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Efeitos do treinamento aeróbio prévio ao infarto do miocárdio na densidade celular e na imunorreatividade da proteína ácida fibrilar glial na amígdala medial póstero-dorsal de ratos com insuficiência cardíaca

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“Foi o tempo que dedicaste à tua rosa que fez
tua rosa tão importante”
(Antoine de Saint Exupéry)

RESUMO

Estudos prévios têm demonstrado que o exercício aeróbio tem efeitos neuroprotetores enquanto o infarto do miocárdio (IM) e a insuficiência cardíaca (IC) podem causar morte neuronal e gliose reativa na amígdala como um todo. O núcleo póstero-dorsal da amígdala medial (MePD) está envolvido com reflexos cardiovasculares, no controle central da resposta simpática/parassimpática. O objetivo desse estudo foi avaliar os efeitos do exercício prévio ao IM/IC na densidade de células neuronais e gliais e na imunorreatividade da proteína ácida fibrilar glial (GFAP-ir) no MePD de ratos machos adultos. Os grupos estudados foram: (1) controle não-manipulado (Cont), (2) ratos sedentários submetidos à cirurgia fictícia de IM (Sed Sham), (3) ratos sedentários submetidos à oclusão cirúrgica da artéria coronária esquerda (Sed IC), (4) ratos submetidos a treinamento aeróbio (corrida em esteira adaptada por 20 min, 3 vezes/semana ao longo de 4 semanas) e à cirurgia sham (T Sham), e (5) ratos também treinados submetidos ao IM (T IC). Os ratos apresentaram melhora significativa do desempenho físico, observados por meio do incremento do tempo e da velocidade no teste de esforço, realizado antes e após o protocolo de treinamento. Os ratos infartados apresentaram área de lesão média de, aproximadamente, 40% da parede do ventrículo esquerdo. Após 5 semanas da indução da IC pelo IM, o número de neurônios e células gliais no MePD foi estimado utilizando a técnica de estereologia por fracionador óptico e a GFAP-ir foi quantificada por densitometria óptica. Os resultados foram obtidos a partir da coluna medial do MePD (MePDm), que apresenta grande quantidade de células densamente agrupadas, no lado direito e esquerdo, ao longo dos aspectos rostral e caudal do MePD. Os resultados mostraram efeito de lateralização hemisférica sobre a densidade de neurônios (maior no MePD direita), mas não houve diferença significativa na avaliação das densidades tanto neuronais e gliais ($p > 0,1$). Por outro lado, a GFAP-ir regional revelou uma interação significativa entre lateralidade hemisférica e condição experimental, onde o grupo Sed IC apresentou aumento da expressão de GFAP no MePD à esquerda em comparação com o grupo Cont e o Sed Sham ($p < 0,01$). Os presentes dados não evidenciaram modificação na densidade celular após treinamento prévio e/ou IM/IC no MePD, mas indicam uma possível reestruturação no citoesqueleto astrocitário após IM/IC.

Palavras-chave: treinamento aeróbio, amígdala estentida, imunorreatividade da GFAP, infarto do miocárdio, densitometria óptica, fracionador óptico.

ABSTRACT

Previous data showed that aerobic exercise has neuroprotective effects whereas myocardial infarction (MI) and heart failure can cause neuronal death and reactive gliosis in the whole amygdala. The posterodorsal medial amygdala (MePD) is involved with cardiovascular reflexes and the central control of sympathetic/parasympathetic responses. Our aim was to study the effects of exercise preconditioning and the MI-induced chronic heart failure (HF) on the neuronal and glial densities and the glial fibrillary acidic protein-immunoreactivity (GFAP-ir) in the MePD of adult male rats. Studied groups were: (1) non-manipulated control (Cont), (2) sedentary rats submitted to a sham MI (Sed Sham), (3) sedentary rats submitted to the surgical occlusion of the left descending coronary artery and MI/CHF (Sed HF), (4) aerobically trained rats (treadmill training for 20 min, 3 times/week along 4 weeks) submitted to a sham MI (T Sham), and (5) aerobically trained rats submitted to MI/HF (T HF). Training protocol improved rats physical performance and MI damaged near 40% of the left ventricle wall. At the end of 5 weeks, the number of neurons and glial cells in the MePD was estimated using the optical fracionator stereological approach and the GFAP-ir was quantified by optical densitometry. Results were obtained from the densely packed cell column from both the right and left MePD and along the anterior and posterior aspects of the MePD. Results showed a hemispheric lateralization effect on the density of neurons (higher in the right MePD), but there were no statistically significant differences due to the experimental condition in both neuronal and glial densities ($p > 0.1$). On the other hand, regional GFAP-ir showed a significant interaction for hemispheric laterality and experimental condition and the Sed HF group had an increased GFAP expression in the left MePD compared to the control and Sed Sham rats ($p < 0.01$). Present novel data did not evidence a modification in cellular density after preconditioning training and/or MI/HF specifically in the MePD, but indicate a possible restructuring in astrocytic cytoskeleton after MI/HF in rats.

Key words: aerobic training, extended amygdala, GFAP immunoreactivity, myocardial infarction, optical densitometry, optical fracionator.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS E SIGLAS

ACe	Núcleo central da amígdala
AL	Amígdala lateral
AMBl	Porção basilar lateral da amígdala
ANOVA	Analysis of variance (análise da variância)
AOI	“Area of interest”
BDNF	Fator neurotrófico derivado do encéfalo
BNST	Núcleo intersticial da estria terminal
DC	Débito cardíaco
FC	Frequência cardíaca
FGF	Fator de crescimento de fibroblastos
FR	Frequência respiratória
GFAP	Glial fibrillary acidic protein (proteína ácida fibrilar glial)
GFAP-ir	Glial fibrillary acidic protein-immunoreactivity (imunorreatividade da proteína ácida fibrilar glial)
HF	Heart Failure
IAM	Infarto agudo do miocárdio
IC	Insuficiência cardíaca
IM	Infarto do miocárdio
MeA	Núcleo medial da amígdala
MeAD	Núcleo ântero-dorsal da amígdala medial
MeAV	Núcleo ântero-ventral da amígdala medial
MePD	Posterodorsal medial amygdala (núcleo póstero-dorsal da amígdala medial)
MePDi	Coluna intermediária do MePD
MePDl	Coluna lateral do MePD
MePDm	Coluna medial do MePD
MePV	Núcleo póstero-ventral da amígdala medial
MI	Myocardial infarction
NGF	Fator de crescimento neural
NTS	Núcleo do trato solitário
OT	Optic tract

PA	Pressão arterial
PFA	Paraformaldehyde
SD	Standard deviation
SN	Sistema nervoso
SNC	Sistema nervoso central
ST	Stria terminalis (estria terminal)
TO	Trato óptico

SUMÁRIO

CAPÍTULO I

1 INTRODUÇÃO

O exercício físico aeróbio induz melhora das funções cognitivas (Gligoroska e Manchevska, 2012) envolvendo diversas formas de aprendizado (Neeper et al., 1995; Molteni et al., 2002; Molteni et al., 2004; van Praag et al., 2005; Kashihara et al., 2009) e memória (Alaei et al., 2008; De Senna et al., 2011; Lin et al., 2012; Bechara et al., 2013) além de reduzir índices de ansiedade e depressão (Martinsen, 2008). Adicionalmente, tem potencial na indução da plasticidade neural por promover angiogênese (Swain et al., 2003; van der Borght et al., 2009; Gligoroska e Manchevska, 2012; Zhang et al., 2013), neurogênese (van Praag et al. 1999; Kim et al., 2003; van Praag et al. 2005; van der Borght et al., 2009; Gligoroska e Manchevska, 2012; Lee et al., 2013; Curlik et al., 2013), modificar morfofisiologicamente as células neurais e aumentar a liberação de fatores tróficos em quantidade e tempo de ação em regiões do sistema nervoso central (SNC) de roedores (Neeper et al., 1996; Gomez Pinilla et al., 1997; Berchtold et al., 2010; Bechara et al., 2013). Pode ainda promover aumento na proliferação celular e modificações na densidade de espinhos dendríticos em regiões como o córtex motor (Adkins et al., 2006), cerebelo (Gonzalez-Burgos et al., 2011), amígdala basolateral, região CA3 hipocampal (Lin et al., 2012), regiões CA1 e giro denteado do hipocampo e estriado de ratos, tanto em condições de normalidade quanto após lesão (Eadie et al., 2005; Wu et al., 2007; Hillman et al., 2008; Curlik et al., 2013; Bechara et al., 2013).

Além disso, a atividade física regular de intensidade moderada tem efeitos favoráveis sobre o encéfalo, incluindo propriedades neuroprotetoras, bem como a redução da morte celular neuronal induzida por isquemia no giro denteado e região CA1 do hipocampo (Sim et al., 2005; Zheng et al., 2006; Scopel et al., 2006; Cechetti et al., 2007). Além disso, impede a morte neuronal e danos induzidos por outros agentes excitotóxicos (Carro et al., 2001). Por outro lado, o infarto do miocárdio (IM) é uma das causas mais comuns de insuficiência cardíaca (IC) e afeta órgãos centrais e periféricos. Estudos demonstram a presença de morte neuronal por apoptose em regiões do encéfalo de ratos após IM (Kaloustian et al., 2008). Isso inclui a amígdala como um todo (Wann et al., 2006) ou, especificamente, os núcleos

lateral e medial da amígdala, a área CA1 e o giro denteado do hipocampo (Wann et al., 2006, Kaloustian et al., 2008) e parte do hipotálamo aos 1, 2 e 7 dias após IM (Kaloustian et al., 2008). Além disso, morte celular e gliose reativa são encontrados na amígdala (como um todo) de ratos 14 dias após o IM (Bae et al., 2010). Esses dados são interessantes, pois refletem a remodelamento estrutural de áreas centrais depois do IM e, mais especificamente, de alguns núcleos da amígdala com funções neuroendócrinas e comportamentais integrativas (Dayas et al., 1999; Rasia-Filho et al., 2000; Marcuzzo et al., 2007; Rasia-Filho et al., 2012).

É notável que o núcleo póstero-dorsal da amígdala medial (MePD) modula comportamentos sociais, respostas ao estresse e faça parte do controle central dos barorreceptores e quimiorreceptores, mediando reflexos e ajustes cardiovasculares que envolvem atividade simpática e parassimpática (Quagliotto et al., 2008; Neckel et al., 2012; Quagliotto et al., 2012). O MePD de ratos tem ligações relevantes diretas e/ou indiretas com outros núcleos da amígdala, do hipotálamo e áreas do tronco cerebral (Canteras et al., 1995; Petrovich et al., 2001; Saha, 2005) que recebem aferências sensoriais cardiovasculares e regulam a freqüência cardíaca (FC) e a pressão arterial (PA) (Turner et al., 1986; Longhurst, 2008) em condições fisiológicas, adaptativas e/ou patológicas (Kubo et al., 2004; Davern e Head, 2011; Quagliotto et al., 2012).

Sabe-se que o treinamento aeróbio diminui o tônus simpático e aumenta a atividade parassimpática (Martins-Pinge, 2011). Nas doenças cardiovasculares como a IC que há excesso de ativação do sistema nervoso simpático e está diretamente relacionado com a morbidade e mortalidade nesta síndrome (Martins-Pinge, 2011). No entanto, atualmente, não é sabido se o treinamento aeróbio prévio e/ou a ocorrência de IM/IC podem alterar o número de neurônios e células gliais ou a imunorreatividade astrocitária no MePD de ratos. O MePD é uma área específica relevante a ser estudada devido ao seu papel no controle cardiovascular central e os presentes resultados experimentais podem ser complementares aos dados publicados anteriormente sobre os efeitos neuroprotetores do treinamento aeróbio (Eadie et al., 2005; Adkins et al., 2006; Scopel et al., 2006; Cechetti et al., 2007; González-Burgos et al., 2011) ou perda celular após o IM/IC (Wann et al., 2006; Kaloustian et al., 2008; Bae et al., 2010), alguns deles envolvendo toda a amígdala ou parte dela.

2 REVISÃO DE LITERATURA – CONTEXTUALIZAÇÃO

2. 1 AMÍGDALA

A amígdala, ou complexo amigdaliano, constitui-se de núcleos subcorticais que se situam no lobo temporal, lateral ao hipotálamo e ventral ao estriado, no encéfalo de mamíferos. Em primatas, é caracterizada como uma massa ovóide de substância cinzenta, localizada na porção terminal e rostral da formação hipocampal, tendo como limite anterior o corno temporal do ventrículo lateral (Rasia-Filho et al., 2000; de Olmos et al., 2004; Rasia-Filho et al., 2004). Em ratos, é constituída por núcleos e subnúcleos que formam uma complexa rede estrutural inter-relacionada e multifuncional, sendo, dessa forma, importante para sobrevivência e adaptação do animal em seu ambiente, pois participa de atividades comportamentais, endócrinas, simpáticas e parassimpáticas, inatas e aprendidas (Rasia-Filho et al., 2000).

Nesses animais, apesar dos limites anatômicos precisos, a classificação de suas subdivisões permanece ainda controversa (Canteras et al., 1995; Swanson e Petrovich, 1998; de Olmos et al., 2004; Dall'Oglio et al., 2008), porém, aceita-se que a amígdala seja dividida em quatro regiões: 1) amígdala "expandida", denominada assim, pois estende-se além de seus limites anatômicos e é formada pelos núcleos medial (MeA) e central (ACe) da amígdala; 2) amígdala com características corticais, subdividida em porção basilar lateral (AMBl) e em porções que se ligam às vias olfatórias e vomeronasal; 3) áreas de transição, localizadas entre a porção ventral dos núcleos da base e a amígdala "expandida"; e 4) núcleos ainda não classificados, constituídos por um grande grupo de células dispersas na substância branca e no interior do BNST (de Olmos et al., 2004; Swanson e Petrovich, 1998).

O MeA é um dos núcleos superficiais do complexo amigdaliano que, ao longo de sua extensão, está em contato direto com a região lateral do trato óptico (TO), ventral à estria terminal (ST) em sua parte mais posterior, em posição medial e posterior ao núcleo do trato olfativo e, tendo como limite posterior, aproximadamente onde surgem os ventrículos cerebrais (de Olmos et al., 2004). Baseado em critérios citoarquitetônicos, hodológicos e funcionais, o MeA tem sido dividido em quatro subnúcleos: núcleo medial ântero-dorsal (MeAD), núcleo medial ântero-ventral

(MeAV), MePD e núcleo medial póstero-ventral (MePV) (Canteras et al., 1995; de Olmos et al., 2004; de Castilhos et al., 2006; Dall'Oglio et al., 2008; Arpini et al., 2010; Brusco et al., 2010).

O MePD localiza-se adjacente ao TO e ventralmente a ST podendo, ainda, ser subdividido, conforme a disposição de seus neurônios, em três colunas paralelas. A primeira coluna é a superficial ou medial (MePDm) e possui muitas células agrupadas compactamente e de tamanho pequeno a médio; a segunda é a coluna intermediária (MePDi) e possui neurônios de tamanho médio; a terceira é a coluna mais "profunda" e lateral (MePDl), também com neurônios de tamanho médio. Assim, a MePDm é a coluna com a maior quantidade de neurônios densamente agrupados, seguida pelas colunas lateral e intermediária, respectivamente (de Castilhos et al., 2006).

2.1.1 Aferências e Eferências da Amígdala Medial Póstero-Dorsal e Participação no Controle Central Cardiovascular

O MePD recebe aferências de diversas regiões encefálicas, como as densas projeções do sistema olfativo, especificamente do bulbo olfativo acessório; de núcleos corticais, como o córtex infralímbico, área pré-límbica e ínsula agranular ventral; da formação hipocampal, como o córtex entorrinal e partes proximal e distal do *subiculum* temporal; de núcleos hipotalâmicos, dentre eles, a área pré-óptica medial, regiões dorsal e medial do núcleo paraventricular, núcleos ventromedial, pré-mamilar ventral e dorsal, arqueado, lateral; de núcleos talâmicos, como os núcleos paraventricular, parataenial, parafascicular, basal do complexo ventromedial e divisão medial do complexo geniculado medial; do tronco encefálico; da divisão medial do BNST; e do núcleo da divisão horizontal da faixa diagonal de Broca, entre outras (McDonald, 1998; Pitkänen et al., 2000). Este subnúcleo também se encontra interconectado com os demais subnúcleos do MeA (Canteras et al., 1995). Além disso, o MePD possui conexões intra-amigdalianas, sendo muitas delas recíprocas, podendo-se destacar as divisões magnocelular e parvocelular do núcleo basal acessório, as divisões medial e ventro-lateral do núcleo lateral, os núcleos cortical anterior e posterior e as divisões medial e lateral da área amígdalo-hipocampal (Ottersen e Ben-Ari, 1979; Ottersen, 1980; de Olmos et al., 2004).

O MePD também envia uma série de eferências a diversas regiões encefálicas, entre as quais estão aquelas para alguns núcleos hipotalâmicos como o posterior, o anterior, o periventricular ântero-ventral, o pré-óptico medial e o pré-mamilar ventral; para áreas corticais, principalmente entorrinal lateral, de transição pós-piriforme, CA1 hipocampal e *subiculum*; áreas do tronco encefálico, para tegmental dorsal e substância cinzenta periaqueductal; para outras regiões, como o BNST, parte ântero-dorsal e posterior principal; e para a substância *innominata* (Canteras et al., 1995; Petrovich et al., 2001).

Os axônios de projeção extra-amigdalianos do MePD utilizam a ST como via eferente principal, indo, a seguir, em direção a regiões telencefálicas ou diencefálicas. Já os axônios que chegam aos outros subnúcleos da MeA (MeAD e MePV), utilizam como via predominante a *"ansa pedicularis"* (via amígdalo-fungal), também em direção as mesmas regiões acima citadas (Swanson e Cowan, 1979; Canteras et al., 1992; Paredes et al., 2000; de Olmos et al., 2004; Pereno et al., 2012).

Os subnúcleos do MeA possuem diferentes funções (revisado em Rasia-Filho et al., 2000). O MePD além de participar da modulação do comportamento reprodutivo masculino, do comportamento social, respostas ao estresse e comportamentos de defesa (revisado em Rasia-Filho et al., 2012), também faz parte do controle central dos reflexos dos barorreceptores e quimiorreceptores. Este subnúcleo pode, ainda, modular ajustes simpáticos e parassimpáticos cardiovasculares (Rasia-Filho, 2006; Quagliotto et al., 2008; Quagliotto et al., 2012; Neckel et al., 2012).

Os ajustes centrais cardiovasculares podem ocorrer por respostas reflexas e/ou por mudanças que precedem ou são concomitantes à ocorrência dos mais diversos reflexos e comportamentos (Rasia-Filho, 2006 e referências deste trabalho). Dois elementos interativos funcionais estão envolvidos no controle central do sistema cardiovascular: um constituído de áreas do SNC que mantém atividade basal ou tônica simpática/parassimpática para o coração e leitos vasculares enquanto o outro se relaciona com informações sensoriais fásicas para modular a atividade neural que atinge os órgãos efetores (Longhurst, 2008 e referências deste capítulo). Neste, por exemplo, reflexos neurais envolvem os barorreceptores

carotídeos e aórticos e as vias neuronais dos quimiorreceptores periféricos, a curto prazo, que convergem principalmente a áreas do tronco encefálico, embora outras regiões do prosencéfalo também sejam relevantes para a resposta cardiovascular final (Longhurst, 2008).

A contribuição com os ajustes neurais e hormonais de variáveis cardiovasculares advém de muitos núcleos do SNC. Dentre elas, pode-se ressaltar as projeções do MeA que inervam áreas previamente envolvidas com a organização central cardiovascular (Canteras et al., 1995; Petrovich et al., 2001), tais como: o ACe, cuja ativação elétrica leva a um aumento na FC, primeiro devido à diminuição da atividade vagal e, posteriormente, a um aumento do tônus simpático (Turner et al., 1986); as células noradrenérgica A1 do bulbo ventrolateral caudal e; as células A2 noradrenérgica do núcleo do trato solitário (NTS) (Canteras et al., 1995; Dayas et al., 1999; Dayas and Day, 2002; Saha and Datta, 2005).

O MePD também inerva as partes neuroendócrinas e simpática/parassimpática relacionadas com a zona periventricular do hipotálamo de ratos (Petrovich et al., 2001), a qual poderia permitir um papel integrador das respostas cardiovasculares em associação com a ocorrência de comportamentos (Quagliotto et al., 2008; Davern et al., 2010; Davern and Head, 2011). Por exemplo, ao se estimular alguns núcleos na amígdala, pode-se gerar taquicardia em ratos e no ser humano, dentro de um conjunto de manifestações relacionadas com o medo (Rasia-Filho et al., 2000). Já em situações de lesão no núcleo central da amígdala de ratos pode-se ter os efeitos contrários aos acima mencionados, como diminuição da FC, por exemplo (Rasia-Filho et al., 2000). Outro estudo, aponta que a estimulação elétrica do MePD pode aumentar a PA em camundongos e que lesões bilaterais, deste mesmo subnúcleo, por agentes neurotóxicos pode atenuar o aumento da PA em ratos espontaneamente hipertensos (Fukumori et al., 2004). Dessa forma, o MePD e suas conexões parecem estar envolvidos na regulação da FC e PA em condições fisiológicas, adaptativas e patológicas (Kubo et al., 2004; Qugliotto et al., 2008).

2.2 Insuficiência Cardíaca e Efeitos no Sistema Nervoso

A IC é uma síndrome clínica complexa, caracterizada pela incapacidade do coração em manter suprimento sanguíneo adequado aos órgãos e tecidos do corpo,

culminando em prejuízo na oferta de oxigênio e nutrientes para o metabolismo energético em geral (Ventura-Clapier et al., 2002; Ventura-Clapier et al., 2007; Ventura-Clapier, 2009; Ventura-Clapier et al., 2011).

A causa mais comum de IC é o IM e constitui uma importante causa de morbidade e mortalidade (Francis et al., 2001). Faz parte de um grupo de doenças chamado de doenças isquêmicas do coração e resulta de uma necrose da musculatura miocárdica devido à deficiência de fluxo sanguíneo e à consequente falta de aporte nutritivo e de oxigênio. A interrupção do fluxo coronário, quase sempre, é devido à oclusão repentina ou à obstrução total de uma artéria coronária (Yavuz, 2008). Essa interrupção ao fluxo sanguíneo na coronária determina a ocorrência da necrose miocárdica e pode envolver toda ou parte da espessura da musculatura ventricular (Pfeffer e Braunwald, 1990).

A expansão do infarto, caracterizada por adelgaçamento e distensão da região infartada, é observada principalmente em grandes infartos transmurais. Podem ser observadas áreas de distensão adicionais e mudanças do padrão contrátil em áreas remotas à área necrótica, associadas a fatores compensatórios da sobrecarga de volume (Pfeffer e Braunwald, 1990). A dilatação ventricular ocorre não apenas na fase precoce do infarto, mas se mantém ao longo do tempo como parte de um processo adaptativo, o que favorece o aumento do volume diastólico final do ventrículo esquerdo e a redução da função sistólica ventricular (Pfeffer e Braunwald, 1990).

As manifestações clínicas mais comuns da IC, em seres humanos, são: dispnéia, fraqueza muscular e fadiga precoce, o que pode limitar a tolerância ao exercício; retenção hídrica, podendo causar congestão pulmonar e edema periférico (Francis et al., 2001; Ventura-Clapier et al., 2002). Todas essas anormalidades podem prejudicar a capacidade funcional e a qualidade de vida dos indivíduos afetados. Já em ratos, modelo animal bastante utilizado para mimetizar a IC, os sinais característicos da síndrome são: lentidão de movimentos, alterações na pelagem, retardo de crescimento, perda de peso corporal e aterações na FC e frequência respiratória (FR) (Zornoff et al., 2009).

Este cenário, característico da IC, pode ser atribuído a alterações estruturais e funcionais tanto nos mecanismos centrais (controle da função cardíaca e

hemodinâmica) como periféricos (fluxo sanguíneo periférico e musculatura esquelética) (Ventura-Clapier et al., 2002; Ventura-Clapier et al., 2007; Ventura-Clapier, 2009; Ventura-Clapier et al., 2011). A ativação dos sistemas neuro-humorais, especialmente o sistema nervoso simpático, está envolvida na progressão e aumento da mortalidade na IC (Negrão e Middlekauff, 2008b; Kishi, 2012). No início da lesão miocárdica, a ativação aguda do sistema nervoso (SN) simpático ocorre como resposta adaptativa e tem como finalidade a restauração ou manutenção dos níveis pressóricos e do débito cardíaco (DC). Em modelos experimentais de IC, a atividade nervosa simpática está presente no início da disfunção ventricular esquerda. Além da hiperativação simpática, ocorre aumento da atividade do sistema renina-angiotensina-aldosterona e dos níveis de vasopressina, como uma tentativa compensatória (Negrão e Middlekauff, 2008a). Essas alterações, que no início da disfunção ventricular compensam o baixo DC e garantem uma pressão de perfusão sanguínea adequada, em longo prazo agravam a disfunção ventricular, contribuindo para a progressão da IC (Mousa et al., 2008; Negrão e Middlekauff, 2008b). Há evidências de que alterações no controle dos barorreflexos arteriais, reflexos cardiopulmonares, quimiorreflexo central e periférico, e diretamente no SNC em núcleos específicos como NTS podem contribuir sobremaneira para essa patologia. Estudos prévios demonstram que a IC provoca redução na sensibilidade barorreflexa (Zucker et al., 2001; Iellamo et al., 2007), aumenta a atividade simpática e altera a FC e a PA (Wang et al., 2004; Wang et al., 2008; Kishi, 2012).

Em contraste, a atividade parassimpática é significativamente reduzida em pacientes com IC crônica com uma menor modulação parassimpática da FC foi observada em seres humanos com IC (Kishi, 2012). Medidas diretas da atividade nervosa simpática, por meio de técnica microneurografia, têm mostrado que a atividade nervosa simpática muscular aumenta progressivamente em pacientes com insuficiência cardíaca moderada e grave (Negrão e Middlekauff, 2008a). Outro sinal comum, característico da IC é a depressão. Esta doença associada ao IM e a IC vem sendo investigada nos últimos anos. Estudos relataram que 65% dos pacientes com IM apresentaram depressão e que 20% desenvolveram depressão mais grave em 18 meses após o infarto (Frassure-Smith e Lespérance, 2003; 2006).

Adicionalmente, alguns poucos estudos descreveram morte neuronal por apoptose em regiões encefálicas do sistema límbico, como na MeA e na região CA1 do hipocampo no 1º dia após IM, na amígdala lateral (AL) e na região CA1 do hipocampo no 2º dia após IM e no córtex frontal 7 dias após o IM (Kaloustian et al., 2008). A amígdala como um todo, ao ser estudada 72 horas (Wann et al. 2006) e 14 dias (Bae, 2010) após o IM, também apresentou sinais de morte apoptótica dos neurônios (Wann et al., 2006; Bae, 2010), porém a presença de gliose reativa ocorreu somente após 14 dias da cirurgia de IM. Por outro lado, mais tardiamente ao IM (2 e 3 semanas após) não foram encontrados marcadores de apoptose na amígdala e no hipocampo de ratos, mas foi observada morte neuronal no hipotálamo e no córtex pré-frontal (Wann et al., 2007).

2.3 Exercício Aeróbio e Benefícios Para o Sistema Nervoso

O treinamento físico promove vários ajustes cardiovasculares, incluindo a remodelação do tecido cardíaco e do músculo esquelético (DiCarlo e Bishop, 1990; Mack et al., 1991). Regula e é capaz de modificar o equilíbrio simpático/parassimpático cardíaco por aumento da atividade parassimpática e diminuição da atividade simpática (de Abreu et al., 2009), promovendo aumento na capacidade do SNC em atender às demandas do sistema cardiovascular (Martins-Pinge, 2011).

Foi proposto que áreas do SNC, que são conhecidas pela sua participação no controle cardiovascular, mostram alteração na atividade em resposta a uma sessão de exercício em ratos treinados (Ichiyama et al., 2002). Há evidências que mostram os benefícios do exercício aeróbio sobre a função cerebral em seres humanos (Laurin et al., 2001; Hillman et al., 2008) e outros animais (Albeck et al., 2006; Stranahan et al., 2010). A prática de atividade física melhora funções cognitivas amplas (Neeper et al., 1995; Molteni et al., 2002; Molteni et al., 2004; van Praag et al., 2005; Alaei et al., 2008; Kashihara et al., 2009; De Senna et al., 2011; Lin et al., 2012; Bechara et al., 2013) e reduz índices de ansiedade e depressão (Martinsen, 2008).

Além disso, está envolvido com a plasticidade cerebral, ou seja, pode induzir angiogênese em regiões do SNC, como, por exemplo, no córtex cerebral (Zhang et al., 2013), córtex motor primário (Swain et al., 2003) e no hipocampo, particularmente no giro denteado (van der Borght et al., 2009) e aumentar a expressão de marcadores de plasticidade sináptica e estrutural do hipocampo, tais como a sinapsina I, neurofilamento 68, proteína associada a microtúbulos tipo 2 (MAP-2) (Ferreira et al., 2011), induzir neurogênese (van Praag et al., 1999; Kim et al., 2003; van Praag et al., 2005; van der Borght et al., 2009; Gligoroska e Manchevska, 2012; Lee et al., 2013; Curlik et al., 2013), modificar morfofisiologicamente as células neurais, e aumentar a liberação de fatores tróficos, como o fator neurotrófico derivado do encéfalo (BDNF), fator de crescimento neural (NGF), o fator de crescimento de fibroblastos (FGF) e seus respectivos mRNAs (Neeper et al., 1996; Gomez Pinilla et al., 1997; Berchtold et al., 2010; Bechara et al., 2013). Pode promover aumento na proliferação celular e modificações na densidade de espinhos dendríticos em regiões como o córtex motor (Adkins et al., 2006), cerebelo (Gonzalez-Burgos et al., 2011), amígdala basolateral, região CA3 (Lin et al., 2012), estriado, regiões CA1 e giro denteado do hipocampo, tanto em condições de normalidade quanto após lesão (Eadie et al., 2005; Wu et al., 2007; Hillman et al., 2008; Curlik et al., 2013; Bechara et al., 2013).

As células da glia, especificamente os astrócitos também são influenciados pelo exercício aeróbio, que é capaz de aumentar a imunorreatividade da proteína fibrilar ácida (GFAP-ir), o número de astrócitos GFAP positivos no córtex frontoparietal e no estriado (Li et al., 2005), e estimular a proliferação de astrócitos na zona subgranular do hipocampo (Uda et al., 2006). Da mesma forma, o exercício foi capaz de aumentar a densidade de astrócitos positivos ao GFAP, a densidade óptica regional, a densidade óptica celular e modificar a morfologia dessas células, na região CA1 do hipocampo (Saur et al., 2013).

Outro efeito da atividade aeróbia é a neuroproteção, isto é, a prática de exercício físico tem capacidade de proteger o SN contra o acidente vascular cerebral isquêmico (Zhang et al., 2013) e doenças neurológicas degenerativas, como a doença de Parkinson (Smith e Zigmond, 2003), doença de Alzheimer (Mirochnic et al., 2009). Um estudo realizado *in vitro* com privação de oxigênio e glicose em fatias do hipocampo de ratos treinados, evidenciou uma menor suscetibilidade à lesão por

isquemia (Cechetti et al., 2007). Da mesma forma, foi evidenciado que o treinamento prévio em esteira foi capaz de reduzir a área de infarto e o edema causado pela oclusão da artéria cerebral média (Wang et al., 2001; Zhang et al., 2013). Além disso, exercício aeróbio prévio a isquemia cerebral, foi capaz de promover melhora da memória (Sim et al., 2005; Cechetti et al., 2012) e redução da morte neuronal por apoptose nas regiões CA1 e giro denteado do hipocampo (Sim et al., 2005).

Porém, sabe-se que este efeito protetor gerado pelo exercício aeróbio depende diretamente da intensidade do mesmo, ou seja, exercícios de intensidade baixa são ineficazes para a proteção, já em intensidades moderadas protegem contra lesões e altas intensidades aumentam a vulnerabilidade para lesão (Scopel et al., 2006; Cechetti et al., 2007). Desta forma, o exercício aeróbio realizado em intensidade moderada, além de prevenir danos neurológicos, também contribui para o aumento do número de neurônios e de sinapses, em regiões como o giro denteado do hipocampo (van Praag et al., 1999; van Praag et al., 2005; Eadie et al., 2005).

2.4 JUSTIFICATIVA

O MePD é uma área específica relevante a ser estudada devido ao seu papel no controle cardiovascular central e participação nos ajustes cardiovasculares simpáticos e parassimpáticos. Sabe-se que as doenças cardiovasculares interferem nestes ajustes, causando uma hiperatividade simpática e, portanto um desequilíbrio simpato-vagal. Como descrito acima, estudos prévios têm demonstrado a presença de morte neuronal apoptótica em regiões do encéfalo relacionadas com o sistema límbico, como amígdala e alguns de seus núcleos e subnúcleos. Já o exercício aeróbio, além de causar benefícios diretos para o SN, como angiogênese, neurogênese e neuroproteção, também interfere no sistema simpático e parassimpático, diminuindo o primeiro e aumentando a atividade do último. No entanto, ainda não é sabido se o treinamento aeróbio prévio e/ou o IM/IC podem alterar o número de neurônios e células gliais ou a GFAP-ir no MePD de ratos.

Desta forma, a contribuição deste trabalho torna-se significativa, visto que os resultados deste estudo podem ser complementares aos dados já publicados sobre os efeitos neuroprotetores do treinamento aeróbio (Eadie et al., 2005; Adkins et al., 2006; Scopel et al., 2006; Cechetti et al., 2007; González-Burgos et al., 2011) e morte neuronal e gliose reativa após o IM/IC (Wann et al., 2006; Kaloustian et al., 2008; Bae et al., 2010), alguns deles envolvendo toda a amígdala ou alguns núcleos da mesma.

2.5 OBJETIVO GERAL

Estudar os efeitos da IC e do exercício prévio ao IM na densidade de neurônios e células da glia do MePD de ratos adultos.

2.6 OBJETIVOS ESPECÍFICOS

1. Verificar se ocorrem alterações na densidade de células neuronais e gliais com a IC e o exercício prévio ao IM, analisar se ocorrem diferenças entre os hemisférios direito e esquerdo, bem como comparar os efeitos nas porções rostral e caudal do MePD;
2. Estudar os efeitos da IC e do exercício prévio ao IM na GFAP-ir regional e celular, comparar os hemisférios direito e esquerdo e os efeitos nas porções rostral e caudal do MePD;
3. Estudar a morfologia dos astrócitos de ratos com IC que realizaram exercício aeróbio prévio ao IM, comparando os hemisférios direito e esquerdo e as porções rostral e caudal do MePD.

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4 ARTIGO

Effect of prior exercise training and myocardial infarction-induced heart failure on the neuronal and glial densities and the GFAP-immunoreactivity in the posterodorsal medial amygdala of rats

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Running title: Cellular density and GFAP-ir in the medial amygdala

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Abstract

Exercise training has neuroprotective effects whereas myocardial infarction (MI) and heart failure (HF) can cause neuronal death and reactive gliosis in the whole amygdala. The posterodorsal medial amygdala (MePD) is involved with cardiovascular reflexes and the central control of sympathetic/parasympathetic responses. Our aim was to study the effects of prior exercise training and of MI-induced HF on the neuronal and glial densities and the glial fibrillary acidic protein-immunoreactivity (GFAP-ir) in the MePD of adult male rats. Animals (n= 5/group) were: control, sedentary submitted to a sham MI (Sed Sham), sedentary submitted to MI/HF (Sed HF), trained on a treadmill and submitted to a sham MI (T Sham) or trained on a treadmill and submitted to MI/HF (T HF). The number of neurons and glial cells in the MePD was estimated using the optical fracionator and the GFAP-ir was quantified by optical densitometry. In the respective groups, treadmill training improved physical performance and MI damaged near 40% of the left ventricle. There was a hemispheric lateralization effect on the density of neurons (higher in the right MePD), but no significant difference in both the neuronal and the glial densities due to experimental condition. Regional GFAP-ir results revealed that the Sed HF group had a higher expression in the left MePD compared to the control and the Sed Sham rats ($p < 0.01$). Present data did not evidence effects of training and MI/HF in the MePD cellular density, but indicate a possible local restructuring of astrocytic cytoskeleton after MI/HF in rats.

Key words: Extended amygdala, optical fracionator, cellular density, astrocytic cytoskeleton.

Introduction

The rat posterodorsal medial amygdala (MePD), a major component of the "extended amygdala" (de Olmos et al., 2004), is an important part of the neural network that modulates the occurrence of social/reproductive behaviors (Newman, 1999; Rasia-Filho et al., 2012b) and stress responses (Marcuzzo et al., 2007; Singewald et al., 2008). The MePD has also relevant connections with various amygdaloid and hypothalamic nuclei and brain stem areas (Canteras et al., 1995; Petrovich et al., 2001; Saha, 2005) that receive cardiovascular sensorial afferences and that can ultimately regulate the heart rate (HR) and arterial blood pressure (AP; Turner et al., 1986; Longhurst, 2008) under daily physiological conditions and in both adaptive or pathological circumstances (Kubo et al., 2004; Davern and Head, 2011; Quagliotto et al., 2012). The MePD alters the baroreceptor- and chemoreceptor-mediated reflexes and the concomitant central sympathetic and/or parasympathetic cardiovascular adjustments (Quagliotto et al., 2008, Neckel et al., 2012; Quagliotto et al., 2012). For example, local microinjections of the major neurotransmitters glutamate and GABA induced, respectively, selective activations of the sympathetic and parasympathetic components of the baroreflex in awake rats (Neckel et al., 2012).

Physical exercise training protects the cardiovascular system by modulating the sympathetic and parasympathetic output to cardiac and vascular functions (Joyner and Green, 2009; Gielen et al., 2010) as well as improves the outcome of acute myocardial infarction (MI; Freimann et al., 2005; Jorge et al., 2011). Regular aerobic exercise or rearing in enriched environments allow the development of motor skills and increase cerebral blood flow (Swain et al., 2003; Eadie et al., 2005), induce cellular proliferation in different brain regions, such as the dentate gyrus (Eadie et al., 2005), cerebellar cortex (Gonzalez-Burgos et al., 2011) and cerebral cortex (Adkins et al., 2006), and enhance memory and learning in rodents (Wu et al., 2007; Liu et al., 2009; Stranahan et al., 2011). Treadmill running prevents the damage and neuronal loss induced by excitotoxic agents (Carro et al., 2001). Physical activity of moderate intensity also has neuroprotective properties, as evidenced by the reduction in the ischemia-induced neuronal cell death in the dentate gyrus (Sim et al., 2005; Zheng et al., 2006; Scopel et al., 2006; Cechetti et al., 2007). On the other

hand, MI is one of the most common causes of chronic heart failure (HF) and affects both central and peripheral organs. Notably, neuronal death can occur due to apoptosis in different brain regions after MI in rats (Kaloustian et al., 2008). That occurred in the whole amygdala 3 days after MI (Wann et al., 2006) or in the lateral and medial nuclei of the amygdala, in the hippocampal CA1 area, in the dentate gyrus (Wann et al., 2006; Kaloustian et al., 2008) and in some hypothalamic nuclei at 1, 2 and 7 days following MI (Kaloustian et al., 2008). Additionally, cell death and reactive gliosis were found in the whole amygdala 14 days after MI in rats (Bae et al., 2010).

These data are interesting because indicate a structural remodeling of brain areas after MI and, more specifically, in different amygdaloid nuclei that have integrative roles in neuroendocrine secretion and behavioral display (Dayas et al., 1999; Rasia-Filho et al., 2000; Marcuzzo et al., 2007; Rasia-Filho et al., 2012a). In this sense, the MePD has an evident role in the modulation of activities that require motor activation, such as the sexual and aggressive behaviors (reviewed in Newman, 1999; Rasia-Filho et al., 2012a), as well as in ongoing cardiovascular adjustments (Quagliotto et al., 2012). However, it is not currently known whether prior exercise training or MI/HF can alter the number of neurons and glial cells or the astrocytic cytoskeleton in the MePD of rats. Interestingly, aerobic training reduces the sympathetic tone at the same time that increases the parasympathetic one (Martins-Pinge, 2011) whereas in cases of HF there is an over-activation of the sympathetic tone occurs, which is directly related to greater disease severity and increased mortality rate (Martins-Pinge, 2011). Given its role on the central cardiovascular control, the MePD is a putative candidate where neuroprotective effects of training (Eadie et al.,2005; Adkins et al., 2006; Scopel et al., 2006; Cechetti et al., 2007; González-Burgos et al., 2011) and cellular loss following MI/HF would occur (Wann et al., 2006, Kaloustian et al., 2008; Bae et al., 2010), as previously reported for the whole amygdala and that included various anatomically and functionally heterogeneous amygdaloid nuclei studied at the same time. Therefore, our aim was to test the effects of exercise training and MI-induced HF on the neuronal and glial densities and the glial fibrillary acidic protein-immunoreactivity (GFAP-ir) in the MePD of adult male rats.

Materials and Methods

Animals

Adult Wistar male rats (3 months-old and weighing 200-250 g) were obtained from a local facility (Federal University of Health Sciences of Porto Alegre, Brazil; UFCSPA) and kept in groups with food and water *ad libitum*. Room temperature was maintained at 22 ± 1ºC in a 12 h light:dark cycle (lights off at 7 p.m.). All efforts were made to minimize the number of animals and their suffering. Animals were manipulated according to international laws for ethical care and use ('European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes', Council of Europe No 123, Strasbourg 1985), conformed to national guidelines and was approved by the local Ethical Committee (UFCSPA protocol no. 053-11).

Twenty-five rats were randomly assigned to the following groups (n= 5 each): (1) non-manipulated control (Cont), (2) sedentary rats submitted to a sham MI procedure (Sed Sham), (3) sedentary rats submitted to the surgical occlusion of the left descending coronary artery to induce MI and subsequent development of HF (Sed HF), (4) exercise trained rats submitted to a sham MI (T Sham), and (5) exercise trained rats submitted to MI and subsequent HF (T HF). Experimental design is shown in Figure 1 and described in details below.

Preconditioning Training Protocol

Rats that composed the two trained groups (T Sham and T HF) were habituated to an adapted motorized rodent treadmill ("Athletic Racer", Brazil) during 1 week to minimize novelty stress. Animals that composed the groups of sham training (Sed Sham and Sed HF) were set on the same experimental room and were placed on the treadmill turned off.

Measurement of the maximum exercise test (ET) was realized in all animals of the trained groups both before the first day of training and at the middle of the exercise protocol. Each rat started running at a low treadmill speed with a pace increasing 5 m/min every 3 min. The point of exhaustion and the time to fatigue (in min) and workload (in m/min) were taken as indexes of capacity for exercise (Rodrigues et al., 2007). The exercise training intensity was set at 60% of the

maximum speed of ET. Treadmill training lasted 20 min/day and was done 3 times/week during 4 weeks (Ben et al., 2009).

Surgical Procedure for Myocardial Infarction

Rats that composed the Sed HF and the T HF groups were anesthetized with intraperitoneal injections of xilazine (12 mg/kg) and ketamine (90 mg/kg), intubated and artificially ventilated (SamWay VR 15, USA) to maintain a respiratory frequency of 60 breaths/min and an oxygen inspired fraction of 100%. The MI was induced as previously described (Pfeffer et al., 1979; Jaenisch et al., 2011). Briefly, the heart was exposed through a left thoracotomy between the fourth and fifth ribs and a mononylon 6-0 suture closed the main left descending coronary artery between 1 and 2 mm distal to the edge of the left atrium. The animals that composed respective sham groups (Sed Sham and T Sham) underwent the same anesthetical and surgical procedure of heart manipulation, but without the coronary manipulation. Afterwards, thoracic chest was closed, skin was sutured, pneumothorax was drained by a continuous aspiration system and animals received ibuprofen (oral administration of 100 mg/0.1 mL every 8 hours during the first postoperative day).

We did not measure directly the final diastolic intraventricular pressure in rats submitted to MI to avoid brain ischemic events that could affect cellular counts in the MePD and lead to misleading results. In another set of experiments, it was clearly observed that rats that underwent the same MI procedure as performed here (and with left ventricle myocardial infarted areas such as those presently observed) have a remarkable development of HF (Vanoli et al., 2004). Indeed, values of MI area higher than 25% and, more evidently, equal or higher than 40% highly correlates with the development of HF (Kclocke et al., 2007; Jaenisch et al., 2011; Tucci, 2011). Our present procedure led to mean MI areas of 39% in the left ventricle (see Results and Fig. 2B).

Stereological Study

Experiments ended after 9 weeks (Figure 1). That is, 4 weeks for the preconditioning training and 5 weeks following the MI induction as well as the respective sham procedures. Then, animals were deeply anesthetized with xilazine

(12 mg/kg ip) and ketamine (90 mg/kg ip) and transcardially perfused with 0.9% saline (150 mL) and heparine (1200 IU/Kg) followed by 4% paraformaldehyde (PFA) in 0.1M phosphate buffer, pH 7.4% (150 mL). After perfusion, the hearts were removed and weighed. For those rats submitted to MI, left ventricles were filled with an insufflating latex balloon and placed in buffered formaldehyde for 24 h for subsequent analysis of the size of the infarction area (Figure 2). The total left ventricle area and the myocardial infarction scar area were manually drawn on the scanned images and measured automatically using a computer program (Image Pro Plus 6.1, Media Cybernetics, USA). The infarction size is expressed as a percentage of the total left ventricle area (Pfeffer et al., 1979; Jaenisch et al., 2011).

Brains were removed, kept in 4% PFA for 24h, immersed in 15% and 30% sucrose solutions, frozen in isopentane and dry ice and maintained at -80ºC until further processing. Brains were sectioned using a cryostat (Leica, Germany) and each alternate serial coronal section of 30 µm-thick were or stained with thionin for the stereological study or processed for the GFAP immunohistochemistry, as described below. The rostro-caudal location of the MePD in the rat brain was based on the description provided by de Olmos et al. (2004) and compared with the schematic images of a rat brain atlas (Paxinos and Watson, 2005). The MePD was located medially to the optic tract (OT) and ventrally to the stria terminalis (ST) in the rat ventral forebrain (Figure 3). Although being continuous areas, the cytoarchitectonical organization of the rostral and the caudal MePD appeared distinct (de Olmos et al., 2004).The rostral aspect of the MePD was arbitrarily set at 2.64 to 2.76 mm posterior to the bregma and the caudal aspect was at 3.36 to 3.48 mm posterior to the bregma (based on Paxinos and Watson, 2005). An evident medial column of cells was found in the MePD near the “molecular layer”, a cell-sparse rim close to the OT (de Olmos et al., 2004; de Castilhos et al., 2006). Both right and left hemispheres were studied apart and we tested for lateralized effects in the cellular components of the MePD (based on Johnston et al., 2008; Morris et al., 2008).

Thionin staining was done as reported elsewhere (Dall’Oglio et al., 2013). Briefly, brain sections were dried at room temperature (RT), immersed in different solutions of ethanol, cleared in xylene, immersed in a solution of 0.25% thionin for 3 min, immersed again in solutions of increasing ethanol concentration, dipped in a

solution of 95% ethanol with 1% acetic acid and absolute xylene and were, finally, covered with synthetic balsam and coverslips.

For the stereological estimation, images were obtained with an Olympus BX61 microscope (400x, Olympus, Japan) attached to a high-resolution digital D172 camera and a computer with the Image Pro Plus software. The neurons in the MePD were identified morphologically by their large, pale nuclei with an evident nucleolus and by the dark cytoplasm that contained Nissl bodies. The glial cells were identified according to their relatively small size and unstained cytoplasm. That is, the nucleolus was used as the counting marker for neurons, and the nucleus was used as the counting marker for glial cells (Figure 3). No precise borders for the anterior, lateral and ventral limits of the MePD could be established along the whole extension of this subnucleus. A critical evaluation about this technical restriction was elaborated by us for both the rat and the human medial amygdala (Rasia-Filho et al., 2002; Dall'Oglio et al., 2013). Therefore, to avoid imprecise data collection and misleading results, cells were counted in the MePD medial cellular column taking the unequivocal presence of OT and the ST as anatomical references for all animals in all experimental groups.

The MePD cellular density (the number of neurons and glial cells) was estimated by the optical fractionator method. Five animals were studied in each experimental group, 1 of 5 serial sections containing the rostro-caudal axis of the MePD was selected, 8-10 sections were studied per rat (3 sections in the rostral aspect and 3 in the caudal aspect of the MePD). Four squared counting frames [called the area of interest (AOI) with 1,203 μm^2] were randomly overlaid onto the medial column of the MePD in each studied section. The cells were counted at different focal planes, including the z axis, throughout the brain slice. Neurons and glia cells overlaying the "including" borders of the counting frame were counted as well as the cells located within the counting frame. Conversely, the cells overlaying the "excluding" borders of the counting frame were not counted (Dall'Oglio et al., 2013; Figure 3).

The numerical density of neurons and glial cells was estimated using the following formula:

$Nv = (1/[a/f.h]) \cdot (\Sigma Q/\Sigma P)$

Where, Nv= estimated numerical density, a/f= area of the counting frame; h= disector height; ΣQ= sum of cells counted and ΣP= sum of analyzed counting frames. The postprocessing thickness of each brain slice was measured, and the section height was used as the disector height (Costa Ferro et al., 2010; Dall'Oglio et al., 2013).

From these data it was calculated the neuronal/glial ratio by dividing the number of neurons per glial cells in the rostral and caudal MePD of each hemisphere and in each experimental group.

GFAP Immunohistochemistry and Quantification

The procedure for GFAP immunohistochemistry was adapted from previous reports (Rasia-Filho et al., 2002; Martinez et al., 2006; Johnson et al., 2008). Briefly, the brain sections were mounted on histological slides, washed and, then, blocked with 2 % bovine serum albumin (BSA) in PBS containing 0.4 % Triton X-100 (PBS-Tx, Sigma Chemical Co., USA) for 30 min, and incubated with polyclonal GFAP antiserum raised in rabbit (Dako, UK) diluted 1:500 in 0.3 % of PBS-Tx for 48 h at 4°C. After being washed with PBS-Tx twice, sections were incubated in anti-rabbit IgG peroxidase-conjugated antibody produced in goat (Sigma, USA) diluted 1:150 in PBS-Tx at RT for 2 h. The reaction was developed by incubating the sections in a medium containing 0.06 % 3,3′-diaminobenzidine (DAB, SigmaChemical Co., USA) dissolved in PBS for 10 min and in the same solution containing 1 µL of 3 % H_2O_2 per mL of DAB medium for 10 min. Sections were rinsed again in PBS, dehydrated in series of increasing ethanol concentrations (70, 90 and 100 %, 2 min each) cleared with xylene and covered with Permount and coverslips. To control for unspecific binding, primary antibody was omitted and replaced by PBS. No staining was evident after that. Furthermore, in order to minimize differences in the staining of astrocytes and in background levels, the brains in both experimental groups were fixed and post-fixed in identical solutions for the same length of time, processed at the same time and incubated in the same immunostaining medium for the same period of time (Saur et al., 2013). As a rule, GFAP-ir appeared similarly as found in other reports (Rasia-Filho et al., 2002; Dall'Oglio et al., 2013).

The intensity of GFAP-ir was measured using semi-quantitative densitometric analysis (Ferraz et al., 2003; Xavier et al., 2005; Martinez et al., 2006) using a BX 50 microscope (200x; Olympus, Japan) coupled to a Motic Images Plus 2.0 camera and

Image Pro Plus software. One of 5 serial sections containing the rostro-caudal axis of the MePD was selected and converted to an 8-bit gray scale (256 gray levels). Five animals were studied in each experimental group (except the Cont and Sed Sham HF groups, where n= 4), 5 sections were studied per rat (2 sections in the rostral aspect and 2 in the caudal aspect of the MePD). This same procedure was done for the right and the left hemispheres. Three AOIs (8,260 μm^2 each) were randomly overlaid on each section. These images were used in the analysis of regional optical density (OD; Saur et al., 2013). For the analysis of cellular OD, one AOI measuring 4.19 μm^2 was placed over the astrocytic soma in each studied image. The regional and the cellular ODs were calculated according the following formula (Martinez et al., 2006):

$OD\ (x,y)= -\log\ [(INT_{(x;y)} – BL)]/(INC – BL)$

Where, OD(x,y) is the optical density at pixel (x,y), INT(x,y) is the intensity at pixel(x,y), BL or black is the intensity generated when no light goes through the material and INC" or incident is the intensity of the incident light. All lighting conditions and magnifications were kept constant during the process of capturing the images. Blood vessels and other artifacts were avoided and the background correction was performed according to the procedure previously described in Xavier et al. (2005).

Additional morphological analysis of GFAP-ir astrocytes was done using the same images employed to measure cellular OD. Three parameters were evaluated: (1) An adaptation of Sholl's concentric circles technique was used for the study of astrocytic ramification (Sholl, 1953; Saur et al., 2013). That is, 7 virtual concentric circles with 50 μm intervals were drawn around each astrocytic cell body. The degree of ramification of each astrocyte was measured by counting the number of times astrocytic processes intersected each virtual circle in both the lateral (i.e. right/left) and central (i.e. superior/inferior) quadrants around the astrocytes. (2) The quantification of primary processes was performed by directly counting the number of astrocytic prolongments extending from the soma in both the lateral and central quadrants. (3) The longest primary process in each quadrant was measured by tracing the process with a manual measurement tool found in the Image Pro Plus software (Saur et al., 2013).

Statistical Analysis

The effectiveness of the training protocol was compared within-group (pre- and post-training performance) and between trained groups using a two-way analysis of variance (ANOVA) test for repeated measures. The infarcted area of the left ventricle was compared between HF groups using the two-tailed Fisher test. The density of neurons and glial cells, the neuronal/glia ratio and the different GFAP-ir parameters were compared in each hemisphere and location in the MePD (rostral and caudal aspects) among the experimental groups using an ANOVA test for repeated measures (considering the data from the right and left hemispheres) followed by the Tukey test or the Bonferroni test. The significant statistical level was set at $p \leq 0.05$.

Results

Training effect and infarction area

The exercise protocol was able to enhance the performance of trained rats (Figure 2A). That is, the statistical analysis revealed that time expend exercising increased significantly [$F(1,7) = 7.25$; $p = 0.03$] and speed on the treadmill showed a clear trend to be improved [$F(1,7) = 4.58$; $p = 0.06$] when comparing the pre-training vs the post-training performance in the T Sham and the T HF groups. There was no interaction between training protocol and the rat experimental condition [$F(1,7) = 0.03$; $p = 0.85$ for time expend; and, $F(1,7) = 0.77$; $p = 0.4$ for speed on the treadmill].

Left ventricular infarction area in the Sed HF and T HF groups did not show statistically significant difference between groups (mean $\pm$ SD = 40 $\pm$ 7 and 38 $\pm$ 6%, respectively; $p = 0.8$; Figure 2B).

Neuronal and glial densities in the right and left MePD

According to the aforementioned including criteria, the thionin staining indeed allowed the identification of MePD neurons with round, fusiform or multiangular cell bodies shapes and the neuronal nucleus with an evident and single nucleolus. Glial cells had a comparatively small size, no nucleolus and an unstained cytoplasm (Figure 3).

The density of neurons in the MePD showed a statistically significant difference due to hemispheric lateralization [$F(1,18)= 56.54$; $p < 0.01$; higher values in the right MePD, Figure 4A], but there were no difference due to the group experimental condition [$F(4,18)= 2.22$; $p= 0.1$] or the interaction of these two factors, i.e., MePD laterality and experimental condition [$F(4,18)= 2.25$; $p = 0.1$; Figure 4B and C].

The density of glial cells in the MePD showed no statistically significant difference due to hemispheric laterality [$F(1,18)= 0.22$; $p = 0.64$], experimental condition [$F(4,18)= 2.31$; $p = 0.1$] or the interaction of laterality and experimental condition [$F(4,18)= 0.43$; $p = 0.1$; Figure 5].

For the density of neurons and glial cells, there were no effect of experimental condition in the rostral or in the caudal parts of the right and the left MePD ($p > 0.05$ in all cases; Figures 4B and C and 5B and C, respectively). Notably, there were more neurons than glial cells either in the rostral or in the caudal MePD, with the neuronal/glia ratio varying from 1.2 to 6.4 and 1.1 to 7.6, respectively. In the rostral MePD there was a statistically significant difference due to hemispheric laterality [$F(1,20)= 17.80$; $p < 0.01$; higher in the right MePD], but no statistically significant difference due to experimental condition [$F(4,20)= 0.19$; $p = 0.93$] or the interaction of laterality and experimental condition [$F(4,20)= 0.72$; $p = 0.58$]. Likewise, in the caudal MePD there was a statistically significant difference due to hemispheric laterality [$F(1,20)= 10.68$; $p < 0.01$; higher in the right MePD], but no statistically significant difference due to experimental condition [$F(4,20)= 1.13$; $p = 0.36$] or the interaction of laterality and experimental condition [$F(4,20)= 0.13$; $p = 0.96$].

GFAP expression in the MePD

GFAP expression was found in the MePD of all experimental groups. Immunoreactive astrocytes were found isolated or in small clusters, with evident cell bodies and various branched prolongments (Figure 6).

The regional GFAP-ir in the MePD showed a statistically significant interaction for hemispheric laterality and experimental condition [$F(4,16)= 4.38$; $p = 0.01$; main differences in the left MePD]. That is, the Sed HF group showed an increased GFAP expression in the left MePD compared to the control and the Sed Sham data ($p <$

0.01 in both cases). T Sham and T HF results were also higher than the control group ($p < 0.05$ in both cases), but did not differ between them ($p > 0.05$; Figure 6).

No statistically significant differences among experimental groups were found in the morphological features of the GFAP-immunoreactive astrocytes (ramification evaluated by the Sholl's concentric circles technique, number of primary processes and length of the primary processes; $p > 0.05$ in all cases, data not shown).

Discussion

Present results based on stereological and immunohistochemical approaches did not evidence a modification in neuronal or glial density in the MePD after exercise training and/or following MI/HF, but indicate a likely restructuring in GFAP-ir and astrocytic cytoskeleton after MI/HF in rats. Because the MI/HF condition did not affect relevant astrocytic morphological parameters, such changes found in the GFAP-ir likely represent a redistribution of intermediate filaments in the already existing astrocytic branches extending within the MePD. These novel data add to the discussion of methodological differences in the evaluation of nervous tissue adaptation to physical exercise and to MI/HF and suggest the existence of different site-specific effects among brain areas that could be involved in the elaboration of goal-oriented motor activity and the central control of cardiovascular responses, as occurs for the rat MePD.

Here, we studied the medially densely-packed column of cells in the MePD to count neurons and glial cells because it was the place where effective neurotransmitters/neuropeptides microinjections initially reached and diffused into the MePD to modify the central cardiovascular control of HR and AP and to induce sympathetic/parasympathetic output changes (Quagliotto et al., 2008; Neckel et al., 2012). This MePD column of cells also showed notable Fos immunoreactivity following stressful stimulation and concomitant increases in HR and AP (Davern et al., 2010; Porter and Hayward, 2011). Taking these data into account, we tested the occurrence of plastic responses in this part of the MePD under the present experimental design. Both exercise training and MI were effective procedures. That is, training improved the performance of the animals in the treadmill protocol along 4 weeks of exercising whereas the induced MI promoted a mean left ventricle infarcted

area correlated with the development of characteristically impaired cardiovascular parameters of HF (Jaenisch et al., 2011). Although the experimental condition of the rats did not affect the density of neurons in the MePD, it was found an inherent hemispheric lateralization difference in this morphological parameter. That is, the right MePD contains more neurons in its medial column of cells than in its left counterpart. Previous works have also shown a hemispheric specialization in neuronal number in the rat MePD (Morris et al., 2008) and compared adult male and female Long-Evans rats using the stereological optical fractionator method to count the number of cells. Higher values were found in males, independently of circulating androgens in these animals, and in both males and females there were more neurons in the left MePD (Morris et al., 2008). In our hands, the MePD of Wistar rats imposes critical difficulties for the exact delimitation of the MePD borders. Without consistency in this crucial condition, the Cavalieri method to estimate total number of cells can not be used. Then, precautionary to avoid potential confusion and unreal results (see a critical discussion in the preface of the rat brain atlas of Kruger et al., 1995), we decided to estimate the neuronal and glial densities in part of the MePD that could be reliably found in all experimental groups. Here, Wistar rats showed another lateralized condition for the number of neurons in the MePD (i.e, higher values in the right hemisphere) and this might mean that more subtle subregion-specific differences can be found in this structure. It has also to be considered that rat strain differences were already reported for the whole medial amygdale, either for morphological data or for subcellular components and responses in local neurons (reviewed in Rasia-Filho et al., 2012b and references therein).

In this same regard, other authors used GFAP immunocytochemistry and found that the right MePD contains more astrocytes than the left MePD in Long Evans adult male rats (Johnson et al., 2008). Here we did not find a hemispheric specialization in the number of glial cells in the medial column of the MePD of Wistar rats among the studied groups, although regional GFAP-ir differences were found in the left MePD of the Sed HF group compared to the control data. Altogether, these findings point to a more complex condition of glial presence and GFAP expression in the MePD of rats of different strains. Taking into consideration the methodological concerns that involve the specific epitope immunodetection in fixed tissue, it is also

possible that direct comparisons of different immunohistochemical studies can be indeed controversial and it would be more appropriate to compare data “within” each experimental set.

We did not evidence a higher number of glial cells in the MePD suggestive of a proliferation of these cells after MI/HF. On the other hand, the higher GFAP-ir observed in the Sed HF group would represent a form of reactive gliosis with increase in intermediate filaments, but not in the number and length of primary processes or the number of prolongments crossing the Sholl concentric circles around the cell body. The beneficial or detrimental effects of this GFAP increase in rats submitted to MI/HF remain to be tested. T HF rats also increased GFAP-ir, but their respective control group presented similar values, which did not allow the determination of the main cause of this enhanced GFAP expression in trained animals.

It is also worth noting that MI consequences were studied after 5 weeks of the experimental procedure, a period of time relevant for the development of HF, and, thus, related with chronic adaptation of the nervous system to this pathological condition. Previously, it was shown brain cellular numerical changes 1-7 days following MI (Wann et al., 2006; Kaloustian et al., 2008). In this same sense, MI-induced cell death was found after 2 weeks in the whole amygdala (Bae et al., 2010). On the other hand, 2 and 3 weeks after MI, there were no apoptosis markers found in the amygdala and hippocampus of rats, although neuronal death could still be observed in the hypothalamus and prefrontal cortex (Wann et al., 2007). Because the amygdaloid complex is composed by different nuclei and subnuclei (Rasia-Filho et al., 2000; Petrovich et al., 2001; de Olmos et al., 2004), it is highly likely that differences in the sensitivity to experimental manipulations can occur on a site-specific basis. That is, within the whole amygdale, the MePD could be a putative candidate to be involved with the neuroplasticity induced by physical exercise and/or MI/HF. Our data did not support such affirmative on a chronic-based protocol. Then, taking the present MePD study, the results obtained after physical training and MI/HT can also indicate that those evident acute neuroprotective and neurodegenerative effects of these testing conditions disappear along weeks in chronic experimental protocols.

Finally, there are some limitations in the present study. In this regard, further studies can be proposed to determine if different subpopulations of intrinsic neurons in the MePD (Choi et al., 2005; Carney et al., 2010) are more or less sensitive to the present experimental procedures. It is also possible that differences can occur in the number of neurons and glial cells after training or MI/HF, but they have to be revealed by additional techniques that test for apoptosis or neurogenesis markers in the MePD, also checking if different compensatory ways could influence the total number of cell counted. Furthermore, it is relevant to test if the MePD of trained rats and the MePD of MI/HF animals maintain the same functional properties or if they can adapt accordingly to new allostatic conditions and alter the central sympathetic and parasympathetic modulation of the cardiovascular system. These working hypotheses require additional methodological approach and open a new venue for research on the structure and function of the rat MePD.

In conclusion, present data did not evidence a modification in cellular density after preconditioning training and/or MI/HF in part of the MePD, but indicate a possible reactive gliosis and restructuring of the astrocytic cytoskeleton after MI/HF in rats.

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Conflict of Interest

The authors declare no actual or potential conflict of interest.

Legends

Figure 1. Experimental design (in weeks and days) involving the preconditioning training and the myocardial infarction. Control animals were not manipulated whereas other control groups were submitted to "sham" procedures during this period. Further details are provided in the text.

Figure 2. **(A)** Training effects, as evaluated by the maximum exercise test, on the time expent and speed on the treadmill of the aerobically trained rats submitted to a sham or effective myocardial infarction. * $p < 0.05$ compared to pre-training values. **(B)** Representative left ventricle frontal view as found in rats after 5 weeks of myocardial infarction. An insufflating latex balloon inside the ventricle allows the visual identification of the infarcted area (dashed lines). IS = Infarct size; in this representative case, approximately 40% of the left ventricle wall. The arrow points to the approximate location of the suture of the left coronary artery.

Figure 3. (Top, Left panel) Schematic diagram of a coronal brain slice showing the posterodorsal medial amygdala (MePD) in the ventral forebrain of rats, in this example at 3.14 mm posterior to the bregma. Adapted from Paxinos and Watson (1995). **(Top, Right panel)** Histological staining used for the identification of the MePD in a matched coronally sliced brain section. The MePD was divided in medial (m), intermediate (i), and lateral (l) cellular columns. Stereological data was obtained from the medial column as illustrated. ST, stria terminalis; OT, optic tract. Scale bar = 300 μm. Reprinted from de Castilhos J., Marcuzzo S., Forti C.D., Frey R.M., Stein D., Achaval M., Rasia-Filho A.A. "Further studies on the rat posterodorsal medial amygdale: Dendritic spine density and effect of 8-OH-DPAT microinjection on male sexual behavior". Brain Res. Bull. 69, 131-139. Copyright (2008) with permission from Elsevier. **(Bottom)** Photomicrographs of thionin-stained cells in the rat MePD at the magnification used for the optical fractionator stereological technique. The overlaid square represents the "area of interest" (1205 μm^2), where cells were counted, with including (solid lines) and excluding (dashed lines) borders. Examples areas shown for a neuron (solid arrow) and a glial cell (dashed arrow). Contrast and background were adjusted using Photoshop 7.0, Adobe (USA). Scale bar = 25 μm.

Figure 4. (A) Values are mean ($\pm$ SD) of the stereological estimation of neuronal density and hemispheric difference found in the whole posterodorsal medial amygdala (MePD) of rats. ** $p < 0.01$ compared to the left MePD. **(B, C)** Mean ($\pm$ SD) of the stereological estimation of the neuronal density found in the rostral **(B)** and caudal **(C)** aspects of the MePD and from the right and left hemispheres of non-manipulated controls, sedentary rats submitted to a sham MI (Sed Sham), sedentary rats submitted to the surgical occlusion of the left coronary artery and effective MI/HF (Sed HF), aerobically trained rats submitted to a sham MI (T Sham) and (5) aerobically trained rats submitted to MI/HF (T HF). Higher values were found in the right MePD compared to the left MePD but there were no statistically significant differences among experimental groups.

Figure 5. (A) Values are mean ($\pm$ SD) of the stereological estimation of the glial density found in whole posterodorsal medial amygdala (MePD) of rats. **(B, C)** Mean

(± SD) of the stereological estimation of the glial cells density found in the rostral **(B)** and caudal **(C)** aspects of the MePD and from the right and left hemispheres of non-manipulated controls, sedentary rats submitted to a sham MI (Sed Sham), sedentary rats submitted to the surgical occlusion of the left coronary artery and effective MI/HF (Sed HF), aerobically trained rats submitted to a sham MI (T Sham) and (5) aerobically trained rats submitted to MI/HF (T HF). No statistically significant differences were found.

Figure 6. (A) Photomicrographs of the GFAP-immunoreactivity (GFAP-ir) pattern in the rat posterodorsal medial amygdala of non-manipulated controls, sedentary rats submitted to a sham MI (Sed Sham), sedentary rats submitted to the surgical occlusion of the left coronary artery and effective MI/HF (Sed HF), aerobically trained rats submitted to a sham MI (T Sham) and (5) aerobically trained rats submitted to MI/HF (T HF). Note the presence of astrocytic cell bodies and prolongments in all experimental groups, but apparently fewer cells in the Sed Sham and Sed HF groups. Contrast and background were adjusted using Photoshop 7.0, Adobe (USA). Scale bar = 50 μm. **(B)** Values are mean (± SD) of regional optical density measurements of GFAP-ir in these same experimental groups. * $p < 0.05$ when compared to the control group data in the left hemisphere; ** $p < 0.01$ compared to the Sed Sham data in the left hemisphere.

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Figure 1

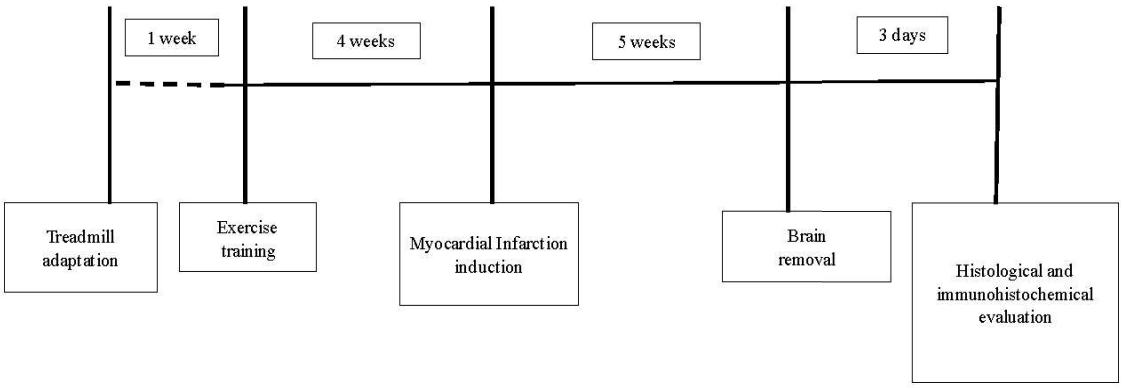
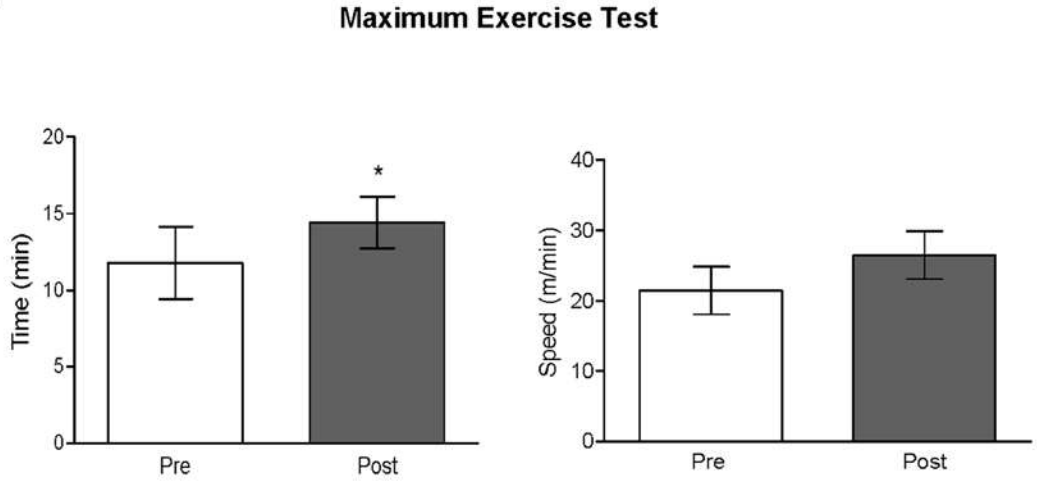

1 week
4 weeks
5 weeks
3 days
Treadmill adaptation
Exercise training
Myocardial Infarction induction
Brain removal
Histological and immunohistochemical evaluation

Figure 2

(A)


Maximum Exercise Test
Time (min)
20
15
10
5
0
*
Pre
Post
Speed (m/min)
40
30
20
10
0
Pre
Post

(B)

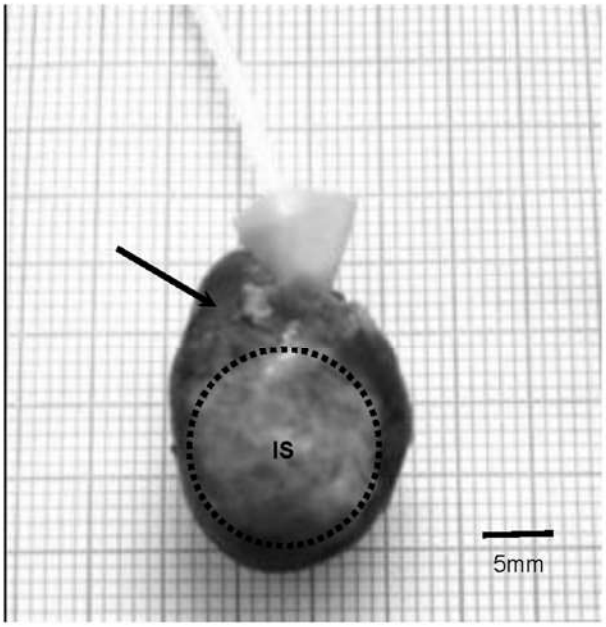

IS
5mm

Figure 3

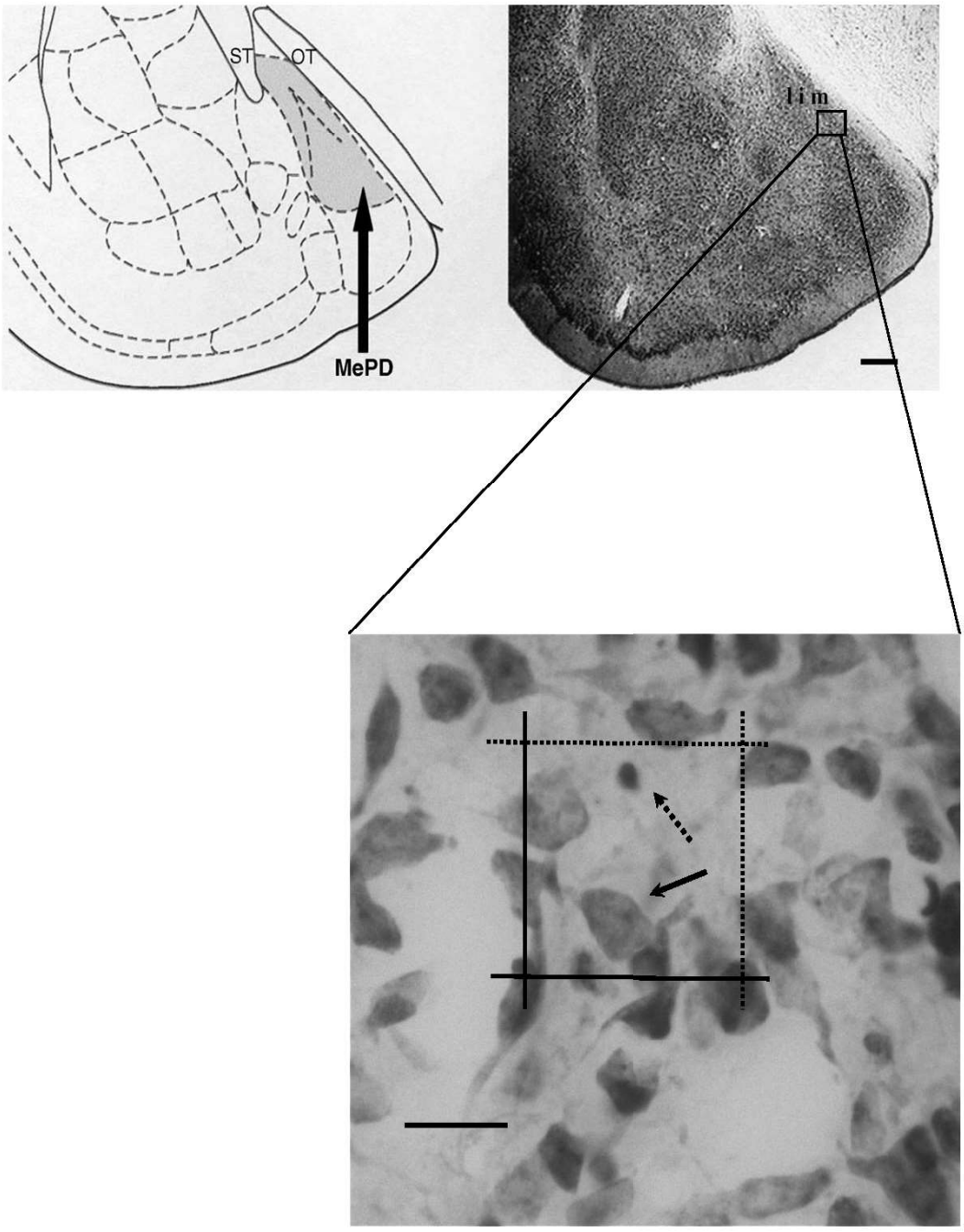


Figure 4

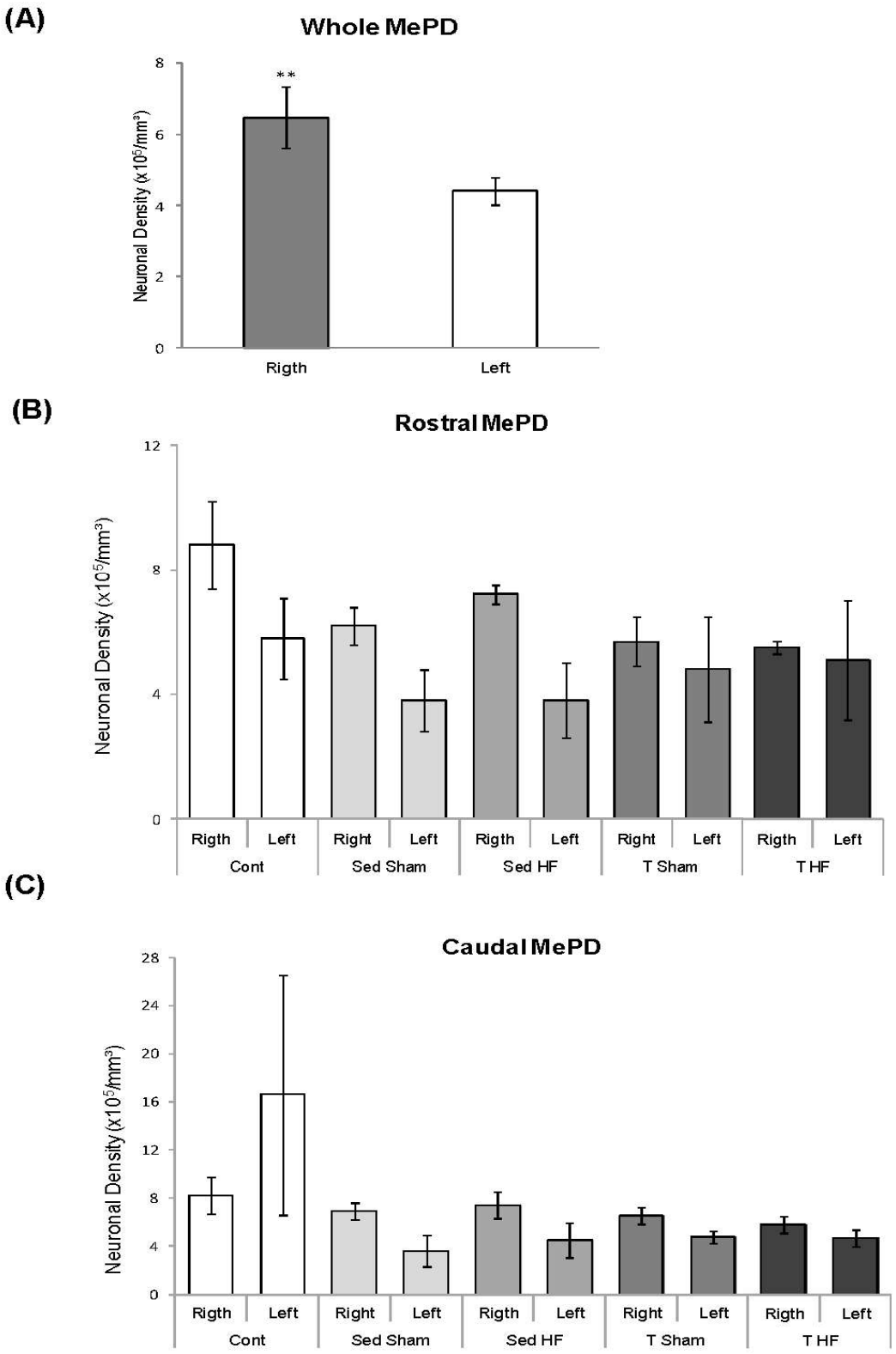
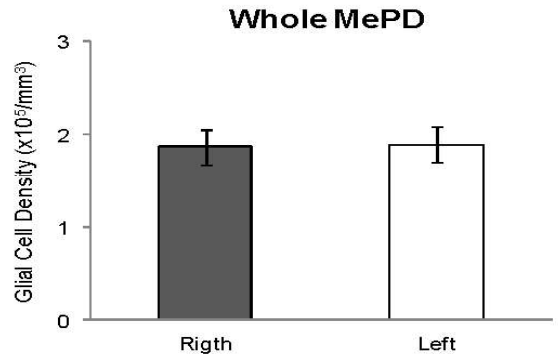
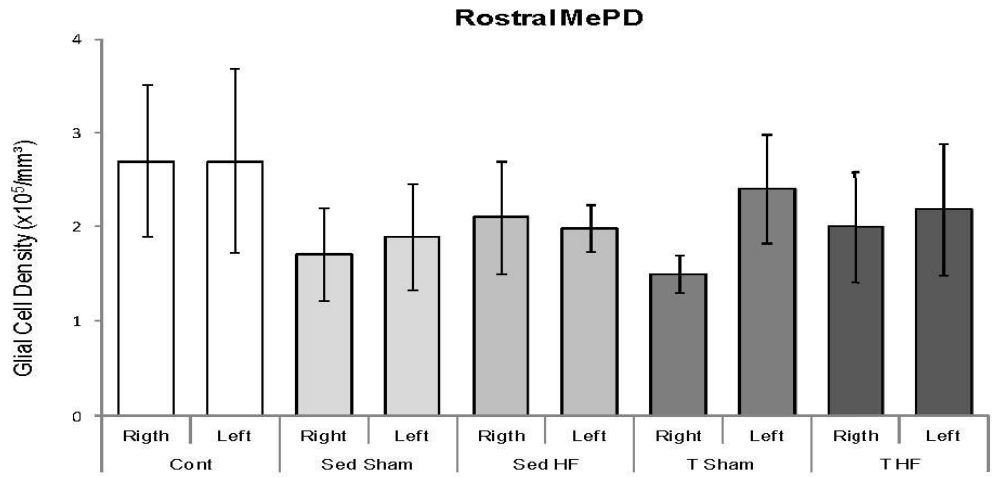

(A)
Whole MePD
Neuronal Density (x10⁵/mm³)
**
Rigth
Left
(B)
Rostral MePD
Neuronal Density (x10⁵/mm³)
Rigth
Left
Right
Left
Cont
Sed Sham
Sed HF
T Sham
T HF
(C)
Caudal MePD
Neuronal Density (x10⁵/mm³)

Figure 5

(A)


Whole MePD
Glial Cell Density (x10⁵/mm³)
3
2
1
0
Rigth
Left

(B)


Rostral MePD
Glial Cell Density (x10⁵/mm³)
4
3
2
1
0
Rigth
Left
Right
Left
Rigth
Left
Right
Left
Rigth
Left
Cont
Sed Sham
Sed HF
T Sham
T HF

(C)

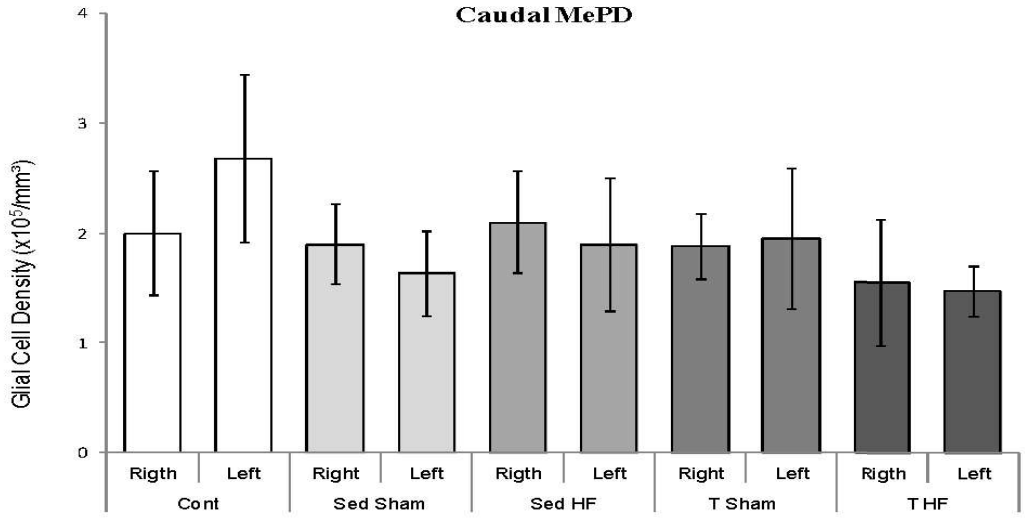
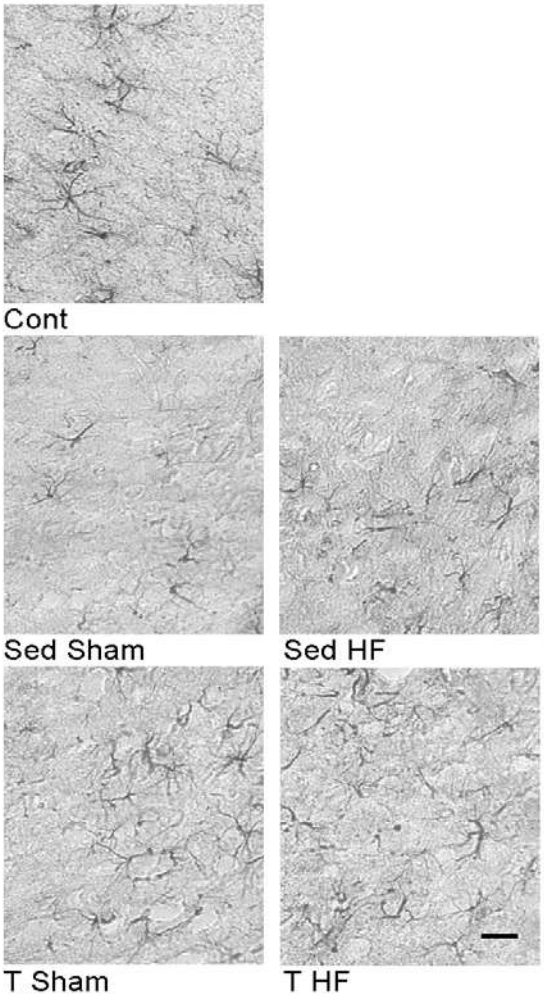
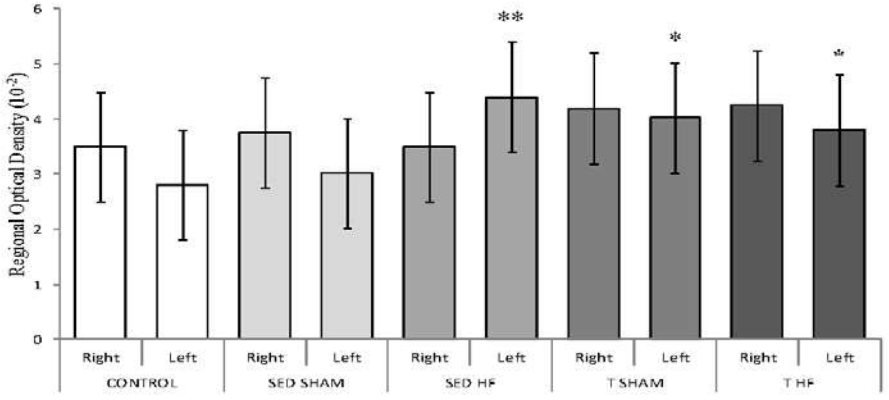

Caudal MePD
Glial Cell Density (x10⁵/mm³)
4
3
2
1
0
Rigth
Left
Right
Left
Rigth
Left
Right
Left
Rigth
Left
Cont
Sed Sham
Sed HF
T Sham
T HF

Figure 6

(A)


Cont
Sed Sham
Sed HF
T Sham
T HF

(B)


Regional Optical Density (10⁻²)
0
1
2
3
4
5
6
**
*
*
Right
Left
CONTROL
SED SHAM
SED HF
T SHAM
T HF

5 CONCLUSÃO GERAL E PERSPECTIVAS

Este trabalho é relevante na medida que ainda não havia sido estudado o MePD após IM e IC em ratos. Nossas hipóteses iniciais eram que ratos com IC apresentariam uma menor densidade de neurônios e maior de células gliais, com efeitos protetores do exercício físico prévio à ocorrência do IM experimental. Ou seja, com o treinamento, esperávamos que o protocolo em questão fosse capaz de reverter a morte neuronal, caso ela ocorresse e, com isso os ratos treinados antes da IC, apresentariam uma maior densidade celular do que os sedentários com IC. Essas hipóteses não se confirmaram no presente modelo experimental e de estudo da densidade neuronal ou glial. Não obstante, foram encontradas diferenças quanto à lateralidade hemisférica, indendepentemente da manipulação experimental, e aumento da GFAP-ir no grupo sedentário com IM/IC. Isto sugere a ocorrência de gliose reativa e possibilidade de rearranjo da quantidade de filamentos intermediários em astrócitos no MePD de ratos.

Com isso, há possibilidade de estudar se há diferenças entre os efeitos agudos e crônicos do IM neste subnúcleo, a morfologia dos neurônios desta região tanto em período agudo quanto após semanas depois do IM, além de trabalhos que incluam marcadores de apoptose e/ou neurogênese local.

ANEXOS

ANEXO A

Normas de formatação do periódico *"Histology and Histopathology"* (2013).

This Journal publishes works in all fields of microscopical morphology; high quality is the overall consideration.

HISTOLOGY AND HISTOPATHOLOGY is an international journal, the purpose of which is to publish original works in English in histology, histopathology and cell biology. Its format is the standard international size of 21 x 27.7 cm. One volume is published every year. Each volume consists of 12 numbers published monthly online. The printed version of the journal includes 4 books every year; each of them compiles 3 numbers previously published online. The **price** per volume, including postage by surface mail and free online access, is 550 euros (or equivalent USD) for 2012. Impact factor: 2.502. Journal Citation Report® 2010, published by Thomson Scientific.

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NOTICE TO CONTRIBUTORS:

1. Two copies of each manuscript and illustrations (no photocopies) should be submitted in English, as hard copies and on disk. Authors should retain one copy, as the Editor cannot accept responsibility for damage or loss of manuscripts. Submission of a paper to **HISTOLOGY AND HISTOPATHOLOGY** must be based on the tacit assurance that no similar paper, except for a preliminary report, has been or will be submitted for publication elsewhere. The decision on acceptance of manuscripts will be made on the basis of a peer review system.
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3. For indexing purposes, a small number of "key words" (no more than 5) must be supplied.
4. **The text** should be preceded by a short summary not exceeding 250 words and should then proceed to sections of Introduction, Materials and methods, Results, Discussion, Acknowledgements and References.
5. Do not divide words at the end of lines. Pages should be numbered consecutively in arabic numerals. Tables, footnotes and figure legends including magnifications, should be submitted on separate sheets. Tables and figures should be referred in the text as (Table 1), (Fig. 1)...
6. **References** to the literature should be cited in the text by the name of the author(s) followed by the year of publication. In cases in which there are more than two authors, only the first is named, followed by "et al."

Examples: Smith (1980) reported that...; (Smith, 1980, 1982); (Smith and Tanaka, 1980); (Smith et al., 1980).

Suffixes a, b, etc., should be used following the year to distinguish two or more papers by the same author(s) published in the same year; example (Smith, 1981a).When two or more references are included in the same bracket, they must be quoted in the chronological order; example (Smith, 1980; Bell et al., 1984).

7. The reference list should be in alphabetical order. References to articles in periodical publications must include: Names and initials of all authors, year of publication, complete title of paper, name of journal (abbreviated in accordance with Index Medicus), number of volume, and first and last page numbers.

Example: Morita T., Suzuki Y. and Churg J. (1973). Structure and development of the glomerular crescent. Am. J. Pathol. 72, 349-368.

Reference to books must include: Name and initials of authors, year of publication, full title, edition, editor, publisher, place of publication and page numbers.

Example: Powell D. and Skrabanek P. (1981). Substance P. In: Gut Hormones. 2nd ed. Bloom S.R. and Polak J.M. (eds). Churchill Livingstone. Edimburgh. pp 396-401.

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Supplying digital images is not a substitute for the press set of figures.

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10. **Electronic submission.** Alternatively, the articles can be submitted by e-mail to Prof Juan F. Madrid, Editor (jfmadrid@um.es) or uploaded to *this page*. The text document must be saved as Word or RTF format. Tables must be included in the text document. Figures must be saved in the formats and at the resolution indicated in point 8 (Illustrations).

ANEXO B

COMISSÃO CIENTÍFICA E COMISSÃO DE PESQUISA E ÉTICA EM SAÚDE

COMISSÃO DE ÉTICA NO USO DE ANIMAIS - CEUA
UFCSPA

A Comissão de Ética no uso de Animais, analisou o Projeto:

Projeto: 11-053 **Versão do Projeto:** **Versão do TCLE:**

Pesquisadores:
ALBERTO ANTÔNIO RASIA FILHO
ALINE DE SOUZA PAGNUSSAT
PEDRO DALL'AGO
ANA PAULA DA SILVA SALAZAR

Título: ESTUDO DA PLASTICIDADE NUERONAL DA AMÍGDALA MEDIAL PÓSTERO-DORSAL DE RATOS COM INSUFICIÊNCIA CARDÍACA E APÓS PROGRAMA DE EXERCÍCIO AERÓBICO.

Este projeto foi aprovado em seus aspectos éticos e metodológicos. Todo e qualquer alteração do projeto, assim com eventos adversos graves, deverão ser comunicados a esta CEUA.

Porto Alegre, 02 de dezembro de 2011.

Pedro R. T. Romão
Coordenador CEUA/UFCSPA