UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM NEUROCIÊNCIAS

Exercício Físico e Estimulação Ambiental Como Estratégias Terapêuticas Após a Hipoperfusão Cerebral Crônica Em Ratos Wistar: Aspectos Neurais, Gliais e Comportamentais

FERNANDA CECHETTI

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Tese de doutorado apresentada ao Programa de Pós-Graduação em Neurociências da Universidade Federal do Rio Grande do Sul como requisito parcial para obtenção do título de doutor em Neurociências

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"A alegria está na luta, na tentativa e no sofrimento envolvido. Não na vitória propriamente dita"

Mahatma Gandhi

Para minha mãe Teresa Foppa e meu pai Dorremi Cechetti

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Sumário

APRESENTAÇÃO

Esta tese está organizada em tópicos: *Introdução, Objetivos, Capítulos* (1 a 4 – referente a artigos científicos), *Discussão, Conclusões, Perspectivas* e *Bibliografia*.

A *Introdução* apresenta o embasamento teórico, que nos levou a formular a proposta de trabalho. O *objetivo geral* e os *objetivos específicos* estão dispostos no corpo da tese, e, especificamente dentro de cada capítulo. Os *capítulos* contêm os artigos científicos, realizados para responder aos objetivos propostos. Todos os experimentos foram desenvolvidos no departamento de Bioquímica-ICBS-UFRGS.

A seção *Discussão* contém uma interpretação geral dos resultados obtidos nos diferentes trabalhos. Os tópicos seguintes, *Conclusões* e *Perspectivas*, abordam as conclusões gerais da tese, bem como possibilidades de futuros trabalhos a partir dos resultados descritos.

O item *Bibliografia* lista as referências citadas na *Introdução* e *Discussão*. As referências utilizadas nos diferentes artigos estão listadas ao final de cada trabalho.

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LISTA DE ABREVIATURAS

AB: Artéria Basilar

ACC: Artéria Carótida Comum

ACoA: Artéria Comunicante Anterior

ACoP: Artéria Comunicante Posterior

Ca^{+2}: Íon Cálcio

CCE: Carótida Comum Esquerda

DA: Demência de Alzheimer

DV: Demência Vascular

EA: Ambiente Enriquecido

EROS: Espécies Reativas de Oxigênio

FSC: Fluxo Sanguíneo Cerebral

GFAP: Proteína Glial Fibrilar Ácida

HEC: Hipoperfusão Encefálica Crônica

M1: Córtex Motor

DNA: Ácido Desoxirribonucléico

2VO: modelo de oclusão de 2 vasos

Resumo

O objetivo geral deste estudo foi verificar os efeitos terapêuticos do enriquecimento ambiental e do exercício físico forçado sobre as conseqüências funcionais e cognitivas da Hipoperfusão Encefálica Crônica (HEC) em ratos *Wistar*. Além disso, padronizamos protocolo alternativo de HEC, a fim de aumentar a taxa de sobrevivência característica deste modelo, juntamente com a análise comportamental e imunohistoquímica do sétimo ao sexto mês após a cirugia, analisando o comportamento de neurônios e astrócitos no hipocampo.

Os resultados mostraram que o protocolo modificado, ou seja, com a oclusão das carótidas realizadas com uma semana de intervalo, apresentam resultados comportamentais e morfológicos similares aos do protocolo padrão; todavia, a taxa de sobrevida dos animais no protocolo modificado é significativamente maior quando comparados ao padrão. Além disso, em ambos os protocolos, os animais hipoperfundidos apresentam um déficit cognitivo no Teste *Water Maze* três meses após a cirurgia, mas os grupos isquêmicos não apresentaram diferenças morfológicas quando comparados ao seus controles em relação a volume total Hipocampal e área Estriatal.

Ao analisar o tempo pós- lesão do déficit cognitivo, verificamos que a HEC causa danos comportamentais a longo prazo, chegando a seis meses após o evento isquêmico. Enquanto a cognição é afetada na fase crônica, as células neuronais e astrogliais são acometidas nas fases mais agudas deste modelo, começando a aparecer morte neuronal (através da quantificação de NeuN) e astrogliose reativa (através da quantificação do imunoconteúdo de GFAP) já nos primeiros dias, permanecendo até aproximadamente 3 meses após a lesão.

Após o dano estabelecido, testamos estratégias terapêuticas com o objetivo de amenizar principalmente o dano cognitivo apresentado pelos animais a partir dos três meses após cirurgia. Inicialmente foi analisado o efeito pré e pós - isquemia do exercício físico forçado através de uma esteira para ratos na memória espacial e de trabalho, através do Water Maze e bioquimicamente no estado oxidativo cerebral. Posteriormente, os mesmos parâmetros foram testados, só que com a intervenção do enriquecimnto ambiental tanto pré quanto pós-isquemia também. O modelo de hipoperfusão cerebral crônica causa como citado anteriormente, um déficit bastante importante na memória e no apredizado. Somado a isso, em relação ao estresse oxidativo, a quantidade de radicais livres e o estado de lipoperoxidação celular encontra-se bastante aumentados no hipocampo de animais submetidos a isquemia, assim como as enzimas antioxidantes.

O protocolo de atividade física regular forçada (3 vezes por semana, durante 20 minutos num período de 3 meses), sobretudo nos grupos pré e pré+pós-isquemia, preveniu os efeitos cognitivos e bioquímicos da HEC. Tal reversão também ocorreu com o uso do enriquecimento ambiental realizado 3 vezes por semana, 1 hora durante 3 meses em todos os grupos estimulados .

Palavras-Chaves: exercício físico, enriquecimento ambiental, hipocampo, hipoperfusão, cognição, estresse oxidativo

ABSTRACT

The aim of this study was to verify the experimental therapeutic effects of environmental enrichment and forced physical exercise on the consequences of cognitive and functional chronic cerebral hypoperfusion (CCH) in *Wistar* rats. To do that, a CCH alternative protocol was standardized, in order to increase the survival rate of this model, together with the behavioral and immunohistochemistry analysis done up to the sixth month after the surgery, analyzing hippocampus neurons and astrocytes.

Results showed that the modified protocol, with occlusion of both carotid arteries performed with a one-week interval, presents morphological and behavioral results similar to the standard protocol; however, the rate of survival after the modified protocol was significantly greater when compared to the standard one. In addition, both protocols produced ischemic animals with cognitive deficits in the *Water Maze* Task, three months after the surgery, however with no gross morphological lesion, as assessed by hippocampal volume and estriatal area. The cognitive deficit in CCH rats is long lasting, reaching six months after the ischemic event.

While the cognition is affected in the chronic phase of hypoperfusion, the neuronal and astrogliais cells are affected in acute phases of this model; neuronal death (through quantification of NeuN) and reactive astrogliosis (through quantification of imunocontent of GFAP) are already present in the first days and remain until approximately 3 months after the lesion.

We tested therapeutic strategies with the aim of alleviating mainly the cognitive damage presented by animals, as from three months after surgery. Initially, the effects of pre- and post-ischemia forced physical exercise in spatial and working memory, through the *Water Maze* Tak and oxidative stress parameters were analyzed. Subsequently, we tested the effects of environmental enrichment, both pre- and post-ischemia.

The model of chronic cerebral hypoperfusion as previously mentioned, causes an important deficit in spatial learning and memory; the free radicals content and cellullar lipid peroxidation is also substantially increased in the hippocampus of animals submitted to hypoperfusion, as well as the antioxidant enzymes. Forced regular physical activity protocol (20-min, 3 times per week during 12 weeks – moderate), especially in groups pre and pre+post-ischemia, warned cognitive and biochemical preventive effects in CCH rats; the same occurred with the use of enrichment environmental performed 1-hr, 3 times per week during 12 weeks in all groups stimulated.

Key-words: physical exercise, environmental enrichment, hippocampus, hypoperfusion, 2VO, spatial memory, oxidative stress.

1. INTRODUÇÃO

1.1 Demências

Estudos sobre o envelhecimento e desordens neurodegenerativas relacionadas com a idade, estão cada vez mais freqüentes, devido ao aumento da incidência destas enfermidades relacionadas ao fenômeno de envelhecimento cerebral. Só no Brasil, 7,1% das pessoas acima de 65 anos são acometidas por algum tipo de demência, sendo a doença de Alzheimer (DA) a de maior incidência, seguida pela demência vascular (DV) (HERRERRA *et al*., 2002). E o que realmente preocupa, é que enquanto a expectativa de vida continuar aumentando no mundo, o número de vítimas das demências cresce proporcionalmente (MATTSON *et al*., 2004).

A distinção entre declínio cognitivo relacionado à idade e provável demência é de grande importância (VANDENBERGUE & TOURNOY, 2004). Dentre os principais fatores de risco estão à baixa escolaridade, o sedentarismo, a hipercolesterolemia, a hipertensão, o diabetes e a homocistinúria, a ocorrência de trauma craniano, além de fatores genéticos e ambientais (JEDRZIEWSKIA *et al*., 2005).

Demência é um termo que descreve uma disfunção cortical e subcortical crônica ou progressiva que resulta num complexo declínio cognitivo. As mudanças cognitivas são comumentes acompanhadas por distúrbios do humor, comportamento e personalidade (RITCHIE & LOVESTONE, 2002). Distúrbios da circulação cerebral têm sido associados com declínio da função cognitiva em idosos e com o desenvolvimento de demência vascular. A isquemia cerebral produz elevados níveis de espécies reativas de oxigênio, iniciando uma cascata de eventos neuropatológicos que podem culminar em doenças neurodegenerativas (KUANG *et al*., 2008).

Tanto no envelhecimento normal como nas demências ocorre uma diminuição do fluxo sanguíneo cerebral (FSC) (FARKAS *et al*., 2004). Alguns trabalhos têm demonstrado que uma acentuada diminuição deste fluxo está relacionado com o aumento das injúrias cognitivas dos pacientes com DA e Demência Vascular (FARKAS *et al*., 2004; RITCHIE *et al*., 2004).

A hipoperfusão encefálica crônica (HEC), que provoca esta redução do fluxo sanguíneo cerebral, é um bom indicador para a progressão da demência vascular, levando a uma disfunção cognitiva e danos neuronais (FARKAS *et al*., 2007). Atualmente, existem vários modelos animais que são utilizados na literatura, com o objetivo de desvendar os processos neurodegenerativos e possíveis estratégias terapêuticas, como por exemplo, o uso de camundongos transgênicos que através de determinadas mutações "mimetiza" a demência de Alzheimer com a deposição progressiva de ß-amilóide.

Neste contexto, o modelo de HEC, inicialmente proposto por DE LA TORRE & FORTIN (1994), vem sendo empregado como um modelo experimental em animais para o estudo da demência vascular, já que a hipoperfusão é um importante achado em humanos acometidos por este mal (KASPAROVA *et al*., 2005; OBRENOVICH *et al*., 2006).

1.1.1 Hipoperfusão Encefálica Crônica

Desordens relacionadas com a circulação cerebral contribuem significativamente para danos neurológicos e psiquiátricos. Uma interrupção no suprimento sanguíneo pode lesionar diversas regiões do encéfalo, enquanto uma moderada, mas persistente

redução do FSC compromete todo o processo relacionado com a memória e contribui para o desenvolvimento de uma demência progressiva (DE LA TORRE, 2002; FARKAS & LUITEN, 2001; MATSUDA, 2001).

Para a reprodução da hipoperfusão encefálica crônica (HEC) que acontece tanto em humanos idosos como em indivíduos com DA, a oclusão permanente bilateral das artérias carótidas comuns em ratos (modelo de 2 vasos – 2VO) tem sido utilizada como modelo experimental (Figura 1). Os trabalhos iniciais revelam que este evento isquêmico resulta numa diminuição do fluxo sanguíneo cortical e hipocampal de 25 a 50 % (PAPPAS *et al*., 1996), podendo causar um déficit cognitivo e dano neuronal semelhante aos efeitos observados na demência vascular (DAVIDSON *et al.,* 2000; KUANG *et al*., 2008). Estudos recentes demonstram que este dano acomete principalmente a área hipocampal, córtex cerebral, substância branca e o sistema visual (FARKAS *et al*., 2007).

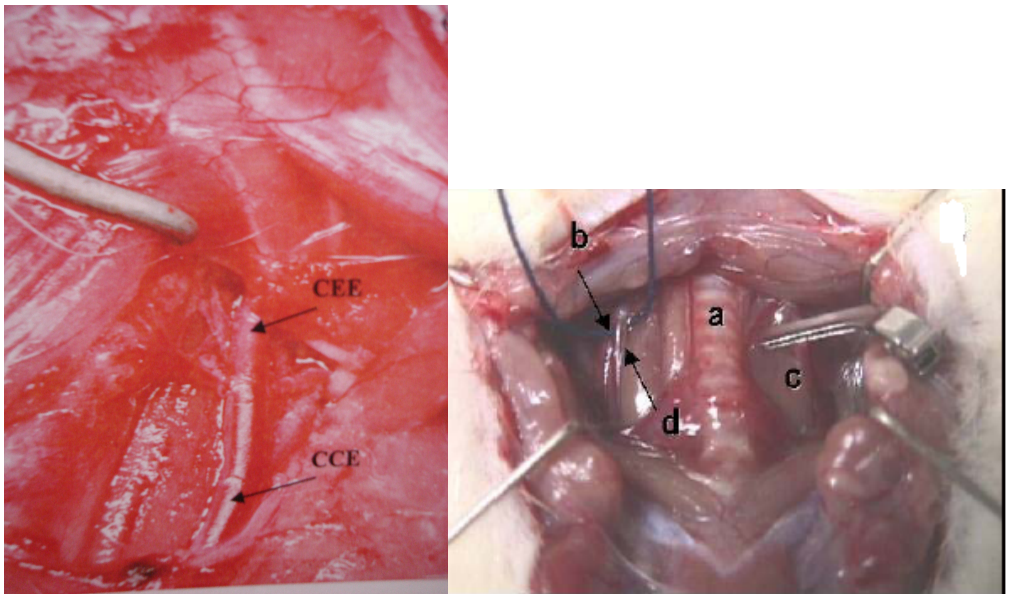

Figura 1. A esquerda foto ilustrando a exposição e identificação de uma das carótidas comuns, neste caso, à esquerda (CCE), após afastamento da musculatura cervical superficial. A direita, imagem similar de cervicotomia mediana anterior, onde a – traquéia; b – artéria carótida comum esquerda reparada com fio cirúrgico e d – nervo vago (extraído de MUMIZ *et al.*, 2004).

O metabolismo energético cerebral apresenta algumas características especiais, como a alta taxa metabólica, reservas energéticas limitadas e uma alta dependência do metabolismo aeróbico da glicose. Por esta razão, o cérebro é mais vulnerável ao dano isquêmico do que os outros tecidos (SIESJO, 1978). A redução da taxa de fluxo sangüíneo e/ou conteúdo arterial de oxigênio pode afetar gravemente a função cerebral, ocasionar alterações bioquímicas e moleculares, e manifestar-se como seqüela neurológica (RODRIGO *et al*., 2005).

O tecido encefálico, submetido à isquemia, passa por uma série de eventos, denominado de "cascata isquêmica' (Figura 2). Em poucos minutos de oclusão vascular, uma seqüência complexa de eventos fisiopatológicos espaciais e temporais acontece em certa ordem, apresentando importantes inter-relações entre si, e perdurando por várias horas ou dias (DURUKAN & TATLISUMAK, 2007). Decorrente da falha energética,

ocorre despolarização neuronal, excessiva liberação e falha na recaptação do neurotransmissor glutamato, aumento dos níveis intracelulares de Ca^{+2}, produção excessiva de espécies reativas de oxigênio (EROS), depleção dos níveis de enzimas antioxidantes, produção de ácido araquidônico e mediadores inflamatórios, além da ativação de segundos mensageiros envolvidos na sinalização da morte celular programada. Em função de todas essas modificações e da ativação de enzimas que danificam a estrutura das membranas celulares, ocorre perda da compartimentalização, abalo da homeostase celular e, finalmente, morte neuronal. Acompanhando as adaptações que acontecem nas células neuronais, também ocorre ativação das células da microglia, astrogliose reativa e rompimento da barreira hematoencefálica (HARUKUNI & BHARDWAJ, 2006; MEHTA *et al*., 2007).

Dentre as mais importantes conseqüências da oclusão permanente das carótidas estão o déficit de memória espacial no labirinto aquático de Morris (PAPPAS *et al*, 1996) e o prejuízo da memória de trabalho no labirinto radial em ratos (OHTA *et al*., 1997). Diminuição de células piramidais na região CA1 do hipocampo e aumento da imunoreatividade a GFAP ocorrem somente aos 190 dias pós-cirurgia (PAPPAS et al., 1996). Outros autores relatam que o fluxo sanguíneo cerebral diminui significativamente cerca de 2 a 10 dias após a cirurgia, voltando ao normal cerca de 90 dias após o evento isquêmico (OHTA *et al*., 1997). Esta redução de fluxo nos capilares cerebrais acarreta numa diminuição significativa de glicose e outros substratos metabólicos (PAPPAS *et al*., 1996). Também um mês após a oclusão, foi encontrada uma diminuição da acetilcolina no estriado e, quatro meses após, esta redução também ocorre no córtex, além de ser mantida no estriado (NI *et al*., 1995).

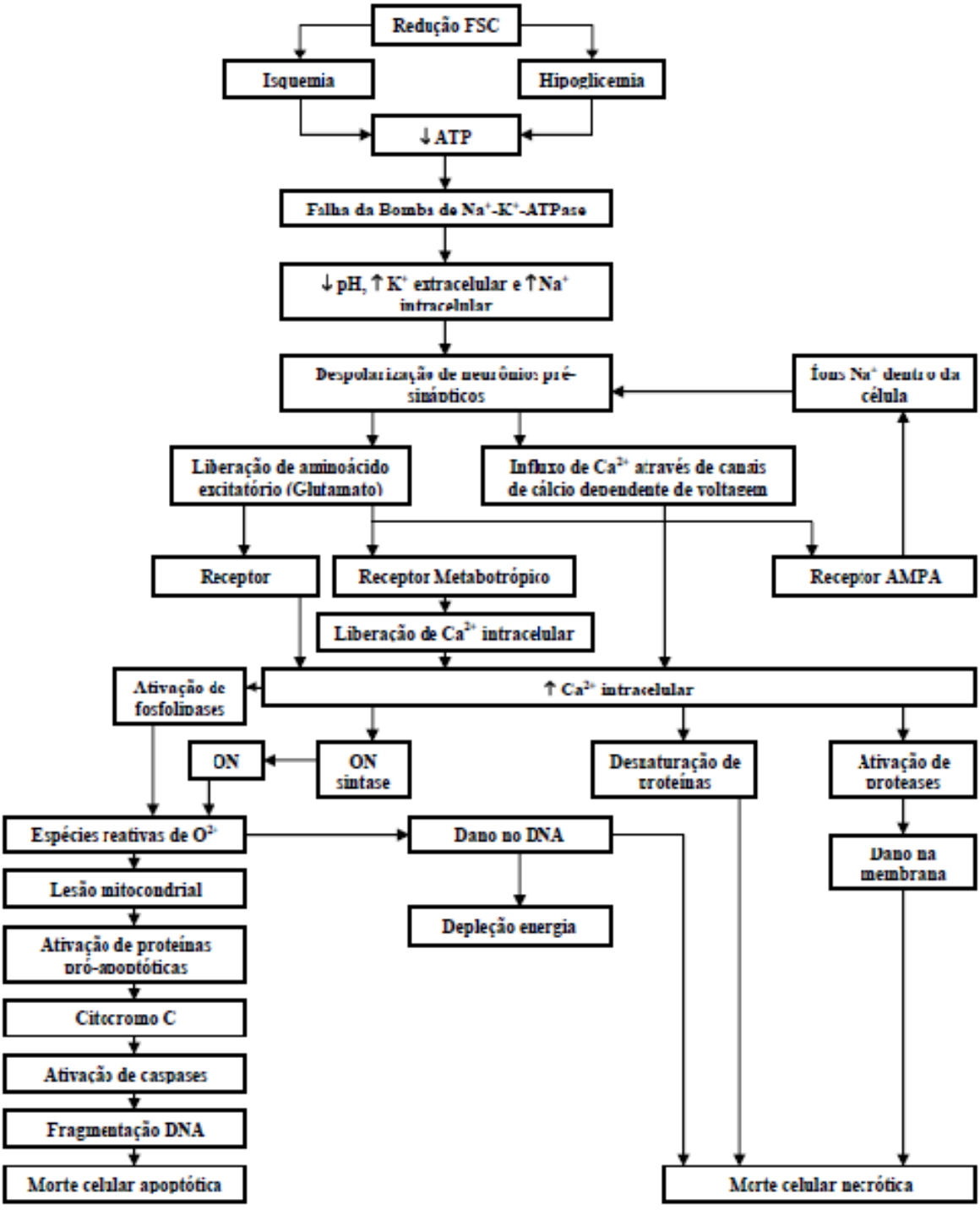

Figura 2 – Cascata Neurotóxica envolvida na Isquemia Cerebral. FSC: fluxo sanguíneo cerebral; ATP: adenosina trifosfato; ON: óxido nítrico; Ca^{+2} : Cálcio (adaptado de WAHLGREN & AHMED, 2004).

Sugere-se que a base neural dos danos vasculares seja a degeneração neuronal de regiões específicas, particularmente em áreas conectadas ao hipocampo. A população neuronal mais afetada são os neurônios colinérgicos do prosencéfalo basal, células que estão diretamente envolvidas com as funções de memória e cognição (KNUSEL & GAO, 1996). A causa da degeneração neste sistema não é conhecida, mas parece ocorrer em paralelo com mudanças que ocorrem no córtex cerebral. Portanto, esta interação entre córtex e prosencéfalo basal podem ser tanto a causa como uma contribuição às mudanças degenerativas dos neurônios colinérgicos (SOFRONIEW & COOPER, 1993).

1.1.2 Fases da HEC e mecanismos compensatórios

Após a oclusão bilateral das artérias carótidas comuns, FARKAS e colaboradores (2007) relatam que há três sucessivas fases bem definidas de hipoperfusão cerebral (Figura 3). A primeira delas é definida como a Fase Aguda, ou seja, aquela que ocorre imediatamente após a oclusão e dura até o segundo ou terceiro dia. Nesta fase, o fluxo sanguíneo cerebral foi subitamente interrompido criando uma situação drástica de hipóxia-isquemia nos tecidos, podendo comprometer a atividade eletrofisiológica do tecido cerebral (MAROSI *et al.*, 2006).

A fase seguinte, chamada de Fase Crônica, pode permanecer até aproximadamente 8 semanas a 3 meses. Neste período, o tecido sofre com uma moderada hipoglicemia somada a uma oliguemia, podendo ser considerada a fase que mais se assemelha com a redução do FSC em humanos dementes. A última fase, também chamada de restituição, corresponde ao período no qual o fluxo sanguíneo volta

a seu estado basal e a hipoperfusão vai cessando lentamente (CHOY *et al*., 2006; FARKAS *et al*., 2007).

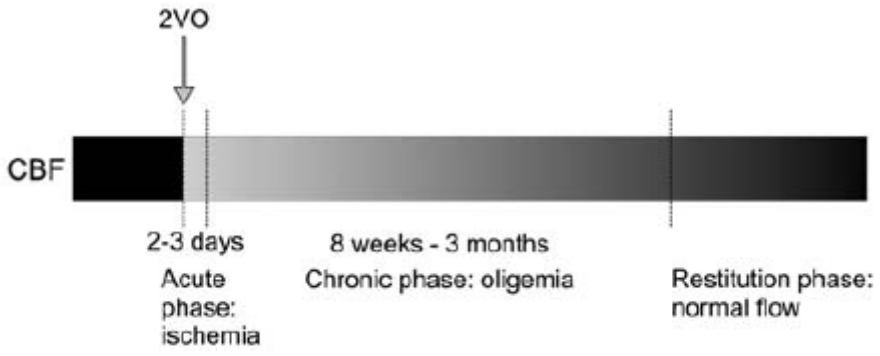

Figura 3 – Fases sucessivas da Hipoperfusão cerebral crônica induzida pela oclusão permanente, bilateral das artérias carótidas comuns (extraído de FARKAS *et al*., 2007)

A principal justificativa para esta restituição do fluxo sanguíneo cerebral nos ratos é que estes animais possuem um Polígono de Willis completo. Com o evento isquêmico causado pelo modelo 2VO a circulação fica reduzida, mas não cessa (FARKAS *et al.,* 2007), principalmente pelo fluxo nas artérias vertebrais e comunicantes posteriores. Somado a isso, outros mecanismos adaptativos são capazes de auxiliar no retorno basal do fluxo sanguíneo cerebral, dentre eles estão a dilatação arterial (CHOY *et al*., 2006; OLDENDORF, 1989), angiogênese, recrutamento de capilares não perfundidos e regulação bioquímica, com o aumento dos níveis de um potente vasodilatador, o óxido nítrico (KEYNES & GARTHWAITE, 2004).

1.2 Estratégias Terapêuticas

Para um completo entendimento do papel de uma patologia cerebrovascular no desenvolvimento de uma disfunção cognitiva e posterior demência, é importante explorar as mudanças metabólicas relacionadas com a hipoperfusão cerebral e sua relação com o dano cognitivo. Somado a isso, o reconhecimento de mecanismos particulares dos eventos causados pela hipoperfusão pode auxiliar na identificação de estratégias terapêuticas eficazes.

A disfunção neuronal, e a conseqüente morte celular, têm sido amenizadas nos animais em experimentação por manipulações que reduzam os níveis de estresse oxidativo, por agentes que mantêm os níveis de ATP e homeostase iônica (MATTSON *et al*., 2000) e que possam reverter o quadro patológico instalado (MILGRAM *et al*., 2006). Dentre as estratégias terapêuticas disponíveis na literatura, as principais são: estratégica farmacológica, intervenção nutricional, enriquecimento ambiental e exercício físico (MILGRAM *et al*., 2006).

A relação entre enriquecimento ambiental/exercício físico e demências vem sendo bastante investigada. Estudos epidemiológicos, principalmente nos últimos anos, têm proposto que altos níveis de estímulos sociais (SCARMEAS *et al*., 2001; WANG *et al*., 2001) ou atividade física (ABBOTT *et al*., 2004) reduzem os riscos de desenvolver a Doença de Alzheimer ou qualquer outro tipo de demência, e que estes pode ser uma estratégia terapêutica contra as perdas funcionais destas patologias (ROLLAND *et al*., 2000).

O exercício físico regular forçado, geralmente aquele realizado em esteiras adaptadas para ratos (Figura 4), beneficia o sistema nervoso central, assim como o sistema músculo-esquelético e cardiovascular. Tem sido proposto que o exercício mantém a integridade cerebrovascular (MC FARLAND, 1963), promovendo a

capilarização (BLACK *et al.,* 1987) e principalmente aumentando as conexões sinápticas (PYSH e WEISS, 1979). Ratos exercitados regularmente durante nove semanas, aumentam seu desempenho cognitivo e reduzem os níveis de lipoperoxidação da membrana e dano oxidativo ao DNA (MATTSON *et al*., 2004). Após um programa de exercícios em camundongos adultos, VAN PRAAG e colaboradores (1999) observaram que, além de melhorar o aprendizado espacial, o exercício aumenta a neurogênese no giro denteado de maneira similar ao enriquecimento ambiental. Segundo RAMSDEN e colaboradores (2003), a atividade física regular causa aumento do aprendizado espacial, ganho significativo na memória e diminuição do declínio da atividade espontânea relacionados com a idade em roedores. Associado a isso, também ocorre um aumento da sinalização serotoninérgica e da estimulação da angiogênese (MATTSON *et al*., 2004).

FIGURA 4 – Foto ilustrando a esteira ergométrica utilizada para a prática de exercício forçado em ratos.

O Enriquecimento Ambiental (EA) é definido como uma combinação de interação social, exercício físico voluntário e exposição continuada a "possibilidades de aprendizagem", que segundo KRECH e colaboradores (1960), pode alterar a estrutura e função do encéfalo em roedores. O protocolo de estimulação por EA é variável. Geralmente, há variações no tamanho das caixas, composição, duração, complexidade social e de estímulos por objetos, e na freqüência da troca destes objetos (Figura 5). Alguns trabalhos utilizam a manutenção permanente dos animais neste ambiente, enquanto outros utilizam a estimulação prévia a um evento isquêmico (BIERNASKIE & CORBETT, 2001; BELAYEV *et al.*, 2003).

Tem sido relatado, nos estudos que envolvem dano encefálico, que estímulos ambientais (cognitivo e social) podem desempenhar papel importante na plasticidade neural hipocampal (VAN PRAAG *et al.*, 1999). Estudos têm demonstrado que ratos mantidos em um ambiente enriquecido após evento isquêmico na vida neonatal e adulta apresentaram melhor aquisição no teste do labirinto aquático de Morris (BRIONES *et al.*, 2000). Ainda, aumento nos níveis de fatores neurotróficos e preservação das células piramidais da região CA1 são resultados que corroboram a efetividade do estímulo ambiental como neuroprotetor. Sabe-se também que o enriquecimento ambiental tem um efeito bastante positivo sobre o processo de envelhecimento, protegendo o hipocampo da perda neuronal e das disfunções relacionadas à idade (MOHAMMED *et al.*, 1993).

FIGURA 5 – Foto ilustrando a gaiola utilizada para o enriquecimento sensorial, cognitivo, motor e social dos ratos.

Estudos prévios realizados em nosso laboratório já confirmam a eficácia tanto do protocolo de atividade física como do enriquecimento ambiental. Em relação a primeira estratégia terapêutica, SCOPEL e colaboradores (2006) demonstraram que o exercício físico causa, de maneira dependente da intensidade, alterações na susceptibilidade hipocampal ao dano isquêmico *in vitro*. O exercício moderado parece proteger células hipocampais do dano isquêmico, já que reduziu o dano celular induzido pela isquemia-reoxigenação *in vitro* em fatias hipocampais de ratos. Quanto a freqüência desta atividade física, CECHETTI e colaboradores. (2007) concluíram que as células hipocampais frente ao exercício moderado apresentam baixa susceptibilidade ao dano isquêmico, de forma dependente da freqüência, e, que o exercício com intensidade moderada realizado três vezes por semana reduz o dano produzido pela isquemia *in vitro*.

O enriquecimento ambiental quando realizado como estratégia terapêutica a fim de amenizar os danos causados pela hipóxia-isquemia neonatal, parece neuroproteger as células hipocampais, recuperando o prejuízo tanto na memória de referência, como de

trabalho, sem alterar o dano morfológico causado pela isquemia (PEREIRA *et al*., 2007). A mesma autora demonstrou em seu outro trabalho que o este protocolo de enriquecimento ambiental, realizado diariamente por um período de 1 hora, diminui os níveis de BDNF e da enzima antioxidante Superóxido Dismutase (SOD), pois os mesmos encontravam-se aumentados após a realização da Hipóxia-isquemia (PEREIRA *et al*., 2009).

Logo, nesta Tese decidimos investigar as conseqüências da estimulação pelo Enriquecimento Ambiental e Exercício Físico Forçado em animais submetidos a Hipoperfusão Cerebral Crônica. Considerando-se a abordagem que será utilizada neste estudo, a **hipótese principal deste trabalho** é que tanto o enriquecimento ambiental como o exercício físico forçado poderão causar efeitos neuroprotetores cognitivos nos animais hipoperfundidos. Ainda, sugere-se que os animais hipoperfundidos apresentarão danos morfológicos e celulares no hipocampo quando comparados aos controles. Alguns dos possíveis mecanismos bioquímicos envolvidos relacionados com os efeitos supracitados serão investigados, com o intuito de contribuir para os conhecimentos relacionados com a hipoperfusão cerebral.

2. OBJETIVOS

2.1 Objetivo Geral

O objetivo geral deste estudo foi verificar os efeitos terapêuticos do enriquecimento ambiental e do exercício físico forçado sobre a recuperação cognitiva e bioquímica de animais expostos à Hipoperfusão Encefálica Crônica.

2.2 Objetivos Específicos

(1) Padronizar um protocolo modificado do modelo padrão de HEC, a fim de aumentar a sobrevivência dos animais deste modelo, através de uma comparação entre o modelo padrão e o modificado em relação a memória espacial no Labirinto Aquático de Morris, volume hipocampal total e área estriatal dos animais– Capítulo 1.

(2) Investigar se os ratos submetidos ao modelo modificado de 2VO apresentam déficit na memória e aprendizado através do Water Maze e teste de Reconhecimento de objetos 6 meses após a cirurgia, e se o dano hipocampal testados através da imunoreatividade para GFAP e para marcador neuronal NeuN acompanham este dano cognitivo. – Capítulo 2;

(3) Analisar o potencial terapêutico do exercício físico forçado sobre o déficit cognitivo e o estado oxidativo cerebral no hipocampo, estriado e córtex cerebral de ratos submetidos ao modelo modificado de hipoperfusão encefálica crônica – Capítulo 3;

(4) Analisar o potencial terapêutico do enriquecimento ambiental sobre o déficit cognitivo e o estado oxidativo cerebral no hipocampo, estriado e córtex cerebral de ratos submetidos ao modelo modificado de hipoperfusão encefálica crônica – Capítulo 4.

3. CAPÍTULO 1

Artigo: The modified 2VO ischemia protocol causes cognitive impairment similar to that induced by the standard method, but with a better survival rate – Publicado no Brazilian Journal of Medical and Biological Research

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The modified 2VO ischemia protocol causes cognitive impairment similar to that induced by the standard method, but with a better survival rate

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Abstract

Permanent bilateral occlusion of the common carotid arteries (2VO) in the rat has been established as a valid experimental model to investigate the effects of chronic cerebral hypoperfusion on cognitive function and neurodegenerative processes. Our aim was to compare the cognitive and morphological outcomes following the standard 2VO procedure, in which there is concomitant artery ligation, with those of a modified protocol, with a 1-week interval between artery occlusions to avoid an abrupt reduction of cerebral blood flow, as assessed by animal performance in the water maze and damage extension to the hippocampus and striatum. Male Wistar rats (N = 47) aged 3 months were subjected to chronic hypoperfusion by permanent bilateral ligation of the common carotid arteries using either the standard or the modified protocol, with the right carotid being the first to be occluded. Three months after the surgical procedure, rat performance in the water maze was assessed to investigate long-term effects on spatial learning and memory and their brains were processed in order to estimate hippocampal volume and striatal area. Both groups of hypoperfused rats showed deficits in reference ($F_{(8,172)}$ = 7.0951, $P < 0.00001$) and working spatial memory [2nd ($F_{(2,44)}$ = 7.6884, $P < 0.001$), 3rd ($F_{(2,44)}$ = 21.481, $P < 0.00001$) and 4th trials ($F_{(2,44)}$ = 28.620, $P < 0.0001$)]; however, no evidence of tissue atrophy was found in the brain structures studied. Despite similar behavioral and morphological outcomes, the rats submitted to the modified protocol showed a significant increase in survival rate, during the 3 months of the experiment ($P < 0.02$).

Key words: Chronic cerebral hypoperfusion; Spatial memory; Hippocampus; Striatum; Water maze

Introduction

Disorders of the cerebral circulation are associated with neurological and psychiatric illnesses. Clinical evidence supports the hypothesis that chronic cerebral hypoperfusion is associated with cognitive decline, both in aging and in neurodegenerative disorders (1-3); in fact, there is a correlation between the severity of memory dysfunction and the decline in cerebral blood flow in Alzheimer's disease, vascular dementia and post-stroke hypoperfusion (4,5).

Permanent bilateral occlusion of both common carotid arteries in rats (2-vessel occlusion, 2VO) has been used to model chronic cerebral hypoperfusion (6); the main findings include histopathological damage and impaired spatial learning function (6-8). This cognitive impairment may be related to progressive loss of hippocampal pyramidal neurons, an association often observed in human aging and dementia states (6).

The hippocampus is highly vulnerable to ischemic insults (7), particularly the CA1 pyramidal cell layer (7,8). Ischemia-induced neuronal degeneration is also observed in other structures, such as the striatum, cerebral cortex and thalamus

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(7-9). In this model, there is an abrupt reduction of whole brain blood flow, ranging from approximately 35–45% in the cortical area to 60% in the hippocampus compared to control levels (4,10-12). This hypoperfusion is believed to sustain a chronic state of moderate hypoglycemia, a pathophysiological condition closely resembling that of reduced cerebral blood flow present in human aging and dementia (6).

Adaptations of the 2VO protocol have been explored in order to refine the experimental model by avoiding the abrupt reduction of cerebral blood flow. A modified procedure permitting the gradual establishment of cerebral hypoperfusion (13) has been proposed, with a 1-week interval between the occlusion of the two common carotid arteries. Consequent cognitive dysfunction was demonstrated regarding object recognition and Y-maze tests (14), but no direct comparison has been made between the two surgical protocols.

However, the conventional 2VO model, although producing interesting results, has a survival rate well below that induced by other lesion methods frequently used in our laboratory (4-vessel occlusion ischemia and neonatal hypoxia-ischemia) (15,16). Additionally, high mortality rates of about 50% have been reported for rats undergoing conventional 2VO (17-19). In order to minimize the number of animals for future experiments and to optimize research efforts, we decided to directly compare survival rates and long-term cognitive and morphological outcomes after applying the standard and modified 2VO protocols, as assessed by reference and working memory tasks in the water maze and damage extension to the hippocampus and striatum.

Material and Methods

Animals

Male Wistar rats aged 3 months were obtained from the Central Animal House of the Institute of Basic Health Sciences, Universidade Federal do Rio Grande do Sul. They were maintained in a temperature-controlled room (21 ± 2°C) on a 12/12-h light/dark cycle, with food and water available *ad libitum*. All procedures were in accordance with the Guide for the Care and Use of Laboratory Animals adopted by the National Institute of Health (USA) and with the Federation of Brazilian Societies for Experimental Biology (FESBE), and were approved by the Research Ethics Committee of Universidade Federal do Rio Grande do Sul.

Animals were randomly assigned to different experimental groups: sham-operated control with manipulation of both carotids at the same time – standard protocol (N = 12); sham-operated control with manipulation of the two carotids with a 1-week interval between procedures – modified protocol (N = 12); ischemic group submitted to occlusion of both carotids at the same surgical time (N = 10), and ischemic group submitted to carotid occlusion with a 1-week interval between procedures (N = 13). Unpaired *t*-tests indicated no significant differences **between** the two control **groups.**

2VO procedure

Rats were anesthetized with halothane; a neck ventral midline incision was made and the common carotid arteries were then exposed and gently separated from the vagus nerve. Rats (N = 10) assigned to the standard 2VO protocol had both arteries concomitantly occluded with 5-0 silk suture. In the modified protocol (N = 13), carotids were occluded with a 1-week interval between interventions, the right common carotid being the first to be assessed and the left one being occluded 1 week later. Sham-operated controls (N = 24) received the same surgical procedures without carotid artery ligation. Animals were randomly assigned to sham or 2VO groups so as to avoid any litter effect.

Morris water maze

Three months after surgery, the rats were submitted to behavioral testing for spatial memory in the Morris water maze. The maze consisted of a black circular pool 200 cm in diameter filled with water (temperature about 23°C, 40 cm in depth) situated in a room with visual cues on the walls. A black platform 10 cm in diameter was submerged in the water (2 cm below the water surface). The pool was conceptually divided into four quadrants and had four points designed as starting positions (N, S, W, or E). Two behavioral protocols, for reference and working memory, were used.

Reference memory protocol

In this task, rats received five training sessions (one session/day) and a probe trial on the 6th day. Each session consisted of four trials with a 15-min intertrial interval. A trial began when the rat was placed in the water at one of the four starting positions, chosen at random, facing the wall. The order of the starting position varied in each trial and any given sequence was not repeated on acquisition phase days. The rat was given 60 s to locate the platform; if the animal did not succeed it was gently guided to the platform and left on it for 10 s. Rats were dried and returned to their home cages after each trial. The latency to find the platform was measured in each trial and the mean latency for each training day was calculated. The probe consisted of a single trial, as described before, with the platform removed. Here, the latency

to reach the original platform position, the number of crossings over that place and the time spent on the target, as well as in the opposite quadrants, were measured (20). Sessions were recorded with a video acquisition system. Videotapes were used by a trained observer using a dedicated software (ANY-maze®). Videos were subsequently placed in randomized order in a separate ANY-maze protocol to be scored by a trained observer blind to the experimental condition using a keyboard-based behavioral tracking system.

Working memory protocol

This protocol consisted of four trials/day on 4 consecutive days, with the location of the platform being changed daily. Each trial was conducted as described in the reference memory protocol, with a 5-min intertrial interval. Latency to find the platform was measured in each trial and the mean latency for each trial along the 4 days was calculated, permitting the observation of the ability of the animals to locate the novel platform position each day (20).

Morphological analysis

Rats were sacrificed 1 day after the completion of the behavioral study. They were anesthetized with chloral hydrate (30%, 10 mL/kg, *ip*) and submitted to transcardiac perfusion with 0.9% saline followed by 4% formaldehyde. Brains were removed and maintained in formaldehyde solution. For the morphological analysis, brains were cryoprotected with a 30% sucrose solution for 2 days and sectioned, and coronal 50-µm thick sections were obtained using a cryostat (Leica).

Hippocampal volume

The volume of the hippocampus was estimated as described below. The 50-µm sections covering the whole hippocampus were mounted on gelatinized glass slides and stained with hematoxylin and eosin; the Image J program (NIH, USA) was used to delineate and estimate the hippocampal and dentate gyrus area (21). The volume of the hippocampus was calculated by the sum of areas multiplied by the section interval according to the Cavalieri method (22). The Ammon's horn volume was calculated as the difference between the volume of the entire hippocampus and the volume of the dentate gyrus (22).

Striatal area

Striatal atrophy was also estimated at the +1.20-mm level from the bregma according to Paxinos and Watson (23); one slice per rat was used. The Image J program (NIH, USA) was used to delineate and estimate the striatal area at that level (21).

Statistical analysis

Behavioral performance in reference memory was analyzed by one-way repeated-measures analysis of variance (ANOVA) with lesion as the independent variable and session as the repeated measure. Data regarding working memory, hippocampus volume and striatum area at the +1.20-mm level from the bregma according to Paxinos and Watson (23), were analyzed by one-way ANOVA. The *post hoc* Duncan test for multiple comparisons was applied when indicated. Fisher exact tests were applied to contingency tables (2 x 2) for comparison of mortality rates. Unpaired *t*-tests indicated no significant differences **between** sham-operated control groups submitted to the standard or modified protocols, **and** therefore only 3 groups will appear in the figures: control, ischemic group-standard protocol and ischemic group-modified protocol.

Data are reported as means ± SEM. Probability values of less than 5% were considered to be significant. All statistical analyses were performed using the Statistica® software package running on a compatible personal computer.

Results

Survival rate

The survival rates obtained after the conventional and modified 2VO protocols were 60 and 92%, respectively, when assessed 24 h after surgery (Table 1). Fisher exact tests revealed a significant decrease of lethality in the group exposed to the modified 2VO protocol compared to the group exposed to the standard protocol ($P < 0.02$).

Behavioral effects

Rats receiving either standard or modified protocols of bilateral common carotid artery occlusion showed a significant impairment of reference memory performance in the water maze compared to control (one-way ANOVA for repeated measures, $F_{(8,172)} = 7.0951$, $P < 0.00001$). Interestingly, the Duncan test for multiple comparisons indicated no significant differences between the standard and modified protocol groups (Figure 1).

One-way ANOVA revealed significant differences in the escape latencies of 2VO animals in the 2nd ($F_{(2,44)}$ = 7.6884, P = 0.00137), 3rd ($F_{(2,44)}$ = 21.481, P = 0.00001) and 4th trials ($F_{(2,44)}$ = 28.620, P = 0.0001) of the working memory task compared to control. There were also no differences between the standard and modified protocols in this task (Figure 2).

Permanent occlusion of the bilateral common carotid arteries did not cause any motor deficit; the mean swimming speed was 26 cm/s for control animals and 24.5 cm/s for ischemic rats.

Lesion extension in the hippocampus and striatum

There were no differences between the control and 2VO groups regarding the total hippocampus (Figure 3A, B and C) or the striatum area at the +1.20-mm level from the bregma according to Paxinos and Watson (23) (Figure 3D, E and F).

Discussion

The present study reports, for the first time, the effects of conventional and modified 2VO protocols on survival rates, spatial water maze memory performance and brain damage in adult Wistar rats. It is shown that the modified protocol, with a 1-week interval between each carotid occlusion, significantly reduced the mortality rate compared with the standard model (Table 1). High mortality rates after the standard procedure have been previously reported. Farkas and colleagues (17) showed a survival rate of 69.23% for the group submitted to hypoperfusion compared to 82% for the control group. Other authors have reported a survival rate of 66.66% (18) and 50% (19) for ischemic groups. However, Institoris et al. (19) managed to significantly improve survival with the administration of a selective cyclooxygenase (COX-2) inhibitor.

Both protocols impaired cognitive function similarly as assessed by the use of spatial memory, hippocampus-dependent, tasks in the water maze (Figures 1 and 2), run 3 months after surgery, and failed to produce measurable lesions in the hippocampus and striatum (Figure 3). Previous studies have reported that 2VO occlusion provokes short- and long-term memory impairments using passive avoidance, Y-maze, eight-arm radial maze tasks (24) and the Morris Water Maze task (25,26).

The reports of morphologic outcomes after 2VO ischemia are conflicting. Some studies have found a direct correlation between cerebral hypoperfusion-induced memory deficit and CA1 cell damage (26-28), and an association of impaired learning performance 3 weeks after the procedure with a significant pyramidal cell loss in the CA1 region (29). However, others have reported no correlation or only a weak one between diminished CA1 neuron number and performance in the Morris maze (30-32). In addition, Murakami et al. (33) reported that mice with chronic cerebral hypoperfusion exhibit learning impairments in the water maze without marked histological alterations in the hippocampus. In summary, a direct link between 2VO ischemia-induced memory failure and the appearance of neuronal damage in the hippocampus has not been established (19).

It is possible that delayed cell death processes were still ongoing in our study, since Farkas et al. (6) reported that CA1 neurons begin to degenerate after several weeks of reduced blood perfusion, and Ni et al. (25) demonstrated that 2VO rats showed loss of cells (30%) and increased glial fibrillary acidic protein (GFAP) density in CA1 only 150 days after surgery. On the other hand, acquisition and performance of both Morris and radial arm mazes might be impaired by dysfunction of neurochemical systems in many brain regions (34,35). The neurobehavioral consequences of chronically reduced cerebral blood flow could provide an insight into the role of reduced cerebral energy metabolism in Alzheimer's dementia, which is characterized by decreased brain glucose metabolism (36,37). It is important to remember that physiological levels of endogenous glutamate can become excitotoxic whenever neuronal energy production is compromised (38). Along this line, we have recently described a significant increase of S100B and GFAP levels, as well as a decrease in glutamate uptake in the hippocampus, in 2VO rats (39).

We suggest that the use of a modified 2VO protocol, with 1 week of interval between the occlusion of each carotid, will allow brain hypoperfusion to gradually develop (13) and result in higher survival rates, as compared with the standard method of concomitant artery occlusion. In addition, Kaliszewski et al. (40) showed that gradual vessel occlusion produces less severe tissue ischemia due to a more effective development of collateral circulation. In conclusion, the modified 2VO protocol may be more useful and reliable, with similar induced cognitive deficits and lower mortality rates, than the standard 2VO procedure.

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Table 1. Survival rate of rats submitted to standard (both arteries concomitantly occluded) and modified protocols (carotids were occluded with a 1-week interval) of bilateral occlusion of the common carotid arteries (2VO).

2VO protocol	Operated rats	Surviving rats	Survival rate	Time of survival
Standard protocol	20	12	60%	3 months
Modified protocol	20	18	90%*	3 months

*P < 0.05 compared to the standard protocol (Fisher exact test).

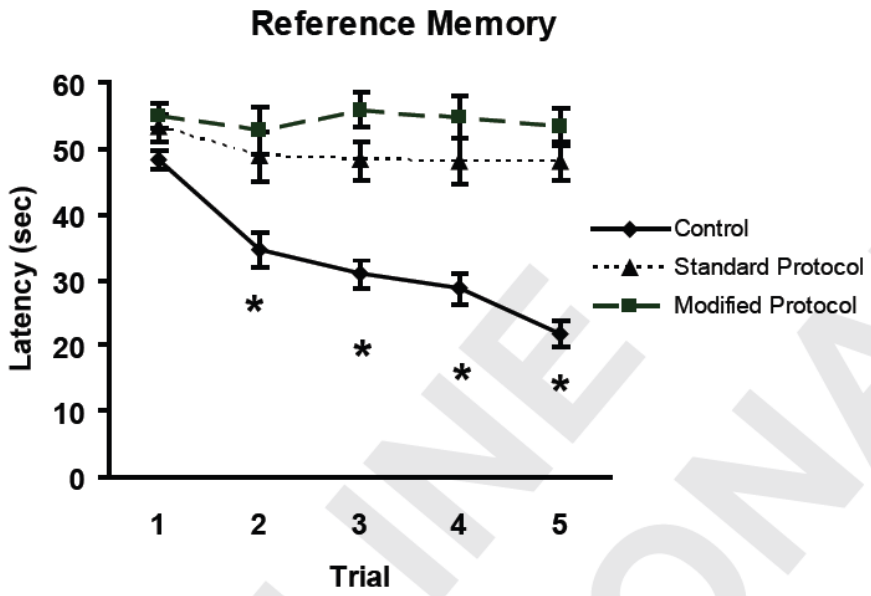

Figure 1. Reference memory performance in the water maze. Data are reported as means ± SEM. *P < 0.05 control group (N = 24) compared to standard (both arteries concomitantly occluded, N = 10) and modified (carotids were occluded with a 1-week interval, N = 13) 2VO protocol groups (repeated measures ANOVA).

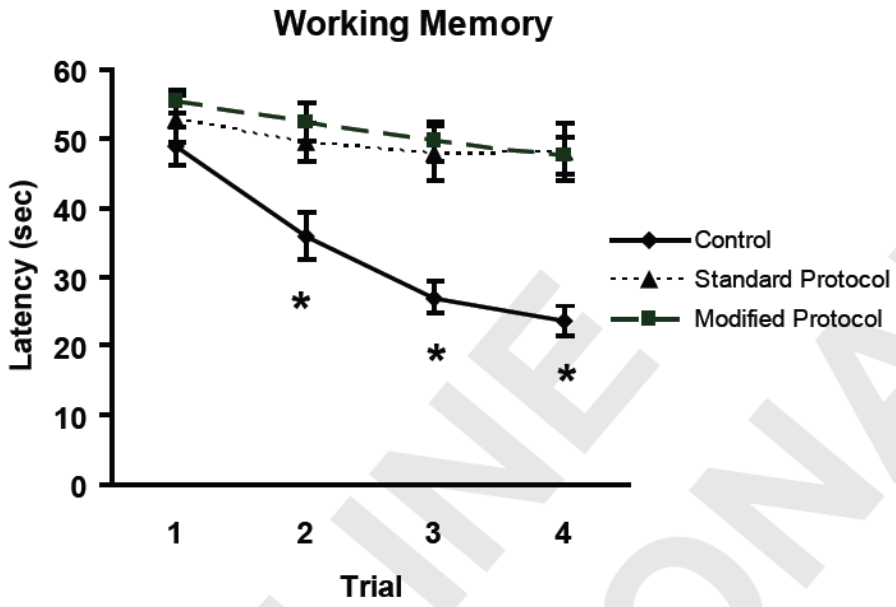

Figure 2. Working memory performance in the water maze. Data are reported as means ± SEM. *P < 0.05 control group (N = 24) compared to standard (both arteries concomitantly occluded, N = 10) and modified (carotids were occluded with a 1-week interval, N = 13) 2VO protocol groups (repeated measure ANOVA followed by the Duncan test).

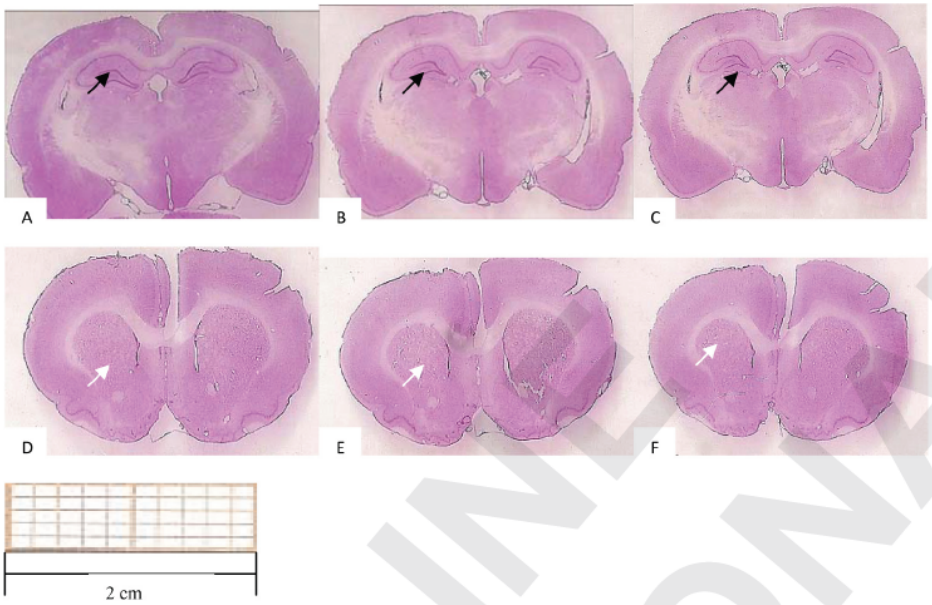

Figure 3. Representative photomicrographs of the rat brain. The hippocampus (black arrows) appears in panels *A* (control), *B* (standard protocol), and *C* (modified protocol). The striatum (white arrows) appears in panels *D* (control), *E* (standard protocol), and *F* (modified protocol).

4. CAPÍTULO 2

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Chronic brain hypoperfusion causes long-term memory impairment associated with early glial activation and neuronal death

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Abstract

Reduction of cerebral blood flow is a prominent risk factor for brain dysfunction, as occurs in some dementia states. In this study, we investigated the effects of permanent occlusion of common carotid arteries (2VO), a well established experimental model of brain ischemia, on memory function, as assessed by reference and working spatial memory protocols and the object recognition task, and on cell damage to the hippocampus, as measured through changes in immunoreactivity for GFAP and the neuronal marker NeuN. The working hypothesis is that metabolic impairment following hypoperfusion will affect neuron and glial function and result in functional damage. Adult male Wistar rats were submitted to the modified 2VO method, with right common carotid artery being first occluded, and tested seven days, three and six months after the ischemic event. A significant cognitive deficit was found in both reference and working spatial memory and object recognition tasks, both three and six months after surgery. Neuronal death and reactive astrogliosis were already present 7 days, and continues to 3 months, after the occlusion. Interestingly, there was no significant reduction in hippocampal volume. Present data suggests that cognitive impairment caused by brain hypoperfusion is long-lasting and depends on cell damage to the hippocampus.

Keywords: chronic cerebral hypoperfusion, 2VO- ischemia, memory impairment, hippocampus, glia, neuron

1. INTRODUCTION

Chronic cerebral hypoperfusion is thought to be an important cause of dementia in patients with cerebrovascular disease (De la Torre, 1994), since there is a correlation between the severity of memory dysfunction and the decline in cerebral blood flow (CBF) in Alzheimer's disease, vascular dementia and post-stroke hypoperfusion (Ohta et al., 1997; Komatani et al., 1988).

Animal models have been developed to study conditions and consequences of chronic reduction in CBF that may occur in humans (De la Torre et al., 1994; Ni et al., 1994; Ohta et al., 1997). The permanent bilateral occlusion of both common carotid arteries in rats (2-vessel occlusion, 2VO) has been widely used (Farkas et al., 2007); it causes an abrupt reduction of whole CBF to approximately 35–45%, in cortical areas, and 60% of control levels in the hippocampus (Ohta et al., 1997; Otori et al., 2003; Tsuchiya et al., 1992; Ulrich et al., 1998).

Progressive cognitive impairment has been reported after 2VO (Ni et al., 1994; Pappas et al., 1996; Farkas et al., 2007; Otori et al., 2003; Sarti et al., 2002) and these behavioral changes are associated to delayed onset of the CA1 cell loss, which is also often observed in human ageing and dementia states (Farkas et al., 2007), gliosis, astrogliosis, cholinergic dysfunction and neuronal apoptosis of pyramidal neurons (Bennett et al., 1998). Considering that high mortality rates, of as much as 50%, for rats undergoing conventional 2VO have already been reported (Farkas et al., 2004; Farkas et al., 2005; Institoris et al., 2007), we have recently tested an alternative protocol in which arteries occlusion was done with one week of interval. It was shown that the alternative method produced similar cognitive impairment with much better survival rates (Cechetti et al., 2010).

Astrocytes are in close morphological and functional relation with neurons and its participation in pathophysiology of several brain disorders is strongly suggested (e.g. Maragakis and Rothstein, 2004). Glial activation in response to injury stimuli commonly involves changes in glial fibrillary acidic protein (GFAP) and S100 calcium binding protein B (S100B) conten, as well as in glutamate levels and metabolism (Vicente et al., 2009). GFAP is a specific astrocyte marker; currently, tissue GFAP increase is taken as an index of astrogliosis associated with conditions of brain injury (Eng et al., 2000; Vicente et al., 2004).

An important experimental question is whether animal models of dementia cause long-lasting and/or progressive cognitive deficits (Bennett et al., 1998; Pappas et al., 1996, Ni et al., 1994;), as dementia states produce in humans. Given that, the aim of present study is to investigate whether rats suffering cerebral hypoperfusion induced by the alternative 2VO protocol will present cognitive impairment for as long as six months after surgery, and if hippocampus tissue damage, as assessed by immunoreactivity for GFAP and the neuronal marker NeuN, will appear. The working hypothesis is that chronic cerebral hypoperfusion causes cognitive impairment associated to hippocampus neuron damage and astroglial activation.

2. RESULTS

2.1. Behavioral Analysis

Ischemic animals surviving 3 and 6 months after 2VO showed impairment of reference memory performance (Figure 1), as compared to controls (One-way ANOVA for repeated measures, 3 months after surgery: $F(1,22)=29.97$, $p<.00001$; 6 months after surgery: $F(4, 80)=16.045$, $p<.00001$). P*ost-hoc* tests indicated that both

ischemic groups had significantly greater latencies to reach the platform position, made less crossings and spent less time in the target quadrant, as compared to controls in the probe trial ($p < .05$, Table 1). Ischemic rats tested seven days after surgery showed no cognitive impairment in this task.

As depicted in Figure 2, performance on the working memory task exhibited the same pattern of results; one-way ANOVA revealed significant differences in escape latency of ischemic animals 3 months after surgery on the 2nd ($F(1,22)=6.68$, $p<.005$) , 3rd ($F(1,22)=32.64$, $p<.001$) and 4th trials ($F(1,22)=58.87$, $p=.0001$), as compared to controls. The same was displayed 6 months after surgery on the 2nd ($F(1,20)=53.46$, $p<.00001$) , 3rd ($F(1,20)=41.84$, $p<.00001$) and 4th trials ($F(1,19)=233.16$, $p<.00001$). Again, rats tested 7 days after surgery had no impairment ($p >.05$ for all trials). Spatial navigation impairments in 2VO rats were not likely biased by any motor deficit, since means of swimming speed were 26cm/s for sham-control animals and 24.5cm/s for ischemic rats.

In the object recognition task (Figures 3), total exploration in training and test sessions were analyzed. Chronic hypoperfusion did not affect the time spent exploring both objects in training [$F(1,35)= .32$, $p> .05$]. In the short-term memory (90 min) both groups tested 3 months ($F(1,11)= 6.758$, $p< .05$) and 6 months ($F(1,11)= 2.47$, $p= .0006$) after 2VO surgery stayed longer with the familiar object, what suggests a memory deficit (Fig. 3A). A similar pattern was observed when the test session was performed 24 h (long term memory) after training where both 2VO groups spent less time with the familiar object (3 months after surgery: $F(1,12)= 2.48$, $p<.005$); 6 months after surgery: $F(1,12)= 2.276$, $p< .001$) (Fig. 3B).

In order to confirm the absence of motor deficits possibly caused by chronic hypoperfusion, animals were exposed to one 3 min-session in the open-field. This

test shows that ischemia did not cause any effect on the numbers of crossings and rearings (data not presented).

2.2 Morphological analysis

Confirming previous results (Cechetti et al., 2010; Pereira et al., 2007), bilateral common carotid artery occlusion caused no major loss of hippocampus tisse, as assessed estimated by total hippocampal and Ammon's horn volumes (Table 2).

2.3. Immunohistochemistry

A representative photomicrography of GFAP and NeuN immunofluorescence of CA1 subfield of hippocampus is shown in Figure 4. Rats sacrificed 7 days after 2VO had a significant increase of GFAP and decrease NeuN immunocontent when compared with your control ($p<.005$).

Three months after surgery, significant change in the NeuN content was observed in rats submitted to chronic cerebral hypoperfusion, showing a decrease in the intensity of the fluorescent signals in CA1 of 2VO rats when compared with control group ($p<.05$). On the other hand, no significant change was observed six months after surgery in the GFAP and NeuN immunofluorescence, as show in Figure 4 (B).

3. DISCUSSION

The permanent bilateral occlusion of rat common carotid arteries (2VO) is a well established model to investigate the effects of chronic cerebral hypoperfusion on cognitive dysfunction and neurodegenerative processes. Progressive cognitive impairment, neuronal loss, cholinergic dysfunction and astrogliosis in the hippocampus have been associated with the 2VO hypoperfusion (Schmidt-Kastner et al., 2005).

Results here presented show consistent memory impairment three and six months, but not 7 days, after 2VO, as assessed by water maze and object recognition tasks. Although morphometry did not show any major loss of hippocampus tissue in ischemic animals, imunohistochemistry revealed GFAP and NeuN changes in the pyramidal layer of hippocampus in the earlier periods, but not 6 months after the event.

Chronic hypoperfusion produced by 2VO affects cognitive function studied in a hippocampus-dependent spatial task, the water maze, both three and six months after surgery (Figures 1 and 2). Previous studies have reported that 2VO provokes short and long term memory impairments using passive avoidance, Y-maze, eight-arm radial maze tasks (Zhao et al., 2005) and Morris Water Maze task (Pappas et al., 1996; Kim et al., 2006; Ni et al., 1994). Interestingly, it was also shown that spatial memory gradually worsened as the survival times extended from 4 up to 20 weeks after vessels ligation (Liu et al., 2007). We are then allowed to suggest that functional neurodegeneration appear as the chronic phase of 2VO-induced cerebral hypoperfusion takes place. However, additional studies are required to establish the onset of impairments, i.e., between 7 days and three months, as well as whether the memory impairment develops exclusively due to the sudden drop in blood flow in the acute phase or worsens in the chronic phase of 2VO (Farkas et al., 2007). Is

important to emphasize that locomotor activity this animals are intact in this model, demonstrated by open field (Table 2) and corroborated by others authors (De Bortoli et al., 2005).

A point to consider is whether learning ability can return to normal levels on the cessation of cerebral hypoperfusion by the compensatory or adaptive mechanisms, such as artery dilation, recruitment of nonperfused capillaries and angiogenesis (Choy et al., 2006; Oldendorf, 1989), occurring in the chronic phase of ischemic event, i.e. up to 3 months post-surgery (Farkas et al., 2007). Our results support that the cognitive deficit remains after six months (24 weeks) of surgery, corroborating previous studies (De Jong et al.,(1999); Pappas et al., (1996). These observations suggest that the 2VO-induced neuronal damage, rather than cerebral hypoperfusion itself, is correlated with memory failure.

Recognition memory confers the ability to discriminate between novel and familiar entities, and lesion experiments with rodents and non-human primates indicate that functional integrity of the temporal lobe, specially the hippocampus, is essential for encoding, storage and expression of this type of memory (Logothetis and Sheinberg 1996; Riesenhuber and Poggio 2002). Although it has been described that object recognition is impaired in the acute model of permanent bilateral carotids occlusion (Sarti et al., 2002a), we here report a cognitive impairment that remains through the chronic phase, six months, of hypoperfusion (Fig 3). These results convincingly support the concept that the chronic phase of 2VO plays a major role in the gradual deterioration of the learning ability, although damage occurring in the acute phase of CBF reduction cannot be categorically excluded.

Histological outcome of 2VO studies are conflicting. Some authors found a direct correlation between cerebral hypoperfusion-induced memory deficit and

neuronal atrophy (De Jong et al., 1999; Nunn and Hodges, 1994; Pappas et al., 1996) or an association of impaired learning performance 3 weeks after the procedure with a significant pyramidal cell loss in the CA1 region (Xiong et al., 2006). However, other studies did not find progressive tissue damage in younger rats with chronic occlusions (De Butte et al., 2002; Plaschke et al., 2001). In addition, Murakami and colleagues (2005) reported that mice with chronic cerebral hypoperfusion exhibit learning impairments in a water maze task without marked histological alterations in the hippocampus.

Morphometric analysis revealed an overall normal architecture in the hippocampus of 2VO rats (Table 2). Schmidt-Karsten et al. (2005) observed that chronic of occlusion of both bilateral common carotid arteries in young adult male Wistar rats, induced a subacute phase of modest reactions in grey matter regions that was followed by recovery in the majority of animals and that white matter areas were more severely affected with more damage to the visual system. Therefore, a direct link between ischemia-induced memory failure and the appearance of neuronal damage in the hippocampus cannot be established (Institoris et al., 2007). A moderate reduction of CBF in the range of 40–60% of control levels is usually not associated with tissue damage macroscopic (Ginsberg, 2003; Marshall et al., 2001). Our results clearly demonstrated that the neuronal damage and reactive astrogliosis happens in the acute phase and continues to approximately 3 months after the occlusion (Fig 4), different cognitive behavior, which is installed in stages more chronic of the model.

GFAP is commonly used as a marker for changes in astroglial cells during brain development and injury (Eng et al., 2000). In fact, injury of the central nervous system, either as a consequence of trauma, disease, genetic disorders, or chemical

insult causes astrocytes to become reactive, a condition characterized by an increase in GFAP (O'Callaghan and Sriram, 2005). It is important to mention that astrocyte reaction is not necessarily accompanied by neuronal death and that astrocytes themselves react to reduction of cerebral blood flow through mechanisms mediated by changes in energy metabolism, oxygen radical formation and/or cytokine production (Vagnozzi et al., 1997; Zimmer et al., 1991). Chronic hypoperfusion can be associated with glial reactions in the absence of obvious neuronal loss in rats (Zimmer et al., 1991). Otherwise, neuronal nuclear antigen (NeuN) antibody recognizes a neuron-specific nuclear protein in the rat brain; some studies indicate that cerebral hypoperfusion disturbs the wiring of the neuronal circuits and the communication between the neurons, which contributes to the learning deficiency (Bennet et al., 1998; Liu et al., 2005).

Differences in the outcome after occlusion in different laboratories may be explained by rat strain, age of animals at the time of occlusion, intrinsic variability of the cerebral vasculature, differences in the age groups, anesthesia, environment temperature at the time of occlusion, housing and diet. Most studies have shown changes in the cerebral white matter which suffered from more severe hypoperfusion during 2VO than the neocortex and hippocampus (Farkas et al., 2004; Tomimoto et al., 2003; Wakita et al., 1994; Schmidt-Karsten et al., 2005).

Chronic hypoperfusion is tolerated by aged rats due to compensatory or adaptive mechanisms, through artery dilation, the recruitment of nonperfused capillaries, angiogenesis, biochemical regulation of the CBF and an enhanced immunocytochemical signal for vascular endothelial growth factor (Farkas et al., 2007). Plaschke and colleagues (2001) using a model of permanent brain vessel occlusion have shown that in such conditions hippocampal energy state recovers

significantly within 2 weeks of occlusion (ATP levels return to 70–80% of pre-ischemic conditions) and CBF tended to return to normal levels after several weeks (Otori et al., 2003). Another factor to be considered is that the tissue aged may seem less dense, although it may contain the same number of cells. Over time, these changes may cause a atrophy neuronal and not a neuronal loss disseminated (Wickelgren, 1996; West et al., 1994).

Concluding, we reported that chronic cerebral hypoperfusion triggered by 2VO causes long lasting cognitive deficits, as assessed by spatial and object recognition tasks for up to six months after the event. Although no major tissue loss was evidenced, immunohistochemistry revealed acute reactive astrogliosis and neuronal death in the hippocampus. More studies are needed to determine the cellular substrate of hypoperfusion cognitive impairment.

4. EXPERIMENTAL PROCEDURES

4.1. Animals

Male Wistar rats, aged 3 months, were obtained from the Central Animal House of the Institute of Basic Health Sciences, Universidade Federal do Rio Grande do Sul. They were maintained in a temperature-controlled room (21 ± 2 °C), on a 12/12 h light/dark cycle, with food and water available *ad libitum*. All procedures were in accordance with the Guide for the Care and Use of Laboratory Animals adopted by National Institute of Health (NIH-USA) and with the Federation of Brazilian Societies for Experimental Biology (FESBE), and were approved by the Committee of Ethics on Research of the University.

4.2. Modified two-vessel occlusion protocol, 2VO, and experimental design

Rats were anesthetized for surgery with halothane; a neck ventral midline incision was made and the common carotid arteries were then exposed and gently separated from the vagus nerve. Carotids were occluded with a one week interval; the right common carotid being the first to be assessed and the left one was occluded one week later (Sarti et al., 2002a; Cechetti et al., 2010). Sham-operated controls received the same surgical procedures without carotid artery ligation. After the procedure, rats were put on a heating pad to maintain body temperature at 37.5±0.5 °C and kept on it until recovery from anesthesia (Lavinsky et al., 2006; Cechetti et 2010). Animals were randomly assigned to sham or 2VO procedures so as to avoid any litter effect.

Experimental groups were as follows: (1) 2VO + 7 days of survival (n=10); (2) 2VO + 3 months of survival (six months of age, n=13); (3) 2VO + 6 months of survival (nine months of age, n=14); (4) sham + 7 days after procedure (n=10); (5) sham with six months of age (n=11); (3); (6) sham with nine months of age (n=8).

4.3. Morris water maze

Seven days, three or six months after the 2VO surgery rats were submitted to behavioral testing for spatial memory in the Morris water maze. The maze consisted of a black circular pool with 200 cm in diameter filled with water (temperature around 23 °C, depth 40 cm) situated in a room with visual cues on the walls. A black platform with 10 cm in diameter was submerged in the water (2 cm below the water surface). The pool was conceptually divided into four quadrants and had four points

designed as starting positions (N, S, W or E) (Morris, 1984). Two behavioral protocols, for reference and working memory, were run (Netto 93; Pereira et al., 2007).

4.3.1. Reference memory Task

In this task rats received five training sessions (one session/day) and a probe trial in the 6th day. Each session consisted of four trials with a 15 min intertrial interval. A trial began when the rat was placed in the water at one of the four starting positions, chosen at random, facing the wall. The order of starting position varied in every trial and any given sequence was not repeated on acquisition phase days. The rat was given 60 s to locate the platform; if the animal did not succeed it was gently guided to the platform and left on it for 10 s. Rats were dried and returned to their home cages after each trial. The latency to find the platform was measured in each trial and the mean latency for every training day was calculated. The probe consisted of a single trial, as described before, with the platform removed. Here, the latency to reach the original platform position, the number of crossings over that place and the time spent in the target, as well as in the opposite quadrants, were measured (Netto et al., 1993, Pereira et al., 2007). Sessions were recorded by a video acquisition system. Videotapes were blinded scored by a trained observer using a dedicated software (ANY-maze®). Videos were subsequently placed in randomized order in a separate ANY-maze protocol for a trained observer to score using a keyboard-based behavioral tracking system, blinded to the experimental condition.

4.3.2. Working memory Task

This protocol consisted of four trials/day, during four consecutive days, with the platform location daily changed. Each trial was conducted as described in the reference memory protocol, with a 5 min intertrial interval. Latency to find the platform was measured in each trial and the mean latency for every trial, along the four days, was calculated, allowing for the observation of the ability of animals in locating the novel platform position in the day (Netto et al., 1993, Pereira et al., 2007).

4.4. Object Recognition Task

The object recognition task was performed according to the protocol recently reviewed (Bevins and Besheer, 2006) and was applied seven days, three or six months after surgery. The apparatus consisted of a glass box (60×45×30 cm) where two similar objects (in shape, texture and size) were positioned equidistant from the sidewalls. Briefly, adult rat were submitted to a habituation period for ten minutes 24 hours before training session. After each trial, the apparatus was cleaned to alleviate olfactory cues. In the second trial, test sessions were run 90 minutes (short term memory) and 24 hours (long term memory) after training, where one of the objects was substituted by a new one. An experimenter registered the time of object exploration, i.e., touching it with paws or exploring it by olfaction with direct contact of the snout (Plamondon et al., 2006). Recognition object index was calculated by the following ratios: time spent exploring the novel object (TN) by the time spent exploring the familiar (TF) and the novel one in the test session (index= TN/ TN + TF). Rodents usually spent less time exploring the familiar object in the test session, which implies that they recognized the object previously presented. Different groups

of rats were used for each intertrial interval (90 min or 24 h; Botton et al., 2010; Costa et al., 2008).

4.5 Open Field

The task was run in a wooden box measuring 60x40x50 cm, with a frontal glass wall, and whose floor was divided by white lines into 12 equal squares. Animals were placed facing the rear left corner of the arena and observed for 3 min. The number of squares crossed with the four paws from one square to another and the number of rearings were indicative of motor activity (Netto et al., 1986; Lavinsky et al., 2003). After each trial, the apparatus was cleaned with an ethanol solution (20%). Rats were submitted seven days, three and six months after the surgery to test. All animals were tested only once.

4.6 Morphological Analysis

Morphology was run, for all experimental groups, after the last behavioral test. Rats were deeply anesthetized with chloral hydrate (30%, 10 ml/kg, i.p.) and submitted to transcardiac perfusion with 0.9% saline followed by 4% formaldehyde. Brains were removed and maintained in formaldehyde solution. For the morphological analysis, brains were cryoprotected with a 30% sucrose solution for two days and then sectioned; coronal 50 µm thickness sections were obtained using a cryostat (Leica).

The volume of hippocampus was estimated by the use of the Cavalieri method (Miki et al., 2005; Rodrigues et al., 2004). Briefly, 50 µm sections covering the whole hippocampus were mounted on gelatinized glass slides and stained with

hematoxylin and eosin; the Image J program (NIH, USA) was utilized to delineate and estimate the hippocampal and dentate gyrus area (Pereira et al., 2007). The volume of hippocampus was calculated by the sum of areas multiplied by the section interval. The Ammon's horn volume was calculated as the diference between the entire hippocampus and dentate gyrus volumes (Rodrigues et al., 2004). Analysis was performed seven days, three and six months after surgery.

4.7 Immunohistochemistry

For the immunohistochemical investigation animals (7 days, 3 and 6 months after surgery) were deeply anesthetized with chloride hydrate (30%, 10 mL/kg, i.p.) and injected with 1000 UI heparin (Cristália, Brazil). They were transcardially perfused through the left ventricle, using a peristaltic pump (Control Company, São Paulo, Brazil) with 100 mL of saline solution followed by 200 mL of fixative solution composed of 4% paraformaldehyde (PFA) (Reagen, Rio de Janeiro, Brazil) in 0.1 M phosphate buffer (PB) pH 7.4 at room temperature. The brains were postfixed in PFA at room temperature for 4 h, kept in 30% sucrose in PBS for 3 days

and then frozen in isopentane and liquid nitrogen. Coronal sections (40 μm) were obtained using a cryostat (Leica, Germany).

Coronal sections from 5 to 6 rats per group were stained for the astrocytic marker rabbit anti-glial fibrillary acidic protein (GFAP, 1:250, Sigma-Aldrich) and for the neuronal marker mouse anti-neuronal-specific nuclear protein (NeuN, 1:250, Chemicon). Secondary antibodies were goat anti-rabbit IgG Alexa 488 (1:500, Molecular Probes) and goat anti-mouse IgG Alexa 555 (1:500, Molecular Probes). Briefly, sections were fixed in 4% PFA, washed in PBS and blocked for 30 min with

3% normal goat serum (Sigma-Aldrich) in PBS with 0.3% Triton-X (PBS-Tx) at room temperature. Then, sections were incubated overnight with primary antibody at 4 °C with PBS-Tx with 3% NGS. In the next day sections were washed in PBS and incubated with secondary fluorescence antibody for 2 h at room temperature in a dark chamber, washed in PBS, mounted and cover slipped with antifading mounting medium PVA-DABCO (Fluka Analytical). A laser scanning confocal microscope (Olympus FV1000) was used to visualize the fluorescent dyes (excitation wavelengths of 488 and 555 nm) in two randomized areas (40X magnification) within the CA1 subfield of hippocampus.

Primary antibodies were omitted in negative controls for immunofluorescence stains. The samples were processed at the same time and incubated within the same medium during the same period. All conditions and magnifications were kept constant during the capture process. ImageJ 1.14u software was used to measure the intensities of the fluorescent signals in areas labeled for NeuN and GFAP after background correction. Each analysis was performed using 4 fields obtained from both hemispheres of 4 coronal sections (2835.4 μm2 – total area analyzed per section). The observer was blind to animals' experimental condition during image analysis.

4.8. Statistical analysis

Behavioral performance in reference memory was analyzed using a one-way repeated-measures analysis of variance (ANOVA) with lesion as independent variable and session as the repeated measure. Variables of the working memory in the Morris Water Maze, as well as volumes of hippocampus and striatum areas were

analyzed by one-way ANOVA, followed by *post-hoc* Duncan's test for multiple comparisons, when necessary. Statistical analysis for object recognition task, morphological analysis and imunohistochemistry measurements were performed by Student t-test (unpaired) and One-Way ANOVA followed by Newman-Keuls Multiple Comparisons test.

Data are expressed as means±SEM. Probability values less than 5% ($p<.05$) were considered significant. Statistical analysis were performed using the *Statistica*® software package running on a compatible personal computer.

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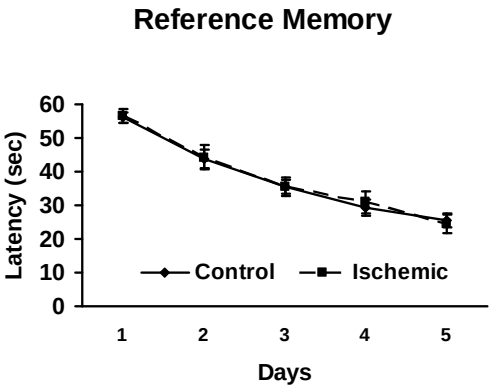
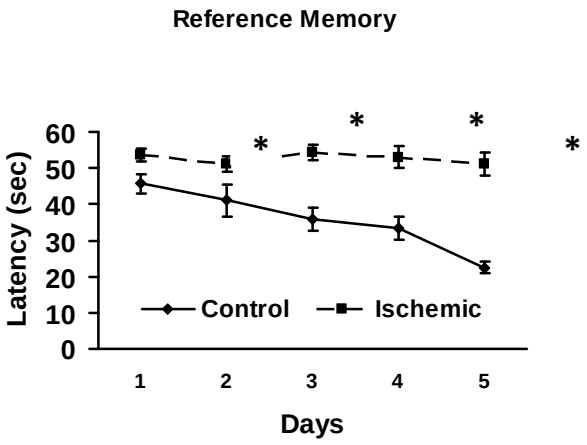
Legends to Figures

Figura 1 – Reference memory task performance in the water maze seven days (A), three months (B) and six months (C) after 2VO surgery. Each line represents mean± standard error (SEM). * Different from sham group (Repeated measures ANOVA, $p<.05$).

A



B



C

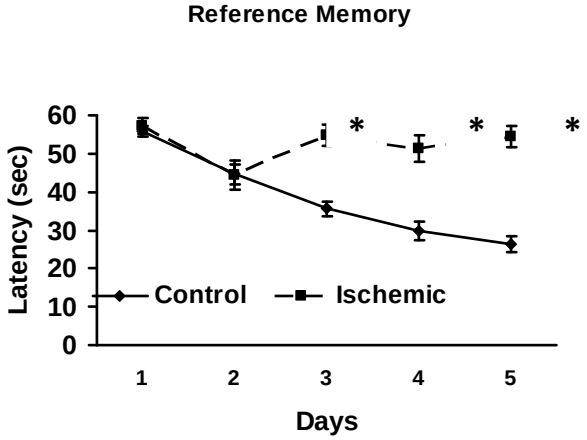
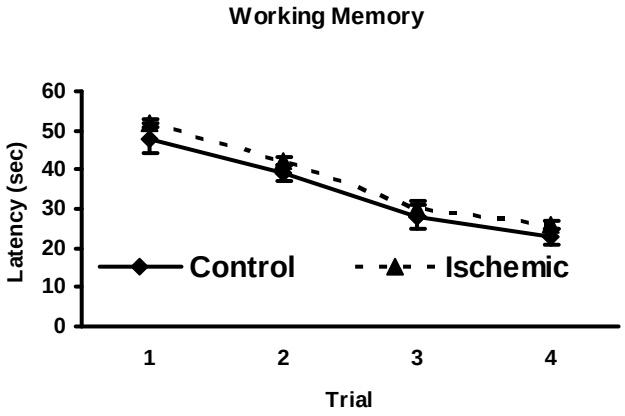

Reference Memory
Latency (sec)
60
50
40
30
20
10
0
Control
Ischemic
*
*
*
1
2
3
4
5
Days

Table 1 – Probe trial of the reference memory task in the Water Maze. Number of platform crossings, latency to find the platform position and the spent time in target quadrant. Data are expressed as means ± S.E.M. *Different from sham and ischemic group (ANOVA followed by Duncan's test, $p < .05$).

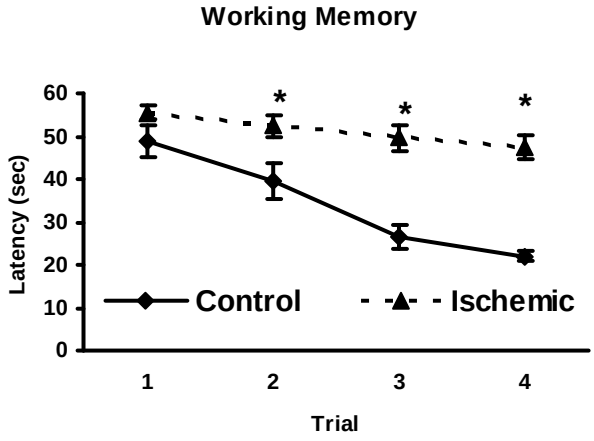
	Crossings	**Latency (sec)**	**Target Quadrant (sec)**
Sham + 3 months	1.4±.4 *	34.6±5 *	19.5±3 *
2VO + 3 months	0.3±0.07	50±4	9.3±1.6
Sham + 6 months	1.6±0.3 *	5.3±3.4 *	16.7±1.7 *
2 VO + 6 months	0.14±0.09	19±4.8	9±1.2

Figura 2 - Working memory task performance in the water maze seven days (A), three months (B) and six months (C) after 2VO surgery. Each line represents mean± standard error of the mean (SEM) * Different from sham group (ANOVA followed by Duncan's test, p<.05).

A



B



C

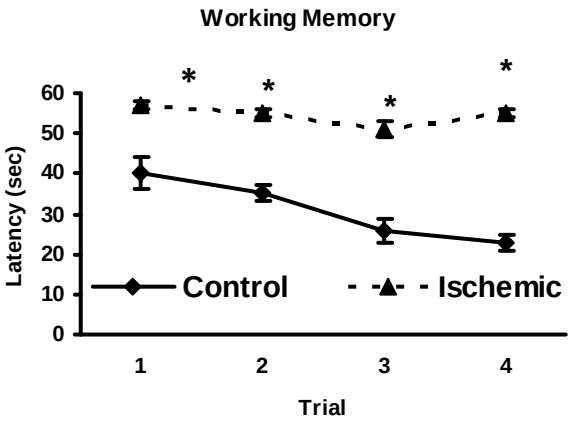
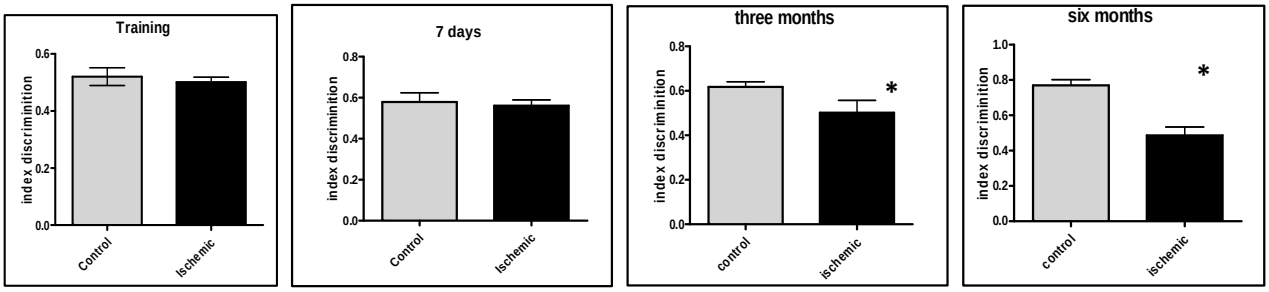

Working Memory
Latency (sec)
60
50
40
30
20
10
0
1
2
3
4
Trial
Control
Ischemic
*
*
*
*

Figura 3- The novel object recognition index obtained seven days, three and six months after after 2VO surgery, (A) short-term memory (90 minutes) and (B) long-term memory (24hs). Results are means ± S.E.M of recognition index calculated by the following ratio: index= (TN/ TN + TF), where TN = time spent exploring novel object at each intertrial interval; TF = time spent exploring the familiar object at each intertrial interval. * Different from sham group (ANOVA followed by Newman Keulls test, p <.05).

A



B

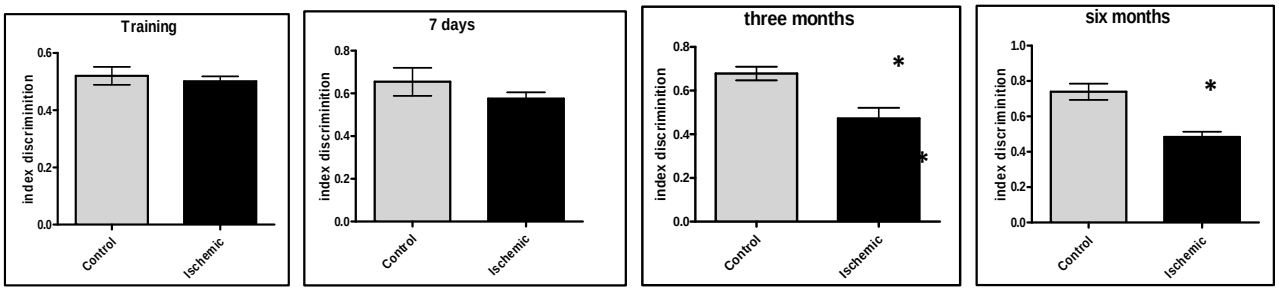


Table 2 – Measurement of hippocampus and Amonn´s Horn volume of rats after 2VO surgery (A). Each value represents mean ± S.E.M. No significant differences between groups were detected (ANOVA). (B) Representative photomicrographs of sham (a) and 2VO (b) rat brain. Scale bar: 500 µm.

A

	Hippocampal volume	**Amonn´s Horn volume**
Sham + 7 days	41±1.1	27±0.9
2 VO + 7 days	39±1	26±0.7
Sham + 3 months	41±0.8	27±0.6
2 VO + 3 months	40±1	26±0.6
Sham + 6 months	41±1.2	27±0.8
2 VO + 6 months	40±1	26±0.8

B

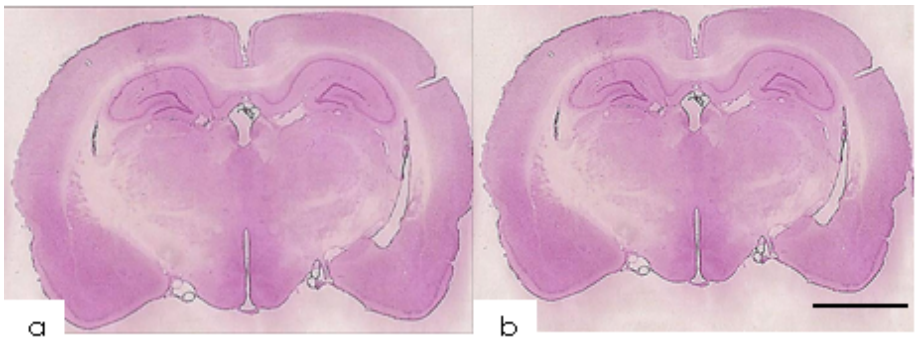
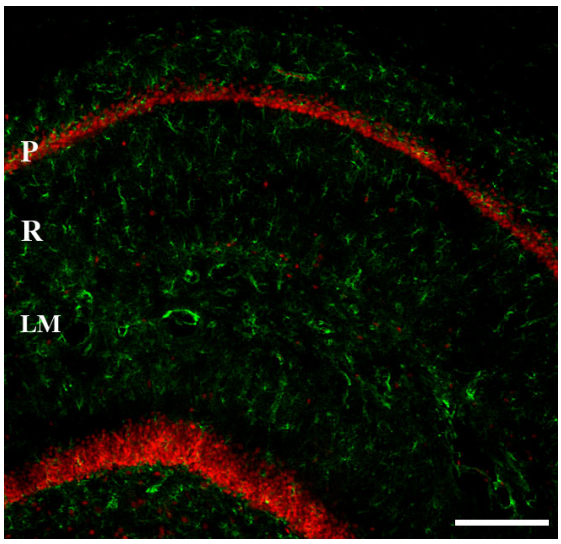


Figure 4 - Intensity of the fluorescent signals in areas labeled for GFAP and NeuN of the CA1 hippocampus from rats submitted to chronic cerebral hypoperfusion 7 days, three and six months after surgery (A). Data expressed as percentage of the control group (mean ± SEM). * Difference between both ischemic and respective control groups (Student's t test, $p<.05$). (B) Representative digitized image of GFAP and NeuN immunofluorescence in the CA1 region of the hippocampus. P: stratum pyramidale; R: stratum radiatum; LM: stratum lacunosum moleculare; Scale bar: 50 µm.

A

Groups	**GFAP**	**NeuN**
2 VO + 7 days	150±16 *	81±9.5 *
2 VO + 3 months	93±16	74±15 *
2 VO + 6 months	97±21	101±21

B



5. CAPÍTULO 3

Artigo: Forced treadmill exercise prevents oxidative stress and memory deficits following chronic cerebral hypoperfusion in the rat - *a ser submetido à Behavioural Brain Research.*

Forced treadmill exercise prevents oxidative stress and memory deficits following chronic cerebral hypoperfusion in the rat

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Abstract

Physical activity impacts the brain oxidative status and behavioral recovery following stroke, however its effects in animals submitted to chronic cerebral hypoperfusion have not been investigated. The aim of this study was to evaluate the therapeutic potential of exercise, as assessed by cognitive activity in the Morris water maze and brain oxidative status, through measurement of macromolecules damage, TBARS levels and total cellular thiols, as well as antioxidant enzymes in hippocampus, striatum and cerebral cortex. Adult male Wistar rats were submitted to the modified permanent bilateral occlusion of the common carotid arteries (2VO) method, with right common carotid artery being first occluded, and tested three months after the ischemic event. The effects of three different exercise protocols were examined: pre-ischemia, post-ischemia and pre+post-ischemia. Physical exercise consisted in sessions of 20-min, 3 times per week during 12 weeks (moderate intensity). Rats were sacrificed after the end of exercise period and cognitive function, in both reference and working spatial memory, and oxidative stress parameters were determined. A significant cognitive deficit was found in both spatial water maze tasks after hypoperfusion; this effect was reversed in 2-VO exercised group. Moreover, hippocampal oxidative damage and antioxidant enzyme activity was regulated by forced regular treadmill. These results suggest that the physical exercise protocol protects against the cognitive and biochemical damage caused by chronic cerebral hypoperfusion.

Keywords: chronic cerebral hypoperfusion, oxidative state, hippocampus, 2VO, water maze.

1. Introduction

Chronic cerebral hypoperfusion has been well characterized as a pathological state contributing to neurodegenerative diseases (Hartman et al., 2005; Masada et al., 1997; Pazos et al., 1999) and a correlation has been established between the severity of memory dysfunction and the decline of cerebral blood flow in Alzheimer's disease, vascular dementia and post-stroke hypoperfusion (Ohta et al., 1997; Komatani et al., 1988). The permanent bilateral occlusion of both common carotid arteries in the rat (2-vessel occlusion, 2VO) causes an abrupt reduction of whole cerebral blood flow (CBF; Farkas et al., 2007), that decreases to approximately 35–45% and 60% of control levels in cortical areas and hippocampus, respectively (Ohta et al., 1997; Otori et al., 2003; Tsuchiya et al., 1992; Ulrich et al., 1998); this is widely accepted as an appropriate experimental model to cerebral hypoperfusion.

The main clinical outcomes of chronic cerebral hypoperfusion are the permanent neural impairments and cognitive decline (Ni et al., 1994; Pappas et al., 1996; Farkas et al., 2007; Otori et al., 2003; Sarti et al., 2002). Accordingly, rodent two-vessel occlusion provokes short and long term memory impairments using passive avoidance, Y-maze, eight-arm radial maze tasks (Zhao et al., 2005) and Morris Water Maze task (Ni et al., 1994; Pappas et al., 1996).

Previous studies revealed that oxidative injury plays a key role on the pathogenesis of neurodegenerative diseases like stroke, Alzheimer's disease and vascular dementia (Chong et al., 2005; Coyle and Puttfarcken, 1993). It is suggested that oxygen free radicals and lipid peroxidation are important for the development of lesions caused by chronic cerebral hypoperfusion in the central nervous system (Markesbery, 1997). Free radicals produce oxidative damage directly to critical biological molecules and, in order to handle that, organisms utilize antioxidant defenses, including superoxide dismutase (SOD), catalase and glutathione peroxidase activities, as well as non-enzymatic antioxidants (as, for example, glutathione, ascorbate and tocopherol) (Halliwell, 1991). These endogenous molecules assist aerobic cells to maintain a reducing state, despite the oxidizing environment (Chung, 2005; Pogocki and Schöneich, 2001). On the other hand, thiols are extraordinarily efficient antioxidants in protecting cells against consequences of free radical damage due to their ability to react with the latter (Atmaca, 2004; Sen, 1998). Both intracellular and extracellular redox states of thiols play a critical role in the

determination of protein structure and function, and regulation of transcription factors activities (Wlodek, 2002).

Experimental therapeutic strategies to alleviate cognitive damage have been tested, including physical activity, and there is evidence that exercise may support brain health and function (Radák et al., 2001), with beneficial effects on learning, long-term potentiation and memory (Van Praag et al., 1999;; Ogonovszky et al., 2005). Consistent to that regular physical activity has been indicated as a therapeutic approach to prevent age-related neurodegenerative diseases (Mattson, 2000).

Although the exact molecular mechanisms through which physical exercise affects brain function are unclear, it has been suggested to activate cellular and molecular pathways that contribute to neuroprotection. Some reports demonstrate a significant increase in antioxidant enzymes activities, what increases resistance against oxidative stress and therefore reduces cell damage (Powers et al., 1994; Leeuweenburgh et al., 1997; Servais et al., 2003), as well as demonstrate that regular exercise attenuates the protein oxidative damage in aged rats (Radák et al., 2001).

Surprisingly, there are just few studies on the effects of exercise on the oxidative status of vulnerable brain regions (Candelario-Jalil et al., 2001) and after excitotoxic events, like brain ischemia. We have recently demonstrated that daily moderate intensity exercise (2 weeks of 20min/day of training; Scopel et al., 2006) and a 12-week of three-times a week treadmill training (Cechetti et al., 2007) reduces *in vitro* ischemia damage to the hippocampus of Wistar rats.

In present study, chronic cerebral hypoperfusion induced by permanent occlusion of bilateral common carotid arteries in the rat was used to evaluate the therapeutic potential of physical activity, as assessed by cognitive activity and brain oxidative status on the hippocampus, striatum and cerebral cortex. Spatial memory deficits in both reference and working memory tasks in the Morris water maze, and parameters of cellular oxidative status, namely free radicals content, index of macromolecules damage, were studied in adult rats receiving 2VO followed by three exercise protocols.

2. Materials and Methods

2.1. Animals

Male Wistar rats were obtained from the Central Animal House of the Institute of Basic Health Sciences, Universidade Federal do Rio Grande do Sul. They were maintained in a temperature-controlled room (21 ± 2 °C), on a 12/12 h light/dark cycle, with food and water available *ad libitum.*

2.2. Surgical procedure-Two-vessel occlusion

Three months-old rats were anesthetized for surgery with halothane; a neck ventral midline incision was made and the common carotid arteries were then exposed and gently separated from the vagus nerve. Both carotids were occluded with 5-0 silk suture, with a one week interval in between; the right common carotid artery being the first to be occluded (Cechetti et al., 2010). Sham-operated controls received the same surgical procedure with no artery ligation. Animals were randomly assigned to sham or 2VO groups, to avoid any litter effect, as following: (1) pre-surgery exercise group (from twenty days to three months of life) (n=9), (2) post-surgery exercise group (from three months until six months of life) (n=9) and (3) pre+post-surgery exercise group (from twenty days until six months of life) (n=10).

All procedures were in accordance with the Guide for the Care and Use of Laboratory Animals adopted by National Institute of Health (USA) and with the Federation of Brazilian Societies for Experimental Biology (FESBE) and were approved by the Committee of Ethics on Research at the Universidade Federal do Rio Grande do Sul.

2.3. Exercise Training

Rats were habituated with the treadmill apparatus to minimize novelty stress and randomly assigned to different experimental groups. The exercise protocol chosen was of 20-min sessions three times per week (Scopel et al., 2006; Cechetti et al., 2007; Ben et al., 2010). Animals in non-exercised (sedentary) groups were left on the treadmill for 5 min without any stimulus to run.

We used a moderate intensity exercise protocol (Ben et al., 2010; Ben et al., 2009; Cechetti et al., 2007; Scopel et al., 2006), i.e., the exercise intensity was set at 60% of animal's maximal oxygen uptake (Brooks & White, 1978). Indirect assessment of oxygen uptake (VO2) peak was carried out for all rats before training, considering their exhaustion. Each animal ran on the treadmill at a low initial speed, followed by increases of 5 m/min every 3 min until the point of exhaustion (i.e., failure of rats to continue running); the time to fatigue (in min) and workload (in m/min) were taken as indexes of exercise capacity that were taken as VO2 max (Brooks and White, 1978; Arida et al., 1999, Scopel et al., 2006; Cechetti et al., 2007).

Animals were adapted to the treadmill by gradually increasing running speed and time, between 17:00 and 19:00 p.m., for 12 weeks, as follows: weeks 1 and 2, at 12 m/min for the first 3min, 24m/min for the next 4min, 36m/ min for the following 6 min, 24 m/min for the following 4 min and 12m/min for the last 3min;weeks 3 to 6, at 24m/min for the first 4min, 36 m/min for the next 12min, and 24m/min for the last 4 min; weeks 7 to 10, at 24 m/min for the first 2 min, 36 m/min for the next 16min, and 24 m/min for the last 2min. By the end, rats were running at 48 m/min, with the first and the last 2-min run at 36 m/min (Scopel et al., 2006; Cechetti et al., 2007).

2.4. Morris water maze

Three months after surgery rats were submitted to behavioral testing for spatial memory in the Morris water maze. It consisted of a black circular pool with 200 cm in diameter filled with water (temperature around 23 °C, depth 40 cm) situated in a room with visual cues on the walls. A black platform with 10 cm in diameter was submerged in the water (2 cm below the water surface). The pool was conceptually divided into four quadrants and had four points designed as starting positions (N, S, W or E) (Morris et al., 1984; Pereira et al., 2007). Two behavioral protocols, for reference and working memory, were utilized.

2.4.1. Reference memory protocol

In this task rats received five training days (sessions) and a probe trial in the 6th day. Each session consisted of four trials with a 15 min intertrial interval. A trial began when the rat was placed in the water at one of the four starting positions, chosen at random, facing the wall. The order of starting position varied in every trial and any given sequence was not repeated on acquisition phase days. The rat was given 60 s to locate the platform; if the animal did not succeed it was gently guided to the platform and left on it for 10 s. Rats were dried and returned to their home cages after each trial. The latency to find the platform was measured in each trial and the mean latency for every training day was calculated. The probe consisted of a single trial, as described before, with the platform removed. Here, the latency to reach the original platform position, the number of crossings over that place and the time spent in the target, as well as in the opposite quadrants, were measured (Netto et al., 1993; Pereira et al., 2007; Cechetti et al., 2010). Videos were subsequently placed in randomized order in a separate ANY-maze protocol to be scored by a trained observer blind to the experimental condition using a keyboard-based behavioral tracking system.

2.4.2. Working memory protocol

This protocol consisted of four trials/day, during four consecutive days, with the platform location daily changed. Each trial was conducted as described in the reference memory protocol, with a 5 min intertrial interval. Latency to find the platform was measured in each trial and the mean latency for every trial, along the four days, was calculated, allowing for the observation of the ability of animals in locating the novel platform position in the day (Netto et al., 1993; Pereira et al., 2007; Cechetti et al., 2010).

2.5. Oxidative State

2.5.1. Free radicals levels

To assess the free radicals content we used 2'-7'-dichlorofluorescein diacetate (DCFH-DA) as a probe (Lebel et al., 1990). An aliquot of the sample was incubated with DCFH-DA (100 μM) at 37oC for 30 min. The reaction was terminated by

chilling the reaction mixture in ice. The formation of the oxidized fluorescent derivative (DCF) was monitored at excitation and emission wavelengths of 488 nm 525 nm, respectively, using a fluorescence spectrophotometer (Hitachi F-2000). The free radicals content was quantified using a DCF standard curve and results were expressed as pmol of DCF formed/mg protein. All procedures were performed in the dark and blanks containing DCFH-DA (no homogenate) were processed for measurement of autofluorescence (Driver et al., 2000; Sriram et al., 1997).

2.5.2. Thiobarbituric acid reactive substances (TBARS)

Lipoperoxidation (LPO) was evaluated by the thiobarbituric acid reactive substances (TBARS) test (Bromont et al., 1989). Aliquots of samples were incubated with 10% trichloroacetic acid (TCA) and 0.67% thiobarbituric acid (TBA). The mixture was heated (30 min) on a boiling water bath. Afterwards, n-butanol was added and the mixture was centrifuged (1000×g for 10 min). The organic phase was collected to measure fluorescence at excitation and emission wavelengths of 515 and 553 nm, respectively. 1,1,3,3-tetramethoxypropane, which is converted to malondialdehyde (MDA), was used as standard. Results are expressed as pmol MDA/mg protein and reported as percentage of control.

2.5.3. Total thiol content

Cellular thiols, as glutathione and protein thiols, were measured. Aliquots of samples were incubated with 100 mM DTNB (final concentration) for 15 min in darkness. Absorbance of the reaction mixture was measured at 412 nm (Khajuria et al., 1999); results are expressed as nmoles SH per mg protein.

2.5.4. Superoxide dismutase (SOD) activity

SOD activity was determined using a RANSOD kit (Randox Labs., USA). This method employs xanthine and xanthine oxidase to generate O2-.that react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to form a red formazan dye which is assayed spectrophotometrically at 505 nm at 37oC. The inhibition on

production of the chromogen is proportional to the activity of SOD present in the sample.

2.5.5. Protein determination

Protein was measured by the Coomassie blue method using bovine serum albumin as standard (Bradford, 1976).

2.6. Statistical analysis

Training days behavioral performance was analyzed using two-way repeated-measures analysis of variance (ANOVA), with lesion and exercise as independent variables and session as the repeated measure. Reference memory probe trial, working memory in the MWM variables and oxidative status were analyzed by two-way ANOVA. All analyses were followed by *post-hoc* Duncan's test for multiple comparisons, whenever indicated. Data are expressed as means±SEM. Probability values less than 5% were considered significant. All statistical analysis was performed using the Statistica® software package running on a compatible personal computer.

3. Results

3.1. Behavioral Effects

Two-way repeated measures ANOVA of reference memory acquisition (Fig.1A) revealed only significant main effects of *lesion* ($F(5,25)=11.80$, $p<.01$), with no significant *treatment* interaction ($F(5,25)=1.5$, $p>.05$) on the latencies to find the platform, in pre-surgery exercise protocol. In post-surgery (Fig.1B) and pre+post-surgery (Fig.1C) protocols, ANOVA showed significant differences in factors *lesion* and *treatment*, where ischemic sedentary groups presented greater latencies than all other animals on sessions 2 to 5 ($p<.05$).

Probe trial analysis showed effects for *lesion* ($F(1, 31) = 6.29$, $p<.01$) and *exercise* ($F(1, 31)=4.17$, $p<.05$) on platform crossings in the post-surgery protocol. *Post-hoc* test indicated that ischemic/sedentary rats had significantly greater latencies to reach the platform position and made fewer crossings than all others animals ($p < .05$, Table 1A). Pre+post-surgery protocol produced differences among ischemic/sedentary and other groups in time spent in the opposite and target quadrants, as well as crossings ($p < .05$, Table 1B). Two-way ANOVA did not reveal any significant difference in pre-surgery group (data not presented).

Analysis of working memory performance demonstrated significant differences in escape latency of ischemic sedentary in post-surgery protocol (Fig.2B) on the 2nd, 3rd and 4th trials ($p<.05$), as compared to other three groups. The same pattern of results appeared after the pre+post-surgery protocol (Fig.2C) ($p<.05$); however there was no differences after pre-surgery exercise protocol ($p >.05$ in all trials) (Fig.2A).

Permanent bilateral carotids occlusion did not cause any swimming deficit; means of swimming speed were 26cm/s for sham-control animals and 24.5cm/s for ischemic rats.

3.2. Oxidative State

3.2.1 Free Radical levels

ANOVA revealed no changes on DCF measurements in the hippocampus, striatum and cerebral cortex after 2VO rats, irrespective of the physical exercise regimen (data not presented).

3.2.2 Thiobarbituric acid reactive substances (TBARS) and total cellular thiols

There were changes of TBARS levels ($F (3, 53)= 4.1228$, $p=.01$) in the hippocampus, as well as on total thiol levels ($F(3, 52)=4.6249$, $p=.01$). Differences were associated to *exercise* factor in the hippocampus in both techniques utilized: TBARS ($F3, 53) = 8.48$, $p=.01$) and thiol levels ($F(3,52)=6.46$, $p=.01$), where ischemic sedentary group was significantly different from all other groups analyzed (Fig.3 A/B).

ANOVA did not reveal any significant difference between groups in striatum and cerebral cortex (data not presented).

3.2.3. Superoxide dismutase activity

No changes appear on SOD activity in the hippocampus of 2VO rats ($F(3,53)= .16$ $p>.05$) nor for the physical activity factor ($F(3,53)= .44$ $p>.05$). The same happened for striatum and cerebral cortex (data not presented).

4. Discussion

Permanent bilateral occlusion of the common carotid arteries (2VO) in rats is an established procedure to investigate the effects of chronic cerebral hypoperfusion on cognitive dysfunction and neurodegenerative processes. (Sarti et al., 2002). We here reported that the forced running paradigm protects from spatial learning and memory deficits in 2VO rats when applied either pre-, post-, or pre+post ischemia. To our knowledge, this is the first study that demonstrates the effects of physical activity administered distinct event-related protocols in this model.

Hippocampus plays an important role on learning and memory processing and is highly sensitive to ischemic insults (Ni et al., 1994; Pappas et al., 1996). Ischemia-induced neuronal degeneration is also observed in other structures, such as striatum, cerebral cortex and the thalamus (Freund et al., 1990; Pereira et al., 2009), but for several reasons the favored brain region for the study of 2VO-induced neurodegeneration is the hippocampus. First of all, it is the area that displays the most characteristic neuropathological damage in Alzheimer's disease. Second, the hippocampus is highly implicated in spatial learning and memory as assessed by the Morris water maze and the 8-arm radial maze. Third, the hippocampus (and particularly its CA1 subfield) is one of the brain regions most sensitive to ischemia. Finally, the distinct laminar structure of the hippocampus and its precisely mapped synaptic connections allow exact cell-type (e.g. pyramidal cells) or layer specific measurements (Farkas et al., 2007)

It has been reported that learning and memory can be influenced by exercise. Animal studies on rats and mice reported better cognitive performance as a result of

increased physical activities (Samorajski et al., 1985; Fordyce and Farrar, 1991a,b; Anderson et al., 2000; Ji et al., 2010). Present results clearly demonstrate that forced running could significantly improve spatial learning and memory in rats after 2VO ischemia, especially when performed after ischemic event.

The majority of studies report positive effects when the exercise is administered after the lesion event (Somani et al., 2005; Jolitha et al., 2006). As for possible mechanisms, it appears that exercise could enhance neurogenesis (Van Praag et al., 1999), up-regulate the expression of trophic factors (Neeper et al., 1996; Ang et al., 2003) and promote long-term potentiation (LTP) (Van Praag et al., 1999). This is significant as it is widely accepted that LTP may serve as a model for some of the molecular and synaptic events underlying memory (Abraham, 2003), including the formation of dendritic spine (Toni et al., 1999). It is also interesting to note that such increased LTP might be the result of increased endogenous neurotrophic factors due to exercise.

The brain tissue is sensitive to oxidative damage and it has been known that 2VO model induces oxidative events, but few studies are performed to reduce oxidative stress in cerebral hypoperfusion (Yanpallevar et al., 2005; Ghoneim et al., 2002).

Oxidative stress, defined as the imbalance between oxidants and antioxidants in favor of oxidants that potentially lead to tissue damage (Polidori et al, 2000). Oxidative metabolism of the brain tissue is high and large lipid contents of myelin sheaths are also a target of reactive oxygen species (ROS) (Choi 1993). Thiols levels, GSH and superoxide dysmutase (SOD) are involved in the antioxidant system and are important for the protection of the brain tissue from oxidative damage.

There are not many papers on the effects of exercise upon oxidative status in the hippocampus, striatum and cerebral cortex, and findings reported on oxidative stress parameters are conflicting; it may be possible that the variability on studies outcomes are caused by biases due to the distinct experimental models kinds and intensities of physical activities protocols (Cechetti et al., 2008; Ramsden et al., 2003; Risedal et al., 1999). The protocol here used (three 20-min sessions per week, during 12 weeks) did not alter the free radical content in hippocampus, however it affected basal TBARS and thiols level.

We observed that lipid peroxidation was significantly increased after chronic cerebral hypoperfusion (Fig. 3A) corroborating previous studies (Aytac et al., 2006; Yanpallewar et al., 2004; Ningaraj and Rao, 1998). Several studies show a direct relationship between impaired performance in cognitive Tasks and disfunction of neurochemical systems in many brain regions (Brandeis et al., 1993; McNamara and Skelton, 1993; Raghavendra et al., 2007).

Excessive generation of (ROS) results in lipid peroxidation of the cell membrane and subsequent damage is reflected by accumulation of MDA, a by-product of lipid peroxidation (Halliwell, 1991). The neurobehavioral consequences of chronically reduced cerebral blood flow could provide insight into the role of reduced cerebral energy metabolism in Alzheimer's dementia, which is characterized by decreased brain glucose metabolism (Simpson et al., 1994, Taylor et al., 1996; Frantseva et al., 2001).

In the present study, occlusion of both carotids caused an increase of thiols activity in hippocampus (Fig.3B). We suggest this effect indicates that brain's antioxidant machinery is activated in response to excessive generation of free radicals, corroborating with numerous studies that address this issue both *in vivo* and *in vitro* (Bannister et al., 1987; Omar and McCord, 1990; Yanpallewar et al., 2004).

Present data also demonstrate that forced regular physical activity was able to prevent the oxidative state and increased antioxidant capacity in the hippocampus after 2VO. The majority of studies on this model of hypoperfusion and oxidative stress demonstrate the effects neuroprotectors of drugs and plant extracts (Yampallewar et al., 2004; 2005); ours is the first to report exercise-induced neuroprotection.

The beneficial effects of endurance training on the redox status of the nervous system of rodents have been reported after swimming (Jolitha et al., 2006), treadmill training (Cosk et al., 2005; Somani et al., 2005), and running wheel training (Suzuki et al., 1993). On the other hand, there are also studies where swimming (Radák et al., 2006) and treadmill (Ozkaya et al., 2002) training did not affect brain antioxidant defenses. It has been proposed that exercise, through its continuous free radical generating effect, thus induce the adaptation of the cellular antioxidant system (Powers et al., 1994; Leeuweenburgh et al., 1997; Servais et al., 2003).

Summarizing, present results demonstrate that chronic cerebral hypoperfusion causes cognitive damage associated to increased lipoperoxidation in the hippocampus, and that moderate treadmill running exercise performed post- and pre+post-ischemia prevents both effects. This implies a neuroprotective action of running exercise over cognitive and biochemical damage caused by cerebral hypoperfusion injury; further studies are needed to investigate the mechanism involved.

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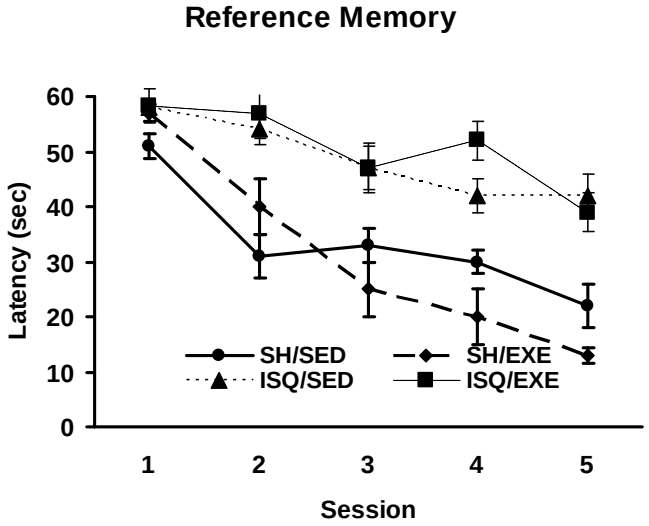
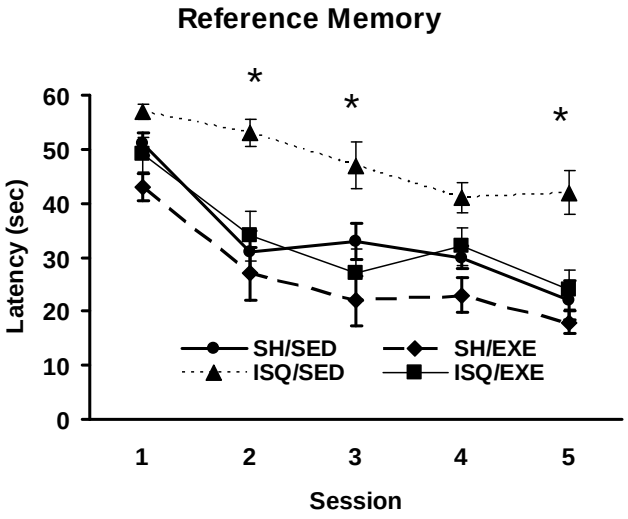
Figura 1 – Performance in the reference memory task in the water maze after pre-surgery exercise (A), post-surgery exercise (B) and pre+post-surgery exercise protocols (C). SH/SED – sham sedentary group, SH/EXE – Sham group submitted to physical activity during three months, three times per week, ISQ/SED - ischemic sedentary group, ISQ/EXE – ischemic group submitted to physical activity during three months, three times per week. Each line represents mean± standard error of mean (SEM). * Significant difference between sedentary ischemic group and all other groups (ANOVA followed by Duncan's test, $p < .05$).

A



B


Reference Memory
*
*
*
60
50
40
30
20
10
0
Latency (sec)
SH/SED
SH/EXE
ISQ/SED
ISQ/EXE
1
2
3
4
5
Session

C

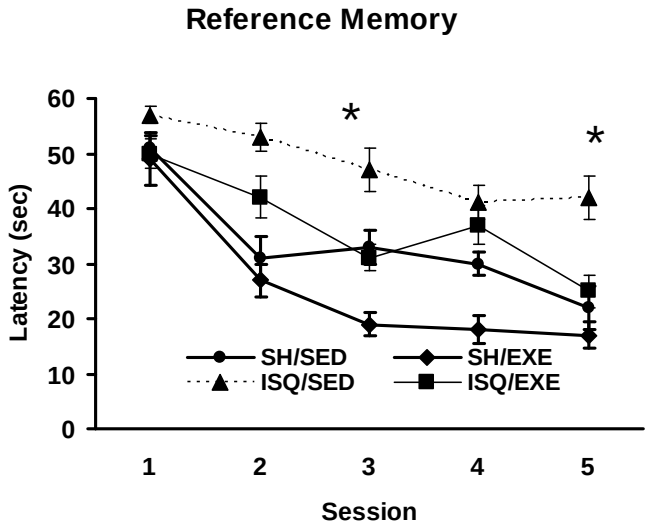

Reference Memory
*
*
60
50
40
30
20
10
0
Latency (sec)
SH/SED
SH/EXE
ISQ/SED
ISQ/EXE
1
2
3
4
5
Session

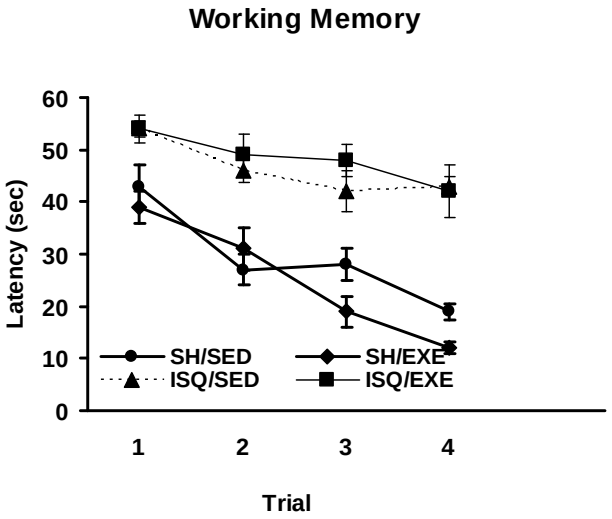
Table 1 – Probe trial of the reference memory protocol: Number of platform crossings, latency to find the platform position, and spent time in the target and opposite quadrant. Panel A: Post – surgery group and Panel B: Pre+post surgery group: SH/SED – control sedentary group, SH/EXE post - control submitted to exercise post-surgery group, ISQ/SED – ischemic sedentary group, ISQ/EXE post - ischemic group submitted to exercise post-surgery, SH/EXE pre-post - control submitted to exercise pre-post-surgery group, ISQ/EXE pre-post - ischemic group submitted to exercise pre-post-surgery. Data represent means ± S.E.M. * Significant difference between sedentary ischemic group and all other groups (Two-way ANOVA, $p<.05$).

A

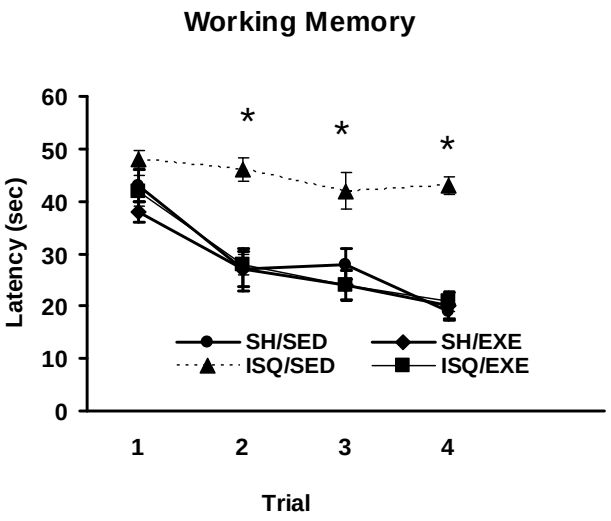
	Crossings	**Latency (sec)**	**Target Quadrant (sec)**
Panel A			
SH/SED	2 ± 0.37	10.1 ± 3.1	18 ± 1.8
SHAM / EXE POST	2.55 ± 0.68	9.1 ± 4	23 ± 3.9
ISQ/SED	0.5 ± 0.2 *	23.4 ± 6.5 *	16.75 ± 2
ISQ/EXE POST	1.8 ± 0.4	9.1 ± 2.8	18.2 ± 2.8
Panel B			
SH/SED	2 ± 0.4	12.11 ± 4.4	18 ± 1.9
SHAM / EXE PRE+POST	2.2 ± 0.4	16.6 ± 5.7	19.3 ± 2.5
ISQ/SED	0.75 ± 0.4 *	17.9 ± 7.2	14.8 ± 2 *
ISQ/EXE PRE+POST	2.3 ± 0.3	18 ± 7.1	24.9 ± 2.3

Figura 2- Performance in the working memory task in the water maze after pre-surgery exercise (A), post-surgery exercise (B) and pre+post-surgery exercise protocols (C). SH/SED –, SH/EXE – sham group submitted to physical activity during three months, three times per week, ISQ/SED - ischemic sedentary group, ISQ/EXE – ischemic group submitted to physical activity during three months, three times per week. Each line represents mean± standard error of mean (SEM). * Significant difference between sedentary ischemic group and all other groups (ANOVA followed by Duncan's test, $p < .05$).

A



B


Working Memory
Latency (sec)
60
50
40
30
20
10
0
*
*
*
SH/SED
SH/EXE
ISQ/SED
ISQ/EXE
1
2
3
4
Trial

C

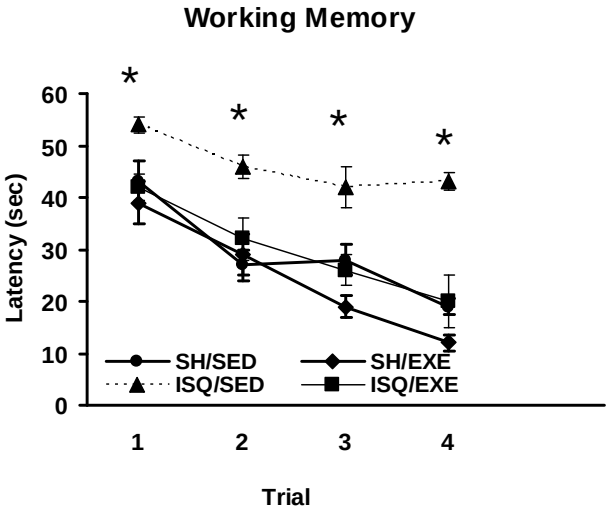
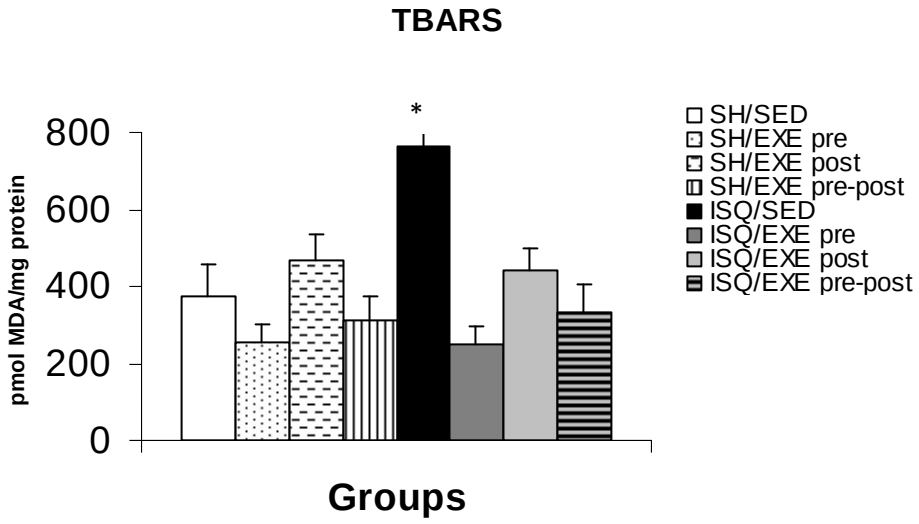
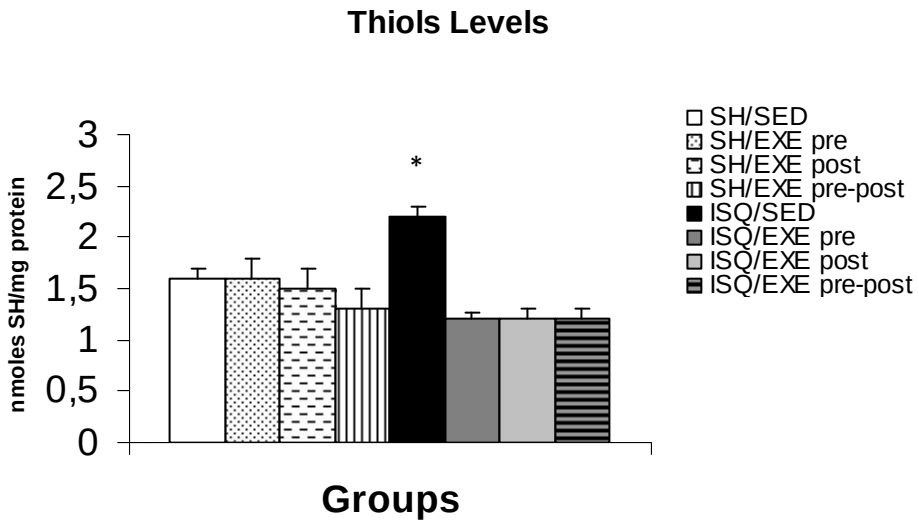

Working Memory
Latency (sec)
60
50
40
30
20
10
0
*
*
*
*
SH/SED
SH/EXE
ISQ/SED
ISQ/EXE
1
2
3
4
Trial

Figure 3 - Thiobarbituric acid reactive substances (TBARS) (panel A) and total cellular thiols (panel B) levels in hippocampus. SH/SED – sham sedentary group, SH/EXE pre – sham submitted to exercise pre-surgery group, SH/EXE post - sham submitted to exercise post-surgery group, SH/EXE pre+post - sham submitted to exercise pre+post-surgery group, ISQ/SED – ischemic sedentary group, ISQ/EXE pre – ischemic group submitted to exercise pre-surgery, ISQ/EXE post - ischemic group submitted to exercise post-surgery, ISQ/EXE pre+post - ischemic group submitted to exercise pre+post-surgery. Results are expressed as mean± standard error of mean (SEM).* Significant difference between sedentary ischemic group and all other groups (Two-Way ANOVA; $p < .05$).

A



B


Thiols Levels
nmoles SH/mg protein
3
2,5
2
1,5
1
0,5
0
*
SH/SED
SH/EXE pre
SH/EXE post
SH/EXE pre-post
ISQ/SED
ISQ/EXE pre
ISQ/EXE post
ISQ/EXE pre-post
Groups

6. CAPÍTULO 4

Artigo: Environmental enrichment prevents behavioral deficits and oxidative stress caused by chronic cerebral hypoperfusion in the rat - a ser submetido para Neurobiology of Learning and Memory

Environmental enrichment prevents behavioral deficits and oxidative stress caused by chronic cerebral hypoperfusion in the rat

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Abstract

Environmental enrichment prevents memory deficits and pro-oxidative activities following acute stroke and hypoxia, however its effects on animals suffering chronic cerebral hypoperfusion have not yet been investigated. The aim of this study was to evaluate the therapeutic potential of environmental enrichment, as assessed by cognitive performance in the Morris water maze and brain oxidative status, through measurement of macromolecules damage, TBARS levels and total cellular thiols, as well as antioxidant enzymes in hippocampus, striatum and cerebral cortex. Adult male Wistar rats were submitted to the modified permanent bilateral occlusion of the common carotid arteries (2VO) method, with right common carotid artery being first occluded, and tested three months after the ischemic event. The effects of three distinct enrichment protocols were examined: pre-ischemia, post-ischemia and pre+post-ischemia. Environmental enrichment consisted in sessions of 1-hr, 3 times per week during 12 weeks. Rats were sacrificed after the end of stimulation period and cognitive function, in both reference and working spatial memory, and oxidative stress parameters were determined. A significant cognitive deficit was found in both spatial tasks after hypoperfusion; this effect was reversed in 2-VO enriched group. Moreover, hippocampal oxidative damage and antioxidant enzyme activity was regulated by environmental enrichment. These results suggest that the stimulation protocol protects against the cognitive and biochemical damage caused by chronic cerebral hypoperfusion.

Keywords: chronic cerebral hypoperfusion, oxidative state, hippocampus, 2VO, water maze.

1. Introduction

Chronic cerebral hypoperfusion (CCH) is associated to several cerebrovascular conditions, including cerebral arteriovenous malformations, dural arteriovenous fistula, artherosclerosis, carotid stenosis/occlusion and cerebral small vessel diseases (Sekhon et al., 1998; Meyer et al., 1999; Hasumi et al., 2007; Hainsworth and Markus, 2008; Aliev et al., 2009). As regards to neurodegeneration and cognitive decline, there is a correlation between the severity of memory dysfunction and the decline in cerebral blood flow in Alzheimer's disease, vascular dementia and post-stroke hypoperfusion (Ohta et al., 1997; Komatani et al., 1988).

Surgical ligation of both common carotid arteries in rats produces a chronic, global hypoperfusion (2-vessel occlusion, 2VO), causing a failure of cerebral perfusion, where ischaemic damage is maximal in hippocampal neurons and in cortical arterial border zones, including deep white matter (Jiwa et al., 2010). Impaired learning and memory were revealed in Morris water maze tasks (Pappas et al. 1996; Vicente et al. 2009;Wang et al. 2010a;Wang et al. 2010b, Cechetti et al., 2010), in radial arm maze and Y-maze (Sarti et al. 2002; Pappas et al. 1996), despite substantial recovery of CBF. Cognitive deficits resulted primarily from white matter histopathology, with relative sparing of the hippocampus (Farkas et al. 2004; Ohta et al. 1997). Some hippocampal changes appeared from ~ 4 weeks, with increased astrocyte density and cell loss in the CA1 area (Bennett et al. 1998; Farkas et al. 2004;Vicente et al. 2009;Pappas et al. 1996).

The brain tissue is sensitive to oxidative damage and previous studies have demonstrated that oxidative injury plays a key role in the pathogenesis of numerous neurodegenerative diseases including stroke, Alzheimer's disease, and vascular

dementia, etc (Chong et al., 2005; Coyle and Puttfarcken, 1993). Oxidative stress, defined as the imbalance between oxidants and antioxidants in favor of oxidant activity that potentially lead to tissue damage (Polidori et al, 2000). Oxidative metabolism of the brain tissue is high and the large lipid content of myelin sheaths are also a target of reactive oxygen species (ROS) (Choi 1993). Enzymes like superoxide dismutase (SOD), catalase and glutathione peroxidase, as well as non-enzymatic antioxidants (as, for example, glutathione, ascorbate and tocopherol) (Halliwell, 1992), may play a protective role in the development of lesions induced by chronic cerebral hypoperfusion in the central nervous system (Markesbery, 1997), protecting cells against the consequences of damage induced by free radicals, due to their ability to react with the latter (Atmaca, 2004; Sen, 1998).

Many therapeutic strategies are emerging to alleviate the cognitive and oxidative damage caused by experimental ischaemia. Environmental enrichment (EE), as a stimulation paradigm, involves a combination of increased social interaction, physical exercise and continuous exposure to learning tasks (Krech, Rosenzweig, & Bennett, 1960) and produces interesting effects. This therapeutic strategie causes improved learning, working and reference spatial memory in rats (Bindu, Rekha, & Kutty, 2005; Escorihuela et al., 1995; Kempermann, Kuhn, & Gage, 1997; Leggio et al., 2005; Nilsson, PerWlieva, Johansson, Orwar, & Eriksson, 1999; Van Praag, Kempermann, & Gage, 2000). The mechanism appears to be mediated by multiple pathways, particular the enhancement of dendrites number, branches and spines, as well as increased glial numbers (Faherty, Kerley, & Smeyne, 2003; Rosenzweig & Bennett, 1996), reduced inflammatory response , enhanced microglial phagocytosis and proteasomal degradation (Ambreé et al., 2006), and increased angiogenesis

(Herring et al., 2008) and neurogenesis in the hippocampus (Auvergne et al., 2002; Brown et al., 2003; Bruel-Jungerman, Laroche, & Rampon, 2005).

Some authors have demonstrate that environmental stimulation attenuated pro-oxidative processes and triggered anti-oxidative defense mechanisms in Alzheimer's disease model (Adlard et al., 2005, Herring et al., 2008). In addition, a long term modulation on oxidative status was also described after focal ischemia, once an increase on SOD content was found both 30 and 60 days after the event (Bidmon et al., 1998).

Surprisingly, there are no studies on the effects of environmental enrichment on oxidative status in hippocampus of rats submitted to the 2VO model of hypoperfusion, one of the most vulnerable brain regions to oxidative stress (Candelario-Jalil et al., 2001) and/or excitotoxic events like.

In this study, permanent occlusion of bilateral common carotid arteries in rats was used to evaluate the therapeutic potential of environmental enrichment on cognitive deficits and brain oxidative status on the hippocampus of 2VO rats. Spatial memory deficit in both reference and working memory tasks in the Morris water maze as well as parameters of cellular oxidative status, namely free radicals content, index of macromolecules damage and antioxidant enzymes were studied on hippocampus, striatum and cerebral cortex from adult rats receiving 2VO model followed by Environmental enrichment pre and post ischemia.

2. Materials and Methods

2.1. Animals

Male Wistar rats were obtained from the Central Animal House of the Institute of Basic Health Sciences, Universidade Federal do Rio Grande do Sul. They were maintained in a temperature-controlled room (21 ± 2 °C), on a 12/12 h light/dark cycle, with food and water available ad libitum.

2.2. Surgical procedure-2VO

Rats were anesthetized for surgery with halothane; a neck ventral midline incision was made and the common carotid arteries were then exposed and gently separated from the vagus nerve. The carotides were occluded with 5-0 silk suture, with one week interval, initially the right common carotid artery was assessed, following by the left artery (Cechetti et al., 2010). Sham-operated controls received the same surgical operation without carotid artery ligation. Animals were randomly assigned to sham or 2VO procedures so as to avoid any litter effect.

All animals in the ischemic group were submitted surgery at three months of age, and sacrificed with six months of life. The animals of the sham group received the same manipulation but without occlusion of the carotid arteries (n=8). Animals were randomly assigned to three experimental groups with yours respective controls that maintained in standard environmental: (1) Environmental enrichment pre-surgery group (twenty days until three months of life) (n=9), (2) Environmental enrichment post-surgery group (three months until six months of life) (n=9) and (3) Environmental enrichment pre and post-surgery group (twenty days after six months of life) (n=10). All procedures were in accordance with the Guide for the Care and Use of Laboratory Animals adopted by National Institute of Health (USA) and with the Federation of Brazilian Societies for Experimental Biology (FESBE) and were

approved by the Committee of Ethics on Research at the Universidade Federal do Rio Grande do Sul.

2.3. Environmental enrichment (EE)

Rats were submitted to enrichment during one hour, three times per week. Although there are reports of EE utilizing 2 h/day (Widman & Rossellini, 1990) and 3 h/day protocols (Feng et al., 2001), we opted for 1 h of session enrichment because it has also proven effective (Gaulke, Horner, Fink, McNamara, & Hicks, 2005, Pereira et al., 2006). The enriched environment consisted of a large cage (40 x 60 x 90 cm) with three floors, ramps, running wheel and several objects with different shapes and textures, modeled as previously described (Diamond, 2001; Widman & Rossellini, 1990). Objects in the cage were changed once a week. Rats groups non-enriched were removed from their home cages to another standard cage during the other animal's enrichment period.

2.4. Morris water maze

Rats were submitted three months after the surgery to behavioral testing for spatial memory in the Morris water maze. The maze consisted of a black circular pool with 200 cm in diameter filled with water (temperature around 23 °C, depth 40 cm) situated in a room with visual cues on the walls. A black platform with 10 cm in diameter was submerged in the water (2 cm below the water surface). The pool was conceptually divided into four quadrants and had four points designed as starting positions (N, S, W or E) (Morris et al., 1984). Two behavioral protocols, for reference and working memory, were utilized.

2.4.1. Reference memory protocol

In this task rats received five training days (sessions) and a probe trial in the 6th day. Each session consisted of four trials with a 15 min intertrial interval. A trial began when the rat was placed in the water at one of the four starting positions, chosen at random, facing the wall. The order of starting position varied in every trial and any given sequence was not repeated on acquisition phase days. The rat was given 60 s to locate the platform; if the animal did not succeed it was gently guided to the platform and left on it for 10 s. Rats were dried and returned to their home cages after each trial. The latency to find the platform was measured in each trial and the mean latency for every training day was calculated. The probe consisted of a single trial, as described before, with the platform removed. Here, the latency to reach the original platform position, the number of crossings over that place and the time spent in the target, as well as in the opposite quadrants, were measured (Netto et al., 1993; Pereira et al., 2007). Videos were subsequently placed in randomized order in a separate ANY-maze protocol to be scored by a trained observer blind to the experimental condition using a keyboard-based behavioral tracking system.

2.4.2. Working memory protocol

This protocol consisted of four trials/day, during four consecutive days, with the platform location daily changed. Each trial was conducted as described in the reference memory protocol, with a 5 min intertrial interval. Latency to find the platform was measured in each trial and the mean latency for every trial, along the four days, was calculated, allowing for the observation of the ability of animals in

locating the novel platform position in the day (Netto et al., 1993; Pereira et al., 2007).

2.5. Oxidative State

2.5.1. Free radicals levels

To assess the free radicals content we used 2'-7'-dichlorofluorescein diacetate (DCFH-DA) as a probe (Lebel et al., 1990). An aliquot of the sample was incubated with DCFH-DA (100 μM) at 37oC for 30 min. The reaction was terminated by chilling the reaction mixture in ice. The formation of the oxidized fluorescent derivative (DCF) was monitored at excitation and emission wavelengths of 488 nm 525 nm, respectively, using a fluorescence spectrophotometer (Hitachi F-2000). The free radicals content was quantified using a DCF standard curve and results were expressed as pmol of DCF formed/mg protein. All procedures were performed in the dark and blanks containing DCFH-DA (no homogenate) were processed for measurement of autofluorescence (Driver et al., 2000; Sriram et al., 1997).

2.5.2. Thiobarbituric acid reactive substances (TBARS)

LPO (lipoperoxidation) was evaluated by thiobarbituric acid reactive substances (TBARS) test (Bromont et al., 1989). Aliquots of samples were incubated with 10% trichloroacetic acid (TCA) and 0.67% thiobarbituric acid (TBA). The mixture was heated (30 min) on a boiling water bath. Afterwards, n-butanol was added and the mixture was centrifuged (1000×g for 10 min). The organic phase was collected to measure fluorescence at excitation and emission wavelengths of 515 and 553 nm,

respectively. 1,1,3,3-tetramethoxypropane, which is converted to malondialdehyde (MDA), was used as standard. Results are expressed as pmol MDA/mg protein and reported as percentage of control.

2.5.3. Total thiol content

Cellular thiols, as glutathione and protein thiols, were measured. Aliquots of samples were incubated with 100 mM DTNB (final concentration) for 15 min in darkness. Absorbance of the reaction mixture was measured at 412 nm (Khajuria et al., 1999); results are expressed as nmoles SH per mg protein.

2.5.4. Superoxide dismutase (SOD) activity

SOD activity was determined using a RANSOD kit (Randox Labs., USA). This method employs xanthine and xanthine oxidase to generate O2-.that react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to form a red formazan dye which is assayed spectrophotometrically at 505 nm at 37oC. The inhibition on production of the chromogen is proportional to the activity of SOD present in the sample.

2.5.5. Protein determination

Protein was measured by the Coomassie blue method using bovine serum albumin as standard (Bradford, 1976).

2.6. Statistical analysis

Behavioral performance in the training days was analyzed using a two-way repeated-measures analysis of variance (ANOVA) with lesion and exercise as the independent variables and session as the repeated measure. Reference memory probe trial, working memory in the MWM variables and oxidative status were analyzed by two-way ANOVA. All analyses were followed by post-hoc Duncan's test for multiple comparisons, when necessary. Data were expressed as means±SEM. Probability values less than 5% were considered significant. All statistical analysis was performed using the Statistica® software package running on a compatible personal computer.

3. Results

3.1. Behavioral Effects

In Morris Water Maze task, in the environmental enrichment pre-surgery group (Fig.1A), Two-way repeated measures ANOVA revealed significant main effects of factor *lesion* and *treatment* interaction ($F(5,24)=5.75$, $p<.01$), where the ischemic group submitted to standard environmental presented greater latencies than other three groups on 2nd, 3rd and 5th sessions ($p<.05$). The same results occurred with the post-EE ($F(5,27)=7.7$, $p<.01$) and pre+post- EE ($F(5,27)=11.2$, $p<.01$) (Fig. 1B/C).

In the probe trial, p*ost-hoc* tests indicated that both standard environmental and pre-EE groups had significantly greater latencies to reach the platform position and spent more time in the opposite quadrant, as compared to groups submitted to EE (p

<.05). In the post-EE and pre-post-EE groups, the time spent in the opposite, target quadrants and number crossings differ among experimental groups for *treatment* effect in the probe trial ($p<.05$) (Table 1).

Two-way ANOVA of working memory task demonstrated positive results. The ischemic group in standard environment was different from other three groups, pre, post and pre+post-EE, on the 2nd, 3rd and 4th trials performance ($p<.05$) (Fig 2A/B/C). Permanent occlusion of both common carotid arteries did not cause any motor deficit; the means of swimming speed were 26cm/s for sham-control animals and 24.5cm/s for ischemic rats.

3.2. Oxidative State

3.2.1 Free Radical levels

ANOVA evidence changes on DCF measures in the hippocampus of *lesion* and *environment* factors ($F(2,41)= 5.41$ $p<.01$), where ischemic sedentary group was significantly different from all other groups analyzed (Fig.3A). No changes on DCF measurements were found in striatum and cerebral cortex after 2VO rats, irrespective of the environment factor (data not presented).

3.2.2 Thiobarbituric acid reactive substances (TBARS) and total cellular thiols

ANOVA revealed an effect of *environment* factor in TBARS levels ($F(2,41)=5.41$, $p<.01$) in the hippocampus. In adittion, significant differences were present in *lesion* and *environment* factors in the same structure ($F(2,41)=6.84$, $p<.01$). Post hoc test showed that ischemic rats maintained in standard environment had greater values as compared others groups ($p<.01$) (Fig 3B). No significant

differences were present on cortex and striatum samples, the same appears for thiols levels in all analyzed structures (data not presented).

3.2.3. Superoxide dismutase activity

Changes appear on SOD activity in the hippocampus of 2VO rats in *lesion* and *environment* factors ($F(2,42)= 3.9$ $p<.05$). Post hoc analysis showed greater values of ischemic rats maintained in standard environment as compared others groups (Fig 3C). No effect was observed in cortex and striatum (data not presented).

4. Discussion

Hippocampus is highly sensitive to ischemic insults, which plays an important role on learning and memory (Ni et al., 1994; Pappas et al., 1996). Permanent bilateral occlusion of the common carotid arteries (2VO) in rats has been established as a procedure to investigate the effects of chronic cerebral hypoperfusion on cognitive dysfunction and neurodegenerative processes. This model is a useful model for studying pathophysiology of learning and memory deficits in human dementia with cerebral circulation impairment, and for assessing therapeutic potential and/or exploring possible mechanisms of anti-dementia (Sarti et al., 2002).

In this study, we report that the environmental stimulation is effective in enhancing spatial learning and memory in rats when applied before and after event ischemic (Fig 1 and 2). Concomitantly, was found changes in the oxidative status of these animals. A recent study in our laboratory has demonstrated the effectiveness of this protocol of stimulation in the hypoxia-ischemia model (Pereira et al., 2009).

Cognitive deficits were described earlier for this experimental model (Vicente et al., 2009, Cechetti et al., 2010) and indicate the adequacy for studying vascular dementia. In addition, it has been reported that learning and memory can be influenced by social stimulation. A recent study confirmed that daily EE prevents HI-induced spatial memory deficit in both reference and working memory tasks in the Morris water maze (Pereira et al., 2007) as well as increase in number and survival of the newly originated cells in the hippocampus (Auvergne et al., 2002, Brown et al., 2003, Bruel-Jungerman et al., 2005) and in number of dendrites (Faherty et al., 2003; Rosenzweig and Bennett, 1996).

Frequent participation in cognitively stimulating intellectual and physical activities is linked to a reduced risk of DA (Friedland et al., 2001, Wilson et al., 2002). In laboratory rodents it is well known that environmental stimulation has a great impact on various behavioural parameters (Olsson & Dahlborn, 2002) and can also improve learning and memory (Frick & Fernandez, 2003; Rampon et al., 2000), with delays behavioural symptoms and disease progression in mouse models of neurodegenerative diseases (Faherty et al., 2005; Lazic et al., 2006).

In this light our results were surprising as several other studies revealed a compensation of spatial memory deficits attributed to enrichment procedures in ischaemia models. Social and cognitive stimulation enhanced the rate of spatial water maze learning and reduced plaque load in female mice DA model (Adlard et al., 2005). The same was true for enriched housed hypoxia-ischemia rats (Pereira et al., 2009). What is more, Faherty et al. (2005) reported the benefits of environmental enrichment in an experimental model of Parkinson's disease.

The brain tissue is sensitive to oxidative damage and it has been known that 2VO model induces oxidative events (Yanpallevar et al., 2005; Ghoneim et al.,

2002), but few studies are performed to reduce oxidative stress in cerebral hypoperfusion (Yanpallevar et al., 2005; Ghoneim et al., 2002). Oxidative stress, generally caused by the increase in levels of free radical, can lead to tissue damage (Polidori et al, 2000). Thiols levels, GSH and superoxide dysmutase (SOD) are involved in the antioxidant system and are important for the protection of the brain tissue from oxidative damage.

Additionally, it has been proposed that oxidative status may mediate cognitive and motor changes arising by the enriched experience (Fernández et al., 2004). Acquisition and performance of both the Morris maze and the object recognitive task can be impaired by dysfunction of neurochemical systems in many brain regions (Brandeis et al., 1993; McNamara and Skelton, 1993). To our knowledge, this is the first study that demonstrates the effects of enrichment environmental administered distinct event-related protocols in this model. The protocol here used (three 1-hr sessions per week, during 12 weeks) alters the free radical content in hippocampus, as well as basal TBARS and SOD activity.

Moreover, it was found that there is a significant increase in the activity of antioxidant enzymes glutathione-transferase and SOD after the combined treatment, EE and antioxidant diet (Opii et al., 2008). Candelario-Jalil et al. (2001) identified increased MnSOD activity only in the hippocampus of gerbils submitted to transient cerebral ischemia at 24, 48 and 72 h of reperfusion. Benzi and Moretti (1995) proposed that the augment of SOD activity might be related to oxidative stress, considering that might allow accumulation of hydrogen peroxide (H_2O_2). This, combined to metal iron, would originate hydroxyl radicals, when H_2O_2 is not effectively transformed into water. In present study the high SOD activity is paralleled by an increase of total free radicals content, as evidenced by DCF and

TBARS levels in the hippocampus (Fig. 3). Similar results were shown by Pereira et al. (2009) in a model of hypoxia-ischemia submitted to stimulation social.

Considering that such effect was prevented in the hippocampus of ischemic rats housed in enriched environment, it might mean that the EE reduces the vulnerability to additional peroxidative injury. There was a strong reduction of free radicals content in hippocampus from ischemic animals submitted to EE.

Cognitive and voluntary physical exercise has been shown to lower peripheral oxidative stress in both humans and animals (Gomez-Cabrera, 2008) and cerebral oxidative damage in aging animals (Cui et al., 2007; Opii et al., 2008), thereby revealing neuro-/cardioprotective abilities (Cechetti et al., 2008, Powers et al., 2004). Herring et al. (2008) showed increased memory function and reduction biomarkers for oxidative damage after four months of continuous and diversified environmental stimulation in mice.

Summarizing, present results demonstrate that chronic cerebral hypoperfusion causes cognitive damage associated to increased ROS and lipoperoxidation in the hippocampus, and that environmental enrichment prevents both effects. This implies a neuroprotective action of social and motor stimulation over cognitive and biochemical damage caused by cerebral hypoperfusion injury; further studies are needed to investigate the mechanism involved.

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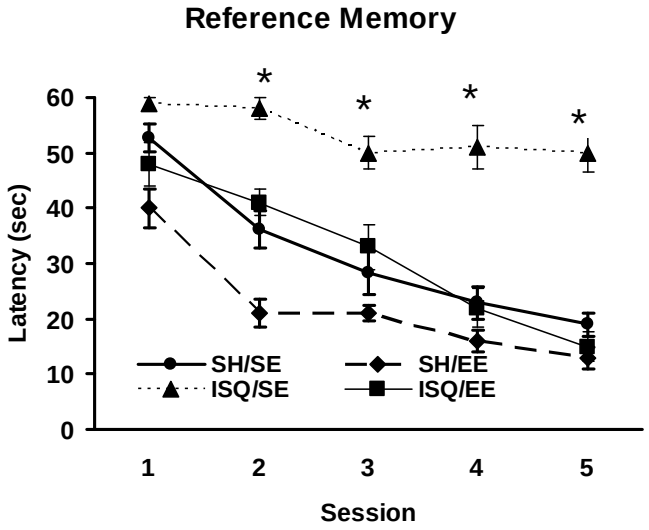
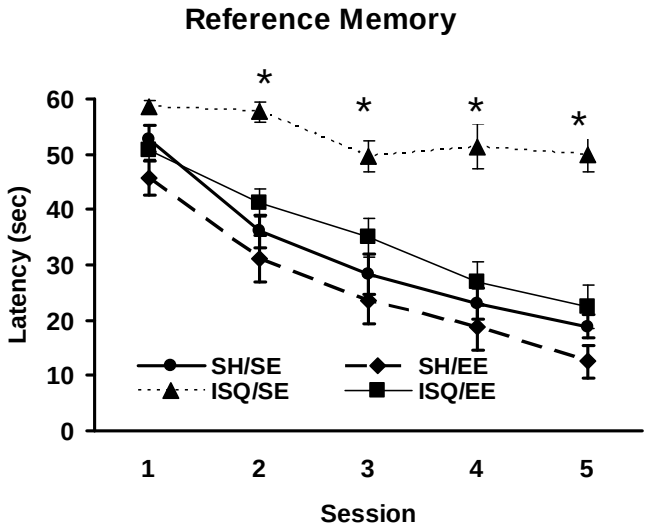
Figura 1 – Performance in the reference memory task in the water maze of environmental enrichment pre-surgery group (A), post-surgery group (B) and pre+post-surgery group (C). SH/SE – control group submitted to standard environment, SH/EE – control group submitted to environmental enrichment during three months, three times per week, ISQ/SE - ischemic group submitted to standard environment, ISQ/EE – ischemic group submitted to environmental enrichment during three months, three times per week. Each line represents mean± standard error of the mean (SEM). * Significant difference between ISQ/SE group and all other groups (ANOVA followed by Duncan's test, $p < .05$).

A



B


Reference Memory
Latency (sec)
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Session
SH/SE
SH/EE
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C

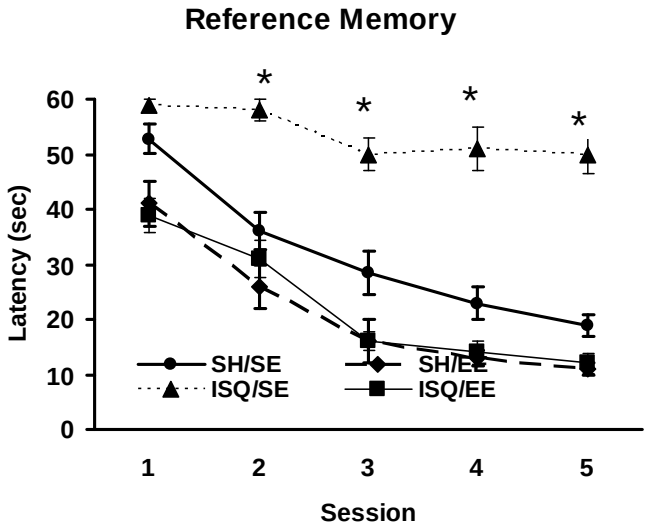
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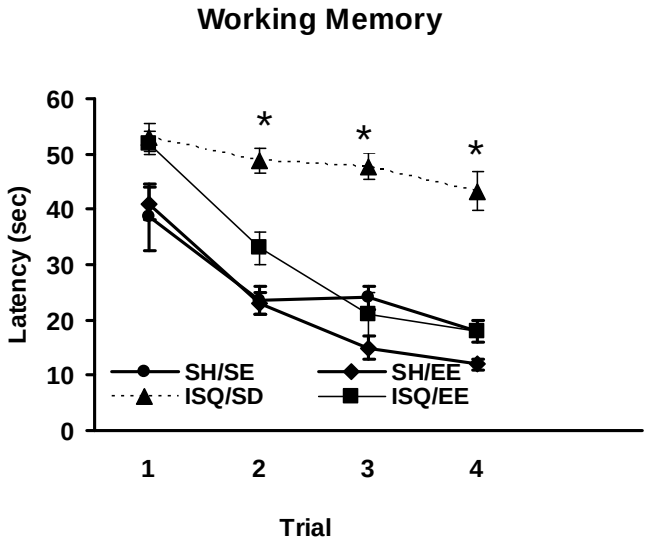
Table 1 – Probe trial of the reference memory task: Number of platform crossings, latency to find the platform position, and time spent in the target and opposite quadrants. (A) Pre –surgery group, (B) Post - surgery group and (C) Pre+post-surgery group. SH/SE – control group submitted to standard environment, SH/EE pre – control submitted to the enriched environment pre-surgery group, SH/EE post - control submitted to the enriched environment post-surgery group, SH/EE Pre+post - control submitted to the enriched environment pre+post-surgery group, ISQ/SE – ischemic submitted to standard environment group, ISQ/EE pre – ischemic group submitted to enriched environment pre-surgery, ISQ/EE post - ischemic group submitted to enriched environment post-surgery, ISQ/EE pre+post - ischemic group submitted to enriched environment pre-post-surgery. Data represent means±S.E.M. * Difference between ISQ/SE with ISQ/EE (Two-way ANOVA, $p<.05$).

	Crossings	**Latency (sec)**	**Target Quadrant (sec)**	**Opposite quadrant(sec)**
Panel A				
SH/SE	2.1 ± 0.3	17.6 ± 5.3	13.1 ± 2.3	25 ± 5
SHAM / EE PRE	2 ± 0.5	33 ± 7	15.6 ± 3.3	15 ± 4.6
ISQ/SE	1.6 ± 0.2	52.3± 6.7 *	16 ± 2	21.25 ± 2.7 *
ISQ/EE PRE	1.7 ± 0.3	21.5 ± 7.3	14.4 ± 4	12.5 ± 1.8
Panel B				
SH/SE	2 ± 0.5	18 ± 4.2	12 ± 1.5	22 ± 2.2
SHAM / EE POST	4 ± 0.9	21,1 ± 5	22 ± 1.7	19.6 ± 3.4
ISQ/SE	0.8 ± 0.2 *	19.2 ± 3.2	14.2 ± 1.6 *	21.3 ± 4

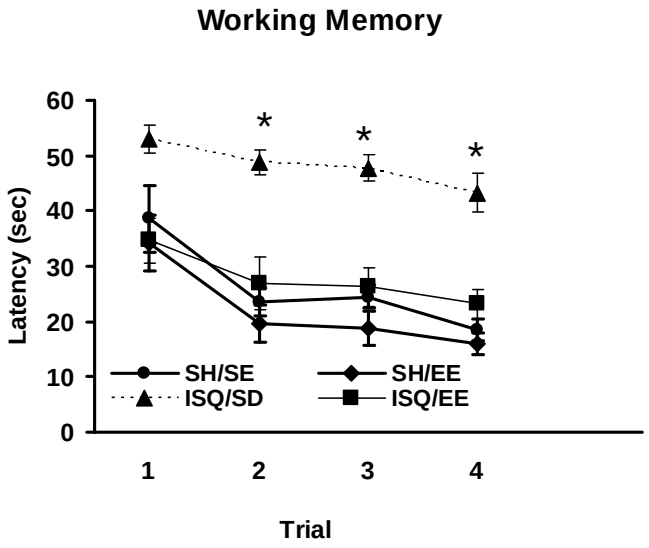
ISQ/EE POST	2.8 ± 1	18.6 ± 1	18.5 ± 3.2	18.4 ± 2.2
Panel C				
SH/SE	2 ± 0.4	16 ± 3.2	11.7 ± 1.6	25 ± 4.9
SHAM / EE PRE+POST	2 ± 0.3	19.1 ± 2.4	20.3 ± 2.9	12 ± 2.2
ISQ/SE	1.79 ± 0.4	17.2 ± 1	14.2 ± 1.7 *	21.2 ± 2.7 *
ISQ/EE PRE+POST	2.1 ± 0.2	19. 9 ± 2.2	22± 2.1	10 ± 2.1

Figura 2- Performance in the working memory task in the water maze environmental enrichment pre-surgery group (A), post-surgery group (B) and pre+post-surgery group (C). SH/SE – control group submitted to standard environmental, SH/EE – control group submitted to environmental enrichment during three months, three times per week, ISQ/SE - ischemic group submitted to standard environment, ISQ/EE – ischemic group submitted to environmental enrichment during three months, three times per week. Each line represents mean± standard error of the mean (SEM). * Significant difference between ISQ/SE group and all other groups (ANOVA followed by Duncan's test, $p < .05$).

A



B


Working Memory
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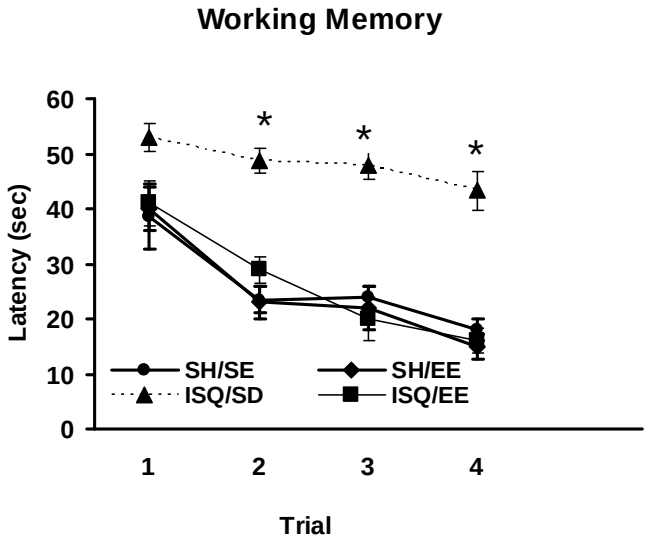
SH/SE
SH/EE
ISQ/SD
ISQ/EE

C


Working Memory
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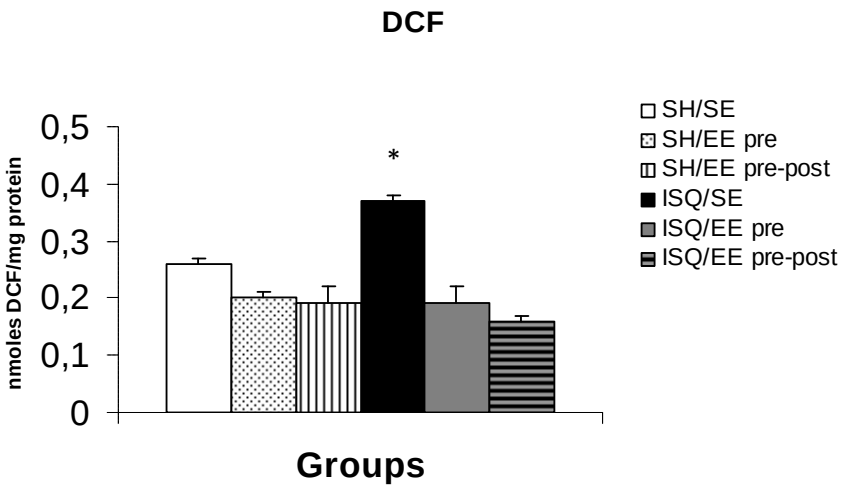
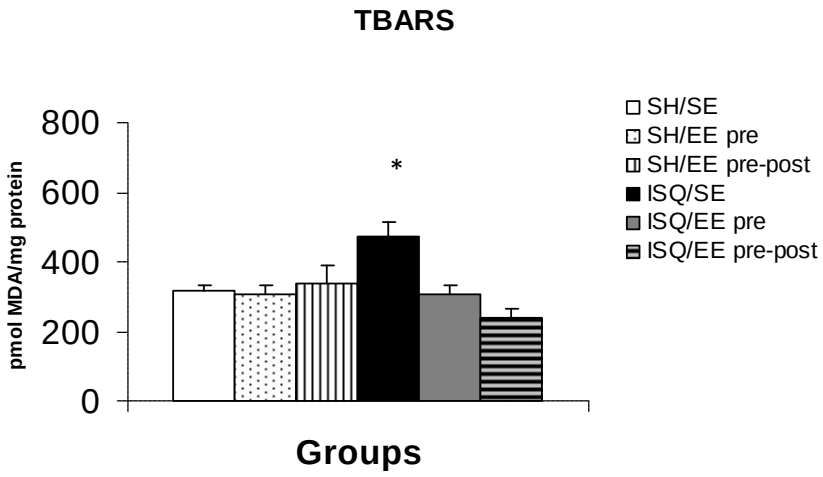
SH/SE
SH/EE
ISQ/SD
ISQ/EE

Figure 3 – Free radical levels (DCF formed) (panel A), Thiobarbituric acid reactive substances (TBARS) (panel B) and Superoxide Dismutase (SOD) activity (panel C) in hippocampus. SH/SE – control group submitted to standard environment, SH/EE pre – control submitted to environmental enrichment pre-surgery group, SH/EE Pre+post - control submitted to environmental enrichment pre+post-surgery group, ISQ/SE – ischemic submitted to standard environment group, ISQ/EE – ischemic group submitted to environmental enrichment, ISQ/EE pre+post - ischemic group submitted to environmental enrichment pre-post-surgery. Results are expressed as mean± standard error of mean (SEM).* Significant difference between standard environment ischemic group and all other groups (Two-Way ANOVA; $p < .05$).

A



B


TBARS
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200
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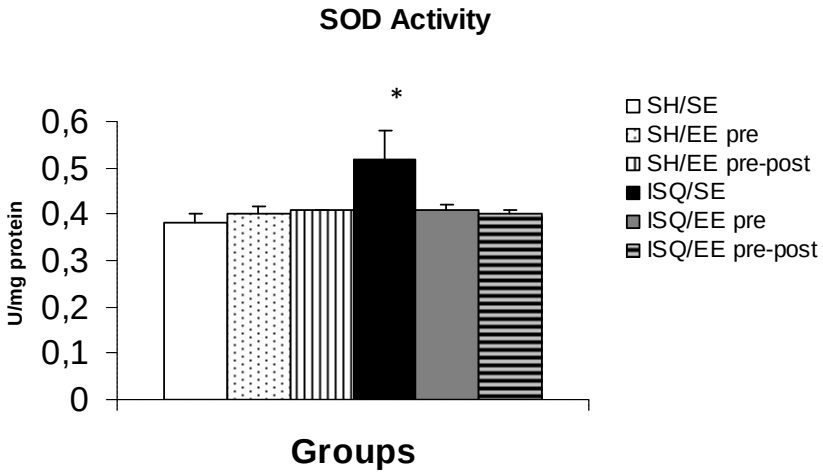
pmol MDA/mg protein
*
Groups
SH/SE
SH/EE pre
SH/EE pre-post
ISQ/SE
ISQ/EE pre
ISQ/EE pre-post

C


SOD Activity
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Groups
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SH/EE pre-post
ISQ/SE
ISQ/EE pre
ISQ/EE pre-post

7. DISCUSSÃO GERAL

Esta tese teve como objetivo principal estudar os efeitos do tratamento através da atividade física regular forçada e do enriquecimento ambiental sobre parâmetros comportamentais (memória de referência e trabalho) e aspectos bioquímicos de ratos *Wistar* submetidos à Hipoperfusão Encefálica Crônica. Sabe-se que a melhora no desempenho do Teste *Water Maze* e outros testes comportamentais como, por exemplo, o *Y-Maze* é fundamental para a demonstração do ganho cognitivo, e que este se encontra bastante prejudicado após isquemias globais do Sistema Nervoso Central (SMITH *et al.*, 1984; FARKAS *et al.*, 2007).

Os resultados do capítulo 1 demonstram através de uma comparação entre o protocolo padrão e o modificado, que a hipoperfusão encefálica crônica três meses após o procedimento cirúrgico, causa déficit tanto na memória de referência como na memória de trabalho; entretanto evidências de atrofia hipocampal e estriatal não foram encontradas. Um dos principais achados neste capítulo foi de que os resultados referentes a cognição e morfologia encontrados nos dois protocolos não diferem, mas a sobrevida dos animais é significativamente maior no protocolo modificado.

A questão da sobrevivência dos animais submetidos a HEC vem sendo tratada por alguns autores, mas a intervenção nestes casos se deu através do uso de medicamentos ou plantas (Farkas *et al.*, 2005; 2006). Por exemplo, Institoris e colaboradores (2007) demonstraram aumento na sobrevida, de 50 para 93% dos ratos isquêmicos, com a administração de um inibidor seletivo da ciclooxigenase (COX-2), uma das enzimas responsáveis pela progressão da injúria isquêmica. Nosso trabalho demonstrou através de uma modificação no protocolo padrão, através do intervalo dado de 1 semana entre a oclusão de uma carótida e outra, um aumento significativo da sobrevida destes animais. Sarti e colaboradores (2002) propuseram este modelo, mas

não chegaram a compará-lo ao modelo padrão a fim de tornar fidedigna a utilização deste novo protocolo experimental.

Podemos observar, através dos resultados dos capítulos 1 e 2, que o modelo modificado de 2 vasos causa um dano cognitivo bastante importante nestes animais, principalmente na fase mais crônica do processo, aos 3 meses após lesão, perdurando até o fim do período de avaliação, isto é, 6 meses após o procedimento. Várias observações clínicas suportam a hipótese de que a hipoperfusão é um dos fatores que mais contribui para o declínio cognitivo, ambos relacionados a idade avançada e também com desordens neurológicas relacionadas com a idade (de la Torre, 2006; Kalaria, 2000). O persistente decréscimo do fluxo sanguíneo cerebral que acontece tanto na DA como na DV apresentam correlação com a severidade dos distúrbios na memória (Komatani *et al.,* 1988).

Estudo prévios corroboram nossos achados, relatando que a oclusão de 2 vasos provoca um prejuízo na memória espacial dos animais tanto a curto quanto a longo prazo através de testes como *Y - Maze, Radial – Maze* e também com o Teste *Water Maze* (Ni *et al*., 1994; Pappas *et al*., 1996; Farkas *et al.,* 2007). Atualmente, está bem estabelecido na literatura que a hipoperfusão compromete o aprendizado em ratos (Liu *et al*., 2005; Shang *et al*., 2005); o que ainda se tenta é estabelecer até quanto tempo após a cirurgia este déficit permanece. Liu e colaboradores (2005) demonstraram que estes danos ainda estavam presentes com 20 semanas pós cirurgia, enquanto nossos experimentos revelam efeitos que perduram até 6 meses após o evento lesivo.

Somado a estes testes comportamentais, outras tarefas cognitivas estão sendo aplicadas ao modelo de 2 vasos, como por exemplo a esquiva inibitória e o *T-Maze*, com o objetivo de analisar a ansiedade destes animais; e uma Tarefa de memória não-

espacial denominada Reconhecimento de Objetos (RO). Neste último, os roedores são apresentados a objetos familiares e novos, sendo esperado que os animais dediquem mais tempo a exploração do objeto novo. Este comportamento típico tem sido utilizado no desenho de um paradigma comportamental conhecido como tarefa de RO (Ennaceur e Delacour, 1988), o qual vem sendo amplamente utilizado para avaliar os mecanismos envolvidos na formação de memórias declarativas (Reed *et al*., 1999; Moses *et al.,* 2005). Existem poucos trabalhos que avaliaram o comportamento dos animais submetidos a HEC nesta tarefa. Sarti e colaboradores (2002) demonstraram em seu estudo que o dano a memória não-espacial permanecia até 90 dias após o evento. Nossos resultados relatam a permanência deste dano cognitivo somados ao déficit da memória espacial, até 180 dias após a isquemia.

Trabalhávamos com a hipótese de que a hipoperfusão cerebral induziria atrofia neural tanto na região hipocampal como no estriado, mas isto não foi confirmado. Os resultados dos capítulos 1 e 2 mostram que não existe relação entre o déficit cognitivo e os danos morfológicos em hipocampo e estriado, corroborando com outros achados que demonstram pobre, ou nenhuma, relação entre a perda de neurônios nesta região com a performance no Water Maze (Jaspers *et al.,* 1990; Lyeth *et al*., 1990; Olsen *et al*., 1994, Murakami *et al*. ,2005). O declínio cognitivo associado com o distúrbio nos parâmetros de energia cerebral, causados pela hipoperfusão já foram relatados (Ueda *et al*., 2000; Plaschke *et al*., 1999, 2001). Alguns estudos não concordam com esta afirmação, relatando haver uma associação direta (De Jong *et al*., 1999; Nunn and Hodges, 1994; Pappas *et al*., 1996). Portanto, não há concordância sobre este aspecto na literatura especializada.

Frente a estes achados, resolvemos analisar microscopicamente o comportamento neuronal e de suas células vizinhas, especificamente os astrócitos.

Atualmente além dos neurônios, tem-se dado atenção especial ao papel de um segundo componente, a astroglia circundante (VICENTE *et al*., 2009), que atua regulando o microambiente extracelular (FELLIN *et al*., 2006). A GFAP é o marcador mais utilizado para identificar a reatividade astrocítica. Se, por um lado sua expressão normalmente está aumentada após lesões do SNC (RIDET *et al.,* 1997), a reatividade astroglial também tem sido reportada como participante da plasticidade neuronal em animais saudáveis (SIREVAAG *et al*., 1991) e após lesão (BRIONES *et al*., 2006, VICENTE *et al.,* 2009). Observamos que os níveis de GFAP encontram-se aumentados no hipocampo principalmente na fase aguda, após o evento isquêmico. Em relação às células neuronais, os níveis apresentam-se diminuídos e obedecem o mesmo comportamento dos astrócitos, apresentando diminuição de sua expressão nas primeiras semanas após o evento, desaparecendo na fase crônica.

Atualmente, sabe-se que existem populações de células que apresentam uma vulnerabilidade diferente em relação a isquemia. O dano depois de uma isquemia global ocorre principalmente na região CA1 do hipocampo e, em menor extensão, em certas sub-regiões do neocórtex, tálamo e estriado (SCHREIBER & BAUDRY, 1995). No hipocampo, as células piramidais da região CA1 são sensíveis, enquanto as células piramidais da região vizinha CA3 e giro denteado são resistentes (YANG *et al*., 2000). Outra característica importante é que nenhum dano significativo é encontrado até 3-4 dias após o evento isquêmico, sendo conhecido este fenômeno como "morte celular tardia" (HSU *et al*., 1994; KIRINO, 2000). No capítulo 2 desta tese, demonstramos uma diminuição da intensidade da imunofluorescência de uma proteína nuclear específica de neurônios (NeuN), principalmente nos primeiros dias após a lesão até aproximadamente 3 meses pós-isquemia, desaparecendo na fase mais crônica do modelo (6 meses).

É importante mencionar que a reação astrocitária ao evento isquêmico não é necessariamente acompanhada por morte neuronal, e que, os astrócitos por si só, podem reagir a redução do fluxo sanguíneo cerebral através de mecanismos mediados por alterações no metabolismo energético, formação de radicais livres e/ou produção de citocinas (VAGNOZZI *et al*., 1997; ZIMMER *et al.,* 1991).

Estudos relacionam uma reação glial associada a perda neuronal em ratos submetidos a hipoperfusão (ZIMMER *et al*., 1991), contribuindo para uma deficiência na memória e aprendizado, principalmente pela interrupção da comunicação entre a circuitaria neuronal (BENNET *et al*., 1998; LIU *et al*., 2005). Nosso trabalho demonstra esta relação aos 3 meses após o evento isquêmico, fase caracterizada como crônica no modelo de 2 vasos, período que mais se assemelha as condições de redução do FSC em humanos idosos e portadores de demência (FARKAS *et al*., 2007).

Após esta fase, o FSC começa a se restabelecer e cabe salientar que a hipoperfusão crônica é tolerada pelos ratos devido a mecanismos compensatórios e adaptativos, como por exemplo, dilatação arterial, recrutamento de capilares não-perfundidos, angiogênese e regulação bioquímica (FARKAS *et al*., 2007; PLASCHKE *et al*., 2001).

Conforme descrito nos capítulos 3 e 4 desta Tese, buscamos verificar os efeitos do exercício físico forçado e do enriquecimento ambiental sobre a memória espacial de referência e de trabalho, assim como no estado oxidativo cerebral. Além do hipocampo, outras estruturas também foram estudadas, como o córtex cerebral e o estriado, mas somente na primeira estrutura obtivemos resultados significativos.

O hipocampo apresenta um papel essencial no processo de aprendizado, mas ao mesmo tempo, conforme citado acima é altamente sensível a eventos isquêmicos (NI *et*

al., 1994; PAPPAS *et al*., 1996). A degeneração neuronal causada pela isquemia também é observada em outras estruturas, como o estriado, córtex cerebral e tálamo (FREUND *et al*., 1990; PEREIRA *et al.,* 2009), mas as alterações mais severas causadas pela hipoperfusão crônica acontecem no hipocampo (FARKAS *et al*., 2007).

Juntamente com o declínio cognitivo e dano celular, a hipoperfusão cerebral crônica causa um dano oxidativo no encéfalo de ratos. Estudos prévios revelam um papel importante na patogênese de desordens neurodegenerativas como na DA e na Demência Vascular (CHONG *et al.,* 2005; COYLE & PUTTFARCKEN, 1993). Sugere-se que os radicais livres e a lipoperoxidação são pontos-chave no desenvolvimento de lesões provocadas pela hipoperfusão no Sistema Nervoso Central (MARKESBERY, 1997), juntamente com a estimulação de defesas antioxidantes (HALLIWELL, 1991).

Nossos dados demonstraram o desequilíbrio causado pela hipoperfusão tanto no estado oxidativo, como também das defesas antioxidantes cerebrais, com o aumento dos níveis de lipoperoxidação e do conteúdo dos tióis, um eficiente antioxidante, aos 3 meses após o evento lesivo, corroborando com alguns autores (AYTAC *et al*., 2006; YANPALLEWAR *et al*., 2004; NINGARAJ & RAO, 1998).

O tecido cerebral hipoperfundido torna-se muito vulnerável ao dano oxidativo (GHONEIM *et al.,* 2002). Acredita-se que os radicais livres sejam os responsáveis pela chamada injúria pós-reperfusão que acontece após o evento isquêmico através dos mecanismos compensatórios do animal; onde estes radicais livres iniciariam a lipoperoxidação, causando danos em componentes macromoleculares das células (GRINGO, 1997; NAKASHIMA *et al*., 1999). YAMPALLEVAR e colaboradores (2005) ao testar alguns parâmetros do estresse oxidativo 2 semanas após a cirurgia,

nenhuma diferença significativa foi encontrada entre os grupos. Portanto, sugere-se que estas alterações bioquímicas se instalem na fase crônica do modelo, juntamente com o déficit cognitivo. Diversos estudos relatam uma direta relação entre diminuição na performance de tarefas comportamentais e disfunções em sistemas neuroquímicos em muitas regiões cerebrais (BRANDEIS *et al*., 1993; MCNAMARA & SKELTON, 1993; RAGHAVENDRA *et al*., 2007).

Portanto, tanto a atividade física quanto o enriquecimento ambiental atenuam significativamente estes efeitos deletérios, sugerindo um papel positivo no aprendizado e memória a longo prazo em ratos hipoperfundidos, confirmando nossa hipótese principal Na literatura não encontramos trabalho prévio que tenha investigado os efeitos destas estratégias terapêuticas pré, pós e pré+pós-isquemia em parâmetros cognitivos e bioquímicos. A maioria dos estudos que envolvem o modelo de hipoperfusão e estresse oxidativo, demonstram o efeito neuroprotetor de fármacos ou plantas (YAMPALLEWAR *et al*., 2004; 2005).

Muitos estudos sugerem que o exercício físico possui a capacidade de produzir efeitos benéficos em certas regiões cerebrais, além de alterações positivas no balanço homeostásico, envolvendo alterações hormonais, metabólicas e imunológicas (RADAK *et al*., 2005). Estudos de mapeamento metabólico sugerem que o hipocampo, o córtex motor e o estriado apresentam alta atividade neuronal durante o exercício, portanto, podem sofrer plasticidade cerebral relacionada à atividade física (VISSING *et al.*, 1996).

Outras evidências têm demonstrado que a intensidade moderada regular do exercício físico, a mesma utilizada neste trabalho, provoca efeitos favoráveis no encéfalo, incluindo propriedades neuroprotetoras. Por exemplo, o exercício em esteira,

por 30 min., 1 vez ao dia, durante 10 dias, aumentou a memória e reduziu a morte celular neuronal induzida pela isquemia no giro denteado de gerbilos (SIM *et al*., 2005). Em adição, corrida na esteira em baixa intensidade previne a perda celular neuronal e o dano induzido por agentes citotóxicos (CARRO *et al*., 2001).

Estudos relatam que o comportamento sedentário está relacionado com o aumento de risco de isquemias cerebrais (GILLUM e INGRAM, 1996). Mesmo com o conhecimento empírico de que o exercício físico tem efeito protetor em doenças isquêmicas cardíacas e cerebrais, poucos estudos têm examinado os efeitos protetores da atividade física contra as doenças cerebrovasculares.

É comum, na prática de centros de reabilitação, o uso de tratamento e programas de treinamento para pacientes que sofreram um processo isquêmico. Estudos recentes sugerem que a intensidade da manipulação, como a duração, o tipo e o tempo de atividade empregada, podem ter profundos efeitos na recuperação destes indivíduos (YANG *et al.,* 2003). Em relação ao tipo de atividade física realizada, resultados interessantes foram descritos por SACCO e colegas (1998) onde observaram as relações entre os benefícios e o tempo de prática de atividade física. A prática de atividade física mais freqüentemente observada foi a caminhada, que quando praticada moderadamente traz benefícios à saúde.

Efeitos positivos da atividade física sobre o estado oxidativo do sistema nervoso central de roedores têm sido reportados para natação (Jolitha *et al*., 2006), treinamento em esteira (Cosk *et al*., 2005; Somani *et al*., 2005) e exercício voluntário realizado nas rodas de corrida (Suzuki *et al*., 1993). Existem muitos estudos que comprovam os efeitos positivos do exercício na saúde cerebral (Radák *et al*., 2001), prevenindo desordens neurodegenerativas relacionadas a idade avançada (Mattson, 2000). Além de demonstrar efeitos positivos sobre o aprendizado e memória

principalmente a longo prazo (Van Praag *et al*., 1999;; Ogonovszky *et al*., 2005), o exercício também contribui de maneira positiva atenuando os danos oxidativos em ratos mais velhos, com a regulação de enzimas antioxidantes tornando as células mais resistentes a eventos danosos (POWERS *et al*., 1994; LEEUWEENBURGH *et al.*, 1997; SERVAIS *et al*., 2003). Por outro lado, são poucos os estudos nos quais a natação e o treinamento em esteira não afetam as defesas antioxidantes cerebrais (RADÁK *et al*., 2006; OZKAYA *et al*., 2002). A maioria dos autores demonstram que a atividade física regular pode induzir uma adaptação do sistema antioxidante celular frente uma contínua geração de radicais livres (POWERS *et al*., 1994; LEEUWEENBURGH *et al.*, 1997; SERVAIS *et al*., 2003).

Da mesma forma, o enriquecimento ambiental traz benefícios interessantes ao funcionamento cerebral. Os mecanismos moleculares e celulares mediadores dos efeitos do ambiente, em particular níveis de atividade física e mental, vem sendo paulatinamente elucidados. Enquanto há diversos estudos focando nos efeitos do enriquecimento ambiental em roedores e outros modelos experimentais (LAVIOLA *et al*., 2008; SALE *et al*., 2009), estudos relevantes com trabalhos em humanos não tem sido discutidos em detalhe (NITHIANANTHARAJAH & HANNAN, 2009). Atualmente, quando o assunto é estimulação social, ambiental e física, muitos autores utilizam o termo "reserva cerebral" e "reserva cognitiva" para explicar um dos possíveis mecanismos dos benefícios cognitivos destas estimulações a nível cerebral. Estes dois termos começaram a ser utilizados na década passada (GRAVES *et al*., 1996; STERN, 2002, 2006), sendo o primeiro conceituado como capacidade cerebral neuroprotetora que pode ser induzido pelo enriquecimento crônico mental e físico (STERN *et al*., 1995; VALENZUELA, 2008; VALENZUELA & SACHDEV, 2006a,b). Além disso, reserva cognitiva é um termo mais específico para o aumento da função cognitiva e mental

como fatores protetores contra demências e outras desordens (ANDEL *et al.,* 2006; LÊ CARRET *et al*., 2005; RICHARDS & DEARY, 2005).

Vários autores indicam que indivíduos que mantém altos níveis de atividade física e mental (mensurados através do nível educacional máximo, história de trabalho, prática de hobbies, entre outros) apresentam menor probabilidade de desenvolver DA e outras demências (revisado por VALENZUELA *et al*., 2008), principalmente pelo fato da função neuroprotetora da reserva cognitiva acumulada durante anos de estímulos (CAAMANO-ISORNA *et al*., 2006; VALENZUELA & SACHDEV, 2006a,b). Somado a isso, um aumento de atividade mental apresenta relação direta com reduzidos níveis de atrofia hipocampal (VALENZUELA *et al*., 2008).

Na pesquisa básica experimental, está bem descrito que esta estratégia terapêutica provoca um ganho significativo na memória espacial tanto de referência como de trabalho e no aprendizado em inúmeros modelos experimentais (BINDU, REKHA, & KUTTY, 2005; ESCORIHUELA *et al*., 1995; KEMPERMANN, KUHN, & GAGE, 1997; PEREIRA *et al*., 2009). Ainda, a estimulação ambiental tem sido apontada como fator importante na prevenção e tratamento de doenças neurodegenerativas em humanos (BRIONES, 2006). O capítulo 4 também comprova este achado no modelo experimental de ratos submetidos ao procedimento de hipoperfusão encefálica crônica, tanto realizado na fase pré quanto pós-isquemia, confirmando uma das hipóteses do trabalho.

Os mecanismos pelos qual isto ocorre parecem envolver uma série de fatores, incluindo aumento no número de dendritos e células gliais fagocitárias (FAHERTY, KERLEY, & SMEYNE, 2003), redução da resposta inflamatória (AMBREÉ *et al.,* 2006), aumento da angiogênese e neurogênese hipocampal (HERRING et al., 2008).

Somado a estes fatores, alguns autores demonstram a eficácia desta combinação de interação social, exercício voluntário e exposição contínua ao aprendizado na atenuação de processos pró-oxidativos, com aumento das defesas antioxidantes em modelos de demência de Alzheimer (ADLARD *et al.,* 2005, HERRING *et al.,* 2008).

Em suma, observamos que o modelo de Hipoperfusão Cerebral Crônica causa um importante prejuízo cognitivo que pode ser avaliado de forma fidedigna utilizando-se o teste do *Water Maze* e Reconhecimento de Objetos em ratos *Wistar*. Além disso, este modelo de isquemia global induziu alterações nas células neuronais da região CA1 do hipocampo e astrócitos vizinhos na fase mais aguda do modelo, sem produzir alterações macroscópicas importantes. Bioquimicamente, o modelo 2VO causa alterações significativas, especificamente sobre a lipoperoxidação e a atividade de substâncias antioxidantes, amenizadas tanto pelo exercício físico regular forçado, como pelo enriquecimento ambiental. Esses resultados ampliam os conhecimentos acerca da isquemia global, sobre as formas de avaliação comportamental, morfológica e bioquímica e sobre os efeitos das estratégias terapêuticas experimentais utilizadas para reabilitação.

8. CONCLUSÕES

Os dados obtidos por meio dos trabalhos desenvolvidos nesta tese permitem concluir que:

- O modelo modificado de Hipoperfusão Encefálica Crônica, no qual as duas carótidas são ocluídas com uma semana de intervalo, apresenta um aumento significativo na sobrevida dos animais e produz déficit cognitivo na Tarefa do *Water Maze* e no Teste de Reconhecimento de Objetos similar aos 3 meses após o evento isquêmico, sem causar danos macroscópicos ao tecido hipocampal e estriatal, quando comparados ao modelo padrão.

- O déficit cognitivo, em tarefas de memória espacial (referência e trabalho) e não-espacial, geralmente se estabelece na fase crônica do modelo, aparecendo aproximadamente aos 3 meses após a cirurgia e perdurando até os 6 meses; diferentemente os danos neuronal e glial acontecem na fase aguda e desaparecem depois de algumas semanas.

- O exercício físico forçado, quando realizado após o evento isquêmico e durante toda a vida do animal foi capaz de reverter o dano cognitivo causado pelo modelo de HEC. Já o enriquecimento ambiental reverteu os efeitos da HEC quando realizado pré-, pós- ou pré+pós isquemia.

- O modelo dos 2 vasos causa dano oxidativo no tecido hipocampal em ratos 3 meses após a isquemia, mensuráveis através do conteúdo de radicais livres, dano a macromoléculas e níveis de enzimas antioxidantes. Tanto a atividade física forçada quanto o enriquecimento ambiental foram capazes de reverter totalmente tais efeitos.

9. PERSPECTIVAS

Os resultados aqui relatados permitem conceber a continuidade dos estudos utilizando este modelo de Hipoperfusão Cerebral. É interessante investigar o possível efeito neuroprotetor tanto da atividade física forçada quanto do enriquecimento ambiental nas alterações neuronais e gliais que acontecem na fase aguda deste modelo. Também, analisar outros parâmetros que possam dar mais suporte para o estudo da relação entre as 2 estratégias terapêuticas aqui estudadas e o estado oxidativo do hipocampo de ratos submetidos a hipoperfusão crônica. Além disso, seria interessante investigar outras variáveis morfológicas possivelmente envolvidas com a hipoperfusão e conseqüente declínio cognitivo, como por exemplo, arborização dendrítica e densidade de espinhos dendríticos nas células piramidais do hipocampo.

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