UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS – BIOQUÍMICA

Efeito da suplementação crônica do extrato de *Paullinia cupana Mart.* em ratos *Wistar* senescentes

TESE DE DOUTORADO

MOARA RODRIGUES MINGORI

Porto Alegre – RS

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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, como requisito obrigatório para a obtenção do título de doutora em Bioquímica.

Orientador: Dr. José Cláudio Fonseca Moreira

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Metafísica? Que metafísica têm aquelas árvores?

A de serem verdes e copadas e de terem ramos

E a de dar fruto na sua hora, o que não nos faz pensar,

A nós, que não sabemos dar por elas.

Mas que melhor metafísica que a delas,

Que é a de não saber para que vivem

Nem saber que o não sabem?

Alberto Caeiro, Heterónimo de Fernando Pessoa - "O Guardador de Rebanhos - Poema V".

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SUMÁRIO

PARTE I

APRESENTAÇÃO

A presente tese de doutorado está organizada em três partes, conforme a seguir:

Parte I: Resumo, *Abstract*, Lista de Abreviaturas, Introdução e Objetivos;

Parte II: Resultados apresentados na forma de 2 artigos científicos, dentre os quais um aceito para publicação e outro submetido (capítulo I e II)

Parte III: Discussão, Conclusões, Perspectivas, Referências Bibliográficas.

i. RESUMO

Durante o envelhecimento há um declínio acentuado na capacidade antioxidante celular principalmente no tecido cerebral, o que leva a um desbalanço na homeostase redox gerando danos oxidativos. O extrato de *Paullinia cupana* Mart possui efeitos metabólicos e propriedades farmacológicas bem descritas. Neste trabalho nós investigamos o efeito do extrato comercial de guaraná (ECG) na função cognitiva, parâmetros bioquímicos e modulação da senescência em ratos *Wistar* de meia-idade. Os animais foram divididos em três grupos de acordo com o tratamento (solução salina: 0,9%, ECG: 21 mg/kg/dia e cafeína: 0,83 mg/kg/dia). As soluções foram administradas diariamente por gavagem. Os testes comportamentais foram realizadas antes e após o tratamento. As análises bioquímicas foram realizadas no Sistema Nervoso Central bem como no rim, fígado e coração. Nossos dados em Campo Aberto demonstraram aumento na atividade exploratória e diminuição no comportamento de ansiedade nos animais que receberam cafeína, esse comportamento não foi observado no grupo ECG. As análises do hipocampo e estriado indicam que a ECG e/ou a cafeína alteraram parâmetros analisados de modo tecido específico. O tratamento crônico com guaraná elevou a atividade enzimática da Glutationa S-transferase assim como os carbonilação de proteínas no tecido cardíaco. O tratamento com *Paullinia cupana* alterou parâmetros hematológicos e reduziu os níveis de gordura gonadal nos animais tratados. Em contraste a administração de cafeína isolada foi capaz de aumentar o imunoconteúdo de *Sirt1* em ambos os tecidos, renal e cardíaco. Nossos dados sugerem que ECG não melhora o desenvolvimento cognitivo, mas modifica a maquinaria de estresse oxidativo e vias de sinalização neurodegenerativa, inibindo moléculas relacionadas a vias de sobrevivência no hipocampo e estriado. Isto pode contribuir para o desenvolvimento de microambientes desfavoráveis no cérebro e distúrbios neurodegenerativos. Além disso, os resultados demonstram que os principais efeitos benéficos remetem a cafeína, além de demonstrar que ao contrário da crença popular, a administração crônica do guaraná não gerou melhora nos parâmetros avaliados referentes a cognição e senescência em ratos de meia idade.

ii. ABSTRACT

During aging there is a marked decline in cellular antioxidant capacity mainly in brain tissue, which leads to an imbalance in redox homeostasis generating oxidative damage. The extract of *Paullinia cupana Mart* has well-described metabolic effects and pharmacological properties. In this work we investigated the effect of commercial guarana extract (CGE) on cognitive function, biochemical parameters and modulation of senescence in middle-aged *Wistar* rats. The animals were divided into three groups according to the treatment (saline, CGE or caffeine). The solutions were administered daily by oral gavage. Behavioral tests were performed before and after treatment. Biochemical analyzes were performed in the Central Nervous System as well as in the kidney, liver and heart. Our data in Open Field showed an increase in the exploratory activity and decrease in the behavior of anxiety in the animals that received caffeine, this behavior was not observed in the CGE group. Hippocampus and striatum analyzes indicate that CGE and/or caffeine altered parameters analyzed in a tissue-specific fashion. Chronic treatment with guarana increased the enzymatic activity of Glutathione S-transferase as well as carbonylation of proteins in cardiac tissue. Treatment with *Paullinia cupana* altered haematological parameters and reduced levels of gonadal fat in treated animals. In contrast, administration of caffeine alone was able to increase the immunocontent of Sirt1 in both renal and cardiac tissues. Our data suggest that CGE does not improve cognitive development but modifies the machinery of oxidative stress and neurodegenerative signaling pathways by inhibiting molecules related to survival pathways in the hippocampus and striatum. This may contribute to the development of unfavorable microenvironments in the brain and neurodegenerative disorders. In addition, the results demonstrate that the main beneficial effects refer to caffeine, in addition to demonstrating that contrary to popular belief, chronic administration of guarana did not improve the parameters evaluated for cognition and senescence in middle-aged rats.

iii. LISTA DE ABREVIATURAS

A1: Receptor de adenosina 1

A2AR: Receptor de adenosina A2AR

AGEs: Produtos de glicação avançada

APP: Proteína precursora amiloide (do inglês, *Amyloid precursor protein*)

ATP: Adenosina trifosfato

DA: Doença de Alzheimer

DNA: Ácido desoxirribonucleico (do inglês, *Deoxyribonucleic acid*)

DP: Doença de Parkinson

ECG: Extrato comercial de guaraná

EROs: Espécies reativas de oxigênio

ERNs: Espécies reativas de nitrogênio

GFAP: Proteína Acídica Fribrilar Glial (do inglês, *Glial fibrillary acidic protein*)

NADPH: Dinucleotídeo de nicotinamida-adenina-fosfato

RAGE: Receptor de produtos finais de glicação avançada (do inglês, *Receptor for advanced glycation endproducts*)

SNC: Sistema Nervoso Central

iv. INTRODUÇÃO

Paullinia cupana Mart.

O guaraná (*Paullinia cupana* Mart.) é uma planta nativa do centro do Amazonas, pertencente à família das *Sapindaceae*. A planta pode atingir 3 metros de altura, suas flores crescem em forma de cacho, os frutos são formados por cápsulas vermelho alaranjadas quando maduras e contém de 1 a 4 sementes que exibem coloração castanho-escuro envolvidas por um arilo farináceo branco (Schimpl, da Silva, Gonçalves, & Mazzafera, 2013). As suas sementes desidratadas têm sido usadas para a elaboração de bebidas medicinais desde tempos pré-colombianos devido sua notória ação como estimulante do Sistema Nervoso Central (SNC) e protetor do trato gastrointestinal. As sementes de guaraná são comercialmente cultivadas exclusivamente no Brasil, sendo utilizadas para a produção de refrigerante industrial e de extratos para uso comercial **Figura 1**. A produção de guaraná no Brasil é praticamente toda voltada para o consumo interno, há uma estimativa de que 70% da produção é absorvida pela indústria do refrigerante e bebidas não alcoólicas (Angelo et al., 2008).

Figura 1. As sementes de *Paullinia cupana* são desidratadas e moídas para a formação de um pó solúvel em água denominado, comercialmente, de extrato de guaraná.

O extrato de guaraná contém cafeína como metilxantina majoritária **Figura 2** (Baratloo et al., 2016). Seu conteúdo pode variar de 2 a 6% no volume total no extrato de guaraná, por esta razão a propriedade estimulante do guaraná frequentemente é atribuída a cafeína. Os polifenois como epicatequinas e catequinas estão presentes em concentrações menores mas possuem alto potencial biológico **Figura 3** (da Silva et al., 2017) (Marques et al., 2016).

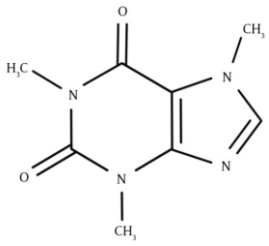

Figura 2. Estrutura química da cafeína

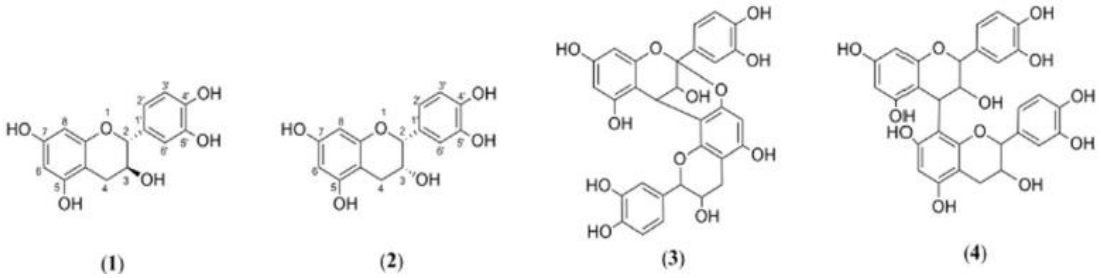

Figura 3. Estruturas químicas das substâncias identificadas no extrato de guaraná (Paullinia cupana). (1) (+) - catequina, (2) (-) - epicatequina, (3) (epi) catequina - tipo A, (4) (epi) catequina Tipo B.

Cafeína

A cafeína é um alcaloide purínico (1,3,7 – Trimetilxantina) encontrado em diversas espécies de plantas como no cacau, nos chás, na erva-mate, no café e no guaraná. O consumo mundial de cafeína, derivada de inúmeras fontes, é estimado em cerca de 70 a 76 mg/pessoa/dia e por isso, essa metilxantina é conhecida como o principal

psicoestimulante mundial (Ashihara, Mizuno, Yokota, & Crozier, 2017). Os mecanismos bioquímicos das ações da cafeína são dose dependentes. Em humanos, 99% da cafeína é absorvida pelo trato gastrointestinal, principalmente no intestino delgado (Fredholm, Bättig, Holmén, Nehlig, & Zvartau, 1999). Posteriormente, a cafeína é metabolizada no fígado, por N-desmetilação, sendo convertida em metabólitos intermediários pelo citocromo P450, em sua maioria é convertida a paraxantina e em menor proporção pode ser convertida nos metabólitos ativos como teofilina e teobromina (Arnaud, 2011).

Quando alcançam a corrente sanguínea, a cafeína e seus metabólitos atravessam as membranas celulares e podem se acumular em diversos órgãos e tecidos, no caso do consumo de altas doses por longo tempo a acumulação de metilxantinas pode ser tóxica (Denaro, Brown, Wilson, Jacob, & Benowitz, 1990). No cérebro, os alvos moleculares da cafeína, em doses não tóxicas são os receptores de adenosina A1 e A2A. Seus mecanismos clássicos de atuação envolvem além do antagonismo aos receptores de adenosina, a inibição da fosfodiesterase gerando os principais efeitos biológicos observados (Pohanka, 2015). Uma das ações mais reconhecidas da cafeína é a capacidade estimulante do SNC e a redução do sono. Esse alcaloide é capaz de atuar no receptor A2AR no núcleo accumbens promovendo a vigília (Lazarus et al., 2011). Dessa forma há relatos na literatura de que a ingestão da cafeína gera melhora no desempenho cognitivo e confere alterações de humor (Kennedy et al., 2017), aumento da resistência física (Richardson & Clarke, 2016) além de causar efeitos antidepressivos e ansiolíticos (Szopa et al., 2016). Além disso vários autores reportam efeitos terapêuticos frente a doenças neurodegenerativas como Parkinson e Alzheimer (Kolahdouzan & Hamadeh, 2017).

Polifenois e compostos antioxidantes

Os polifenois são os compostos bioativos mais abundantes na dieta humana, derivados em sua maioria de plantas, estão presentes em grande quantidade em frutas, verduras, cereais, chás, cafés e vinhos (Malongane, McGaw, & Mudau, 2017). A estrutura química dos polifenois é um fator crítico que influencia a sua biodisponibilidade. Nos alimentos, os polifenois são encontrados como polímeros ou em formas glicosiladas e agliconas. A maioria dos polifenois não pode ser absorvida diretamente após o consumo mas precisa ser metabolizada por uma série de reações enzimáticas através da microbiota intestinal. O último grupo hidrolisa glicosídeos em agliconas e os degrada a simples ácidos fenólicos. Esta atividade é de grande importância para a ação biológica dos polifenois, a fim de produzir um certo número de metabolitos específicos com propriedades terapêuticas. Os anéis aromáticos presente nos polifenois possuem capacidade de absorção de luz, esta característica garante a diversidade de coloração apresentado pelas plantas conferindo propriedades atrativas na propagação ou dispersão de sementes além de inibição da herbivoria (Corcoran, McKay, & Blumberg, 2012) .

Atualmente o consumo diário de polifenois já ultrapassa o consumo de suplementos como vitamina C e carotenoides porém os estudos direcionados a compostos que contém polifenois avança de forma mais lenta devido a diversidade e complexidade de suas estruturas químicas (Scalbert, Manach, Morand, Rémésy, & Jiménez, 2005). O papel da nutrição e suplementação antioxidante como fonte de moléculas bioativas capazes de prevenir doenças crônicas ligadas ao envelhecimento despertou o interesse da comunidade científica nos últimos anos (Scalbert et al., 2005) (Abate, Marziano, Rungratanawanich, Memo, & Uberti, 2017). Assim, têm sido descritas inúmeras atividades biológicas para esses metabólitos.

Apesar do emprego terapêutico de extratos de plantas contendo polifenois ser vasto na dieta humana, em muitos casos, ainda é empírico. Muitos extratos consumidos diariamente necessitam de maiores estudos para comprovar seus efeitos a longo prazo. Embora alguns resultados tenham mostrado que os polifenois possam apresentar efeito mutagênico, em geral são considerados benéficos (Ruiz-Pérez et al., 2014) (Lin et al., 2014). A maioria dos efeitos biológicos descritos são a neutralização de espécies oxidantes, como o ânion superóxido e radical hidroxila, porém outros estudos indicam modulação da atividade enzimática, inibição da proliferação celular além de efeitos antibióticos e anti-inflamatórios (Galleano, Verstraeten, Oteiza, & Fraga, 2010). As propriedades antioxidantes dos polifenois se devem a sua configuração estrutural complexa constituída de ligações duplas conjugadas além dos grupos carbonil e inúmeros grupos hidroxil que permitem estabilidade entre os elétrons (Corcoran et al., 2012). A sinalização redox fisiológica envolve reações de ajustes contínuos que levam a fosforilações e desfosforilações de proteínas, oxidação de sulfetos e oxidação de NADPH. Em condições normais, a produção de espécies reativas durante o metabolismo aeróbico é regulada pelo sistema antioxidante, que age como sequestrador e neutralizador de espécies reativas. Os problemas surgem quando a produção de espécies reativas excede a capacidade de resposta do sistema antioxidante, gerando oxidação de proteínas, dano ao DNA e lipoperoxidação com generalizada disfunção celular e morte, eventos comuns no contexto patológico ou de envelhecimento. Assim, a comunidade científica tem buscado compostos antioxidantes capazes de reverter ou atenuar estes processos para preservar a função celular e prolongar a sobrevivência (Morrone et al., 2016).

Durante o envelhecimento ocorre um aumento nos níveis de estresse oxidativo que está relacionado ao desenvolvimento de doenças cardiovasculares e neurodegenerativas (Höhn et al., 2017). Nesse contexto, compostos ricos em polifenóis

vem sendo amplamente estudados, catequinas provenientes do chá verde demonstraram efeitos terapêuticos por inibição da oxidação da lipoproteína de baixa densidade implicando em benefícios frente a arteriosclerose (Suzuki-Sugihara et al., 2016) e resultados sugerem que a ingestão de polifenois de cacau pode modular mediadores inflamatórios em pacientes com alto risco de doença cardiovascular (Monagas et al., 2009). Modelos *in vitro* também demonstram dados promissores para polifenois extraídos da cana de açúcar que apresentaram capacidade de modular fatores de transcrição pró-inflamatórios em células de câncer do cólon (Bucio-Noble, Kautto, Krisp, Ball, & Molloy, 2018). Da mesma forma, polifenois derivados da cúrcuma, uvas, chá verde e açai podem contribuir na diminuição de mediadores pró-inflamatórios em pessoas com risco de inflamação crônica (Ford et al., 2016). Implicações em relação a atividades analgésicas de polifenois obtidos a partir de folhas de *Vitis vinífera* também são relatadas em roedores (Aouey, Samet, Fetoui, Simmonds, & Bouaziz, 2016). Propriedades antioxidantes e anti-inflamatórias de *Croton cajucara Benth* nativa da Amazonia tem sido reportados em ensaios de edema em roedores (Nascimento et al., 2017). E recentemente pesquisadores demonstraram que a suplementação com curcumina induziu melhora nas alterações do metabolismo lipídico, com acentuada redução de tecido adiposo e o dano oxidativo associado à privação de hormônio na menopausa como o estrogênio (Morrone et al., 2016). Estudos demonstraram que os polifenois provenientes da suplemementação com chá preto (catequinas) conferem aumento das defesas antioxidantes no plasma de roedores avaliados em diferentes idades, pontuando os efeitos benéficos deste composto durante o envelhecimento observados pelo aumento de grupamentos tiois totais em roedores de meia idade (12 meses) e idade avançada (24 meses) quando comparados com animais jovens (Kumar & Rizvi, 2017).

O extrato do guaraná contém altas quantidade de cafeína e de polifenois como catequinas, epicatequinas e taninos (Schimpl et al., 2013). Por esta razão, o composto despertou interesse científico nos últimos anos devido à variedade de moléculas com potencial bioativo e seu amplo alcance de consumo por parte da população. Alguns trabalhos têm apontando para uma ação bacteriostática do guaraná frente a bactérias gran-negativas e gran-positivas (Basile et al., 2013). Além disso o guaraná desempenha papeis importantes frente a doenças vasculares contribuindo para a diminuição da oxidação nos níveis de LDL-colesterol e reduzindo o dano ao DNA em linfócitos, sendo a catequina o princípio ativo responsável por estes efeitos (Yonekura et al., 2016). Também já foram relatadas ações anti-agregadora de plaquetas e antitrombóticas (Subbiah & Yunker, 2008), contra a obesidade (Opala, Rzymski, Pischel, Wilczak, & Wozniak, 2006) antimutagênicas e anticarcinogênicas (Fukumasu et al., 2006) do extrato de guaraná. Outros estudos têm demonstrado que *Paullinia cupana* apresenta propriedades protetoras contra a toxicidade hepática causada por tetracloreto de carbono (Kober et al., 2016). O composto também apresentou capacidade de melhorar a memória em testes preliminares (Ruchel, Braun, et al., 2016), além disso seu papel em desordens de ansiedade mostraram efeitos antidepressivos e psicoestimulantes (Otobone et al., 2007) (Roncon, Biesdorf de Almeida, Klein, de Mello, & Audi, 2011). Em concordância há estudos que apontam suas propriedades estimuladoras em casos de fadiga crônica (de Oliveira Campos et al., 2011), como acreditavam os adeptos ao consumo desta planta na medicina popular. Mais concretamente, estudos recentes vêm relatando a capacidade antioxidante e na prevenção de doenças neurodegenerativas, demonstrando a capacidade do extrato de guaraná em prevenir a formação de produtos de glicação avançada (AGEs) e agregação do peptídio β-amilóide *in vitro* (L. a. S. Bittencourt et al., 2014) . Corroborando com estes dados, o extrato de guaraná mostrou efeitos protetores em linhagem de células de neuroblastoma

SH-SY5Y durante ensaios de citotoxicidade induzido pela droga rotenona, demonstrando possível atividade farmacológica para doença de Parkinson (de Oliveira et al., 2011).

Envelhecimento e Estresse oxidativo

O envelhecimento, definido como um fenômeno biológico marcado pelo acumulo de alterações deletérias que afetam os organismos vivos com o avançar da idade, também está associado ao desenvolvimento de patologias como arteriosclerose, câncer, diabetes e doenças neurodegenerativas (van der Flier et al., 2018). Há inúmeras teorias que se propõem a explicar a origem do envelhecimento, porém a teoria dos Radicais Livres, explica de fato os processos básicos relacionados a senescência e reúne mais consenso entre os gerontologistas (HARMAN, 1956). Essa teoria elucida o envelhecimento com um único processo comum, modificável por fatores genéticos e ambientais, nos quais os radicais livres ou espécies reativas derivadas do oxigênio (ERO) ou nitrogênio (ERN) quando não detoxificados corretamente por falha no sistema antioxidante são responsáveis pelo dano associado à idade em nível celular e tecidual. O desbalanço entre as reações de oxidação e redução que acontecem no metabolismo é prejudicial por causar alterações físico-químicas nas moléculas biológicas alterando a funcionalidade original. Neste processo, ERO e ERN incluem radicais livres conhecidos como: radical superóxido (O_2), peróxido de hidrogênio (H_2O_2), radical hidroxil (OH), óxido nítrico (NO) dentre outros associados a alterações de estrutura e permeabilidade de membranas (Di Meo, Reed, Venditti, & Victor, 2016). O acúmulo de radicais endógenos gera modificação oxidativa de moléculas biológicas importantes na homeostase celular como lipídios, proteínas e DNA, contribuindo para o envelhecimento e consequentemente morte (Finkel & Holbrook, 2000). Estas espécies reativas derivam de inúmeras fontes, incluindo fontes endógenas como mitocôndria, peroxissomos, citocromo P450, monoamino oxidases e

fontes exógenas como poluentes e toxinas de modo geral. Embora os ERO em excesso possam ser mediadores de diversas doenças, a sua concentração em quantidades baixas é essencial e fisiologicamente benéfica ao organismo, desempenhando importante papel de defesa contra infeções e na resposta inflamatória (Viña, Borras, Abdelaziz, Garcia-Valles, & Gomez-Cabrera, 2013). Sob condições normais, a produção de espécies reativas durante o metabolismo aeróbico é regulada pelo sistema antioxidante, que age como sequestrador e neutralizador de espécies reativas. O sistema antioxidante é composto por dois tipos de barreiras, uma enzimática, constituída pelas enzimas superóxido dismutase (SOD), glutationa peroxidase, glutarredoxinas, tiorredoxinas e catalase, e outra não-enzimática, constituída, por exemplo, por vitaminas e polifenois (Tönnies & Trushina, 2017).

No contexto redox algumas organelas desempenham papéis fundamentais como os peroxissomos e as mitocôndrias, ambos são fontes de produção de ERO e representam uma grande parte do consumo de oxigênio em tecidos metabolicamente ativos, como o fígado (Boveris, Oshino, & Chance, 1972). As mitocôndrias são envolvidas em processos essenciais como metabolismo energético, produção de defesas antioxidantes e controle do ciclo celular em células eucarióticas (Nunnari & Suomalainen, 2012). Durante os processos de fosforilação oxidativa realizados dentro da mitocôndria, um dos passos primordiais para a produção de moléculas de ATP é a redução do oxigênio molecular, tal processamento gera subprodutos tóxicos durante o metabolismo normal, como o radical superóxido (O_2) ou peróxido de hidrogênio (H_2O_2). Contudo a mitocôndria também possui mecanismos antioxidantes de detoxificação destes radicais como a presença de enzimas como SOD, catalase, glutationa S-transferase, Glutationa redutase, Glutahiona peroxidase além de defesas não-enzimáticas: o acúmulo de NADPH, vitamina C e coenzima Q que tem funções importante na eliminação de ROS (Starkov, 2008). A

atuação disfuncional da mitocôndria em contextos patológicos ou de envelhecimento gera ROS em excesso e perpetua o dano celular (Weidinger & Kozlov, 2015).

A deficiência nas enzimas antioxidantes aumenta a susceptibilidade a doenças relacionadas ao envelhecimento como diabetes, câncer ou aterosclerose (Lankin & Tikhaze, 2017). Além disso, o SNC é particularmente susceptível aos danos oxidativos em decorrência do envelhecimento devido a sua alta taxa metabólica e alta composição lipídica. Inclusive, sua constituição estrutural rica em ferro e cobre, favorece a geração de radicais livres através da reação de Fenton (Riederer et al., 1989). Tem sido relatado extensivamente na literatura que a diminuição de defesas antioxidante e aumento do estresse oxidativo leva ao desenvolvimento de doenças neurodegenerativas como doença de Alzheimer (DA) (Huang, Zhang, & Chen, 2016) e doença de Parkinson (DP) (Ilic, Jovanovic, Jovicic, & Tomovic, 1999) (Mancuso et al., 2007) bem como isquemia e reperfusão (Molyneux, Glyn, & Ward, 2002).

A superprodução de espécies reativas de oxigênio (ERO) é considerada um fator bem inicial da patologia mas que pode perdurar até os estágios finais, estudos demonstram que íons metálicos livres no SNC que estão presente nas placas senis podem se ligar a oligômeros de beta-amiloide e persistir o status oxidativo cada vez mais tóxico para o ambiente neuronal (Collin, Cheignon, & Hureau, 2018). Alteração da homeostase de outros metais incluindo cobre, zinco, magnésio, manganês e alumínio também estão relacionados com a geração de estresse oxidativo e agregação do peptídeo β-amilóide e da proteína tau. Foi relatado, por exemplo, que o zinco se liga à APP e afeta o seu processamento, assim como o zinco, alumínio, ferro e cobre se ligam diretamente ao peptídeo β-amilóide e promovem a sua agregação. Outros estudos indicam que o próprio peptídeo β-amilóide pode gerar espécies reativas na presença de ferro e cobre (Tönnies & Trushina, 2017).

Baseados nas evidências acerca da teoria dos radicais livres, onde a acumulação de estresse oxidativo possui efeito principal na progressão do envelhecimento, vários pesquisadores têm realizado estudos buscando reforço nas defesas antioxidantes com o intuito de atenuar ou prolongar o envelhecimento de forma saudável. A administração de compostos antioxidantes tem papel importante nestes estudos. Além disso pesquisas focadas na identificação de biomarcadores precoces frente a doenças neurodegenerativas como Alzheimer, aumentaram drasticamente, com o finalidade de melhorar a compreensão dos mecanismos que desencadeiam patologias. Danos oxidativos em lipídios e proteínas neuronais são características importantes na etiologia da doença e estão ligados ao estresse oxidativo estabelecido no início da doença. A identificação precoce dessas alterações redox podem garantir maior sucesso na interrupção da patologia (Cheignon et al., 2018). A presença de marcadores inflamatórios em cérebros de pacientes com doenças neurodegenerativas aponta para uma relação entre estresse oxidativo e inflamação na etiologia das doenças. Estudos relatam RAGE, ou receptor para produtos finais de glicação avançada, como um receptor extracelular multi-ligante, que desencadeia a transcrição NF-κB dependente de citocinas, e também a produção de espécies reativas comumente associada à diabetes e outros estados inflamatórios crônicos ligados ao envelhecimento. Um dos ligantes descritos atualmente de RAGE é o peptídeo β-amilóide, liberado no processo de desenvolvimento da DA, mas também pode se ligar a demais produtos oriundos da glicação oxidativa de carboidratos (Ray, Juranek, & Rai, 2016). O RAGE possui ligantes clássicos como AGEs (*advanced glycation end-products*) e o peptídio β-amilóide. Em situações de desequilíbrio redox, a ativação deste receptor está relacionada com a ativação e progressão de inflamação e vias pró-apoptóticas (Piras et al., 2016). Recentemente, estudos indicam uma forte participação do RAGE na demência em DA, visto que observou-se a ativação de muitas vias de sinalização

neurotóxicas associadas com degeneração celular a partir da ligação do peptídeo β-amiloide ao RAGE (Yan et al., 2012). Outros estudos apontam o RAGE como mediador para o transporte do peptídeo β-amilóide através da barreira hematoencefálica, aumentando assim a sua deposição no tecido neuronal, e também um aumento no imunoconteúdo de RAGE foi associado com o aumento de outros marcadores para a DA (Candela et al., 2010).

Desde que estudos surgiram mostrando a capacidade antioxidante do extrato de *Paullinia cupana*, vários autores começaram a sugerir o uso da suplementação com este composto como forma de prevenção ou tratamento contra doenças relacionadas ao envelhecimento ou neurodegeneração.

Paullinia cupana, extresse oxidativo e envelhecimento

Devido ao aumento da expectativa de vida, a ocorrência de distúrbios relacionados ao envelhecimento que causam comprometimento cognitivo e motor está aumentando, incluindo doenças neurodegenerativas como a doença de Alzheimer, demência vascular e doenças cardiovasculares (Burlá, Camarano, Kanso, Fernandes, & Nunes, 2013). Devido a importante relação entre o estresse oxidativo e as doenças neurodegenerativas, pesquisas sugerem o uso de antioxidantes para o tratamento e prevenção destas doenças. Extratos antioxidante ricos em substâncias bioativas são compostos naturais, e por apresentarem características de neutralização das espécies reativas, seu uso tem sido amplamente proposto contra os distúrbios do envelhecimento e doenças neurodegenerativas.

Os componentes do extrato de *Paullinia cupana*, capazes de atravessar a barreira hematoencefálica, já demonstraram efeito protetor frente a lipoperoxidação de membranas celulares *in vitro* (Veloso et al., 2018). O uso deste extrato já foi sugerido

como forma de prevenção e/ou tratamento da DA (L. a. S. Bittencourt et al., 2014), em contraste alguns estudos correlacionaram o extrato da semente e o estabelecimento de morte neuronal oxidativa (Zeidán-Chuliá et al., 2013). O guaraná tem sido apontado como uma fonte fitoquímica promissora no manejo da hiperlipidemia e distúrbios cognitivos (Ruchel et al., 2017). A composição do extrato de guaraná é bastante ampla, de fato, contém uma variedade de moléculas redox-ativa que apresentam efeitos dualísticos, ora protegendo contra a ação de espécies reativas, ora promovendo o dano oxidativo. A sinergia dos componentes presentes no extrato de guaraná são capazes de modular a sinalização redox mas maiores investigações são necessárias. Alguns efeitos relatados na literatura relacionam a habilidade desse extrato em neutralizar formas tóxicas de nitrogênio e outros radicais livres (L. S. Bittencourt et al., 2013). A administração de guaraná foi capaz de inibir a proliferação de células de câncer coloretal através de indução de apoptose (Cadoná et al., 2015).

Melhorar a compreensão dos fatores que aceleram o declínio cognitivo e identificar compostos eficazes que possam atenuar os processos de envelhecimento ou neurodegeneração são considerados grandes desafios da medicina moderna. Isto requer estudos científicos adicionais sobre os seus efeitos e indica a grande importância farmacológica dos constituintes vegetais (Youdim & Joseph, 2001). Devido à presença de uma diversidade de compostos secundários no guaraná, há um enorme leque de propriedades biológicas potenciais. No entanto, devido à sua baixa biodisponibilidade e metabolismo extensivo no processo de digestão, seu efeito antioxidante *in vivo* é incerto. Raríssimos estudos *in vivo* têm relatado os efeitos do extrato de guaraná através da administração crônica e prolongada (Cechella et al., 2014; Ferrini, Hlaing, Chan, & Artaza, 2015) apesar de seu alto consumo pela população brasileira. A maioria dos

estudos na literatura utilizam animais jovens e doses únicas para investigar seus efeitos (Boeck et al., 2009; Brothers, Marchalant, & Wenk, 2010).

v. OBJETIVOS

Justificativa e apresentação dos objetivos:

Considerando: i) A relevância da modulação do status redox como principal mecanismo no envelhecimento celular particularmente em estruturas relacionadas ao comportamento cognitivo e emocional no SNC; ii) A importância da restauração das defesas antioxidantes; iii) O crescente número de trabalhos epidemiológicos e experimentais relacionando os efeitos do consumo de extratos naturais ricos em polifenois com potencial de mitigar fenômenos associados à idade; iv) A escassez de estudos sobre os efeitos a longo prazo do extrato de *Paullinia cupana* em parâmetros de envelhecimento.

O objetivo central da tese foi avaliar os efeitos do consumo crônico de *Paullinia cupana* no SNC e nos principais órgãos do metabolismo intermediário de animais de meia idade. Para atingir tal objetivo, traçamos os seguintes objetivos específicos:

a) Investigar a modulação dos parâmetros bioquímicos nas regiões do hipocampo e estriado (SNC) e no fígado, rim e coração de ratos de *Wistar* de meia idade submetidos a administração crônica de *Paullinia cupana*.

b) Avaliar os parâmetros comportamentais via teste de Campo Aberto e Reconhecimento de Objetos antes e depois do consumo crônico do extrato.

c) Avaliar o imunoconteúdo e localização por imunofluorescência das proteínas 1) Marcador de antígeno nuclear neuronal (NeuN), Proteína acídica fribrilar glial (GFAP) 2) Receptor de produtos de glicação avançada (RAGE) e Proteína precursora β-amiloide (APP) no SNC.

d) Investigar vias de sinalização p-AKT e p- ERK no SNC no hipocampo e estriado de ratos Wistar de meia idade após consumo do extrato de *Paullinia cupana*.

e) Avaliar o imunoconteúdo das proteínas da família das Sirtuínas: Sirt1 e Sirt3 nos órgãos periféricos: fígado, rim e coração.

PARTE II

RESULTADOS

CAPÍTULO I

Effect of *Paullinia cupana Mart.* commercial extract during the aging of middle age *Wistar* rats: differential effects on the hippocampus and striatum

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ORIGINAL PAPER

Effect of *Paullinia cupana* Mart. Commercial Extract During the Aging of Middle Age *Wistar* Rats: Differential Effects on the Hippocampus and Striatum

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Abstract During aging, there is a marked decline in the antioxidant capacity of brain tissue, leading to a gradual loss of the antioxidant/oxidant balance, which causes oxidative damage. The effects of *Paullinia cupana* Mart. extract, which is described as being rich in caffeine and many polyphenol compounds, on the central nervous system have not been extensively investigated. The aim of this study was to therefore investigate the effect of a commercial guarana extract (CGE) on cognitive function, oxidative stress, and brain homeostasis proteins related to cognitive injury and senescence in middle age, male *Wistar* rats. Animals were randomly assigned to a group according to their treatment (saline, CGE, or caffeine). Solutions were administered daily by oral gavage for 6 months. Open field and novel object recognition tasks were performed before and after treatment. Biochemical analyses were carried out on the hippocampus and striatum. Our open field data showed an increase in exploratory activity and a decrease in anxiety-like behavior with caffeine but not with the CGE treatment. In the CGE-treated group, catalase activity decreased in the hippocampus and increased in the striatum. Analyses of the hippocampus and striatum indicate that CGE and/or caffeine altered some of the analyzed parameters in a tissue-specific manner. Our data suggest that CGE intake does not improve cognitive development, but modifies the oxidative stress machinery and neurodegenerative-signaling pathway, inhibiting pro-survival pathway molecules in the hippocampus and striatum. This may contribute to the development of unfavorable microenvironments in the brain and neurodegenerative disorders.

Keywords *Paullinia cupana* commercial extract · Caffeine · Aging · Neurodegenerative diseases · Polyphenols · Neuroprotection

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Introduction

The number of people older than 65 years has risen sharply in Europe, North America, and South America. In Brazil, the population aged over 60 years has increased by 7.5% between 2009 and 2011 and reached 1.8 million, according to Burlá et al. [1]. Owing to an increase in life expectancy, the occurrence of age-related disorders that cause cognitive and motor impairment is increasing, including neurodegenerative diseases like Alzheimer's disease, vascular dementia, and cardiovascular diseases [2]. A better understanding

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of the factors that accelerate this cognitive decline is needed in order to prevent diseases or develop effective treatments that can attenuate neurodegenerative processes in the search for healthier aging. In recent decades, the scientific community has been engaged in studies to reverse or reduce age-related neuronal death or damage by investigating molecules or genes that encode proteins that suppress oxidative stress, stabilize cellular calcium homeostasis, and diminish mitochondrial dysfunction caused by aging and reverse neurodegenerative biochemical reactions [3, 4].

In this context, antioxidant compounds are being extensively investigated owing to their protective properties in cells modeling inflammatory and oxidative processes related to aging [5]. These substances are rich in redox-active molecules, such as polyphenols, which modulate behavioral and age-related cognitive processes [6]. This necessitates further scientific studies on their effects and indicates the marked pharmacological importance of plant constituents [7].

Guarana (*Paullinia cupana* Mart.) is a native plant from the center of the Amazon, which is rich in caffeine and polyphenols [8]. Guarana extract and its derivatives have been widely used as stimulants of the central nervous system (CNS) owing to their high caffeine content [9]. In addition to methylxanthines like caffeine, guarana extract also contains theobromine, theophylline, epicatechins, catechins, tannins, saponins, and proanthocyanidins. In this sense, the therapeutic properties of guarana can be assigned to one or more of its major components [10, 11]. However, because of the presence of a wide variety of secondary metabolites, many potential biological properties of guarana have not been fully explored. More specifically, recent studies have revealed its antioxidant [12] and antiaggregatory β-amyloid peptide capacities [13]. It has also been used in antiobesity studies owing to its connection to food satiety [14], and the guarana seed extract has been researched in aging studies [15]. Other studies have shown how guarana has the ability to improve memory [16], although its antidepressant effects have not been widely researched [17].

There is a consensus in the literature about the decline of antioxidant functions in almost all aged mammals. However, antioxidant compound administration may modulate or ameliorate this process. In contrast, few animal studies have reported the effects of guarana extract, which is rich in polyphenols and methylxanthines, during age-related changes [15, 18]. Most studies have used young animals to investigate this class of compounds (*e.g.*, guarana and caffeine) [19, 20]. Considering the above-mentioned information, this study was performed to investigate the effects of a chronic administration of CGE to middle age *Wistar* rats on parameters related to oxidative stress, cognitive injury, senescence, and behavior in the striatum and hippocampus.

Methods

Animals

Thirty six-month-old male adult rats, weighing 415 ± 6.51 g (mean ± standard error of mean (SEM)), were randomly selected from our animal facility. They were group housed (four animals per cage) in home cages ($65 \times 25 \times 15$ cm) with sawdust covered floors and kept in a controlled environment (lights on between 07:00 and 19:00 h, 22 ± 2 °C, cage cleaning twice a week, free access to standard lab rat chow and water). After a two-week adaptation period, an open field behavioral task was carried out between 10:00 and 15:00 h. Subsequent to the open field task, animals were randomized into experimental groups: caffeine (as present in the respective CGE volume), CGE, and (saline control, $n = 10$ per group). The experimental solutions were administered daily by oral gavage for 6 months. After this period, animals were subjected to an open field task and body weight variation was measured (Supplementary Fig. 1).

Ethical Aspects

All animal treatments were approved by the Institutional Ethical Committee (Universidade Federal do Rio Grande do Sul, Number 27.686) and followed the recommendations of the International Council for Laboratory Animal Sciences (ICLAS). All efforts were taken to minimize pain and discomfort. This article does not contain any studies with human participants.

Experimental Groups

After the first behavioral task, experimental solutions were administered daily by oral gavage for 6 months. The experimental solutions were prepared on the day of use by dissolving the powder in 0.9% saline solution.

Control group (saline): gavage of 1 mL of 0.9% saline/body weight (kg)/day. Sodium chloride is an electrolyte widely used in standard solutions for experimentation in rodents [21–25]. The saline solution at a 0.9% concentration is an isotonic solution, providing electrolytes to maintain plasma tonicity, contributing to an adequate osmotic regulation and humidifying the mucous membranes more effectively due to the methodology of administration of our oral gavage treatment.

Guarana-treated group: gavage of 21 mg of *Paullinia cupana* powder/body weight (kg)/day, also named CGE.

Caffeine-treated group: gavage of 0.84 mg of caffeine powder/body weight (kg)/day, also named CGE standard solution.

The specific doses used during treatments were defined according to recommendations made for daily CGE human consumption, suggesting 3 g of guarana for individuals weighting 70 kg, since each 1 g of guarana contains 40 mg of caffeine. The ECG dose used in this study were half of the manufacturer's recommended dose, since (3 g/70 kg/day)/2 = 21 mg/kg/day. Caffeine administration, since 1 g ECG having 40 mg caffeine, we have (0.021*40) = 0.84 mg/kg/day. Caffeine dose was delineated obeying the proportion of manufacturer, on the presence of caffeine in the CGE, but it was calculated in relation to the chosen CGE dose (21 mg/kg/day). This caffeine dose was used as in internal control of the concentration of caffeine in the chosen CGE dose.

Paullinia cupana Mart. powder (guarana; Pharmaceutical Industrial Laboratory—LIFAR Porto Alegre, Brazil) and caffeine powder (Sigma, St. Louis, MO, USA) were stored dry and protected from light at 20 °C until solution preparations.

Determination of Guarana Bioactive Compounds

The guarana bioactive compounds were determined previously and estimated in this work according to Bittencourt et al. [13]: 100 mg dry weight guarana extract contains 0.14 mg/g theobromine, 34.19 mg/g caffeine, 3.76 mg/g catechins, and 4.05 mg/g epicatechins (Supplementary Table 1).

A liquid chromatographic analysis of guarana powder was performed as described by Sousa et al. [26], using a Shimadzu Prominence HPLC system (Kyoto, Japan) coupled to an SPD-20A UV/VIS detector. The stationary phase was a Kromasil RP-18 column (Eternity-5-C18 250×4.6 mm *i.d.*, 5 µm particle size) guarded by a Phenomenex precolumn (2×4 mm *i.d.*, 10 µm particle size). The mobile phase consisted of water:acetonitrile:methanol:ethyl acetate:acetic acid (89:6:1:3:1). The flow rate was 1.0 mL/min and the injection volume was 20 µL. The detection wavelength was 274 nm and the analysis was carried out at room temperature. Caffeine anhydrous (99%), (+)-catechin hydrate (99%), (−)-epicatechin (90%), theobromine (99%), and theophylline anhydrous (99%) were used as external standards. They were dissolved in 0.1 M HCl:methanol (77:33, v/v) and diluted to obtain a concentration between 0.1 and 20.0 µg/mL. These solutions were filtered through a 0.45-mm membrane filter (HVLP; Millipore, São Paulo, Brazil). The linear equations were $y = 60{,}984x + 13{,}333$ ($r_2 = 0.9994$), $y = 49{,}426x + 8691$ ($r_2 = 0.9998$), $y = 25{,}872x + 94$ ($r_2 = 0.9996$), $y = 12{,}769x + 306$ ($r_2 = 0.9999$), and $y = 12{,}721x + 947$ ($r_2 = 0.9998$) for theobromine, theophylline, caffeine, catechins, and epicatechin, respectively.

Behavioral Tasks

Before and after the administration period (6 months), each rat was evaluated in open field behavioral and novel object recognition tasks.

Open Field Task

The open field task explores the conflict between rodents' innate fear of the open center area of the arena versus their desire to explore a new environment. Animals were individually and gently placed in an apparatus made of black painted Plexiglas measuring 50×50 cm, surrounded by 50-cm-high walls. All tests were recorded with ANY-maze video tracking software (Stoelting Co., Wood Dale, IL, USA). Behavioral parameters were evaluated from these recordings and the total distance travelled in the central and peripheral areas (m) was analyzed as general locomotor activity criteria, according to Ménard et al. [27]; time (sec) spent in peripheral and central areas (m) and mean speed (m/sec) were analyzed for assessing environmental exploration and anxiety-related behaviors [28].

Novel Object Recognition Task

The novel object recognition task was modified from that reported by Pagnussat et al. [29]. This task evaluates non-spatial memory in rodents through quantifying object exploration and delimiting memory settings. Briefly, rats were placed in the open field 24 h before the training session and were allowed to explore it for 10 minutes. A 5 minute training session allowed the animal to explore the apparatus containing two similar objects (A and A'), consisting of two glass bottles of identical size (15 cm in height), differing only in shape and color. The 5-minute test sessions were performed either for 3 h (for short-term memory) or 24 h (for long-term memory) after training. During the test session, a novel object (a metal bottle, named object B) was inserted instead of an A object. The discrimination index was calculated as the time spent exploring the novel object divided by the time spent exploring both objects (novel and familiar). The exploring time was characterized as the rat touching the object with their nose or forepaws. During all novel object recognition tasks, rats were gently placed in a standardized area, facing the wall. The apparatus and objects were cleaned with 30% ethanol between trials to eliminate odor cues.

Sample Collection

A week after the behavioral tests, animals were euthanized by decapitation in random order using a small animal guillotine (Insight, São Paulo, Brazil) and the trunk blood was collected in tubes without anticoagulants. Serum was obtained by centrifugation at 1000×*g* for 10 min. Afterwards, the brains were removed and the hippocampus and the striatum were rapidly dissected and stored at −70 °C. Part of these structures ($n=6$ per group) were used for oxidative status evaluation and western blotting quantifications and the remnant structures ($n=4$ per group) were used for immunofluorescence assessment.

Biochemical Analysis

Serum samples were used for the determination of biochemical parameters. Hepatic function was analyzed using Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) assessments as markers of toxicity. Renal function was analyzed by determining urea and creatinine levels. All assays were carried out using commercial kits (Labtest, Diagnostica S.A., Minas Gerais, Brazil).

Oxidative Status Assessment

For all oxidative status evaluations, the hippocampus and striatum were homogenized in ice-cold 50 mM potassium phosphate buffer (PPB; pH 7.4) containing 1 mM Ethylenediaminetetraacetic acid (EDTA). The homogenate samples were centrifuged at 1000×*g* for 10 min at 4 °C and the supernatant was used. For data normalization, homogenate protein content was determined according to Lowry et al. [30].

Enzyme Activity Measurements

Catalase Activity Catalase activity was determined upon establishing the rate of H_2O_2 degradation by spectrophotometric analysis at 240 nm (25 °C) in a reaction containing 20 mM H_2O_2, 0.1% Triton X-100, 10 mM PPB (pH 7.0), and approximately 1 µg of protein. The results are expressed as units of catalase/mg of protein [31].

Superoxide Dismutase Activity Superoxide dismutase activity was determined by quantifying the inhibition of superoxide-dependent adrenaline autoxidation to adrenochrome formation [32]. In brief, the protein content in each sample was quantified and the inhibition of superoxide-dependent adrenaline autoxidation in a reaction buffer containing 1 mM adrenaline, 50 mM glycine, NaOH (pH 10.2), and 1 mM catalase was monitored using a spectrophotometer at 480 nm (32 °C). The inhibition of the production of the chromogen is proportional to the activity of superoxide dismutase present in the sample. Results are expressed as units of superoxide dismutase/mg of protein.

Glutathione Peroxidase Activity Glutathione peroxidase activity was determined according to Wendel [33], with modifications. The reaction was carried out at 37 °C in a solution containing 20 mM PPB (pH 7.4), 1 mM EDTA, 0.8 mM sodium azide, 5 mM NADPH, 40 mM glutathione, and 40 U glutathione reductase per mL. The glutathione peroxidase activity was measured at 340 nm using tert-butylhydroperoxide as substrate. The contribution of spontaneous NADPH oxidation was subtracted from the overall reaction. Glutathione peroxidase activity is expressed as µmol NADPH oxidized per min per mg of protein (µmol oxidized NADPH/min)/mg protein).

Glutathione S-transferase Activity Glutathione S-transferase activity was measured using 1-chloro-2,4-dinitrobenzene as a substrate [34]. Briefly, the tissues were homogenized in 50 mM PPB (pH 6.5) and centrifuged at 4000×*g* for 15 min at 4 °C. The supernatant was centrifuged at 16 000×*g* for 1 h at 4 °C. The pellet was discarded and the supernatant was used as the enzyme source. The reaction mixture contained 250 µL of 0.3 M PPB (pH 6.5, 200 mL), 30 mM 2,4-dinitrochlorobenzene (0.03 mL), 30 mM glutathione (0.03 mL) and the appropriate enzyme source, 2.5 µL sample. This reaction was initiated by the addition of glutathione and the absorbance was read at 340 nm against the reagent (blank). The activity is expressed as units of glutathione S-transferase/mL. One unit of glutathione S-transferase is defined as the amount of enzyme required to produce 1 µmol of conjugated glutathione S-transferase/2,4-dinitrochlorobenzene/min.

Oxidative Damage Markers

Lipid Peroxidation Thiobarbituric acid reactive species were quantified by an acid heating reaction with thiobarbituric acid [35]. Briefly, after precipitation with 10% trichloroacetic acid, the supernatant was mixed with 0.67% 2-thiobarbituric acid and heated in boiling water for 25 min. The samples were sedimented and the absorbance of the supernatant was read by spectrophotometry at 535 nm. The results are expressed as nmol/mg of protein.

Protein Carbonyl Formation Protein carbonyl formation is a widely adopted parameter for oxidative damage of proteins. The carbonyl groups were determined as an index of protein oxidative damage based on the reaction with dinitrophenylhydrazine [36]. In short, the proteins precipitated in 20% thiobarbituric acid were washed with ethanol, ethyl acetate and re-dissolved in dinitrophenylhydrazine. Then,

the absorbance was read in a spectrophotometer at 370 nm. The carbonyl content is expressed as nmol/mg of protein.

Sulfhydryl Groups Quantification Total reduced sulfhydryl groups in samples provided the oxidative status of thiol groups [37]. Briefly, 60 µg of the sample was diluted in 10 mM PPB and 5,5′-dithionitrobis-2-nitrobenzoic acid for 60 min at room temperature. The reduction in sulfhydryl groups was read in a spectrophotometer at 412 nm and the results are expressed as µmol/mg of protein.

Western Blotting Experiments

To perform immunoblotting experiments, tissues ($n=6$ per group) were homogenized with Radioimmunoprecipitation assay buffer 1x (RIPA, 246 mM Tris–HCl pH 8, 150 mM NaCl, Octylphenoxy poly(ethyleneoxy)ethanol 1% (IGEPAL), 0.5% biliary salts, 0.1% Sodium dodecyl sulfate (SDS), and 1 µg/mL leupeptin), centrifuged at 14,000×*g* for 10 min and supernatant proteins were measured according to Lowry et al. [25]. Thirty microgram of protein was fractionated per well by SDS polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes with a Trans-Blot semi-dry electrophoretic transfer cell (Bio-Rad, Hercules, CA, USA).

Protein loading and electroblotting efficiency were verified through Ponceau S staining and the membranes were washed with a Tris-Tween buffer solution (Tris 100 mM, pH 7.5, 0.9% NaCl, and 0.1% Tween-20). Membranes were incubated overnight at 4 °C with each primary antibody: [anti-Glial fibrillary acid protein (GFAP), anti-Neuronal nuclear antigen marker (NeuN), anti-Receptor for advanced glycation endproduct (RAGE), anti-β-amyloid precursor protein (APP), anti-Phosphorylated extracellular signal regulated kinase 1/2 (pERK1/2), anti-Extracellular signal regulated kinase 1/2 (ERK 1/2), anti-Phosphorylated p38 mitogen-activated kinase (pp38MAPK), anti-p38 mitogen-activated kinase (p38MAPK), anti-Phosphorylated Jun-amino-terminal kinase (JNK), anti-Jun-amino-terminal kinase (pJNK), anti-Protein kinase B (Akt), anti- phosphorylated kinase B (pAkt), and anti-β-actin; 1:1000 dilution range] and subsequently washed with the Tris-Tween buffer solution. Anti-rabbit or anti-mouse IgG peroxidase-linked secondary antibodies were incubated for 2 h on a shaker and then washed. The immunoreactivity was detected by enhanced chemiluminescence using SuperSignal West Pico Chemiluminescent Substrate containing luminol/enhancer and stable peroxide buffer (Thermo Scientific, Rockford, IL USA). The densitometric analysis of the films was performed using ImageJ software. Blots were developed to be linear in the range used for densitometry. All results are expressed as a relative ratio to β-actin.

Immunofluorescence Staining

Tissue Collection and Sample Preparation For this analysis, four rats per group were anesthetized using ketamine/xylazine (75 and 10 mg/kg, respectively, intraperitoneally) and perfused transcardially, according to Heimfarth [38], with modifications. Briefly, 30 µm sections were incubated for 48 h with rabbit polyclonal anti-GFAP, mouse anti-NeuN (clone A60), mouse anti-β-amyloid, and rabbit anti-RAGE, in a 1:500 dilution, respectively, in PBS containing 0.3% TritonX-100 and 2% bovine serum albumin (BSA). The negative controls were performed without primary antibodies. After washing with PBS several times, tissue sections were incubated with anti-rabbit Alexa 488 and anti-mouse Alexa 555, both diluted 1:500 in 0.3% Triton X-100 and 2% BSA for 1 h at room temperature. After washing the samples with PBS, the slices were mounted with FluorSave Reagent covered with coverslips and sealed with nail polish. All images were obtained with an Olympus IX-81 confocal FV-1000 microscope and analyzed with Olympus Fluoview software.

Statistical Analysis

Body weight variation, enzyme activity, oxidative damage markers, immunocontent, and localization were analyzed using a one-way analysis of variance (ANOVA; group as the main factor) with Tukey's *post hoc* analysis, when appropriate. The behavioral task data were analyzed using a two-way ANOVA (groups and treatment period—pre and post gavage administrations, as factors) with Tukey's *post hoc* analysis, when appropriate. Data are expressed as means ± SEM or as a percent of the control. Significance levels for all measures were set at $p \leq 0.05$. Statistical Package for the Social Sciences (SPSS, SPSS Inc., Chicago, IL) version 18.0 was used for the statistical analyses.

Results

Guarana Composition

We initially determined the composition of the guarana powder used for the present study through liquid chromatographic separation. The composition of the guarana bioactive compound used was caffeine (34.19 ± 1.26 mg/g), theobromine (0.14 ± 0.01 mg/g), (+)-catechin (3.76 ± 0.12 mg/g), and (−)-epicatechin (4.05 ± 0.16 mg/g) (Supplementary Table 1). These results were in accordance with that reported in [13].

Systemic Toxicity Parameters

Next, we addressed whether CGE (21 mg/kg/day) and/or the experimental internal standard control, caffeine (0.84 mg/kg/day), chronically administered to rats daily for 6 months could cause systemic toxicity. Therefore, we recorded the body weight of rats and analyzed biochemical markers (*e.g.*, ALT, AST, creatinine, and urea). CGE or caffeine did not affect the body weight of rats, compared to the values reported for the control group ($p > 0.05$, Fig. 1a). In addition, no hepatic (ALT and AST) ($p > 0.05$, Fig. 1b) or renal (creatinine and urea) toxicity markers were altered ($p > 0.05$, Fig. 1c).

Behavioral Tasks

Open Field Task

In an attempt to evaluate the effects of CGE exposure and/or aging on behavioral parameters, we analyzed each rat in an open field test before the treatment (6-month-old rats; pre-treatment) and repeated during the post-treatment period (12-month-old rats).

In the open field task, a treatment period effect was observed, since animals of all groups (control, caffeine, and CGE) during the post-treatment period spent more time in peripheral and less time in central areas (Fig. 2a, b) when compared to that reported for the same animals during the pre-treatment period. Moreover, during the post-treatment experiments, all animals presented lower mean speeds (Fig. 2c) and lesser peripheral and central area distances travelled (Fig. 2d, e) than that reported for the same animals during the pre-treatment period. These results show that the effects between the pre-treatment and post-treatment periods were not related to CGE or caffeine administration, but were probably associated with natural aging degeneration.

In an attempt to search for a group (control, caffeine, and CGE) effect for behavioral parameters, we analyzed the saline, caffeine, and CGE-treated groups in an open field task in the post-treatment period. The results showed that

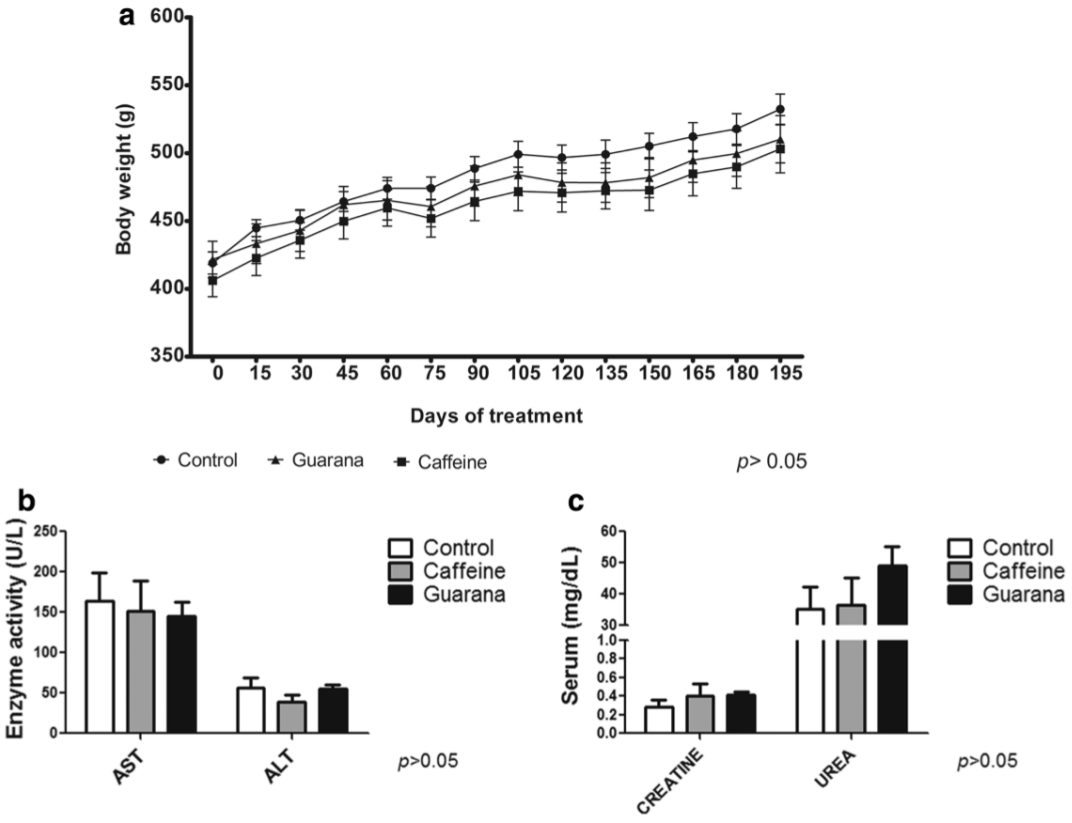

Fig. 1 Body weight variation and systemic toxicity parameters. **a** shows body weight variation of middle age male *Wistar* rats treated with guarana and caffeine solutions for 6 months. A one-way ANOVA indicated no statistical differences among groups ($p > 0.05$). **b, c** show serum parameters. Figure 1b shows AST and ALT activity. Figure 1c shows creatine and urea concentrations. Data are expressed as means ± SEM (commercial guarana extract (CGE), caffeine, and control groups). A one-way ANOVA indicated no statistical differences among groups ($p > 0.05$)

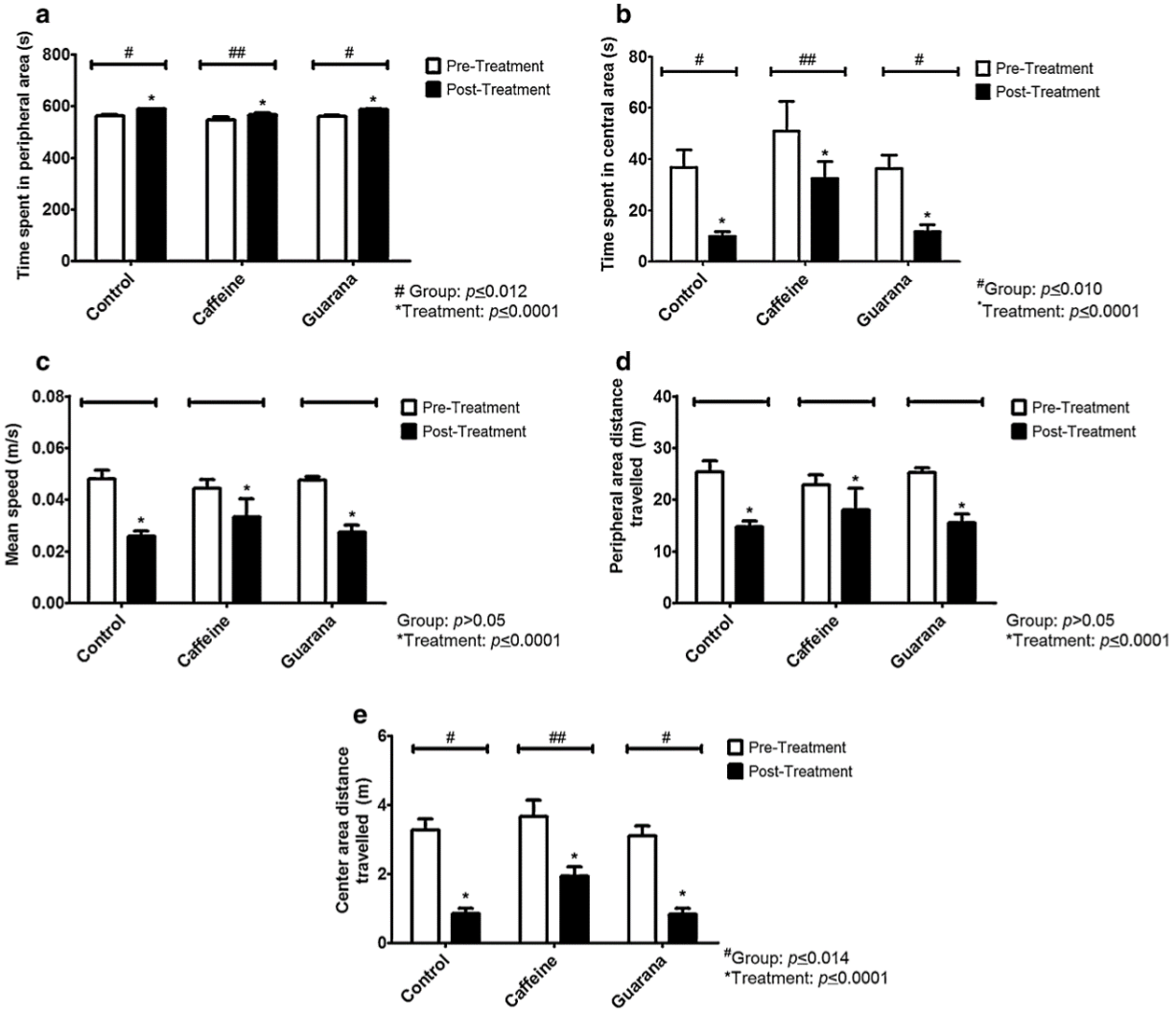

Fig. 2 Locomotive activity in open field tests. Figure displays locomotive activity in the open field test for middle age male *Wistar* rats treated with CGE and its standard solution (caffeine) over 6 months. Animals were tested for 10 min. Data is presented as means ± SEM. The different symbols indicate statistical differences among groups ($p \leq 0.05$). **a** displays the results of the time spent in periphery area. A group effect was demonstrated, since control and CGE treated groups spent more time in the peripheral area than the caffeine treated group. A treatment period effect was also revealed as animals spent more time in peripheral areas in the post-treatment period than animals in the pre-treatment period. An interaction was observed, as the control group spent more time during the post-treatment period in the periphery area than the caffeine treated group during pre-treatment period. **b** displays the results of the time spent in the central area. A group effect was demonstrated, as the caffeine treated group spent more time in the central area than the control and CGE treated groups. A treatment period effect was also revealed, as animals spent more time in the central area during the pre-treatment period than animals in the post-treatment period. An interaction was observed, as the caffeine treated group spent more time in the central area during the post-treatment period than the control group during the pre-treatment period. **c** displays the mean speed results. No group effect was found for mean speed. A treatment period effect was revealed, as animals presented higher mean speeds during the pre-treatment period than animals during the post-treatment period. An interaction was observed, as all pre-treatment groups displayed higher mean speeds than control and CGE post-treatment groups. **d** displays the peripheral area distance traveled. No group effect was found in the peripheral area distance traveled. A treatment period effect was revealed, as animals presented higher peripheral area distance traveled during the pre-treatment period than animals during the post-treatment period. An interaction was observed, as control and CGE treated groups displayed higher peripheral area distance traveled during the pre-treatment period than the control and CGE treated groups during the post-treatment period. **e** displays the center area distance traveled. A group effect was found, as the caffeine-treated group presented a higher center area distance travelled than control and CGE treated groups did. A treatment period effect was revealed, as all pre-treatment animals displayed a higher distance traveled in the center area than post-treatment groups. An interaction was observed, as CGE and control post-treatment groups displayed a lower center area distance travelled than control and caffeine pre-treatment groups

the animals in the control and CGE-treated groups spent more time in the peripheral area than that reported for the caffeine-treated group; consequently, the control and CGE-treated rats spent less time in the central area compared to that reported for the caffeine-treated group (Fig. 2a, b). As shown in Fig. 2, the CGE or caffeine treatment did not alter the animal mean speed (Fig. 2c), or the periphery and central distance traveled (Fig. 2d, e) when compared to that reported for the control groups showing that chronic administration of CGE did not alter locomotor activity.

Novel Object Recognition Task

After the open field task, animals were subjected to a novel object recognition task for short-term memory (3 h) and long-term memory (24 h) evaluation before and after the 6 months of treatment (*i.e.*, daily oral gavage, 1 mL/kg body weight of saline, 21 mg/kg body weight/day of CGE, or 0.84 mg/kg body weight/day of caffeine). CGE or caffeine did not affect the discrimination index; therefore, a chronic exposure to CGE and caffeine did not improve the short-term or long-term memory ($p > 0.05$) (Supplementary Fig. 1).

Redox Parameters

Next, we addressed whether chronic treatment with CGE or caffeine disrupts the redox homeostasis of striatum or hippocampus cells. For these analyses, enzymatic activity (e.g., catalase, superoxide dismutase, glutathione peroxidase, and glutathione S-transferase) and oxidative damage markers (e.g., protein carbonyl formation, sulfhydryl groups, and lipid peroxidation) were quantified (Table 1).

In the hippocampus, a group effect was observed for the catalase activity. Catalase activity in the CGE-treated group decreased compared to that in the control or caffeine treated groups (Table 1). No difference was observed in superoxide dismutase, glutathione peroxidase, or glutathione S-transferase activity in the hippocampus of treated rats. Furthermore, lipid peroxidation, sulfhydryl content, and protein carbonylation levels were unaltered in the hippocampus of CGE- and caffeine-treated rats (Table 1).

In the striatum, CGE increased catalase activity compared to that reported for the control group (Tukey's *post hoc* test, $p \leq 0.05$). No difference was observed in superoxide dismutase and glutathione peroxidase activity in the striatum of treated rats. Moreover, CGE- and caffeine-treated groups exhibited higher glutathione S-transferase activity compared to that reported for the control group. Additionally, a group effect was observed for the striatum protein carbonyl formation, since the CGE-treated group showed higher protein carbonyl formation than the control group did. However, no difference was found between CGE- and caffeine-treated rats in the striatum protein carbonylation levels (Table 1).

Reinforcing the oxidative imbalance in the hippocampus and striatum of CGE- or caffeine-exposed rats, we observed upregulated catalase levels in the hippocampus and downregulated catalase levels in the striatum, as demonstrated by western blot analysis using specific antibodies (Fig. 3a, b).

Neuronal Nuclear Antigen Marker (NeuN), Glial Fibrillary Acid Protein (GFAP), Receptor for Advanced Glycation Endproduct (RAGE), and β-Amyloid Precursor Protein (APP) Immunocontent

In an attempt to evaluate the effects of CGE or caffeine exposure on cell damage, the immunocontent of proteins related to cellular homeostasis, cognitive injury, and senescence (NeuN), (GFAP), (RAGE) and (APP) were quantified in the hippocampus (Fig. 4a) and striatum (Fig. 4b) of middle age rats.

Table 1 Oxidative enzymatic activities and damage markers in hippocampus and striatum

	Hippocampus			Striatum		
	Control	Caffeine	Guarana	Control	Caffeine	Guarana
Number of treated rats (n)	6	6	6	6	6	6
Enzymatic defenses						
GST activity (U GST/mg protein)	16.29 ± 1.45	17.39 ± 1.32	16.62 ± 1.32	23.46 ± 1.50[a]	27.69 ± 0.68[b]	28.20 ± 0.84[b]
Enzymatic antioxidant defenses						
SOD activity (U SOD/mg protein)	18.71 ± 1.09	20.40 ± 0.65	17.39 ± 0.61	11.14 ± 1.42	15.36 ± 0.80	16.25 ± 2.12
CAT activity (U CAT/mg protein)	3.97 ± 0.34[a]	3.75 ± 0.98[a]	1.15 ± 0.30[b]	6.67 ± 1.18[a]	9.35 ± 0.95[a,b]	13.51 ± 2.00[b]
GPx activity (U GPx/mg protein)	49.49 ± 4.70	74.93 ± 29.09	80.55 ± 17.48	60.47 ± 26.68	121.28 ± 20.54	65.81 ± 11.66
Oxidative damage markers						
TBARS level (nmol/mg protein)	0.42 ± 0.05	0.48 ± 0.04	0.59 ± 0.07	0.65 ± 0.13	1.15 ± 0.35	0.98 ± 0.14
Carbonyl level (nmol/mg protein)	5.22 ± 0.49	3.90 ± 0.44	3.75 ± 0.38	1.74 ± 0.21[a]	2.33 ± 0.18[a,b]	2.42 ± 0.16[b]
SH content (umol/mg protein)	41.54 ± 2.72	38.17 ± 0.88	37.74 ± 0.58	27.79 ± 2.35	29.93 ± 1.04	30.37 ± 1.17

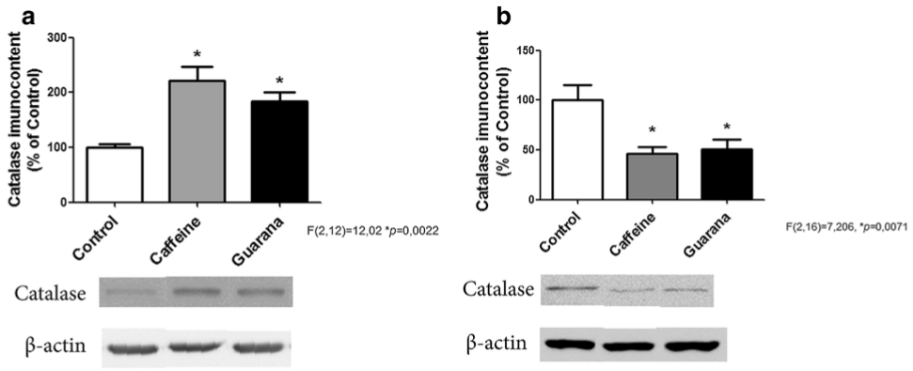

Fig. 3 Catalase enzyme immunocontent in hippocampus and striatum. Figure exhibits the catalase enzyme immunocontent in the hippocampus (*panel A*) and striatum (*panel B*) of middle age male *Wistar* rats treated with CGE and caffeine for 6 months. Data are expressed as a percent of the control (means ± SEM) and normalized by β-actin. The *different symbols* indicate statistical differences among groups ($p \leq 0.05$). **a** Catalase immunocontent was increased in the hippocampus of middle age rats chronically treated with caffeine and CGE. **b** Catalase immunocontent was decreased in the striatum of middle age rats chronically treated with caffeine

In the hippocampus, western blot and immunofluorescence confocal microscopic analyses showed increased immunoreactivity of the RAGE protein in the CGE-treated group. There was no change in the immunocontent of RAGE in the control or caffeine-treated group. Furthermore, the results showed increased APP levels in the hippocampus of the CGE-treated group and in the internal standard caffeine-treated group when compared to that reported for the control group (Fig. 4a). Moreover, the immunocytochemistry staining results show preserved GFAP and NeuN in the hippocampus of CGE- or caffeine-exposed rats when compared to the control. In line with these findings, the western blot analysis showed unaltered immunocontent of the biomarkers, further supporting the absence of reactive astrocytes and neuronal death in the hippocampus of middle age rats exposed to CGE or caffeine (Fig. 4a).

In the striatum, western blot and immunofluorescence confocal microscopic analyses showed a decrease in immunoreactivity of GFAP in CGE-treated rats compared to that reported for the control and caffeine-treated groups. Furthermore, APP immunoreactivity increased in the caffeine-treated groups' striatum when compared to that reported for the control and CGE-treated groups. These results show that CGE could prevent the increase in APP immunocontent induced by the caffeine treatment. No effects were observed in NeuN or RAGE immunocontent in the striatum of treated rats (Fig. 4b).

Signaling Pathways

In an attempt to understand signaling mechanisms following chronic exposure to CGE or caffeine, we initially evaluated the Akt/PKB pathway. Akt is known to be activated by phosphorylation of Thr308 [39, 40]. Western blot analysis with anti-totalAkt and anti-phosphoAkt (Thr308) antibodies showed a decrease in phosphoAkt/totalAkt immunoreactivity in the hippocampus of middle age rats treated with CGE and caffeine, when compared to that reported for the control group (Fig. 5a). No difference was observed in phosphoAkt/totalAkt immunoreactivity in the striatum of treated rats (Fig. 5e).

Next, we focused on the MAPK pathway downstream of CGE and caffeine signaling. A western blot analysis with antibodies against total and phosphorylated/activated forms of MAPKs showed decreased phosphor-Erk1/2/total Erk1/2 ratios in the striatum of middle age rats treated with CGE and caffeine (Fig. 5h) and decreased phospho-p38 MAPK/total-p38 MAPK ratios in the caffeine-treated group, compatible with the inhibition of the kinase activity. However, phosphor-JNK/total JNK ratios were not altered in the hippocampus or striatum (Fig. 5b, f).

Discussion

CGE, obtained from dried seeds of *Paullinia cupana* Mart., is rich in caffeine and other active compounds. CGE contains flavonoids, catechins, epicatechins, and tannins, substances that also have psychostimulant properties [41] and is known as a nutritional supplement for the improvement of intellectual and physical capacities. Initially, the CGE bioactive compounds were determined, and a high concentration of methylxanthines (reiterating its stimulant action) and a low concentration of epicatechins and catechins polyphenols were noted. In the literature, the majority of the effects of guarana extract were attributable to the caffeine compounds. However, polyphenols present in this extract

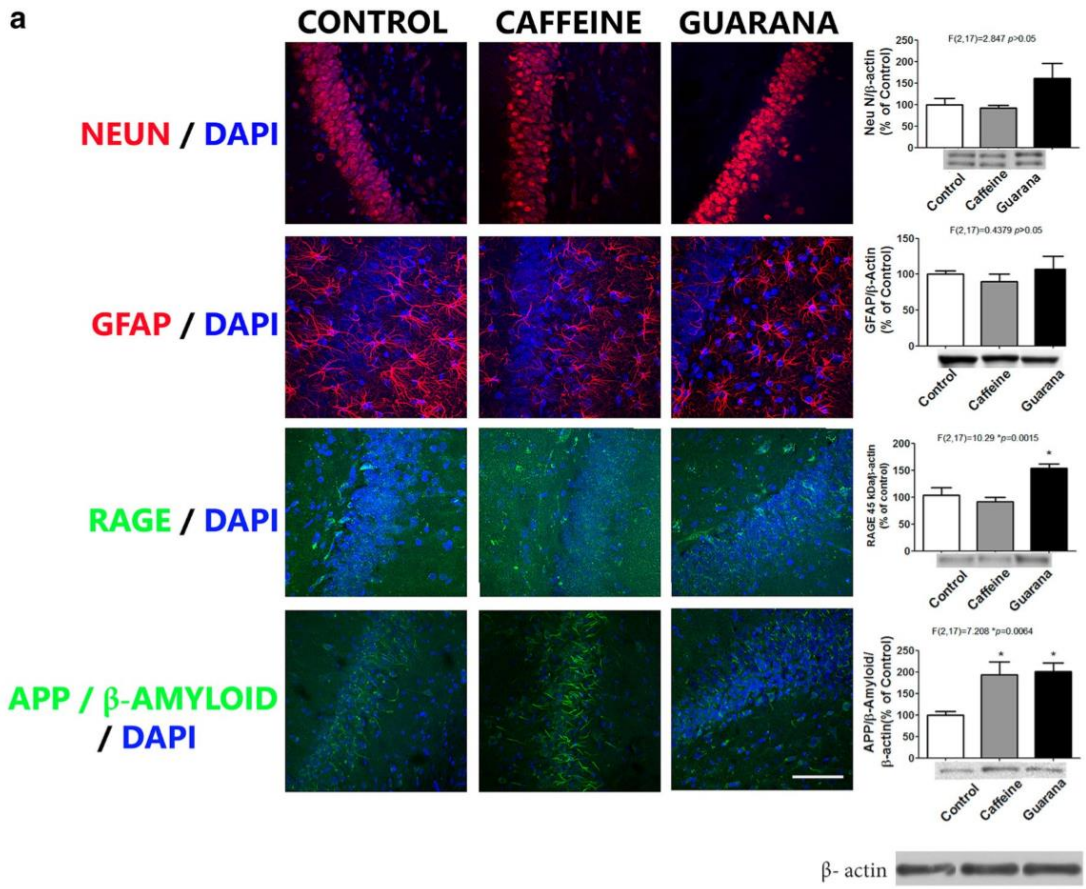

Fig. 4 Proteins related to homeostasis, cognitive injury, and senescence immunocontent in the hippocampus and striatum. Figure shows the immunocontent of some proteins related to homeostasis, cognitive injury, and senescence in the hippocampus (**a**) and striatum (**b**) of middle age male *Wistar* rats treated with CGE and caffeine for 6 months. Data expressed as a percent of the control (means ± SEM). The *different symbols* indicate statistical differences among groups ($p \leq 0.05$). Red dye NeuN and GFAP. Green dye RAGE and APP. Blue dye DAPI. Images were captured from the same brain structure, but not necessarily from the same brain slice. **a** Hippocampus analysis no effects were found in NeuN or GFAP expressions. RAGE displayed increased immunoreactivity in the hippocampus of animals treated with CGE, compared to other groups. APP displayed increased immunoreactivity in the hippocampus of animals treated with CGE and caffeine, compared to the control group. **b** Striatum analysis no effects were found in NeuN or RAGE expressions. GFAP displayed decreased immunoreactivity in the striatum of animals treated with CGE, compared to control and caffeine treated groups. APP displayed increased immunoreactivity in the striatum of animals treated with caffeine, compared to control and CGE treated groups. *NeuN* neuronal specific nuclear protein marker, *GFAP* glial fibrillary acid protein, *RAGE* receptor for advanced glycation endproduct, *APP* β-Amyloid precursor protein. *Scale bar* 50 µm

also have many biological effects. To better understand the effect of these compounds (caffeine or polyphenols), caffeine was administered at the same concentration as that present in CGE, acting as an internal experimental standard for CGE. Our results suggest that caffeine is the major active constituent in CGE but it cannot be responsible for all of the observed changes in behavior and biochemistry parameters.

In this study, we initially established whether CGE (21 mg/kg/day) and/or the experimental internal standard control, caffeine (0.84 mg/kg/day), chronically administered to rats for 6 months could cause systemic toxicity. The results showed that CGE and caffeine treatments did not cause any renal or hepatic toxicity. These results corroborated with the results of other reports that doses of 30 mg/kg showed no toxicity in an animal model [42]. Furthermore, during CGE and caffeine administration, we recorded the body weight of the rats daily, and neither of the treatment affected the weight of the rats when compared to that reported for the control group. These results suggest that CGE and caffeine did not cause systemic toxicity. Moreover, the absence of weight loss in CGE-treated rats indicates that CGE was not thermogenic at the doses used in this study. This report contradicts another study that

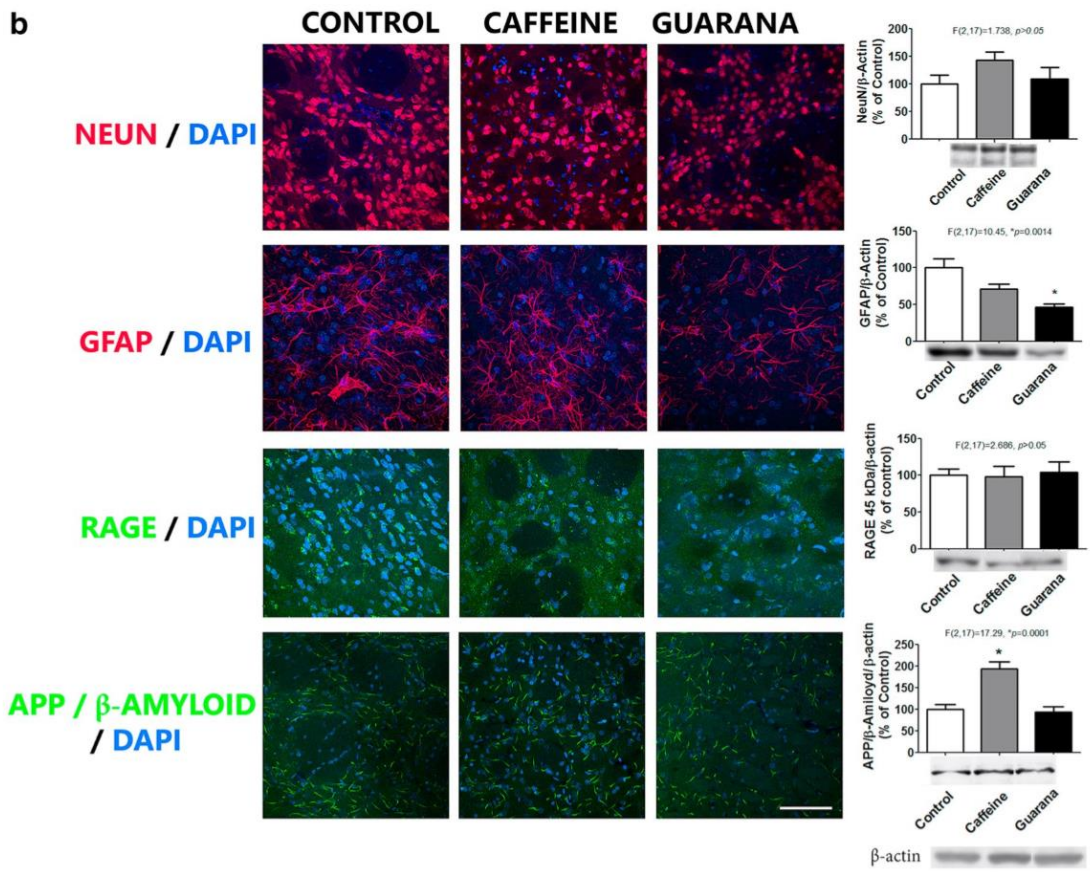

Fig. 4 (continued)

showed an increase in energy expenditure and weight loss of patients consuming guarana [43].

Experimental and clinical studies have shown that CGE could affect memory, cognitive, and physical performances [44, 45]. Nonetheless, further research in this area is warranted, because of the high consumption of caffeinated beverages, which have been shown to have activity relevant to brain function. Therefore, in this work, we initially focused on understanding the role of chronic consumption of CGE and/or caffeine on anxiety-like behavior, locomotion, and cognitive parameters.

The open field task measured autonomous behavior focused on locomotion and emotional activity in the rodents, and their memory was evaluated by the novel object recognition task. In this study, some behavioral parameters were explored for testing of chronic consumption of CGE, since the aging of the rats could alter memory, cognition, locomotion activity, or anxious-like behavior. The sequence of behavioral tasks was performed before the treatment (6-month-old rats) and repeated during the post-treatment period (12-month-old rats), aiming for a better interpretation of the results.

In the open field data interpretation, any experimental procedure able to decrease the time spent in the central area of the apparatus is considered an anxiogenic manipulation, while the opposite is considered an anxiolytic outcome [46]. Our open field results showed an increase in the time spent in the central area of the apparatus by animals chronically treated with caffeine, indicating a reduction in the anxiety of these animals, in accordance with Caravan et al. [47]. Surprisingly, CGE treatment was not able to alter the time spent in the central area of the apparatus in a significant way. This result demonstrates a group effect and it shows that CGE could inhibit the reduction in anxiety provoked by caffeine. Çakır et al. [48] reported that the increased anxiety-like behavior under stress conditions improved with acute and predominantly chronic caffeine pre-treatments. Based on this, we can infer that other compounds present in CGE may be masking the effect observed for caffeine on the emotional performance of these animals. Other CGE bioactive compounds could inhibit the anxiolytic effect of caffeine.

Next, we focused on understanding if a chronic consumption of CGE or caffeine could alter the rat's memory

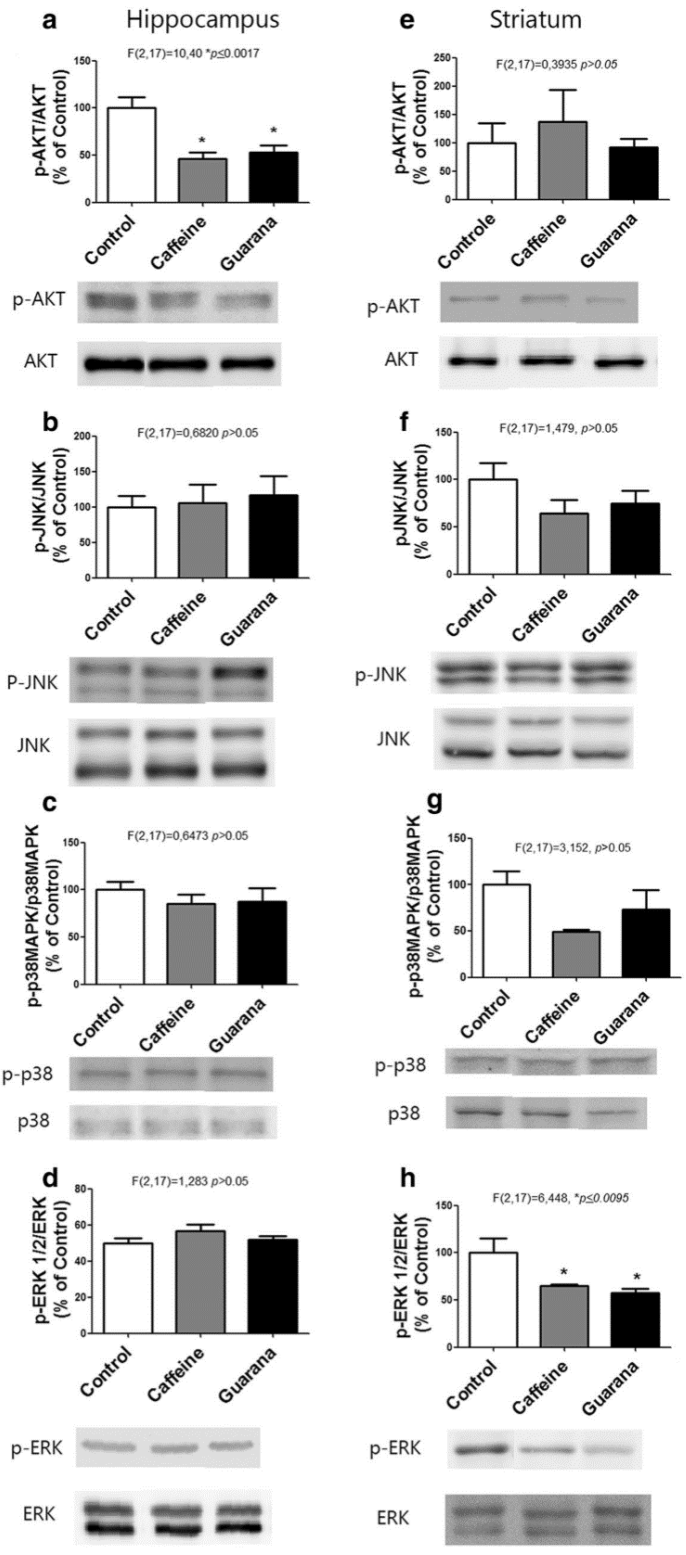

Fig. 5 MAPK pathway western blots of the hippocampus and striatum. Figure displays densitometric results for the MAPK pathway western blots in the hippocampus (*left column*) and striatum (*right column*) of middle age male *Wistar* rats treated with CGE and caffeine for 6 months. Data expressed as a percent of the control (means ± SEM). The *different symbols* indicate statistical differences among groups ($p \leq 0.05$). **a** AKT phosphorylation levels were decreased in the hippocampus of middle age animals treated with CGE and caffeine, compared to the control group. No effects were observed in the hippocampus JNK (**b**), p38 (**c**), or ERK phosphorylation (**d**) levels of the animals treated with CGE and caffeine. In the striatum, no effects were observed in AKT phosphorylation (**e**), JNK (**f**), or p38 (**g**) levels. ERK phosphorylation (**h**) was decreased in the striatum of animals treated with CGE and caffeine. *pAkt* phosphorylated kinase B, *Akt* protein kinase B, *pJNK* phosphorylated Jun-amino-terminal kinase, *JNK* Jun-amino-terminal kinase, *p-p38* phosphorylated p-38 enzymes, *p38* p38 enzymes, *pERK1/2* phosphorylated extracellular signal regulated kinase 1/2, *ERK* extracellular signal regulated kinase

or locomotor activity. Our results show that neither treatment (CGE and caffeine) affected the short- or long-term memory in the novel object recognition task. Moreover, CGE treatment was not able to improve locomotor activity, distance traveled, or mean speed. Therefore, we can conclude that chronic CGE treatment does not improve locomotor behavior, cognition, or memory in middle age rats, in agreement with the results of Otobone et al. [49]. The behavior parameters analyzed show that the CGE treatment could block the anxiolytic effect of caffeine, indicating that other compounds of CGE present important biological actions.

Despite the compelling evidence of the marked susceptibility of the middle age brain to the effects of natural products, the effects of chronic exposure to CGE or caffeine on the normal function of different brain structures remain to be clarified. Previous studies indicated that the hippocampus and striatum, structures involved in learning and memory, cognitive, emotional, and motor functions, are susceptible to aging [50–52] and effects of natural products [53]. Therefore, elucidation of the biochemical mechanisms of CGE in the striatum and hippocampus is a relevant issue.

Previous publications from our group state that CGE treatment presented antioxidant properties against different sources of free radical production, likely related to its methylxanthine and polyphenols content [13, 54]. In this regard, we found that disruption of redox homeostasis in the hippocampus and striatum could affect the brain impairment observed in middle age rats, chronically exposed to CGE. Modest antioxidant defenses, higher oxygen consumption, and percentage of lipid composition make the brain vulnerable to redox impairments. As a result, oxidative stress can alter neurotransmission, neuronal function, and overall brain activity [55–57]. Oxidative imbalance has also been implicated in anxiety disorders [58, 59]. The disruption of redox homeostasis in the CGE- and caffeine-treated groups could be correlated with an alteration of anxiety-like behavior observed in the treated animals.

CGE and/or caffeine induced oxidative stress in a tissue-specific manner. A relevant point to be raised concerns the role of catalase activity in CGE or caffeine actions in CNS. Catalase is an enzyme that metabolizes H_2O_2, releasing water and oxygen, and present in low levels in most brain structures [57]. The metabolic dynamics in the brain generates a considerable amount of H_2O_2, an important indicator of brain homeostasis. Our findings indicate that in the hippocampus of the CGE-treated rats, there was a decrease in catalase activity compared to that in the control and caffeine-treated groups and an increase in catalase immunocontent. However, the hippocampus of caffeine-treated rats exhibited an increase in the immunocontent of catalase without an expected increase in enzyme activity. In the striatum, catalase activity increased in CGE-treated rats while its immunocontent decreased. In contrast, animals chronically treated with caffeine exerted no change in striatal catalase activity; however, it was observed a reduction in catalase immunocontent. Therefore, chronic consumption of CGE could alter the amount of H_2O_2 in the brain of treated rats in a tissue-specific manner and disturb the H_2O_2 signaling. H_2O_2 is an important signaling molecule for synaptic and neuronal activity in the CNS [60] and a disturbance in H_2O_2 homeostasis is associated with brain diseases [61]. Biological system enzymes are often subject to modulation in their activity regardless of protein expression because antioxidant enzymes may be regulated by short-term (e.g. acute increase in the enzyme activity due to an allosteric or substrate activation) or long-term activation (e.g. increased expression due to a chronic condition of unbalanced reactive species production), giving them a dynamic and special regulation. Our research group have shown that CAT enzymatic regulation by Retinoic Acid was independent of mechanisms of protein expression and even transcriptional [62]. Therefore, it is not surprising to see an increase in the enzymatic expression and a decrease in the immunocontent due to the different types of regulation that CAT is subjected to. However, it is plausible to suggest that the ECG extract administration could be associated with an adaptive response, altering the pattern of antioxidant capacity for different components of the CNS involved in mood and emotional regulation, since the administration of ECG did not have any beneficial effects during the aging of male "*Wistar* rats". This effect is named "antioxidative stress", a machinery of homeostatic reactions in cells that govern the amount of allowable antioxidant activity [63]. That way, it should be stressed that the antioxidants can not distinguish between the radicals that play a beneficial physiological role and those that originate ROS. Several natural compounds have been described in the literature with similar effects and failed to obtain beneficial results, displaying an "antioxidative stress" potential in addition to oxidative stress in the brain [12, 64].

Furthermore, our present findings show that CGE and caffeine could alter the catalase immunocontent in a tissue-specific manner. The modification of antioxidant enzyme level is a common cell adaptive mechanism to endure ROS production. To protect against oxidative stress, cells have developed complex signaling cascades to detoxify potentially harmful substances and maintain cellular redox homeostasis. The transcription factor, Nrf2, is largely responsible for the basal and inducible expression of these proteins involved in oxidative stress response, drug metabolism, and cellular protection. The central role of Nrf2 in cell survival and in neurodegenerative diseases has been well established [65–67].

Oxidative stress is the most severe consequence of the imbalance between the production of different types

of ROS and the action of antioxidant defenses. Despite the increase in the activity of the enzymatic antioxidant defense molecule catalase in the striatum of CGE-treated rats, our results show an increase in protein carbonylation. Therefore, the increase in the catalase activity was not enough to prevent the protein damage caused by the CGE compounds, possibly because other ROS may be attacking this structure in the CGE-treated rats. These chemical groups are produced in the lateral chains of protein oxidation events by different sources of radical species, such as superoxide, hydroxyl radical, and peroxyl radicals, among others [68]. This oxidative damage promotes the generation and accumulation of highly cytotoxic intra- and/or intermolecular crosslinks that might disturb cellular signaling [57].

Our biochemical data for the striatum of CGE-and caffeine-treated rats also showed increased enzymatic detoxification defenses by glutathione S-transferase activity, clearly indicating the effect of the major compound of both treatments, leading to the stimulation of phase II detoxification enzymes. Glutathione S-transferase has the capacity to mediate the combination of reduced glutathione and a variety of xenobiotics for the purpose of detoxification [69]. This effect can be attributed to a moderate toxicity profile of these treatments in the CNS, as the striatum is a sensitive structure to this modulation.

An imbalance in ROS production can disrupt the brain homeostasis via several mechanisms of action. Increasing evidence suggests that oxidative stress is a key modulator of the biochemical changes that lead to the activation of the apoptotic process and neuronal cell death in neurodegenerative diseases [70, 71]. Previous studies have shown that Akt and MAPKs are two important signaling pathways involved in oxidative stress-induced cell injury and H_2O_2-induced oxidative stress [72, 73]. Thus, the misregulation of H_2O_2 production observed in the brain of middle age *Wistar* rats after the chronic supplementation of CGE or caffeine may also have an effect on these two important cell-signaling pathways.

Our present findings show that in the striatum, chronic exposure to CGE or caffeine misregulated the MAPK cascade, resulting in dephosphorylation/inhibition of the ERK1/2 and p38MAPK pathways without altering JNK activity. These findings could represent a disrupted cellular homeostasis of neural cells as MAPK phosphorylation/dephosphorylation plays critical roles in brain function, modulating protein-mediated processes related to proliferation, differentiation, and functioning of cells. However, our results show no alteration of the MAPK pathway in the hippocampus, but a decrease in Akt phosphorylation in middle age rats chronically treated with CGE and caffeine when compared to that reported for the control group. Akt is known as protein kinase B (PKB), which plays a critical mediation role in disorders associated with longevity in cellular metabolism, oxidative resistance, and cell survival [74].

The dephosphorylation/inhibition of the ERK1/2 and Akt pathways seems to be associated with the caffeine content present in CGE treatments, because both groups exhibited the same effect. Cechella et al. [18] reported that caffeine could reduce Akt phosphorylation levels, previously increased by physical activity, in middle age rats. In addition, another study reported that caffeine exhibits an inhibitory effect on the Akt and ERK pathways in microglia BV2 cells stimulated by lipopolysaccharides [75].

To better understand the effects of these CGE and/or caffeine treatments on brain structure, we also investigated the status of some proteins related to CNS homeostasis, cognitive injury, and senescence in the striatum and hippocampus of middle age *Wistar* rats. Recent findings associate alteration of redox homeostasis with RAGE upregulation/activation [76] and amyloid-β peptide formation [77]. Our results show an increase in the RAGE immunocontent of the hippocampus cells in the CGE-treated group. RAGE activation can be involved in neuronal damage due to the overproduction of toxic ROS, cytokines, and pro-inflammatory molecules [78]. The accumulation of RAGE ligands promotes oxidative stress, progressive neuronal dysfunction, and neurodegeneration [79, 80]. Furthermore, RAGE plays an important role in the production and metabolism of amyloid-β peptide [81].

In line with these findings, we demonstrated that CGE and caffeine treatment increased APP levels in hippocampal neuronal cell surfaces. Such findings suggest that caffeine can suppress APP internalization in hippocampus neurons in both treatments. This result suggests that caffeine exerts protective effects against amyloidogenic processing of APP in the hippocampus. Surprisingly, in the striatum, some compound present in CGE blocked this protective effect of caffeine in APP internalization. The caffeine results were in accordance with the findings of Li et al. [82]; they reported that caffeine could inhibit the internalization of APP in the endolysosomes, reducing the process of amyloid-β peptide formation in vitro.

Next, to evaluate the effects of CGE and caffeine exposure on cell damage, we assayed the striatum and hippocampus of treated rats for GFAP and NeuN by immunofluorescence confocal microscopy. GFAP is an astrocyte cytoskeletal protein overexpressed in response to stressor signals [83]. NeuN is a neuron-specific nuclear protein used as a marker of live neurons [84]. We observed a decrease in the number of striatum GFAP-positive cells, without a change in neuronal marker immunocontent. However, further evaluation is required to directly correlate this lower astrocytic number with the increased antioxidant load and/or possible oxidative damage in the striatum. Additionally, no effects were found in the hippocampal NeuN and GFAP

immunocontent. These results indicate that CGE treatment, but not caffeine, decreased GFAP immunocontent without altering NeuN, indicating that astrocytes could be more susceptible to CGE compounds than neurons and that these effects are attributable to other substances present in the CGE rather than caffeine.

Conclusions

We observed that CGE and/or the experimental internal standard control (caffeine) chronically administered to rats over 6 months was capable of modifying behavioral parameters and biochemical markers in a tissue-specific manner. To our knowledge, this is the first study reporting the effects of CGE treatment from adulthood to senescence; CGE was found to affect oxidative stress parameters in the hippocampus and striatum. These data suggest that caffeine and other compounds present in guarana could shift the balance between oxidative stress and neurodegenerative signaling in the CNS, toward a striatal and hippocampal inhibition of the pro-survival pathways, which may be favorable to neurodegenerative microenvironments and disorders.

Several effects of the CGE treatment could be assigned to caffeine compounds; however, other bioactive substances present in CGE could alter the redox homeostasis and brain function. In this work, it was observed that the CGE minority compounds could inhibit the anxiolytic and protective effect of caffeine in APP internalization. Furthermore, CGE minority compounds act on astrocyte cells rather than neurons. Therefore, we could infer that astrocytes are more susceptible to CGE compounds than neurons.

Contrary to popular belief, these findings demonstrate that chronic supplementation with guarana extract was not effective against oxidative stress and did not provide any cognitive benefit during the 6 months aging of the Wistar rat model. Guarana treatment inhibited beneficial effects found in the caffeine group, and in several results the effects of guarana probably resulted from its caffeine content. These results indicate that antioxidant extract supplementation may have no effect or even damage brain health status, inviting additional studies to further characterize the potential role of CGE bioactive compounds in the disruption of brain homeostasis observed in CGE-treated rats".

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Author Contributions All authors listed above participated in the study to a significant extent. Moara Rodrigues Mingori, Fares Zeidán-Chuliá, José Cláudio Fonseca Moreira, and Daniel Pens Gelain conceived and designed the experiments. Moara Rodrigues Mingori, Luana Heimfarth, Karla Suzana Moresco, Henrique Mautone Gomes, Jeferson Delgado, and Sabrina Roncato performed the experiments. Moara Rodrigues Mingori, Luana Heimfarth, Charles Francisco Ferreira, and José Cláudio Fonseca Moreira worked on the analysis and interpretation of the data, writing, and intellectual content of the article. All authors read and approved the submitted manuscript.

Compliance with Ethical Standards

Conflict of interest Luana Heimfarth has received a postdoctoral scholarship from the National Council for Scientific and Technological Development (CAPES/FAPERGS, Brazil). Moara Rodrigues Mingori and Karla Suzana Moresco have received doctoral scholarships from the National Coordination for Improvement of Higher Education Personnel (CAPES, Brazil). Henrique Mautone Gomes has received a PROBIT/FAPERGS graduation scholarship. Jeferson Delgado and Sabrina Roncato have received PROPESQ/UFRGS graduation scholarships. None of the authors declare a conflict of interest related to this study, whether financial or otherwise.

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RESULTADOS

CAPÍTULO II

Paullinia cupana Mart. seed extract increase Glutathione S transferase and decrease carbonylation in the heart of aged rats

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Abstract: The accumulation of reactive oxygen species (ROS) generated by intracellular metabolic processes is accentuated during aging. We investigated the hypothesis that consumption of antioxidant compounds may curb the effects of aging by studying the effects of Guarana seed extract on oxidative stress, hematological parameters, thermogenic profile, sirtuin proteins related to longevity, and senescence in middle-aged male Wistar rats. The animals were randomized into 3 treatment groups (saline, commercial guarana extract (CGE) or caffeine), administrated daily by oral-gavage for a period of six months. Biochemical analyses were carried out on the hearts, livers and kidneys. CGE administration resulted in increased circulating lymphocytes and decreased neutrophils. Glutathione S-transferase (GST) activity in the cardiac tissue was increased and there was a decrease of gonadal fat in CGE group. Furthermore, CGE conferred a reduction in gonadal fat, while it did not modify the expression of longevity markers, such as sirtuin 1 (SIRT1). In contrast, caffeine treatment showed a protective effect on cardiac and hepatic tissues, as well as an increase in SIRT1 immunocontent levels. These findings suggest that the CGE has immunostimulatory and thermogenic effects on the rodents' systemic performance, but the beneficial effects of longevity are attributed only to caffeine.

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Opposed Reviewers:

Effects of *Paullinia cupana Mart.* seed extract in the heart of aged rats: increased glutathione S-transferase activity and decreased carbonylation

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Abbreviations: AMPK: Activated protein kinase; **CAT:** Catalase; **CGE:** Commercial Guarana Extract; **DNPH:** Dinitrophenylhydrazine; **EDTA:** Ethylenediaminetetraacetic acid; **GPx**: Glutathione peroxidase; **GST:** Glutathione S-transferase; **H_2O_2**: Hydrogen peroxide; Hb: Hemoglobin; **IGEPAL:** 1% Octylphenoxypolyethoxyethanol; **MCHC:** Mean corpuscular hemoglobin concentration percentage; **MCV:** Mean corpuscular volume; **NADPH:** Reduced form of Nicotinamide adenine dinucleotide phosphate; **pH**: Potential of hydrogen; **RBCC:** Red blood cell count; **RIPA:** 1X Radioimmunoprecipitation assay; **ROS:** Reactive oxygen species; **SDS:** 0.1% sodium dodecylsulfate; **SEM:** Standard error of mean; **SIRT 1:** Sirtuin 1 protein; **SOD:** Superoxide dismutase; **TBARS:** Thiobarbituric acid reactive species; **TPP:** Total plasma proteins.

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HIGHLIGHTS

1. Guarana chronic administration increased circulating lymphocytes and decreased neutrophils.
2. Redox changes through the activation of detoxification pathways were induced in the cardiac tissue by chronic guarana intake.
3. Both guarana and caffeine compounds demonstrated thermogenic effects, reducing gonadal fat after chronic administration.
4. Caffeine treatment increased Sirtuin 1 immunocontent on cardiac and hepatic tissues.

*Abstract

ABSTRACT

The accumulation of reactive oxygen species (ROS) generated by intracellular metabolic processes is accentuated during aging. We investigated the hypothesis that consumption of antioxidant compounds may curb the effects of aging by studying the effects of Guarana seed extract on oxidative stress, hematological parameters, thermogenic profile, sirtuin proteins related to longevity, and senescence in middle-aged male *Wistar* rats. The animals were randomized into 3 treatment groups (saline, commercial guarana extract (CGE) or caffeine), administrated daily by oral-gavage for a period of six months. Biochemical analyses were carried out on the hearts, livers and kidneys. CGE administration resulted in increased circulating lymphocytes and decreased neutrophils. Glutathione S-transferase (GST) activity in the cardiac tissue was increased and there was a decrease of gonadal fat in CGE group. Furthermore, CGE conferred a reduction in gonadal fat, while it did not modify the expression of longevity markers, such as sirtuin 1 (SIRT1). In contrast, caffeine treatment showed a protective effect on cardiac and hepatic tissues, as well as an increase in SIRT1 immunocontent levels. These findings suggest that the CGE has immunostimulatory and thermogenic effects on the rodents' systemic performance, but the beneficial effects of longevity are attributed only to caffeine.

Keywords: *Paullinia cupana* commercial extract; caffeine; aging; neurodegenerative diseases; polyphenols; neuroprotection.

1. INTRODUCTION

In spite of the fundamental role of redox signaling in cellular homeostasis, the pro-oxidant-antioxidant balance is substantially altered during aging because the recycling of several forms of free radicals is compromised. Oxidative stress, induced by reactive oxygen species (ROS), plays an important role in the aging process [1]. Research demonstrates increased ROS generation in several tissues, such as brain, liver, kidney and heart, during aging, and indicates that peripheral enzymatic and non-enzymatic defense systems try to limit cell damage during this modulation [2-4]. Additionally, many authors have highlighted the therapeutic potential of nutritional antioxidants for health protection again age-induced oxidative stress [2, 5].

Paullinia cupana (guarana) is a native Amazon plant recognized for its stimulating effects due to its high levels of caffeine and polyphenols [6]. Guarana is vastly cultivated in Brazil to supply the markets for use in energy drinks, soft drinks, nutritional supplements and cosmetics [7]. The seeds have been used, in the form of aqueous or ethanolic extracts, due to their higher caffeine content than the seeds of *Arabica coffee* [6]. Caffeine is the main constituent of guarana seeds, and is considered responsible for many of the biological activities of the extract. However, the seeds also contain other active molecules, such as theobromine and many polyphenols (*e.g.* catechin and epicatechin). This combination of molecules present in guarana seed powder may be responsible for its protective properties against aging [8].

Studies have demonstrated the effects of guarana on the modulation of oxidative stress [9] [10], atherosclerosis [11], cardiotoxicity [12], and cardioprotection [13], as well as *diabetes mellitus* and obesity [14]. The diverse components of guarana seed extract are being analyzed in relation to its antioxidant activity. Although the

antioxidant activity of the seed extract is usually associated with low-mass compounds, such as phenols, other components like polysaccharides have shown high activity for the reduction of hydroxyl radicals [15]. In an *in-vivo* study with carbon tetrachloride as a liver damage agent, Kober and collaborators demonstrated that guarana is able to reduce liver damage, conferring protection through the inhibition of DNA damage [16].

Although some studies on the possible protective effects of guarana and its phenolic compounds have been carried out, there are no reports on the effect of *Paullinia cupana* against age-induced oxidative stress. Studies of herbal compounds often disregard systemic effects caused by the constituent compounds. Given the consumption of guarana powder as a food supplement, the aim of this work was to investigate the possible effects of chronic treatment with guarana seed extract on oxidative and hematological parameters in liver, kidney, heart, and blood tissue samples from male older *Wistar* rats treated with a daily supplement.

2. MATERIALS AND METHODS

2.1 Materials

Guarana seed extract (Pharmaceutical Industrial Laboratory – LIFAR Porto Alegre, Brazil) and caffeine powder (Sigma, St. Louis, MO, USA) were conserved dry and away from light at 20 °C until the solutions were prepared. Both the guarana and caffeine solutions were prepared on the day of use by dilution in 0.9% saline solution.

The determination of bioactive compounds in guarana extract was previously performed by Bittencourt *et al*. [8]. The concentrations of bioactive compounds in a 100-mg (dry weight) sample of guarana extract were estimated as follows: 0.14 mg/g of

theobromine, 34.19 mg/g of caffeine, 3.76 mg/g of catechins, and 4.05 mg/g of epicatechins.

2.2 Animals and treatments

Thirty 6-month-old male adult *Wistar* rats, weighing 415±6.51 grams (mean±standard error of mean [SEM]), were randomly selected from our animal facility. They were group housed (four animals per cage) in home cages, (65 × 25 × 15 cm^3) with sawdust-covered floors and kept in a controlled environment (lights on between 07:00 and 19:00 hours, temperature at 22±2 °C, cage cleaning twice a week, free access to standard lab rat chow and water). At the beginning of the experiments, the young animals were 6 months old and received guarana uninterruptedly for 6 months. After a two-week acclimatization period, the animals were divided into 3 groups and then started to receive daily treatments by oral gavage: 21 mg of guarana seed extract /body weight (kg)/day (Guarana group); 0.84 mg of caffeine powder/body weight (kg)/day (Caffeine group); or 1 mL of 0.9% saline/body weight (kg)/day (Control group).

2.3 Ethical Aspects

All animal treatments were approved by the Institutional Ethical Committee (Federal University of Rio Grande do Sul, Number 27.686) and followed the recommendations of the International Council for Laboratory Animal Sciences (ICLAS). All efforts were taken to minimize pain or discomfort. This article does not contain any studies with human participants.

2.4 *Blood sampling and measurement of hematological parameters*

Six months after treatment, rats were anesthetized and blood samples were collected directly from the heart using a cardiac puncture syringe and stored in

ethylenediaminetetraacetic acid (EDTA)-containing tubes. Blood samples were then investigated for different biochemical parameters. Red blood cell count (RBCC), hemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration percentage (MCHC), total plasma proteins (TPP), leukocytes, segmented neutrophils, eosinophils, monocytes and lymphocytes were assayed using an automated full blood count analyzer (Sysmex America Inc., Lincolnshire, IL60069, USA).

2.5 Tissue samples preparation

After cardiac blood collection, animals were euthanized by decapitation in random order using a small animal guillotine (Insight, São Paulo, Brazil). The hearts, livers and kidneys were then removed and rapidly dissected and stored at −70 °C for further analysis (Figure 1). The heart, liver and kidney tissues (n=6 per group) were used for oxidative status evaluation and western blotting. Additionally, visceral, gonadal and brown fat depots were dissected and weighted.

2.6. Oxidative Status Assessment

For all oxidative status evaluations, heart, kidney and liver tissues were homogenized in ice-cold 50 mM potassium phosphate buffer (pH 7.4) containing 1 mM EDTA. The homogenate samples were centrifuged at 1000 ×g for 10 min at 4 °C and the supernatant parts was used. For data normalization, homogenate protein content was determined according to Lowry [17].

2.6.1 Antioxidant enzymatic activities

The activities of the antioxidant enzymes catalase, superoxide dismutase and glutathione peroxidase were determined by spectrophotometric analysis of the heart, kidney and liver homogenates.

Catalase (CAT) activity was determined upon establishing the rate of decrease in the absorbance of hydrogen peroxide (H_2O_2) at 240 nm (25 °C, approximately 1 µg of protein). The results are expressed as units of catalase/mg of protein [18].

Superoxide dismutase (SOD) activity was determined by quantifying the inhibition of superoxide-dependent adrenaline autoxidation to adrenochrome formation. This inhibition in the production of the chromogen is proportional to the activity of superoxide dismutase present in the sample. Results are expressed as units of superoxide dismutase/mg of protein [19].

Glutathione peroxidase (GPx) activity was determined by measuring the rate of NADPH oxidation at 349 nm using tert-butylhydroperoxide as the substrate, as previously described [20]. Values are expressed as µmol NADPH oxidized per minute per mg of protein ([µmol oxidized NADPH/min]/mg protein).

2.6.2 Detoxifying enzymatic activity

Glutathione S-transferase (GST) activity was measured using 1-chloro-2,4-dinitrobenzene as substrate [20]. This reaction was initiated by the addition of glutathione and the absorbance was read at 340 nm against the reagent (blank). The activity is expressed as units of glutathione S-transferase/mL. One unit of GST is defined as the amount of enzyme required to produce 1 µmol of conjugated glutathione S-transferase/2,4-dinitrochlorobenzene/minute.

2.6.3 Oxidative damage markers

To measure the rate of oxidative damage to which proteins are subjected, carbonyl groups were determined based on their reaction with dinitrophenylhydrazine (DNPH), as previously described [21]. The carbonyl content is expressed as nmol/mg of protein. Thiobarbituric acid reactive species (TBARS) generated by lipoperoxidation

were quantified by an acid-heating reaction between thiobarbituric acid and lipoperoxides [22]. The quantity of TBARS was determined by spectrophotometry at 535 nm. The results are expressed as nmol/mg of protein.

The oxidative status of thiol groups was measured by the reduction of sulfhydryl groups in the samples tested [23]. Samples (60 µg) were diluted in 10 mM phosphate buffer and 5,5'-dithionitrobis-2-nitrobenzoic acid buffer for 60 min at room temperature. The reduction in sulfhydryl groups was determined by spectrophotometry at 412 nm, and the results are expressed as µmol/mg of protein.

2.6.4 Western blotting

The tissues of each group (n = 6 per group) were collected and homogenized with 1X Radioimmunoprecipitation assay (RIPA) buffer (246 mM Tris–HCl pH 8, 150 mM NaCl, 1% octylphenoxypolyethoxyethanol (IGEPAL), 0.5% biliary salts, 0.1% sodium dodecylsulfate (SDS) and 1 µg/mL leupeptin). Protein (40 µg) was fractionated per well by SDS polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose membranes with Trans-Blot Semi-Dry Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA, USA). Membranes were incubated overnight at 4 °C with each primary antibody (Sirtuin 1, Sirtuin 3 and β-actin; 1:1000 dilution range) and subsequently washed with Tris-Tween buffer Solution. Anti-rabbit or anti-mouse IgG peroxidase-linked secondary antibodies were incubated for 2 hours on a shaker and then washed. The immunoreactivity was detected by enhanced chemiluminescence using SuperSignal West Pico Chemiluminescent kit (Thermo Scientific; Luminol/Enhancer and Stable Peroxide Buffer, Rockford, IL USA). Densitometric analysis of the films was performed using Image J. software. Blots were developed to be linear in the range used for densitometry. All results are expressed as a relative ratio to β-actin.

2.6.5 Statistical analysis

For data processing, the database double entry and review were performed using SPSS, version 18.0. [SPSS Inc. Released 2009. PASW Statistics for Windows, Version 18.0. Chicago: SPSS Inc.].

Continuous variables were expressed as mean and standard error of mean (SEM) or median and 95% Confidence Interval (CI), as defined by Shapiro-Wilk test. Categorical variables were described by absolute (*n*) and relative frequencies (*n*%), or as percentage mean of control (% mean of control±SEM). A One-way Analysis of Variance (ANOVA) with Tukey *post hoc* test, or Kruskal-Wallis test with Dunn *post hoc* test was applied for comparing means between continuous variables. Spearman's correlations were carried out among groups and enzymatic measurements. The significance level adopted for all analyses was set at 5%.

3. RESULTS

The specific doses of *Paullinia cupana Mart.* (21 mg/kg/day) used during treatments were defined according to human recommendations by the manufacturer in Brazil (Pharmaceutical Industrial Laboratory – LIFAR Porto Alegre/RS, Brazil), in a 1:2 ratio adaptation for rodent consumption. The caffeine dose (0.84 mg/kg/day) was equivalent to the amount of caffeine contained in the guarana dose defined. According to the manufacturer, every 1 g of guarana powder provides 40 mg of caffeine. Six months after chronic guarana and caffeine treatment, no difference in body weight was found among groups [data not shown].

The blood count results indicated no difference in RBCC, HB, hematocrit, TPP, MCV, MCHC, leukocytes, eosinophils and monocytes among the groups (Table 1).

However, there was a decrease in segmental neutrophils [One-Way ANOVA, $F(2,9)=6.442$, $p=0.018$] and an increase in lymphocytes [Kruskal-Wallis χ^2 (2)=18.8192, $p=0.017$] in animals that were chronically administered guarana seed extract, when compared to the control and caffeine groups (Tukey *post hoc* test, $p = 0.016$ and Dunn *post hoc* test $p = 0.015$, respectively).

Biochemical analyses were carried out on the rodents' kidney, liver and heart tissues. The defense enzymatic activity and oxidative damage measurements are displayed in Table 2. The GST enzymatic activity in the cardiac tissue was elevated in animals chronically treated with guarana extract as compared to both other groups [One-Way ANOVA, $F(2,15)=4.985$, $p = 0.022$, Tukey *post hoc* test $p \leq 0.05$]. Additionally, guarana-treated animals also presented a decrease in protein carbonylation in the cardiac tissue, when compared to the control and caffeine groups [One-Way ANOVA, $F(2,15)=5.668$, $p = 0.015$, Tukey *post hoc* test $p \leq 0.05$]. No additional variations were found in any other tissues and measurements among the three analyzed groups ($p > 0.05$).

Spearman's correlations were carried out among the experimental groups and biochemical markers (enzymatic activity and oxidative damage measurements). These results are displayed in Table 3. Briefly, positive correlations were found between the caffeine and guarana groups with regard to cardiac GST activity, renal GST and GPx activities, and hepatic TBARS level, GST and CAT activities. On the other hand, negative correlations were found between the caffeine and guarana groups with regard to TBARS and SH contents in the kidney.

Due to its thermogenic profile, the possible effects of guarana extract were evaluated in the body fat distribution of the animals and these results are presented in Figure 1. There was a significant decrease in gonadal fat in guarana- and caffeine-

consuming animals, in comparison to the control group [One-Way ANOVA, $F(2,15)=15.888$, $p \leq 0.0001$, Tukey *post hoc* test $p \leq 0.05$] (Figure 1B).

Moreover, a study also demonstrated the cardio-protective effects of guarana extract, indicating its potential in the treatment of thrombosis and cardiovascular diseases [24]. For this purpose, a quantitative evaluation of the immunocontent levels of SIRT1 and SIRT3 proteins was performed on all tissues (liver, kidney and heart). These results are shown in Figure 2. In fact, SIRT1 immunocontent levels were elevated in the cardiac and hepatic tissues of animals consuming only caffeine for 6 months [One-Way ANOVA, $F(2,15)=9.255$, $p = 0.02$, Tukey *post hoc* test $p \leq 0.05$ and $F(2,15)=10.816$, $p \leq 0.001$, Tukey *post hoc* test $p \leq 0.05$, respectively], when compared to the control group.

4. DISCUSSION

Blood cell counts are an important index of the physiological and pathological profiles of mammals. The parameters evaluated in this study were red blood cell, hemoglobin, total plasma protein, platelet, neutrophil, eosinophil, monocyte and lymphocyte counts. Our findings revealed a relative lymphocytosis, or proliferation of lymphocytes, induced by chronic administration of guarana extract, indicating the potential immunostimulatory action of guarana. This result may be attributed to an immune response of rodents to *Paullinia cupana* extract, which led to the mobilization of immunocompetent cells. The implication of this finding is that guarana seed extract shows an immunogenic effect when administered chronically. This corroborates a previous *in vitro* study that demonstrated the protective effect of guarana extract in the

model of DNA damage in lymphocytes [9]. In contrast, the decrease in segmental neutrophils is a cytotoxicity indication in neutrophil lineages.

Neutrophils are the first defense line in any microbial infection and are often significantly elevated in acute inflammation. Lymphocytes, on the other hand, produce antibodies that bind to pathogens for direct elimination, and are more involved in defense against intracellular pathogens and tumors. The neutropenic effects of guarana extract could suggest that the extract has a detrimental effect on this first line of defense of body, while its effect on lymphocyte stimulation suggests hematopoietic immune stimulation. These results correlate with findings reported on the medicinal plant *Hunteria umbellate*, used in folk medicine for the treatment of ulcers, diabetes and obesity. The aqueous seed extract of *Hunteria umbellate* was able to increase lymphocyte counts and reduce levels of circulating neutrophils in rodents [25]. Blood parameters evaluated in caffeinated animals, showed that the lymphocyte count remained unchanged compared to the guarana group. This result corroborates the findings of Dulson and Bishop, who investigated the effects of caffeine on lymphocyte - activation in blood samples from young adults who consumed caffeine. Their results demonstrated that the intake of low concentrations of caffeine did not affect the activation of lymphocytes in humans within 1 hour after consumption [26]. Thus, other components of guarana extract besides caffeine, like catechins and epicatechins, may promote the action of growth factors related to lymphocyte production, while inhibiting growth factors associated with neutrophil production. Further studies are needed to acquire more information on the effect of guarana on lymphocytes and neutrophils.

The administration of antioxidant compounds, such as guarana, may alter the activities of enzymes such as GST, an important enzyme in xenobiotics biotransformation and degradation of endogenous compounds [27]. In animals

chronically supplemented with guarana, positive stimulation of GST activity in the heart tissue prevented an increase in protein carbonylation. This indicates the protein carbonylation-reducing activity of guarana by stimulation of the xenobiotics detoxification-signaling pathway in cardiac tissue. Another study reported that guarana was able to reduce cholesterol and low-density lipoprotein to basal levels in rats with high cholesterol levels, conferring intermediate protection to cardiac tissue [28]. On the other hand, these effects were not seen in the animals that received only caffeine, indicating that activation of the detoxification route is due to the effect of other compounds present in the extract of guarana, like the flavonoids.

Previous studies reported that *Paullinia cupana* extract has a thermogenic effect due to its caffeine content, as well as other polyphenols. Dysfunctions in the regulation of body temperature and energy balance homeostasis are worse during the aging process, and can result in obesity or thermal instability. For this reason, it was decided to investigate the volume of brown adipose tissue in the animals after consumption of the extract, but no difference was observed among the groups. Additionally, this research addressed the white fat evaluation, revealing that both experimental groups (guarana and caffeine) had lower gonadal fat, when compared to the control group. The total fat of these animals is decreased mainly by the effect of both extracts on gonadal fat. These findings corroborate the thermogenic effect of *Paullinia cupana* extract reported in other studies, which already indicated guarana as a potent complement in weight loss and energy expenditure [29]. Taken together, our data highlights that chronic administration of guarana was able to reverse the accumulation of gonadal fat related to aging.

The natural aging process is also associated with a decrease in testosterone levels and an increase in adipose tissues in rodents older than 12 months [30]. Studies

demonstrate a direct relationship between the increase in circulating testosterone levels and the decrease in gonadal fat in male rats [31]. Additionally, the increase in testosterone may decrease plasma markers associated with obesity or increased body fat [32]. Our findings demonstrate that the consumption of guarana led to a reduction of gonadal fat in elderly animals. Furthermore, it is reported in the literature that guarana, even at low concentrations, is able to raise plasma testosterone levels in rats treated with the extract for 56 days [33]. A possible explanation for the results in our chronic administration model is that guarana may be modulating the adipose hypothalamic-pituitary-gonadal (HPG) axis, which is involved in maintaining body weight, fat distribution and reproductive function.

Besides the accumulation of gonadal fat, the aging process is related to systemic and metabolic impairment. In general, the reduction of cardiac and hepatic capacity is remarkable. In these tissues, protein acetylation events play an important role in the metabolic regulation of homeostasis. SIRT1 is responsible for the reversible acetylation of proteins and is highlighted in many studies as a molecule regulating energy metabolism and anti-aging. Surprisingly, our results demonstrate that there was an increase in the immunocontent of SIRT1 in the liver and heart of caffeine-treated animals, when compared to the control, and this effect was not observed in animals treated with guarana. Our findings demonstrate that chronic administration of low doses of caffeine was able to increase the immunocontent of SIRT1 in the liver and heart of treated animals. These results correlate with those of other studies, which demonstrated that red wine components (polyphenols) are able to positively modulate SIRT1 [34]. Surprisingly, this protective effect was not observed in animals treated with *Paullinia cupana*. In the literature, studies show that caffeine can induce higher SIRT3 levels by promoting lipid metabolism [35]. *In-vitro* administration of low doses of caffeine was

also able to activate SIRT1 in cultured adipose tissue-derived adult stem (ADAS) and bone marrow cells [36]. Our findings indicate the protective effect of caffeine by positively modulation of the expression of sirtuins, which are lower in aging processes. Other compounds presented in guarana seed extract appear to inhibit this protective effect of caffeine in the cardiac and hepatic tissues of animals supplemented with this compound.

5. CONCLUSION

Modulations in blood cell (lymphocyte and neutrophil) counts of animals that chronically consumed guarana seed extract indicate that this compound has a hematopoietic immunostimulatory effect. This effect may be due to polyphenolic compounds alone, or in combination with caffeine - the major compound of this extract. It should be noted that caffeine alone was not able to induce these effects. Administration of *Paullinia cupana* modulated redox changes through the activation of detoxification pathways in the cardiac tissue. Additionally, chronic guarana administration conferred a reduction in gonadal fat, but did not modify the expression of longevity markers, such as SIRT1. On the other hand, caffeine treatment presented a protective effect on cardiac and hepatic tissues, represented by an increase in SIRT1 immunocontent levels in these tissues of animals consuming only caffeine for 6 months. These findings suggest that the consumption of guarana extract has immunostimulatory and thermogenic effects on the rodents' systemic performance, but the beneficial effects of longevity are attributed only to caffeine, when this is administered alone.

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Figure Titles:

Figure 1. Brown, gonadal and visceral fat masses of *Wistar* rats subjected to control, caffeine or guarana treatment. Data displayed as mean ± standard error of mean or median and percentiles. One-Way Analysis of Variance with Tukey *post hoc* test or Kruskal-Wallis with Dunn *post hoc* test. * indicate significant differences among experimental groups. Significance set as $p \leq 0.05$ for all analyses.

Figure 2. SIRT-1 and SIRT-3/β-actin (% of control) in heart, kidney and liver tissues of *Wistar* rats subjected to control, caffeine or guarana treatment. Data displayed as mean ± standard error of mean or median and percentiles. One-Way Analysis of Variance with Tukey *post hoc* test or Kruskal-Wallis with Dunn *post hoc* test. * indicate significant differences among experimental groups. Significance set as $p \leq 0.05$ for all analyses.

Table 1

Table 1. Comparisons of blood cell analyses among experimental groups.

Groups	**Control *n*=4**	**Caffeine *n*=5**	**Guarana *n*=5**	***p* Value***
RBC (x106/µL) – mean±SEM	7.14±0.84	8.11±0.20	7.63±0.28	0.375
Hemoglobin (g/dL) – mean±SEM	14.50±0.29	15.11±0.12	14.18±0.49	0.184
Hematocrit – mean±SEM	41.88±1.85	41.60±0.68	38.80±1.59	0.265
MCV – median[95%CI]	53.88[36.19–86.21]	49.30[42.55–59.20]	50.00[46.14-52.41]	0.088
MCHC – mean±SEM	34.70±0.87	34.57±1.70	36.55±0.82	0.468
TPP – mean±SEM	69.00±1.91	69.20±2.24	66.20±0.66	0.406
Leukocytes – mean±SEM	4866.25±977.64	3526.00±603.81	3340.00±833.43	0.398
Segmented neutrophils – mean±SEM**	41.38±4.33[a]	36.25±2.15[ab]	27.13±0.97[b]	**0.018**
Eosinophils – median[95%CI]**	1.25[-1.93–5.68]	0.50[-0.42–1.42]	0.00[0.00–0.00]	0.241
Monocytes – median[95%CI]**	0.00[-3.82–7.32]	0.00[-0.27–0.52]	0.00[0.00–0.00]	0.573
Lymphocytes – median[95%CI]**	48.00[20.01–5.74][a]	64.00[56.24–70.01][ab]	72.75[69.80–75.95][b]	**0.017**

Legend: CI: Confidence Interval. RBCC: red blood cells count, MCV: mean corpuscular volume, MCHC: mean corpuscular hemoglobin concentration, TPP: Total plasma protein. Data presented as mean±SEM or as median and 95% Confidence Interval. **Guarana group *n*=4 for these analyzes. *One-Way Analysis of Variance with Tukey *post hoc* test or Kruskal-Wallis with Dunn *post hoc* test. [ab]Different letters indicate significant differences among experimental groups. Significance set as $p \leq 0.05$ for all analyzes.

Table 2

Table 2. Enzymatic activities and oxidative damage markers in the liver, kidney and heart.

Variable	Heart *n*=6 per group			*p*	Kidney *n*=6 per group			*p*	Liver *n*=6 per group			*p*
	Control	Caffeine	Guarana		Control	Caffeine	Guarana		Control	Caffeine	Guarana	
Enzymatic defenses												
GST activity (U GST/mgprotein)	1.18±0.24[a]	1.89±0.26[ab]	2.66±0.10[b]	**0.02**	9.00±0.36	10.31±0.71	10.07±0.24	0.16	43.93 [39.68–51.61]	50.84 [38.29–69.89]	59.41 [50.17–66.02]	0.06
Enzymatic antioxidant defenses												
SOD activity (U SOD/mgprotein)	13.25±0.74	13.47±1.20	15.29±1.11	0.34	13.25±0.74	13.47±1.20	15.29±1.11	0.34	11.54 [9.12–12.88]	11.89 [10.32–12.96]	11.29 [10.15–12.45]	0.81
CAT activity (U CAT/mgprotein)	166.10±29.67	117.63±17.30	237.11±45.71	0.06	340.01±68.19	346.39±31.30	417.40±50.53	0.52	121.44 [111.11–158.06]	124.36 [84.69–214.15]	203.28 [134.46–248.77]	0.17
GPx activity (U GPx/mgprotein)	269.21±24.36	264.20±17.98	258.06±18.62	0.93	94.52±7.77	112.34±16.21	113.82±4.59	0.06	171.22±42.51	235.77±27.43	246.52±40.13	0.33
Oxidative damage markers												
TBARS level (nmol/mgprotein)	22.66±6.97	29.40±3.96	14.83±3.32	0.15	6.67±0.62	6.13±0.62	4.96±0.65	0.18	0.29 [0.23–0.39]	0.37 [-0.16–1.49]	0.41 [0.32–0.66]	0.12
Carbonyl level (nmol/mgprotein)	2.76±0.66[a]	2.61±0.39[a]	0.77±0.24[b]	**0.01**	3.86±0.34	5.72±0.70	4.78±0.36	0.06	7.51 [-0.05–22.86]	8.95 [-0.05–28.27]	15.69 [6.65–24.54]	0.49
SH content (nmol/mgprotein)	79.74±3.36	75.01±2.61	89.15±7.15	0.14	63.60±3.33	52.20±6.19	49.35±4.10	0.11	35.59±1.96	39.47±2.41	38.24±1.11	0.36

Legend: GST=glutathione S-transferase; SOD=superoxide dismutase; CAT=catalase, GPx=glutathione peroxidase; TBARS=thiobarbituric acid reactive substances (lipid peroxidation quantification); SH=sulfhydryl groups; U=unity. Data presented as mean±SEM or as median[95%CI]. SEM: Standard Error of Mean. CI: Confidence Interval. One-Way Analysis of Variance with Tukey *post hoc* test or Kruskal-Wallis with Dunn *post hoc* test. [ab]Different letters indicate significant differences in the specific structure among experimental groups. Significance set as $p \leq 0.05$ for all analyzes.

Table 3

Table 3. Correlations among enzymatic activities and oxidative damage markers in liver, kidney and heart tissues.

Variable	Experimental Groups	
	r_S	p
Heart		
GST activity (U GST/mgprotein)	**0.53**	**0.02**
SOD activity (U SOD/mgprotein)	0.41	0.09
CAT activity (U CAT/mgprotein)	0.21	0.40
GPx activity (U GPx/mgprotein)	-0.04	0.88
TBARS level (nmol/mgprotein)	-0.18	0.47
Carbonyl level (nmol/mgprotein)	**-0.63**	**0.01**
SH content (nmol/mgprotein)	**0.17**	**0.50**
Kidney		
GST activity (U GST/mgprotein)	**0.48**	**0.04**
SOD activity (U SOD/mgprotein)	0.41	0.09
CAT activity (U CAT/mgprotein)	0.30	0.22
GPx activity (U GPx/mgprotein)	**0.66**	**0.001**
TBARS level (nmol/mgprotein)	**-0.44**	**0.07**
Carbonyl level (nmol/mgprotein)	0.37	0.13
SH content (nmol/mgprotein)	**-0.53**	**0.03**
Liver		
GST activity (U GST/mgprotein)	**0.58**	**0.01**
SOD activity (U SOD/mgprotein)	0.01	0.96
CAT activity (U CAT/mgprotein)	**0.42**	**0.08**
GPx activity (U GPx/mgprotein)	0.28	0.27
TBARS level (nmol/mgprotein)	**0.50**	**0.04**
Carbonyl level (nmol/mgprotein)	0.29	0.25
SH content (nmol/mgprotein)	0.24	0.35

Legend: GST=glutathione S-transferase; SOD=superoxide dismutase; CAT=catalase, GPx=glutathione peroxidase; TBARS=thiobarbituric acid reactive substances (lipid peroxidation quantification); SH=sulfhydryl groups; U=unity. r_S=Spearman's correlation coefficient. Experimental Groups: (1) Control, (2) Caffeine, (3) Guarana. p=significance. Spearman correlations. Significance set as $p \leq 0.05$ for all analyzes.

Figure 1
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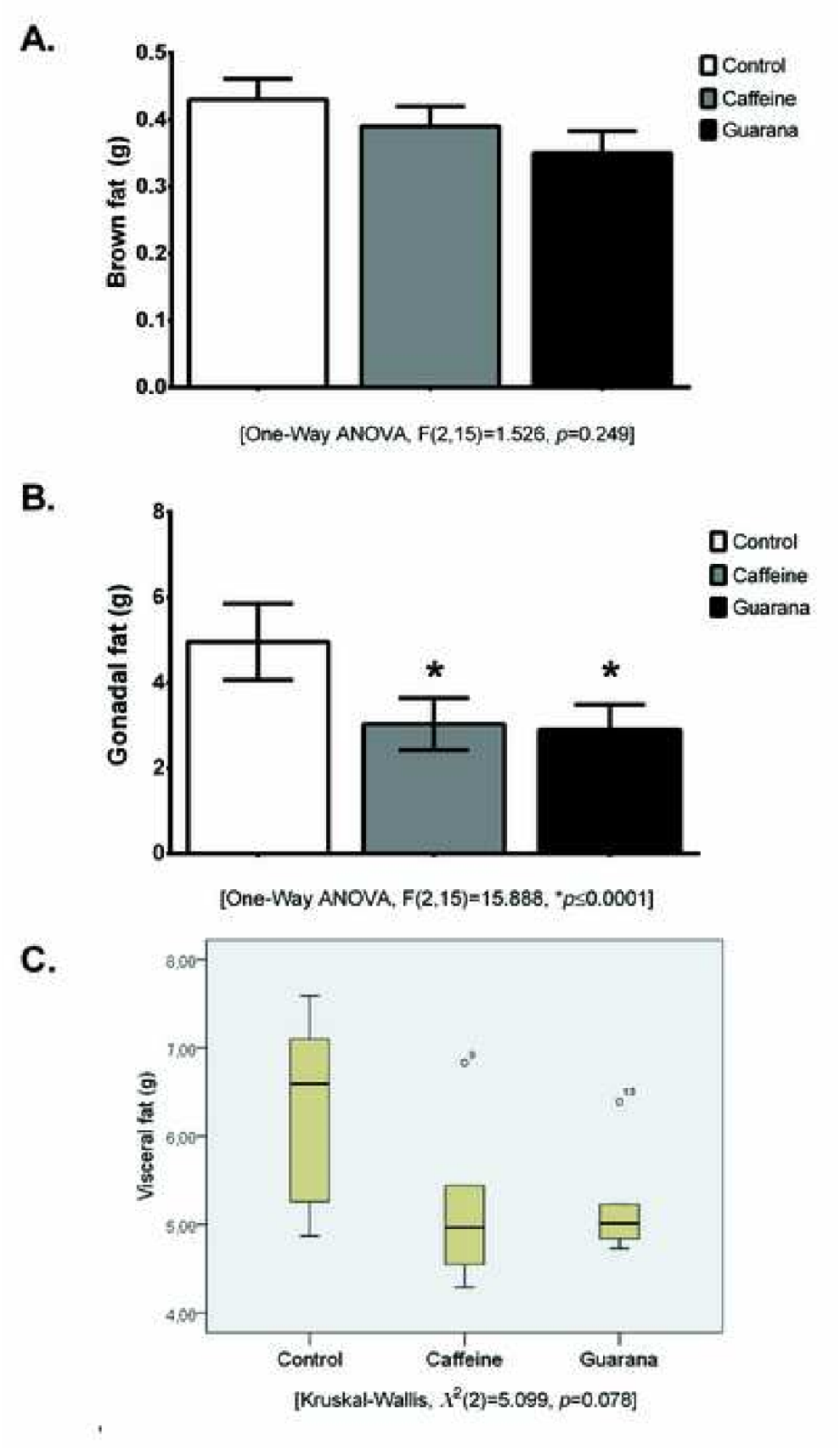

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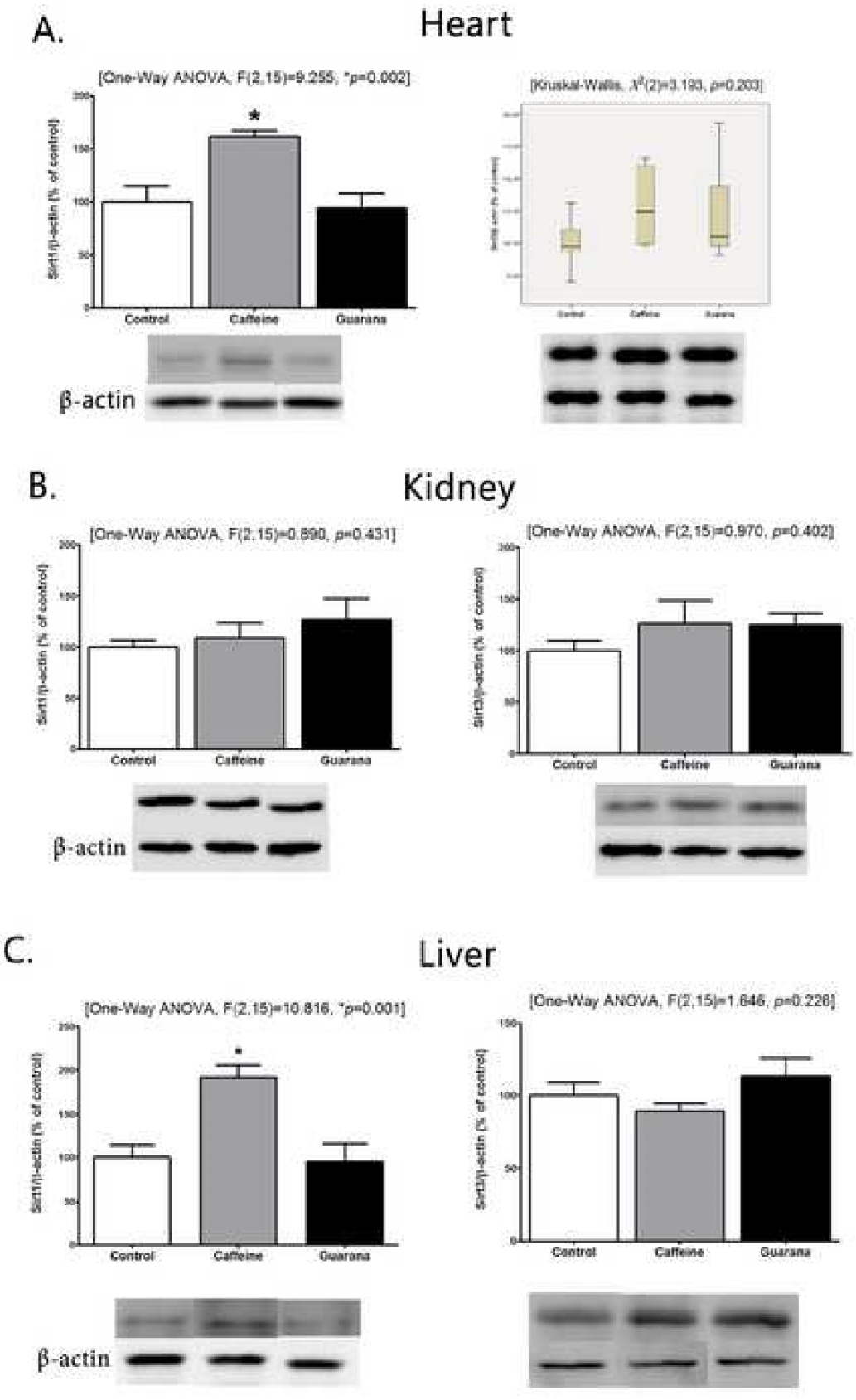

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PARTE III

DISCUSSÃO

Grande parte da relação entre guaraná e sua capacidade de modulação de rotas via neutralização de espécies reativas e diminuição de estresse oxidativo está relacionada com a sua caracterização redox ativa, porém a maioria dos estudos utilizam dosagens baixas e de curta duração sem atentar para os efeitos do consumo a longo prazo.

Dependendo do microambiente em que se encontra, o extrato de guaraná em doses baixas a moderadas pode atuar de maneira antioxidante, combatendo espécies reativas, ou de maneira pró-oxidante em altas concentrações, sendo a fonte geradora de estresse oxidativo. Em pacientes portadores de alguma condição tóxica ou patológica, o guaraná parecem diminuir os efeitos deletérios, visto que interagem com um microambiente alterado fisiológica e bioquimicamente dessa forma parece atuar aumentando as defesa antioxidante. Uma vez que a suplementação com guaraná é feita em indivíduos considerados saudáveis, os efeitos encontrados são contraditórios, ora estudos relatam um aumento preventivo das defesas antioxidantes ora pode desencadear efeitos citotóxicos.

Por muitos anos, o uso de extrato de guaraná foi sugerido como suplementação antioxidante, com propriedades estimulantes e anti-fadiga (Kennedy et al., 2008). Mais recentemente, a suplementação com o extrato das sementes passou a ser indicada no tratamento de enfermidades bacterianas, já que o consumo deste composto estava associado com uma menor incidência destas infecções (Basile et al., 2013). Além disso, passou a ser utilizado popularmente para o tratamento de doenças renais, cardíacas, para problemas estomacais, controle do funcionamento do intestino e do apetite. Essas indicações podem ser facilmente verificadas nos sites de revenda no Brasil, em farmácias brasileiras e até mesmo em outros países. Esses efeitos podem ser vistos apenas como um estratégia de venda e não são objetos de intensos estudos e comprovações científicas.

Os efeitos do guaraná são atribuídos diretamente a seu elevado teor de cafeína. Sendo assim suas ações farmacológicas advindas da cafeína incluem estimulação do SNC (por aumento de liberação de catecolaminas), diurese, hiperglicemia, estimulação cardíaca, vasodilatação coronariana e periférica, vasoconstrição vascular dentre outros (Fisone, Borgkvist, & Usiello, 2004). No entanto o guaraná conta em sua composição com diversas classes de polifenois como catequina e epicatequinas, que podem ser responsáveis pelo sua função na neutralização de ROS. Assim, seu principal mecanismo de ação é a neutralização de espécies reativas de oxigênio e redução da lipoperoxidação (L. S. Bittencourt et al., 2013).

Por outro lado, muitos trabalhos indicam atuação pró-oxidante, investigações *In vitro* demonstraram que o guaraná é capaz de induzir genotoxicidade e efeitos mutagênicos em ratos (Santa Maria, Lopez, Diaz, Muñoz-Mingarro, & Pozuelo, 1998) estudos adicionais relatam que a suplementação com guaraná visando a diminuição de peso corporal deve ser evitada em pacientes com hipertensão ou condições de comprometimento cardíaco devido a ação direta da cafeína (Hess & Sullivan, 2005). Um estudo singular avaliou os efeitos cognitivos, motores e parâmetros de ansiedade em ratos jovens e velhos após administração crônica de guaraná durante 12 meses porém não encontraram mudanças significativas nos resultados em comparação ao grupo controle (Mattei, Dias, Espínola, Carlini, & Barros, 1998). Um estudo recente elucidou o efeito do extrato de guaraná em células de neuroblastoma *SH-SY5Y* indicando citotoxicidade em concentrações de 10 to 40 ug/mL, tais concentrações causaram apoptose e necrose com acentuada liberação de caspase 3 ativada via efeitos pro-oxidantes do extrato de guaraná (Zeidán-Chuliá et al., 2013). Estes efeitos pro-oxidantes do guaraná, acontecem provavelmente, devido ao excessiva neutralização de ROS intracelular devido ao seu alto potencial antioxidante, o que leva a interrupção da homeostase redox celular dos sistemas

biológicos e, portanto, gera um microambiente redox desfavorável as funções de proliferação e as vias de sinalização relacionadas à sobrevivência. Esse conceito chamado de "estresse antioxidante" retrata que um forte componente antioxidante pode gerar um desequilíbrio redox tão nocivo quanto o estresse oxidativo, pela interrupção da sinalização celular gerada pelas espécies restivas (Poljsak & Milisav, 2012). Portanto, o conceito de estresse antioxidante seria particularmente relevante no caso de consumo excessivo de suplementos nutricionais ou extratos com alto potencial antioxidante que vem sendo utilizadas indiscriminadamente, sem prescrição médica. A planta nativa brasileira, guaraná, poderia ser um exemplo disso, tradicionalmente usado como estimulante e afrodisíaco também já demonstrou efeito anti-oxidante além de prevenir a glicação protéica (L. a. S. Bittencourt et al., 2014) porém poucos estudos abordam o efeito deste composto administrado cronicamente. Um dos principais componentes do extrato de guaraná, a cafeína, antagonista de receptores de adenosina, teve seu consumo indicado como potencial modulador do declínio cognitivo associado à idade, através da proteção do sistema antioxidante endógeno cerebral (Abreu, Silva-Oliveira, Moraes, Pereira, & Moraes-Santos, 2011) mas seus efeitos aliados a outros componentes presentes no extrato de guaraná tem sido pouco abordados. Embora a cafeína seja considerada o principal ingrediente ativo do extrato de guaraná, a presença de outras substâncias como composto fenólicos deve ser considerado, uma vez que os efeitos agudos e a longo prazo do consumo do extrato tendo efeitos sinérgicos entre seus componentes não são bem conhecidos ou estudados.

Em geral, ROS são capazes de reagir com diversos componentes celulares (DNA, carboidratos, proteínas e lipídios) de forma destrutiva. Nos processos de envelhecimento e neurodegeneração, acredita-se que a deficiência neurológica está intrinsecamente ligada ao dano neuronal por apoptose desencadeada por ROS. Dentre estas, estão a DA como a

causa mais comum de demência na atualidade e doença de Parkinson. Trabalhos relatam a relação entre produtos de glicação avançada (AGEs) e formação/processamento do peptídio tóxico β-amiloide na DA via sinalização de RAGE (Batkulwar et al., 2018). O peptídeo β-amilóide também possui um papel importante no processo da DA, e pode sofrer oligomerização com a formação de placas senis que levam a disfunção sináptica. A APP pode, alternativamente, ser processada através da via não amiloidogênica onde a clivagem inicial é por α-secretase, membros da família ADAM (metaloprotease), o que impede a formação do peptídio Aβ (Kitazawa, Medeiros, & Laferla, 2012). Suplementação com doses terapêuticas do extrato de guaraná demostraram melhorar a memória e a tomada de decisão em humanos (Pomportes, Davranche, Brisswalter, Hays, & Brisswalter, 2015). Além disso o guaraná foi capaz de inibir a agregação do peptídio β-amilóide, a geração de produtos finais de glicação avançada (AGEs) e prevenir a citotoxicidade induzida por acroleína em células de neuroblastoma (L. a. S. Bittencourt et al., 2014).

Suplementação com polifenois encontrados no guaraná como epicatequina também modula a sinalização celular, incluindo a via MAP quinase, que está envolvida na proliferação celular (Shih, Chen, Lin, Liu, & Kao, 2016) (Cho et al., 2009). A sinalização de polifenois como epicatequina galato, *in vitro*, foi capaz de inibir a agregação de proteínas amiloidogênicas, *α*-synucleína, and β-amiloide, revelando um papel importante no tratamento de patologias neurodegenerativas (Bieschke et al., 2010). Além disso epicatequina é capaz de melhorar a inflamação através do sequestro da subunidade p65 do fator de transcrição NF-KB e inibir a secreção de citocinas em linhagens de células de câncer de próstata (Mukherjee, Siddiqui, Dayal, Ayoub, & Malathi, 2014). Em conjunto, esses estudos indicam que os polifenois presentes no extrato de guaraná tem múltiplos efeitos celulares, são capaz de diminuir o risco de

iniciação e progressão do tumor e podem ser úteis na prevenção de doenças amiloidogênicas. Embora muitos autores sugiram os benefícios dos polifenois na prevenção do déficit cognitivo, uma meta-análise revelou ser inconclusiva a relação entre a suplementação antioxidante, funções cognitivas e humor, necessitando de maiores estudos epidemiológicos para conferir validação (Camfield, Stough, Farrimond, & Scholey, 2014).

Neste trabalho nossos resultados sugerem que a cafeína é o maior constituinte ativo do guaraná, mas não pode ser responsável por todas as mudanças nos parâmetros observados.

O completo perfil redox após a administração crônica do extrato comercial de *Paullinia cupana*, para detectar mudanças em ROS, atividades antioxidantes enzimáticas e não-enzimáticas (L. a. S. Bittencourt et al., 2014) e alterações oxidativas em proteínas e lipídios que podem correlacionar-se com mudanças características do envelhecimento em regiões específicas do sistema nervoso e nos principais órgãos do sistema intermediário. Análises dos parâmetros bioquímicos apresentaram efeitos fortemente antioxidantes induzidos pelo extrato de guaraná. A administração do extrato de guaraná foi capaz de ativar a via de detoxificação no SNC modulando a atividade da glutationa S-transferase e catalase, o que indica que o consumo do extrato de guaraná afetou a sinalização de peróxido de hidrogênio nas estruturas analisadas, uma vez que o peróxido é um importante sinalizador da atividade neuronal e sinapse no SNC (Ohashi et al., 2016). A rápida modulação de enzimas antioxidantes é um mecanismo adaptativo celular para suportar a produção de ROS. Apesar do aumento da atividade enzimática antioxidante e não-antioxidante no estriado de ratos que receberam guaraná, isso não foi capaz de impedir o aumento na carbonilação de proteínas nesta estrutura. Este dano oxidativo promove a geração e acumulação ROS intra ou extracelular que podem perturbar a

sinalização celular e são eventos característicos do processo de envelhecimento (Halliwell, 2006).

Observamos que apenas a administração de cafeína durante o envelhecimento foi capaz de modificar parâmetros comportamentais demonstrando redução do comportamento ansioso dos animais enquanto o grupo guaraná não apresentou efeito, esse efeito parece estar associado aos outros componentes bioativos presentes no guaraná que inibem os efeitos causados pela cafeína. Corroborando com os resultados, neste trabalho investigamos rotas de sinalização alteradas e verificou-se uma inibição da fosforilação de ERK1/2 e p38MAPK no estriado de animais tratados com guaraná e cafeína.

Nossos resultados mostraram que a sinergia dos componentes presentes no guaraná podem mudar o equilíbrio entre estresse oxidativo e sinalização neurodegenerativa no SNC, em direção a uma inibição das vias de sobrevivência da células (ERK1/2 e p38MAPK) no estriado. A inibição de AKT no hipocampo indica diminuição na resistência oxidativa e interrupção redox nesta estrutura (Mattson, Duan, & Maswood, 2002). Da mesma forma, os tratamentos com guaraná e cafeína foram capazes de alterar proteínas relacionadas à homeostase, lesão cognitiva e senescência no sistema nervoso destes animais. No hipocampo houve um aumento de imunoconteúdo de RAGE nos animais que receberam guaraná, demonstrando indícios de dano neuronal devido à superprodução de ROS, citocinas e pró-inflamatórios moléculas (Ray et al., 2016). Quando avaliamos a homeostase de astrócitos e neurônios, ambos os tratamentos não afetaram as células neuronais em nenhuma das estruturas investigadas, porém os astrócitos estriatais mostraram maior sensibilidade frente ao extrato de guaraná, este efeito pode ser atribuído a outras substâncias presentes no guaraná como compostos fenólicos. Finalizando os efeitos observados no SNC e cientes que RAGE desempenha

um papel importante na produção e metabolismo do peptídio β-amilóide, neste trabalho demonstramos que no hipocampo, tanto o tratamento com guaraná quanto o com cafeína aumentaram os níveis da Proteína precursora amiloide (APP) na superfície de células neuronais. Essas descobertas sugerem que a cafeína pode suprimir a internalização da APP nos neurônios do hipocampo em ambos os tratamentos. Este resultado sugere que a cafeína exerce efeitos protetores contra o processamento amiloidogênico da APP no hipocampo (Li et al., 2015). Surpreendentemente, no estriado, outros compostos presentes no guaraná parecem bloquear esse efeito protetor de cafeína na internalização da APP.

Posteriormente, investigamos os efeitos sistêmicos do extrato de guaraná e observamos através de parâmetros sanguíneos uma indução de linfócitos indicando um efeito imunoestimulante deste composto. Este resultado pode ser uma resposta imune dos roedores frente a ingestão crônica de *Paullinia cupana*, que levou à mobilização de células imunocompetentes frente a carga excessivamente antioxidante.

A implicação deste achado é que o extrato de sementes de guaraná mostrou um efeito imunogênico durante o tratamento crônico, o que corrobora um estudo anterior *in vitro* que demonstrou um efeito imuno-estimulante do extrato de guaraná no modelo de linfocitose estimulada (Yonekura et al., 2016). Além disso a diminuição dos neutrófilos segmentares também é indicação de citotoxicidade. Os neutrófilos são a primeira linha de defesa contra infecções apresentam aumento elevado frente a inflamação aguda, enquanto os linfócitos, por outro lado, produzem anticorpos e estão mais envolvidos na defesa contra patógenos intracelulares e defesa tumoral. Os efeitos neutropênicos do extrato de guaraná poderiam sugerir um efeito prejudicial sobre esta primeira linha de defesa do corpo, enquanto seu efeito na estimulação de linfócitos sugere uma estimulação hematopoiética. Estes resultados corroboram os achados relatados com a planta medicinal

Hunteria umbellate que foi capaz de aumentar a contagem de linfócitos e reduzir os níveis de circulação neutrófilos em roedores (Adeneye, Adeyemi, Agbaje, & Banjo, 2010). Os linfócitos permaneceram inalterados nas análises realizadas nos animais que receberam apenas cafeína. Este resultado corrobora os achados de Dulson e Bishop que investigaram os efeitos da cafeína em ativação de linfócitos em amostras de sangue de jovens adultos que consumiram cafeína, demonstrando que a ingestão de baixas concentrações de cafeína não afetou a ativação de linfócitos em humanos uma hora após o consumo (Dulson & Bishop, 2016). Assim, outros componentes do extrato de guaraná como catequinas e epicatequinas além da cafeína podem promover a ação de fatores de crescimento relacionados à produção de linfócitos, enquanto inibe fatores de crescimento associados à produção de neutrófilos. São necessários mais estudos abordando mais informações sobre o efeito do guaraná em linfócitos e neutrófilos. A administração de compostos antioxidantes, como guaraná, pode alterar atividades enzimáticas, como GST, uma enzima importante na biotransformação de xenobióticos e degradação de compostos endógenos (O'Brien, Kruh, & Tew, 2000). A estimulação da atividade de GST no tecido cardíaco foi capaz de prevenir o aumento carbonilação de proteínas de animais cronicamente suplementados com guaraná, indicando que por combater a carbonilação de proteínas o ECG pode estar estimulando a sinalização de desintoxicação tecidual. Esses dados estão de acordo com estudos anteriores onde o guaraná foi capaz de reduzir o colesterol total e o LDL-colesterol até níveis basais, conferindo proteção ao tecido cardíaco (Ruchel, Rezer, et al., 2016). A cafeína não apresentou esse efeito indicando que a ativação da rota de desintoxicação se deve ao efeito de outros compostos presentes no extrato de guaraná, como os polifenois ou pela sinergia dos compostos na geração de alto efeito antioxidante. Estudos relatam que o extrato de *Paullinia cupana* tem efeito termogênico devido ao seu teor de cafeína. Disfunções no equilíbrio térmico e energético

se agravam durante o processo de envelhecimento e podem resultar em obesidade ou instabilidade térmica. Observamos que ambos os grupos experimentais (guaraná e cafeína) apresentaram redução na gordura gonadal, mas não nos demais tipos de tecido adiposo, esses resultados corroboram com o efeito termogênico do extrato de Paullinia cupana visto em outros estudos que já indicaram guaraná como um complemento potente em perda de peso e gasto energético (Bérubé-Parent, Pelletier, Doré, & Tremblay, 2005). Contudo, nossos dados destacam que a administração crônica de guaraná foi capaz de reverter o acúmulo de gordura gonadal relacionada ao envelhecimento. O processo de envelhecimento está associado a uma diminuição dos níveis de testosterona e aumento dos tecidos adiposos em roedores com idade superior a 12 meses (Guan et al., 2011). Estudos demonstram que há uma relação direta entre aumento dos níveis de testosterona circulante com uma diminuição da gordura gonadal em ratos machos (Turner, Morley, Atanassova, Swanston, & Sharpe, 2000). Da mesma forma, a adição de testosterona pode diminuir os marcadores plasmáticos associados à obesidade ou aumento da gordura corporal (Jockenhövel et al., 1997). Uma possível explicação para a redução de gordura gonadal nos animais idosos, seria que a administração crônica de guaraná poderia estar causando a redução da gordura via estimulação do hormônio testosterona circulante. Estudos demonstram que o guaraná, mesmo em baixas concentrações, é capaz de aumentar os níveis plasmáticos de testosterona em ratos previamente tratados com o extrato durante 56 dias (Leite, Wada, Monteiro, Predes, & Dolder, 2011) [33]. Assim, os dados parecem indicar que o guaraná pode estar modulando o eixo envolvido na manutenção do peso corporal, distribuição de gordura e função reprodutiva em nosso modelo de administração crônica.

Além disso, o envelhecimento está relacionado ao comprometimento sistêmico e metabólico. Em geral, a redução da capacidade cardíaca e hepática é notável. Nesses

tecidos, um grupo de proteínas de acetilação desempenham um papel importante na regulação metabólica da homeostase. Sirt1 é responsável pela acetilação reversível de proteínas e tem sido destacada em muitos estudos como uma molécula reguladora do metabolismo energético e antienvelhecimento. Nossos resultados demonstram que houve um aumento no imunoconteúdo de Sirt1 no fígado e no coração de animais tratados com cafeína, quando comparado ao controle, e esse efeito não foi observado em animais tratados com guaraná. Nossas descobertas demonstram que baixas doses de cafeína administradas durante longos períodos, podem aumentar o imunoconteúdo de Sirt1 no fígado e coração de animais tratados. Estes resultados corroboram com pesquisas anteriormente realizadas indicando que os componentes do vinho tinto (polifenois) foram capazes de modular positivamente Sirt1 (Bagul, Dinda, & Banerjee, 2015).

Efeitos protetores não foram observados em animais tratados com *Paullinia cupana*. Na literatura, estudos mostram que a cafeína pode induzir a produção de Sirt3 ativando a lipólise (Zhang et al., 2015). Administração *in vitro* de baixas doses de cafeína também foi capaz de ativar Sirt1 em células tronco derivadas de adipócitos e células de medula óssea (Su et al., 2013).

Nossas descobertas apontam para o efeito protetor da cafeína, modulando positivamente a expressão de sirtuínas. Outros componentes contidos no extrato de sementes de guaraná parecem inibir esse efeito protetor da cafeína no coração e tecido hepático de animais suplementados com este composto.

CONCLUSÕES

Concluindo com base nos resultados apresentados neste trabalho, é possível identificar os efeitos de *Paullinia cupana* envolvendo a geração de espécies reativas tanto no envelhecimento quanto na modulação de marcadores neurodegenerativos como o RAGE. Também conclui-se haver outros mecanismos de modulação de marcadores de doenças neurodegenerativas pelo extrato de guaraná, não envolvendo a geração de ambiente pró-oxidante, mas sim via estresse antioxidante. O extrato tem efeito imunomodulador possivelmente devido ao seu conteúdo polifenólico pois a cafeína sozinha não conseguiu induzir esses efeitos. O consumo do extrato de guaraná foi capaz de reduzir gordura gonadal nos animais suplementados, mas os efeitos benéficos de longevidade foram atribuídos apenas à cafeína quando administrada sozinho. A maioria dos efeitos observados do extrato de guaraná resultaram do seu teor de cafeína.

Estes resultados indicam que a suplementação de extrato antioxidante pode prejudicar as vias de sinalização oxidativa cerebral e sistêmica convidando estudos adicionais para caracterizar o papel potencial de compostos bioativos deste extrato.

PERSPECTIVAS

Finalizar investigação do efeito de *Paullinia cupana* em modelos *in vitro* (Cultura primária de astrócitos):

- Análises morfológicas: Investigar a modulação causada pelo extrato de *Paullinia cupana* nas culturas primárias de células astrocitárias por microscopia de contraste de fase e imunofluorescência (β-tub III, MAP2, Actina e GFAP, RhoA) na presença do inibidor MK-801.
- Avaliar o imunoconteúdo das proteínas GFAP e RhoA e Nrf2.
- Avaliar os parâmetros bioquímicos: Enzimáticos por SOD, CAT, GPX e GST e dano tecidual por Sulfidril, Carbonil e TBA.
- Investigar mecanismos mediados por glutamato através de ensaio de Captação de ^{3}H-glutamato em culturas de astrócitos.

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