alkyl chain of AAs and the phenolic ring are responsible for its antioxidant capacity (Trevisan et al. 2006). Moreover, in vivo studies have also shown that AAs display anti-inflammatory, anticancer (Sun et al. 2006), antimicrobial (Mamidyala et al. 2013), and gastroprotective properties (Morais et al. 2010). AAs also protect against epidermal thickening induced by ultraviolet rays (Kim et al. 2013). Enzymatic assays have demonstrated that AAs can hinder the production of superoxide radicals and uric acid by xanthine oxidase (Masuoka and Kubo 2004), and inhibit cyclooxygenase, lipoxygenase (Ha and Kubo 2005), tyrosinase (Kubo et al. 1994), and histone acetyltransferase activities (Dekker and Haisma 2009; Hemshekhar et al. 2011). Noteworthy, additional evidence regarding the safety profile of AAs has demonstrated that oral administration of these compounds at a single dose of 2000 mg/kg is non-toxic. Successive administrations for 30 days at 300, 600, or 1000 mg/kg did not show any genotoxic effect, although slight hematologic effects were observed at the two higher doses, suggesting a safety condition during doses until 300 mg/kg (Carvalho et al. 2011).

All biochemical studies cited above suggest a potential therapeutic use of AAs, particularly for pathological conditions, wherein inflammation and OS occur. Recent in vitro studies, using dopaminergic cell lineages, have demonstrated that AA 6-pentadecylsalicylic acid was able to reduce neuronal damage, modulating histone hyperacetylation induced by pesticides, such as dieldrin and paraquat (Song et al. 2010, 2011). This type of AA was also able to rescue the abnormal motor neuron phenotype in cells derived from patients with amyotrophic lateral sclerosis (Egawa et al. 2012). Nevertheless, none of these studies were carried out in order to test the antioxidant effects of the systemic administration of AAs on the central nervous system under conditions of impaired redox balance. Hydroalcoholic extract of the cashew *Anacardium occidentale* Linn was able to counteract the oxidative stress caused by the pesticide rotenone (Linard-Medeiros et al. 2015). However, the bioactive substances that might be involved with these effects on oxidative stress were not investigated yet. In the present study, we hypothesize that when taken orally, AAs extracted from cashew nuts can exert a preventive effect on rotenone-induced behavioral and lipoperoxidation changes in the nigrostriatal system and cerebral cortex of rats.

Material and Methods

Animals

In the present study, the experiments were conducted with adult male Wistar rats *(Rattus norvegicus* var. Albinus) weighing 250 to 300 g, from the Department of Physiology and Pharmacology, Federal University of Pernambuco (UFPE). The animals were maintained under standard

environmental conditions (12-h light/dark cycle; light on 6 am) and temperature (22 ± 2 °C) and fed with a balanced commercial chow (Presence®, Purina, Brazil) and water ad libitum. Experimental protocols were approved by the Ethics Committee on Animal Use of the Federal University of Pernambuco, Brazil, under license no. 23076.014558/2011-55, according to the standards recommended by the Brazilian College for Animal Experimentation.

Drugs and Solvents

Rotenone was purchased from Sigma (St. Louis, MO, USA) and dissolved (3 mg/mL) in dimethyl sulfoxide (DMSO) (10% *w/v* in sunflower oil, pH 7.4) for 5 days prior to the beginning of treatment. During the treatment period, the rotenone solution was stored in a refrigerator (6 °C).

Cashews (*A. occidentale*) were harvested at the Embrapa Tropical Agroindustry Experimental Station, located in Paraipaba, Ceará, Brazil, during the 2009 summer season. The fruit came from a commercial cultivar (CCP-76) whose genetic material is maintained in Embrapa's germplasm bank. The fresh cashew fruit, supplied by Dr. Edy Sousa de Brito (Embrapa, Fortaleza, Brazil), was manually separated from the nuts. The AAs used in this study were extracted from the CNSL, provided by Dr. Maria Teresa Salles Trevisan—Federal University of Ceará, Fortaleza, Brazil. The plant extract preparation and the chemical characterization of these compounds was previously described by Trevisan et al. (2006). Briefly, 10 g of cashew nuts, apple, and fibers was extracted twice with hexane in a Soxhlet device. Thereafter, the lipid fraction was extracted again using methanol. The purification of AAs, cardanols, and cardols from CNSL was carried out according to the following steps: AAs were isolated as their calcium salts, the acid-free CNSL was treated with ammonia and extracted with hexane/ethyl acetate (98:2) to separate the mono-phenolic cardanol components. Then, it was proceeded a new extraction with ethyl acetate/hexane (80:20) to yield the cardols. The separation using analytical HPLC was done using a Hewlett-Packard (HP) 1090 liquid chromatography fitted with a C-18, reversed phase (5 μm) column (25 cm × 4 mm ID; Latek, Eppelheim, Germany). For the mobile phase, 2% acetic acid was used in double distilled water (solvent A) and methanol (solvent B) over a total run time of 40 min: initially 50:50 solvents A and B followed by an increase in solvent B to 100% for 20 min, held isocratically for a further 20 min at a flow rate of 1.0 mL/min. Phenolic compounds in the eluent were detected with a UV-diode-array detector (HP1040 M) set at 250, 278, and 315 nm. The amount of phenolic compounds in the extracts and alkyl phenol fractions was determined using calibration curves, generated with authentic standards in duplicate. It was measured the UV absorption at kmax as a function of concentration in the range 0.025–4.0 mM.

The chemical structure of the AAs present in the mix is illustrated in Fig. 1b. This mix was stored at − 16 °C. Immediately before use, it was dissolved in a Tween 80 solution (2% *w/v* in water). The wide dose range of the AAs (1–100 mg/kg/day) was chosen for identification of the minimum dose necessary for their beneficial effects on oxidant or behavior changes induced by rotenone, considering that the sensitivity of these parameters to deleterious exposure of this pesticide is not similar (Greenamyre et al. 2010). The short time course, dose (3 mg/kg/day), and route chosen for rotenone administration have been recognized as effective for inducing motor and cognitive impairment, as well as OS and neurochemical effects in the SN and striatum, similar to the early stages of PD (for review, see Johnson and Bobrovskaya 2015).

Treatments

The animals were randomly divided into nine experimental groups and treated over five consecutive days ($n = 6$–18 animals/group). The daily treatment with the drugs was carried out in the morning, between 8 and 9 am. Rats from group 1 ($n = 15$ rats) received Tween 80 (2% *w/v* in water) by gavage (1 mL/kg) and 1 h after, vehicle (sunflower oil 90% *w/v* + DMSO 10% *w/v*; s.c.). Group 2 ($n = 15$ rats) received Tween 80 (2% *w/v*) by gavage and 1 h after, rotenone (3 mg/kg/day, s.c.). Groups 3–8 ($n = 15$ rats) were treated with AAs at 1, 3, 10, 25, 50, or 100 mg/kg/day by gavage and 1 h after, rotenone (3 mg/kg/day, s.c.). Twenty-four hours after the last day of treatment, the rats were evaluated by behavioral tests. Group 9 ($n = 6$ rats) was treated with AAs alone at 100 mg/kg/day by gavage, in order to test whether this compound per se is able to act on superoxide dismutase activity. In the majority of the rats (95%), this 5-day rotenone treatment did not induce mortality. For analysis of gene expression of the cytoplasmic Cu/Zn superoxide dismutase (SOD-1) and mitochondrial MnSOD (SOD-2), an additional 12 animals were used. These animals were treated with the vehicle sunflower oil 90% *w/v* ($n = 6$) or AAs alone, diluted in sunflower oil at the dose 100 mg/kg/day

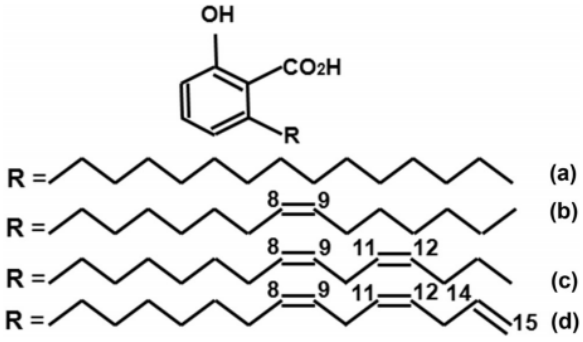

Fig. 1 The chemical structure of the four anacardic acids contained in the mix used in the present study. a = 15:0 anacardic acid; b = 15:1 anacardic acid; c = 15:2 anacardic acid 2; d = 15:3 anacardic acid

($n = 6$). For analysis of a direct effect of AAs on complex 1 of mitochondrial chain, 12 animals were used.

Behavioral Tests

Behavioral tests were also conducted in the morning between 8 and 11 am, starting with the control groups and followed by the AAs-treated groups. Between the administration of the last drug and prior to the behavioral tests, the animals were habituated for 24 h to the environment of the test room. The tests were carried out in the following sequence: open field test, T-maze test, and rotarod test. On the second day, the T-maze test was repeated.

Exploratory and General Locomotor Activity

To quantify exploratory and general locomotor activity, the open field test was carried out according to Gould et al. (2009). Each rat was placed by itself in the center of an open field arena (a circular wooden box with a diameter of 100 cm and 40 cm high, with a floor divided into 20 equal squares). The frequency of locomotion (number of times that each animal entered into the delimited regions with its four paws), rearing frequency (number of times that the animal stood on its hind legs, with the trunk perpendicular to the floor of the arena), immobility time (time the animal stood without making any movement), and time to start of movement (time for the animal to start moving out of the center of the open field arena) were assessed for 5 min. The open field arena was cleaned with 5% ethanol solution before each test, with each animal being placed in the arena only after the smell of the alcohol had vanished.

The Elevated T-Maze Test

To measure spatial memory performance, the elevated T-maze test was carried out according to Kumar et al. (2006), who used this test in order to evaluate limbic and cognitive memory performance in experimental models of basal ganglia impairment. The elevated T-maze consisted of one open arm (50 × 10 cm) and two closed arms of the same dimensions, facing each other and a central area (10 × 10 cm) connecting the arms. The walls of the closed arms were 40 cm tall. The rats were placed individually at the end of the open arm facing away from the central area. The time taken by the animal to move from the open arm to either of the closed arms is called the initial latency. Rats were allowed to explore the maze for 30 s after acquisition of the initial latency, and were then returned to their cages. The learning retention latency was evaluated again the next day. The percentage of memory retention was calculated using the following formula: (initial latency − latency repetition) × 100/initial latency.

Rotarod Test

In order to quantify motor deficiency, the rotarod test was used at a fixed speed, according to Monville et al. (2006). To perform this test, the animal was placed with all four paws on a bar with a diameter of 7.0 cm and set 25 cm above the floor. The bar rotated at a speed of 25 rpm. Before being submitted to the different treatments, the rats were trained in two sessions of 180 s each for habituation. Animals were placed on the rotating bar and the time of stay on the bar was recorded. A cutoff time of 180 s was maintained throughout the experiment. The average results were recorded as time of fall. Immediately after the behavioral tests, the animals were euthanized for biochemical assays.

Biochemical Studies

Tissue Dissection and Homogenization

For biochemical analysis of lipoperoxidation and total superoxide dismutase (t-SOD) activity, pooled tissue from three independent control and experimental animal groups containing five animals/group were used (total $n = 45$ animals /per each experiment). After behavioral assessments, the animals were anesthetized and then decapitated. The brains were removed and placed on ice. The striatum, cortex, and SN were dissected and weighed. After weighing, the pooled tissue of each region was homogenized in a Potter-Elvehjem glass homogenizer with phosphate-buffered saline (PBS 1:10 *w*/*v*), to which butyl hidroxy toluene (BHT, 0.004%) had been added to prevent autoxidation of the samples. The homogenate was centrifuged at 10,000×*g* for 30 min at 4 °C and an aliquot of supernatant was separated for biochemical analysis. Each individual experiment was carried out in triplicate.

Measurement of Lipid Peroxidation

Quantitative measurement of lipid peroxidation was performed according to Buege and Aust (1978). The amount of malonaldehyde (MDA) present in the samples was quantified by the reaction with thiobarbituric acid (TBA). Aliquots of 500 μL of the supernatant were added to 1 mL of a TBA solution: 0.38% (*w*/w) TBA, 250 mL of 1 N hydrochloric acid, 15% (*w*/*w*) trichloroacetic acid, and 20 mL of 2% (*w*/*v*) ethanolic BHT. The solution was heated at 100 °C for 15 min, followed by cooling in an ice bath. Then, 1.5 mL of *n*-butanol was added. The mixture was shaken and centrifuged at 3000×*g* for 20 min. After centrifugation, the upper phase was collected and analyzed in a spectrophotometer (CARY 3E UV-Visible Spectrophotometer Varian, Inc., Brazil) at 532 nm. The results were expressed as nanomole per milligram of protein, using a standard curve generated with different concentrations of a 1,1,3,3-tetramethoxypropane solution.

Effects of AAs on Rotenone-Induced Inhibition in the Complex 1 of the Mitochondrial Respiratory Chain

In order to check if the antioxidant effect of AAs could involve a direct effect on the complex 1 of the respiratory chain, we carried out assays using isolated mitochondria in 12 adult animals used in the control group. The animals were sacrificed by decapitation. Immediately after sacrifice, the SN and cerebral cortex were dissected on ice. Individual tissues of cerebral cortex and pooled tissue of three SN per experiment were homogenized in a buffer solution containing 225 mM mannitol, 75 mM sucrose, 4 mM HEPES, 2 mM taurine, and 0.5 mM EGTA, pH 7.4. The homogenates were centrifuged at 2500 rpm (461 g) for 5 min at 4 °C. Then, the supernatants were centrifuged at 11,500 rpm (9760 g) and the pellets were re-suspended in a buffer containing 120 mM KCl, 4 mM HEPES, 5 mM K_2HPO_4, and 0.2% BSA (*w*/*v*) (pH 7.4). The mitochondrias were kept on ice until the moment of the assay (Lagranha et al. 2010). Total protein quantification was done according to Bradford assay. In order to quantify the O_2 consumption, samples of mitochondria containing 1 mg protein/mL were incubated in the same buffer described above, in a 600 SL chamber connected to a Clark-type oxygen electrode (Hansatech Instruments, Pentney King's Lynn, UK) at 28 °C. The basal condition of mitochondrial respiration was assessed using Complex I substrates (10 mM glutamate + 2 mM malate + 2.5 mM EGTA). Then, 1.8 μM rotenone was added to the solution, followed by AAs 0.4 or 1.5 μM (diluted in ethanol) 1.5 min after. The O_2 consumption (nmol/min/mg protein) was assessed during these three experimental steps. Three independent experiments were carried out.

Total Superoxide Dismutase Activity

Total SOD enzymatic activity in the SN, cortex, and striatum was determined according to Misra and Fridovich (1972). This method relies on the ability of t-SOD to inhibit epinephrine oxidation. Briefly, 100 μL of the homogenate was added to 880 μL of a carbonate buffer (0.05 M, pH 10.2, 0.1 mM ethylene-diamino-tetracetic acid). Twenty microliters of epinephrine (30 mM in 0.05% acetic acid) was added to the mixture and the reading was performed in a spectrophotometer (CARY 3E UV-Visible Spectrophotometer Varian, Inc., Brazil) at 480 nm. The enzymatic activity is expressed as the amount of enzyme that inhibits 50% of the epinephrine oxidation; equal to 1 unit.

Analysis of SOD-1 and SOD-2 Gene Expression in the Substantia Nigra and Striatum

For analysis of gene expression of the cytoplasmic Cu/ZnSOD (SOD-1) and the mitochondrial MnSOD (SOD-2), 12 animals were used. Animals were divided in two groups according to the treatment. The gavage was performed during a period of 5 days. The control group was treated with the vehicle sunflower oil 90% *w*/*v* ($n = 6$) and the other group was treated with only AAs diluted in sunflower oil at the dose 100 mg/kg/day ($n = 6$). After anesthesia with isoflurane, the animals were decapitated. Then, the SN and striatum were dissected on ice, put in sterile eppendorfs and kept in − 80 °C deep freezer until the analysis. The RNA was extracted from SN and striatum using TRIzol® protocol (Invitrogen®). Five hundred nanograms of RNA was used for real-time PCR analysis. PCR was performed in a 15-μL reaction mixture, containing 7.5 μL 2× SYBR Green Reaction Mix (Invitrogen®), 0.3 μL each primer (10 pmol), 0.3 μL Super Script III RT/Platinum Taq Mix (10 pmol/μL), sample, and DEPC water according to the protocol described by Barroso et al. (2017). The following gene-specific rat primers were used: SOD-1: sense-AGCATTCCATCATTGGCCGT, antisense-CGCAATCCCAATCACTCCAC; SOD-2: sense-GCGACCTACGTGAACAATCT, antisense-CAGCAACT CTCCTTTGGGTT. The data were normalized using the Beta-2 microglobulin (B2M) as housekeeping gene: sense-CTTGAATTTGGGGAGTTTTCTG, antisense-TGACCGTGATCTTTCTGGTG. Reactions were performed using Rotor-Gene Q Real-Time PCR System (Qiagen®).

Statistical Analysis

The results are expressed as mean ± standard error of the mean (SEM). The difference between groups was assessed by one-way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison post-hoc test to determine the significance level. Statistical analysis was performed using Graph Pad Prism® 5.0 (GraphPad Software, Inc., La Jolla, CA 92037, USA). *P* values less than 0.05 were considered statistically significant.

Results

Effect of AAs on Locomotor Activity

Administration of only rotenone for 5 days significantly reduced (~ 80%) the number of crossings in the ambulatory activity (Fig. 2a; $F = 41.91$ and $P < 0.0001$) and rearings (Fig. 2b; $F = 67.97$ and $P < 0.0001$). Rotenone also increased the duration of immobility (Fig. 2c; $F = 78.36$ and $P < 0.0001$) and time to start movement (Fig. 2d; $F = 53.62$ and $P < 0.0001$) of rats in the open field test.

Treatment with AAs at the doses 25, 50, and 100 mg/kg/day significantly reduced deficits induced by rotenone ($P < 0.0001$) in both ambulatory activity and rearings. At the dose 100 mg/kg/day, AAs increased the ambulatory activity when compared to the control condition. A

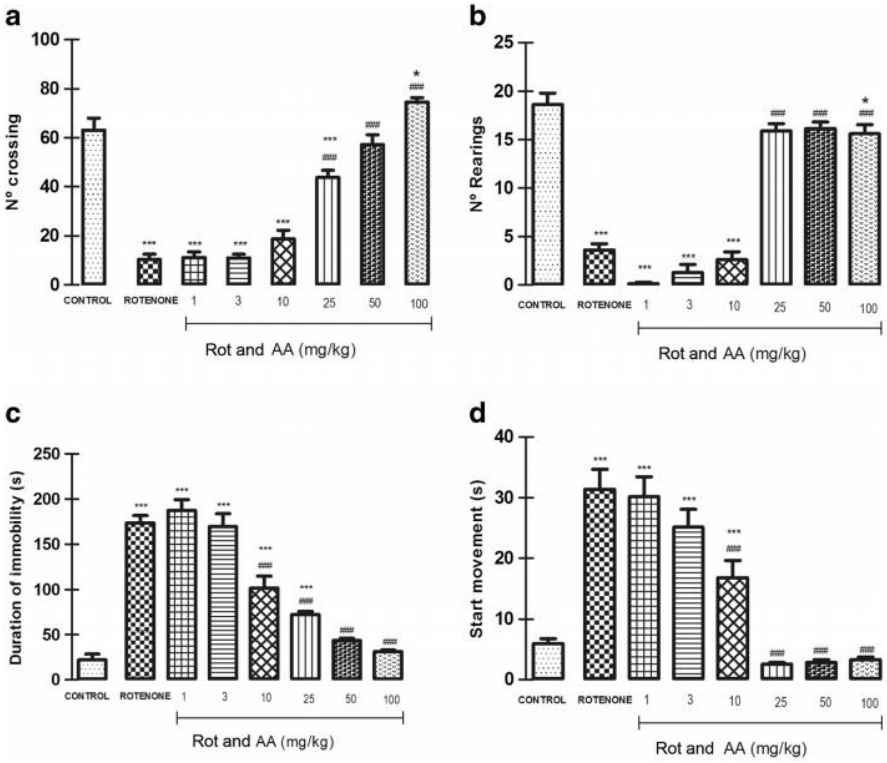

Fig. 2 Animals' locomotor activity. Effects of treatment with AAs (1, 3, 10, 25, 50, and 100 mg/kg/day) on the rats' performance in the open field test. Rats received 3 mg/kg/day rotenone for 5 days ($n = 8$ per group). **a** Total ambulatory activity; **b** rearings; **c** duration of immobility; **d** time for starting movement. Each bar represents a mean ± SEM. $^{***}P < 0.0001$, $^{*}P < 0.05$ vs. control group. $^{\#\#\#}P < 0.0001$, $^{\#}P < 0.05$ vs. rotenone group. One-way analysis of variance followed by Newman-Keuls Multiple Comparison test

significant dose-dependent decrease in the immobility time and the latency to start movement was observed when AAs were administered from the dose 10 mg/kg/day (F = 78.36 and $P < 0.0001$; Fig. 2c, d), and the values matched the control levels between 25 and 100 mg/kg/day.

Effect of AAs on Memory Performance in the Elevated T-Maze Test

Treatment with rotenone caused marked memory loss, as shown by the significant decrease (~90%; F = 76.93 and $P < 0.0001$) in the percentage of memory retention compared with the control group. Daily treatment with AAs (1, 3, 10, 25, 50, and 100 mg/kg/day) 1 h before the administration of rotenone increased the percentage of memory retention in rats, when compared with treatment of rotenone alone (F = 76.93 and $P < 0.0001$) (Fig. 3). At the doses 10, 25, 50, and 100 mg/kg/day, AAs treatment was able to recover memory retention completely when compared to the control condition (F = 76.93 and $P < 0.0001$).

Effect of AAs on Rotarod Test

Rotenone caused a similar decrease (~90%) in animal permanence in the rotarod test. Daily treatment with AAs (3, 10, 25, 50, and 100 mg/kg/day) for 5 days significantly increased permanency time in the rotarod, compared to the rotenone group (F = 17.97 and $P < 0.0001$). The effect of 100 mg/kg/day AAs on the permanency time in the rotarod was similar to that observed in the control condition (Fig. 4).

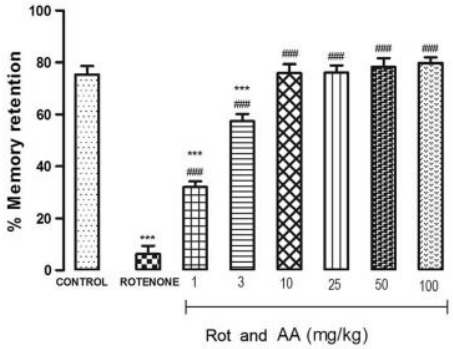

Fig. 3 Animals' memory performance. Effects of treatment with AAs (1, 3, 10, 25, 50, and 100 mg/kg/day) on rats' performance in the elevated T-maze test. Rats received 3 mg/kg/day rotenone for 5 days ($n = 8$ per group). Each bar represents a mean ± SEM. $^{***}P < 0.0001$ vs. control group. $^{\#\#\#}P < 0.0001$ vs. rotenone group. One-way analysis of variance followed by Newman-Keuls Multiple Comparison test

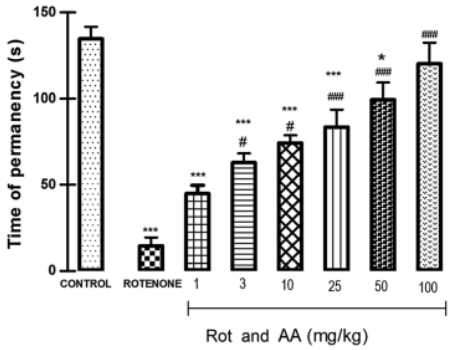

Fig. 4 Rats' performance in the Rotarod. Effect of AAs treatment (1, 3, 10, 25, 50, and 100 mg/kg/day) on rats' performance in the rotarod test. Rats received rotenone 3 mg/kg/day rotenone for 5 days ($n = 8$ per group). Each bar represents a mean ± SEM. $^{***}P < 0.0001$, $^{*}P < 0.05$ vs. control group. $^{\#\#\#}P < 0.0001$, $^{\#}P < 0.05$ vs. rotenone group. One-way analysis of variance followed by Newman-Keuls Multiple Comparison test

Effect of AAs on Brain Lipoperoxidation and Mitochondrial Respiratory Chain

The administration of rotenone for 5 days induced lipoperoxidation, as indicated by the significant increase (~ 26-fold) of MDA levels in the SN, cortex, and striatum, in comparison to the control group. Treatment with AAs (1, 3, 10, 25, 50, and 100 mg/kg/day) 1 h before the administration of rotenone attenuated lipoperoxidation levels in the three regions analyzed. In the SN (Fig. 5a), 1 and 3 mg/kg/day were able to reduce the effect induced by rotenone by about 10-fold. Doses between 10 and 100 mg/kg/day produced lipoperoxidation levels that were similar to and almost matched the control condition ($F = 3681$ and $P < 0.00001$ vs rotenone alone). In the cerebral cortex (Fig. 5b), a dose-dependent effect was induced by AAs, and the values matched the control levels between 10 and 100 mg/kg/day ($F = 726.2$ and $P < 0.00001$ vs rotenone alone). In the striatum (Fig. 5c), a tendency for a dose-dependent effect was also observed, particularly from 3 to 50 mg/kg/day ($F = 518.7$ and $P < 0.00001$). In all the regions, the best preventive doses of AA were 25 and 50 mg/kg/day.

The antioxidant effect of AAs involves a direct effect on the complex 1 of the mitochondrial respiratory chain. In the SN (Fig. 6a), the basal O_2 consumption (mean ± SD) was 2.44 ± 0.98 nmol/min/mg protein. This consumption was reduced by 1.8 μM rotenone to 1.14 ± 0.38 nmol/min/mg protein ($P = 0.05$). AAs at both concentrations were able to completely reverse rotenone-induced inhibition of O_2 consumption (5.01 ± 1.32 nmol/min/mg protein at 0.4 μM ($P < 0.0001$) and 4.03 ± 0.58 nmol/min/mg protein at 1.5 μM ($P = 0.006$)). At 0.4 μM, the O_2 consumption was higher than in the basal condition ($P = 0.004$).

In the cerebral cortex (Fig. 6b), the basal O_2 consumption was 5.03 ± 1.2 nmol/min/mg protein, which was reduced by

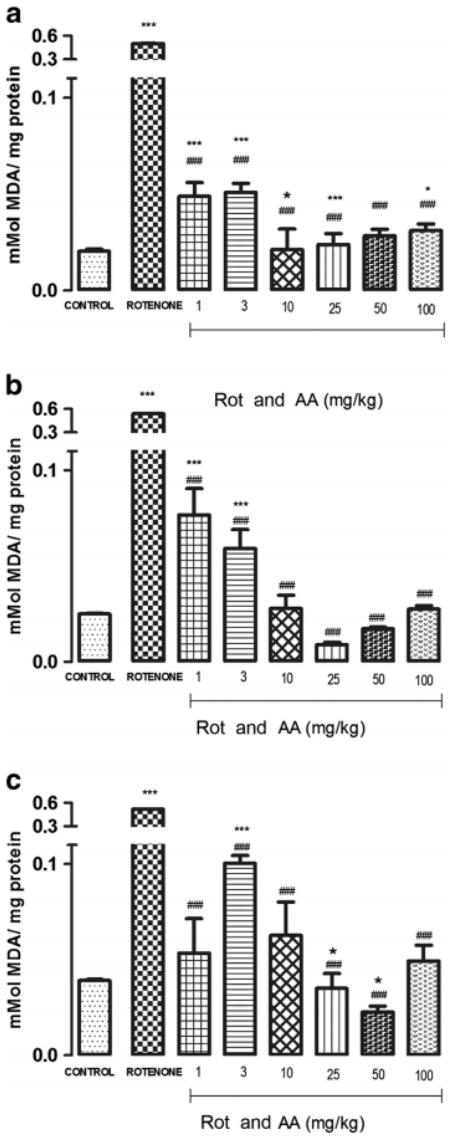

Fig. 5 Lipoperoxidation assay. Levels of thiobarbituric acid-reactant substances (TBARS) in the substantia nigra (**a**), cerebral cortex (**b**), and striatum (**c**) of rats from the control and experimental groups. Rats were treated with either rotenone alone or rotenone coupled with AAs (1, 3, 10, 25, 50, and 100 mg/kg/day). Each bar represents a mean ± SEM. $^{***}P < 0.00001$, $^{*}P < 0.05$ vs. control group. $^{\#\#\#}P < 0.00001$, $^{\#}P < 0.05$ vs. rotenone alone group. One-way analysis of variance followed by Newman-Keuls Multiple Comparison test. ($n = 10$ per group)

rotenone to 1.98 ± 0.56 nmol/min/mg protein ($P = 0.039$). At both concentrations, AAs reversed the effect induced by rotenone (6.96 ± 2.40 nmol/min/mg protein at 0.4 μM ($P = 0.006$) and 8.68 ± 3.31 nmol/min/mg protein at 1.5 μM ($P < 0.0001$)). At this later concentration, AAs significantly increased the O_2 consumption compared to the basal condition ($P = 0.04$).

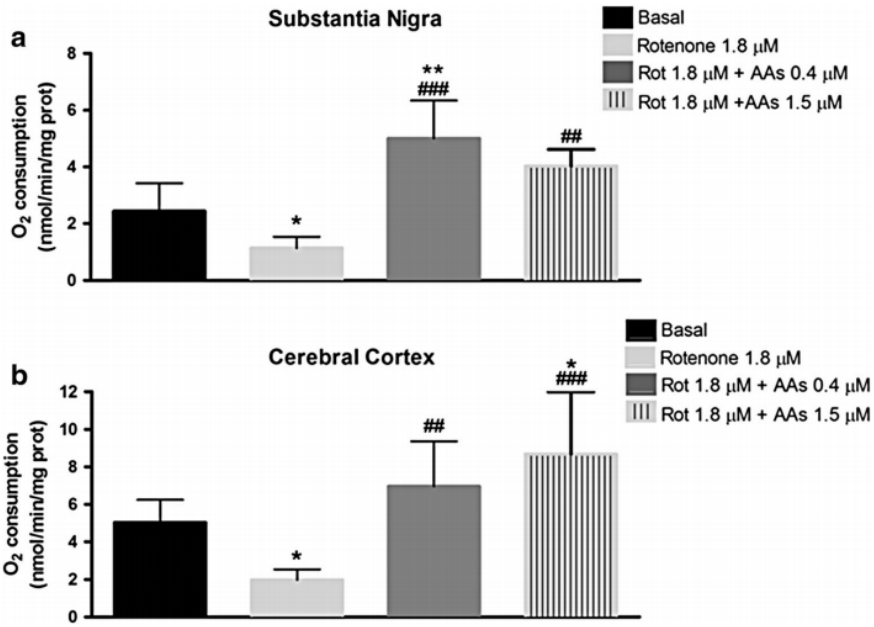

Fig. 6 Isolated mitochondria assay. Oxygen consumption (nmol/min/mg protein) in isolated mitochondria from substantia nigra (**a**) and cerebral cortex (**b**) at basal state, after 1.8 μM rotenone application or after 1.8 μM rotenone followed by 0.4 or 1.5 μM AAs 1.5 min after. Each bar represents a mean ± SD. $^{**}P < 0.01$, $^{*}P < 0.05$ vs. basal condition. $^{###}P < 0.0001$, $^{##}P < 0.01$ vs. rotenone alone. One-way analysis of variance followed by Newman-Keuls Multiple Comparison test. ($n = 10$ per group)

Effect of AAs on t-SOD Activity and SOD-1 or SOD-2 Gene Expression in the SN and Striatum

Rotenone alone produced a decrease in t-SOD activity when compared with the control group in the SN and cerebral cortex (Fig. 7a, b), but not in the striatum (Fig. 7c). Treatment with AAs (25, 50, and 100 mg/kg/day) plus rotenone significantly increased the activity of this antioxidant enzyme in all regions analyzed: Fig. 7a; ($F = 95.12$); Fig. 7b ($F = 111.1$); Fig. 7c ($F = 81.37$) and $P < 0.0001$. Doses of 25 and 50 mg/kg/day AAs were equally effective in elevating activity of t-SOD in the SN and cerebral cortex, while 100 mg/kg/day significantly increased this effect compared to lower doses (Fig. 7a, $F = 95.12$ and 6B, $F = 111.1$; $P < 0.0001$). In the striatum, a dose-dependent effect was detected between the doses 25 and 50 mg/kg/day; however, no additional increase was induced by AAs at 100 mg/kg/day (Fig. 6c). In order to clarify mechanisms involved in this effect on t-SOD, one animal group was treated with AAs at 100 mg/kg/day in the absence of rotenone. As can be seen in Fig. 7, the treatment with AAs was also able to increase t-SOD activity, compared to the control condition. This increase varied according to the region and corresponded to about 80% in the SN, 53% in the cerebral cortex, and 230% in the striatum.

As depicted in the Fig. 8, a significant increase in the gene expression of SOD-1 (~2490-fold; $P < 0.000001$) and SOD-2 (190-fold; $P < 0.000001$) was detected in the striatum of the group treated with AAs 100 mg/kg/day in the absence of rotenone, compared to the control condition. In the SN, increased SOD-2 gene expression was detected (2.63-fold the control condition; $P \leq 0.05$), while no intergroup difference was found for SOD-1 gene in this nucleus.

Discussion

Although the pathogenic mechanisms underlying pesticide-induced neurotoxicity are considered multifactorial (Kanthasamy et al. 2012), OS in particular contributes to this injury, increasing the probability of developing PD (Sherer et al. 2003; Norazit et al. 2012; Sanders and Greenamyren 2013). The present study tested the hypothesis that AAs can exert a preventive antioxidant action upon the rat nigrostriatal system and cerebral cortex, and are able to preclude motor and cognitive behavioral deficits induced by the pesticide rotenone.

It is well established that rotenone can modify various aspects of behavior: inducing neurodegeneration and neuroinflammation in the SN, reproducing the pathological hallmarks of PD (Alam and Schmidt 2002; Sherer et al. 2003; Johnson and Bobrovskaya 2015; von Wrangel et al. 2015). In line with the rotenone-related risks to one's health state, it is noteworthy that humans can be affected by rotenone through oral, dermal, and inhalation routes (Finlayson et al. 2012). In rodents, Sherer et al. (2003) reported that 2.75 mg/kg rotenone via subcutaneous route for 7 days is enough to induce focal neuron loss in both SN and striatum, accompanied by behavioral changes similar to the early stages of PD. Adopting a similar experimental model, the present study reproduced the rotenone-induced OS and behavior impairment. This corroborated the initial hypothesis, demonstrating a beneficial effect of AAs in all parameters analyzed.

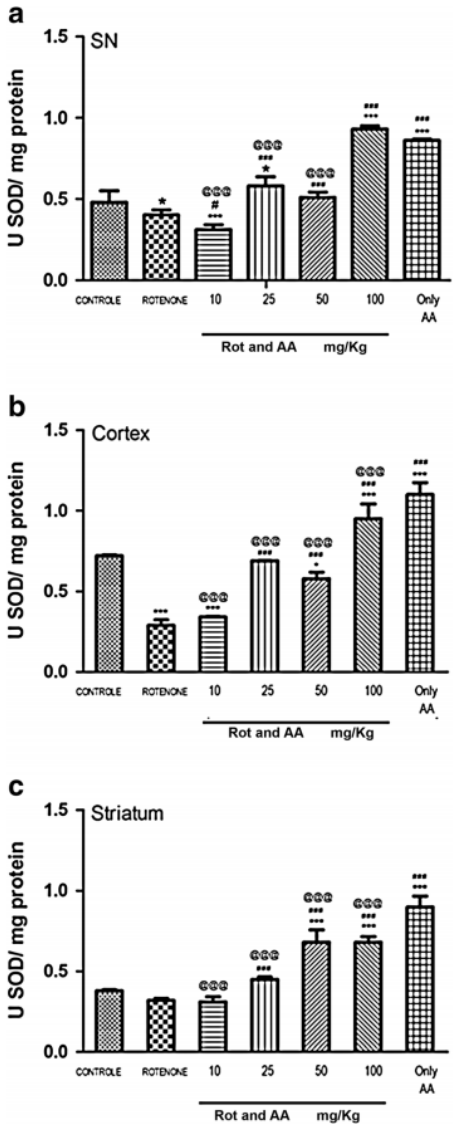

Fig. 7 Superoxide dismutase activity. Total superoxide dismutase (t-SOD) activity in the substantia nigra (**a**), cerebral cortex (**b**), and striatum (**c**) of rats in the control and experimental groups treated with rotenone alone, or rotenone coupled with AAs (10, 25, 50, and 100 mg/kg/day), or animals that received only AAs at the dose 100 mg/kg/day. Each bar represents a mean ± SEM. $^{***}P < 0.00001$, $^{*}P < 0.05$ vs. control group. $^{\#\#\#}P < 0.00001$, $^{\#}P < 0.05$ vs. rotenone group. @@@$P < 0.00001$ compared to AAs 100 mg/kg/day in the presence or absence of rotenone. One-way analysis of variance followed by Newman-Keuls Multiple Comparison test ($n = 15$ per group)

Preventive Action of Anacardic Acids on Rotenone-Induced Behavioral Impairment

Motor and cognitive behavior reflects the coordinated function of a large portion of the neural network, involving sensoriomotor and integrative processes. Dysfunctions in these systems are important biomarkers associated with different stages of PD (Morais et al. 2012; Sanders and Greenamyren 2013). In the present study, rotenone taken over 5 days reduced about 90% the time of permanence in the rotarod, as well as the memory retention time, compared to the control condition. Pretreatment with AAs at doses of 1 or 3 mg/kg/day was enough to improve these parameters to around 3- and 5-fold, respectively. Nevertheless, the dose-dependent effects of AAs in these tests were distinct. In the elevated T-maze, AAs at 10 mg/kg/day completely reversed rotenone-induced effects, whereas in the rotarod test, the maximum effect was only observed at ten times this (100 mg/kg/day). Thus, two different pathways related to the midbrain dopaminergic systems were differentially affected by AAs. The mesocorticolimbic system involved in the working memory (Groenewegen 2003) was more sensitive to the effects of AAs, than the nigrostriatal system. This latter is mainly associated with motor performance, especially under conditions that require balance (rotarod test) (Graybiel 2000, Groenewegen 2003; Monville et al. 2006).

Early studies along with a recent investigation utilizing optogenetics for monitoring animal locomotion in an open field demonstrated that this motor activity requires the activation of the direct and indirect pathways in the basal ganglia complex (Roesler et al. 1999; Graybiel 2000; Gould et al. 2009; Freeze et al. 2013). Therefore, the open field test was used in the present study as a measure of exploratory behavior and general activity related to neural circuits associated to these regions. Number of crossings assesses the ambulatory movement; rearing, the exploratory behavior; and the latency to start the movement mainly reflects animal motivation. The administration of AAs in a dose range of 10 to 100 mg/kg/day was able to prevent rotenone-induced decline on these parameters in a dose-dependent manner, reinforcing the efficacy of this bioactive substance in restoring the activity of neural circuits whose function is impaired in PD.

Anacardic Acids Attenuated Rotenone-Induced Oxidative Stress Modulating Mitochondria Respiration Chain, Superoxide Dismutase Gene Expression, and Activity

AAs treatment almost completely blunted lipoperoxidation provoked by rotenone in the lowest dose of 1 mg/kg/day. This protective effect was similar in order of magnitude among SN, cerebral cortex, and striatum (~92, 87, and 90% reduction, respectively) and occurred with all doses used. These findings are in part similar to findings that have recently been reported for systemic administration (50 mg/kg/day) of ferulic acid (a hidroxycinamic acid highly abundant in the seeds and leaves of many plants), or Glycyrrhizic acid (a saponin obtained from licorice root, in a rotenone model of PD) (Ojha et al. 2015, 2016). Nevertheless, the antioxidant

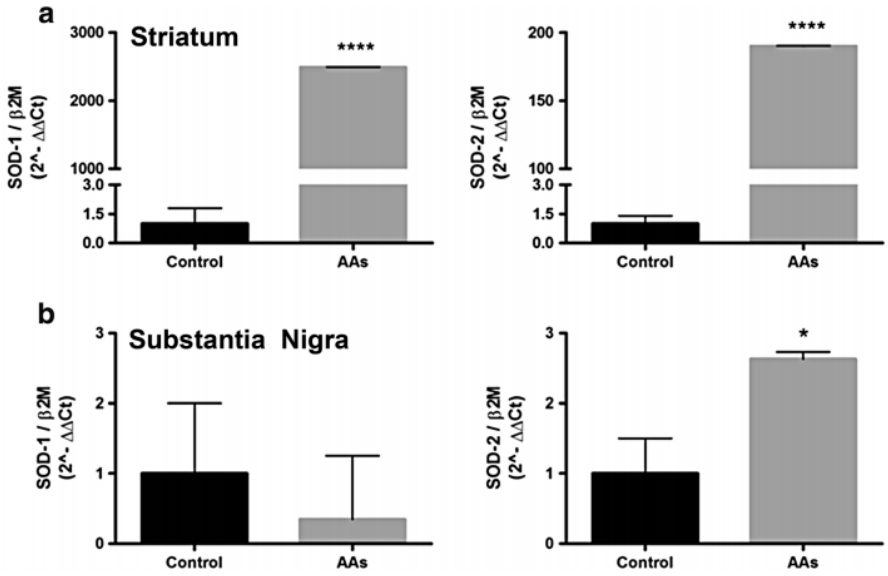

Fig. 8 SOD-1 and SOD-2 gene expression. mRNA levels of the cytoplasmic Zn/Cu SOD (SOD-1) and mitochondrial MnSOD (SOD-2) in the striatum (**a**) or substantia nigra (**b**) of control group or experimental group treated with AAs at the dose 100 mg/kg/day. The values illustrate the relative expression levels of each transcript by the threshold cycle comparative method (2^-Ct) using β-2 microglobulin (B2M) as a housekeeping gene. *$P < 0.05$; ****$P < 0.000001$ compared with the respective controls (Student's t test; $N = 5$ animals per group)

efficacy of pretreatment with ferulic acid was reduced in the dose of 80 mg/kg/day when administered for 7 days before the toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine hydrochloride (MPTP), another model of PD. With respect to the cerebral cortex, the antioxidant action of AAs up to a dose of 100 mg/kg/day was more protective against OS than ascorbic acid, as ascorbic acid induced pro-oxidative effects in this brain region when taken by oral route in doses of 60 and 120 mg/kg/day (Mendes-da-Silva et al. 2014).

The effects of AAs on rotenone-induced lipoperoxidation were at least in part due to a stimulatory action on the t-SOD enzyme. These results were supported by the fact that the AAs were effective in elevating t-SOD activity even in the absence of rotenone. To our knowledge, this is the first evidence of a modulatory effect of these compounds per se on this antioxidant enzyme, providing a novel molecular mechanism of action of AAs. The screening of natural products with ability to mimic or enhance SOD activity has been investigated by some authors, especially considering that this enzyme can be impaired under some degenerative conditions and cannot be efficient when administered via oral route (Kim et al. 1994). Activation of ethanolic extracts from various fruits, vegetables, and mushrooms on SOD activity in the human erythrocyte has been reported. For example, increases of 40 and 48% in t-SOD/activity were induced by extracts obtained from onions and watermelons, respectively. Additionally, at least 8% in this enzymatic function was provoked by soybean sprouts (Kim et al. 1994). Previous evidence also indicates that a polyphenol curcumin supplement in the diet of 6- and 24-month-old rats resulted in significant increases in SOD activity in various brain regions of rats (Bala et al. 2006), as well as in the *Drosophila*, where the increase ranged from 8.4 to 32% according to gender (Shen et al. 2013). In the present study, the increase in the t-SOD activity promoted by AA at 100 mg/kg/day was more expressive and corresponded to ~ 80% in the SN, ~ 53% in the cerebral cortex, and ~ 230% in the striatum, indicating a differential sensitivity of this enzyme according to the region. These results are in part due to a direct and differential effect of AAs on SOD gene expression. In the striatum, the gene expression of both SOD-1 and SOD-2 was increased by a factor of 2490- and 190-fold, respectively. In the SN, only the levels of mitochondrial SOD-2 gene were increased and by a factor of 2.6-fold compared to that found in the control group. Such findings suggest beneficial effects of AAs, not only restricted to the degenerative condition herein analyzed but also to other disorders that involve OS. Studies on regulation of SOD genes have shown that gene coding for Cu/ZnSOD (SOD-1) are especially modified on alteration in the coding region, while regulatory elements of the MnSOD (SOD-2) gene reside in both the non-coding and coding regions. In addition, changes associated with SOD-2 usually modify its expression levels, as well as protein function (review in Miao and St Clair 2009). The mechanisms involved in the action of AAs on SOD-1 or SOD-2 gene expression is not clear at this moment, but encourages future studies considering the importance of this action for a wide range of neurodegenerative disorders involving the basal ganglia.

Besides SOD activity and gene expression, the antioxidant action of AAs observed in our study was also due to a direct effect of these substances on the mitochondrial respiratory chain. The ability of AAs to completely reverse the rotenone-induced inhibition on complex I demonstrated another mechanism of action by which AA exert antioxidant effects. This data reinforces our initial hypothesis that these

substances could display a potential neuroprotective action in the central nervous system. Moreover, it is important to consider the presence of alkyl side chains, which confer to AAs an ability to inhibit the generation of superoxide anions and uric acid by xanthine oxidase (Trevisan et al. 2006). Moreover, it should be taken into account the pharmacological action of AAs as a non-selective inhibitor of histone acetyltransferase (HAT). It has been demonstrated that rotenone treatment and ROS production can increase histone acetylation as one of the mechanisms involved in hepatic cellular damage (Choudhury et al. 2010). In addition, in a cell culture model of Parkinson's disease, using the rat mesencephalic dopaminergic cell line (N27 cells), inhibition of HAT by the anacardic acid 6-pentadecylsalicylic acid (present in our AAs mix) protected against neuronal cell death induced by the paraquat or dieldrin pesticides (Song et al. 2010, 2011). Our laboratory is currently investigating epigenetic mechanisms involved in rotenone-induced neurodegeneration to address this issue.

Conclusion

The present findings demonstrate for the first time that systemic administration of AAs exerts protective action on an experimental model of Parkinson's disease. As observed in our research protocol, sufficient amounts of the AAs were able to cross the brain-blood barrier and reach the central nervous system to display positive effects. Therefore indicating that there was an adequate AAs bioavailability. Beneficial effects of AAs were evident, given their ability to reduce rotenone-induced behavioral impairment, as well as OS in the nigrostriatal system. Our results also introduce novel mechanisms of AAs action, such as a direct effect on mitochondrial respiratory chain and the upregulation of SOD gene expression, which consist of potential pharmacological targets of these alkyl phenols. As a result, new perspectives on potential therapeutic use of AAs in neurodegenerative disorders should be considered.

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Analyzed and/or discussed the data: CFB Medeiros-Linard, BLS Andrade-da-Costa, RLopes Augusto, CJ Lagranha, SSL Lafayette, AGWanderley

Wrote the paper: CFB Medeiros-Linard, BLS Andrade-da-Costa, SSL Lafayette

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Compliance with Ethical Standards

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

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ANEXO A – CARTA DE APROVAÇÃO PELA CEUA da UFPE

Universidade Federal de Pernambuco
Centro de Ciências Biológicas

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Recife, 08 de abril de 2015.

Ofício nº 27/15

Da Comissão de Ética no Uso de Animais (CEUA) da UFPE
Para: **Prof.ª Belmira Lara da Silveira Andrade da Costa**
Departamento de Fisiologia e Farmacologia – CCB
Universidade Federal de Pernambuco
Processo nº 23076.005404/2015-04

Os membros da Comissão de Ética no Uso de Animais do Centro de Ciências Biológicas da Universidade Federal de Pernambuco (CEUA-UFPE) avaliaram seu projeto de pesquisa intitulado, **"Efeitos moleculares e epigenéticos envolvidos na ação neuroprotetora do ácido anacárdico em modelo de doença de Parkinson".**

Concluímos que os procedimentos descritos para a utilização experimental dos animais encontram-se de acordo com as normas sugeridas pelo Colégio Brasileiro para Experimentação Animal e com as normas internacionais estabelecidas pelo National Institute of Health Guide for Care and Use of Laboratory Animals as quais são adotadas como critérios de avaliação e julgamento pela CEUA-UFPE.

Encontra-se de acordo com as normas vigentes no Brasil, especialmente a Lei 11.794 de 08 de outubro de 2008, que trata da questão do uso de animais para fins científicos e didáticos.

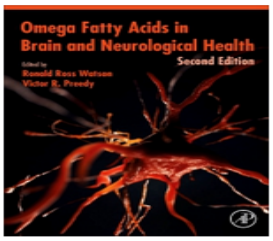
Diante do exposto, emitimos **parecer favorável** aos protocolos experimentais a serem realizados.

Origem dos animais: Biotério; Animais: ratos; Linhagem: *Wistar*; Idade: adultos (entre 90 e 120 dias); Peso: 250-300g (adultos); Sexo: machos; Número total de animais previsto no protocolo: 120.

Atenciosamente,

Prof. Dr. Pedro V. Carelli
Presidente da CEUA / CCB - UFPE
SIAPE 1801584

ANEXO B – CAPÍTULO DE LIVRO



Omega Fatty Acids in Brain and Neurological Health
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CHAPTER

29

Epigenetic Effects of Omega-3 Fatty Acids on Neurons and Astrocytes During Brain Development and Senescence

Belmira Lara da Silveira Andrade-da-Costa[*], *Alinny Rosendo Isaac*[*,†], *Ricielle Lopes Augusto*[*], *Raphael Fabricio de Souza*[*,‡], *Hércules Rezende Freitas*[†], *Ricardo Augusto de Melo Reis*[†]

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EPIGENETIC MECHANISMS RELATED TO NEURON AND ASTROCYTE DEVELOPMENT

Epigenetic can be defined by environmental influences on changes in gene expression (active versus inactive genes) that do not involve changes to the underlying DNA sequence. Therefore, epigenetic remodeling is required during early stages of brain development, especially for cell genesis, proliferation, and differentiation, inducing switching off or maintaining active genes necessary to convert pluripotent in specialized cells.[1–3] This process involves DNA methylation, chromatin remodeling, histone posttranslational modifications, and regulation mediated by noncoding RNAs, including microRNAs (miRNAs), and long noncoding RNAs (lncRNAs). These mechanisms act as modulatory players of programmed events, which are not directly dependent on the inherited DNA sequence.[4–6]

One of the most studied epigenetic mechanisms is DNA methylation, which can exert an important role in gene silencing during ontogeny. Such a process consists in the covalent

ANEXO C – COLABORADORA EM ARTIGO CIENTIFICO

Neuroscience Research 145 (2019) 1–9

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Review article

Endurance training on rodent brain antioxidant capacity: A meta-analysis

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ABSTRACT

The influence of physical exercise on brain antioxidant defense mechanisms has been studied. Nevertheless, the effect of training volume on the brain's redox balance remains unclear. In this meta-analysis, we compared the effect of training volume on antioxidant enzymatic resource and lipid peroxidation on various brain regions. The activities of the enzymes glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase (CAT) and the levels of thiobarbituric acid reactive substances (TBARS) were also evaluated. The effects of training periods (weeks) and exercise duration were compared. Meta-analysis revealed that protocols over 8 weeks were associated with an increase in SOD ($p = 0.0008$) and CAT activities ($p = 0.0001$). Exercise durations for 30 and 60 min were associated with higher CAT activity ($p = 0.04$). Joint analysis revealed that moderate physical exercise over 4 and 8 weeks promoted a healthy enzymatic balance. However, high volumes of exercise over 8 weeks were associated with the increased antioxidant enzymatic activity, indicating higher reactive oxygen species (ROS) levels. The data also indicated that there is still limited research and inaccurate information, on the safety conditions of training periods that simulate tests of ultra resistance in humans.

Contents

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ANEXO D – COLABORADORA EM ARTIGO CIENTIFICO

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Low omega-6/omega-3 ratio in a maternal protein-deficient diet promotes histone-3 changes in progeny neural cells and favors leukemia inhibitory factor gene transcription☆

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Abstract

Omega-3 (n-3) fatty acids modulate epigenetic changes critical to genesis and differentiation of neural cells. Conversely, maternal protein-malnutrition can negatively modify these changes. This study investigated whether a low n-6/n-3 ratio in a maternal diet could favor histone-3 (H3) modifications, gene transcription and differentiation in the offspring neural cells even under protein-deficiency. Female rats fed a control (Ct), or 3 types of multideficient diets differing in protein levels or linoleic/alpha-linolenic fatty acid ratios (RBD, RBD-C, RBD-SO) from 30 days prior to mating and during pregnancy. Cerebral cortex tissue and cortical cultures of progeny embryonic neurons and postnatal astrocytes were analyzed. H3K9 acetylation and H3K27 or H3K4 di-methylation levels were assessed by flow cytometry and/or immunocytochemistry. In astrocyte cultures and cortical tissue, the GFAP protein levels were assessed. Glial derived neurotrophic factor (GDNF) and leukemia inhibitory factor (LIF) gene expression were evaluated in the cortical tissue. GFAP levels were similar in astrocytes of Ct, RBD and RBD-C, but 65% lower in RBD-SO group. Higher levels of H3K9Ac were found in the neurons and H3K4Me2 in the astrocytes of the RBD group. No intergroup difference in the cortical GDNF mRNA expression or the H3K27Me2 levels in astrocytes was detected. LIF mRNA levels were higher in the RDB (P=.002) or RBD-C (P=.004) groups than in the control. The findings indicate the importance of dietary n-3 availability for the brain, even under a protein-deficient condition, inducing Histone modifications and increasing LIF gene transcription, involved in neural cell differentiation and reactivity.

Keywords: Protein malnutrition; Histone post-translational changes; Astrocytes; Neurons; Leukemia inhibitory factor; GDNF

1. Introduction

A growing body of evidence has shown that the status of maternal undernutrition can modify the epigenetic state of the fetal genome, inducing developmental adaptations usually referred as fetal programming [1,2]. Therefore, targeting epigenetic changes with dietary supplementation of essential nutrients has been proposed as a potential therapeutic approach [2].

Histone post-translational modifications such as acetylation and methylation are important epigenetic mechanisms, given their role on chromatin remodeling during transcriptional activity regulation. Low levels of histone acetylation and high levels of di- or tri-methylated H3K27 have been shown to cause a reduction in gene transcription. On the other hand, increased acetylation and methylation at residues H3K4, H3K9, H3K14 and H3K36 have been shown to induce gene transcription [3]. Adequate levels of histone acetylation depend on a

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ANEXO E – COLABORADORA EM ARTIGO EM FASE DE REVISÃO

Elsevier Editorial System(tm) for **Neuroscience**

Manuscript Number: NSC-18-598
Title: Maternal omega-3 deficiency differentially alters gene expression profile in the substantia nigra and striatum of rat progeny: impact on neurodegeneration
Article Type: Research Paper
Section/Category: Developmental Neuroscience
Keywords: Gene expression; Omega 3 deficiency; LIF; PINK-1; calbindin-1; Aldh1a1.

Corresponding Author: Professor Belmira Lara da Silveira Andrade-da- Costa, Ph.D.
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First Author: Emerson Alexandre N Silva, Master degree
Order of Authors: Emerson Alexandre N Silva, Master degree; Alinny R Isaac, Master degree; **Ricielle L Augusto,** Master degree; Giselle Machado M Moreno, Master degree; Ingrid P Mendonça, Biomedical; Rhowena Jane B de Matos, PhD; Marcos André S de Souza, Biologist ; Renata C da Silva, Technician ; Marcelo Cairrão A Rodrigues, PhD; Belmira Lara da Silveira Andrade-da-Costa, Ph.D.

Abstract: Maternal n-3 fatty acid deficiency induces a differential vulnerability in the substantia nigra (SN) and striatum of the young progeny. This study hypothesized that this effect involve changes in the expression of astrocyte markers and a reduced gene expression of neuroprotective factors such as calbindin-1, glial-derived neurotrophic factor (GDNF), insulin-like growth factor (IGF-1), leukemia inhibitory factor (LIF), Aldehyde dehydrogenase (Aldh1a1) and the serine/threonine protein kinase (PINK-1) in the SN of neonates. Female adult rats were fed from 60 days prior to mating and during gestation on control or n-3 deficient diets (n-3D group). Reduced tyrosine hydroxylase levels in the SN (~20%) and phosphorylated-GFAP in both regions (~30%) were found in the n-3D offspring. PINK-1 gene expression was increased (~30%) in the striatum of n-3D group compared to control, while no intergroup difference was evident for GDNF mRNA levels in both regions. LIF and calbindin-1 genes were less expressed (36 and 52% respectively) in the SN of n-3D neonates, but not in the striatum. Higher levels of IGF-1 gene were found in both regions while the Aldh1a1 gene was increase in the SN of the n-3D group. The findings suggest that a differential vulnerability of the progeny`s SN and striatum owing to a maternal n-3 deficiency may involve the striatum's ability to maintain or increase the gene transcription of neuroprotective molecules, such as LIF, calbindin-1, GDNF, IGF-1 and PINK-1. Reduced LIF and calbindin mRNA levels and increased Aldh1a1 and IGF-1 expression in the SN indicate novel molecular mechanisms induced by n-3 deficiency.
Keywords: Gene expression; Omega 3 deficiency; LIF; PINK-1; calbindin-1; Aldh1a1

Graphical abstract