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

EFEITOS BENÉFICOS DO ENRIQUECIMENTO AMBIENTAL PRECOCE NOS DÉFICITS MOTORES E NA PLASTICIDADE NA MEDULA ESPINHAL EM RATOS SUBMETIDOS A UM MODELO DE PARALISIA CEREBRAL

Dissertação de Mestrado

Marília Rossato Marques

Porto Alegre

2013

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Marília Rossato Marques

Orientadora: Prof[a]. Dr[a]. Simone Marcuzzo

Dissertação apresentada ao Programa de Pós-Graduação em Neurociências, da Universidade Federal do Rio Grande do Sul, como requisito parcial para obtenção do grau de Mestre.

Porto Alegre

2013

AGRADECIMENTOS

À UFRGS, ao Instituto de Ciências Básicas da Saúde (ICBS) e ao Laboratório de Histofisiologia Comparada, pela estrutura disponibilizada para a realização deste estudo.

Ao CNPq, pela bolsa de estudos concedida neste período.

À Professora Doutora Simone Marcuzzo, pelo acolhimento, orientação, paciência e conhecimento que permitiram a concretização deste trabalho.

À Professora Doutora Matilde Achaval, pelo conhecimento e apoio científico.

Ao Programa de Pós-Graduação em Neurociências pela oportunidade e à Professora Doutora Carla Dalmaz e secretárias Andrea Aquino Ferreira e Sara Talice Santos Bastos pela disponibilidade em ajudar.

Aos colegas do Laboratório de Histofisiologia Comparada, em especial Felipe Stigger, Francele Piazza, Ethiane Segabinazi, Otávio Augustin e Bruno Gomes pelo companheirismo, empenho e animação na realização deste trabalho.

À colega e técnica de laboratório Sílvia Barbosa, pelos ensinamentos, incentivo e ajuda.

À colega e amiga Pamela Bagatini, pela amizade, confiança, preocupação e risadas nos momentos mais impróprios.

Aos amigos “especiais” da Neurociências Cícero Garcia, Fabrício Nicola e Itiana Menezes pelo apoio, conversas e risadas que tornaram esses dois anos mais animados.

Às minhas ex-professoras e amigas Ana Lucia Cervi Prado e Caren Bernardi pelo incentivo e exemplo de amor à Fisioterapia e Neurociências.

Aos amigos de infância e faculdade, pela amizade, incentivo e compreensão da minha ausência em muitos momentos.

Às amigas Bianca Piccolli, Candissa Silva, Fabiana Flores e Viviane Forgiarini que acompanharam "de perto" este período. Obrigada pelo companheirismo e incansáveis risadas.

Aos meus pais, pessoas que eu mais amo e admiro, agradeço pelo incentivo, compreensão, dedicação e amor.

“Quando você partir em direção à Ítaca,

Que a sua jornada seja longa,

Repleta de aventuras, plena de conhecimento.

(...) Não perca Ítaca de vista,

Pois chegar lá é o seu destino."

Konstantinos Kavafis

SUMÁRIO

RESUMO

A Paralisia Cerebral (PC) é causada por lesões não progressivas no encéfalo em desenvolvimento gerando comprometimentos secundários normalmente acompanhados de mudanças morfológicas, bioquímicas e fisiológicas no sistema neuromuscular. O enriquecimento ambiental (EA) é uma combinação de estímulos de interação social, atividade física e aprendizagem que busca mimetizar a intervenção fisioterápica. Assim, o objetivo desta dissertação foi estudar se o EA é capaz de prevenir o estabelecimento de comprometimentos motores em um modelo de PC em ratos. Foram utilizadas ratas Wistar grávidas para indução do modelo experimental de PC. Os filhotes machos foram divididos em 4 grupos: controle (grupo CT), animais mantidos no enriquecimento ambiental (grupo EA), animais com paralisia cerebral (grupo PC) e animais com paralisia cerebral mantidos em enriquecimento ambiental (grupo PC-EA). A avaliação das habilidades motoras foi realizada no vigésimo nono dia pós-natal utilizando os seguintes testes: Campo Aberto, Rotarod, Escada Horizontal, Barra Estreita e comprimento da passada. As análises histológicas avaliaram a área média das fibras do músculo sóleo, a área média dos somas dos motoneurônios e expressão de sinaptofisina no corno ventral da medula espinhal. O EA mostrou ser capaz de prevenir os déficits motores, porém não reverteu a atrofia das fibras musculares observadas nos animais PC. Além disso, foi verificado um aumento na área média dos motoneurônios e um aumento na expressão de sinaptofisina no corno ventral da medula espinhal de animais com PC mantidos em EA, em relação aos que viveram em ambiente padrão. Dessa forma, o incremento de estímulos proporcionado pelo EA pode prevenir a instalação de déficits motores e alterações histológicas em um modelo de PC em ratos.

ABSTRACT

Cerebral Palsy (CP) results from nonprogressive lesions in the immature brain generating secondary impairments usually accompanied by morphological, biochemical and physiological changes in neuromuscular system. Environmental enrichment (EE) is a combination of stimuli including social interaction, physical activity and learning experiences that seeks to mimic the physiotherapy intervention. Therefore, the aim of this study was to verify whether EE is able to prevent the establishment of motor impairment in a rat model of CP. Pregnant Wistar rats were used to induce the experimental model of CP associating the maternal exposure to low doses of bacterial endotoxin, perinatal anoxia and sensorimotor restriction of the pups. Pups were divided into 4 groups: control (CT group), animals reared in environmental enrichment (EE group), animals submitted to cerebral palsy model (CP group) and animals submitted to cerebral palsy model reared in environmental enrichment (CP-EE group). The assessment of motor skills was held on the twenty-ninth post natal day using the following tests: Open Field Test, Rotarod, Horizontal Ladder, Narrow Suspended Bar and stride length. The histological analysis evaluated the mean cross-sectional area (CSA) of the soleus muscle fibers, the mean CSA of motoneuronal somata and expression of synaptophysin in the ventral horn of the spinal cord. EE was able to prevent the motor deficits, however did not reverse the muscle atrophy observed in CP animals. Furthermore, there was an average increase in the mean area of motoneurons and an increase in the expression of synaptophysin in the ventral horn of the spinal cord of animals submitted to CP model reared in EE in relation to CP animals reared in a standard environment. Hereupon, the stimulus increment provided by EE can prevent the onset of motor deficits and histological changes in a CP rat model.

LISTA DE ABREVIATURAS

EA	Enriquecimento Ambiental
LPV	Leucomalácia Periventricular
PC	Paralisia Cerebral
SN	Sistema Nervoso
SNC	Sistema Nervoso Central

ARTIGO

AOI	Area of interest
CSA	Cross-sectional area
CT group	Control group
CP	Cerebral Palsy
CP group	Cerebral Palsy group
CP-EE group	Cerebral palsy group reared in an enriched environment
EE	Environmental enrichment
EE group	Environmental Enriched group
G17	17^{th} day of gestation
OD	Optical Densitometry
P0	Day of birth
PBS	Phosphate Buffer saline
PBS-Tx	Phosphate Buffer saline with 0.4% Triton X-100

PB	Phosphate Buffer
PBS-Tx	Triton X-100

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1. INTRODUÇÃO

1.1 Paralisia Cerebral

A Paralisia Cerebral (PC) pode ser definida como um grupo de desordens causadas por lesões não progressivas no encéfalo do feto ou em estágios iniciais do desenvolvimento da criança, gerando comprometimentos no movimento e na postura e, conseqüentemente, uma limitação de atividade. Estes distúrbios motores podem ser acompanhadas por epilepsia, prejuízos sensoriais e limitação cognitiva (BAX *et al*, 2005; ZARRINKALAM *et al*, 2010), o que contribui para o isolamento social (BALANDIN; BERG; WALLER, 2006) e interfere nas atividades (HORSMAN *et al*, 2010). A incidência de PC na Europa e nos Estados Unidos é de 2 à 2,6 casos a cada 1000 nascidos vivos (ODDING; ROEBROECK; STAM, 2006; YEARGIN-ALLSOPP *et al*, 2008), destacando-se por ser a causa mais comum de comprometimento motor na infância (JOHNSON, 2002).

Uma das primeiras descrições na PC foi feita em 1843 pelo médico ortopedista William Little, que relacionou os seus sintomas a uma lesão no encéfalo da criança, chamada por ele de “deformidade cerebral”. O neurologista e psiquiatra Sigmund Freud publicou entre 1891 e 1897 diversos volumes intitulados “Paralisia Cerebral”, nos quais estabeleceu um sistema de classificação da patologia utilizado até hoje: doenças congênitas (pré-parto), doenças adquiridas durante o nascimento (intra-parto) e doenças adquiridas no período pós-natal (pós-parto) (JOHNSTON; HOON, 2006; PANTELIADIS; PANTELIADIS; VASSILYADI, 2012).

Cerca de 70 a 80% dos casos registrados de PC ocorrem no período pré-natal, tendo como principais etiologias a asfixia, infecções (rubéola, citomegalovírus, toxoplasmose) e traumas, enquanto no período pós-natal as causas mais comuns são meningite bacteriana, encefalite viral, hemorragia intracraniana, icterícia neonatal e traumas (REDDIHOUGH; COLLINS, 2003; JOHNSTON; HOON, 2006; KRIGGER, 2006; MCINTYRE *et al*, 2012). A idade gestacional é outro fator interferente na incidência de PC; quanto maior a idade gestacional menor a incidência de PC: 14,6% de crianças com esta patologia nasceram com menos de 28 semanas de gestação, 6,2% entre 28 e 31 semanas, de 0,4% a 0,7% entre 32 e 36 semanas e 0,1% nasceram a termo (HIMPENS *et al*, 2008).

Devido a essa diversidade de fatores de risco para a PC e a possibilidade de ocorrerem em diferentes estágios de maturidade do encéfalo, a magnitude, a extensão e a localização da lesão encefálica são variáveis (KOMAN; SMITH; SHILT, 2004), gerando uma ampla variabilidade de manifestações clínicas. Uma forma de classificação da PC se dá pela distribuição topográfica dessas manifestações, sendo utilizados os termos hemiplegia para o comprometimento de um hemicorpo, monoplegia (um membro afetado), diplegia (envolvimento maior dos membros inferiores), triplegia (três membros afetados) e quadriplegia (todos os membros afetados) (DELGADO; ALBRIGHT, 2003; JONES, 2006). Conforme a gravidade dos sintomas, a PC pode ser classificada em leve, moderada e severa, baseando-se no grau de limitação funcional observado clinicamente (OEFFINGER *et al*, 2004).

A PC ainda pode ser classificada considerando-se o tipo de distúrbio motor (STANLEY; BLAIR; ALBERMAN, 2000): a espasticidade (desequilíbrio entre o sistema sensório-motor associado à hiperreflexia e ao aumento do tônus muscular) (LANCE, 2000), a discinesia (caracterizada por tônus muscular flutuante conferindo

uma lentidão nos movimentos voluntários e presença de movimentos involuntários em períodos de estresse) (DELGADO; ALBRIGHT, 2003), a ataxia (comprometimento da coordenação motora), a hipotonia (diminuição do tônus muscular) (REDDIHOUGH; COLLINS, 2003) e a mista (envolvendo espasticidade e discinesia) (GORTER *et al*, 2004).

A lesão encefálica da PC inclui uma variedade de lesões neuropatológicas como a leucomalácia periventricular (LPV), a hemorragia com extensão intraventricular e a lesão do córtex cerebral, núcleos da base, tálamo e cerebelo (KADHIM *et al*, 2005). A LPV é a perda de substância branca encefálica como resultado da vulnerabilidade dos oligodendrócitos imaturos principalmente a eventos isquêmicos (VOLPE, 2001; JOHNSTON; HOON, 2006). Além da isquemia, o dano encefálico perinatal é influenciado por respostas inflamatórias infecciosas ou não no período embrionário ou neonatal (LEVITON; PANETH, 1990; NELSON *et al*, 1998; NELSON; CHANG, 2008), ativando células do sistema imunológico que atravessam a barreira hematoencefálica e geram danos diretos ou indiretos, através da ativação de células locais como a microglia e os astrócitos (DAMMAN; HAGBERG; LEVITON, 2001).

Os comprometimentos motores característicos em crianças com PC são influenciados em parte pelo atraso no desaparecimento de determinados reflexos primitivos, interferindo no desenvolvimento do controle motor fino e grosso (BERKER; YALÇIN, 2008). O reflexo de Moro, por exemplo, que raramente está presente em crianças sem patologias após os seis meses de idade, é normalmente observado crianças com PC após este período (TAYLOR, 2001). Além da persistência de reflexos, os comprometimentos motores são também decorrentes da alteração do tônus muscular, como a espasticidade. A espasticidade é o comprometimento mais comum e é causada pela lesão dos motoneurônios superiores que perdem a capacidade de enviar ‘inputs’

inibitórios descendentes através do trato reticuloespinal e outros sistemas, aumentando a excitabilidade dos neurônios gama e alfa, levando ao aumento do tônus muscular (KOMAN; SMITH; SHILT, 2004). A associação destes fatores contribui para o estabelecimento de comprometimentos motores, levando a alterações posturais (KRIGGER, 2006) e, consequentemente, à limitação da criança com PC em suas atividades (BRÆNDVÍK *et al*, 2012).

1.1.1 Alterações musculoesqueléticas na Paralisia Cerebral

Apesar da patologia do sistema nervoso central (SNC) na PC ser definida como uma encefalopatia estática por ser causada por lesões não progressivas no encéfalo imaturo, seus comprometimentos secundários como contraturas, dor e subluxações podem ser progressivos (KRIGGER, 2006).

Os comprometimentos motores decorrentes da PC normalmente evoluem com mudanças morfológicas, bioquímicas e fisiológicas no sistema neuromuscular, associadas a uma reorganização do córtex motor após a lesão (GOLDSTEIN, 2004; KESAR *et al*, 2012) . Além de gerar espasticidade, a lesão dos motoneurônios superiores diminui os ‘inputs’ corticais ao trato reticuloespinal e corticoespinal, comprometendo o controle motor, reduzindo o número de unidades motoras e gerando fraqueza e alterações no controle muscular (KOMAN; SMITH; SHILT, 2004).

Em crianças com PC, a área média das fibras musculares, maior determinante da produção de força no músculo esquelético, encontra-se reduzida em músculos com contratura (BARRETT; LICHTWARK, 2010), resultando em fraqueza muscular que é exacerbada pela redução da inervação muscular (MOCKFORD; CAULTON, 2010). Também ocorre uma transição no fenótipo das fibras musculares, havendo uma

predominância de fibras lentas (MARBINI *et al*, 2002) caracterizadas por terem menos força de contração. Além disso, outro contribuinte para a perda de força muscular e a redução da qualidade de contração muscular é o acúmulo de tecido adiposo entre as células musculares, e isso está relacionado ao menor nível de atividade física dessas crianças (HILTON *et al*, 2008; JOHNSON *et al*, 2009).

Outra característica encontrada em crianças com PC é o baixo nível de densidade mineral óssea, principalmente quando há a associação de PC, déficit cognitivo e epilepsia (COPPOLA *et al*, 2012), podendo levar a ocorrência de fraturas. A literatura reporta uma incidência de fraturas em 4% das crianças com PC moderada ou grave ao ano (STEVENSON *et al*, 2006), havendo uma redução de 77% na densidade mineral óssea no osso fêmur destas crianças (HENDERSON *et al*, 2002).

Estas alterações musculoesqueléticas na PC contribuem para o estabelecimento de comprometimentos na marcha como diminuição da velocidade durante a caminhada, menor comprimento da passada e maior tempo gasto durante o apoio dos dois membros inferiores (ABEL & DAMIANO, 1996). Além disso, há uma redução da eficiência do movimento e aumento do gasto energético e fadiga durante a realização de tarefas (MALTAIS *et al*, 2005a; MALTAIS *et al*, 2005b; PICCININI *et al*, 2007; KURZ; STUBERG; DEJONG, 2010).

1.2 Enriquecimento Ambiental

O conceito de enriquecimento ambiental (EA) foi inicialmente proposto por Donald Hebb, quando permitiu que alguns ratos de laboratório explorassem livremente

a sua casa. Hebb percebeu que estes animais manifestaram posteriormente maior habilidade na resolução de problemas do que aqueles que foram mantidos em casas moradia típicas de laboratório (NITHIANANTHARAJAH; HANNAN, 1947). Desde então, diversos estudos começaram a serem realizados com o objetivo de compreender os efeitos do EA.

O EA consiste de uma combinação de estímulos: interação social, atividade física e aprendizagem. O ambiente enriquecido é utilizado em experimentos com modelos animais, como uma intervenção comportamental (PLANE *et al*, 2008), e também em locais que mantêm animais domésticos ou silvestres (LEONE *et al*, 2007). Neste último caso, o EA proporciona aos animais características ambientais biologicamente relevantes que estimulam e encorajam comportamentos naturais (MELLEN; MACPHEE, 2001) e reduzem o estresse (FAIRHURST *et al*, 2011) e respostas de medo (MEEHAN; MENCH, 2002), aumentando a qualidade de vida destes animais (BARONCELLI *et al*, 2010).

O EA busca mimetizar a intervenção fisioterápica (KLINE *et al*, 2007; SOZDA *et al*, 2010), expondo modelos animais de doenças à ambientes que permitem uma intensa exploração e interação sensorial, física e social (NITHIANANTHARAJAH; HANNAN, 2006). Para ratos e camundongos, este ambiente é criado utilizando-se caixas-moradia maiores que as caixas-moradia padrão de laboratório, contendo objetos para exploração e atividade física voluntária, como rodas de exercício e escadas de acesso a diferentes andares do ambiente (VAN PRAAG; KEMPERMANN; GAGE, 2000). Outra característica é a exposição de um maior número de animais em relação às caixas-padrão, favorecendo o estímulo social. Esta diversidade de características permite uma grande variação entre os protocolos de EA (CUTULI *et al*, 2011), modificando o tamanho do ambiente, os objetos de estímulo e a

freqüência de mudança deles e o número de animais expostos (BENNETT *et al*, 2006). O tempo de exposição dos animais ao EA é outra variável, podendo permanecer algumas horas por dia (RAMPON *et al*, 2000; FRICK *et al*, 2003a), ou permanentemente, sendo neste caso o ambiente de moradia destes animais (FRICK *et al*, 2003b; BENNETT *et al*, 2006).

A utilização do ambiente enriquecido pode influenciar o peso corporal dos animais, havendo uma redução do peso provavelmente devido à maior atividade durante o período claro, enquanto os animais mantidos em caixas-padrão normalmente realizam menos atividades (ZAIAS *et al*, 2008). Além de poder interferir no peso corporal dos animais, o EA tem grande influencia sobre a estrutura e função do SN (SCOTTO-LOMASSESE, 2000). Diante de modelos experimentais de diversas patologias, o EA tem proporcionado efeitos benéficos na aprendizagem e memória (LEGGIO *et al*, 2005; BRUEL-JUNGERMAN; LAROCHE; RAMPON, 2005; GOSHEN *et al*, 2009; VALERO *et al*, 2011), na plasticidade encefálica e na recuperação cognitiva (JANKOWSKY *et al*, 2005; PANG; HANNAN, 2013) e funcional (RAMPON *et al*, 2000; VAN PRAAG; KEMPERMANN; GAGE, 2000; DIAMOND, 2001; JADAVJI; KOLB; METZ, 2006) e ainda mostra um potencial neuroprotetor à determinadas doenças como as neurodegenerativas (WILL *et al*, 2004; NITHIANANTHARAJAH; HANNAN, 2006; LAVIOLA *et al*, 2008). Já em doenças isquêmicas, o EA apresenta resultados positivos na modulação da excitotoxicidade glutamatérgica, atenuando a lesão oxidativa e a degeneração nervosa (BRIONES; ROGOZINSKA; WOODS, 2011). Esses efeitos do EA parecem ser influenciados pelo gênero; por exemplo, em tarefas motoras, machos apresentam maior coordenação motora após a exposição ao EA, enquanto fêmeas obtêm melhores resultados em tarefas de cognição espacial (SAUCIER; YAGER; ARMSTRONG, 2010).

Apesar dos mecanismos moleculares que envolvem a plasticidade encefálica estrutural e funcional induzida pelo EA não serem totalmente conhecidos (HU *et al*, 2010), à nível celular o EA pode promover um aumento do tamanho neuronal e uma maior arborização dendrítica e densidade de espinhos dendríticos (BEAUQUIS *et al*, 2010; ROJAS *et al*, 2013), estimulando mudanças na morfologia sináptica e atividade neuronal (HUANG *et al*, 2006). Alterações nas células gliais também são observadas pelo uso do EA, havendo um aumento da arborização astrocítica e uma menor densidade de microglia, que tipicamente está presente após lesões encefálicas (KOLB *et al*, 1998), além de ocorrerem menos mudanças patológicas nos astrócitos em patologias como a Doença de Alzheimer (BEAUQUIS *et al*, 2013). Ainda é observado um aumento no número de capilares sanguíneos no encéfalo (BEAUQUIS *et al*, 2010), assim como há uma maior atividade metabólica provavelmente pelo maior número de mitocôndrias (WHISHAW, 1998).

O córtex cerebral de animais expostos ao EA apresenta uma maior espessura e há um aumento no peso encefálico (HUANG *et al*, 2006), assim como a neurogênese encontra-se em maior atividade no hipocampo (FABEL *et al*, 2009; VALERO *et al*, 2011; LIU; HE; YU, 2012; SPEISMAN *et al*, 2013). O EA também aumenta os níveis de neurotrofinas (ICKES *et al*, 2000; PHAM *et al*, 2002), sinaptofisina (FRICK; FERNANDEZ, 2003; NITHIANANTHARAJAH; HANNAN, 2004), fator de crescimento neural e expressão do gene CREB (TORASDOTTER *et al*, 1998; WILLIAMS *et al*, 2001) no giro denteado do hipocampo. O estriado, estrutura encefálica importante nos comportamentos motores e motivados, também é induzido a mudanças pelo EA, ocorrendo alterações na expressão de genes responsáveis pelo metabolismo celular, sinalização intracelular e proliferação e diferenciação de células (THIRIET *et al*, 2008).

1.3 Justificativa

As lesões no encéfalo imaturo interferem no controle motor e postural que resultam em um atraso na aquisição de habilidades motoras básicas. A PC é a causa mais comum de déficit motor em crianças e estas tendem a ter um estilo de vida sedentário, independente da idade, piorando o déficit motor já existente.

O período até os primeiros dois anos de vida de uma criança é crítico para o desenvolvimento do SN, pois processos importantes estão ocorrendo, como a neurogênese, gliogênese, sinaptogênese e mielinização de axônios. Processos esses que dependem de atividade motora para serem estabelecidos. Diante do quadro clínico da PC, o estímulo à promoção de saúde através da atividade física e convívio comunitário tem sido reconhecido como um fator importante para a qualidade de vida e prevenção de complicações secundárias em indivíduos com PC. Essas características de estímulos podem ser observadas no EA proposto em modelos experimentais com animais.

Assim, sendo a PC uma patologia com comprometimentos secundários progressivos em que se preconiza a estimulação terapêutica precoce, estudar a influência do EA em um modelo experimental de PC é interessante a fim de possibilitar o entendimento dos mecanismos biológicos relacionados a esta intervenção.

2. OBJETIVOS

2.1 Objetivo geral

Avaliar se o enriquecimento ambiental precoce previne a instalação das alterações funcionais e sensoriomotoras em um modelo experimental de paralisia cerebral em ratos.

2.2 Objetivos específicos

- Avaliar os efeitos comportamentais motores do enriquecimento ambiental precoce em um modelo de PC: análise da atividade locomotora espontânea, da habilidade motora e da marcha.

- Avaliar os efeitos morfológicos do enriquecimento ambiental precoce em um modelo de PC: análise do músculo sóleo e da medula espinhal.

3. MÉTODOS E RESULTADOS

3.1. Artigo: **Beneficial effects of early environmental enrichment on motor development and spinal cord plasticity in a rat model of cerebral palsy.** Marília Rossato Marques, Felipe Stigger, Ethiane Segabinazi, Otávio Américo Augustin, Sílvia Barbosa, Francele Valente Piazza, Matilde Achaval, Simone Marcuzzo.

Beneficial effects of early environmental enrichment on motor development and spinal cord plasticity in a rat model of cerebral palsy

Marília Rossato Marques[a,b], Felipe Stigger[a,b], Ethiane Segabinazi[b], Otávio Américo Augustin[b], Sílvia Barbosa[b], Francele Valente Piazza[a,b], Matilde Achaval[a,b], Simone Marcuzzo[a,b].

[a]Programa de Pós-Graduação em Neurociências, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Sarmento Leite 500, CEP: 90050-170, Porto Alegre, RS, Brazil.

[b]Laboratório de Histofisiologia Comparada, Departamento de Ciências Morfológicas, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Sarmento Leite 500, CEP: 90050-170, Porto Alegre, RS, Brazil.

Corresponding author:

Simone Marcuzzo, PhD

Laboratório de Histofisiologia Comparada, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul

Rua Sarmento Leite 500, CEP: 90050-170, Porto Alegre, RS, Brazil

Tel.: +55 51 33083624 Fax: +55 51 33083092 E-mail address: simone.marcuzzo@ufrgs.br

ABSTRACT

Cerebral Palsy (CP) results from nonprogressive lesions in the immature brain generating secondary impairments usually accompanied by morphological, biochemical and physiological changes in neuromuscular system. Environmental enrichment (EE) is a combination of stimuli including social interaction, physical activity and learning experiences that seeks to mimic the physiotherapy intervention. Therefore, the aim of this study was to verify whether EE is able to prevent the establishment of motor impairment in a rat model of CP. Pregnant Wistar rats were used to induce the experimental model of CP associating the maternal exposure to low doses of bacterial endotoxin, perinatal anoxia and sensorimotor restriction of the pups. Pups were divided into 4 groups: control (CT group), animals reared in environmental enrichment (EE group), animals submitted to cerebral palsy model (CP group) and animals submitted to cerebral palsy model reared in environmental enrichment (CP-EE group). The assessment of motor skills was held on the twenty-ninth post natal day using the following tests: Open Field Test, Rotarod, Horizontal Ladder, Narrow Suspended Bar and stride length. The histological analysis evaluated the mean cross-sectional area (CSA) of the soleus muscle fibers, the mean CSA of motoneuronal somata and expression of synaptophysin in the ventral horn of the spinal cord. EE was able to prevent the motor deficits, however did not reverse the muscle atrophy observed in CP animals. Furthermore, there was an average increase in the mean area of motoneurons and an increase in the expression of synaptophysin in the ventral horn of the spinal cord of animals submitted to CP model reared in EE in relation to CP animals reared in a standard environment. Hereupon, the stimulus increment provided by EE can prevent the onset of motor deficits and histological changes in a CP rat model.

INTRODUCTION

Cerebral palsy (CP) is a static encephalopathy resulting from nonprogressive lesions or anomalies in the immature brain (Bax *et al*, 2005; Zarrinkalam *et al*, 2010) being the most common cause of physical disability affecting children (Johnson, 2002). Although the lesion is not progressive in CP, secondary motor impairment associated with abnormal motor patterns and postures (Krigger, 2006) persists throughout the lifespan and interferes in children's normal development (Damiano *et al*, 2009), contributing to activity limitations (Brændvík *et al*, 2012).

The motor impairment normally is accompanied by morphologic, biochemical and physiological changes in the neuromuscular system (Goldstein, 2004; Kesar *et al*, 2012). These musculoskeletal challenges contribute to alterations in the gait pattern, such as slower speed, shorter stride length and more time spent in double support while walking (Abel & Damiano, 1996). Furthermore, children with CP have a reduction in movement efficiency, an increase in metabolic energy spent for walking (Maltais *et al*, 2005a; Maltais *et al*, 2005b; Piccinini *et al*, 2007; Kurz *et al*, 2010) and a more sedentary lifestyle (Wrotniak *et al*, 2006) that contributes to a decline in mobility-related activities (Jahnsen *et al*, 2004). Less mobility in CP is related to increased motor deficits and decreased physical conditioning (Damiano *et al*, 2006).

Rehabilitation in CP disease consists of improving mobility, preventing deformity and helping children to learn the needed skills for daily life. The therapy is often recommended throughout early childhood while the nervous and musculoskeletal systems are the most adaptable and the normal neuromotor development can be facilitated (Koman *et al*, 2004; Damiano *et al*, 2006; Berker & Yalçin, 2008). The

therapy should support the development of cognitive, sensory, visual and musculoskeletal systems, involving play activities to enhance social integration (Berker & Yalçin, 2008).

These characteristics of therapy are found in the environmental enrichment (EE) that provides physical activity, learning experiences, increased somatosensorial inputs and social interaction (Nithianantharajah & Hannan, 2006). It induces plastic changes in the brain and recovery of sensorimotor function and memory impairment in several models of pathology (Spires *et al*, 2004; Schneider *et* al, 2006; Pang *et al*, 2010; Fairhurst *et al*, 2011; Rojas *et al*, 2013; Beauquis *et al*, 2013). The EE may be considered a rodent correlate of theses therapeutic interventions in humans (Kline *et al*, 2007; Sozda *et al*, 2010).

The CP rat model that consists in the association of maternal exposure to low doses of bacterial endotoxin, perinatal anoxia and sensorimotor restriction of the pups reproduces behavioral and damage characteristics that closely resemble the pattern described in CP (Stigger *et al*, 2011a). Although there is a consensus that early intervention is more likely to improve the physical condition of patients with CP, the neurobiological mechanisms responsible for possible functional improvements are poorly understood. Therefore, the aim of this study was to investigate if the early exposure to an enriched environment could prevent the acquisition of the motor impairment induced in a rodent model of CP and investigate the biological substrate involved in it.

MATERIALS AND METHODS

All procedures were approved by the Ethical Committee at the Universidade Federal do Rio Grande do Sul (Nº 23594). The animal care followed the recommendations of the Brazilian Society for Neuroscience, Committee of the School of Veterinary Surgery, University of Buenos Aires and International Brain Research Organization (IBRO) and are in compliance with the National Institute of Health's Guidelines for Care and Use of Laboratory Animals (publication nº 85-23, revised 1985). Food and water were available *ad libitum* and the animals were maintained on a 12:12h light/dark cycle in a temperature-controlled environment (20 ± 1ºC), according to the Brazilian law that regulates animal use for didactic-scientific practice. All efforts were done to minimize animal suffering as well as to reduce the number of animals.

Experimental Animals

The cerebral palsy model was induced as previously described (for details see Stigger *et al*, 2011a), and consisted in the association of maternal exposure to low doses of bacterial endotoxin (lipopolysaccharide), perinatal anoxia and sensorimotor restriction of the pups. Pregnant Wistar rats (n = 10) were obtained from a local breeding colony (Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Brazil) and prior to experiments, they were left undisturbed for 7 days. The pups were divided in four groups as follows: control group (CT group), animals exposure to environmental enrichment (EE group), cerebral palsy group (CP group) and

CP exposure to environmental enrichment (CP-EE group). Fig. 1 shows the time line of the experimental procedures.

Environmental Enrichment

The CT and CP groups were housed in standard laboratory cages (3 or 4 animals/cage) whereas the EE and CP-EE were placed in groups of eight animals in a complex enriched environment. This enriched environment consisted of a large cage (50 x 50 x 50 cm) with three floors, ramps, plastic tubes, one running wheel and several objects with different shapes and textures. The objects were rearranged every day and renewed every week to favour animals' exploratory behaviour. The rat pup weights were evaluated in P1, P10, P20 and P29.

Motor skills assessment

At P29, the animals were submitted to motor skills evaluations. Spontaneous locomotor activities were evaluated using an Open Field, the motor balance and coordination was assessed using a Rotarod and the hind-limb sensorimotor function was examined with the Horizontal Ladder and Narrow Suspended Bar.

Open Field test

The rats were evaluated in a 40 cm x 50 cm x 60 cm box, in which the floor was divided into 12 squares, and then filmed with a digital camcorder (DCR-SR47, Sony, Japan) for 3 min. The number of crossings from one square to another was counted.

Rotarod

The animals were placed in a Rotarod (Insight, Brazil) with 60 mm diameter textured rod, 75 mm in length, rotating at a speed of 25 rpm. Each animal was tested 5 times with a 2 minutes interval between each trial and the maximum duration of the test was 3 minutes. The time spent by the animal on the Rotarod was considered as the latency to fall.

Horizontal ladder and Narrow suspended bar

The horizontal ladder is a ladder with 5 cm in width, with parallel metal rungs (2 cm a part) and the suspended bar is a rectangular bar with 2,5 cm in width. The apparatuses have 100 cm in length and were positioned 30 cm from the floor. Motor skills were assessed based on the rat ability to walk on these apparatus. The animal was placed in one extremity of the ladder or bar and walked until the other extremity, entering in a darkened goal box (Kline *et al*, 2010). The animals were filmed 3 times

with a digital camera (Sony; DCR-SR47, USA) and during the entire course of the tests total number of hind limb step errors was counted. It was considered as an error when the hind limb of the animal slipped or was not placed on the bar or the metal rungs.

Gait testing

Walking pattern was evaluated at P29 and consisted in measure the hind paw stride length (sum of the stance and the swing phases of the gait cycle). For this, the rats walked with their painted hind feet along a 100-cm-long, 8.5-cm-wide track covered with a white sheet of paper. The stride length of each rat was obtained from the mean values of three consecutive footprints each side (Marcuzzo *et al*, 2008).

Histological, morphometrical and immunoistochemical analysis

At P29, after motor testing, rats were deeply anesthetized with sodium thiopental (50mg/kg, i.p.; Cristália, Brazil), injected with 1000 IU heparin (Cristália, Brazil) and euthanized by transcardiac perfusion with 200 mL of saline solution, followed by 250 mL of a solution containing 4% paraformaldehyde (Reagen, Brazil) diluted in 0.1 M phosphate buffer (PB; pH 7.4) at room temperature.

Soleus analysis

Right soleus muscle was dissected free from surrounding connective tissue and post fixed with 0,5% glutaraldehyde (Sigma, USA). Small samples (2 x 1 mm) of the central part of the muscles were collected and washed in PB and post fixed in 1% OsO_4 (Sigma, USA) in PB for 1 h and then rinsed again in PB and dehydrated in a graded series of alcohol and propylene oxide (Electron Microscopy Sciences, USA), embedded in resin (Durcupan, ACM-Fluka, Switzerland), maintained in a vacuum for 24 h and, then, polymerized for 48 h at 60ºC. Transverse semithin sections (1 μm) were obtained using an ultramicrotome (RMC; PT-x, USA) and stained with 1% toluidine blue (Merck, Germany) in 1% sodium tetraborate (Ecibra, Brazil). Images of the muscles were captured and digitalized (initially 20x and further amplified 200% for analysis) using a Nikon Eclipse E-600 microscope (Japan) coupled to a digital camera. For morphometric measurement, a set of 6 images was chosen using random sampling of one slice and the mean fiber cross-sectional area (CSA) was estimated with the software Image Pro Plus 6.0 (Media Cybernetics, Rockville, MD, USA) using a point-counting technique as described by Marcuzzo *et al* (2008). It was used an area of interest (AOI) with 47988.28 μm², a grid with a density of one point per 143.5 μm².

Spinal cord analysis - Motoneuronal analysis

The spinal cord segments at L4-5 level were removed after cautions laminectomy. The L4 segments level were post fixed in the same fixative solution

described for soleus. Transversal sections of the segment (200 µm) were cut using a vibratome (Leica, Germany). Four samples were embedded in resin blocks, mantained in vacuum and, afterwards, polymerized like described for the muscles. One of the samples was randomly selected and transverse-semithin sections (1 µm) were obtained using an ultramicrotome (RMC; PT-x, USA). Every 10 µm, one section was collected and stained with 1% toluidine blue in 1% sodium tetraborate. Images of the left ventral horn were captured and digitalized (initially 20x and further amplified 200% for analysis). The CSA of the motoneurons in which the nucleolus was visible was estimated by the point-couting technique, using a grid with a density of one point per 43.99 µm².

Spinal cord analysis - Immunoistochemical procedure and optical densitometry (OD)

The L5 level of spinal cord was post fixed in a solution containing 4% paraformaldehyde in 0.1M PB for 4 h and cryoprotected by immersion in a 15% and 30% sucrose (Synth, Brazil) in PB at 4ºC until they sank. After, the segments of spinal cord were quickly frozen in isopentane (Merck, Germany) cooled in liquid nitrogen and kept in a freezer (-70ºC) for further analyses. Transversal sections (40 µm) were cut using a cryostat (CM1850, Leica, Germany) at -20ºC and collected in a PB saline (PBS), pH 7.4. The free-floating sections were washed in PBS, pre-treated with 3% hydrogen peroxide for 30 min, washed again in PBS and after in PBS containing 0.4% Triton X-100 (PBS-Tx) for 15 min, treated with 2% bovine serum albumin (In lab, Brazil) in PBS-Tx for 30 min and incubated with primary polyclonal rabbit Anti-Synaptophysin antibody (1:200; Chemicon-Millipore, USA) for 48 h at 4ºC. The

sections were again washed in PBS-Tx and incubated in the secondary Anti-Rabbit IgG-Peroxidase antibody (1:500; Sigma Chemical Co, USA) for 2 h at room temperature. The reaction was revealed in a medium containing 0.06% 3.3-diaminobenzidine (DAB, Sigma Chemical Co, USA) dissolved in PBS for 10 min and after in 0.06% DAB with 2 μL of 3% hydrogen peroxide for 10 min. Finally, the sections were washed in PBS, dehydrated in ethanol, cleared with xylene and covered with Balsam (Merck, Germany) and coverslips. Control sections were prepared omitting the primary antibody.

To measure the intensity of the synaptophysin immunoreaction was used a semi-quantitative densitometric analysis. Digitalized images of the ventral horn of the spinal cords were obtained with a Nikon Optiphot-2 microscope (200x, Tokyo, Japan) coupled to a Micrometric camera (Accu Scope, Commack, NY, USA). With the software Image Pro Plus 6.0, the images were converted to an 8-bit gray scale (0-255 gray levels). The OD was obtained from mean of 2 squares measuring 3762.62 μm^2 each one (area of interest, AOI). All of the lighting conditions and magnifications were held constant. Both the left and right ventral horn of spinal cord were used (10 measurements for each rat). The background correction and the optical density were calculated like described for Xavier *et al* (2005).

Statistical analysis

Two-way analysis of variance (ANOVA) was used to compare the difference between the groups. All analyses were followed by *post hoc* Tukey test and probability values less than 5% were considered significant. Data were expressed as means ± SEM. Statistical analysis was performed using the Statistica Software package.

RESULTS

Physical examination

Two-way ANOVA results examining the body weight revealed significant effects of CP at P1 ($p = 0.00001$), CP and CP-EE at P10 ($p = 0.001$ and $p = 0.04$, respectively) and CP at P20 ($p = 0.00001$). The body weight significantly reduced at P1 in CP animals when compared to EE ($p = 0.007$) and in CP-EE group in relation to CT ($p = 0.006$) and EE ($p = 0.0003$). At P10, CP-EE body weight was significant decreased in relation to EE ($p = 0.002$). At P20 lower body weight was seen in CP group in relation to EE ($p = 0.002$) and CP-EE compared to CT ($p = 0.009$) and EE ($p = 0.0002$). Despite a lower weight have been found in CP and CP-EE animals throughout the experiment, no significant difference in the average weight was found in the evaluation at P29 (Table 1).

Motor Skills

The animal's spontaneous locomotor activities evaluated in 3 minutes by two-way ANOVA revealed significant effects of CP ($p = 0.004$) in the Open Field test, but no difference between the groups. When the first minute was separately evaluated, the CP group had less locomotor activities that EE group ($p = 0.0008$). Although not

significantly different, CP-EE animals had a better performance when compared to CP animals in total three minutes evaluated (Table 2).

Regarding the motor balance and coordination assessed by the time spent by the animal on the Rotarod (latency to fall), two-way ANOVA revealed effect of EE ($p = 0.0002$). This test showed better performance of CP-EE when compared to CP animals ($p = 0.04$). CP had no significant difference from CT ($p = 0.92$), but they spent significantly less time in the apparatus compared to EE group ($p = 0.003$), and EE and CP-EE group had equally good performances ($p = 0.75$) (Table 3).

Two-way ANOVA results regarding the hind-limb sensorimotor function showed, on Horizontal Ladder, effects of CP ($p = 0.000001$), EE ($p = 0.000001$) and PC-EE ($p = 0.000001$) and, on Narrow Suspended Bar, effects of CP ($p = 0.0005$), EE ($p = 0.00006$) and PC-EE ($p = 0.005$). In both tests, the CP-EE group performed significantly better than CP ($p = 0.0001$) and similar to CT ($p = 0.33$ in Horizontal Ladder and $p = 0.94$ in Narrow Suspended Bar) and EE animals ($p = 0.96$ and $p = 0.94$, respectively) (Table 3).

Gait testing

The walking pattern was evaluated with the measure of the hind paw stride length. Effects of CP ($p = 0.0004$), EE ($p = 0.007$) and PC-EE ($p = 0.02$) were revealed by two-way ANOVA. CP had significantly shorter stride length (6.79 cm ± 0.19) than CT (8.20 cm ± 0.20; $p = 0.0005$) and EE (8.30 cm ± 0.24; $p = 0.0004$). The EE (7.97 cm ± 0.24) was able to improve the stride length of the CP group ($p = 0.004$) (Fig. 2).

Soleus analysis

The CSA measure in soleus evaluated by two-way ANOVA revealed significant effects of EE ($p = 0.00001$). In CP group, the CSA (288.25 μm²) revealed significant decrease when compared to CT group (723.57 μm²; $p = 0.0007$). This atrophy was also detected in CP-EE group (340.85 μm²) when compared with CT ($p = 0.002$). The CP-EE showed similar soleus CSA of CP group ($p = 0.92$) (Fig. 3A e 3B). The frequency histogram (Fig. 3C) demonstrated that CP and CP-EE groups had a higher percentage of atrophied fibers (0-300 μm²) (75.55% and 70.12%, respectively) than CT (12.60%; $p = 0.0003$ and $p = 0.0005$, respectively) and EE (11.48%; $\rho = 0.0003$ and $p = 0.0005$, respectively). Fibers within 300-600 μm² interval were similar percentage in all groups (33.69% in CT, 36.31% in EE, 22.84% in CP and 20.00% in CP-EE). This situation inverted in fibers within 600-900 μm² and 900-1200 μm², CP (1.42% and 0.19%, respectively) and CP-EE group (6.52% and 2.65%, respectively) had fewer percentage ($\rho < 0.02$) than CT (17.05% and 16.68%, respectively) and EE (30.02% and 30.51%, respectively). The groups did not show differences in the percentage of fibers within 1200-1500 μm² ($p > 0.18$).

Spinal cord analysis - Motoneuronal analysis

The two-way ANOVA showed significant effects of CP ($p = 0.03$) and EE ($p = 0.004$). The EE was able to increase the mean size of motoneuronal somata of CP-EE (996.08 μm² ± 36.27) group in relation to CP ($p = 0.04$). CP-EE was considered similar

to CT ($p = 0.88$) and EE ($p = 0.82$). Despite this beneficial effect, the mean CSA of the motoneuron somata was significantly decreased in CP group (791.89 $\mu m^2 \pm 30.72$) in relation to EE (1050.70 $\mu m^2 \pm 62.91$; $p = 0.006$) only, but not significantly different from CT (947.61 $\mu m^2 \pm 25.22$; $p = 0.15$) (Fig. 4).

Spinal cord analysis - Immunoistochemical procedure and optical densitometry

The OD of the ventral horn of spinal cord revealed significant effects of EE ($p = 0.01$). Lower synaptophysin immunoreactivity was seen in CP (0.124 ± 0.002) compared to CP-EE (0.149 ± 0.004; $p = 0.02$) (Fig. 5).

DISCUSSION

Children with CP frequently experience impaired growth, and poor nutritional status is correlated with increased health care utilization and decreased participation in normal activities (Samson-Fang *et al*, 2002). In this study, CP groups (CP and CP-EE) had a significant lower weight gain compared to control groups (CT and EE) and a subtle difference at P29. This data is comparable with another CP rat model in which sensorimotor restriction was employed (Strata *et al*, 2004; Marcuzzo *et al*, 2010). Additionally, the EE did not reverse the weight loss of CP animals, probably due to the physical enrichment, as demonstrated in Zaias *et al* (2008). Maybe a nutritional supplementation is required to supply this lower weight gain similarly to the proposed treatment for children with CP that improves nutritional status and have a positive effect on growth and motor skills (Campanozzi *et al*, 2007).

With reference to the motor deficits that children with CP have, non-pharmacological treatments are used to maintain or improve joint range of motion, facilitate or strengthen weak muscles, inhibit or weaken spastic agonist muscles, provide support and improve muscle strength (Koman *et al*, 2004). The EE is considered a rodent correlate of therapeutic intervention that involves physical activity, learning experiences and social interaction (Kline *et al*, 2007; Sozda *et al*, 2010; Nithianantharajah & Hannan, 2006). In this study, we demonstrated that EE can prevent the motor deficits development in CP rats. The CP animals housed in EE showed better performance on motor skills evaluated with Rotarod, Horizontal Ladder and Narrow Suspended Bar tests. These results are similar to others experimental pathologies in which the EE restored motor skills (Risedal *et al*, 2002; Jadavji *et al*, 2006; Kline *et al*, 2007; Hoffman *et al*, 2008; Kline *et al*, 2010). In the Open Field test, although not statistically significant, it was found a tendency in CP group to have less spontaneous locomotor activities and CP-EE to have more locomotion than CP, but both groups were less active than control groups. This evaluation is not a specific motor test, so others parameters may be interfering in these data. For example, isolation-reared rats show spontaneous hyperactivity in response to a novel environment (Schrijver *et al*, 2002) and animals housed in EE had a locomotor habituation to novelty with a lower basal activity in relation to animals housed in an impoverished condition (Benaroya-Milshtein *et al*, 2004; Brenes *et al*, 2009). Therefore, this test did not show evident motor impairment, as seen in other evaluations. Similar data were observed in Nilsson *et al* (1993) that did not found difference in the first ten minutes in an Open Field test between animals housed in EE conditions and animals housed in impoverished conditions.

The motor deficits observed in CP children are normally associated with gait impairment (Berker *et al*, 2008). The stride length was used to evaluate the walking pattern. CP rats reared in EE had an improvement in stride length when compared to CP rats housed in standard cages, suggesting a better motor control (Brown *et al*, 2003). Similar data were found in a treadmill training used in a CP model involving perinatal anoxia and sensorimotor restriction. In this case, the treadmill was used to restore the deficit established after the induction of the CP model (Marcuzzo *et al*, 2008), differently of our study which sought to prevent the establishment of deficits.

In order to study possible neurobiological mechanisms involved in the functional improvement (motor skills and walking pattern) observed, histological analyses of soleus muscle and spinal cord were realized. The muscles evaluation consisted of measure the CSA of soleus muscle fibers, a postural muscle that presents marked changes after a period of disuse (Thomason & Booth, 1990). In CP animals, soleus had fibers' atrophy and the EE could not prevent this atrophy. CP animals housed in EE had a tendency to have soleus fibers with larger CSA than CP group housed in standard cages, but this difference was not significant. Probably, to increment the CSA fiber in soleus muscle is necessary a specific motor training associated to complex environment adopted in our study. Accordingly, the treadmill training (a task-specific motor strategy) restored the soleus atrophy in a rat CP model (Marcuzzo *et al*, 2008). In humans, the treadmill or cycling are activity-based strategies that include repetition of various cyclical motions to take advantage of existing or available "motor programs". These activities can also be designed to address other specific aspects of motor performance such as endurance and coordination in addition to strength (Damiano *et al*, 2006). Moreover, considering the process of development of locomotion that includes the phase of *pivoting* during the first week of life, phase of *crawling* on the second week

and the phase of walking on the third week, the pups had just a efficient locomotion from 16 days of life, when the exploration of the EE turned more significant (Altman & Sudarshan, 1975). Perhaps, if the animals had remained at the EE for longer than 28 days, the stimulation of this environment could be more efficient to prevent or restore the muscular atrophy.

The motoneuron CSA showed no significant reduction in CP animals compared to CT animals, differently of Stigger *et al* (2011b) that found a reduction of motoneurons CSA in the same CP rat model. However, in this study, the motoneurons were evaluated at 52 days of life and in our study the animals were younger (29 days of life). Perhaps the greatest deficits are not fully established until 1 month of age, indicating that the deficits can be progressive even after the induction model. Kerai *et al* (1995) showed similar results that indicate that the growth rate of soleus motoneurons during the first 3 postnatal weeks is not affected by transiently blocking neuromuscular transmission shortly after birth. However, at later stages (10 weeks), a significant loss in motoneurons occurs (Greensmith & Vrbová, 1992). Nevertheless, the CP animals housed in EE had an increase in motoneuron soma size when compared to CP reared in standard housing. This indicates that an increment in motor activity, as EE, was efficient to prevent the motoneuron reduction caused for the disuse.

Finally, to assess the synaptic activity in ventral horn of the spinal cord, where the cell bodies of alpha motor neurons are located, the synaptophysin immunoistochemistry was used. The synaptophysin is a presynaptic vesicular protein found in all nerve terminals and useful in the identification of axonal nerve terminals and synapses (Walaas *et al*, 1988). Furthemore, the overexpression of synaptophysin results in an increased neurotransmitter release (Alder *et al*, 1995). The densitometric analysis of the imunoreaction showed higher expression of synaptophysin in CP animals

reared in EE compared to CP rats housed in standard house. This could mean that CP-EE had more active axonal nerve terminals. Although not statistically significant, there was a slight decrease in CP group synaptophysin expression compared to CT and EE animals. Besides, EE group had a tendency to demonstrate more expression of synaptophysin in relation to CT. This tendency could also be justified by the greatest deficits are not fully established to show significant differences in synaptic activity between CP and healthy animals. But the difference found between CP and CP-EE demonstrated that EE can influence the synaptophysin expression and indicate that could increase the synaptic activity. Moreover, the upregulation of synaptophysin might play an important role in neuronal plasticity (Chou *et al*, 2002).

These results suggest that the early stimulation with an enriched environment is able to prevent deficits on motor skills and histological alterations in a CP rat model. The present study is the first, to our knowledge, to examine the effects of the EE in typical motor deficits in a CP model and to verify the morphological background linked to the motor improvements. Moreover, this study highlights the importance of an early therapeutic intervention in order to prevent further motor impairments and the necessity of a global intervention in child with CP.

Acknowledgments

This work was supported by Brazilian funding agencies: CNPq, CAPES and FAPERGS. M. R. Marques is supported by a scholarship from CNPq and M. Achaval is a CNPq investigator.

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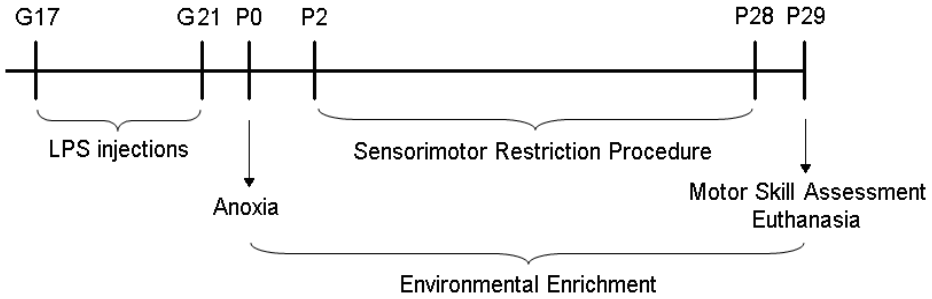

Fig. 1**.** Time line of the experimental procedures. Pregnant rats were injected with LPS or saline twice a day (12 h interval) stating from the 17th day of gestation (G17) until the end of gestation. At the day of birth (P0) pups experienced one episode of anoxia for 20 min. The sensorimotor restriction procedures were performed daily (16 h day) from P2 to P28 (Stigger *et al*, 2011a). The animals were housed in the environmental enrichment from P0 to P29 day, when the motor skill was assessed.

Table 1: Body weight

Group	Body weight			
	P1	P10	P20	P29
CT	6.67 ± 0.19	20.99 ± 0.53	38.90 ± 1.50	66.84 ± 3.72
EE	6.96 ± 0.17	23.16 ± 1.64	43.22 ± 2.39	65.41 ± 4.65
CP	6.10 ± 0.22 *	19.66 ± 0.71	33.33 ± 0.86 *	58.87 ± 1.37
CP-EE	5.78 ± 0.14 **	17.04 ± 1.12 *	30.45 ± 1.93 **	61.16 ± 3.15

Data are expressed as means of the body weight (in grams) ± SEM. * Significantly different from EE ($p < 0.01$). ** Significantly different from CT and EE ($p < 0.01$).

Table 2:
Number of square crossed on the Open Field test

Group	Number of square crossed			
	1st minute	2nd minute	3rd minute	Total
CT	17.08 ± 2.21	13.33 ± 2.51	11.33 ± 1.69	41.75 ± 5.36
EE	23.03 ± 2.41	14.25 ± 2.09	7.00 ± 1.73	44.28 ± 4.40
CP	10.33 ± 1.65 *	10.20 ± 1.90	5.10 ± 1.79	25.63 ± 5.05
CP-EE	15.68 ± 1.70	12.87 ± 2.40	9.21 ± 2.20	37.76 ± 5.90

Data are expressed as means ± SEM. * Significantly different from EE group ($p < 0.01$).

Table 3:
Motor skill assessment

Group	Motor Skills		
	Rotarod	Horizontal Ladder	Narrow Suspended Bar
CT	62.90 ± 8.58	4.08 ± 0.55	1.00 ± 0.20
EE	132.50 ± 16.60	1.88 ± 0.50	0.13 ± 0.07
CP	48.54 ± 17.53 *	21.07 ± 2.86 ***	4.73 ± 1.17 ***
CP-EE	110.33 ± 19.64 **	0.95 ± 0.30 **	0.58 ± 0.24 **

Motor skill assessment. Rotarod data are expressed as means of latency to fall ± SEM. Horizontal Ladder and Narrow Suspended Bar data are expressed as means of hind limb step errors ± SEM. * Significantly different from EE ($p < 0.01$) ** Significantly different from CP ($p < 0.05$). *** Significantly different from CT, EE and CP-EE ($p < 0.001$).

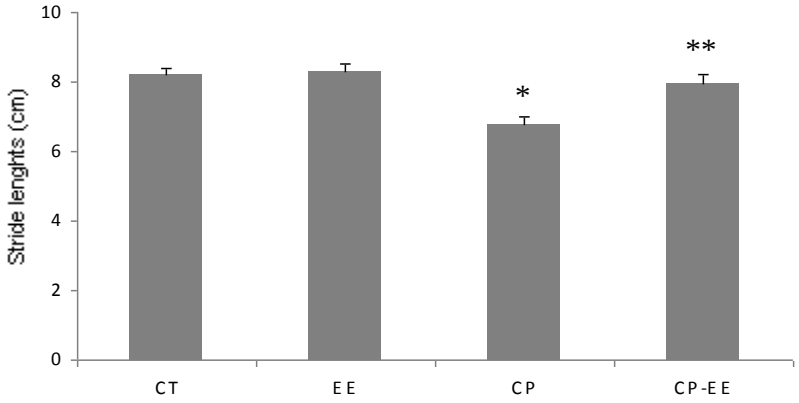

Fig. 2: Gait testing. Stride lengths in groups. Data are expressed as means of the stride lenghts (in cm) ± SEM. * Significantly different from CT and EE ($p < 0.01$). ** Significantly different from CP ($p < 0.01$).

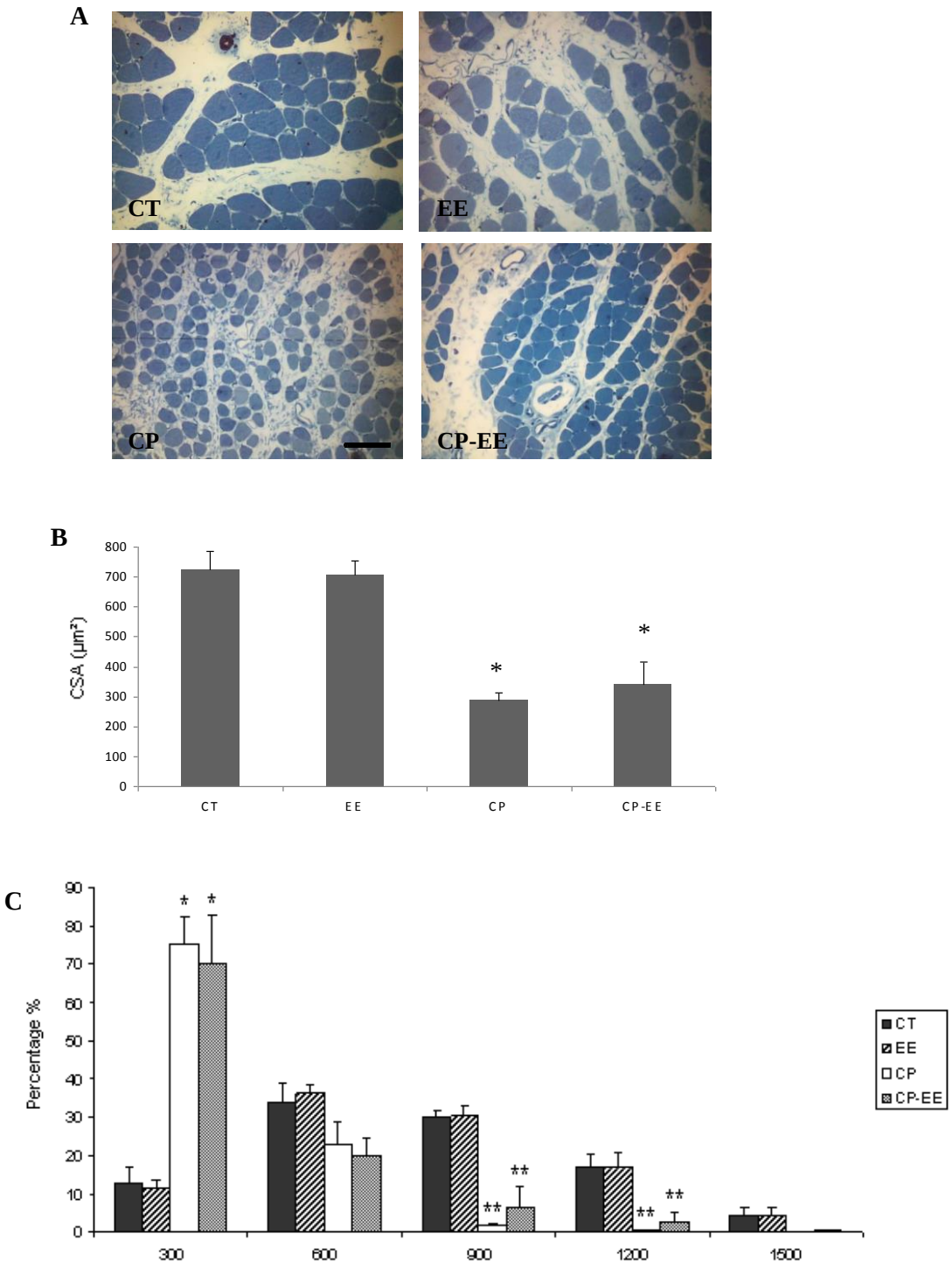

Fig. 3: Effects of EE on the morphometric parameters of soleus muscle. (A) Digitalized images of transverse-semithin sections (1 µm) in all groups (captured at 20x). Scale bar = 50 µm. (B) Cross-sectional fibers (1 µm²) on soleus muscle. * Significantly different from CT and EE groups ($p < 0.01$). (C) Frequency histograms of soleus fiber cross-sectional area. Data are expressed as means ± SEM. * Significantly different from CT and EE groups ($p < 0.001$). ** Significantly different from CT and EE groups ($p < 0.02$).

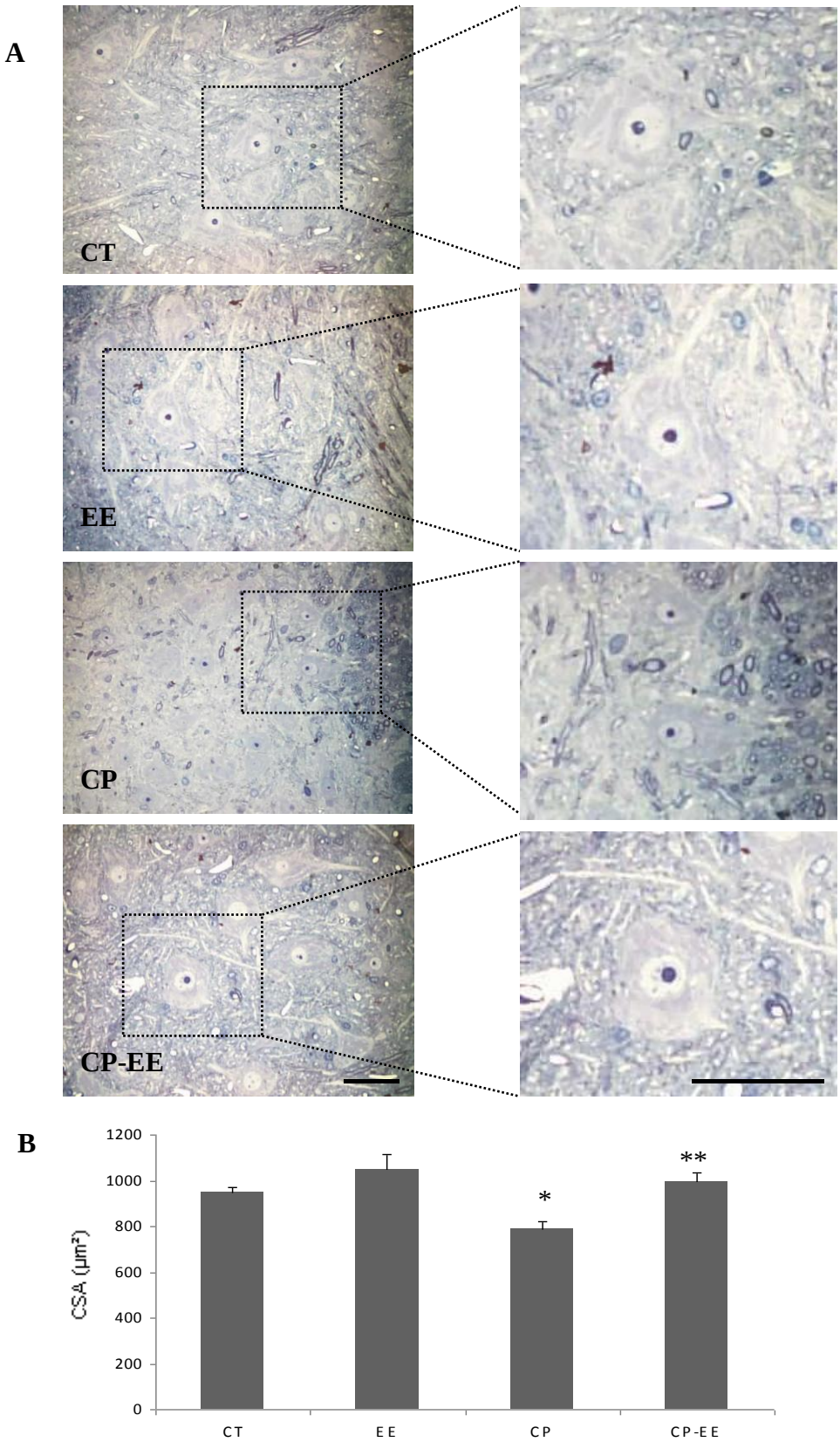

Fig. 4: Motoneuron analysis in the ventral horn of the spinal cord. **A.** Representative photomicrographs of motoneurons from ventral horn in all experimental groups (captured at 20x – left – and amplified 200% - right). Scale bar = 50 µm. **B.** CSA of motoneurons are expressed as means ± SEM. * Significantly different from EE ($p < 0.01$). ** Significantly different from CP ($p < 0.05$).

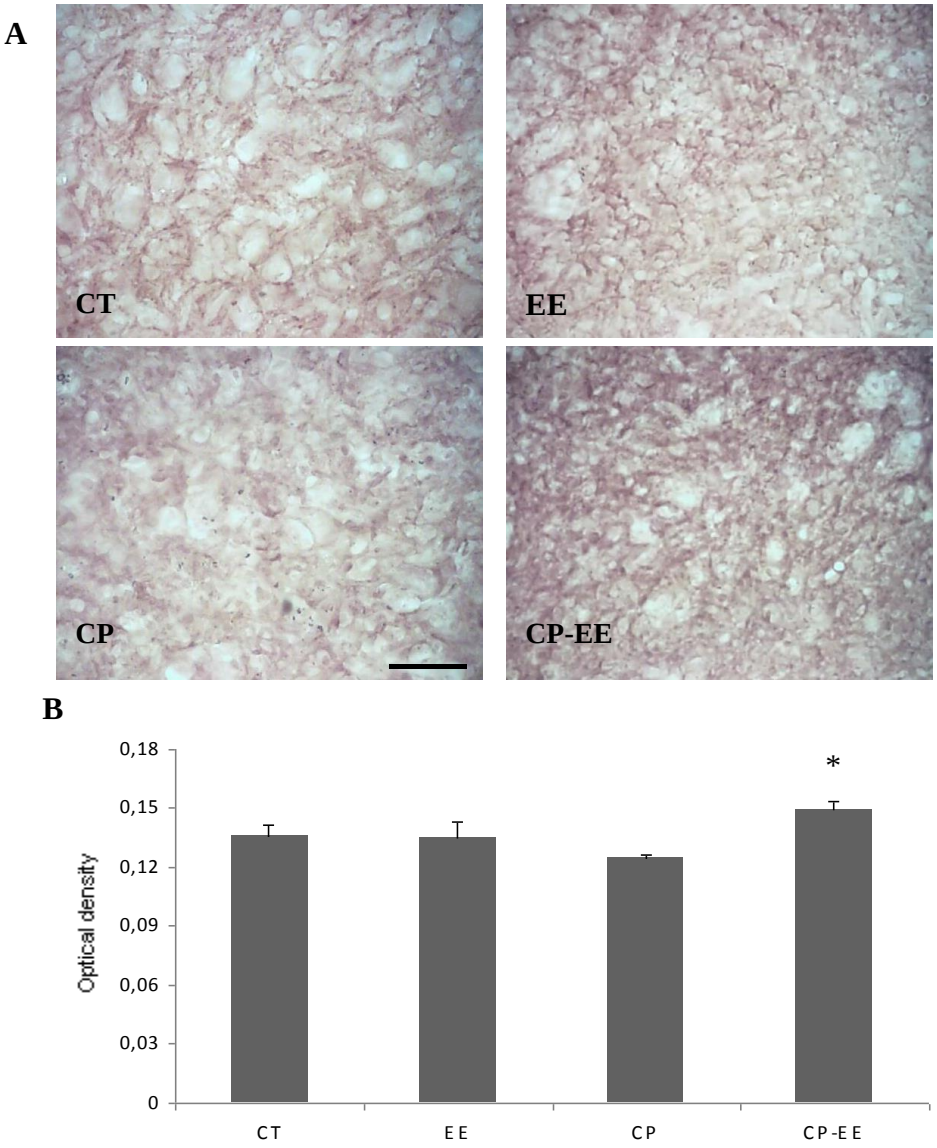

Fig. 5: Synaptophysin immunoreactivity in the ventral horn of the spinal cord. **A.** Representative photomicrographs of synaptophysin in ventral horn in all experimental groups (captured at 20x). Scale bar = 100 μm. **B.** Optical density measurements are expressed as means ± SEM. * Significantly different from CP ($p < 0.05$).

4. DISCUSSÃO GERAL

Embora a PC seja uma encefalopatia estática que resulta de lesões não progressivas no SNC, secundariamente são estabelecidos comprometimentos motores que persistem durante toda a vida e interferem no desenvolvimento normal da criança (BAX *et al*, 2005; KRIGGER, 2006; DAMIANO; ALTER; CHAMBERS, 2009; ZARRINKALAM *et al*, 2010). A reabilitação na PC é preconizada nas fases iniciais da infância, enquanto o SN e o sistema musculoesquelético são mais suscetíveis a adaptações e o desenvolvimento neuromotor normal pode ser facilitado (KOMAN; SMITH; SHILT, 2004; DAMIANO, 2006; BERKER; YALÇIN, 2008). O EA proporciona uma combinação de interação social, atividade física e aprendizagem, que aumentam a possibilidade de estímulos aos animais e é muito utilizada em modelos experimentais de diversas patologias (KLINE *et al*, 2007; SOZDA *et al*, 2010). A presente dissertação teve como objetivo estudar os efeitos do EA precoce na instalação de déficits motores e alterações morfológicas em um modelo de PC em ratos.

Assim como crianças com PC que normalmente apresentam menor crescimento corporal (SAMSON-FANG *et al*, 2002), modelos experimentais de PC com ratos que utilizam restrição sensoriomotora apresentam menores pesos corporais comparados aos animais controle (STRATA *et al*, 2004; MARCUZZO *et al*, 2010). Criar os animais no EA não evitou esta alteração de peso nos animais submetidos ao modelo de PC, embora este ambiente pudesse até promover uma diminuição de peso, devido a maior possibilidade de realizarem atividades físicas (ZAIAS *et al*, 2008). A associação do EA a outras terapias, inclusive a suplementação nutricional, poderia prevenir esta perda de

peso corporal, contribuindo para o desenvolvimento e a aquisição de habilidades motoras (CAMPANOZZI *et al*, 2007).

Por outro lado, o EA foi capaz de prevenir a instalação de déficits motores típicos da PC. Os animais que viveram em EA apresentarem uma tendência a maior atividade locomotora que aqueles com PC que viveram em caixas padrão de biotério. Devido ao teste utilizado (Campo aberto) não ser específico para avaliação motora, outros parâmetros podem ter interferido nos resultados como a hiperatividade espontânea em animais que vivem em ambiente empobrecido (SCHRIJVER *et al*, 2002) e a habituação locomotora nos que vivem no ambiente enriquecido (BENAROYA-MILSHTEIN *et al*, 2004; BRENES; PADILLA; FORNAGUERA, 2009) quando expostos a um ambiente novo. Nos testes específicos para a avaliação das habilidades motoras (Rotarod, Escada Horizontal e Barra Estreita Suspensa), o EA mostrou ser capaz de prevenir os déficits, assim como observado em estudos envolvendo outras patologias (RISEDAL *et al*, 2002; JADAVJI; KOLB; METZ, 2006; KLINE *et al*, 2007; HOFFMAN *et al*, 2008; KLINE *et al*, 2010). Apesar de não ser um treino específico para locomoção, este ambiente também preveniu alterações no comprometimento da marcha em animais submetidos ao modelo de PC, de forma semelhante a resultados obtidos com o treino em esteira que reverteu estes comprometimentos (MARCUZZO *et al*, 2008).

Com o objetivo de entender os mecanismos neurobiológicos envolvidos nestas recuperações funcionais, foram realizadas análises histológicas do músculo sóleo e medula espinhal. Observou-se uma atrofia muscular nos animais submetidos ao modelo de PC, utilizando-se a medida da área de secção transversal das fibras do músculo sóleo. O EA não preveniu estas alterações, provavelmente pela não especificidade da terapia proposta. Marcuzzo *et al* (2008), utilizou o treino em esteira como um treinamento

motor específico e observou uma recuperação desta atrofia no músculo sóleo. Resultados semelhantes também são observados em sujeitos com PC que realizam um treino em esteira ou bicicleta como terapia (DAMIANO, 2006). Outro fator que possivelmente tenha influenciado nos resultados observados no presente estudo, é o curto período de exposição especificamente locomotora dos animais ao EA. Considerando o processo de desenvolvimento dos ratos, a locomoção torna-se eficiente, permitindo uma atividade exploratória, a partir dos 16 dias de vida (ALTMAN; SUDARSHAN, 1975). Um maior tempo de exposição ao EA poderia prevenir ou restaurar esta atrofia muscular.

A análise da área média do soma dos motoneurônios do corno ventral da medula espinhal não apresentou diminuição nos animais submetidos ao modelo de PC em relação aos animais controles, diferentemente do observado em Stigger *et al* (2011). A diferença de idade nos animais avaliados no presente estudo (29 dias de vida) e em Stigger *et al* (2011) (52 dias de vida) pode ser uma explicação para essa diferença nos resultados. É possível que os déficits observados neste modelo não estejam totalmente estabelecidos até o primeiro mês de vida dos animais, indicando também que os déficits podem ser progressivos, aparecendo mesmo após a indução do modelo. Apesar de não ser significativa a diferença entre os animais submetidos ao modelo de PC e os controles, o EA nos animais com PC promoveu um aumento da área média do soma dos motoneurônios em relação ao grupo PC, provavelmente pelo maior exigência de atividade motora exigida pelo ambiente enriquecido.

A expressão de sinaptofisina, uma proteína vesicular pré-sináptica encontrada em terminais nervosos (WALAAS; BROWNING; GREENGARD, 1988), não encontrou-se diminuída significativamente no corno ventral da medula espinhal dos animais submetidos ao modelo de PC quando comparados aos controles, sugerindo não

haver alteração na atividade sináptica nesta região. De forma semelhante ao observado na área média dos motoneurônios, provavelmente a atividade sináptica não esteja ainda comprometida devido a não instalação completa dos déficits do modelo de PC. Porém o EA aumentou a expressão de sinaptofisina em animais submetidos ao modelo de PC em relação ao grupo PC mantido em ambiente padrão, indicando que a estimulação promovida pelo ambiente enriquecido aumenta a atividade sináptica, podendo influenciar na plasticidade neuronal (CHOU *et al*, 2002).

5. CONCLUSÃO

Os achados deste estudo permitem concluir que o enriquecimento ambiental precoce pode prevenir déficits motores através da modificação do tamanho dos motoneurônios e de um incremento na atividade sináptica em um modelo de PC em ratos. Estes resultados evidenciam a importância da intervenção terapêutica precoce na PC buscando prevenir a instalação dos comprometimentos motores secundários à lesão.

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