UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
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Efeito do exercício físico materno sobre parâmetros bioquímicos e comportamentais em ratos Wistar submetidos à hipóxia-isquemia neonatal

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Porto Alegre, 15 de Maio de 2015

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

Albert Einstein (1879-1955)

Agradecimentos

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Sumário

Parte I

Resumo

A encefalopatia causada pela hipóxia-isquemia durante o período neonatal é frequente e extremamente prejudicial para o encéfalo do recém-nascido. O estresse oxidativo é um componente da sua fisiopatologia e está relacionado à morte celular encontrada em animais submetidos ao modelo de hipóxia-isquemia, o que resulta em danos às células neurais, expresso em problemas cognitivos e alterações comportamentais. O exercício físico durante a gestação possui potencial terapêutico, considerando os benefícios que ele produz tanto para a mãe quanto para o feto. Nosso grupo demonstrou o efeito neuroprotetor que a natação materna apresenta para o filhote de ratos, através de uma regulação antioxidante positiva e biogênese mitocondrial. Com isso o objetivo deste trabalho foi avaliar se a adaptação proporcionada pelo exercício materno é capaz de amenizar os efeitos prejudiciais da hipóxia-isquemia neonatal e se eles persistem até a fase adulta, se manifestando através de alterações comportamentais. Ratas Wistar adultas realizaram o protocolo de natação materna antes e durante a gestação. Os filhotes dessas mães foram submetidos ao modelo de hipóxia-isquemia 7 dias após o seu nascimento, permanecendo na caixa com cuidados maternos até completarem 21 dias de vida, quando ocorreu o desmame e uma parte dos animais foi eutanasiada. Cerebelo, córtex parietal, hipocampo e estriado foram dissecados e utilizados para a avaliação dos parâmetros de estresse oxidativo. A outra parte dos animais permaneceu no biotério até a fase adulta, quando realizaram os testes comportamentais juntamente com a quantificação dos níveis de BDNF nas mesmas estruturas. Os animais que foram submetidos ao modelo de hipóxia-isquemia apresentaram uma modulação antioxidante dependente de estrutura juntamente com um aumento da atividade motora no campo aberto. Nas estruturas do hipocampo e córtex parietal foi observada uma diminuição dos níveis de espécies reativas, enquanto no estriado ocorreu um aumento estatisticamente significativo. O protocolo de nado materno apresentou uma melhora na memória de longo prazo no reconhecimento de objetos, porém, essa modulação não foi suficiente para deter os efeitos deletérios da encefalopatia neonatal induzida pela hipóxia-isquemia. Os níveis de BDNF não foram significativamente alterados em nenhum grupo experimental. Nossos dados sugerem que a hipóxia-isquemia produz efeitos deletérios sobre o encéfalo, demonstrados por alterações bioquímicas e comportamentais, que não puderam ser prevenidas pelo exercício de natação materno.

Abstract

Encephalopathy caused by hypoxia-ischemia in the neonatal period is frequent and extremely harmful to the brain of the newborn. Oxidative stress is a component of the pathophysiology and is linked to cell death found in animals subjected to hypoxia-ischemia model, which results in damage to neural cells, expressed as cognitive impairment and behavioral changes. Exercise during pregnancy has therapeutic potential, considering the benefits it produces for both mother and fetus. Our group demonstrated the neuroprotective effect that maternal swimming presents to the offspring of rats, through a positive antioxidant regulation and mitochondrial biogenesis induction. Therefore, the objective of this study was to evaluate whether the adaptation provided by maternal exercise can minimize the harmful effects of neonatal hypoxia-ischemia and if they persist into adulthood, manifesting through behavioral changes. Adult female Wistar rats performed the maternal swimming protocol before and during pregnancy. The pups delivered were submitted to the model of hypoxia-ischemia 7 days after birth, remaining in the box with maternal care until they are 21 days of life, when they were weaning and a part of the animals was euthanized. Cerebellum, parietal cortex, hippocampus, and striatum were dissected and used for the evaluation of oxidative stress parameters. The other part of the animals remained in the animal facility until adulthood, when performed behavioral tests along with the quantification of BDNF levels in the same structures. The animals which were subjected to hypoxia-ischemia model presented an antioxidant modulation dependent structure, along with an increase in motor activity in the open field. In hippocampal and parietal cortex structures were observed a decrease in the levels of reactive species, while the striatum had a statistically significant increase. Maternal swimming protocol showed an improvement in long-term memory evaluated by object recognition task, however, this modulation was not enough to stop the deleterious effects of neonatal encephalopathy induced by hypoxia-ischemia. BDNF levels were not significantly altered in any experimental group. Our data suggest that hypoxia-ischemia produces deleterious effects on the brain, demonstrated by biochemical and behavioral changes, which could not be prevented by maternal swimming exercise.

Lista de abreviaturas

AKT ..... proteína cinase B

AMPK ..... proteína cinase ativada por AMP

BDNF ..... fator neurotrófico derivado do encéfalo

CA1 ..... região 1 do Corno de Amon

CAT ..... catalase

DCF ..... diclorofluoresceína

ERK 1/2 ..... proteína cinase regulada por sinal extracelular 1 e 2

ERN ..... espécies reativas de nitrogênio

ERO ..... espécies reativas de oxigênio

GPx ..... glutationa-peroxidase

GSH ..... glutationa reduzida

H_2O_2 ..... peróxido de hidrogênio

HI ..... hipóxia-isquemia

HIF-1 ..... fator induzido por hipóxia-1

HOCl ..... ácido hipocloroso

HSF ..... fator de transcrição das proteínas de choque térmico

JNK ..... c-jun N-terminal proteína cinase

KEAP-1 ..... Kelch-like ECH-associated protein 1

MAPK ..... proteína cinase ativada por mitógenos

NF-κβ ..... fator de transcrição nuclear kappa beta

NGF ..... fator de crescimento neural

$NO^{\bullet}$ ..... óxido nítrico

NOS ..... óxido-nítrico-sintase

Nrf2 ..... fator eritroide nuclear 2

$O_2^{\bullet -}$ ..... superóxido

$^{\bullet}OH$ ..... radical hidroxil

$ONOO^-$ ..... peroxinitrito

p38 ..... p38 MAP-cinase

p53 ..... proteína supressora de tumor p53

PGC1-α ..... coativador de transcrição 1α do receptor ativado por proliferação peroxissomal

PON 2/3 ..... paraoxonase isoformas 2 e 3

Sirt1 ..... sirtuína-1

SNC ..... sistema nervoso central

SOD ..... superóxido-dismutase

TNFα ..... fator de necrose tumoral α

TRAP ..... potencial antioxidante total não enzimático

TrkB ..... receptor tirosina-cinase B

Trx ..... tiorredoxina

I. Introdução

1. Exercício físico

O termo atividade física está relacionado a qualquer movimento produzido pelos músculos esqueléticos com gasto energético, o que inclui atividades voluntárias, comuns do cotidiano e lazer. O exercício físico pode ser classificado como uma atividade física planejada, de forma repetida e regular, com diferentes objetivos que variam de acordo com o trabalho realizado (Caspersen et al., 1985). O tempo ideal recomendado pela Sociedade Brasileira de Medicina do Esporte para atingir os benefícios do exercício físico é de 150 minutos por semana, de forma contínua ou intercalada. O mais recomendado é 30 minutos, 5 vezes por semana, ou 3 dias na semana com sessões de 50 minutos.

O exercício físico promove adaptações metabólicas em diversos órgãos, o que faz com que ele seja reconhecido como potencial ferramenta terapêutica, podendo ser utilizado na prevenção e no tratamento de algumas doenças como obesidade, diabetes mellitus tipo 2, problemas cardíacos, depressão, osteoporose, entre outras (Dishman et al., 2006; Powell et al., 2011; Wolff et al., 2011). A prática de exercício físico aeróbico aumenta a captação de glicose, promove angiogênese, e modula respostas inflamatórias (Cotman et al., 2007; Dishman et al., 2006; Itoh et al., 2011; Nicklas et al., 2005; Ratey e Loehr, 2011).

A atividade muscular necessária para a realização do exercício físico moderado exige o aumento do consumo de oxigênio pela mitocôndria, a fim de suprir a demanda de ATP; o que traz como consequência o aumento da

atividade da cadeia transportadora de elétrons, levando a uma maior produção de espécies reativas de oxigênio (ERO), podendo causar um desequilíbrio redox celular (Radak et al., 2008). Para manter a homeostase redox, a célula utiliza um sistema de defesa antioxidante, induzindo o aumento da atividade de suas principais enzimas, evitando assim que ocorram danos a proteínas, lipídios e/ou DNA (Elokda et al., 2010; Radak et al., 2008; Somani et al., 1995; Tsou et al., 2015).

O desempenho em tarefas que avaliam o aprendizado e a memória também é melhorado em animais que realizam exercício físico. Acredita-se que o aumento da neurogênese e de fatores neurotróficos, tais como o fator neurotrófico derivado do encéfalo (BDNF) e o fator de crescimento neuronal (NGF), esteja associado a esse efeito neuroprotetor (Dishman et al., 2006; Gerecke et al., 2012; Wu et al., 2011). Estudos em ratos submetidos ao exercício em esteira rolante mostraram um aumento no crescimento de espinhos dendríticos em algumas regiões corticais (Hwang et al., 2010) juntamente com uma melhora no aprendizado/memória e uma maior vascularização cerebral (Cotman e Berchtold, 2002; van Praag et al., 1999; Vaynman et al., 2004).

Trabalhos na literatura mostram que o exercício físico pode contribuir para a recuperação pós-isquêmica em ratos. Choi et al. (2013) mostraram que o exercício em esteira durante um período curto, pós-isquemia, pode fornecer uma estratégia útil para a recuperação das complicações cerebrais causadas pela lesão da hipóxia-isquemia (HI) cerebral, diminuindo a fragmentação de DNA e inibindo a expressão da caspase-3.

As mudanças induzidas pelo exercício são consequência de modulações em algumas cascatas de sinalização (Figura 1). Além de alterações nas concentrações de cálcio, o exercício físico altera a atividade de algumas vias de sinalização, mediadas por proteína cinase ativada por mitógenos (MAPK), proteína cinase B (Akt), proteína cinase regulada por sinal extracelular 1 e 2 (ERK 1/2), p38 MAP-cinase (p38) e c-jun N-terminal proteína cinase (JNK), resultando em diferentes tipos de ações de acordo com o seu respectivo alvo (Ji et al., 2004; Sakamoto et al., 2004; Vina et al., 2014).

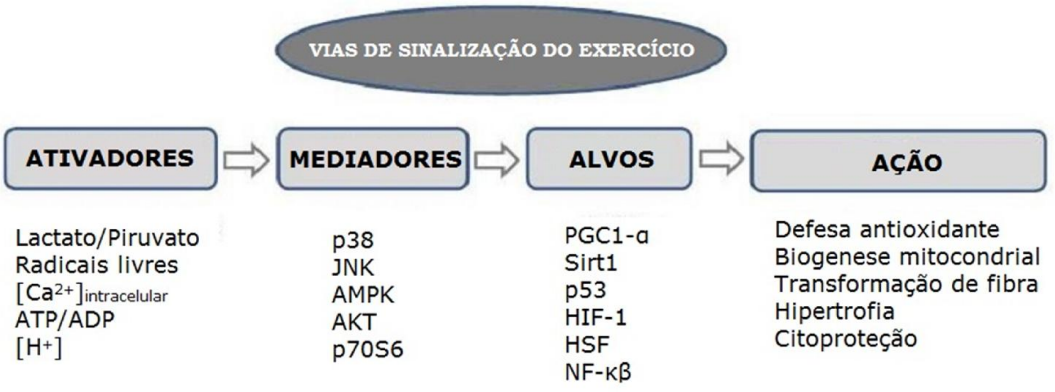

Figura 1- Vias de sinalização celular envolvidas na adaptação metabólica em musculo esquelético induzida pelo exercício físico. Adaptado de Vina et al. (2014). Abreviaturas: p38: p38 MAP-cinase, JNK: c-jun N-terminal proteína cinase, AMPK: proteína cinase ativada por AMP, AKT: proteína cinase B, PGC1-α: coativador de transcrição 1α do receptor ativado por proliferação peroxissomal, Sirt1: sirtuína-1, p53: proteína supressora de tumor p53, HIF-1: fator induzido por hipóxia-1, HSF: fator de transcrição das proteínas de choque térmico, NF-κβ: fator de transcrição nuclear kappa beta.

1.1. Exercício físico materno

Estudos sobre a prática de exercício físico durante a gravidez são necessários para elucidar e informar melhor a população sobre os riscos e benefícios que ela proporciona tanto para a mãe quanto para o feto em desenvolvimento, uma vez que é um período extremamente crítico no desenvolvimento dos órgãos e sistemas, tornando-os mais vulneráveis a fatores externos como a dieta da mãe, hormônios e estresse (Meaney e Aitken, 1985). O exercício físico de intensidade moderada durante a gravidez reduz o risco de pré-eclâmpsia, diabetes gestacional, nascimento prematuro, melhora a tolerância à dor e reduz o ganho de peso corporal materno (Gaston e Cramp, 2011). O exercício aquático é o mais recomendado para as gestantes em função do controle do peso corporal materno, da manutenção da temperatura corporal e do baixo impacto nas articulações (Hartmann e Bung, 1999; Lynch et al., 2003).

A fim de esclarecer o efeito do exercício materno sobre a prole, alguns modelos de nado "involuntário" em ratos têm sido utilizados, demonstrando efeitos benéficos para a mãe e para a prole. A avaliação do exercício de natação materno demonstrou uma melhora na fase de aquisição e retenção de memória na tarefa do labirinto aquático de Morris, assim como o aumento de células hipocampais na região 1 do Corno de Amon (CA1) e no giro denteado de filhotes de ratas que realizaram exercício durante a gravidez (Akhavan et al., 2008; Lee et al., 2006). Nosso grupo demonstrou que o exercício de nado materno induz uma programação bioquímica no encéfalo dos filhotes, induz biogênese mitocondrial e apresenta uma modulação antioxidante com o aumento da atividade da superóxido-dismutase (SOD), catalase (CAT) e

glutationa-peroxidase (GPx), juntamente com a melhora do potencial antioxidante total não enzimático (TRAP). Foi detectada também a produção de espécies reativas pela oxidação da diclorofluoresceína (DCF), associada ao aumento de produtos da oxidação do óxido nítrico (NO˙); porém, não foram observadas alterações nos níveis de carbonilas e produtos de lipoperoxidação, indicando uma programação positiva no metabolismo oxidativo do encéfalo dos filhotes cujas mães sofreram o protocolo de nado forçado (Marcelino et al., 2013).

Em um trabalho utilizando o modelo de exercício aquático em ratos adultos, ocorreu a prevenção da liberação excessiva de glutamato na fenda sináptica, evitando a excitotoxicidade, aliado à diminuição de marcadores de apoptose em cultura de fatias de hipocampo de roedores (Mourao et al., 2014). A literatura tem mostrado que mesmo doenças neurodegenerativas graves podem ser prevenidas pelo exercício materno. Um modelo murino transgênico para a doença de Alzheimer foi submetido a exercício voluntário em roda de corrida durante a prenhez, e a prole desses camundongos apresentou uma redução no desenvolvimento das placas de peptídeo β-amiloide, do processo inflamatório, do estresse oxidativo, da disfunção neurovascular e um aumento de marcadores de plasticidade cerebral (Herring et al., 2012).

2. Encefalopatia hipóxico-isquêmica

Uma das causas mais comum de encefalopatia perinatal é a HI neonatal, com uma frequência de aproximadamente 1-6:1000 recém-nascidos (du Plessis e Volpe, 2002), causando sequelas como: paralisia cerebral, retardo mental, epilepsia, alterações sensoriais, motoras e transtornos de aprendizado (de Paula et al., 2009; Volpe, 2001), assim como deficiência em algumas

habilidades cognitivas e alguns tipos de memória (Arteni et al., 2003; Arteni et al., 2010; Brockmann et al., 2013; Pereira et al., 2007). As causas mais comuns da HI são: interrupção do fluxo sanguíneo umbilical, dificuldade de troca de gases pela placenta, prematuridade do neonato e estresse durante o parto (Procianoy e Silveira, 2001). A imaturidade do sistema nervoso central (SNC) dos filhotes que sofrem esse processo é um dos fatores mais agravantes para a ocorrência de danos, que podem ocorrer devido a uma "janela de suscetibilidade" criada pela comunicação entre a excitotoxicidade, mecanismos inflamatórios e estresse oxidativo (Ferriero, 2004).

Por volta de 1980 ainda não havia nenhum modelo experimental para estudar os efeitos da HI perinatal em roedores, apenas o efeito da obstrução da carótida e privação de oxigênio em ratos adultos. Na década de 80, Rice et al. (1981) desenvolveu um modelo adaptado de Levine (1960), utilizando ratos de 7 dias pós-natal. Este modelo tem sido utilizado por muitos autores a fim de elucidar os efeitos da asfixia perinatal em diversos parâmetros como alterações moleculares, alterações cognitivas, morfológicas, motoras e bioquímicas (Arteni et al., 2003; Ikeda, 2008). A lesão que o protocolo de HI causa no encéfalo dos filhotes é unilateral e ipsilateral à obstrução da carótida. Os dados obtidos podem ajudar a compreender as consequências desse fenômeno em humanos recém-nascidos que sofreram essa encefalopatia.

A perda de funcionalidade dos neurônios e o comprometimento de suas funções básicas como a despolarização e a repolarização são os principais indícios de morte celular induzida pela HI. Um dos mecanismos responsáveis pela ativação de apoptose em células afetadas é uma alteração na permeabilidade da mitocôndria, com a abertura de poros permanentes de

membrana liberando o citocromo *c* e ativando a via das caspases (Blomgren e Hagberg, 2006; Feng et al., 2003; Wang et al., 2009). A ativação dessas cascatas bioquímicas é consequência da privação de oxigênio que ocorre durante um período curto de tempo, variando de acordo com o modelo estudado; sendo seguido por um período de reperfusão, caracterizado pela ativação da cadeia transportadora de elétrons e da fosforilação oxidativa, o que pode gerar um aumento da glicólise e produção excessiva de ATP, associado ao possível aumento de ERO (Blomgren e Hagberg, 2006; Duarte et al., 2003; Ferriero, 2004).

3. Estresse oxidativo

A formação de um radical livre ocorre quando uma molécula ganha ou perde um elétron, resultando em um elétron desemparelhado em seu orbital mais externo, o que lhe confere alta reatividade e o potencial de extração de um elétron das biomoléculas próximas a fim de completar sua estrutura eletrônica. O termo espécie reativa é mais geral e engloba radicais livre e alguns compostos não radicalares que podem gerar radicais livres. ERO, tais como o superóxido ($O_2^{\bullet -}$), o peróxido de hidrogênio (H_2O_2), o radical hidroxil ($^{\bullet}OH$); e espécies reativas de nitrogênio (ERN), como o $NO^{\bullet}$ e o peroxinitrito ($ONOO^-$) são as principais espécies reativas encontradas nos sistemas biológicos (Halliwell e Gutteridge, 2007). A produção de radicais livres é comum nos sistemas biológicos, sendo provenientes do escape de elétrons na cadeia respiratória mitocondrial, do retículo endoplasmático e de diversas reações enzimáticas, tais como a óxido-nítrico-sintase (NOS), xantina-oxidase, NADPH-oxidase, mieloperoxidase e citocromo-P450. Além disso, fontes exógenas também podem induzir a síntese de espécies reativas, tais como a radiação

ionizante, ultravioleta e alguns compostos tóxicos presentes na fumaça e como contaminantes de alimentos (Commoner et al., 1954; Cooke et al., 2003).

Um dos exemplos mais primitivos, em uma análise evolutiva, de uma reação mediada por espécies reativas que gera um potencial radical livre causador de dano oxidativo é a reação de Fenton (Fenton et al., 1964; Gutowski e Kowalczyk, 2013). Ela pode ocorrer *in vivo* quando H_2O_2 reage com Fe^{2+} formando o radical $^{\bullet}OH$ (Halliwell, 1982), um dos radicais mais danosos e deletérios aos sistemas biológicos devido a sua meia vida curta, e a falta de enzimas antioxidantes que possam atuar sobre essa espécie. A produção de espécies reativas no SNC pode levar à morte celular, estando associada a doenças neurodegenerativas como Alzheimer e Parkinson (Ahlskog, 1990; Bowling e Beal, 1995; Dubinina et al., 2015; Seet et al., 2010). Porém, ela também está envolvida em processos fisiológicos como o envelhecimento, a sobrevivência celular, a imunidade, a inflamação, a vasodilatação, a neurotransmissão (Halliwell, 2011; Hultqvist et al., 2009) e a sinalização de autofagia (Lee et al., 2012).

A fim de controlar o ambiente oxidante altamente reativo, os sistemas biológicos utilizam defesas antioxidantes enzimáticos e não-enzimáticos. As enzimas antioxidantes mais importantes que realizam essa função sobre as ERO são a SOD, a CAT e a GPx. As moléculas antioxidantes não-enzimáticas mais importantes e responsáveis pelo controle da população de espécies reativas são a glutationa reduzida (GSH), a tiorredoxina (Trx), o ácido ascórbico (vitamina C) e os flavonoides, que se oxidam e estabilizam as moléculas altamente reativas (Finkel e Holbrook, 2000; Halliwell, 2006; Nordberg e Arner, 2001). O mecanismo simplificado de ação antioxidante em combate a ERO

endógenas proveniente da cadeia transporta de elétrons se dá pela ação primária da SOD catalisando a dismutação do $O_2^{\bullet-}$ em H_2O_2, que pode ser convertido à água pela CAT ou pela GPx, oxidando duas moléculas de GSH. A glutationa oxidada é recuperada à forma reduzida pela enzima glutationa-redutase, consumindo NADPH para isso. Quando os sistemas antioxidantes não conseguem conter a demanda de produção de espécies reativas ocorre o chamado estresse oxidativo, podendo trazer como consequência o dano de macromoléculas como lipídeos, proteínas e DNA (Finkel e Holbrook, 2000) (Fig. 2).

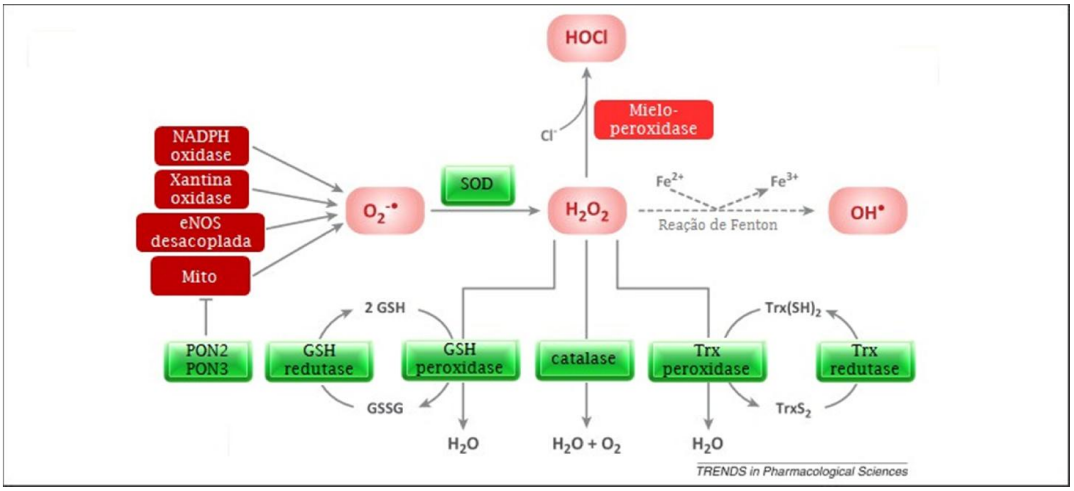

Figura 2 – Metabolismo de EROs. Adaptado de Li et al. (2013). Abreviaturas: NOS: óxido-nítrico-sintase, PON 2/3: paraoxonase isoformas 2 e 3, GHS: glutationa reduzida, O_2•: superóxido, SOD: superóxido-dismutase, HOCl: ácido hipocloroso, H_2O_2: peróxido de hidrogênio, •OH: radical hidroxil, Trx: tiorredoxina.

Em resposta a um aumento da concentração de espécies reativas, o perfil transcricional celular pode ser alterado, direcionando a sobrevivência ou morte das células afetadas. Um dos fatores de transcrição que é alterado em

resposta ao estresse oxidativo é o fator eritroide nuclear 2 (Nrf2). Ele está diretamente relacionado com o controle transcricional de enzimas como SOD, GPx e CAT em resposta ao aumento nos níveis de ERO (An et al., 2005; Itoh et al., 2010). Quando ocorre um insulto oxidativo, o Nrf2, que está no citoplasma na forma inativa ligado a ao repressor Kelch-like ECH-associated protein 1 (KEAP-1), é fosforilado e se desloca até o núcleo onde se liga a regiões promotoras de genes que codificam as enzimas antioxidantes mencionadas anteriormente (Leinonen et al., 2014; Zhang, 2006). Esse é um dos mecanismos propostos para as adaptações metabólicas induzidas pelo exercício físico em músculo e cérebro (Muthusamy et al., 2012; Tsou et al., 2015; Zhang, 2006). Nesse contexto, os efeitos benéficos do exercício físico moderado estão diretamente relacionados à produção de ERO e ERN durante o exercício (Sachdev e Davies, 2008), tanto que o bloqueio da ação de radicais livres pelo consumo excessivo de antioxidantes impede a instalação dessas adaptações (Gomez-Cabrera et al., 2008a; Gomez-Cabrera et al., 2008b).

4. Memória e BDNF

As regiões e estruturas do SNC estão diretamente relacionadas com funções como sensação, ação e emoção, em sistemas interligados por bilhões de neurônios que se comunicam através de sinapses. Uma das funções mais estudadas do encéfalo é a memória, principalmente seus mecanismos de aprendizado, consolidação, evocação, extinção e retenção de informações. Segundo Purves et al. (2004), o termo aprendizado é dado ao processo pelo qual a nova informação é adquirida, sendo observada através da mudança do comportamento. Já o termo memória englobaria decodificação, estocagem e recuperação da informação que foi aprendida.

A memória pode ser classificada levando em consideração diversos aspectos. Uma das classificações mais utilizadas compreende a forma de expressão dessa memória: declarativa é a armazenagem de material disponível no consciente, enquanto a não-declarativa não esta disponível no consciente. Uma subdivisão dessas está relacionada à sua natureza: associativa ou não-associativa. Cada uma delas pode ser detectada com diferentes tipos de tarefas, onde são verificadas alterações em comportamentos já padronizados de acordo com cada modelo animal (Izquierdo e Cavalheiro, 1976) (Fig. 3). Uma das estruturas mais estudadas relacionadas à memória e outros tipos de comportamentos é o hipocampo, mais especificamente a região CA1 na qual ocorre uma maior plasticidade e esta envolvida com a memória declarativa em mamíferos (Izquierdo et al., 2002; Squire, 1992; Taubenfeld et al., 1999).

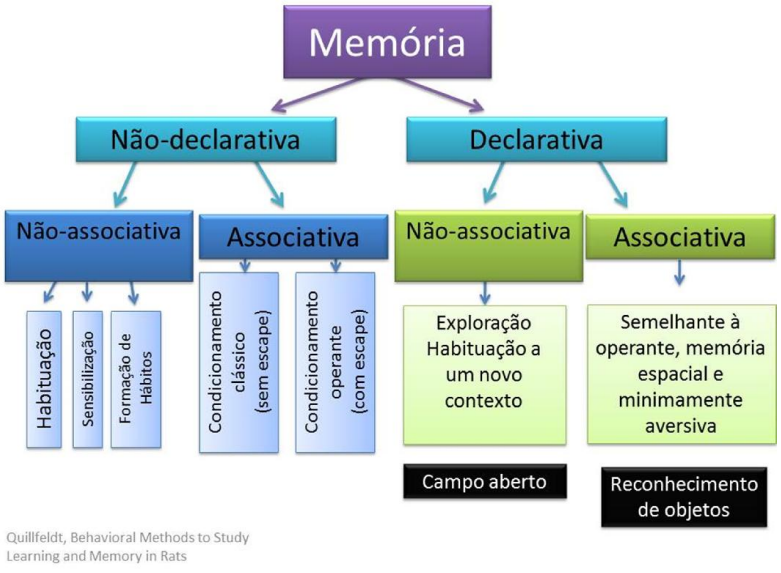

Figura 3 – Classificação da memória e tarefas comportamentais. Adaptado de (Quillfeldt, 2010)

O tempo de duração é outro critério para classificar a memória. De acordo com Alonso et al. (2002) a memória da maioria das espécies pode ser dividida em: memória de curta duração, que perdura de minutos até 1-3 horas e é caracterizada pela independência da síntese de proteínas e de RNA; e a memória de longa duração, que dura um longo período de tempo, variando de algumas horas até dias e semanas, sendo dependente da síntese de proteínas e de RNA. Uma das vias mais estudadas que se acredita estar envolvida na síntese proteica responsável pela plasticidade do hipocampo e memória de longa duração é a vida da MAPK/ERK (Kelleher et al., 2004).

Segundo Quillfeldt (2010), alguns testes são utilizados para observar a consolidação e evocação de diferentes tipos de memórias, através de alterações de comportamento, juntamente com um possível efeito do tratamento que esse animal foi submetido, comparando-o com um controle. O teste de campo aberto avalia possíveis alterações na atividade motora basal, taxa de exploração inata e diferentes níveis de ansiedade (Netto et al., 1986). A tarefa de reconhecimento de objetos (Ennaceur e Delacour, 1988) é um teste que envolve diferentes operações cognitivas como percepção, discriminação, identificação e comparação, associando assim a memória de reconhecimento de um objeto e a memória espacial (Quillfeldt, 2010).

O BDNF é uma proteína dimérica pequena da família das neurotrofinas e sua estrutura está relacionada com diversas funções como o crescimento dendrítico e axonal, diferenciação neuronal, sobrevivência celular e memória, atuando via ligação ao receptor tirosina-cinase B (TrkB) (Chen et al., 2013; Hofer e Barde, 1988; Hohn et al., 1990; Kenchappa et al., 2010; Murer et al., 2001; Pruunsild et al., 2007; Snider, 1994). O BDNF é sintetizado como uma

pró-proteína, o pró-BDNF, que possui atividade oposta à proteína madura, podendo induzir morte celular via ativação de receptores p75 (Lu et al., 2005).

Alguns estudos relacionam o papel do BDNF como necessário para a formação tanto da memória de curta quando de longa duração, via ativação da cascata de sinalização mediada por ERK1/2 (Alonso et al., 2002). O BDNF maduro também apresenta um papel importante na proteção do SNC de neonatos submetidos ao modelo de HI contra a perda de tecido, através de um bloqueio da ativação da caspase-3 mediada pela via da ERK (Han e Holtzman, 2000).

Um déficit cognitivo é encontrado em ratos adultos submetidos ao modelo de HI neonatal, observados através de um baixo desempenho no labirinto aquático de Morris assim como no teste de reconhecimento de objetos (Pereira et al., 2007; Pereira et al., 2008). Por outro lado, o exercício de natação materno em ratos promove uma melhora na aquisição e retenção da memória nos filhotes, avaliada na tarefa do labirinto aquático de Morris, bem como sinais de neurogênese hipocampal nas regiões CA1 e giro denteado e aumento do mRNA do BDNF (Akhavan et al., 2008; Lee et al., 2006).

II. Objetivos

1. Objetivos gerais

Sabe-se que o exercício físico durante a gestação apresenta efeitos benéficos para os filhotes e que a encefalopatia causada pela HI neonatal demanda estudos sobre possíveis novas estratégias terapêuticas. Com isso o objetivo deste trabalho é avaliar se os benefícios proporcionados pelo exercício materno antes e durante a gestação no SNC dos filhotes é suficiente para proteger contra as lesões causadas pela HI induzida pelo modelo de Levine-Rice, tanto em parâmetros de estresse oxidativo como em parâmetros comportamentais e de concentração de BNDF em machos adultos.

2. Objetivos específicos

- ✓ Avaliar os parâmetros de estresse oxidativo através dos seguintes ensaios bioquímicos: a produção de espécies reativas pela técnica de oxidação do DCF, a concentração de GSH, e as atividades de enzimas antioxidantes (SOD, GPx) em algumas estruturas do SNC (hipocampo, estriado, córtex parietal e cerebelo) de ratos submetidos ao modelo de HI neonatal, cujas mães realizaram natação antes e durante a gestação.
- ✓ Avaliar o efeito do exercício físico materno sobre parâmetros comportamentais (testes de campo aberto, reconhecimento de objetos) em ratos submetidos ao modelo de HI neonatal.
- ✓ Determinar a concentração de BDNF maduro nas estruturas do encéfalo (cerebelo, córtex parietal, hipocampo, estriado) de filhotes submetidos ao modelo de HI neonatal, e previamente ao exercício materno de natação.

Parte II

Capítulo I

Effect of maternal exercise on biochemical parameters in rats submitted to neonatal hypoxia-ischemia

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Effect of maternal exercise on biochemical parameters in rats submitted to neonatal hypoxia-ischemia

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Abstract

Hypoxia-ischemia (HI) encephalopathy results in severe damage to brain, disrupting motor, cognitive, and learning abilities. Physiopathology includes oxidative stress, allied to mitochondria energy production failure, glutamatergic excitotoxicity, and cell death. Clinical neonatal HI has been reproduced in an animal model developed by Levine-Rice, by the occlusion of the right carotid, ensuring a reliable model of study and allowing the development of therapeutic strategies. In this context, moderate physical exercise during pregnancy, a critical period to brain development, brings many benefits for both, mother and fetus. We previous demonstrated, through the maternal swimming model in Wistar rats, an antioxidant upregulation and a mitochondrial biogenesis in offspring's brain, which should provide neuroprotection against neonatal HI insults. Swimming exercise was performed one week before and during pregnancy, in controlled environment. The offspring was submitted to HI on postnatal day 7, and the brain samples for biochemical assays obtained in the weaning. It was observed a positive modulation in the activities of antioxidant enzymes in hippocampus, striatum, and cerebellum of pups delivered from exercised mothers and subjected to neonatal HI. Reactive species levels were reduced in hippocampus and parietal cortex, while were significantly augmented in striatum of rats submitted to HI, with or without maternal exercise associated. Reduced glutathione (GSH) was increased only in cerebellum from HI-rats subjected to maternal exercised. Considering the scenery presented, we concluded that HI elicited a neurometabolic adaptation in both brain hemispheres, particularly in hippocampus, parietal cortex, and cerebellum; while striatum appears to be most damaged. The protocol of aerobic maternal exercise was not enough to fully prevent HI-induced brain damages.

Highlights

- Long-term hypoxia-ischemia improves brain antioxidants parameters.
- Hypoxia-ischemia increased levels of reactive species in offspring's striatum.
- Maternal exercise did not prevent brain oxidative changes induced by hypoxia-ischemia.

Key words: Maternal swimming exercise; Antioxidant status; Reactive species; hypoxia-Ischemia; Neurometabolic adaptation.

Abbreviations

CHI	Control + Hypoxia-ischemia
CNS	Central nervous system
CS	Control + Sham
DCF	Dichlorofluorescein
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycoltetraacetic acid
EHI	Maternal Exercise + hypoxia-ischemia
ES	Maternal Exercise + Sham
GPx	Glutathione peroxidase
GSH	Reduced glutathione
H_2DCFDA	Dichlorofluorescein diacetate
H_2O_2	Hydrogen peroxide
HI	Hypoxia-ischemia
PBS	Phosphate-buffer saline
PMSF	Phenylmethylsulfonyl fluoride
PND	Postnatal day
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SOD	Superoxide dismutase

Introduction

One of the most common causes of fetal encephalopathy is the neonatal hypoxia-ischemia (HI), leading to sensory, motor, and learning disabilities; even cerebral palsy, mental retardation, and epilepsy (de Paula et al., 2009; Rivkin and Volpe, 1993). This condition could be a result of umbilical blood flow cessation, interruption of gas exchange by the placenta, premature birth, or stress during childbirth (Procianoy and Silveira, 2001). The immaturity of the central nervous system (CNS) of pups who suffer this insult is one of the most aggravating factors for the extent of injury, which may be a consequence of a "window of susceptibility" created by the synergism between excitotoxicity, oxidative stress, and inflammatory mechanisms (Ferriero, 2004).

In order to study the mechanisms involved in brain injury induced by hypoxic-ischemic condition, Rice et al. (1981) developed a model, adapted from Levine (1960) using 7 days postnatal rats submitted to unilateral carotid occlusion with an incision at the neck, isolating and blocking the right carotid with a surgical wire, followed by a period of hypoxia (8% oxygen and 92% nitrogen), maintaining the physiological temperature. This model has been used by many authors to further elucidate the effects of perinatal asphyxia on molecular mechanisms, cognitive, morphological, biochemical, and motor changes (Arteni et al., 2003; Ikeda, 2008; Levine, 1960; Rice et al., 1981). Neonatal asphyxia can facilitate the formation of a pro-oxidative environment (Fatemi et al., 2009). Some authors show the accumulation of hydrogen peroxide (Alkan et al., 2008; Dringen et al., 2005; Lafemina et al., 2006; Sarco et al., 2000; Sheldon et al., 2007), as well as the

regulation of transcription factors responsible by antioxidant enzymes expression, as consequences of hypoxia-ischemia (Guglielmotto et al., 2009; Ishida et al., 2001).

An effective therapy to fully prevent or treat the sequelae left by HI is still far from close. Aerobic physical exercise has been recognized as an important therapeutic modality that can assist in the treatment of chronic diseases such as obesity, type 2 diabetes, heart problems, depression, and osteoporosis (Dishman et al., 2006; Powell et al., 2011; Wolff et al., 2011). In addition to promoting metabolic programming in the CNS, such as increased glucose uptake, oxidative capacity, and electron transport chain activity in some brain regions (Dishman et al., 2006; Gokbuget et al., 2011), exercise could lead to enlarged angiogenesis, cell proliferation and neurogenesis, enhanced synaptic plasticity, and modulation of inflammatory responses (Cotman et al., 2007; Itoh et al., 2011; Ratey and Loehr, 2011). Literature reports that physical exercise may contribute to post-ischemic recovery in mice submitted to treadmill (Choi et al., 2013; Park et al., 2013).

Pregnancy is the most critical period for individual development when its organs and systems are growing and specializing, which makes them more vulnerable to environmental factors as the mother's diet, lifestyle, stress, and hormones (Desai et al., 2015; Meaney and Aitken, 1985; Pereira and Martel, 2014). It has been shown that physical exercise of moderate intensity during pregnancy present benefits to mother and child, such as a reduced risk of pre-eclampsia, gestational diabetes, and premature birth, as well as improved pain tolerance and reduced maternal body weight gain (Gaston and Cramp, 2011). The aquatic exercise is mostly recommended for pregnant due to the control of maternal body weight, body temperature, and the low impact on joints (Hartmann and Bung, 1999; Lynch et al., 2003).

To evaluate the effects of exercise during gestational period, animal models of "involuntary" swimming have been used, demonstrating positive neural adaptations. Offspring born from exercised rats shows a better performance in the acquisition and retention phases in the Morris water maze task, associated to increased number of hippocampal cells, especially in CA1 and dentate gyrus regions (Akhavan et al., 2008; Lee et al., 2006). In agreement, we demonstrated a potential brain biochemical programming in pups subjected to maternal swimming, verified by mitochondrial biogenesis and antioxidant upregulation, probably induced by reactive species (Marcelino et al., 2013). Voluntary wheel running exercise appears to be effective against the hippocampal neurons loss induced by postnatal HI, in the offspring from rats trained during pregnancy, emerging a potential neuroprotective role of aerobic exercise (Akhavan et al., 2012). In addition Mourão et al. (2014) recently demonstrated that aquatic exercise performed by rodents is able to prevent the release of glutamate in the synaptic cleft, and decreased some pro-apoptotic factors in hippocampal slices exposed to *in vitro* HI. Considering the well established oxidative damage induced by HI model, our objective in this study was to evaluate the neuroprotective potential of maternal physical exercise on brain from offspring submitted to postnatal HI.

Materials and methods

1. Animals and reagents

Adult female (65 animals) and males (33 animals) Wistar rats were obtained from the Central Animal House of Departamento de Bioquímica, Instituto de Ciências

Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. The offspring was kept with its mother in a single box until weaning at 21 days. The animals were maintained on a 12/12-hour light/dark cycle in an air-conditioned colony room at a constant temperature (22±1ºC). They had free access to water and a 20% (w/w) protein commercial chow.

Experiments were approved by the local animal ethics commission (Comissão de Ética no Uso de Animais/Universidade Federal do Rio Grande do Sul-CEUA/UFRGS) under the number 23670, and follows national animal rights regulations (Law 11.794/2008), the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication No. 80-23, revised 1996) and Directive 2010/63/EU. We further attest that all efforts were made to minimize the number of animals used and their suffering.

All chemicals were obtained from Sigma Chemical Co., St. Louis, MO, USA.

2. Experimental Design

Female Wistar rats (approximate body weight = 250 g) were initially divided into the following groups: (1) sedentary control group, which were placed in contact with the stress of the aquatic environment, without swimming; (2) the exercised group, which were subjected to swimming exercise for one week previous to mating, and during the pregnancy. To mating, one male rat was placed in contact with two females for 12 hours. Pregnancy was diagnosed by the presence of vaginal plug. The pregnant rats were submitted to the exercise protocol during all the pregnancy. From the 20st day after the onset of pregnancy, the rats were observed twice a day (9h and 18h), to verify the litter's birth. The day corresponding to the birth of offspring

is defined as postnatal day (PND) 0 (Fig. 1A). We used one pup for each offspring for each technique, in order to eliminate the litter effect (total = 85 animals).

The offspring, on PND7, was subjected to hypoxia-ischemia model. From this point, rats were divided into the following 4 groups: (1) control + sham (CS), (2) control + hypoxia-ischemia (CHI), (3) maternal exercise + sham (ES), or (4) maternal exercise + hypoxia-ischemia (EHI). The pups weaning occurred in PN21, when they were euthanized and the brain was isolated and dissected into cerebellum, parietal cortex, hippocampus, and striatum, which were analyzed as ipsilateral and contralateral hemisphere from carotid obstruction (Fig. 1B).

2.1. Maternal swimming protocol

The maternal exercise protocol was adapted from Lee et al. (2006), as described in Marcelino et al. (2013). The rats were divided into control and exercised groups. In the exercised group, rats were submitted to swimming in a pool filled with 32±1ºC water on 5day/week for 4 weeks. Each swimming session lasted for 30 minutes, and always took place between 9 and 12 a.m. Each rat was isolated for the swim, which was conducted using an apparatus designed specifically for rat swimming. Within this apparatus, each room measures 30x30x90 cm (widthxlengthxdepth), preventing the animals from touching the bottom of the tank. The animals were left free to swim, without any extra weight, and were gently stimulated to swimming. This protocol has moderate intensity. Control rats were immersed in water, carefully dried, and returned to the housing boxes.

2.2. Model of hypoxia-ischemia (HI)

The method of Levine (1960) further modified by Rice et al. (1981), producing a unilateral brain lesion in the offspring (PND7). The pups, anesthetized by air

halothane, suffered an incision on the ventral surface of the neck (parallel and lateral to the trachea), facilitating access to the right carotid artery, thus being isolated from the vague and permanently occluded with surgical thread. Animals submitted to surgery were placed 15 minutes under a heating lamp for post-surgical recovery and then were returned to their mothers. After 2h of maternal care, the pups were exposed to 90 minutes of hypoxia atmosphere with a standard mixture of 8% oxygen and 92% nitrogen in a 1500 mL chamber partially immersed in a water bath at 37°C (Arteni et al., 2003; Rodrigues et al., 2004). Shortly after the hypoxic ischemic procedure, the animals returned to their mothers in their respective cages. Controls were sham-operated, i.e., subjected to the effect of anesthetic and suffered the same incision and isolation of the carotid artery, but without the obstruction or even coming into contact with the environment of hypoxia.

3. Biochemical assays

3.1. Sample preparation

Cerebellum, parietal cortex, hippocampus, and striatum were stored at -80ºC until use in biochemical techniques, when the tissue was homogenized in 10 volumes (1:10, w/v) of phosphate-buffer saline (PBS) pH 7.4, containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1mM ethyleneglycoltetraacetic acid (EGTA). The homogenates used were from individual animals; samples were never pooled. Homogenates were centrifuged at 1000xg for 10 minutes at 4ºC, and nuclei and cell debris were discarded. The pellet was discarded and the supernatant was taken for biochemical assays.

3.2. Oxidation of dichlorofluorescein (DCF assay)

Production of reactive species was assessed using the dichlorofluorescein (DCF) oxidation method (LeBel et al., 1992). Briefly, 60 µL of biologic sample was incubated at 37ºC, in the dark, for 30 minutes, with the addition of 240 µL 2',7'-dichlorofluorescein diacetate (H_2DCFDA) in a 96-well plate. H_2DCFDA is cleaved by cellular esterases to form H_2DCF. This is oxidized by reactive oxygen/nitrogen species present in the sample, producing a fluorescent compound, DCF. DCF oxidation was measured fluorimetrically using a wavelength of 488 nm excitation and 525 nm emission. A standard curve, using standard DCF (0.25-10 mM), was performed in parallel with the samples. Results were calculated as nmol/mg protein.

3.3. Activity of antioxidant enzymes

Superoxide dismutase (SOD) (EC 1.15.1.1) activity was evaluated by qualifying the inhibition superoxide-dependent autoxidation of epinephrine, verifying the absorbance of the samples at 480 nm (Misra and Fridovich, 1972). Considering the protocol used in sample preparation, we measured total SOD activity, expressed as the amount of enzyme that inhibits the oxidation of epinephrine by 50%, which is equal to 1 unit. The data were calculated as units/mg protein.

Glutathione peroxidase (GPx) (EC 1.11.1.9) activity was measured according to the method described by (Wendel, 1981) using *tert*-butyl hydroperoxide as the substrate. NADPH disappearance was monitored spectrophotometrically at 340 nm in a medium containing 2mM GSH, 0,15 U/mL glutathione reductase (EC 1.8.1.7), 0.4 mM azide, 0.5 mM *tert*-butyl hydroperoxide, and 0.1 mM NADPH. One GPx unit is defined as 1 µmol NADPH consumed per minute, with the specific activity calculated as units/mg protein.

3.4. Reduced glutathione concentration

Reduced glutathione (GSH) concentration was measured according to (Browne and Armstrong, 1998), where GSH reacts with the fluorophore *o*-phthaldialdehyde. The proteins in supernatant were initially precipitated with meta-phosphoric acid (1:1, v/v), and centrifuged at 5.000xg for 10 minutes at 25ºC. A total of 50 µL supernatant was incubated at room temperature for 15 minutes with 15 µL of 7.5 mM *o*-phthaldialdehyde and 235 µL of 120 mM sodium phosphate buffer (pH 8.0) containing 5mM ethylenediaminetetraacetic acid (EDTA). A blank sample was run in parallel. Fluorescence was measured using excitation and emission wavelengths of 350 and 420 nm, respectively. A calibration curve was prepared with standard GSH (0.001-1mM) and the concentrations were calculated as nmol/mg protein.

3.5. Protein determination

Protein concentration was measured by the method of Lowry et al. (1951), using bovine serum albumin as standard.

4. Statistical analysis

Statistical analysis was performed using two-way ANOVA followed by Tukey post-test for multiple comparisons, when F was significant. Data were presented as mean ± SEM. Data were analyzed by GraphPad Prism program 6. Data were considered statistically significant if $p<0.05$.

Results

Hypoxia-ischemia improved antioxidant activity and reduced reactive species levels in hippocampus of rats

DCF oxidation was used as an indicator of reactive oxygen (ROS) and nitrogen species (RNS) levels. It was observed a decrease in the oxidation of DCF in the hippocampus from HI groups (CHI and EHI) in the hemispheres contralateral and ipsilateral to the obstruction of the carotid artery, when compared with the respective SHAM group (contralateral: $F(1,20)=208.7$, $p<0.0001$; ipsilateral: $F(1,20)=216.7$, $p<0.0001$) (Fig. 2A). Two-way ANOVA showed a significant interaction between the two variables (HI and maternal exercise), when it is analyzed the activity of SOD in the hemisphere contralateral to the obstruction of the carotid artery (interaction: $F(1,19)=22.05$, $p=0.0002$; HI: $F(1,19)=0.07928$, $p=0.7813$; exercise: $F(1,19)=0.01188$, $p=0.9144$), while exercise and HI isolated induced enhanced SOD activity, as showed by Tukey post-hoc test. SOD activity in the ipsilateral hippocampus was not affected ($F(1,20)=0.01729$, $p=0.8967$) (Fig. 2B). The GPx activity was increased in the groups HI (CHI and EHI) when compared with their respective sham groups, showing an effect only in the variable HI in two-way ANOVA in contralateral ($F(1,19)=22.28$, $p=0.0001$) and ipsilateral hemispheres ($F(1,18)=17.03$, $p=0.0006$) (Fig. 2C). There was no statistically significant change in the concentration of GSH in this structure (contralateral: $F(1,20)=1.540$, $p=0.2290$; ipsilateral: $F(1,20)=0.03521$, $p=0.8530$) (Fig. 2D).

Hypoxia-ischemia modulates reactive species levels and GPx activity in the parietal cortex

Similarly to observed in hippocampus, DCF oxidation was reduced in parietal cortex by HI in the hemisphere ipsilateral to arterial occlusion, as showed by two-way

ANOVA ($F(1,20)=9.614$, $p=0.0056$). Contralateral parietal cortex was not affected ($F(1,19)=0.1380$, $p=0.7144$) (Fig. 3A). Despite the reduction in reactive species levels, there was no statistically significant change in SOD activity in this structure (contralateral: $F(1,20)=0.02541$, $p=0.8750$; ipsilateral: $F(1,20)=0.0002030$, $p=0.9888$) (Fig. 3B). On the other hand, HI groups presented a significant reduction in GPx activity (contralateral: $F(1,17)=24.03$, $p=0.0001$; ipsilateral: $F(1,15)=13.81$, $p=0.0021$) (Fig. 3C). The alteration in GPx activity appear not to be related to changes in GSH concentration (contralateral: $F(1,20)=0.6406$, $p=0.4329$; ipsilateral: $F(1,19)=0.1386$, $p=0.7137$) (Fig. 3D).

Hypoxia-ischemia increases the levels of reactive species and SOD activity in the striatum of rats

Different from hippocampus and parietal cortex, HI induced an increase in reactive species levels in striatum, evidenced by two-way ANOVA analyzes of DCF oxidation (contralateral: $F(1,20)=196.8$, $p<0.0001$; ipsilateral: $F(1,20)=167.0$, $p<0.0001$) (Fig. 4A). Similarly to hippocampus, SOD activity was upregulated in both hemispheres from HI encephalopatic animals (contralateral: $F(1,16)=5.268$; $p=0.0356$; ipsilateral: $F(1,16)=13.48$, $p=0.0021$) (Fig. 4B). There was no statistically significant change in GPx activity (contralateral: $F(1,19)=0.0591$, $p=0.8105$); ipsilateral: $F(1,19)=0.1473$, $p=0.7054$) (Fig. 4C), nor the total concentration of GSH (contralateral: $F(1,18)=1.90$, $p=0.1852$; ipsilateral: $F(1,19)=0.934$, $p=0.3459$) (Fig. 4D) in the striatum of rats subjected to maternal swimming and/or neonatal HI model.

Glutathione peroxidase and glutathione were increased in cerebellum from rats submitted to maternal swimming and postnatal hypoxia-ischemia

Fig. 5A shows that there was no alteration in cerebellar DCF oxidation (contralateral: $F(1,20)=0.2104$, $p=0.6514$; ipsilateral: $F(1,20)=1.193$, $p=0.2877$). SOD activity did not show any statistic significant alteration in this structure, evaluated by two way ANOVA (contralateral: $F(1,20)=0.4637$, $p=0.5037$; ipsilateral: $F(1,20)=0.02276$, $p=0.8816$) (Fig. 5B). The hemisphere contralateral to arterial occlusion had an increased GPx activity in the EHI group when compared with the ES group, indicated by a change in the variable HI in two-way ANOVA ($F(1,18)=8.865$, $p=0.0081$), while the ipsilateral hemisphere was not altered ($F(1,19)=0.1473$, $p=0,7054$) (Fig. 5C). GSH levels were also increased by HI and maternal exercise, when compared to CS group, in the contralateral hemisphere ($F(1,20)=9.630$, $p=0.0056$). In the ipsilateral cerebellum, we verified an interaction between HI and maternal exercise ($F(1,20)=7.224$, $p=0.0142$), associated to a non-significant increase in GSH levels by maternal exercise and HI when the factors are isolated (HI: $F(1,20)=0.2370$, $p=0.6317$; exercise: $F(1,20)=0.8867$, $p=0.3576$) (Fig. 5D).

Discussion

Brain insult caused by HI encephalopathy is well established in the literature, evidenced by extensive tissue injury in the ipsilateral carotid occlusion structures, such as cortex, hippocampus, and striatum, as was observed in our study, affecting motor, cognitive, and learning abilities (Levine, 1960; Pereira et al., 2007; Rice et al., 1981; Vannucci and Vannucci, 2005). Biochemically, the neurodegeneration results of a complex set of factors, including mitochondrial injury leading to energy failure,

glutamatergic excitotoxicity, high levels of reactive species and the activation of apoptotic pathways (Perlman, 2006). In addition, rodents subjected to HI present alterations on antioxidant enzymes, mainly SOD and GPx (Alkan et al., 2008; Almli et al., 2000; Arteni et al., 2003; Baburamani et al., 2012; Blomgren and Hagberg, 2006; Pereira et al., 2007; Rodrigues et al., 2004; Sarco et al., 2000; Vannucci and Hagberg, 2004). In our study, a moderate intensity swimming protocol was performed one week before, and during pregnancy by mothers, in order to induce neurometabolic adaptations in the offspring (Marcelino et al., 2013), being able to prevent brain insult provoked by HI performed in 7 day-old rats. Oxidative stress parameters were evaluated 14 days after that, in order to find out the long-term effect of HI encephalopathy. Reactive species was reduced in hippocampus verified in contralateral and ipsilateral structures of HI animals, probably as a result of SOD and GPx upregulation. Parietal cortex also present a reduction in DCF oxidation, however GPx was inhibited in both hemispheres. Striatum, on the other hand, present an increment in reactive species levels, associated to an increase SOD activity in the ipsilateral hemisphere. Finally, cerebellum presented augmented levels of GSH in parallel to GPx activation in the contralateral structure from HIE rats, suggesting a positive adaptation in the antioxidant system. The diverse modulation verified in different structures of the CNS, concerning to reactive species levels and antioxidant enzymes activities, probably impacts its physiological and biochemical functions, representing a long-term adaptation to acute oxidative insult caused by hypoxic-ischemic process established in the PND7. This pattern is already reported in literature for chronic hypoxic-rats, showing differential adaptive responses in glycolytic and TCA cycle enzymes in different brain structures (Lai et al., 2003).

Hippocampus is one of the structures most studied, probably because injury caused by HI brings several behavior impairs on spatial and working memory in adult rats (Arteni et al., 2003; Ikeda, 2008; Pereira et al., 2007). One of the consequences of HI injury to the nervous system is a prominent oxidative stress due to the vulnerability of the brain in newborn rats (Alkan et al., 2008; Sarco et al., 2000). Some studies mention the accumulation of hydrogen peroxide (H_2O_2) generated after the insult, responsible for oxidative stress and cell death (Alkan et al., 2008; Hyslop et al., 1995; Penna et al., 2014), the same reactive specie responsible by DCF oxidation (LeBel et al., 1992). This initial increase of ROS elicited by HI process (Ikonomidou and Kaindl, 2011; Lafemina et al., 2006; Olmez and Ozyurt, 2012) can be the factor responsible for the activation of antioxidant enzymes found in our experiments, probably caused by upregulation of its expression (Dayalan Naidu et al., 2015; Jaiswal, 2004; Kang and Li Ji, 2012; Osburn and Kensler, 2008; Sachdev and Davies, 2008). Considering that SOD and GPx are the most important ROS eliminating enzymes (Halliwell and Gutteridge, 2007), it explains the reduced DCF oxidation found in the hippocampus of rats undergo HI. An interaction between exercise and HI variable in the hemisphere contralateral to the carotid occlusion on SOD activity was also observed, suggesting that, at least in part, maternal swimming might prevent HI effect on pups' hippocampus. Some studies with different exercise models show an increase in the activity and amount of SOD in the hippocampal structure through several mechanisms, including increased regulatory nuclear transcription factors that regulate the activity of this enzyme (Hosseinzadeh et al., 2013; Kim et al., 2015; Marosi et al., 2012; Souza et al., 2013) . This effect could have been transmitted to the fetus through the exercise before and during pregnancy, being observed in CS groups in the contralateral hemisphere (Fig 2B).

Additionally, offspring delivered from mothers exercised during pregnancy presents an activated hippocampal SOD, demonstrating the persistence of metabolic adaptation verified in PND7 rats in previous results (Marcelino et al., 2013).

Parietal cortex is responsible by accuracy of the movement, motor control and adaptation (Penhune and Steele, 2012), and is clearly injured in patients affected by a severe form of perinatal hypoxic-ischemic encephalopathy (Bregant et al., 2013). In a similar way, rat parietal cortex is intensely affected by HI, which presents increased apoptosis demonstrated by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) positive cells and caspase-3 assay performed in rat pups subjected to right common carotid artery ligation and hypoxia for 2 h (Kilicdag et al., 2014). We previous shown that maternal exercise strongly improves antioxidant network and mitochondrial biogenesis (Marcelino et al., 2013) in this cortical structure. Interestingly, DCF oxidation and GPx activity were reduced in the ipsilateral hemisphere, in our animal model conditions, which seems contradictory in the beginning. However, the system responsible by ROS detoxification was not entirely evaluated, what limits our conclusions about parietal cortex.

Striatum is also damaged by HI, as demonstrated by Martin et al. (1997), in one-week-old piglets subjected to 30 minutes of hypoxia and then seven minutes of airway occlusion, presenting a serious neuronal damage in putamen and caudate nucleus, compatible with necrosis induced by oxidative stress in the first hours of reperfusion (Martin et al., 2000). In agreement, we observed an increment in DCF oxidation, probably caused by augmented H_2O_2, the residual ROS left by increased SOD and unaltered GPx activity observed in striatum from PND21-rats submitted to HI on PND7. In the HI protocol, after a period of oxygen deprivation, the brain of the offspring undergoes reperfusion phase where a large input of O_2 occurs, increasing

the activity of the chain transports electrons which will lead to increase production of ROS (Armogida et al., 2012), as observed in our work. We hypothesized that striatum was not able to adapt to initial oxidative status induced by HI model, different from hippocampus of the same rats. In agreement, Omata et al. (2006) have shown the induction of hypoxic tolerance after hypoxic preconditioning is region-specific, since striatum did not improves its antioxidant and stress proteins expression, while frontal cortex was able to react. A potential explanation is the high susceptibility to oxidative stress verified in the striatum, when compared to hippocampus and cerebellum, probably related to high iron content (Mitra et al., 2014), the mediator of Fenton reaction (Halliwell and Gutteridge, 2007). The insult demonstrated in striatum could be related to HI encephalopathy consequences, considering its functions on behavior, related to motor sequence learning (Penhune and Steele, 2012), associative learning (Liljeholm and O'Doherty, 2012), and consolidation of memory (Albouy et al., 2008).

Cerebellum, besides its classical functions, as the maintenance of the balance and posture, and the coordination of voluntary movements; it has been related to motor learning and control (Penhune and Steele, 2012; Shmuelof and Krakauer, 2011). In our experimental conditions, HI minor affected the cerebellar tissue. We detected increased GSH levels and GPx activity in the contralateral hemisphere subjected to HI, in the offspring from exercised mothers, suggesting a long-term antioxidant protection. We might theorize that the postnatal development of cerebellum, which comprehends dendritogenesis, synaptogenesis and cellular proliferation, differentiation and migration (Benitez et al., 2014), might be associated to biochemical adaptation. Acutely, mitochondrial enzymes in rat cerebellar tissue were inhibited by HI, in an oxidative stress-dependent mechanism (Clarkson et al.,

2007), also verified by *in vitro* conditions (Scorziello et al., 2001; Scorziello et al., 2004), which could be the trigger of the adaptations found in our experiments.

It is known that oxidative stress is one of the characteristics of early lesion process after injury caused by HI in the brain of adult and young rats, thus becoming a prime target for neuroprotective strategies (Blomgren and Hagberg, 2006). The CNS of puppies is still immature and extremely susceptible to redox adjustments in the maternal gestation period. Our group demonstrated that maternal exercise promotes a protective metabolic programming concerning to antioxidant status in the brain of pups (Marcelino et al., 2013). Unfortunately, present data shows that the benefits of maternal aerobic exercise were not enough to prevent insult caused by neonatal HI. Both the carotid blockage, ipsilateral and contralateral, showed a modulation in the activity of antioxidant enzymes and altered ROS levels after injury caused by the Levine-Rice HI model, which were not fully prevented by maternal swimming.

Conclusion

We observed a marked metabolic adaptation in the brain of rats 14 days after HI protocol establishment, specially the hippocampus, parietal cortex, and cerebellum, damaging in a major extension the striatum through pro-oxidant effect found only in this structure. The neurometabolic adaptation was observed in the contralateral and the ipsilateral hemisphere to carotid occlusion. Finally, maternal swimming exercise was not able to totally prevent the oxidative insult mediated by HI neonatal protocol.

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Conflict of interest

The authors declare no conflicts of interest or competing interest.

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Figure Subtitles

Fig. 1. Experimental design to mother (A) and offspring (B).

Fig. 2. Effect of maternal swimming exercise on oxidative stress parameters measured in hippocampus of pups subjected to neonatal HI: dichlorofluorescein oxidation (A), superoxide dismutase (B), glutathione peroxidase (C), and total levels of reduced glutathione (D). The white bar represents surgical Sham and black bar represents HI group. Results are expressed as mean ± S.E.M. for 5-6 animals in each group, ***p<0.001 (Two-Way ANOVA, followed by Tukey's test). DCF: dichlorofluorescein, GPx: glutathione peroxidase, GSH: reduced glutathione, HI: hypoxia-ischemia, SOD: superoxide dismutase, ME: maternal exercise.

Fig. 3. Effect of maternal swimming exercise on oxidative stress parameters measured in parietal cortex of pups subjected to neonatal HI: dichlorofluorescein oxidation (A), superoxide dismutase (B), glutathione peroxidase (C), and total levels of reduced glutathione (D). The white bar represents surgical Sham and black bar represents HI group. Results are expressed as mean ± S.E.M. for 4-6 animals in each group, **p<0.01 and ***p<0.001 (Two-Way ANOVA, followed by Tukey's test). DCF: dichlorofluorescein, GPx: glutathione peroxidase, GSH: reduced glutathione, HI: hypoxia-ischemia, SOD: superoxide dismutase, ME: maternal exercise.

Fig. 4. Effect of maternal swimming exercise on oxidative stress parameters measured in striatum of pups subjected to neonatal HI: dichlorofluorescein oxidation (A), superoxide dismutase (B), glutathione peroxidase (C), and total levels of reduced glutathione (D). The white bar represents surgical Sham and black bar represents HI group. Results are expressed as mean ± S.E.M. for 4-6 animals in each group, **p<0.01 and ***p<0.001 (Two-Way ANOVA, followed by Tukey's test). DCF: dichlorofluorescein, GPx: glutathione peroxidase, GSH: reduced glutathione, HI: hypoxia-ischemia, SOD: superoxide dismutase, ME: maternal exercise.

Fig. 5. Effect of maternal swimming exercise on oxidative stress parameters measured in cerebellum of pups subjected to neonatal HI: dichlorofluorescein oxidation (A), superoxide dismutase (B), glutathione peroxidase (C) and total levels of reduced glutathione (D). The white bar represents surgical Sham and black bar represents HI group. Results are expressed as mean ± S.E.M. for 4-6 animals in each group, **p<0.01 (Two-Way ANOVA, followed by Tukey's test). DCF: dichlorofluorescein, GPx: glutathione peroxidase, GSH: reduced glutathione, HI: hypoxia-ischemia, SOD: superoxide dismutase, ME: maternal exercise.

Fig. 1

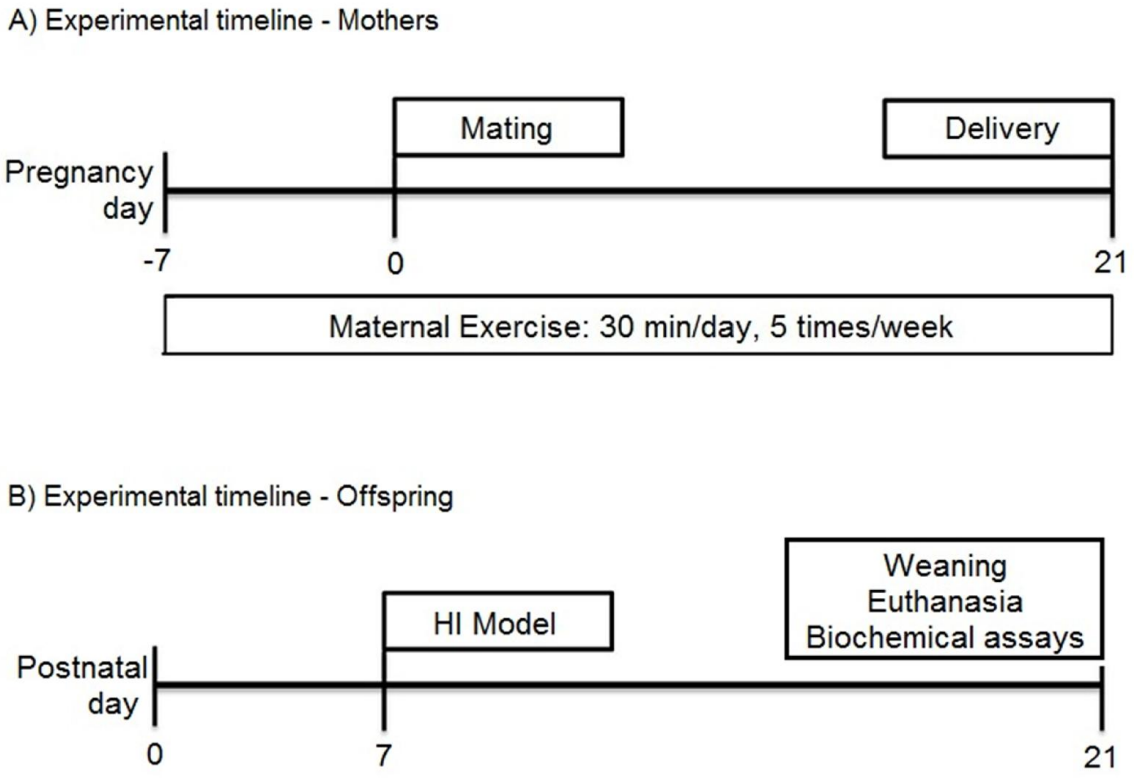
A) Experimental timeline - Mothers
Mating
Delivery
Pregnancy day
-7
0
21
Maternal Exercise: 30 min/day, 5 times/week
B) Experimental timeline - Offspring
Weaning
Euthanasia
Biochemical assays
HI Model
Postnatal day
0
7
21

Fig. 2

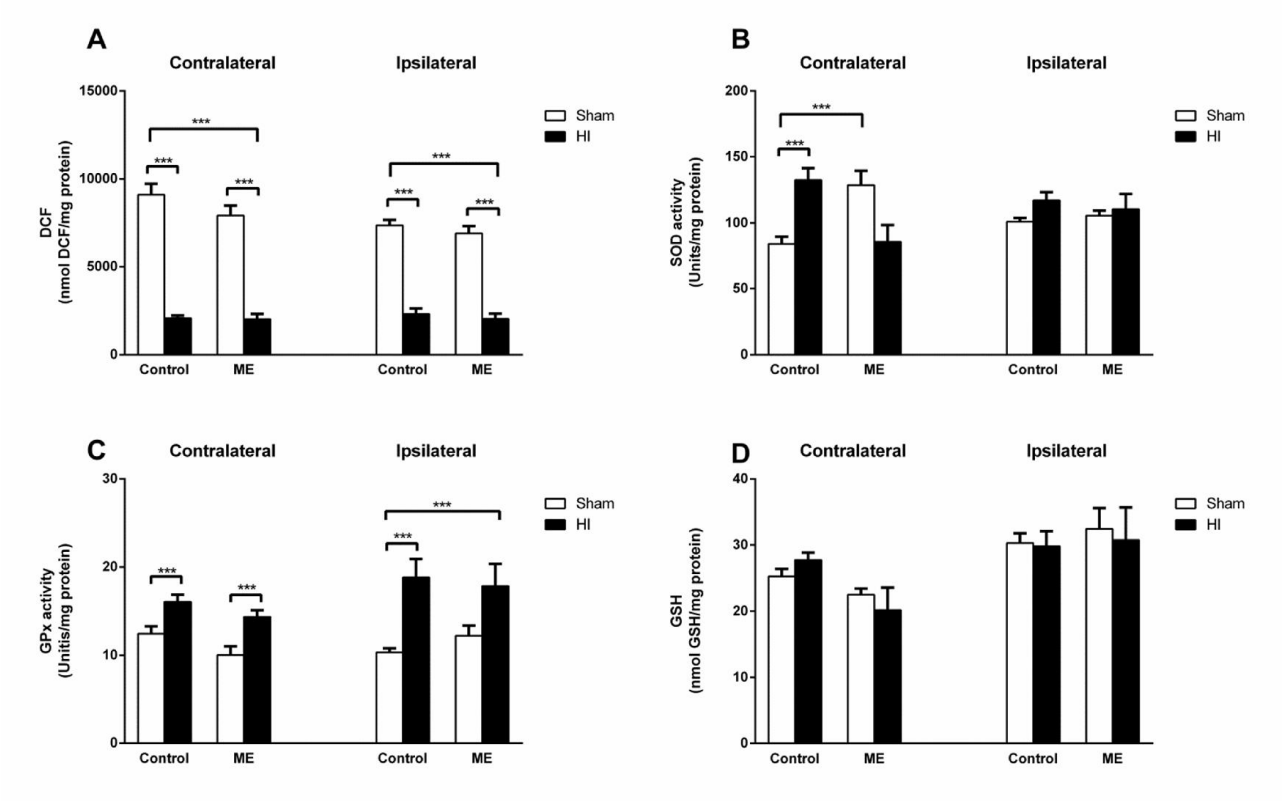
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Fig. 3

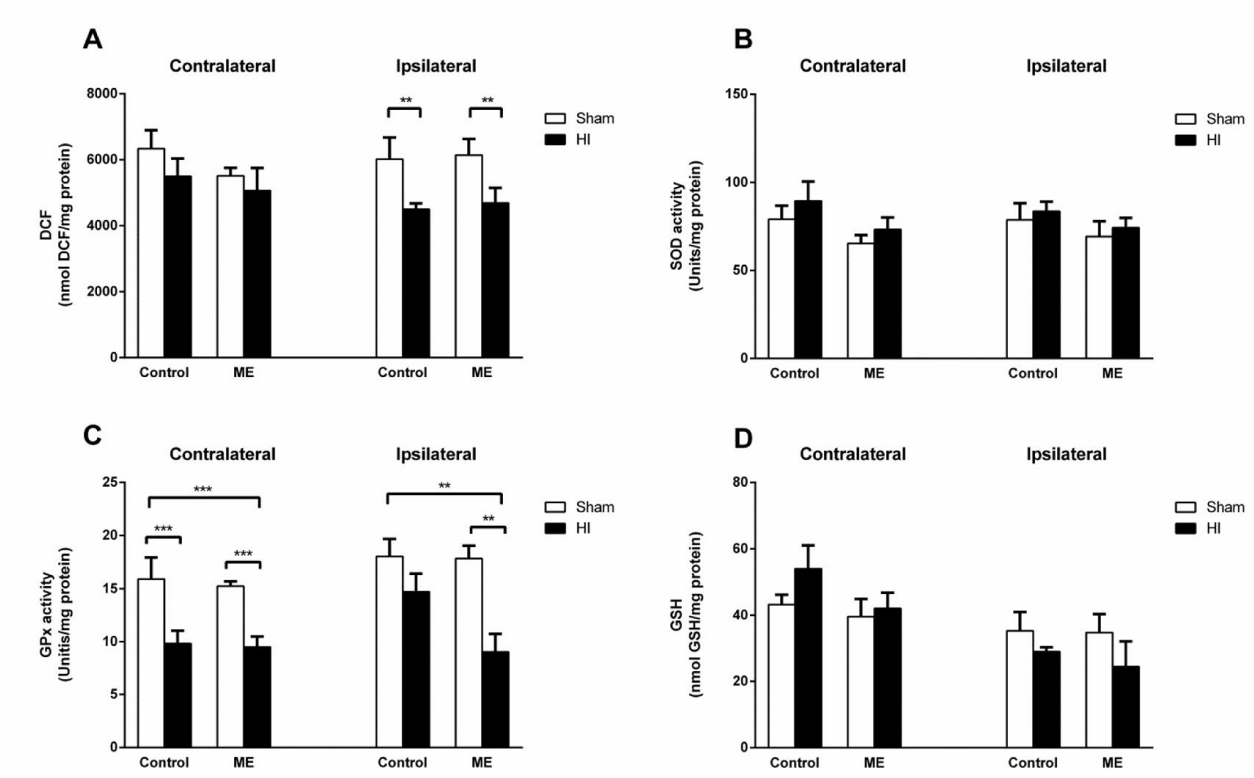
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Fig. 4

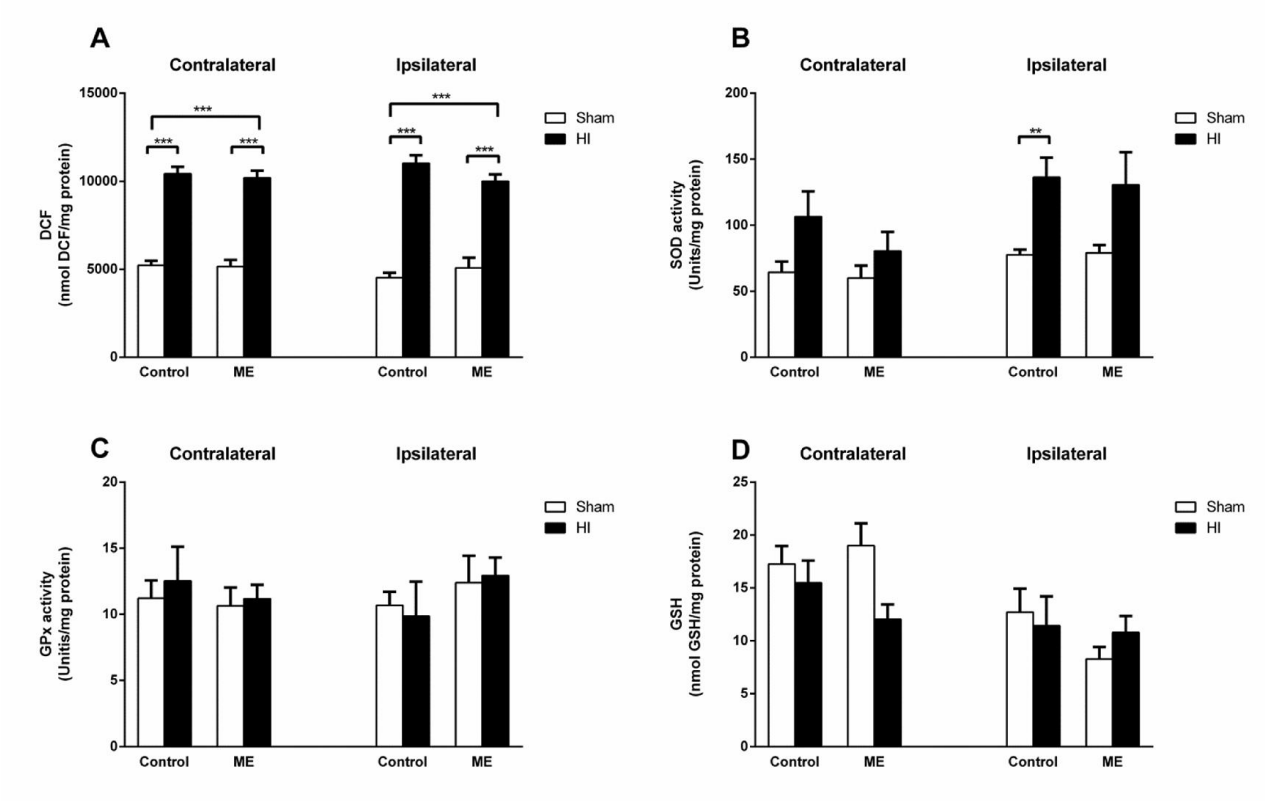
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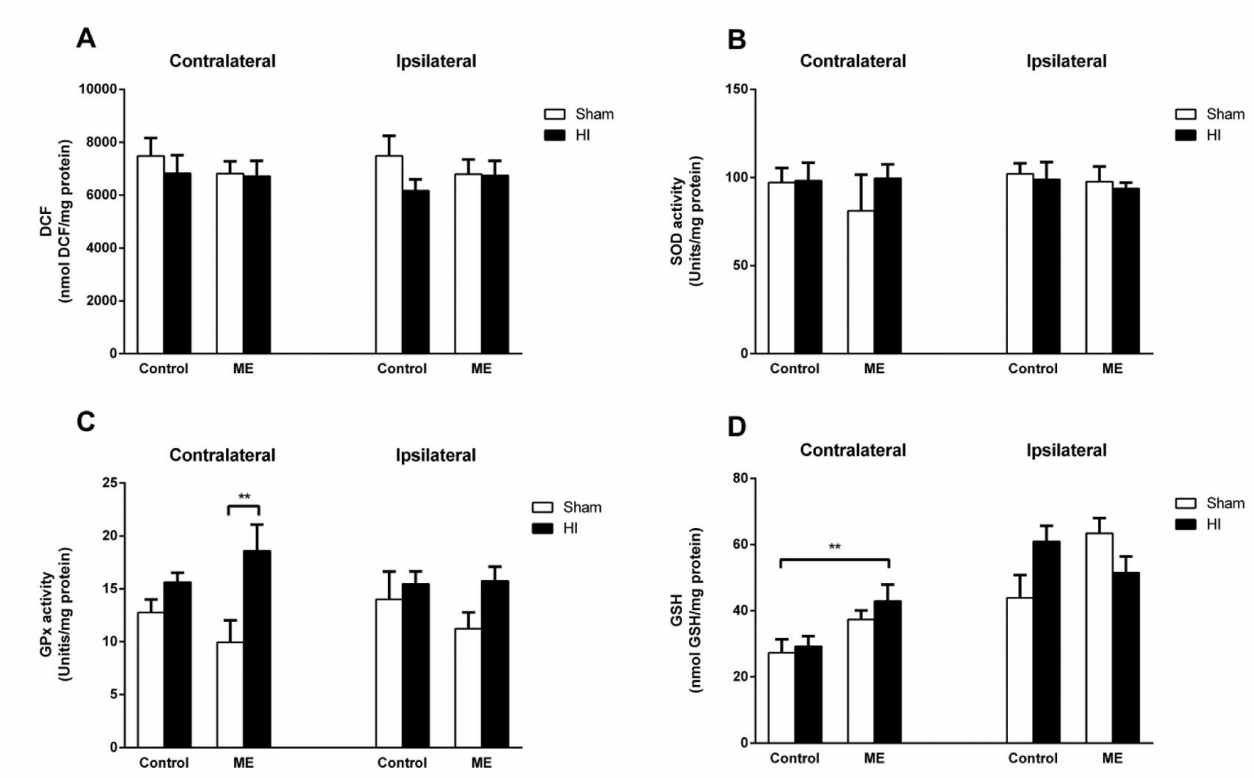
Fig. 5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Capítulo II

Neonatal hypoxia-ischemia alters motor activity and object recognition memory in rats subject to maternal swimming

Status: a ser submetido para publicação

Neonatal hypoxia-ischemia alters motor activity and object recognition memory in rats subject to maternal swimming

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Abstract

One of the most common causes of neonatal encephalopathy is hypoxia-ischemia (HI). The injury of the central nervous system comprises several mechanisms, including inflammatory, excitotoxicity, and oxidative stress that could lead to cell death and cognitive impairment. Exercise during pregnancy is a potential therapeutic tool due to the benefits it offers to both, mother and fetus. Swimming during pregnancy elicited a strong metabolic programming in the brain's offspring, evidenced by increased antioxidant enzymes activity, mitochondrial biogenesis, and hippocampal neurogenesis. This article aims to evaluate whether the benefits of maternal exercise are capable of prevent the injury caused by neonatal HI. Female adult Wistar rats swim before and during pregnancy (30 min/day, 5 days/week, 4 weeks). The offspring was submitted to HI protocol in the 7^{th} day after birth, and performed the behavioral tests in adulthood. It was observed an increase in motor activity in the open field test in the rats submitted to HI, which was not prevented by maternal exercise. The rats subjected to maternal swimming present a tendency of improvement in long-term memory in the object recognition task, which was significantly reversed by neonatal HI encephalopathy. BDNF levels were measured in cerebellum, parietal cortex, hippocampus and striatum. We did not find any significant change, suggesting that HI or maternal exercise effects were BDNF-independent. We concluded that the neonatal HI augmented motor activity in the pups, and reversed the improved object recognition memory showed by maternal exercise group, suggesting that the possible beneficial effect of maternal swimming was not enough to prevent the damage caused by HI.

Highlights

- Rats subjected to neonatal hypoxia-ischemia increased motor activity in the adulthood
- Pups whose mothers swam during pregnancy had a trend of improvement in memory in object recognition task
- Neonatal hypoxia-ischemia reverted the benefits of maternal exercise in memory
- Memory effects in maternal exercised-rats were BDNF-independent

Key words: Maternal swimming exercise, neonatal hypoxia-ischemia, BDNF, open field test, object recognition test

Abbreviation

BDNF	Brain-Derived Neurotrophic Factor
CHI	Control + hypoxia-ischemia
CNS	Central nervous system
CS	Control + sham
EHI	Maternal exercise + hypoxia-ischemia
ES	Maternal exercise + sham
HI	Hypoxia-ischemia
ME	Maternal exercise
PND	Postnatal day

Introduction

Neonatal hypoxia-ischemia (HI) results in a harmful encephalopathy, occurring in a frequency of approximately 1-6 per 1000 newborns (du Plessis and Volpe, 2002), impairing some types of memory and leading to reduced cognitive skills (Arteni et al., 2003; Arteni et al., 2010; Brockmann et al., 2013; Pereira et al., 2007). These behavioral changes can be directly related to decreased volume and atrophy of certain brain structures such as the hippocampus (Arteni et al., 2010; Fan et al., 2006; Gerstner et al., 2009; Mestriner et al., 2013), and probably grounded in metabolic insult evidenced by excitotoxicity, oxidative stress, and inflammatory mechanisms (Ferriero, 2004). The animal model of Levine (1960) adapted by Rice et al. (1981) is the most widely used to study the effects of HI injury during the perinatal period. In response to the damage caused by HI induction, several neurotrophic signaling factors upregulate in order to restores normal cell function or induces metabolic adaptations. Chen et al. (2013) reports that brain-derived neurotrophic factor (BDNF) is one of these potent brain defense indicators, although its neuroprotective effects are not proven in human beings (Pruunsild et al., 2007).

Considering the complex pathophysiology of HI-encephalopathy and the lack of drugs with full curative activity, the development of neuroprotective therapeutic strategies are required. Aerobic physical activity has been recognized as an important therapeutic modality, which can assist the treatment of chronic diseases (Dishman et al., 2006; Powell et al., 2011; Wolff et al., 2011). In addition to stimulates increased antioxidant capacity in some structures of the central nervous system (CNS) (Dishman et al., 2006; McCloskey et al., 2001), aerobic exercise improves behavioral parameters in animal models and arouses hippocampal neurogenesis (Dishman et al., 2006; Greenwood et al., 2003; Ji et al., 2014). In addition, Erickson

et al. (2011) found a direct relationship between BDNF serum levels and the volume of the anterior hippocampus in humans, and both are increased by aerobic exercise. It has been shown that animal models submitted to voluntary wheel running present enhanced cell proliferation, BDNF levels in hippocampus, and improved learning memory (Gibbons et al., 2014; Yau et al., 2012; Yu et al., 2014). Fascinatingly, mouse underwent treadmill present a better post-ischemic recovery (Choi et al., 2013; Park et al., 2013). Choi (2013) also showed that rats subjected to Levine-Rice model of hypoxia-ischemia performed moderate exercise on a treadmill and exhibited a decrease on the DNA fragments.

In view of physical exercise has proven to be a potential neuroprotective strategy, we wonder whether the exercise during pregnancy would have the same effect in the offspring. We found promising results, evaluating antioxidant status and mitochondriogenesis in brain of young rats delivered from swimming exercised mothers (Marcelino et al., 2013). Cerebellum, parietal cortex, and hippocampus from pups presented enhanced activity of enzymatic and non-enzymatic antioxidants, accompanied by increased mitochondrion mass and membrane potential, compatible with biogenesis (Marcelino et al., 2013). A similar maternal swimming model has shown considerable improvement in Morris water maze performance by rat pups as well as an increased number of neurons in hippocampus (Akhavan et al., 2008; Lee et al., 2006). Recently,(Akhavan et al., 2012) reported that voluntary wheel running exercise in mothers reduced hippocampal neurons loss elicited by postnatal HI. In face of this innovative strategy, the main objective of this study was to evaluate whether the benefits provided by exercise during pregnancy are able to prevent the injuries caused by neonatal HI in the offspring, evaluating behavioral parameters in

adulthood, as well as the total concentration of BDNF in cerebellum, parietal cortex, hippocampus, and striatum.

Materials and methods

1. Animals

Adult female (30 animals) and males (15 animals) Wistar rats were obtained from the Central Animal House of Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. The offspring was kept with its mother in a single box until weaning at 21 days. The animals were maintained on a 12/12-hour light/dark cycle in an air-conditioned colony room at a constant temperature (22±1ºC). They had free access to water and a 20% (w/w) protein commercial chow. Experiments were approved by the local animal ethics commission (Comissão de Ética no Uso de Animais/Universidade Federal do Rio Grande do Sul- CEUA/UFRGS) under the number 23670, and follows national animal rights regulations (Law 11.794/2008), the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication No. 80-23, revised 1996) and Directive 2010/63/EU. We further attest that all efforts were made to minimize the number of animals used and their suffering.

2. Experimental design

Female Wistar rats (approximate body weight = 250 g) were initially divided into the following groups: (1) sedentary control group, which were placed in contact with the stress of the aquatic environment, without swimming; (2) the exercised group, which were subjected to swimming exercise for one week previous to mating, and during

the pregnancy. To mating, one male rat was placed in contact with two females for 12 hours. Pregnancy was diagnosed by the presence of vaginal plug. The pregnant rats were submitted to the exercise protocol during all the pregnancy. From the 20st day after the onset of pregnancy, the rats were observed twice a day (9h and 18h), to verify the litter's birth. The day corresponding to the birth of offspring is defined as postnatal day (PND) 0 (Fig. 1A). The offspring, on PND7, was subjected to hypoxia-ischemia model. From this point, rats were divided into the following 4 groups: (1) control + sham (CS), (2) control + hypoxia-ischemia (CHI), (3) maternal exercise + sham (ES), or (4) maternal exercise + hypoxia-ischemia (EHI). The pups weaning occurred in PND21, and the rats where reallocated in the house box in number of 5 until the behavioral tests, in PND60. Twenty-four hours after the end of memory tests, the animals were euthanized and the brain was isolated and dissected into cerebellum, parietal cortex, hippocampus, and striatum. Samples were stored at -80°C until used in the quantification of mature BDNF by immunoassay (Fig. 1B). In order to reduce the litter effect, we used one-two pups from each offspring (total = 86 animals).

2.1. Maternal swimming protocol

The maternal exercise protocol was adapted from Lee et al. (2006), as described in Marcelino et al. (2013). The rats were divided into control and exercised groups. In the exercised group, rats were submitted to swimming in a pool filled with 32±1ºC water on 5 day/week for 4 weeks. Each swimming session lasted for 30 minutes, and always took place between 9 and 12 a.m. Each rat was isolated for the swim, which was conducted using an apparatus designed specifically for rat swimming. Within this apparatus, each room measures 30x30x90 cm (widthxlengthxdepth), preventing the animals from touching the bottom of the tank. The animals were left free to swim,

without any extra weight, and were gently stimulated to swimming. This protocol has moderate intensity. Control rats were immersed in water, carefully dried, and returned to the housing boxes.

2.2. Model of hypoxia-ischemia (HI)

The method of Levine (1960) further modified by Rice et al. (1981), producing a unilateral brain lesion in the offspring (PND7). The pups, anesthetized by air halothane, suffered an incision on the ventral surface of the neck (parallel and lateral to the trachea), facilitating access to the right carotid artery, thus being isolated from the vague and permanently occluded with surgical thread. Animals submitted to surgery were placed 15 minutes under a heating lamp for post-surgical recovery and then were returned to their mothers. After 2h of maternal care, the pups were exposed to 90 minutes of hypoxia atmosphere with a standard mixture of 8% oxygen and 92% nitrogen in a 1500 mL chamber partially immersed in a water bath at 37°C (Arteni et al., 2003; Rodrigues et al., 2004). Shortly after the hypoxic ischemic procedure, the animals returned to their mothers in their respective cages. Controls were sham-operated, i.e., subjected to the effect of anesthetic and suffered the same incision and isolation of the carotid artery, but without the obstruction or even coming into contact with the environment of hypoxia.

3. Behavioral tests

Behavior tests were carried out in properly acclimatized rooms with a constant temperature of 21 ± 1°C and controlled lighting. A habituation was performed so the animals get used to the environment room for 1 hour before the experiments. The tests were all conducted in the period day.

3.1. Open field task

The motor performance test was conducted in the open field task (Netto et al., 1986). The main objective of this test was to evaluate possible changes in motor behavior in animals subjected HI model in the perinatal period whose mothers had performed the involuntary swimming protocol, through the analysis of variables: total distance traveled, number of crossings, mobility and activity time. The apparatus consists of a box dimensions 40X50X60 cm with the background divided into 12 equal quadrants. The rats were always placed in the same starting position in the opposite corner where the objects in the object recognition test were placed, and left free to explore for 5 min. After performing the task the animal was removed, returning to the housing box; and the apparatus cleaned with ethanol, repeating this procedure for all animals.

3.2. Object recognition task

The object recognition task evaluates declarative memory (Ennaceur and Delacour, 1988). The memory test was performed one day after the open field test, in the same apparatus, and was carried out in two stages: training and testing. In the first stage, each animal was exposed to two identical objects (object A and object A') and we recorded the time spent on each of them, lasting 5 min for each rat. In the next stage, a similar protocol was repeated, replacing one of the objects (object A and object B). The time spent exploring the two objects was recorded. This experiment aims to measure the exploitation time of the novel object in the test session, performed 24 hours after training, considered a long-term memory index. Data were analyzed through the second session exploitation index: (object B - object A)/(object B + object A) (Mostafa et al., 2002; Rojas et al., 2013).

4. Mature BDNF concentration

Mature BDNF protein was assessed using the E-Max ELISA kit (Promega) according to the manufacturer's recommendations (Matté et al., 2009). Briefly, brain structures were individually homogenized (1:10 w:v) in lysis buffer containing: 137 mM NaCl, 20 mM Tris–HCl (pH 8.0), Igepal (1%), glycerol (10%), 1 mM phenylmethanesulfonyl fluoride (PMSF), 0.5 mM sodium vanadate, 0.1 mM EDTA, and 0.1 mM EGTA, and centrifuged for 3 min at 14,000 rpm at 4 °C. Supernatant was diluted (1:5 v/v) in sample buffer and incubated on a 96-well flat-bottom plates previously coated with anti-BDNF monoclonal antibody and blocked with Block and Sample buffer. After sample incubation, plates were incubated with polyclonal anti-human antibody for 2 h and horseradish peroxidase for 1 h. Then color reaction with tetramethylbenzidine was quantified in a plate reader at 450 nm. The standard BDNF curve, ranging from 0 to 500 pg/mL, was performed in each plate.

5. Protein determination

Protein concentration was measured by the method of Lowry et al. (1951), using bovine serum albumin as standard.

6. Statistical analysis

A normality test D'Agostino-Pearson was conducted first. Considering the model with two variables (maternal exercise and HI), two-way ANOVA was used, and, when F-test was significant, we performed Tukey's multiple comparison test for parametric data. The data were presented as mean ± SEM. It was used the program GraphPad 6 for the purposes of the calculation. The differences were statistically significant if $p < 0.05$.

Results

Hypoxia-ischemia increased motor activity in the Open field test

Through the open field test was possible to analyze the motor activity of rats. Two-way ANOVA followed by Tukey's post hoc showed an increase in the total distance traveled during the experiment in CHI and EHI groups compared to their respective controls (CS and ES), demonstrating the effect of HI variable ($F(1,82)=26.30$, $p<0.0001$) (Fig. 2A). The same motor profile could be observed in the following variables: time mobile ($F(1,82)=14.83$, $p=0.0002$) (Fig. 2B), time active ($F(1,82)=14.85$, $p = 0.0002$) (Fig. 2C) and number of crossings ($F(1,79)=28.50$, $p<0.0001$) (Fig 2D). These data indicate a change in motor activity in pups subjected to neonatal HI, whose mothers performed swimming during pregnancy or remained sedentary.

HI reversed the improvement in object recognition memory observed in the offspring from exercised mothers

Recognition memory evaluated by object recognition task uses the natural tendency of rats to explore the novel object. Increased locomotion observed in open field test for HI animals was confirmed in the object recognition test, supported by a higher distance traveled in the apparatus when compared to controls ($F(1,73)=10.89$, $p=0.0015$) (Fig. 3A). In the test session, all groups explored object A, already presented in the training session, in a similar way ($F(1,73)=0.2146$, $p=0.6446$) (Fig. 3B), however, ES group spent a longer time exploring the object B when compared to

the CS group, thereby characterizing a statistical significance in the variable exercise showed by two-way ANOVA analysis ($F(1,17)=5.796$, $p=0.0187$) (Fig. 3C). The inhibition of the positive effect elicited by maternal physical activity in the new object discrimination rate ($F(1,67)=1.184$, $p=0.2804$) demonstrated in HIE group evidenced the harmful effect of HI protocol on long-term memory ($F(1,67)=5.172$, $p=0.0262$) (Fig. 3D).

Object recognition memory effect is BDNF-independent

Mature BDNF was detected by ELISA immunoassay technique. Striatum, ipsilateral to the carotid obstruction, presented an interaction between the two variables HI and maternal exercise ($F(1,20)=5.478$, $p=0.0287$), while the contralateral hemisphere to the carotid obstruction was not affected ($F(1,20)=3.871$, $p=0.0632$) (Fig. 4D). There was no statistically significant change observed in the two-way ANOVA analyses on cerebellum (contralateral $F(1,20)=0.0106$, $p=0.9189$; ipsilateral $F(1,20)=0.0751$, $p=0.7869$) (Fig. 4A); parietal cortex (contralateral $F(1,20)=2.181$, $p=0.1553$; ipsilateral $F(1,20)=3.168$, $p=0.0716$) (Fig. 4B), and hippocampus (contralateral $F(1,20)=0.0213$, $p=0.8854$; ipsilateral $F(1,18)=0.5850$, $p=0.4543$) (Fig. 4C).

Discussion

HI encephalopathy, induced in neonate rats by right carotid occlusion followed by a hypoxic environment, elicits extensive damage to hippocampus, cortex and striatum affecting sensorimotor function, precipitating learning and memory disabilities (Levine, 1960; Rice et al., 1981; Vannucci and Hagberg, 2004). In order to establish a non-pharmacologic strategy to prevent mnemonic prejudice, adult female Wistar rats underwent swimming exercise five days/week during four weeks, starting one week previous to mating. The offspring experienced the HI protocol on the 7th day of life, and was evaluated in the tasks on the 60th day of life. We observed a high locomotor activity in HI-rats, which was not prevented by maternal aerobic exercise. Otherwise, maternal swimming seems to improve object recognition long-term memory in the offspring, in a mechanism apparently BDNF-independent. The worthwhile effect elicited by *in utero* intervention was reversed by hypoxic-ischemic condition, evidencing its interference in learning/memory.

It is known that CNS injuries cause significant metabolic and behavioral changes. In adulthood, these animals showed a metabolic adaptation to injury caused by deprivation of oxygen and nutrients, but it was not enough to prevent the changes in motor function. In the open field task, evaluated 53 days after HI insult, we observed that animals submitted to perinatal HI model developed a higher motor activity when compared to control groups, evidenced by the total distance traveled during observation of 5 min experiment, number of line crossings in the apparatus, as well as mobile and active time, which is consistent with the literature (Delcour et al., 2012a; Lubics et al., 2005; Rojas et al., 2013; Sanches et al., 2015; Tejkalova et al., 2007). Motor hyperactivity induced by HI was reversed by long-term administration of caspase inhibitor, measured in the open field task seven weeks after the insult (Han

et al., 2014). In this regard, the atrophy of certain brain structures such as the hippocampi (Arteni et al., 2010; Fan et al., 2006; Gerstner et al., 2009; Weis et al., 2014) may explain the results observed in the groups submitted to HI model. Maternal exercise did not alter motor activity, which is in accord with literature (Bustamante et al., 2013).

The tendency for new object preference was measured through the object recognition task, and the discrimination between novel and familiar objects, spending more time exploring the novel than the familiar object, was taken as a memory index, which is consistent with the literature (Akhavan et al., 2013; Lee et al., 2006; Robinson and Bucci, 2014), suggesting that swimming maternal exercise could modulates the cellular mechanisms of memory that relies on hippocampus and perirhinal cortex, repercussing in adulthood. Voluntary or involuntary physical exercise increases learning and memory in rats, as well as the density of hippocampal CA1 region (Inoue et al., 2014; Okamoto et al., 2012; Uysal et al., 2015). Furthermore, the positive memory modulation was not observed in EHI group, suggesting suppression by HI insult. The perinatal brain injury causes a difficulty to retrieve information on the new object recognition task, affecting the long-term memory (Delcour et al., 2012b). Literature shows that neonatal HI induced a significant learning impairment on the object recognition test in rats, performed 3 weeks after insult protocol, when and environmental enrichment reversed the memory changes (Cuellar-Rodriguez et al., 2013). In agreement, (Rojas et al., 2013) reported an increment in locomotion and an impaired object discrimination in object recognition task, in rats evaluated 9 weeks after neonatal HI. In the same study, aversive memory was also impaired by oxygen/nutrient deprivation, as well as dendritic spines density, which were not entirely reversed by environmental

enrichment. In a similar way, we also found a statistically significant deleterious effect of HI, which reverse a positive benefit of maternal exercise on object recognition task. Meanwhile, the age of the rats used in our experiments was different of the studies conducted by (Cuellar-Rodriguez et al., 2013) and (Rojas et al., 2013), and might explain the diverse results. Also the increased hyperactivity observed in open field and object recognition by increasing the total distance traveled found in our results, may explain the low performance of these animals on time exploring the objects.

According to the literature pro and mature BDNF possess diverse functions concerning neural system (Chen et al., 2013; Greenberg et al., 2009; Yang et al., 2009). Mature BDNF has potent effects on neuron differentiation, synaptic plasticity, regulating complex behaviors, while proBNDF can be critical in the neuronal cell death (Chen et al., 2013; Kenchappa et al., 2010). Our results showed BDNF concentration was not changed in any of the brain structures evaluated, presenting similar levels in both hemispheres with greater levels in cerebellum and hippocampus when compared to parietal cortex and striatum. In contrast, literature reports (Akhavan et al., 2013; Uysal et al., 2011; Uysal et al., 2015) showed the central role for BDNF mediating the effects of voluntary maternal exercise in the offspring. Some studies have shown that this neurotrophic factor protects against injuries caused by HI (Han and Holtzman, 2000; Hill et al., 1995; Mehmet et al., 1994; Pulera et al., 1998; Sidhu et al., 1997), being indicated even as a possible treatment against CNS damage.

In conclusion, object recognition memory could be ameliorated by aerobic maternal exercise in a BDNF-independent pathway, implying in adulthood repercussion of a non-pharmacological therapy. Furthermore, neonate HI encephalopathy reversed positive mnemonic effects established by maternal exercise and elicits

hyperlocomotor activity in the offspring. The development of innovative therapies to neonatal HI encephalopathy is required, and *in utero* interventions could bring long-term benefits. Possibly we need to merge a plethora of therapies to face such complex encephalopathy.

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Conflict of interest

The authors declare no conflicts of interest or competing interest.

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Figure Subtitles

Fig. 1. Experimental design to mother (A) and offspring (B).

Fig. 2. Open field test performed on adult offspring who underwent neonatal hypoxia-ischemia, and whose mothers experienced the involuntary swimming exercise. The variables evaluated were: total distance traveled (A), mobility time (B), activity time (C) and number of crossings (D). White bar represents surgical Sham and black bar represents HI group. Results are expressed as mean ± S.E.M. for 20-22 animals in each group, ***p<0.001 (Two-Way ANOVA, followed by Tukey's test). HI: hypoxia-ischemia, ME: maternal exercise

Fig. 3. Object recognition test performed on adult offspring who underwent neonatal hypoxia-ischemia, and whose mothers experienced the involuntary swimming exercise. The variables evaluated were: total distance traveled (A), time spent exploring object A (B), time spent exploring object B (C), and exploitation index (D). White bar represents surgical Sham and black bar represents HI group. Results are expressed as mean ± S.E.M. for 22 animals in each group, *p<0.05 and **p<0.01 and (Two-Way ANOVA, followed by Tukey's test). HI: hypoxia-ischemia, ME: maternal exercise

Fig. 4. Mature BDNF concentration measured on adult offspring who underwent neonatal hypoxia-ischemia, and whose mothers experienced the involuntary swimming exercise, euthanized 24h after object recognition test: cerebellum (A), parietal cortex (B), hippocampus (C), and striatum (D). White bar represents surgical Sham and black bar represents HI group. Results are expressed as mean ± S.E.M. for 6 animals in each group (Two-Way ANOVA, followed by Tukey's test). HI: hypoxia-ischemia, ME: maternal exercise.

Fig. 1

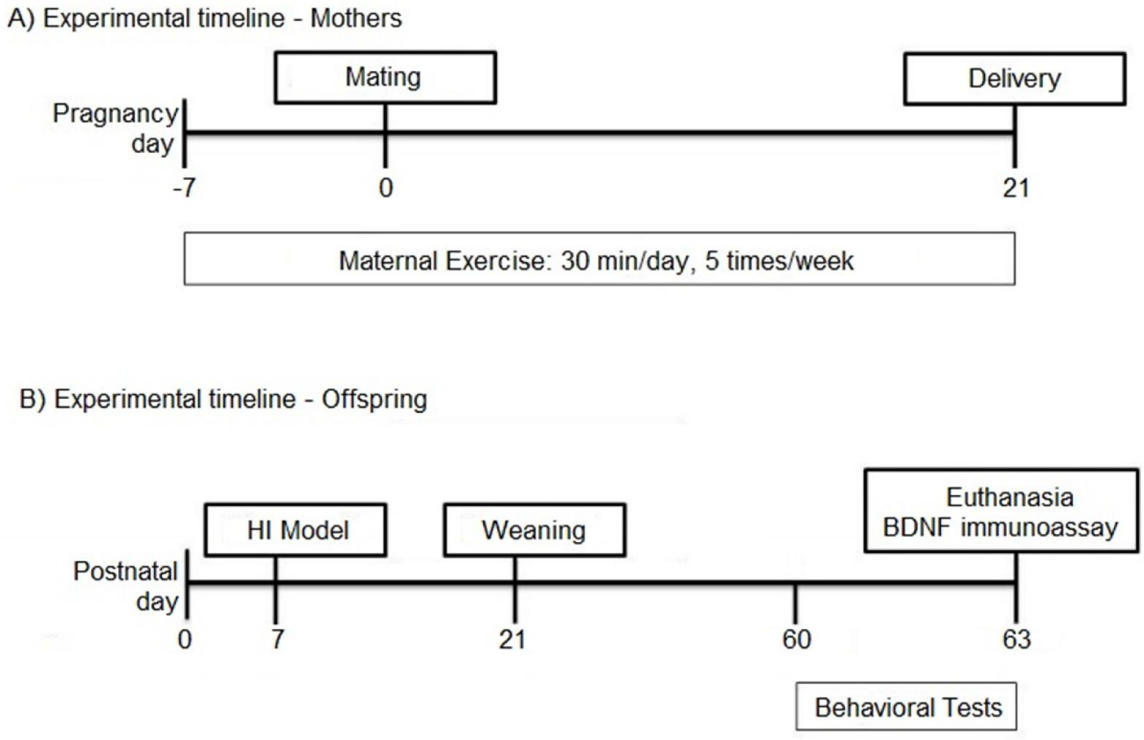

A) Experimental timeline - Mothers
Mating
Delivery
Pragnancy day
-7
0
21
Maternal Exercise: 30 min/day, 5 times/week
B) Experimental timeline - Offspring
HI Model
Weaning
Euthanasia
BDNF immunoassay
Postnatal day
0
7
21
60
63
Behavioral Tests

Fig. 2

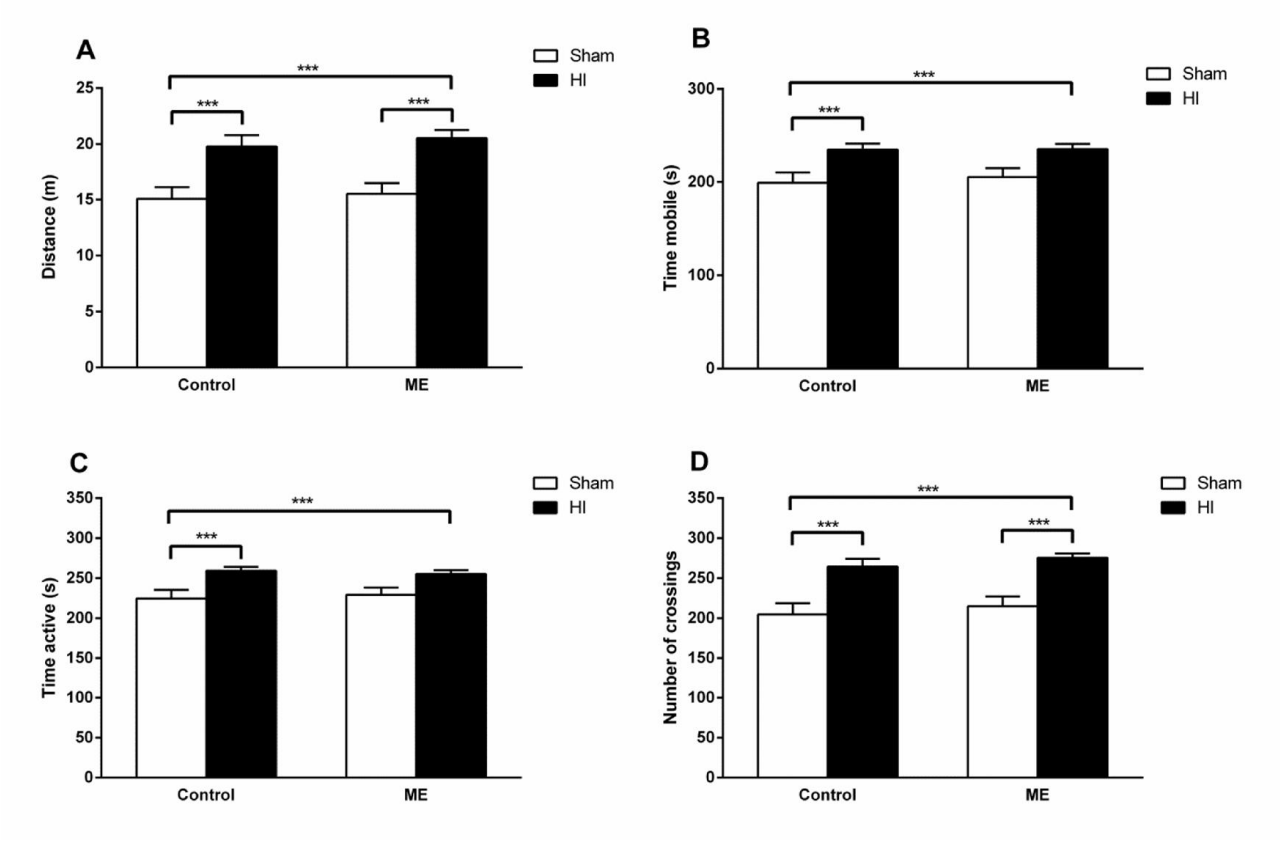

A
Distance (m)
25
20
15
10
5
0
***
***
***
Sham
HI
Control
ME
B
Time mobile (s)
300
200
100
0
***
***
Sham
HI
Control
ME
C
Time active (s)
350
300
250
200
150
100
50
0
***
***
Sham
HI
Control
ME
D
Number of crossings
350
300
250
200
150
100
50
0
***
***
***
Sham
HI
Control
ME

Fig. 3

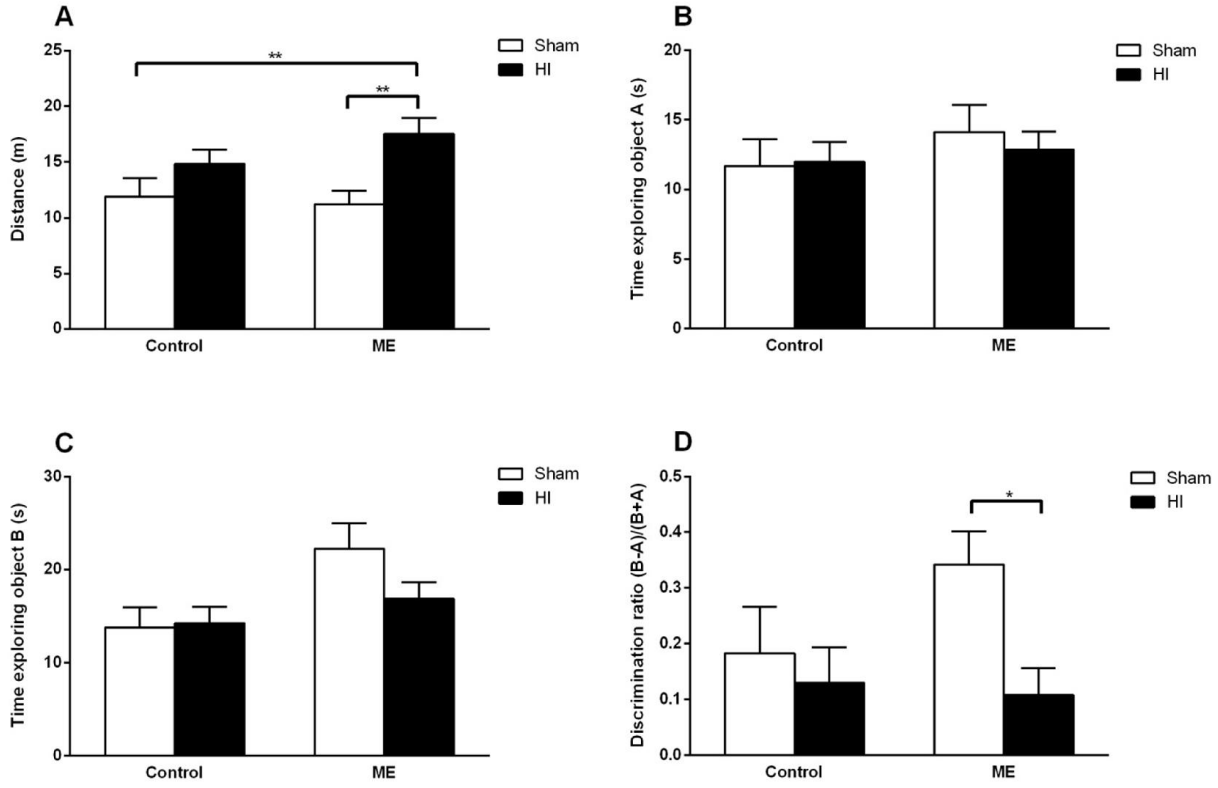
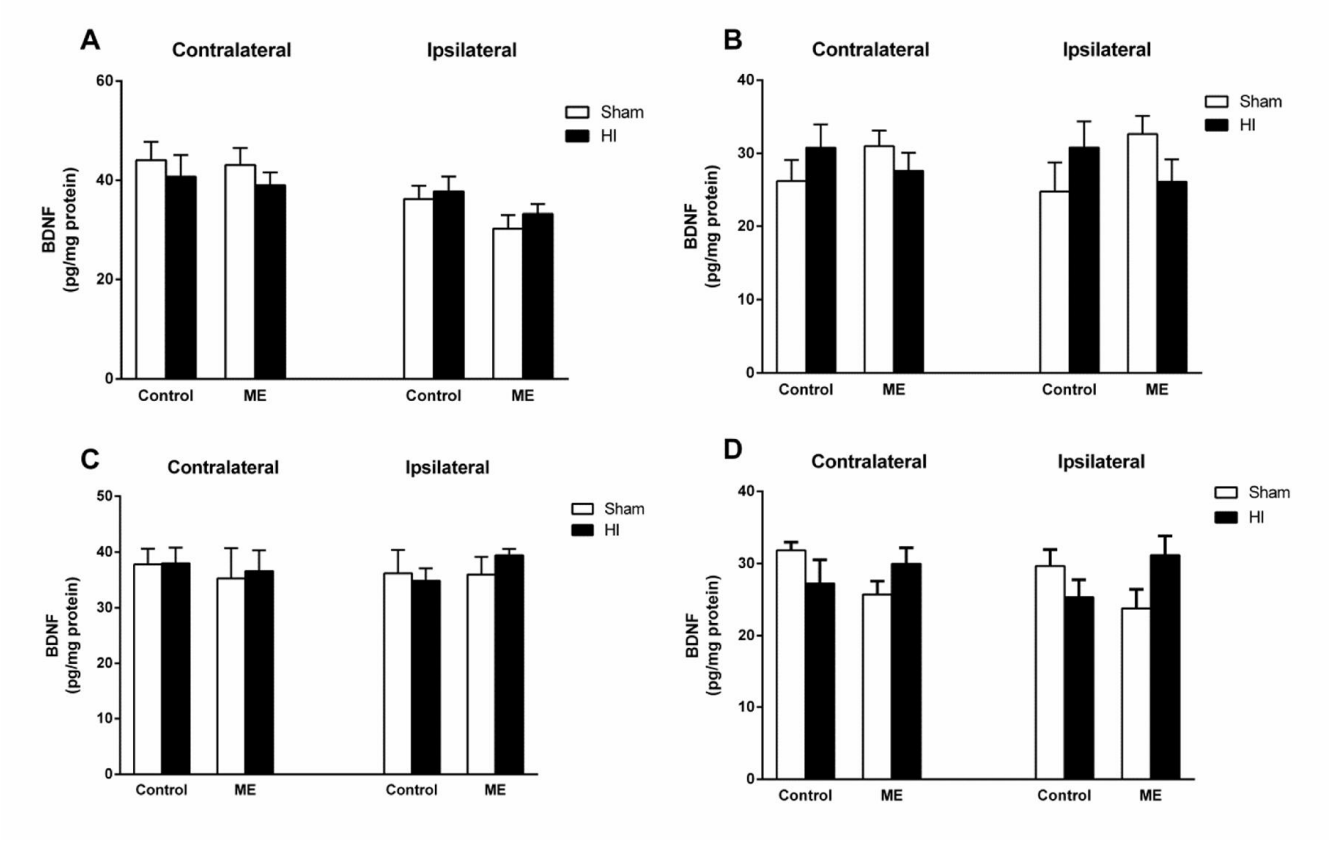

A
Distance (m)
25
20
15
10
5
0
**
**
Sham
HI
Control
ME
B
Time exploring object A (s)
20
15
10
5
0
Sham
HI
Control
ME
C
Time exploring object B (s)
30
20
10
0
Sham
HI
Control
ME
D
Discrimination ratio (B-A)/(B+A)
0.5
0.4
0.3
0.2
0.1
0.0
*
Sham
HI
Control
ME

Fig. 4


A
Contralateral
Ipsilateral
BDNF (pg/mg protein)
Sham
HI
Control
ME
B
C
D

Parte III

III. Discussão

Devido à frequência significativa de casos de neonatos com encefalopatias causadas pela privação total ou parcial de oxigênio e nutrientes para o SNC de recém-nascido, as pesquisas que buscam a descoberta de mecanismos fisiopatológicos e ferramentas terapêuticas são bastante relevantes. Estudos que utilizam o modelo de Levine adaptado por Rice e colaboradores, em 1981, em ratos de 7 dias (Rice et al., 1981), mostram uma evidente lesão tecidual no hemisfério ipsilateral a oclusão da carótida nas estruturas do córtex, hipocampo e estriado, ocasionando alterações metabólicas e cognitivas (Levine, 1960; Pereira et al., 2007; Rice et al., 1981; Vannucci e Vannucci, 2005). As cascatas de reações bioquímicas que ocasionam a morte celular nas estruturas do encéfalo em ratos submetidos ao modelo de HI envolvem uma falha na demanda energética pela disfunção mitocondrial, excesso de glutamato nas fendas sinápticas, ativação de cascatas relacionada a apoptose e morte celular programada, uma resposta inflamatória e um aumento do estresse oxidativo (Ferriero, 2001; Ferriero, 2004; Perlman, 2006; Vannucci e Hagberg, 2004). Essas alterações estruturais, bioquímicas e comportamentais foram observadas neste trabalho e os resultados encontrados ajudam a explicar os possíveis motivos e consequências dessa lesão nesses animais.

A realização de natação pelas ratas prenhez aumenta a produção de espécies reativas no feto, estimulando adaptações metabólicas no sistema de defesa antioxidante e biogênese mitocondrial em estruturas encefálicas (Marcelino et al., 2013). Esses efeitos, considerados sinais de neuroproteção, poderiam auxiliar a combater o estresse oxidativo causado pela modelo de HI,

evitando um efeito agudo durante os primeiros momentos da lesão ou um efeito adaptativo que se prolonga até a fase adulta. Essa foi a premissa para a realização desse estudo.

A estrutura do hipocampo é uma das mais estudadas, pois o dano causado pela HI pode trazer diversas alterações do comportamento a longo-prazo, na memória espacial e de trabalho em ratos adultos (Arteni et al., 2003; Pereira et al., 2007). Uma das consequências da lesão no sistema nervoso é o estresse oxidativo devido à vulnerabilidade do encéfalo em ratos neonatos (Alkan et al., 2008; Sarco et al., 2000). Alguns trabalhos citam o acúmulo de H_2O_2como uma das ERO gerada após o insulto, responsável pelo estresse oxidativo e morte celular que ocorre no tecido, devido a grande concentração de ferro livre, facilitando a reação de Fenton e consequente liberação do •OH (Alkan et al., 2008; Hyslop et al., 1995; Penna et al., 2014; Sarco et al., 2000; Sheldon et al., 2007). Esse aumento do H_2O_2 pode ser o fator responsável pelo aumento da atividade da GPx encontrado nos grupos HI em ambos os hemisférios, uma vez que essa enzima é uma das principais responsáveis pela eliminação desta ERO (Dringen et al., 2005). Esses dados são corroborados pela diminuição da oxidação do DCF, indicando que possivelmente o aumento da atividade da GPx foi suficiente para reduzir os níveis dessa ERO, que pode ter sido gerada agudamente pelo processo hipóxico-isquêmico no encéfalo dos neonatos com 7 dias de vida e quando o hipocampo foi analisado com 21 dias de vida apresentou esse perfil de adaptação. Também foi observada uma interação entre a variável exercício materno e HI no hemisfério contralateral a obstrução da carótida na atividade da SOD. O grupo Sham-operado apresentou um aumento da atividade da SOD no grupo exercitado, o que está

de acordo com o que nosso grupo havia descrito em trabalho anterior (Marcelino et al., 2013).

A exatidão do movimento, o controle motor e a tomada de decisão baseado em pistas visuais é responsabilidade do córtex parietal (Drew e Marigold, 2015; Penhune e Steele, 2012), o qual é uma das estruturas mais afetadas pelo protocolo de HI (Bregant et al., 2013). Após o bloqueio da oxigenação do SNC, acredita-se que uma cascata de eventos neurotóxicos ocorre como, por exemplo, a formação de ERN pela NOS (Delivoria-Papadopoulos e Mishra, 1998; Liu et al., 2014). Ferriero et al. (1995) demonstraram que, destruindo os neurônios que continham NOS um dia antes da lesão hipóxico-isquêmica, os ratos apresentavam uma redução da lesão tecidual nessa região. Embora tenhamos mostrado um aumento da rede antioxidante nessa estrutura nos filhotes cujas mães realizaram natação durante a gravidez (Marcelino et al., 2013), a atividade da GPx juntamente com a oxidação do DCF foram diminuídas nos ratos que sofreram HI, independente do fator exercício materno. A falta de elementos que avaliem o metabolismo das ERN nesse estudo limitam nossas conclusões nessa estrutura cerebral.

Assim como o hipocampo e o córtex parietal, o estriado também foi afetado, apresentando alterações no seu status redox. Essa estrutura é um dos núcleos da base responsável pelo controle de movimento, além de estar envolvido em algumas funções cognitivas como memória de trabalho (Baydyuk e Xu, 2014; Bolam et al., 2000; Voytek e Knight, 2010). Ele apresentou um perfil oxidativo oposto ao hipocampo, com um possível aumento dos níveis de espécies reativas, detectado pela técnica de oxidação do DCF, bem como um aumento da atividade da SOD, o que pode ser um efeito adaptativo na tentativa

de detoxificação das ERO. No protocolo de HI, após o período de privação de oxigênio, o encéfalo dos filhotes passa por uma fase de reperfusão onde ocorre uma grande entrada de O_2, aumentando a atividade da cadeia transportadora de elétrons que faz com que aumente a produção de ERO (Armogida et al., 2012). Como uma adaptação a este excesso de $O_2^{\cdot -}$ formado, a atividade da SOD pode ter sido aumentada. Alguns trabalhos mostram que o aumento da expressão da SOD no encéfalo de ratos adultos reduz os danos teciduais causados pela HI (Yang et al., 1994).

O cerebelo tem se mostrado envolvido em outras funções além de coordenação motora, tônus muscular e regulação do reflexo, como em processos cognitivos relacionados ao aprendizado motor (Hashimoto et al., 2015; Penhune e Steele, 2012; Shmuelof e Krakauer, 2011). Os nossos resultados mostraram que a lesão proporcionada pelo modelo de HI não causou muitos danos nessa estrutura, sem apresentar alterações drásticas no perfil antioxidante no cerebelo dos filhotes. A alteração que foi encontrada nessa estrutura foi uma modulação positiva na concentração de GSH juntamente com o aumento da atividade da GPx no hemisfério contralateral a obstrução da carótida em filhotes submetidos à HI provenientes do grupo cujas mães realizaram natação durante a gestação. Esse seria o sinal de uma possível adaptação dessa estrutura durante seu desenvolvimento (Benitez et al., 2014).

Sabe-se que lesões que atingem o SNC podem estar acompanhadas de alterações neuroquímicas significativas, tais como as encontradas nesse estudo, que podem refletir no comportamento e memória do animal. Os ratos

submetidos ao modelo de HI apresentam uma alteração na atividade motora seguida de outros comprometimentos cognitivos (Vannucci e Hagberg, 2004). Existem alguns estudos que associam a substância branca cortical e o seu comprometimento tecidual a uma alteração direta na atividade motora, e isso pode ser uma das justificativas para a diferença encontrada nos grupos submetidos ao protocolo de HI (Delcour et al., 2012; Inder et al., 1999; Vry et al., 2008). A fim de impedir a instalação dos danos cognitivos, foi utilizado o modelo de natação materna nos filhotes que sofreram HI no período perinatal, avaliando se a adaptação metabólica permaneceu até a fase adulta.

O teste de campo aberto demonstrou que os animais submetidos ao modelo de HI perinatal desenvolveram uma maior atividade motora com relação aos grupos controle, medida por meio da distância total percorrida durante os 5 min de experimento, número de crossings, tempo de atividade e tempo ativo, estando de acordo com alguns trabalhados da literatura (Lubics et al., 2005; Rojas et al., 2013; Sanches et al., 2015; Tejkalova et al., 2007). Esse tipo de comportamento pode indicar uma maior exploração espontânea e hiperatividade motora (Balduini et al., 2000; Row et al., 2002). Não foram observadas alterações significativas no número de *rearings* e *groomings*, conforme mostra Delcour et al. (2012). Uma das possíveis explicações para o comportamento de hiperatividade pode ser devido ao aumento da atividade das caspases, como resultado do dano causado pela HI, trazendo como consequência uma lesão no hipocampo o que altera a atividade motora desses animais (Han et al., 2014; Praag et al., 1994). O exercício materno não alterou a atividade motora, concordando com dados da literatura (Bustamante et al., 2013).

Através do teste de reconhecimento de objetos foi possível observar uma maior preferência de exploração do objeto novo pelos animais cujas mães realizaram o protocolo de natação durante a prenhez. Essa tendência observada nos resultados condiz com os dados da literatura (Akhavan et al., 2013; Lee et al., 2006; Robinson e Bucci, 2014), sugerindo que a natação durante a gravidez pode modular alguns mecanismos celulares responsáveis pela memória e esse efeito permanece até a fase adulta desses animais. Trabalhos mostram que o exercício físico, tanto voluntário quanto involuntário, aumenta o aprendizado e a memória de ratos, bem como o aumento na densidade celular na região CA1 do hipocampo (Inoue et al., 2014; Okamoto et al., 2012; Uysal et al., 2015). Todavia, essa alteração não foi observada no grupo submetido ao protocolo de HI, indicando um possível efeito de reversão causado pela lesão no SNC. Um aumento da atividade locomotora e um comprometimento na discriminação do objeto novo no teste de reconhecimento de objetos também foram observados em ratos nove semanas após a HI (Rojas et al., 2013), o que pode justificar ao menor tempo de exploração encontrado nesses animais. Não foram encontradas diferenças no tempo de exploração dos objetos A e B separadamente nos grupos controle em relação aos dois protocolos tratados, porém, quando é analisado o índice de retenção de memória através da taxa de discriminação do objeto A e B, aparece uma tendência no aumento da preferência de exploração do objeto novo nos grupos controles em relação aos grupos que sofreram o protocolo de HI.

O BDNF apresenta diversas funções relacionadas ao SNC (Chen et al., 2013; Greenberg et al., 2009; Yang et al., 2009). De acordo com a literatura o BDNF pode estar presente nos neurônios de duas formas: na forma madura

onde está relacionado com diferenciação de neurônios, plasticidade sináptica, regulação de comportamentos complexos e na forma de pró-BDNF, a qual tem um papel critico na morte neuronal (Chen et al., 2013; Kenchappa et al., 2010; Lu et al., 2005). Nossos dados mostram que a concentração de BDNF não foi alterada em nenhuma das estruturas do SNC, apresentando valores maiores em cerebelo e hipocampo, quando comparados com o córtex parietal e o estriado. Alguns trabalhos mostram que esse fator neurotrófico protege contra as lesões causadas pela HI (Han e Holtzman, 2000; Hill et al., 1995; Mehmet et al., 1994; Pulera et al., 1998; Sidhu et al., 1997) sendo indicado até mesmo como um possível tratamento contra os danos no encéfalo.

IV. Conclusão

O protocolo de HI gerou um extenso dano tecidual em cerebelo, córtex parietal, hipocampo e estriado. As avaliações de estresse oxidativo em ratos de 21 dias, mostraram adaptações metabólicas tanto no hemisfério contralateral quanto no ipsilateral à obstrução da carótida. Na idade adulta, os animais submetidos ao modelo de HI neonatal apresentaram alterações na atividade motora e exploratória, compatíveis com hiperatividade.

O exercício físico aeróbico antes e durante a gravidez aumentou a memória de longo-prazo no reconhecimento de objetos nos filhotes, em um mecanismo aparentemente independente de BDNF, porém não foi suficiente para expressar o mesmo efeito nos animais submetidos ao modelo de HI.

Em conclusão, nossos dados demonstram que o dano encefálico causado pela HI não foram prevenidas de forma satisfatória pelo exercício de natação materno. Mais estudos são necessários a fim de identificar novas estratégias terapêuticas para o tratamento da encefalopatia hipóxico-isquêmica.

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VI. Anexos

Guide for Authors – Brain Research

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Divide your article into clearly defined and numbered sections (e.g. Abstract, 1. Introduction, 2. Results, 3. Discussion, 4. Experimental Procedure, Acknowledgements, References). Subsections should be numbered 1.1 (then 1.1.1, 1.1.2, ...), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to " the text" . Any subsection may be given a brief heading. Each heading should appear on its own separate line.

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State the objectives of the work and provide relevant background information. Published studies should be described concisely, and be cited appropriately.

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The results should be described clearly and in logical order without extended discussion of their significance. Results should usually be presented descriptively and be supplemented by photographs or diagrams.

Discussion

The results of the research should be discussed in the context of other relevant published work; Extensive citations and discussion of published literature should be avoided. The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion section.

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The abstract should state briefly (in no more than 250 words) the purpose of the research, the principal results and major conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. For this reason, References should be avoided, but if essential, then cite the author(s) and year(s). Also, non-standard or uncommon abbreviations should be avoided, but if essential they must be defined at their first mention in the abstract itself.

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