

**UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
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**Avaliação do estado redox e de parâmetros gliais no hipocampo de
ratos submetidos a exercício físico moderado e restrição calórica**

KATIANE SANTIN

Orientador

Prof. Carlos Alberto Gonçalves

Co-orientadora

Profa. Christianne Salbego

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**“Toda a nossa ciência, comparada com a realidade,
é primitiva e infantil – e, no entanto, é a
coisa mais preciosa que temos.”**

Albert Einstein

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Parte I

Resumo

O desequilíbrio entre defesas antioxidantes e espécies reativas, conhecido como estresse oxidativo, tem sido relacionado com o desenvolvimento de doenças neurodegenerativas. Diversos estudos indicam que o exercício físico traz benefícios à saúde, inclusive para o sistema nervoso central, que estariam relacionados à adaptação das defesas antioxidantes. Além disso, pesquisas sugerem que o exercício associado a uma condição de restrição calórica pode reduzir tanto a incidência quanto a severidade de desordens neurológicas. O objetivo deste trabalho foi avaliar a influência de exercício físico e restrição calórica no estado redox e em parâmetros gliais no hipocampo de ratos. Quarenta ratos Wistar machos, com idade de aproximadamente 60 dias, foram divididos em 4 grupos: alimentados *ad libitum* e sedentários (AS), alimentados *ad libitum* e exercitados (AE), em restrição calórica e sedentários (RS) e em restrição calórica e exercitados (RE). Os animais foram sacrificados após 3 meses de restrição calórica (30% da ingestão de ração) e exercício físico (de intensidade moderada em esteira), sendo o hipocampo cirurgicamente removido para avaliação dos parâmetros de interesse. Foram realizadas também análises bioquímicas séricas. Os grupos AE, RS and RE apresentaram um aumento nos níveis de glutathiona reduzida (GSH) e na reatividade antioxidante total (TAR). Os níveis de nitratos/nitritos diminuíram somente no grupo RE. Foi observado um decréscimo no conteúdo de proteínas carboniladas nos grupos AE, RS e RE, enquanto nenhuma modificação foi detectada no ensaio de TBARS (espécies reativas ao ácido tiobarbitúrico). Não houve diferenças no potencial antioxidante reativo total (TRAP), na atividade da superóxido dismutase (SOD), no conteúdo de S100B e GFAP, entretanto, a restrição calórica foi capaz de aumentar a captação de glutamato (grupos RS e RE) e a atividade da glutamina sintetase (GS) (grupo RS). O exercício físico, a restrição calórica e a combinação de ambos são capazes de atenuar o dano oxidativo no hipocampo, possivelmente através da modulação de parâmetros gliais, podendo ser utilizados como estratégias na prevenção de doenças neurodegenerativas.

Abstract

Imbalanced redox status has been related to neurodegenerative disease development. Some studies demonstrate that physical activity produces antioxidant adaptations that have health benefits, including in the nervous system. Additionally, available data suggest exercise and a caloric restriction regimen may reduce both the incidence and severity of neurological disorders. Therefore, our aim was to compare hippocampal redox status and glial parameters among sedentary, trained, caloric-restricted sedentary and caloric-restricted trained rats. Main methods: Forty male adult rats were divided into 4 groups: ad libitum-fed sedentary (AS), ad libitum-fed exercise training (AE), calorie-restricted sedentary (RS) and calorie-restricted exercise training (RE). The caloric restriction (decrease of 30% in food intake) and exercise training (moderate in a treadmill) were carried out for 3 months. Thereafter hippocampus was surgically removed, and redox and glial parameters were assessed. Key findings: Increases in reduced glutathione (GSH) levels and total antioxidant reactivity (TAR) were observed for AE, RS and RE groups. The nitrite/nitrate levels decreased only at RE. We found a decrease in carbonyl content for the AE, RS and RE groups, while no modifications were detected in thiobarbituric acid reactive substances (TBARS). Total reactive antioxidant potential (TRAP), superoxide dismutase (SOD) activity, S100B and glial fibrillary acid protein (GFAP) content did not change, but caloric restriction was able to increase glutamine synthetase (GS) activity (RS group) and glutamate uptake (RS and RE groups). Significance: Exercise training, caloric restriction and both combined can decrease oxidative damage in the hippocampus, possibly involving modulation of astroglial function, and could be used as a strategy for the prevention of neurodegenerative diseases.

Lista de Abreviaturas

AMPA – Ácido α -amino-3-hidróxi-5-metil-4-isoxazolenopropionato (α -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate)

AST - Aspartato aminotransferase (Aspartate aminotransferase Transaminase)

DNA – Ácido Desoxirribonucleico (Deoxyribonucleic Acid)

ER – Espécies Reativas (Reactive Species)

GFAP – Proteína Glial Fibrilar Ácida (Glial Fibrillary Acid Protein)

GPx- Glutationa Peroxidase

GS – Glutamina Sintetase

GSH – Glutationa

GSSG – Glutationa Oxidada

H₂O₂ – Peróxido de Hidrogênio

4-HNE – 4-Hidroxinonenal

NMDA – N-Metil-D-Aspartato

NO[•] – Óxido Nítrico

nNOS – Óxido Nítrico Sintase Neuronal

NT – Neurotransmissor

O₂^{•-} – Ânion superóxido

ONOO^{•-} - peroxinitrito

[•]OH – Radical hidroxil

RC – Restrição Calórica

SNC – Sistema Nervoso Central

SOD – Superóxido Dismutase

TBARS – Espécies Reativas ao Ácido Tiobarbitúrico

Introdução

1. Estresse Oxidativo

Espécies reativas (ER) é um termo genérico usado para definir espécies (átomos, íons ou moléculas) derivadas do oxigênio e nitrogênio molecular que são reativas *per se*, ou que podem ser facilmente convertidas em espécies reativas; algumas ER são radicais livres (Matsuo and Kaneko, 2000).

As ER podem ser geradas por fontes endógenas ou exógenas. Entre as principais fontes endógenas estão a cadeia mitocondrial transportadora de elétrons, a degradação de ácidos graxos nos peroxissomas, os mecanismos de detoxificação mediados pelo complexo enzimático P-450 e o processo de fagocitose; dentre as fontes exógenas estão as radiações, o cigarro e os solventes orgânicos (Gutteridge and Halliwell, 2000).

A geração de ER *in vivo* é um processo fisiológico que ocorre de maneira controlada pelos mecanismos antioxidantes celulares. Quando há um desequilíbrio oxidante x antioxidante, ou seja, geração descontrolada de ER, diminuição das defesas antioxidantes, ou ambos, ocorre a oxidação de biomoléculas, como ácidos nucléicos, proteínas e lipídeos, o que pode alterar a informação genética, desnaturar proteínas, inativar enzimas e desorganizar biomembranas, processo este denominado estresse oxidativo (Sies, 1991).

A mudança mais significativa que ocorre durante o exercício físico é um aumento na taxa metabólica, ligado a um aumento no consumo de oxigênio. Um

fluxo de oxigênio maior que a capacidade mitocondrial pode provocar “vazamento” de elétrons na cadeia transportadora, levando a um aumento da geração de ER, contribuindo para o estresse oxidativo (Jenkins, 1993). No entanto, alguns estudos sugerem que com o treinamento físico ocorre uma adaptação do sistema antioxidante celular, aumentando a atividade de enzimas antioxidantes, o que reduziria o dano oxidativo (Powers et al., 1994, Leeuwenburgh et al., 1997, Radak et al., 2008).

Além dos antioxidantes enzimáticos, existe uma segunda categoria de antioxidantes, denominados não-enzimáticos, representada pelas vitaminas, que não podem ser sintetizadas ou induzidas e devem ser absorvidas através de uma alimentação balanceada (Berger, 2005). Então, a nutrição possui um impacto significativo no sistema antioxidante celular. Estudos demonstraram que animais submetidos à restrição calórica, mas não mal-nutridos, apresentaram menos dano mediado por ER, incluindo uma diminuição da peroxidação lipídica e perda da fluidez de membrana, de proteínas com dano oxidativo (proteínas carboniladas), e do dano oxidativo ao DNA (Merry, 2002, Barja, 2004).

1.1 Equilíbrio Redox no Sistema Nervoso Central

A demanda de oxigênio do cérebro humano é estimada em cerca de 20% do oxigênio consumido por todo o corpo, mesmo o cérebro representando 2% do peso corporal (Somani and Husain, 2000). O cérebro está entre os maiores órgãos geradores de ER e, comparado com outros órgãos, é especialmente vulnerável ao

estresse oxidativo por estar inadequadamente equipado com defesas antioxidantes e por conter lipídeos com ácidos graxos insaturados em abundância, alvo da peroxidação lipídica, o que poderia ajudar a esclarecer o dano oxidativo observado no decorrer de doenças neurodegenerativas (Dringen et al., 2000, Halliwell, 2006).

Aproximadamente de 2 a 4% do oxigênio consumido por mitocôndria é desviado para formar ânion superóxido ($O_2^{\bullet-}$). A enzima superóxido dismutase (SOD) converte o superóxido em peróxido de hidrogênio (H_2O_2), que é subsequentemente convertido em água e oxigênio molecular pela glutathione peroxidase (GPx) ou pela catalase (Dringen et al., 2000). Foi demonstrado que a atividade da catalase no cérebro é baixa e confinada aos peroxissomos (Gaunt and de Duve, 1976).

A GPx corresponde a uma família de enzimas contendo selênio (Brigelius-Flohe, 1999), que remove o H_2O_2 , por reduzir esta ER numa reação acoplada com a oxidação da glutathione (GSH), um tripeptídeo formado por glutamato, cisteína e glicina, contendo grupamento tiol.

A glutathione oxidada (GSSG) consiste em duas GSH ligadas por uma ponte dissulfeto, que pode ser reconvertida em GSH pelo complexo de enzimas glutathione reductase (Halliwell, 2006). A GSH é um dos maiores antioxidantes no sistema nervoso central (SNC) (Dringen, 2000b) (Figura 1) com uma concentração em torno de 2-3 mM, que é bem maior que a do sangue e a do líquido (Cooper and Kristal, 1997).

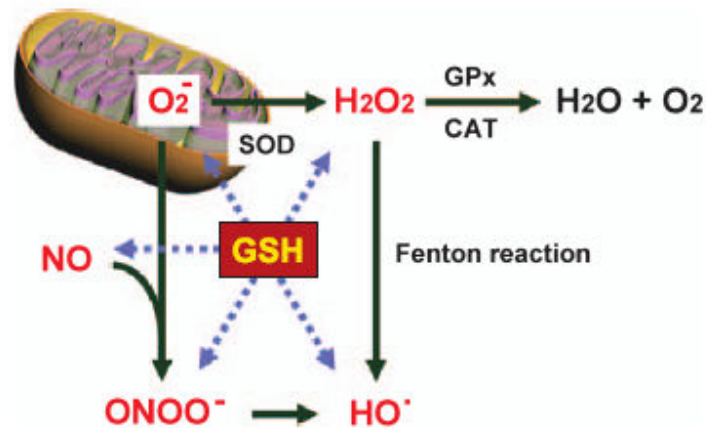


Figura 1: Proteção da GSH contra estresse oxidativo. Adaptado de Aoyama et al. 2008

Quando não removido, o H_2O_2 pode reagir com o ferro, encontrado de forma abundante ligado a proteínas no SNC (Zecca et al., 2004), via Reação de Fenton e formar radicais hidroxil (HO^\bullet), que podem desencadear a peroxidação lipídica (Youdim et al., 1989) .

O SNC também possui em abundância o óxido nítrico (NO^\bullet) produzido pela óxido nítrico sintase neuronal (nNOS) (Zhou and Zhu, 2009), que atua como vasodilatador e neurotransmissor não-clássico (Garthwaite, 2008). O superóxido e o NO^\bullet isolados não são tóxicos *in vivo*, mas quando combinados podem reagir, gerando um oxidante fortemente tóxico, o peroxinitrito (ONOO^\bullet). A presença de ONOO^\bullet leva à oxidação de proteínas, lipídeos, e DNA, bem como à nitração de aminoácidos, principalmente tirosina, e rapidamente inativa as enzimas mitocondriais resultando em falha da produção de energia (Pacher et al., 2007).

A peroxidação lipídica induzida por ER leva à conversão de ácidos graxos poliinsaturados em aldeídos altamente reativos como o 4-hidroxinonenal (4-HNE).

4-HNE pode danificar facilmente os transportadores de glutamato, diminuindo a sua remoção da fenda sináptica (Mattson and Chan, 2003). 4-HNE também inibe a atividade enzimática de GPx, aumentando por consequência os níveis de H₂O₂ (Bosch-Morell et al., 1999).

O esqueleto protéico e as cadeias laterais da maioria das proteínas apresentam aminoácidos suscetíveis à oxidação, porém a adição não-enzimática de aldeídos e cetonas a resíduos de aminoácidos específicos (carbonilação) constitui a maior e mais comum alteração oxidativa a proteínas (Berlett and Stadtman, 1997).

Diversas ER (incluindo ONOO⁻) podem diminuir a captação de glutamato pelas células gliais e inativar a enzima glutamina sintetase (GS), prejudicando a conversão de glutamato em glutamina (Aksenov et al., 1997).

2. Sistema Nervoso Central

O sistema nervoso central (SNC) é composto por dois tipos celulares – neurônios e células gliais (Jenssen, 2004). Os neurônios foram por muito tempo considerados os elementos celulares responsáveis pelo processamento da informação, enquanto que as células gliais eram reconhecidas apenas pelo seu papel de suporte inerte para os neurônios (Volterra and Meldolesi, 2005). Atualmente sabe-se que as células da glia atuam como um componente ativo em funções cerebrais essenciais (Van Eldik and Wainwright, 2003).

As células da glia são divididas em dois grupos principais: a macroglia, composta de astrócitos, oligodendrócitos e células ependimárias; e a microglia, composta de células fagocíticas envolvidas em resposta inflamatória (Perea and Araque, 2005).

Os astrócitos constituem aproximadamente 50% de toda massa cerebral, sendo então, as células gliais mais abundantes do SNC (Gee and Keller, 2005) (Figura 2). Eles são divididos em dois tipos celulares: os protoplasmáticos, na substância cinzenta (freqüentemente ramificados e com largas expansões); e os fibrosos, na substância branca (células cilíndricas e longas, com menos ramificações) (Young, 1991).

Existe um novo conceito em transmissão sináptica, a sinapse de três elementos: onde os astrócitos trocam informações com neurônios pré e pós-sinápticos e participam da neurotransmissão como elementos regulatórios dinâmicos (Perea and Araque, 2005). Astrócitos e neurônios possuem então, um sofisticado sistema de comunicação recíproca que pode regular a liberação de neurotransmissores, a excitabilidade neuronal e a transmissão sináptica (Carmignoto, 2000).

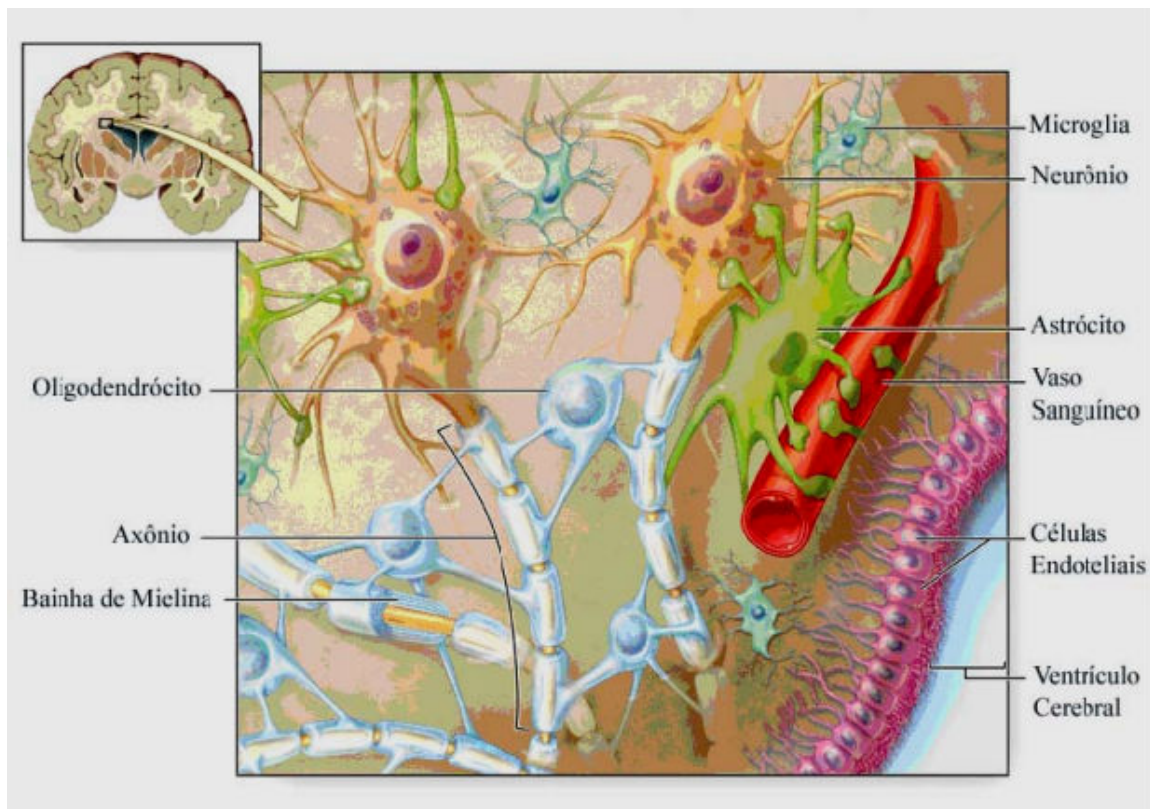


Figura 2: Os principais grupos celulares do SNC e suas inter-relações.

Adaptado de www.indiana.edu

2.1 Marcadores Gliais em Astrócitos

Os astrócitos participam de vários processos fisiológicos e metabólicos responsáveis por manter a homeostase do SNC. Estas células possuem três proteínas específicas usadas como marcadoras: a S100B; a GFAP (proteína glial fibrilar ácida); e a glutamina sintetase (responsável pela formação de glutamina a partir de glutamato). Insultos físicos e metabólicos provocam alterações rápidas nas células gliais, processo conhecido como astrogliose reativa, caracterizada por

um aumento na expressão dos marcadores gliais citados anteriormente (Baydas et al., 2003).

2.1.1 S100B

A S100B é uma proteína de 21 kDa, ligante de cálcio do tipo EF-hand (hélice-loop-hélice). Essa proteína foi isolada há mais de 40 anos (Moore, 1965) a partir de extrato de cérebro bovino e a denominação “S100” é devida a sua solubilidade em sulfato de amônio 100% (Van Eldik and Wainwright, 2003).

Estruturalmente, a proteína S100 forma homodímeros constituídos de duas subunidades β unidos por pontes dissulfeto e capazes de se ligarem a proteínas alvos (Donato, 2003).

A S100B é produzida e secretada principalmente por astrócitos e exerce efeitos parácrinos em neurônios e microglia, e autócrinos em astrócitos (Ponath et al., 2007). Entretanto, outras fontes extracerebrais como, células adiposas, também podem secretar S100B. O mecanismo de secreção de S100B ainda não foi identificado (Rothermundt et al., 2003).

A S100B possui funções intra e extracelulares. Intracelularmente, a S100B inibe a fosforilação de diversas proteínas, tais como GFAP, p53, entre outras; está envolvida na regulação do metabolismo energético cerebral; modula a proliferação e a diferenciação de neurônios e células gliais. Além disso, a S100B regula a homeostase do Ca^{2+} , interage com muitas funções imunológicas do SNC

e influencia a integridade do citoesqueleto (Donato, 2003, Rothermundt et al., 2003).

Extracelularmente, a proteína S100B pode ter efeitos tróficos ou tóxicos, dependendo da concentração secretada. Em baixas concentrações (doses nM), a S100B exerce efeito neurotrófico, promovendo o crescimento de neuritos, aumentando a sobrevivência de neurônios durante o desenvolvimento e após dano ao SNC (Tramontina et al., 2002, Gottfried et al., 2003, Tramontina et al., 2006a) e protegendo neurônios contra a excitotoxicidade do glutamato (Ahlemeyer et al., 2000, Tramontina et al., 2006a). Este efeito trófico também é exercido em astrócitos.

Em altas concentrações (doses μM), a S100B exerce um efeito neurotóxico por induzir a apoptose através de mecanismo dependente da liberação de citocinas pró-inflamatórias como a interleucina-6, interleucina-1 β e fator de necrose tumoral- α na glia e interleucina-6 em neurônios (Van Eldik and Wainwright, 2003) e através de estímulo de secreção de óxido nítrico por astrócitos e microglia (Donato, 2001).

2.1.2 Proteína Glial Fibrilar Ácida (GFAP)

A GFAP é uma proteína estrutural e constitui a subunidade protéica de filamentos intermediários do tipo III do citoesqueleto glial (Rodnight et al., 1997, Gomes et al., 1999).

Inicialmente isolada de lesões cerebrais (placas) de pacientes com esclerose múltipla (Eng et al., 2000), a GFAP é reconhecida e amplamente utilizada como um marcador de astrócitos e de tumores de linhagem astrocítica (Pekny and Pekna, 2004). Durante o desenvolvimento do SNC e na gliose reativa ocorre um aumento na expressão de GFAP (Gomes et al., 1999).

Estruturalmente, a GFAP é um polímero que consiste em uma região amino-terminal não-helicoidal altamente básica, uma região carboxi-terminal não-helicoidal e responsável pela ligação entre os monômeros e uma região central formada por uma α -hélice, cuja sequência de aminoácidos é conservada em relação a outras proteínas filamentosas intermediárias. Diferenças na estrutura dessas proteínas são normalmente evidenciadas nos aminoácidos da região amino-terminal (Alberts et al., 2002).

A polimerização da GFAP envolve diversos passos. Inicialmente, um dímero é formado através da pareação entre dois monômeros paralelos. Posteriormente, dímeros anti-paralelos interagem através de resíduos na região central formando um tetrâmero ou protofilamento e, finalmente, vários protofilamentos se unem dando origem ao polímero (Rodnight et al., 1997).

A fosforilação de sítios específicos de proteínas de filamentos intermediários, como a GFAP, regula o equilíbrio dinâmico entre a sua forma polimerizada e despolimerizada, desempenhando um importante papel na mitose (Rodnight et al., 1997).

2.2 Transmissão Glutamatérgica

O aminoácido L-glutamato é o principal neurotransmissor (NT) excitatório do SNC de mamíferos e exerce um importante papel na plasticidade neural e neurotoxicidade (Nakanishi, 1992). O glutamato apresenta diversas funções, incluindo indução e eliminação de sinapses, migração, diferenciação e morte celular durante o desenvolvimento do SNC e, provavelmente, está envolvido em muitos aspectos funcionais, como cognição, memória e aprendizagem (Danbolt, 2001).

O SNC contém uma grande quantidade de glutamato, mas somente uma pequena fração deste NT ($\cong 1\mu\text{M}$) está presente no espaço extracelular. As maiores concentrações são encontradas no interior dos terminais nervosos (Danbolt, 2001).

O glutamato exerce seus efeitos através da interação com receptores de glutamato localizados na superfície das células neuronais e gliais (Figura 2). Então o glutamato ativa receptores ionotrópicos (NMDA, AMPA e Kainato) - que são canais iônicos permeáveis a cátions; e receptores metabotrópicos (mGluRs 1-8) que estão acoplados à proteína G (Matute, 2006).

2.2.1 Captação de Glutamato e Atividade da Glutamina Sintetase

O glutamato, após sua síntese, é estocado pelo sistema de transporte presente nas vesículas que se encontram no terminal pré-sináptico. Quando

ocorre a despolarização dos terminais sinápticos glutamatérgicos, o glutamato, é então, liberado para a fenda sináptica com a função de interagir com seus receptores ionotrópicos e/ou metabotrópicos que estão localizados nas membranas pré e pós-sinápticas, bem como nas membranas gliais (Gallo and Ghiani, 2000). O glutamato é removido da fenda sináptica principalmente por sistemas de transporte que são dependentes de sódio, localizados nos neurônios e, principalmente, nas células gliais (Figura 3) (Robinson and Dowd, 1997, Anderson and Swanson, 2000, Danbolt, 2001, Gottfried et al., 2002).

A captação de glutamato pelo astrócito é a mais eficiente para manter normal a concentração extracelular de glutamato. No astrócito, o glutamato é convertido em glutamina pela enzima glutamina sintetase. A glutamina é liberada pelos astrócitos e captada pelos neurônios para ser novamente convertida em glutamato, sendo este ciclo conhecido como glutamina-glutamato (Matthews et al., 2005).

O glutamato pode ser ainda, transportado através de transportadores dependentes de sódio: GLAST (EAAT1), GLT-1 (EAAT2), EAAC1 (EAAT3), EAAT4 e EAAT5 (Amara and Fontana, 2002). Os transportadores presentes na membrana das células gliais são de fato os responsáveis pela manutenção de baixos níveis extracelulares de glutamato, de forma a garantir a homeostase celular (Anderson and Swanson, 2000, Amara and Fontana, 2002, Porciuncula et al., 2004)

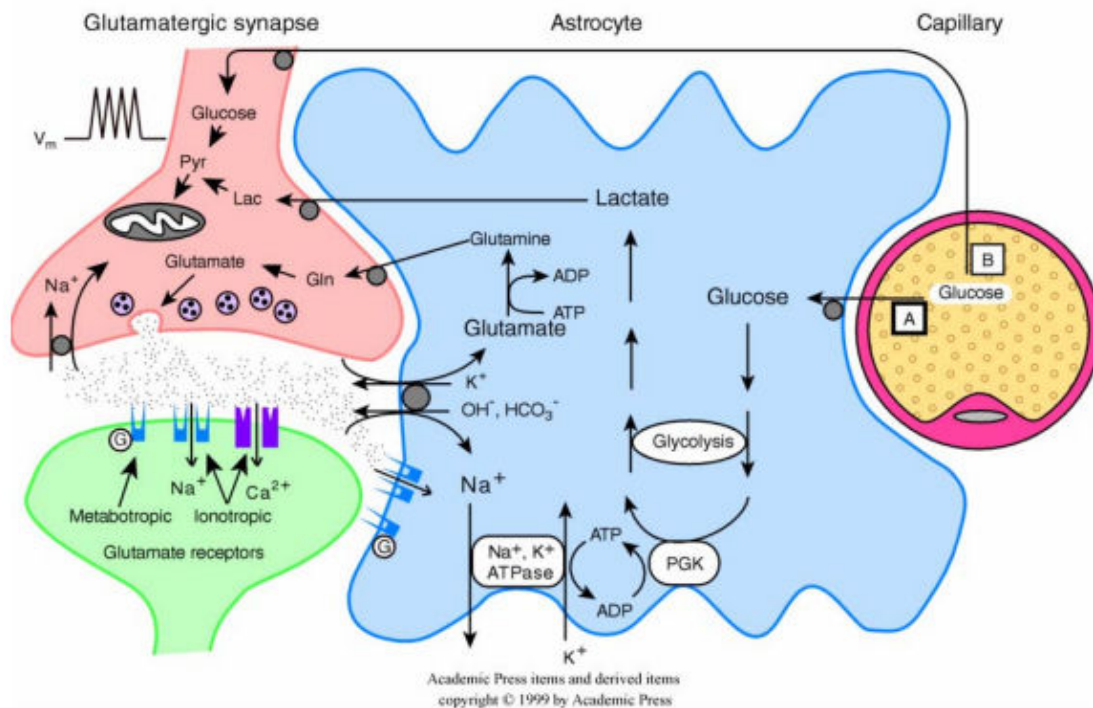


Figura 3: Representação esquemática de uma sinapse glutamatérgica.

Adaptado de Magistretti et al. 1999

A concentração de glutamato no espaço extracelular determina o grau de ativação de seus receptores, por isso, é essencial manter níveis extracelulares normais deste NT. A ativação excessiva ou prolongada dos receptores de glutamato leva à morte celular por um processo denominado excitotoxicidade, o que faz do glutamato, quando em altas concentrações, uma potente neurotoxina. Alguns estudos também demonstraram que o glutamato pode ser tóxico também para células gliais, incluindo astrócitos e oligodendrócitos (Matute et al., 2006).

A excitotoxicidade do glutamato está associada à patogênese de diversas doenças neurológicas, tais como: epilepsia, isquemia cerebral, esclerose múltipla, doenças neurodegenerativas e outras (Anderson and Swanson, 2000).

3. Exercício físico

Diversos estudos epidemiológicos e em animais demonstram que o exercício físico regular atua de forma periférica melhorando a saúde cardiovascular, a densidade mineral óssea e reduzindo fatores de risco para doenças como câncer, diabetes, hipertensão, isquemia e infarto agudo do miocárdio. Adicionalmente, o exercício físico influencia de forma elementar a função cerebral por atuar em alvos no sistema nervoso central (SNC) que melhoram a aprendizagem e memória, aliviam a depressão e previnem o declínio cognitivo e a demência (Nichol et al., 2007), observados durante o envelhecimento, em decorrência de doenças neurodegenerativas como doença de Alzheimer, Parkinson, Huntington e também, em decorrência de acidente vascular cerebral (Prolla and Mattson, 2001, Kramer and Erickson, 2007).

As mudanças estruturais e funcionais induzidas pelo exercício são estudadas em várias regiões cerebrais, sendo a região do hipocampo uma das mais relatadas (Cotman et al., 2007). O hipocampo é uma estrutura localizada nos lobos temporais do cérebro, considerada a principal sede da memória e importante componente do sistema límbico (Bear et al., 2002). O exercício físico pode aumentar a neurogênese no hipocampo (van Praag et al., 1999b), aumentar a produção de fatores neurotróficos derivados do SNC (Neeper et al., 1996) e melhorar a aprendizagem e memória (van Praag et al., 1999a).

Os mecanismos moleculares exatos pelos quais o exercício afeta a função cerebral não estão completamente elucidados (Sutoo and Akiyama, 2003). Embora existam dados controversos, decorrentes de distintas metodologias empregadas, vem sendo demonstrado que o exercício físico pode ativar vias celulares e moleculares que contribuem para neuroproteção (Cotman and Berchtold, 2002).

4. Restrição Calórica

O aumento da expectativa de vida em resposta a uma restrição moderada do suprimento alimentar, é comum em uma grande variedade de organismos, incluindo leveduras, invertebrados como nematódeos e drosófilas, e mamíferos, como roedores e, possivelmente, humanos (Partridge and Brand, 2005, Holloszy and Fontana, 2007, Cruzen and Colman, 2009).

O primeiro estudo científico mundialmente reconhecido por aumentar a expectativa de vida através de uma dieta restritiva foi publicado em 1935. Neste estudo, os pesquisadores viram que alimentando ratos com uma dieta contendo 20% de celulose não digestível, a expectativa de vida média e máxima foi incrivelmente aumentada (McCay, 1935).

Inicialmente, os estudos sobre RC permaneceram focados na questão da expectativa de vida aumentada, posteriormente, foi dada atenção ao estudo dos marcadores biológicos de doenças relacionadas ao envelhecimento como câncer, doença cardiovascular e desordens neurodegenerativas (Swindell, 2008).

Uma restrição calórica moderada, sem atingir estado de desnutrição, de 30 a 40% menos que uma alimentação *ad libitum* durante toda a vida, pode diminuir a incidência de diabetes melitus tipo 2 (Astrup et al., 2001), desordens neurodegenerativas (Patel et al., 2005), doença cardiovascular (Fontana 2004), perda auditiva (Someya et al., 2007), atenuar a atrofia muscular relacionada ao envelhecimento (McKiernan et al., 2004) e aumentar a longevidade de roedores e primatas (Masoro, 2003, 2005).

Pesquisas sugerem que um decréscimo na ingestão de calorias pode desacelerar as mudanças moleculares no SNC relacionadas ao envelhecimento e que levam a um aumento de proteína glial fibrilar ácida (GFAP) e dano oxidativo a proteínas e ao DNA (Dubey et al., 1996, Morgan et al., 1997).

Uma melhor compreensão dos mecanismos celulares e moleculares pelos quais a restrição calórica e a atividade física influenciam a função e a estrutura cerebral poderia conduzir ao desenvolvimento de novos agentes terapêuticos que mimetizem os efeitos benéficos observados em decorrência de um estilo de vida saudável, no qual estas práticas são fundamentais.

Objetivo Geral

Avaliar o estado redox e parâmetros gliais no hipocampo de ratos submetidos a exercício físico moderado e restrição calórica.

Objetivos Específicos

- Analisar no hipocampo os efeitos do exercício físico moderado e da restrição calórica sobre: conteúdo de GSH, níveis de nitratos e nitritos, potencial antioxidante não enzimático, atividade da superóxido dismutase, carbonilação de proteínas e peroxidação lipídica.
- Avaliar o conteúdo de GFAP e S100B em fatias hipocampais de ratos submetidos a exercício físico moderado e restrição calórica;
- Avaliar a captação de glutamato e a atividade da glutamina sintetase em fatias hipocampais de ratos submetidos a exercício físico moderado e restrição calórica;

Parte II

Artigo a ser submetido à revista:

Life Sciences

Moderate exercise training and chronic caloric restriction modulate redox status in rat hippocampus

Katiane Santin^{a*}, Ricardo Fagundes da Rocha^a, Fernanda Cechetti^a, Marina Concli Leite^a, André Quincozes-Santos^a, Daniela Fraga de Souza^a, Patrícia Nardin^a, Letícia Rodrigues^{a,b}, José Cláudio Fonseca Moreira^a, Carmem Gottfried^{a,b}, Christiane Gazzana Salbego^a, Carlos Alberto Gonçalves^{a,b}

^aDepartament of Biochemistry, Institute of Health Basic Sciences (ICBS), Federal University of Rio Grande do Sul (UFRGS)

^bPost-graduation Program of Neurosciences, Institute of Health Basic Sciences (ICBS), Federal University of Rio Grande do Sul (UFRGS)

*Corresponding author

Katiane Santin

Rua Ramiro Barcelos, número 2600-anexo, 90035-003, Porto Alegre, RS, Brazil
Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde (ICBS),
Universidade Federal do Rio Grande do Sul (UFRGS)

Phone: +55 51 33085566/ +55 51 33085412

Fax: +55 51 33085540

E-mail address: katianesantin@yahoo.com.br

Abstract

Imbalanced redox status has been related to neurodegenerative disease development. Some studies demonstrate that physical activity produces antioxidant adaptations that have health benefits, including in the nervous system. Additionally, available data suggest exercise and a caloric restriction regimen may reduce both the incidence and severity of neurological disorders. Therefore, our aim was to compare hippocampal redox status and glial parameters among sedentary, trained, caloric-restricted sedentary and caloric-restricted trained rats. Main methods: Forty male adult rats were divided into 4 groups: ad libitum-fed sedentary (AS), ad libitum-fed exercise training (AE), calorie-restricted sedentary (RS) and calorie-restricted exercise training (RE). The caloric restriction (decrease of 30% in food intake) and exercise training (moderate in a treadmill) were carried out for 3 months. Thereafter hippocampus was surgically removed, and redox and glial parameters were assessed. Key findings: Increases in reduced glutathione (GSH) levels and total antioxidant reactivity (TAR) were observed for AE, RS and RE groups. The nitrite/nitrate levels decreased only at RE. We found a decrease in carbonyl content for the AE, RS and RE groups, while no modifications were detected in thiobarbituric acid reactive substances (TBARS). Total reactive antioxidant potential (TRAP), superoxide dismutase (SOD) activity, S100B and glial fibrillary acid protein (GFAP) content did not change, but caloric restriction was able to increase glutamine synthetase (GS) activity (RS group) and glutamate uptake (RS and RE groups). Significance: Exercise training, caloric restriction and both combined can decrease oxidative damage in the hippocampus, possibly involving

modulation of astroglial function, and could be used as a strategy for the prevention of neurodegenerative diseases.

Key Words:

Caloric restriction

Exercise training

Glia

Oxidative stress

Introduction

Much evidence suggests that aging and neurological disorders are associated with oxidative stress (Halliwell, 2001, Barja, 2004, Sinclair, 2005). The central nervous system (CNS) is prone to oxidative damage, since it presents a higher O₂ uptake (VO₂) than other organs/tissues, has lower antioxidant enzyme activity and contains large amounts of unsaturated fatty acids, which are targets for peroxidation (Dringen, 2000a, Halliwell, 2006). The main redox defense at the neural level is glutathione (GSH), and its precursors are provided mainly by astrocytes in the CNS (Dringen, 2000b).

Astrocytes are also the major glial cell responsible for glutamate removal from the synaptic cleft (Magistretti and Pellerin, 1999, Anderson and Swanson, 2000) and its conversion, through glutamine synthetase (GS) catalysis, into glutamine for replacement in the neurons (Bak et al., 2006). Several reactive oxygen/nitrogen species (RS) can decrease the uptake of glutamate by glial cells and inactivate glutamine synthetase (Aksenov et al., 1997). Moreover, oxidative stress can generate neural damage and promote excitatory amino acid release, creating a “vicious cycle” (Mailly et al., 1999). High glutamate release or failure in glutamate uptake by astrocytes, can lead to excessive and prolonged increases in intracellular free calcium (Ca⁺⁺) and sodium (Na⁺), yielding excitotoxicity and often brain cell death by necrosis (Matute et al., 2006). Raised Ca⁺⁺ levels can interfere with mitochondrial function, increasing superoxide radical ([•]O₂⁻) production and activating neuronal nitric oxide synthase (nNOS) enzyme (Halliwell, 2006, Zhou and Zhu, 2009). nNOS catalyzes nitric oxide (NO[•]) synthesis, which then diffuses

through the brain to exert its functional roles (Garthwaite, 2008). Despite the physiological functions of NO^{*}, in excessive amounts it can react with ⁻O₂ leading to peroxynitrite (ONOO⁻) formation, which can damage proteins by nitration (Pacher et al., 2007).

The activity of astrocytes is commonly related to two protein markers: glial fibrillary acid protein (GFAP) and S100B. The former is the major intermediate filament protein in mature astrocytes (Rodnight et al., 1997) and its increased expression is observed in astrogliosis (O'Callaghan and Sriram, 2005). The S100B protein is a Ca⁺⁺ binding protein, expressed and secreted by astrocytes, that has a trophic activity in neuron and glial cells with implications in neuronal survival (Van Eldik and Wainwright, 2003, Tramontina et al., 2006b). Nonetheless, S100B overproduction by activated glia can lead to exacerbation of neuronal dysfunction and inflammation (Donato et al., 2009).

Caloric restriction (CR) increases the maximum and the mean life spans of laboratory rodents, suppresses a wide variety of time-related diseases, and modulates (preventing or delaying) much of the physiological changes associated with aging (Mattson et al., 2001, Masoro, 2005). Physical exercise also exerts a number of beneficial effects, including an increase in median life span (Mattson, 2000). Several reports indicate that CR and regular exercise modulate cellular antioxidant defenses and protect against free radical damage, suggesting that the beneficial adaptations could be mediated by their effects on the redox balance (Alessio and Goldfarb, 1988, Kim et al., 1996, Radak et al., 2007). Therefore, our

aim was to compare hippocampal redox status and glial parameters among sedentary, trained, calorie-restricted sedentary and calorie-restricted trained rats.

Material and Methods

Chemicals

N-methyl-D-glucamine, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and all other reagents were purchased from Sigma Chemical CO (St. Louis, MO); L-[3H]glutamate (specific activity 30 Ci.mmol⁻¹) was purchased from Amersham International, UK.

Animals

All experiments were approved by the Local Animal Care Committee. Experiments were carried out with 40 male 60-day-old Wistar rats obtained from our breeding colony. The animals were kept under standard laboratory conditions (12 hours light/dark, 22 ± 2 °C) with water ad libitum. Animals were weight matched and divided into four experimental groups (n = 10 to each): ad libitum-fed sedentary (AS); ad libitum-fed exercise (AE); calorie-restricted sedentary (RS) and calorie-restricted exercise (RE).

Training protocol

Rats were habituated with the treadmill apparatus to minimize novelty stress. Moderate exercise training was conducted as previously described (Cechetti et al., 2007). Briefly, running sessions consisted of 20 minutes (min), three times a week

for twelve weeks, on an adapted motorized rodent treadmill (INBRAMED TK01®, Brazil) at 60 % of their maximal oxygen uptake (VO_{2max}) (Brooks and White, 1978). The oxygen uptake peak (VO_{2peak}) was measured in all animals, indirectly before training. All rats ran on a treadmill at a low initial speed followed by increases in $5 \text{ m}\cdot\text{min}^{-1}$ speed every 3 min until the exhaustion point. The time to fatigue (in min) and workload (in $\text{m}\cdot\text{min}^{-1}$) were recorded as indexes of capacity for exercise, which was taken as VO_{2max} (Cechetti et al., 2007). Neither electric shock nor physical prodding was used in this study.

Caloric Restriction Diet

All the animals received a regular laboratory chow (Nuvilab-CR1® from Nuvital, Brazil), as presented in Table 1. The caloric restriction diet was conducted as previously described (Ribeiro et al., 2009). Briefly, the treatment was progressive, being initiated at 10 % restriction in the first week, changing to 20 % at the second week and to 30 % at the third week, maintained until the end of the experiment (at week twelve). Hence, the rats on the RS and RE were provided with an amount of food equivalent to 70 % of that consumed by rats in the AS and AE groups, respectively. The food intake was monitored daily, and the animals were weighed weekly (Horska et al., 1999, Chang et al., 2007).

Biochemical Analysis

Forty-eight hours after the last training session, animals were overnight-starved (12 hours fasting) and anesthetized with an intramuscular injection of ketamine and xylazine (75 and 10 mg.Kg⁻¹, respectively). Blood samples were obtained from intracardiac puncture, and the animals were killed by decapitation. The blood samples were kept at room temperature (25 °C) for 30 min and then centrifuged at 1000 G for 5 min. The serum was collected and the biochemical analyses were carried out on the same day, in a Multi-test Analyzer (Labmax 240® from Labtest, Brazil), using specific kits supplied by Labtest: total protein, C-reactive protein (CRP), aspartate aminotransferase transaminase (AST), alanine aminotransferase transaminase (ALT), creatinine, urea, glucose, triacylglycerol, total cholesterol, high density lipoprotein (HDL) and low density lipoprotein (LDL).

Hippocampal dissection

The brains were removed and placed in cold saline medium with the following composition (in mM): NaCl 120, KCl 2, CaCl₂ 1, MgSO₄ 1, HEPES 1, KH₂PO₄ 1 and glucose 10 adjusted to pH 7.4 and previously aerated with oxygen (O₂). The hippocampi were quickly dissected out and one hemisphere of each animal was transformed in transverse sections (300 μm) using a McIlwain tissue chopper, while the other one was stored at -70 °C for subsequent analysis.

Glutathione content

Reduced GSH content was determined as previously described (Browne and Armstrong, 1998). Briefly, slices were homogenized in sodium phosphate buffer (0.1 M, pH 8.0) containing 0.005 M EDTA and protein was precipitated with 1.7 % meta-phosphoric acid. Supernatant was assayed with o-phthalaldehyde (1 mg.mL⁻¹ of methanol) at room temperature for 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 and 420 nm, respectively. A calibration curve was performed with standard GSH solutions (0-500 μM). GSH concentrations were calculated as nmol.mg protein⁻¹.

TRAP and TAR

The non-enzymatic antioxidant potential of the reproductive tract structures was estimated by the total reactive antioxidant potential (TRAP) and total antioxidant reactivity (TAR) (Lissi et al., 1995). The reaction was initiated by adding luminol (5-Amino-2,3-dihydro-1,4-phthalazinedione, 4 mM)- an external probe for monitoring radical production – and AAPH (2,2'-Azobis-2-methylpropanamide-dihydrochloride, 10 mM) – a free radical source that produces peroxy radical at a constant rate – in glycine buffer (0.1 M, pH 8.6) at room temperature, resulting in a steady luminescence emission (system counts). Chemiluminescence was read with a liquid scintillation counter (Wallace 1409®) as counts per minutes. Sample addition decreases the luminescence proportionately to its antioxidant potential. The luminescence emission was followed for 40 min after the addition of the sample (100 μg of protein) in a TRAP protocol, and the area under the curve (AUC)

was quantified (Dresch et al., 2009). The TAR was calculated and expressed as a ratio of the luminescence without sample for the first luminescence after sample addition.

Superoxide Dismutase Activity

Superoxide dismutase (E.C.1.15.1.1) activity was assessed by quantifying the inhibition of superoxide-dependent adrenaline auto-oxidation in a spectrophotometer at 480 nm, as previously described (Misra and Fridovich, 1972). Results were expressed as Units SOD.mg protein⁻¹.

Nitrite-nitrate Levels

The NO[•] metabolites, nitrites (NO₂⁻) and nitrates (NO₃⁻) were determined as previously described (Hevel and Marletta, 1994). Five hundred µl of supernatant were deproteinized with 20 µl 25 % (w/v) trichloroacetic acid and centrifuged at 1800 X g. The supernatant was immediately neutralized with 35 µl 2 M potassium bicarbonate and used for the quantification of NO₂⁻ and NO₃⁻. NO₃⁻ was reduced to NO₂⁻ by nitrate reductase. The total NO₂⁻ in the incubation was measured by a colorimetric assay at 540 nm based on the Griess reaction. A standard curve was performed in the same way using sodium nitrate (0–80 µM). Results were expressed as µM NO₂⁻.mg protein⁻¹.

Measurement of protein Carbonyls

The oxidative damage to proteins was measured by the quantification of carbonyl groups, based on a reaction with dinitrophenylhydrazine, as previously described (Levine et al., 1990). Proteins were precipitated by the addition of 20% trichloroacetic acid and redissolved in dinitrophenylhydrazine and the absorbance read in a spectrophotometer at 370 nm. Results were expressed as nmol carbonyl.mg protein⁻¹.

TBARS

As an index of lipid peroxidation, the formation of thiobarbituric acid reactive species (TBARS) was monitored during an acid-heating reaction, which is widely adopted for measurement of lipid redox state, as previously described (Draper and Hadley, 1990). The samples were mixed with 0.6 ml of 10 % trichloroacetic acid and 0.5 ml of 0.67 % thiobarbituric acid, and then heated in a boiling water bath for 25 min. Thiobarbituric acid reactive species were determined by the absorbance in a spectrophotometer at 532 nm. Results were expressed as thiobarbituric acid reactive species.mg protein⁻¹.

GFAP Measurement

Enzyme-linked immunosorbent assay was carried out for measurement of GFAP content, as previously described (Tramontina et al., 2007). Briefly, the microtiter plate was coated with 100 µL samples containing 500 ng of protein for 24 hours at 4 °C. Incubation with a polyclonal anti-GFAP from rabbit for 1 hour was followed by

incubation with a secondary antibody conjugated with peroxidase or 1 hour, at room temperature. A colorimetric reaction with o-phenylenediamine was measured at 493 nm. The standard human GFAP (from Calbiochem) curve ranged from 0.1 to 5 ng.mL⁻¹.

S100B Measurement

An enzyme-linked immunosorbent assay was carried out for measurement of intracellular S100B content, as previously described (Leite et al., 2008). Briefly, 50 µL of sample (5-10 ng.µl⁻¹ of total protein) plus 50 µl of Tris buffer were incubated for 2 hours on a microtiter plate previously coated with anti-S100B monoclonal antibody (SH-B1, from Sigma). Anti-S100 polyclonal antibody (from DAKO) was incubated for 30 min and then peroxidase-conjugated anti-rabbit antibody was added for a further 30 min. The color reaction with o-phenylenediamine was measured at 492 nm. The standard S100B curve ranged from 0.002 to 1 ng.ml⁻¹.

Glutamate uptake assay

Hippocampal slices were transferred immediately to 24-well culture plates, each well containing 0.3 mL of physiological medium and only one slice. Glutamate uptake was measured as previously described (Thomazi et al., 2004) with some modifications. Medium was replaced by Hank's balanced salt solution (HBSS) containing (in mM): NaCl 137; Na₂HPO₄ 0.63, NaHCO₃ 4.17, KH₂PO₄ 0.44, KCl 5.36, CaCl₂ 1.26, MgSO₄ 0.41, MgCl₂ 0.41 and glucose 5.55, in pH 7.4. The assay was started by the addition of 0.1 mM L-glutamate and 0.66 Ci.mL⁻¹ L-[2,3-

^3H] glutamate. Incubation was stopped after 10 min by removal of the medium and rinsing the slices twice with ice-cold HBSS. Slices were then lysed in a solution containing 0.5 M NaOH. Final glutamate uptake was obtained by discounting non-specific uptake from specific uptake in assays carried out in sodium-free medium, prepared by replacing NaCl with choline chloride in the HBSS. Radioactivity was measured with a scintillation counter and the results were expressed as $\eta\text{mol.mg protein}^{-1}.\text{min}^{-1}$.

Glutamine synthetase activity

The enzymatic assay was performed, as previously described (dos Santos et al., 2006). Homogenized tissue samples (0.1 mL) were added to 0.1 mL of reaction mixture containing (in mM): MgCl_2 10, L-glutamate 50; imidazole-HCl 100 buffer, pH 7.4; 2-mercaptoethanol 10; hydroxylamine-HCl 50; ATP 10 and incubated for 15 min at 37 °C. The reaction was stopped by the addition of 0.4 mL of a solution containing (in mM): ferric chloride 370; HCl 670; trichloroacetic acid 200. After centrifugation, the supernatant was measured at 530 ηm and compared to the absorbance generated by standard quantities of glutamylhydroxamate treated with ferric chloride reagent. The results were expressed as $\text{mmol.mg protein}^{-1}.\text{hour}^{-1}$.

Mitochondrial viability assay

Mitochondrial activity was evaluated by the colorimetric 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method. Briefly, after a recovery period, slices were incubated in a medium, containing $45 \mu\text{g.ml}^{-1}$ MTT, for 45min at 37°C.

Active mitochondrial dehydrogenases of living cells cause cleavage and reduction of the soluble yellow MTT dye to the insoluble purple formazan, which was extracted in dimethyl sulfoxide (DMSO) (Mosmann, 1983). The optical density was measured at 570 and 630 nm, and the net A570 – A630 was taken as an index of cell viability (Siqueira et al., 2004).

Protein Content

The total protein content was determined by the modified method of Lowry (Peterson, 1977), using bovine serum albumin (BSA) as standard.

Statistical analysis

Data are expressed as mean \pm standard error of mean (SE). The values were considered significant when $p \leq 0.05$. Differences in experimental groups were determined by one-way ANOVA followed by the post hoc Newman-Keuls' test when indicated.

Results

Changes in body weight gain and serum biochemical parameters during physical training and caloric restriction

The performances of the two rat groups (AE and RE) submitted to physical exercise during 12 weeks are shown in Fig 1A. Their running capacity improved during the first 4 weeks of training and remained stable afterwards. No significant

difference in exercise performance was observed in these groups, independently of *ad libitum* or restricted feeding. Body weight gain at the end of the experimental protocol is shown in Fig 1B. Rats submitted to caloric restriction, sedentary or not, had a decrease of 27% in body weight gain during these 12 weeks. Table 2 shows serum biochemical parameters for the four groups. No differences were observed in glycemia, total proteinemia or levels of reactive-C protein, indicating a good health state in all groups. LDL content decreased in rats submitted to caloric restriction, independent of training/sedentary protocol. No change was observed in HDL content. Total cholesterol decreased in rats submitted to caloric restriction and exercise (RE) compared to *ad libitum* fed rats. No significant changes were observed in triacylglycerol content among groups. Serum aspartate aminotransferase transaminase (AST) activity was lower in rats submitted to physical training (AE and RE) than the AS group. No change was detected in serum alanine aminotransferase transaminase (ALT). Urea levels decreased in rats submitted to caloric restriction, but creatinine levels were not different.

Physical exercise and caloric restriction affect the hippocampal redox status

The glutathione content in the hippocampus increased after caloric restriction and/or physical exercise (Fig 2A). Moreover, using a chemiluminiscent assay with luminol we found that total antioxidant reactivity (TAR) also increased after caloric restriction and/or physical exercise (Fig 2B), but total reactive antioxidant potential (TRAP) was not different among groups (data not shown). It is also important to mention that hippocampal SOD activity was also not different (data not shown).

Interestingly, the NO[•] content was reduced in rats submitted to the combination of caloric restriction and physical training (Fig 2C).

Two parameters were investigated to evaluate hippocampal oxidative damage, for instance lipid peroxidation and protein carbonylation. Lipid peroxidation did not differ among groups (Fig 3A), but caloric restriction and/or physical exercise decreased the content of protein carbonylation (Fig 3B).

Astroglial protein markers under the influence of caloric restriction and physical exercise

Assuming that glutathione is predominantly astroglial, we investigated alterations in two specific markers for these cells; GFAP and S100B. No differences were observed in the hippocampal immunocentents of GFAP or S100B among the experimental groups (Fig 4A and 4B, respectively).

Astroglial activity measured by glutamate uptake and glutamine synthetase in hippocampal slices

Two functional parameters of astroglial activity were investigated in hippocampal slices; glutamate uptake (Fig 5A) and glutamine synthetase (Fig 5B). Only the caloric restriction by itself (RS group) was able to induce an increase in GS activity, while the hippocampal glutamate uptake was increased by caloric restriction independently of physical activity (RS and RE groups). In order to confirm the equivalent viability of ex-vivo hippocampal slices we measured MTT reduction capacity and found no differences among the studied groups (Fig 5C).

Discussion

As life expectancy increases, the incidence of age-related neurodegenerative conditions such as Alzheimer's disease, Parkinson's disease and stroke has increased, the last being the most prevalent (Mattson, 2000). In addition to benefiting learning and memory, extensive research demonstrates that exercise has neuroprotective effects, reducing brain injury and delaying onset of neurodegenerative diseases (van Praag et al., 1999a, Cotman et al., 2007) while caloric restriction lowers the incidence of several other age-related diseases and is highly associated with increased longevity (Prolla and Mattson, 2001). Chronic exercise, through an adaptative response, is able to improve the antioxidant system, inhibiting the extension of the oxidative insult induced by acute exercise (Powers et al., 1994, Leeuwenburgh et al., 1997, Servais et al., 2003). Calorie-restricted animals display less RS-mediated damage (Merry, 2002, Barja, 2004), which may be related to a resistance to stress-induced apoptosis (Hiona and Leeuwenburgh, 2004).

Our study investigated the effects of moderate exercise training and chronic caloric restriction on hippocampus glial parameters and the CNS redox status of rats, since reactive oxygen/nitrogen species have been related to aging and neurodegenerative disease processes.

Differences in protocols results in different levels of oxidative stress, however the data are still controversial. The variability in results reported probably arises from differences in the exercise protocol (voluntary vs forced), the kind of exercise (running vs swimming) in combination with intensity (in forced exercise models)

and duration of exercise exposure (acute vs chronic) (Cotman et al., 2007). Indeed, most benefits have been associated with longer-term exercise (3-12 weeks) (van Praag et al., 2005, Schweitzer et al., 2006, O'Callaghan et al., 2007). It has been demonstrated that performance of moderate intensity exercise (treadmill training protocol) with a frequency of three times a week, for 12-weeks, reduces damage in hippocampal slices from Wistar rats that were submitted to an in vitro ischemia protocol, suggesting exercise-induced neuroprotection (Cechetti et al., 2007).

The CR increases longevity, independently of whether protein intake is also reduced or not, providing evidence that energy intake could play a greater role in life extension than protein intake reduction (Masoro, 2005). A 30% reduction in calorie intake extends the life spans of rats and mice by 30-40%, and maintenance of this regimen for 2-4 months results in lower levels of oxidative stress in the hippocampus, compared to mice fed ad libitum, indicating that suppression of oxidative stress may be one mechanism underlying the neuroprotective effect of caloric restriction (Mattson, 2000).

As an indirect measure of training status, we observed that the rats' performance improved during the 3 months of running training, in agreement with other studies (Radak et al., 2005, Radak et al., 2006). Moreover, the CR-fed rats (RS and RE) presented a lower body mass gain than ad libitum-fed rats (AS and AE), in accordance with other reports (Horska et al., 1999, Wanagat et al., 1999). To ensure that the animals were not undernourished or physically exhausted, we performed biochemical serum analysis. The CR-fed rats, RS and RE, showed benefits in health, demonstrating lower levels of total cholesterol and LDL

cholesterol than ad libitum-fed rats. Additionally, the exercised rats, AE and RE, displayed lower AST activities than the sedentary, AS group, suggesting that the exercise training program used in this experiment results in lower muscular tissue damage (Nelson and Cox, 2005). Although creatinine levels were not different, we found decreased urea serum levels in CR-fed rats, indicating that renal glutaminase activity may be altered in these animals, as observed in a prior study (Ribeiro et al., 2009). Since no differences in other parameters were observed, rats were deemed to be healthy.

GSH is an important redox sensor for most cell types, and plays a crucial role as a redox buffer in the central nervous system (Aoyama et al., 2008). Previous studies have shown that GSH declines with aging (Maher, 2005) and that GSH deficiency is involved in neurodegenerative diseases (Sian et al., 1994, Christen, 2000). The three interventions studied were each able to increase GSH levels and, probably as a consequence, the TAR. TRAP and the TAR are parameters of total non-enzymatic capacity, although TAR is more related to antioxidant quality while TRAP is more related to antioxidant amount (Lissi et al., 1995).

Our study demonstrated that non-enzymatic adaptation may be responsible for the reduced damage, as opposed to enzymatic adaptation, since, in the present study, no modifications were found in SOD activity. Somani and coworkers also found no changes in hippocampal SOD activity after 7.5-weeks of exercise (Somani et al., 1995). In contrast, Devi and Kiran found increased SOD activity in the hippocampus after 4 months of swimming exercise, compared to controls, while Asku and coworkers demonstrated a decreased SOD activity when regular

treadmill exercise was performed at different strengths (Devi and Kiran, 2004, Aksu et al., 2009). Different results in these studies may be due to the differences in type, duration and intensity of the exercise.

Protein carbonyl content, a measure of protein oxidation, increases with age most rapidly in the hippocampus and striatum (Dubey et al., 1996), regions associated with significant losses in function due to the aging process. To evaluate oxidative damage in the hippocampus, we measured the lipid peroxidation by TBARS and protein oxidation by carbonyl content. Although no differences were seen for TBARS, the exercise training and caloric restriction, and both combined, decreased carbonyl contents, indicating an attenuation of aging that is in agreement with other caloric restriction studies (Dubey et al., 1996, Forster et al., 2000). Other authors found similar results for TBARS following chronic exercise (Coskun et al., 2005, Aksu et al., 2009).

NO[•] plays important roles in central nervous system, participating in neurogenesis, neuron differentiation and development, memory and neuroprotection (Garthwaite, 2008). In addition, NO[•] can react very fast with $\cdot\text{O}_2^-$ to form peroxynitrite (ONOO⁻), which can directly oxidize and nitrate proteins, lipids and DNA (Alvarez and Radi, 2003). NO₂⁻/NO₃⁻ levels are a good indication of NO[•] production, since these molecules are the metabolism end products of NO[•] (Levine, 2002, Halliwell and Whiteman, 2004). The decreased NO₂⁻/NO₃⁻ levels observed following the combination of the two approaches could indicate that only regular exercise associated with chronic caloric restriction are effective in reducing NO[•]. Interestingly, Asku and coworkers did not find any change in brain (prefrontal

cortex, striatum and hippocampus) nitrate-nitrite levels after chronic treadmill exercise by itself (Aksu et al., 2009).

Astrocytes are closely associated with neurons in glutamatergic transmission and, consequently, with synaptic plasticity and neuroprotection (Chen and Swanson, 2003, Tramontina et al., 2006b). In the present study, we investigated important astroglial functions such as glutamate uptake (which avoids excitotoxicity damage that could lead to neuronal death) (Danbolt, 2001), and the ability to convert glutamate into glutamine via glutamine synthetase activity. Although only caloric restriction by itself (RS group) was able to increase GS activity, this intervention, associated or not to exercise, showed an increase in glutamate uptake from extracellular media due to modulation in activity and/or the amount of glutamate transport, enhancing one of the most important functions of astrocytes. This is in agreement with prior work (Ribeiro et al., 2009).

After injury of the CNS, either as a result of trauma, disease, genetic disorders or chemical insult, astrocytes become reactive, termed astrogliosis, and this activation is characterized by an increase in GFAP (Eng et al., 2000). Increasing evidence indicates that S100B exerts functional roles by acting as an intracellular regulator and an extracellular signal (Donato et al., 2009). S100B is secreted by an unknown mechanism and has dual effects: at nanomolar levels, S100B stimulates neurite growth and promotes neuronal survival, and at micromolar levels this protein produces undesirable events such as neuronal apoptosis (Van Eldik and Wainwright, 2003, Donato et al., 2009). High levels of brain tissue S100B have been found in neurodegenerative disorders, including Alzheimer's disease (Griffin

et al., 1998). In the present study, GFAP and S100B contents were not influenced by caloric restriction and/or exercise, indicating absence of astrogliosis and non predisposition to apoptosis (O'Callaghan and Sriram, 2005).

Conclusion

The present study demonstrated an improvement in antioxidant system following exercise training, caloric restriction and both in combination, leading to a significant modulation of astroglial functions. Moreover, caloric restriction improved glutamate uptake and glutamine synthetase activity, which could be related to a lower risk of excitotoxicity. These findings provide new insights into how caloric restriction, allied with regular physical activity, could be a strategy for the prevention of neurodegenerative diseases.

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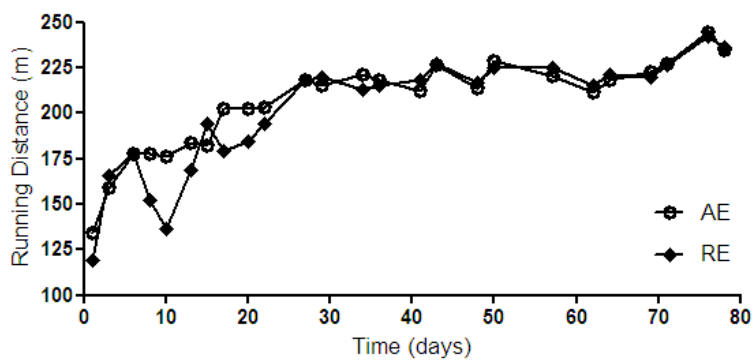
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Figures and captions

Figura 1

(A)



(B)

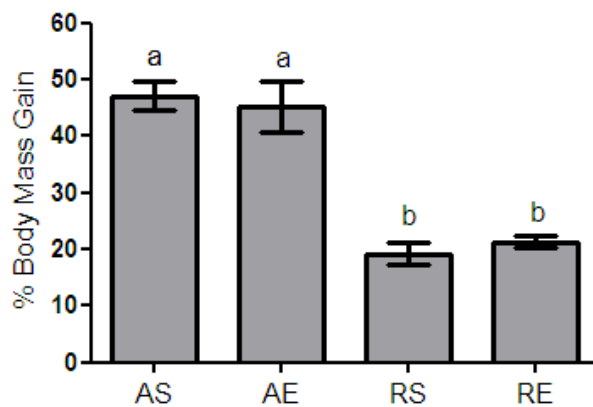
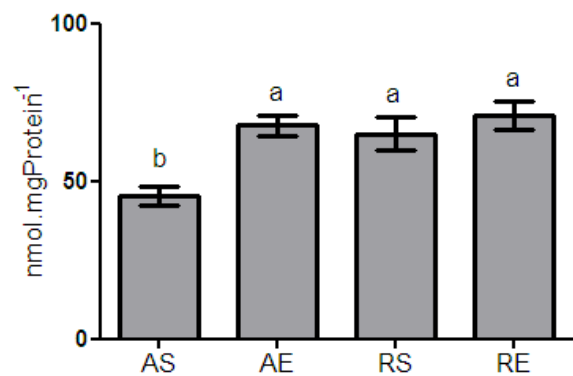


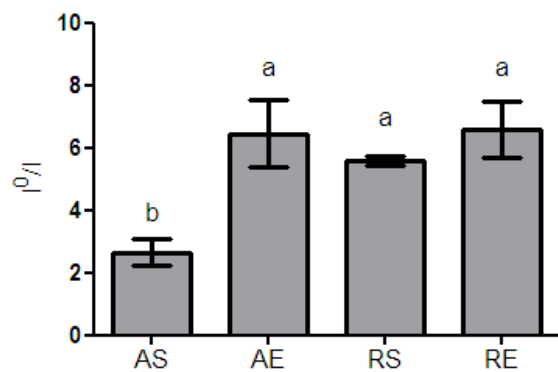
Fig. 1. A: Rats performance throughout experiment period (n = 10 for each group), evaluated only on ad libitum exercised (AE) and restricted exercised (RE) groups; Data are expressed as mean; B: Body mass gain after experiment period (n = 10 for each group). Data are expressed as mean \pm standard error of mean; (a) different of restricted groups (RS and RE), (b) different of ad libitum groups (AS and AE); Differences were determined with Anova one way followed by Newman Keul's pos hoc and the accepted significance level was $p \leq 0.05$.

Figura 2

(A)



(B)



(C)

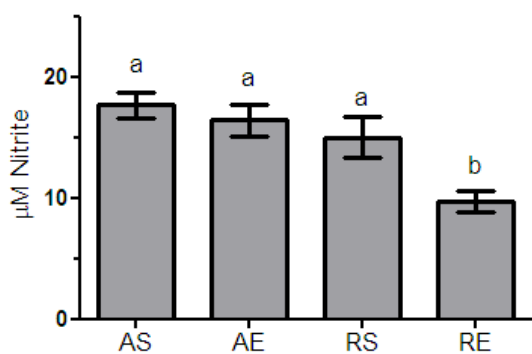
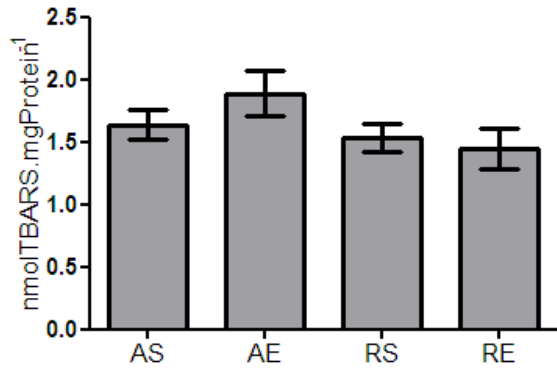


Fig. 2. A: Reduced glutathione (GSH) content; (a) different of ad libitum sedentary (AS), (b) different of ad libitum exercised (AE), of restricted sedentary (RS) and of restricted exercised (RE); B: Total antioxidant reactivity (TAR); (a) different of ad libitum sedentary (AS), (b) different of ad libitum exercised (AE), of restricted sedentary (RS) and of restricted exercised (RE); C: Nitrite (NO²⁻) and nitrate (NO³⁻) levels. (a) different of restricted exercised (RE), (b) different of ad libitum sedentary (AS), of ad libitum exercised (AE) and of restricted sedentary (RS); Data are expressed as mean ± standard error of mean (n = 5 for each group); Differences were determined with Anova one way followed by Newman Keul's pos hoc and the accepted significance level was $p \leq 0.05$.

Figura 3

(A)



(B)

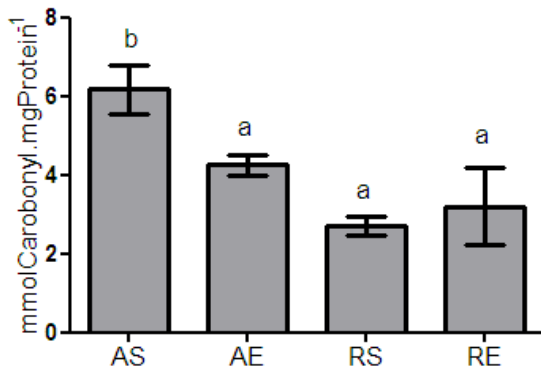
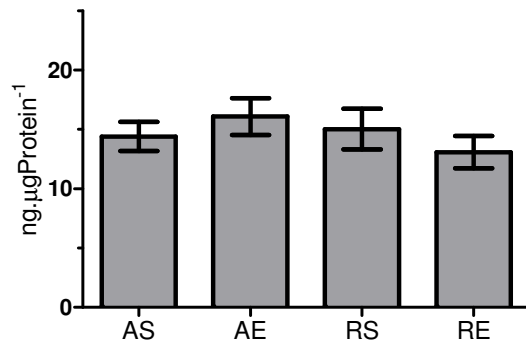


Fig. 3. A: Thiobarbituric acid reactive species (TBARS) content; B: Protein carbonyl content; Data are expressed as mean \pm standard error of mean (n = 5 for each group); (a) different of ad libitum sedentary (AS), (b) different of ad libitum exercised (AE), of restricted sedentary (RS) and of restricted exercised (RE); Differences were determined with Anova one way followed by Newman Keul's pos hoc and the accepted significance level was $p \leq 0.05$.

Figura 4

(A)



(B)

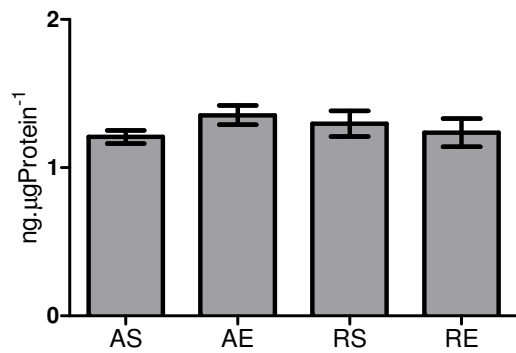
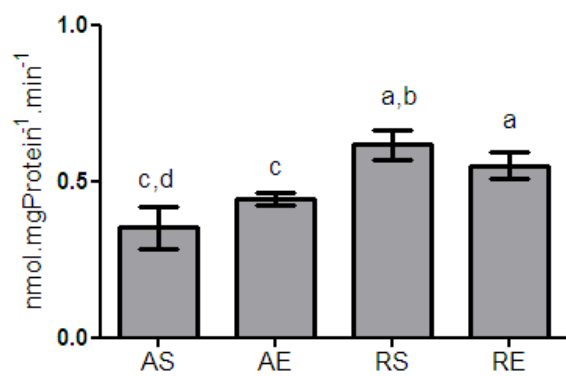


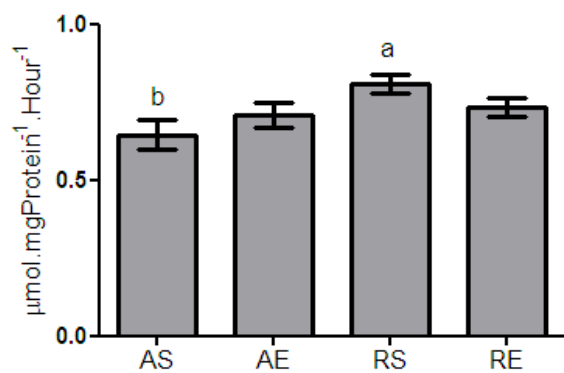
Fig. 4. A: Glial fibrillary acid protein (GFAP) content; B: S100B content; Data are expressed as mean \pm standard error of mean (n = 5 for each group); Differences were determined with Anova one way followed by Newman Keul's pos hoc and the accepted significance level was $p \leq 0.05$.

Figura 5

(A)



(B)



(C)

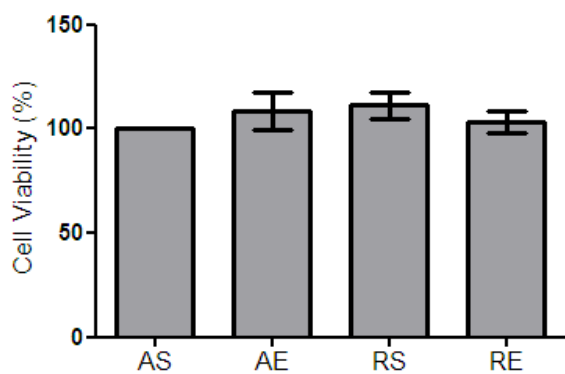


Fig. 5. A: Glutamate uptake; (a) different of ad libitum sedentary (AS), (b) different of ad libitum exercised (AE), (c) different of restricted sedentary (RS) and (d) different of restricted exercised (RE) B: Glutamine synthetase activity; (a) different of ad libitum sedentary (AS), (b) different of restricted sedentary (RS), C: Cell viability; Data are expressed as mean \pm standard error of mean (n = 5 for each group);; Differences were determined with Anova one way followed by Newman Keul's pos hoc and the accepted significance level was $p \leq 0.05$.

Table 1. Composition of the laboratory chow

Composition	(g/Kg)
Total fat	110
Sunflower oil	5
Proteins	220
Fibers	30
Ash	60
Vitamins	20
Carbohydrates	520

Commercial nonpurified diet, Nuvilab-CR1 (PR – Brazil)

Table 2. Serum biochemistry

	AS	AE	RS	RE
Glucose (mg.dL ⁻¹)	222 ± 4	186 ± 29	219 ± 3	214 ± 11
Cholesterol (mg.dL ⁻¹)	69 ^a ± 4	75 ^a ± 5	60 ^b ± 4	56 ^b ± 2
Triacylglycerol (mg.dL ⁻¹)	63 ± 13	58 ± 15	65 ± 10	61 ± 5
HDL (mg.dL ⁻¹)	27 ± 0,6	30 ± 1,3	27 ± 1,4	24 ± 0,4
LDL (mg.dL ⁻¹)	32 ^a ± 2	34 ^a ± 4	20 ^b ± 1	19 ^b ± 2
Creatinine (mg.dL ⁻¹)	0,58 ± 0,05	0,60 ± 0,04	0,56 ± 0,02	0,51 ± 0,04
Urea (mg.dL ⁻¹)	62 ^a ± 2,3	57 ^a ± 2,6	45 ^b ± 2,4	46 ^b ± 2,6
Total Proteins (g.dL ⁻¹)	6,2 ± 0,3	6,2 ± 0,2	6,1 ± 0,1	6,1 ± 0,2
C-Reactive Protein (mg.dL ⁻¹)	3,6 ± 0,1	3,3 ± 0,2	3,5 ± 0,1	3,5 ± 0,1
AST (U.L ⁻¹)	112 ^c ± 5	86 ^d ± 1	95 ^d ± 2	87 ^d ± 1
ALT (U.L ⁻¹)	55 ± 2	50 ± 1	51 ± 4	49 ± 4

Data are expressed as mean ± standard error of mean (n=5 for each group). (a) different of restricted groups (RS and RE), (b) different of ad libitum groups (AS and AE), (c) different of ad libitum exercised (AE), of restricted sedentary (RS) and of restricted exercised (RE), (d) different of ad libitum sedentary (AS). Differences were determined with Anova one way followed by Newman Keul's pos hoc and the accepted significance level was p≤0,05.

Parte III

Discussão

Durante os últimos anos, observou-se um aumento da expectativa de vida média da população mundial acompanhado do aumento na prevalência de doenças neurodegenerativas, como a doença de Alzheimer, a doença de Parkinson, e a isquemia cerebral. Com isso, houve um aumento do interesse público e científico nos efeitos benéficos da atividade física e da dieta visando a manutenção cognitiva e a saúde mental na longevidade (Mattson, 2000, Kramer and Erickson, 2007).

O exercício físico praticado regularmente, através de uma resposta adaptativa, é capaz de melhorar as defesas antioxidantes, aumentando a atividade de enzimas antioxidantes e o sistema GSH, dessa forma diminuindo a extensão do dano oxidativo observado durante sessões agudas de treinamento físico (Powers et al., 1994, Leeuwenburgh et al., 1997, Servais et al., 2003).

Estudos com ratos e camundongos mantidos em restrição calórica demonstraram uma diminuição de dano relacionado a espécies reativas, incluindo uma menor peroxidação lipídica, menor dano oxidativo a proteínas e ao DNA (Merry, 2002, Barja, 2004), o que poderia estar relacionado a uma maior resistência à apoptose, induzida por adaptação ao estresse (Hiona and Leeuwenburgh, 2004).

O objetivo deste estudo foi investigar os efeitos de protocolos de treinamento físico moderado e restrição calórica sobre o estado redox e parâmetros gliais no hipocampo de ratos, uma vez que o estresse oxidativo está

relacionado com o desenvolvimento de desordens neurodegenerativas e com o envelhecimento. Os principais resultados são apresentados na figura 4.

Os pesquisadores têm usado diferentes protocolos de treinamento (forçado vs voluntário), que também variam em tempo de treinamento (semanas vs meses), duração das sessões, intensidade e tipo de atividade (corrida vs nado) (Cotman et al., 2007). Recentemente, foi desenvolvido um protocolo de exercício físico moderado em esteira, com sessões três vezes por semana durante doze semanas, capaz de reduzir o dano a fatias hipocâmpais submetidas a um protocolo de isquemia *in vitro*, sugerindo neuroproteção induzida pelo exercício (Cechetti et al., 2007).

Os efeitos benéficos de um regime de restrição calórica são devidos à restrição da ingestão de calorias, ao contrário da restrição de um componente alimentar específico (gorduras, por exemplo) ou da redução de algum componente alimentar tóxico (aditivos, conservantes, por exemplo) (Masoro, 1998, 2000). Uma redução de 30% na ingestão calórica demonstrou aumento na longevidade de ratos e camundongos, e a manutenção desta condição resultou em níveis menores de estresse oxidativo no hipocampo destes animais quando comparados com outros alimentados *ad libitum* (Mattson, 2000). Estes estudos sugerem que a supressão do estresse oxidativo pode ser um dos mecanismos pelos quais a restrição calórica induza neuroproteção.

Neste estudo foi observada uma melhora na *performance* dos ratos submetidos ao treinamento físico moderado em esteira, o que também foi observado em outros estudos (Radak et al., 2005, Radak et al., 2006). Os animais

submetidos à restrição calórica de 30% apresentaram um ganho de massa corporal aproximadamente 27% menor que os animais alimentados *ad libitum*, corroborando com estudos prévios (Horska et al., 1999, Wanagat et al., 1999).

Para assegurar que os animais utilizados neste estudo não estavam fisicamente exauridos ou desnutridos foram realizadas análises bioquímicas séricas. Os ratos submetidos à restrição calórica apresentaram níveis menores de colesterol total e LDL colesterol que os ratos alimentados *ad libitum*, demonstrando benefícios da restrição calórica à saúde de forma geral. Os ratos exercitados apresentaram níveis menores de atividade da transaminase aspartato aminotransferase (AST) quando comparados ao grupo controle sedentário, sugerindo que o programa de exercício físico utilizado resultou em menor dano muscular (Nelson and Cox, 2005). Os níveis de creatinina entre os grupos não foram significativamente diferentes, assegurando a manutenção da função renal, porém foram encontrados níveis séricos diminuídos de uréia, podendo vir a ser objeto de futura investigação da atividade da glutaminase renal. Este resultado também está de acordo com estudo prévio (Ribeiro et al., 2009). Os demais parâmetros estudados (glicose, triglicérides, HDL, proteínas totais, proteína C reativa, alanina aminotransferase), não apresentaram alteração, indicando que os animais estavam saudáveis.

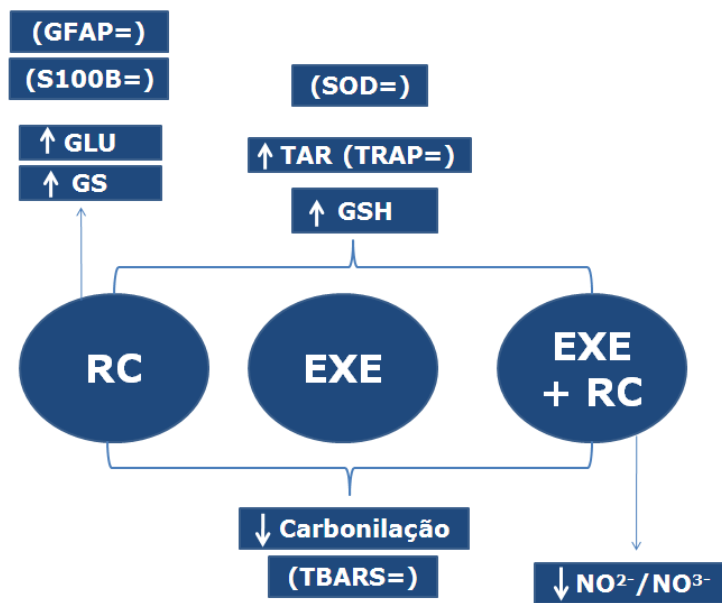


Figura 4: Principais resultados do estudo.

O sistema glutaciona é um importante sensor redox para a maioria das células, e desempenha um papel fundamental no sistema nervoso central como um tampão redox (Aoyama et al., 2008). Estudos mostraram um declínio do conteúdo de GSH com o envelhecimento (Maher, 2005) e a sua deficiência está intimamente relacionada a processos neurodegenerativos (Sian et al., 1994, Christen, 2000). O exercício físico, a restrição calórica e a combinação das duas intervenções aumentaram os níveis de glutaciona e, provavelmente em consequência, a reatividade antioxidante total. O potencial antioxidante reativo total (TRAP) e a reatividade antioxidante total (TAR) são parâmetros de capacidade antioxidante não enzimática, mas o ensaio TAR estaria mais relacionado com a qualidade antioxidante, enquanto o TRAP à quantidade antioxidante (Lissi et al., 1995). Então, apesar de não ter sido observada melhora

na quantidade de defesas antioxidantes não enzimáticas (TRAP), as intervenções (exercício, restrição e ambos) melhoraram a qualidade antioxidante não enzimática (TAR).

Nosso estudo demonstrou que uma adaptação não enzimática pode ser responsável por diminuir o dano oxidativo, ao contrário de uma adaptação enzimática, já que neste estudo não foram encontradas modificações na atividade da SOD. Somani e colaboradores também não encontraram mudanças na atividade da SOD hipocampal após 7,5 semanas de exercício (Somani et al., 1995). Devi e Kiran, por outro lado, reportaram um aumento da atividade desta enzima no hipocampo após 4 meses de nado, enquanto Asku e colaboradores evidenciaram uma diminuição da atividade da SOD com exercício regular em esteira sob diferentes intensidades (Devi and Kiran, 2004, Aksu et al., 2009). Os diferentes resultados destes estudos podem ser atribuídos a diferenças no tipo, duração e intensidade dos protocolos de exercício aplicados.

O conteúdo de proteínas carboniladas, tomado como medida da oxidação protéica, aumenta com o envelhecimento mais rapidamente no hipocampo e no estriado, regiões associadas com perdas significativas de função durante o processo de envelhecimento (Dubey et al., 1996). Para mensurar o dano oxidativo no hipocampo, foi avaliada a peroxidação lipídica através de TBARS e a oxidação protéica através do conteúdo de proteínas carboniladas. O exercício físico, a restrição calórica e a combinação dos dois tratamentos não causaram nenhuma alteração no ensaio TBARS, mas foram aptos a diminuir o conteúdo de proteínas carboniladas. Estes resultados poderiam estar associados de forma positiva com a

atenuação do processo de envelhecimento, o que está de acordo com outros estudos sobre restrição calórica (Dubey et al., 1996, Forster et al., 2000). Outros pesquisadores encontraram resultados similares para o TBARS com exercício físico regular (Coskun et al., 2005, Aksu et al., 2009).

O óxido nítrico desempenha um papel importante no SNC, incluindo neurogênese, diferenciação e desenvolvimento neuronal, memória e neuroproteção (Ignarro, 2002, Garthwaite, 2008). No entanto, o NO^\cdot pode reagir rapidamente com o ânion superóxido formando peroxinitrito, o qual pode causar oxidação e nitração em proteínas, lipídeos e DNA (Alvarez and Radi, 2003). Os níveis de nitratos e nitritos são um bom indicativo da produção de óxido nítrico, já que são produtos finais do seu metabolismo (Levine, 2002, Halliwell and Whiteman, 2004). Um decréscimo nos níveis de $\text{NO}_2^-/\text{NO}_3^-$ observado com a combinação das duas intervenções pode indicar que somente o exercício físico regular em associação à restrição calórica crônica é capaz de causar esta diminuição. Aksu e colaboradores não encontraram nenhuma alteração nos níveis de $\text{NO}_2^-/\text{NO}_3^-$ no cérebro (córtex pré-frontal, estriado e hipocampo) de ratos submetidos somente a exercício regular em esteira (Aksu et al., 2009).

Os astrócitos possuem uma íntima relação com os neurônios na transmissão glutamatérgica, por consequência, na plasticidade sináptica e neuroproteção (Chen and Swanson, 2003, Tramontina et al., 2006a). No presente estudo, foram investigadas funções astrogliais importantes como a captação de glutamato, essencial para evitar o fenômeno de excitotoxicidade que poderia levar a morte neuronal (Danbolt, 2001) e a habilidade em converter glutamato em

glutamina através da glutamina sintetase (GS). Somente a restrição calórica demonstrou aumento na atividade da GS, e esta intervenção, associada ou não ao exercício, também foi capaz de aumentar a captação de glutamato do meio extracelular. Este resultado pode ser atribuído à modulação da atividade e/ou um aumento nos transportadores de glutamato localizados nos astrócitos. Assim, a restrição calórica estaria melhorando uma das principais funções dos astrócitos que é a manutenção adequada do metabolismo do glutamato. Estes resultados estão de acordo com um estudo anterior (Ribeiro et al., 2009).

Para a avaliação da captação de glutamato e da atividade da GS, indicadores da atividade astrogliar, foram utilizadas fatias hipocâmpais. Com o propósito de assegurar a integridade destas fatias entre os grupos estudados foi realizado o ensaio de viabilidade mitocondrial (MTT), onde não foram encontradas diferenças, validando os resultados obtidos.

Uma vez que a glutatona é predominantemente astrogliar e que, a restrição calórica e o exercício físico aumentaram os níveis de GSH, foram investigadas alterações em dois marcadores específicos destas células – GFAP e S100B. O processo de astrogliose resultante de dano no SNC por trauma, doença, desordem genética ou insulto químico, é evidenciado por um aumento de GFAP (Eng et al., 2000). Da mesma forma, altos níveis de S100B no tecido cerebral foram encontrados em casos de doenças neurodegenerativas, incluindo Alzheimer (Griffin et al., 1998). No presente estudo, o conteúdo de GFAP e S100B não foi influenciado pela restrição calórica nem pelo exercício físico, indicando

ausência de astrogliose e ausência de pré-disposição à apoptose (O'Callaghan and Sriram, 2005).

Resumindo, o presente estudo demonstrou uma melhora no sistema antioxidante não enzimático e uma diminuição do dano oxidativo protéico através de restrição calórica crônica e exercício físico moderado. Somente a combinação das duas intervenções foi capaz de diminuir os níveis de nitratos e nitritos. A restrição calórica, possivelmente através de modulação da função astrogliar, foi capaz de promover uma melhora na captação de glutamato e na atividade da glutamina sintetase, o que pode ser relacionado a um menor risco de excitotoxicidade. Estes resultados nos dão novas perspectivas para compreender os mecanismos pelos quais a restrição calórica aliada ao exercício físico pode prevenir doenças neurodegenerativas e promover a longevidade.

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