UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS - BIOQUÍMICA

EFEITO NEUROPROTETOR DO EXERCÍCIO FÍSICO EM RATAS ADULTAS OVARIECTOMIZADAS

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Dissertação apresentada ao Programa de Pós-Graduação em Ciências Biológicas - Bioquímica, ICBS da Universidade Federal do Rio Grande do Sul como requisito parcial à obtenção do grau de Mestre em Bioquímica

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À minha mãe pelo incentivo constante,

paciência e apoio em toda minha vida,

dedicação e amor incondicional.

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A vida é uma peça de teatro que não permite ensaios. Por isso, cante, chore, dance, ria e viva intensamente, antes que a cortina se feche e a peça termine sem aplausos.

(Charles Chaplin)

RESUMO

Considerando que a deficiência hormonal causada pela ovariectomia promove alterações nas atividades das enzimas Na^+,K^+-ATPase, acetilcolinesterase (AChE) e ectonucleotidases e pode prejudicar a memória em ratas, no presente trabalho nós investigamos a influência do exercício físico sobre a ativação da Na^+,K^+-ATPase e acetilcolinesterase em hipocampo e córtex cerebral causada pela ovariectomia em ratas adultas, bem como sobre a hidrólise de nucleotídeos de adenosina no córtex cerebral e no soro. Também investigamos o efeito do exercício sobre a memória espacial e aversiva em ratas adultas ovariectomizadas. Ratas Wistar adultas foram divididas em quatro grupos: sham (submetidas à cirurgia sem a remoção dos ovários), exercício, ovariectomizadas (Ovx) e Ovx+exercício. Trinta dias após a cirurgia, os animais foram submetidos a um mês de exercício físico por 20 min, três vezes por semana. Logo após, as ratas foram decapitados, o soro coletado e o hipocampo e córtex cerebral dissecados, ou foram submetidas às tarefas de esquiva inibitória e labirinto aquático de Morris. Os dados demonstraram que o exercício físico reverte a ativação das atividades da Na^+,K^+-ATPase e AChE em hipocampo e córtex cerebral de ratas ovariectomizadas. A ovariectomia diminuiu a hidrólise de AMP no córtex cerebral e não alterou a hidrólise de nucleotídeos de adenosina sérica. Exercício *per se* diminuiu a hidrólise de ADP e AMP em córtex cerebral. Resultados também mostraram que ratas ovariectomizadas apresentaram prejuízo na memória aversiva e espacial (memória de referência e de trabalho), quando comparadas ao grupo controle (sham). Confirmando nossa hipótese, o déficit na memória foi revertido pelo exercício físico. Nossos achados mostram que a ovariectomia prejudica significativamente a memória/aprendizado aversiva e espacial e altera as enzimas Na^+,K^+-ATPase e AChE, e que o exercício físico preveniu tais efeitos. Esses dados sugerem que o exercício físico pode se mostrar uma estratégia importante para minimizar déficits cognitivos encontrados em mulheres pós-menopáusicas.

ABSTRACT

Hormone deficiency following ovariectomy causes activation of Na+,K+-ATPase and acetylcholinesterase (AChE), that has been related to cognitive deficits in experimental animals, and memory impairment. Considering that physical exercise presents neuroprotector effects, we decide to investigate whether exercise training would affect enzyme activation in hippocampus and cerebral cortex, as well as adenosine nucleotide hydrolysis in synaptosomes from cerebral cortex of ovariectomized rats, and ovariectomy-induced memory deficits in inhibitory avoidance and Morris water maze tasks. Female adult Wistar rats were assigned to one of the following groups: sham (submitted to surgery without removal of the ovaries), exercise, ovariectomized (Ovx) and Ovx plus exercise. Thirty days after surgery, animals were submitted to one month of exercise training for 20 min, three times per week. After, rats were euthanized, blood serum was collected and hippocampus and cerebral cortex were dissected, or rats were tested in inhibitory avoidance and Morris water maze tasks. Data demonstrated that exercise reversed the activation of Na+,K+-ATPase and AChE activities both in hippocampus and cerebral cortex of ovariectomized rats. Ovariectomy decreased AMP hydrolysis in cerebral cortex and did not alter adenosine nucleotides hydrolysis in blood serum. Exercise per se decreased ADP and AMP hydrolysis in cerebral cortex. On the other hand, AMP hydrolysis in blood serum was increased by exercise in ovariectomized adult rats. Results also show that ovariectomized rats presented impairment in aversive memory and spatial navigation, both in reference and working memory protocols, when compared to sham group (control). Confirming our hypothesis, ovariectomized rats submitted to exercise had those impairments prevented. We conclude that ovariectomy significantly impairs aversive and spatial reference learning/memory and related enzymes, and that physical exercise prevents such effects. These findings support that physical exercise might constitute an important strategy to minimize cognitive deficits found in postmenopausal women.

SUMÁRIO

LISTA DE ABREVIATURAS

ACh	acetilcolina
AChE	acetilcolinesterase
AChT	colina acetiltransferase
AMP	adenosina monofosfato
ADP	adenosina difosfato
ATP	adenosina trifosfato
BDNF	fator neurotrófico derivado do cérebro
BUChE	butirilcolinesterase
CAMKII	proteína cinase calmodulina II
CREB	elemento responsivo de ligação por proteína
FSH	hormônio folículo estimulante
GnRH	hormônio liberador de gonadotrofinas
Ovx	ovariectomia
LH	hormônio luteinizante
LTP	potenciação de longa duração
MAPK	proteína cinase ativadora de mitose
MDA	malondialdeído
NGF	fator de crescimento do nervo
NMDA	receptor N-metil-D-aspartato
NTPDase	nucleosídeo trifosfato difosfohidrolases
PKA	proteína cinase dependente de AMPc
PKC-δ	proteína cinase C

ROS	espécies reativas de oxigênio
SNC	sistema nervoso central
TRH	terapia de reposição hormonal
TrkB	tirosina cinase B

LISTA DE FIGURAS

I. INTRODUÇÃO

1. Menopausa

No último século houve um aumento dramático na expectativa de vida em países industrializados. Anteriormente ao século XX, as mulheres morriam antes dos 50 anos; hoje em dia, contudo, a expectativa de vida aumentou para mais de 80 anos, enquanto que a média da idade para a menopausa continua a mesma. Portanto, muitas mulheres irão passar mais de 3 décadas de suas vidas na pós-menopausa (Wise 2001). A partir disso, tem ocorrido um intenso interesse na prevenção e amenização dos efeitos causados pela menopausa.

Mulheres adultas com ciclo reprodutivo normal secretam muitos compostos esteroidais em grande quantidade pelos ovários, como os estrógenos e as progesteronas, e não esteroidais, como inibinas, relaxinas, ativinas e folistatinas. Existem três estrógenos presentes no plasma feminino, o estriol, a estrona e o estradiol, sendo o 17β-estradiol o mais importante deles (Rodrigues et al. 1999).

A função ovariana é regulada pelo eixo hipotálamo-hipófisário-gonadal, a partir da secreção das gonadotrofinas hipofisárias, o hormônio luteinizante (LH) e o hormônio folículo estimulante (FSH), os quais estão sob o controle da secreção do hormônio liberador de gonadotrofinas (GnRH), produzido e liberado pelo hipotálamo. Um dos fatores de regulação deste hormônio é o mecanismo de retro-alimentação positiva e negativa exercido pelas substâncias ovarianas e pelas gonadotrofinas presentes na corrente sanguínea que variam durante o ciclo menstrual. Na menopausa esses mecanismos são abolidos

devido às baixas concentrações de estrógeno e progesterona e de substâncias ovarianas não esteroidais (Messinis 2006).

O estrógeno possui ações não reprodutivas em diversos sistemas fisiológicos, incluindo o ósseo, o cardiovascular, o imunitário e o sistema nervoso central (SNC) (Wise et al. 2001b; Wise 2002b). No cérebro, os estrógenos promovem um efeito neuroprotetor (Dubal e Wise 2001), e a sua falta permite que mulheres na menopausa sejam mais vulneráveis a distúrbios neurodegenerativos, tais como as doenças de Alzheimer e Parkinson, e acidente vascular cerebral (Zhang et al. 1998a; Wise et al. 2001b). Sabe-se também, que a ovariectomia, o modelo animal mais utilizado de supressão hormonal ovariana (Savonenko e Markowska 2003), prejudica a memória na tarefa do labirinto aquático de Morris (Monteiro et al. 2005a; Monteiro et al. 2008).

A terapia de reposição hormonal (TRH) tem sido usada por algumas mulheres na pós-menopausa como uma forma de substituição dos estrógenos endógenos. Estudos sugerem que a TRH melhora a memória em mulheres com a doença de Parkinson (Shulman 2002), já outros mostram que essa terapia pode ser responsável pelo desenvolvimento de tumores e pelo aumento no risco de doenças cardiovasculares (Miquel et al. 2006b). A partir dessas evidências, são necessários estudos utilizando alternativas não hormonais para amenizar os efeitos causados pela menopausa.

2. *Exercício Físico e Neuroproteção*

Nas últimas duas décadas, evidências têm mostrado que o exercício físico pode beneficiar as funções cognitivas (Kramer et al. 2005), tais como a capacidade de induzir a plasticidade sináptica (Farmer et al. 2004; Vaynman et al. 2004b; Christie et al. 2008) melhorar a memória/aprendizado (van Praag et al. 1999a; Farmer et al. 2004; Vaynman et al. 2004b; van Praag et al. 2005), promover a vascularização cerebral (Cotman e Berchtold 2002; Pereira et al. 2007), facilitar a recuperação após injúria cerebral (Griesbach et al. 2007), bem como diminuir o declínio mental associado com o avanço da idade e o risco para doenças neurodegenerativas (Cotman e Berchtold 2002; Kramer et al. 2005).

Dados na literatura mostram que o exercício físico exerce um efeito neuroprotetor em casos de lesão hipocampal, aumentando a expressão de fatores neurotróficos, os quais estão envolvidos na sobrevivência neuronal, diferenciação, alteração da plasticidade sináptica/memória e altera os níveis de neurotransmissores e o metabolismo neuronal no hipocampo de ratos (Berchtold et al. 2001; Cotman e Berchtold 2002; Sim et al. 2004; Griesbach et al. 2007).

A neurogênese no hipocampo é um dos fatores que influenciam o aprendizado e a memória, e parece possuir um papel central sobre o efeito neuroprotetor do exercício físico (Sim et al. 2004). A neurogênese em adultos se refere ao crescimento de novos neurônios, os quais podem mediar o aumento na plasticidade sináptica e aumentar o aprendizado (van Praag et al. 1999a). Algumas regiões do cérebro de mamíferos contêm células progenitoras ativas, que dão origem a novos neurônios e à glia (van Praag et al. 1999b;

Ernst et al. 2006). A neurogênese ocorre no giro denteado do hipocampo e na zona subventricular adjacente ao ventrículo lateral, podendo também ocorrer no neocórtex (Ernst et al. 2006).

Já foi observado que a neurogênese em ratos adultos aumenta três dias após o início do exercício físico, embora esse aumento seja mais expressivo após uma semana de exercício (Ernst et al. 2006). No estudo de van Praag e colaboradores (1999b), ratas com acesso livre à corrida em roda por 2 a 4 meses aumentam mais de duas vezes o número de células novas na zona subgranular do giro denteado do hipocampo. Nesse estudo a corrida também aumentou o desempenho na tarefa do labirinto aquático de Morris e a potenciação de longa duração (LTP) nas sinapses do giro denteado.

A molécula mais fortemente relacionada ao aumento da neurogênese induzida pelo exercício é o fator neurotrófico derivado do cérebro (BDNF). O BDNF tem uma importância fundamental no cérebro por promover a sobrevivência e regeneração neuronal (Vaynman et al. 2004b; Ernst et al. 2006), sendo importante para a sinaptogênese e neurogênese, especialmente no hipocampo, que é uma estrutura com alta plasticidade e associada com função cognitiva, incluindo memória e aprendizagem (Berchtold et al. 2001; Kramer et al. 2005).

O exercício aumenta os níveis de RNAm do BDNF e a proteína BDNF (Neeper et al. 1996; Russo-Neustadt et al. 1999; Cotman e Berchtold 2002; Vaynman et al. 2003; Vaynman et al. 2004a; Vaynman et al. 2004b; Radak et al. 2006). Neeper e colaboradores (1996) relataram que a corrida voluntária na roda aumenta os níveis de RNAm de BDNF no hipocampo. Estudos mostram que mudanças no RNAm de BDNF, encontradas principalmente no giro

denteado, hilo e região CA3 do hipocampo de ratos foram mantidas várias semanas após o exercício (Neeper et al. 1996; Russo-Neustadt et al. 1999).

Um aumento da expressão de BDNF resultante do exercício físico pode afetar a plasticidade sináptica nos terminais pré- ou pós-sinápticos (Figura 1). Os sinais de transdução do BDNF são mediados primariamente através do receptor tirosina cinase B (TrkB), o qual tem sua expressão aumentada pelo exercício físico. A ativação do receptor TrkB nos terminais pré- ou pós-sinápticos resulta numa ativação de vários genes, como as proteínas cinases ativadoras de mitose I e II (MAPKI e MAPKII), proteína cinase C (PKC-δ) e proteína cinase calmodulina II (CaMKII). Nos terminais pré-sinápticos, o exercício físico também pode atuar na sinapsina, sinaptotagmina e sintaxina para modular a liberação de neurotransmissores. Nos terminais pós-sinápticos, os efeitos do exercício físico podem ser mediados através do influxo de Ca^{2+} via receptor N-metil-D-aspartato (NMDA), o qual tem sua expressão aumentada pelo exercício físico. O receptor NMDA pode ativar a cascata de MAPK via CaMK. A MAPK ativada pode atuar no núcleo, como na transcrição da proteína de ligação ao elemento responsivo ao AMPc (CREB), o qual também é aumentado pelo exercício físico (Molteni et al. 2002).

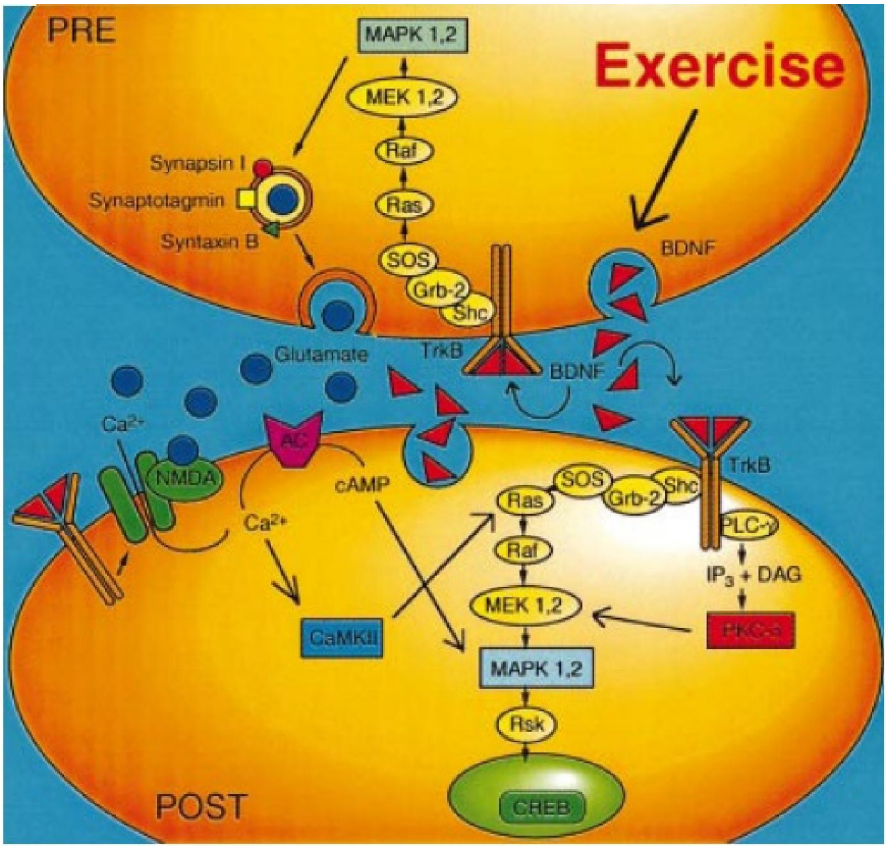


Figura 1. Mecanismos pelos quais o exercício físico pode modular a plasticidade no hipocampo (Molteni et al. 2002).

Evidências indicam que alterações no volume hipocampal podem estar relacionadas com o declínio da memória e das funções cerebrais (Kramer et al. 2007). Sabe-se que após a 5ª década de vida, ocorre um declínio no volume hipocampal de 1 a 2% em indivíduos saudáveis, aumentando este percentual em doenças neurodegenerativas (Mungas et al. 2005). Erickson e colaboradores (2009) sugeriram que maiores níveis de capacidade aeróbia estão associados a maiores volumes hipocampais, o que estaria levando a um aumento da memória espacial em humanos idosos e uma diminuição do risco de desenvolver doenças neurodegenerativas.

Os mecanismos exatos pelos quais o exercício físico exerce seus efeitos benéficos ainda não estão totalmente esclarecidos. No entanto, mais estudos são necessários para elucidar tais mecanismos.

3. Na^+,K^+-ATPase

A Na^+,K^+-ATPase (E.C. 3.6.1.37) é uma proteína integral de membrana presente em grande quantidade no tecido cerebral (Hansen e Clausen 1988). É responsável pelo transporte ativo de três íons Na^+ para o meio extracelular e de dois íons K^+ para o meio intracelular no SNC, mantendo o gradiente iônico necessário para a excitabilidade neuronal e a regulação do volume celular (Kaplan 2002). Atua regulando o fluxo de íons e o transporte de moléculas ligadas ao cotransporte de Na^+, como aminoácidos, glicose e neurotransmissores (Horisberger et al. 1991; Lees 1991; Lees 1993) (Figura 2). Essa enzima consome 40% - 50% do ATP cerebral (Erecinska e Silver 1994) e é alterada por radicais livres (Lees 1991; Kurella et al. 1999; Wang et al. 2003) e pela fluidez da membrana (Morel et al. 1998; Rauchova et al. 1999; Chakraborty et al. 2003).

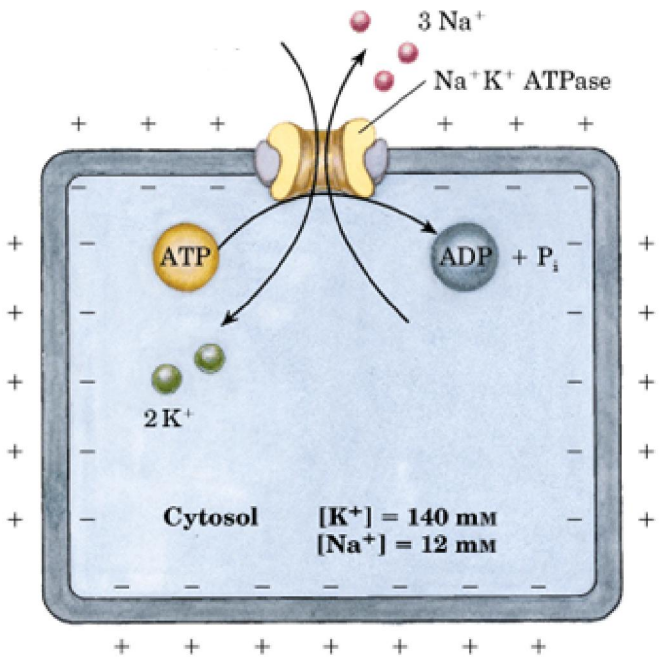


Figura 2. Representação esquemática da Na^+,K^+-ATPase.

A Na^+,K^+-ATPase apresenta uma estrutura oligomérica composta por duas subunidades α transmembranas, que contém sítios de ligação para os íons Na^+, K^+, ATP e glicosídeos cardíacos; duas subunidades β regulatórias, na forma de glicoproteínas, e uma subunidade γ, que está associada ao dímero $\alpha\beta$ (Mobasheri et al. 2000; Kaplan 2002).

O mecanismo pelo qual a Na^+,K^+-ATPase exerce sua função é dependente de fosforilação. A subunidade α pode ser fosforilada ou desfosforilada em um resíduo de ácido aspártico, estabilizando a estrutura em duas conformações, E1 e E2 (Kaplan 2002; Jorgensen et al. 2003). Na conformação E1, ocorre a ligação de três íons Na^+ que são transportados para o meio extracelular como resultado da transferência de um grupo fosfato do ATP para o sítio ativo da enzima, ocorrendo a conversão da Na^+,K^+-ATPase para o estado E2. Na conformação E2, os sítios fixadores de íons têm alta

afinidade por K^+, ocorrendo assim, a ligação de dois íons K^+ na face extracelular, provocando a desfosforilação da enzima e o transporte desses íons para o meio intracelular. A enzima, agora sem o grupamento fosfato em seu sítio ativo, não é estável na forma E2, voltando à forma E1, que tem alta afinidade por Na^+, dando início a um novo ciclo (Mobasheri et al. 2000; Kaplan 2002; Jorgensen et al. 2003).

Evidências sugerem que a Na^+,K^+-ATPase está envolvida nos mecanismos de memória em ratos (Brunelli et al. 1997; Wyse et al. 2004) e que sua inibição pode ocasionar prejuízo no funcionamento normal do SNC, como em doenças neurodegenerativas (Lees 1993; Yu 2003; Lima et al. 2008), incluindo a doença de Alzheimer (Hattori et al. 1998; Dickey et al. 2005).

Estudos realizados em nosso laboratório mostraram que a ovariectomia aumenta a atividade da Na^+,K^+-ATPase em hipocampo e córtex cerebral de ratas adultas (Monteiro et al. 2005b).

4. Acetilcolinesterase

A acetilcolina é um neurotransmissor sintetizado pela condensação de acetato e colina pela enzima colina acetiltransferase no citoplasma do terminal sináptico e armazenada em vesículas no neurônio pré-sináptico (Prado et al. 2002). A acetilcolina é liberada na fenda sináptica e liga-se a seus receptores na membrana pós-sináptica, tendo sua ação finalizada por colinesterases presentes na sinapse (Massoulie 2002) (Figura 3).

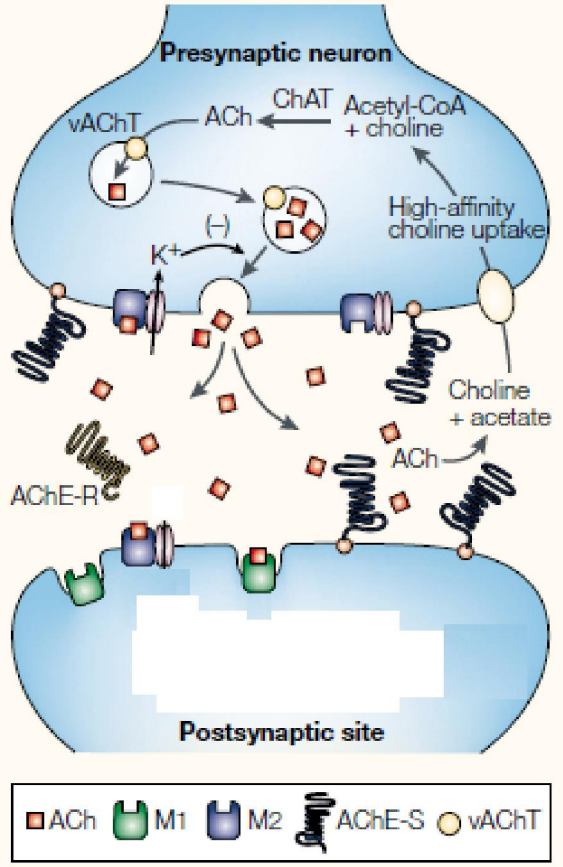


Figura 3. Representação esquemática da atividade da AChE (adaptado de (Soreq e Seidman 2001). ACh, acetilcolina; M1, receptor M1; M2, receptor M2; AChE-S, acetilcolinesterase; vAChT, colina acetiltransferase.

As colinesterases, incluindo a acetilcolinesterase (AChE) e a butirilcolinesterase (BUChE), são enzimas que fazem parte do sistema colinérgico, o qual apresenta um papel importante na função cognitiva (Everitt e Robbins 1997). Estas enzimas diferem basicamente quanto às propriedades cinética, aos inibidores seletivos, à distribuição tecidual e à especificidade por substratos (Massoulie et al. 1993).

A AChE (E.C. 3.1.1.7) está presente em maior concentração no SNC, na junção neuromuscular e na membrana de eritrócitos, hidrolisando

preferencialmente acetilcolina (Massoulie et al. 1993; Aldunate et al. 2004). Esta enzima contribui para a integridade e a permeabilidade da membrana sináptica durante a neurotransmissão e a condução (Grafius et al. 1971) e tem sido implicada em ações colinérgicas e não colinérgicas, como a influência na transmissão sináptica dopaminérgica e glutamatérgica (Zimmerman e Soreq 2006).

Há evidências mostrando que a AChE está alterada em cérebro de pacientes com a doença de Alzheimer (Atack et al. 1986; Bowen e Davison 1986; Fishman et al. 1986). A diminuição da neurotransmissão colinérgica observada em pacientes com essa doença, contribui para o déficit cognitivo e os distúrbios comportamentais associados a ela (Ballard et al. 2005).

Sabe-se que tanto a privação quanto a reposição estrogênica afeta o sistema colinérgico em uma variedade de regiões cerebrais (Simpkins et al. 1997) e estudos realizados em nosso laboratório mostram que a AChE está aumentada em cérebro de ratas adultas ovariectomizadas, o que sugere uma redução na concentração de acetilcolina, levando a hipoatividade colinérgica em condições de privação de estrogênio (Monteiro et al. 2005b).

5. Ectonucleotidases

O ATP (adenosina trifosfato), assim como a acetilcolina, é considerado uma substância de sinalização extracelular no SNC e em outros tecidos. Ambos podem ser co-armazenados em vesículas sinápticas e co-liberados de nervos colinérgicos. O ATP e a acetilcolina não podem ser reciclados diretamente, primeiro precisam ser degradados a adenosina e colina,

respectivamente, para então serem transportados novamente para dentro da célula.

O ATP e outros nucleosídeos extracelulares tri- e difosfatos podem ser hidrolisados pelas NTPDases (nucleosídeo trifosfato difosfoidrolases), as quais são enzimas que hidrolisam ATP e ADP, presentes em vários tecidos, incluindo o sistema vascular (Ralevic e Burnstock 2003) e o SNC de várias espécies (Sarkis et al. 1995). O AMP produzido é subseqüentemente hidrolisado a adenosina por uma 5'-ectonucleotidase (CD73, EC 3.1.3.5), que constitui um passo limitante de velocidade destas reações (Zimmermann 1992; Battastini et al. 1995) (Figura 4).

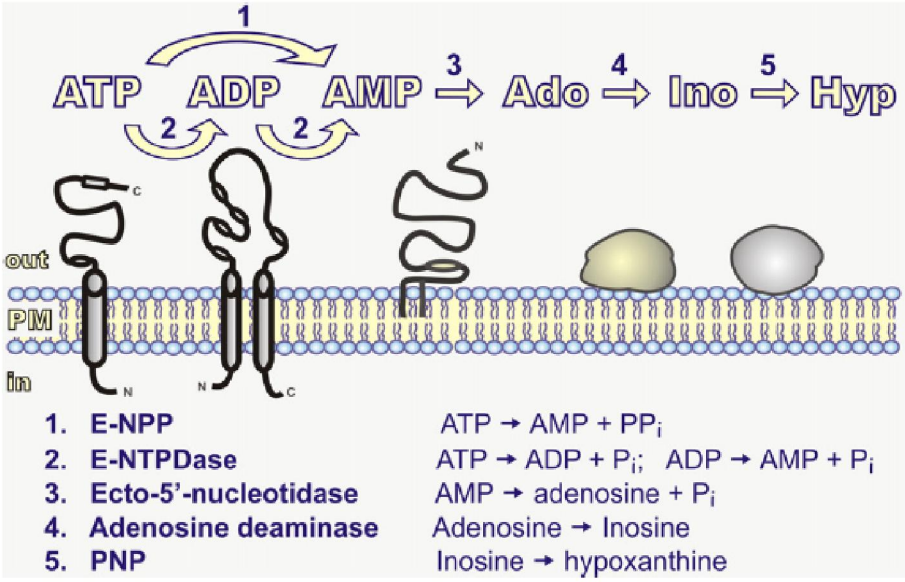


Figura 4. Representação esquemática da hidrólise de nucleotídeos (Yegutkin 2008).

Os nucleotídeos podem ser liberados por células neuronais, gliais e vasculares e exercem suas funções por meio de receptores ionotrópicos (P2X)

ou metabotrópicos (P2Y). O ATP extracelular e seus produtos, ADP e adenosina, possuem efeitos em vários processos biológicos (Agteresch et al. 1999). No cérebro, os nucleotídeos atuam na neurotransmissão, modulando a liberação de transmissores, a comunicação neurônio-glia, promovendo desenvolvimento neural, o sistema imunitário inato e o controle vascular (Zimmermann 2006).

O ATP extracelular pode atuar como vasodilatador ou vasoconstritor dependendo da sua concentração e de receptores. O ADP induz mudanças na forma e agregação das plaquetas via interação com os receptores plaquetários $P2Y_{12}$. O nucleosídeo adenosina inibe a agregação plaquetária (Kawashima et al. 2000), é capaz de atuar como vasodilatador (Marshall 2000) e também é considerada um importante agente neuroprotetor e neuromodulador do SNC (Bonan et al. 2001; Ribeiro et al. 2003), regulando a transmissão sináptica e a excitabilidade neuronal (Latini e Pedata 2001).

Tem sido sugerido que a privação de hormônios esteróides pode modular a expressão e atividade das ecto-ATPases no hipocampo e no núcleo caudado (Nedeljkovic et al. 2000), e foi mostrado uma menor expressão dos receptores de adenosina em resposta à Ovx em cérebro total (Rose'Meyer et al. 2003).

II. OBJETIVOS

Objetivo geral

Considerando que a ovariectomia prejudica a memória espacial e altera as atividades da Na^+,K^+-ATPase, AChE e ectonucleotidases cerebrais e que o exercício físico promove alterações em componentes relacionados com os processos de memória, o presente estudo teve como *objetivo geral* estudar a influência do exercício físico sobre algumas alterações nos parâmetros bioquímicos e comportamentais em ratas adultas submetidas à ovariectomia bilateral com conseqüente depleção estrogênica endógena.

Capítulo I

1) Avaliar a influência do exercício físico sobre o aumento na atividade da AChE cerebral causado pela ovariectomia em ratas adultas;

2) Verificar a atividade da Na^+,K^+-ATPase em cérebro de ratas adultas ovariectomizadas submetidas ao exercício físico;

3) Avaliar os efeitos da ovariectomia e do exercício físico sobre a atividade das ectonucleotidases em córtex cerebral e soro de ratas adultas.

Capítulo II

1) Verificar o efeito do exercício físico sobre o prejuízo na memória na tarefa de labirinto aquático causado pela ovariectomia em ratas;

2) Verificar o efeito da ovariectomia sobre a memória na tarefa de esquiva inibitória em ratas, bem como a influência do exercício sobre as possíveis alterações nessa tarefa.

III. METODOLOGIA E RESULTADOS

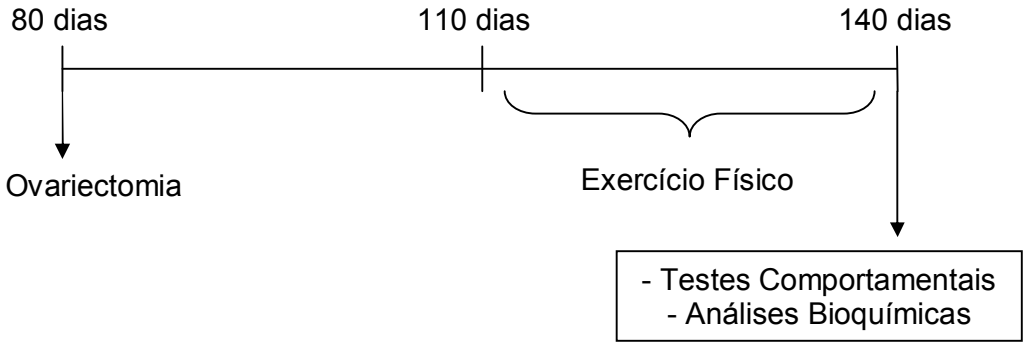
MODELOS EXPERIMENTAIS

Os capítulos I e II serão apresentados na forma de artigos científicos, os quais apresentam os mesmos desenhos experimentais no que se refere aos modelos de ovariectomia e de exercício físico.

Ovariectomia: ratas Wistar de três meses foram divididas em quatro grupos: (1) sham, submetidas à cirurgia sem remoção dos ovários, (2) exercício, (3) ovariectomizadas, com remoção bilateral dos ovários (Ovx) e (4) Ovx + exercício.

A ovariectomia foi realizada através da remoção ovariana bilateral utilizando como anestésicos Ketamina (90 mg/kg) e Xilazina (10 mg/kg) para eliminação de esteróides endógenos ovarianos (Waynforth e Flecknell 1992).

Exercício físico: o treinamento foi realizado em esteira adaptada para ratos, após um mês da cirurgia (pós-treinamento). O treinamento foi realizado a 60% do $VO2_{máx}$ por 20 min, três vezes por semana, durante um mês. Finalizando o treinamento as ratas foram avaliadas nos testes de comportamento ou decapitadas com guilhotina para as análises bioquímicas.



Capítulo I

ARTIGO 1

Exercise effects on activities of Na^+,K^+-ATPase, acetylcholinesterase and adenine nucleotides hydrolysis in ovariectomized rats

Juliana Ben, Flávia M.S. Soares, Fernanda Cechetti, Fernanda C. Vuaden, Carla D. Bonan, Carlos A. Netto, Angela T.S. Wyse

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BRAIN RESEARCH

Research Report

Exercise effects on activities of Na^+,K^+-ATPase, acetylcholinesterase and adenine nucleotides hydrolysis in ovariectomized rats

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ABSTRACT

Hormone deficiency following ovariectomy causes activation of Na^+,K^+-ATPase and acetylcholinesterase (AChE) that has been related to cognitive deficits in experimental animals. Considering that physical exercise presents neuroprotector effects, we decide to investigate whether exercise training would affect enzyme activation in hippocampus and cerebral cortex, as well as adenosine nucleotide hydrolysis in synaptosomes from cerebral cortex of ovariectomized rats. Female adult Wistar rats were assigned to one of the following groups: sham (submitted to surgery without removal of the ovaries), exercise, ovariectomized (Ovx) and Ovx plus exercise. Thirty days after surgery, animals were submitted to one month of exercise training, three times per week. After, rats were euthanized, blood serum was collected and hippocampus and cerebral cortex were dissected. Data demonstrated that exercise reversed the activation of Na^+,K^+-ATPase and AChE activities both in hippocampus and cerebral cortex of ovariectomized rats. Ovariectomy decreased AMP hydrolysis in cerebral cortex and did not alter adenine nucleotides hydrolysis in blood serum. Exercise per se decreased ADP and AMP hydrolysis in cerebral cortex. On the other hand, AMP hydrolysis in blood serum was increased by exercise in ovariectomized adult rats. Present data support that physical exercise might have beneficial effects and constitute a therapeutic alternative to hormone replacement therapy for estrogen deprivation.

1. Introduction

Estrogen displays important roles beyond the reproductive system, such as trophic and protective role in the adult brain (Wise et al., 2001b, 2002) and it has been shown that estrogen deprivation is implicated in the pathogenesis of Alzheimer's disease and cerebral ischemia (van Duijn, 1999; Zhang et al., 1998). Studies have suggested that post-menopausal women

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are more vulnerable to such diseases and to cognitive deficits (Green and Simpkins, 2000; Wise et al., 2001a,b). Hormone replacement therapy (HRT), in the form of estrogen and progesterone or estrogen alone, has been used to treat menopause symptoms. However, due to the possible side effects of HRT, such as breast cancer and increased risk of thromboembolic accidents, there is a growing demand for alternatives for the treatment of pathological processes and symptoms associated with menopause (Miquel et al., 2006); physical exercise has been proposed as an alternative therapeutic tool.

Evidence suggests that exercise may support brain health and function; consistent to that, there are studies indicating that physical activity may reduce age-induced cognitive decline and it is recommended as a therapeutic strategy to prevent, or recover from, neurodegenerative diseases (Kramer et al., 1999; Mattson, 2000). In this context, it has been shown that exercise increases levels of brain-derived neurotrophic factor (BDNF) and other growth factors that stimulate neurogenesis, increases resistance to brain insult and was proposed to improve learning and performance (Cotman and Berchtold, 2002; van Praag et al., 2005). Studies suggest that dynamic physical exercise produces elevated regional cerebral blood flow (CBF), alterations in endogenous peptides and neurotransmitters, and increases amino acid transport through the blood brain barrier (Hollmann et al., 1994; Ide et al., 1999). Although the exact molecular mechanisms by which physical exercise affects brain function are unclear, it has been suggested that it might activate cellular and molecular pathways that contribute to neuroprotection (Cotman and Berchtold, 2002; van Praag et al., 2005).

Na^+,K^+-ATPase (E.C 3.6.1.37) is responsible for the generation of membrane potential through the active transport of sodium and potassium ions. This enzyme is necessary to maintain the ionic gradient for neuronal excitability, consuming about 40–50% of the ATP generated in brain cells (Erecinska and Silver, 1994). Na^+,K^+-ATPase activity is inhibited by free radicals and its activity is reduced in cerebral ischemia (Wyse et al., 2000) and in neurodegenerative diseases as Alzheimer's disease (Gómez-Ramos and Morán, 1997; Hattori et al., 1998). In addition, we have recently demonstrated that ovariectomy increases Na^+, K^+-ATPase activity in synaptic plasma membranes of rat hippocampus (Monteiro et al., 2005b).

ATP and acetylcholine serve as extracellular signaling substances in the nervous system and in other tissues. They can even be co-stored within synaptic vesicles and co-released from cholinergic nerves. Neither ACh nor ATP can be directly recycled. They must first be degraded to either choline or adenosine and those substances are transported back into cells. Acetylcholine is specifically hydrolyzed by acetylcholinesterase (AChE) (E.C. 3.1.1.7). This enzyme has been implicated in cholinergic and non-cholinergic actions that may play a role in neurodegenerative diseases (Cummings, 2000); it has been also shown that AChE per se activates neuronal cell death (Calder'on et al., 1998). On the other hand, it is known that estrogen withdrawal and replacement affect the cholinergic system in a variety of brain regions (Gibbs and Aggarwal, 1998; Simpkins et al., 1997). In addition, we have shown that ovariectomized female adult rats present an increase of brain AChE activity (Monteiro et al., 2005b, 2007).

ATP and the other extracellular nucleoside tri- and diphosphates can be hydrolyzed by NTPDases (nucleoside triphosphate diphosphohydrolases), which are enzymes that hydrolyze ATP and ADP, and are present in many tissue, including the vascular system (Ralevic and Burnstock, 2003) and central nervous system (SNC) of several species (Sarkis et al., 1995). The AMP produced is subsequently hydrolyzed to adenosine by an ecto-5′-nucleotidase (CD73, EC 3.1.3.5), which constitutes the rate-limiting step in this pathway (Battastini et al., 1995; Zimmermann, 1992). Extracellular ATP and its breakdown products, ADP and adenosine, have pronounced effects in a variety of biological processes (Agteresch et al., 1999). It has been suggested that steroid hormone deprivation can modulate the expression and activity of an ecto-ATPase of hippocampus and caudate nucleus (Nedeljkovic et al., 2000), and it demonstrated a down regulation in adenosine receptors in response to ovariectomy using total brain (Rose'Meyer et al., 2003).

In the present study we investigated the influence of physical exercise on the effects elicited by ovariectomy on Na^+, K^+-ATPase, AChE activities and adenine nucleotide hydrolysis in hippocampus and/or cerebral cortex of ovariectomized rats, respectively. The working hypothesis is that exercise would reverse the effects of ovariectomy over enzyme activities.

2. Results

The effect of exercise on Na^+,K^+-ATPase in female adult Wistar rats is shown in Fig. 1. Animals subjected to ovariectomy presented a significant increase (14%) of cerebral cortex Na^+,K^+-ATPase activity (Panel 1A) and exercise reversed the stimulation caused by ovariectomy [$F(3,33)=5.17$; $p<0.01$]. Panel 1B shows that Na^+,K^+-ATPase activity was significantly increased (11%) in hippocampus of rats subjected to ovariectomy, which was reversed by exercise [$F(3,30)=3.49$; $p<0.05$]. Exercise per se did not alter Na^+,K^+-ATPase activity, with exception of hippocampal Na^+,K^+-ATPase, that had a tendency to increase with exercise.

The effect of exercise on acetylcholinesterase in female adult Wistar rats is showed in Fig. 2. Fig. 2a shows that animals subjected to ovariectomy presented a significant increase (42%) of cerebral cortex AChE activity and exercise reversed such effects [$F(3,39)=5.21$; $p<0.01$]. Fig. 2b shows that AChE activity was significantly increased (29%) in hippocampus of rats subjected to ovariectomy, and that effect was reversed by exercise [$F(3,43)=7.77$; $p<0.001$]. Exercise per se did not alter AChE activity.

The effects of ovariectomy and exercise on hydrolysis of ATP, ADP and AMP in synaptosomes from cerebral cortex and blood serum of female adult Wistar rats are shown in Table 1. When compared to sham group, Ovx group did not show any significant difference in ATP hydrolysis and have a tendency to decrease ADP hydrolysis in cerebral cortex. Animals submitted to exercise per se or exercise and Ovx did not show significant changes in ATP, but decreased significantly ADP hydrolysis in this same cerebral structure [$F(3,15)=3.67$; $p<0.05$]. Results demonstrated a decrease in AMP hydrolysis in the cerebral cortex of exercised, ovariectomized, and ovariectomized rats submitted to exercise [$F(3,14)=3.87$; $p<0.05$] when compared to the sham group (Table 1).

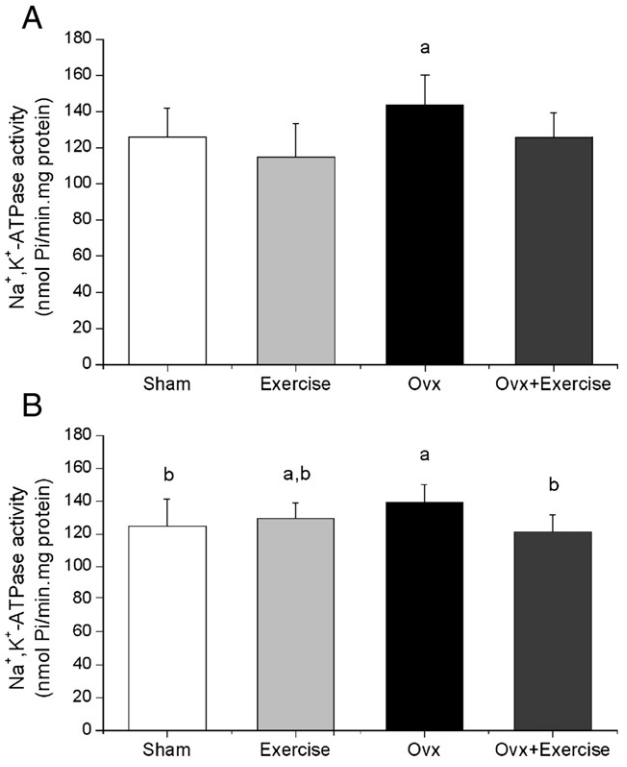


Fig. 1 – Effect of ovariectomy and exercise on Na^+,K^+-ATPase activity in cerebral cortex (a) and hippocampus (b) of female adults rats. Data are expressed as mean ± S.D. for 8 to 10 animals in each group. [a]$p<0.05$ compared to sham group and [b]$p<0.05$ compared to Ovx group (Duncan's multiple range test). Ovx—ovariectomized.

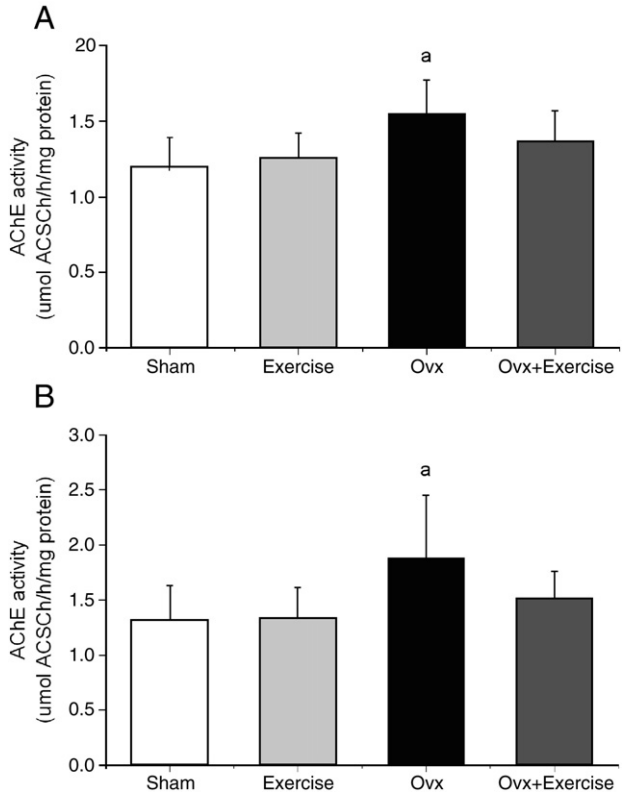


Fig. 2 – Effect of ovariectomy and exercise on acetylcholinesterase activity in cerebral cortex (a) and hippocampus (b) of female adult rats. Data are expressed as mean ± S.D. for 10 to 13 animals in each group. [a]$p<0.01$ compared to sham group (Duncan's multiple range test). Ovx—ovariectomized.

In blood serum, Ovx or exercise group did not show any significant difference in ATP and ADP hydrolysis compared to sham group. However, results show an increase in AMP hydrolysis in the blood serum of ovariectomized rats submitted to exercise [$F(3,13)=4.74$; $p<0.05$] when compared to the sham group (Table 1).

We observed that the animal weight gain was increased by ovariectomy [$F(3,43)=6.04$; $p<0.01$] (Table 2). As can be observed in this table, exercise reversed body weight gain of ovariectomized rats.

3. Discussion

A growing number of studies indicate the brain as one of the body organs that suffers from the loss of estrogen in menopause and that damage from stroke and neurodegeneration in dementia may be retarded by estrogenic actions (McEwen, 2002). It has also been shown that post-menopausal estrogen replacement therapy reduces the risk and delay in the onset of these diseases (van Duijn, 1999; Yaffe et al., 1998). On the other hand, evidence showed that estrogen plus progestin therapy to postmenopausal women increased the risk for dementia in women aged 65 years or older and did not improve cognitive impairment in these women (Shumaker et al., 2003).

In the present study, we investigated the influence of exercise on the activation of hippocampal and cerebral cortex Na^+,K^+-ATPase and AChE activities caused by ovariectomy, as well as on nucleotide hydrolysis in cerebral cortex and blood serum of Ovx rats. We used this animal model of steroid hormone deprivation because ovariectomy is considered the

Table 1 – Effect of ovariectomy and exercise on nucleotide hydrolysis in synaptosomes from cerebral cortex and in serum of female adult rats.

Groups	ATP	ADP	AMP
Cerebral cortex (nmol P/min/mg)			
Sham	86.85 ± 7.55	40.45 ± 9.60	19.39 ± 2.80
Exercise	90.68 ± 12.10	26.96 ± 4.80*	14.38 ± 3.34*
Ovx	88.87 ± 13.54	36.53 ± 8.35*#	15.42 ± 1.49*
Ovx + exercise	76.47 ± 16.77	27.19 ± 5.69*	15.60 ± 1.69*
Serum (nmol P/min/mg)			
Sham	14.45 ± 2.75	14.46 ± 1.70	12.26 ± 1.38
Exercise	14.90 ± 5.55	16.36 ± 4.11	13.34 ± 0.46
Ovx	14.29 ± 1.28	12.54 ± 2.89	11.93 ± 1.08
Ovx + exercise	12.37 ± 1.07	12.47 ± 2.16	16.70 ± 3.74*

Data are expressed as mean ± S.D. for 4 to 6 animals in each group. *$p<0.05$ compared to sham group (Duncan's multiple range test), #$p<0.05$ compared to exercise and Ovx + exercise group.

Table 2 – Effect of ovariectomy and exercise on body weight of female adult rats.

Groups	Body weight (g)	Body weight (g)
	First day of experiment	After 30 days of exercise
Sham	187.69 ± 12.77	222.54 ± 20.56
Exercise	189.08 ± 12.73	216.00 ± 8.43
Ovx	189.17 ± 10.44	239.83 ± 9.70*
Ovx + exercise	189.60 ± 10.05	227.60 ± 14.22

Data are presented as mean ± S.D. for 10 to 13 rats in each group. Ovariectomized rats were significantly different from sham groups after 30 days of training *$p < 0.01$ compared to other groups (ANOVA).

most common animal model of postmenopausal changes in adult female rats (Savonenko and Markowska, 2003). The hippocampus and cerebral cortex were studied because these cerebral structures are associated with memory modulation (Daniel and Dohanich, 2001) and ovariectomized rats present memory impairments (Monteiro et al., 2005a; Singh et al., 1994).

Our results showed that ovariectomy significantly increased Na^+,K^+-ATPase and AChE activities (Figs. 1 and 2) in hippocampus and cerebral cortex of female rats submitted to ovariectomy. These results are in agreement with our previous studies showing that hippocampal Na^+,K^+-ATPase and AChE activities are increased in ovariectomized rats (Monteiro et al., 2005b, 2007). We also observed that exercise per se was unable to affect the enzyme activities. Interestingly, exercise markedly reversed the action of ovariectomy on Na^+,K^+-ATPase and AChE activities in hippocampus and cerebral cortex of ovariectomized rats (Figs. 1 and 2).

The exact mechanism of reversal of Na^+,K^+-ATPase activities by exercise is unknown. However, the activity of Na^+,K^+-ATPase can be modulated by several mechanisms. It has also been shown that the stimulation of Na^+,K^+-ATPase activity is associated with a decrease in membrane fluidity (Levin et al., 1990) and lipid peroxidation (Nanji et al., 1994). In this context, it is known that regular exercise increases resistance against reactive oxygen species (ROS) (Alessio et al., 1988) and decreases the accumulation of oxidative protein and DNA damage (Leeuwenburgh et al., 1998; Radak et al., 1999). In addition, chronic exercise also decreases the malonaldehyde (MDA) level in brain, a measure of oxidative damage to lipids, indicating a possible beneficial effect of chronic exercise on brain functioning and protection from oxidative damage (Liu et al., 2000). Also, there are data showing that diverse signal transduction pathways, leading to the formation of different mediators and the activation of a variety of kinases, regulate Na^+,K^+-ATPase activity (Bertorello and Katz, 1995). Based on this, chronic exercise also can modify signal transduction pathways that seem to be involved in the brain plasticity (Molteni et al., 2002). On the other hand, exercise may increase neurogenesis in hippocampus (van Praag et al., 1999) under estrogen-deprived conditions and that may be useful in improving brain function in climacteric women (Ji et al., 2008). Na^+,K^+-ATPase activity can also be modulated by changes in the intracellular sodium concentration (Inoue and Matsui, 1991). Another possibility could be the increase in synthesis or decreases in degradation of Na^+,K^+-ATPase.

In present study, exercise also was able to reverse the increase in AChE activity caused by ovariectomy. The reversal of AChE activity probably indicates that exercise can affect brain cholinergic mechanisms, since stimulation of AChE activity provoked an enhanced acetylcholine (ACh) hydrolysis and choline reuptake, reducing cholinergic activity in the CNS (Okuda et al., 2000). Previous study showed that soy isoflavones prevented the increase in AChE activity caused by ovariectomy (Monteiro et al., 2007) and it had been suggested that soy phytoestrogens may function as estrogen agonists in regulating choline acetyltransferase and nerve growth factor in brain of female rats (Pan et al., 1999). In this line, exercise can revert the increase in AChE activity of ovariectomized rats by increasing the number of cholinergic neurons, which also express the nerve growth factor (NGF) receptor, and exercise can also increase gene expression of NGF (Ang et al., 2003). So far we do not know the exact underlying mechanism through which AChE activity is modified in our study.

We also observed in this study that Ovx treatment significantly decreases AMP, and did not alter ADP and ATP hydrolysis in the cerebral cortex. Exercise did not prevent Ovx effect. On the other hand, exercise per se significantly decreased AMP and ADP hydrolysis. Since 5′-nucleotidase activity is involved in the hydrolysis of AMP to adenosine in the synaptic cleft (Battastini et al., 1995; Zimmermann, 1992), a decrease in this enzyme may have occurred as consequence of Ovx and/or exercise. Probably in our study the levels of adenosine, an important neuroprotective and neuromodulator agent (Bonan et al., 2001; Ribeiro et al., 2003), are lower in all tested groups when compared to sham group. In agreement with this idea, Rose'Meyer and colleagues (2003) demonstrated a down regulation in adenosine receptors in response to Ovx using total brain. However, controversial effects on the control of nucleotide levels have been promoted by Ovx or exercise. Rucker et al. (2005) showed that Ovx increases 5′-nucleotidase in synaptosomes from cerebral cortex, whereas it did not alter NTPDase activity. In addition, Dworak and colleagues (2007) did not observe any difference in brain AMP, ADP, and ATP concentrations after moderate and exhaustive treadmill exercise. Our results suggest that exercise is able to decrease the enzyme activities involved in nucleotide levels control, leading to a decrease in adenosine levels, which could suggest that the beneficial effects induced by exercise are not related to a neuroprotective action of adenosine in brain. However, it is important to consider that the different effects induced by exercise on nucleotide hydrolysis could be related with differences in exercise intensity and brain structures investigated.

Considering that the ratio nucleotides/nucleoside in the circulation could present some changes that could evoke responses in both CNS and circulatory system, in the present study we also investigated serum nucleotide hydrolysis. Results show that ATP and ADP hydrolysis were not altered in none group when compared to sham (control), suggesting that NTPDase activity in the blood serum did not change. Results also demonstrated an increase in AMP hydrolysis in the blood serum of ovariectomized rats submitted to exercise, this may result in an increase in 5′-nucleotidase activity in blood serum. Adenosine, an inhibitor of the platelet aggregation (Cristalli et al., 1995), may be increased in response to exercise in

ovariectomized rats, this can be a compensation in situations of increased platelet activity, and cardiovascular risk, such as postmenopausal conditions (Pochmann et al., 2004).

Our previous study showed that ovariectomy significantly decreases (98%) the estradiol levels in all ovariectomized rats, confirming the efficacy of the surgical procedure of ovariectomy (Monteiro et al., 2007). We also observed that the animal weight gain was increased by ovariectomy and exercise reverted weight body gain of ovariectomized rats. These findings are in agreement with Saengsirisuwan and colleagues (2009), that suggested an increased energy expenditure by exercise training, which could prevent fat accumulation and/or enhanced fat utilization of Ovx rats.

In summary, in the present study we demonstrated that exercise significantly reverses the action of ovariectomy on Na^+,K^+-ATPase and AChE activities in hippocampus and cerebral cortex of female adult rats. Ovariectomy decreased AMP hydrolysis in cerebral cortex and did not alter adenine nucleotide hydrolysis in blood serum. Exercise per se decreased the ADP and AMP hydrolysis and did not alter effects of ovariectomy in cerebral cortex, but increased the AMP hydrolysis in blood serum of ovariectomized adult rats. Based on these findings we could suggest that exercise may have a protective role against the damage brain and increased platelet activity caused by the loss of estrogen during menopause. Present data support that physical exercise might constitute a therapeutic alternative to hormone replacement therapy for estrogen deprivation.

4. Experimental procedures

4.1. *Animals and reagents*

Female adult Wistar rats (3 months, 180–210 g BW) were obtained from the Central Animal House of the Department of Biochemistry, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. Animals were maintained on a 12/12 h light/dark cycle in an air-conditioned constant temperature (22±1 °C) colony room, with free access to water. Animal care followed the official governmental guidelines in compliance with the Federation of Brazilian Societies for Experimental Biology and was approved by the Ethics Committee of the Federal Rio Grande do Sul, Brazil. All chemicals were purchased from Sigma Chemical Co., St. Louis, MO, USA.

Animals were randomly assigned to one of the following groups: (n=11-15): sham (only submitted to surgery without removing of ovaries), exercise, ovariectomized (Ovx), and Ovx plus exercise. Animals were ovariectomized by the surgical removal of both ovaries under ketamine anesthesia (90 mg/kg) and xylazine (10 mg/kg) intraperitoneous (i.p.) to eliminate endogenous ovarian steroids (Waynforth and Flecknell, 1992). One month after the surgery, animals were submitted to exercise.

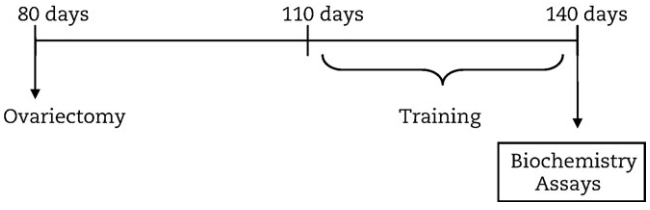
4.2. *Exercise training*

Rats were habituated to the treadmill apparatus to minimize novelty stress and randomly assigned to different experimental groups (n=11-15 in each group): non-exercised (sedentary-control group) and exercised during 20 min, 3 times a week. The exercise training consisted of running sessions on an adapted motorized rodent treadmill (INBRAMED TK 01, Porto Alegre, Brazil) at 60% of their maximal oxygen uptake (Brooks and White, 1978), a moderate exercise. Measurement of oxygen uptake (VO2) peak was carried out in all animals, indirectly before training, considering the exhaustion. Each rat ran on the treadmill at a low initial speed followed by increases in speed of 5 m/min every 3 min until the point of exhaustion (i.e., failure of the rats to continue running) and the time to fatigue (in min) and workload (in m/min) were taken as indexes of capacity for exercise, that was taken as VO2 max. (Arida et al., 1999; Brooks and White, 1978).

Selected animals that initially refused to run were encouraged by gently tapping their backs. Neither electric shock nor physical prodding was used in this study (Cechetti et al., 2007). The control group was transported to the experimental room and handled exactly as the experimental animals and were maintained in the turned off treadmill for 5 min without being forced to run (Scopel et al., 2006).

The animals were adapted to the treadmill by gradually increasing running speed and time, as follows: week 1, at 18 m/min for the first 3 min, 24 m/min for the next 3 min, 36 m/min for the following 6 min, 24 m/min for the following 3 min and 18 m/min for the last 3 min; week 2, at 18 m/min for the first 3 min, 36 m/min for the next 12 min, and 18 m/min for the last 3 min; weeks 3 and 4, at 18 m/min for the first 3 min, 48 m/min for the next 14 min, and 18 m/min for the last 3 min (Cechetti et al., 2007).

Approximately 12 h after the last exercise session, rats were euthanized by decapitation without anesthesia and the brain was immediately isolated, washed with saline solution and the cerebral cortex and hippocampus were dissected; and the blood was collected.



4.3. *Na^+,K^+-ATPase activity assay*

For determination of Na^+,K^+-ATPase activity, the hippocampus and cerebral cortex were homogenized in 10 vol. 0.32 mM sucrose solution containing 5.0 mM HEPES and 1.0 mM EDTA, pH 7.4.

The reaction mixture for Na^+,K^+-ATPase activity assay contained 5.0 mM $MgCl_2$, 80.0 mM NaCl, 20.0 mM KCl and 40.0 mM Tris–HCl, pH 7.4, in a final volume of 200 μL. The reaction was initiated by the addition of ATP. Controls were carried out under the same conditions with the addition of 1.0 mM ouabain. Na^+,K^+-ATPase activity was calculated by the difference between the two assays, as previously described (Wyse et al., 2000). Released inorganic phosphate (Pi) was

measured by the method of (Chan et al., 1986). Specific activity of the enzyme was expressed as nmol Pi released per min per mg of protein. All samples were run in duplicate.

4.4. AChE activity assay

For the AChE assay, the hippocampus and cerebral cortex were homogenized in 10 volumes 0.1 mM potassium phosphate buffer, pH 7.5, and centrifuged for 10 min at 1000×*g*. The supernatant was used for the enzymatic AChE analyses.

Acetylcholinesterase activity was determined according to Ellman et al. (1961), with some modifications (Villescas et al., 1981). Hydrolysis rates were measured at acetylthiocholine (S) concentration of 0.8 mM in 1 mL assay solutions with 30 mM phosphate buffer, pH 7.5, and 1.0 mM 5,5′-dithiobis-(2-nitrobenzoic Acid) (DTNB) at 25 °C. Fifty microliters of rat hippocampus and cerebral cortex supernatant was added to the reaction mixture and preincubated for 3 min. The hydrolysis was monitored by the formation of the thiolate dianion of DTNB at 412 nm for 2–3 min (intervals of 30 s). Specific enzyme activity was expressed as μmol ASCh per hour per milligram of protein. All samples were run in duplicate.

4.5. Adenine nucleotide hydrolysis

4.5.1. Subcellular fractionation

Cerebral cortex was removed and placed in ice-cold isolation medium (320 mM sucrose, 5 mM HEPES, pH 7.5 and 0.1 mM EDTA) and were cut longitudinally. The cerebral cortex was gently homogenized in 10 volumes, respectively, of ice-cold isolation medium with a motor-driven Teflon-glass homogenizer and synaptosomes were isolated as previously described (Nagy and Delgado-Escueta, 1984). Briefly, 0.5 ml of crude mitochondrial fraction was mixed with 4.0 ml of an 8.5% Percoll solution and layered onto an isoosmotic Percoll/sucrose discontinuous gradient (10/16%). The synaptosomes that banded at the 10/16% Percoll interface were collected with wide tip disposable plastic transfer pipettes. The synaptosomal fractions were washed twice at 15,000×*g* for 20 min with the same ice-cold medium to remove the contaminating Percoll and the synaptosome pellet was resuspended to a final protein concentration of approximately 0.5 mg/ml. The material was prepared fresh daily and maintained at 0–4 °C throughout preparation.

4.5.2. Isolation of blood serum fraction

Blood samples were drawn after decapitation of rats and were soon centrifuged in plastic tubes at 3000 rpm for 10 min at 20 °C. The serum samples obtained were then stored on ice and immediately used in the experiments (Stefanello et al., 2003).

4.5.3. Measurement of synaptosome ATP, ADP and AMP hydrolyses

The reaction medium used to assay the ATP and ADP hydrolysis was essentially as described previously (Battastini et al., 1991). The reaction medium contained 5.0 mM KCl, 1.5 mM $CaCl_2$, 0.1 mM EDTA, 10 mM glucose, 225 mM sucrose and 45 mM Tris–HCl buffer, pH 8.0, in a final volume of 200 ml. The synaptosome preparation (10–20 μg protein) was added to the reaction mixture and preincubated for 10 min at 37 °C. The reaction was initiated by the addition of ATP or ADP to a final concentration of 1.0 mM and the reaction was stopped by the addition of 200 μl 10% trichloroacetic acid (TCA). The released inorganic phosphate (Pi) was measured as previously described (Chan et al., 1986). The reaction medium used to assay the 5′-nucleotidase activity (AMP hydrolysis) contained 10 mM $MgCl_2$, 0.1 M Tris–HCl, pH 7.0 and 0.15 M sucrose in a final volume of 200 ml (Heymann et al., 1984). The synaptosome preparation (10–20 μg protein) was preincubated for 10 min at 37 °C. The reaction was initiated by the addition of AMP to a final concentration of 1.0 mM and was stopped by the addition of 200 ml 10% TCA; the released inorganic phosphate (Pi) was measured as previously described (Chan et al., 1986). Controls with the addition of the enzyme preparation after addition of TCA were used to correct non-enzymatic hydrolysis of the substrates. All samples were run in triplicate. Specific enzyme activity was expressed as nmol Pi released per min per mg of protein.

4.5.4. Measurement of blood serum ATP, ADP and AMP hydrolyses

ATP and ADP hydrolyses were determined using a modification of the method described by Yegutkin (1997) according to Delwing et al. (2006) The reaction mixture containing 3 mM ATP, ADP or AMP as substrate, 112.5 mM Tris–HCl, pH 8.0, was incubated with approximately 1.0 mg of serum protein at 37 °C for 40 min in a final volume of 200 μl. The reaction was stopped by the addition of 200 μl of 10% TCA. The samples were chilled on ice and the amount of inorganic phosphate (Pi) released was measured as described by (Chan et al., 1986). In order to correct non-enzymatic hydrolysis, we performed controls by adding the serum after the reaction was stopped with TCA. All samples were centrifuged at 5000*g* for 5 min to eliminate precipitated protein and the supernatant was used for the colorimetric assay. All samples were assayed in triplicate. Specific enzyme activity was expressed as nmol Pi released per min per mg of protein.

4.6. Protein determination

Protein was measured by the Comassie Blue method according to Bradford, using bovine serum albumin as standard (Bradford, 1976).

4.7. Statistical analysis

All assays were performed in duplicate and the mean was used for statistical analysis. Data were analyzed by one way ANOVA followed by the Duncan multiple test when *F*-test was significant. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software using a PC-compatible computer. Values of $p<0.05$ were considered to be significant.

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Capítulo II

ARTIGO 2

Running exercise effects on spatial and avoidance tasks in ovariectomized rats

Juliana Ben, Flávia M.S. Soares, Fernanda Cechetti, Carlos A. Netto and Angela T.S. Wyse

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Running exercise effects on spatial and avoidance tasks in ovariectomized rats

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ABSTRACT

We investigated whether physical exercise would affect ovariectomy-induced memory deficits in inhibitory avoidance and Morris water maze tasks. Female adult Wistar rats were assigned to one of the following groups: sham (submitted to surgery without removal of the ovaries), exercise, ovariectomized (Ovx) and Ovx plus exercise. Thirty days after ovariectomy or sham surgery, animals were submitted to one month of treadmill exercise training for 20 min, three times per week. After, rats were tested in inhibitory avoidance and Morris water maze tasks in order to verify ovariectomy effects on aversive and spatial memory performance. Results show that ovariectomized rats presented impairment in aversive memory and spatial navigation, both in reference and working memory protocols, when compared to sham group (control). Confirming our hypothesis, ovariectomized rats submitted to exercise had those impairments prevented. We conclude that ovariectomy significantly impairs aversive and spatial reference learning/memory and that physical exercise prevents such effects. These findings support that physical exercise might constitute an important strategy to minimize cognitive deficits found in postmenopausal women.

Keywords: Ovariectomy; Physical Exercise; Running; Water Maze Task; Inhibitory Avoidance Task

1. Introduction

Estrogens exert important actions in the reproductive system, as well as in the adult brain (Wise et al. 2001c; Wise 2002a). Estrogen deprivation is implicated in the pathogenesis of Alzheimer's disease and cerebral ischemia (Zhang et al. 1998b; van Duijn 1999) and studies have suggested that post-menopausal women are more vulnerable to cognitive deficits (Green and Simpkins 2000; Wise et al. 2001a; Wise et al. 2001c).

Hormone replacement therapy (HRT) has been used to treat menopause symptoms, however the occurrence of side effects like increased incidence of breast cancer and thromboembolic accidents points to the need of alternative treatments of signs and symptoms associated with menopause (Miquel et al. 2006a).

The effects of ovariectomy on cognition in experimental animals have been studied in an attempt to model the human condition. Some reports show that ovariectomy might impair spatial memory in rats (Singh et al. 1994; Monteiro et al. 2005a), although the mechanisms involved in such impairments are not clearly established. It was shown that estrogen improves memory retention (Fader et al. 1999; Sandstrom and Williams 2004) and promotes synaptic plasticity and modulates neurotransmission (Baum 2005; Bora et al. 2005; Pinkerton and Henderson 2005).

Regular physical activity has been related to improvement of cognitive function in humans and rats (Kramer 1999; Cotman and Berchtold 2002; Sutoo and Akiyama 2003; Berchtold et al. 2005). Physical exercise causes a variety of morphological effects (Arida et al. 2004), that can lead to evident changes in

brain structure and function (Booth and Lees 2006). Running exercise not only stimulates brain cells releasing more trophic factors and neurogenesis to maintain healthier brain function, but also protects against brain insults or reverses the injury consequences in rats (Wu et al. 2007).

Clinical studies show that aerobic activity improves learning and task acquisition, increases the secretion of key neurochemicals associated with synaptic plasticity and promotes the development of new neuronal architecture (Hillman et al. 2008). An increase in cell proliferation and cell survival in the dentate gyrus of the hippocampus is one of the most consistently observed effects of exercise training (van Praag et al. 1999a; van Praag et al. 1999b; Trejo et al. 2001). These newborn cells might facilitate learning and memory (Kempermann et al. 2004; Hillman et al. 2008). The process of adult neurogenesis is known to be influenced by a variety of factors, such as aging (Kuhn et al. 1996), inflammation (Ekdahl et al. 2003), stress (Karten et al. 2005), enriched environment (Kempermann et al. 1997) and physical exercise (van Praag et al. 1999b).

Beneficial effects of running exercise, such as enhanced neurogenesis (van Praag et al. 1999b; Fabel et al. 2003), long-term potentiation (LTP) (van Praag et al. 1999a), synaptic plasticity (Farmer et al. 2004), and hippocampus-dependent learning and memory (van Praag et al. 2005) have been related to the elevated expressions of neurotrophic factors, including brain-derived neurotrophic factor (BDNF) (Neeper et al. 1995; Adlard et al. 2005; Berchtold et al. 2005; Huang et al. 2006; Vaynman et al. 2006), fibroblast growth factor 2 (FGF-2) (Gomez-Pinilla et al. 1997), and insulin-like growth factor 1 (IGF-1) (Trejo et al. 2001). Among them, BDNF has emerged as a major regulator of

activity-dependent synaptic plasticity (Xu et al. 2000), as well as of neuronal survival and differentiation (Sairanen et al. 2005),

In the present work we investigated whether physical exercise would affect cognitive performance of rats subjected to ovariectomy, as measured by spatial and aversive memory tasks. The working hypothesis is that physical activity would prevent the memory impairments caused by ovariectomy.

2. Materials and methods

2.1. Animals and reagents

Female adult Wistar rats (3 months, 170–210 g BW) were obtained from the Central Animal House of the Department of Biochemistry, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. Animals were maintained on a 12/12 h light/dark cycle in an air-conditioned constant temperature (22±1°C) colony room, with free access to water. The NIH “Guide for the Care and Use of Laboratory Animals” (NIH publication No. 80-23, revised 1996) and the official governmental guidelines in compliance with the Federação das Sociedades Brasileiras de Biologia Experimental were followed in all experiments. The study was approved by the Ethics Committee of the Universidade Federal do Rio Grande do Sul, Brazil. All chemicals were purchased from Sigma Chemical Co., St Louis, MO, USA.

Animals were randomly assigned to one of the following groups: 1) sham (only submitted to surgery without removing of ovaries); 2) exercise; 3)

ovariectomy and, 4) ovariectomy followed by exercise. Animals were ovariectomized by the surgical removal of both ovaries under intraperitoneal (i.p.) ketamine anesthesia (90 mg/kg) and xylazine (10 mg/kg) to eliminate endogenous ovarian steroids (Waynforth and Flecknell 1992). One month after the surgery, animals were submitted to exercise training (Ben et al. 2009).

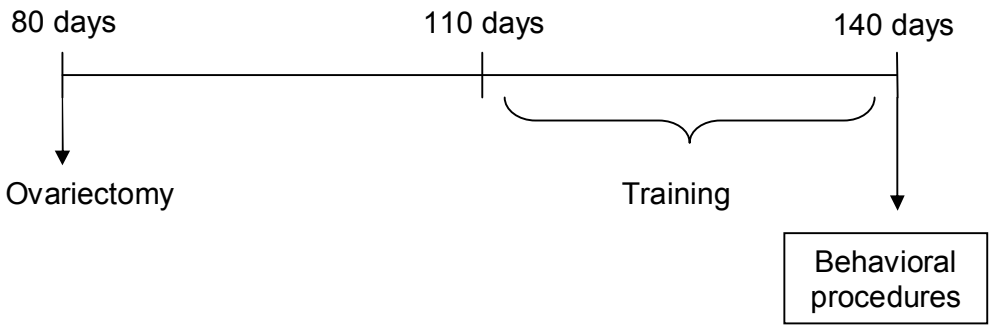
2.2. Exercise training

Rats were habituated with the treadmill apparatus to minimize novelty stress and randomly assigned to different experimental groups (n=10-15 in each group): non-exercised (sedentary - control group) and exercised during 20 min, 3 times a week. The exercise training consisted of running sessions on an adapted motorized rodent treadmill (INBRAMED TK 01, Porto Alegre, Brazil) at 60% of their maximal oxygen uptake (Brooks and White 1978). Measurement of oxygen uptake (VO_2) peak was carried out in all animals, indirectly before training, considering the exhaustion. Each rat ran on the treadmill at a low initial speed followed by increases in speed of 5 m/min every 3 min until the point of exhaustion (i.e., failure of the rats to continue running) and the time to fatigue (in min) and workload (in m/min) were taken as indexes of VO_{2max}. (Brooks and White 1978; Arida et al. 1999).

Animals that initially refused to run were encouraged by gently tapping their backs. Neither electric shock nor physical prodding was used in this study. Animals from the control group were transported to the experimental room and handled exactly as the experimental ones and maintained in the turned off treadmill for 5 min (Scopel et al. 2006).

Animals were adapted to the treadmill by gradually increasing running speed and time, as follows: week 1, at 18 m/min for the first 3 min, 24 m/min for the next 2 min, 36 or 48 m/min for the following 10 min, 24 m/min for the following 2 min and 18 m/min for the last 3 min; week 2, at 18 m/min for the first 3 min, 24 m/min for the next 1 min, 36 or 48 m/min for the following 12 min, 24 m/min for the following 1 min and 18 m/min for the last 3 min; weeks 3 and 4, at 18 m/min for the first 3 min, 36 or 48 m/min for the next 14 min, and 18 m/min for the last 3 min.

Twelve hours after the last exercise session rats started behavioral testing.



2.3. Behavioral procedures

On the 140th day of life, animals were subjected to behavioral testing, as described below.

2.3.1. Step-down inhibitory avoidance

Animals were subjected to training and test sessions in a step down inhibitory avoidance task with an interval of 24 h in between (Izquierdo and Medina 1997) . This task involves learning not to step down from a platform in order to avoid a mild footshock (Izquierdo and Medina 1997). The task was carried out in an automatically operated, brightly illuminated box. The left extreme of the grid was covered by a 7.0 cm wide, 2.5 cm high formic platform. Animals were placed on the platform and their latencies to step-down, placing their four paws on the grid (42.0 × 25.0 cm grid of parallel 0.1 cm caliber stainless steel bars spaced 1.0 cm apart), No foot shock was used in test session and step-down latency (with a ceiling of 180 s) was used as a measure of memory retention, as previously described (Wyse et al. 2004).

2.3.2. Morris water maze

We used the Morris water maze, an apparatus widely employed for the study of spatial learning and memory tasks that depend on hippocampal function (Morris et al. 1982; Netto et al. 1993; D'Hooge and De Deyn 2001). The water maze consisted of a black round tank, 200 cm in diameter and 100 cm high, filled to a depth of 50 cm with water, maintained at constant temperature of 23°C. The tank was theoretically divided into four equal quadrants for the purpose of analysis. Several distal visual cues were placed on the walls of the room. Trials were recorded by a video camera mounted above the center of the tank.

2.3.2.1. Reference memory task

The task consisted of six training and one test session. In the acquisition phase, rats had daily sessions of four trials per day for 6 days to find the platform, submerged 2 cm under the water surface, placed on the center of one of the quadrants of the tank during all training days. For each trial, the rat was placed in water facing tank wall, in one of the four starting locations (N, S, W and E). The order of starting position varied in every trial and any given sequence was not repeated on acquisition phase days. Rats were allowed to search for the platform during 60 s and, in the case of failing to find it, they were gently guided to it; all animals were permitted to remain on the platform for 10 s. Latency to find the platform was measured in each trial. The interval between trials was 15-20 min. One day after the last training trial, each rat was subjected to a probe trial in which the platform was removed. We measured four parameters, namely latency to cross on the location of the platform, the number of target crossings and the time spent in target (the quadrant in which the platform was located in the training sessions) and opposite quadrants. These parameters were taken as a measure for spatial memory (Netto et al. 1993). In order to detect motor impairments that could affect performance in experimental groups, the swimming speed was calculated by taking the distance traveled in the first 15 s of the probe trial.

2.3.2.2. Working memory task

After 1 week, the working memory version of Morris water maze was performed. The task consisted of four consecutive trials per day, with a 30-s

inter-trial interval, when the animals were placed in the tank facing the wall and allowed to search for the submerged platform, positioned on the center of one of the quadrants. Platform position changed every subsequent day during the four testing days. Latencies to find the platform in every first, second, third and fourth trials were calculated considering all testing days so to assess working memory performance (Netto et al. 1993).

2.4. Statistical analysis

Reference memory training and working memory data were analyzed by repeated measure analysis of variance (ANOVA) and data from the probe trial parameters were analyzed by one-way ANOVA; post hoc Duncan multiple range test was run when indicated. Descriptive statistics data were expressed as mean ± SEM.

Differences between test and training latency differences on inhibitory avoidance task were assessed by Kruskal-Wallis test followed by Dunn test, Descriptive statistics is expressed as median (interval interquartile).

All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software, in a PC-compatible computer; $p<0.05$ was considered significant.

3. Results

3.1. Step-down inhibitory avoidance

Non-parametric analyses showed that there was no significant difference in the latency to step down from the platform during training. On test session, ovariectomized rats presented shorter step-down latencies than sham animals (p<0.0001); exercise *per se* did not alter this behavioral parameter in sham animals, but prevented the decrease in step-down latency of ovariectomized rats (Fig. 1, Kruskal-Wallis test, x^2(3)=20.56; p<0.0001).

3.2. Morris water maze task

3.2.1. Reference memory task

Ovariectomized animals showed lower ability to find the platform and learn its location in the 3rd , 5th and 6th days of training ([F(3,45)=6.793; p<0.001], [F(3,45)=5.111; p<0.01] and [F(3,45)=5.262; p<0.01] respectively) (Fig. 2). The probe trial was run 24 h after acquisition phase, when four parameters were evaluated: latency to cross and the number of crossings on the location of the platform, time spent in target and opposite quadrants (Table 1). Ovariectomy did not affect the number of crossings on the former platform location [F(3,45)=0.321; p>0.05], the time spent in target quadrant [F(3,45)=1.764; p>0.05] nor the latency to cross over the location of the platform [F(3,45)=1.643; p>0.05], when compared to sham groups. However, ovariectomized rats showed an enhancement of time spent in the opposite quadrant [F(3,45)=3.564; p<0.05]. Results show that exercise *per se* did not alter any behavioral parameter studied in sham animals, but prevented the increase in latency to find the platform in 3rd , 5th and 6th days of training in

ovariectomized rats. In addition, exercise reversed the enhancement of time spent in opposite quadrant on the probe trial. There was no difference in swim speed among groups, mean speed for all groups was 26.5 cm/s.

3.2.2. Working memory task

It is shown that ovariectomy affected rat performance in the working memory version of Morris water maze in the 2nd, 3rd and 4th trials ([$F(3,192)=3.263$; $p<0.05$], [$F(3,192)=8.183$; $p<0.001$] and [$F(3,192)=10.045$; $p<0.001$] respectively) (Fig. 3), as compared to sham group, with no change in the 1st trial [$F(3,192)=0.516$; $p>0.05$]. Exercise *per se* did not alter any behavioral parameter studied in sham animals, but prevented the increase in latency to find the platform in the 2nd, 3rd and 4th trials in ovariectomized rats.

We also observed that the animal weight gain was increased by ovariectomy and exercise also prevented such effect [$F(3,50)=4.512$; $p<0.01$] (Table 2).

4. Discussion

The present study investigated the effect of ovariectomy, the most common animal model of hormone deprivation in adult female rats (Savonenko and Markowska 2003), on spatial navigation tasks in the Morris water maze and on aversive memory in the inhibitory avoidance, as well as the influence of physical exercise on such effects. Results show that ovariectomized rats are

impaired in inhibitory avoidance performance (Fig. 1), as well as in the acquisition phase (Fig. 2) and probe trial (Table 1) of reference memory task. In the working memory protocol, ovariectomized rats were also impaired in the 2nd, 3rd and 4th trials (Fig. 3). Treadmill running exercise did not affect task performance *per se*, i.e., when compared to controls (sham), but prevented the memory impairment caused by ovariectomy. To our knowledge, this is the first report showing that physical exercise is able to reverse ovariectomy-dependent memory impairments in the rat.

This is in agreement with previous studies showing that ovariectomized rats present spatial memory impairment (Singh et al. 1994; Monteiro et al. 2005a). The lack of difference between gonadally intact (sham) and ovariectomized groups during the training in inhibitory avoidance and in the first days of training on Morris water maze, suggests that gonodal estrogens are not biasing our results. Additionally, the probe trial, in which ovariectomized rats performed poorly than controls, provided a measure of reference memory that was independent of motor performance, since swim speed did not vary between groups.

It has been recognized that treadmill exercise training improves both passive avoidance and Morris water maze performances in rats (Alaei et al. 2006; Ang et al. 2006; Huang et al. 2006) suggesting that treadmill exercise with careful handling does not induce significant stress in rats (Chen et al. 2008). As for the possible mechanism of action, treadmill exercise in rats during 4 weeks significantly facilitated passive avoidance performance and down-regulated the serotonin (5-HT) system in some brain regions associated with aversive learning, hippocampus and amygdala (Chen et al. 2008).

It has been demonstrated that aged runners not only showed enhanced acquisition in the Morris water maze but also displayed, with bromodeoxyuridine labeling, more newborn neurons in the dentate gyrus than age-matched sedentary controls (van Praag et al. 2005). Other studies have reported exercise-induced neuron proliferation in the dentate gyrus of young and aged animals (van Praag et al. 1999b; Kim et al. 2004b). Summing up, cognitive effects of physical exercise possibly involve hippocampal neurogenesis (van Praag et al. 1999b; Fabel et al. 2003; During and Cao 2006), reduced oxidative stress (Ogonovszky et al. 2005; Radak et al. 2006), and increased BDNF levels (Neeper et al. 1995; Berchtold et al. 2005; Huang et al. 2006; Vaynman et al. 2006). Recently we have shown that running exercise reverses the increase in Na^+,K^+-ATPase and acetylcholinesterase activities in brain of rats subjected to ovariectomy (Ben et al. 2009).

It has been suggested that reduced expression of BDNF and protein cleavage, as tissue plasminogen activator, and the expression of neurotrophic receptors such as the tyrosine kinase receptor B may underlie age-related deficits in LTP, learning and memory, and hippocampal function in rodents. Exercise increases cognitive performance in both young and aged animals and increases mRNA and protein levels of BDNF, which may be contributing to exercise induced neurogenesis in the dentate gyrus (Kramer et al. 2006b).

A recent study showed that voluntary running increases cell proliferation in the hippocampus of ovariectomized mice and this neurogenesis is closely related to learning and memory (Jin et al. 2008). Voluntary running exercise after OVX increased the numbers of BrdU- and Ki-67-positive cells (markers of proliferation), and DCX- and calretinin-immunoreactive cells (markers of

differentiation) in the hippocampus of ovariectomized mice (Jin et al. 2008), suggesting that exercise may increase the birth of new neurons in the hippocampus under estrogen-deprived conditions (Jin et al. 2008).

Human studies report that fitness training have a positive influence on cognition; although fitness training broadly influenced a variety of cognitive processes, the largest positive effects were observed for executive control processes such as planning, scheduling, working memory, inhibitory processes, and multitasking (Kramer et al. 2006b). Interestingly, these are many of the processes that show substantial age-related decline (Hillman et al. 2008).

Aerobically trained older adults showed increased activity in the frontal and parietal regions of the brain, which are thought to be involved in efficient attentional control and performance on a test of executive functioning, and reduced activity in the dorsal region of the anterior cingulate cortex, a region thought to be sensitive to behavioral conflict or to the need for increased cognitive control (Colcombe et al. 2004). Older adults undergoing aerobic training showed a significant increase in gray matter volume in regions of the frontal and superior temporal lobe, compared with controls (Kramer et al. 2006b). These results suggest that even relatively short exercise interventions can begin to restore some of the losses in brain volume associated with normal aging (Kramer et al. 2006b). Such increases in brain volume have previously been shown to be predictive of performance in older adults (Erickson et al. 2007; Marks et al. 2007). In addition, a large prospective clinical study of older women showed that higher levels of long-term regular physical activity were strongly associated with higher levels of cognitive function and less cognitive decline (Weuve et al. 2004).

Our results also showed that physical exercise prevented the weight gain in ovariectomized rats (Table 2). It is reported that Ovx in rodents leads to weight gain and fat deposition probably caused by an increase in food intake; conversely exercise would promote a reduction in this weight gain, possibly due to a decrease in fat deposits in Ovx mice (Wu et al. 2008; Pighon et al. 2009). Exercise can also prevent weight gain in peri- and postmenopausal women, however factors related to menopause and aging make weight maintenance a challenge (Kohrt 2009).

In conclusion, the present study reports an impairment of spatial navigation and of aversive memory caused by ovariectomy in Wistar rats and that these effects were prevented by treadmill running exercise. The beneficial exercise effect is not task-specific, pointing to a broad action on cognitive performance. Considering that hormone deprivation might impair cognition in human beings, our results support that physical exercise could be an effective strategy to minimize cognitive deficits found in postmenopausal women.

Disclosure

Authors report that there are no actual or potential conflicts of interest that could inappropriately influence their work, including financial, personal or other relationships with other people or organizations within three years of beginning the work submitted. There was no additional funding.

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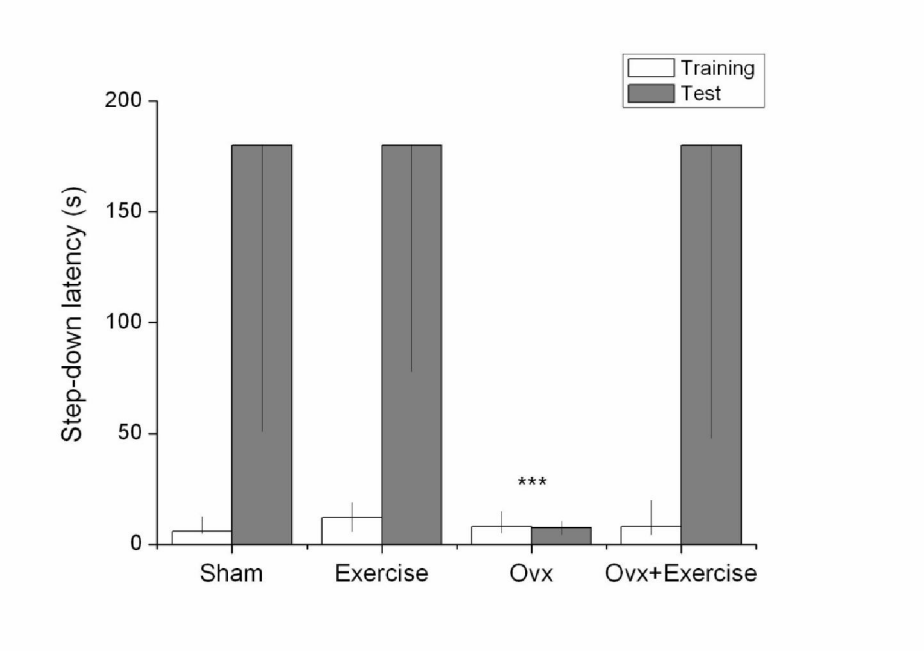

Fig. 1. Effect of ovariectomy and exercise on step-down inhibitory avoidance performance. Data are median (interquartile range) of 11–13 animals in each group. ***Different from the control group ($p<0.001$).

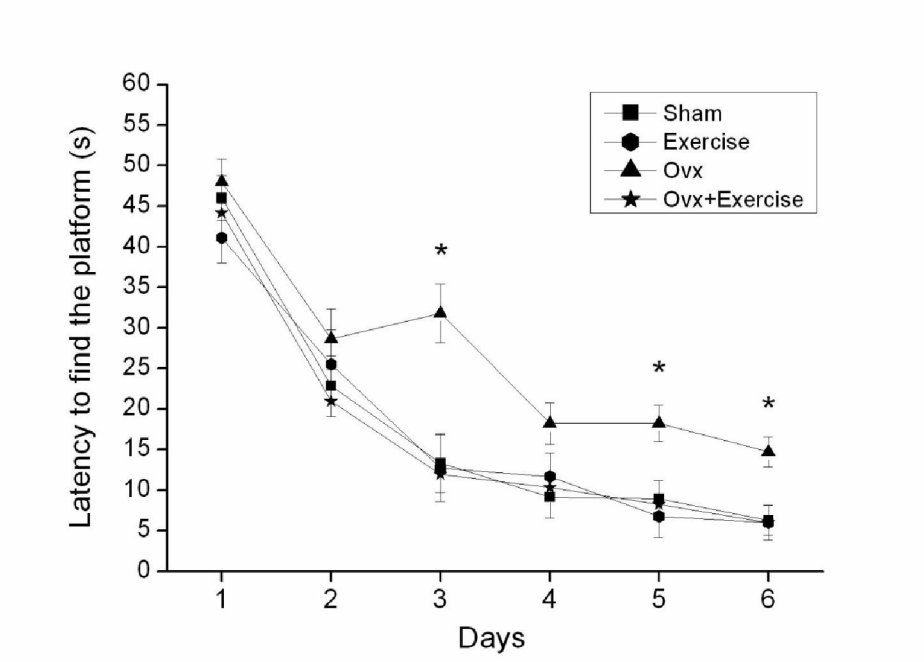

Fig. 2. Effects of ovariectomy and exercise on performance in the reference memory protocol in spatial working memory task. Data are expressed as mean

± S.E.M. for 10-13 animals in each group. *p<0.01 different from sham, exercise and Ovx+exercise groups (ANOVA). Ovx = ovariectomized.

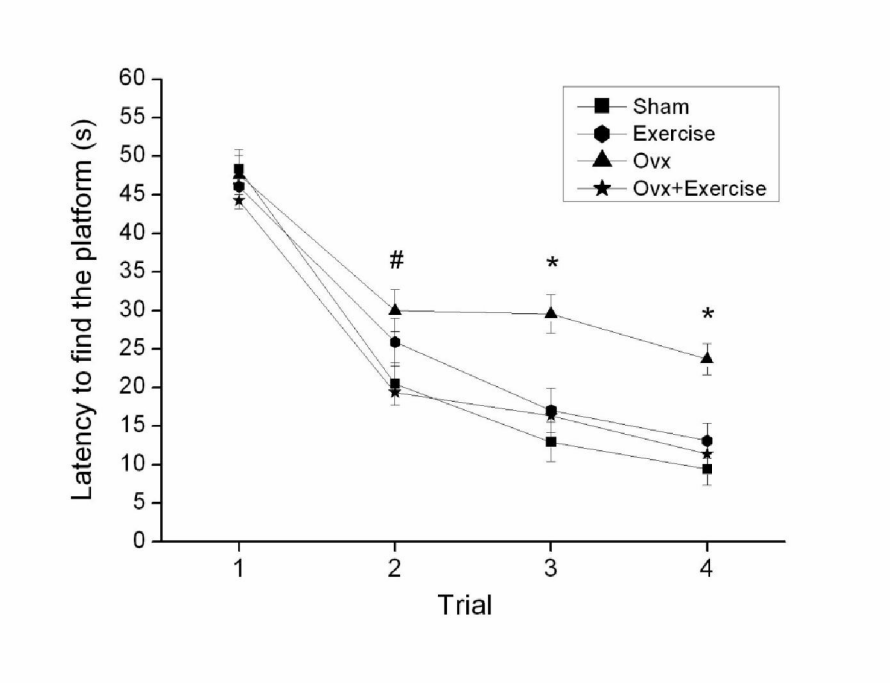

Fig. 3. Effect of ovariectomy on performance in the working memory version of Morris water maze spatial task. Data are latencies to find the platform on each trial during the four testing days and are expressed as mean ± S.E.M for 10-13 animals in each group. Ovx = ovariectomized.

* different from sham, exercise and Ovx+exercise groups (p<0.001).

different from sham and Ovx+exercise groups (p<0.05).

Table 1

Effects of ovariectomy and exercise on performance of spatial memory test session parameters of Morris water maze (s).

Groups	Time spend to cross the platform	Number of crossings on the platform
Sham	10.53 ± 3.08	4.85 ± 0.60
Exercise	9.30 ± 3.51	4.90 ± 0.68
Ovx	16.38 ± 3.08	4.15 ± 0.60
Ovx+Exercise	7,08 ± 3.08	4.46 ± 0.60

Groups	Time spend in the target quadrant	Time spend in the opposite quadrant
Sham	40.62 ± 2.05	5.15 ± 1.31
Exercise	41.20 ± 2.34	5.90 ± 1.50
Ovx	35.15 ± 2.05	10.69 ± 1.31*
Ovx+Exercise	37.54 ± 2.05	6.31 ± 1.31

Data are expressed as mean ± S.E.M. for 10-13 animals in each group. There was no significant difference between groups, except in the time spend in the opposite quadrant, *p<0.05 (ANOVA). Ovx = ovariectomized.

Table 2

Effect of ovariectomy and exercise on body weight of female adult rats.

Groups	Body weight (g) First day of experiment	Body weight (g) After 30 days of exercise
Sham	184.93 ± 13.88	222.67 ± 23.08
Exercise	187.36 ± 11.80	217.18 ± 7.73
Ovx	189.14 ± 11.92	240.29 ± 11.20*
Ovx+Exercise	189.36 ± 11.72	226.93 ± 18.38

Data are presented as mean ± S.D. for 11 to 15 rats in each group. Ovariectomized rats (Ovx) were significantly different from sham groups after 30 days of treatment *p<0.01 (ANOVA) and exercise reverted this effect.

IV. DISCUSSÃO

A menopausa tem sido associada com perda de memória e com o desenvolvimento de disfunções cognitivas (Henderson 1997). A falta de produção de estrógenos pelos ovários em mulheres pós-menopáusicas provoca alterações em diversos órgãos, incluindo o cérebro (Wise 2002b). Evidências sugerem que o estrogênio está envolvido na plasticidade neuronal (Woolley e McEwen 1993; Foy et al. 1999). Neste contexto, evidências mostram que o estrogênio aumenta a LTP (Warren et al. 1995; Foy et al. 1999), aumenta a neurogênese (Tanapat et al. 1999) e é capaz retardar o dano cerebral causado por doenças neurodegenerativas (McEwen 2002).

No presente trabalho, nós investigamos a influência do exercício físico sobre a ativação da Na^+,K^+-ATPase e da acetilcolinesterase em hipocampo e córtex cerebral causada pela ovariectomia em ratas adultas, bem como sobre a hidrólise de nucleotídeos de adenosina no córtex cerebral e no soro. Também investigamos o efeito do exercício sobre a memória espacial e aversiva em ratas adultas ovariectomizadas. Nós utilizamos esse modelo de privação de estrogênio porque a ovariectomia é considerada o modelo mais comum para mimetizar os efeitos da pós-menopausa em ratas adultas (Savonenko e Markowska 2003). O hipocampo e o córtex cerebral foram analisados por estarem intimamente associados com a modulação da memória (Daniel e Dohanich 2001) e sabe-se que ratas ovariectomizadas podem apresentar prejuízo na memória (Singh et al. 1994; Monteiro et al. 2005a; Monteiro et al. 2008).

Nossos resultados mostraram que a ovariectomia aumentou significativamente as atividades da Na^+,K^+-ATPase e da acetilcolinesterase no hipocampo e córtex cerebral de ratas. Estes resultados estão de acordo com estudos prévios de nosso laboratório, os quais também mostraram um aumento na atividade dessas enzimas em ratas ovariectomizadas (Monteiro et al. 2005b; Monteiro et al. 2005c; Monteiro et al. 2007). O exercício físico *per se* não alterou a atividade dessas enzimas, mas foi capaz de reverter a ação da ovariectomia sobre as atividades da Na^+,K^+-ATPase e da acetilcolinesterase no hipocampo e córtex cerebral de ratas adultas.

O mecanismo pelo qual o exercício físico reverte os efeitos da ovariectomia sobre a atividade da Na^+,K^+-ATPase não está esclarecido. Contudo a atividade dessa enzima pode ser modulada por diversos mecanismos. A enzima pode ser alterada por produtos de lipoperoxidação e por alterações na fluidez da membrana plasmática (Morel et al. 1998; Rauchova et al. 1999; Chakraborty et al. 2003). Sabe-se que o exercício físico regular aumenta a resistência contra espécies reativas de oxigênio (ROS) (Alessio et al. 1988) e diminui o acúmulo de proteínas oxidadas e o dano ao DNA (Leeuwenburgh et al. 1998; Radak et al. 1999). O exercício físico crônico também diminui os níveis de malondialdeido (MDA) no cérebro, uma medida de dano oxidativo aos lipídios, indicando um possível efeito benéfico do exercício crônico sobre a função cerebral e na proteção contra o dano oxidativo (Liu et al. 2000). Diversas vias de transdução de sinal também podem regular a atividade da Na^+,K^+-ATPase, como a formação de diferentes mediadores e a ativação de diversas cinases (Bertorello e Katz 1995). O exercício físico crônico pode modificar as vias de transdução de sinais que parecem estar envolvidos com a

plasticidade cerebral (Molteni et al. 2002). A Na^+,K^+-ATPase também pode ser modulada por mudanças na concentração intracelular de sódio (Inoue e Matsui 1991). Outra possibilidade poderia ser o aumento da síntese ou a diminuição da degradação da enzima.

Em nosso trabalho, o exercício físico também foi capaz de reverter o aumento da atividade da AChE causada pela Ovx, o que pode indicar uma influência do exercício sobre o sistema colinérgico cerebral. A estimulação da atividade da AChE provoca um aumento da hidrólise de acetilcolina (ACh) e recaptação de colina, reduzindo a atividade colinérgica no SNC (Okuda et al. 2000). Estudos anteriores mostraram que isoflavonas de soja previnem o aumento da atividade da AChE causado pela Ovx (Monteiro et al. 2007), sendo sugerido que fitoestrógenos de soja devem funcionar como um agonista de estrogênio, regulando a colina acetiltransferase (AChT) e fator de crescimento do nervo (NGF) no cérebro de ratas adultas (Pan et al. 1999). Neste contexto, o exercício físico pode reverter o aumento da atividade da AChE em ratas ovariectomizadas por aumentar o número de neurônios colinérgicos, os quais expressam receptores de NGF (Conner et al. 2009). Essa hipótese está de acordo com Ang e colaboradores (2003) que mostram um aumento da expressão de genes de NGF com o exercício físico. No entanto, nós não sabemos o exato mecanismo pelo qual a atividade da AChE está modificada em nosso estudo.

Também observamos em nosso estudo que a Ovx diminuiu significativamente a hidrólise de AMP e não alterou as hidrólises de ADP e ATP no córtex cerebral. O exercício físico não reverteu tal efeito, mas diminuiu significativamente as hidrólises do AMP e ADP. A atividade da 5'-nucleotidase

está envolvida na hidrólise de AMP à adenosina na fenda sináptica (Zimmermann 1992; Battastini et al. 1995), uma diminuição dessa enzima deve ter ocorrido como conseqüência da Ovx e/ou exercício físico. Em nosso estudo os níveis de adenosina, um importante agente neuroprotetor e neuromodulador (Bonan et al. 2001; Ribeiro et al. 2003), provavelmente estão mais baixos em todos os grupos testados quando comparados ao controle (sham). Neste contexto, Rose'Meyer e colaboradores (2003) demonstraram um menor padrão de expressão dos receptores de adenosina em cérebro total de ratas submetidas à Ovx. Contudo, efeitos controversos nos níveis de nucleotídeos têm sido promovidos por Ovx e exercício físico. Rucker e colaboradores (2005) mostraram que a Ovx aumentou a 5'nucleotidase em sinaptossomas de córtex cerebral, entretanto não alterou a atividade da NTPDase. Dworak e colaboradores (2007) não observaram nenhuma alteração nas concentrações de AMP, ADP e ATP após exercício em esteira moderado e exaustivo. Nossos resultados sugerem que o exercício físico é capaz de diminuir a atividade das enzimas envolvidas no controle dos níveis de nucleotídeos, promovendo uma diminuição dos níveis de adenosina, o que se pode concluir é que os efeitos benéficos do exercício físico não estão relacionados à ação neuroprotetora da adenosina no cérebro. Contudo, é importante considerar que os diferentes efeitos do exercício físico na hidrólise de nucleotídeos podem estar relacionados às diferentes intensidades e duração do exercício, bem como na estrutura cerebral investigada.

Considerando que a razão nucleotídeos/nucleosídeos na circulação pode apresentar algumas mudanças que podem provocar respostas no SNC e no sistema circulatório, no presente estudo também investigamos a hidrólise de

nucleotídeos no soro sanguíneo. Os resultados mostraram que a hidrólise de ADP e ATP não foi alterada em nenhum grupo quando comparados ao controle, sugerindo que a atividade da NTPDase no soro sanguíneo não foi alterada. Os resultados também mostraram um aumento na hidrólise de AMP no soro sanguíneo de ratas ovariectomizadas submetidas ao exercício físico, o que pode resultar no aumento da atividade da 5'-nucleotidase sérica. A adenosina, um inibidor de agregação plaquetária (Cristalli et al. 1995), pode estar aumentada em resposta ao exercício físico em ratas ovariectomizadas, o que pode ser uma compensação em situações de aumento da atividade plaquetária e risco cardiovascular, como observado em algumas mulheres pós-menopáusicas.

No presente estudo também investigamos o efeito da ovariectomia sobre a navegação espacial no labirinto aquático de Morris e sobre a memória aversiva na esquiva inibitória, assim como a influência do exercício físico em tais tarefas. Foi observado que a Ovx prejudicou o desempenho na tarefa de esquiva inibitória, na fase de aquisição e teste da memória de referência no labirinto aquático de Morris. No protocolo de memória de trabalho, as ratas ovariectomizadas apresentaram um prejuízo nas tentativas 2, 3 e 4. O exercício físico *per se* não alterou o desempenho em nenhuma tarefa analisada quando comparado ao controle (sham), mas preveniu o prejuízo causado pela Ovx.

Outros estudos também mostraram um prejuízo de memória em ratas ovariectomizadas (Singh et al. 1994; Monteiro et al. 2005a; Monteiro et al. 2008). Não houve diferença entre os grupos controle e Ovx durante o treino na esquiva inibitória e nos primeiros dias de treinamento no labirinto aquático de Morris. O desempenho motor não foi afetado pela Ovx, visto que a velocidade

do nado não variou entre os grupos na tarefa de labirinto aquático de Morris. Esses resultados corroboram com estudos prévios realizados em nosso laboratório que mostraram que a Ovx não alterou a atividade motora no campo aberto (Monteiro et al. 2005a; Monteiro et al. 2008).

Tem sido mostrado que o exercício físico em esteira aumenta o desempenho tanto em esquiva inibitória quanto no labirinto aquático de Morris (Alaei et al. 2006; Ang et al. 2006; Huang et al. 2006), sugerindo que o exercício em esteira não induz estresse significativo nos ratos (Chen et al. 2008).

No presente estudo os testes comportamentais não foram influenciados pelo exercício *per se*, apesar de muitos estudos relatarem o benefício do exercício físico nas funções cognitivas. Esses resultados controversos podem estar relacionados com as diferentes durações e intensidades dos exercícios físicos realizados, assim como a idade dos animais utilizados.

A memória na esquiva inibitória requer receptores de glutamato, proteína cinase dependente de AMPc (PKA), MAPK em determinadas regiões cerebrais, como o córtex cerebral (Barros et al. 2000). E sabe-se que o exercício é capaz de alterar esses componentes (Molteni et al. 2002), podendo dessa maneira estar contribuindo para o melhor desempenho na tarefa de esquiva inibitória encontrado nas ratas Ovx submetidas ao exercício em nosso trabalho.

O exercício físico em esteira por quatro semanas facilitou significativamente o desempenho na esquiva inibitória e apresentou uma diminuição do padrão de expressão do sistema serotoninérgico em algumas regiões cerebrais associadas com o aprendizado aversivo, como o hipocampo e amígdala (Chen et al. 2008). Este pode ser outro possível mecanismo pelo

qual as ratas ovariectomizadas submetidas ao exercício melhoraram o desempenho na tarefa de esquiva inibitória.

Foi demonstrado que ratos de 19 meses de idade que correm mostram um aumento na aquisição no labirinto aquático de Morris, mas também apresentam um maior crescimento neuronal no giro denteado que os controles sedentários (van Praag et al. 2005). Outros estudos observaram uma indução da proliferação neuronal pelo exercício físico no giro denteado de animais jovens e com idade avançada (van Praag et al. 1999b; Kim et al. 2004a). Possivelmente, os efeitos cognitivos do exercício físico estão relacionados com a neurogênese hipocampal (van Praag et al. 1999b; Fabel et al. 2003; During e Cao 2006), com a redução do estresse oxidativo (Ogonovszky et al. 2005; Radak et al. 2006), e com o aumento dos níveis de BDNF (Neeper et al. 1995; Berchtold et al. 2005; Huang et al. 2006; Vaynman et al. 2006). Neste trabalho nós mostramos que o exercício físico em esteira reverte o aumento das atividades da Na^+,K^+-ATPase e da AChE em cérebro de ratas submetidas à Ovx, enzimas relacionadas com os processos de memória (Brunelli et al. 1997; Wyse et al. 2004; Ballard et al. 2005).

Tem sido sugerido que a redução da expressão de BDNF e quebra de proteínas, como o ativador do plasminogênio tecidual, e a expressão de receptores neurotróficos, como o receptor de tirosina quinase B, devem estar envolvidos nos déficits na LTP, memória e aprendizado, e funções hipocampais associados com a idade de roedores. O exercício físico aumenta o desempenho cognitivo em animais jovens e idosos e aumenta o mRNA e os níveis de proteína de BDNF, o que pode estar contribuindo para a neurogênese no giro denteado induzida pelo exercício físico (Kramer et al. 2006a).

Um recente estudo mostrou que a corrida voluntária aumentou a proliferação celular no hipocampo de ratas ovariectomizadas e que essa neurogênese está intimamente relacionada com a memória e o aprendizado (Jin et al. 2008). Estudos mostraram que a corrida voluntária após a Ovx aumentou os números de células BrdU- e Ki67-positivas (marcadores de proliferação), e de células DCX- e calretinina-imunoreativas (marcadores de diferenciação) no hipocampo de ratas ovariectomizadas, sugerindo que o exercício físico deve aumentar o surgimento de novos neurônios no hipocampo em condições de privação de estrogênio (Jin et al. 2008).

Estudos com humanos mostraram que o exercício físico exerce uma influência positiva na cognição, entretanto o maior efeito positivo foi observado em processos de controle executivo, como planejar, relacionar, memória de trabalho, processos inibitórios e múltiplas tarefas (Kramer et al. 2006a). Muitos desses processos apresentam um declínio substancial com o envelhecimento (Hillman et al. 2008).

Indivíduos idosos treinados aerobicamente mostraram aumento da atividade das regiões frontal e parietal do cérebro, as quais estão envolvidas no controle da concentração e desempenho no teste de função executiva eficientes, e redução da atividade na região dorsal do córtex cingulado anterior, região sensível ao comportamento de conflito ou à necessidade de aumento do controle cognitivo (Colcombe et al. 2004). O treinamento aeróbico em idosos promove um aumento significativo no volume de massa cinzenta nas regiões do lobo frontal e temporal superior, comparados aos controles (Kramer et al. 2006a). Esses resultados sugerem que, mesmo com intervenções curtas de exercício físico, as perdas no volume cerebral associadas com o

envelhecimento podem começar a ser restauradas (Kramer et al. 2006a). Tais aumentos do volume cerebral foram previamente associados com o aumento do desempenho em idosos (Erickson et al. 2007; Marks et al. 2007). Um estudo clínico mostrou que mulheres idosas que foram submetidas a altos níveis de atividade física regular apresentaram altos níveis de função cognitiva e um menor declínio cognitivo (Weuve et al. 2004).

Nesse estudo, também observamos que a Ovx aumentou o ganho de peso dos animais e que o exercício físico reverteu este efeito. Saengsirisuwan e colaboradores (2009) sugeriram que o aumento no gasto energético pelo exercício físico pode prevenir o acúmulo de gordura e/ou aumentar a utilização de lipídios pelas ratas ovariectomizadas (Wu et al. 2008; Pighon et al. 2009).

✓ Nossos achados mostram que a ovariectomia diminui a hidrólise do AMP cerebral; o exercício físico não reverte tal efeito e diminui a hidrólise de AMP e ADP. Por outro lado, a ovariectomia prejudica significativamente a memória/aprendizado nas tarefas aversiva e espacial e altera as atividades das enzimas Na^+,K^+-ATPase e AChE, e o exercício físico reverte tais efeitos. Esses dados sugerem que o exercício físico pode se mostrar uma estratégia importante para minimizar déficits cognitivos encontrados em mulheres pós-menopáusicas.

V. CONCLUSÕES

A ovariectomia em ratas adultas:

✓ Aumentou a atividade da Na^+,K^+-ATPase em hipocampo e córtex cerebral;

✓ Aumentou a atividade da AChE em hipocampo e córtex cerebral;

✓ Diminuiu a hidrólise de AMP em sinaptossoma de córtex cerebral e não alterou a hidrólise de nucleotídeos de adenosina no soro sanguíneo;

✓ Prejudicou o desempenho na tarefa de labirinto aquático de Morris, apresentando déficit na memória de referência e de trabalho;

✓ Prejudicou o desempenho na tarefa de esquiva inibitória, apresentando déficit na memória aversiva;

✓ Aumentou o ganho de peso corporal.

O exercício em ratas adultas:

✓ Reverteu o aumento da Na^+,K^+-ATPase em hipocampo e córtex cerebral causado pela Ovx;

✓ Reverteu o aumento da atividade da AChE em hipocampo e córtex cerebral causado pela Ovx;

✓ Diminuiu a hidrólise de AMP e ADP e não alterou os efeitos causados no córtex cerebral pela Ovx;

✓ Aumentou a hidrólise de AMP em soro sanguíneo de ratas ovariectomizadas;

✓ Reverteu o prejuízo de memória espacial na tarefa de labirinto aquático de Morris em ratas ovariectomizadas;

✓ Reverteu o prejuízo de memória aversiva na tarefa de esquiva inibitória em ratas ovariectomizadas;

✓ Reverteu o aumento de peso corporal causado pela Ovx.

VI. PERSPECTIVAS

1) Estudar os mecanismos de alteração das atividades da Na^+,K^+-ATPase, AChE e ectonucleotidases, bem como do déficit de memória causados pela Ovx e o efeito neuroprotetor do exercício físico;

2) Determinar os níveis de BDNF no cérebro de animais submetidos à Ovx com ou sem exercício físico;

3) Avaliar se o exercício físico realizado anteriormente à Ovx também previne as alterações bioquímicas e comportamentais causadas por esse modelo experimental.

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