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WELLINGTON DE ALMEIDA

EFEITOS DO EXERCÍCIO ACROBÁTICO SOBRE PARÂMETROS COGNITIVOS E PLASTICIDADE HIPOCAMPAL E ESTRIATAL EM RATOS SUBMETIDOS À HIPÓXIA-ISQUEMIA NEONATAL

PORTO ALEGRE - RS
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Dissertação apresentada como requisito parcial à obtenção do título de Mestre em Neurociências pelo Programa de Pós-graduação em Neurociências da Universidade Federal do Rio Grande do Sul – UFRGS.

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LISTA DE ABREVIATURAS

ATP	adenosina trifostato
Ca^{2+}	íons cálcio
DPN	dia pós-natal
HIE	hipóxia-isquemia encefálica
LPV	leucomalácia periventricular
Na^{+}, K^{+}ATPase	transportador de Na^{+}, K^{+} dependente de ATP
NMDA	receptores N-metil D-Aspartato
O_2	gás oxigênio
ROS	espécies reativas de oxigênio
SNC	sistema nervoso central

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RESUMO

A hipóxia-isquemia encefálica (HIE) neonatal pode levar a graves disfunções cognitivas e motoras. Programas de reabilitação física são amplamente utilizados com o intuito de favorecer o desenvolvimento motor e reduzir problemas musculoesqueléticos decorrentes da HIE. Neste contexto, a aprendizagem motora, que pode ser realizada através de exercícios acrobáticos, surge como opção terapêutica coadjuvante frente aos déficits causados pela HIE. Em experimentos com animais, o exercício acrobático já é estudado como forma de tratamento frente aos déficits motores e musculoesqueléticos causados pela HIE, no entanto, os déficits cognitivos ainda não foram avaliados após a aplicação desta modalidade terapêutica. Desta forma, o objetivo deste estudo foi avaliar os efeitos do protocolo de exercício físico acrobático sobre a memória e a atrofia no hipocampo e estriado de ratos *Wistar* machos submetidos à HIE no período neonatal. Aos sete dias de vida dos animais, foi realizado o modelo de HIE de Rice-Vannucci e após o desmame, os mesmos foram separados nos seguintes grupos: controle não exercitado (CTSED); controle submetido ao treinamento acrobático (CTACR); animais submetidos ao modelo de hipóxia-isquemia não exercitados (HISED) e animais submetidos ao modelo de hipóxia-isquemia e ao treinamento acrobático (HIACR). Após 4 semanas de exposição ao protocolo de treinamento acrobático, os animais foram submetidos aos testes de reconhecimento de objetos e labirinto aquático de Morris; após, os encéfalos foram coletados para análise da atrofia do hipocampo e do estriado, além da quantificação dos níveis de BDNF nas mesmas estruturas. Os resultados obtidos mostraram déficit na memória espacial causada pela HIE, nas avaliações realizadas no labirinto aquático de Morris e também no teste de reconhecimento de objetos (RO), no entanto, no teste do RO os animais HIACR tiveram um melhor desempenho quando comparados com HISED. A análise histológica do hipocampo e do estriado mostrou atrofia no hemisfério ipsilateral à lesão, que não foi revertida pelo exercício acrobático. Em relação à quantificação do BDNF, não houve diferença em ambas as estruturas e hemisférios cerebrais. Desta forma, este estudo sugere que o exercício acrobático pode ser uma estratégia coadjuvante promissora para o tratamento de déficits cognitivos relacionados à HIE, no entanto mais estudos são necessários para melhor compreender os possíveis mecanismos relacionados a este efeito benéfico.

Palavras-chave: Asfixia perinatal, treinamento de habilidade, neurodesenvolvimento, exercício físico.

ABSTRACT

Neonatal hypoxia-ischemia (HI) can lead to severe cognitive and motor dysfunction in survivors. Physical rehabilitation programs are widely used in order to promote motor development and reduce musculoskeletal problems. In this context, motor learning, which can be performed through acrobatic exercises, appears as a therapeutic option to manage with the deficits caused by HI. In the experimental context, acrobatic exercise has been already studied as a form of treatment for motor and musculoskeletal deficits caused by HI, however, cognitive deficits have not yet been evaluated after the application of this therapeutic modality. Thus, the objective of this study was to evaluate the effects of acrobatic physical exercise protocol on memory, and hippocampal and striatal size of male *Wistar* rats submitted to hypoxic-ischemic insult in the neonatal period. At seven days of age, the pups were submitted to the Rice-Vannucci hypoxia-ischemia model and after weaning, they were separated into the following groups: non-exercised control (CTSED); control submitted to acrobatic training (CTACR); non-exercised submitted to HI insult (HISED) and animals submitted to HI and acrobatic training (HIACR). After five weeks of acrobatic training protocol, the animals were submitted to the novel-object recognition task (NOR) and the Morris water maze (WM), and after, the brains were collected for analysis of the atrophy in hippocampus and striatum, as well as the quantification of BDNF levels in these structures. The results showed deficits in the spatial memory caused by HI, in the WM and NOR evaluations, however, in the NOR evaluation the HIACR had a better performance when compared to HISED. The results of the histological analysis of the hippocampus and the striatum show atrophy in the hemisphere ipsilateral to the lesion, which was not reversed by acrobatic exercise. The quantification of BDNF showed no significant difference in both brain structures and hemispheres. Thus, this study suggests that acrobatic exercise may be a promising adjuvant strategy for the treatment of cognitive deficits related to HIE, however more studies are needed to understand the possible mechanisms related to this beneficial effect.

Key-words: Perinatal asphyxia, physical exercise, neurodevelopment, motor skill learning.

1 INTRODUÇÃO

1.1 HIPÓXIA-ISQUEMIA ENCEFÁLICA NEONATAL

A hipóxia-isquemia encefálica (HIE) neonatal é uma condição clínica que pode ocorrer antes, durante ou após o nascimento, sendo caracterizada pela redução do fluxo sanguíneo para o encéfalo, com consequente redução das quantidades de oxigênio e glicose disponíveis (HABERNY *et al.*, 2002; GOPAGONDANAHALLI *et al.*, 2016). A HIE é o principal fator de risco para o desenvolvimento da encefalopatia da prematuridade, que é um termo descritivo para uma síndrome de disfunção cerebral global de amplo espectro (DAVIES *et al.*, 2012). Os danos cerebrais decorrentes da HIE ainda permanecem como um importante problema de saúde, sendo que a estimativa da sua incidência varia de 1,3 a 1,7 para cada 1000 nascidos vivos a termo (KURINCZUK *et al.*, 2010; ARTEAGA *et al.*, 2017). Apesar dos avanços nos cuidados obstétricos e neonatais, a incidência se mantém, sendo que a HIE pode levar a graves disfunções cognitivas e motoras aos sobreviventes (KIM, G. S. *et al.*, 2014; MILLAR *et al.*, 2017).

Sabe-se que a partir da década de 1990 houve um aumento na sobrevivência de recém-nascidos, principalmente prematuros nascidos após a 23º semana de gestação, devido a melhora nos cuidados pré-natais e progressos na tecnologia de terapia respiratória e intensiva assistida (ROBERTSON; IWATA, 2007). Essa dimuição das taxas de mortalidade dos prematuros consequentemente levou a um aumento na freqüência de distúrbios motores e cognitivos ligados à lesão encefálica nos períodos perinatal e pós-natal (O'SHEA *et al.*, 1998; LARROQUE *et al.*, 2004; FELLMAN *et al.*, 2009; MILLAR *et al.*, 2017).

Em humanos, o desenvolvimento encefálico continua durante os primeiros anos de vida, sendo portanto muito susceptível a lesões nos períodos pré, peri e pós-natal precoce (BASS, 1999; KRIGGER, 2006). Ainda, estudos sugerem que respostas inflamatórias não específicas e outros tipos de estresse durante o período pré-natal são importantes fatores de risco para o desenvolvimento da encéfalopatia hipóxico-isquêmica (DEAN *et al.*, 2015). Complicações durante o parto, restrições do crescimento intrauterino, doenças pulmonares, anemia ou redução da pressão sanguínea sistêmica fetal, também podem contribuir para a ocorrência de anóxia, asfixia, isquemia ou HIE (O'SHEA, 2002; COQ *et al.*, 2016; PAMENTER, 2016).

O diagnóstico clínico de hipóxia-isquemia neonatal baseia-se em dois critérios distintos, um deles consiste em evidências de depressão neurológica e cardiorrespiratória (obtenção de menos de 7 no índice de Apgar aos 5 minutos após o nascimento) (MACLENNAN, 2000). A escala ou índice de Apgar é um teste desenvolvido pela médica norte-mericana Virginia Apgar, que consiste na avaliação de 5 sinais objetivos do recém-nascido (frequência cardíaca, respiração, tônus muscular, irritabilidade reflexa e cor da pele), atribuindo-se a cada um dos sinais uma pontuação de 0 a 2, o somatório da pontuação (no mínimo 0 e no máximo 10) resultará no Índice de Apgar (APGAR, 1966). Outro critério consiste em acidemia (definido como um pH do sangue arterial inferior a 7), pois o termo asfixia é definido experimentalmente como trocas gasosas respiratórias prejudicadas, acompanhadas do desenvolvimento de acidose metabólica (MACLENNAN, 2000).

O diagnóstico da HIE pode ser realizado com base na presença de um tônus muscular anormal, desenvolvimento motor lento, déficits posturais e persistência de reflexos primitivos (KRIGGER, 2006). Os distúrbios motores são frequentemente acompanhados por perturbações da função sensorial, cognição, comunicação e comportamento, além de estar associada a problemas secundários como a epilepsia e alterações musculoesqueléticas (MCLEAN; FERRIERO, 2004; ROSENBAUM *et al.*, 2007; COQ *et al.*, 2016). No entanto, a maioria das crianças com HIE não apresentam os sinais clínicos precocemente. Em muitos casos, os déficits decorrentes dessa encefalopatia tornam-se identificáveis a medida que os pais percebem um atraso no desenvolvimento cognitivo de seus filhos em relação as demais crianças (BADAWI; KEOGH, 2013).

Atualmente, exames de ressonância magnética e ultrassonografias têm auxiliado no diagnóstico dessa patologia (SALAS *et al.*, 2018). Os achados mais comuns em tais exames de imagem são a compactação ou redução das fibras nervosas, dilatação ventricular, hemorragia intraventricular, hematomas subdurais ou anormalidades da substância cinzenta (WEIERINK *et al.*, 2013; REID *et al.*, 2014; LENNARTSSON *et al.*, 2015; SALAS *et al.*, 2018).

A dilatação dos ventrículos é um dos primeiros sinais nos exames de imagem que indicam presença de uma anormalidade cerebral durante o período pré-natal (LEITNER *et al.*, 2004; GAREL; ALBERTI, 2006). Esse aumento está frequentemente associado com a degeneração da substância branca que está ao seu redor, uma condição

conhecida como leucomalácia periventricular (LPV) (GAREL; ALBERTI, 2006; COQ *et al.*, 2008; GIRARD *et al.*, 2009).

O aumento da liberação de citocinas pró-inflamatórias no encéfalo fetal devido a infecções maternas no período pré-natal pode ser responsável pela dilatação ventricular e pela LPV (NALETILIC *et al.*, 2009; PANG *et al.*, 2010; MALLARD *et al.*, 2014). As citocinas são liberadas pela microglia e causam danos a células precursoras de oligodendrócitos, uma vez que neste período há uma alta proliferação, migração e a maturação destas células. Consequentemente, pode ocorrer uma falha na diferenciação dos oligodendrócitos e na formação da bainha de mielina, levando a hipomielinização e redução da substância branca periventricular (FOLLETT *et al.*, 2000; PANG *et al.*, 2005; PANG *et al.*, 2010; BACK; ROSENBERG, 2014).

A pesquisa com modelos animais é crucial para a compreensão das respostas frente a lesões que acometem o sistema nervoso central (SNC), como é o caso da HIE, e para o desenvolvimento de novas terapias (CLOWRY *et al.*, 2014). Roedores são animais de fácil manejo e amplamente utilizados em estudos experimentais (CLOWRY *et al.*, 2014). O desenvolvimento do sistema nervoso em roedores têm considerável maturação pós-natal, o que difere dos seres humanos (RICE; BARONE, 2000). Além disso, cabe citar que a HIE está associada a vários fatores de risco, achados fisiopatológicos, sinais e sintomas. Apesar dessas limitações, vários estudos vêm sendo desenvolvidos na tentativa de reproduzir em animais as características observadas nestes pacientes e visando o estabelecimento de novas estratégias terapêuticas (CHOI *et al.*, 2011; YU *et al.*, 2013; COQ *et al.*, 2016).

O modelo de hipóxia-isquemia em roedores proposto por (RICE *et al.*, 1981), conhecido como modelo de Rice-Vannucci, é amplamente utilizado como modelo de encefalopatia hipóxico-isquêmica neonatal (PEREIRA *et al.*, 2007). Neste modelo é realizada uma isquemia unilateral através da oclusão da artéria carótida comum (direita ou esquerda) no 7º dia pó-natal (7º DPN), seguido pela exposição dos animais a um ambiente hipóxico, com 8% de oxigênio (O_2). Estudos utilizando este modelo são capazes de reproduzir os déficits funcionais e neuropatológicos também observados em neonatos humanos acometidos pela HIE neonatal (ARTENI *et al.*, 2003; PEREIRA *et al.*, 2007; PEREIRA *et al.*, 2008; MIGUEL *et al.*, 2017).

1.2 FISIOPATOLOGIA DA HIPÓXIA-ISQUEMIA ENCEFÁLICA NEONATAL

Os mecanismos patogênicos da lesão causada pela HI foram classificados em três fases: falha primária de energia que ocorre devido à redução do fluxo global de oxigênio e glicose e consequente falha no processo energético; fase secundária, que ocorre devido à reoxigenação e reperfusão e, por fim, fase terciária em que os eventos anteriores podem piorar e a inflamação resultante tornar-se crônica (DIXON *et al.*, 2015).

Após o insulto hipóxico-isquêmico, há uma rápida depleção de adenosina trifostato (ATP) devido à diminuição da fosforilação oxidativa. Embora a célula mude para o metabolismo anaeróbico, isso é energeticamente ineficiente e resulta na falha da bomba Na^+ / K^+ dependente de ATP (Na^+, K^+ -ATPase), que é uma proteína de membrana essencial que desempenha um papel na manutenção do potencial de membrana em células excitáveis (EDWARDS *et al.*, 2013; ARTEAGA *et al.*, 2017). Tais falhas podem promover um aumento da liberação do glutamato no espaço extracelular, levando a um fenômeno conhecido como excitotoxicidade glutamatérgica (HABERNY *et al.*, 2002).

A via de produção, liberação e recaptação do glutamato é uma das maiores vias metabólicas do encéfalo. A remoção deste neurotransmissor da fenda sináptica depende de transportadores específicos presentes, em sua maioria, nas células da glia. Nestas células o glutamato é convertido em glutamina, esta por sua vez é levada novamente aos neurônios e utilizada para nova síntese de glutamato, sendo todo este processo dependente da maquinaria celular intacta e com funcionamento normal, o que é prejudicado após a ocorrência da HIE (MCLEAN; FERRIERO, 2004).

A excitotocicidade é consequência da entrada de íons cálcio (Ca^{2+}) nos neurônios através de receptores N-metil D-Aspartato (NMDA) o que promove a produção de óxido nítrico e ativação de proteases e fosfolipases. Além disso, a super ativação glutamatérgica permite a entrada excessiva de água e sódio na célula, desencadeando uma série de eventos que aumentam a permeabilidade da membrana e geram radicais livres, assim, estes processos levam a formação de edema celular e à consequente morte celular (HABERNY *et al.*, 2002; ANDRADE *et al.*, 2009), além da ativação da microglia e formação de uma cicatriz glial astrocitária no local da lesão (KOHLHAUSER *et al.*, 1999; CAI *et al.*, 2001; MARCUZZO *et al.*, 2010).

A reoxigenação e reperfusão subsequentes levam à recuperação parcial do metabolismo oxidativo e desencadeiam um aumento na produção de espécies reativas de oxigênio (ROS), níveis mais altos de cálcio intracelular e disfunção mitocondrial. Há também um aumento na expressão de genes pró-inflamatórios e morte celular tardia, sendo que estes processos prejudiciais podem ser exacerbados na fase terciária, que pode durar de dias a meses (figura 1) (ARTEAGA *et al.*, 2017).

Além das alterações moleculares, em estudos prévios utilizando o modelo de HIE de Levine-Rice, foi observado déficit na memória espacial, utilizando o teste do labirinto aquático de Morris e também na memória aversiva, utilizando o teste da esquiva inibitória (ARTENI *et al.*, 2003; PEREIRA *et al.*, 2007). Outros diversos prejuízos podem ser causados pela HIE, como diminuição do volume hipocampal e do estriado na região ipsilateral à lesão e redução do peso encefálico (PEREIRA *et al.*, 2008), além da diminuição da densidade de espinhos dendríticos no hipocampo ipsilateral à lesão (ROJAS *et al.*, 2013). Ainda, alterações bioquímicas como a diminuição da atividade da enzima Na^+, K^+-ATPase no estriado, córtex (CARLETTI *et al.*, 2012) e também no hipocampo (WEIS *et al.*, 2011).

Em particular, sabe-se que a região do hipocampo é vulnerável à excitotoxicidade e aos radicais livres após a exposição ao glutamato, devido à grande presença de receptores glutamatérgicos nesta região (BARTSCH *et al.*, 2015). Além disso, o estriado e a substância branca no encéfalo são áreas que parecem ser especialmente vulneráveis a uma redução nas concentrações de oxigênio (VAN DE BERG *et al.*, 2002; COQ *et al.*, 2016).

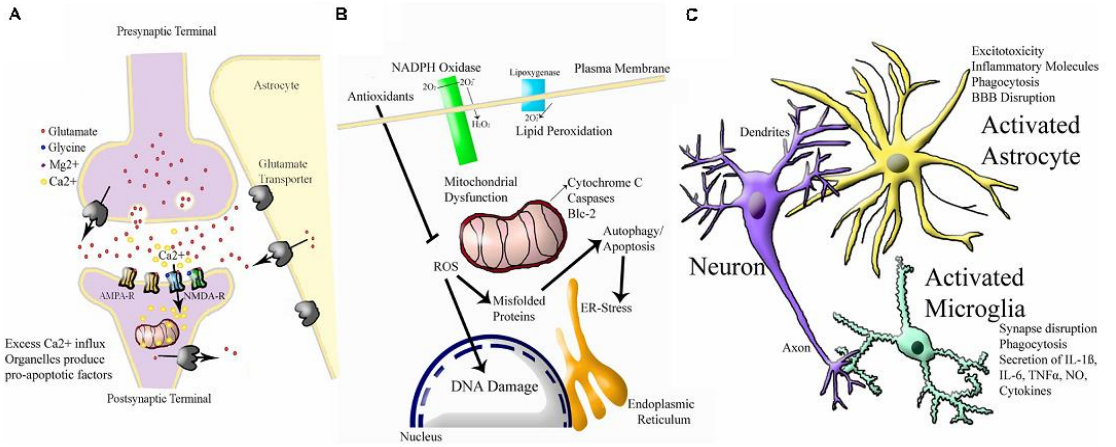

Figura 1. Esquema simplificado da cascata de eventos que ocorre após a HIE. (A) Excitotoxicidade. Ca^{2+} = íon cálcio, Mg^{2+} = íon magnésio AMPA-R = receptor alfa-amino-3-hidroxi-metil-5-4-isoxazolpropiónico, NMDA-R = receptor N-metil-D-aspartato. (B) Estresse oxidativo. São mostrados os radicais livres. NADPH $^{+}$ Nicotinamida Adenina Dinucleotideo Fosfato Hidrogenio, O_2 = oxigenio, O−2 = oxigênio radical livre com carga negativa, H_2O_2 = peróxido de hidrogênio, ROS = ROS1 receptor de tirosina quinase expresso pelo gene ROS1, Blc = proteína de leucemia de células B, ER = retículo endoplasmático, DNA = acido desoxirribonucleico. (C) Inflamação. BBB = Barreira hematoencefálica, IL-6 = interleucina 6, IL-1B = interleucina 1 beta, TNFalpha = fator de necrose tumoral alfa, NO = oxido nítrico (MILLAR *et al.*, 2017).

Proteínas da família das neurotrofinas também tem sua expressão alterada frente a lesões hipóxico-isquêmicas, como é o caso do fator neurotrófico derivado do encéfalo (BDNF), que é considerada uma proteína chave que suporta o crescimento, desenvolvimento e sobrevivência de neurônios (LEE *et al.*, 2002). O BDNF e o seu receptor *tropomyosin receptor kinase B* (TrkB), também já são conhecidos por estarem intimamente associados com a formação da espinhos dendríticos em neurônios do hipocampo, uma importante região relacionada com a memória (JI *et al.*, 2005).

A isquemia encefálica global ou transitória pode levar a um aumento na expressão do gene do BDNF (KOKAIA *et al.*, 1995; TSUKAHARA *et al.*, 1998). Isso também foi observado no modelo de HI de Rice-Vannucci, onde o BDNF teve seus níveis aumentados no hipocampo ipsilateral à lesão (PEREIRA *et al.*, 2009; DENIZ *et al.*, 2018a). A normalização na expressão de BDNF observada no modelo de HIE, utilizando um modelo de enriquecimento ambiental, foi associada à melhora em parâmetros cognitivos dos animais (PEREIRA *et al.*, 2009).

Neurogênese, aumento da densidade sináptica, assim como de proteínas envolvidas no funcionamento da sinapse e de fatores neurotróficos ocorrem especialmente no hipocampo, mas também no estriado e no córtex, sendo também associados a um melhor desempenho nas tarefas de memória (ICKES *et al.*, 2000; LAMBERT *et al.*, 2005; PEREIRA *et al.*, 2008).

A proteína sinaptofisina está localizada nas vesículas pré-sinápticas sendo relacionada à função sináptica e à neuroplasticidade nos neurônios do hipocampo (TARSA; GODA, 2002), podendo ser um importante indicador de alterações plásticas nessa estrutura encefálica (GRIVA *et al.*, 2017; DENIZ *et al.*, 2018a). Estudos utilizando o mesmo modelo de HIE não encontraram diminuição na expressão dessa proteína no hipocampo ipsilateral à lesão, mesmo que se tenha observado uma diminuição no número de neurônios (ZHAO *et al.*, 2012; DENIZ *et al.*, 2018a), indicando a tentativa do tecido de preservar sua função.

1.3 ESTRATÉGIAS TERAPÊUTICAS PARA A HIPÓXIA-ISQUEMIA ENCEFÁLICA NEONATAL

Como citado anteriormente, a HIE pode ser causada por danos nos períodos pré, peri e pós-natal. Assim, o acompanhamento médico e da equipe multidisciplinar durante a gestação e parto é importante para prevenção de anormalidades e detecção precoce de eventuais problemas (O'SHEA *et al.*, 1998; OHSHIMA *et al.*, 2012; DAVIES *et al.*, 2012; MILLAR *et al.*, 2017). Em gestações com crescimento fetal anormal ou risco de prematuridade utiliza-se a terapia com glicocorticóides para aumentar a chance de sobrevivência fetal e diminuir a ocorrência da HIE (LEVITON *et al.*, 1999; O'SHEA; DOYLE, 2001; O'SHEA, 2002; MILLAR *et al.*, 2017). Após o nascimento, uma ampla variedade de terapias é utilizada em pacientes com essa condição clínica e suas consequências, devido ao amplo espectro da fisiopatologia causada pela HIE (MATTHEWS; BALABAN, 2009; CHAN; MILLER, 2014; GILSON *et al.*, 2014). Em ralação aos distúrbios motores, como para o tratamento da espasticidade, são utilizados medicamentos como o baclofen e os benzodiazepínicos (TEIVE *et al.*, 1998; LEITE; PRADO, 2004; GOYAL *et al.*, 2016).

Como alternativa frente à utilização de medicamentos, programas de reabilitação física também são amplamente utilizados em pacientes com HIE, no intuito de favorecer o desenvolvimento motor, reduzir a espasticidade e problemas musculoesqueléticos, aumentar a força muscular, flexibilidade articular e a coordenação motora. Para isso são utilizadas técnicas que utilizam estímulos táteis, proprioceptivos e cinestésicos que favorecem o padrão normal de movimento (LEITE; PRADO, 2004; DAVIES *et al.*, 2012; CHIU; ADA, 2016).

No âmbito experimental da pesquisa básica, diferentes metodologias de exercícios físicos e estímulos ambientais são relatados na literatura de acordo com o objetivo proposto por cada estudo, dentre eles o enriquecimento ambiental, exercícios em esteira e o exercício acrobático (JONES *et al.*, 1999; SAMPAIO-BAPTISTA *et al.*, 2013).

O enriquecimento ambiental (EA) é uma estratégia que utiliza interação social, resolução de tarefas e exercício físico, sendo considerado um eficiente neuroprotetor e capaz de promover a recuperação de déficits de memória espacial e aversiva causados pela HIE (PEREIRA *et al.*, 2007; ROJAS *et al.*, 2013; ROJAS *et al.*, 2015; DIAZ *et al.*, 2016). Por outro lado, a utilização do exercício em esteira frente à HIE também tem bons resultados, levando a uma redução de morte neuronal no hipocampo e estriado, além de melhora na aprendizagem espacial (PARK *et al.*, 2013; CHOI *et al.*, 2013; KIM *et al.*, 2017).

O protocolo de exercícios acrobáticos surge com uma opção intermediária em relação ao EA e ao clássico exercício em esteira, pois tem a presença de estímulos ambientais, com diferentes tipos de obstáculos que estimulam o aprendizado, memória, equilíbrio entre outras funções motoras e cognitivas, sendo formado por uma série de tarefas repetidas destinadas a incentivar a resolução de problemas e a melhorar a coordenação motora (BLACK *et al.*, 1990; JONES *et al.*, 1999; TAMAKOSHI *et al.*, 2014). E além dessa estimulação cognitiva, possui o estímulo ao exercício, uma vez que os animais precisam atravessar a pista de treino, composta por obstáculos como cordas, grades, escada de cordas e barra estreita (figura 2).

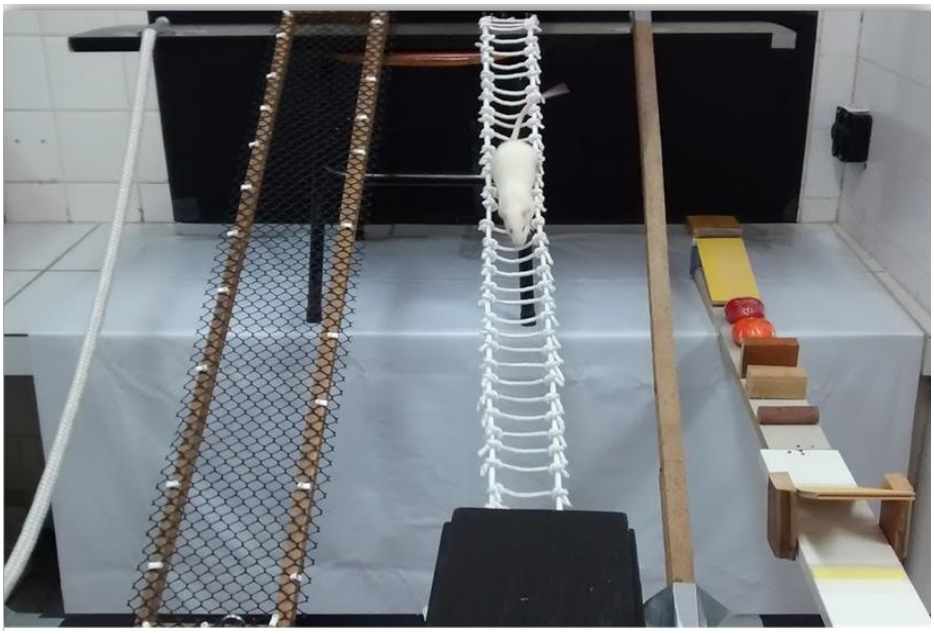

Figura 2. Conjunto de aparatos utilizados para o treinamento acrobático. É possível observar os diferentes meios utilizados para estimular o equilíbrio e a coordenação motora dos animais durante a fase de treinos (imagem do autor).

Um estudo experimental que comparou diferentes tipos de exercício em ratos jovens revelou que tanto o exercício acrobático quanto o exercício em esteira induziram mudanças na expressão de proteínas sinápticas, como a sinaptofisina (GARCIA *et al.*, 2012). Neste estudo as principais alterações ocorridas no protocolo de esteira foram observadas no circuito cerebelo-tálamo-cortical, responsável pelo aprendizado e movimentos rítmicos automatizados (GARCIA *et al.*, 2012). Por outro lado, o protocolo que utilizou circuito acrobático requer mais planejamento e maior recrutamento dos circuitos núcleos da base-tálamo-corticais (GARCIA *et al.*, 2012; SALAME *et al.*, 2016). Ainda, o exercício acrobático pode levar a mudanças na sinaptogênese, ativação e proteção celular, além da função mitocondrial e vascular (GUTIERREZ *et al.*, 2018).

Frente a patologias que acometem o sistema nervoso, como a HI, o exercício físico surge como opção terapêutica, possuindo ações benéficas e tendo a capacidade de melhorar estruturalmente e funcionalmente o sistema nervoso (CHANG *et al.*, 2014; KIM, K. *et al.*, 2014). Porém, poucos estudos avaliaram os efeitos benéficos do exercício acrobático em modelos animais, principalmente avaliando aspectos cognitivos (GUTIERREZ *et al.*, 2018).

Em um recente estudo desenvolvido pelo nosso grupo, o exercício acrobático foi capaz de reverter os déficits motores causados pela HIE, sem promover alterações morfológicas relacionadas ao controle motor (CONFORTIM *et al.*, 2018). No entanto,

avaliações cognitivas dos animais submetidos ao modelo de HIE e treinados com a modalidade de exercícios acrobáticos ainda não foi realizada.

Assim, levando em consideração que a HIE é uma condição multifatorial e que necessita de uma estratégia terapêutica mais abrangente; que estudos experimentais mostram resultados mais satisfatórios dos exercícios acrobáticos em relação à aprendizagem de tarefas complexas; e ainda, que os dados já obtidos pelo nosso grupo indicam que esta modalidade de exercício é mais efetiva em relação ao exercício em esteira na reversão dos déficits motores causados pelo modelo de HIE, este trabalho justifica-se na medida em que busca avaliar a função cognitiva de animais submetidos a um modelo de HIE tratados com a modalidade de exercícios acrobáticos.

2 OBJETIVO GERAL

O objetivo deste estudo foi avaliar os efeitos do protocolo de exercício físico acrobáticos sobre a memória, hipocampo e corpo estriado de ratos *Wistar* machos submetidos a um evento hipóxico-isquêmico encefálico no período neonatal.

3 OBJETIVOS ESPECÍFICOS

Analisar o efeito do protocolo de exercício físico acrobático em ratos *Wistar* machos submetidos à hipóxia-isquemia encefálica neonatal avaliando:

a) O desempenho em relação à memória de curta duração;

b) O desempenho em relação à memória de trabalho e de referência;

c) Marcador de plasticidade no hipocampo e no estriado;

d) Estimativa da área do estriado e do hipocampo;

4 ARTIGO

EFFECTS OF ACROBATIC EXERCISE ON COGNITIVE PARAMETERS AND HYPOCAMPAL AND STRIATAL PLASTICITY IN RATS SUBMITTED TO NEONATAL HYPOXIA-ISCHEMIA

Wellington de Almeida, Heloísa Deola Confortim, Bruna Ferrary Deniz, Patrícia Maidana Miguel, Loise Bronauth, Milene Cardoso Vieira, Adriana Souza dos Santos, Lenir Orlandi Pereira.

INTRODUCTION

The perinatal hypoxia-ischemia (HI) is a clinical condition that may occur before, during or after birth and it is characterized by reduced blood flow to the brain with consequent decrease of available oxygen and glucose levels (HABERNY *et al.*, 2002; GOPAGONDANAHALLI *et al.*, 2016). The brain damage arising from the HI is an important health problem and the estimated incidence varies from 1.3 to 1.7 for every 1000 live-born infants (KURINCZUK *et al.*, 2010; ARTEAGA *et al.*, 2017). HI may lead to severe cognitive and motor dysfunction and, despite advances in obstetric and neonatal care, its incidence remains high (MILLAR *et al.*, 2017).

The development of therapeutic strategies and the comprehension about the injury mechanisms heavily depends on the use of animal models. These studies are crucial for the understanding of the central nervous system's (CNS) responses to injuries like HI (CLOWRY *et al.*, 2014). Several studies have been developed in an attempt to reproduce in animals the characteristics observed in these patients, aiming to establish new therapeutic strategies (CHOI *et al.*, 2011; YU *et al.*, 2013; COQ *et al.*, 2016; MILLAR *et al.*, 2017).

The model of HI in rodents proposed by (LEVINE, 1960), modified by (RICE *et al.*, 1981), is widely used as a model of neonatal hypoxic-ischemic encephalopathy (PEREIRA *et al.*, 2007). In this model, unilateral ischemia is performed by occlusion of the common carotid artery (right or left) on the 7^{th} day of the birth (7^{th} PND), followed by exposure of the animals to a hypoxic environment, with 8% oxygen (O_2). Studies using this model are able to reproduce the functional and neuropathological deficits observed in human neonates affected by neonatal HI (ARTENI *et al.*, 2003; PEREIRA

et al., 2007; PEREIRA *et al.*, 2008; MIGUEL *et al.*, 2017). It has been identified spatial memory deficit observed using Morris's water maze test, aversive memory deficit in the inhibitory avoidance test and impairment in novel object recognition memory task (ARTENI *et al.*, 2003; PEREIRA *et al.*, 2007; ROJAS *et al.*, 2013; DENIZ *et al.*, 2018a). Brain damage was also observed, such as decreased hippocampal and striatal volume in the ipsilateral hemisphere to the lesion and reduction of the encephalic weight (PEREIRA *et al.*, 2009), reduction of dendritic spine density in the hippocampus ipsilateral to the lesion (ROJAS *et al.*, 2013). In addition, biochemical changes such as decreased activity of the Na^+, K^+-ATPase enzyme in the striatum, cortex (CARLETTI *et al.*, 2012) and in the hippocampus (WEIS *et al.*, 2011; DENIZ *et al.*, 2018b) also have been found. In particular, the hippocampus region is vulnerable to excitotoxicity and free radicals due to the large presence of glutamatergic receptors in this region (BARTSCH *et al.*, 2015). In addition, the striatum and white matter in the brain are areas that appear to be especially vulnerable to a reduction in oxygen concentrations (VAN DE BERG *et al.*, 2002; COQ *et al.*, 2016).

The neurotrophins proteins family have their expression altered after hypoxic-ischemic lesions, as the brain-derived neurotrophic factor (BDNF) (PEREIRA *et al.*, 2009; DENIZ *et al.*, 2018a), which is considered a key protein that supports the growth, development and survival of neurons (LEE *et al.*, 2002). Besides, BDNF and its TrkB receptor are known to be closely associated with the formation of dendritic spines in hippocampal neurons (JI *et al.*, 2005). Other neuroplastic changes such as increase neurogenesis and synaptic density occur especially in the hippocampus, but also in the striatum and are frequently associated with better performance in memory tasks (ICKES *et al.*, 2000).

As previously mentioned HI can be caused by damage in the pre, peri and postnatal periods. Thus, medical follow-up and the multidisciplinary team during gestation and delivery are important for the prevention of abnormalities and early detection of possible problems (O'SHEA, 2002; OHSHIMA *et al.*, 2012; DAVIES *et al.*, 2012; MILLAR *et al.*, 2017).

An alternative to the use of medications, physical rehabilitation programs are widely used in patients with HI and its consequences, such as cerebral palsy, in order to promote motor development, reduce spasticity and musculoskeletal problems, increase muscle strength, flexibility articulation and motor coordination. For this, techniques that

use tactile, proprioceptive and kinesthetic stimuli that favor the normal pattern of movement are used (LEITE; PRADO, 2004; DAVIES *et al.*, 2012; CHIU; ADA, 2016). Different physical exercise methodologies are reported in the literature according to the objective proposed by each study, like environmental enrichment (EE), treadmill training and acrobatic exercise (JONES *et al.*, 1999; SAMPAIO-BAPTISTA *et al.*, 2013; ROJAS *et al.*, 2015; CONFORTIM *et al.*, 2018).

Environmental enrichment (EE) is a strategy that uses social interaction, resolution tasks and physical exercise, being considered an efficient neuroprotector and able to promote the recovery of spatial and aversive memory deficits caused by HIE (PEREIRA *et al.*, 2007; ROJAS *et al.*, 2013; ROJAS *et al.*, 2015; DIAZ *et al.*, 2016). On the other hand, the use of treadmill training for treatment of HIE also has good results, has reduction of neuronal death in the hippocampus and in the striatum, and improvement of spatial learning (CHOI *et al.*, 2013; PARK *et al.*, 2016; KIM *et al.*, 2017).

The protocol of acrobatic exercises arises with an intermediate option in relation to EE and the classic treadmill training, because it has the presence of environmental stimuli, with different types of obstacles that stimulate the learning, memory, balance between motor and cognitive functions, being which is composed of a series of repetitive tasks designed to encourage problem solving and improve motor coordination (BLACK *et al.*, 1990; JONES *et al.*, 1999; TAMAKOSHI *et al.*, 2014). An experimental study comparing different types of exercise in young rats revealed that both acrobatic exercise and treadmill exercise induced changes in the synaptic proteins expression, such as synaptophysin on the striatum. The main alterations in the treadmill protocol were observed in the cerebellum-thalamic-cortical circuit, responsible for learning and automated rhythmic movements (GARCIA *et al.*, 2012). In opposition, the protocol that used acrobatic circuitry required more planning and more recruitment of the basal ganglia-thalamic-cortical circuits (GARCIA *et al.*, 2012; SALAME *et al.*, 2016) and promoted changes in synaptogenesis, cellular activation and protection, besides mitochondrial and vascular function (GUTIERREZ *et al.*, 2018). Physical exercise appears as a therapeutic option to pathologies that affect the nervous system, such as HI, having beneficial actions and ameliorating the structure and function of the nervous system (CHANG *et al.*, 2014; KIM, K. *et al.*, 2014). However, few studies have evaluated the beneficial effects of acrobatic exercise on animal models.

In this way, considering that HIE is a complex condition that requires therapeutic strategies that cover more aspects of pathology, and the literature shows only the effects of physical exercise on treadmill versus HI and beyond that experimental studies show more satisfactory results of acrobatic exercises due to the learning of complex tasks, this study aims to evaluate the cognitive function and hippocampal and striatal damage in animals submitted to a model of HI treated with acrobatic exercise modality.

MATERIALS AND METHODS

Animals

Experimental procedures were approved by the Research Ethics Committee of the *Universidade Federal do Rio Grande do Sul, Brazil* (n. 29230). All experiments were performed in accordance with the Federation of Brazilian Societies for Experimental Biology and the Guide for the Care and Use of Laboratory Animals adopted by National Institute of Health (USA) and the Arouca Law (Law nº 11.794/2008). Initially, pregnant *Wistar* rats were obtained from the *Centro de Reprodução e Experimentacão de Animais de Laboratório* (*Universidade Federal do Rio Grande do Sul*, Brazil). They were maintained in standard boxes, in a temperature-controlled room (approximately 22ºC), on a 12-h light/dark cycle with food and water available *ad libitum*. At 7th postnatal day (PND), male pup rats were randomly divided into two groups, control (CT) and hypoxia-ischemia (HI). After the HI procedure, pups remained with their dams, until weaning (PND 21). At 22nd PND, animals were separated in four experimental groups as follows: control group non-exercised (CTSED), control group submitted to acrobatic training (CTACR), HI group non-exercised (HISED) and HI group submitted to acrobatic training (HIACR).

Hypoxia–ischemia procedure

The HI model, described by Rice-Vanucci and colleagues (RICE *et al.*, 1981), was utilized to produce unilateral brain injury in neonate rats. At 7th PND, pups were anesthetized with halothane 2–4%. Through a ventral neck incision, the left common carotid was isolated and permanently occluded by a surgical thread of silk 4.0. Animals

were maintained in controlled temperature to recover for 15 min and then returned to their dams. After recovery period of two hours with their dams, pups were exposed to hypoxic atmosphere (8% oxygen and 92% nitrogen, 5 L/minute flow) for 90 min in a chamber partially immersed in a 37 ºC water bath in order to maintain body temperature. Control animals were "sham-operated", they received manipulation, anesthesia and neck incision, but did not suffer arterial occlusion or exposure to hypoxic atmosphere. Following the HI procedure, animals were returned to their respective home cages where they were maintained until weaning, at PND 21 (MIGUEL *et al.*, 2017; DENIZ *et al.*, 2018a).

Acrobatic training

The acrobatic training protocol involves the presence of different types of obstacles, like the grid platform, rope ladder, bars, rope and barriers, that stimulate motor learning and other cognitive functions. This series of tasks was designed to encourage problem-solving and motor coordination providing challenges for brain functions (BLACK *et al.*, 1990; TAMAKOSHI *et al.*, 2014).

At 22nd PND began the training, when the animals were adapted to the circuit; they were conducted to travel the circuit two times per day for five consecutive days, in order to alleviate the stress caused by a new environment and to learn the route. Over the following four weeks, the animals performed the training three times a week, with six repetitions of the circuit per day. The level of difficulty increased progressively during the training as described by Black et al., (1990) and (CONFORTIM *et al.*, 2018). Experimenters, with slight manual stimuli, occasionally assisted some animals. The non-exercised animals were maintained in the same room where the animals performed the protocol training and they were transferred to individual cages for 20 minutes. The training protocol started after weaning and had a total duration of five weeks, being carried until approximately PND 60. The time spent by each animal to undertake the circuit was registered.

Behavioral tasks

Novel object recognition task

This test was performed at 60th PND, after the training period (N=12/group). The novel object recognition task was used to evaluate visual learning and memory based on rat natural propensity to explore novelty. To perform this test, 1 day before, the animals were habituated to the empty arena (with no objects) for 5 minutes. This arena consists a wooden box 50 X 50 X 39 (length x height x depth) and the floor was marked into 12 equal quadrants. A day later, in the first session, each rat was placed on the apparatus with two identical objects for 5 min. To evaluate the short-term memory, the second session was carried out after a 5-minute interval (PEREIRA *et al.*, 2008). In the second session, the rats were placed back on the apparatus with one familiar and one new object. The time exploring each object was evaluated for 5 minutes. Time spent investigating each object was manually scored and a preference index for the new object was utilized to evaluate memory deficits in the second session (test). The index was calculated as the time difference between the exploration of the new object to the familiar object, divided by the sum of the time exploring both objects (B-A/B+A, being B the new and A the familiar object) (ROJAS *et al.*, 2013; DENIZ *et al.*, 2018a).

Morris water maze

In the following day after the novel object recognition task, animals were submitted to the Morris Water maze task to evaluate spatial memory (N=12/group). The apparatus is composed by a 117 cm diameter circular pool filled with water at 21°C. The testing room contained distinct visual cues and the water pool was virtually divided into 4 quadrants. A circular platform was 2 cm below the water surface. For the memory evaluation two different protocols were used as follows.

Reference memory protocol

This protocol was performed as previously described by Pereira et al., 2007 and Deniz et al., 2018. Brifly, the rats received 5 training days (sessions) (4 trials/ day, 20 minutes of intertrial interval) and a probe trial on the 6th day. The rat was given 60 s to locate the platform; if the animal did not succeed it was gently guided to the platform and left on it for 10 s. The latency to reach the platform was measured in each trial and

the mean latency for every training day was calculated. In the probe trial, the platform was removed, and the following variables were observed: the latency to reach the platform area, the number of crossings in the platform area and the time spent in the target and in the opposite quadrant (PEREIRA *et al.*, 2007; DENIZ *et al.*, 2018a).

Working memory protocol

This test was performed according Pereira et al., 2007, in this protocol, 4 trials/day were performed, during four consecutive days, with the platform location changed daily. Each trial was conducted as described in the reference memory protocol, but with an intertrial interval of 5 min. The working memory was evaluated by the average latency to find the platform in each trial for each animal, allowing observing the ability of the animals in locating the novel position of the platform in the day.

Tissue collection

One day after the behavioral tests, a set of rats were anesthetized with thiopental and then perfused transcardiacally with 0.9% saline and buffered 4% paraformaldehyde (pH 7.4) solutions using a peristaltic pump. Brains were dissected, fixed during 4 h in the same fixative solution, cryoprotected in 15 and 30% sucrose at 4 °C, frozen in liquid nitrogen and stored at − 80 °C until analysis.

Morphological analysis

Hippocampal atrophy

To investigate the damage on the hippocampus, were used 2 images of dorsal hippocampus per rat, based on the Miguel et al., (2017). The brains were sectioned using a cryostat and were made slices (30 μm) with 240 μm interval were mounted on gelatinized glass slides what were stained with toluidine blue 5% (tolonium chloride) (Vetec fine chemistry ltda, Rio de Janeiro, Brazil). The dorsal hippocampus was analyzed between coordinates -3.12 mm and -3.48 mm of the Paxinos Atlas (PAXINOS; WATSON, 1998; PEREIRA *et al.*, 2008).The measurements were performed by blinded and trained evaluator, using the software Image Pro-Plus 6.0

(Media Cybernetics Inc., Rockville, MD, USA). The hippocampal atrophy was calculated by relating the right hemisphere (contralateral to the lesion) with the left hemisphere area (ipsilateral to the lesion).

Striatal atrophy

According to Pereira et al., 2008, striatal area was measured to estimate the extent of damage in this region. For this, four sections per rat were taken at the level -0.20 and -0.8 mm from bregma according to Paxinos and Watson (1986). The slices were stained with toluidine blue 5% (Vetec química fina ltda, Rio de Janeiro, Brazil) and the Image Pro-Plus 6.0 program (Media Cybernetics Inc., Rockville, MD, USA) was utilized to delineate and estimate striatal damage (PEREIRA *et al.*, 2008). Following the same procedure used for hippocampus, it was estimated the striatum atrophy.

BDNF levels

After training period, at 60th PDN, 6-7 animals/group were euthanized by decapitation and the hippocampi and striatum were quickly dissected and placed in liquid nitrogen. The samples were stored at -80°C until the biochemical assay (Deniz et al., 2018). BDNF protein was assessed using the E-Max ELISA kit (Promega, USA), according to manufacturer's recommendations. For this, hippocampus were individually homogenized in lysis buffer (containing, in mM: 137 NaCl, 20 Tris–HCl pH 8.0, Igepal 1%, glycerol 10%, 1 PMSF, 0.5 sodium vanadate, 0.1 EDTA and 0.1 EGTA) and centrifuged at 11,200 rpm at 4 °C during 30 min. Supernatant was diluted in sample buffer and incubated on 96-well flat-botton plates previously coated with anti-BDNF monoclonal antibody. After, plates were incubated with polyclonal antibody for 2 h and horseradish peroxidase for 1 h. Subsequently, color reaction with tetramethyl benzidine was quantified in a plate reader at 450 nm; the standard BDNF curve ranged from 0 to 500 pg/mL (Pereira et al., 2009). Total quantification of proteins was made using Bradford method (BRADFORD, 1976). The data is represented as a percentage in relation to CTSED group.

Statistical analysis

Two-way analysis of variance (ANOVA) was performed, with *lesion* and *treatment* as factors, followed by Tukey test, when appropriated, to novel object recognition task, Morris water maze task and data from morphological analysis and BNDF assays. Behavioral performance in the training days of the water maze was analyzed using a two-way repeated-measures analysis of variance (ANOVA). All statistical tests were performed using the Statistic software package running on a compatible personal computer; differences were considered statistically significant whenever $p<0.05$.

RESULTS

Novel-object recognition

Two-way ANOVA revealed significant effect of *lesion* factor ($F(1,47)=5.07$, $p<0.05$) and *training* factor ($F(1,47)=5.06$, $p<0.05$). Tukey's post hoc evidenced that HISED group had lower novel-object preference index when compared to all control groups and HIACR group. HIACR group had similar preference index compared to controls, showing a recovery of object recognition memory (Fig. 1). In the first session, no differences were observed in the time exploring the two objects.

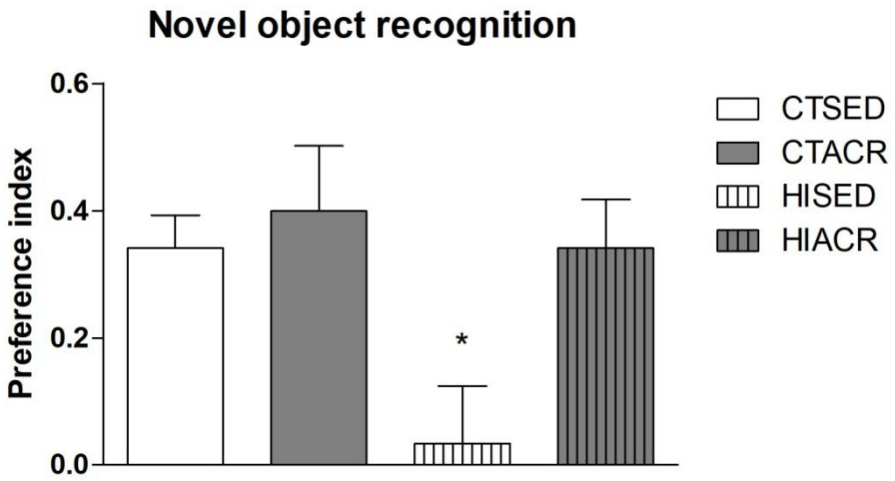

Fig. 1. Novel Object Recognition task performed by the animals at the 61th PND. Data represent the preference index (difference in exploration time of the new object divided by the total time spent exploring the two objects in the second session). * different from all other groups. Data are expressed by mean ± S.E.M. Two-way ANOVA followed by Tukey's test, $p<0.05$. N= 11-14 animals/ group. CTSED: control sedentary. CTACR: control that performed acrobatic training. HISED hypoxic–ischemic sedentary. HIACR: hypoxic–ischemic that performed acrobatic training.

Morris water maze

Reference memory

Two-way repeated-measures ANOVA was used to analyzed the latency to find the platform in the acquisition phase of Morris water maze task: the analysis revealed significant effect on *lesion* ($F(1,47)=19.89$, $p<0.05$), and *day* factors ($F(4,188)=36.85$, $p<0.05$) without effect on *training* factor ($F(1,47)=1.39$, $p=0.24$) and *lesion*training*day* interaction ($F(4,188)=0.51$, $p=0.72$). Control groups took less time to find the platform compared to HI animals but all groups presented a learning curve during training days. Tukey's test revealed that CTSED group lowered their latency since the second day; CTACR, HIACR and HISED groups took less time to find the platform starting in the third day (Fig. 2). These results demonstrate a spatial memory impairment consequent to HI without recovery effect in trained animals.

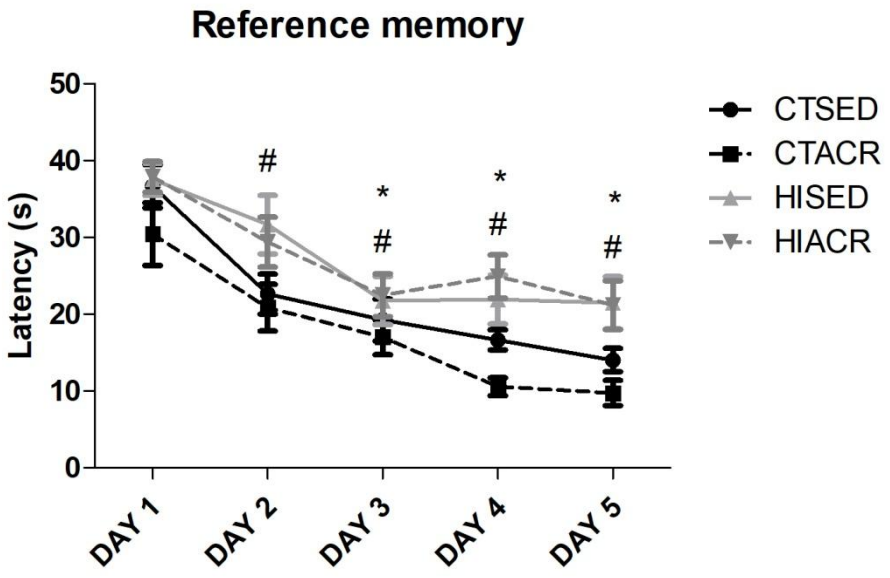

Fig. 2. Morris Water Maze – reference memory protocol made by animals starting one day after object recognition task. # CTSED different from day 1. * Difference of CTACR, HISED and HIACR to their day 1. Behavioral performance in the training days was evaluated to repeated Two-way ANOVA followed by Tukey's test, $p<0.05$. Two-way ANOVA reveled *lesion* effect ($F(1,47)=19.89$, $p<0.05$), but not *training* or interaction *lesion* training* effect. Data are expressed by mean ± S.E.M. N= 11-14 animals/ group. CTSED: control sedentary. CTACR: control that performed acrobatic training. HISED hypoxic–ischemic sedentary. HIACR: hypoxic–ischemic that performed acrobatic training.

Two-way ANOVA followed by Tukey's test was also performed to detect possible differences in each training day: in the days 2, 4 and 5, significant *lesion* effect was identified ($F(1,47)=7.30$, $p<0.05$, $F(1,47)=16.52$, $p<0.05$, and $F(1,47)=12.35$, $p<0.05$ respectively) without differences on *training* ($F(1,47)=1.14$, $p=0.28$, $F(1,47)=0.40$, $p=0.52$, and ($F(1,47)=0.72$, $p=0.39$ respectively) and *lesion*training* ($F(1,47)=0.061$, $p=0.93$, $F(1,47)=3.54$, $p=0.06$, and $F(1,47)=0.55$, $p=0.46$ respectively).

In the probe trial, two-way ANOVA and Tukey's test demonstrated a *lesion* effect in the latency ($F(1,47)=5.98$, $p<0.05$): HI groups took more time to achieve the platform area (Fig. 3A) with no difference on *training* ($F(1,47)=1.01$, $p=0.31$) and *lesion*training* interaction ($F(1,47)=0.11$, $p=0.73$). In the opposed quadrant a significant effect was only observed in the interaction of the factors *lesion*training* ($F(1,47)=4.20$, $p=0.04$) (Fig. 3D), with no effect in *lesion* ($F(1,47)=4.20$, $p=0.26$) and *training* ($F(1,47)=0.12$, $p=0.72$); Tukey's test did not confirm this effect. Considering the crossings in the platform area, there was no effects on *lesion* ($F(1,47)=0.0002$, $p=0.98$), *training* ($F(1,47)=1.91$, $p=0.17$) and *lesion*training* interaction ($F(1,47)=0.14$, $p=0.70$) (Fig 3B). No effect was observed on the time spent in the target area *lesion*

(F(1,47)=0.52, p=0.47), *training* (F(1,47)=2.94, p=0.09), *lesion*training* (F(1,47)=0.05, p=0.82) (Fig. 3C).

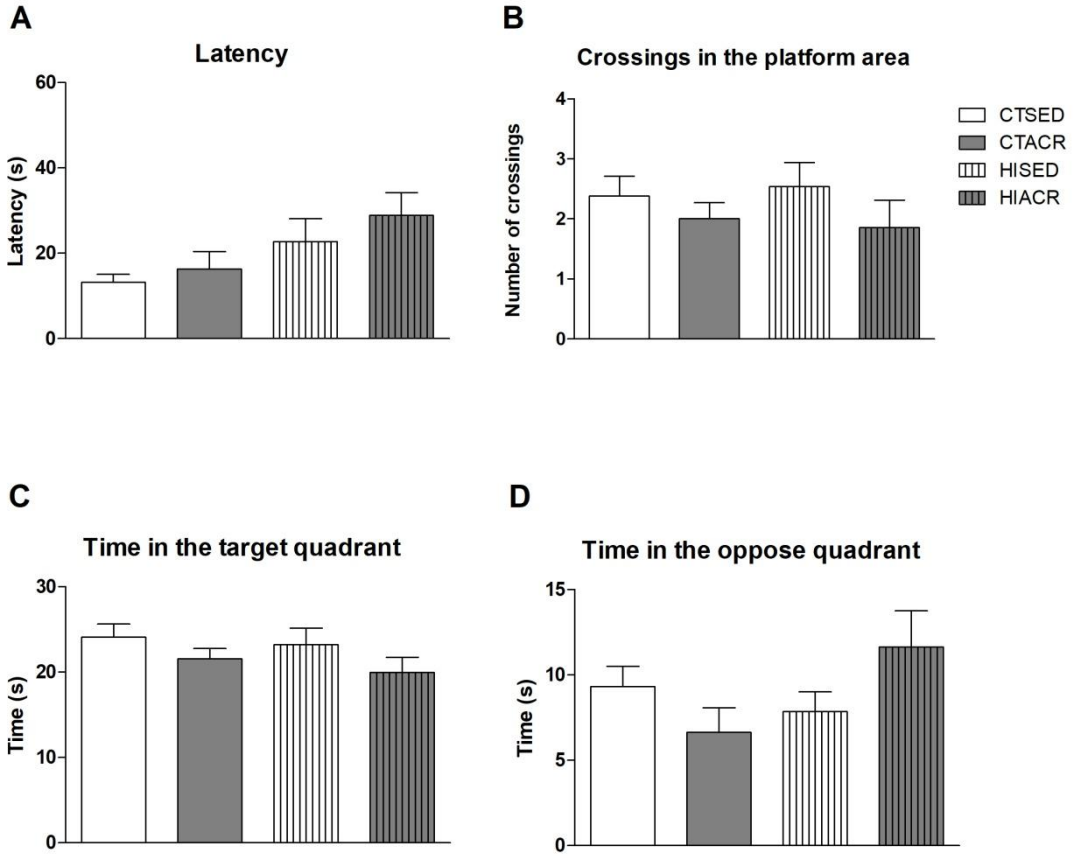

Fig. 3. Morris Water Maze - Probe trial of the reference memory performed by the animals 24 hours after the end of the reference protocol. A) Latency to find platform. B) Number of platform crossings. C) Time in the target quadrant. D) Time in the opposed quadrant. *Lesion* effect was observed only in Latency (F(1,47)=5.98, p<0.05). Data are expressed by mean ± S.E.M. Two-way ANOVA followed by Tukey's test, p<0.05. N= 11-14 animals/ group. CTSED: control sedentary. CTACR: control that performed acrobatic training. HISED hypoxic–ischemic sedentary. HIACR: hypoxic–ischemic that performed acrobatic training.

Working memory

Two-way ANOVA followed by Tukey's test was used to analyze the latency to find the platform in the working memory protocol (Fig. 4). There were significant differences in the escape latency for *lesion*, in the 3rd and 4th trials (F(1, 47)=5.16, p < 0.05 and F(1, 47)=10.60, p < 0.05, respectively); HI animals had greater latencies than CTs. There was no *training* or *lesion*training* interaction effect. These results

demonstrate working memory impairment consequent to HI without recovery effect after acrobatic training.

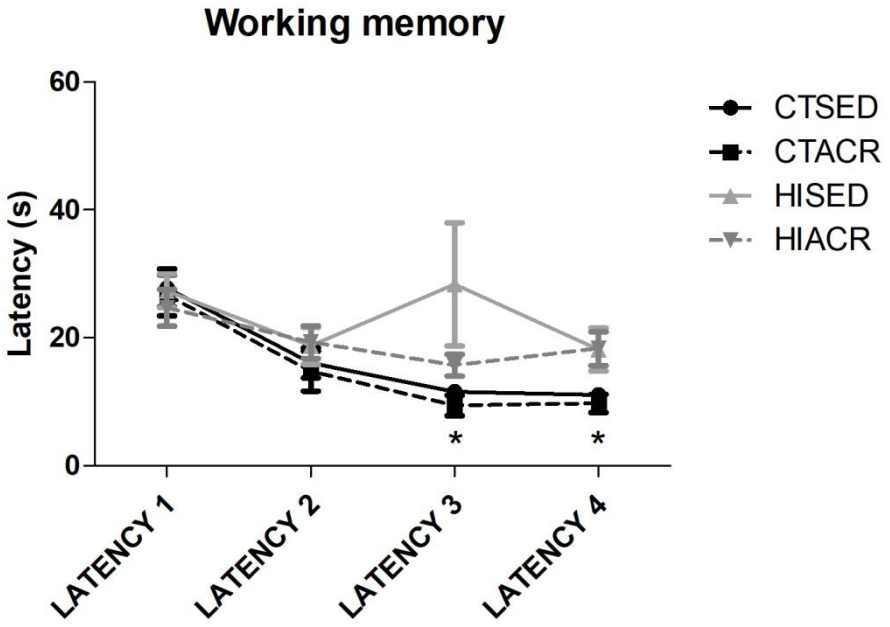

Fig. 4. Morris Water Maze – working memory protocol. * significant differences in the latency to find platform for *lesion* in the 3rd (F(1, 47)=5.16, p < 0.05) and 4th (F(1, 47)=10.60, p < 0.05) trials. Two-way ANOVA, p<0.05. Data are expressed by mean ± S.E.M. N= 11-14 animals/ group. CTSED: control sedentary. CTACR: control that performed acrobatic training. HISED hypoxic–ischemic sedentary. HIACR: hypoxic–ischemic that performed acrobatic training.

Morphological analysis

Hippocampal Atrophy

Two-way ANOVA followed by Tukey's test was used to analyze the estimative of atrophy of the left hemisphere in relation to the right hemisphere. A significant effect by *lesion* was observed (F(1,11)=12.95, P<0.05), without *training* or *lesion*training* interaction effect (Table 1). Again, these results indicate a HI-induced hippocampal atrophy with no recovery effect after acrobatic training.

Striatum Atrophy

As performed for hippocampus, two-way ANOVA followed by Tukey's test was used to analyze the estimative of atrophy in the striatum. There was significant difference in atrophy estimation by *lesion* (F(1,15)=21.62, P<0.05) (Table 1). There was

no *training* or *lesion*training* interaction effect. These results demonstrate damage caused by HI without significant training effect. Interestingly, striatal atrophy was around 50% and hippocampal atrophy reached 70% in HI group.

	Atrophy of the Striatum	Atrophy of the hippocampus
CTSED	3.33 ± 3.46	-9.89 ± 10.13
CTACR	16.36 ± 0.74	3.05 ± 4.02
HISED	54.06 ± 13.32	70.38 ± 19.91
HIACR	41.02 ± 8.34	36.80 ± 19.59

Table 1. Atrophy of striatum and hippocampus. Two-way ANOVA, $p<0.05$. Data are expressed by mean ± S.E.M. N= 3-5 animals/ group. CTSED: control sedentary. CTACR: control that performed acrobatic training. HISED hypoxic–ischemic sedentary. HIACR: hypoxic–ischemic that performed acrobatic training. Main effect of ANOVA revealed that both HI groups had a major atrophy in striatum and hippocampus ipsilateral to arterial occlusion.

BDNF Levels

Two-way ANOVA was adopted to analyze BDNF Levels in Hippocampus and Striatum in both hemispheres. There were no differences in BDNF quantification on right or left hippocampus (Fig. 5). Taken the levels of BDNF in the striatum, a tendency was identified on *lesion* factor ($p=0.06$) in the left striatum revealing higher levels of BDNF in HI groups (Fig. 6).

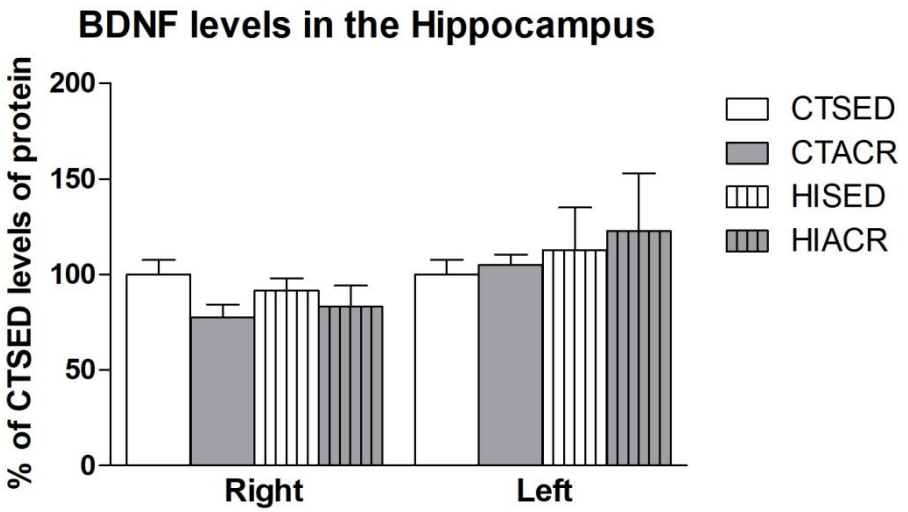

Fig. 5. BDNF levels in the right and left side of Hippocampus. Two-way ANOVA, $p<0.05$. Data are expressed by mean ± S.E.M. N= 6-7 animals/ group. CTSED: control sedentary. CTACR: control that performed acrobatic training. HISED hypoxic–ischemic sedentary. HIACR: hypoxic–ischemic that performed acrobatic training.

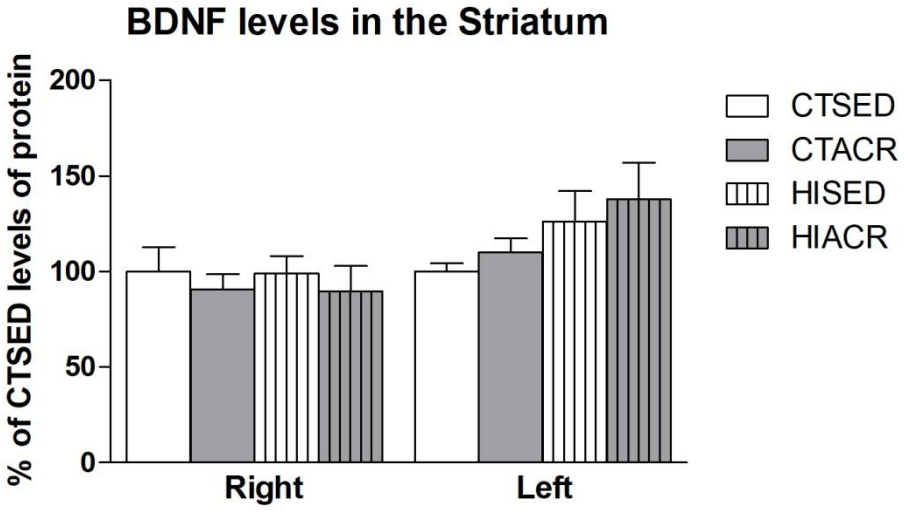

Fig. 6. BDNF levels in the right and left side of Striatum. Two-way ANOVA, $p<0.05$. Data are expressed by mean ± S.E.M. N= 6-7 animals/ group. CTSED: control sedentary. CTACR: control that performed acrobatic training. HISED hypoxic–ischemic sedentary. HIACR: hypoxic–ischemic that performed acrobatic training.

DISCUSSION

Motor training has capacity to induce plastic changes in nervous tissue, besides to promote recovery after brain injury (TAMAKOSHI *et al.*, 2014; GONZALEZ-TAPIA *et al.*, 2016; KIDA *et al.*, 2016; GONZALEZ-TAPIA *et al.*, 2017). Thus, the present study proposed to investigate the ability of the acrobatic training in ameliorating the memory and decrease brain lesion, associated to induce plasticity of animals submitted to HI model. Our results show HI-induced memory deficit, as expected. The acrobatic training partially reverted memory deficits caused by the HI insult without decreasing the damage in the structures evaluated, besides BDNF evaluation did not show difference in the hippocampus and in the striatum, suggesting other plastic mechanism involved in this recovery.

A short-term memory or declarative memory is dependent of the striatum and a previous study demonstrated improving in plasticity on this structure after acrobatic training (TAMAKOSHI *et al.*, 2014). Then we decided to investigate this type of memory more related to the striatum, using the novel-object recognition task. Hypoxia-ischemia procedure resulted in deficits in the short-term memory. This deficits have already observed in previous studies using the same HI model (PEREIRA *et al.*, 2008; ROJAS *et al.*, 2013; DENIZ *et al.*, 2018a), and this result can be explained by the damage in striatum and hippocampus that are well described in this HI model. The animals submitted to acrobatic training and the HI procedure did not present this deficit, indicating a positive effect related to training.

In a study that used EE as a treatment strategy against HI, recognition memory recovery was also observed, but not in aversive memory evaluated in the inhibitory avoidance test (ROJAS *et al.*, 2013). Another study also identified the increase of synaptophysin in the striatum after acrobatic training, indicating plastic alterations in this structure (TAMAKOSHI *et al.*, 2014). Thus, we can suggest that the best performance observed in the novel-object recognition task identified in our evaluations may have occurred due to this greater plasticity in the striatum as a result of acrobatic training.

Morris Water maze task is an important test to evaluate spatial memory. In this study, in accordance with literature, the HI animals presented deficit in spatial memory and working memory evaluated by the Morris water maze task (PEREIRA *et al.*, 2007;

PEREIRA *et al.*, 2008; DENIZ *et al.*, 2018a). This result can be related to HI damage in the brain regions, particularly the hippocampus (PEREIRA *et al.*, 2007; PEREIRA *et al.*, 2008; MIGUEL *et al.*, 2017), since this is a crucial structure for spatial learning (D'HOOGE; DE DEYN, 2001; DENIZ *et al.*, 2018a). However, the acrobatic exercise did not reverse the deficit in spatial memory evaluated by this task. It's possible that this treatment wasn't able to reverse the damage in the hippocampus since this region is very important for this type of memory.

In addition, the acrobatic exercise began later in the 22nd PND and could not prevent damage, since this injury is very prominent and was already installed.

However, in a similar way to that identified by our study, previous studies developed with EE only identified partial recovery in working memory, and this recovery was observed only in young animals, but not in adults (Pereira et al., 2009).

Another parameter to identify structural recovery in HI is the evaluation of the atrophy. This measurement was performed in the hippocampus and striatum, which are memory-related brain regions.

The hippocampal and striatal memory systems are thought to operate independently and in parallel, supporting cognitive memory and habits, respectively (FERBINTEANU, 2016). The striatum is part of a structure called the basal ganglia. The dorsal striatum, along with the ventral striatum, serve as the major input and output neurons of this brain area (SOMOGYI *et al.*, 1981; GOODMAN; PACKARD, 2015).

Moreover, the striatum have a crucial role in learning and memory, coordinating space exploration and mediating the updating of information, since functional inactivation of this structure alters behavioral flexibility and recognition memory in mice (QIAO *et al.*, 2017).

In the present study we found atrophy in both regions which has already been described after HI procedure (MIGUEL *et al.*, 2015). In relation to these structures, a previous study developed by Miguel et al., 2015, find the major damage on the hippocampus in relation to the striatum. Such finding was here corroborated which may partially explain the better performance in novel-object recognition task. Notwithstanding, damage on hippocampus was around 70 per cent and is important to note that this region is very vulnerable to excitotoxicity (BARTSCH *et al.*, 2015) that is one of the main mechanisms in the pathophysiology of the HI injury. Yet, in comparison, the damage in the striatum area was less prominent and this could partially

explain the cognitive improvement in the novel-object task by the acrobatic training. Another study that used EE as a therapeutic strategy showed only a partial recovery of brain atrophy after HI when the treatment was early (started 24 hours after injury), showing that HI leads to severe atrophy and difficult reversion (SCHUCH *et al.*, 2016).

Furthermore, the performance in behavioral tasks and cerebral damage extension are lateralized and is known that the right hemisphere is more vulnerable to neonatal cerebral HI (ARTENI *et al.*, 2010). In the present study, the HI was made in the left hemisphere and this could also be related to the better performance observed in novel-object recognition task.

It is important to note that the exposure of animals to an different environment can generate an exploratory behavior that allows the process of familiarization with the new environment, being this exploratory motor behavior closely related to the striatum function (YAMIN *et al.*, 2013). Furthermore, acrobatic training involves motor learning and exposure to a different environment throughout the training weeks (CONFORTIM *et al.*, 2018). These factors may also have contributed to an increase in synaptic plasticity in the striatum, which has already been observed in previous studies (TAMAKOSHI *et al.*, 2014). Thus, recovery of recognition memory in the HIACR group may be directly related to the type of motor / cognitive training provided by acrobatic exercise.

Still looking to understand the mechanisms involved in this improvement on recognition memory, we decided to investigate the BDNF levels in hippocampus and striatum. Surprisingly, we did not find difference in this neurotrophic factor in both structures. Statistical analysis showed only a tendency to increase BDNF levels in striatum of the HI animals. This up-regulation was already been related as a preservation strategy after HI damage (PEREIRA *et al.*, 2009; DENIZ *et al.*, 2018a) and also related to cognitive improvement in response to exercise (CHEN *et al.*, 2017).

However, our study did not find such changes either related to HIE or to training. This result may have been observed due to loss of the expression window of this protein since it is already known that BDNF expression can change over time, thus associated to ephemeral effects, or transitory plastic alterations (MEGA *et al.*, 2018).

In conclusion, this study suggests that motor learning can be a promissor coadjuvant strategy to the treatment of cognitive deficits related to HI, but additional

studies are necessary to understand in greater depth the mechanisms of action responsible for this beneficial effect.

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5 DISCUSSÃO

O treinamento motor tem capacidade de induzir alterações plásticas no tecido nervoso, além de promover a recuperação após lesão encefálica (TAMAKOSHI *et al.*, 2014; GONZALEZ-TAPIA *et al.*, 2016; KIDA *et al.*, 2016; GONZALEZ-TAPIA *et al.*, 2017). Assim, o presente estudo se propôs a investigar a capacidade do treinamento acrobático em melhorar a memória e diminuir a lesão encefálica, associado à melhora de parâmetros relacionados à plasticidade de animais submetidos ao modelo HIE. Como esperado, nossos resultados mostram déficit de memória induzido pela HIE. O treinamento acrobático reverteu parcialmente os déficits de memória causados pela lesão, sem diminuir o dano nas estruturas avaliadas, além disso, a avaliação do BDNF não mostrou diferença no hipocampo e no estriado, sugerindo outro mecanismo plástico envolvido nessa recuperação.

A memória de reconhecimento ou memória declarativa é dependente do estriado e um estudo anterior demonstrou melhora na plasticidade dessa estrutura após o treinamento acrobático (TAMAKOSHI *et al.*, 2014). Assim, decidimos investigar esse tipo de memória mais relacionada ao corpo estriado, usando a tarefa de reconhecimento de objetos. O procedimento de HIE resultou em déficits na memória de curto prazo. Esses déficits já foram observados em estudos anteriores utilizando o mesmo modelo (PEREIRA *et al.*, 2008; ROJAS *et al.*, 2013; DENIZ *et al.*, 2018a), sendo que este resultado pode ser explicado pelos danos no estriado e no hipocampo que já foram bem descritos após HIE. Os animais submetidos à HIE e ao treinamento acrobático não apresentaram esse déficit, indicando um efeito benéfico relacionado ao treinamento. Em um estudo que utilizou EA como estratégia de tratamento frente à HIE, também foi observada a recuperação da memória de reconhecimento, mas não na memória aversiva avaliada no teste da esquiva inibitória (ROJAS *et al.*, 2013). Outro estudo também identificou o aumento de sinaptofisina no estriado após a realização de treinamento acrobático, indicando alterações plásticas nesta estrutura (TAMAKOSHI *et al.*, 2014).

Assim, podemos sugerir que o melhor desempenho observado no teste da memória de reconhecimento identificado em nossas avaliações pode ter ocorrido devido a esta maior plasticidade no estriado em decorrência do treinamento acrobático.

O labirinto aquático de Morris é um teste importante para avaliar a memória espacial. Neste estudo, os animais que sofreram HIE apresentaram déficit na memória

espacial e na memória de trabalho, assim como já relatado em trabalhos prévios (PEREIRA *et al.*, 2007; PEREIRA *et al.*, 2008; DENIZ *et al.*, 2018a). Este resultado pode estar relacionado ao dano causado pela lesão em diversas regiões e, particularmente, no hipocampo (PEREIRA *et al.*, 2007; PEREIRA *et al.*, 2008; MIGUEL *et al.*, 2017), uma vez que esta é uma estrutura crucial para a aprendizagem espacial (D'HOOGE; DE DEYN, 2001; DENIZ *et al.*, 2018a). Entretanto, o exercício acrobático não reverteu o déficit na memória espacial avaliado por esta tarefa. Provavelmente, por não ter sido capaz de reverter os danos no hipocampo, já que essa região é muito importante para esse tipo de memória. Além disso, o exercício acrobático começou mais tarde, no DPN 22 e não pôde evitar tais danos, uma vez que esta lesão é muito proeminente e já estava instalada.

Ainda, de forma semelhante ao identificado por nosso estudo, trabalhos prévios desenvolvidos com EA identificaram apenas recuperação parcial na memória de trabalho, sendo esta recuperação observada apenas em animais jovens, mas não em adultos (PEREIRA *et al.*, 2009). Outro parâmetro para identificar a recuperação estrutural na HIE é a avaliação da atrofia. Esta avaliação foi realizada no hipocampo e no estriado, que são regiões encefálicas relacionadas à memória.

Acredita-se que os sistemas de memória do hipocampo e do estriado operam de forma independente e em paralelo, apoiando a memória cognitiva e a memória episódica, respectivamente (FERBINTEANU, 2016). O corpo estriado faz parte dos chamados núcleos da base. O estriado dorsal, juntamente com o estriado ventral, servem como as principais vias de entrada e saída de informação dessa área do encéfalo (SOMOGYI *et al.*, 1981; GOODMAN; PACKARD, 2015). Ainda, o estriado desempenha um papel crucial no aprendizado e na memória, coordenando a exploração espacial e mediando a atualização das informações, uma vez que inativação funcional desta estrutura altera a flexibilidade comportamental e a memória de reconhecimento em roedores (QIAO *et al.*, 2017).

Como esperado, no presente estudo encontramos atrofia no hipocampo e estriado. Em relação a essas estruturas, um estudo anterior desenvolvido por Miguel et al., 2015, encontrou o maior dano no hipocampo em relação ao corpo estriado. Não obstante, os nossos resultados mostram um dano de cerca de 70% no hipocampo, sendo importante notar que essa região é muito vulnerável à excitotoxicidade (BARTSCH *et al.*, 2015), que é um dos principais mecanismos na fisiopatologia da lesão causada pela

HIE. No entanto, o dano na área do estriado foi menos proeminente e isso também poderia explicar parcialmente a melhora cognitiva na tarefa de reconhecimento de objetos, causada pelo treinamento acrobático. Ainda, outro estudo que utilizou EA como estratégia terapêutica obsevou apenas uma recuperação parcial da atrofia no encéfalo após HIE quando o tratamento foi precoce (iniciado 24h após a lesão), mostrando que a HIE leva a atrofia severa e de difícil reversão (SCHUCH *et al.*, 2016).

Além disso, a extensão de dano encefálico e o desempenho em tarefas comportamentais podem ser diferentes de acordo com o hemisfério em que ocorre a lesão, sendo que o hemisfério direito é mais vulnerável ao dano causado ela HIE neonatal (ARTENI *et al.*, 2010). No presente estudo, a HIE foi realizada no hemisfério esquerdo e isso também pode estar relacionado ao melhor desempenho observado na tarefa de reconhecimento de objetos.

É importante notar que a exposição de animais a um ambiente diferente pode gerar um comportamento exploratório que possibilita o processo de familiarização com o novo ambiente, sendo este comportamento motor exploratório intimamente relacionado à função do estriado (YAMIN *et al.*, 2013). Ainda, o treinamento acrobático envolve a aprendizagem motora e a exposição a um ambiente diferente ao longo das semanas de treino (CONFORTIM *et al.*, 2018). Esses fatores também podem ter contribuído para um aumento na plasticidade sináptica no estriado, o que já foi observado em estudos prévios (TAMAKOSHI *et al.*, 2014). Assim, a recuperação da memória de reconhecimento no grupo HIACR pode estar relacionada diretamente ao tipo de treino motor/cognitivo propiciado pelo exercício acrobático.

Ainda, buscando entender os mecanismos envolvidos nessa melhora na memória de reconhecimento, decidimos investigar os níveis de BDNF no hipocampo e estriado dos animais. Surpreendentemente, não encontramos diferença neste fator neurotrófico em ambas as estruturas. A análise estatística mostrou apenas uma tendência de aumento dos níveis de BDNF no estriado dos animais HI. Esse aumento já foi indicado como uma estratégia de preservação após a HIE (PEREIRA *et al.*, 2009; DENIZ *et al.*, 2018a) e também já foi relacionada à melhora cognitiva na resposta ao exercício (CHEN *et al.*, 2017). No entanto, nosso estudo não encontrou tais alterações relacionadas à HIE ou ao treinamento. Este resultado pode ter sido observado devido à perda da janela de expressão desta proteína, uma vez que, já se sabe que a expressão do BDNF pode mudar

ao longo do tempo, estando assim associada a efeitos efêmeros ou alterações plásticas transitórias (MEGA *et al.*, 2018).

Em conclusão, este estudo sugere que a aprendizagem motora pode ser uma estratégia coadjuvante promissora para o tratamento de déficits cognitivos relacionados à HIE, podendo ser facilmente adaptados na prática clínica, especialmente na reabilitação motora em crianças, uma vez que este tipo de exercício envolve tarefas que estimulam a aprendizagem, podendo também ser associadas a jogos ou atividades lúdicas (SIDAWAY *et al.*, 2012). No entanto, estudos adicionais são necessários para compreender em maior profundidade os mecanismos de ação responsáveis por esse efeito benéfico.

6 CONCLUSÕES

- A hipóxia-isquemia encefálica neonatal leva a prejuízo cognitivo em avaliações realizadas no labirinto aquático de Morris e teste de reconhecimento de objetos em ratos adultos jovens;

- A hipóxia-isquemia encefálica neonatal causa atrofia no estriado e no hipocampo ipsilaterais à lesão em animais adultos jovens;

- A hipóxia-isquemia encefálica neonatal parece aumentar os níveis de BDNF no estriado ipsilateral à lesão nos animais adultos jovens;

- O exercício acrobático promove recuperação da memória de reconhecimento de objetos em animais submetidos ao modelo de hipóxia-isquemia encefálica neonatal.

7 PERSPECTIVAS

- Avaliação da plasticidade sináptica por meio da marcação da proteína sinaptofisina no hipocampo e estriado;

- Quantificação de perda neuronal pela marcação de neurônios utilizando o anticorpo para NeuN, no hipocampo e estriado;

- Quantificação da expressão do receptor de BDNF / Trkβ no hipocampo e estriado;

- Mensuração do volume do hipocampo e estriado para uma quantificação mais refinada do dano causado nessas estruturas e possíveis efeitos do treinamento acrobático.

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