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O efeito protetor do Exercício Físico e da suplementação com Cafeína nas convulsões e dano oxidativo induzidos por pentilenotetrazol em ratos.

TESE DE DOUTORADO

Mauren Assis de Souza

Santa Maria, RS, Brasil
2012

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Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de **Doutor em Ciências Biológicas: Bioquímica Toxicológica.**

Orientador:Prof. Dr. Luiz Fernando Freire Royes
Co-Orientadora: Prof[a]. Dr[a]. Michele Rechia Fighera

Santa Maria, RS, Brasil
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Universidade Federal de Santa Maria
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A Comissão Examinadora, abaixo assinada,
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DEDICATÓRIA

Dedico esta caminhada a minha mãe e a minha avó que sempre acreditaram e incentivaram esta jornada.

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A minha Grande e querida família do BioEx....

Esta família é muito unida

E também muito ouriçada

Brigam por qualquer razão

Mas acabam pedindo perdão...

Que grande alegria poder ter convivido e ter uma história com cada um desses grandes amigos, que por coincidência foram também colegas de laboratório. 2012 está sendo um ano de grandes mudanças para a maioria de nós, e isso fez com que nos aproximássemos ainda mais. Com vocês aprendi a ser uma menina que fala ao invés de pronunciar barulhos não identificáveis não é Maurício. Pensar que não serão mais vocês meus companheiros de lab, e pós lab faz dar aquele aperto, mas feliz fico por ter compartilhado muitas e ótimos momentos ao lado de vocês. Podia escrever muitas páginas no dialeto BioEx só pra agradecer a cada um de vocês e dizer o quanto vocês são importantes pra mim, mas nesse momento uma avalanche de recordações me impede de organizar tudo isso.

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EPÍGRAFE

“Uma vida sem desafios não vale a pena ser vivida.”

Sócrates

RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica
Universidade Federal de Santa Maria, RS, Brasil

O efeito protetor do Exercício Físico e da suplementação com Cafeína nas convulsões e dano oxidativo induzido por pentilenotetrazol em ratos.

Autor: Mauren Assis de Souza
Orientador: Luiz Fernando Freire Royes
Co-Orientadora: Michele Rechia Fighera
Local e data da defesa: Santa Maria, 05 de outubro de 2012.

As crises convulsivas constituem a principal manifestação clínica da epilepsia. A epilepsia é uma condição neurológica crônica com incidência de 1 % na população em geral sendo que cerca de 20 a 30% dos pacientes apresentam-se refratários ao tratamento com as drogas antiepiléticas disponíveis. Existem evidências para a participação das espécies reativas de oxigênio (EROs) na fisiopatologia das epilepsias, entretanto determinar o seu papel é difícil, uma vez que, o estresse oxidativo pode ser causa ou consequência das crises epilépticas.
Considerando o grande número de pacientes refratários ao tratamento disponível, e que o dano oxidativo parece ser um importante fator envolvido nas crises, terapias alternativas que aumentem as defesas antioxidantes e/ou diminuam o dano oxidativo podem se tornar importantes adjuvantes no tratamento das crises epilépticas, como o exercício físico e a administração de cafeína. Neste sentido o presente trabalho teve como objetivo investigar os efeitos da atividade física e da suplementação com cafeína nas convulsões comportamentais e eletroencefalográficas (EEG), bem como nas alterações dos parâmetros oxidativos induzidos por pentilenotetrazol (PTZ) em ratos.
No estudo 1 demonstrou-se que o exercício físico (natação 6 semanas) atenuou a latência e a duração das convulsões generalizadas induzidas pela administração de PTZ (45 mg / kg i.p) e atenuou o aumento da amplitude das ondas eletroencefalográficas induzidas por PTZ (30, 45 e 60 mg/Kg i.p). Análise de correlação de Pearson revelou que a proteção do exercício físico contra as convulsões correlaciona-se com conteúdo de tióis não protéicos (TNP), atividade da enzima Na^+,K^+-ATPase e manutenção da captação de glutamato. O exercício físico aumentou a atividade da superóxido dismutase (SOD) e o conteúdo de TNP *per se* atenuando a produção de EROs *per se*. Além disso, o exercício físico protegeu contra a neutoxicidade induzida por PTZ caracterizada aqui pela produção de EROs, peroxidação lipídica (LPO), carbolinação de proteínas, diminuição no conteúdo de TNP inibição da atividade da SOD e catalase (CAT), e a inibição da captação de glutamato.
No estudo 2 verificou-se que a administração prolongada de cafeína (6 mg/Kg, 15 dias p.o), mas não a administração aguda diminuiu o tempo gasto nas convulsões tônico-clônico generalizadas e atenuou o aumento da amplitude EEG induzida por PTZ (60 mg/Kg i.p). Além disso, verificou-se que a administração prolongada de cafeína aumentou o conteúdo de glutationa reduzida (GSH) *per se* e protegeu do aumento da LPO, produção de EROs e inibição da atividade da enzima Na^+,K^+-ATPase induzida por PTZ. A infusão de L-butionina sulfoximina (BSO, 3,2 micromol / site icv), um inibidor da síntese de GSH, dois dias antes da administração de PTZ reverteu o efeito anticonvulsivante da cafeína frente as convulsões e dano oxidativo induzidos por PTZ.
Além disso, um estudo subsequente revelou que a administração prolongada de cafeína juntamente com exercício físico durante 4 semanas aumentou a latência para a primeira convulsão mioclônica e primeira generalizada, além de diminuir a duração das convulsões generalizadas induzidas pela administração de PTZ (60 mg / kg i.p).
Considerando os dados apresentados no presente estudo, conclui-se que o exercício físico e a suplementação prolongada com cafeína atenuam as convulsões induzidas por PTZ, por modular positivamente o sistema antioxidante e manter a atividade da enzima Na^+,K^+-ATPase em ratos.

Palavras-chave: Convulsões, pentilenotetrazol, dano oxidativo, exercício físico, cafeína.

ABSTRACT

Doctoral Thesis
Graduate Program in Biology Science: Toxicological Biochemistry
Federal University of Santa Maria, RS, Brazil

The protective effect of Exercise and Caffeine supplementation on oxidative damage and seizures induced by pentylenetetrazol in rats.

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Place and date of defense: Santa Maria, October 5th, 2012.

Seizures are the main clinical manifestation of epilepsy. Epilepsy is a common neurological disorder with an incidence of 1% in the general population, with about 20 to 30% of patients are refractory to treatment with antiepileptic drugs available. There is evidence for the involvement of reactive oxygen species (ROS) in the pathophysiology of epilepsy, however, determines their role is difficult since oxidative stress can be a cause or consequence of epileptic seizures.

Considering the large number of patients refractory to available treatment, and that oxidative damage appears to be an important factor involved in crises, alternative therapies that enhance antioxidant defenses and / or decrease the oxidative damage may become important adjuvants in the treatment of epileptic seizures, as exercise and caffeine administration. In this sense the present work aimed to investigate the effects of physical activity and caffeine supplementation on behavioral and electroencephalographic (EEG) seizures, as well as on changes in oxidative parameters induced by pentylenetetrazol (PTZ) in rats.

The first study showed that physical exercise (swimming for 6 weeks) attenuated the onset and duration of generalized seizures induced by administration of PTZ (45 mg / kg i.p) and attenuated the increase in amplitude of EEG waves induced by PTZ (30, 45 and 60 mg / kg i.p). The Pearson correlation analysis revealed that the protection of physical training against seizures, correlates with the content of non-protein thiol (NPT), Na^+,K^+-ATPase activity and glutamate uptake. Exercise increased the activity of superoxide dismutase (SOD) and the content of the NPT *per se*. Moreover, physical exercise protect against PTZ-induced neutoxicity, characterized here by ROS production, lipid peroxidation (LPO), protein carbonylation, decreased the content of TNP inhibition of SOD and catalase (CAT) and inhibition of glutamate uptake.

The second study, showed that prolonged administration of caffeine (6 mg / kg, 15 days po), but not acute administration decreased the duration of generalized tonic-clonic seizures and attenuated the increase in EEG amplitude induced by PTZ (60 mg / kg i.p). Moreover, prolonged administration of caffeine increased content of reduced glutathione (GSH) *per se* and protected against increased LPO, ROS and the inhibition of Na^+,K^+-ATPase activity induced by PTZ. Infusion of L-buthioninesulfoximine (BSO, 3.2 micromol / site, i.c.v), an inhibitor of GSH synthesis, two days before the injection of PTZ, reversed the anticonvulsant effect of caffeine on seizures and oxidative damage induced by PTZ.

In addition, a subsequent study has revealed that the prolonged administration of caffeine along with exercise for 4 weeks increased the latency to first myoclonic seizure and first generalized beyond decreased the time spend on generalized seizures induced by the administration of PTZ (60 mg / kg i.p).

Considering the data presented in this study, conclude that physical exercise and supplementation with caffeine attenuates seizures by positively modulating the antioxidant system and maintain Na^+,K^+-ATPase activity.

Keywords: Seizures, pentylenotetrazol, oxidative damage, exercise, caffeine.

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LISTA DE ABREVIATURAS E SIGLAS

4-HNE	4-hidroxinonenal
AMPc	3',5'- adenosina monofosfato cíclico
BDNF	Fator neurotrófico derivado do encéfalo
BSO	Butionina Sulfoximina
CAT	Catalase
CREB	Proteína responsiva a adenosina monofosfato cíclico
DA	Doenças de Alzheimer
DAE	Drogas antiepiléticas
DH	Doenças de Huntington
DNA	Ácido desoxirribonucleico
DP	Doenças de Parkinson
EEG	Eletroencefalográfica
EM	Esclerose múltipla
ERN	Espécies reativas de nitrogênio
ERO	Espécies reativas de oxigênio
$GABA_A$	Receptor do ácido gama-aminobutírico do tipo A
GPx	Glutationa Peroxidase
GR	Glutationa Redutase
GSH	Glutationa reduzida
GSSG	Glutationa oxidada
GSNO	S-nitrosoglutationa
GST	Glutationa-S-transferase
i.p	Intraperitoneal
i.v	Intravenosa
LPO	Peroxidação lipídica
MAPK	Proteína Quinase Ativada por Mitógeno

MDA	Malondialdeído
MPTP	Poro de transição mitocondrial
NMDA	N-Metil-D-Aspartato
NO	Óxido nítrico
Nrf2	Fator nuclear relacionado ao eritróide-2
PGC-1α	Receptor ativado por proliferador de peroxissoma
PKA	Proteína quinase A
p.o	Via oral
PTZ	Pentilenotetrazol
s.c	Subcutânea
SNC	Sistema nervoso central
SOD	Superóxido dismutase
TBARS	Espécies reativas ao ácido tiobarbitúrico
TNP	Tióis não protéicos

SUMÁRIO

INTRODUÇÃO

Epilepsia

1.1.1 Definição

A epilepsia foi provavelmente descrita pela primeira vez nos antigos escritos egípcios, aproximadamente 2000 a.C, e foi um tema popular dos estudiosos gregos e romanos. A doença "sagrada", como foi frequentemente chamada, foi relacionada com forças sobrenaturais e considerada uma manifestação dos deuses e espíritos. Em 400 a.C., Hipócrates já havia escrito um texto médico desmistificando o tema, propondo que a epilepsia não era sagrada nem divina, e sim, uma doença relacionada ao cérebro, com possível origem hereditária (MOREIRA, 2004).

A era moderna da epilepsia começou com os escritos de Jackson que estabeleceu as bases neuroanatômicas para os fenômenos epilépticos no final dos anos de 1870 (JACKSON, 1879). A introdução da eletroencefalografia em 1930 favoreceu a compreensão das bases neurofisiológicas do distúrbio, facilitando o desenvolvimento de tratamentos farmacológicos para os vários tipos de epilepsia (GASTAUT, 1950).

A epilepsia é uma condição caracterizada por crises epilépticas espontâneas e recorrentes, devido a um substrato neurológico que possibilite o surgimento de novas crises, nas quais ocorrem uma atividade elétrica encefálica hipersincrônica e paroxística (FISHER; KETTL, 2005), sendo as convulsões caracterizadas como a manifestação motora das crises.

A epilepsia representa uma doença heterogenea que tem diversas etiologias, padrões electrofisiológicos e comportamentais, sem, no entanto, deixar de responder ao tratamento farmacológico. Como tal, a sua patogenia é multifactorial pois qualquer perturbação neurológica, lesional ou funcional é susceptível de desencadear o aparecimento de convulsões. Pode ter uma componente genético, pode ser desencadeada por alterações desconhecidas na atividade neural, por alterações na neurotransmissão ou ainda por estímulos ambientais que não causam ataques em cérebros normais (Goodkin *et al.*, 2002; Podell, 2004).

Em relação ao início das convulsões, elas podem ser classificadas como focais e generalizadas (Tabela 1). As Crises focais são definidas como crises epilépticas que se originam em redes limitadas à um hemisfério cerebral, no qual apresentam padrões de propagação semelhantes entre as crises, os quais podem envolver o hemisfério contralateral. As crises generalizadas são consideradas como originárias em algum ponto do encéfalo e que rapidamente ativam redes neuronais bilaterais, mas não necessariamente todo o córtex cerebral. Ainda, as crises generalizadas podem ser assimétricas e semelhantes às crises focais, porém se diferem por não apresentar um padrão entre cada crise (BERG et al., 2009).

Tabela 1. Classificação de crises[a]

Crises generalizadas
Tônico-clônicas (em qualquer combinação)
Ausência
Típica
Atípica
Ausência com características especiais
Ausência mioclônica
Mioclonias palpebrais
Mioclônica
Mioclônica
Mioclônica atônica
Mioclônica tônica
Clônica
Tônica
Atônica
Crises focais
Desconhecido
Espasmos epilépticos

[a]Crises que não podem ser claramente diagnosticadas em uma das categorias anteriores devem ser consideradas não classificadas até que informações permitam o seu diagnóstico preciso. No entanto, esta não é considerada uma categoria de classificação.

Tabela 1. Classificação das crises epiléticas. Fonte: Berg et al., 2010.

A epilepsia afeta aproximadamente 50 milhões de pessoas em todo o mundo (STRINE et al., 2005). A cada ano somam-se aproximadamente dois milhões de casos, sendo que esta condição acomete pessoas de todas as raças, sexos e condições socioeconômicas. Estima-se que 80% das pessoas com epilepsia vivam em países em desenvolvimento, onde 60-90% destas não recebem nenhum tipo de tratamento (SANDER; SHORVON, 1996; MEINARDI et al., 2001; REYNOLDS, 2002).

Nos países da América Latina, a incidência das epilepsias tem variado entre 78-190 novos casos por 100.000 habitantes por ano, e a prevalência média é de aproximadamente 18 casos por 1.000 habitantes, podendo variar de acordo com os métodos de investigação em cada país (Figura 1, BURNEO; TELLEZ-ZENTENO; WIEBE, 2005).

O elevado número de pessoas com epilepsia nos países em desenvolvimento tem sido atribuído a aspectos socioeconômicos e a problemas de saúde pública, geralmente em decorrência de uma assistência pré-natal e materna deficiente, do alto índice de nascimentos de prematuros, desnutrição, traumas durante o parto, crises febris na infância, infecções e neurocisticercose (FERNANDES et al., 1992; PAL; CARPIO; SANDER, 2000; ROMAN et al., 2000; SCOTT et al., 2001; BURNEO; TELLEZ-ZENTENO; WIEBE, 2005). Apesar do crescente número e variedade de drogas antiepilépticas (DAE), mais de 30% dos casos são clinicamente classificados como refratários e, muitas vezes, não responsivos em mais de três tentativas terapêuticas, com DAE apropriadamente escolhidas (ELGER, 2003). A refratariedade é definida como a falha de dois esquemas terapêuticos bem tolerados e apropriadamente escolhidos, sejam eles de mono ou politerapia farmacológica, para que o paciente fique livre de crises. O paciente é considerado livre de crises se apresentar um período de 12 meses sem crises ou três vezes o período de intervalo entre as crises, sendo escolhido como critério o que resultar em maior período (KWAN; BRODIE, 2010).

No caso de pacientes refratários, algumas terapias alternativas podem ser adotadas, como a cirurgia para a retirada do foco epiléptico, dieta cetogênica e estimulação do nervo vago (SHNEKER; FOUNTAIN, 2003). Uma vez que um grande número de pacientes com epilepsia permanece refratário ao tratamento medicamentoso, surge a importância do estudo da fisiopatologia dos diferentes tipos de crises convulsivas e de síndromes eletroclínicas, para assim, desenvolverem-se novas terapias com potencial anticonvulsivante.

Neste sentido, os modelos experimentais são de grande valia para o estudo da fisiopatologia envolvida nestas manifestações, assim como para a identificação de novas terapias antiepilépticas.

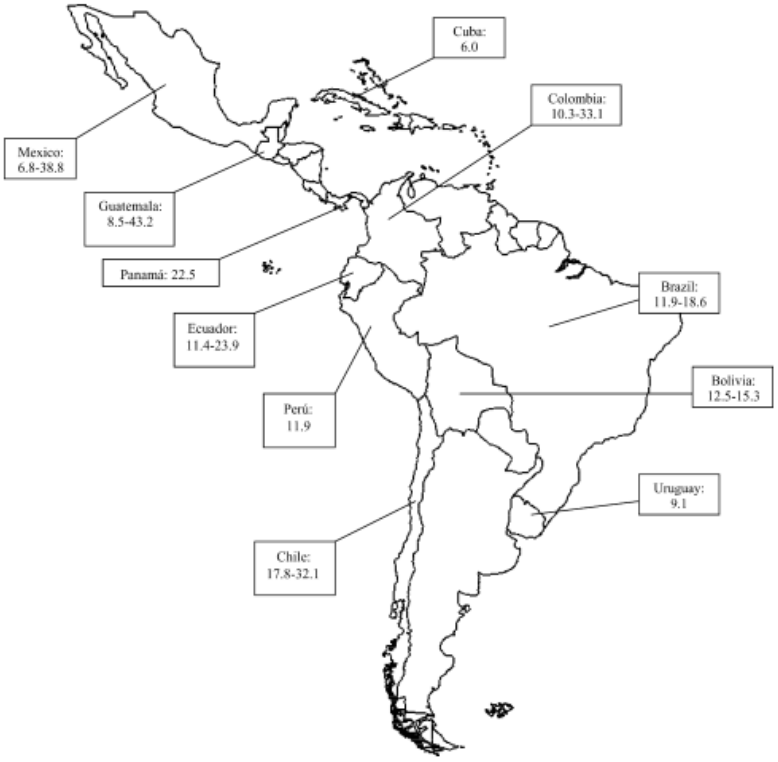

Figura 1: Prevalência de epilepsia em diferentes países da América Latina. Fonte: BURNEO et al., 2005.

1.1.2 Modelos experimentais de epilepsias e convulsões

Os modelos experimentais em animais para convulsões e epilepsia têm desempenhado um papel fundamental no avanço da compreensão dos mecanismos básicos subjacentes de ictogênese e epileptogênese. Dessa forma, esses estudos têm sido fundamentais para a descoberta e desenvolvimento pré-clínico de novas DAE (LOSCHER, 2011). Durante as últimas décadas foram desenvolvidos diversos modelos experimentais de epilepsias e convulsões (FIGURA 2 PURPURA; SHOFER, 1972; LOSCHER, 2011). Os vários modelos animais podem ser atribuídos a diferentes categorias, por exemplo, modelos com crises espontâneas induzidas por alterações genéticas ou modelos com crises espontâneas induzidas química ou eletricamente; modelos com crises únicas e modelos com crises parciais e/ou modelos de convulsões generalizadas (LOSCHER et al., 1999; LOSCHER; SCHMIDT, 2011).

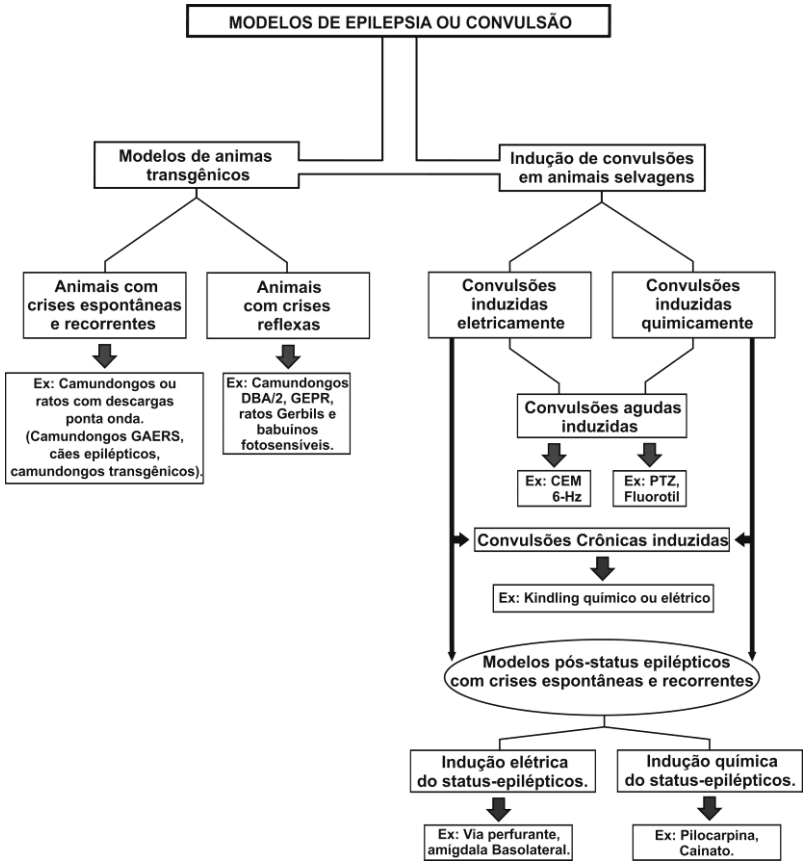

Figura 2. Uma visão geral dos modelos de epilepsia ou convulsões epilépticas. Fonte: Adaptado de Loscher W 2011.

Dentre os modelos de convulsão generalizada, destaca-se o modelo de convulsão induzido por pentilenotetrazol (PTZ), também conhecido como pentetrazol e metrazol. Este estimulante do SNC é amplamente usado experimentalmente para estudar crises convulsivas generalizadas tônico-clônicas, crises de ausência e para identificar fármacos que possam alterar a susceptibilidade a convulsões (SCHMIDT; LEPPIK, 1988; LOSCHER et al., 1999; WHITE et al., 2008). Além disso, o PTZ é uma droga ansiogênica que pode exercer efeitos positivos sobre a cognição (JUNG; DOUSSARD-LEFAUCHEUX, 2002; RUEDA; FLOREZ; MARTINEZ-CUE, 2008).

O mecanismo de ação do PTZ é por antagonizar os receptores $GABA_A$ que, por inibir as correntes de cloreto associadas a este mesmo canal, potencializa a neurotransmissão excitatória e desencadeia as convulsões (RAMANJANEYULU; TICKU, 1984; KUPFERBERG, 2001).

O PTZ pode ser administrado através de infusão por via intravenosa (i.v), via intraperitoneal (i.p) ou subcutânea (s.c), em doses convulsivantes ou subconvulsivantes,

podendo ser avaliado a latência para a primeira convulsão mioclônica, latência para a primeira generalizada tônico-clônica e a duração das convulsões (ZIENOWICZ et al., 2005; YILMAZ et al., 2007; REHNI; SINGH; KUMAR, 2008).

Existem evidências para a participação das EROs na fisiopatologia das epilepsias, entretanto determinar o seu papel é difícil, uma vez que, o estresse oxidativo pode ser causa ou consequência das crises epiléticas (WALDBAUM; PATEL, 2010). Entretanto, alguns autores descreveram que a produção de EROs é uma consequência de crises epiléticas agudas, como as induzidas por PTZ (BASHKATOVA et al., 2000; MURASHIMA; YOSHII; SUZUKI, 2000). Contudo, as crises epiléticas também podem ser iniciadas por paradigmas que aumentem a produção de EROs, como acidente vascular encefálico, exposição a oxigênio hiperbárico e breves períodos de isquemia-reperfusão (JENSEN et al., 1992; ELAYAN et al., 2000; VELIOGLU et al., 2001).

Estresse Oxidativo

Qualquer átomo ou molécula que contenha um ou mais elétrons desemparelhados são chamados de radicais livres (SOUTHORN; POWIS, 1988; HALLIWELL, 1989). Os radicais livres são produzidos como um subproduto do metabolismo fisiológico, principalmente no final do processo respiratório na mitocôndria, dentre eles o radical superóxido (O_2•) e o radical hidroxil (OH•). A produção de O_2• se dá principalmente no complexo I (NADH desidrogenase) e complexo III (ubiquinonacitocromo c oxidase) onde a coenzima FMN e o ubiquinol respectivamente, reduzem o oxigênio univalentemente formando o radical (NAVARRO; TORREJON, 2007).

Outras moléculas, tais como o peróxido de hidrogênio (H_2O_2) produzido enzimaticamente pela ação da superóxido dismutase (SOD) e peroxinitrito (ONOO-), embora não sejam radicais livres, são altamente reativas e podem levar a produção dos radicais livres por meio de várias reações químicas. Assim o H_2O_2, reagindo com o cobre (Cu^{+1}) ou ferro (Fe^{+2}), na reação de Fenton e ainda a partir do O_2• + H_2O_2 na reação de Haber – Weiss pode formar OH• (LIANG, C. et al., 2000). Juntas, essas moléculas são chamadas de EROs e as espécies reativas de nitrogênio (ERNs). As EROs e ERNs são por sua vez compostos altamente reativos que buscam estabilidade durante sua breve existência, reagindo com a

matéria circundante, desta maneira causando danos às membranas celulares, proteínas e DNA (HALLIWELL, 2012).

Níveis fisiológicos de EROs/ERN podem ser eliminados por um sistema antioxidante enzimático e não enzimático (NAVARRO; BOVERIS, 2008). Entretanto, um aumento na produção de EROs, uma diminuição na eficiência dos sistemas antioxidantes ou ambos podem levar ao estresse oxidativo, que é caracterizado por uma oxidação de biomoléculas com consequente perda de suas funções biológicas (HALLIWELL; WHITEMAN, 2004).

O sistema antioxidante enzimático é composto principalmente pelas enzimas SOD, Glutationa Peroxidase (GPx), e Catalase (CAT), e o sistema antioxidante não-enzimático é representado pelas vitaminas A, C, E e D, e a glutationa reduzida (GSH) (HALLIWELL, 1989; GUTTERIDGE, 1995).

Em relação ao sistema enzimático, existem dois tipos de SOD, as que contêm cobre e zinco no sítio ativo e são encontradas principalmente no citoplasma, mas também nos peroxissomos, lisossomos e espaço intermembrana da mitocôndria, e as que contêm manganês no sítio ativo, que são encontradas na matriz mitocondrial (CHANCE; SIES; BOVERIS, 1979). A SOD catalisa a dismutação do O_2•, formando o H_2O_2. Depois de formado, o H_2O_2 é degradado pela GPx, uma enzima que está presente em duas formas, a que utiliza o selênio como co-fator, encontrada tanto na mitocôndria como no citosol, e a que não utiliza selênio como co-fator, encontrada apenas no citosol. A GPx catalisa a reação de hidroperóxidos com a GSH, formando glutationa oxidada (GSSG) e o produto da redução do hidroperóxido (MILLS, 1960). Fisiologicamente, a GPx funciona acoplada a glutationa redutase (GR), que por sua vez, catalisa a redução de GSSG para GSH, utilizando NADPH como coenzima (MAIORINO; GREGOLIN; URSINI, 1990).

Além disso, a GSH reage com vários metabólitos endógenos e xenobióticos mediadas pela glutationa-S-transferase (GST) para formar dissulfuretos mistos, que são exportados para o exterior da célula (FANG, 2004). A GSH é um antioxidante importante no cérebro, com uma concentração de aproximadamente 2-3 mM (DRINGEN, 2000), sendo que a maior parte do GSH celular (85-90%) está presente no citosol, com o restante em muitas organelas (incluindo a matriz mitocondrial, núcleo e peroxissomas).

Este tripeptídeo exerce as suas funções através de vários mecanismos (Fang, Y. Z. 2002), podendo reagir não enzimaticamente com o O_2• (WINTERBOURN; METODIEWA, 1994), OH• (CLANCY et al., 1994) e ONOO- (KOPPAL; DRAKE; BUTTERFIELD, 1999). A GSH também serve como um reservatório de óxido nítrico (NO) endógeno para formar S-nitrosoglutationa (GSNO) (SINGH et al., 1996). A GSNO pode liberar NO sob certas

condições, com efeitos biológicos, enquanto GSNO tem um efeito protetor no cérebro em condições de estresse (RAUHALA; LIN; CHIUEH, 1998). Além disso, não há defesa enzimática conhecida contra OH•, tornando GSH o único composto capaz de detoxificar estes radicais (BAINS, 1997).

Outra acão da GSH se d por atuar como um co-factor essencial para as enzimas GPx, GR, GST como descrito anteriormente. Além disso, é uma importante forma de armazenamento do aminoácido cisteína, que tem efeitos neurotóxicos mediados pela produção de EROs, aumento de glutamato extracelular e ativação dos receptores N-Metil-D-aspartato (NMDA) (JANAKY et al., 2000). Sob condições de estresse oxidativo, a GSH pode levar a formação reversível de dissulfetos mistos entre os grupos tiol das proteínas (S-glutationilação), um processo crítico para prevenir a oxidação irreversível de proteínas (GIUSTARINI et al., 2004). Assim, a GSH modula uma variedade de funções de proteínas através de S-glutationilação. Outra possível ação da GSH é como neuromodulador dos receptores NMDA, podendo atuar como agonista ou antagonista (JANAKY et al., 1999). Além disso, a GSH também é necessária para a proliferação celular e diferenciação neuronal (SAGARA; MAKINO, 2008).

As EROs têm uma vida curta e sua recombinação química é quase imediata (RICE-EVANS et al., 1993). Visto isso, torna-se muito difícil a quantificação imediata dos mesmos, o que torna bem aceito a medição de seus produtos. A determinação da formação de grupos carbonila (R-C=O) é um método bastante utilizado para avaliar o dano das EROs às proteínas. Particularmente os aminoácidos histidina, arginina e lisina que são os principais alvos das EROs (PRATICO; DELANTY, 2000).

Por sua vez, a peroxidação lipídica (LPO) é um processo fisiológico e contínuo que ocorre nas membranas celulares. Além de ser um fator de renovação da membrana este processo é essencial para a síntese de prostaglandinas e leucotrienos. No entanto, este processo pode se tornar tóxico quando as defesas antioxidantes são insuficientes ou quando há uma produção intensa de EROs (HALLIWELL; GUTTERIDGE, 1995). A LPO produz aldeídos, gases hidrocarbonados e vários resíduos químicos, como o malondialdeído (MDA), dienos conjugados e 4-hidroxinonenal (4-HNE) (HOTZ; HOET; LAUWERYS, 1987). Desta forma, esta reação pode ser estimada pela medida de seus produtos, e é utilizada para medir indiretamente a produção de radicais livres (HOTZ; HOET; LAUWERYS, 1987).

Em comparação com outros órgãos, o cérebro é particularmente vulnerável ao estresse oxidativo, pois possui menor atividade das enzimas SOD, CAT e GPx e menor quantidade de GSH (DRINGEN, 2000; GUPTA; DATTA; SHUKLA, 2000), por utilizar grandes

quantidades de oxigênio e por conter uma grande quantidade de ferro e de ácidos graxos poliissaturados (DRINGEN, 2000; JOVANOVIC, 2011).

O estresse oxidativo está envolvido na patogênese de uma série de condições neurológicas e doenças neurodegenerativas, incluindo a doença de Alzheimer, doença de Parkinson, esclerose lateral amiotrófica e epilepsia (PERRY et al., 2002; MIGLIORE et al., 2005; ASHRAFI et al., 2007).

Em relação à epilepsia, o início e a propagação de uma convulsão podem ocorrer como resultado de um desequilíbrio entre mecanismos excitatórios e inibitórios de uma rede interligada de neurônios (MCCORMICK; CONTRERAS, 2001). A excitação prolongada dos neurônios durante as convulsões pode levar ao aumento da liberação de neurotransmissores excitatórios, incluindo o aspartato e o glutamato, e aumento do influxo de íons cálcio através dos receptores NMDA e canais de cálcio dependente de voltagem (ZATTA; TOGNON; CARAMPIN, 2003; ERCEGOVAC et al., 2010). Dessa forma, os níveis elevados de cálcio intracelular podem levar a excitotoxicidade induzida por disfunção mitocondrial, aumento na liberação de citocinas, aumento na produção de EROs/ERN, reversão dos transportadores sódio/cálcio ativação de cascatas bioquímicas que provocam a morte neuronal (FERRIERO, 2005).

Em modelos experimentais geneticamente propensos ao desenvolvimento de epilepsia, foi demonstrado uma diminuição da atividade da GPx e da razão GSH/GSSG, aumento da LPO e oxidação de proteínas (SHIN et al., 2008a). O modelo de crise espontânea induzido por cainato é particularmente útil para o estudo da evolução, propagação e consequências patológicas da descarga epiléptica no sistema límbico. A administração de cainato aumenta produção de O_2• e dano oxidativo ao DNA, disfunção mitocondrial e apoptose principalmente nas regiões de CA1, CA3 e no giro dentado do hipocampo (KIM; HAN, 2000; PATEL, R. N. et al., 2005; LIANG, L. P.; PATEL, 2006). Além disso, ocorre aumento da produção de MDA e diminuição nos níveis de GSH (ONG et al., 2000; LIANG, L. P.; PATEL, 2006; SHIN et al., 2008b; SHIN et al., 2008a).

Outro modelo de crise espontânea amplamente utilizado em roedores para estudar a fisiopatologia das convulsões é o modelo da pilocarpina (CAVALHEIRO et al., 1992; DE FREITAS et al., 2003; DE FREITAS et al., 2010). Neste modelo, as alterações comportamentais e eletroencefalográficas são semelhantes à Epilepsia do Lobo Temporal (TURSKI et al., 1983). Essas alterações são acompanhadas por LPO, aumento do conteúdo de nitrito e diminuição nos níveis de GSH no hipocampo, estriado e córtex (FREITAS et al., 2004).

O aumento em marcadores de dano oxidativo é demonstrado também em modelos de crise única como as convulsões induzidas por PTZ, onde reduções significativas de GSH, e cisteína, bem como o aumento de proteínas carboniladas e LPO são observados (PATSOUKIS et al., 2004; RAMBO et al., 2009). Além disso, as convulsões induzidas por PTZ causam um aumento de nitrito bem como redução na atividade de enzimas antioxidantes como a SOD e a GPx. A ativação excessiva de receptores de glutamato e estresse oxidativo podem ser eventos sequenciais, bem como paralelos, que convergem como uma via final comum para a vulnerabilidade neuronal (COYLE; PUTTFARCKEN, 1993).

Considerando o grande número de pacientes refratários ao tratamento disponível, e que o dano oxidativo parece ser um importante fator envolvido nas crises, terapias alternativas que aumentem as defesas antioxidantes e/ou diminuam o dano oxidativo podem se tornar importantes adjuvantes no tratamento das crises epilépticas, como o exercício físico e a cafeína (RADAK et al., 2007; ARIDA et al., 2009; EL YACOUBI ey al., 2008; LOSCHER, 2011).

Terapias Adjuvantes

1.3.1 Exercício Físico

Os efeitos do exercício físico sobre a saúde geral são bem descritos, principalmente sobre o sistema cardiovascular e metabolismo (POWELL; PAFFENBARGER, 1985; BOOTH; CHAKRAVARTHY; SPANGENBURG, 2002). Além disso, nas últimas décadas os efeitos benéficos da atividade física sobre o sistema nervoso central (SNC) têm sido evidenciados, sugerindo um papel protetor contra uma variedade de distúrbios psicológicos e neurológicos (HILLMAN; ERICKSON; KRAMER, 2008; VAN PRAAG, 2009).

Estudos clínicos e epidemiológicos indicam que o exercício pode melhorar os sintomas de estresse e depressão (SALMON, 2001; BROSSE et al., 2002; CALLAGHAN, 2004), o declínio cognitivo relacionado à idade (LAURIN et al., 2001; COLCOMBE et al., 2004; MCAULEY, E.; KRAMER; COLCOMBE, 2004) e reduzir o risco de demência (FRATIGLIONI; PAILLARD-BORG; WINBLAD, 2004; LARSON et al., 2006). O exercício físico também se mostrou benéfico em uma série de modelos de doenças do SNC, como,

Esclerose Múltipla (EM), doenças de Huntington (DH), Parkinson (DP), Alzheimer (DA) e epilepsia (ARIDA et al., 1999; LAURIN et al., 2001; TILLERSON et al., 2003; KOHL et al., 2007; BENEDETTI et al., 2009). No entanto, os mecanismos subjacentes a estes efeitos benéficos são pouco compreendidos.

Alguns trabalhos têm se direcionado ao entendimento das bases neurobiológicas dos benefícios associados ao exercício físico. Dessa forma, vários autores mostraram que o exercício físico voluntário e aeróbico forçado aumenta a expressão do fator neurotrófico derivado do encéfalo (BDNF) (NEEPER et al., 1995; VAN PRAAG et al., 1999; RADAK et al., 2001; ANG et al., 2006; DING et al., 2006; RADAK et al., 2006; DIETRICH; ANDREWS; HORVATH, 2008) e que, a ativação desse fator trófico está relacionada com a sobrevivência e diferenciação celular, assim como,com o aumento da resistência ao estresse oxidativo (GUO; MATTSON, 2000; KLUMPP; LIPOWSKY, 2005; LEEDS et al., 2005). De fato, estudos em modelos experimentais mostraram que a atividade física aumenta a expressão de BDNF (GRIESBACH; HOVDA; GOMEZ-PINILLA, 2009) e modula positivamente o sistema antioxidante, aumentando o conteúdo e/ou atividade das enzimas SOD, CAT e GPx tanto no músculo como no cérebro de ratos (RAMBO et al., 2009; STEINER; PHILBERT, 2011).

Além disso, estudos mostraram o envolvimento do BDNF com a via de ativação do fator de transcrição responsivo a adenosina monofosfato cíclico (CREB) (FINKBEINER et al., 1997). Nesse contexto, Lee e colaboradores observaram que a administração de BNDF causa um aumento significativo das convulsões induzidas por pilocarpina e dos níveis de EROs, assim como, uma redução na expressão de enzimas antioxidantes e de PGC-1α em animais transgênicos com diminuição da expressão de CREB, sugerindo que este elemento é importante na neuroproteção contra o dano oxidativo (LEE, B. et al., 2009). De fato, vários estudos mostram que o CREB induz a expressão de PGC-1α (HERZIG et al., 2001; LEE, W. J. et al., 2006; ST. PIERRE et al., 2006) e regula a expressão das enzimas antioxidantes, incluindo a GPx e a SOD (BEDOGNI et al., 2003; ST. PIERRE et al.2006).

Nesse sentido, terapias que modulem positivamente o sistema BDNF/CREB/PGC-1α e consequentemente o sistema antioxidante podem ser importantes ferramentas no tratamento de diversas doenças do SNC, como por exemplo a epilepsia.

1.3.1.1 Exercício e Epilepsia

Pessoas com epilepsia são frequentemente aconselhadas a evitar exercícios vigorosos para evitar a manifestação das convulsões, embora existam raros casos de convulsões induzidas pela prática do exercício físico. A maioria dos estudos têm geralmente mostrado que a atividade física pode diminuir a frequência de crises, bem como melhorar a eficiência cardiovascular e aspectos psicológicos associados a pessoas com epilepsia (HEISE J, 2002; ARIDA et al., 2009).

Os efeitos positivos do exercício na epilepsia são principalmente baseados em questionários e / ou estudos (ROTH et al., 1994; STEINHOFF et al., 1996) e por meio de ensaios padronizados de resistência física (STEINHOFF et al., 1996; JALAVA; SILLANPAA, 1997). Apenas poucos estudos em humanos têm examinado o efeito de um programa de exercício físico em pessoas com epilepsia.

Em um estudo realizado por Nakken e colaboradores (1997) foi verificado que a atividade epileptiforme interictal registrada por eletroencefalograma permanece inalterada ou diminui a frequência durante ou imediatamente após o exercício físico aeróbico (NAKKEN et al., 1997). De acordo com estes dados, foi verificado que ocorrem menos convulsões durante a atividade mental e física quando comparado com períodos de descanso (CORDOVA; NAVAS; ESCANERO, 1993), sugerindo que uma maior atenção e vigilância durante a atividade física pode reduzir a ocorrência de convulsões (KUIJER, 1980). Um estudo em mulheres com epilepsia refratária mostrou que o treinamento físico aeróbio diminuiu o número de crises durante o período de exercício (ERIKSEN et al., 1994). Nesse contexto, outro estudo mostrou nenhum impacto na frequência das crises após um programa de exercícios de 12 semanas (MCAULEY, J. W. et al., 2001). Além disso, Vancini e colaboradores (2010) verificaram que o exercício físico exaustivo não induz convulsões (VANCINI et al., 2010).

Estudos experimentais também têm demonstrado um efeito benéfico do exercício físico em animais com epilepsia onde o treinamento físico retarda o desenvolvimento de crises espontâneas antes e durante a indução de crises por estimulação elétrica (ARIDA; DE JESUS VIEIRA; CAVALHEIRO, 1998), bem como reduz a frequência de crises induzidas por pilocarpina (ARIDA et al., 1999). Além disso, estudos metabólicos, eletrofisiológicos e imunohistoquímicos também encontraram efeitos positivos do exercício em modelos experimentais de epilepsia (ARIDA et al., 2003; ARIDA et al., 2007). Neste sentido, tanto o

exercício voluntário como em esteira ou natação oferecem proteção contras as crises induzidas por ácido domóico, cainato e pilocarpina (CARROLL et al., 2001; SETKOWITZ E MAZUR, 2006; REISS et al., 2009). Além disso, um programa de natação de 6 semanas atenuou as convulsões e o dano oxidativo induzido por PTZ em ratos (RAMBO et al., 2009).

Levando-se em consideração que o exercício pode exercer ações benéficas, como a redução de susceptibilidade a convulsão e melhora da qualidade de vida nas pessoas com epilepsia (ARIDA et al., 2010), o exercício físico pode ser um candidato potencial para ser integrado a terapia convencional para o tratamento desta patologia.

Cafeína

A cafeína (1,3,7-trimetilxantina), pertence ao grupo das purinas alcalóides, e é um psicoestimulante comumente consumido em todo o mundo. Esta trimetilxantina é encontrada em bebidas como café, mate, chá e refrigerantes, assim como em chocolates. Também é utilizada pela indústria farmacêutica na composição de analgésicos, estimulantes do apetite e alguns antivirais (GILBERT et al., 1976; BERNSTEIN et al., 2002).

A história da cafeína está associada com a história do café. O café é a principal fonte de cafeína e provém de uma árvore do gênero *Coffeae.* Dentre as várias espécies conhecidas as mais comercializadas são *Coffea arábica* e *Coffea canephora,* sendo popularmente conhecidas como arábica e robusta, respectivamente (FREDHOLM, 2011).

Alguns antropólogos acreditam que o primeiro uso da cafeína derivada de plantas, remonta a 600 mil anos a.C. (Idade da Pedra), no entanto, acredita-se que a descoberta do cafeeiro tenha ocorrido na Etiópia, em torno de 700 a.C., onde a planta crescia naturalmente. Apesar disso, as primeiras plantações de café denominadas "*Kaweh*" apareceram na península Arábica no século XIV, e eram usadas como alimento, na fabricação de vinho, como remédio e para fazer uma bebida árabe denominada "*qahwa*", conhecida por prevenir o sono (FREDHOLM, 2011).

Embora os árabes tenham cultivado o cafeeiro e tenham preparado bebidas de seu grão, somente no século XIV que o processo de torração foi criado. Somente quando isso aconteceu que o uso do café difundiu-se através do Iémen e dos países árabes para o resto do mundo (FREDHOLM, 2011).

Entre os muçulmanos, a necessidade de uma bebida social foi preenchida pelo café, sendo um excelente substituto das bebidas alcoólicas. A bebida foi consumida tanto em casa como em estabelecimentos comerciais (Cafés). O desenvolvimento de casas de café em centros intelectuais foi percebido como uma ameaça às autoridades, sendo o hábito de tomar café condenado pela ortodoxia islâmica. Entretanto, posteriormente, chegou a ser considerado como algo providencial para rezar sem cair em sonolência (FREDHOLM, 2011).

Na Europa, o café apareceu no século XVI sendo introduzido, principalmente, pelos espanhóis e holandeses, no período das grandes navegações e descobertas. Entretanto, o café era consumido de maneira restrita e a bebida nobre era o chá. Inicialmente, o café encontrou uma forte oposição em alguns países protestantes, como a Alemanha, Áustria e Suíça, nações essas que chegaram mesmo a punir o comércio e o seu consumo (FREDHOLM, 2011).

Em 1736, surgem as primeiras plantações na América Latina, principalmente em Porto Rico e cerca de 20 anos depois já era seu principal produto de exportação (RIGOULOUT et al., 2003). Atualmente, o café é cultivado em mais de cinquenta países, sendo o Brasil e a Colômbia os maiores produtores, juntos produzem 39% dos grãos consumidos no mundo (WEINBERG E BEALER, 2001).

Em 1819, o químico alemão Ferdin Runge Friedlieb isolou pela primeira vez um extrato relativamente puro de cafeína, o qual foi chamado de "Kaffebase" (ou seja, uma base que existe no café). Em 1821, a cafeína um pó cristalino branco de gosto amargo foi isolada por químicos franceses (FREDHOLM, 2011).

1.4.1 Metabolismo

O metabolismo da cafeína é caracterizado por uma rápida e completa absorção gastrointestinal (WALTON, K.; DORNE; RENWICK, 2001), sendo que após a administração oral a absorção ocorre em 45 minutos (CHVASTA; COOKE, 1971; BLANCHARD; SAWERS, 1983).

A meia vida, tempo necessário para que sua concentração plasmática diminua pela metade, varia entre as faixas etárias, na gravidez, em combinação com alguns medicamentos e com a integridade hepática. Em adultos saudáveis a meia vida é de aproximadamente 3-4 horas, enquanto que em ratos é mais curta (cerca de uma hora). Mulheres que usam anticoncepcionais a meia vida é de 5-10 horas e naquelas em gestação de 9-11 horas. Já em

recém-nascidos a meia-vida é de 30 horas. Nos indivíduos com doença hepática, a meia-vida da cafeína pode chegar até 96 horas. O metabolismo da cafeína pode ser alterado por outros compostos, por exemplo, o consumo de tabaco diminui a meia-vida da cafeína (FREDHOLM et al., 1999).

A cafeína distribui-se nos tecidos e é encontrada em todos os fluidos corporais, incluindo o plasma, fluido cérebro-espinhal, saliva, bile, sêmen, leite materno, sangue do cordão umbilical, bem como todos os órgãos, entretanto não há o acúmulo de cafeína e/ou seus metabólitos (ARNAUD, 1976). Em ratos adultos, por exemplo, a concentração de cafeína no plasma é semelhante à encontrada no líquido cérebro-espinhal (LIU et al., 2006).

A distribuição tecidual após uma hora de administração intravenosa em coelhos mostrou que a razão de concentração de cafeína tecido/sangue foi de aproximadamente 1. Exceto no tecido adiposo, adrenais, fígado e bile, onde as razões foram 0,2; 0,6; 1,5; e 2,7; respectivamente (BEACH et al., 1985).

O metabolismo de primeira passagem é mínimo (YESAIR; BRANFMAN; CALLAHAN, 1984) ou inexistente em humanos (ARNAUD et al., 1983). As enzimas do citocromo P-450 são as responsáveis pelo metabolismo da cafeína no fígado, sendo degradada em dimetilxantinas, como a paraxantina, teobromina e teofilina. Cada um destes metabólitos tem suas funções no organismo, sendo excretados na urina após metabolizados. Nos humanos, a paraxantina é o metabólito predominante (72 a 80%), enquanto em roedores, apesar da paraxantina ser o metabólito plasmático predominante, os níveis de teofilina também estão elevados.

1.4.2 Mecanismo de ação da Cafeína

A cafeína em baixas concentrações (μM) pode antagonizar todos os receptores de adenosina (A_1, A_{2A}, A_{2B} and A_3), sendo que a maior parte de suas ações é mediada pela inibição dos receptores de alta afinidade A_1 e A_{2A}, (FREDHOLM et al., 1999). Estes, são receptores acoplados a proteína G que afetam muitas vias de sinalização intracelular, incluindo a via do AMPc. A ativação dos receptores dos subtipos A_1 e A_3 leva a uma inibição da enzima adenilatociclase através de uma proteína G inibitória, diminuindo os níveis intracelulares de AMPc. Entretanto a ativação dos receptores A_{2A} e A_{2B} estimula a

adenilatociclase através de uma proteína G estimulatória aumentando os níveis intracelulares de AMPc (OLAH; STILES, 1995).

A cafeína é normalmente ingerida de uma forma crônica, sendo que seus efeitos em longo prazo podem ser diferentes dos efeitos da administração aguda, especialmente em relação à sua ação sobre os receptores de adenosina (JACOBSON et al., 1996). Isso pode se dar em função de um aumento na expressão dos receptores do tipo A_1 e A_{2A} ou por sensibilização dos receptores de adenosina (BIAGGIONI et al., 1991; JACOBSON et al., 1996).

Em altas concentrações, a cafeína pode elevar os níveis intracelulares de AMPc através da inibição da fosfodiesterase 4 (FREDHOLM et al., 1999). Em concentrações na faixa de mM a cafeína pode mobilizar cálcio dos estoques do retículo endoplasmático através dos receptores IP_3 e de rianodina (FREDHOLM; HEDQVIST, 1980; FREDHOLM et al., 1999).

A maioria dos estudos discute uma relação entre o efeito neuroprotetor da cafeína e antagonismo dos receptores A_{2A}, que levaria a uma diminuição da liberação de neurotransmissores excitatórios (KALDA et al., 2006), porém um aumento na expressão dos receptores A_1, cuja ativação pode significar neuroproteção ou um aumento das defesas antioxidantes, poderia ser um mecanismo alternativo (CUNHA et al., 2006). Desta forma, em modelos experimentais, o tratamento com cafeína tem demonstrado efeito neuroprotetor contra neurotoxicidade induzida por β-amilóide ou MPTP (XU et al., 2002; DALL'IGNA et al., 2003; ARENDASH et al., 2006; DALL'IGNA et al., 2007).

Além do efeito direto sobre os receptores de adenosina, alguns outros efeitos têm sido evidenciados após a administração de cafeína. Jones e colaboradores (2008) verificaram um aumento na expressão e na atividade da enzima citocromo oxidase (Cox) no estriado após a administração aguda de cafeína e esses efeitos foram associados ao antagonismo dos receptores A_{2A} (JONES et al., 2008). Desde que a ativação destes receptores aumenta a atividade de proteínas kinases (PKA e MAPK) que podem aumentar a ligação de fatores nucleares repressores da ativação gênica de Cox, antagonistas deste receptor podem inibir a repressão da ativação gênica desta enzima (JONES et al., 2008).

Além disso, a cafeína pode agir via ativação da expressão gênica do fator nuclear relacionado ao eritróide-2 (Nrf2), que ocorre na mesma região promotora da Cox e é um fator de transcrição sensível ao estado redox. A estimulação da via Nrf2 resulta em aumento no mecanismo endógeno contra dano oxidativo e eletrofílico, caracterizado pelo aumento na expressão de GST, que suporta seu potencial neuroprotetor (CHAN et al., 2000;

SCHWARZSCHILD; CHEN; ASCHERIO, 2002; ASCHERIO et al., 2004; HARVEY; SEIB; LUCKE, 2009). Além disso, Cavin e colaboradores (2008) sugerem que o café medeia a estimulação da via Nrf2 resultando em um aumento dos mecanismos de defesa endógenos, como o aumento na expressão das enzimas que regulam a síntese de GSH (CAVIN et al., 2008).

Além dos efeitos citados até o momento, alguns estudos têm mostrado que a ação da cafeína pode não necessariamente depender do antagonismo dos receptores de adenosina. Han e colaboradores (2009) encontraram que a cafeína aumenta a produção de fluído cérebro-espinhal e a expressão da Na^+,K^+-ATPase em células endoteliais em animais tratados cronicamente com cafeína, podendo este ser um dos mecanismos pelo qual a cafeína protege contra DA (HAN et al., 2009; WOSTYN et al., 2011).

Nesse contexto, se demonstrou que a cafeína diminui a produção de PGE_2 induzida por lipopolissacarídeo em cultura de células microgliais (FIEBICH et al., 2000), bem como atenua a perda de células e previne a apoptose induzida por MPP^+ em cultura neurônios granulares cerebelares, sendo que esse efeito não parece ser dependente do antagonismo dos receptores A_1 e A_{2A} (KAVITA et al., 2010).

Alguns estudos também demonstraram que a cafeína e seus metabólitos apresentam efeitos antioxidantes *in vitro*, sendo inclusive capaz de reagir diretamente com OH (SHI; DALAL; JAIN, 1991; LEE, C., 2000; GOMEZ-RUIZ; LEAKE; AMES, 2007). A maioria dos estudos sobre cafeína e estresse oxidativo foram feitos *in vitro*, entretanto, outros estudos sugerem que a cafeína pode aumentar a atividade de enzimas antioxidantes *in vivo* (ROSSOWSKA; NAKAMOTO, 1994; MUKHOPADHYAY; MONDAL; PODDAR, 2003).

Nesse sentido, Aoyama e colaboradores (AOYAMA et al., 2011) verificaram que a cafeína aumenta a síntese de GSH, podendo este ser um novo mecanismo pelo qual esta metilxantina apresenta efeito neuroprotetor (AOYAMA et al., 2011). A GSH é o tiol reduzido mais abundante que desenvolve uma importante função como antioxidante no SNC (DRINGEN; GUTTERER; HIRRLINGER, 2000). De fato, algumas doenças neurodegenerativas apresentam níveis reduzidos de GSH, dentre elas DP, DA e epilepsia (SIAN et al., 1994; RAMASSAMY et al., 2000; MUELLER et al., 2001).

1.4.3 Cafeína e Epilepsia

Embora o papel neuroprotetor da cafeína esteja bem descrito, principalmente para DA e DP, seu papel nas convulsões ainda é controverso. Existem estudos mostrando que a cafeína pode atuar como proconvulsivante ou anticonvulsivante, sendo que este efeito parece ser dependente da dose e do tempo de administração (JOHANSSON et al., 1996; HALLER; MEIER; OLSON, 2005).

Nesse contexto, alguns estudos evidenciam que a administração de altas doses de cafeína (acima de 150 mg/Kg) pode induzir convulsões tanto em humanos quanto em modelos experimentais (CHU, 1981; MARANGOS et al., 1981; NEHLIG; DAVAL; DEBRY, 1992; HALLER; MEIER; OLSON, 2005).

Além disso, a administração desta trimetilxantina diminui o limiar e aumenta a incidência de convulsões tônicas induzidas por PTZ e aminofililina (CZUCZWAR et al., 1987; CUTRUFO et al., 1992). Da mesma forma, a cafeína reduz o limiar e prolonga a duração das convulsões induzidas por estímulos elétricos em ratos (ALBERTSON; JOY; STARK, 1983; CZUCZWAR et al., 1990; FRANCIS; FOCHTMANN, 1994; GASIOR et al., 1996), bem como, prolonga a duração das convulsões em terapia de eletrochoque para depressão profunda (STERN et al., 1999).

Neste sentido, alguns estudos têm demonstrado que a administração de cafeína atenua o efeito protetor de algumas DAE como a carbamazepina, fenobarbital, fenitoína, valproato e topiramato, bem como, reduziu os efeitos anticonvulsivantes dodiazepam nas convulsões induzidas por PTZ em ratos (KULKARNI; JOSEPH; DAVID, 1991). Entretanto, a administração de cafeína não reduz a eficácia das AED de nova geração como lamotrigina, tiagabina, e oxcarbazepina. Da mesma forma, não foi observado efeito da cafeína sobre a atividade anticonvulsivante da etossuximida e valproato (CHROSCINSKA-KRAWCZYK et al., 2011).

O conceito de que o uso de metilxantinas pode potencializar as convulsões tem sido desafiado por diversos autores que argumentam que os estimulantes do SNC atuam como proconvulsivante somente em doses supramáximas (GEORGIEV; JOHANSSON; FREDHOLM, 1993; JOHANSSON et al., 1996; LOSCHER, 2009).

Visto isso, o tratamento com cafeína (60-70 mg/kg/dia durante 2 semanas) reduziu as convulsões induzidas por NMDA, bicuculina, e PTZ em camundongos, sem alterações na expressão dos receptores A_1 e A_{2A} e $GABA_A$ (GEORGIEV; JOHANSSON; FREDHOLM,

1993; JOHANSSON et al., 1996). Estudos anteriores sugerem que o efeito neuroprotetor exercido pela cafeína dá-se pelo aumento na expressão dos receptores A_1 de adenosina (DAVAL et al., 1989; JOHANSSON et al., 1993). No entanto, a exposição prolongada a uma dose baixa de cafeína não tem nenhum efeito, quer sobre o número de receptores de adenosina (GEORGIEV; JOHANSSON; FREDHOLM, 1993; JOHANSSON et al., 1996) ou sobre o número de receptores $GABA_A$ (JOHANSSON et al., 1996). Neste sentido, El Yacoubi sugere uma possível participação do antagonismo dos receptores A_{2A} (EL YACOUBI et al, 2008).

Desta forma, camundongos transgênicos que demonstram uma diminuição na expressão dos receptores do tipo A_{2A} são parcialmente resistentes a convulsões induzidas por PTZ ou pela retirada do etanol (El YACOUBI et al. 2001, 2008). A administração de cafeína (0,3 g/l na água), durante 14 dias reduziu as convulsões induzidas por PTZ em camundongos (EL YACOUBI et al.2008).

No modelo experimental de epilepsia do lobo temporal, a administração de cafeína, ofereceu neuroproteção completa contra a perda neuronal na região CA1 do hipocampo, entretanto, não ofereceu proteção contra o aparecimento de convulsões (RIGOULOUT 2003).

Em um modelo de morte súbita em epilepsia (SUDEP), a administração aguda de cafeína (40 mg/kg i.p) aumentou o tempo de sobrevivência dos camundongos (SHEN; LI; BOISON, 2010). Esse efeito protetor pode ser explicado pelo antagonismo dos receptores de adenosina, já que após as convulsões o aumento excessivo da liberação de adenosina pode induzir insuficiência respiratória e cardíaca por hiperestimulação dos receptores de adenosina do tronco cerebral (SHEN; LI; BOISON, 2010).

Dessa forma, considerando que a cafeína e o exercício físico apresentam efeito protetor frente ao dano oxidativo induzido em modelos experimentais de doenças neurodegenerativas e epilepsia, torna-se importante investigar seus efeitos sobre as convulsões e o dano oxidativo induzidos por PTZ.

Objetivo Geral

Investigar os efeitos do exercício físico e da suplementação com cafeína nas convulsões comportamentais e alterações eletroencefalográficas, bem como nas alterações dos parâmetros oxidativos induzidos por pentilenotetrazol em ratos.

1.5.1 Objetivos Específicos

- Avaliar o efeito do exercício físico na gênese das convulsões comportamentais e alterações eletroencefalográficas induzidas por PTZ, bem como sobre diferentes parâmetros oxidativos.

- Avaliar o efeito da suplementação de cafeína nas convulsões comportamentais e eletroencefalográficas induzidas por PTZ, bem como sobre diferentes parâmetros oxidativos.

- Avaliar o efeito do exercício físico associado à suplementação com cafeína nas convulsões comportamentais e eletroencefalográficas induzidos por pentilenotetrazol em ratos.

ARTIGO–TREINAMENTO DE NATAÇÃO PREVINE A INIBIÇÃO DA ATIVIDADE DA Na^+,K^+-ATPase, CONVULSÕES E ESTRESSE OXIDATIVO INDUZIDOS POR PENTILENOTETRAZOL

Título Original

Swimming training prevents pentylenetetrazol-induced inhibition of Na^+,K^+-TPase activity, seizures, and oxidative stress.

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FULL-LENGTH ORIGINAL RESEARCH

Swimming training prevents pentylenetetrazol-induced inhibition of Na^+, K^+-ATPase activity, seizures, and oxidative stress

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Summary

Purpose: In the present study we decided to investigate whether physical exercise protects against the electrographic, oxidative, and neurochemical alterations induced by subthreshold to severe convulsive doses of pentyltetrazole (PTZ).

Methods: The effect of swimming training (6 weeks) on convulsive behavior induced by PTZ (30, 45, and 60 mg/kg, i.p.) was measured and different electrographic electroencephalography (EEG) frequencies obtained from freely moving rats. After EEG recordings, reactive oxygen species (ROS) generation, nonprotein sulfhydryl (NPS), protein carbonyl, thiobarbituric acid-reactive substances (TBARS), superoxide dismutase (SOD), catalase (CAT), Na^+, K^+-ATPase activity, and glutamate uptake were measured in the cerebral cortex of rats.

Results: We showed that physical training increased latency and attenuated the duration of generalized seizures induced by administration of PTZ (45 mg/kg). EEG recordings showed that physical exercise decreased the spike amplitude after PTZ administration (all doses). Pearson's correlation analysis revealed that protection of physical training against PTZ-induced seizures strongly correlated with NPS content, Na^+, K^+-ATPase activity, and glutamate-uptake maintenance. Physical training also increased SOD activity, NPS content, attenuated ROS generation per se, and was effective against inhibition of Na^+, K^+-ATPase activity induced by a subthreshold convulsive dose of PTZ (30 mg/kg). In addition, physical training protected against 2′,7′-dichlorofluorescein diacetate (DCFH-DA) oxidation, TBARS and protein carbonyl increase, decrease of NPS content, inhibition of SOD and catalase, and inhibition glutamate uptake induced by PTZ.

Conclusions: These data suggest that effective protection of selected targets for free radical damage, such as Na^+, K^+-ATPase, elicited by physical training protects against the increase of neuronal excitability and oxidative damage induced by PTZ.

KEY WORDS: Seizure, Oxidative damage, Physical exercise, Pentylenetetrazol, Na^+, K^+-ATPase.

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Epilepsy is a common and chronic neurologic disorder constituting a large group of neurologic diseases, with an incidence of 0.5–1% in the general population (Andrade & Minassian, 2007). Many studies have suggested that a cascade of biologic events, including generation of reactive oxygen species (ROS), underlie the development and propagation of epilepsy (Patsoukis et al., 2005). In fact, oxidative stress in the central nervous system (CNS) has been shown in several rodent models of experimental epilepsy, such as the amygdala kindling model (Frantseva et al., 2000), the kainic acid model (Gluck et al., 2000), the pentylenetetrazol (PTZ) kindling model (Gupta et al., 2003), and in acute PTZ-induced seizure (Patsoukis et al., 2004).

PTZ is a blocker of the chloride ionophore complex to the γ-aminobutyric acid $(GABA)_A$ receptor (Huang et al., 2001) that has convulsant effects after repeated or single-dose administration and also affects several neurotransmitter systems, such as the GABAergic and glutamatergic systems (Jensen et al., 1991; Psarropoulou et al., 1994; Thomsen, 1999; Walsh et al., 1999). In addition, previous studies from our group have demonstrated that striatal injection of PTZ-induced convulsive activity is accompanied by ROS generation and inhibition of local Na^+, K^+-ATPase activity (Oliveira et al., 2004; Ribeiro et al., 2005; Fighera et al., 2006). These experimental data reinforce the assumption that inhibition of some selected targets for free radicals increases cellular excitability (Jamme et al., 1995; Danbolt, 2001; Prigol et al., 2007).

Recently, a substantial body of evidence has suggested that regular exercise has the capacity to beneficially effect certain brain functions and plays an important preventive and therapeutic role in oxidative stress–associated diseases, including ischemic heart disease, type 2 diabetes, Alzheimer's disease, and Parkinson's disease (Kiraly & Kiraly, 2005; Lazarevic et al., 2006; Belardinelli et al., 2007; Khedr et al., 2007). Accordingly, studies have shown that animals and humans clearly undergo significant adaptive responses to regular endurance exercise that involve greatly increased endurance capacity, which is permitted by dramatic mitochondrial biogenesis, reduction of oxidant production, and increase of antioxidant defenses (Packer & Cadenas, 2007; Sachdev & Davies, 2008). In fact, data on the effect of physical exercise on the brain indicate that, under certain conditions, physical exercise can attenuate oxidative stress-related damage in the brain causing improved brain function (Alessio et al., 1988; Radak et al., 2001; Cotman & Engesser-Cesar, 2002). Furthermore, regular physical exercise plays a preventive role against lifestyle-dependent diseases, and molecular mechanisms behind this favorable effect could be linked to redox homeostasis, a free radical–related adaptation mechanism (Radak et al., 2008).

Although the favorable effect of physical exercise on general health is unquestionable, fitness programs in patients with epilepsy are still a matter of controversy. Clinical investigations have demonstrated that there is a reduction in the number of seizures after physical training programs (Denio et al., 1989; Nakken et al., 1990; Eriksen et al., 1994); however, a significant number of patients with epilepsy believe that physical exercise increases likelihood of a seizure and are advised by family, friends, and even their physicians to avoid exercise (Steinhoff et al., 1996). Therefore, epileptic patients leading sedentary lives have shown greater body weight, and significantly poorer muscle strength and respiratory capacity than people taking part in regular exercise (Jalava & Sillanpaa, 1997).

On the other hand, experimental findings in animal models of epilepsy, such as temporal lobe epilepsy and kindling development, reveal that physical exercise increases the amount of stimulation necessary to reach the convulsive threshold (Arida et al., 1998), attenuates the frequency of seizures, and decreases susceptibility to subsequently evoked seizures in the pilocarpine model of epilepsy (Arida et al., 1999; Setkowicz & Mazur, 2006). In addition, it has been demonstrated that physical exercise promotes positive plastic changes in the hippocampal formation of rats with epilepsy (Arida et al., 2007a).

These facts clearly indicate that physical exercise may ameliorate the course of epileptic activity in the brain. However, very little information is available regarding the exact role of free radicals in its development and the improvement induced by physical exercise in an experimental model of epilepsy induced by PTZ. Therefore, in the present study we aimed to investigate whether physical exercise protects against the electrographic, oxidative, and neurochemical alterations induced by subthreshold, moderate, and severe convulsive doses of PTZ (30–60 mg/kg).

Materials and Methods

Animal and reagents

All experiments involving the animals were conducted in conformance with the policy statement of the American College of Sports Medicine. In the present study 90-day-old male Wistar rats, weighing 250–300 g at the beginning and 400–450 g at the end of the experimental period were used. During this period, the animals were maintained in a controlled environment (12:12 h light–dark cycle, 24 ± 1°C, 55% relative humidity) with free access to food (Guabi, Santa Maria, Brazil) and water. Animal utilization protocols followed the Official Government Ethics guidelines and were approved by the University Ethics Committee. All efforts were made to reduce the number of animals used, as well as to minimize their suffering. All other reagents were purchased from Sigma (St Louis, MO, U.S.A.).

Adaptation to the water

All the rats were adapted to the water before the beginning of the experiment. The adaptation consisted of keeping the animals in shallow water at 32°C between 9:00 and 11:00 a.m. The adaptation period proceeded during the entire experimental period. The purpose of the adaptation was to reduce stress without promoting a physical training adaptation.

Training protocol and lactate threshold assay

The use of swimming rats as a model of exercise presents advantages over treadmill running, since swimming is a natural ability of the rats and this avoids the selection of animals, which is necessary in experimental protocols using treadmill running (Arida et al., 1999). For exercise training, the rats were randomly assigned to the following groups: trained/saline (0.9% NaCl, 1 ml/kg, i.p., n = 8), trained/PTZ (30 mg/kg; n = 8), trained/PTZ (45 mg/kg; n = 8), and trained/PTZ (60 mg/kg; n = 8). The training period lasted 6 week and consisted of 60-min daily sessions five times per week. The training tank used for this study was 80 cm in length, 50 cm in width, and 90 cm in depth, and swimming was always performed in water at a temperature of 32°C between 9:00 and 11:00 a.m. During the first week of training, all animals underwent a swimming adaptation period without weights. After the swimming adaptation period, the rats were subjected to swimming training with a work load (5% of body weight) to improve endurance (Gobatto et al., 2001). At the same time of the training session, sedentary rats were placed in a separate tank with shallow water (5 cm in depth) at 32°C, 5 days/week without the work load (5% of body weight). After 6 weeks, sedentary rats received saline (0.9% NaCl, 1 ml/kg, i.p., n = 8–10) or PTZ (30, 45 or 60 mg/kg, i.p., n = 8–10 in each group) and were used as controls.

After 6 weeks of training, a test protocol was used to determine the lactate threshold (LT) in sedentary (n = 6) and trained rats (n = 6). The LT test was carried out according to the protocol described by Marquezi et al. (2003) and consisted of swimming exercises with progressive overload through weights attached to the animal's tail, corresponding to 4%, 5%, 6%, 7%, and 8% of body weight of each animal for 3-min periods, separated by 1-min resting periods. During the resting periods, 25-μl blood samples were collected from the tail vein into heparinized capillary tubes for determination of lactate concentration. The LT for each animal was calculated based on the point of inflection of the graph when plotting lactate concentration against the corresponding exercise workload.

Surgical procedure

All animals were submitted to surgery 24 h after the last session of training. In brief, the rats were deeply anesthetized with Equithesin (1% phenobarbital, 2% magnesium sulfate, 4% chloral hydrate, 42% propylene glycol, 11% ethanol; 3 ml/kg, i.p.). Two screw electrodes were placed bilaterally over the parietal cortex, along with a ground lead positioned over the nasal sinus. The electrodes were connected to a multipin socket fixed to the skull with acrylic cement. The experiments were performed 5 days after surgery.

Seizure evaluation

Seizures were monitored in all animals by electrocorticographic recording. On the day of the experiments, each animal was transferred to an acrylic glass cage (25 × 25 × 40 cm) and allowed to adapt for 20 min before electroencephalography (EEG) recording. The rat was then connected to the lead socket in a swivel inside a Faraday's cage, and the EEG was recorded using a digital encephalographer (Neuromap EQSA260, Neuromap LTDA, Itajubá, MG, Brazil). EEG signals were amplified, filtered (0.1–70.0 Hz, bandpass), digitalized (sampling rate 256 Hz), and stored in a personal computer for off-line analysis. Routinely, a 10-min baseline recording was obtained to establish an adequate control period. After baseline recording, the sedentary and trained animals received an injection of saline (0.9% NaCl, 1 ml/kg, i.p.) and/or PTZ (30, 45 and 60 mg/kg, i.p.). The animals were observed for the appearance of generalized tonic–clonic convulsive episodes for 20 min according to Ferraro et al. (1999), who describes clonic convulsions as episodes characterized by typical partial clonic activity affecting the face, head, vibrissae, and forelimbs. Generalized convulsive episodes were considered as generalized whole-body clonus involving all four limbs and tail, rearing, and wild running and jumping, followed by sudden loss of upright posture and autonomic signs, such as hypersalivation and defecation, respectively. During the 20-min observation period, the latencies for the first generalized tonic–clonic convulsions were measured. EEG recordings were visually analyzed for seizure activity, which were defined by the occurrence of the following alterations in the recording leads (McColl et al., 2003): isolated sharp waves (≥1.5 × baseline); multiple sharp waves (≥2 × baseline) in brief spindle episodes (≥1 s ≥ 5 s); multiple sharp waves (≥2 × baseline) in long spindle episodes (≥5 s); spikes (≥2 × baseline) plus slow waves; multispikes (≥2 × baseline, ≥3 spikes/complex) plus slow waves; and major seizure (repetitive spikes plus slow waves obliterating background rhythm, ≥5 s). For quantitative analysis of EEG amplitude, we averaged EEG amplitude over the 20-min of observation.

Sample processing

After the behavioral evaluation (20 min after PTZ administration), the animals were killed by decapitation and their brain was exposed by removing the parietal bone. After decapitation, the whole brain was removed

and placed in ice-cold Krebs Cerebral Henseleit buffer containing 124 mM NaCl, 5 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 23 mM $NaHCO_3$, 3 mM HEPES, and 10 mM D-glucose, and the pH was adjusted to 7.4 with 95% O_2/5% CO_2. Cerebral cortex slices (0.4 mm) were obtained by transversal cuts using a McIlwain chopper. The time duration between decapitation and preparation of the slices was 2–3 min, and during this period the brain was immersed in the ice-cold medium.

Estimation of ROS generation

Generation of ROS was estimated with the fluorescent probe, 2′,7′-dichlorofluorescein diacetate (DCFH-DA), as described by Ali et al. (1992). Briefly, the slice from cerebral cortex was homogenized in 2.5 ml of saline solution (0.9% NaCl). Aliquots of 2.5 ml were incubated in the presence of DCFH-DA (5 μM) at 37°C for 60 min. The DCFH-DA is enzymatically hydrolyzed by intracellular esterases to form nonfluorescent DCFH, which is then rapidly oxidized to form highly fluorescent 2′,7′-dichlorofluorescein (DCF) in the presence of ROS. DCF fluorescence intensity is proportional to the amount of ROS that is formed. Fluorescence was measured using excitation and emission wavelengths of 480 and 535 nm, respectively. A calibration curve was established with standard DCF (0.1 nM to 1 μM), and ROS levels were expressed as percentages of control.

Nonprotein sulfhydril (NPS) levels

The levels of NPS in slices of cerebral cortex were determined in the presence of 50 mM Tris–Cl, pH 7.4. Free –SH groups were determined according to Ellman and Lysko (1967). Incubation at 37°C was initiated by the addition of the thiol compounds. Aliquots of the reaction mixture (100 μl) were checked for the amount of –SH groups at 412 nm after 90–120 min of addition of color reagent 5′5′-dithio-bis (2-nitrobenzoic) acid (DTNB).

Measurement of protein carbonyl

For the protein carbonyl assay, a slice from the cerebral cortex was homogenized in 10 volumes (w/v) of 10 mM Tris–HCl buffer pH 7.4 using a glass homogenizer, and its carbonyl protein content was determined by the method described by Levine et al. (1990) adapted for brain tissue (Oliveira et al., 2004).

Measurement of thiobarbituric acid-reactive substances (TBARS) content

For the TBARS assay, a slice from cerebral cortex was homogenized in ultra-purified water, and the TBA reagent (15% of trichloroacetic acid, 0.375% of thiobarbituric acid, and 2.5% v/v of HCl) was added. After 30 min of incubation, samples were centrifuged (3000***g***, 15 min) and then TBARS levels were measured at 532 nm (Ríos & Santamaría, 1991).

Superoxide dismutase (SOD) and catalase (CAT) activity

To verify SOD and CAT activity, a slice from the cerebral cortex was adequately homogenized in 40 volumes (w/v) with Tris–HCl 10 mM (pH 7.4), and an assay was performed according to the methods of Misra and Fridovich (1972) and Aebi (1984) respectively. The SOD activity was expressed as units/g of protein, and CAT activity was expressed in units (1 U decomposes 1 μmol of H_2O_2 per minute at pH 7.0 at 25°C).

Measurement of Na^+, K^+-ATPase activity

The measurement of Na^+, K^+-ATPase activity was performed in the same kind of homogenate used for determination of the protein carbonyl content. The enzyme assay was performed according to Wyse et al. (2000).

Glutamate uptake

For the glutamate uptake measurement, the slices of cerebral cortex were previously maintained in a pregassed (carbogen) artificial cerebrospinal fluid for 15 min containing (in mM): 137 NaCl, 0.63 Na_2HPO_4, 4.17 $NaHCO_3$, 5.36 KCl, 0.44 KH_2PO_4, 1.26 $CaCl_2$, 0.41 $MgSO_4$, 0.49 $MgCl_2$, and 5.55 glucose (pH 7.2). Glutamate uptake was performed according to Frizzo et al. (2002) with few modifications. Briefly, uptake was carried out at 35°C by adding 100 μM of unlabeled glutamate and 1 μM [^{3}H] glutamate. The reaction was stopped after 7 min by washing two times with 1 ml cold buffer, immediately followed by addition of 0.5 N NaOH, which was kept overnight. Sodium independent uptake was determined by using choline chloride instead of sodium chloride, which was subtracted from the total uptake to obtain the sodium dependent uptake. Incorporated radioactivity was determined with a Packard scintillator (TRI CARB 2100 TR).

Protein determination

Protein content was measured colorimetrically by the method of Bradford (1976) using bovine serum albumin (1 mg/ml) as a standard.

Statistical analysis

Statistical analysis was carried out by one- or two-way analysis of variance (ANOVA), and only F-values of $p < 0.05$ are presented. Post hoc analysis was carried out, when appropriate, by the Student–Newman–Keuls test. All data are expressed as mean ± SEM.

Results

Effects of physical exercise on lactate threshold and body weight

In the present investigation, we showed a significant increase in total body weight in sedentary versus trained rats along the 6 weeks of swimming training [$F(1,14) = 15.09$; $p < 0.05$; Fig. 1A]. In addition, statistical analysis

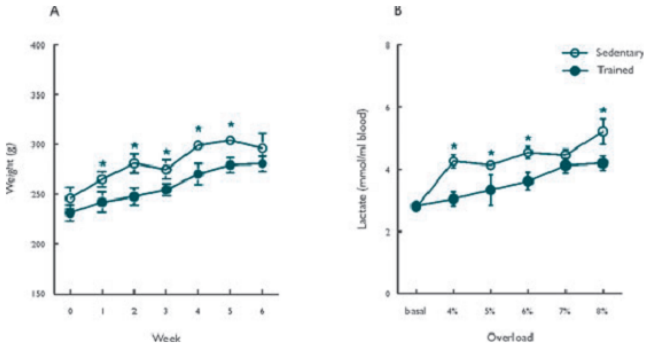

Figure 1.
Effect of 6 weeks of swimming training on body weight (**A**) and lactate threshold assay (**B**). *p < 0.05 compared with sedentary group (F test for simple effect). Data mean + SEM for n = 6 in each group.
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showed a clear stabilization of the blood lactate concentration in the trained group when compared with the sedentary group for the lactate threshold assay [F(1,14) = 10.89; p < 0.05; Fig. 1B] indicating that the training program increased aerobic resistance of the animals.

Effects of physical training on PTZ-induced behavioral convulsions and epileptiform EEG activity

Figs 2A, B show the effect of physical training on the latency and duration of generalized tonic–clonic convulsions induced by PTZ (45 or 60 mg/kg). Partitioning of the sum of squares into trend components revealed that administration of PTZ induced convulsive activity linearly with the dose given. The 6 weeks of swimming training increased the latency for the first convulsive episode induced by the moderate convulsive dose of PTZ (45 mg/kg) [F(2,44) = 1,33; p < 0.05; Fig. 2A] and attenuated the duration of convulsive episodes induced by PTZ (45 and 60 mg/kg) [F(2,44) = 10,75; p < 0.05; Fig. 2B].

Electroencephalographic recordings revealed similar EEG signals between the trained and sedentary groups before and after saline administration (Fig. 3A, panels I–II), suggesting that the protocol of physical training used here did not elicit detectable alterations in surface basal EEG. Injection of PTZ at 30 mg/kg caused only minor behavioral and EEG alterations (Fig. 3B, panel III), but injection of PTZ (45 or 60 mg/kg) caused the appearance of generalized tonic–clonic seizures characterized by the appearance of 2–3 Hz high-amplitude activity in the recording leads (Figs 3C, D, panels V and VII, respectively). Of note, physical training decreased the occurrence of EEG seizure activity induced by all doses of PTZ (Figs 3B, C, and D, panels IV, VI, and VIII, respectively). In addition, statistical analysis showed that swimming training had no effect on baseline EEG amplitude (Fig. 3E) but decreased the increase in EEG wave amplitude induced by administration of all doses of PTZ [F(3,44) = 4,55; p < 0.05; Fig. 3F].

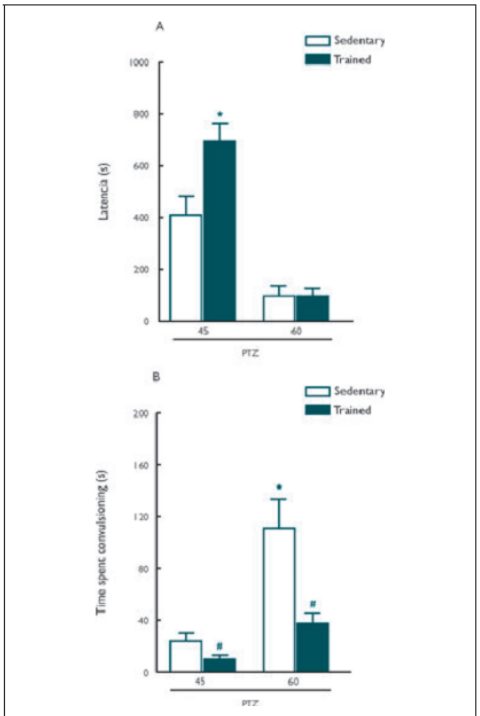

Figure 2.
Effect of physical training on latency (**A**) and duration (**B**) of generalized tonic–clonic convulsions induced by PTZ (45 and 60 mg/kg, i.p.). *p < 0.05 compared with sedentary-PTZ group (45 mg/kg, i.p.). #p < 0.05 compared to sedentary-PTZ group (45 and 60 mg/kg, i.p.) Data are mean + SEM for n = 8–10 in each group.
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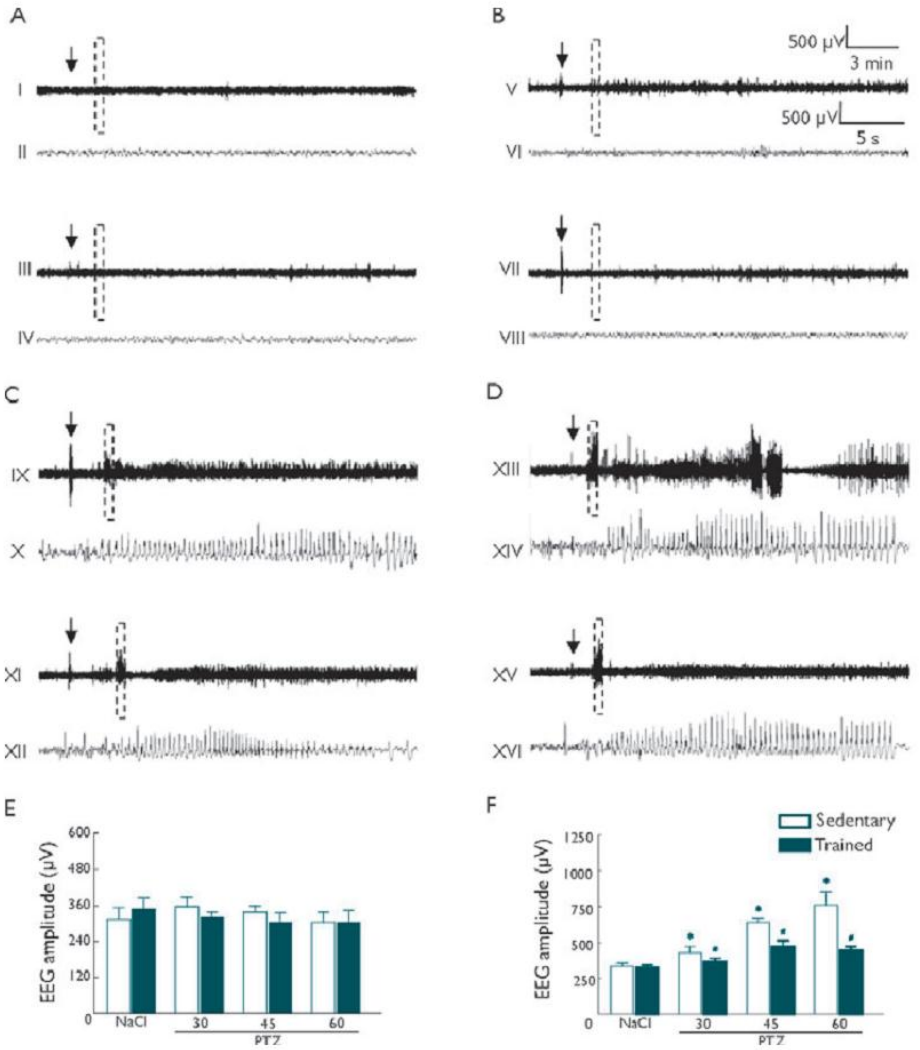

Figure 3.

Representative electroencephalographic recordings demonstrate the discharge sequence observed in sedentary (A-I) and trained group (A-II) after systemic administration after saline (0.9% NaCl, 1 ml/kg, i.p.). Typical electroencephalographic recordings variations after administration of the subthreshold to severe convulsive dose of PTZ (30–60 mg/kg, i.p.) in sedentary (B-III; 3C-V; 3D-VII) and physical training group (3B-IV; 3C-VI; 3D-VIII). The arrow indicates NaCl or PTZ (30, 45 or 60 mg/kg, i.p.) administration. Calibration bars 500 μV and 3 min. Figs. 3E, F show the effect of physical training on EEG amplitude before (**A**) and after saline (0.9% NaCl, 1 ml/kg, i.p.) or PTZ (30–60 mg/kg, i.p.) (**B**). *$p < 0.05$ compared with NaCl group. $^{\#}p < 0.05$ compared to sedentary-PTZ group (30–60 mg/kg, i.p.) Data are mean + SEM for n = 8–10 in each group.

Table 1. Effect of swimming training on 2′,7′-dichlorofluorescein (DCF) oxidation, protein carbonyl, and TBARS content after injection of PTZ (30, 45, and 60 mg/kg, i.p.)

	DCFH-DA oxidation (% of control)		Protein carbonyl content (nmol/mg protein)		TBARS content (nmol MDA/mg protein)	
Treatment (mg/kg)	Sedentary	Trained	Sedentary	Trained	Sedentary	Trained
NaCl	100 ± 5.0	−16.33 ± 8.2[a]	6.6 ± 0.7	6.01 ± 0.2	57.45 ± 17.7	40.10 ± 10.6
PTZ-30	50.01 ± 5.5[c]	10.21 ± 5.1[c]	7.7 ± 0. 6	5.2 ± 0.4	63.37 ± 10.7	55.90 ± 14.4
PTZ-45	51.96 ± 6.1[c]	11.16 ± 4.8[b]	8.9 ± 0.6[c]	6.2 ± 0.3[b]	68.9 ± 15.6	54.2 ± 10.5
PTZ-60	49..47 ± 4.2[c]	10.05 ± 4.3[b]	10.4 ± 0.6[c]	6.0 ± 0.5[b]	118.2 ± 20.5[c]	66.2 ± 13.9[b]

Data are mean ± SEM for n = 8–10 in each group. MDA, malondialdeide.
[a]$p < 0.05$ compared to NaCl-sedentary group.
[b]$p < 0.05$ compared with sedentary-PTZ groups (30–60 mg/kg, i.p.) (Student–Newman–Keuls test).
[c]$p < 0.05$ compared to NaCl-sedentary group.

Epileptiform activity–induced alteration in ROS generation and the antioxidant status in cerebral cortex: effects of physical training

Several parameters that indicate the antioxidant status and the generation of oxidative stress in the cell were determined in cortical homogenates. These include DCFH-DA oxidation, lipid peroxidation (TBARS), protein carbonyl, NPS content, and CAT and SOD activities.

The effect of injection of PTZ (30, 45, and 60 mg/kg, i.p.) and swimming training on DCFH-DA oxidation, protein carbonyl, and lipid peroxidation (TBARS) is showed in Table 1. Statistical analysis revealed that physical training attenuated DCFH-DA oxidation [$F(1,64) = 35.17$; $p < 0.05$] per se, and prevented the increase in DCFH-DA oxidation induced by all doses of PTZ [$F(1,30) = 5.17$; $p < 0.05$]. This training protocol also protected against TBARS [$F(1,64) = 14.98$; $p < 0.05$] and protein carbonyl increase [$F(3,64) = 2.17$; $p < 0.05$] induced by higher doses of PTZ (45 and 60 mg/kg).

In addition, statistical analysis showed that physical training increased SOD activity [$F(1,64) = 31.69$; $p < 0.05$; Fig. 4A] per se, and protected against its inhibition after administration of the fully convulsant dose of PTZ (60 mg/kg) [$F(3,64) = 3.90$; $p < 0.05$]. Moreover, administration of PTZ at 45 or 60 mg/kg induced a significant decrease in CAT activity [$F(3,64) = 4.89$; $p < 0.05$], an effect prevented by physical training [$F(3,64) = 2.73$; $p < 0.05$; Fig. 4B]. Furthermore, physical training increased NPS content [$F(1,64) = 22.24$; $p < 0.05$] per se, and protected against the decrease in NPS induced by higher doses of PTZ (45 and 60 mg/kg) [$F(3,64) = 5.87$; $p < 0.05$] (Fig. 4C).

Effects of physical training on PTZ-induced glutamate uptake and inhibition of Na^+,K^+-ATPase activity

Considering that alterations in the redox state of regulatory sulfhydryl groups in selected targets, such as Na^+, K^+-ATPase (Morel et al., 1998) and glutamate transporters

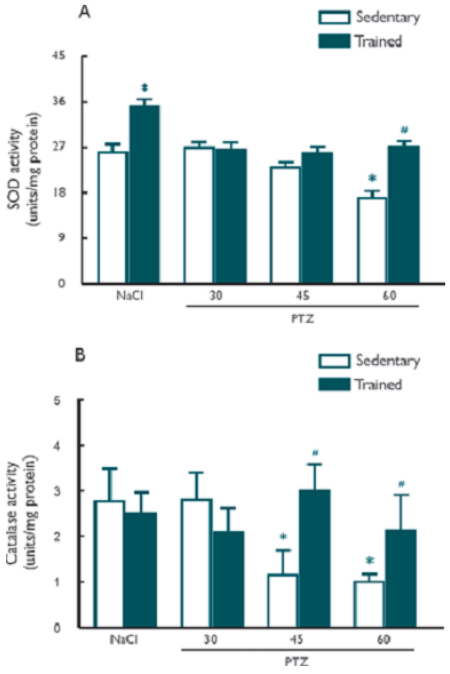

Figure 4.
Effect of physical training and PTZ injection on SOD (**A**) and catalase (**B**) activity ex vivo. Data are mean ± SEM for n = 8–10 in each group. ‡$p < 0.05$ compared to NaCl-sedentary group. #$p < 0.05$ compared with sedentary-PTZ groups (45–60 mg/kg, i.p.). *$p < 0.05$ compared to NaCl-sedentary group. (Student–Newman–Keuls test).
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Table 2. Effect of swimming training on nonprotein sulfhydryl groups, Na^+, K^+-ATPase activity and, [^{3}H] Glutamate uptake after injection of PTZ (30, 45, and 60 mg/kg, i.p.)

	Free –SH group content (μmol/mg protein)		Na^+, K^+-ATPase activity (nmol Pi/mg protein/min)		[^{3}H] Glutamate uptake (pmol/mg protein/min)	
Treatment (mg/kg)	Sedentary	Trained	Sedentary	Trained	Sedentary	Trained
NaCl	1.21 ± 0.02	2.79 ± 0.02[a]	141.3 ± 13.0	156.4 ± 12.9	101.3 ± 13.0	89.20 ± 15.0
PTZ-30	1.09 ± 0.06	2.02 ± 0.02	108.8 ± 8.3[c]	139.9 ± 10.0[b]	81.27 ± 8.3	90.25 ± 13.9
PTZ-45	0.96 ± 0.01[c]	2.16 ± 0.01[b]	88.8 ± 7.8[c]	131.1 ± 10.1[b]	69.80 ± 7.8	73.10 ± 10.1
PTZ-60	0.47 ± 0.02[c]	1.53 ± 0.02[b]	79.7 ± 6.2[c]	153.6 ± 8.7[b]	37.62 ± 6.3[c]	64.5 ± 8.7[b]

Data are mean ± SEM for n = 8–10 in each group.
[a] $p < 0.05$ compared to NaCl-sedentary group.
[b] $p < 0.05$ compared with sedentary-PTZ groups (30–60 mg/kg, i.p.) (Student–Newman–Keuls test).
[c] $p < 0.05$ compared to NaCl-sedentary group.

(Trotti et al., 1997), increases cellular excitability and facilitates the appearance or propagation of convulsions (Ames, 2000), we investigated the effect of physical training and PTZ administration on Na^+, K^+-ATPase activity and [^{3}H]-glutamate uptake (Table 2). Statistical analysis showed that administration of PTZ at all doses used decreased Na^+, K^+-ATPase activity [$F(3,64) = 4.72$; $p < 0.05$], and that physical training was protective against Na^+, K^+-ATPase activity inhibition induced by PTZ [$F(3,64) = 1.14$; $p < 0.05$]. Moreover, statistical analysis showed that administration of PTZ at 60 mg/kg decreased [^{3}H]-glutamate uptake [$F(3,64) = 3.85$; $p < 0.05$)] and that physical training prevented this effect [$F(3,64) = 1.27$; $p < 0.05$].

Correlation analysis of the duration of convulsive episodes with biochemical parameters

Correlation analysis (Pearson's correlation analysis) revealed that the duration of convulsive episodes induced by severe convulsive dose of PTZ (60 mg/kg) did not correlate with TBARS production ($r = 0.418$; $p < 0.303$), protein carbonylation ($r = 0.302$; $p < 0.453$), or DCFH-DA oxidation ($r = 0.450$; $p < 0.263$) (data not shown). On the other hand, Pearson's correlation analysis revealed that the duration of convulsive episodes elicited by this dose of PTZ strongly correlated with free –SH-group oxidation ($r = 0.912$; $p < 0.007$); Na^+, K^+-ATPase activity ($r = 0.844$; $p < 0.05$); and glutamate-uptake inhibition ($r = 0.934$; $p < 0.001$) (Fig. 5). Furthermore, we showed a negative correlation between duration of convulsive episodes after injection of PTZ (60 mg/kg) with free –SH-group oxidation ($r = 0.931$ $p < 0.001$); Na^+, K^+-ATPase activity ($r = 0.812$; $p < 0.05$); and glutamate uptake inhibition ($r = 0.921$; $p < 0.001$) in trained rats.

Discussion

In the current study we have confirmed and extended our previous findings that PTZ elicits behavioral and electrographic seizures and increases reactive species generation (ROS) in vivo (Oliveira et al., 2004; Ribeiro et al., 2005; Fighera et al., 2006), and have shown for the first time that a 6 weeks of swimming training protocol affords significant alteration in the antioxidant status and is effective in attenuating convulsions and neurochemical alterations elicited by PTZ.

Concerns about increased seizure frequency and its potential for injury have led to overprotective measures by many health care professionals (Bjorholt et al., 1990; Arida et al., 2003; Wong & Wirrell, 2006). In this context, the protection exerted by physical training on epileptiform activity and neurochemical alterations induced by PTZ is of particular interest because PTZ-induced seizures are an important model of myoclonic and generalized tonic–clonic seizures, which are utilized in the routine test for screening anticonvulsants (Swinyard et al., 1987) and because it supports the idea that physical training may prevent seizures induced by different agents. In this context, a significant body of evidence has demonstrated that training performed before and during the kindling procedure slows amygdala kindling development (Arida et al., 1998,2007b) and decreases susceptibility (Setkowicz & Mazur, 2006) and frequency of spontaneous recurrent seizures in the pilocarpine model of epilepsy in rats (Arida et al., 1999; 2004). On the other hand, although there is convincing evidence of the positive role of physical exercise in reducing the frequency and severity of seizures in several models of epilepsy (McAuley et al., 2001; Sutoo & Akiyama, 2003; Howard et al., 2004), the mechanism underlying this protective effect has not been clearly investigated.

In the present study, we showed that 20 min after administration of moderate and severe doses of PTZ (45 and 60 mg/kg, i.p.), several parameters that indicate the anti-oxidant status and generation of oxidative stress in cortical homogenates were affected. The occurrence of DCFH-DA oxidation, lipid peroxidation (TBARS), increase in protein carbonyl, decrease in NPS content, and inhibition of catalase and SOD activity after single doses

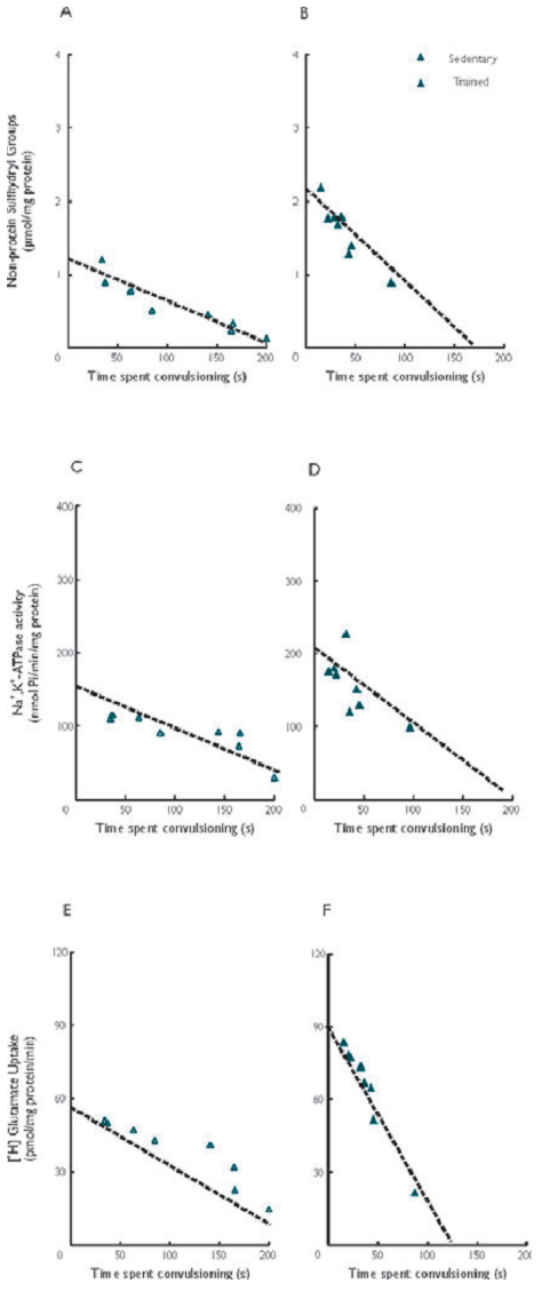

Figure 5.
Duration of convulsive episodes induced by PTZ (60 mg/kg, i.p.) correlates with free –SH-group oxidation (**A**); Na^+, K^+-ATPase activity (**C**); and glutamate-uptake inhibition (**E**) in sedentary group. Pearson's correlation coefficient also showed a negative correlation between duration of convulsive episodes after injection of PTZ (60 mg/kg, i.p.) with free –SH-group oxidation (**B**); Na^+, K^+-ATPase activity; (**D**) and glutamate-uptake inhibition (**F**) in a trained group of rats. Data are individual values for n = 7 in each group.
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of PTZ suggest that epileptic seizures elicited by this convulsant agent were accompanied by increase of oxidative stress. However, the cause–effect relationship between these events is difficult to postulate, since we found no correlation between duration of PTZ-induced convulsion and total protein carbonylation, TBARS production, or DCFH-DA oxidation. If such a cause–effect relationship between convulsions and these neurochemical parameters existed, a significant positive correlation between these variables should be found. Nevertheless, one might also consider the possibility that selected targets, which could not contribute significantly to the content observed, could be responsible for the convulsant action exerted by PTZ. In this context, it has been demonstrated that, in some cases, such as Alzheimer's disease, only selected proteins show increases in the levels of carbonylation (Castegna et al., 2002). With respect to the effects of the subconvulsive dose of PTZ (30 mg/kg, i.p.) in cerebral cortex, the data from the present study suggest that changes in this structure could be related to the specific function of PTZ as a selective blocker of the chloride ionophore complex (Psarropoulou et al., 1994; Walsh et al., 1999) or to cellular thiol homeostasis (Patsoukis et al., 2004) before the appearance of seizures.

On the other hand, the results presented in this report showed that the duration of convulsive episodes elicited by PTZ (60 mg/kg, i.p.) strongly correlated with free –SH-group oxidation; Na^+, K^+-ATPase activity; and glutamate-uptake inhibition, suggesting that convulsive activity and neurochemical parameters are closely linked events. These experimental findings also suggest that the significant correlation observed previously may be related to a thiol redox-affected function of *N*-methy-D-aspartate (NMDA) and $GABA_A$ receptors (Amato et al., 1999; Sanchez et al., 2000). Furthermore, the PTZ-induced excitability proposed in this report is in accordance with previously reported data showing that a single convulsive dose of PTZ results in significant changes in many parameters such $GABA_A$ receptor density and function (Psarropoulou et al., 1994; Walsh et al., 1999), whole brain hydroxyl radicals (Rauca et al., 1999), free fatty acids, and glutathione peroxidase activity in specific brain areas (Eraković et al., 2003).

In the current study, the electrographic and behavioral recordings indicate that physical training had an effect on the generation and duration of generalized seizures induced by PTZ (45 and 60 mg/kg, i.p.). These experimental findings suggest that important changes in the brain induced by physical training (Sutoo & Akiyama, 2003) might affect the susceptibility to seizurogenic stimuli or events followed by spontaneous epileptiform activity elicited by PTZ. In this context, considering that Na^+,K^+-ATPase enzyme plays a pivotal role in cellular ionic gradient maintenance and is particularly sensitive to reactive species (Morel et al., 1998; Petrushanko et al., 2006), we suggest that the maintenance of Na^+, K^+-ATPase activity induced by physical training might protect against enhance neuronal excitability induced by PTZ. In fact, in the present study we showed a strongly negative correlation between duration of PTZ-induced convulsive episodes with free –SH-group oxidation, Na^+, K^+-ATPase activity, and glutamate-uptake inhibition in trained group of rats.

The results presented in this report also showed that administration of a moderate to severe convulsive dose of PTZ (45 and 60 mg/kg, i.p.) inhibited Na^+, K^+-ATPase activity, whereas a severe convulsive dose of PTZ (60 mg/kg, i.p.) inhibited glutamate uptake. Although the exact mechanism through which PTZ reduces glutamate uptake is still unknown, it is tempting to propose that the reduction of glutamate uptake by PTZ could be related directly or indirectly to PTZ-induced oxidative stress. In this line of thinking, oxidation of regulatory sulfhydryl groups in glutamate transporters decrease its activity (Trotti et al., 1997) and a reduction in Na^+,K^+-ATPase activity also decreases glutamate uptake, since it depends on Na^+ gradients across cell membrane. This is particularly important considering that the activity of glutamate transporters can be impaired by several indirect mechanism, including ROS formation and reduction of Na^+,K^+-ATPase activity (Volterra et al., 1994; Nanitsos et al., 2004). However, further in-depth studies are necessary to definitively establish the mechanisms involved.

In the present study, we showed that physical training was effective against PTZ-induced TBARS formation, protein carbonylation, DCFH-DA, and free –SH-group oxidation. In addition, the present protocol of training protected against PTZ- induced inhibition of SOD and CAT activity. These results agree with a substantial body of evidence that suggests adaptive responses to regular and moderate endurance exercise involving an increase of antioxidant defenses, a reduction of basal production of oxidants, and a reduction of radical leak during oxidative phosphorylation (Packer & Cadenas, 2007). Accordingly, a recent study has demonstrated that moderate exercise significantly decreases the age-associated development of oxidative damage in mice, increases lifespan, prevents deterioration of mitochondrial function, and even improves behavioral performance (Navarro et al., 2004). Furthermore, Radak et al. (2006) suggest that exercise-induced production of ROS plays a role in the induction of antioxidants, and DNA repair and protein degrading enzymes, resulting in decreased incidence of oxidative stress.

Considering that regular exercise leads to the development of compensatory responses to oxidative stress (Salo et al., 1991; Viguie et al., 1993; Leeuwenburgh & Heinecke, 2001) and that failure of some selected targets, such as Na^+,K^+-ATPase, may increase cellular excitability and facilitate the appearance or propagation of convulsions (Patel et al., 2004), we suggest that the increase of antioxidant defenses and reduction of basal production of oxidants elicited by this physical training may protect

against Na^+, K^+-ATPase inhibition induced by PTZ. In fact, results presented in this report showed that this protocol of swimming training increased NPS content and SOD activity, protected against ROS generation per se, and was effective against Na^+, K^+-ATPase inhibition elicited by a subthreshold convulsive dose of PTZ (30 mg/kg, i.p.). Accordingly, the negative correlation between the duration of convulsive episodes after injection of PTZ (60 mg/kg, i.p.) with free –SH-group oxidation, Na^+, K^+-ATPase activity, and glutamate-uptake inhibition in a trained group of rats reinforce the assumption that physical training displays antioxidant properties and protects against the epileptiform activity–induced alterations in ROS generation in cerebral cortex.

With respect to the difference in body weight between sedentary and trained rats, we think that such difference may be explained by changes in body composition. For instance, a decrease in subcutaneous adipose tissue of trained rats may explain why body mass was smaller in this group. However, we have not determined body composition in the present study, and, therefore, this explanation remains speculative in nature, and further studies are necessary to determine the mechanisms involved.

In conclusion, the present study reports that PTZ administration induces convulsive behavior following excitotoxic damage in vivo and that previous physical training protects against these deleterious effects. The results showing specific molecular systems modulated by exercise also provide a framework to guide further studies to examine the mechanisms by which exercise alters neuronal functions. Therefore, these experimental findings suggest that physical training may be a new therapeutic approach to control acute and chronic excitotoxicity, including seizure activity.

Acknowledgments

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We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines. In addition, we would like to state that all authors have seen and approved the study and that no part of the work has been published or is under consideration for publication elsewhere. Moreover, the present study was supported by government funding and has no financial or other relationship that might lead to a conflict of interest. We also would like to declare that all experiments were carried out according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996, and that the University Ethics Committee approved the respective protocols.

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MANUSCRITO –ATIVIDADE ANTIOXIDANTE INDUZIDA POR BAIXAS DOSES DE CAFEÍNA ATENUA AS CONVULSÕES E DANO OXIDATIVO INDUZIDOS POR PENTILENOTETRAZOL EM RATOS.

Título Original

Antioxidant activity elicited by low dose of caffeine attenuates pentylenetetrazol-induced seizures and oxidative damage in rats.

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Periódico

Physiology and Behaviour

Antioxidant activity elicited by low dose of caffeine attenuates pentylenetetrazol-induced seizures and oxidative damage in rats.

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Abstract

Although caffeine supplementation has been a documented beneficial effect on neurological disorders, its implications for epilepsy are still controversial. Thus, the aim of this study was to investigate the effects of 2-week caffeine supplementation (6mg/kg; p.o.) on seizures and neurochemical alterations induced by pentylenetetrazol (PTZ 60 mg/kg i.p). Statistical analyses showed that long-term but not acute caffeine administration decreased the duration of PTZ-induced seizures in adult male Wistar rats as recorded by cortical electroencephalographic (EEG) and behavioral analysis. The quantification of EEG recordings also revealed that caffeine supplementation protected against PTZ-induced wave amplitude increase. Neurochemical analyses revealed that caffeine supplementation increased glutathione (GSH) content *per se* and protected against the increase in the levels of thiobarbituric acid reactive substances (TBARS) and oxidized diclorofluoresceine diacetate (DCFH-DA) as well as the decrease in GSH content and Na^+,K^+-ATPase activity induced by PTZ. Our data also showed that the infusion of L-buthionine sulfoximine (BSO; 3.2 µmol/site i.c.v), an inhibitor of GSH synthesis, 2 days before PTZ injection reverted the anticonvulsant effect elicited by caffeine. BSO infusion also decreased GSH content and Na^+,K^+-ATPase activity, however, it increased DCFH-DA oxidation and TBARS *per se* and reverted the protective effect exerted by caffeine. Results presented in this report extend the neuroprotective effects of low long-term caffeine exposure to epileptic damage and suggest that the increase in the cerebral GSH content elicited by caffeine supplementation may provide a new therapeutic approach to the control of the seizure activity.

Key words: Caffeine, seizures, oxidative damage, GSH, Na^+, K^+-ATPase activity, BSO.

1. Introduction

Epilepsy is a neurological disorder characterized by recurrent episodes of seizures due to an imbalance between cerebral excitability and inhibition with a tendency towards uncontrolled excitability [1]. Currently, around 50 million people worldwide have active epilepsy with continuing seizures that need treatment. Despite the increasing number and variety of anti-epileptic drugs, morethan 30% of the cases of epilepsy are medically intractable [2]. In this sense, animal models for seizures and epilepsy have played a key role in advancing our comprehension of ictogenesis basic mechanisms underlying epileptogenesis [3]. More important than understanding the mechanism of seizure is the possibility of devising novel strategies to treat epilepsy, which may also offer some additional insights in key mechanism processes. Other research groups have focused on the study of alternative therapies such as caffeine, which reduce seizures and related brain damage [4, 5].

Caffeine (1,3,7-trimethylxanthine), which belongs to the group of purine alkaloids, is the most commonly and widely ingested psychoactive substance. Caffeine is found in beverages such as coffee, tea, and many soft drinks as well as in chocolate products and desiccated coconut. Structurally, caffeine is similar to adenosine, an endogenous neuromodulator, and binds to adenosine receptor to act as a nonselective antagonist [6]. Experimental and clinical studies have indicated that chronic caffeine supplementation provides neuroprotective effects against several neurological disorders, including Alzheimer's and Parkinson's diseases by the blockade of adenosine receptors [7-10]. On the other hand, caffeine-mediated neuroprotection is not exclusively attributed to the blockade of adenosine receptor.

Caffeine may have other effects against neurodegeneration in the CNS, although the precise entity is still elusive. In addition, it is important to consider that the potential benefits of this compound for patients with convulsive disorders remain poorly defined. While some authors suggest a proconvulsant role of acute caffeine treatment [11, 12], others suggest that caffeine may be an anticonvulsant agent [5, 13, 14]. The basis for this discrepancy is unknown; however, methodological differences may account for the apparent disagreement among findings. Important methodological considerations that may have impacted the results of various studies include the convulsant agents administered, the drug doses studied, the administration routes, the animal model species, and the parameters evaluated.

The brain is one of the major organs that generates large amounts of reactive oxygen species (ROS). Compared with other organs, the brain is especially vulnerable to oxidative stress

because of its lower antioxidant enzyme activities, since it contains high quantities of lipids with unsaturated fatty acids, which are targets of lipid peroxidation [15]. Under normal conditions, the brain can equilibrate the generated ROS with its own antioxidant defense. In this context, glutathione (GSH) is the most abundant thiol-reducing agent that plays a critical role as a major antioxidant in the CNS [16]. In line with this view, a growing number of studies have demonstrated that oxidative stress facilities the appearance and/or propagation of seizures in several models of epilepsy [17, 18]. Accordingly, experimental findings from our research group have demonstrated that the inhibition of some selected target for oxidative stress such as Na^+,K^+-ATPase may lead to neuronal excitability and appearance of convulsion in model of seizure elicited by PTZ [19-21]. This is particularly important considering the fact that this ion-motive pump plays a key role in regulating and controlling nerve excitability [22]. Based on the hypothesis that the selected targets are involved in epilepsy, an alternative approach to the treatment of this disorder would be the use of neuroprotective therapy to prevent or slow down seizure progression.

Over the last years, accumulating evidence has suggested a potential antioxidant role of caffeine [23-26]. The suggestions are largely based on chemical studies showing it to be able to scavenge ROS, particularly the hydroxyl radical ($OH^{\cdot}$), known to be generated in the body by many physiologic reactions involving oxygen utilization [27, 28]. Additionally, caffeine has been shown to prevent Fenton's reaction-induced oxidation of glutathione [27], a major antioxidant reserve in many tissues, including CNS. However, the effect of caffeine against ROS inducing oxidative stress especially in the brain where oxidative stress has been implicated in the genesis and propagation of seizures has not been studied to date. Therefore, since it has been proposed that at least part of the neuroprotective effects of caffeine are due to antioxidant effects [25, 26] and that oxidative stress facilitates the appearance and propagation of seizures in several experimental models of epilepsy [29, 30], we evaluated the effect of caffeine supplementation on PTZ-induced electrographic and neurochemical alterations in cerebral cortex of rats.

2. Materials and Methods

2.1 Animals and reagents

Adult male Wistar rats (270–300 g) were used in the present study. Rats were housed four to a cage. Light and temperature were controlled (12-h light/dark cycle, 22±1 ◦C, 55% relative humidity) and rats had free access to food (Guabi, Santa Maria, Brazil) and water. All experimental protocols were designed to keep the number of animals used to a minimum as well as to keep them from suffering. All experimental protocols were conducted in accordance with national and international legislation (National Council for Control of Animal Experimentation (CONCEA) and of U.S. Public Health Service's Policy on Human Care and Use of Laboratory Animals-PHS Policy), and approved by the Ethics Committee for animal research at the Federal University of Santa Maria. Behavioral tests were conducted during the light phase of the cycle (between 10:00 AM and 4:00 PM). All reagents were purchased from Sigma (St Louis, MO, U.S.A.). Caffeine anhydrous was dissolved in water and L-buthionine sulfoximine (BSO) and PTZ were dissolved in 0.9% physiological saline.

2.2 Study design

The study design is summarized in Figure 1 and consisted of two experiments. The experiments were as follows.

Experiment 1: in order to determine the role of caffeine on the electrographic, and neurochemical alterations in cerebral cortex of rats induced by convulsive dose of PTZ (60 mg/kg, i.p), animals were supplemented with caffeine (6 mg/Kg) [31] or its vehicle (water) by intragastric gavage (p.o.) for 15 days. In the present study, we also evaluated the participation of glutathione pathway on electroencephalographic and neurochemical alterations exerted by caffeine in this model of seizure. For this propose, a subset of animals was supplemented with caffeine (6 mg/Kg) for 15 days. On the days 14 and 15 of caffeine treatment, another subset of animals received intracerebroventricular infusion of L-buthionine sulfoximine (BSO; 3.2 µmol/site i.c.v), an inhibitor of GSH synthesis .Twenty four hours after the last administration of BSO, animals were injected with a convulsive dose of PTZ (60 mg/kg, i.p) as described in Figure 1.

Experiment 2: To evaluate if the acute caffeine administration protects against PTZ-induced electrographic seizures and neurochemical alterations, a subset of animals was treated with caffeine (6 mg/Kg) 60 min before the injection of a convulsive dose of PTZ (60 mg/kg, i.p). After a 20 min seizure evaluation, animals were killed and parietal cortex removed for biochemical analyses.

2.3 Surgical procedure

For the electroencephalographic recordings (EEG), all animals were subjected to surgery. In brief, animals were anesthetized with Equithesin (1% phenobarbital, 2% magnesium sulfate, 4% chloral hydrate, 42% propylene glycol, 11% ethanol; 3 ml/kg, i.p.) and placed in a rodent stereotaxic apparatus. For the EEG recordings, two screw electrodes were placed bilaterally over the parietal cortex along with a ground lead positioned over the nasal sinus. The electrodes were connected to a multipin socket fixed to the skull with acrylic cement. For intracerebroventricular infusion of BSO (3.2 µmol/site i.c.v), a cannula was positioned on the right ventricle (coordinates relative to bregma: AP 0 mm; ML 1.5 mm; 2.5 mm from the dura) [32]. Ceftriaxone (200 mg/kg, i.p.) was administered immediately before the surgical procedure. The behavioral and EEG evaluation were performed 4 days after surgery.

2.4 Seizure evaluation

Seizures were monitored in all animals by EEG recording. On the day of the experiments, each animal was transferred to an acrylic glass cage (25 X 25 X 40 cm) and allowed to adapt for 20 min before EEG recording. The rat was then connected to the lead socket in a swivel inside a Faraday's cage, and the EEG was recorded using a digital encephalographer (Neuromap EQSA260, Neuromap LTDA, Itajaú, MG, Brazil). EEG signals were amplified, filtered (0.1–70.0 Hz, band pass), digitalized (sampling rate 256 Hz), and stored in a personal computer for off-line analysis. Routinely, a 10 min baseline recording was obtained to establish an adequate control period. After baseline recording animals received an injection of saline (0.9% NaCl, 1 ml/kg, i.p.) or PTZ (60 mg/kg, i.p.). The animals were observed for the appearance of clonic and generalized tonic–clonic convulsive episodes for 20 min according to [33], who describes clonic convulsions as episodes characterized by typical partial clonic activity affecting the face, head, vibrissae, and forelimbs. Generalized

convulsive episodes were considered as generalized whole-body clonus involving all four limbs and tail, rearing, and wild running and jumping, followed by sudden loss of upright posture and autonomic signs, such as hypersalivation and defecation, respectively. During the 20min observation period, latencies for the first clonic and generalized tonic–clonic convulsions were measured. EEG recordings were visually analyzed for seizure activity, which were defined by the occurrence of the following alterations in the recording leads [34]: isolated sharp waves (≥ 1.5 x baseline); multiple sharp waves (≥ 2x baseline) in brief spindle episodes (≥ 1s ≥ 5 s); multiple sharp waves (≥ 2x baseline) in long spindle episodes (≥ 5 s); spikes (≥ 2 x baseline) plus slow waves; multispikes (≥ 2 x baseline, ≥3 spikes/complex) plus slow waves; and major seizure (repetitive spikes plus slow waves obliterating background rhythm, ≥ 5 s). For quantitative analysis of EEG amplitude, we averaged EEG amplitude over the 20min of observation.

2.5 Sample processing

Immediately after the seizure evaluation period, animals were killed by decapitation and their brains were exposed by the removal of the parietal bone. The cerebral cortex was quickly dissected on an inverted ice-cold Petri dish and the material was stored at -80^{o} C for subsequent biochemical analyses. Samples were prepared according to the guidelines for each technique, as described below.

2.6 Measurement of TBARS content

Thiobarbituric Acid Reactive Substances (TBARS) content was estimated in a medium containing 0.2 ml of cortex homogenate, 0.1 ml of 8.1% SDS, 0.4 ml of acetic acid buffer (500 mM, pH 3.4), and 0.75 ml of 0.81% (TBA). The mixture was finally made up to 2 ml with type I ultrapure water and heated at 95 °C for 90 min in a water bath using a glass ball as a condenser. After cooling to room temperature, absorbance was measured in the supernatant at 532 nm [35].

2.7 Isolation of rat brain mitochondria for Oxidized Diclorofluoresceine (DCFH) Level Determination

Rat cortex mitochondria were isolated as described by [36] with some modifications. Firstly, the cerebral cortex was quickly removed from the rat skull and homogenized in a buffer containing (in mM): 100 sucrose, 10 EDTA, 100 Tris-HCl, and 46 KCl (pH 7.4). After homogenization, the resulting suspension was centrifuged for 3 min at 2,000 g (4° C) to obtain a low speed supernatant fraction (S1). S1 was centrifuged for 20 min at 12,000 g (4° C). The pellet was re-suspended in a buffer containing (in mM): 100 sucrose, 10 EDTA, 100 Tris-HCl, 46 KCl and bovine serum albumin (BSA, 0.5%; pH 7.4) and re-centrifuged for 10 min at 12,000 g (4° C). The supernatant was decanted and the final pellet re-suspended in a buffer containing (in mM): 70 sucrose, 0.02 EDTA, 20 Tris-HCl, 230 mannitol, 1 K_2HPO_4, to yield a protein concentration of 30-40 mg/mL.

2.8 Oxidized Diclorofluoresceine (DCFH) Level Determination

The levels of DCFH were determined as an index of the peroxide production by the cellular components. This experimental method of analysis is based on the deacetylation of the probe DCFH-DA and its sub-sequent oxidation by reactive species to DCFH, a highly fluorescent compound [37]. Fractions of cortex mitochondria (350 µg/µl) were added to a medium containing buffer III and DCFH-DA (1 mM). After DCFH-DA addition, the fluorescence measurement procedure started (excitation at 488 nm and emission at 525 nm, and both slit widths used were at 1.5 nm). DCFH oxidation was determined using a standard curve of DCF and results were corrected by the protein content.

2.9 GSH levels

The levels of GSH were determined fluorometrically as described by [38], using 0-phthaladehyde (OPA) as fluorophore. Briefly, cortex was homogenized in 0.1 M $HClO_4$. Homogenates were centrifuged at 2500 g for 10 min and the low-speed supernatants were separated for measurement of GSH. Supernatant (100 µl) was incubated with 100 µl of OPA (0.1% in methanol) and 1.8 ml of 0.1 M phosphate buffer (pH 8.0) for 15 min at room temperature in the dark. Fluorescence was measured with a fluorescence spectrophotometer at excitation wavelength of 350 nm and at emission wavelength of 420 nm. GSH levels were expressed as nmol GSH/g of tissue.

2.10 Na^+,K^+-ATPase activity measurements

Assay of Na^+,K^+-ATPase activity was performed according to Wise [39]. Briefly, the reaction medium consisted of 30 mM Tris–HCl buffer (pH 7.4), 0.1 mM EDTA, 50 mM NaCl, 5 mM KCl, 6 mM MgCl2, and 50 µg of protein in the presence or absence of the Na^+,K^+-ATPase inhibitor ouabain (1 mM), in a final volume of 320 µL.

The reaction was started by the addition of adenosine triphosphate (ATP) to a final concentration of 5 mM. After 30 min at 37ºC, the reaction was stopped by the addition of 70 µL of trichloroacetic acid (TCA, 50%). Saturating substrate concentrations were used and the reaction was linear with protein and time. The amount of inorganic phosphate released was quantified by the colorimetric method described by Fiske and Subbarow [40]. The Na^+,K^+-ATPase activity was calculated by subtracting the ouabain-sensitive activity from the overall activity (in the absence of ouabain).

2.11 Protein determination

Protein content was measured colorimetrically by the method of [41] using bovine serum albumin (1 mg/mL) as standard.

2.12 Statistical analysis

Data from *ex-vivo* TBARS, GSH levels, DCFH and Na^+,K^+-ATPase activity determinations were analyzed by three-way ANOVA (analysis of variance) and were expressed as mean ± S.E.M. Latency to first clonic and generalized tonic-clonic seizures were analyzed by Scheirer-Ray-Hare test and expressed as median ± interquartile range. A probability of $p< 0.05$ was considered significant.

3. Results

Figure 2 shows the effect of a two week caffeine supplementation (6 mg/kg) on behavioral seizures induced by PTZ (60 mg/kg). Statistical analyses revealed that the caffeine treatment did not alter the latency periods for the first myoclonic jerk [U=24; p>0.05 Fig. 2A] or first generalized tonic-clonic seizures [U=24; p>0.05; Fig. 2B]. However, it decreased the time spent in generalized tonic-clonic seizure [U=10; p<0.05; Fig. 2C] induced by the

convulsive dose of PTZ. The behavior repertoire observed after PTZ injection occurred concomitantly with electrographically recorded seizures: myoclonic jerks were characterized by multiple sharp waves in brief spindle episodes, whereas generalized seizures were characterized by the appearance of 2–3 Hz high-amplitude activity (Fig.4A-B, E and F). The quantification of electroencephalographic wave amplitude revealed that all groups increased EEG amplitude after PTZ administration [$F(1,13)=23,93$; $p< 0.05$, Fig. 2J]. However, caffeine atenuates the increase in wave amplitude after the injection of PTZ (60 mg/kg; i.p.) [$F(1,13)=5,98$; $p< 0.05$].

Considering that the oxidative stress facilitates the appearance and/or propagation of seizures in several models of epilepsy [19, 20] and that caffeine has been shown antioxidant effects [25, 26], we decided to investigate the effects of caffeine supplementation on oxidative stress induced by PTZ, characterized here by DCFH-DA oxidation, TBARS content, and GSH levels in cerebral cortex of rats. The results presented in this report revealed that caffeine supplementation increased GSH content [$F(1,27)=5.54$; $p< 0.05$; Fig. 3A] *per se* and protected against PTZ-induced GSH decrease [$F(1,27)=5.54$;$p< 0.05$; Fig. 3A]. In addition, statistical analyses revealed that caffeine supplementation prevented against PTZ-induced DCFH-DA oxidation [$F(1,27)=4.28$; $p< 0.05$ Fig. 3B] and TBARS content increase [$F(1,27)=4.56$; $p<0.05$ Fig. 3C]. The caffeine supplementation also protected against PTZ-induced Na^+,K^+-ATPase activity inhibition [$F(1,27)=8.76$; $p< 0.01$]. These experimental data suggest that alterations in the redox state of regulatory sulfhydryl groups in selected targets such as Na^+,K^+-ATPase activity [42] increased cellular excitability and that chronic administration of caffeine prevented such an effect.

In the present study, we also evaluated the participation of glutathione pathway on electroencephalographic and neurochemical alterations exerted by caffeine in this model of seizure. Behavioral and EEG recordings revealed that caffeine supplementation decreased the time spent in generalized tonic-clonic seizures induced by PTZ ($U= 8,15$; $p< 0.05$, Fig. 4E) and that infusion of BSO (3.2 µmol/5 µl i.c.v) 2 days before PTZ injection reverted the anticonvulsant effect elicited by caffeine ($U=8,15$ $p<0,05$, Fig. 4E). The quantification of electroencephalographic wave amplitude revealed that the infusion of BSO altered the effect exerted by caffeine supplementation characterized here by EEG wave amplitude increase after PTZ injection ($F(1,26)=4.52$; $p<0.05$ Fig. 4L). Neurochemical analyses also revealed that the BSO (3.2 µmol/5µli.c.v) infusion decreased GSH content [$F(1,54)=26.73$; $p< 0.01$ Fig. 5A] and Na^+,K^+-ATPase activity[$F(1,54)=9.15$; $p< 0.01$ Fig. 5D], whereas DCFH-DA oxidation [$F(1,54)=5.18$; $p< 0.01$] and TBARS content *per se* [$F(1,54)=18,85$; $p< 0.01$ Fig. 5D] were

increased . In addition, BSO reverted the protective effect exerted by caffeine against PTZ-induced GSH decrease [F(1,54)=11.54; $p< 0.05$], Na^+,K^+-ATPase activity inhibition [F(1,54)=13.43; $p< 0.05$], as well as DCFH-DA oxidation[(1,54)=13.03; $p< 0.05$] and TBARS increase [F(1,54)=16.93; $p< 0,05$].

In the present study we investigated the role of the acute caffeine administration on PTZ-induced electrographic seizures as well as neurochemical alterations. Figure 6 shows that the acute caffeine administration one hour before PTZ administration (6 mg/kg) had no effect on the latency periods for the first myoclonic jerk [U=10.5; $p>0.05$], the first generalized tonic-clonic seizure [U=9.5; p>0.05] or the time spent in generalized tonic-clonic seizure [U=11; $p>0.05$]. Behavioral seizures were accompanied by EEG recording observed after PTZ injection. EEG recordings revealed that PTZ treatment increase EEG amplitude [F(1,11)=21,48; $p<0.05$] the acute caffeine administration had no effect on wave amplitude increase elicited by PTZ (Figure 6J). Accordingly, neurochemical analyses revealed that the acute caffeine administration did not protect against the increase of DCFH-DA oxidation [F(1,25)=0.14; $p> 0.05$ Fig. 7B], TBARS content [F(1,25)=0.05; $p> 0.05$, Fig. 7C], as well as Na^+,K^+-ATPase activity inhibition [F(1,25)=0.38;$p> 0.05$ Fig. 7D] and GSH level decrease [F(1,25)=0.46; $p> 0.05$, Fig. 7A] induced by the injection of convulsive dose of PTZ.

4. Discussion

In the present study, we confirmed and extended our previous findings that PTZ elicits behavioral seizures, electrographic seizures, and oxidative stress [19, 20]. The idea that the selected target dysfunction may play a critical role in oxidative stress during seizure onset [20, 43, 44] was supported by our findings of decreased Na^+, K^+-ATPase activity, GSH levels, increased lipid peroxidation, and DCFH-DA oxidation in cerebral cortex of rats after PTZ-induced seizures.

The results presented in this report also revealed that long-term caffeine administration (6 mg/kg) attenuates EEG alterations and decreases generalized tonic-clonic seizures induced by PTZ. Furthermore, our data reveled that caffeine supplementation increases GSH content *per se* and the infusion of BSO, an inhibitor of GSH synthesis, and reverts the protective effect of caffeine against toxicity elicited by PTZ characterized here by EEG seizures, Na+, K+-ATPase activity inhibition, GSH decrease, increased lipid peroxidation, and DCFH-DA oxidation. The results presented in this report also showed that

the acute caffeine administration (6 mg/kg) had no effect on seizures and did not protect against the increase of oxidative stress and Na^+, K^+-ATPase activity inhibition induced by injection of convulsive dose of PTZ. These experimental data reinforce the idea that adaptive long-lasting neurochemical and behavioral responses are usually different from the acute drug effect [45-47]. Furthermore, the protection exerted by caffeine supplementation on the epileptic activity and neurochemical alterations induced by PTZ is of particular interest because PTZ-induced seizure is an important model of myoclonic and generalized tonic–clonic seizures, which is used in the routine test for screening anticonvulsants [48].

Caffeine is one of the most favorable psycho stimulant in beverages or foods for motor activation, mood chances, information processing, and cognitive/performances [31]. Considering that caffeine is structurally similar to adenosine, an endogenous inhibitory neuromodulator, most of the studies have suggested that caffeine has neuroprotective effects as an adenosine receptor antagonist [49-51]. Although epidemiological studies have indicated that caffeine consumption is negatively correlated with the incidence of some neurological diseases [8, 52, 53], anecdotally, caffeinated beverages are “known” to lower seizure thresholds in patients with epilepsy [54]. However, due to the lack of well-designed, randomized, and placebo-controlled clinical trials, this concept has been challenged [55]. While clinical trials have demonstrated that higher doses of antagonist of adenosine receptor ($A1_R$) rolofylline induce seizures in patients with renal failure [56], another study with 116,363 women revealed that caffeine ingestion was not associated with an increased risk of epilepsy [57]. In this context, the understanding of the mechanisms involved in the caffeine-related control of seizure is important since caffeine holds the second position in consumption among all beverages followed by water, and people from all over the world consume approximately 500 billion cupsof coffee annually [58].

In experimental animals the caffeine administration at dose of 0.3 g/L per day over a period of two weeks (resulting in plasma levels of caffeine in the range of 6 to 14μM corresponding to chronic caffeine in human) reduced NMDA, bicucculine and PTZ-induced seizures in mice in the absence of changes in A_1, A_{2A}, or $GABA_A$ receptors [59]. Considering that chronic but not acute caffeine administration attenuates EEG seizures elicited by PTZ, we suggest that another mechanism underlies caffeine’s ability to induce anticonvulsant effect. In this context, more recent observations have demonstrated that caffeine also acts as an antioxidant [60]. This idea is largely based on chemical studies showing it to be able to scavenge ROS, particularly the hydroxyl radical (OH•) and ambient with physiologic reactions involving oxygen utilization [28, 61]. The interaction of OH• with caffeine results in

its oxidative de-methylation generating partially N-methylated xanthines such as theobromine, paraxanthine, and theophylline [62, 63]. In addition, studies have shown that the antioxidant effect of caffeine is similar to that of glutathione and higher than that of ascorbic acid [28]. This antioxidant effect protects humans against health disorders associated with ROS generation such as Alzheimer's and Parkinson's diseases [64, 65].

In the present study the occurrence of DCFH-DA oxidation, TBARS increase, decrease in GSH content, and Na^+,K^+-ATPase activity inhibition after PTZ injection suggests that epileptic seizures elicited by this convulsant agent were accompanied by an increase of oxidative stress. In addition, the increase of ROS production attacks the unsaturated bonds of membrane fatty acids leading to an autocatalytic process called membrane lipid peroxidation, which may impair the function of several membrane transport proteins including Na^+,K^+-ATP-ase [66].Thus, the alteration in the redox state of regulatory sulfhydryl groups in selected targets such as Na^+,K^+-ATPase activity might increase cellular excitability[67]. Accordingly, considering that GSH is the major determinant of the cellular redox state protecting SH-groups of proteins from oxidation and restoring Na^+,K^+-ATPase activity [68, 69], the failure of Na^+, K^+-ATPase activity caused by the infusion of the inhibitor of GSH synthesis (BSO) in this report may be due to the weak action of the antioxidant on the membrane enzyme. In fact, the depletion of GSH results in the inhibition of the Na^+,K^+-ATPase activity [70] and increase of lipid peroxidation in models of seizures induced by PTZ (Kumar and Gandhimathi, 2010). Furthermore, it has been demonstrated that the intracerebroventricularly administered GSH inhibited PTZ induced convulsions in mice [71] and protected against seizure episodes induced by diphenyl diselenide in rat pups by reducing oxidative stress [72].

In line with this view, results presented in this report also revealed that caffeine supplementation increased GSH content *per se*. Considering that caffeine supplementation leads to the development of compensatory responses to oxidative stress induced by experimental model of Alzheimer's and Parkinson's diseases [64]; [73] and GSH protects against free radical-induced Na^+,K^+-ATPase inhibition [74], we suggest that the increase of antioxidant defenses (GSH) in this protocol of caffeine supplementation may protect against Na^+,K^+-ATPase inhibition induced by PTZ. In fact, the infusion of BSO (an GSH inhibitor synthesis) decreased GSH content, Na^+,K^+-ATPase activity and increased DCFH-DA oxidation *per se* as well as reverted the protective effect exerted by caffeine against PTZ-induced EEG seizures.

In conclusion, the present study reports that PTZ administration induces convulsive behavior following excitotoxic damage *in vivo* and that caffeine supplementation protects

against these deleterious effects. The results showing specific molecular systems modulated by caffeine also provide a framework to guide further studies to examine the mechanisms by which this compound alters neuronal functions. Therefore, although further studies are necessary to determine the mechanisms involved in this protective action exerted by caffeine, these experimental findings suggest that the administration of low doses of caffeine may be a new therapeutic approach to control acute and chronic excitotoxicity including seizure activity.

5. References

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Figure Legends

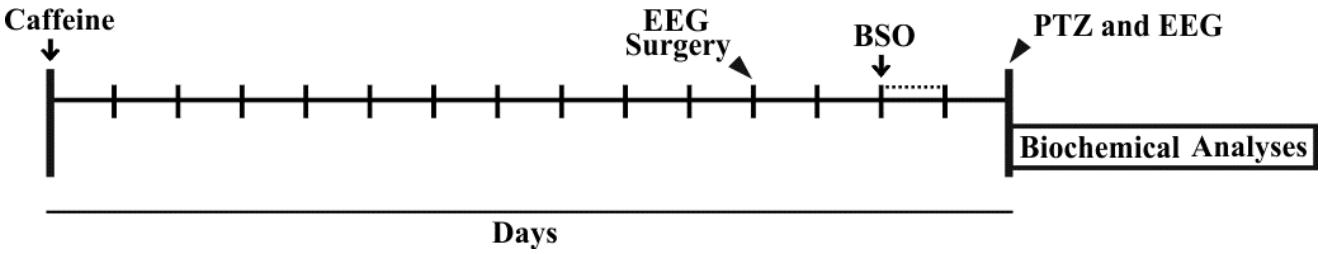

Figure 1. Representation of experimental desigin. The animals were treated wifh caffeine (6 mg/Kg p.o) during fifteen days. On the twenth day, the animal were submitted to a surgery for electrodes and/or cannula implantments. In the experiment with BSO, animal received an i.c.v infusion (3.2 umol/5 ul) on fourteenth and fifteenth day. On the sixteenth day animals were connected to EEG and injected with PTZ (60 mg/Kg i.p) or saline 0.9% and twenty minutes after were killed to biochemical analyses.

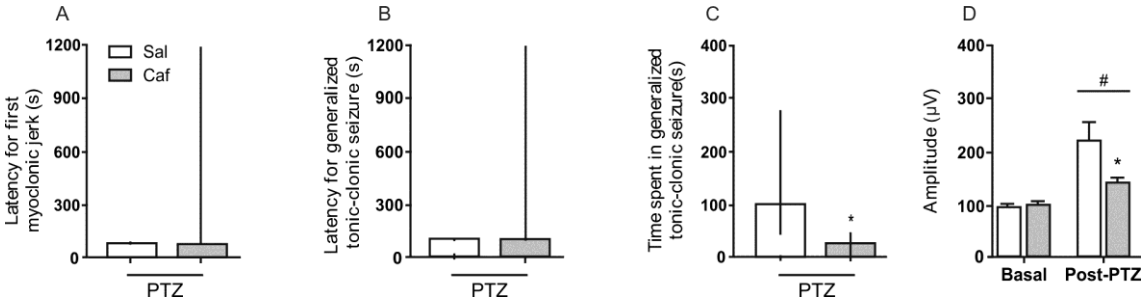

Figure 2. Effect of long-term caffeine administration (6 mg/kg, p.o.) on the convulsive behavior induced by PTZ (60 mg/kg, i.p.). (A) Latency for first clonic seizure; (B) latency for generalized tonic-clonic seizure; (C) time spent in generalized tonic-clonic seizure ; (D) wave amplitude quantification. Data are presented as median and interquartile range Mann-Whitney test (A-C) and data are presented as the mean ± S.E.M One-Way Anova * $p< 0.05$ compared with PTZ-treated group, # $p< 0.05$ compared with basal period (n= 7-8).

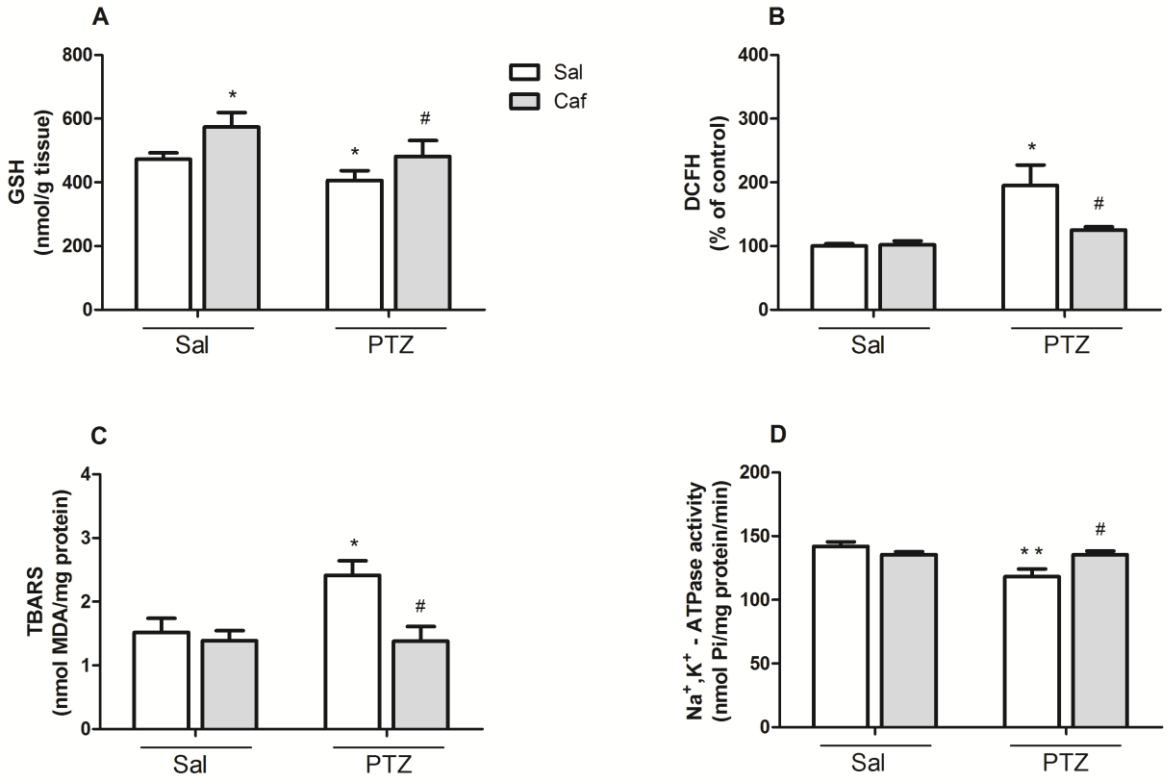

Figure 3. Effect of cronic caffeine administration (6 mg/kg, p.o.) on the oxidative damage induced by PTZ (60 mg/kg, i.p.). The effect of caffeine and PTZ on GSH content (A), DCFH oxidation (B), TBARS content (C) and Na^+, K^+, ATPase activity. Data are presented as the mean ± S.E.M Two-Way Anova * $p<0.05$ compared with vehicle-treated group, ** $p< 0.01$ compared with vehicle-treated group, # $p<0.05$ compared with PTZ-treated group (n= 7-8).

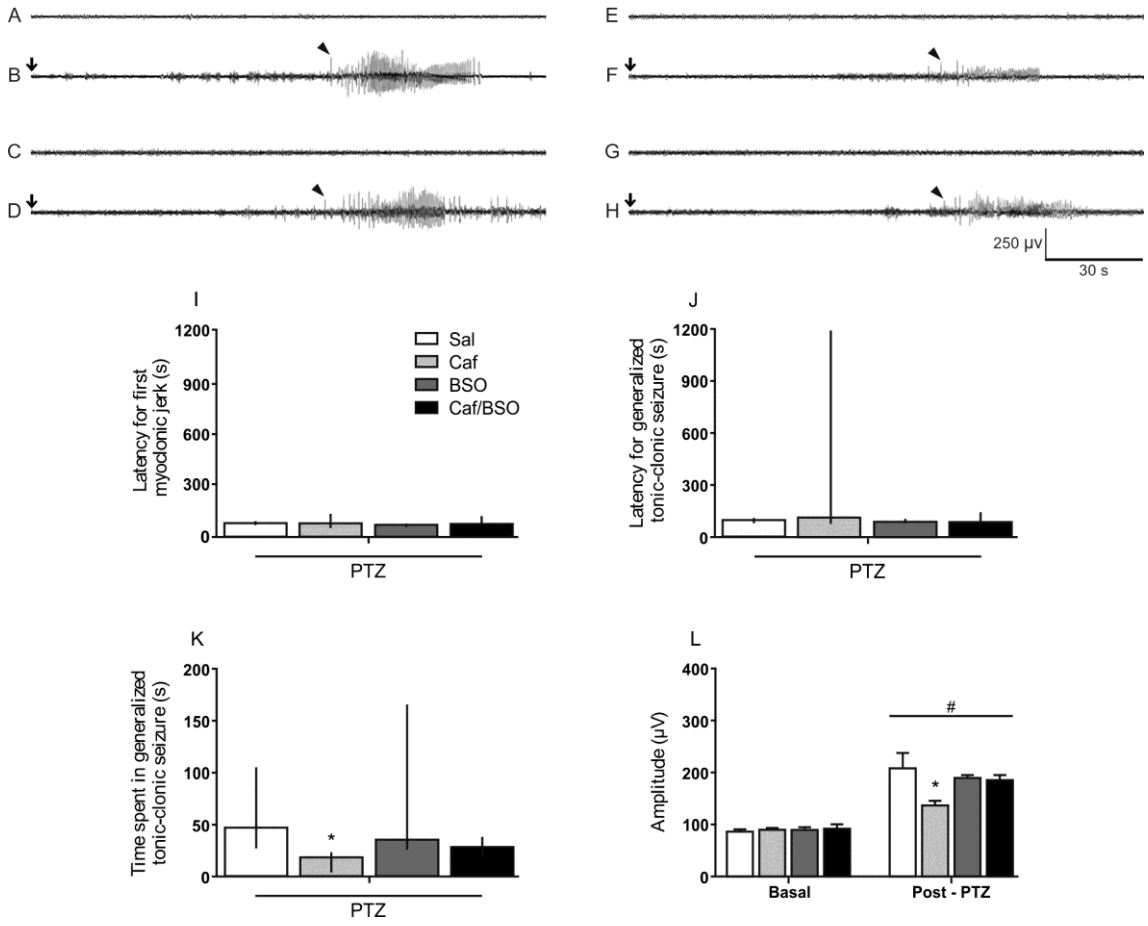

Figure 4. Effect of BSO on the neuroprotective effect of caffeine against behavioral and electroencephalographic seizures induced by PTZ (60 mg/kg, i.p.). Representative electroencephalographic recordings of animals treated with Vehicle (A-B), BSO (C-D) caffeine (E-F) and caffeine plus BSO (G-H) after PTZ injection (B – D - F and H). Arrows indicate PTZ injection; arrowheads indicate the first clonic seizure. Data from (I) Latency for first clonic seizure; (J) latency for generalized tonic-clonic seizure; (K) time spent in generalized tonic-clonic seizure are presented as median and interquartile range. Data from (L) wave amplitude quantification are presented as the mean ± S.E.M. * $p<0.05$ compared with PTZ-treated group # $p<0.05$ compared with basal period (Scheirer-ray-hare and Three-way Anova test, n=7=8).

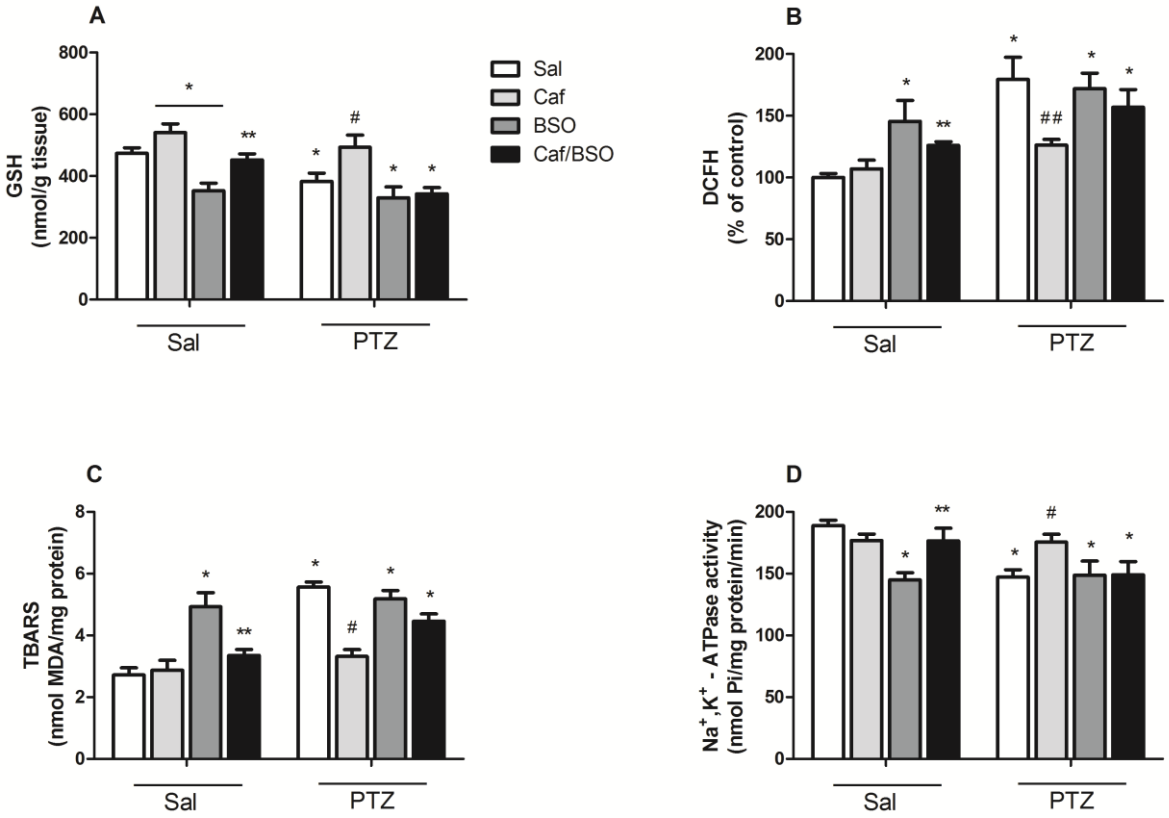

Figure 5. Effect of BSO on the neuroprotective effect of long-term caffeine (6 mg/Kg p.o) against oxidative damage induced by PTZ (60 mg/kg, i.p.). The effect of caffeine and PTZ on GSH content (A), DCFH oxidation (B), TBARS content (C) and Na^{+}, K^{+}, ATPase activity. Data are presented as the mean ± S.E.M Three-Way Anova * $p< 0.05$ compared with vehicle treated group ** $p<0.05$ compared with BSO treated group, # $p<0.05$ compared with PTZ-treated group (n= 6-8).

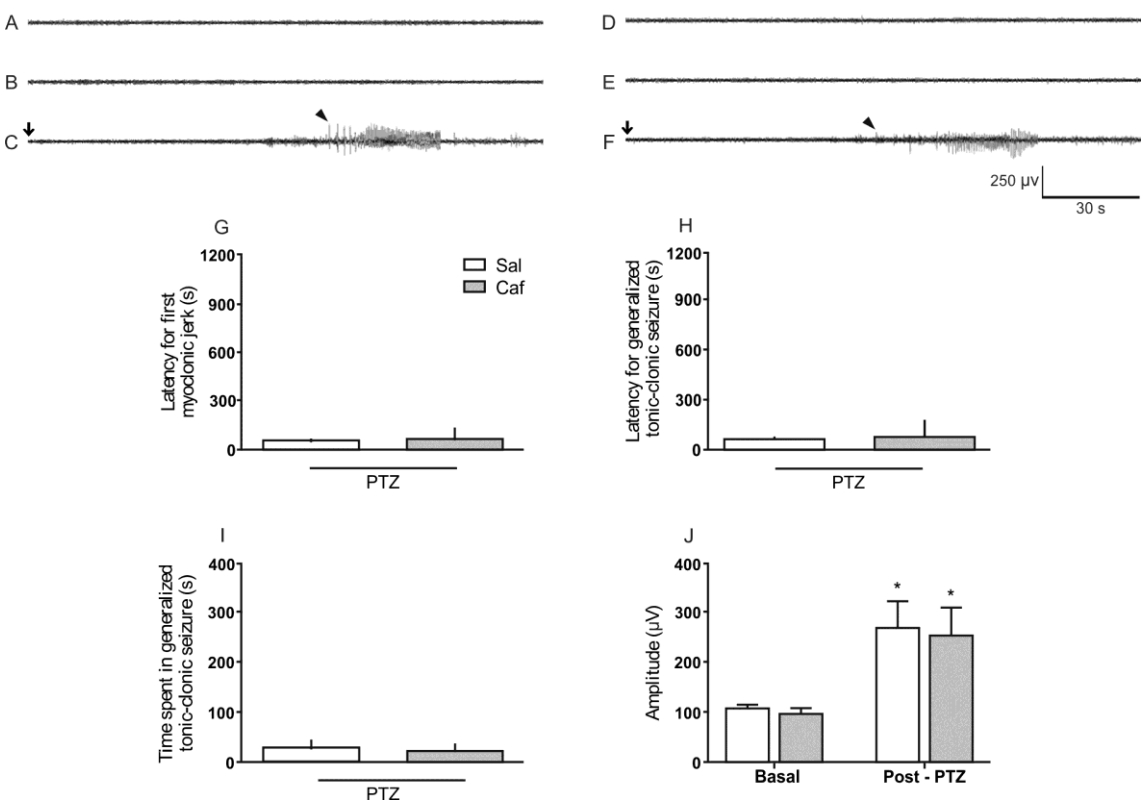

Figure 6. Effect of acute caffeine administration (6 mg/kg, p.o.) against behavioral and electroencephalographic seizures induced by PTZ (60 mg/kg, i.p.). Representative electroencephalographic recordings of animals treated with Vehicle (A-C) or caffeine after PTZ injection (C and F), (A and D) basal period (B and E) vehicle or caffeine treatment before PTZ administration. Arrows indicate PTZ injection; arrowheads indicate the first clonic seizure. Data from (G) Latency for first clonic seizure; (H) latency for generalized tonic-clonic seizure; (I) time spent in generalized tonic-clonic seizure are presented as median and interquartile range. Data from (J) wave amplitude quantification are presented as the mean ± S.E.M. * $p<0.05$ compared with PTZ-treated group # $p<0.05$ compared with caffeine-treated group (Mann-Whitney test and One-way Anova test, n=7=8).

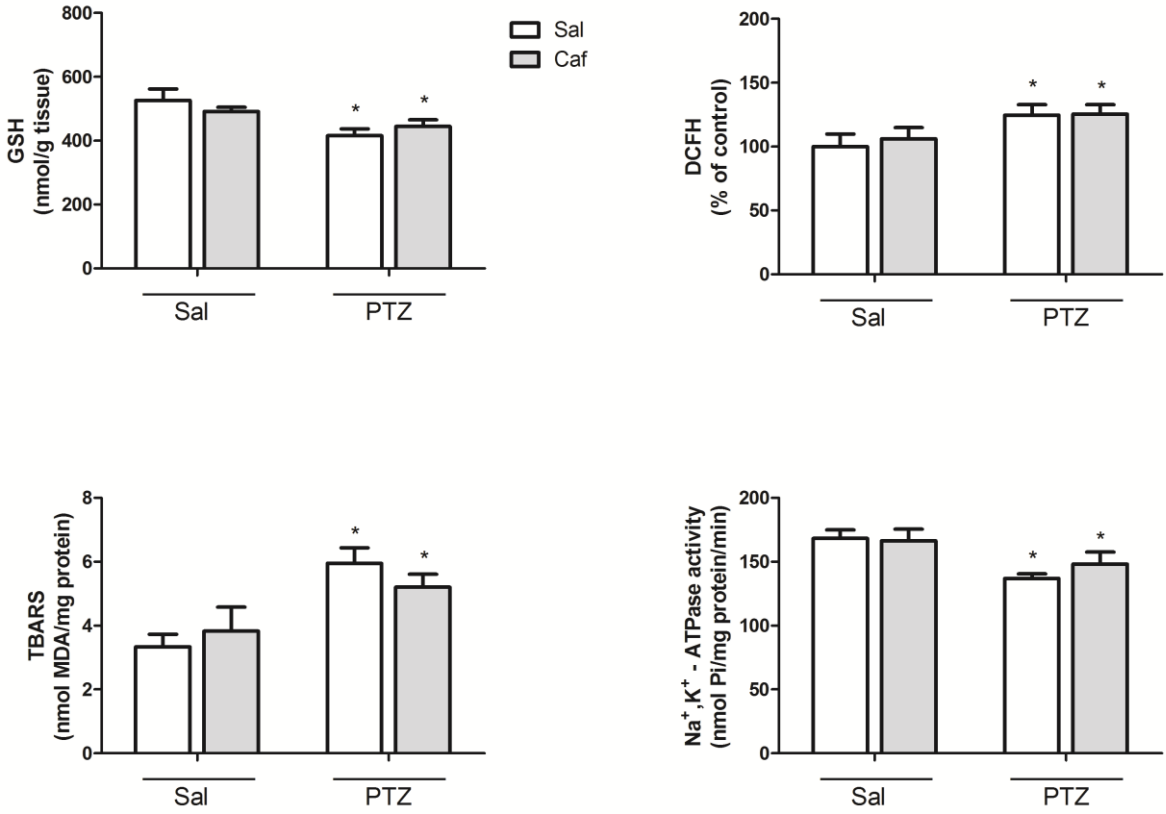

Figure 7. Effect of acute caffeine administration (6 mg/kg, p.o.) on the oxidative damage induced by PTZ (60 mg/kg, i.p.). The effect of caffeine and PTZ on GSH content (A), DCFH oxidation (B), TBARS content (C) and Na^+, K^+, ATPase activity. Data are presented as the mean ± S.E.M Two-Way Anova * $p<0.05$ compared with vehicle-treated group (n= 7-8).

MANUSCRITO EM PREPARACÃO

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Introdução

Estudos na área de epilepsia e modelos experimentais de convulsões em animais têm desempenhado um papel fundamental no avanço da compreensão dos mecanismos básicos de ictogênese e epileptogênese (Loscher, 2011). Dessa forma, esses estudos têm sido fundamentais para a descoberta e desenvolvimento de novas terapias antiepilépticas (Loscher, 2011). Durante as últimas décadas, foram desenvolvidos diversos modelos experimentais de convulsões (Purpura and Shofer, 1972, Loscher et al., 1999), como por exemplo, o modelo de convulsão induzido por pentilenotetrazol (PTZ) (Loscher and Schmidt, 1988, Loscher et al., 1999, White et al., 2008).

Muitos estudos sugerem o envolvimento de uma cascata de eventos biológicos, incluindo a geração de espécies reativas de oxigênio (EROs), subjacentes ao desenvolvimento e propagação da epilepsia e convulsões induzidas por PTZ (Shin et al., 2011, Azam et al., 2012).

Nesse contexto, o SNC apresenta vários mecanismos na tentativa de reduzir os potenciais efeitos fisiopatológicos da EROs, entre eles,o aumento na expressão de genes das enzimas antioxidantes. Dentre esses genes, alguns são expressos regularmente, enquanto outros são induzidos em resposta ao aumento do estresse oxidativo (Ishii et al., 2000).

O elemento de ligação de resposta do AMPc (CREB) é um fator de transcrição que parece ser essencial para o desenvolvimento normal do CNS e na regulação da detoxificação de EROs. Alguns estudos mostram que o CREB regula uma classe de genes de antioxidantes através da expressão de um fator nuclear (PGC-1α) (Herzig et al., 2001, Lee et al., 2005, St-Pierre et al., 2006), que está envolvido na expressão de enzimas antioxidantes, como a GPx1 e SOD2 (St-Pierre et al., 2006).

Além disso, pesquisas mostram que alguns fatores neurotróficos, como o fator neurotrófico derivado do cérebro (BDNF), ativam o CREB (Finkbeiner et al., 1997). O BDNF esta implicado na sobrevivência e diferenciação celular, assim como, no aumento da resistência ao estresse oxidativo (Guo and Mattson, 2000, Klumpp et al., 2006, Boutahar et al., 2010). De fato, a ativação da via BDNF/CREB protege das convulsões, aumento dos níveis de EROs e da redução na expressão de PGC-1a (Lee et al., 2009).

No entanto, ensaios clínicos prévios revelaram inúmeros efeitos colaterais das neurotrofinas, bem como, a sua pobre penetração na barreira hemato-encefálica, dificultando o uso terapêutico destas proteínas (Allen and Dawbarn, 2006). Portanto, muitos estudos estão

sendo realizados para encontrar um agente com propriedades terapêuticas que possa agir na via BNDF/CREB (Hooper and Scott, 2005, Choi et al., 2011).

O exercício físico é uma intervenção de baixo custo, não invasiva com vários resultados benéficos, incluindo o aumento de respostas anti-inflamatórias e antioxidantes (Linke et al., 2005), e o aumento no conteúdo de fatores neurotróficos, especialmente o BDNF (Radak et al., 2006, Dietrich et al., 2008). Além disso, o exercício físico protege das crises epilépticas em pacientes (REF) e em diferentes modelos de convulsões (Arida et al., 2009), assim como do dano oxidativo induzido por PTZ (Rambo et al., 2009).

A cafeína (1,3,7-trimethylxanthine), que pertence ao grupo dos alcalóides, tem efeito protetor em diferentes modelos de doenças neurológicas, como por exemplo, a epilepsia (Chen et al., 2001, Arendash et al., 2006, Dall'Igna et al., 2007). Neste contexto, foi observado que a suplementação de cafeína, em baixas doses, atenua as convulsões e o estresse oxidativo induzidos pelo PTZ, assim como, aumenta os níveis de BDNF e CREB (El Yacoubi et al., 2011, Porciuncula et al., 2012).

Considerando que a atividade física e a cafeína exerceram um efeito protetor nas convulsões e dano oxidativo induzido por PTZ (artigos 1 e 2, respectivamente), e que a via BDNF/CREB/PGC1-a pode ser um importante mecanismo de regulação das EROs, decidimos avaliar os efeitos do exercício físico associado a suplementação com cafeína na expressão de fatores de transcrição responsáveis pela regulação das enzimas detoxificantes de EROs.

Objetivos:

Avaliar o efeito do exercício físico e da suplementação com cafeína:

1. Nas convulsões comportamentais e eletroencefalográficas induzidas por PTZ
2. Na expressão de BDNF, CREB e PGC1-α
3. No conteúdo de GSH e atividade da GPx
4. Na atividade e expressão da SOD
5. No conteúdo de TBARS e carbonilação proteica

Materiais e Métodos

Animais e reagentes

Foram utilizados ratos Wistar machos fornecidos pelo Biotério Central da UFSM, com peso variando entre 270 – 300g, mantidos em ciclo claro-escuro de 12 horas a temperatura de 22 ± 1°C, com alimento e água *ad libitum.* Todos os protocolos serão submetidos à avaliação pelo comitê de ética da Universidade Federal de Santa Maria. O número de animais utilizados será o mínimo possível de modo a fornecer efeitos consistentes de nossos resultados. Os procedimentos experimentais foram realizadas durante a fase clara do ciclo (9:00 - 16:00). Todos os reagentes foram adquiridos da Sigma (St Louis, MO, EUA). A cafeína anidra foi solubilizada em água e o PTZ foi dissolvido em solução salina fisiológica 0,9%.

Protocolo de exercício físico e suplementação com cafeína

O exercício de natação em estilo livre foi realizado em um tambor de plástico circular (diâmetro, 120 cm; profundidade, 90 cm) cheio com água mantido a uma temperatura de 32 ± 2°C. O exercício consistiu de natação diária por 50 minutos, cinco dias por semana, durante 4 semanas. Durante os três primeiros dias de exercício, todos os animais foram submetidos a um período de adaptação de natação sem pesos. Após o período de adaptação da natação, os ratos foram submetidos ao exercício de natação com uma carga de trabalho (5% do peso corporal), para melhorar a resistência (Gobatto et al., 2001). O tratamento com cafeína na dose de 6 mg/kg por via oral (gavage) foi realizado durante todo o protocolo de treinamento.

Procedimento Cirúrgico

Para os registros eletroencefalográficos (EEG), todos os animais foram submetidos ao procedimento cirúrgico para implantação de eletrodos. Em resumo, os animais foram anestesiados com equitesina (fenobarbital 1%, sulfato de magnésio 2%, hidrato de cloral 4%, propileno glicol 42%, etanol 11%, 3 ml / kg, ip) e fixados no aparelho estereotáxico para roedores. Para os registros de EEG, dois eletrodos foram colocados bilateralmente sobre o córtex parietal, juntamente com um cabo de massa posicionada sobre o seio nasal. Os eletrodos foram conectados a uma tomada de multipino fixado ao crânio com acrílico.

Ceftriaxona (200 mg / kg, ip) foi administrado imediatamente antes do procedimento cirúrgico. A avaliação comportamental e EEG foram realizadas 4 dias depois da cirurgia.

Avaliação das convulsões

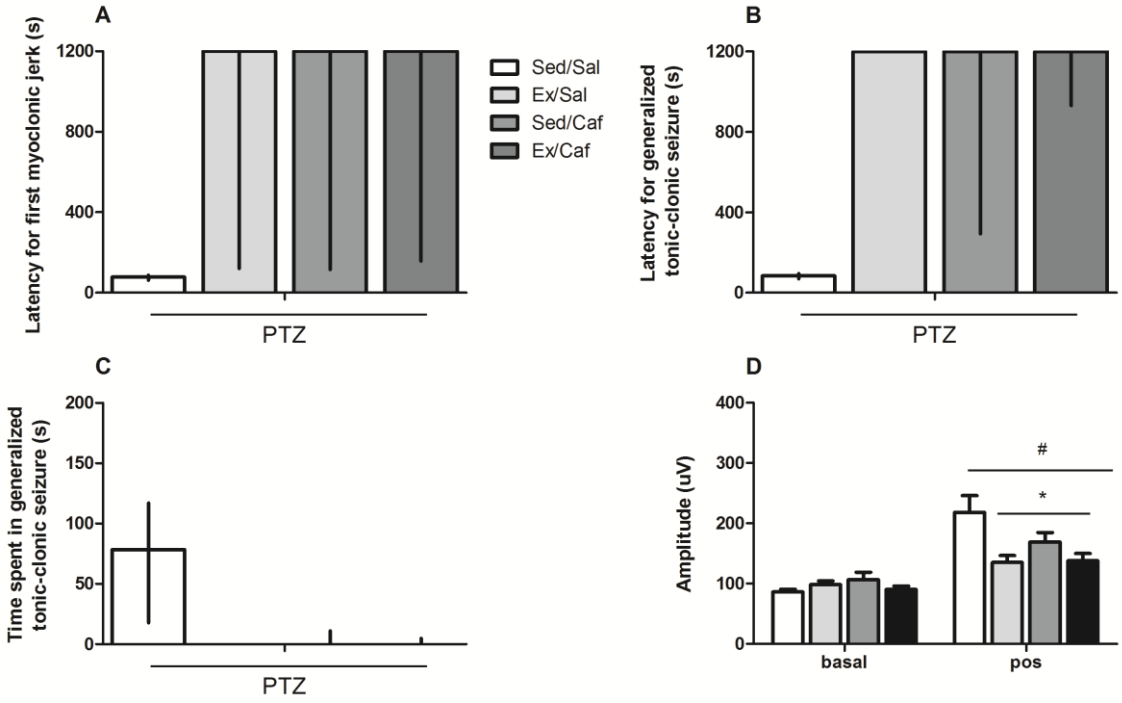
Após 4 dias de recuperação, os animais serão conectados ao aparelho eletroencefalográfo digital (Neuromap EQSA260, Neuromap LTDA, Brasil). Será feito um registro da atividade eletroencefalográfica basal por 10 minutos e logo após os animais serão injetados com cloreto de sódio (NaCl) ou PTZ (60mg/kg) e observados durante 20 minutos.

O sistema de eletroencefalografia, os protocolos cirúrgicos e de registro da atividade eletroencefalográfica assim como os critérios para análise das convulsões são descritos detalhadamente em estudos prévios de nosso grupo (Oliveira et al., 2008). Em resumo, os animais que estarão em caixas de acrílico divididas em 9 áreas iguais serão observados quanto ao aparecimento de comportamentos convulsivos. A latência para o primeiro episódio convulsivo (caracterizada pelo aparecimento de mioclônias) e a soma da duração de todas as convulsões apresentadas durante o período de avaliação (tempo total convulsionando) será cronometrado. Os sinais eletroencefalográficos serão amplificados, filtrados, digitalizados e gravados em um computador pessoal para posterior análise. Após o registro eletroencefalográfico e/ou a avaliação comportamental os animais serão eutanasiados por decapitação e o córtex cerebral será obtido para realização das análises neuroquímicas, conforme descrito abaixo.

Resultados obtidos até o momento:

A análise estatística demonstrou que os animais submetidos ao protocolo de exercício físico aumentaram a latência para a primeira convulsão mioclôncica [U=15,41; p<0,05] e primeira convulsão generalizada tônico-clônica [U=19,02; p<0,05] bem como diminui a duração das convulsões [U=18,01; p<0,05], além de prevenir o aumento de amplitude das ondas após a administração de PTZ [F(1,22)=3,9; p<0,05]. Da mesma o tratamento com cafeína aumentou a latência para a primeira convulsão mioclônica [U=15,41; p<0,05] e primeira convulsão generalizada tônico-clônica [U=19,02; p<0,05], diminuiu a duração das convulsões [U=18,01; p<0,05] e atenuou o aumento de amplitude de onda induzido por PTZ [F(1,22)=2,34; p<0,05]. O tratamento com exercício físico mais a suplementação com cafeína

também demonstrou o mesmo efeito protetor frente as convulsões, aumentando a latência para a primeira convulsão mioclônica [U=15,41; p<0,05], primeira convulsão generalizada tônico-clônica [U=19,02; p<0,05] bem como diminuiu a duração das convulsões [U=18,01; p<0,05] e atenuou o aumento de amplitude de onda induzido por PTZ [F(1,22)=3,6; p<0,05].



Perspectivas do Estudo:

Os resultados obtidos até o momento indicam um efeito protetor tanto do exercício físico quanto da suplementação com cafeína frente as convulsões induzidas por PTZ, entretanto não houve um efeito aditivo entre os dois tratamentos. Considerando estudos prévios do grupo e da literatura de que o exercício físico e a cafeína modulam positivamente o sistema antioxidante e protegem do dano oxidativo induzido por PTZ nós temos como perspectivas desse estudo:

- Analisar a expressão de BDNF, CREB e PGC1-α
- Analisar conteúdo de GSH e atividade da GPx
- Analisar a atividade e expressão da SOD
- Analisar o conteúdo de TBARS e carbonilação protéica.

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DISCUSSÃO

As convulsões são a principal manifestação clínica da epilepsia (Fischer et al., 2005) e apesar do crescente número e variedade de DAE, mais de 30% dos casos são clinicamente classificados como refratários ao tratamento (ELGER, 2003).

Existem evidências para a participação das EROs na fisiopatologia das epilepsias, entretanto, determinar o seu papel é difícil, uma vez que o estresse oxidativo pode ser causa ou consequência das crises epilépticas (WALDBAUM; PATEL, 2010).

Dessa forma, considerando o grande número de pacientes refratários ao tratamento disponível, e que o dano oxidativo parece ser um importante fator envolvido nas crises, terapias alternativas que aumentem as defesas antioxidantes e/ou diminuam o dano oxidativo podem se tornar importantes adjuvantes no tratamento das crises epilépticas. Neste sentido, programas de exercício físico têm apresentado efeito protetor tanto em pacientes quanto em modelos experimentais de epilepsia (ARIDA et al., 2009).

De fato, os resultados apresentados no primeiro estudo demonstraram que seis semanas de protocolo de treino de natação proporciona alteração no status antioxidante e é eficaz em atenuar convulsões e alterações neuroquímicas induzidas pela administração de PTZ.

As avaliações comportamentais e eletroencefalográficas indicaram que o treinamento físico teve um efeito protetor sobre a geração e duração de crises generalizadas induzidas por PTZ (45 e 60 mg / kg, i.p.). Estes dados sugerem que as alterações na neuroplasticidade induzidas pelo treinamento físico podem diminuir a suscetibilidade a crises convulsivas (SUTOO; AKIYAMA, 2003). Além disso, a administração de doses sub efetivas e efetivas de PTZ (45 e 60 mg / kg, ip) aumentaram os níveis de oxidação de DCFH-DA, TBARS, carbonilação protéica, assim como inibiram a atividade da SOD e CAT, sugerindo que as convulsões foram acompanhadas por um aumento do estresse oxidativo. No entanto, é difícil estabelecer uma relação causa-efeito entre estes eventos, já que não houve correlação entre a duração das convulsões e carbonilação proteica, produção de TBARS ou oxidação de DCFH-DA.

No presente estudo, também foi observado que o treinamento físico aumentou os níveis de TNP e a atividade da SOD *per se*, bem como protegeu do aumento da formação de TBARS, carbonilação de proteínas, oxidação DCFH-DA e TNP induzidas pelas convulsões.

Além disso, o exercício físico protegeu da inibição da atividade das enzimas SOD e CAT. Estes resultados concordam com algumas evidências que sugerem que as respostas adaptativas ao exercício moderado envolvem um aumento das defesas antioxidantes e uma redução de produção basal de oxidantes (RADAK et al., 2006; PACKER; CADENAS; DAVIES, 2008). Navarro e colaboradores (2004) demonstraram que o exercício físico moderado diminui o dano oxidativo, aumenta a expectativa de vida e melhora o desempenho comportamental associado ao envelhecimento em ratos (NAVARRO et al., 2004).

Além disso, os resultados apresentados neste estudo mostraram que a administração de doses subconvulsivantes, e convulsivantes de PTZ (30, 45 e 60 mg / kg, ip) inibiram a atividade da Na^+,K^+-ATPase, a captação de glutamato (60 mg / kg, ip) e diminuiram o conteúdo de TNP (45 e 60 mg / kg, ip).

Tendo em vista que a enzima Na^+, K^+-ATPase desempenha um papel fundamental na manutenção do gradiente ionico celular e é particularmente sensível às espécies reativas (MOREL et al., 1998; PETRUSHANKO et al., 2006), sugere-se que o manutenção da atividade Na^+,K^+-ATPase induzida pelo treinamento físico pode proteger do aumento da excitabilidade neuronal induzida pela administração de PTZ. De fato, o presente estudo mostrou uma forte correlação negativa entre a duração das convulsões e a oxidação de TNP, atividade da Na^+,K^+-ATPase e inibição da captação de glutamato nos ratos treinados e sedentários, sugerindo que a convulsão e os parâmetros neuroquímicos são eventos que estão correlacionados.

Embora o mecanismo exato pelo qual PTZ reduz a captação de glutamato ainda não esteja totalmente esclarecido, sugere-se que possa ter relação, direta ou indiretamente, com o estresse oxidativo induzido pela administração de PTZ. Neste contexto, a oxidação de grupos SH em transportadores de glutamato pode diminuir sua atividade (TROTTI et al., 1997), assim como, uma redução da atividade da Na^+,K^+-ATPase, também pode diminuir a captação de glutamato, já que esta é dependente de gradiente de Na^+ através da membrana celular (KANAI 2003). Isso é particularmente importante considerando que a atividade dos transportadores de glutamato pode ser afetada por vários mecanismo, incluindo formação de EROs e redução da atividade da Na^+,K^+-ATPase (VOLTERRA et al., 1994; NANITSOS et al., 2004). No entanto, mais estudos são necessários para estabelecer definitivamente os mecanismos envolvidos.

Considerando que o exercício regular leva ao desenvolvimento de respostas compensatórias ao estresse oxidativo (VIGUIE et al., 1993; LEICHTWEIS et al., 2001) e que a falha de alguns alvos selecionados, tais como Na^+,K^+-ATPase, pode aumentar a

excitabilidade celular e facilitar o aparecimento ou a propagação das convulsões (PATEL, M., 2004), sugere-se que o aumento das defesas antioxidantes e redução da produção basal de oxidantes suscitados por este treinamento físico pode proteger contra a inibição da atividade da enzima Na^+,K^+-ATPase induzida por PTZ.

Portanto, como o exercício físico promoveu um aumento da resistência ao estresse oxidativo e reduziu as crises neste modelo de convulsão, pode-se considerar que a prática de atividade física, tanto de maneira profilática como terapêutica, pode ter um impacto positivo para auxiliar no tratamento da epilepsia.

Dessa forma, visto que as convulsões estão relacionadas com o dano oxidativo, compostos com potencial efeito antioxidante podem auxiliar no controle das crises, entre elas podemos citar a suplementação com a cafeína (AOYAMA et al., 2011).

Neste sentido o segundo estudo deste trabalho mostrou que a suplementação com baixas doses de cafeína atenuou as convulsões comportamentais e alterações eletroencefalográficas e o dano oxidativo induzidos por PTZ, bem como, aumentou o conteúdo de GSH *per se.* Além disso, a infusão de BSO, um inibidor da síntese de GSH, reverteu o efeito protetor da cafeína nas convulsões e nos parâmetros neuroquímicos, caracterizados pela diminuição na atividade da Na^+,K^+-ATPase, nos níveis de GSH , no aumento da LPO e na oxidação DCFH-DA induzidos por PTZ.

Embora o tratamento prolongado com a cafeína apresentou efeitos protetores, a administração de forma aguda desta xantina (6 mg / kg) não alterou as convulsões e os parâmetros neuroquímicos induzidos por PTZ, sugerindo que os efeitos protetores induzidos pela cafeína são devido ao tratamento crônico.

Embora os estudos epidemiológicos tenham indicado que o consumo de cafeína está negativamente correlacionado com a incidência de algumas doenças neurológicas (MAIA E DE MENDONÇA 2002; ASCHERIO; MUNGER; SIMON, 2010), seus efeitos na epilepsia ainda permanecem controversos. Enquanto alguns estudos evidenciam que a administração de altas doses de cafeína diminui o limiar para as convulsões induzidas por PTZ (CZUCZWAR et al., 1987; CUTRUFO et al., 1992) e anula o efeito protetor de algumas DAE (CHROSCINSKA-KRAWCZYK et al.), outros estudos sugerem um efeito neuroprotetor para a cafeína em modelos de epilepsia (SHEN; LI; BOISON; EL YACOUBI et al., 2008).

Ainda que não se descarte que a ação protetora da cafeína neste estudo seja por atuar como antagonista dos receptores de adenosina, sugere-se que seu efeito neuroprotetor se de por atenuar o dano oxidativo e prevenir a inibição da atividade da enzima Na^+,K^+-ATPase. Neste contexto, alguns trabalhos mostram que a cafeína também age como um antioxidante

(NOSCHANG et al., 2009; LEON-CARMONA; GALANO, 2011) pode proteger de doenças associadas a produção de EROs, como DA, DP e epilepsia (ROSSO et al 2008; EL YACOUBI et al., 2008; PRASANTHI et al., 2010). De acordo com esses dados, neste trabalho também foi observado que a suplementação de cafeína aumentou o conteúdo de GSH *per se*, sugerindo um potencial antioxidante. Considerando-se que a suplementação de cafeína induz ao desenvolvimento de respostas compensatórias ao estresse oxidativo em modelos de doenças neurodegenerativas (NOBRE et al., 2010) e que a GSH protege da inibição da Na^+,K^+-ATPase induzida por radicais livres (TSAKIRIS et al., 2000), sugere-se que o aumento da GSH pode ser a responsável por proteger a Na^+,K^+-ATPase da inibição induzida por PTZ. Reforçando esta hipótese, foi observado que a administração de BSO *per se*diminuiu o conteúdo de GSH, aumentou a oxidação DCFH-DA e inibiu a atividade Na^+,K^+-ATPase, assim como, reverteu o efeito protetor da cafeína nas convulsões induzidas por PTZ.

Nesse contexto, dados da literatura mostram que a depleção de GSH causa uma inibição da enzima Na^+,K^+-ATPase em cultura de células neuronais (PETRUSHANKO et al., 2006) e que a GSH administrada intracerebroventricular inibe as convulsões induzidas por PTZ (ABE et al., 2000). Da mesma forma, Prigol e colaboradores (2011) mostraram que a administração de GSH (i.c.v) protegeu das convulsões e do dano oxidativo induzidos por diseleneto (PRIGOL et al., 2011).

Em resumo, o presente trabalho demonstrou que a proteção oferecida tanto pelo exercício físico quanto pela cafeína frente as convulsões pode ser devido a manutenção do estado redox e da atividade da enzima Na^+,K^+-ATPase.

CONCLUSÃO

De acordo com os resultados obtidos pode-se concluir que:

O exercício físico modula positivamente o sistema antioxidante e protege das convulsões e dano oxidativo induzido por PTZ.

A administração prolongada de cafeína modula positivamente o sistema antioxidante (aumento nos níveis de GSH) sendo que este pode ser um dos mecanismos pelo qual ela protege das convulsões e dano oxidativo induzido por PTZ.

O exercício físico associado a administração prolongada de cafeína não oferecem proteção aditiva frente as convulsões e dano oxidativo induzido por PTZ. Provavelmente pelo fato de os dois modularem o estado redox neuronal.

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