UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM NEUROCIÊNCIAS

EFEITO DE DIFERENTES PROTOCOLOS DE EXERCÍCIO FÍSICO SOBRE A FUNÇÃO MOTORA E A MORFOLOGIA DE ESTRUTURAS ASSOCIADAS AO MOVIMENTO EM RATOS SUBMETIDOS À HIPÓXIA-ISQUEMIA ENCEFÁLICA NEONATAL

HELOISA DEOLA CONFORTIM

Porto Alegre

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Tese de Doutorado apresentada como requisito parcial à obtenção do título de Doutor em Neurociências pelo Programa de Pós-Graduação *Stricto Sensu* em Neurociências da Universidade Federal do Rio Grande do Sul.

HELOISA DEOLA CONFORTIM

Orientadora: Profa. Dra. Lenir Orlandi Pereira Silva

Porto Alegre

2019

EPÍGRAFE

“A persistência é o caminho do êxito”.

Charles Chaplin

AGRADECIMENTOS

Ainda estou em dúvida se meu desafio maior foi escrever a tese em si, ou utilizar apenas alguns parágrafos para agradecer a todos que tiveram um papel tão importante para que ela pudesse ser concluída.

Irei iniciar essa sessão agradecendo aos meus pais. Eles são e sempre foram meu alicerce em todos os sentidos, sempre ao meu lado (mesmo que distantes fisicamente) me apoiando e incentivando, mesmo sem saber exatamente o que eu estava fazendo em alguns momentos. Sei que muitas vezes vocês prefeririam que eu estivesse aí pertinho de vocês, mas agradeço pela compreensão e confiança depositadas em mim sempre. Agradeço imensamente também pela ajuda e amor incondicional. Muito obrigada por tudo. Amo muito vocês.

Aos demais membros da família pelo apoio e palavras de incentivo durante estes anos. Especialmente a tia Marinita, por ter me presenteado com o livro Kandel para que eu estudasse para a seleção do doutorado, com certeza esse presente auxiliou muito para o resultado positivo que tive nesta etapa e será muito útil ainda. Também a agradeço a minha madrinha Maricilda pelo apoio e valorização de sempre. Obrigada.

No âmbito acadêmico muitas pessoas foram essenciais para que todo este trabalho pudesse ser realizado. Gostaria de começar agradecendo a professora Lígia, minha eterna co-orientadora. Graças ao empurrãozinho dela e ao primeiro contato com a professora Lenir, conheci a UFRGS e o PPG Neurociências ao qual tenho orgulho de fazer parte. Meus eternos agradecimentos a você, tu és e sempre será um exemplo de professora, orientadora e principalmente de pessoa para mim. Obrigada.

Na sequência preciso agradecer a professora Lenir, por ter aberto as portas do LG e ter me dado a oportunidade de conviver e aprender com esse grupo sem igual! Sempre brincamos que o nosso grupo é o melhor e acredito mesmo nisso, tenho muito orgulho de dizer que fiz e farei parte dele para sempre. Agradeço especialmente a professora Lenir, pela paciência, pelo incentivo, pela ajuda, pelas críticas construtivas e pelos ensinamentos de sempre, aprendi muito contigo e espero que essa parceria seja eterna. Obrigada.

Ainda em relação ao grupo, o que falar do LG, inicialmente éramos nós quatro. Ramiro, Bru, Pati e eu. Trabalhar e aprender com vocês diariamente foi um privilégio.

Sentirei muitas saudades. Mesmo nos piores problemas, acordando às 6 da manhã (nas His do Ramiro) a gente sempre ria e conseguia resolver! Obrigada pelas sessões de terapia em grupo e por enxugarem minhas lágrimas muitas vezes!! Obrigada por me acolherem assim tão bem e por serem meus irmãos aqui! Amo vocês!

Depois nosso grupo foi se renovando e chegaram Wellington, as nossas ICs maravilhosas Loise, Bruna e Milene e por último a Ethi e a Drika. Não tenho palavras suficientes para agradecer pela ajuda que vocês me deram não só no trabalho, mas na vida!! Todos são exemplos para mim, cada um com suas personalidades me tocaram de alguma forma e foram imprescindíveis para que essa tese fosse finalizada. Nunca me esquecerei de vocês. Obrigada.

Aos demais colegas de laboratório Filipe, Christiano, Fran e André que mesmo não sendo exatamente do mesmo grupo sempre estavam por ali dando auxílio ou proporcionando uma boa risada. Ah e obrigada por me ensinarem a tomar café também! Agradeço especialmente ao Filipe, por ter se tornado meu irmão em vários momentos e pela parceria de estudo e de carona em várias disciplinas. Obrigada.

Aos técnicos Silvia e Maikel. Além de sempre estarem dispostos a ajudar nos mais variados assuntos, vocês se tornaram grandes amigos. Quero guardar para sempre como mais um presente que a UFRGS me deu. Obrigada. Quero agradecer também as técnicas do Centro de microscopia eletrônica pela atenção e cordialidade durante o período que trabalhei no Campus do Vale. Obrigada também a todos os outros colegas, técnicos ou professores que deram uma dica, uma palavra amiga ou uma ajuda pontual em algum desses momentos.

Preciso agradecer também a CAPES pela bolsa de doutorado, a UFRGS e ao PPG Neurociências pela oportunidade, e aos professores aos quais tive a oportunidade de partilhar conhecimentos. Em especial a prof. Simone pelas dicas, ajuda, desabafos e amizade de sempre. Obrigada.

Encerrando os agradecimentos acadêmicos gostaria de agradecer a minha relatora Prof. Dra. Tais Malysz e aos demais membros da banca Prof. Dra. Ionara Siqueira e Prof. Dr. Pedro Dal Lago pelas considerações e pelos ensinamentos transmitidos durante a minha defesa, com toda certeza não enriqueceram somente o meu trabalho, mas também propagaram dicas e experiências muito úteis a todos os presentes. Obrigada.

Gostaria de agradecer ainda as minhas amigas de Marmeleiro-PR especialmente a Talita, pelo apoio e momentos de descontração de sempre. A minha amiga Diandra por ter se mudado para Porto Alegre comigo (quase isso), por sempre estar presente nos momentos mais tensos (qualificações e defesas) e pela parceria de sempre. Tu sabes que sempre estará no meu coração. Te amo. Obrigada.

A minha família aqui em Porto Alegre, meus sogros Mari e Lupe, pela acolhida, pelo abrigo quando me mudei para o RS, pela ajuda e incentivo de sempre. Obrigada.

A Patrícia, pelo apoio e incentivo semanal, ao qual me ajudou muito principalmente nestes últimos momentos. Muito obrigada.

Agradecimentos também ao Jimi, Cindy, Lilica e Pepeu, os pets da família, pelos momentos de descontração e alegria que me proporcionaram em meio a tensão durante este período. Agradeço especialmente ao Jimi, meu filhote de 4 patas, por sempre me receber feliz e por me fazer companhia nos momentos de estudo! Tu és um animalzinho muito especial!

Meu último e mais profundo agradecimento só poderia ser dedicado ao meu companheiro André. Agradeço imensamente por ter estado ao meu lado durante essa caminhada (nada fácil) e por fazer parte dela ativamente, estando presente desde os experimentos nos fins de semana até o encorajamento diário que só tu sabes fazer!! Agradeço ainda por aguentar minhas ausências em vários momentos e por nunca ter deixado de acreditar em mim, quando muitas vezes nem eu acreditei. Tu és o melhor companheiro que eu poderia ter!! Essa vitória com certeza é nossa!! Muito obrigada!! Te amo.

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AMPA: receptores alfa-amino-3-hidroxi-metil-5-4-isoxazolpropiônico

AVC: acidente vascular encefálico

BDNF: fator neurotrófico derivado do encéfalo

BHE: barreira hematoencefálica

CT: grupo controle não exercitado

CTT: grupo controle submetido ao treinamento em esteira

CTA: grupo controle submetido ao treinamento acrobático

DPN: dia pós-natal

GFAP: proteína glial fibrilar ácida

HIE: hipóxia-isquemia encefálica

HIEA: grupo submetido à hipóxia-isquemia encefálica submetido ao treinamento acrobático

HIET: grupo submetido à hipóxia-isquemia encefálica submetido ao treinamento em esteira

IML: núcleo intermediolateral

IN: interneurônios

JNMs: junções neuromusculares

MMP: metaloproteinase de matriz

MN: motoneurônios

M1: córtex motor primário

NMDA: receptores N-metil-D-aspartato

NP: neurônios piramidais

PMRT: Pasta Matrix Reaching Task

SNC: sistema nervoso central

SYP: sinaptofisina

TCE: trato corticoespinal

VEGF: fator de crescimento endotelial vascular

VTA: área tegmental ventral

RESUMO

A hipóxia-isquemia encefálica (HIE) neonatal é uma das principais causas de mortalidade e morbidade neonatal, contribui para a incapacidade geral, levando ao comprometimento motor e de estruturas relacionadas. Os modelos animais disponíveis para mimetizar essa patologia mostram resultados contraditórios, especialmente relacionados à função motora, dificultando assim a translação dos resultados encontrados. Da mesma forma, estudos pré-clínicos que avaliem os benefícios de protocolos de exercício que já são utilizados na clínica como forma de tratamento para esta patologia, ainda são escassos. Nesse contexto, o objetivo deste estudo foi avaliar os efeitos de diferentes protocolos de exercício físico (aeróbico e acrobático) sobre a função motora, o córtex sensoriomotor, o trato corticoespinal, medula espinal, junções neuromusculares e músculo esquelético em ratos *Wistar* machos submetidos a um evento hipóxico-isquêmico encefálico no período neonatal. O protocolo experimental teve início no 7º dia pós-natal (DPN), quando ratos Wistar machos foram submetidos à HIE neonatal de acordo com o modelo proposto por Rice-Vannuci. A partir do 22º DPN até o 60º DPN, foram realizados os protocolos de exercício (aeróbico ou acrobático) e foram definidos então seis grupos experimentais: grupo controle não exercitado (CT), grupo controle submetido a treinamento em esteira (CTT), grupo controle submetido a treinamento acrobático (CTA), grupo HIE não exercitado (HIE), grupo HIE submetido ao treinamento em esteira (HIET) e grupo HIE submetido ao treinamento acrobático (HIEA). Após o fim do treinamento, a função motora geral dos animais foi avaliada através dos testes: Campo aberto, Escada horizontal e Rotarod. Também foram avaliados a capacidade de utilização, a preferência e a força dos membros anteriores dos animais através do Pasta Matrix Reaching Task (PMRT). Após a realização dos testes comportamentais, os animais foram eutanasiados e os encéfalos, fragmentos da medula espinal, nervo isquiático e músculo plantar foram coletados para análise morfológica. Também foi realizada a técnica de imunohistoquímica com marcação das proteínas sinaptofisina (SYP), NeuN e proteína glial fibrilar ácida (GFAP) no córtex motor primário. Nossos principais resultados mostraram que a HIE causou atrofia cerebral, déficits de aprendizado, comprometimento na locomoção observado no teste da escada horizontal e menor utilização/preferência do membro afetado pela lesão demonstrado pelo teste PMRT.

Em relação à plasticidade celular, foi possível observar um aumento no número de astrócitos e da proteína SYP no córtex motor nos ratos HIE no lado ipsilateral à lesão. Não encontramos alterações morfológicas no córtex sensoriomotor, no nervo isquiático, na medula espinal, nas junções neuromusculares e no músculo esquelético dos animais submetidos ao modelo de HIE neonatal, tampouco efeitos do exercício físico sobre esses parâmetros. Contudo, um efeito benéfico do treinamento acrobático foi evidenciado nos animais HIE, demonstrado pela melhora na locomoção avaliada no teste da Escada horizontal e uma diminuição da atrofia cerebral. Esses resultados mostram evidências pré-clínicas de que o exercício acrobático pode ser uma boa opção terapêutica após eventos de HIE neonatal, sendo responsável pela melhora de aspectos cognitivos e motores.

Palavras chave: asfixia perinatal, lesão cerebral, treino de aprendizado motor, trato corticoespinal, neurodesenvolvimento.

ABSTRACT

The neonatal hypoxia-ischemia encephalopathy (HIE) is the major cause of neonatal mortality and morbidity, contributing to an overall disability, leading to motor impairment and damage to related structures. The animal models available to mimic this pathology show contradictory results, especially related to motor function, making it difficult to transition the results to humans. Similarly, preclinical studies that evaluate the benefits of exercise protocols that are already used in the clinic as a form of treatment for this pathology are still scarce. In this context, the aim of this study was to evaluate the effects of different physical exercise protocols (aerobic and acrobatic) on motor function, sensorimotor cortex, corticospinal tract, spinal cord, neuromuscular junctions and skeletal muscle in male Wistar rats submitted to a hypoxic-ischemic event in the neonatal period. The experimental protocol started on the 7th postnatal day (PND) when male Wistar rats were submitted to neonatal HIE according to the model proposed by Rice-Vannuci. From the 22nd PND to the 60th PND, the exercise protocols (aerobic and acrobatic) were performed and six experimental groups were defined: control group non-exercised (CT), control group submitted to treadmill training (CTT), control group submitted to acrobatic training (CTA), HIE group non-exercised (HIE), HIE group submitted to treadmill training (HIET) and HIE group submitted to acrobatic training (HIEA). After the end of the training, the general motor function of the animals was evaluated through the tests: Open field, horizontal ladder and Rotarod. The utilization, preference, and strength of the animals' forelimbs were also evaluated through the Matrix Reaching Task (PMRT). After the end of the behavioral tests, the animals were euthanized and brains, spinal cord, sciatic nerve, and plantar muscle fragments were collected for morphological analysis. The immunohistochemistry technique with the labeling of synaptophysin (SYP), NeuN and glial fibrillary acidic protein (GFAP) was also performed in the primary motor cortex. Our main results showed that HIE caused cerebral atrophy, learning deficits, impairment in locomotion observed in the horizontal ladder test, and lower use/preference of forelimb affected by the lesion demonstrated by the PMRT test. Regarding cell plasticity, it was possible to observe an increase in the number of astrocytes and SYP protein in the motor cortex in HIE rats on the ipsilateral side to the lesion. We did not find morphological changes in the sensorimotor cortex, sciatic nerve,

spinal cord, neuromuscular junctions and skeletal muscle of the animals submitted to the neonatal HIE model, also were not observed physical exercise effects on these parameters. However, a beneficial effect of acrobatic training was evidenced in the HIE animals, demonstrated by the improvement in locomotion evaluated in the Horizontal Ladder test and a decrease in cerebral atrophy. These results show preclinical evidence that acrobatic exercise may be a good therapeutic option after neonatal HIE events, and is responsible for the improvement of cognitive and motor aspects.

Keywords: perinatal asphyxia, brain lesion, motor skills learning, corticoespinal tract, neurodevelopment.

1. INTRODUÇÃO

1.1 Hipóxia-isquemia encefálica neonatal

A hipóxia-isquemia encefálica (HIE) neonatal é uma disfunção cerebral causada por uma redução no suprimento de oxigênio para o encéfalo e outros órgãos (hipóxia), agravada pelo baixo fluxo sanguíneo. Esta disfunção leva a danos cerebrais e está frequentemente associada a deficiências no neurodesenvolvimento (GLASS; FERRIERO, 2007). Considerada a principal causa de mortalidade em crianças, a HIE é responsável por 23% de todas as mortes neonatais em todo o mundo (SCHUMP, 2018). Dados atuais indicam que de um a três a cada 1.000 nascidos vivos são afetados por essa disfunção em países desenvolvidos (HAGBERG et al., 2015). Grande parte desses recém-nascidos morre no período pós-natal (15%-20%) e um adicional de 25% desenvolvem sequelas neuropsicológicas graves e permanentes como: desatenção, deficiência visual, disfunção perceptiva, disfunção executiva, hiperatividade, epilepsia e presença de déficits sensório-motores, incluindo atraso nos marcos do desenvolvimento, problemas na postura, marcha e presença de movimentos involuntários (HOPKINS; HAALAND, 2004; ANDERSON; ARCINIEGAS, 2010; LAI; YANG, 2011; ADHIKARI; RAO, 2017). A literatura relata ainda que indivíduos acometidos por lesões cerebrais hipóxico-isquêmicas podem apresentar comprometimento em tratos neurais, especialmente os responsáveis pelo controle motor, como o trato corticoespinal (TCE) (LEE et al., 2012).

Em humanos, os eventos hipóxico-isquêmicos têm como principais fatores etiológicos a prematuridade do neonato, dificuldades de expulsão do feto, sofrimento fetal, desnutrição da mãe, interrupção transitória do fluxo sanguíneo umbilical, descolamento placentário ou fortes contrações uterinas que podem ocasionar insuficiência na troca de gases pela placenta (PROCIANOY; SILVEIRA, 2001, KURINCZUK et al., 2010). Atualmente, o diagnóstico precoce da HIE neonatal depende da observação dos sintomas e sinais clínicos, utilizando uma combinação de tomografia computadorizada, ressonância magnética, ultrassonografia e eletroencefalograma. Porém na maioria dos casos o diagnóstico só consegue ser realizado posteriormente, após o aparecimento de sintomas mais graves, normalmente identificados na idade escolar das crianças, dificultando assim o tratamento devido à progressão dos danos (LV et al., 2015).

Devido ao alto consumo energético, o encéfalo possui grande sensibilidade a alterações no suprimento de oxigênio, sendo um dos órgãos mais susceptíveis a esses episódios hipóxico-isquêmicos. Bebês que sobrevivem ao insulto inicial causado pela HIE exibem danos cerebrais macroscópicos que podem ser detectados através de exames de ressonância magnética. Na literatura há relatos de diferentes regiões anatômicas afetadas por esse insulto nos indivíduos acometidos, mas a maioria dos trabalhos relata comprometimento no córtex cerebral, núcleos da base e tálamo (MILLAR et al., 2017). A área do córtex e núcleos da base danificados durante a lesão inicial é diretamente preditiva das deficiências de linguagem e motoras observadas nesses pacientes (BARNETT et al., 2002; STEINMAN et al., 2009; MARTINEZ-BIARGE et al., 2011;).

As lesões cerebrais são causadas principalmente pela acentuada morte celular nessas regiões encefálicas, que ocorrem devido a uma cascata de eventos. O insulto hipóxico-isquêmico inicial é caracterizado por uma diminuição do aporte sanguíneo e este é o fator desencadeante de lesão neuronal, pois as células se tornam incapazes de suprir suas demandas metabólicas (NOVAK et al., 2018). Após este evento inicial, ocorre um período de reperfusão dos tecidos cerebrais, e com isso há uma restauração transitória da função metabólica celular. Este período é seguido por uma diminuição do aporte de glicose e uma deficiência em fosfatos de alta energia (ATP) que resultam em uma lesão secundária (DISTEFANO; PRATICO, 2010).

Em resposta a essas lesões celulares, os aminoácidos excitatórios glutamato e aspartato são liberados no encéfalo de maneira exacerbada, exercendo um efeito excitotóxico (BURD et al., 2016). O glutamato é considerado o principal neurotransmissor excitatório do sistema nervoso central (SNC), sendo responsável por 90% das sinapses excitatórias. Em estados não patológicos, ao final da neurotransmissão os astrócitos recaptam esse neurotransmissor da fenda sináptica, evitando assim a sua permanência no espaço extracelular (HAWKINS et al., 2009). Devido a falha energética decorrente da HIE, esse processo de recaptação fica prejudicado, podendo causar assim uma hiperativação desses receptores glutamatérgicos (MILLAR et al., 2017). Essa ativação exacerbada principalmente dos receptores N-metil-D-aspartato (NMDA) e alfa-amino-3-hidroxi-metil-5-4-isoxazolpropiônico (AMPA) encontrados em neurônios e precursores oligodendrogliais resultam no acúmulo de cálcio intracelular, causando morte celular

e ativação microglial com a consequente liberação de fatores prejudiciais para as células neurais vizinhas (GALLUZI et al., 2009). A insuficiência mitocondrial e a liberação de proteínas pró-apoptóticas resultam também em apoptose das células nervosas. Ocorre ainda a geração de espécies reativas de oxigênio prejudicando ainda mais a estrutura e função celular, agravando os danos neuronais (HALLIWELL, 1992). Por último, ocorre inflamação induzida pela ativação da microglia e dos macrófagos com a consequente produção e liberação de citocinas pró-inflamatórias, quimiocinas, proteases, fatores do complemento, aminoácidos excitotóxicos, espécies reativas de oxigênio e óxido nítrico contribuindo ainda mais para o agravo da lesão cerebral secundária (EKLIND et al., 2001; NOVAK et al., 2018) (Figura 1).

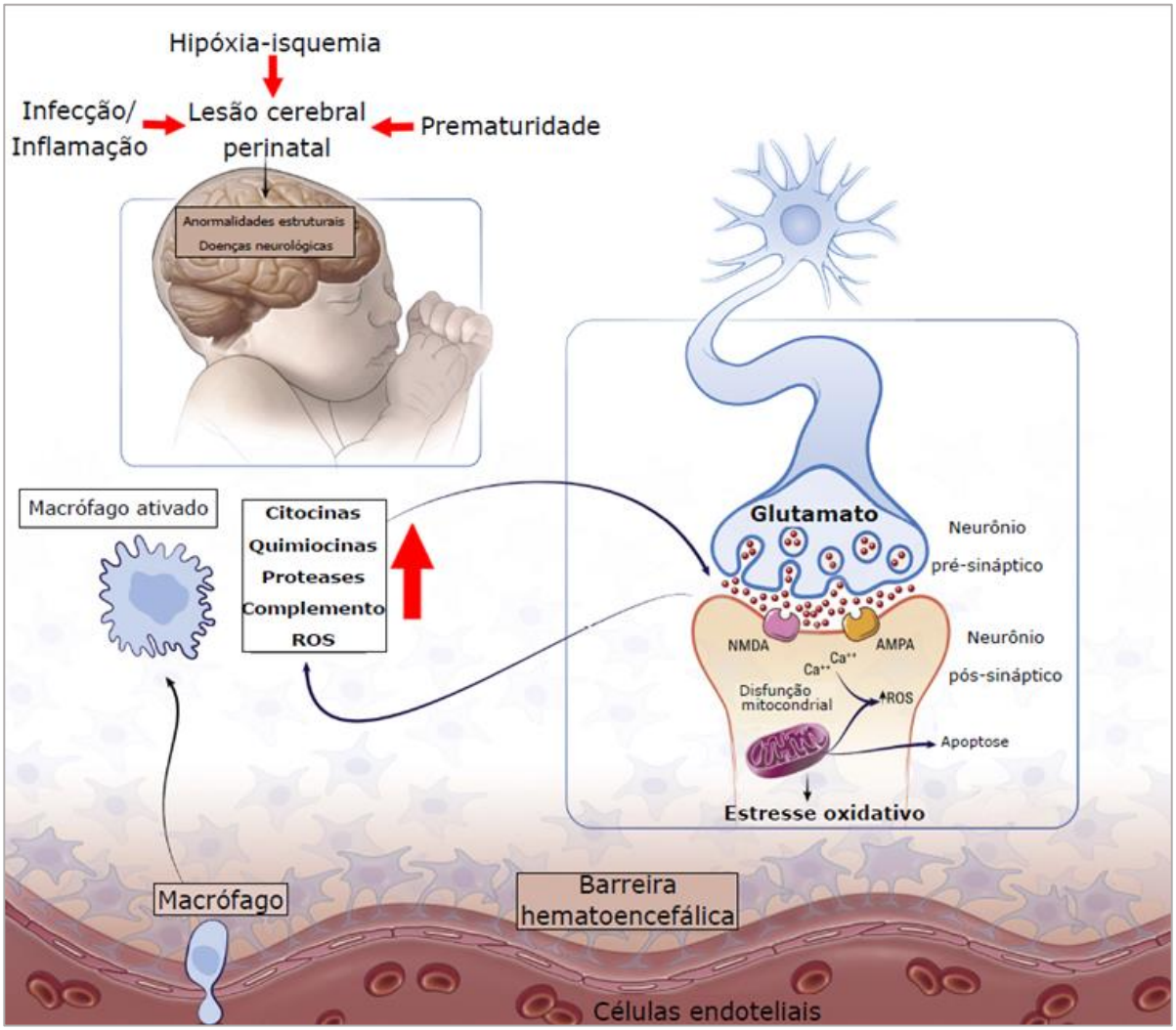

Figura 1. Principais mecanismos envolvidos na patogênese da HIE. A figura mostra as principais vias envolvidas na lesão cerebral perinatal marcadas pela excitotoxicidade glutamatérgica, geração de espécies reativas de oxigênio e inflamação (Adaptado de NOVAK et al., 2018).

Os principais mecanismos envolvidos na patogênese da HIE são responsáveis em conjunto pelo extenso dano ao tecido nervoso observado, sendo determinante para o comprometimento funcional encontrado nos indivíduos acometidos por esse tipo de lesão.

1.2 O modelo de Rice-Vannucci

Para entender com mais detalhes os mecanismos envolvidos na patologia da HIE e estudar possíveis terapias, vários modelos animais são utilizados para mimetizar essa condição. Destaca-se neste campo o modelo proposto por Rice e colegas (1981) comumente referido como modelo de Rice-Vannucci. O modelo Rice-Vannucci é amplamente utilizado experimentalmente pois reproduz a lesão no hipocampo, estriado e córtex cerebral encontrada nos recém-nascidos humanos, no hemisfério ipsilateral à oclusão arterial (PEREIRA et al., 2007). Este modelo consiste na oclusão unilateral da artéria carótida comum dos animais, podendo ser realizada do lado esquerdo ou direito dependendo do estudo, seguido de recuperação com as mães por aproximadamente 1h. Posteriormente, os animais são expostos a um ambiente hipóxico com 8% oxigênio por períodos que variam de 1 a 3 horas (MIILLAR et al., 2017). Uma representação esquemática deste modelo é apresentada na Figura 2.

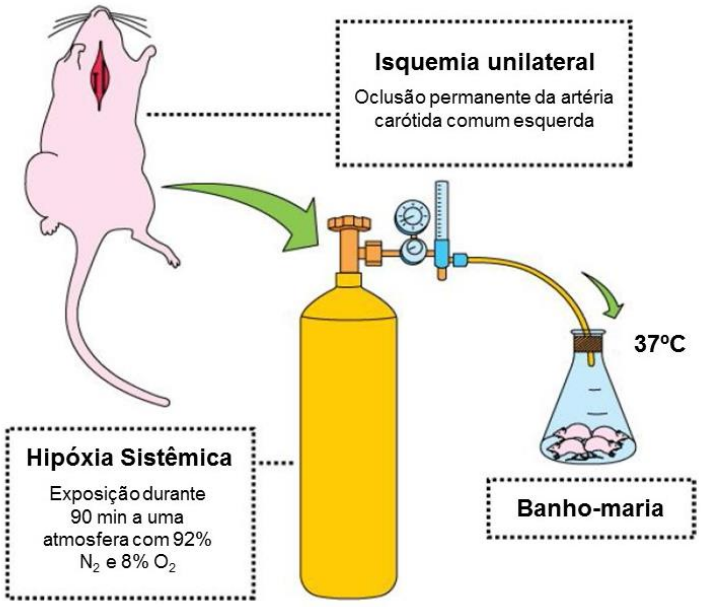

Figura 2. Representação esquemática do modelo de Rice-Vannucci para realização da hipóxia-isquemia encefálica neonatal em roedores.

Considerando que o encéfalo dos ratos aos sete dias de vida é histologicamente similar ao de crianças recém-nascidas a termo, este modelo permite que as alterações morfológicas, bioquímicas e cognitivas encontradas nos humanos sejam reproduzidas de maneira fidedigna (SANDERS et al., 2005, ARTENI et al., 2003; IKEDA et al., 2008). Da mesma forma que em humanos, a lesão por HIE ocasiona diversos prejuízos funcionais, bioquímicos e morfológicos aos animais afetados. Diversos estudos demonstram déficits cognitivos, principalmente relacionados à memória de animais HIE em diferentes tarefas comportamentais como o teste do labirinto aquático de Morris (IKEDA et al., 2001; PEREIRA et al., 2007, 2008; TATA et al., 2015; CARLETTI et al., 2016), o teste de reconhecimento de objetos (ROJAS et al., 2013; MARCELINO et al., 2016), a tarefa de esquiva inibitória (CARLETTI et al., 2012; ROJAS et al., 2013; SANCHES et al., 2013) e o teste do Ox-maze (ROJAS et al., 2015). Alguns estudos também identificaram hiperatividade e déficit de atenção neste modelo (MIGUEL et al., 2015, 2017; DENIZ et al., 2018). Esses resultados demonstram que o comprometimento cognitivo nos animais HIE está muito bem estabelecido.

Entretanto, quando investigamos os achados relacionados à função motora causados por esse modelo, os resultados são conflitantes. Uma série de estudos não identificou comprometimento motor nos animais HIE. Rojas e colegas (2013) não encontraram diferenças entre os grupos HIE e controle no teste do rotarod. Miguel et al. (2015) e Arteaga et al. (2017) utilizando o teste de campo aberto e Schuch et al. (2016a) utilizando o teste do cilindro, também não identificaram disfunção motora nos animais HIE. Contudo, outros estudos demostraram que o déficit motor é evidente neste modelo. Utilizando o teste rotarod, Griva et al. (2017) e Dai et al. (2017) encontraram déficit de equilíbrio em animais HIE. O comprometimento motor também foi demonstrado nos testes do cilindro e no teste de caminhada em escada horizontal em um estudo realizado por Durán-Carabali et al. (2017). Pak et al. (2018) evidenciaram disfunção sensório-motora, mesmo após semanas de indução da HIE, utilizando várias tarefas motoras como: o teste de campo aberto, teste de cilindro, rotarod e avaliação da marcha pelo aparato conhecido como Catwalk. Provavelmente esses achados contraditórios em relação à função motora são encontrados na literatura pois a HIE não resulta em uma lesão cerebral uniforme, ela causa danos variáveis a diferentes estruturas cerebrais, depende da gravidade e duração do

insulto, bem como do estágio de desenvolvimento do encéfalo quando este evento ocorre.

Uma das regiões mais afetadas pelo modelo de HIE neonatal e por consequência uma das regiões mais estudadas é o hipocampo. Esse comprometimento hipocampal é extensivamente demonstrado na literatura através de dados de atrofia e diminuição do número de células dessa região, e corrobora com os desfechos desfavoráveis encontrados em relação a déficits cognitivos, especificamente nos processos relacionados à memória (SOUZA et al., 2014; DOMNICK et al., 2015; ROJAS et al., 2015; CARLETTI et al. 2016; DIAZ et al., 2016; GRIVA et al., 2017; ODORCYK et al., 2018; CHÁVEZ VALDEZ et al., 2018).

Buscando entender com mais detalhes os mecanismos pelos quais esses danos celulares ocorrem nessas determinadas regiões, marcadores celulares específicos podem ser utilizados experimentalmente. Um marcador muito utilizado para a marcação e análise de neurônios em quase todo o sistema nervoso dos vertebrados é a proteína NeuN. Essa proteína é expressa de forma estável durante estágios específicos do desenvolvimento, e encontra-se nos núcleos e no citoplasma perinuclear da maioria dos neurônios do SNC dos mamíferos, portanto, é considerada um marcador confiável de neurônios maduros (DUAN et al., 2016). Em estudos experimentais com o modelo de HIE em ratos, já foi possível observar diminuição dessa proteína nos animais afetados, indicando assim uma diminuição no número de neurônios nesses animais (DEMAREST et al., 2016; KIM, et al., 2017).

Outro tipo celular que sofre alterações nos eventos hipóxico-isquêmicos são os astrócitos. No SNC, os astrócitos são o tipo celular predominante e eles desempenham funções vitais, incluindo a formação e manutenção da barreira hematoencefálica, atuam na sinaptogênese, na neurotransmissão e na regulação metabólica (MOLOFSKY, et al., 2015). Para identificar alterações nesse tipo celular, diversas proteínas podem ser empregadas como a S-100β, a metaloproteinase de matriz (MMP) e a proteína glial fibrilar ácida (GFAP). A GFAP é considerada um dos melhores marcadores para a ativação de astrócitos após lesão ou estresse no SNC (ZHANG et al., 2017). Ela é uma proteína do filamento intermediário do citoesqueleto dos astrócitos e indica o estado fisiológico e patológico dos mesmos. Estudos recentes mostraram que a GFAP sérica indica danos cerebrais isquêmicos em recém-nascidos (FLORIO et al., 2010). Mudanças nas concentrações séricas de GFAP podem indicar

alterações nos astrócitos no tecido cerebral, mas também já foram identificados danos à barreira hematoencefálica no encéfalo de animais HIE através da utilização desse marcador (DIAZ et al., 2016). Os astrócitos, classicamente eram considerados apenas como células de suporte para os neurônios, mas estão emergindo como elementos cruciais na fisiologia cerebral sendo responsáveis por regular a atividade neuronal, a transmissão e a plasticidade sináptica (PÉREZ-ALVAREZ; ARAQUE, 2013).

A plasticidade do SNC também é afetada pela HIE. A neuroplasticidade refere-se à capacidade biológica inerentemente dinâmica do SNC de sofrer maturação, mudar estrutural e funcionalmente em resposta a variadas experiências ou adaptar-se após algum tipo de lesão. Essas adaptações são alcançadas pela modulação de subconjuntos de mecanismos genéticos, moleculares e celulares e influenciam na dinâmica das conexões sinápticas, podendo levar à formação de novos circuitos neurais, culminando em ganho ou perda de comportamento ou função (ISMAIL et al., 2017). Entre esses acontecimentos que podem indicar plasticidade encefálica, e por consequência são utilizados experimentalmente para mapear esse tipo de evento, pode-se citar: a neurogênese, a apoptose, a sinaptogênese, a poda sináptica, a mudança no padrão de ativação de regiões encefálicas, aumento na ramificação dendrítica, e mudanças relacionadas a células gliais (HARA, 2015; LIU et al., 2017).

Embora o encéfalo imaturo seja mais suscetível a estímulos externos comparado ao indivíduo adulto, um evento hipóxico-isquêmico para o neonato interrompe a modelagem das vias motoras centrais e pode afetar a plasticidade normal do desenvolvimento, alterando assim a neurotransmissão, a sinalização celular, a função e a conectividade neural (ROCHA-FERREIRA; HRISTOVA, 2016). Para avaliar as possíveis alterações na plasticidade do tecido nervoso após um evento lesivo uma proteína muito empregada é a sinaptofisina (SYP). A SYP é uma glicoproteína transmembrana encontrada em pequenas vesículas pré-sinápticas das células nervosas. Dados da literatura sugerem que a SYP é um importante marcador sináptico, que permite obter dados confiáveis sobre a organização morfológica das estruturas sinápticas no SNC (KOLOS et al., 2015). Uma redução significativa da expressão dessa proteína no hipocampo dorsal do hemisfério ipsilateral à lesão, induzida pelo modelo de Rice-Vannuci, já foi identificada em ratos e esse efeito indica perda sináptica nesses animais, que pode resultar em déficits funcionais (GRIVA et al., 2017).

De acordo com o exposto é possível perceber que o dano morfológico, caracterizado pela atrofia cerebral e alterações celulares no encéfalo de animais submetidos à encefalopatia hipóxico-isquêmica neonatal está bem estabelecido, especialmente em áreas relacionadas a memória e cognição. Entretanto, quando buscamos resultados em relação a áreas motoras, e especificamente a função motora dos animais submetidos a esse modelo de HIE, a literatura é escassa e não existe consenso. Até o momento, poucos estudos focaram na avaliação de estruturas cerebrais como o córtex motor, e há também escassez de avaliações de estruturas periféricas, como por exemplo nervos e músculos, estruturas efetoras essenciais para o desenvolvimento do movimento.

Mesmo que nenhuma expressiva vulnerabilidade tenha sido identificada nessas áreas motoras, como exposto anteriormente, uma atrofia cerebral geral já é bem estabelecida na literatura e foi relatada no modelo de HIE em regiões como córtex cerebral, corpo caloso e estriado (PEREIRA et al., 2007, 2008; MIGUEL et al., 2015; SCHUCH et al., 2016a; CARLETTI et al., 2016). Do mesmo modo, déficits na função da medula espinal e redução na área de fibras musculares também já foram relatados em alguns estudos (BELLOT et al., 2014; DURÁN-CARABALI et al., 2017).

Devido a essa diversidade de sequelas que eventos hipóxico-isquêmicos podem causar e as opções terapêuticas limitadas, conhecer e entender os efeitos e mecanismos envolvidos em possíveis tratamentos que possam aliviar os sintomas, melhorar a capacidade funcional e a qualidade de vida dos indivíduos que sofreram a HIE neonatal é de suma importância (ANDERSON; ARCINIEGAS, 2010; VAN VELTHOVEN et al., 2010). Crianças que apresentam comprometimentos motores e cognitivos severos causam ainda grande carga mental e econômica para a família e para a sociedade. Apesar de vários estudos relacionados ao tema, a lesão cerebral neonatal permanece ainda como uma condição devastadora, com desfechos desfavoráveis nos pacientes acometidos. Nesse contexto, opções terapêuticas não farmacológicas são amplamente estudadas em diferentes patologias mimetizadas em modelos animais e mostram resultados satisfatórios. Entre essas modalidades terapêuticas podemos citar o enriquecimento ambiental e o exercício físico.

1.3 O exercício físico como opção terapêutica

A literatura mostra uma relação direta da prática de exercícios físicos com uma melhora estrutural e funcional do sistema neuromuscular, sendo responsáveis por reduzir o estresse oxidativo e aumentar a recuperação do encéfalo e do sistema muscular (COELHO et al., 2013; CHANG et al., 2014). Assim, o exercício físico tem sido utilizado como uma das opções terapêuticas frente a doenças que acometem o sistema nervoso. O efeito benéfico do exercício físico já foi demonstrado frente a diferentes patologias como a epilepsia (GOMES et al., 2014), a paralisia cerebral (KIM et al., 2014), a isquemia cerebral global (LOVATEL et al., 2014), o traumatismo crânio-encefálico (JACOTTE-SIMANCAS et al., 2014), a hemorragia intracerebral (TAMAKOSHI et al., 2014) e também após lesão medular (AHMED; WIERASZKO, 2011).

Diferentes modalidades de exercícios físicos são adaptadas para roedores, de acordo com o objetivo proposto por cada estudo. Alguns exemplos são mostrados na Figura 3. Entre os protocolos mais utilizados estão: os exercícios aeróbicos, como as rodas de corrida voluntária, nado forçado e corrida em esteira (LOVATEL et al., 2014; LLORENS-MARTÍN et al., 2016; MIFLIN et al., 2018; MEGA et al., 2018); os exercícios anaeróbicos, também chamados de força ou resistidos, que agregam uma carga ao exercício (GOMES et al., 2014; MIRON et al., 2018); os ambientes enriquecidos que podem integrar diferentes atividades como o exercício voluntário, a interação social e estímulos sensoriais (PEREIRA et al., 2008; DIAZ et al., 2012; ROJAS et al., 2013; SOARES et al., 2015) e os exercícios acrobáticos, que envolvem circuitos de treinamento que incentivam prioritariamente o aprendizado motor e o equilíbrio (JONES et al., 1999; AHMED; WIERASKO, 2011; SAMPAIO-BAPTISTA et al., 2013).

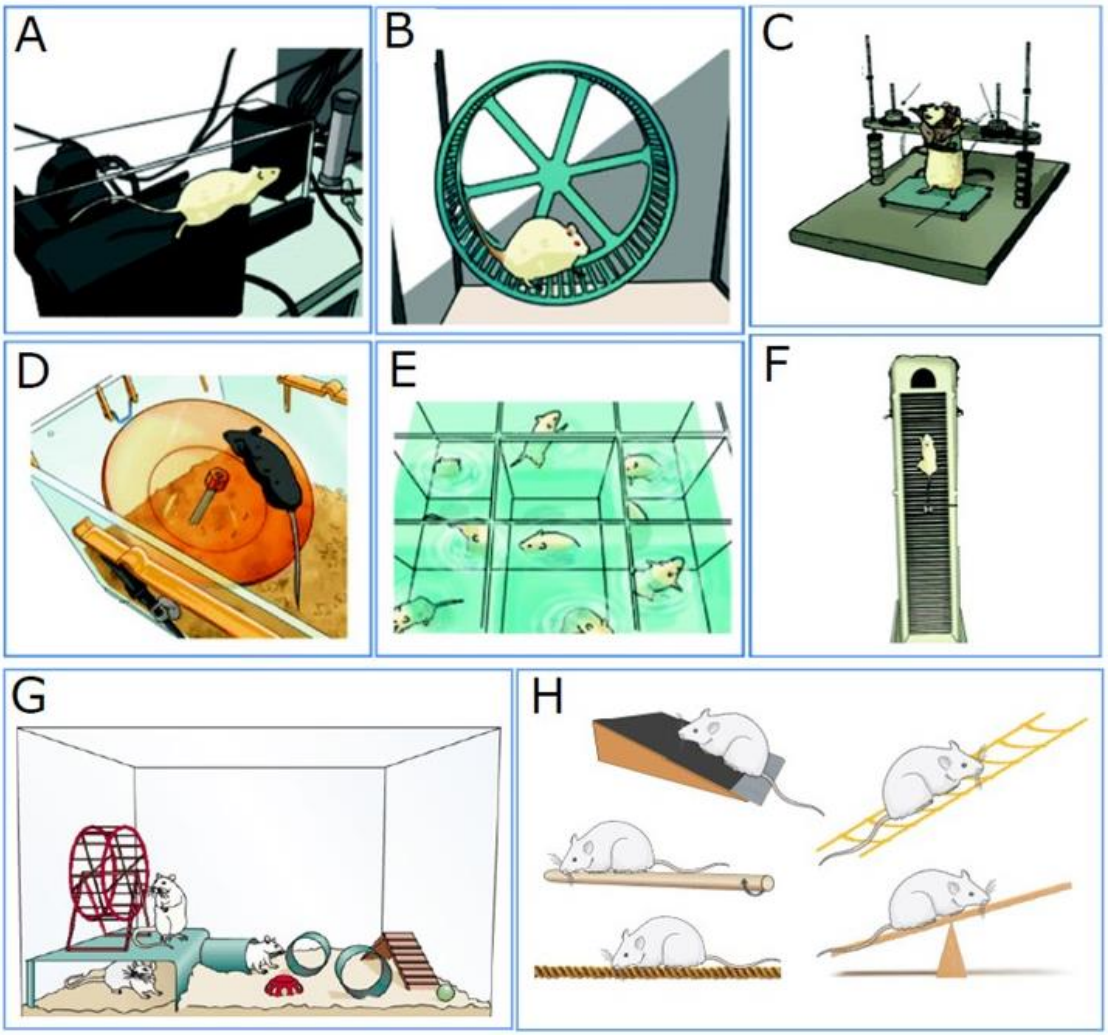

Figura 3. Diferentes tipos de exercícios utilizados em modelos animais. (A) Corrida em esteira. (B) Roda de livre acesso. (C) Treino de levantamento de peso do membro posterior. (D) Roda de corrida com contagem de voltas. (E) Nado forçado. (F) Escada horizontal com peso acoplado à cauda. (G) Enriquecimento ambiental. (H) Alguns obstáculos utilizados no treinamento acrobático, como rampas, escadas, cordas e gangorras. (Adaptado de DÖBRÖSSY; DUNNETT, 2001; SEO, et al., 2014).

A prática de exercícios em esteira é um dos protocolos mais utilizados em pesquisas que utilizam roedores como modelo experimental. Este protocolo de exercício se baseia na corrida em uma esteira similar a utilizada em academias, que é adaptada no laboratório para a utilização pelos animais. A velocidade e o tempo da corrida são determinados pelo pesquisador e podem ser adaptados de acordo com o objetivo do estudo (CECHETTI et al., 2007; SIM, 2014). Em estudos utilizando animais submetidos à HIE que realizaram exercício físico em esteira, os resultados mostram efeitos benéficos como redução da morte neuronal no hipocampo, substância negra e núcleo estriado, preservação da função sensório-motora e melhoria da capacidade de aprendizado espacial (CHOI et al., 2013; PARK et al., 2013). Já foi demonstrado ainda

que os exercícios aeróbicos são capazes de promover angiogênese podendo auxiliar então na recuperação de eventos isquêmicos (HEO et al., 2014).

Uma modalidade menos estudada experimentalmente até o momento, porém muito promissora são os exercícios acrobáticos, também chamados de treino de aprendizado motor. Esta modalidade de treinamento é formada por uma sequência de tarefas que estimulam a coordenação motora, o equilíbrio e a capacidade de resolução de problemas (BLACK et al., 1990). O exercício acrobático é uma modalidade interessante, especialmente na reabilitação motora em crianças, porque este tipo de exercício envolve tarefas que estimulam novas aprendizagens, desafios e jogos e que podem ser facilmente adaptados na clínica (SIDAWAY et al., 2012). Para alcançar essas novas habilidades motoras utilizadas para realizar o treinamento acrobático, é necessário um refinamento do movimento e dois circuitos cerebrais parecem estar envolvidos nesse processo: o cortico-cerebelo-tálamo-cortical e cortico-estriado-tálamo-cortical (GONZÁLEZ-BURGOS, 2015; SALAME et al., 2016). Já foi mostrado que esse tipo de atividade promove a formação e estabilização de novas sinapses na região do córtex motor primário (M1) e, portanto, o surgimento de novos padrões motores (BOSTAN et al., 2013; GONZÁLEZ-TAPIA et al., 2016). Já foi demonstrado ainda que este tipo de exercício é responsável por promover sinaptogênese, plasticidade estrutural e alterações gliais, especialmente no córtex motor (JONES et al., 1999; KLEIM et al., 2004, 2007; GARCIA et al., 2012; SAMPAIO-BAPTISTA et al., 2013; KIDA et al. 2016). Além disso, o córtex pré-frontal responsável pela retificação de informações relacionadas à execução motora; favorece o aprendizado motor e também é recrutado nessa atividade (HONDA et al., 1998; GRAFTON et al., 2002; GONZÁLEZ-TAPIA et al., 2016).

Em estudo recente publicado por Garcia et al. (2012) o efeito dos exercícios aeróbicos e acrobáticos foram comparados em ratos controle e mostraram resultados intrigantes. Foi possível observar que áreas diferentes do encéfalo são ativadas e modificadas dependendo do tipo de exercício que é executado pelos animais. Os exercícios acrobáticos foram responsáveis por induzir alterações na expressão de proteínas sinápticas e estruturais, principalmente no córtex motor e estriado, o que pode estar relacionado com a aprendizagem de tarefas motoras complexas. Já o exercício em esteira promoveu mudanças especialmente no cerebelo, relacionado assim a tarefas aprendidas e automáticas.

Em geral, a maioria dos estudos que utilizam exercício físico como tratamento/terapia focaliza a atenção em exercícios aeróbicos. Os poucos estudos envolvendo o exercício em animais após um evento lesivo por HIE, utilizaram o protocolo de esteira como terapia e observaram melhora da aprendizagem espacial, preservação da função sensório-motora inibição da apoptose no hipocampo, neurogênese e estimulação da oligodendrogênese, mostrando assim um impacto positivo do exercício físico após esse tipo de evento (PARK et al., 2013; CHOI et al., 2013; KIM et al., 2017; PAK et al., 2018). Em relação ao exercício acrobático, mesmo ele sendo considerado uma modalidade encorajadora, especialmente na reabilitação motora em crianças, ao nosso conhecimento não há relatos na literatura sobre o uso desse tipo de exercício como uma proposta de tratamento após um evento hipóxico-isquêmico. Cabe ainda ressaltar que uma lacuna adicional nos estudos com HIE neonatal é o fato de que a função motora e o dano tecidual a estruturas relacionadas a esta função em roedores é expressivamente diferente dos casos HIE em humanos. Porém, até o momento, não existem subsídios teóricos para compreender tais resultados.

1.4 A via corticoespinal, junções neuromusculares, músculo e função motora

Os humanos possuem um sistema complexo de vias ascendentes e descendentes que permitem a comunicação entre o encéfalo e o restante do corpo. Essas vias são chamadas de tratos do SNC (JAVED; LUI, 2018). O TCE é a principal via neuronal que fornece a função motora voluntária, permitindo especificamente o movimento das extremidades distais. Este trato origina-se principalmente dos córtices frontoparietais, incluindo o M1, a área motora secundária e o córtex somatossensorial e à medida que percorre o tronco encefálico, a maioria de suas fibras decussa para o lado contralateral (NATALI; BORDONI, 2018).

Em roedores, esse trato também forma um circuito cruzado responsável pelo controle dos movimentos voluntários dos quatro membros e é composto inicialmente por neurônios piramidais que se originam da camada V do córtex motor, projetando-se principalmente para o lado contralateral da medula espinal conectando-se então a neurônios motores através de uma via multissináptica (Figura 4) (VAN VELTHOVEN et al., 2010; CARMEL; MARTIN, 2014; WELNIARZ et al., 2015). Os primeiros

neurônios motores superiores compõem as fibras nervosas responsáveis pela comunicação entre o encéfalo e a medula espinal (JAVED; LUI, 2018).

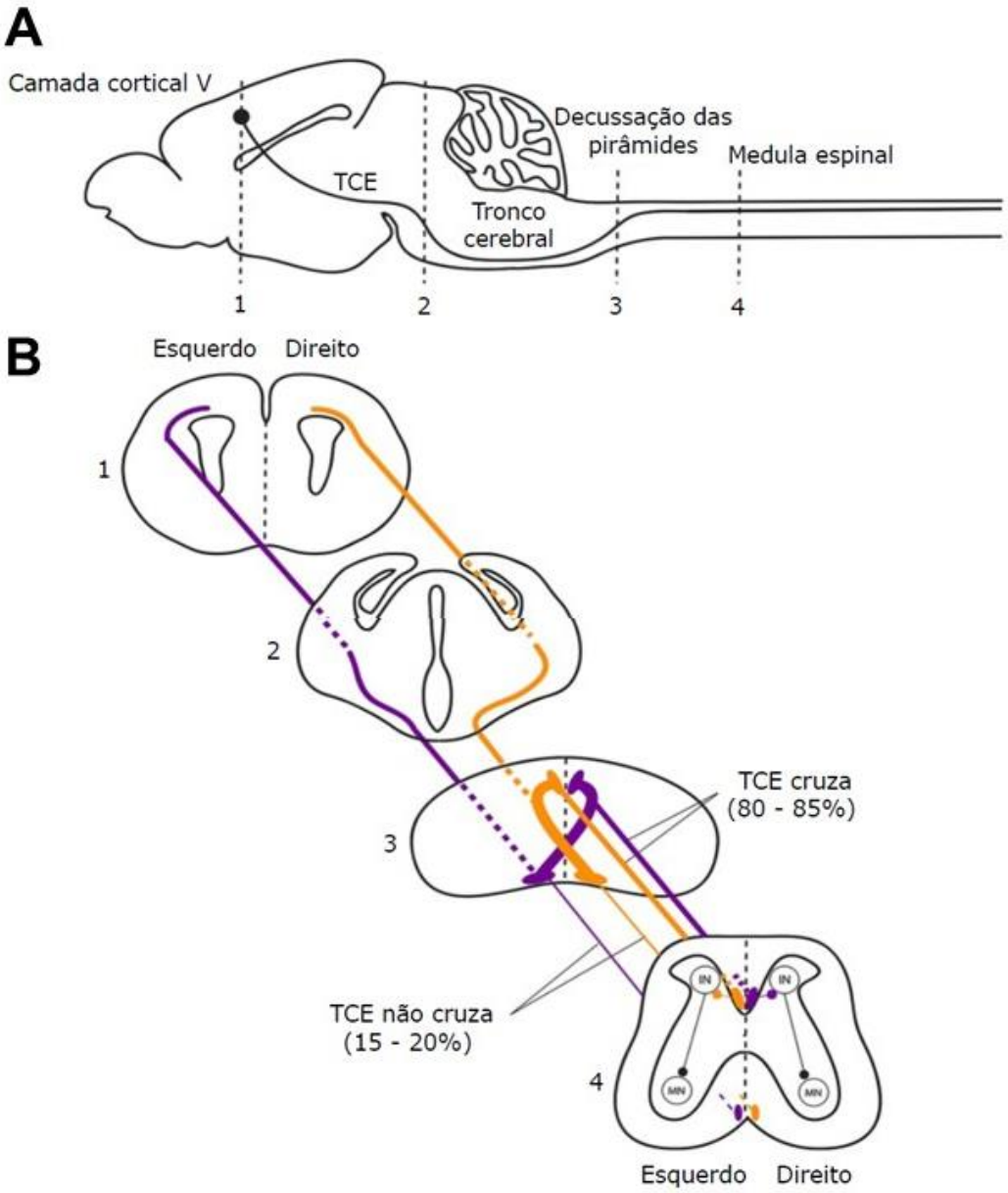

Figura 4. O trato corticoespinal em roedores. (A) Visão sagital do SNC e do TCE. (B) Vista coronal da trajetória do TCE. TCE: trato corticoespinal; IN: interneurônios; MN: motoneurônios. (Adaptado de WELNIARZ et al., 2015).

A região M1, considerado o início do TCE, desempenha um papel fundamental no controle motor volitivo e na aprendizagem de habilidades motoras. Os neurônios dessa região estão organizados em seis camadas de diferentes populações neuronais, sendo cerca de 80% neurônios glutamatérgicos excitatórios e 20% interneurônios (LEV; WHITE, 1997). Além do glutamato, considerado o principal neurotransmissor da região M1, diversos neuromoduladores participam do controle dessa região encefálica, destacando-se a acetilcolina e as monoaminas, como a dopamina. A dopamina cortical é principalmente fornecida pelos neurônios que se projetam da área tegmental ventral (VTA) para o córtex. A via mesocortical do VTA para o córtex pré-frontal tem sido bem estudada, especialmente no contexto do processamento cognitivo, porém evidências crescentes apontam para uma modulação direta do controle motor através dessa inervação dopaminérgica na região do córtex motor (VITRAC; BENOIT- MARAND, 2017). Os neurônios da região dessa região estão agrupados em grupos funcionais e atuam em uma rede interconectada altamente específica que processa e transfere as informações aferentes para estruturas corticais e subcorticais, como o estriado e a medula espinal e (Figura 5) (VITRAC; BENOIT- MARAND, 2017).

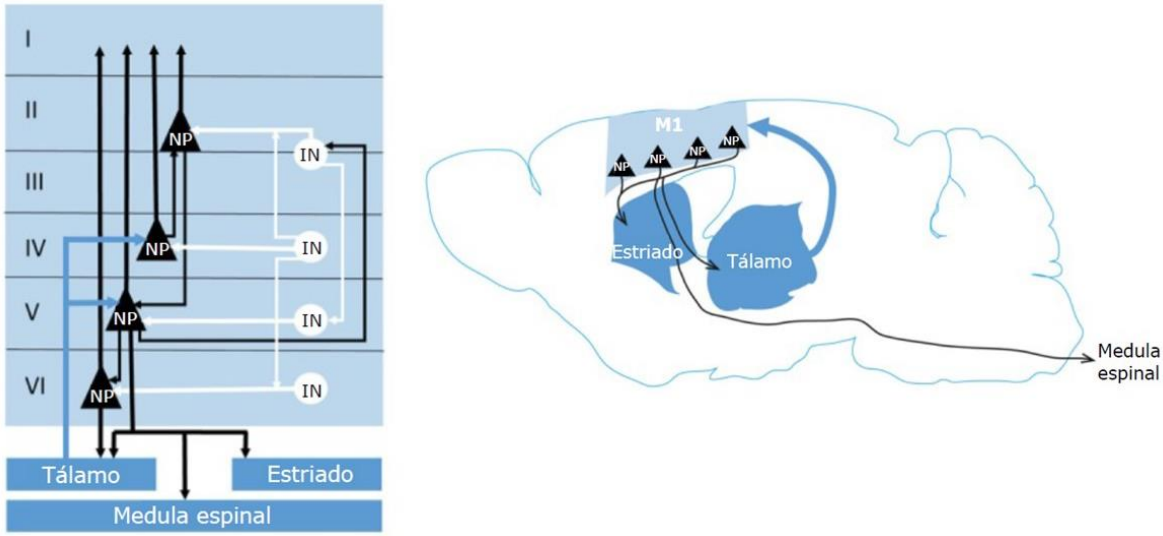

Figura 5. Representação clássica das redes motoras cortico-subcorticais e intracorticais em roedores. IN: interneurônios; NP: neurônios piramidais; M1: córtex motor primário (Adaptado de VITRAC; BENOIT-MARAND, 2017).

A medula espinal é considerada a estrutura responsável pela comunicação entre o encéfalo e o sistema nervoso periférico, sendo considerada um centro para

circuitos neuronais que integram e coordenam funções sensoriais, autonômicas e motoras complexas (ELMONEM et al. 2007). Essa estrutura fica alojada dentro da coluna vertebral, cercada pelas meninges. É anatomicamente alongada, composta por substância cinzenta central e externamente por substância branca. A substância cinzenta da medula espinal é uma estrutura em forma de H, observada em seção transversal, como duas metades simétricas conectadas por uma ponte estreita ou comissura através da qual é possível observar o canal central. A proporção de substância cinzenta em relação à substância branca varia acentuadamente nos diferentes níveis da medula espinal, sendo nos níveis torácicos menor do que nos alargamentos cervicais e lombossacrais (BICAN et al., 2013). Histologicamente, a medula espinal pode ser ainda dividida em segmentos, chamados de camadas ou lâminas. As lâminas I – VI constituem o corno dorsal (posterior) e as lâminas VII – IX o corno ventral (anterior) (Figura 6).

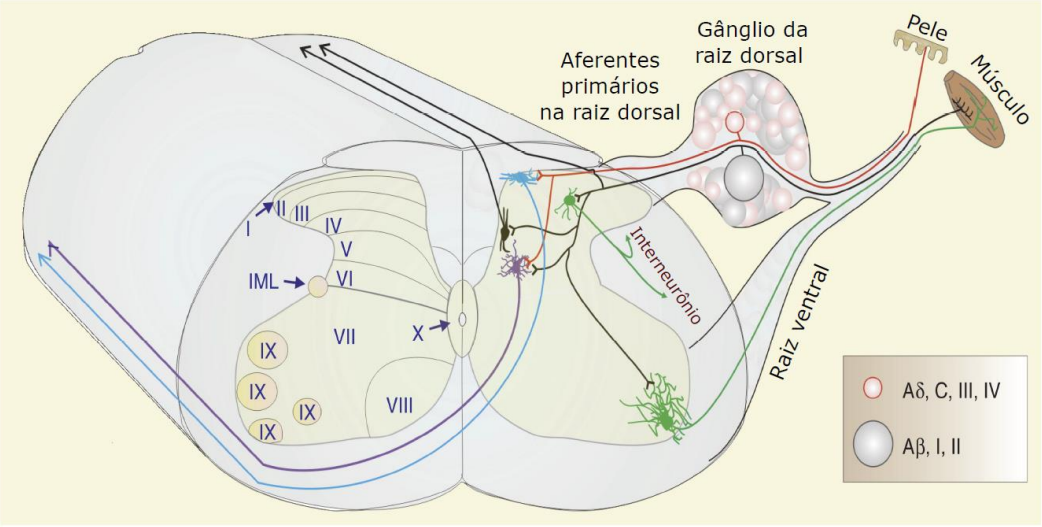

Figura 6. Organização segmentar da medula espinal. A lâmina IX contém os vários reservatórios de motoneurônios (núcleos) cujos axônios, juntamente com os neurônios pré-ganglionares simpáticos localizados no núcleo intermediolateral (IML), saem através de raízes ventrais (Adaptado de HOCHMAN et al., 2007).

Os motoneurônios são encontrados em colunas longitudinais na lâmina IX e enviam axônios para fora da raiz ventral para inervar as fibras musculares esqueléticas através das junções neuromusculares (JNMs). O SNC, em última instância, depende da integração sináptica no nível do motoneurônio para produzir as contrações musculares apropriadas, de modo que os motoneurônios são chamados de "caminho final comum" para o processamento motor (HOCHMAN, et al., 2007).

As JNMs por sua vez representam uma subclasse importante de sinapses no sistema nervoso dos mamíferos e desempenham um papel fundamental na transferência de informações desses neurônios motores inferiores para o músculo esquelético através de nervos motores mielinizados (JONES et al., 2017). Os axônios que controlam essas células musculares esqueléticas surgem então dos corpos celulares localizados centralmente dos neurônios motores na região cortical e viajam, geralmente não ramificados, até os músculos-alvo (SLATER, 2017). À medida que cada axônio motor mielinizado atinge seu músculo alvo, ele se divide abundantemente em 20 a 100 fibras terminais não mielinizadas, cada uma das quais sendo responsável por inervar uma única fibra muscular. A combinação das fibras terminais de um axônio motor e as fibras musculares que eles servem é chamada de unidade motora. As fibras terminais das JNMs são muito finas (0,1 µm) e formam numerosas varicosidades ("botões"), geralmente de 1 a 5 µm de diâmetro, a partir das quais o transmissor é liberado, nessa região também são encontrados canais de potássio (K^+) e sódio (Na^+), que irão controlar a duração e a amplitude do potencial de ação (Figura 7B) (HIRSCH, 2007; SLATER, 2017).

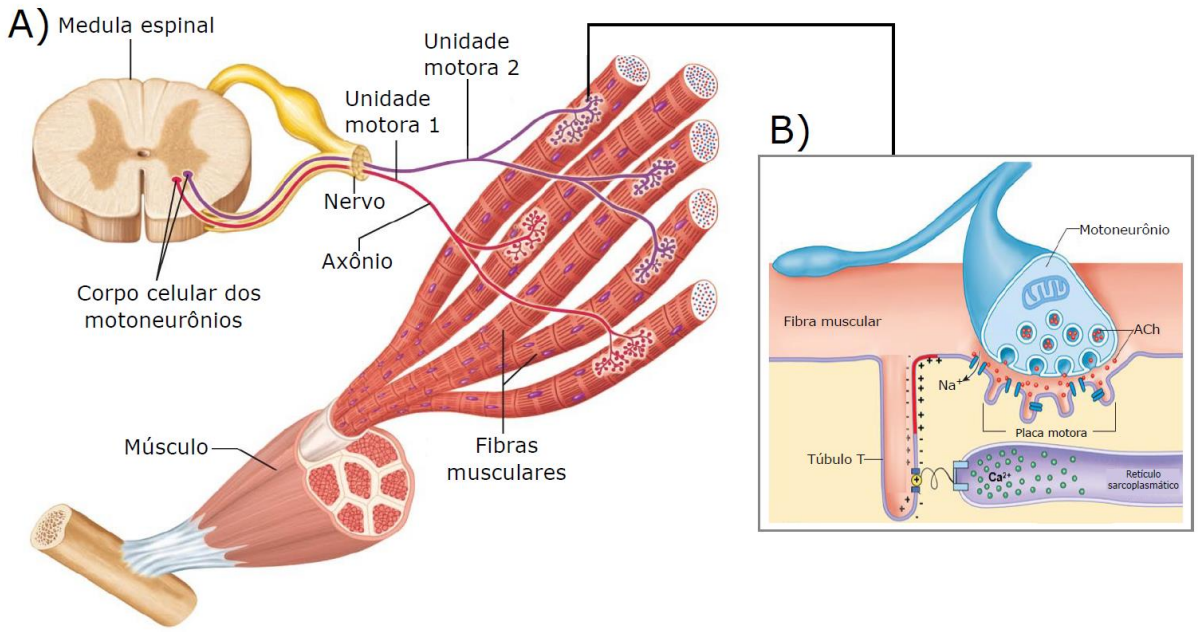

Figura 7. Estrutura das fibras musculares e junções neuromusculares. A) Caminho percorrido pelos motoneurônios da medula até o músculo, formando a junção neuromuscular. B) Junção neuromuscular em detalhe. ACh: acetilcolina. (Adaptado de © Pearson Education, Inc, 2009 e 2012).

Os músculos também desempenham um importante papel na função motora, uma vez que são responsáveis pela execução do movimento e possuem uma conexão

com a medula espinal por meio dos nervos periféricos (WOLFE, 2006). A musculatura esquelética é um dos tecidos mais dinâmicos e plásticos do corpo e contribui significativamente para múltiplas funções. Do ponto de vista mecânico, a principal função do músculo esquelético é converter energia química em energia mecânica para gerar força e potência, mantendo a postura e produzindo movimento. Do ponto de vista metabólico, os papéis do músculo esquelético incluem uma contribuição para o metabolismo energético basal, produção de calor para a manutenção da temperatura e o consumo da maioria do oxigênio e combustível usado durante a atividade física ou exercício (WOLFE, 2006).

A arquitetura do músculo esquelético é caracterizada por um arranjo de fibras musculares muito particular associado a tecido conjuntivo. As fibras musculares são multinucleadas e pós-mitóticas (Figura 7A). São encontradas associadas às fibras musculares, as células satélites, que são células-tronco adultas presentes no músculo esquelético. Essas células estão localizadas entre o sarcolema e a lâmina basal e contribuem para o crescimento, reparo e regeneração muscular (FRONTERA et al., 2015; CHAPMAN et al., 2016). As fibras musculares podem ainda ser classificadas de acordo com o tipo, seguindo alguns critérios como: cor das fibras musculares; propriedades contráteis das unidades motoras em resposta à estimulação elétrica, grau de fatigabilidade, predomínio de certas vias metabólicas ou enzimáticas, reação histoquímica enzimática, manipulação de cálcio pelo retículo sarcoplasmático, expressão de isoforma proteica, dentre outros. A classificação mais utilizada atualmente para os músculos adultos inclui três tipos de fibras: tipo I (lenta, oxidativa e resistente à fadiga), IIA (rápida, oxidativa e com propriedades metabólicas intermediárias) e IIx (mais rápida, glicolítica e fatigável) (SCHIAFFINO, REGGIANI, 2001; FRONTERA et al., 2015). Esse padrão dos tipos de fibras musculares pode ser influenciado por fatores externos ao músculo, como níveis hormonais e padrão de atividade dos nervos periféricos, sendo então importante seu conhecimento frente a patologias ou diferentes intensidades de exercício.

O conhecimento do TCE é de extrema importância em muitos cenários clínicos. A preservação e/ou recuperação desse trato são necessárias para a função motora que pode ser prejudicada após uma lesão cerebral, sendo que mudanças em padrões morfológicos em quaisquer destas estruturas podem influenciar diretamente na resposta funcional e no desempenho motor dos indivíduos afetados.

2 JUSTIFICATIVA E HIPÓTESE

JUSTIFICATIVA

Considerando que:

1) A hipóxia-isquemia encefálica é um evento lesivo para o sistema nervoso que leva a déficits estruturais e funcionais;

2) Pouco se sabe sobre as alterações morfológicas no trato corticoespinal, medula espinal, junções neuromusculares e músculo esquelético após a HIE;

3) Existe relação direta da prática de exercícios físicos com a melhora de sintomas frente a diferentes patologias do sistema nervoso;

4) A literatura mostra somente os efeitos do exercício físico em esteira frente à HIE;

5) Estudos experimentais mostram resultados mais satisfatórios dos exercícios acrobáticos em relação à aprendizagem de tarefas mais complexas;

6) Não são conhecidos os efeitos dos exercícios acrobáticos em filhotes submetidos à hipóxia-isquemia encefálica, *torna-se importante conhecer os efeitos dos exercícios acrobáticos frente à hipóxia-isquemia neonatal, uma vez que esse tipo de terapia pode ser adaptada à prática clínica para melhoria da qualidade de vida em crianças que sofreram este evento lesivo.*

HIPÓTESE

A hipótese do presente trabalho é que a HIE neonatal causará prejuízos motores e afetará as estruturas investigadas. Acreditamos também que o exercício físico trará benefícios, sendo o exercício acrobático o mais eficaz para reverter os déficits.

3 OBJETIVOS

3.1 Objetivo geral

O objetivo deste estudo foi avaliar os efeitos de diferentes protocolos de exercício físico (aeróbico e acrobático) sobre a função motora, o córtex sensoriomotor, medula espinal, nervo isquiático, junções neuromusculares e músculo esquelético em ratos *Wistar* machos submetidos a um evento hipóxico-isquêmico encefálico no período neonatal.

3.2 Objetivos específicos

Investigar os efeitos de diferentes protocolos de exercício físico (aeróbico e acrobático) em ratos *Wistar* machos submetidos à hipóxia-isquemia encefálica neonatal avaliando:

- A locomoção e o comportamento exploratório através do teste do campo aberto e a coordenação e o equilíbrio através do teste rotarod;
- A capacidade motora através da caminhada em escada horizontal e, mais especificamente, a motricidade dos membros anteriores através do teste Pasta Matrix Reaching Task;
- O volume da lesão encefálica através de avaliação macroscópica;
- A densidade neuronal de neurônios no córtex sensoriomotor;
- A quantificação e mensuração dos motoneurônios da medula espinal na região (L4-L5);
- A morfometria das junções neuromusculares, nervo isquiático e das fibras musculares do músculo plantar;
- A condição celular tecidual de neurônios e células da glia e parâmetros de plasticidade no córtex motor primário através de marcadores celulares.

4 RESULTADOS

4.1 ARTIGO I

A primeira parte dos resultados foi publicada no periódico *Brain Research*, sob a forma de um artigo intitulado: *"**Neonatal hypoxia-ischemia caused mild motor dysfunction, recovered by acrobatic training, without affecting morphological structures involved in motor control in rats**".* O referido artigo segue-se abaixo.

Brain Research 1707 (2019) 27–44

Contents lists available at ScienceDirect

Brain Research

journal homepage: www.elsevier.com/locate/brainres

Research report

Neonatal hypoxia-ischemia caused mild motor dysfunction, recovered by acrobatic training, without affecting morphological structures involved in motor control in rats

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HIGHLIGHTS

- Exercise was able to reverse the hyperactivity after the neonatal hypoxia-ischemia.
- The acrobatic training decreased brain atrophy caused by neonatal hypoxia-ischemia.
- The acrobatic exercise caused locomotion improvement after the hypoxia-ischemia.
- Neonatal hypoxia-ischemia did not impact on morphology in motor control structures.

ARTICLE INFO

Keywords:
Perinatal asphyxia
Motor skills training
Corticospinal tract
Neurodevelopment

ABSTRACT

The aim of this study was to evaluated motor function and morphological aspects of the components involved in motor control (sensorimotor cortex, spinal cord, sciatic nerve, neuromuscular junctions and skeletal muscle) in male Wistar rats exposed to a model of neonatal hypoxic-ischemic encephalopathy (HIE) and the possible influence of different physical exercise protocols – treadmill and acrobatic. Male Wistar rats at the 7th post-natal day (PND) were submitted to the HIE model and from the 22nd until 60th PND the exercise protocols (treadmill or acrobatic training) were running. After the training, the animals were evaluated in Open Field, Ladder Rung Walking and Rotarod tasks and after samples of the motor control components were collected. Our results evidenced that the acrobatic training reversed the hyperactivity and anxiety, caused locomotion improvement and decreased brain atrophy in HIE animals. We did not find morphological differences on sensorimotor cortex, spinal cord, sciatic nerve, neuromuscular junctions and skeletal muscle in the animals submitted to HIE model. These intriguing data support the statement of the Rice-Vannucci model does not seem to reproduce, in structures involved in control function, the damage found in humans that suffer HIE. Regarding the protocols of exercise, we proposed that the acrobatic exercise could be a good therapeutic option especially in children affected by neonatal HIE and can be responsible for good results in cognitive and motor aspects.

1. Introduction

Neonatal hypoxic-ischemic encephalopathy (HIE) is a common cause of brain damage, which are frequently associated with neurodevelopmental disabilities (Glass and Ferriero, 2007). This event can be a consequence of a transient interruption of umbilical blood flow, placental detachment or strong uterine contractions (Kurinczuk et al., 2010). The HIE is considered the main cause of mortality in children and affects three per 1000 live full-term births leading to life-long functional impairments (Hagberg et al., 2015). These consequences

Abbreviations: BBB, blood-brain barrier; BDNF, Brain-derived neurotrophic factor; CST, corticospinal tract; HIE, hypoxic-ischemic encephalopathy; NMJs, neuromuscular junctions; VEGF, vascular endothelial growth factor; VO2, evaluation of peak oxygen uptake

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https://doi.org/10.1016/j.brainres.2018.11.021
Received 30 July 2018; Received in revised form 29 October 2018; Accepted 14 November 2018
Available online 15 November 2018
0006-8993/

frequently involve mental retardation, visual impairment, perceptual dysfunction, hyperactivity, epilepsy and sensory-motor deficits including delay of developmental milestones, problems in posture, gait and presence of involuntary movements (Anderson and Arciniegas, 2010; Lai and Yang, 2011; Hopkins and Haaland, 2004; Adhikari and Rao, 2017). The literature reports also that patients with hypoxic-ischemic brain injury may present lesions in the neural tracts, especially those responsible for motor control such as corticospinal tract (CST) (Lee et al., 2012). As it is possible to observe, the damage caused by the HIE can vary greatly among the affected individuals.

In order to understand the mechanisms involved in the HIE pathology and to study possible therapies, several animal models are used to mimic this condition. It is highlighted in this field the model proposed by Rice and colleagues (1981) commonly referred to as the Rice-Vannucci model. This model is widely used because it reproduces the unilateral lesion in the hippocampus, striatum and cerebral cortex found in human neonates in the hemisphere ipsilateral to arterial occlusion (Pereira et al., 2007). Also, extensive evidences have demonstrated cognitive impairments in HIE animals in different behavioral tasks such as water maze test (Ikeda et al., 2001; Pereira et al., 2007, 2008; Tata et al., 2015; Carletti et al., 2016), novel object recognition (Rojas et al., 2013; Marcelino et al., 2016) and inhibitory avoidance (Rojas et al., 2013; Carletti et al., 2012; Sanches et al., 2013). Some studies also identified hyperactivity and attention-deficit in this model (Miguel et al., 2015, 2017; Deniz et al., 2018).

However, regarding the findings of motor function caused by the HIE model, results are conflicting. A range of studies did not identify motor impairment in HIE animals. Rojas and colleagues (2013) did not find differences between HIE and control groups in the rotarod test. Miguel et al. (2015) and Arteaga et al. (2017) used the open field test and Schuch et al. (2016b) utilized the cylinder test and, in the same way, they did not identify motor dysfunction in HIE animals. In opposition, other studies showed that the motor deficit is evident in this model. Utilizing the rotarod test, Griva et al. (2017) and Dai et al. (2017) found balance deficit in HIE animals. Motor impairment was also demonstrated in the cylinder and ladder-walking tests in a study performed by Durán-Carabali et al. (2017). In addition, Pak et al. (2018) evidenced sensory-motor dysfunction, even after weeks of hypoxic-ischemic induction, utilizing several motor tasks: open field test, cylinder test, rotarod, and catwalk. Probably, these contradictory findings are established due to the diverse HIE-induced nervous tissue injury.

It is extensively demonstrated the severe hippocampal damage in neonatal HIE model, which explains unfavorable outcomes in relation to cognitive impairments, specifically on memory processes (Souza et al., 2014; Domnick et al., 2015; Rojas et al., 2015; Carletti et al., 2016; Diaz et al., 2016; Griva et al., 2017; Odorcyk et al., 2018; Chavez-Valdez et al., 2018). Regarding the motor function, few studies have focused on the evaluation of brain structures such as motor cortex and there are also scarce studies on peripheral structures for example nerves and muscles. Even though no particular vulnerability has been identified in motor areas, a general brain atrophy has been reported in HIE model in regions such as cerebral cortex, corpus callosum and striatum (Pereira et al., 2007, 2008; Miguel et al., 2015; Schuch et al., 2016a; Carletti et al., 2016). Moreover, deficits of spinal cord functions and reduction in muscle fibers area have been reported (Bellot et al., 2014; Durán-Carabali et al., 2017). The question that opens up from this information is why this generalized lesion does not seem to affect motor function as clearly as it occurs in humans. And additionally, it is doubtful why there are no evidences on the impact of HIE in other structures involved in motor function, as neuromuscular junctions and peripheral nerve.

Despite several studies related to the topic, the neonatal brain injury remains a devastating condition, with poor outcomes in affected patients. Non-pharmacological therapeutic options have been widely studied in HIE animal models. The literature shows a direct relationship between the practice of physical exercises and the structural and functional improvement of the neuromuscular disturbs, such as cerebral palsy, traumatic brain injury and global cerebral ischemia (Kim et al., 2014; Jacotte-Simancas et al., 2015; Lovatel et al., 2014). In general, most of the studies focus attention on aerobic exercises mainly using the treadmill. The few studies involving exercise in HIE animals demonstrated spatial learning improvement, sensory-motor function preservation, apoptosis inhibition in the hippocampus, neurogenesis, and stimulation of oligodendrogenesis (Park et al., 2013; Choi et al., 2013; Kim et al., 2017; Pak et al., 2018). A hopeful modality known as acrobatic training is a sequence of tasks that stimulate motor coordination and the problem-solving capacity, which is responsible for promoting synaptogenesis, structural plasticity and glial changes especially, in the motor cortex (Black et al., 1990; Jones et al., 1999; Kleim et al., 2004, 2007; Garcia et al., 2012; Sampaio-Baptista et al., 2013; Kida et al., 2016). The acrobatic exercise is an encouraging modality, especially in children's motor rehabilitation because this type of exercise involves tasks that stimulate new learning, challenges and games and that can be adapted in the clinic; to our knowledge, there are no reports in the literature of the use of this type of exercise as a treatment proposal for neonatal HIE.

Thus, the aim of this study was to investigate: the motor function and morphological aspects of sensorimotor cortex, spinal cord, sciatic nerve, neuromuscular junctions and skeletal muscle in male Wistar rats submitted to neonatal hypoxia-ischemia and the possible therapeutic effect of two different physical exercise protocols – treadmill and acrobatic training. Some points give support to this investigation: first, the findings about motor function changes after by the HIE model are conflicting, second, few studies have focused on evaluation of brain structures such as motor cortex and there are scarce studies on peripheral structures involved in motor function and, finally, the exercise seems to be a promising therapy in the neonatal HIE pathology but there are no study using acrobatic exercise in neonatal HIE. Our hypothesis is that the neonatal HIE will cause motor impairments and will affect the structures investigated. We also believe that physical exercise will bring benefits, with acrobatic exercise being the most effective in reversing deficits.

2. Results

2.1. Training performance

Student's *t*-test demonstrated that no differences was identified between CTT and HIET groups in relation to the permanence on the treadmill (CTT: 961.7 ± 45.8, HIET: 955.5 ± 40.4) and VO2 max (CTT: 17.8 ± 0.8, HIET: 17.8 ± 0.7). Concerning the acrobatic training, during phases two and three, it was possible to observe differences between the CTA and HIEA groups. The HIEA group performed the course faster than the CT animals in these phases ($p < 0.05$). The results are shown in Table 1.

In relation to the body weight, the Two-way ANOVA identified a lesion effect ($F(1,105) = 10.0$, $p < 0.05$). The animals of the HIE groups presented lower body weight in relation to the controls independently of the exercise training (means of body weight: control group: 285.7 and HIE group: 273.9).

Table 1
Time to perform the acrobatic training in different phases.

Time to perform	CTA	HIEA
Acrobatic training – Phase 1(s)	554.5 ± 33.9	510.1 ± 31.1
Acrobatic training – Phase 2(s)	634.6 ± 30.3	548.6 ± 29.3*
Acrobatic training – Phase 3(s)	621.8 ± 28.8	538.4 ± 25.7*
Acrobatic training – Phase 4(s)	592.0 ± 28.2	535.0 ± 29.3

* Indicated the HIEA group is different from CTA group.

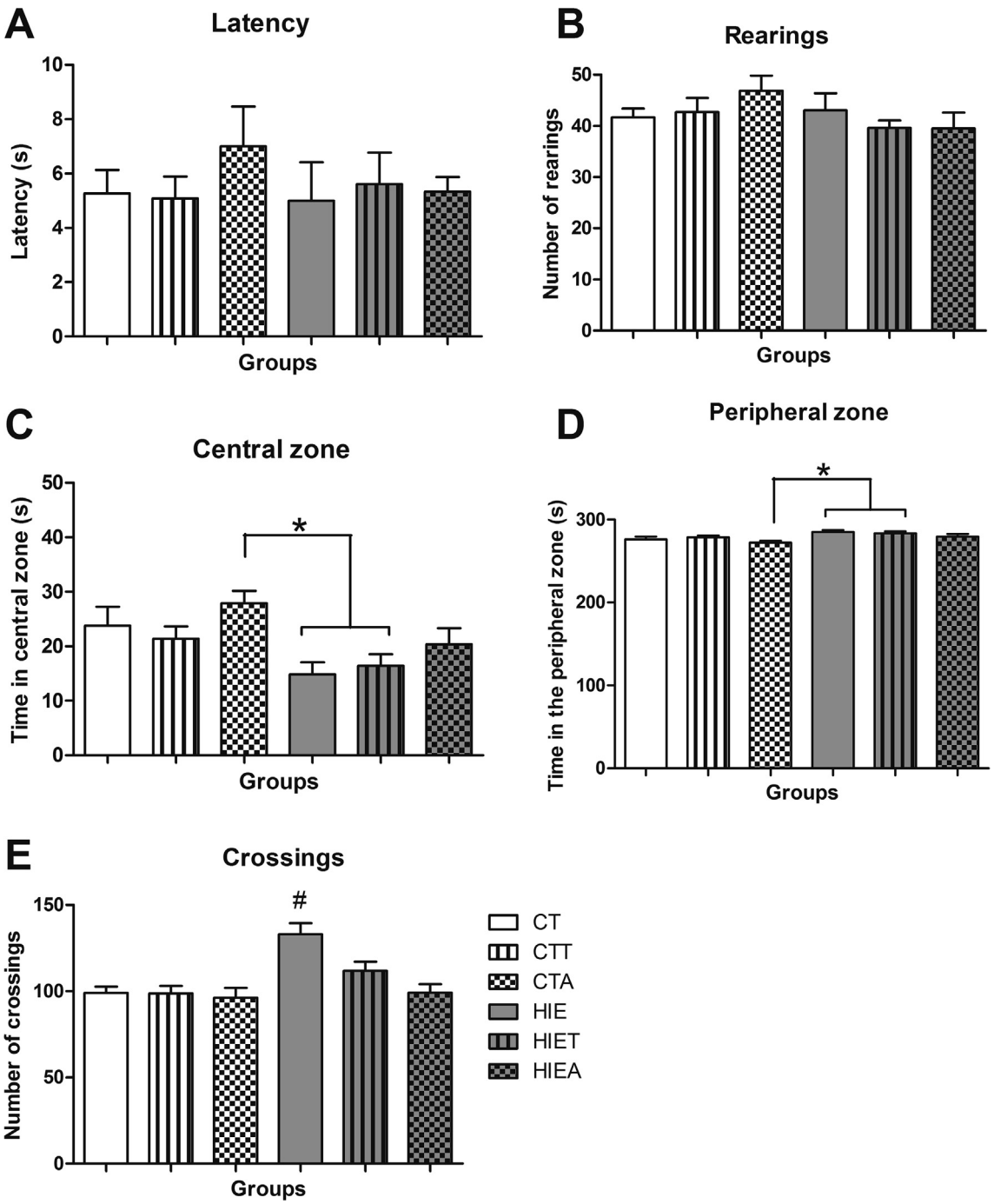

Fig. 1. Open Field task. Latency to leave the first quadrant (A). Number of rearings (B). Time in central zone (C). Time in peripheral zone (D). Number of crossings (E). *different from CTA group and #different from all other groups ($p < 0.05$). Values expressed as mean ± standard error. Two-way ANOVA followed by Tukey's post hoc. N = 9–13 animals/group.

2.2. Behavioral tasks

2.2.1. Open field

Two-way ANOVA demonstrated a lesion effect on the time spent in the central ($F(1,64) = 11.26$, $p < 0.05$) and the peripheral areas ($F(1,64) = 11.27$, $p < 0.05$). Tukey's post hoc indicated that HIE and HIET groups remained a shorter time in the central zone and consequently a longer time in the periphery of the apparatus, when compared to the CTA group. In relation to number of crossings, Two-way ANOVA identified a lesion*training interaction effect ($F(2,64) = 4.48$, $p < 0.05$). Tukey's test revealed that HIE group had a greater number of crossings when compared to all the other groups. No further differences were observed in the other variables: latency and rearings (Fig. 1).

2.2.2. The ladder rung walking test

The statistical analysis indicated a lesion effect on the total number of errors in day 1 ($F(1,62) = 21.64$, $p < 0.05$) and a lesion F ($1,62) = 12.98$, $p < 0.05$) and training effect ($F(2,62) = 3.41$, $p < 0.05$) in total number of errors in day 2. Tukey's post hoc identified that HIE and HIET had a greater number of errors in the first day of the test when compared to all CT groups. In the second day, the post hoc showed that the HIE and HIET groups presented a greater number of errors when compared to the CTA group. It was also possible to

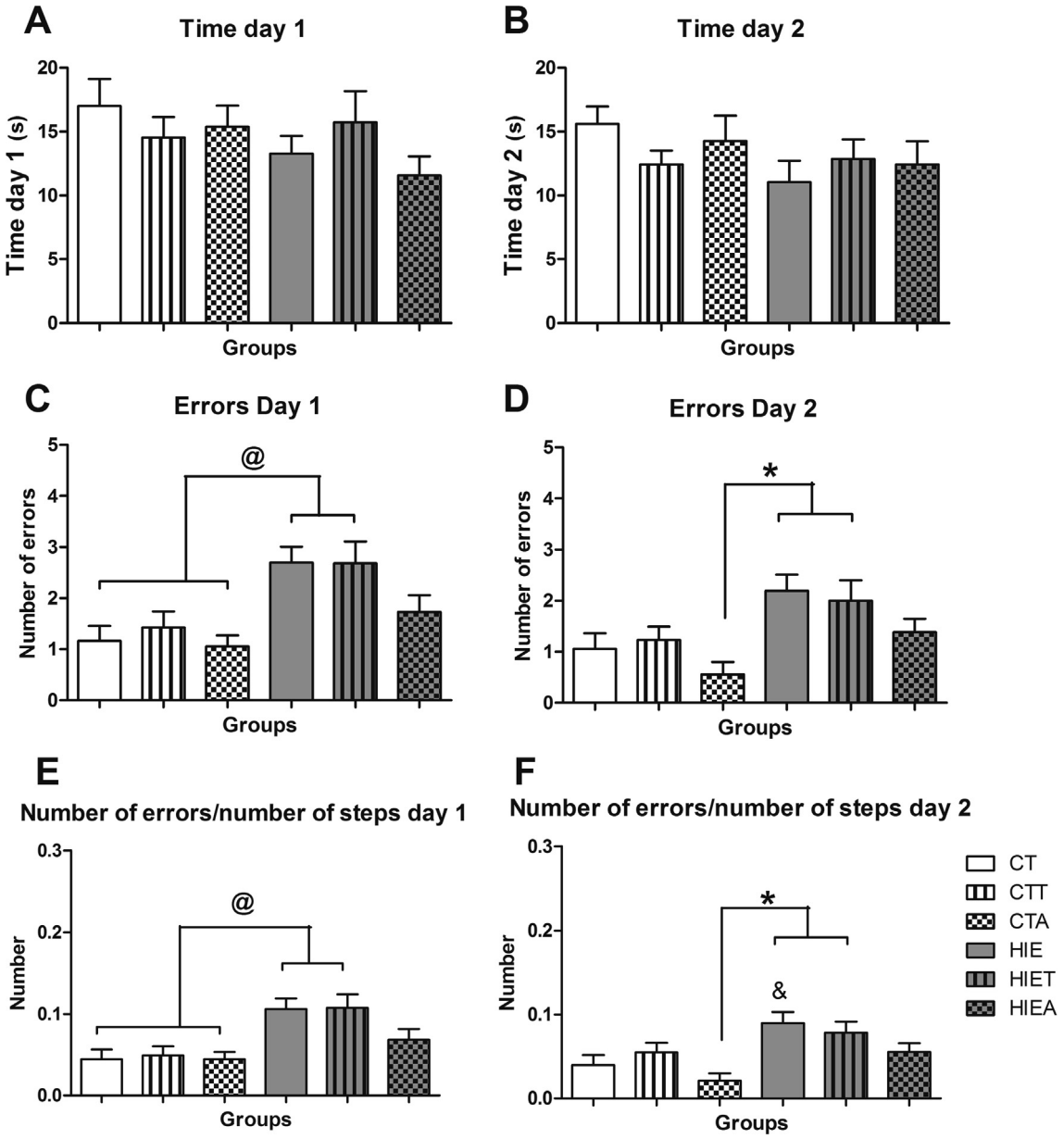

Fig. 2. The Ladder Rung Walking Test. Time day 1 (A). Time day 2 (B). Errors day 1 (C). Errors day 2 (D). Number of errors/number of steps day 1 (E). Number of errors/number of steps day 2 (F). *different from CTA group; @different from all CT groups; &different from CT group ($p < 0.05$). Values expressed as mean ± standard error. Two-way ANOVA followed by Tukey's post hoc. N = 9–13 animals/group.

identify a lesion effect in the ratio of number of errors to number of steps in day 1 ($F(1,62) = 19.36$, $p < 0.05$) and, in day 2, a lesion F ($1,62) = 13.62$, $p < 0.05$) and training effects ($F(2,62) = 3.46$, $p < 0.05$). Tukey's post hoc identified that HIE and HIET had a greater number of errors/number of steps in the day 1 when compared to all CT groups. In the day 2, the HIE and HIET groups presented increase ratio of errors, compared to the CTA group; additionally, increased in the number of errors/number of steps was also observed when compared HIE with CT group. The HIEA had similar errors incidence compared to CT groups. In relation to the time to perform the ladder, no statistical differences were found in both days (Fig. 2).

2.2.3. Rotarod test

Statistical analysis did not evidence differences consequent to lesion or training in the variables analyzed in the Rotarod test (Fig. 3).

2.3. Brain atrophy

Two-way ANOVA revealed significant effect of lesion factor in hemisphere atrophy (left compared to right hemisphere) in all analyzes performed: top view ($F(1,30) = 20.71$, $p < 0.05$), coronal view (F ($1,30) = 28.59$, $p < 0.05$) and cortex ($F(1,30) = 32.51$, $p < 0.05$). On the top view, Tukey's test demonstrated that the HIET group presented a higher percentage of atrophy when compared to all CT groups, and the HIE group had a higher percentage of atrophy when compared to the CTT group. Regarding the percentage of atrophy analyzed in coronal view and in the cortex, post hoc shows that the HIE and HIET groups present greater atrophy when compared to all CT groups (Fig. 4).

2.4. Cortex neuronal quantification

No significant differences were found consequent to lesion or

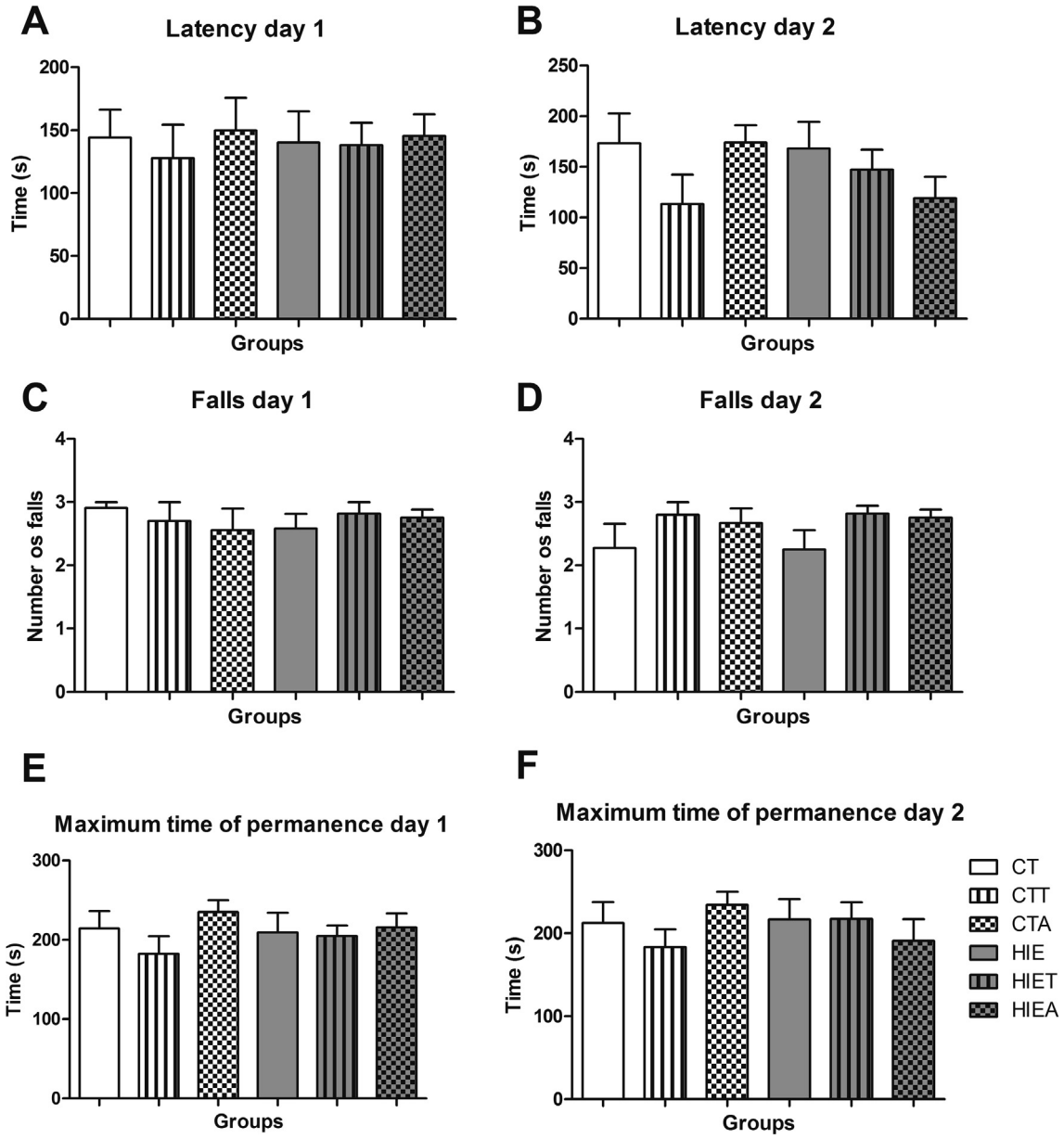

Fig. 3. Rotarod test. Latency day 1 (A). Latency day 2 (B). Falls day 1 (C). Falls day 2 (D). Maximum time of permanence day 1 (E). Maximum time of permanence day 2 (F). Values expressed as mean ± standard error. Two-way ANOVA followed by Tukey's post hoc. N = 9–12 animals/group.

training in the neuronal quantification in the cortex in both hemispheres (Fig. 5).

2.5. Motoneurons analysis

The count and area of motoneurons in the spinal cord were measured and no significant differences were found consequent to lesion or training (Fig. 6).

2.6. Sciatic nerve analysis

No effect was identified on the lesion factor. Two-way ANOVA showed a training effect in the right side on number of fibers ($F(2,30) = 3.49$, $p < 0.05$) and the axons area ($F(1,64) = 3.88$, $p < 0.05$). Tukey's post hoc indicated that HIEA group presents a bigger axon area when compared to the HIE group. Regarding the left side data, Two-way ANOVA demonstrated a training effect in myelin sheath ($F(2,30) = 5.02$, $p < 0.05$). Tukey's test evidenced that HIEA group has a thicker myelin sheath when compared to the CT group (Fig. 7).

2.7. Neuromuscular junction area

No significant differences were found consequent to lesion or training on the neuromuscular junction's area (Fig. 8).

2.8. Muscle fiber and nucleus quantification

In plantar muscle analysis, no significant differences were found consequent to lesion or training on the number of muscle fibers or nucleus (Fig. 9).

3. Discussion

This study was proposed to investigate the motor function and the morphological aspects of components involved in motor control in male

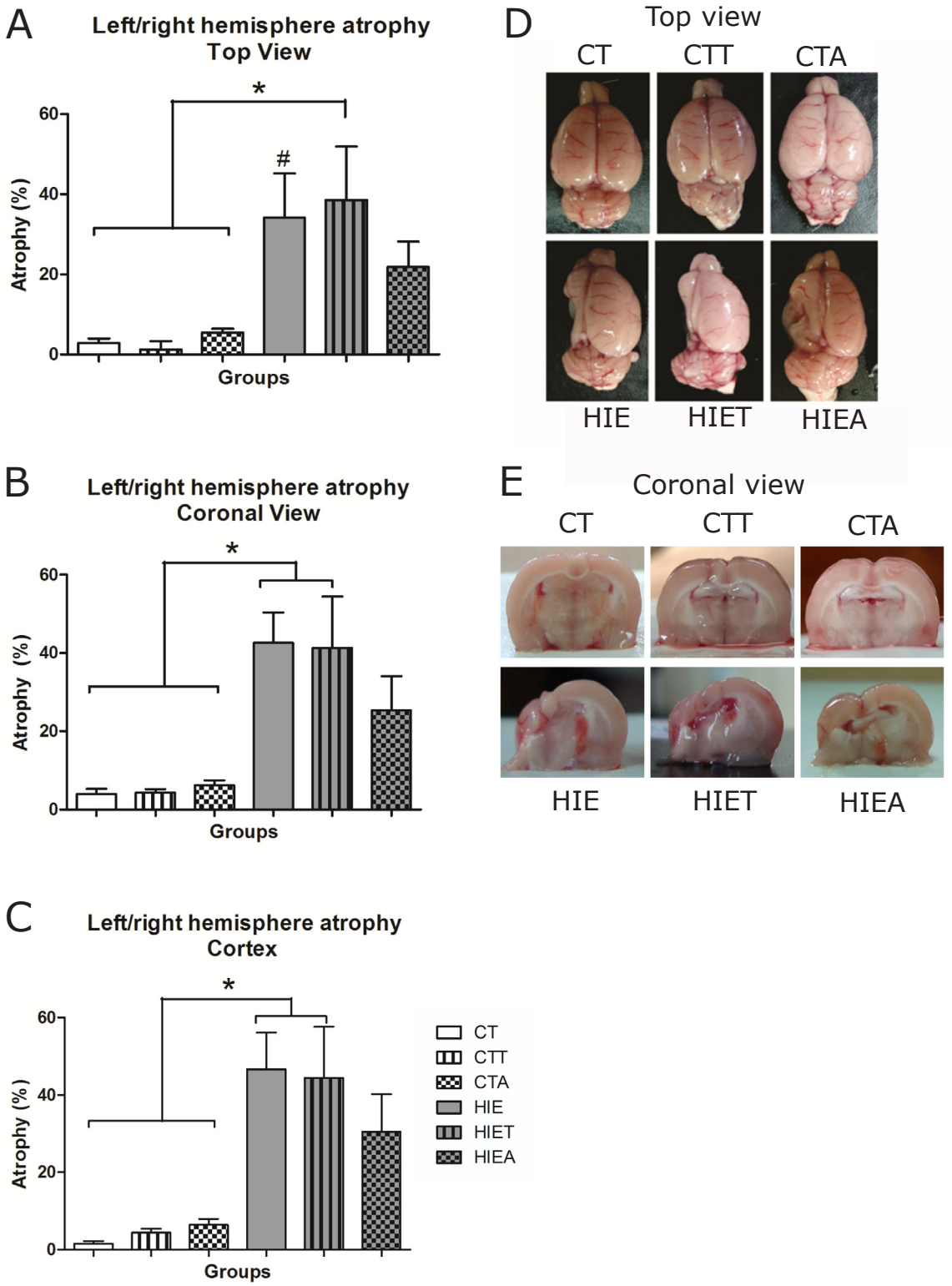

Fig. 4. Percentage of brain atrophy of the left hemisphere in relation to the right. Percentage of atrophy of the hemispheres – top view (A). Percentage of atrophy of the hemispheres – coronal view (B). Percentage of atrophy of the cortex (C). Illustrative images of the brains in top view (D). Illustrative images of the brains in coronal view (E). *different from all CT groups and #HIE group is different from CTT group ($p < 0.05$). Values expressed as mean ± standard error. Two-way ANOVA followed by Tukey's post hoc. N = 6 animals/group.

Wistar rats submitted to neonatal hypoxia-ischemia and the possible therapeutic effect of the treadmill and acrobatic training. Our main findings showed that the acrobatic training was able to reverse the hyperactivity and anxiety, caused locomotion improvement and decreased brain atrophy in HIE animals. As expected, the acrobatic training seemed to be more efficient when compared to the treadmill training and this study bring evidence that this type of exercise can be a good therapeutic choice, especially in children affected by neonatal HIE neonatal. However, contrary to our initial hypothesis, we did not find morphological differences in the sensorimotor cortex, spinal cord,

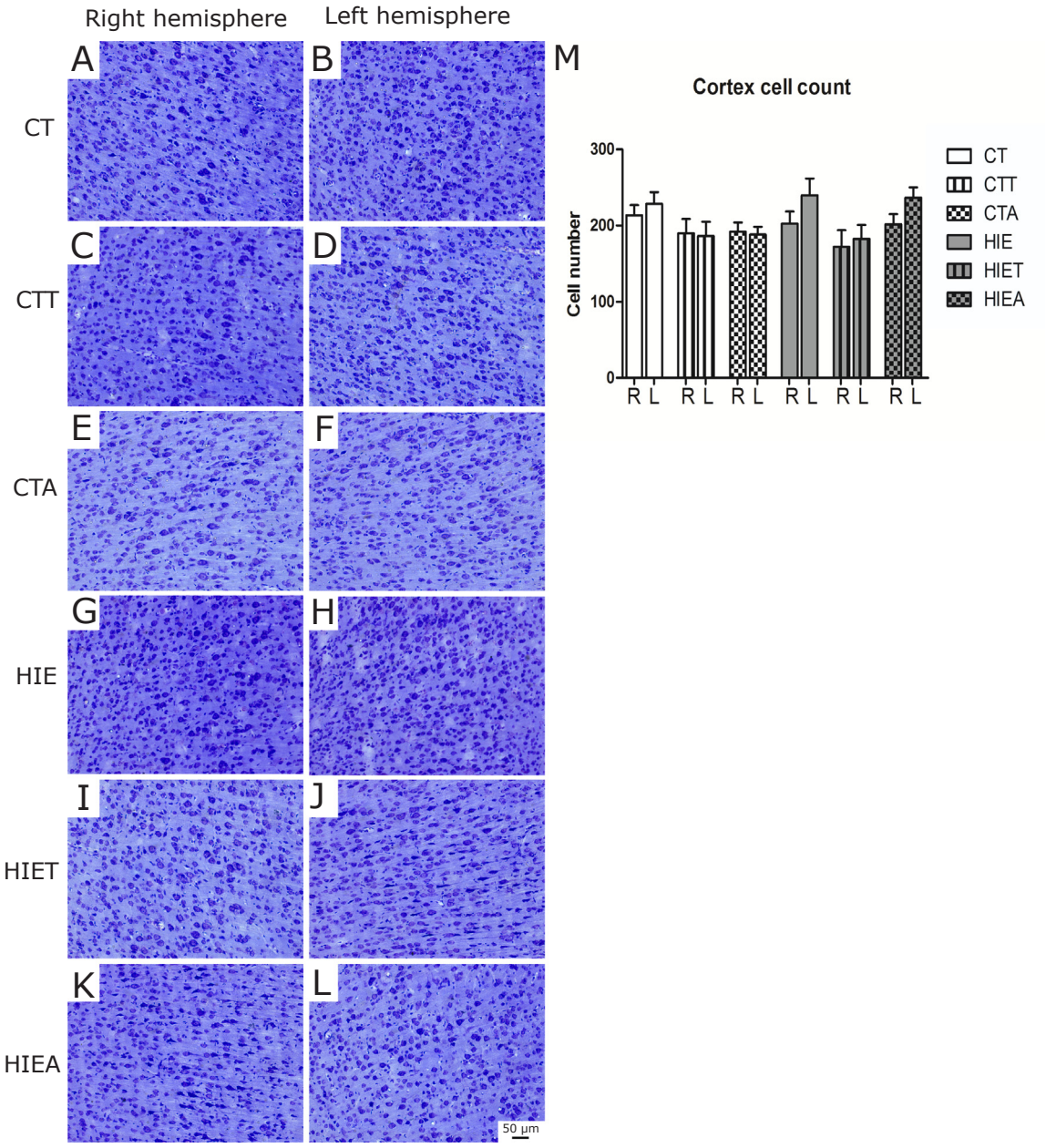

Fig. 5. Neuronal quantification in cortex. Representative images of groups CT (A-B), CTT (C-D), CTA (E-F), HIE (G-H), HIET (I-J), HIEA (K-L). Cortex cell count (M). R = right hemisphere and L = left hemisphere. Values expressed as mean ± standard error. Two-way ANOVA followed by Tukey's post hoc. N = 5–6 animals/group.

sciatic nerve, neuromuscular junctions and skeletal muscle in the animals submitted to neonatal HIE model.

3.1. Animals submitted to neonatal HIE successfully performed physical training

In animal models, different types of exercise demonstrated benefits in front of several pathologies that affect the central nervous system. Concerning aerobic exercises effects, interesting results are evidenced. It has been identified that physical exercise on treadmill prevented the microvascular alterations induced by chronic cerebral hypoperfusion (Leardini-Tristão et al., 2017). This type of exercise also modulated glial cells functions in the dentate gyrus of rats following global cerebral ischemia (Lovatel et al., 2014). In addition, treadmill training-induced plasticity in spinal motoneurons and sciatic nerve in a model of cerebral palsy (Stigger et al., 2011). In studies using animals submitted to HIE that performed physical exercise on a treadmill, the results showed a reduction of neuronal death in the hippocampus, substantia nigra, and striatum, preserving the sensory-motor function and improving spatial learning capacity (Choi et al., 2013, Park et al., 2013). Although, the acrobatic training utilized in this study, also called motor skill learning, has been less studied in front of brain pathologies, but it is already known that its practice is responsible for the activation of important regions of the brain. In a study published by Garcia et al. (2012) the effect of exercise on a treadmill or acrobatic were compared in control animals and they showed that the treadmill exercise promoted changes specifically in the cerebellum, a region related to learned and automatic tasks. On the other hand, the acrobatic exercises were responsible for inducing alterations in the expression of synaptic and structural proteins, especially in the motor cortex and striatum, which may be related to the learning of complex motor tasks. To achieve novel motor skills to perform the acrobatic training a refinement of movement is necessary

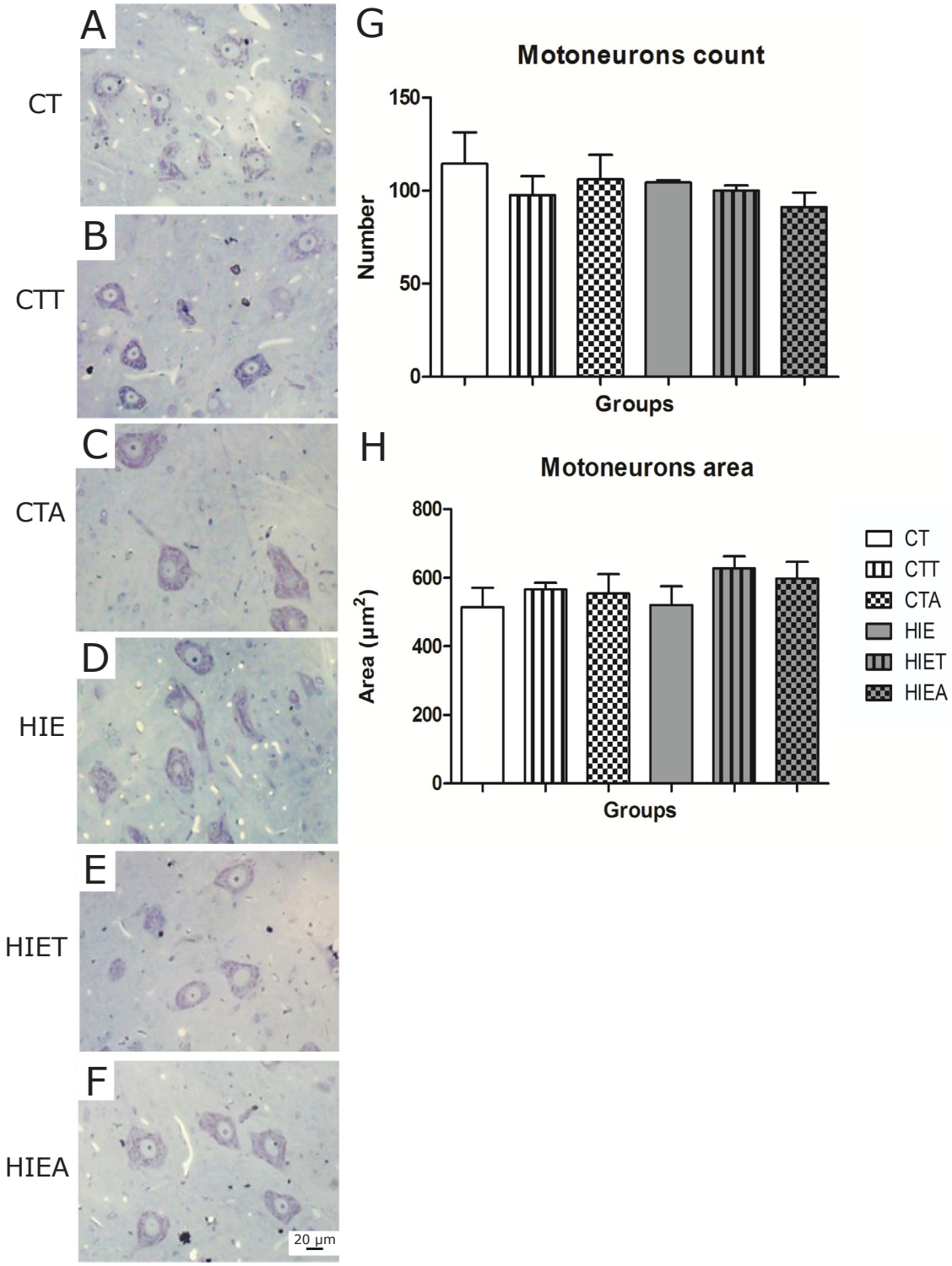

Fig. 6. Motoneurons of spinal cord analysis. Representative images of groups CT (A), CTT (B), CTA (C), HIE (D), HIET (E), HIEA (F). Motoneurons count (G). Motoneurons area (H). Values expressed as mean ± standard error. Two-way ANOVA followed by Tukey's post hoc. N = 5–6 animals/group.

and two brain circuits seem to be involved in this process: the cortico-cerebello-thalamo-cortical and cortico-striatum-thalamo-cortical (González-Burgos, 2015; Salame et al., 2016). It has already been shown that this type of activity promotes the formation and stabilization of new synapses in the motor cortex (M1) and therefore the emergence of new motor patterns (Bostan et al., 2013; González-Tapia et al., 2016). The prefrontal cortex has been associated with the rectification of information related to motor execution; favoring progressive motor learning and it is also recruited in this activity (Honda et al., 1998; Grafton et al., 2002; González-Tapia et al., 2016). Because of motor skills training involves changes in brain regions directly related to motor control, we believe that this type of exercise could be a good therapeutic option for the HIE model. Rehabilitation options that involve challenges, games and new environments are very interesting especially for children because it facilitates the compliance to treatment favoring better results.

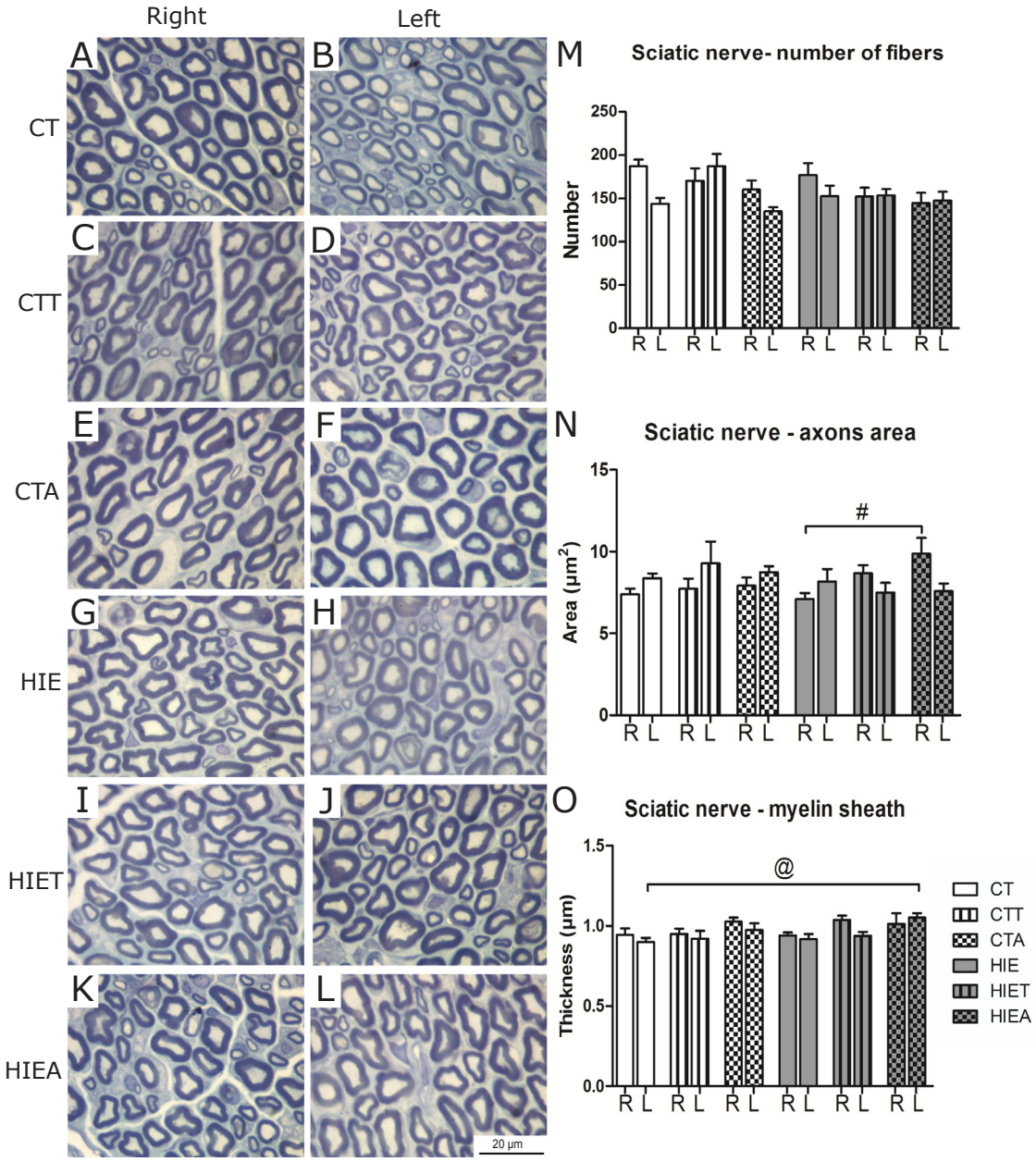

Fig. 7. Sciatic nerve analysis. Representative images of groups CT (A-B), CTT (C-D), CTA (E-F), HIE (G-H), HIET (I-J), HIEA (K-L). Number of fibers (M). Axons area (N). Myelin sheath (O). R = right side and L = left side. #HIA group are different from HIE group on the right side and @HIEA group is different from CT group on the left side ($p < 0.05$). Values expressed as mean ± standard error. Two-way ANOVA followed by Tukey's post hoc. N = 6 animals/group.

An initial concern of our study was whether the HIE animals would perform the exercises, especially the acrobatic. However, our results showed that the HIE animals did not have difficulties to perform any of the two types of exercise, so there was a regularity between the groups. It is still noticeable in phases 2 and 3 of the acrobatic training that HIE groups had a faster performance than the control animals, which may be indicative of hyperactivity or motor impulsivity, a characteristic already evidenced by our group in this model of HIE (Miguel et al., 2015, 2017; Deniz et al., 2018). This regularity in the training evidenced between groups provides a good way of comparing the results, making them replicable and consistent.

3.2. Acrobatic exercise reversed hyperactivity and anxiety-like behavior caused by the neonatal hypoxia-ischemia

The open field test is a convenient test to measure general locomotion and also anxiety-like behaviors have been observed in this task. In rodents, anxiety-like behavior in the open field is triggered by two factors: the tests are performed individually and in a new environment to the animal (Prut and Belzung, 2003). In the present study, we found that the HIE group had more crossings than the all other groups in this test. Besides, the HIE and HIET groups stayed more time in the peripheral zone of the apparatus, when compared to the HIEA group. These results indicate hyperactivity and anxiety-like behavior promoted by the neonatal HIE that was reversed by the exercise, especially the acrobatic training.

Hyperactivity characteristics and anxiety-like behavior have been reported in other studies that use the same HIE model (Rojas et al., 2013; Carletti et al., 2012; Deniz et al., 2018). Miguel and colleagues (2015, 2017) suggested that the model of HIE proposed by Rice-Vannuci could be considered as a model of attention-deficit/hyperactivity disorder, which would explain the hyperactivity seen in HIE animals. It

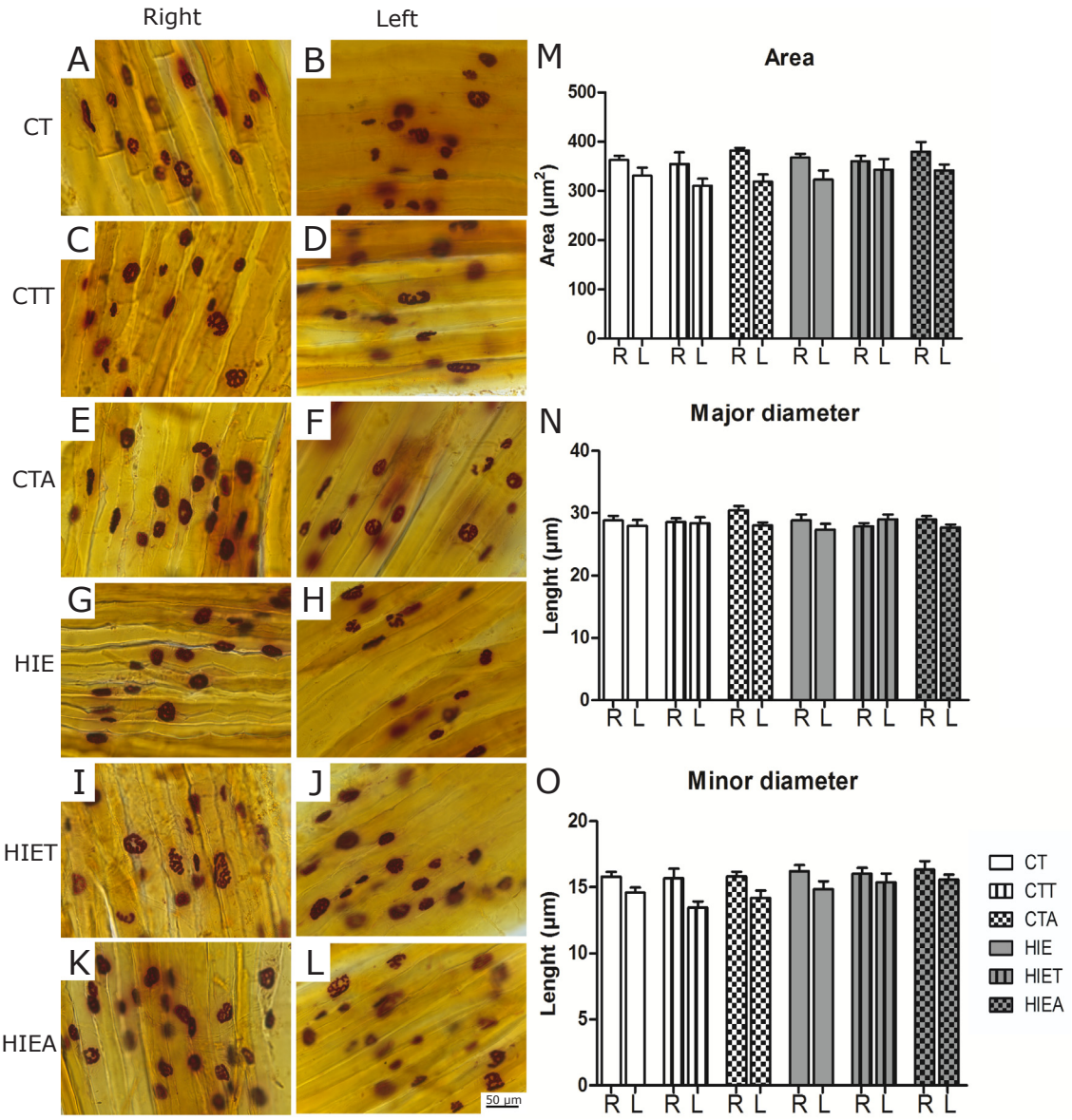

Fig. 8. Neuromuscular junctions analysis. Representative images of groups CT (A-B), CTT (C-D), CTA (E-F), HIE (G-H), HIET (I-J), HIEA (K-L). Neuromuscular junctions area (M). Major diameter (N). Minor diameter (O). R = right side and L = left side. Values expressed as mean ± standard error. Two-way ANOVA followed by Tukey's post hoc. N = 6 animals/group.

is important to consider that the hyperactive characteristics found in the HIE group can be a risk behavior, since an exacerbated activity can lead to negative consequences for the animal such as meeting with a predator, for example.

In relation to protector-effect of exercise, it is widely known that physical training exerts psychological and physical benefits. As seen in humans, a range of studies has shown that the practice of exercise is responsible for decreasing the anxiety levels in rodents (Binder et al., 2004; Duman et al., 2008; Uysal et al., 2015). In the present study, the acrobatic training was the main responsible for reversing both, hyperactivity and anxiety behavior, following the neonatal HIE. A possible explanation for the decrease in hyperactivity and anxiety in animals performing acrobatic exercise may be associated with non-aversion to the new environment since these animals were stimulated and trained in different environments and challenges each week. Cognitive processes used in this test, influence how animals acquire, store and recall information, and sustain behaviors such as deciding where to look for food, when they should hide, or with whom to mate (Guillette et al., 2015). Thus, changes in exploratory behavior identified in HIE group can negatively influence their development, for that reason the behavioral change caused by the exercise in this model brings benefits directly related to the survival of the animals. Future studies might evaluate the anxiety behavior using specific tasks to provide additional data on the potential of physical exercise to improve emotional parameters.

3.3. Acrobatic training recovered motor function after the neonatal hypoxia-ischemia

In this work, the tests used specifically for assessing motor function were the ladder rung walking test and the rotarod. The horizontal ladder rung walking task has previously been described as a sensitive test to evaluate both forelimb and hindlimb use in rats after different types of brain injury lesions (Metz and Whishaw, 2002). Our findings indicate that this test seems to be sensitive also for the HIE injury since the lesioned rats had a greater number of errors in this task. We reaffirmed this result when we performed the ratio of the number of errors according to the number of steps performed in the course and the

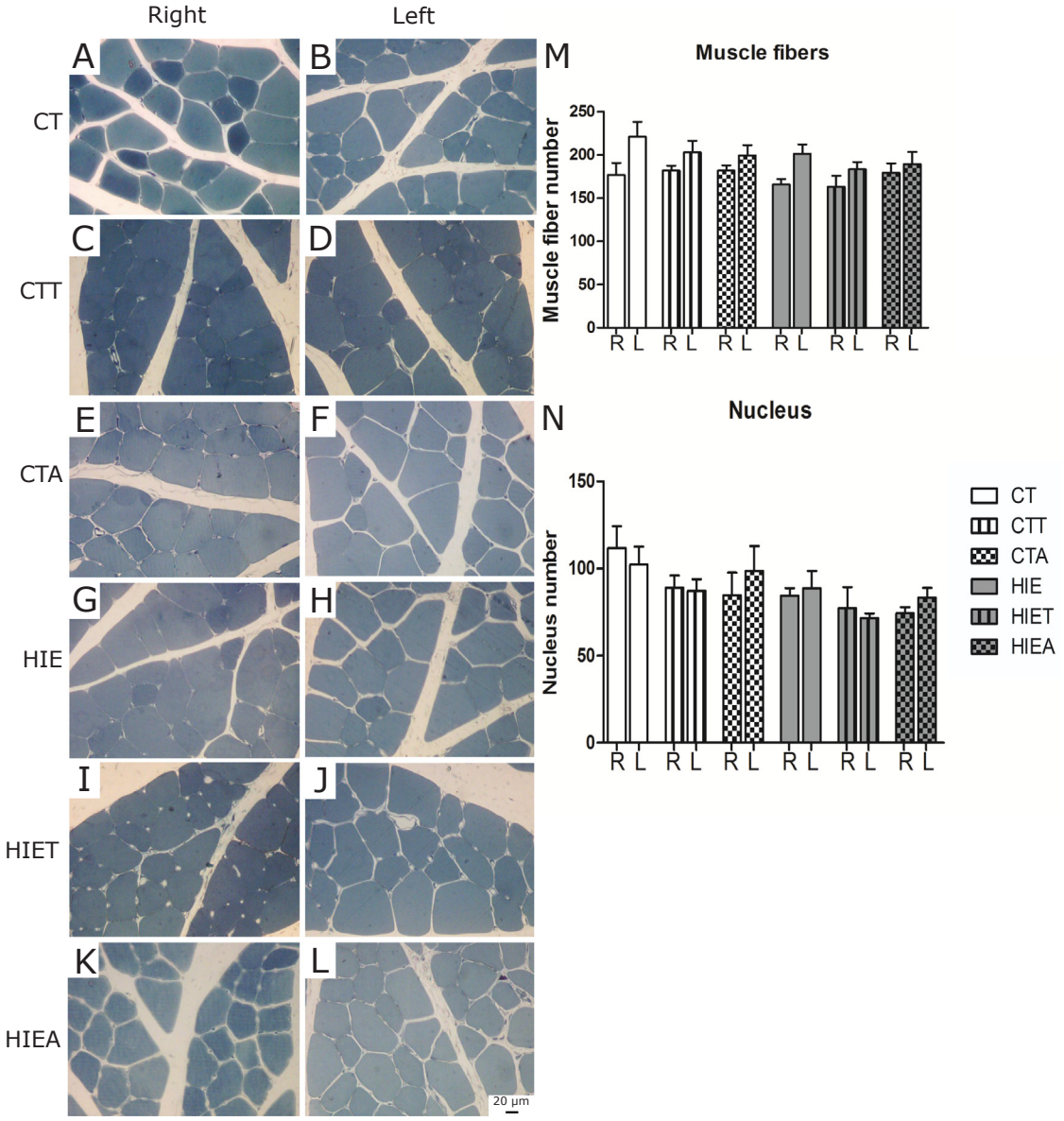

Fig. 9. Muscle analysis. Representative images of groups CT (A-B), CTT (C-D), CTA (E-F), HIE (G-H), HIET (I-J), HIEA (K-L). Number of muscle fibers (M). Number of nucleus (N). R = right side and L = left side. Values expressed as mean ± standard error. Two-way ANOVA followed by Tukey's post hoc. N = 6 animals/group.

finding was confirmed. The motor impairment showed in the ladder-walking test was also identified in studies performed by Durán-Carabali et al. (2017) and Schuch et al. (2016b). Interesting, our results showed that the acrobatic exercise produced an effective therapeutic effect recovering motor function in HIE animals, even with the exercise protocol starting two weeks after the injury. This indicates that this type of exercise seems to be a good treatment option for the individuals affected by this pathology even when they cannot start an early intervention. It is important to highlight that this activity is proposed to mimic stimulation techniques that have already been used in physical therapy. Then, these results reinforced the use of these protocols of treatment because they are inexpensive and non-pharmacological options that can demonstrate satisfactory results on neurological disorders. It has been established that the interaction between the person, the environment, and new tasks can change how our movements are established causing motor development changes (Sigmundsson et al., 2017). For the motor skill learning, as occurred in acrobatic training, current evidences has been demonstrated increasing in the connections between certain areas of the brain and consequently promotes plasticity (Kida et al., 2016). Exploring this strategy of rehabilitation, Sidaway and colleagues (2012) stated that the therapists should manipulate difficulty the task parameters applicated in the clinic's environment, in order to generate an appropriate level of functional difficulty for each patient, thus obtaining optimize motor learning. Kline et al. (2016) demonstrated also that the use of combination therapies can be more beneficial after traumatic brain injuries. Findings of the present study call attention to the importance of translational researches. The pre-clinical trials could contribute to justifying the use of non-pharmacological intervention, proving its functional benefits and demonstrating the neurobiological targets involved.

The other task used in this work in order to evaluate the animal's motor coordination was the rotarod. This test was designed to evaluate maximal motor performance and is widely used to evaluate the motor balance of rodents (Shiotsuki et al., 2010). We found no differences between groups regarding motor coordination evaluated in this test. There is no consensus in the literature about motor impairments in the HIE model measured by the rotarod. Griva et al. (2017) and Dai et al. (2017) found balance deficit in HIE animals but Rojas and colleagues (2013) did not find differences between HIE and controls animals. The rotarod test is more sensitive in detecting alterations in the cerebellum

(Shiotsuki et al., 2010). However, this structure seems to be less affected by the HIE model, since there are no reports of atrophy in this structure in the literature and we did not observe differences in animals balance during acrobatic training; which suggests an explanation about the contrasting results observed in this test.

Summarizing, the neonatal HIE model produced mild negative effects on the motor function of the animals, without prejudicing the balance. We also showed that acrobatic exercises were able to re-establish the motor function, being an effective therapy in this model. Particularly considering the inconsistency on the motor function results, the question that remains is why motor control seems not to be affected so intensely in this model, even with the presence of extensive cerebral atrophy as already demonstrated in previous studies. Therefore, the next step of this study investigated the morphology of components involved in motor control in order to identify the reasons for these divergent results.

3.4. Cerebral atrophy caused by neonatal HIE decreased in animals that performed acrobatic exercise

In rodents, the CST forms a crossed circuit responsible to control voluntary movements of the four limbs. This tract is composed initially of pyramidal neurons originate from layer V in the motor cortex, projecting mainly to the contralateral side of the spinal cord and connecting to motor neurons through a multisynaptic pathway (van Velthoven et al., 2010; Carmel and Martin, 2014; Welniarz et al., 2015). Additionally, peripheral structures such as nerves and muscles also play an important role in motor function, since they are responsible for the movement's execution and have a direct connection with the corticospinal pathway through the peripheral nerves. In order to detect possible changes in some of these components resulted of the neonatal HIE that could help to understand the results found on motor function, we aimed to develop an exploratory study of the main components of the corticospinal pathway and peripheral structures associated to the motor control (Fig. 10).

Firstly, we performed a gross measurement investigating the total brain atrophy. Our results evidenced that the neonatal HIE caused considerable damage in the hemisphere ipsilateral to arterial occlusion and the acrobatic training was able to reduce this atrophy. It has been extensively reported a severe atrophy in the hemisphere ipsilateral to the injury (Pereira et al., 2007, 2008; Miguel et al., 2015; Schuch et al., 2016a; Carletti et al., 2016). Interestingly, the acrobatic exercise prevented the advance of the atrophy in this model; even with the therapy starting two weeks after the injury. In this time point, the acute phase of neonatal HIE had been already been occurred causing cell death, and the exercise seems to alleviate the injury progression, maybe exerting neuroplastic effects. The functional results previously showed in this study also indicate that the exercise is capable to recover the motor function in the HIE animals. In view of these findings, some hypotheses can be stated in order to understand the mechanisms involved in this protection caused by the exercise. It has been well established that the maintenance of vascular structure is critical for brain function and it can be disturbed in a range of pathologies that affect the blood supply or the nervous tissue microenvironment (Abbott et al., 2010). In this context, the physical exercise practice promotes angiogenesis and this process is considered a prerequisite for many forms of neural and behavioral plasticity, particularly on pathological conditions (Berggren et al., 2014). The literature also showed that the physical exercise is able to decrease blood–brain barrier (BBB) dysfunction by improvement of astrocyte function after ischemic stroke (Wang et al., 2014). The combination of these two factors may be decisive for the found exercise benefits observed in the current analysis. Previous study conducted by our research group has already shown that the neonatal HIE model is responsible for changes in the BBB making this barrier less efficient (Diaz et al., 2016). The acrobatic exercise may have restored the BBB function by establishing a favorable microenvironment for angiogenesis in the affected hemisphere. This hypothesis is founded by the current evidences showing that the exercise practice increase vascular endothelial growth factor (VEGF) and brain-derived neurotrophic factor (BDNF) in cortex and hippocampus. Additionally, when the exercise is voluntary, this increase can be more expressive (Uysal et al., 2015). This pathway could explain how the exercise partially protect the brain of HIE animals, by reestablishing oxygen levels and consequently preventing the injury advance. We can also to propose that the modulation of plasticity mechanisms have been considered in studies with exercise and other non-pharmacological therapeutic strategies. Rojas and colleagues (2013) reported that the environmental enrichment was responsible for to increase the dendritic spine density in HIE rats. Studies in healthy rodents subjected to different exercise protocols have also shown increases in dendritic spine density in regions including the hippocampus, entorhinal cortex and cerebellum (Stranahan et al., 2007; Petzinger et al., 2013). The modulation of the dendritic spines is very important to plasticity and changes in these spines can be associated to a reformulation in communication pathways ensuring the efficiency in motor control (Lüscher et al., 2000). Thus, we can propose that the exercise exerts a protective effect promoting a favorable environment for preventing the cell death and/or preserving the surviving cells.

Another interesting fact that needs to be taken into account in our results is the divergent therapeutic effect found in the different types of exercise: treadmill and acrobatic. Our results evidenced a better effect of acrobatic exercise in motor function and reversion of atrophy. Evidences showed that in ischemic rats, low-intensity exercise could improve synaptic plasticity better than high-intensity exercise by promoting an increase in BDNF levels in the hippocampus (Shih et al., 2013). Perhaps, aerobic exercise performed on the treadmill, even at a moderate intensity, can cause more stress on the animals, thus masking the exercise benefits in the present study. Taken together, we can suggest that more attention should be deserved for motor skills learning training as a coadjutant in the processes of neurological rehabilitation in human patients with HIE, particularly in children.

3.5. The neonatal hypoxia-ischemia did not cause morphological alterations in the motor control components

In relation to other variables evaluated, we did not find additional differences in neuronal quantification in the cortex, motoneurons number and area in the spinal cord, sciatic nerve analysis, neuromuscular junctions and muscle fibers of the plantar muscle in rats following neonatal HIE. Even without finding any effects of HIE in any of the regions analyzed, in the sciatic nerve was possible to identify an exercise effect, evidenced by an increase in axons area and myelin sheath in the animals that performed acrobatic training. These findings can be discussed together since they are interdependent.

As cited above, severe brain atrophy is commonly reported in the neonatal HIE model (Pereira et al., 2007; Carletti et al. 2012). Nevertheless, when we look more closely at the morphological studies, we could observe that the percentage of atrophy varies according to each region, being larger in the hippocampus and not so significant in the cortex (Miguel et al., 2015). Hippocampus is a structure selectively vulnerable to a variety of metabolic and cytotoxic insults, and a possible explanation for this vulnerability seems to be related to the distribution of the glutamatergic receptors in this region (Choi and Rothman, 1990). Excitotoxicity mechanisms, as well as oxidative stress related to increases in glutamate levels and then play crucial roles in neuronal injuries implicated in the pathogenesis of many neurologic disorders, including perinatal brain injury associated with hypoxia-ischemia (Burd et al., 2016). The study of Miguel and colleagues (2015) identified the loss of 50% of the hippocampal volume and the cortical atrophy was around 20% in rats. These findings corroborate the data of the present study that show a similar cortex neuronal density in both control and HIE groups.

The specific vulnerability of the hippocampus justifies the

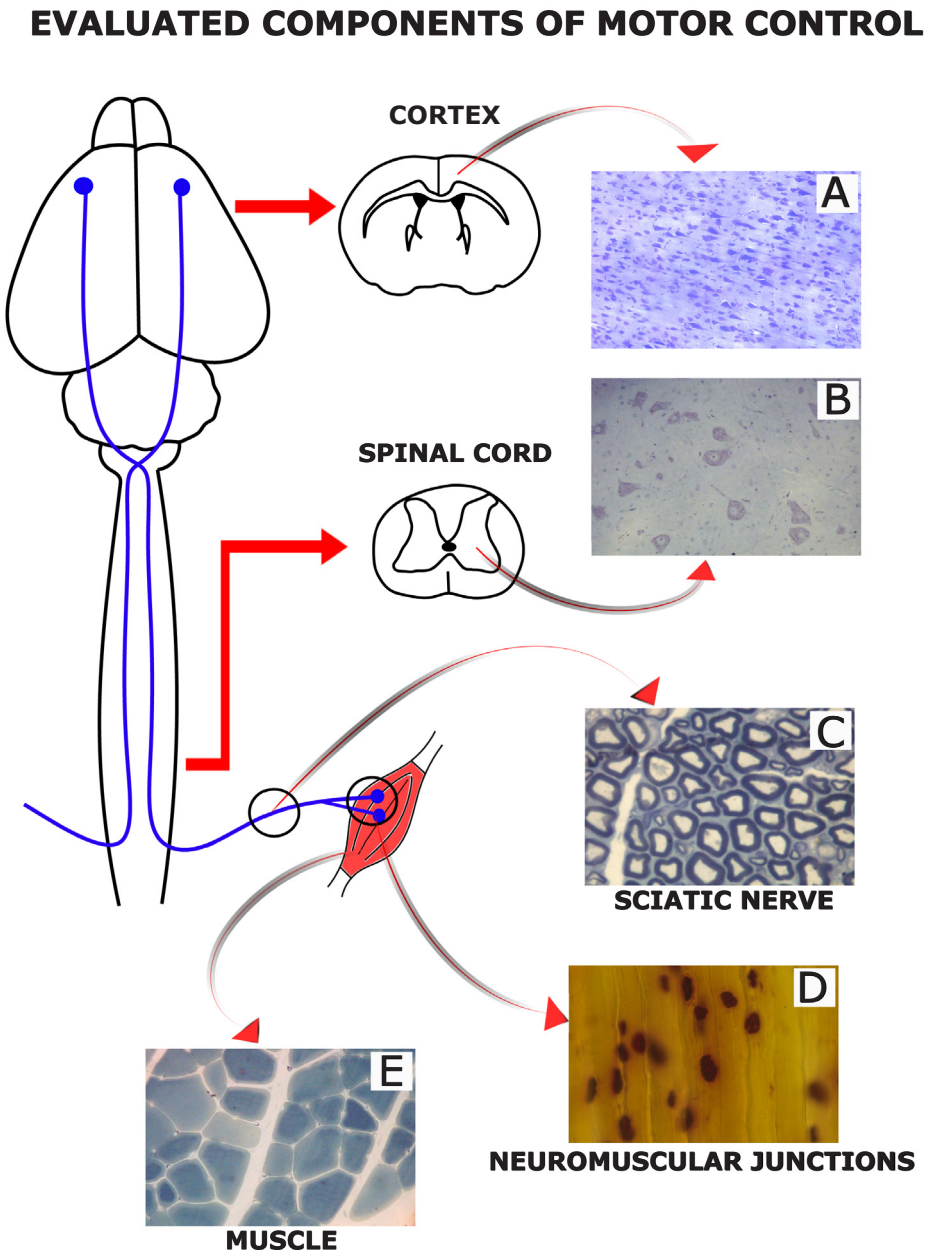

Fig. 10. Evaluated components of motor control. Representative photomicrographs of CT group of all regions evaluated in this study was shown in this scheme. Sensorimotor cortex (A). Spinal cord (B). Sciatic nerve (C). Neuromuscular junctions (D). Plantar muscle (E).

agreement of findings in this structure in several studies of neonatal brain damage. Conversely, there is no consensus about the injury in cortex, spinal cord or even other elements of motor function in HIE rats. Contrary to our results, a study performed by Bellot and colleagues (2014) reported bioaminergic disturbances in the brainstem and the spinal cord of HIE mice and Durán-Carabali et al. (2017) reported reduction in muscle fibers area in rats in the same HIE model. It is well described that the HIE-induced injury can be described as a spectrum, ranging from “moderate” to “severe” (Rumajogee et al., 2016). Such variability is mainly based on the critical period of the development since the brain in this phase is developing and as consequence, the neuronal plasticity is enhanced (Johnston et al., 2009). The nervous system of young animals recovers more quickly and effectively than in adult because it is considered more “plastic” (James-Roberts, 1979), justifying the preservation of some brain structures in HIE rats. Additionally, other possible reason of plastic response in neonates can be the efficient vascularization in this period. After the carotid occlusion in the HIE model, a compensatory effect has been observed due to the presence of the circle of Willis, which allows communication between the brain hemispheres (Cook, 1965; Dorr et al., 2007; Rumajogee et al., 2016). This spontaneous response was more described in a recent study performed by Edwards et al. (2017) which identified a retrograde perfusion through two routes: firstly, via facial, orbital, and meningeal branches, originating from the external carotid artery; and secondly, via collateral blood flow through the external carotid artery by anastomotic branches with the superior and inferior thyroidal or occipital arteries. Consequently, these alterations in perfusion can explain the inconsistencies associated with the Rice-Vannucci procedure. Possibly this blood flow compensation, together with the particular plasticity of the period, may contribute to the variable findings on nervous tissue in newborns rats. In general, the morphological findings in the HIE model indicate the idea that structures related to the motor function in the central nervous system and in the peripheral components are not the major affected. In accordance with the morphological findings, also the

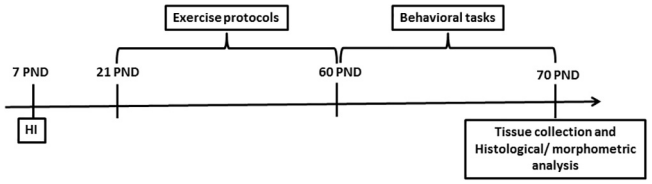

Fig. 11. Experimental design. At the 7th PND, rats were submitted to hypoxia–ischemia. At 21st PND, the animals were divided into 6 groups: CT, CTT, CTA, HIE, HIET and HIEA. The exercised groups started the training in different modalities. From the 60th PND animals performed behavioral tasks and after they were euthanized for tissue collection.

motor function also does not suffer expressive changes. Even though the Rice-Vannuci model has been considered a model of perinatal asphyxia, maybe it is necessary to evaluate if this model is in fact adequate for studying the motor dysfunctions, as seen in cerebral palsy. In humans, the common finding of the cerebral palsy is disturbances in motor functions by the presence of spastic movements (Aarts et al., 2010). However, in HIE animals the presence of spasticity is not observed (Robinson et al., 2013). In opposition, rats submitted to HIE do not demonstrate difficulties to perform common motor skills such as locomotion, feeding, and swimming and these are evidenced through the different tests performed in this model (Pereira et al., 2008; Miguel et al., 2015; Schuch et al., 2016a; Carletti et al., 2016; Diaz et al., 2016). This statement is confirmed in the present study, since the animals did not have difficulties of locomotion in the open field test and for performing the physical training. The divergent functional and morphological impact of a hypoxic-ischemic event in rodents and humans could be carefully considered because the structures of motor control in both species demonstrate some differences. For example, a recent review had already reported that rodents have fewer direct connections between the motor cortex and motor neurons in the ventral horn of the spinal cord and less number of axons that decussate in the medulla to the contralateral side (Rumajogee et al., 2016). However, the Rice-Vannuci model has successfully been used to mimic cognitive dysfunctions observed in humans that suffered perinatal asphyxia and this filed there is a clear consensus of its use and application.

In our study, we found no injuries in the central nervous system (cortex and spinal cord). Naturally, we also did not observe alterations in the nerve, neuromuscular junction, and skeletal muscle; in other words, the movement effectors were not affected since the central control pathway remained preserved. In addition, the HIE model did not cause damage to the structures involved in motor control and, consequently, the protector effect of exercise cannot be seen in these structures.

4. Conclusions

In conclusion, our results evidenced that the acrobatic training was able to reverse the hyperactivity and anxiety, caused locomotion improvement and decreased brain atrophy in HIE animals. Surprisingly, we did not find morphological alterations in the sensorimotor cortex, spinal cord, sciatic nerve, neuromuscular junctions and skeletal muscle in the animals submitted to neonatal HIE model. These findings demonstrated that the acrobatic exercise could be a good therapeutic option in humans affected by neonatal HIE, especially children and was responsible to improve cognitive and motor aspects.

To our knowledge, for the first time it was performed a wide exploratory evaluation of the morphology of the main components related to the motor function in the neonatal HIE model. More specific studies to evaluate proteins involved in the plasticity responsible for the maintenance of these vital functions should be investigated in more detail in order to explain the neurobiology of neonatal HIE and the mechanisms involved in the beneficial effect of the acrobatic exercise in this model.

5. Experimental procedures

5.1. Animals

Firstly, pregnant *Wistar* rats with approximately 60 days of life were obtained from the Central Animal House of the Institute of Basic Health Sciences of the Universidade Federal do Rio Grande do Sul. They were maintained under standard conditions of light (12-h light/dark cycle) and temperature (22 ± 1°C), with food and water available *ad libitum*. After the birth, on the 7th PND, the pups were randomly assigned into two groups: control and HIE. After HIE procedure, the pups remained with their mother in a minimum number of six and maximum of eight per litter, until the weaning (PND 21). At the 22nd PND, the animals were separated into six experimental groups according to the modality of training and HIE procedure: control group non-exercised (CT), control group submitted to treadmill training (CTT), control group submitted to acrobatic training (CTA), HIE group non-exercised (HIE), HIE group submitted to treadmill training (HIET) and HIE group submitted to acrobatic training (HIEA). For the accomplishment of this study, 38 pregnant and 150 male pup rats were used. A timeline of the experimental procedures is depicted in Fig. 11. The Ethics Committee on Animal Experimentation of the Universidade Federal Rio Grande do Sul, Brazil approved this study (n. 29230). All procedures 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 n° 11.794/2008).

5.2. Hypoxia-ischemia procedure

Induction of neonatal HIE was performed using the experimental model for neonates of Rice et al. (1981). At PND 7, animals were initially anesthetized with 2–4% halothane. A longitudinal neck incision was made allowing the left common carotid to be identified and permanently occluded with surgical thread of silk 4.0. After recovery period in their cages for two hours, the pups were placed in a chamber (1500 ml) in a water bath at 37° C, in groups of five, for exposure to the hypoxic atmosphere (8% oxygen and 92% of nitrogen) for 90 min, and then returned to their original home cages. The control animals were "sham-operated", since these animals were also submitted to surgical incision, but there was no occlusion of the left common carotid artery and no exposure to the hypoxic atmosphere (Miguel et al., 2017; Diaz et al., 2016).

5.3. Exercise protocols

One day after weaning (PND 22), animals started the two different exercise protocols and the training lasted five weeks, until approximately PND 60.

5.3.1. Treadmill training

The exercise in treadmill is widely cited in literature (Stigger et al., 2011; Cechetti et al., 2012; Choi et al., 2013; Heo et al., 2014). The protocol used in this study was based on the methodology proposed by Cechetti et al. (2012), using a moderate intensity physical exercise. Initially, the animals underwent an adaptation period, when they were placed on the treadmill turned off for 5 days. The exercise intensity was defined at 60% of the animal's maximum oxygen uptake (Brooks and White, 1978). The indirect evaluation of peak oxygen uptake (VO2) was performed for all rats that performed the exercise in treadmill before start training: animals ran in a treadmill initially at a low speed followed by speed increases in 5 m/min every 3 min until the point of exhaustion (i.e., failure of the rats to continue running). The time to fatigue (in min) and workload (in m/min) were taken as indexes of capacity for exercise that was considered as VO2 max. After VO2 max was defined, the exercises on the treadmill were performed three times

a week, with a total duration of 20 min per day and the velocity was based on 60% of VO2 performed previously.

5.3.2. Acrobatic training

Acrobatic exercises consist of a series of tasks designed to encourage problem-solving and motor coordination. This type of exercise usually involves the presence of different types of obstacles providing challenges in cognitive and motor functions (Black et al., 1990; Ahmed et al., 2011; Bonetti et al., 2011; Tamakoshi et al., 2014). The acrobatic exercise protocol used in this work was based on the model described by Black et al. (1990) and adapted by Jones et al. (1999). Basically, the training performed by the animals consisted of a circuit of obstacles containing seesaws, stairs, ropes with different thicknesses, rockers, bridges among others. The animals were first adapted to the circuit. During the adaptation, animals were conducted to move through a 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. After adaptation, the animals started the training, performed three times a week, with six circuit repetitions/day. The first circuit used to the training consisted of a rope, a narrow bar, a rope ladder, a grid and a circuit with obstacles, involving ramps and a seesaw. The level of difficulty was increased progressively during training through the change of the circuit in the different weeks of training. The new circuit featured thinner ropes, larger spaces between stair steps, higher obstacles, and a narrower bar. Another factor of difficulty was the utilization of the different inclinations of the circuit, forcing the animals to use the balance. Details of the acrobatic circuit used are shown in Fig. 12. In the first week, the same adaptation circuit was used. In the second week, the increased difficulty occurred through the inclination of the same circuit, where the animals performed the circuit going down. In the third week, all circuit was changed by more difficult tasks, like thinner ropes, more spaced ladders, and a narrower bar. In the last week, it was used the same circuit of the third week but now inclined, forcing the animals to make the circuit on the way up (Black et al., 1990; Kleim et al., 1996). Animals received slight manual stimuli, when necessary. The time spent in each trial of the acrobatic circuit was counted when the animal was placed on the first obstacle of the circuit and was finalized when the animal finished the last obstacle. The presented data shows the average time in seconds that each animal took to complete the 6 passages through the circuit throughout the training. The non-exercised animals were taken to the same room where the animals performed the exercises, and they were transferred to individual cages for 20 min.

5.4. Behavioral tasks

5.4.1. Open field

After the exercise period, in the 60th PND, animals were submitted to the Open Field test (animals/group: CT: 11, CTT: 13, CTA: 9, HIE: 12, HIET: 13, HIEA: 12). This task was used to assess motor function and exploratory behavior (Tatem et al., 2014). The apparatus utilized is formed by a wood square arena divided into 12 equal quadrants of 50 X 50 cm, with 40 cm walls (Carletti et al., 2012). The animals were placed facing the corner wall of the apparatus and their free exploration was evaluated for 5 min. The latency to leave the first quadrant, the number of rearings, the number of crossings and the time spent in the central and in the peripheral areas were evaluated. The test was recorded for posterior analysis and all procedures were evaluated by a blind experimenter.

5.4.2. The ladder rung walking test

The Ladder Rung Walking Test proposed by Metz and Whishaw (2002) was used to evaluate the motor capacity of the anterior and posterior limbs of animals. The apparatus used to perform the test consisted of two transparent acrylic side walls (100 cm long × 20 cm high) with metal rods (3 mm in diameter) inserted with one cm interval. The day after the open field (61th PND) animals were challenged to cross the ladder twice a day for three consecutive days. In order to evaluate the motor coordination and to prevent the spatial learning of the circuit, the position of the rods was modified at each session. A video camera was positioned in a way that the body and foot positions could be shot simultaneously for further analysis. An error was considered when the limb completely missed a rung, i.e. did not touch it, and a fall occurred. The test was recorded for posterior analysis and all procedures were evaluated by a blind investigator. The time spent for the animal to cross the ladder and the total number of errors from forelimbs and hindlimb were recorded (animals/group: CT: 9, CTT: 12, CTA: 9, HIE: 13, HIET: 12, HIEA: 13).

5.4.3. Rotarod test

Rota-rod apparatus was used to evaluate motor coordination of rats (Insight®, Brazil). After the Ladder Rung Walking test (64th PND), animals were exposed to one habituation session for 3 min in the apparatus on slow velocity (20 rpm). After, two test days were performed: rats were placed on rotating drums (3 cm diameter) with acceleration from 16 to 40 rpm over a 5 min period (animals/group: CT: 11, CTT: 10, CTA: 9, HIE: 12, HIET: 11, HIEA: 12). The latency of the first downfall, number of falls (maximum 3) and time of permanence in the apparatus were recorded (Rojas et al., 2013).

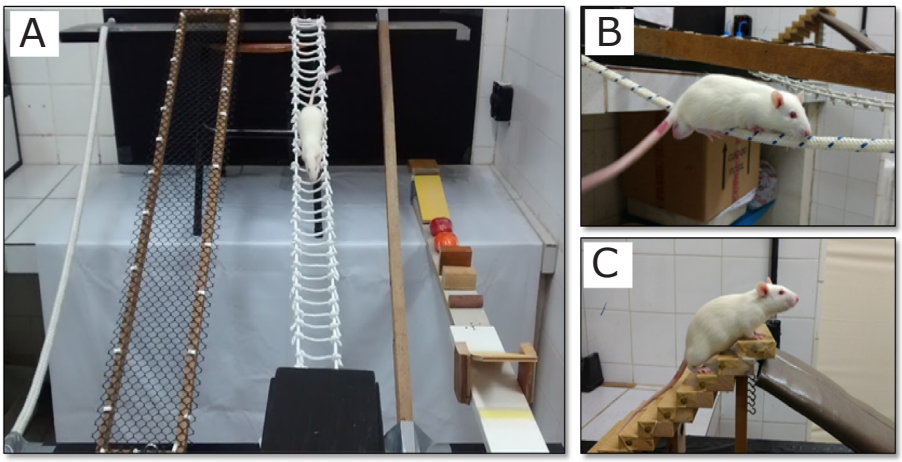

Fig. 12. Demonstrative figure of the apparatus used for acrobatic training. Apparatus used during the first two weeks of training (A). Adaptations used in second apparatus to increase the difficulty of training, thinner rope (B) and higher obstacles in (C).

5.5. Tissue collection

After behavioral tests (66th PND), the animals were weighed. A set of animals were anesthetized with thiopental and then perfused transcardially with 0.9% saline solution followed by 4% paraformaldehyde with phosphate buffer (PB). Brains, fragments of the spinal cord (lumbar region L4-L5), sciatic nerve and plantar muscle were collected and post-fixed in 0.5% glutaraldehyde (Sigma, USA). Brains then were cryoprotected with 15% and 30% sucrose solution and frozen in liquid nitrogen and stored at −80 °C until analysis. The other samples remained in the fixative until processing. The other animals were decapitated; brains were collected and photographed to measure brain atrophy.

5.6. Brain atrophy

The method used to measure the brain atrophy was based on the Miguel et al., (2017). The brains were photographed initially in top view. After, using a scalpel, it was made a median coronal section in cerebrum and a new photograph was made to evaluate another hemisphere area and a cortical measure (N = 6 animals/group). These measurements were made evaluating the following structures: the total size of the right and left hemispheres (top and coronal) and cortical area (coronal view). For delineating and calculating the areas were used the Image Pro-plus Software; two images per view were analyzed for each rat. The result of each rat's hemisphere area was the mean of the areas from the two images. A percentage of brain atrophy for each animal was calculated by relating the right hemisphere (contralateral to the lesion) with the left hemisphere (ipsilateral to the lesion) areas.

5.7. Histological and morphometric analysis

5.7.1. Cortex neuronal quantification

Brains previously cryoprotected were cut using a cryostat (N = 5–6 animals/group). The transition between M1 and S1HL (Bregma: −0.92 a −1.4 mm) was used as reference for delimitation of the area of interest (AOI) of the cortex. Pyramidal neurons of the layer V were evaluate. Every 100 μm, one slice was collected (20 μm thickness). After the collection, the slices were rehydrated and stained with 5% cresyl violet (Sigma-Aldrich, St Louis, MO, USA), immediately dehydrated, cleared, and mounted with Permount (Fisher Scientific®, USA). For the quantification of neurons in somatosensory/motor cortex, six images of each hemisphere (Olympus BX40 microscope – 200× magnification) were randomly chosen for each animal (AOI: 44853.85 μm2) (Marcuzzo et al., 2010; Dos Santos et al., 2017). The results of this analysis were demonstrated through a total number of neurons in the AOI (animals/group: CT: 5, CTT: 6, CTA: 6, HIE: 5, HIET: 6, HIEA: 6).

5.7.2. Motoneurons analysis

After fixation in glutaraldehyde, samples of spinal cord were washed in PB and postfixed in 1% OsO4 (Sigma, USA) in PB for 1 h. The samples were washed with PB again and dehydrated of alcohol and acetone (Electron Microscopy Sciences, USA). Afterward, embedded in resin blocks (Durcupan, ACM-Fluka, Switzerland), maintained in the vacuum for 24 h, and, subsequently, polymerized for 48 h at 60 °C. For motoneurons analysis, transverse-semithin sections (1 μm) were obtained using an ultramicrotome (MT 6000-XL, RMC, Tucson, USA. Every 10 μm, one section was collected and stained with 1% toluidine blue (Merck, Germany). Twenty images of the ventral horn of the spinal cord (lamina IX) were captured (100x magnification) per animal (Olympus BX40 microscope – AOI: 163775.9 μm2), being 10 of each side. Area and number of motoneurons were estimated (Marcuzzo et al., 2008) (animals/group: CT: 5, CTT: 5, CTA: 6, HIE: 5, HIET: 6, HIEA: 5).

5.7.3. Sciatic nerve analysis

The same fixation and formation of resin blocks described above for spinal cord were utilized for sciatic nerve. After, transverse-semithin sections (1 μm) were obtained, using an ultramicrotome and stained with 1% toluidine blue. Six images of each nerve per animal (right and left side) were captured and digitalized an Olympus BX40 microscope (initially 1000x magnification and further amplified 100x for analysis). Three random images from the periphery and three random images from the center of the nerve were obtained. All fibers located inside an AOI were analyzed (1208.031 μm2). Morphometric measurements included fibers number, axons area average and myelin sheath thickness average (N = 6 animals/group) (Stigger et al., 2011).

5.7.4. Neuromuscular junction area

For the analysis of neuromuscular junctions (NMJs), the plantar muscle was cutting manually with stainless steel blades into three or four slices (longitudinally) and was submitted to nonspecific esterase reaction technique (Lehrer and Ornstein, 1959). The slices were incubated in an oven at 37 °C until the reaction becomes positive. Images of the NMJs were captured at 400x magnification in Imager M2 Zeiss (Germany). Measurements of area, major and minor diameter of 50 NMJs per animal were obtained for each animal (AOI: 90866.26) (N = 6 animals/group) (Confortim et al., 2015).

5.7.5. Muscle fiber and nucleus quantification

The same fixation and formation of resin blocks described above for sciatic nerve were utilized in samples of plantar muscle. Then, samples of plantar muscle were also cut in transverse-semithin sections (1 μm). Every 6 μm, one slice was collected and stained with 1% toluidine blue. After, ten images of the plantar muscle were captured and digitalized (Olympus BX40 microscope – 200× magnification). For estimating the number of muscle fibers and nucleus, a set of ten images of each side (right and left side) was randomly chosen (AOI: 44853.85 μm^2) (N = 6 animals/group) (Meireles et al., 2017). Details about all regions evaluated and representative figures are shown in Fig. 10.

5.7.6. Programs and Image analyzes

All morphological and morphometric analysis were performed using the Image Pro Plus 6.0® software (Media Cybernetics, USA). Experienced and previously trained experimenters performed all analysis, and they were all blind for the experimental groups.

5.8. Statistical analysis

Two-way ANOVA followed by Tukey's post hoc test, with lesion and training as factors, were used for body weight, Open field, Rotarod test, The Ladder Rung Walking Test, brain atrophy, neuronal quantification in the cortex, motoneurons analysis, sciatic nerve analysis, muscle fibers quantification and neuromuscular junction area. Permanence time of the treadmill during VO2 test, VO2 max and time spent for the acrobatic training were compared using the Student's *t*-test. The data were expressed as mean ± SEM. The Statistic© software package was used, and differences were considered significant when $p < 0.05$.

6. Contributors

Bruna Ferrary Deniz, Wellington de Almeida, Patrícia Maidana Miguel, Loise Bronauth, Milene Cardoso Vieira and Bruna Chaves de Oliveira conducted the experimental procedures; Heloísa Deola Confortim and Lenir Orlandi Pereira conceived the experiment and wrote the manuscript.

Acknowledgment

We are very grateful to PhD Matilde Achaval, founder of the Laboratório Histofisiologia Comparada, main responsible for laboratory's structure used in this project. We would also like to thank Silvia Barbosa for the technical support at all stages of this work.

Funding

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance code 001, CNPq and FAPERGS.

Declarations of interest

None.

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4.2 ARTIGO II

A segunda parte dos resultados será submetida ao periódico *Experimental Neurology*, sob a forma de um artigo intitulado: **"*Forelimbs function and cellular plasticity in the motor cortex are altered by a model of neonatal hypoxia-ischemia without reversion through exercise*"**. O referido artigo segue-se abaixo.

FORELIMBS FUNCTION AND CELLULAR PLASTICITY IN THE MOTOR CORTEX ARE ALTERED BY A MODEL OF NEONATAL HYPOXIA-ISCHEMIA WITHOUT REVERSION BY EXERCISE

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ABSTRACT

The neonatal hypoxic-ischemic encephalopathy is still the major cause of neonatal mortality and morbidity, contributing overall disability, especially related to the motor dysfunction and structures related. Exercise protocols may ameliorate neurological impairment and have been shown to be a good therapeutic strategy against this pathology in the clinic. However, experimental studies evaluating the benefits of this type of therapy are still scarce. In this context, the aim of this study was to investigate the fine motor function of forelimbs, the expression of proteins involved in cellular morphology and plasticity in the motor cortex in male Wistar rats submitted to neonatal hypoxia-ischemia, and that posteriorly performed two different physical exercise protocols – treadmill or acrobatic training. Wistar rats were submitted to the HIE model at the 7th postnatal day (PND). After, from the 22nd until 60th PND the exercise protocols (aerobic or acrobatic) were performed. After the end of training, the animals were evaluated in the Pasta Matrix Reaching Task (PMRT) to investigate the use of the forelimbs. The brains were collected to investigate the proteins synaptophysin, NeuN, and GFAP trough immunohistochemistry in the primary motor cortex (M1). Our main finding showed deficits in HIE animals to learn the PMRT. As expected, the HIE animals used less the affected forelimb in the task and consequently showed minor success when to use this forelimb in all phases of the test. Additionally, the HIE animals present more SYP and a higher number of astrocytes when compared to control animals in the M1 in the hemisphere ipsilateral to the lesion. There were no benefits effects of exercises in these parameters. In conclusion, our findings demonstrated that the PMRT could be considered a good test to evaluate the motor function of animals submitted to neonatal hypoxia-ischemia, making it possible to demonstrate the fine motor dysfunction of the forelimbs of these animals. In relation to the cell plasticity, we demonstrate the role of astrocytes and synaptophysin in the M1 in this model and the relation to these proteins with the motor function impaired. However, more studies are needed to elucidate these different findings related to motor function and new strategies to treat them.

Keywords: pasta matrix reaching task, brain lesion, acrobatic training, neurodevelopment

Abbreviations: CNS: central nervous system, GFAP: glial fibrillary acidic protein, HIE: hypoxic-ischemic encephalopathy, MRI: magnetic resonance imaging, M1: primary motor cortex, PMRT: The Pasta Matrix Reaching Task, SYP: synaptophysin, VO2: maximum oxygen consumption

1. INTRODUCTION

The perinatal asphyxia is the major cause of neonatal mortality and morbidity, representing the single greatest contribution to overall disability worldwide (Aslam et al., 2014). This clinical situation has as consequence a brain dysfunction caused by a reduction in oxygen supply (hypoxia), aggravated by low blood flow to the brain, and is named hypoxic-ischemic encephalopathy (HIE) (Wassink et al., 2019; Moral et al., 2019). The mechanisms involved in the damage caused by HIE include a cascade of events like inflammation, excitotoxic amino acids release, and oxidative stress, leading to apoptosis and tissue necrosis (Grandvuillemin et al., 2017). These events cause severe brain damage that is linked to a high risk of permanent neurological deficits when affecting newborns (Steinman et al., 2009; Martinez-Biarge et al., 2011).

Currently, the early diagnosis of HIE involves an evaluation of Apgar scores, neurological clinical status and markers of multi-system organ function (Lee et al, 2008). Additionally, the Health professionals use techniques of image analysis such as computed tomography, magnetic resonance imaging (MRI), ultrasonography, and electroencephalogram for auxiliary in the diagnostic (Groenendaal; de Vries, 2016). Infants who survive the initial insult caused by HIE exhibit macroscopic brain damage in structural images, usually identified by MRI scans. In the literature, there are reports of different anatomical regions affected by this insult in the patients, but most studies described impairment in the basal ganglia, thalamus and cerebral cortex (Millar et al., 2017). The primary motor cortex (M1) plays a key role in volitional motor control and motor skills learning (Lev, White, 1997). Consequently, the areas of cortex damaged during the initial lesion are directly predictive of the language and motor function deficits observed in these patients (Barnett et al., 2002; Steinman et al., 2009;

Martinez-Biarge et al., 2011). Motor cortical damage and consequent motor dysfunction are very prevalent in patients affected by this type of lesion. However, this motor disability found frequently in patients is not fully mimicked in the models available to study this pathology.

In the Rice-Vannuci model, the animals submitted to HIE present facility to perform tasks that involves motor skills, such as running and swimming, demonstrating that they do not have severe motor deficits (Pereira et al., 2008; Diaz et al., 2016; Deniz et al., 2018; Confortim et al., 2019). These findings call attention because the injured regions in the model resemble that found in humans and the extent of the lesion is severe. However, when motor tasks are applied in this model, the results are inconsistent and vary widely; showing from animals without any motor alteration to some very compromised ones (Rojas et al., 2013; Schuch et al., 2016; Griva et al., 2017; Dai et al., 2017; Durán-Carabali et al., 2017; Pak et al., 2018). In addition, it is important to consider that more sensitive motor tests that can demonstrate the real severity of these motor deficits is still poorly studied in HIE models. The investigation of plasticity mechanisms involved in these differences, when compared humans affected by this insult with the animal model, is also necessary.

A hypoxic-ischemic event in the immature brain can disrupt central motor pathways and may affect normal developmental plasticity, thereby altering neurotransmission, cellular signaling, function and neural connectivity (Rocha-Ferreira; Hristova, 2016). To evaluate the changes in the plasticity of nervous tissue after damaging events, the investigation of specific proteins are employed. The literature suggests that the proteins synaptophysin (SYP), NeuN, and glial fibrillary acidic protein (GFAP) are important markers that allow to obtain data about the morphological organization of synaptic structures, changes in mature neurons and astrocyte activation, respectively (Kolos et al., 2015; Duan et al., 2016; Zhang et al., 2017). Alterations in these markers have already been identified in rats after the HIE procedure and these changes can be related to the functional deficits and brain tissue alterations found in these animals (Demarest et al., 2016; Diaz et al., 2016; Kim et al., 2017; Griva et al. 2017). In many cases of brain injury, motor physical therapy is recommended to restore motor function (Babcock et al., 2009); the therapeutic options

involve techniques that stimulate the coordination, retraining in activities of daily living, pain management, cognitive and behavioral therapies (Khan et al., 2003). When this type of method specifically involves physical exercise, experimental studies have shown satisfactory results like sensory-motor function preservation, neurogenesis, apoptosis inhibition and oligodendrogenesis (Garcia et al., 2012; Park et al., 2013; Choi et al., 2013; Tamakoshi et al., 2014; Kim et al., 2017; Pak et al., 2018). We have previously demonstrated that modality of exercise named acrobatic training was responsible for general locomotion improvement and decreased brain atrophy in HIE animals. Meanwhile, in the same work, we did not find morphological alterations in the structures related to motor function in the injured animals (Confortim et al., 2019).

How is this structural preservation occurring in HIE animals and how severe is the motor impairment in this model need to be elucidated. Additionally, the therapeutic impact of acrobatic exercise in this model could be better investigated. In this context, the aim of this study was to evaluate the fine motor function of forelimbs and proteins involved in cellular morphology and plasticity in the M1 in male Wistar rats submitted to neonatal hypoxia-ischemia and trained in two different physical exercise protocols – treadmill or acrobatic training. Our hypothesis is that the neonatal HIE would cause motor impairments in the affected forelimb and that we will find cellular and plastic alterations in injured animals.

2. EXPERIMENTAL PROCEDURES

2.1 Animals

Male Wistar rats were obtained from the Central Animal House of the Institute of Basic Health Sciences of the Universidade Federal do Rio Grande do Sul and they were maintained under standard conditions of light (12-h light/dark cycle) and temperature (22±1◦C), with food and water available ad libitum. At 7th postnatal day (PND), the animals were randomly divided into two groups according to HIE procedure: control or HIE. After the HIE, the pups were returned to their cages and remained with their mothers until the weaning (PND 21). After this period, at the 22nd PND, the animals were assigned into six experimental groups according to the modality of training and HIE procedure. Group names: control group non-exercised (CT), control

group submitted to treadmill training (CTT), control group submitted to acrobatic training (CTA), HIE group non-exercised (HIE), HIE group submitted to treadmill training (HIET) and HIE group submitted to acrobatic training (HIEA). An experimental timeline to more details was demonstrated in Fig. 1. All procedures 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). The Ethics Committee on Animal Experimentation of the Universidade Federal Rio Grande do Sul, Brazil approved this study (n. 29230).

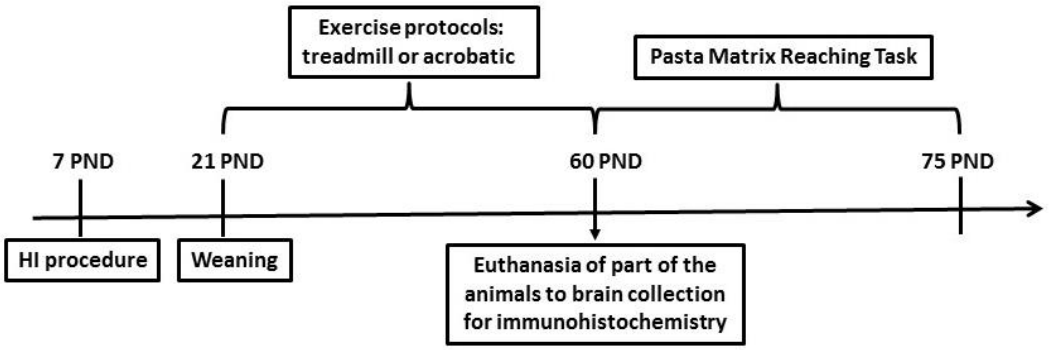

Figure 1. Experimental timeline. Rats were submitted to hypoxia-ischemia at the 7th PND. At 21st PND, the animals were divided into 6 groups: CT, CTT, CTA, HIE, HIET, and HIEA and started the exercise training in different modalities. Part of animals was euthanized in the 60th PND and brains were collected for immunohistochemistry. Other animals performed the Pasta Matrix Reaching Task.

2.2 Hypoxia-ischemia procedure

The model proposed by Rice and colleagues (1981) commonly referred to as the Rice-Vannucci model was employed in this study. In PND 7, the animals were anesthetized with 2-4% halothane and the left common carotid artery was identified through a longitudinal incision in the neck, isolated from the nerve and vein, and permanently occluded with 4.0 silk surgical thread. After two hours (recovery period) in their cages, groups of five pups were placed in a chamber (1500 ml) and exposed to a hypoxic atmosphere (humid nitrogen-oxygen mixture - 92 and 8%, respectively)

for 90 minutes with the chamber is partially immersed in a water bath at 37° C to maintain a constant thermal environment. Control animals were operated by simulation, i.e. they were submitted to anesthesia and surgical cervical incision, but did not receive arterial occlusion or hypoxic atmosphere exposure (Miguel et al., 2017; Deniz et al., 2018).

2.3 Exercise Protocols

At 22nd PND (after weaning), the animals started the exercise protocols. The training lasted five weeks, with an approximate duration of up to the PND 60.

2.3.1 Treadmill Training

The protocol used in this study established a moderate-intensity physical exercise and was based on the methodology proposed by Cechetti et al. (2012). Firstly, the animals were submitted to an adaptation period on the treadmill switched off for 5 days. Exercise intensity was defined as 60% of the maximum oxygen consumption of the animal (Brooks and White, 1978). The indirect evaluation of maximum oxygen consumption (VO2) was performed according to the protocol used by Confortim et al. (2019). Following the VO2 max definition, treadmill exercises were performed three times a week, with a total duration of 20 minutes per day.

2.3.2 Acrobatic Training

The acrobatic exercise protocol used in this work was based on the model described by Black et al. (1990) and adapted by Jones et al. (1999) and was previously described in details in Confortim et al (2019). Basically, the training performed by the animals consisted of an obstacle course containing swings, stairs, ropes with different thicknesses, rockers, bridges among others. The animals were first adapted to the circuit. After the adaptation, the animals started the training, performed three times a week, with six repetitions of circuit/day. The level of difficulty increased progressively during training by changing the circuit and using different slopes, thus forcing the animals to use the balance. The animals received slight manual stimulation when necessary. After the completion of the six repetitions of each animal in the circuit, all

obstacles and platforms were cleaned with ethanol 30%. The non-exercised animals were taken to the same room where the animals performed the exercises and were transferred to individual cages for 20 minutes.

2.4 Pasta Matrix Reaching Task

The Pasta Matrix Reaching Task (PMRT) was proposed by Ballerman et al., (2001) as a test to investigate the use of the forelimb of rats after spinal cord injury. Through this test, it is possible to evaluate the motricity of the forelimbs, including movement, adjustments of distances and directions, laterality and strength. The task has already been applied to models of stroke, and has been useful in assessing functional outcome in other upper extremity injury models (Kerr and Tennant, 2014). In this study, to be able to perform the test, after the exercise training, the animals started a food restriction (15-20g /rat/ day) three days before the test started and continued in food restriction during all duration of phases of PMRT (N= 8-12 animals/group). Additionally, during this period, pieces of pasta were placed in the animal cages to avoid neophobic responses (Kerr and Tennant, 2014). The apparatus used in the task consisted of a transparent acrylic box 150 mm wide, 250 mm long and 480 mm high to house the animal. A 1 cm wide slit was located in front of the box. Adjacent to the slit was a shelf 64 mm wide, 40 mm high and 120 mm wide. The shelf contained a series of holes, 10 lines deep by 15 lines wide. The rows of holes were separated by 4 mm. Pieces of pasta (spaghetti) were cut, inserted vertically into the holes in the shelf and extended 2.5 cm above the shelf. The shelf containing the mass was fixed to the front of the apparatus box by a clamp so that it could be easily removed for refilling during the different phases of the test.

Habituation

In the first stage of habituation, the animals were allowed to explore the apparatus. The rats were placed inside the box with the shelf containing the pieces of dough placed inside the apparatus along with the animals. They remained in the box for 5 to 10 minutes for two consecutive days. This phase was important to evaluate some type of avoid or aversion to pasta.

In the second stage of habituation, the apparatus was used in the normal conformation, with the shelf containing the pieces of dough attached to the outside of the apparatus. As a form of reward, pieces of dough were also located inside the box. At this stage of the task, rats were encouraged to collect pieces of dough from the opening in front of the box. The animals reached 10 min or a total of 10 times, which occurred first. This phase was performed for two consecutive days.

Training phase

After the habituation sessions, the animals started the training. During this phase, the apparatus was used in the same way utilized during the second phase of habituation (with the shelf completely filled with pasta). The animal was considered able to perform the first phase of the test when it was able to pick up/break 10 pieces of pasta for two consecutive days within a total time of 10 minutes. In this phase, the number of days necessary for learning these criteria and the time spent to pick up/break 10 pieces of pasta in each day was noted. The total day's number required to learn the task varied between the animals. Animals that not learned were excluded from the task.

Test phase

Three types of test were performed on three consecutive days. Test 1: the apparatus was used in the same conformation utilized in training phase with the shelf full with pieces of pasta. Test 2: the apparatus was used in the same conformation utilized in test 1; however, the shelf was filled placed with pieces of pasta only on the left side, forcing the animal to use preferentially the right forelimb (affected by HIE). Test 3: the apparatus was used in the same conformation utilized in test 2; however, the shelf was filled placed with pieces of pasta only on the right side, forcing the animal to use preferentially the left forelimb. Each animal was placed in the test apparatus for 15 minutes on each test day. All days of the test were recorded to posterior analyze. The following variables were evaluated during the different tests: the number of pieces of pasta broken in each test, the number of successful attempts (when the animal is able to pick up/break the pieces of pasta) and the number of total attempts of pick

up/break the pasta (successful and unsuccessful) with each of the forelimbs (right and left).

2.5 Tissue collection

Another part of the animals, which did not perform the test, were euthanized one day after the end of exercise training for the collection of the brain. These animals were anesthetized with thiopental solution and perfused transcardially with 0.9% saline solution followed by 4% paraformaldehyde with phosphate buffer (PB). The brains were rapidly collected and post-fixed in 4% paraformaldehyde at room temperature for 4 h. Posteriorly were cryoprotected with 15% and 30% sucrose solution a 4°C. Then, the brains were cooled in isopentane (Merck, Germany), frozen in liquid nitrogen and stored at -80ºC for further analyses. Coronal sections (30 µm thick) were obtained using a cryostat (CM1850, Leica, Germany) at −20 °C. Slices were containing the M1 region (Bregma: −0.92 a −1.4 mm) was serially collected in a previously gelatinized slides (Piazza et al., 2013). Every 120 µm, one slice was collected and processed for immunostaining (described below).

2.6 Synaptophysin, NeuN, and GFAP immunohistochemistry

For the investigation of markers SYP, GFAP, and NeuN thought immunohistochemistry, the brain sections previously cut into microtome were washed in PBS solution. The antigen retrieval was realized only for NeuN trough heating for 20 min in 0.01 M citrate buffer at 92°C. After cool down of NeuN slides, the endogen peroxidase was blocked with 3% hydrogen peroxide for 30 min in all slides. Sections were washed with PBS followed by PBS-Tx and incubated with a solution of BSA plus the monoclonal mouse anti-SYP antibody (1:250; Sigma), monoclonal mouse anti-NeuN antibody (1:1000; Millipore) or polyclonal rabbit anti-GFAP antibody (1:500, Dako) for 48h at 4°C. Posteriorly, the slices were washed with PBS-Tx and incubated with secondary antibody rabbit anti-mouse IgG conjugated with peroxidase (1:500; Sigma-Aldrich) for SYP and NeuN or antibody goat anti-rabbit IgG conjugated with peroxidase (1:500; Sigma-Aldrich) for GFAP for 2h at room temperature. To revelation, the DAB Enhanced Liquid Substrate System (3,3’ diaminobenzidine

tetrahydrochloride) was used according to the manufacturer's instructions (Sigma, USA). Finally, the sections were washed in PBS, dehydrated in ethanol, cleared with xylene and mounted with Permount (Fisher Scientific®, USA) (Deniz et al., 2018).

2.7 Optical densitometry and cell count

To realize the optic densitometry for SYP, NeuN, and GFAP, one image from each hemisphere containing the region of interest (M1) was captured at 400x magnification in Imager M2 Zeiss (Germany) (4 sections/animal). After the photodocumentation, all images were converted to an 8-bit grayscale and optical density was measured with the support of Image-Pro Plus Software 6.0 (Media Cybernetics, USA) in two areas of interest per animal (AOI: 23904,22 μm^2). The number of animals for these analyses were N=5–6 animals/group for SYP and GFAP, and N=3–4 animals/group for NeuN. In order to perform a complementary analysis, we also performed the count of cells labeled by NeuN and GFAP. For this, we used the same images and area of interest used previously for densitometry and all the cells marked within these two areas were counted. The average of cells counted per animal was used as an estimative of cells density.

2.8 Statistical analysis

Two-way ANOVA with lesion and training as factors were used. The data were expressed as mean ± S.E.M. The Statistic© software package was used, and differences were considered significant when $p<0.05$.

3. RESULTS

3.1 Pasta Matrix Reaching Task

3.1.1 Training phase

The statistical analysis indicated a lesion effect in the training phase in the variables: number of days necessary to learn the task ($F(1,55) = 5.62$, $p<0.05$) and in the time spent to broken 10 pastas in each trial ($F(1,55) = 8.59$, $p<0.05$). These results

indicated that the HIE animals take longer to learn the test, and even after they learn they take more time in each trial to break the 10 pastas regardless of the exercise performed (Fig. 2).

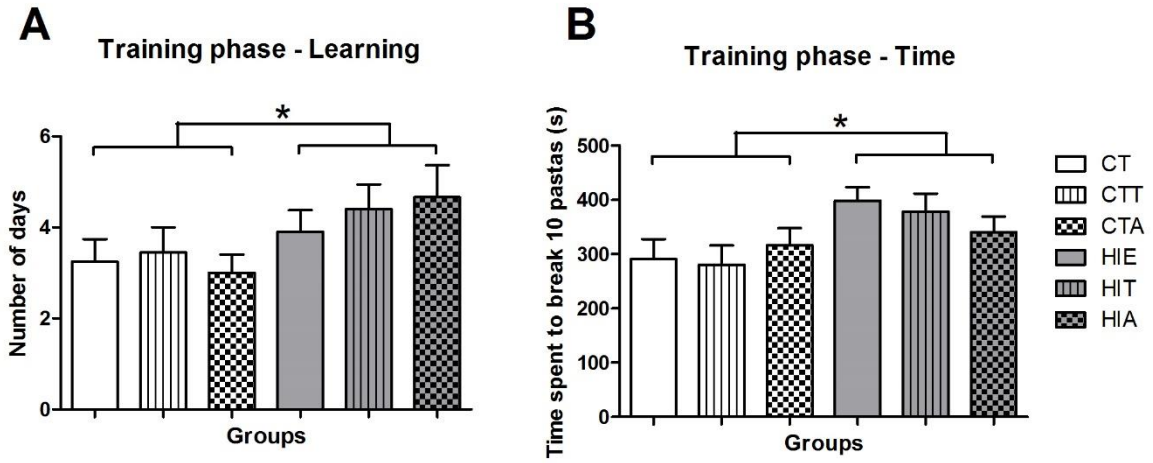

Figure 2. Training phase of Pasta Matrix Reaching Task. (A) Number of days necessary to learning the task. (B) Time spent to broken 10 pastas in training phase. *HIEs different from CTs groups ($p<0.05$). Values expressed as mean ± standard error. Two-way ANOVA. N= 8–12 animals/group.

3.1.2 Test phase

The number of pieces of pasta was counted in the end of each phase of the test and no significant differences were found consequent to lesion or training in none of the phases (Fig. 3).

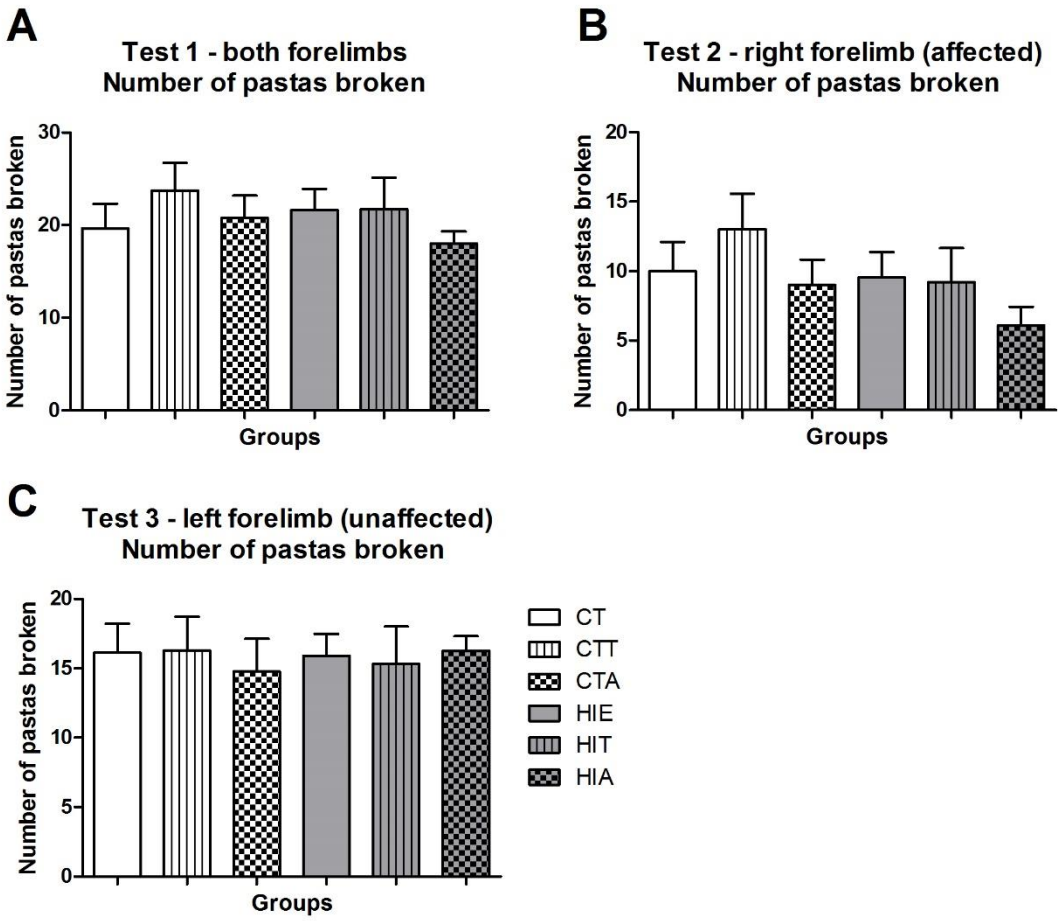

Figure 3. Number of pastas broken in each phase of Pasta Matrix Reaching Task. (A) Test 1. (B) Test 2. (C) Test 3. Values expressed as mean ± standard error. Two-way ANOVA. N= 8–12 animals/group.

3.1.3 Test 1

In test 1, when the animals could use both forelimbs to broken the pieces of pasta the Two-way ANOVA showed a lesion effect with an increase in the number of total attempts utilizing the left forelimb (unaffected) in the HIE animals ($F(1,55) = 5.63$, $p<0.05$). The effect of lesion also was identified with a lower success percentage in the right forelimb ($F(1,54) = 5.02$, $p<0.05$) in the HIE animals. A lower preference of the right forelimb utilization ($F(1,55) = 4.62$, $p<0.05$) and consequently a larger preference of utilization of left forelimb ($F(1,55) = 4.62$, $p<0.05$) in the HIE animals also were

identified. Additionally, the HIE animals showed minor success in this task when using the forelimb affected in relation to all successful attempts when compared the CT animals independently of the exercise training (F (1,55) = 3.85, p<0.05) (Fig. 4).

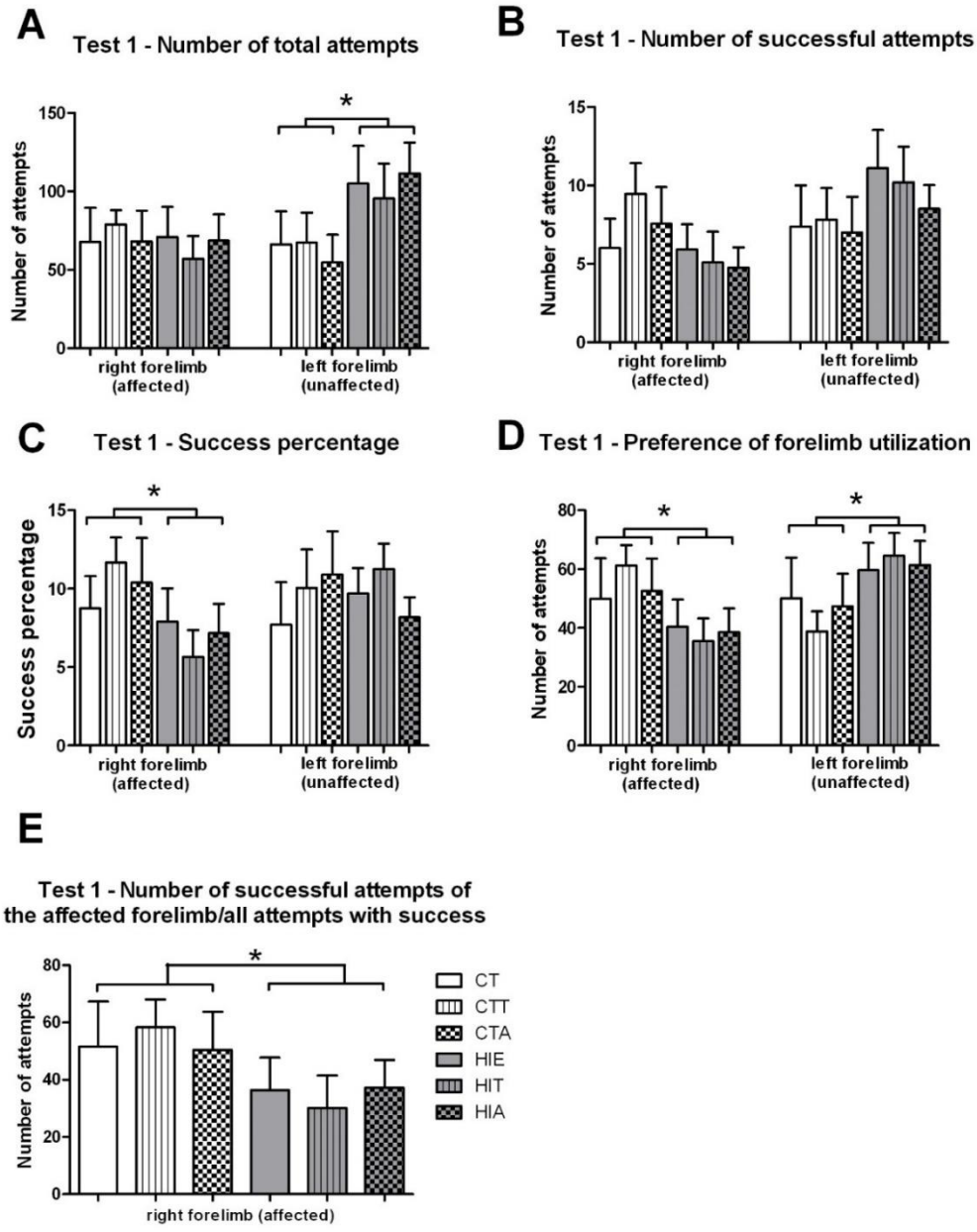

Figure 4. Test 1 of Pasta Matrix Reaching Task. (A) Number of total attempts. (B) Number of successful attempts. (C) Success percentage. (D) Preference of forelimb utilization. (E) Number of successful attempts of the affected forelimb/all attempts with success. *HIEs different from CTs groups (p<0.05). Values expressed as mean ± standard error. Two-way ANOVA. N= 8–12 animals/group.

3.1.4 Test 2

In test 2, when the animals have forced to use preferentially the right forelimb (affected by HIE) in the task, the statistical analysis showed again a lesion effect. We identified a major number of total attempts utilizing the left forelimb (unaffected) ($F(1,55) = 5.24$, $p<0.05$) and a bigger success percentage in the left forelimb ($F(1,54) = 5.23$, $p<0.05$) in the HIE animals. Similar to test 1, the HIE animals showed minor success in this task when using the forelimb affected by hypoxia-ischemia in relation to all successful attempts when compared the CT animals independently of the exercise training ($F(1,55) = 4,08$, $p<0.05$) (Fig. 5).

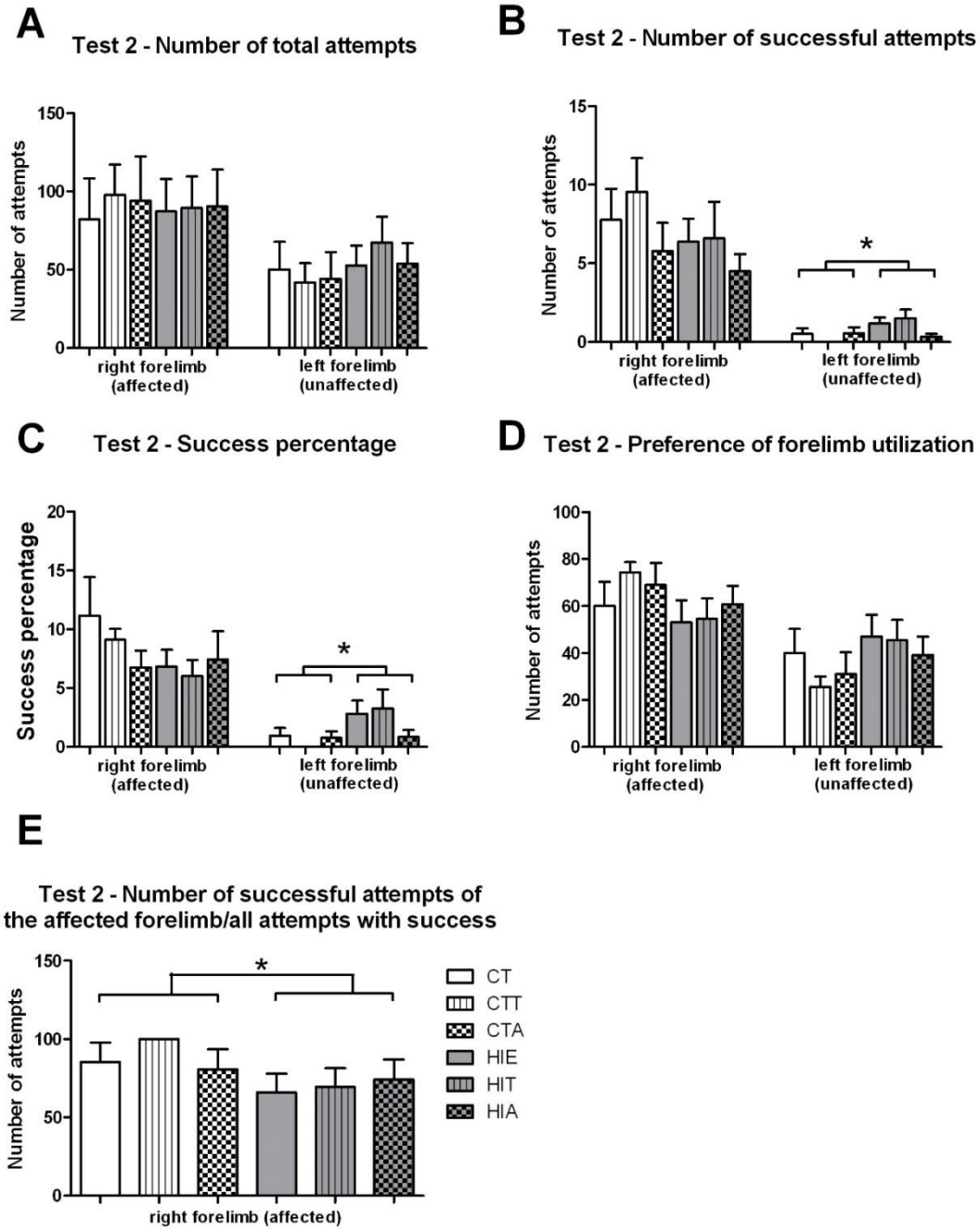

Figure 5. Test 2 of Pasta Matrix Reaching Task. (A) Number of total attempts. (B) Number of successful attempts. (C) Success percentage. (D) Preference of forelimb utilization. (E) Number of successful attempts of the affected forelimb/all attempts with success. *HIEs different from CTs groups ($p<0.05$). Values expressed as mean ± standard error. Two-way ANOVA. N= 8–12 animals/group.

3.1.5 Test 3

In test 3, when the animals have forced to use preferentially the left forelimb, Two-way ANOVA showed a lesion effect. The number of total attempts was lower in the HIE animals when compared to CT animals in the right forelimb ($F(1,55) = 5.41$, $p<0.05$). In the same way, in test 2, observed a lower preference of the right forelimb utilization ($F(1,55) = 7.47$, $p<0.05$) and consequently a larger preference of utilization of left forelimb ($F(1,55) = 7.47$, $p<0.05$) were identified in the HIE animals. Also, the HIE animals showed minor success in this task phase when using the forelimb affected in relation to all successful attempts when compared the CT animals independently of the exercise training ($F(1,55) = 7.70$, $p<0.05$) (Fig. 6).

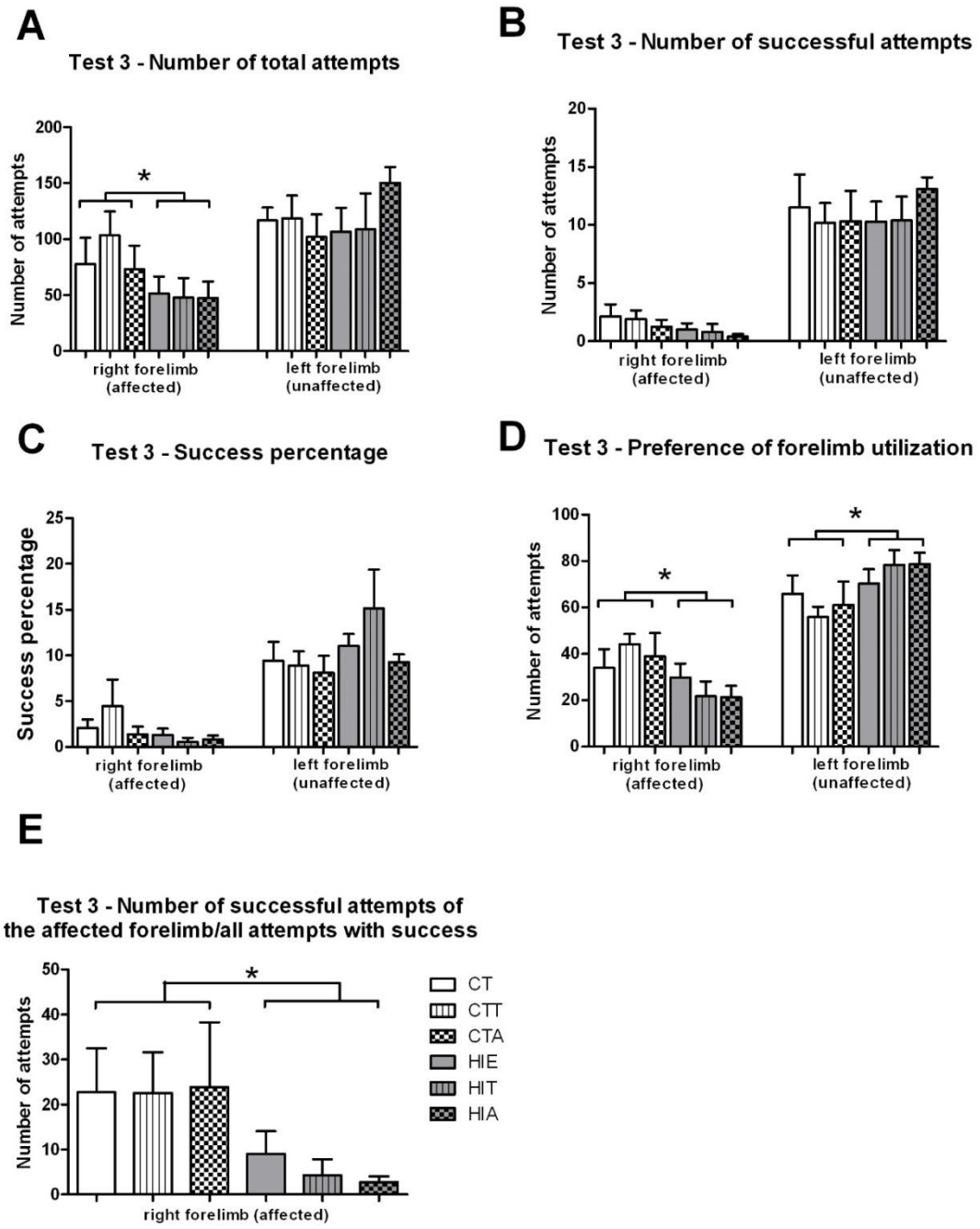

Figure 6. Test 3 of Pasta Matrix Reaching Task. (A) Number of total attempts. (B) Number of successful attempts. (C) Success percentage. (D) Preference of forelimb utilization. (E) Number of successful attempts of the affected forelimb/all attempts with success. *HIEs different from CTs groups ($p<0.05$). Values expressed as mean ± standard error. Two-way ANOVA. N= 8–12 animals/group

3.2 NeuN optical density and neuron count

Two-way ANOVA analysis did not evidence differences consequent to lesion or training in NeuN optical density (Fig. 7A). In relation to the neuron count, the statistical analysis also did not evidence differences in any these factors (Fig. 7B).

3.3 GFAP optical density and astrocyte count

Statistical analysis did not evidence differences consequent to lesion or training in GFAP optical density (Fig. 7C). In relation to the astrocyte count, Two-way ANOVA demonstrated a lesion effect in the left hemisphere ($F(1,29) = 10.8$, $p<0.05$), indicating that the HIE animals present a higher number of astrocytes than the control animals in the hemisphere ipsilateral to lesion (Fig. 7D). No effects of the exercise were identified in these parameters.

3.4 Synaptophysin optical density

Two-way ANOVA demonstrated a lesion effect in the left hemisphere ($F(1,29) = 4.23$, $p<0.05$). This result indicating that the HIE animals present more SYP than the control animals in the hemisphere ipsilateral to the lesion, regardless of the training performed (Fig. 7E).

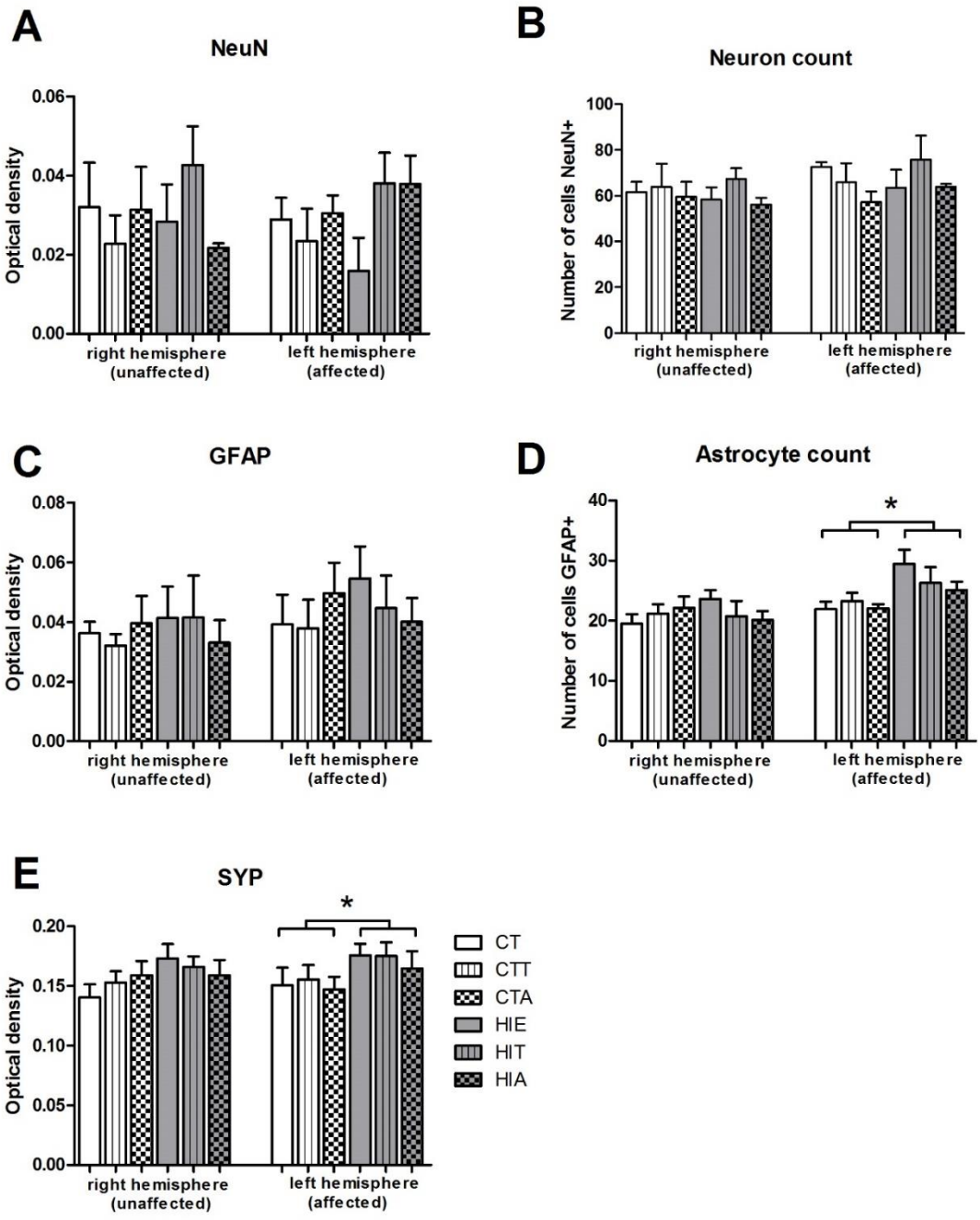

Figure 7. Immunohistochemistry of NeuN, GFAP and SYP. (A) NeuN optical density. (B) Neuron count. (C) GFAP optical density. (D) Astrocyte count. (E) SYP optical density. *HIEs different from CTs groups ($p<0.05$). Values expressed as mean ± standard error. Two-way ANOVA. N=5-6 animals/group to GFAP and SYP and N=3-4 animals/group for NeuN.

4. DISCUSSION

The current study sought to investigate the fine motor function of forelimbs and some proteins involved in cellular morphology and plasticity in the M1 in male Wistar rats submitted to neonatal hypoxia-ischemia and the possible therapeutic effect of two different physical exercise protocols – treadmill and acrobatic training. Our main findings showed learning deficits in HIE animals since they took more time to execute the task PMRT. Additionally, the HIE animals demonstrated motor dysfunction since they used less the affected forelimb and consequently showed minor success when to use this forelimb in all phases of the test. In relation to morphological findings, the HIE animals present more SYP and a higher number of astrocytes when compared to control animals in the M1 in the hemisphere ipsilateral to the lesion. Contrary to our initial hypothesis there were no benefits effects of both exercises in these parameters.

4.1 Learning deficits were found in animals submitted to neonatal HIE

The most commonly used model of neonatal HIE involves permanent unilateral occlusion of a common carotid artery and transient systemic hypoxia (Koehler et al., 2018). In this study we adopted the Rice-Vannucci model (Rice et al., 1981) which is very useful because reproduces the unilateral lesion in the hippocampus, striatum, and cerebral cortex as found in human neonates, making it one of the most accepted models to mimic this condition (Pereira et al., 2007). Cognitive deficits are currently reported: Deniz and collaborators (2018) and Carletti et al. (2016) observed memory deficits in HIE rats in the Morris water maze; in a study of Miguel et al. (2017), the animals were tested in a task of Attentional set-shifting and it was observed that HIE animals needed more trials to reach the criterion of six consecutive correct trials in one phase of the test, proving the cognitive deficits in comparison to controls animals. Learning and memory deficits after HIE injury also were found by Rojas and colleagues (2013; 2015) through the Ox-maze and object recognition task.

The task utilized in this study was the PMRT. In this test, the training phase is the first and can be considered the most important phase. This stage involves cognitive processing that is crucial for animals to perform the task correctly in the following

phases (Ballerman et al., 2001). Interestingly, in this phase was possible to identify a learning deficit in HIE animals, indicated by more days necessary to learn the test compared to control animals and more time in each trial to break the 10 pieces of pasta (criterion used to consider the animal able to go to the test). These results are probably related to the brain areas preferentially affected by the HIE model. It is a consensus in the literature that the hippocampus is extremely affected by the HIE neonatal model, being one of the regions that suffer the greatest atrophy (Miguel et al., 2015). One of the reasons for this vulnerability seems to be related to the large quantity of the glutamatergic receptors in this brain region (Choi and Rothman, 1990).

The initial hypoxic-ischemic insult is characterized by a decrease in the blood supply and this is the triggering factor of neuronal injury since the cells become incapable of fulfilling their metabolic demands (Novak et al., 2018). This period is followed by a decrease in glucose supply and a deficiency in high-energy phosphates (ATP) that result in a secondary lesion (Distefano; Praticò, 2010). In response to these cellular lesions, the excitatory amino acids glutamate and aspartate are release exacerbated in the brain, exerting an excitotoxic effect (Burd et al., 2016). Due to the energetic failure caused by HIE, the process of these neurotransmitters re-uptake is also impaired, thus causing a hyperactivation of these glutamatergic receptors (Millar et al., 2017). These exacerbated activation receptors result in the accumulation of intracellular calcium, causing death cellular and microglial activation with the consequent release of harmful factors to neighboring neural cells (Galluzi et al., 2009). This prevalence of these receptors in this region directly related to cognitive processing explains this learning deficit already well consolidated in the model.

It is important to remember that even with an impaired learning, HIE groups reached the necessary criteria to go to the other phases of the test, i.e., they learned the task. Thus, we believe that the PMRT test can be considered a good test for the evaluation of motor conditions of the forelimb in this HIE model neonatal.

4.2 The neonatal hypoxia-ischemia caused motor impairments in the affected forelimb on Pasta Matrix Reaching Task

Forelimbs assessments are critical to examine motor system status, understanding motor learning and recovery after damage in rats (Sloan et al., 2015). The PMRT was proposed initially by Ballerman et al., (2001) as a test to investigate the use of the forelimbs of rats after spinal cord injury. Through this test, it is possible to evaluate the motricity of the forelimbs, including reaching, movement, adjustments of distances and directions, laterality and strength. The task has already been applied to models of stroke, and have been useful in assessing functional outcome in other upper extremity injury models (Kerr and Tennant, 2014). In the present study, we choose this task with the purpose of evaluating more precisely the forelimbs preference/use in animals submitted to HIE and the possible therapeutic effect of two types of exercise. The results showed that no significant differences between groups in the count of pasta pieces broken in all phases of the test. However, when we evaluate in more details (observing the utilization of each forelimb) the different test phases, the results showed that the HIE animals presented lower preference of the affected forelimb utilization and consequently lower success when this forelimb to was used in all phases of the test. Interestingly, in phase 2, when it was more comfortable to use the right forelimb (affected by HIE), the HIE animals continued used the left forelimb, evidencing the incapacity to use the affected forelimb. Contrary to expected, no beneficial effects were found in any types of exercise used in this study.

Similar results have already been found previously in other models of brain injury that used this same task. In a hemi-Parkinson analog model, when rats were tested by PMRT, no difference was identified in the number of pieces of pasta obtained by the animals when allowed to use both forelimbs. However, when the rats were forced to use the impaired forelimb, the group submitted to lesion showed impaired compared to the control group (Metz et al., 2001). Mice submitted to focal infarcts of the sensorimotor cortex showed deficits in skilled reaching on the PMRT as compared to sham operates. Before lesion induction, the majority of mice were able to reach pasta pieces located anterior and far lateral to the reaching aperture (Tennant; Jones, 2009).

To our knowledge, this is the first study that uses the PMRT to evaluate the motor function of rats in a model of HIE. The present findings bring a particular contribution in relation to motor function taken the discrepancy found in the Rice-Vannucci Model (Rojas et al., 2013; Miguel et al., 2015; Arteaga et al., 2017; Griva et al., 2017; Dai et al., 2017; Durán-Carabali et al., 2017; Pak et al., 2018; Confortim et al., 2019). We can to propose that the discrepancies can be avoided by adopting more specific and adequate tests. Illustrating that problem, in our previous work, it was not possible to observe motor alterations in behavior tests that evaluation gross motor function, such as open field and rotarod, however, when using the horizontal ladder test, the deficit caused by HIE was identified (Confortim et al., 2019). Thus, we believe that the PMRT test is a good choice for animal motor evaluation after induction of neonatal hypoxia-ischemia. In order to try to understand in more detail the possible mechanisms that may explain this fine motor deficit found in this test, the next step of this work involves the evaluation of some proteins related to cellular plasticity in the M1, a region directly related to the motor function.

4.3 Animals submitted to neonatal HIE presented a higher number of astrocytes and more Synaptophysin expression in the ipsilateral primary motor cortex

In a previous study, our group demonstrated that animals submitted to the same HIE model proposed in this study did not present morphological differences on the sensorimotor cortex, spinal cord, sciatic nerve, neuromuscular junctions, and skeletal muscle (Confortim et al., 2019). However, in tests that evaluate fine motor function, it is possible to identify a dysfunction caused by the neonatal hypoxia-ischemia. To understand the structural preservation in the motor pathway and what relation of this preservation with the motor outcomes in this model needs we chose to identify possible cells and/or mechanisms of plasticity related to this structural preservation in the motor pathway. We evaluated through immunohistochemistry, the neurons density (NeuN), the astrocytes (GFAP) and possible synaptic alterations (SYP) in M1 ipsilateral and contralateral to the lesion. Our results show that the HIE animals present more SYP expression and a higher number of astrocytes ($GFAP^+$ cells marked) than the control animals in the hemisphere ipsilateral to the lesion in the M1, without exercise effect.

The M1 is a key structure for the control of voluntary movements and motor skills learning. The elaboration of appropriate responses to different behavioral situations is directly connected to the ability to select appropriate motor responses in accordance with the environment (Vitrac; Benoit-Marand, 2017). In humans, this region is strongly affected by neonatal hypoxia-ischemia and trigger several motor deficits (Barnett et al., 2002; Steinman et al., 2009; Martinez-Biarge et al., 2011). However, in the Rice-Vannuci model, this brain region seems less affected, when we compare the results found in the hippocampus, for example. This assertion is confirmed when it was not possible to observe a difference in the number of neurons marked by the NeuN protein in this encephalic region in this study. This result also had previously been described by cresyl violet marking in the sensorimotor cortex region in this same model, indicating a possible preservation of this region (Confortim et al., 2009). A different result can be observed in other brain regions; Deniz et al. (2018) showed that HIE rats diminished neuronal density in the hippocampus ipsilateral to the lesion and Kim et al., (2017) demonstrated significantly NeuN decreased in the HI group in the contralateral subventricular zone.

Interestingly, although no neuronal loss in the M1 has been observed, a greater number of astrocytes were identified in the HIE animals. This disturbance in neuron-glia interaction may be responsible for the functional deficit found in this study. When a neurological injury or neuroplasticity process occurs, astrocytes become reactive in order to protect and/or regulate the synaptic microenvironment of cells (Coleman et al., 2004; Dong, Greenough, 2004). An increase in GFAP expression may indicate hyperplasia or hypertrophy and may be followed by changes in the number or in the astrocyte ramifications (Sofroniew; Vinters, 2010). High levels of GFAP also have often been associated with a mechanical barrier, interfering negatively in the function of CNS cells (Burda; Sofroniew, 2014). Previous studies showed the increase of GFAP expression after the different models of neonatal HIE in different brain regions (Salmaso et al., 2012; Kim et al., 2018). The expression of this protein did not alter in this study. However, we found a higher number of cells $GFAP^{+}$. The effect of lesion also was identified by an increase of astrocytes number in the HIE animals. The result can be related to the functional deficit, since the astrocytes alterations can cause

changes in neuron function even though there was no reduction in the number of these cells. In order to correlate these findings, we analyze the protein SYP in order to identify changes in plasticity in this region in consequence of neonatal HIE or exercise. The SYP is a transmembrane glycoprotein found in small presynaptic vesicles of the nerve cells and in microvesicles of the neuroendocrine cells. Literature data suggest that SYP is an important synaptic marker being widely used for analysis of plasticity (Kolos et al., 2015). Our results showed that the HIE animals presented more SYP than the control animals in the M1 ipsilateral to the lesion, without the exercise effect. The literature provides contradictory data about the effect of neonatal HIE on SYP expression. Previous studies using the same animal model of HIE have shown no alterations of SYP in hippocampus or showed a decrease of its expression in the brainstem (Zhao et al., 2012; Revuelta et al., 2017; Deniz et al., 2018). Contrary, a decrease in SYP immunoreactivity in the hippocampus have been identified in other studies (Markostamou et al., 2016; Griva et al., 2017). Griva and collaborators (2017) propose that the discrepancies observed in the literature indicate that synaptic structural and functional changes induced by HIE might vary among different brain regions and be dependent on the degree of brain maturity at the time of the HI insult. The increase in SYP content observed could reflect some compensatory synaptic changes in the cortex, results already observed by Tuor et al. (2001). In normal development, the synapse formation is major than pruning at early ages, however, this period it is following by a decline in the number of synapses, in consequence of selection, maturation, formation and synapse stabilization of neuronal circuits (Tang et al., 2014; Riccomagno et al., 2015). Apparently, the HIE disrupts this synaptic pruning, maintaining a greater number of synapses in the ipsilateral M1 to injury. This plastic alteration may also be correlated with the presence of motor deficits found in PMRT, because many synapses may end up disturbing the correct functioning of that region. However, more studies are needed to elucidate the mechanisms of plasticity involved in this apparent neuronal preservation of this important motor area and in that fine motor deficit found in this model of HIE.

4.4 Physical exercise did not show beneficial effects on the parameters evaluated

To conclude this discussion, we need to raise the question of the non-beneficial effect of physical exercise in this work. The physical exercise as a form of therapy against pathologies that affect the central nervous system is widely used and demonstrates satisfactory results in the experimental and clinical studies (Kim et al., 2014; Lovatel et al., 2014; Jacotte-Simancas et al., 2015). However, contrary to our initial hypothesis, none of the exercise modalities used in this study was able to alter the parameters analyzed in rats. We initially believed that the exercise would assist in the learning of HIE animals during the training phase. However, as previously seen, it was not possible to identify such benefits. At this point, we believe that since the lesion severely affects the regions involved in cognitive processing, the training proposed here might not be enough to modulate the changes needed to reverse this cognitive deficit. Another result that caught our attention is that we did not find effects of the exercises in reversing the motor deficits found in forelimbs of HIE animals in the PMRT. Our group had already demonstrated motor dysfunction in the horizontal ladder task in the same model, and in this case, the acrobatic exercise was capable to reverse these deficits (Confortim et al., 2019). We believe that the exercises used as training in this study mainly use/force the hindlimbs of the animals. Perhaps for this is not possible to observe the effects of training on this specific task.

In relation to the results related to cellular plasticity evaluation, it was also not possible to observe the benefits of any of the types of exercise performed and this is a result that goes against expectations. There is a consensus in the literature that the exercise induces plasticity, especially in the hippocampus (Knaepen et al., 2010). Several evidences demonstrated that physical exercise is responsible to increase neurogenesis, cell proliferation and dendritic branching (Cassilhas et al., 2016). In studies that utilized the exercise specifically after the neonatal HIE event, the results identified benefices in the spatial learning, sensory-motor function, decrease in the brain atrophy, apoptosis inhibition, neurogenesis and oligodendrogenesis activation (Park et al., 2013; Choi et al., 2013; Kim et al., 2017; Pak et al., 2018; Confortim et al., 2019). Some points may be raised to try to explain why exercise was not effective to aid plasticity in this study. We first believe that this divergence may be related to the

encephalic region evaluated in these studies. The studies cited above have evaluated the corpus callosum, substantia nigra, striatum, subventricular zone and hippocampus after HIE. To our knowledge, this is the first study to evaluate the effect of exercise on M1 after this type of lesion. As previously stated, this brain region seems not to be so affected by this insult, since it was not possible to identify neuronal loss in this region. With this, we cannot observe the effect of exercise as well. Another point to be raised is the duration and intensity of the training stimulus to which the animals were subjected in these different studies. In our study we chose a stimulus considered in the literature as mild to moderate, lasting on average 20 minutes a day and being performed for 4 weeks for both modalities, mimicking a therapeutic form of training. On the other hand, Pak et al. (2018) used a protocol to run on a treadmill performed 30 min once a day for 12 weeks and in a protocol of Kim et al. (2017), the rats were trained on a treadmill from P22 to the 8th week after birth. These two examples show that the intensity and duration of the exercise were different, which may also explain the divergence of the results.

We need to consider that the literature involving studies evaluating the effect of different types of exercise against HIE is still scarce, which makes it difficult to understand the effects of this type of strategy. We believe that more studies need to be done to clarify the mechanisms involved in the benefits found by this type of treatment widely used in patients in the clinic.

5. CONCLUSIONS

As a conclusion, our main results showed the neonatal HIE caused learning deficit and motor dysfunction on the affected forelimb in the PMRT. Additionally, the HIE animals present more SYP and a higher number of astrocytes in the M1 in the hemisphere ipsilateral to the lesion. Contrary to our initial hypothesis no benefits effects of exercises were found in these parameters. These findings demonstrated that the PMRT could be considered a good test to evaluate the motor function of animals submitted to neonatal hypoxia-ischemia, making it possible to demonstrate the fine motor dysfunction of the forelimbs of these animals. In relation to the cell plasticity, we

demonstrate the role of astrocytes and synaptophysin in the M1 in this model and the relation to these proteins with the motor function impaired. However, more studies are needed to elucidate these different findings related to motor function and new strategies to treat them.

CONTRIBUTORS

Bruna Ferrary Deniz, Wellington de Almeida, Patrícia Maidana Miguel, Adriana Souza dos Santos, Ethiane Segabinazi and Loise Bronauth conducted the experimental procedures; Heloisa Deola Confortim and Lenir Orlandi Pereira conceived the experiment and wrote the manuscript.

ACKNOWLEDGMENT

We are very grateful to PhD Matilde Achaval, founder of the Laboratório de Histofisiologia Comparada, main responsible for laboratory's structure used in this project. We would also like to thank Silvia Barbosa and Maikel Oliveira for the technical support at all stages of this work.

FUNDING

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance code 001, CNPq and FAPERGS.

DECLARATIONS OF INTEREST: none

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5 DISCUSSÃO GERAL

Este estudo foi proposto para investigar a função motora, aspectos morfológicos e proteínas relacionadas à morfologia celular e/ou plasticidade de estruturas envolvidas no controle motor em ratos Wistar machos submetidos à hipóxia-isquemia neonatal, e o possível efeito terapêutico de dois diferentes protocolos de exercícios físicos – aeróbico e acrobático. Nossos principais resultados mostraram que a hipóxia-isquemia neonatal causou atrofia cerebral, déficits de aprendizado, comprometimento na locomoção e menor utilização/preferência do membro afetado para o desenvolvimento da tarefa Pasta Matrix Reaching Task. Em relação à plasticidade celular, foi possível observar ainda um aumento na proteína sinaptofisina e no número de astrócitos no córtex motor dos ratos HIE no lado ipsilateral à lesão. Ao contrário da nossa hipótese inicial, não encontramos diferenças morfológicas no córtex sensoriomotor, no nervo isquiático, na medula espinal, nas junções neuromusculares e no músculo esquelético dos animais submetidos ao modelo de HIE neonatal. Um efeito benéfico do treinamento acrobático foi ainda evidenciado, demonstrado pela melhora na locomoção e diminuição da atrofia cerebral nos animais HIE que realizaram esta modalidade de exercício.

5.1 Animais submetidos à HIE neonatal realizaram com sucesso os diferentes tipos de exercício

Uma preocupação inicial de nosso estudo estava relacionada ao desempenho dos animais HIE em relação ao desenvolvimento dos diferentes protocolos de exercício físico propostos, especialmente os exercícios acrobáticos. No entanto, nossos resultados mostraram que os animais HIE não tiveram dificuldades em realizar nenhum dos dois tipos de exercício, havendo uma regularidade de desempenho entre os grupos. Foi perceptível ainda nas fases dois e três do treinamento acrobático (as fases mais avançadas e por consequência mais difíceis) que os grupos HIE realizaram o percurso mais rápido que os animais controles. Este resultado pode ser um indicativo de hiperatividade ou impulsividade, característica já evidenciada por nosso grupo de pesquisa neste modelo de HIE (MIGUEL et al., 2015, 2017; DENIZ et al.,

2018). Essa regularidade no treinamento evidenciada entre os grupos forneceu uma boa maneira de comparar os resultados, tornando-os replicáveis e consistentes.

Na literatura, diferentes tipos de exercício são utilizados experimentalmente e apresentam efeitos protetores frente a diferentes patologias que afetam o SNC. Em relação aos efeitos dos exercícios aeróbicos, principalmente os protocolos realizados em esteira, resultados benéficos são evidenciados. Este tipo de exercício foi capaz de modular funções das células gliais no giro denteado após isquemia cerebral global, preveniu alterações microvasculares induzidas por hipoperfusão cerebral crônica e foi responsável por induzir plasticidade em motoneurônios e no nervo isquiático em um modelo de paralisia cerebral (STIGGER et al., 2011; LOVATEL et al., 2014; LEARDINI-TRISTÃO et al., 2017). Em estudos utilizando animais submetidos ao mesmo modelo de HIE neonatal utilizado neste trabalho e que realizaram exercício físico em esteira também revelam resultados benéficos, mostrando redução da morte neuronal no hipocampo, substância negra e estriado, preservação da função sensório-motora e melhoria na capacidade de aprendizagem espacial (CHOI et al., 2013; PARK et al., 2013). Embora o treinamento acrobático utilizado neste estudo tenha sido menos estudado diante de patologias cerebrais, sabe-se que sua prática também é responsável pela ativação de regiões importantes do encéfalo. Em estudo publicado por Garcia et al. (2012) o efeito do exercício em esteira e do exercício acrobático foram comparados em animais sem nenhum tipo de lesão. Os resultados mostraram que esses diferentes tipos de exercício são responsáveis pela ativação de diferentes regiões encefálicas. O exercício em esteira promoveu alterações especificamente no cerebelo. Já os exercícios acrobáticos foram responsáveis por induzir alterações na expressão de proteínas sinápticas e estruturais no córtex motor e estriado. Como o treinamento acrobático, também conhecido como treinamento de habilidades motoras, envolve mudanças em regiões cerebrais diretamente relacionadas ao controle motor, acreditamos que este tipo de exercício poderia ser uma boa opção terapêutica frente ao modelo de HIE neonatal. Ainda neste contexto, opções de reabilitação que envolvem desafios, jogos e a mudança de ambientes são muito interessantes especialmente para a terapia com crianças, pois facilitam a adesão ao tratamento, favorecendo assim melhores resultados. Adicionalmente, este tipo de

modalidade é uma opção de baixo custo e que pode ser facilmente adaptada aos diferentes ambientes disponíveis pelos profissionais da saúde.

5.2 O exercício acrobático reverteu a hiperatividade e o comportamento semelhante à ansiedade causados pela hipóxia-isquemia neonatal

Iniciamos nossa investigação através da utilização de testes comportamentais que avaliassem a função motora de forma mais abrangente. O teste de campo aberto é um teste conveniente para avaliar a locomoção geral e também comportamentos semelhantes à ansiedade. Em roedores, o comportamento de ansiedade no campo aberto é desencadeado por dois fatores: o primeiro é o isolamento, uma vez que os animais realizam o teste individualmente e o segundo se deve ao novo ambiente enfrentado pelos animais durante a duração do teste (PRUT; BELZUNG, 2003). No presente estudo, não foi possível evidenciar déficits de locomoção causados pela HIE através da utilização deste teste comportamental. Porém, evidenciamos que o grupo HIE obteve um maior número de cruzamentos no teste do campo aberto quando comparado a todos os outros grupos. Além disso, os grupos HIE e HIET ficaram mais tempo na periferia do aparato, quando comparados ao grupo HIEA. Esses resultados indicam hiperatividade e comportamento semelhante à ansiedade promovidos pela HIE neonatal e revertidos pelo exercício acrobático.

Características de hiperatividade e comportamento semelhante à ansiedade já foram relatados previamente em outros estudos que utilizaram o mesmo modelo de HIE (CARLETTI et al., 2012; ROJAS et al., 2013; DENIZ et al., 2018). Miguel e colaboradores (2015, 2017) sugeriram ainda que o modelo de HIE proposto por Rice-Vannuci poderia ser considerado como um modelo de transtorno de déficit de atenção/hiperatividade, o que vai ao encontro do comportamento de hiperatividade observado nos animais HIE. É importante considerar que as características de hiperatividade encontradas no grupo HIE podem ser um comportamento de risco para os roedores, uma vez que uma atividade exacerbada pode levar a consequências negativas para o animal, como o encontro com um predador, por exemplo.

Em relação ao efeito protetor do exercício, é amplamente difundido que o treinamento físico exerce benefícios físicos e também psicológicos. Assim como visto em humanos, uma série de estudos mostra que a prática de exercício é responsável por diminuir os níveis de ansiedade em roedores (BINDER et al., 2004; DUMAN et al., 2008; UYSAL et al., 2018). No presente estudo, o treinamento acrobático foi o principal responsável por reverter tanto o comportamento de hiperatividade quanto o de ansiedade, após o insulto causado pela HIE neonatal. Uma possível explicação para a diminuição da hiperatividade e ansiedade nos animais que realizam exercícios acrobáticos pode estar associada a não aversão ao novo ambiente, uma vez que esses animais foram estimulados e treinados em diferentes ambientes e desafios durante o protocolo de exercício.

Os processos cognitivos usados neste teste influenciam ainda a maneira como os animais adquirem, armazenam e lembram informações e sustentam comportamentos como decidir onde procurar por comida, quando devem se esconder de um predador ou com quem acasalar (GUILLETTE et al., 2015). Assim, mudanças nesse comportamento exploratório inato que foram identificadas no grupo HIE podem influenciar negativamente o seu desenvolvimento. Em contrapartida a alteração comportamental causada pelo exercício neste modelo traz benefícios diretamente relacionados à sobrevivência desses animais. Contudo, estudos futuros devem ser realizados a fim de avaliar o comportamento de ansiedade usando tarefas mais sensíveis e assim fornecer dados adicionais sobre o potencial do exercício acrobático para melhorar os parâmetros emocionais.

5.3 A hipóxia-isquemia neonatal causou déficits motores e de aprendizado e o treinamento acrobático reverteu parte destes déficits

Como não conseguimos encontrar déficits motores oriundos da HIE no teste de Campo Aberto, considerado um teste para avaliar comportamento locomotor geral, posteriormente utilizamos testes mais específicos para avaliar a função motora dos animais afim de identificar déficits provenientes da HIE e o possível efeito do exercício. Os testes adicionais adotados foram: o rotarod, o teste da escada horizontal e o Pasta

Matrix Reaching Task (PMRT). Para avaliar a coordenação motora dos animais, utilizamos o teste do rotarod. Este teste foi projetado inicialmente para avaliar o desempenho motor e é amplamente utilizado para avaliar o equilíbrio de roedores (SHIOTSUKI et al., 2010). Não encontramos diferenças entre os grupos quanto à coordenação motora nos parâmetros analisados neste teste resultantes da HIE e nem efeitos do exercício físico. Não há consenso na literatura sobre comprometimento motor no modelo HIE medido pelo teste do rotarod, consequentemente os resultados encontrados são divergentes. Griva et al. (2017) e Dai et al. (2017) encontraram déficit de equilíbrio em animais submetidos a HIE. Porém, Rojas e colaboradores (2013) não encontraram diferenças entre os animais HIE e os controles utilizando esse mesmo teste. Segundo Shiotsuki et al. (2010) o teste rotarod é mais sensível para detecção de alterações no cerebelo. No entanto, essa estrutura parece ser menos afetada pelo modelo HIE que utilizamos, uma vez que não há relatos de atrofia nessa estrutura na literatura e não observamos diferenças no equilíbrio dos animais durante o treinamento acrobático. Estas evidências sugerem uma possível explicação sobre os resultados contrastantes observados neste teste.

A tarefa da escada horizontal foi utilizada para avaliação da utilização dos membros anteriores e posteriores dos animais após o evento hipóxico-isquêmico. Esta tarefa foi proposta inicialmente para avaliação dos membros em ratos após diferentes tipos de lesão cerebral (METZ; WISHAW, 2002). Nossos achados indicam que este teste parece ser sensível também para a lesão HIE, uma vez que os ratos lesionados apresentaram um maior número de erros nesta tarefa quando comparados ao grupo controle. O comprometimento motor apresentado no teste da escada horizontal também foi identificado em estudos prévios realizados por Durán-Carabali et al. (2017) e Schuch et al. (2016b) utilizando este mesmo modelo de HIE. Nossos resultados mostraram ainda que o exercício acrobático produziu um efeito benéfico recuperando a função motora nos animais HIE, mesmo com o protocolo de exercícios começando duas semanas após a lesão. Esse resultado indica que esse tipo de exercício parece ser uma boa opção de tratamento para os indivíduos afetados por essa patologia, mesmo quando não se consegue iniciar uma intervenção terapêutica precoce, ou quando esses indivíduos são diagnosticados mais tardiamente.

É importante ressaltar ainda que esta atividade é proposta para mimetizar técnicas de estimulação já utilizadas na clínica, por profissionais de saúde, especialmente fisioterapeutas. Esses resultados reforçam então o uso desses protocolos de tratamento, por serem opções não-farmacológicas, fáceis de se reproduzir no ambiente clínico e que podem demonstrar resultados satisfatórios para o tratamento de distúrbios neurológicos. Sigmundson et al. (2017) estabelece que a interação entre a pessoa, o ambiente e novas tarefas pode mudar a maneira como os movimentos são estabelecidos, causando mudanças no desenvolvimento motor. Evidências atuais demonstram que para a aprendizagem de novas habilidades motoras, como ocorre durante o treinamento acrobático, ocorre aumento nas conexões entre certas áreas do encéfalo e consequentemente plasticidade (KIDA et al., 2016). Explorando ainda essa estratégia de reabilitação, Sidaway e colaboradores (2012) afirmam que os terapeutas devem manipular a dificuldade dos parâmetros da tarefa aplicada no ambiente clínico, a fim de gerar um nível adequado de dificuldade funcional para cada paciente, obtendo assim otimização na aprendizagem motora e melhores resultados. Kline et al. (2016) demonstraram também que o uso de terapias combinadas pode ser mais benéfico após lesões cerebrais traumáticas. Os achados do presente estudo chamam a atenção para a importância das pesquisas translacionais. Ensaios pré-clínicos podem contribuir para justificar o uso de intervenções não-farmacológicas que já são utilizadas ou que ainda estão em fase de teste, comprovando assim seus benefícios funcionais e demonstrando os alvos neurobiológicos envolvidos.

A fim de confirmar os déficits motores encontrados no teste da escada horizontal e o efeito do exercício acrobático, optamos por avaliar com mais precisão a capacidade motora e a preferência/uso dos membros anteriores dos animais submetidos à HIE através do teste PMRT. Segundo Sloan e colaboradores (2015), as avaliações dos membros anteriores são críticas para examinar o status do sistema motor, entender o aprendizado motor e a recuperação após o dano em ratos. A tarefa PMRT foi proposta inicialmente por Ballerman et al., (2001) como um teste para investigar o uso dos membros anteriores de ratos após lesão medular. Através deste teste, é possível avaliar a motricidade dos membros anteriores, incluindo movimento,

ajustes de distâncias e direções, lateralidade e força. A tarefa já foi aplicada também a modelos de acidente vascular cerebral (AVC) e tem sido útil na avaliação do resultado funcional em outros modelos de lesão da extremidade anterior (KERR; TENNANT, 2014).

Esta tarefa é caracterizada inicialmente por uma fase de treinamento. Esta fase envolve a aprendizagem dos animais e a capacidade para realizar corretamente as fases seguintes do teste (BALLERMAN et al., 2001). Se o animal não é capaz de entender o que deve ser feito neste estágio, o teste perde sua capacidade de identificar os déficits motores, tornando-se inútil. Nesta fase foi possível identificar um déficit de aprendizado nos animais com HIE em nosso estudo, indicado por mais dias necessários para aprender o teste em comparação aos animais controle e por mais tempo em cada tentativa para quebrar os 10 pedaços de macarrão (critério usado para considerar o animal capacitado para a realização do teste). Esse déficit cognitivo encontrado nos animais HIE já era esperado, pois déficits de aprendizado e memória são encontrados frequentemente neste modelo de HIE neonatal. Deniz e colaboradores (2018) e Carletti et al. (2016) observaram déficits de memória em ratos submetidos a HIE no labirinto aquático de Morris. Os resultados desses estudos mostraram que os animais HIE necessitaram de um maior número de dias para aprender a tarefa e apresentaram maior latência durante a fase de aprendizagem, respectivamente. Em um estudo de Miguel et al. (2017) que utilizaram o mesmo modelo de HIE, os animais foram testados em uma tarefa de atenção, o Attentional set-shifting, e foi possível observar que animais HIE necessitaram de um maior número de tentativas para atingir o critério de seis execuções corretas consecutivas em uma das fases do teste, comprovando assim os déficits cognitivos em comparação aos animais controles. Déficits de aprendizado e memória após a lesão por HIE também foram encontrados por Rojas e colaboradores (2013; 2015) através da tarefa do Ox-maze e do reconhecimento de objetos.

Esses resultados provavelmente estão relacionados às áreas do encéfalo preferencialmente afetadas pelo modelo de HIE. É consenso na literatura que o hipocampo é extremamente afetado pelo modelo HIE neonatal, sendo uma das regiões que sofrem maior atrofia (MIGUEL et al., 2015). Uma das razões para essa

vulnerabilidade parece estar relacionada à grande quantidade de receptores glutamatérgicos nessa região do encéfalo (CHOI; ROTHMAN, 1990). O insulto hipóxico-isquêmico inicial é caracterizado por uma diminuição do suprimento sanguíneo e este é o fator desencadeante da lesão neuronal, uma vez que as células se tornam incapazes de satisfazer suas demandas metabólicas (NOVAK et al., 2018). Este período é seguido por uma diminuição no suprimento de glicose e uma deficiência em fosfatos de alta energia (ATP) que resultam em uma lesão secundária (DISTEFANO; PRATICO, 2010). Em resposta a essas lesões celulares, os aminoácidos excitatórios glutamato e aspartato são liberados de maneira exacerbada no encéfalo, exercendo assim um efeito excitotóxico (BURD et al., 2016). Devido à falha energética causada pela HIE, o processo de recaptação desses neurotransmissores também é prejudicado, causando hiperativação desses receptores glutamatérgicos (MILLAR et al., 2017). Essa hiperativação resulta em acúmulo de cálcio intracelular, causando morte celular e microglial, e consequente liberação de fatores prejudiciais às células neurais vizinhas (GALLUZI et al., 2009). Essa prevalência desses receptores nessa região diretamente relacionada ao processamento cognitivo explica esse déficit de aprendizagem já bem consolidado no modelo. É importante lembrar que, mesmo com o atraso no aprendizado encontrado nos grupos HIE, todos os animais conseguiram alcançar os critérios necessários para passar às outras fases do teste. Assim, acreditamos que o teste de PMRT pode ser considerado um bom teste para a avaliação das condições motoras do membro anterior neste modelo de HIE neonatal.

Em relação à função motora especificamente, os resultados evidenciados pela tarefa PMRT mostraram que não houve diferenças significativas entre os grupos na contagem de pedaços de macarrão quebrados em nenhuma fase do teste. No entanto, quando avaliamos mais detalhadamente essas diferentes fases do teste, os resultados evidenciaram que os animais HIE apresentaram menor preferência da utilização do membro anterior afetado pela lesão e, consequentemente, menor sucesso quando este membro foi utilizado em todas as fases do teste. Curiosamente, na fase dois, quando era mais confortável a utilização da pata anterior direita (afetada pela HIE) para quebrar os pedaços de macarrão, os animais HIE continuaram usando

a pata anterior esquerda, evidenciando assim a incapacidade de usar a pata anterior afetada. Ao contrário de nossa hipótese inicial, não foram encontrados efeitos benéficos do exercício neste teste.

Resultados semelhantes já foram encontrados anteriormente em outros modelos de lesão cerebral que utilizaram essa mesma tarefa para avaliar a função motora fina dos animais após diferentes tipos de lesão encefálica. Em um modelo de hemi-Parkinson, quando os ratos foram testados através do PMRT, não foram encontradas diferenças no número médio de pedaços de macarrão quebrados pelos animais quando era permitido usar ambos os membros anteriores. No entanto, no mesmo estudo, quando os ratos foram forçados a utilizar o membro anterior comprometido, o grupo submetido à lesão mostrou-se prejudicado em relação ao grupo controle (METZ et al., 2001). Camundongos submetidos à isquemia focal no córtex sensoriomotor também mostraram déficits de alcance na tarefa PMRT. Antes da indução da lesão, a maioria dos camundongos era capaz de alcançar pedaços de macarrão localizados anteriormente e lateralmente à abertura do aparato. Após a indução da lesão, a extensão em que os camundongos conseguiram alcançar os pedaços de macarrão da região anterior e, principalmente na região lateral da matriz do aparato foi limitada (TENNANT; JONES, 2009). A tarefa do PMRT também já foi usada previamente como reabilitação em camundongos jovens e idosos após indução de AVC (TENNANT et al., 2015). Após lesões isquêmicas focais, camundongos idosos apresentam déficits semelhantes aos observados em sobreviventes de AVC humano, incluindo deficiências no uso coordenado e habilidade da extremidade superior contralateral. Os resultados encontrados por Tennant et al. (2015) sugerem que o treinamento específico utilizando a tarefa PMRT pode ser uma maneira especialmente eficaz de induzir uma melhora comportamental das extremidades superiores em camundongos sobreviventes de AVC e conduz a plasticidade em áreas pré-motoras no encéfalo de camundongos jovens.

Para o nosso conhecimento, este é o primeiro trabalho que usa a tarefa PMRT para avaliar a função motora de ratos em um modelo de HIE. O uso de novas tarefas que avaliam a condição motora de maneira mais cuidadosa, como neste modelo, se faz necessário, uma vez que ainda existe uma discrepância em relação ao

comprometimento motor no modelo Rice-Vannucci (ROJAS et al., 2013; MIGUEL et al., 2015; ARTEAGA et al., 2017; GRIVA et al., 2017; DAI et al., 2017; DURÁN-CARABALI et al., 2017; PAK et al., 2018). Os resultados encontrados neste trabalho mostram que apesar de haver essa discrepância, déficits motores podem ser demonstrados através de testes mais específicos. Assim, acreditamos que o teste de PMRT é uma boa escolha para identificar os déficits motores causados nos animais após a indução de hipóxia-isquemia neonatal, pois mimetiza o mesmo déficit motor encontrado em humanos em patologias que atingem o encéfalo de maneira unilateral.

Resumindo os resultados comportamentais, o modelo de HIE neonatal produziu efeitos negativos sobre a aprendizagem e a função motora dos animais, especialmente em relação à utilização do membro anterior afetado pela lesão, sem prejuízo para o equilíbrio. Mostramos também que os exercícios acrobáticos foram capazes de restabelecer a função motora no teste da escada horizontal, porém não encontramos efeito benéfico de nenhum dos tipos de exercício no teste PMRT. Considerando a inconsistência nos resultados encontrados em relação à função motora e em relação à capacidade terapêutica do exercício, o próximo passo deste estudo foi investigar a morfologia dos componentes envolvidos no controle motor, a fim de identificar possíveis explicações para esses resultados divergentes.

5.4 O exercício acrobático diminuiu a atrofia cerebral causada pela HIE neonatal

Com o intuito de detectar possíveis alterações em componentes do TCE, desenvolvemos um estudo exploratório dos principais componentes da via corticospinal e das estruturas periféricas associadas à motricidade. Primeiramente, realizamos uma medida bruta investigando a atrofia encefálica total. Nossos resultados evidenciaram que a HIE neonatal causou danos consideráveis no hemisfério ipsilateral à oclusão arterial e o treinamento acrobático foi capaz de reduzir essa atrofia. A extensa atrofia no hemisfério ipsilateral à lesão é demonstrada expressivamente na literatura neste modelo de HIE (PEREIRA et al., 2007, 2008; MIGUEL et al., 2015; SCHUCH et al., 2016a; CARLETTI et al., 2016). Curiosamente, o exercício acrobático impediu o avanço da atrofia, mesmo com a terapia começando

duas semanas após a lesão. Neste momento, a fase aguda da HIE neonatal já havia ocorrido causando morte celular, e o exercício parece ter aliviado a progressão da lesão, parecendo exercer efeitos de neuroplasticidade. Os resultados funcionais apresentados anteriormente neste estudo também indicaram que o exercício acrobático foi capaz de recuperar parte da função motora nos animais HIE.

Diante desses achados, algumas hipóteses podem ser colocadas para compreender os mecanismos envolvidos nessa proteção provocada pelo exercício. Está bem estabelecido que a manutenção da estrutura vascular é um ponto crítico para uma boa função encefálica. Sabe-se também que esta vasculatura pode ser perturbada em uma variedade de patologias que afetam o suprimento sanguíneo ou o microambiente do tecido nervoso (ABBOT et al., 2010). Nesse contexto, a prática de exercícios físicos é responsável por promover angiogênese e esse processo é considerado um pré-requisito para muitas formas de plasticidade neural e comportamental, particularmente em condições patológicas (BERGGREN et al., 2014). A literatura também mostra também que o exercício físico é capaz de diminuir a disfunção da barreira hematoencefálica (BHE), melhorando a função astrocitária após um AVC isquêmico (WANG et al., 2014). A combinação desses dois fatores pode ser decisiva para os benefícios do exercício encontrados neste trabalho. Um estudo anterior conduzido por nosso grupo de pesquisa já mostrou que este modelo de HIE neonatal é responsável por alterações na BHE, tornando essa barreira menos eficiente (DIAZ et al., 2017). O exercício acrobático pode ter restaurado a função da BHE, estabelecendo um microambiente favorável para a angiogênese no hemisfério afetado. Esta hipótese é fundamentada por evidências atuais que mostram que a prática de exercício físico aumenta o fator de crescimento endotelial vascular (VEGF) e o fator neurotrófico derivado do encéfalo (BDNF) no córtex e no hipocampo. Além disso, estudos mostram que quando o exercício é voluntário, esse aumento pode ser mais expressivo (UYSAL et al., 2015). Essa via poderia explicar como o exercício protege parcialmente o encéfalo dos animais HIE, restabelecendo os níveis de oxigênio e, consequentemente, prevenindo a progressão da lesão. A modulação desses mecanismos de plasticidade também já foi demonstrada em estudos que envolvem exercícios e outras estratégias terapêuticas não-farmacológicas. Rojas e

colaboradores (2013) relataram que o enriquecimento ambiental foi responsável por aumentar a densidade dos espinhos dendríticos em ratos HIE. Estudos em roedores saudáveis submetidos a diferentes protocolos de exercícios também mostraram aumento na densidade dos espinhos dendríticos em regiões como o hipocampo, o córtex entorrinal e o cerebelo (STRANAHAN et al., 2007; PETZINGER et al., 2013). A modulação desses espinhos dendríticos é muito importante para a plasticidade e alterações nestes espinhos podem estar associadas a uma reformulação nas vias de comunicação, garantindo assim uma maior eficiência no controle motor (LÜSCHER et al., 2000). Assim, propomos que o exercício exerça um efeito protetor promovendo um ambiente favorável para prevenir a morte celular e/ou preservar as células sobreviventes.

Outro fato interessante que precisa ser levado em consideração em nossos achados é o efeito terapêutico divergente encontrado nos diferentes tipos de exercício testados: aeróbico e acrobático. Nossos resultados evidenciaram um efeito benéfico do exercício acrobático na função motora e na reversão da atrofia quando comparado ao exercício em esteira, um tipo de exercício aeróbico. Evidências mostram que em ratos após isquemia, o exercício de baixa intensidade poderia ser melhor para a plasticidade sináptica do que o exercício de alta intensidade, promovendo um aumento mais significativo nos níveis de BDNF no hipocampo (SHIH et al., 2013). Talvez, o exercício aeróbico realizado na esteira, mesmo em intensidade moderada, possa causar mais estresse aos animais, mascarando assim os benefícios do exercício no presente estudo. Em conjunto, podemos sugerir que mais atenção deve ser dada para o treinamento de aprendizagem de habilidades motoras como coadjuvante nos processos de reabilitação neurológica em pacientes humanos com HIE, particularmente em crianças, visto que os resultados encontrados neste estudo fornecem evidências de que ele apresenta resultados mais satisfatórios, quando comparados aos exercícios aeróbicos.

5.5 A hipóxia-isquemia neonatal não causou alterações morfológicas nos componentes do controle motor

Em relação à exploração mais detalhada dos componentes da via corticoespinal e do controle motor, não encontramos diferenças na quantificação neuronal no córtex, no número e na área de motoneurônios da medula espinal, na análise do nervo isquiático, nas junções neuromusculares e nas fibras musculares do músculo plantar de ratos após HIE neonatal. Essas descobertas serão discutidas juntas, uma vez que são interdependentes.

Como já citado acima, uma extensa atrofia cerebral é comumente relatada no modelo de HIE neonatal (PEREIRA et al, 2007; CARLETTI et al. 2012). No entanto, quando analisamos com mais detalhes os estudos morfológicos já realizados, podemos observar que a porcentagem de atrofia varia de acordo com cada região, sendo maior no hipocampo e não tão significativa no córtex (MIGUEL et al., 2015). O hipocampo é uma estrutura seletivamente vulnerável a uma variedade de insultos metabólicos e citotóxicos, e uma possível explicação para essa vulnerabilidade como descrito anteriormente, parece estar relacionada à distribuição dos receptores glutamatérgicos nessa região (CHOI; ROTHMAN, 1990). Os mecanismos de excitotoxicidade, assim como o estresse oxidativo relacionado ao aumento dos níveis de glutamato, desempenham papéis cruciais nas lesões neuronais implicadas na patogênese de muitos distúrbios neurológicos, incluindo a lesão cerebral perinatal associada à hipóxia-isquemia (BURD et al., 2016). O estudo de Miguel e colaboradores (2015) identificou perda de 50% do volume do hipocampo neste modelo de HIE em comparação a uma atrofia cortical em torno de 20%. Esses achados corroboram com os dados do presente estudo que mostram uma densidade neuronal do córtex semelhante entre os grupos controle e HIE.

A vulnerabilidade específica do hipocampo justifica os achados nessa estrutura em vários estudos sobre danos cerebrais neonatais. Por outro lado, não há consenso sobre a lesão no córtex, na medula espinal ou mesmo em outros elementos relacionados à função motora em ratos após HIE. Divergindo dos nossos resultados, um estudo realizado por Bellot e colaboradores (2014) relatou distúrbios bioaminérgicos no tronco encefálico e na medula espinal de camundongos HIE.

Durán-Carabali et al. (2017) relataram também uma redução na área de fibras musculares em ratos neste mesmo modelo.

A lesão induzida por HIE pode ser descrita como um espectro, variando de “moderada” a “severa” (RUMAJOGEE et al., 2016). Tal variabilidade é baseada principalmente no período crítico em que ela ocorre, uma vez que o encéfalo nessa fase está se desenvolvendo e, como consequência, a plasticidade neuronal é aumentada nesta fase (JOHNSTON, 2009). O sistema nervoso de animais jovens se recupera mais rapidamente e efetivamente do que o dos adultos, porque é considerado mais “plástico, justificando a preservação de algumas estruturas cerebrais em ratos HIE (JAMES-ROBERTS, 1979). Além disso, outro possível motivo da resposta plástica em neonatos pode ser a vascularização eficiente nesse período.

Após a oclusão da artéria carótida no modelo HIE, um efeito compensatório foi observado devido à presença do círculo de Willis, que permite a comunicação entre os dois hemisférios cerebrais (COOK, 1965; DORR et al., 2007; RUMAJOGEE et al., 2016). Essa compensação espontânea foi melhor descrita em um estudo recente realizado por Edwards et al. (2017) que identificaram uma perfusão retrógrada através de duas vias principais: primeiramente, via ramos faciais, orbitais e meníngeos, originados da artéria carótida externa; e em segundo lugar, através de um fluxo sanguíneo colateral através da artéria carótida externa por ramos anastomóticos com as artérias tireoidianas ou occipitais superiores e inferiores. Consequentemente, essas alterações na perfusão podem explicar as inconsistências associadas ao procedimento criado por Rice-Vannucci. Possivelmente, essa compensação do fluxo sanguíneo, juntamente com a plasticidade específica do período, pode contribuir para os achados variáveis no tecido nervoso nos ratos recém-nascidos.

Em resumo, os achados morfológicos encontrados neste trabalho indicam que as estruturas relacionadas à função motora no sistema nervoso central e nos componentes periféricos não são as principais estruturas afetadas. Indo de encontro com os achados morfológicos, a função motora também sofre alterações variáveis dentro dos diferentes estudos. Embora o modelo de Rice-Vannuci tenha sido considerado um modelo de asfixia perinatal, talvez seja necessário avaliar se esse modelo é de fato adequado para estudar as disfunções motoras, da mesma forma que

são vistas na paralisia cerebral. Em humanos, o achado mais comum da paralisia cerebral é o distúrbio nas funções motoras pela presença de movimentos espásticos (AARTS et al., 2010). No entanto, nos animais HIE a presença de espasticidade não é observada (ROBINSON, 2013). Os ratos submetidos à HIE não demonstram também dificuldades para realizar habilidades motoras comuns, como locomoção, alimentação e natação, e estes resultados são evidenciados através dos diferentes testes realizados neste modelo (PEREIRA et al., 2008; MIGUEL et al., 2015; SCHUCH et al., 2016a; CARLETTI et al., 2016; DIAZ et al, 2016, DENIZ et al., 2018). Essa afirmação é ratificada no presente estudo, uma vez que os animais não apresentaram dificuldades de locomoção, mesmo apresentando déficits motores sutis, e conseguiram realizar os diferentes protocolos de exercício físico. O impacto funcional e morfológico divergente desse evento hipóxico-isquêmico em roedores e humanos deve ser cuidadosamente considerado, porque as estruturas envolvidas no controle motor em ambas as espécies demonstram também algumas diferenças. Uma revisão recente relata que os roedores têm menos conexões diretas entre o córtex motor e os neurônios motores no corno anterior da medula espinal e também um menor número de axônios que decussam na medula para o lado contralateral, o que talvez possa explicar em partes as diferenças encontradas (RUMAJOGGE et al., 2016).

Em nosso estudo, não encontramos lesões no sistema nervoso central (córtex e medula espinhal). Naturalmente, também não observamos alterações no nervo, nas junções neuromusculares e no músculo esquelético. Em outras palavras, os efetores do movimento também não foram afetados, uma vez que a via central de controle permaneceu preservada. Ainda, o modelo HIE não causou danos às estruturas envolvidas no controle motor e, consequentemente, o efeito protetor do exercício não pode ser visto nessas estruturas. Com os achados até o momento não conseguimos responder como esta preservação estrutural ocorre na via motora e qual a relação desta preservação com os diferentes resultados motores encontrados neste modelo. Assim o próximo passo deste trabalho foi investigar possíveis células e/ou mecanismos de plasticidade no córtex motor, considerado o início da via motora, a fim de buscar uma explicação para os resultados encontrados até o momento.

5.6 Animais submetidos à HIE neonatal apresentaram maior número de astrócitos e mais sinaptofisina no córtex motor primário ipsilateral à lesão

Para identificar possíveis células e/ou mecanismos de plasticidade relacionados a esta preservação estrutural na via motora e uma possível explicação para a divergência relacionada ao comprometimento motor e ao efeito do exercício frente à patologia causada pela HIE, avaliamos através da imunohistoquímica, os neurônios (NeuN), os astrócitos (GFAP) e possíveis alterações sinápticas (SYP) no M1 ipsilateral e contralateral à lesão. Nossos resultados mostraram que os animais HIE apresentam mais SYP e maior número de astrócitos (células marcadas por $GFAP^{+}$) do que os animais controles no hemisfério ipsilateral à lesão no M1, sem efeito de exercício.

O M1 é uma estrutura chave para o controle de movimentos voluntários e na aprendizagem de habilidades motoras. A elaboração de respostas adequadas a diferentes situações comportamentais está diretamente ligada à capacidade dessa região de selecionar respostas motoras apropriadas de acordo com o ambiente (VITRAC; BENOIT-MARAND, 2017). Em humanos, essa região é fortemente afetada pela hipóxia-isquemia neonatal, desencadeando assim déficits motores expressivos (BARNETT et al., 2002; STEINMAN et al., 2009; MARTINEZ-BIARGE et al., 2011). No entanto, no modelo Rice-Vannuci, essa região do encéfalo parece menos afetada, quando comparamos os resultados encontrados no hipocampo, por exemplo. Essa afirmação é confirmada quando não foi possível observar diferenças no número de neurônios nesta região encefálica, nem através da técnica de Nissl e nem pela marcação mais específica através da imunohistoquímica com NeuN neste estudo.

Ao contrário dos nossos achados, a maioria dos estudos que analisaram a densidade neuronal encontraram uma diminuição nos neurônios devido à HIE neonatal em diferentes regiões cerebrais. Deniz et al. (2018) mostraram que ratos HIE diminuíram a densidade neuronal no hipocampo ipsilateral à lesão. Kim et al., (2017) e Kim et al. (2018) demonstraram uma redução significativa da proteína NeuN no grupo HIE na zona subventricular contralateral e no córtex cerebral, respectivamente. Comparado com o grupo controle, os níveis de expressão da proteína NeuN também foram reduzidos no encéfalo nos resultados encontrados por Qiao et al. (2018). Esses

achados nos levaram a investigar outras populações de células cerebrais, a fim de buscar explicações para as alterações funcionais encontradas em nosso estudo.

Embora nenhuma perda neuronal no M1 tenha sido observada no modelo HIE, um número maior de astrócitos foi identificado nesses animais através da imunohistoquímica para GFAP. Esse distúrbio na interação neurônio-glia pode ser um dos mecanismos responsáveis pelos déficits funcionais encontrados. A GFAP é considerada um dos melhores marcadores para a ativação de astrócitos após lesão ou estresse no SNC (THEODOSIS; POULAIN, 2008; ZHANG et al., 2017). Quando ocorre um dano neurológico ou um processo que envolve neuroplasticidade, os astrócitos se tornam reativos para proteger e/ou regular o microambiente sináptico das células do SNC (COLEMAN et al., 2004; DONG, GREENOUGH, 2004). Um aumento na expressão da GFAP pode indicar hiperplasia ou hipertrofia e pode ser seguido por alterações no número ou na arborização dendrítica (SOFRONIEW; VINTERS, 2010). Altos níveis de GFAP também têm sido frequentemente associados à formação de uma barreira mecânica, interferindo então negativamente nas funções celulares do SNC (BURDA; SOFRONIEW, 2014). Estudos anteriores mostram aumento da expressão de GFAP após a indução de HIE neonatal em diferentes modelos, em diferentes regiões do encéfalo (SALMASO et al., 2012; KIM et al., 2018). A expressão desta proteína não se alterou neste estudo. No entanto, encontramos um maior número de células $GFAP^+$. O aumento do número de astrócitos pode ser um indicativo da lesão causada pela HIE e pode estar relacionado também ao déficit funcional, já que alterações nos astrócitos podem causar alterações na função neuronal, mesmo não ocorrendo redução no número dessas células.

A fim de correlacionar esses achados, analisamos também a proteína SYP para identificar alterações na plasticidade nessa região em consequência da HIE ou do exercício. A SYP é uma glicoproteína transmembrana encontrada em pequenas vesículas pré-sinápticas das células nervosas e nas microvesículas das células neuroendócrinas, e é amplamente utilizada para análise de plasticidade (KOLOS et al., 2015). Em relação à análise da SYP, nossos resultados mostraram que os animais dos grupos HIE apresentaram mais SYP do que os animais controles no M1 ipsilateral à lesão, sem efeito do exercício. A literatura fornece dados contraditórios sobre o efeito

da HIE neonatal na expressão de SYP. Estudos prévios utilizando o mesmo modelo animal não mostraram alterações de SYP no hipocampo ou mostraram diminuição de sua expressão no tronco cerebral (ZHAO et al., 2012; REVUELTA et al., 2017; DENIZ et al., 2018). Em contrapartida, uma diminuição de SYP foi identificada no hipocampo em outros estudos (MARKOSTAMOU et al., 2016; GRIVA et al., 2017). Griva e colaboradores (2017) propõem que as discrepâncias observadas na literatura indicam que as alterações sinápticas estruturais e funcionais induzidas pela HIE podem variar entre diferentes regiões cerebrais e ser dependentes do grau de maturidade cerebral no momento da lesão. O aumento no conteúdo de SYP observado em nosso trabalho poderia refletir alterações sinápticas compensatórias no córtex, para tentar suprir os efeitos da lesão, resultados já observados por Tuor et al. (2001). Durante o desenvolvimento normal, a formação de sinapses é maior que a poda sináptica em idades precoces, no entanto, este período é seguida de declínio no número de sinapses, em consequência da seleção, maturação, formação e estabilização de sinapses nos circuitos neuronais (TANG et al., 2014; RICCOMAGNO et al., 2015). Aparentemente, a HIE pode interromper essa poda sináptica, mantendo um número maior de sinapses no M1 ipsilateral à lesão. Essa alteração plástica também pode estar correlacionada com a presença dos déficits motores encontrados, pois um número exacerbado de sinapses pode acabar atrapalhando o funcionamento correto daquela região encefálica, no caso o córtex motor, região diretamente relacionada ao controle dos movimentos dos membros. No entanto, mais estudos são necessários para elucidar os demais mecanismos de plasticidade envolvidos nessa preservação neuronal desta importante área motora encontrada neste modelo de HIE.

5.7 O exercício físico não exerceu efeitos benéficos sobre a aprendizagem e sobre a plasticidade celular

Para concluir esta discussão, precisamos levantar a questão do não aparecimento de efeitos benéficos do exercício em relação a aprendizagem e a plasticidade neste trabalho. Inicialmente, acreditávamos que o exercício ajudaria na aprendizagem dos animais da HIE durante a fase de treinamento na tarefa PMRT. No

entanto, como visto anteriormente, não foi possível identificar tais benefícios. Nesse ponto, acreditamos que, como a lesão por HIE afeta gravemente as regiões envolvidas no processamento cognitivo, o treinamento aqui proposto pode não ser suficiente para modular as mudanças necessárias para reverter esse déficit cognitivo. Talvez um protocolo de exercício com uma duração maior, tivesse um resultado mais efetivo sobre esses parâmetros. Outro resultado que chamou nossa atenção é que não encontramos efeitos dos exercícios na reversão do déficit motor encontrado nos membros anteriores dos animais HIE na tarefa PMRT. Acreditamos que uma possível explicação para este resultado é que os exercícios utilizados como treinamento neste estudo utilizam principalmente os membros posteriores dos animais. Talvez por isso não seja possível observar os efeitos do treinamento nessa tarefa específica.

Em relação aos resultados da avaliação da plasticidade celular, também não foi possível observar os benefícios de nenhum dos tipos de exercícios realizados e esse é também é um resultado contrário às expectativas. É consenso na literatura que o exercício induz a plasticidade, principalmente em regiões relacionadas à aprendizagem, como o hipocampo (KNAEPEN et al., 2010). Várias evidências demonstram que tanto o exercício físico forçado quanto o voluntário são responsáveis por aumento na neurogênese, na proliferação celular e na ramificação dendrítica (CASSILHAS et al., 2016). Em estudos que utilizaram o exercício especificamente após um evento de HIE neonatal, os resultados identificaram benefícios na aprendizagem espacial, função sensório-motora, inibição da apoptose, ativação da neurogênese e oligodendrogênese (PARK et al., 2013; CHOI et al., 2013; KIM et al., 2017; PAK et al., 2018).

Alguns pontos podem ser levantados para tentar explicar por que o exercício não foi eficaz para promover a plasticidade neste estudo. Primeiro, acreditamos que essa divergência pode estar relacionada à região encefálica avaliada nesses estudos. Os estudos citados acima avaliaram o corpo caloso, substância negra, estriado, zona subventricular e hipocampo após HIE. Para nosso conhecimento, este é o primeiro estudo a avaliar o efeito do exercício na morfologia da região M1 após a realização do modelo de HIE. Como dito em vários momentos desta discussão, esta região do encéfalo parece não ser tão afetada por este insulto. Com isso, não podemos observar

o efeito do exercício também. Outro ponto a ser levantado é a duração e a intensidade do estímulo de treinamento ao qual os animais foram submetidos nesses diferentes estudos. Em nosso estudo, optamos por um estímulo considerado na literatura como leve a moderado, com duração média de 20 minutos por dia, em dias intercalados e sendo realizado por quatro semanas. Dessa maneira, procuramos imitar uma forma terapêutica de treinamento. Por outro lado, Pak et al. (2018) usaram um protocolo de corrida em esteira realizada durante 30 minutos uma vez ao dia por 12 semanas. No protocolo de Kim et al. (2017), os ratos foram treinados também em esteira do 22º DPN até a 8ª semana após o nascimento. Esses dois exemplos mostram que a intensidade e a duração do exercício foram diferentes, mais intensas, o que também pode explicar a divergência dos resultados.

De qualquer forma, a literatura envolvendo estudos que avaliam o efeito de diferentes tipos de exercício após a HIE ainda é escassa, o que dificulta a compreensão mais detalhada desse tipo de estratégia. Acreditamos que mais estudos precisam ser realizados para esclarecer os demais mecanismos envolvidos nesses benefícios encontrados nesse tipo de tratamento que já é amplamente utilizado em pacientes da clínica e demonstra resultados satisfatórios.

6 CONCLUSÕES

Os resultados obtidos nesta tese nos permitem concluir que:

- Em nível funcional, a HIE neonatal causou déficits motores gerais e alterou a motricidade dos membros anteriores afetados;
- Contraditoriamente, o modelo de HIE neonatal não causou alterações morfológicas no córtex sensoriomotor, medula espinal, nervo isquiático, junções neuromusculares e músculo esquelético;
- Em relação à plasticidade celular, os animais HIE apresentam maior número de astrócitos e maior concentração de sinaptofisina no córtex motor primário no hemisfério ipsilateral à lesão;
- Adicionalmente, evidenciamos hiperatividade, ansiedade e déficits de aprendizagem nos animais HIE;
- Como forma de terapia, nossos resultados evidenciaram que o treinamento acrobático causou melhora na locomoção e diminuição da atrofia cerebral nos animais HIE;
- O exercício acrobático foi ainda responsável por reverter a hiperatividade e a ansiedade causada pela HIE neonatal;
- Não encontramos evidências de benefícios expressivos com o protocolo de exercício aeróbico sobre os parâmetros analisados neste estudo;

Esses achados demonstram que mesmo não tendo exercido efeito sobre todos os parâmetros analisados, o exercício acrobático parece ser uma boa opção terapêutica em humanos acometidos pela HIE neonatal, principalmente em crianças, pois foi responsável por melhorar aspectos cognitivos e motores a nível experimental.

7 PERSPECTIVAS

Considerando os achados do presente trabalho, nossas perspectivas são:

1. Avaliar com mais detalhes a influência do exercício acrobático sobre aspectos cognitivos e de memória frente ao modelo de hipóxia-isquemia neonatal;
2. Avaliar os diferentes tipos de fibras musculares a fim de identificar possíveis alterações causadas pela lesão ou pelo exercício físico, com foco no exercício acrobático;
3. Avaliar possíveis remodelações celulares e de plasticidade na medula espinal dos animais submetidos à hipóxia-isquemia neonatal e ao exercício, para auxiliar na compreensão da preservação estrutural encontrada na via motora;
4. Avaliar os receptores de dopamina D1 e D2 no córtex motor dos animais submetidos à HIE e ao exercício, uma vez que essa via está relacionada ao aprendizado motor.

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Porto Alegre, Quinta-Feira, 11 de Junho de 2015

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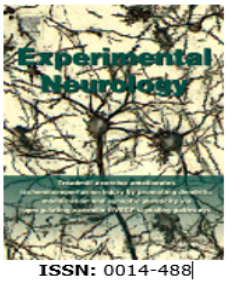

ISSN: 0014-488

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