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

EFEITOS MORFOFUNCIONAIS DA ASSOCIAÇÃO DA ANOXIA PÓS-NATAL E DA RESTRIÇÃO SENSÓRIO-MOTORA: IMPLICAÇÕES PARA UM MODELO DE PARALISIA CEREBRAL EM RATOS E EFEITOS DO EXERCÍCIO FÍSICO

TESE DE DOUTORADO

Simone Marcuzzo

Porto Alegre

2009

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SUMÁRIO

ABREVIATURAS

PC	Paralisia cerebral
SNC	Sistema nervoso central
CIUR	Crescimento intra-uterino retardado
MN	Motoneurônio
P	Dia pós-natal
SR	Restrição sensório-motora (*sensorimotor restriction)*
PA	Anoxia perinatal *(perinatal anoxia)*
PA-SR	Anoxia perinatal combinada à restrição sensório-motora (*perinatal anoxia associated to sensorimotor restriction)*
S1	Córtex Somatossensorial

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RESUMO

A paralisia cerebral (PC) é um grupo desordens do movimento e da postura, atribuídos a insultos durante o desenvolvimento do encéfalo. Em ratos, déficits motores semelhantes à PC podem ser induzidos por imobilização dos membros posteriores (restrição sensório-motora; SR), associada ou não à anoxia perinatal (PA). Além disso, PA e SR têm efeitos deletérios distintos, porém complementares, sobre a organização dos mapas dos membros posteriores no córtex somatossensorial (S1). Investigamos se o treinamento de marcha em esteira ergométrica poderia ter efeitos benéficos sobre déficits consequentes à PA, à SR, ou a uma combinação de ambos. Ratos Wistar machos recém-nascidos foram divididos em quatro grupos: controle (CT), anóxico (PA), submetidos à restrição sensório-motora (SR) e submetidos a anoxia e restrição sensório-motora (PA-SR). Os ratos foram expostos à PA nos dois primeiros dias pós-natais (P0 e P1) e/ou restrição dos membros posteriores do P2 ao P28, 16 h/dia. Ratos controle e experimentais foram submetidos a treinamento em esteira ergométrica do P31 ao P52. O peso corporal e o padrão de marcha (tamanho da passada e ângulo do pé) foram avaliados durante o treinamento. A área média de secção transversal e a densidade de fibras do músculo sóleo foram medidas. A PA, por si só, não causou alterações da marcha ou déficits musculares. No entanto, esses parâmetros foram aumentados em ratos PA treinados. Animais submetidos à SR, com ou sem PA, apresentaram déficits no ganho de peso corporal, diminuição do tamanho da passada, aumento do ângulo do pé e atrofia de fibras do sóleo. No grupo SR, o treinamento melhorou o ganho de peso corporal e tamanho da passada, e diminuiu o percentual das fibras atróficas. No entanto, no grupo PA-SR, o treinamento melhorou somente o comprimento da passada. Na segunda parte deste estudo, investigou-se se a PA, a SR ou uma combinação de ambas produzem alterações no desenvolvimento sensório-motor. Marcos do desenvolvimento (endireitamento na superfície, aversão à queda, estabilidade em superfície inclinada, reação de colocação dos membros inferiores, sobressalto auditivo, abertura dos olhos), foram avaliados diariamente do P3 ao P14 (durante o período de imobilização). Habilidades motoras (caminhada na escada horizontal e caminhada na barra estreita) foram avaliadas semanalmente (do P31 ao P52). Além disso, no P52 foram medidas a espessura de S1, do córtex cerebelar e do corpo caloso, e os números de células neuronais e gliais em S1 foram contados. Nas avaliações desenvolvimentais, a SR (com ou sem PA) atrasou a estabilidade em superfície inclinada e acelerou o aparecimento da reação de colocação. As habilidades motoras foram significativamente prejudicadas em animais SR (PA ou não). Medidas de espessura não apresentaram diferenças significativas entre os grupos. A contagem de células em S1 mostrou que a PA, associada ou não à SR, aumentou o número de células gliais, enquanto a SR sozinha, reduziu o número de células neuronais. Finalmente, ratos PA-SR tiveram um aumento no tamanho dos somas neuronais. Conclui-se que o treinamento em esteira ergométrica foi capaz de melhorar a marcha em animais submetidos a SR e PA-SR. Além disso, a SR prejudicou a aquisição de marcos do desenvolvimento e das habilidades motoras. Além disso, ambos grupos SR e PA apresentaram alterações histológicas em S1, que podem ter contribuído para os déficits sensório-motores.

ABSTRACT

Cerebral palsy (CP) is a group of movement and posture disorders attributed to insults in the developing brain. In rats, CP-like motor deficits can be induced by early hind limb immobilization (sensorimotor restriction; SR), associated or not with perinatal anoxia (PA). Also, PA and SR have distinct but additional deleterious effects on the organization of hind limb somatosensory cortical maps. In the first part of this study, we investigated whether treadmill locomotor training could have beneficial effects on deficits consequent to PA, SR or a combination of both. Newborn male Wistar rats were divided into four groups: control (CT), anoxic (PA), sensorimotor-restricted (SR) and anoxic and sensorimotor-restricted (PA-SR). Rats were exposed to PA in the first two postnatal days (P0 and P1) and/or hind-limb SR from P2 to P28 for 16 h/day. Control and experimental rats underwent treadmill training from P31 to P52. Body weight and walking pattern (stride length and foot angle) were measured during treadmill locomotor training. Soleus muscle cross-sectional mean area and fiber density were measured. PA per se did not cause gait or muscle deficits. However, these parameters were increased in trained PA rats. SR animals, either with or without PA, showed deficits in body weight gain, decreased stride length, wider foot angle and soleus fiber atrophy. In the SR group, treadmill training improved body weight gain and stride length, and decreased the percentage of the atrophic fibers. However, in the PA-SR group, training improved stride length only. In the second part of this study, we addressed the question whether PA, early SR and a combination of both produce alterations on sensorimotor development. Developmental milestones (surface righting, cliff aversion, stability on an incline surface, proprioceptive placing, auditory startle, eye opening) were assessed daily from P3 to P14 (during immobilization period). Motor skills (horizontal ladder and beam walking tests) were evaluated weekly (from P31 to P52). Also, on P52 were measured the thickness of S1, cerebellar cortex and corpus callosum, and the neuronal and glial cell numbers in the S1 were counted. In developmental assessments, SR (with or without PA) delayed the stability on an incline surface and hastened placing reflex appearance. The motor skills were significantly impaired in SR animals (PA or not). Tickness measurements did not present differences between groups. Quantitative histology in S1 showed that PA, either alone or associated with SR, increased the number of glial cells, while SR alone reduced neuronal cell numbers. Finally, the combination of PA and SR increased the size of neuronal somata. We conclude that treadmill training were able to improve the gait in SR and PA-SR animals. Also, SR impaired the achievement of developmental milestones and motor skills. Moreover, both SR and PA induced histological alterations in S1, which may have contributed to the sensorimotor deficits.

1. INTRODUÇÃO

1.1. Paralisia Cerebral

A paralisia cerebral (PC) compreende um grupo de desordens do movimento e da postura que causam limitação funcional, e são atribuídas a distúrbios não-progressivos que ocorrem do sistema nervoso central (SNC) fetal ou da criança em desenvolvimento (BAX et al., 2005; BLAIR; WATSON, 2005). O dano pode ocorrer ainda no útero, durante o nascimento ou nos primeiros dois anos de vida (KOMAN et al., 2004). A incidência da PC tem permanecido estável nos últimos 40 anos (PANETH et al., 2006), porém a prevalência da PC, de aproximadamente 2,5 por 1.000 nascidos vivos, tem aumentado tanto pelo aumento da sobrevivência de nascidos pré-termo quanto pelo aumento da sobrevida geral dos pacientes, que com frequência atingem a vida adulta (KEOGH; BADAWI, 2006). Isso se deve ao avanço nos cuidados perinatais, que causou um aumento da sobrevivência de recém-nascidos criticamente doentes (DODGE, 2008). A maioria dos casos, 70 a 80%, são adquiridos no período pré-natal, originados por uma grande variedade de causas, muitas delas desconhecidas. As condições adversas ao nascimento, como asfixia, compreendem apenas uma minoria de casos, cerca de 6%, aí incluídas complicações obstétricas do trabalho de parto (KRIGGER, 2006). Condições perinatais que aumentam o risco de desenvolvimento da PC incluem: nascimento com menos de 32 semanas de gestação, peso menor que 2.500 g e retardo no crescimento intrauterino (CIUR), hemorragia intracraniana e traumas tocúrgicos. As causas pós-natais mais comuns de PC são: meningite, encefalite, icterícia neonatal, acidente de trânsito, quedas e abuso infantil; e perfazem 10 a 20% dos casos de PC (KRIGGER, 2006).

O diagnóstico da PC é clínico, sendo os principais achados: o atraso na aquisição dos marcos do desenvolvimento, o tônus muscular anormal, a hiperreflexia, e a ausência de

evidência de um diagnóstico mais específico (DODGE, 2008). Além disso, há uma grande quantidade de morbidades associadas: a epilepsia está presente em 20 a 40% dos casos; a sensibilidade é alterada em metade das crianças; a dor crônica é relatada por mais de 25% dos adultos; mais de 80% tem algum prejuízo da fala; quase 75% das crianças têm baixa acuidade visual; metade das crianças tem problemas gastrointestinais e de alimentação; prejuízo no crescimento corporal ocorre em 25% das crianças com PC, e aproximadamente metade tem déficits cognitivos (ODDING et al., 2006).

A natureza ampla e inclusiva do termo PC causa um problema para classificá-la. Tradicionalmente, tem sido utilizado o sistema de classificação que combina o tipo predominante de anormalidade motora com a distribuição dessa anormalidade (Tabela 1; DODGE, 2008).

Tabela 1. Tipos de Paralisia Cerebral (adaptado de Dodge, 2008).

Espástica	Hemiplegia (envolvimento de um hemicorpo) Diplegia (envolvimento maior das extremidades inferiores) Quadriplegia (envolvimento de todo corpo)
Discinética	Coreoatetose Distônica
Hipotônica	Mista

A espasticidade e as desordens motoras mistas somam mais de 85% dos tipos de PC, já a PC discinética é muito menos comum (GRAHAM; SELBER, 2003). A

espasticidade é um desequilíbrio do sistema sensório-motor caracterizada por hiperreflexia e aumento da resposta muscular ao alongamento aplicado, positivamente correlacionado com a taxa de alongamento e dependente da velocidade (LANCE, 1990). A espasticidade é um componente da síndrome do motoneurônio (MN) superior, composta de tônus muscular aumentado, reflexos hiperativos, fraqueza muscular e falta de coordenação motora (GOLDSTEIN, 2001). O desenvolvimento da espasticidade é causado pela perda da inibição dos impulsos supra-espinhais, que, por sua vez, produzem a hiperexcitabilidade dos arcos reflexos espinhais segmentares (GOLDSTEIN, 2001). Quanto ao prognóstico, crianças com PC do tipo hemiplégico e diplégico habitualmente podem caminhar, enquanto aquelas com quadriplegia raramente o fazem, e aquelas com PC discinética possuem uma chance intermediária de deambulação (DODGE, 2008).

A lesão patológica encefálica mais prevalente identificada na PC é a leucomalácia periventricular (perda de substância branca encefálica), que resulta da vulnerabilidade dos oligodendrócitos imaturos antes da 32ª semana de gestação (JOHNSTON; HOON, 2006). A asfixia perinatal em crianças a termo causa um padrão diferente das lesões encefálicas vistas em lesões pré-termo. Esse tipo de insulto causa lesões seletivas no córtex encefálico, núcleos da base e tronco encefálico (FOLKERTH, 2005; JOHNSTON; HOON, 2006). Embora os músculos e os nervos periféricos não sejam lesados inicialmente, a doença normalmente evolui com mudanças morfológicas e bioquímicas no sistema neuromuscular (GOLDSTEIN, 2004). As anormalidades motoras, como a espasticidade e a fraqueza muscular, podem levar à rigidez articular e à inabilidade para deambular (KOMAN et al., 2004; KRIGGER, 2006). Em crianças com PC, os músculos esqueléticos não relaxam durante a atividade, devido à espasticidade. Além disso, a atividade já se encontra reduzida pela própria fraqueza muscular e a falta de equilíbrio (GRAHAM; SELBER, 2003). A

incapacidade motora piora o condicionamento físico que, por sua vez, agrava a incapacidade (GRAHAM; SELBER, 2003; DAMIANO, 2006; Figura 1).

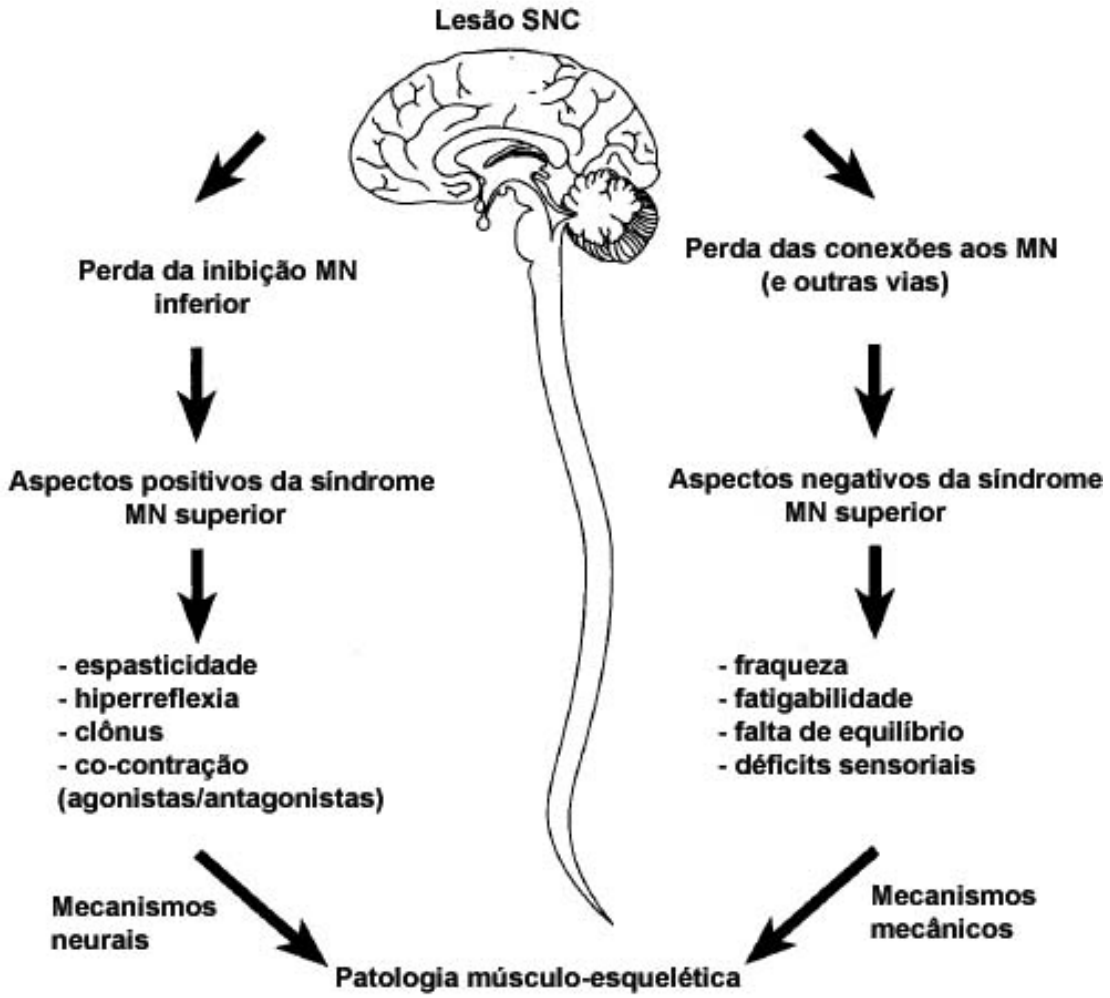

Figura 1. Diagrama mostrando a patologia músculo-esquelética na PC. A patologia do SNC na PC é definida como encefalopatia estática. Dada a grande variabilidade de localização e severidade das lesões, as síndromes clínicas são extremamente variáveis. Em termos motores, a PC resulta em uma lesão do MN superior que causa uma série de aspectos positivos e negativos que interagem (mecanismos neurais somam-se a mecanismos mecânicos) para produzir a patologia músculo-esquelética (adaptado de Graham; Selber, 2003).

Análises histopatológicas de biópsias de músculos dos membros inferiores de pacientes com PC mostraram anormalidades no tamanho das fibras, transição de fenótipo lento para rápido (ITO et al., 1996; MARBINI et al., 2002) e atrofia das fibras (MARBINI et al., 2002). Além disso, o sistema muscular apresenta proliferação de matriz extracelular, aumento da rigidez, e propriedades mecânicas inferiores do material extracelular (FORAN et al., 2005). Os ossos e extremidades podem apresentar alterações de comprimento (NOVACHECK; GAGE, 2007).

1.2. Modelos Animais

A validade de um modelo de PC em roedores se deve à facilidade do manejo em laboratório, e à maturação pós-natal rápida, o que permite observar o desenvolvimento de comportamentos, como a aquisição da marcha (ROOHEY et al., 1997; RICE; BARONE, 2000). A hipóxia-isquemia perinatal tem sido bastante utilizada para causar insultos no encéfalo imaturo (JANSEN; LOW, 1996; HOEGER et al., 2000; ZHURAVIN et al., 2004; LUBICS et al., 2005; ROBINSON et al., 2005). Entretanto, apesar da hipóxia-isquemia perinatal em roedores causar atrofia de regiões encefálicas, como o estriado, o córtex sensório-motor e o hipocampo dorsal no lado ipsilateral à lesão (JANSEN; LOW, 1996), as alterações motoras resultantes são sutis e transitórias (JANSEN; LOW, 1996; HOEGER et al., 2000; ZHURAVIN et al., 2004; LUBICS et al., 2005; ROBINSON et al., 2005). Em geral, roedores se recuperam muito bem após esses insultos e os déficits motores podem ser mínimos e, portanto, difíceis de serem avaliados (WRIGHT; RANG, 1990). Há uma discrepância entre a existência de lesão encefálica e a falta de anormalidades locomotoras e

posturais em ratos semelhantes à PC em humanos, devido a organização e atribuições diferentes do sistema córtico-espinhal em humanos e roedores (EYRE, 2007).

Evidências em humanos sugerem também que respostas inflamatórias fetais desencadeadas por infecções maternas, como a corioamnionite bacteriana subclínica, podem contribuir para o dano encefálico perinatal (NELSON; CHANG, 2008). Insultos aplicados nos períodos intra-uterino e pós-natal, usando injeção de lipopolissacarídeo, induzem resposta inflamatória e causam dano da substância branca encefálica, porém não causam alterações dos marcos do desenvolvimento ou em testes motores no campo aberto e *rotarod* (POGGI et al., 2007; ROBERSON et al., 2006). A associação dos dois insultos (injeção pré-natal de lipopolissacarídeo e hipóxia-isquemia neonatal) causou déficits motores menos sutis, medidos no campo aberto e *rotarod,* e lesões corticais e subcorticais mais extensas (GIRARD et al., 2009).

No entanto, apesar desses modelos contribuírem para o entendimento da patogênese central da PC e seus múltiplos e complexos aspectos, as características clínicas motoras e de anormalidades observáveis da marcha têm sido mais bem observados em modelos de desuso dos membros posteriores (WALTON et al., 1992; WESTERGA; GRAMSBERGEN, 1993; CANU; FALEMPIN, 1998). Em ratos, a restrição dos movimentos em certos períodos críticos do desenvolvimento, como a suspensão pela cauda entre o 13º e o 31º dia pós-natal, causa prejuízo no desempenho locomotor até a idade adulta (WALTON et al., 1992).

O modelo utilizado no presente estudo foi integralmente baseado naquele criado por Strata e cols. (2004) e Coq e cols. (2008). No intuito de causar um desuso dos membros inferiores, semelhante à falta de movimento que ocorre na PC no início do desenvolvimento motor, associado ao insulto anóxico perinatal, aqueles autores submeteram ratos recém-

nascidos a 2 episódios de anoxia de 12 min cada, no primeiro dia do nascimento (P0) e no dia seguinte (P1). Para o procedimento de anoxia os filhotes foram colocados em uma câmara de anoxia e foram expostos a um fluxo contínuo de nitrogênio a 100%. A câmara foi mantida a 37 ºC para manter a temperatura corporal dos ratos lactentes. Após o procedimento, os filhotes foram colocados em condições atmosféricas normais, ressucitados e assim que a cor da pele e a respiração voltassem ao normal, eram recolocados em suas caixas. Esse procedimento causou uma mortalidade em torno de 5% (STRATA et al., 2004). Do P2 ao P28, foi feita a restrição sensório-motora (SR) por 16 horas por dia, nas 8 horas restantes foi permitido a livre movimentação dos animais. Para a SR, os membros posteriores são contidos juntos com uma fita adesiva em posição estendida mantida com uma moldura feita de um material moldável (epóxi), que permite somente movimentos limitados na região do quadril (Figura 2). Esse procedimento foi bem tolerado pelos filhotes e não prejudicou a eliminação de urina e fezes, nem os cuidados maternos (STRATA et al., 2004).

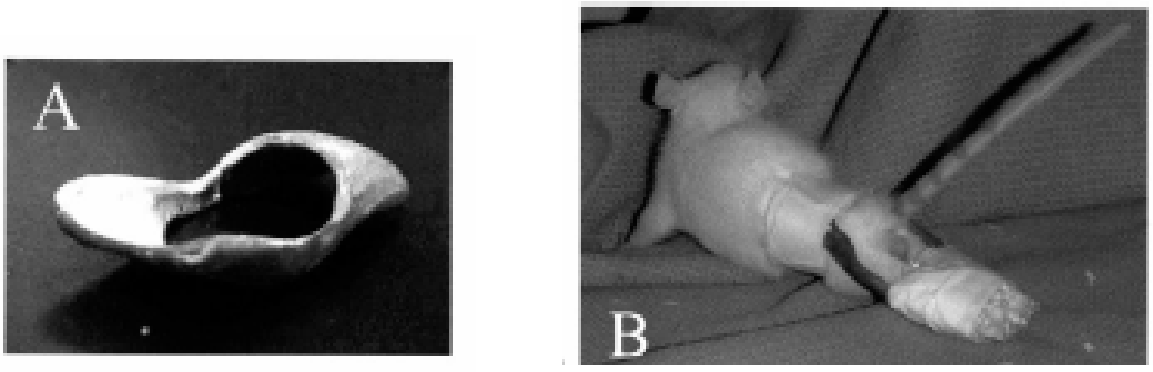

Figura 2. A) Moldura utilizada para a restrição sensório-motora dos membros posteriores. Uma parte fica em contato com o dorso do animal, na altura dos quadris, e a outra fica em contato com a parte anterior das pernas. B) Filhotes com os membros contidos em posição estendida com utilização de fitas adesivas e a moldura (adaptado de Strata et al., 2004).

Nesse trabalho foi mostrado que a imobilização precoce dos membros posteriores, i.e., a SR, combinada ou não a anoxia perinatal (PA), foi capaz de produzir efeitos duradouros, como uma diminuição da velocidade de crescimento, aumento do tônus muscular dos membros inferiores, padrões anormais da marcha, atrofia muscular, e degeneração das articulações do tornozelo e joelho. Estes autores demonstraram ainda uma representação distorcida dos membros posteriores nos córtices motor (Figura 3) e somatossensorial (S1), que seriam semelhantes àquelas alterações encontradas em crianças com PC. Quanto à PA, os dois episódios de anoxia causaram somente leves alterações no tônus muscular, desempenho motor ligeiramente alterado e sutis mudanças na organização cortical.

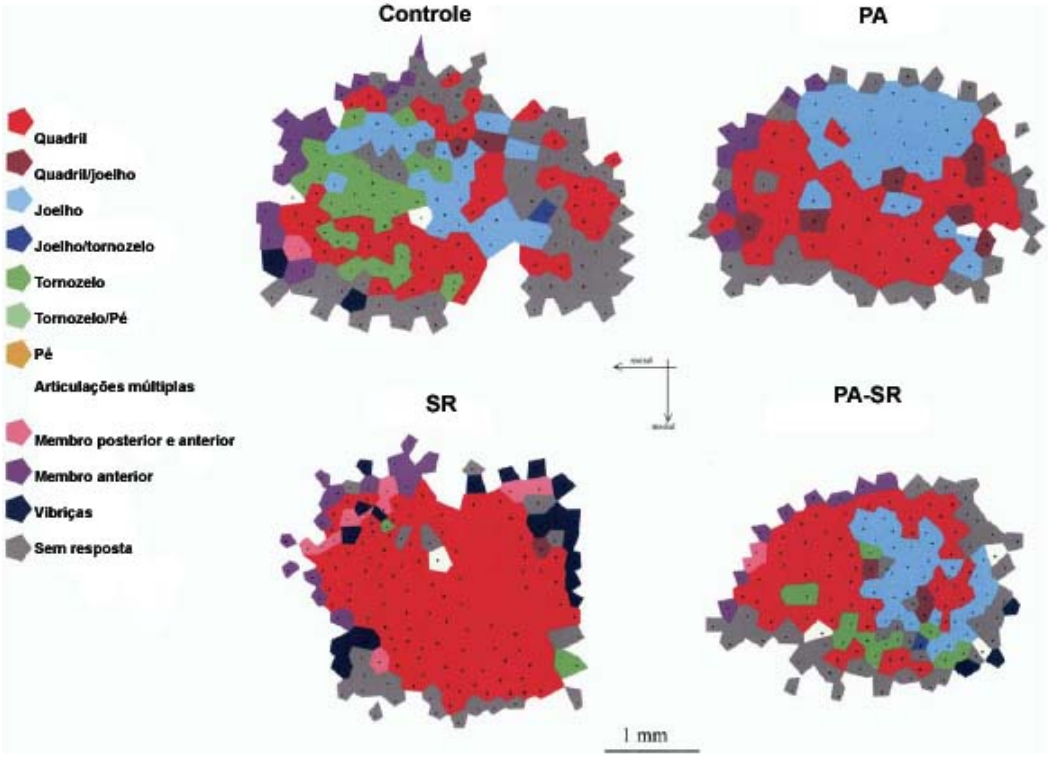

Figura 3. Representação dos membros inferiores no córtex motor de ratos controle e submetidos à anoxia perinatal (PA), restrição sensório-motora (SR) e anoxia perinatal e restrição sensório-motora (PA-SR). Os quatro painéis mostram os mapas de representação dos membros inferiores no córtex motor de um rato representativo de cada grupo. A maior alteração de topografia no córtex motor foi vista no grupo SR, mais de 80% da área do córtex motor evocou contração do quadril (adaptado de Strata et al., 2004).

1.3. Alterações periféricas e centrais da restrição de movimentos em período crítico

O período de maturação de S1 é caracterizado pelo estabelecimento dos mapas somatotópicos corticais, da emergência de conexões corticais de longo alcance, elevada plasticidade dependente da experiência e movimentos músculo-esqueléticos descordenados espontâneos (KHAZIPOV et al., 2004). A organização de S1 é largamente determinada pela atividade aferente, mas a plasticidade encefálica perinatal aumenta a vulnerabilidade a experiências sensório-motoras adversas, levando ao desenvolvimento e comportamento anormais (ANANG; SCALZO, 2000). Entradas sensoriais são, portanto, componentes essenciais na função, controle e desenvolvimento motores. Crianças normais produzem um grande e rico repertório de movimentos espontâneos na vida fetal até o final da metade do primeiro ano de vida. Crianças com PC apresentam movimentos escassos, monótonos, estereotipados e com falta de complexidade, variação e fluência (HADDERS-ALGRA, 2004).

Geralmente, doenças que afetam a motricidade no início da vida produzem efeitos deletérios sobre a maturação do sistema motor, já que processos dependentes da atividade motora são imprescindíveis para refinar as conexões e estabelecer o padrão futuro de especificidade topográfica e de conexões do sistema motor (EYRE, 2007; MARTIN et al., 2007). Na PC, à lesão encefálica inicial, soma-se o comprometimento da movimentação motora normal no início da vida, que pode agravar os déficits motores. Apesar da lesão encefálica não ser progressiva, o quadro motor geralmente piora com o tempo (GRAHAM; SELBER, 2003), causando mais inabilidade (DAMIANO, 2006). Lesões perinatais em crianças causam uma mudança observável na mobilidade espontânea (FERRARI et al., 1990), porém, somente mais tarde são observados os sinais neurológicos característicos da

PC (EYRE, 2007). A intervenção terapêutica deve concentrar-se em impulsionar atividade ao sistema residual à lesão com o objetivo de reforçar conexões sinápticas apropriadas (CLOWRY, 2007) e prevenir danos adicionais (DAMIANO, 2006).

Além disso, as mudanças músculo-esqueléticas podem contribuir para os impulsos sensoriais anormais ao encéfalo, resultando em informações sensoriais aberrantes e repetitivas, contribuindo para a reorganização deletéria dos córtices S1 e motor, e assim, em função motora deficiente (COQ et al., 2008). Porém, déficits sensoriais subjacentes a déficits motores são frequentemente ignorados na PC (COOPER et al., 1995), apesar de já demonstrada reorganização de S1 após lesão encefálica perinatal em crianças (CLAYTON et al., 2003; CHU et al., 2000).

Em roedores, o desenvolvimento da locomoção se dá nas primeiras duas semanas de vida pós-natal, e o padrão de marcha adulto é estabelecido em torno do 15º dia de vida (EYRE, 2007; CLOWRY, 2007, Figura 4). Estudos em que a atividade muscular foi reduzida na primeira e segunda semana pós-natal causam modificações periféricas e centrais de longo prazo, tais como, atraso do desenvolvimento muscular e redução da eliminação da inervação polineuronal na placa motora (GREENSMITH et al., 1998), redução do número de MN medulares (GREENSMITH; VRBOVÁ, 1992), atraso na maturação de MN (PASTOR et al., 2003), aumento no número de neurônios córtico-espinhais no córtex encefálico (HUTTENLOCHER, BONNIER, 1991). Um modelo de deprivação sensorial, obtido pela suspensão dos membros inferiores em ratos, reduziu a área cortical destinada aos membros inferiores, enquanto aumentaram os campos receptivos (LANGLET et al., 1999) e os níveis de RNAm de neurotrofinas em S1 (DUPONT et al., 2005).

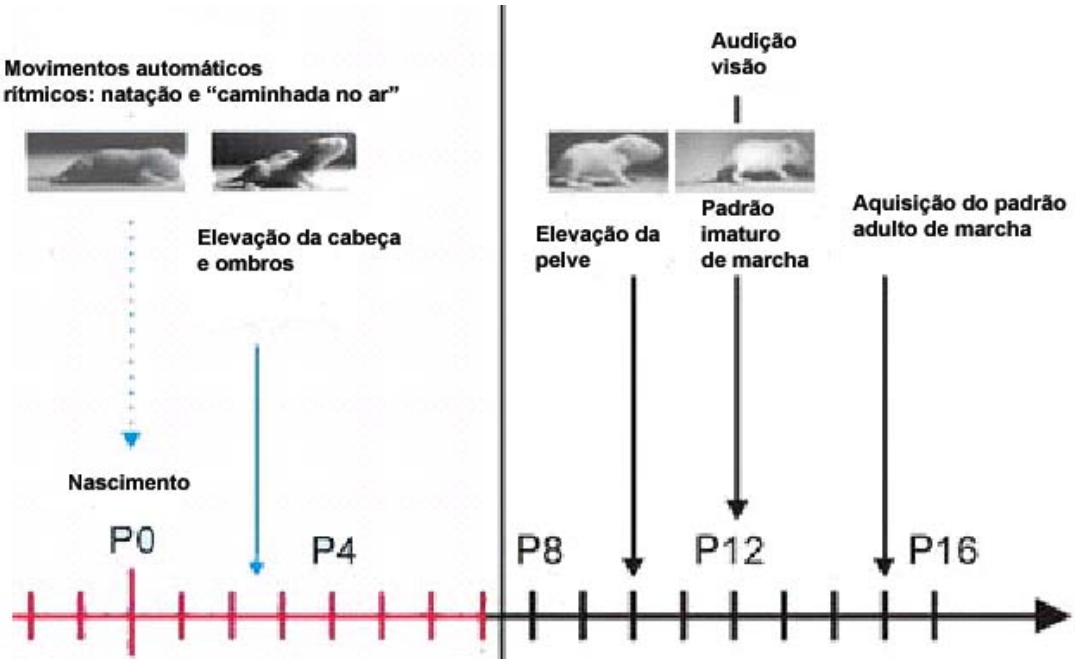

Figura 4. Desenvolvimento da marcha em ratos. Ao nascimento os movimentos são automáticos e rítmicos, porém são observáveis quando a gravidade é reduzida (na água – movimentos de natação, e quando o animal é suspenso – "caminhada no ar"). Entre o P2-P3 os animais conseguem elevar a cabeça e os ombros. No P10 conseguem elevar a pelve. No P12 o padrão de marcha é imaturo, desenvolvem também a audição e a visão. No P15 adquirem o padrão maduro de marcha (adaptado de Clarac et al., 2004).

Utilizando o modelo de asfixia e desuso, Coq e cols. (2008) demonstraram que a PA não produziu alterações dos mapas corticais, porém a SR dos membros posteriores causou desorganização topográfica da representação dos membros posteriores em S1. Ratos imobilizados apresentaram mapas podais degenerados, com campos receptivos maiores e aumentada responsividade cortical à estimulação táctil. Esta desorganização aumentou quando desuso e PA foram associados, e a combinação de ambos os procedimentos causou diminuição das áreas corticais devotadas ao pé inteiro. Este modelo mostrou que a experiência motora precoce do animal tem um papel importante no desenvolvimento motor normal e que a restrição dos membros posteriores, associada ou não à PA, contribui para a gênese das anormalidades de movimento (STRATA et al., 2004). No entanto, não está claro

se a representação cortical aberrante ocorre através de mudanças intrínsecas na organização celular do córtex, ou devida a mudanças nos níveis inferiores (espino-talâmicos) de processamento sensorial.

1.4. Exercício Físico

Características comuns da marcha na PC incluem diminuição da velocidade, diminuição do comprimento da passada e/ou ritmo e diminuição da habilidade de aumentar velocidade sob demanda (EAGLETON et al., 2004; Figura 5). Há correlação direta entre a força produzida pelos músculos dos membros inferiores e a eficiência da marcha e habilidade motora em crianças com PC, sugerindo que a fraqueza dos músculos dos membros inferiores pode ser um dos fatores que limitam essas habilidades (KRAMER; ANN MACPHAIL, 1994).

Figura 5. Padrão de marcha típico apresentados por crianças com PC do tipo diplegia espástica. Em crianças com PC, a marcha equina ou caminhada na ponta dos pés é anormalidade mais prevalente (QUINBY et al., 2005).

Não há cura para a PC, mas um melhor condicionamento físico leva a melhor saúde, e à prevenção ou redução de prejuízos secundários (DAMIANO, 2006). Nos últimos anos, estratégias baseadas em atividades físicas, como o treinamento de marcha em esteira ergométrica, mostraram-se capazes de melhorar as habilidades motoras e deambulatórias de crianças com PC, criando um consenso sobre a importância dessa atividade na fisioterapia nestes pacientes (RICHARDS et al., 1997; CHERNG et al., 2007; BEGNOCH; PITETTI, 2007; BORGGRAEFE et al., 2008). A prática da locomoção com o auxílio da esteira ergométrica pode ajudar a fortalecer os músculos dos membros inferiores e ativar o sistema de controle da locomoção enquanto a criança pratica um comportamento específico da tarefa que se objetiva melhorar (RICHARDS et al., 1997).

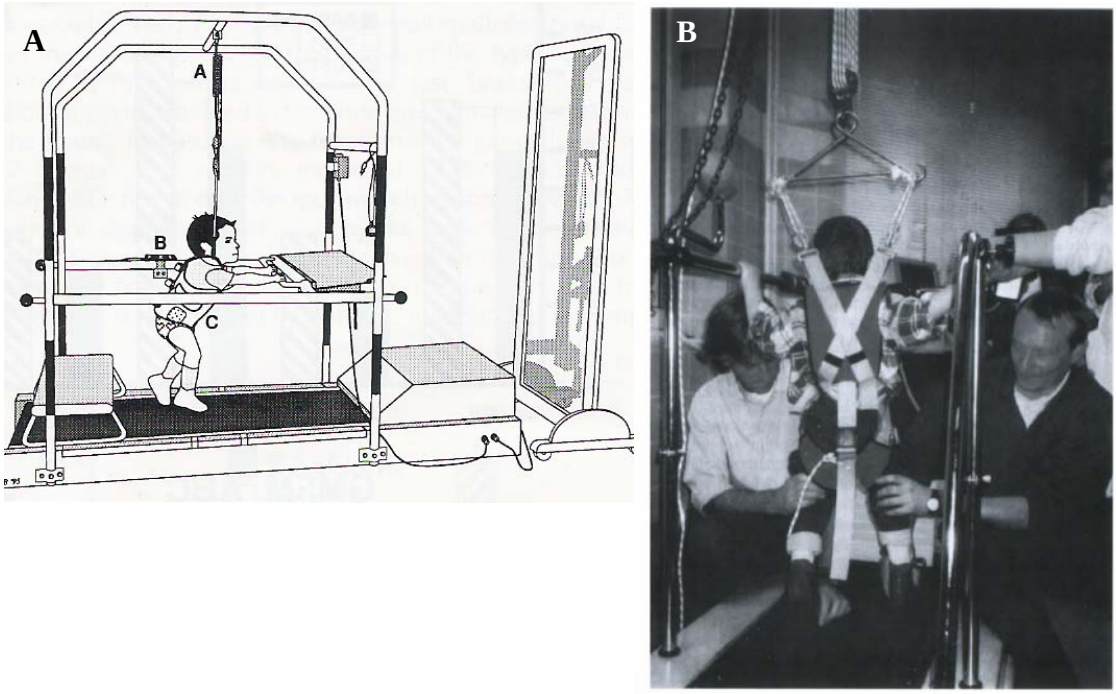

Figura 6. (A) Esquema e (B) fotografia do treino de marcha em esteira em crianças com PC utilizando suporte de peso corporal e aparato que consiste em um cinto que sustenta uma porcentagem do peso corporal, facilitando o treinamento de marcha, já que elas não caminham. Em B fisioterapeutas estão auxiliando a realização da marcha (RICHARDS et al., 1997; SCHINDL et al., 2000).

1.5. Justificativa

A PC é uma doença de características motoras ainda bastante prevalente em nosso meio. Ela causa prejuízos funcionais importantes, levando a limitação das atividades de vida diária e dependência funcional.

Os modelos geralmente estudados que procuram simular a PC em ratos (hipóxia-isquemia, exposição à lipopolissacarídeo) não causam as alterações motoras típicas da PC. O modelo em roedores que associa a anoxia perinatal e a restrição sensório-motora se aproxima mais do fenótipo motor da PC. No entanto, esse modelo ainda requer maior descrição quanto as suas implicações funcionais e possíveis alterações histopatológicas. Bem como os possíveis mecanismos de recuperação após a exposição desse modelo a intervenções terapêuticas.

A maioria dos estudos de prejuízos motores em modelos animais tem utilizado intervenções terapêuticas com o intuito de clarificar os mecanismos de recuperação funcional (RAMIC et al., 2006; ENGESSER-CESAR et al., 2007; ILHA et al., 2008). Além disso, adicionar dados à caracterização do modelo originalmente proposto por Strata e cols. (2004) e Coq e cols. (2008), requer que esse modelo seja testado e estudado quanto às intervenções correntes na fisioterapia usualmente indicadas na PC. Com este intuito, na primeira fase deste trabalho buscou-se evidenciar o conjunto de alterações histológicas periféricas do modelo em ratos de alterações motoras semelhantes à PC, originalmente descrito por Strata e cols. (2004) e Coq e cols. (2008) e a hipótese de que o exercício em esteira ergométrica, aplicado a ratos submetidos à SR, com ou sem PA, pode auxiliar na recuperação de anormalidades da marcha e da histologia do músculo sóleo. Os resultados dessa primeira hipótese são apresentados na forma de artigo (primeiro artigo).

Como parte do esforço em entender o papel da anoxia perinatal e do desuso precoce no desenvolvimento de um modelo de PC, na segunda etapa deste estudo foi realizado um conjunto de observações acerca do desenvolvimento neurológico dos animais e seu desempenho em testes funcionais, e buscamos examinar se esse modelo poderia apresentar alterações histológicas centrais, em nível do SNC. Os resultados dessa segunda hipótese de trabalho são apresentados na forma de artigo (segundo artigo).

O estabelecimento desse modelo animal como um modelo adequado de PC pode auxiliar no entendimento dos mecanismos patológicos dessa enfermidade, assim como no desenvolvimento de estratégias terapêuticas mais eficazes.

3. OBJETIVOS

3. Objetivos

- evidenciar o conjunto de alterações funcionais e histológicas periféricas do modelo em ratos de alterações motoras semelhantes à PC;

- estudar se essas alterações podem ser revertidas pelo exercício em esteira ergométrica,

- analisar o desenvolvimento neurológico dos animais e seu desempenho em testes funcionais,

- examinar se esse modelo poderia apresentar alterações histológicas centrais, em nível do SNC.

4. RESULTADOS

4. Resultados

4.1. Resultados do primeiro artigo: Beneficial effects of treadmill training in a cerebral palsy-like rodent model: walking pattern and soleus quantitative histology. Brain Res. 2008;1222:129-40.

Nesse primeiro estudo, os animais submetidos a PA, SR e PA-SR foram expostos ao treinamento de marcha em esteira ergométrica. O treinamento foi realizado 5 dias por semana, por 3 semanas. Os animais foram pesados semanalmente durante o treinamento. Duas variáveis de marcha foram selecionadas para serem estudadas, o comprimento da passada e o ângulo do pé em relação ao solo no contato inicial da fase de apoio da marcha. A histologia do sóleo foi estudada já que ele é recrutado durante a marcha na esteira (DUDLEY et al., 1982; ROY et al., 1985), além de ser um músculo postural e apresentar mudanças após períodos de desuso (THOMASON; BOOTH, 1990). Além disso, seu desenvolvimento em uma posição encurtada pode levar ao aumento do ângulo do pé e diminuição do tamanho da passada.

Os resultados mostraram que a PA, sozinha, não causou perda de peso, alterações da marcha ou alterações histológicas musculares. Entretanto, o peso, o comprimento da passada e a área transversal das fibras do músculo sóleo aumentaram nos ratos PA submetidos ao treinamento físico. Em acordo com resultados prévios, a SR, associada ou não com PA, causou diminuição do ganho de peso e alterações na marcha, aqui mostradas pelo tamanho reduzido do comprimento da passada e por um maior ângulo do pé ao contato inicial na fase de apoio da marcha, acompanhados por degenerações histológicas do músculo sóleo. O exercício de esteira de baixa intensidade, iniciado após as alterações já

estarem presentes, isto é, depois do término do período de SR, melhorou o comprimento da passada em ambos os grupos SR, associados ou não à PA, aumentou o ganho de peso e diminuiu o grau de alteração morfológica do músculo sóleo, no grupo SR apenas. Apesar do treinamento mudar estes parâmetros, o ângulo do pé permaneceu inalterado com o treinamento.

Esse foi o terceiro estudo que utilizou o modelo de características semelhantes à PC que combina a PA e a SR. Nossos resultados estão de acordo com os resultados prévios (STRATA et al., 2004; COQ et al., 2008) que mostram que esse modelo produz efeitos de longo prazo no sistema locomotor, em níveis funcionais e histopatológicos. A restrição sensório-motora foi a responsável pelos déficits motores, porém a anoxia pode ter contribuído para um fenótipo mais complexo que ainda requer maior caracterização nesse modelo. O treinamento locomotor de baixa intensidade melhorou alguns, mas não todos os parâmetros funcionais e histológicos e de possível importância clínica na PC em humanos. Além disso, esse modelo provou ser uma estratégia útil para se estudar não somente a doença, mas também possíveis mecanismos de recuperação após a intervenção física.

4.2. PRIMEIRO ARTIGO

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BRAIN RESEARCH

Research Report

Beneficial effects of treadmill training in a cerebral palsy-like rodent model: Walking pattern and soleus quantitative histology

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ABSTRACT

The aim of the present study was to investigate whether treadmill locomotor training could have beneficial effects on deficits consequent to perinatal anoxia, sensorimotor restriction or a combination of both. Fifty-six newborn male Wistar rats were divided into four groups: control, anoxic, sensorimotor-restricted and anoxic–sensorimotor-restricted. Rats were exposed to anoxia in the first two postnatal days (P0 and P1) and/or hind-limb sensorimotor restriction from P2 to P28 for 16 h/day. Control and experimental rats underwent treadmill training for three weeks (from P31 to P52). Body weight and walking patterns (stride length and foot angle) were measured weekly during treadmill locomotor training. Soleus muscle cross-sectional mean area and fiber density were measured using planar morphometry. Anoxia per se did not cause gait or muscle deficits. Body weight, stride length and soleus fiber cross-sectional mean area, however, were increased in trained anoxic rats. Sensorimotor-restricted animals, either with or without perinatal anoxia, showed deficits in body weight gain, decreased stride length, wider foot angle and soleus fiber atrophy. In the sensorimotor-restricted group, treadmill training improved body weight gain and stride length, and decreased the percentage of the atrophic fibers. However, in the anoxic–sensorimotor-restricted group, training improved stride length only. Three weeks of treadmill training were able to improve stride length in restricted and anoxic-restricted animals, although body weight deficit and the degree of degradation in muscle histology were reduced only in the restricted group.

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1. Introduction

Cerebral palsy (CP) describes a group of movement and posture disorders that cause activity limitation, which are attributed to non-progressive disturbances that occur in the developing fetal or infant brain (Bax et al., 2005). The motor disability leads to deconditioning that, in turn, worsens the level of disability (Damiano, 2006). Secondary to the brain lesion, changes in length and/or structure in the muscles and bones of the extremities occur (Novacheck and Gage, 2007). Histological analyses of muscle biopsies from patients with CP showed abnormalities in muscle fiber size, slow-to-fast phenotypic transition (Ito et al., 1996; Marbini et al., 2002) and fiber atrophy (Marbini et al., 2002).

There is no cure for CP (Damiano, 2006; Jones et al., 2007), but better physical conditioning leads to better health and prevention or reduction of secondary impairments (Damiano, 2006). In the last few years, activity-based strategies, such as treadmill training, were shown to improve motor and ambulatory skills in children with CP (Richards et al., 1997; Cherng et al., 2007; Begnoch and Pitetti, 2007). However, as yet there are no studies which demonstrate the effects of treadmill training in appropriate CP and/or CP-like animal models.

Perinatal injury models in rodents including hypoxic–ischemic insults induce only subtle and transient motor alterations (Hoeger et al., 2000; Zhuravin et al., 2004; Lubics et al., 2005; Robinson et al., 2005). On the other hand, Strata et al. (2004) and Coq et al. (2008) showed that the postnatal sensorimotor restriction (SR), either associated with or without perinatal anoxia (PA), can produce long-lasting effects such as reduced body growth rate, increased muscular tone, abnormal walking patterns, muscle fiber atrophy, mild to moderate ankle and knee joint degeneration, and distorted hind-limb representation in the primary motor and somatosensory cortices, which could show similarities to those observed in children with CP.

Most studies of motor impairments on animal models have employed therapeutic interventions in order to shed light on the mechanisms of functional recovery (Gómez-Pinilla et al., 2001; Ying et al., 2005; Ramic et al., 2006; Engesser-Cesar et al., 2007; Ilha et al., 2008). With this aim, the present study examines whether treadmill training applied in rats exposed to SR, with or without PA, can help to recover the resulting alteration in gait and muscle histology (Fig. 1). Our results show that PA alone does not cause weight loss, gait alterations and muscle histology alterations. However, body weight, stride length and soleus fiber cross-sectional area, were increased in trained PA rats. In accordance with previous results, SR, associated or not to PA caused reduced weight gain, and alterations in gait, here shown by reduced stride length and wider foot angle at initial contact, accompanied by soleus histological degradations. The low-intensity treadmill training used in the present work was shown to improve stride length in both SR groups, PA or non-PA, increased body weight gain and decreased the degradation degree in soleus, solely in SR group. Although the training changed these parameters, the foot angle remained unchanged with training.

Table 1 – Body weight

	P31	P38	P45	P52
CT	83.68±2.97	124.21±4.16	153.45±4.59	181.14±5.22
TrCT	83.67±1.85	124.60±1.79	155.21±3.16	187.48±3.16
PA	77.86±3.94	113.06±5.18	143.91±6.13	173.56±5.12
TrPA	84.10±3.63	122.12±5.26	157.04±7.24[b]	186.17±7.95[b]
SR	65.59±3.82[a]	102.08±5.44[a]	129.90±8.29[a]	155.32±8.14[a]
TrSR	64.95±3.26[a]	102.08±4.52[a]	135.68±6.90[a]	164.40±7.33[a,c]
PA-SR	65.87±2.07[a]	104.80±2.54[a]	140.35±4.54[a]	165.10±3.32[a]
TrPA-SR	61.90±2.66[a]	95.67±1.35[a,d]	132.46±2.95[a,d]	163.92±2.77[a]

Three-way repeated measures ANOVA revealed significant effects of the factor restriction ($F(1,48)=32.505$; $P<.001$) and time ($F(3,144)=2541.56$; $P<.001$) on body weights, with a significant training × time interaction ($F(3,144)=3.337$; $P<.05$).

[a] Significantly different from control (CT), $P<.001$.

[b] Significantly different from anoxic (PA), $P<.001$.

[c] Significantly different from restricted (SR), $P=.007$.

[d] Significantly different from anoxic-restricted (PA-SR), $P<.03$.

2. Results

2.1. Body weight

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 accordance with a previous study (Strata et al., 2004), restricted animals (SR and PA-SR) displayed significantly lower body weights than those of

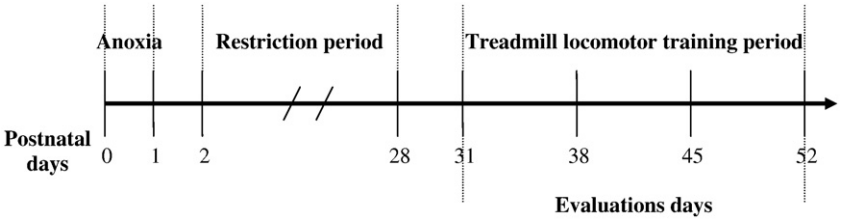

Fig. 1 – Time line of the experimental procedures. The perinatal anoxia (100% nitrogen at 9 L/min for 12 min) was performed at P0 and P1 postnatal days. The sensorimotor restriction procedures were performed daily (16 h/day) from P2 to P28. The treadmill training (5 days/week for 3 weeks) started on P31. Walking pattern evaluation was performed on P31, P38, P45 and P52. The histological procedures were made on P52.

control group (CT) in all evaluations ($P<.001$, Table 1). The training significantly improved the body growth rate in: 1) the trained anoxic group (TrPA) compared to the untrained anoxic group (PA) in the third and fourth weeks ($P<.001$); 2) the trained restricted group (TrSR) compared to the untrained restricted group (SR) in the last week ($P=.007$); while decreasing the body growth rate in the trained anoxic-restricted group (TrPA-SR) compared to the untrained anoxic-restricted group (PA-SR) in the second and third evaluations ($P<.03$), although the body weight gain of TrPA-SR group matched that of the PA-SR group in the last week.

2.2. *Gait, stride length and foot angle*

Sensorimotor-restricted rats, with or without PA, presented an elevation of the hindquarters during posture and walking, due to an abnormal extension of both ankle and knee joints. As compensation, rats showed back hunching in the lumbar and sacral segments, forcing their tails to the ground and their bellies up from the ground (Fig. 2). The gross alterations of gait seen in these groups were reduced principally in third and forth weeks in both untrained and trained groups. In spite of the better performance, the differences between CT and both SR rats (PA or non-PA) remained.

In order to establish the effects of inducing the CP-like model and the potential benefits of treadmill training, two objective measures were used: stride length and foot angle. Stride length is the sum of the stance and the swing phases of the gait cycle and decrease in stride length may reflect rigidity in the hind-limb joints. A wider foot angle at initial contact suggests greater rigidity of the foot in the plantar flexed position and causes toe walking. Visual examination failed to reveal any great differences between untrained and trained rats. But stride length measurement proved to be useful in assessing both the changes caused by the induction of the model and the benefits of training (Fig. 3).

Restricted groups, PA or non-PA, had shorter stride lengths ($P<.002$, Table 2). The training significantly improved the stride length (Table 2) in: 1) TrCT at the third evaluation ($P<.01$); 2) TrPA at the second and following evaluations ($P<.03$); 3) both the TrSR and TrPA-SR groups at the third evaluation ($P=.009$ and $P<.001$, respectively). In the last evaluation, only TrPA-SR group sustained this improvement ($P<.001$, compared to PA-SR group), although stride length mean values were slightly greater in TrSR than SR groups (11.03 ± 0.41 and 10.56 ± 0.48, respectively).

The vertical displacement of the ankle-foot juncture at initial contact was significantly increased in SR and PA-SR groups in previous study (Strata et al., 2004). Similarly, we also found that SR animals, PA or non-PA, had significantly wider foot angles (Fig. 2). The treadmill training did not change the foot angle (Table 3).

2.3. *Histology and morphometry of soleus muscle fibers*

The soleus muscle was selected for study because it is extensively recruited during treadmill running (Dudley et al., 1982; Roy et al., 1985). Furthermore, the soleus is a postural muscle and displays marked changes after a period of disuse (Thomason and Booth, 1990). Its development in a shorted position, may lead to a wider foot angle at the initial foot contact with a walking surface, thus reducing stride length.

In the CT and PA groups, the soleus muscle fibers were regular and polygonal in shape and there was minimal variation in size. In the SR and PA-SR groups, the fibers were found to be smaller, more variable in size, with a more rounded profile and with more interstitial connective tissue. Treadmill training did not change the morphological aspect of

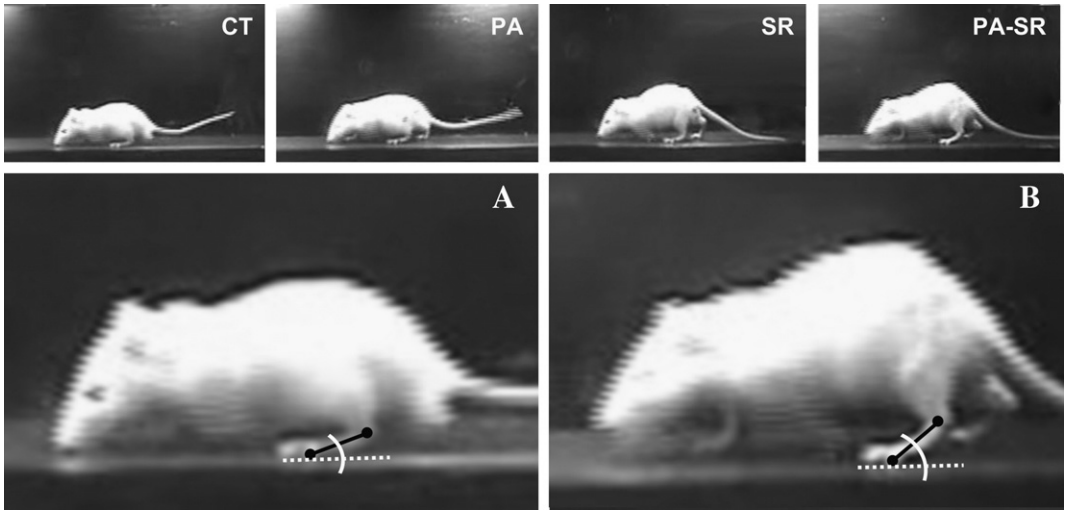

Fig. 2 – Figure showing representative images of the sagittal plane of the control and experimental groups in the second week after the restrained period (P38). In the SR and PA-SR groups the hindquarters are elevated, due to an abnormal extension of both the ankle and knee joints and, in compensation, the rats showed back hunching, forcing their tails to the ground and their bellies up from the ground. These aspects were not seen in the CT and PA groups. Representative images of the CT and PA (A) and SR and PA-SR (B) groups showing how the measurements were made (angle formed by the intersection between the line connecting the lateral malleolus with metatarsus–fifth toe junction and the line parallel to ground, adapted from Varejão et al. (2002).

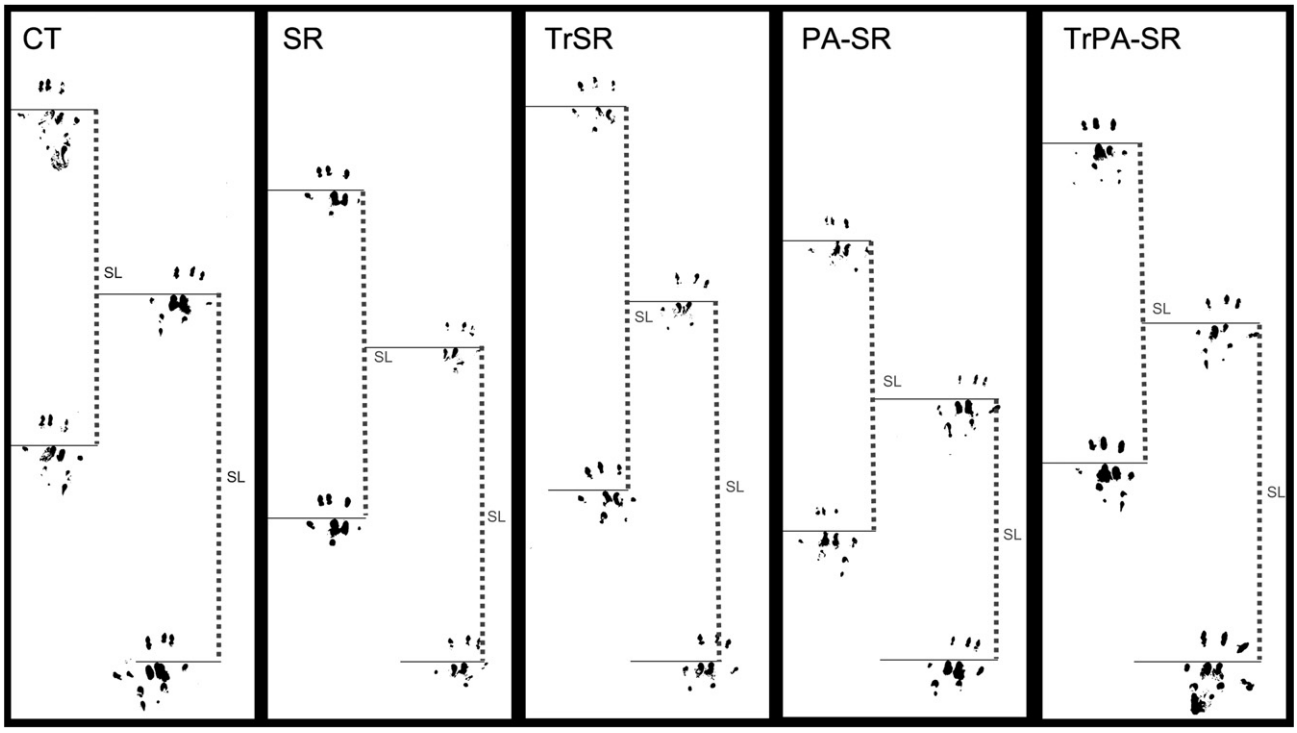

Fig. 3 – Image showing representative stride lengths (SL) for rats belonging to the following groups: CT, SR, TrSR, PA-SR and TrPA-SR in the last week of the training (P52). The figure shows how the measurement was made (distance from metatarsus to metatarsus of the same limb). In SR and PA-SR, note the shorter stride length compared to CT. Note that in TrSR and TrPA-SR this measurement was longer.

muscle fibers in the TrCT, TrPA and TrPA-SR rats, although the training did modify the soleus histology of TrSR rats. The fibers in this latter group were larger in size and had more polygonal aspect; there was also less interstitial connective tissue in the muscle when compared to the SR group (Fig. 4). ANOVA indicated effects of *restriction* ($P<.001$) and *training* ($P=.01$) on mean fiber area. Mean fiber areas were smaller in both restricted groups, trained or untrained (SR, PA-SR, TrSR and TrPA-SR; $P<.001$; Fig. 5A). Training significantly increased mean fiber area in TrPA and in TrSR groups (compared to PA and SR; respectively, $P=.03$).

As a consequence of the reduction in the mean fiber area, there was an increase in fiber density (fibers/mm^2), i. e.; more fibers occupied the muscle area studied. ANOVA revealed significant effects for the factors *restriction* ($P<.001$) and *training* ($P=.02$) on fiber density. The *post hoc* analysis showed that both groups of restrained rats, trained or untrained, had higher fiber densities (SR, PA-SR, TrSR and TrPA-SR; $P<.001$;

Table 2 – Stride length

	P31	P38	P45	P52
CT	9.90±.56	11.51±.32	11.86±.31	12.62±.36
TrCT	9.34±.49	11.22±.50	13.07±.63[b]	12.63±.86
PA	9.81±.46	10.64±.31	10.88±.54	12.09±.39
TrPA	9.57±.32	11.57±.27[c]	13.05±.28[a,c]	13.27±.20[c]
SR	7.55±.27[a]	8.88±.45[a]	10.09±.20[a]	10.56±.48[a]
TrSR	7.75±.24[a]	9.78±.35[a]	11.31±.44[d]	11.03±.41[a]
PA-SR	7.62±.41[a]	9.40±.20[a]	9.39±.40[a]	9.79±.61[a]
TrPA-SR	7.61±.53[a]	9.97±.32[a]	11.63±.39[e]	11.83±.40[e]

Three-way repeated measures ANOVA revealed significant effects of the factors restriction (F (1,48)=60.282; $P<.001$), training (F (1,48)=10.514; $P=.002$), and time (F (3,144)=141.50; $P<.001$) on stride length, with a significant training×time interaction (F (3,144)=11.08; $P<.001$).
[a] Significantly different from control (CT), $P<.002$.
[b] Significantly different from control (CT), $P<.01$.
[c] Significantly different from anoxic (PA), $P<.03$.
[d] Significantly different from restricted (SR), $P=.009$.
[e] Significantly different from anoxic-restricted (PA-SR), $P<.001$.

Table 3 – Foot angle

	P31	P38	P45	P52
CT	23.45±1.58	22.29±1.12	21.75±0.79	19.84±0.93
TrCT	19.90±1.87	23.55±1.64	22.28±1.13	23.85±1.56
PA	22.15±0.76	20.09±1.29	21.02±0.66	21.42±1.43
TrPA	22.21±0.57	21.12±0.96	22.10±1.28	20.87±1.11
SR	46.52±4.26[a]	32.95±3.07[a]	32.38±2.62[a]	28.14±2.86[a]
TrSR	46.98±3.18[a]	33.11±2.17[a]	32.94±1.46[a]	25.48±1.73[a]
PA-SR	48.32±1.45[a]	33.21±2.68[a]	31.38±2.56[a]	27.17±2.34[a]
TrPA-SR	39.15±2.66[a]	33.04±1.58[a]	28.63±1.61[a]	27.48±2.31[a]

Three-way repeated measures ANOVA revealed significant effects of the factors restriction (F (1,48)=145.38; $P<.001$) and time (F (3,144)=56.29; $P<.001$) on foot angle, with a significant restriction×time interaction (F (3, 144)=51.96; $P<.001$) and anoxia×restriction×training×time interaction (F (3, 144)=4.14; $P=.007$).
[a] Significantly different from control (CT), $P<.008$.

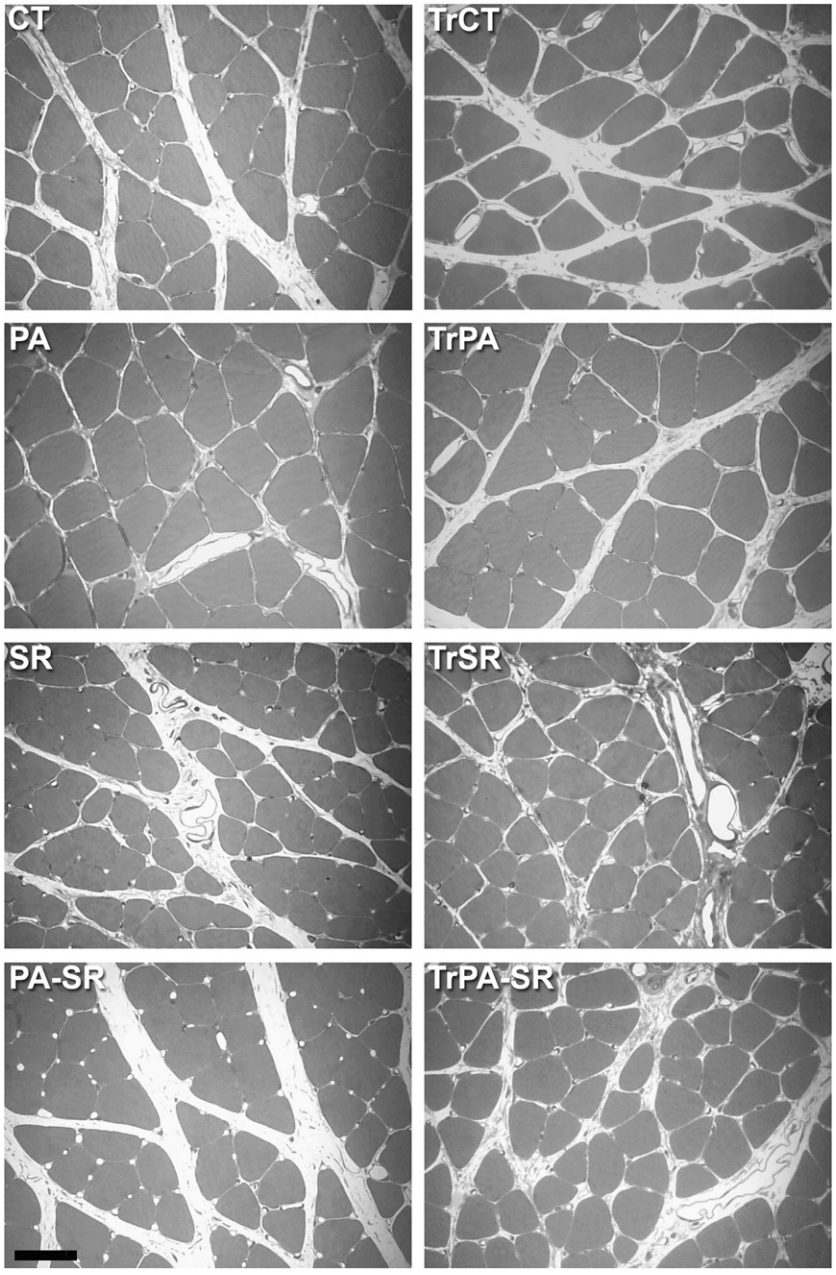

Fig. 4 – Digitized images of transverse-semithin sections (1 μm) obtained from soleus muscles after anoxia, sensorimotor restriction, anoxia plus sensorimotor restriction and 3 weeks of treadmill locomotor training. Left panel corresponds to untrained groups: CT, PA, SR and PA-SR. Right panel corresponds to trained groups: TrCT, TrPA, TrSR and TrPA-SR. Note in CT, TrCT, PA, and TrPA both regular and polygonal aspects of fibers with minimal size variation. In SR, PA-SR, and TrPA-SR note smaller fiber sizes, more size variation and more rounded profile and an increase in interstitial connective tissue. In TrSR note a more polygonal fiber aspect with a decrease in interstitial connective tissue and an increase in fiber size, compared to SR. Scale bar corresponds to 50 μm.

Fig. 5B). Locomotor training significantly decreased soleus fiber density in TrSR compared to SR group ($P = .02$), due to the increase in the area of the fibers.

The mean cross-sectional fiber areas were also analyzed in frequency histograms. The SR group had a higher percentage (12%) of atrophied fibers (0–500 μm^2) than all groups ($P < .002$). This pattern was significantly modified in TrSR group by treadmill training ($P < .001$; Fig. 6C).

Both restricted groups (SR and PA-SR) had a higher percentage (56 and 61%, respectively) of fibers within 501–1000 μm^2 interval, while CT and PA groups, had lower percentages (16 and 27%, respectively). This situation was inverted in fibers within the 1501–2000 μm^2 range, where both restricted groups (SR and PA-SR) had fewer (2 and 5%, respectively) while CT and PA had more (30% and 28%, respectively). Although ANOVA failed to show significant effect from training in frequency histograms,

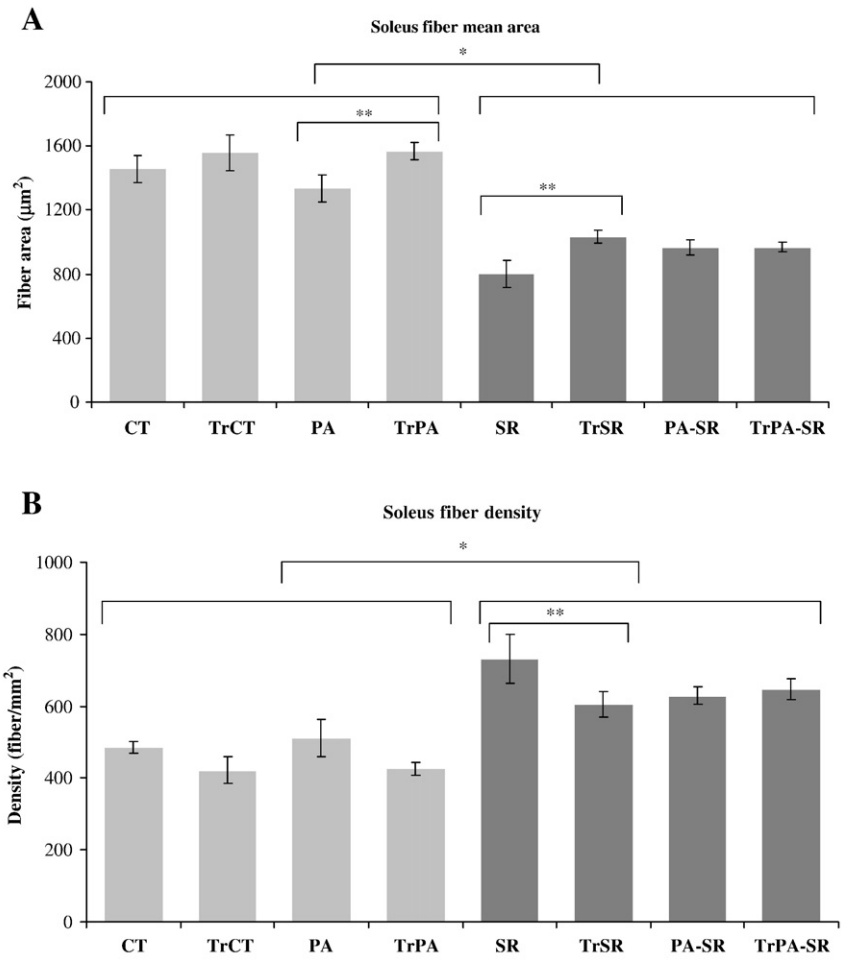

Fig. 5 – Effects of treadmill training on the morphometric parameters of soleus muscle cross-sectional fibers. Graphs show the results of planar morphometry of the soleus: (A) soleus mean fiber area and (B) soleus fiber density. Data are expressed as means and SEM. *significantly different $P<.05$. **significantly different $P\leq.03$.

there was a tendency toward an increase in fiber size with training. Training decreased the percentage of 501–1000 μm^2 fibers in TrPA group compared to PA group (27 to 8%, Fig. 6B). With 1001–1500 μm^2 fibers, training improved this interval in TrSR group compared to SR group (30% to 42%, Fig. 6C). The PA group and both groups of restrained rats, trained or untrained, had lower percentages (5% in PA and 0% for the others) of 2001–2500 μm^2 fibers, while CT, TrCT and TrPA groups had 12–14% (Fig. 6B).

In summary, the sensorimotor restriction, in both groups, SR and PA-SR, caused important soleus atrophy, and the treadmill training produced a decrease in the percentage of atrophic fibers in the TrSR group.

3. Discussion

Our study showed the effects of treadmill locomotor training in the walking pattern and muscle tissue in a CP-like model of rats exposed to neonatal asphyxia and chronic disuse. First, PA alone did not cause deficits in evaluated parameters, but in both restrained and anoxic-restrained groups, the body weights and the stride lengths decreased, the foot angles were wider, and atrophy of soleus muscle was evident. Our work corroborates the results from previous studies that showed markedly lower body weights, abnormal gait pattern (see Strata et al., 2004) and muscle atrophy (see Coq et al., 2008) in rats exposed to SR, with or without PA. Second, the treadmill training led to improvements in body weight, stride length and soleus histology in animals exposed to PA or SR alone. However, in the PA-SR rats only stride length changed positively.

3.1. *Effects of anoxia, sensorimotor restriction and a combination of both*

In the present study, no significant differences was found in terms of body weight, stride length, foot angle and soleus histology between rats exposed to anoxia and controls. Similarly, Coq et al. (2008), using the same anoxia protocol as Strata et al. (2004), also failed to find morphological alterations in triceps surae, though they found hypertrophy in the hamstrings, and mild atrophy in the quadriceps. Furthermore, Strata et al. (2004) identified subtle alterations with PA, such as mild increases in muscular tone accompanied by modest differences in walking patterns. Strata et al. (2004) suggested that the PA protocol used (two episodes of anoxia, at P0 and P1

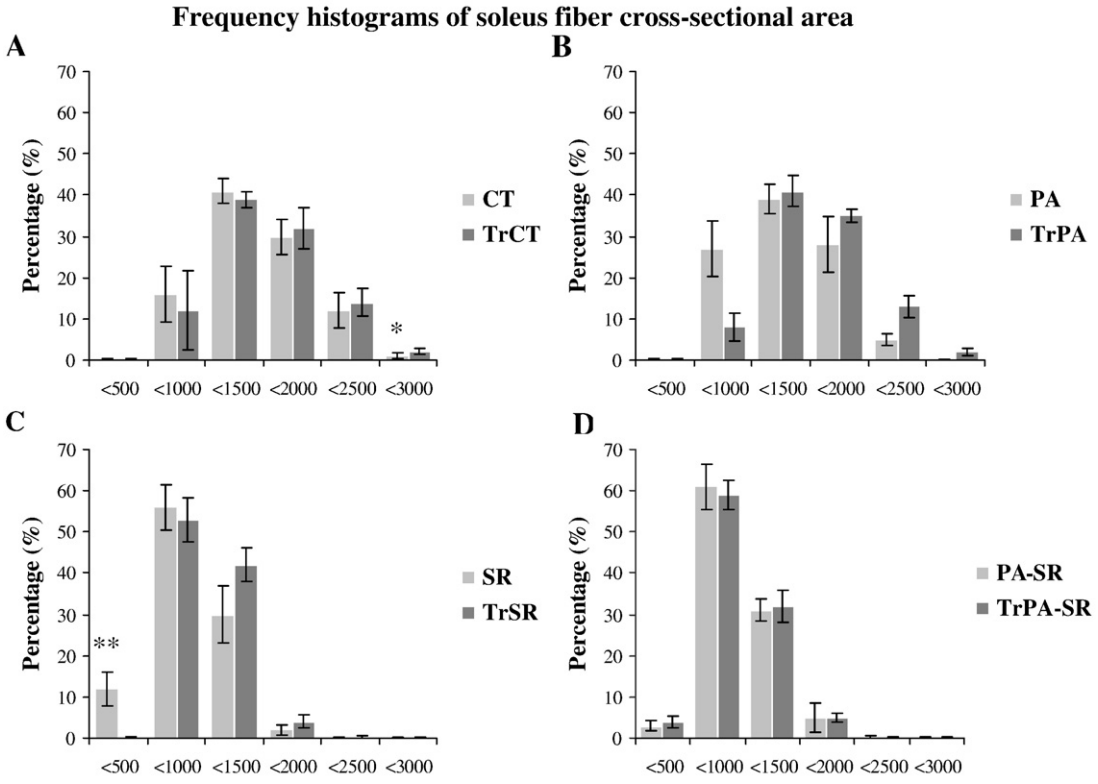

Fig. 6 – Comparison of the effect of treadmill training on soleus fiber cross-sectional area distribution. (A) CT × TrCT; (B) PA × TrPA; (C) SR × TrSR; (D) PA-SR × TrPA-SR. Note the effect of training in the highest range (2501–3000 μm^2) in CT group (A); on 501–1000 and 2001–2500 μm^2 fibers in PA group (B) and on the lower ranges (0–500 μm^2, solely seen in SR group) and 1001–1500 μm^2 in SR group. *: P<.05, **: P<.001.

for 12 min each epoch), was probably not the most suitable, since the PA-induced motor dysfunctions did not completely match the symptoms of CP.

There has been little success at producing such an animal model of chronic spasticity, because there is no frequently occurring natural disease in animals and, after experimental injury, the animals tend to die or recover (Wright and Rang, 1990). Hypertonia has been shown in hypoxic–ischemic injuries in rabbits (Derrick et al., 2004; Drobyshevsky et al., 2007). In rats, several studies employing a wide range of hypoxic–ischemic insults have shown motor deficits, such as delayed development (Zhuravin et al., 2004) and impaired motor coordination (Lubics et al., 2005) that can be partially compensated with age.

On the other hand, PA may cause disruption of spinal circuits. In rats, 3 h of hypoxia on the first postnatal day significantly shortens the lumbar motoneuron dendrites evaluated on P14 (Takahashi et al., 1999). Also in rats, in utero anoxia at term increases apoptosis in the intermediate zone and dorsal horn of lumbar spinal cord as evaluated at P8 (de Louw et al., 2002). In humans, perinatal asphyxia causes severe neuronal necrosis in lumbosacral segments (Sladky and Rorke, 1986; Clancy et al., 1989).

The development of the corticospinal tract in rats occurs during postnatal development (Donatelle, 1977; Schreyer and Jones, 1988) and activity has a significant influence on motoneuron development (Inglis et al., 2000) and locomotion pattern (Westerga and Gramsbergen, 1993). Unilateral forelimb disuse (P5–P30) results in increased corticospinal connections in the ipsilateral motor cortex possibly caused by maintenance of transient fibers that otherwise would be eliminated by normal sensory feedback (Huttenlocher and Bonnier, 1991). Moreover, in cats, forelimb muscle paralysis between postnatal weeks 3 and 7 produces abnormal development of corticospinal terminations in maturity, and deficits in paw prehension (Martin et al., 2004).

Our results showed that SR alone and the SR associated to the PA caused reduced body weight gain, alteration in gait, such as decrease in stride length and wider foot angle, and soleus muscle atrophy. In accordance with Strata et al. (2004), our results also showed that the chronic disuse in these two groups (SR and PA-SR) causes an abnormal walking pattern, the rats extended their hind limbs posteriorly, exhibited reduced joints movements and elevated hindquarters.

In children with CP, equinus gait or “toe walking” is one of the most prevalent abnormalities. Initially, the equinus is dynamic, but even with treatment (orthoses, physical therapy and/or botulinum toxin injections), a fixed contracture often develops (Wren et al., 2004). The fixed contracture can in turn provide abnormal sensory information and reinforces abnormal movement. In rats, severe gait deterioration has been shown in disuse models that provide abnormal sensory inputs to the brain which, in turn, cause degraded motor function (Coq et al., 2008).

Disruption of motor activity during the development also produces modifications to the peripheral nervous system. Immobilization of soleus muscle in a shortened position, in distinct periods, either when the innervation is polyneuronal (from P6 to P12) or monosynaptic (P17 to P23), produced delays in the postnatal maturation of the soleus in the first period, while in the second period of immobilization there was more fast twitch fibers and altered expression of the myosin heavy chain isoforms (Picquet et al., 1998). Transient disruption of neuromuscular transmission in neonatal rats with α-bungarotoxin treatment resulted in a decrease in the tension developed by soleus muscle, in the size and number of muscle fibers and loss of motoneurons in the more caudal part of the spinal cord in adulthood (Greensmith et al., 1996, 1997). Moreover, in developing animals, muscle fiber mechanical activity could be an important retrograde signal for the maturation of innervation, and without this stimulus nerve terminals may remain immature (Greensmith et al., 1998).

Together, these data suggest that the SR protocol may have caused severe alterations in the developing neuromuscular system. Additionally, SR was found to have a fundamental role in producing a more typical phenotype of CP-like motor disorders. In rats, this strategy provides abnormal sensorimotor experience that prejudices the development of the motor system and thus produces an impaired motor performance which is similar to that found in children following immature brain injuries. This highlights the importance of early intervention in order to prevent further degradation caused by abnormal sensorimotor experience. However, in this CP-like model, PA can produce a more favorable environment for development of motor disorders caused by SR. The role of PA in association with SR needs to be further investigated.

3.2. *Effects of treadmill training on anoxia, sensorimotor restriction and a combination of both*

The training had beneficial effects on body weight gain, stride length and soleus fiber area in trained anoxic rats when compared to untrained anoxic rats. This effect can be explained by the fact that PA rats presented only slight deficits in those parameters, although there was no significant difference between the PA and CT rats. In contrast, in a previous study, the PA rats gained more weight at later stages (Strata et al., 2004). The differences between our results and those from Strata's could be due to gender- and species-specific responses (we used male Wistar rats, while Strata et al. (2004) used male and female Sprague–Dawley rats).

The increased body weight and soleus fiber cross-sectional area induced by exercise in the PA rats may be due to a combination of enhanced nutritional intake and increased muscle contraction. Choe et al. (2006) found that low-intensity exercise following acute stroke in adult rats increased dietary intake as well as soleus, plantaris and gastrocnemius muscle weights.

Treadmill training enhanced gains in body weight, and stride length and diminished the degree of degradation in soleus muscle, though there was no change to the foot angle in TrSR rats. Treadmill training in TrPA-SR group, despite leading to decreased body weight in the second and third weeks, increased the stride length from the second week, while neither the foot angle nor the soleus muscle were altered. In the last week, body weight gain was matched to untrained PA-SR rats.

Adult rats, whose hind limbs were unloaded for 2 weeks and then underwent running exercise, were found to have fast-to-slow transition soleus muscle fibers, decreased atrophy levels and increased oxidative enzyme activity of soleus fibers (Ishihara et al., 2004). In another study, treadmill training improved the immobilization-induced muscle fiber histochemical alterations and the range of the ankle motion in adult rats after a 2-week immobilization period (Sakakima et al., 2004). While the above mentioned studies dealt with adult rats, the present study was concerned with chronic disuse during the developmental period. As previously mentioned, it is possible that the SR impairs the maturation of the muscle innervation during a critical period causing permanent histological alterations. Hence, the degree of recovery may be different.

Even though the degree of the atrophy was statistically similar in both the SR and PA-SR groups, it was slightly greater in the SR group (see Fig. 5). The reaction of muscle tissue to treadmill training was also different as the TrSR soleus showed changes with training. Maybe the improvement was more evident in TrSR due to the greater degree of atrophy in the SR compared to PA-SR.

Strata et al. (2004) speculated that anoxia impairs learning circuits. This could lead to less aberrant motor learning in PA-SR rats compared to SR-only rats. On the other hand, PA associated to SR may cause more complex modifications in the spinal cord and soleus innervation than SR alone. In PA-SR rats, Coq et al. (2008) found that asphyxia could worsen the already deleterious effects of disuse during development, causing atrophy in several muscles involved in knee extension and flexion, and ankle flexion.

Nevertheless, training in experimental groups resulted in functional (gait) improvements as shown by the increase in stride length. It appears that the success of rehabilitative strategies is highly task-specific, and strategies which closely simulate the functional situation of walking are the most effective way to improve the locomotion parameters (Barbeau and Rossignol, 1994; Edgerton et al., 1997; de Leon et al., 1998). Stride length is one determinant parameter of gait speed and its increase could be the consequence of improved motor control (Brown et al., 2003).

We chose to start training in this period (after the restriction period, at P31) because we wanted to see the effects of exercise on chronic motor impairment. Additionally, the low-intensity training protocol used in our study was designed to increase both hind-limb rhythmical activity and sensory inputs. It may be more beneficial to start treadmill training as early as possible in order to prevent the development of motor degradation. Further studies are required to more precisely identify the therapeutic window during which greater benefit can be obtained.

However, it is possible that there are changes in neural substrates underlying the behavioral improvements. The recovery of treadmill stepping in treadmill-trained rats that underwent complete spinal transection as neonates was associated with significant changes in the cellular properties of motoneurons and muscle spindle afferents (Petruska et al.,

2007). Wheel running following moderate T9 contusion improved stepping ability and serotonin fiber length caudal to the lesion in the running groups (Engesser-Cesar et al., 2007). Furthermore, physical activity can be used to induce trophic factors with beneficial effects for neuronal health and plasticity (Gómez-Pinilla et al., 2001; Ying et al., 2005). Animals that had paralyzed soleus muscle via intramuscular injection of botulinum toxin and remain sedentary, brain-derived neurotrophic factor and synapsin I mRNAs were reduced below control levels in the spinal cord and soleus muscle (Gómez-Pinilla et al., 2002). Exercise restores levels of neurotrophins and synaptic plasticity following spinal cord injury in adult rats (Ying et al., 2005). These data suggest that post-injury neuromuscular activity is capable of maintaining normal levels of neurotrophins in the neuromuscular system and the potential for neuroplasticity.

3.3. *Concluding remarks*

This is the third study, to our knowledge, to use an animal model with CP-like characteristics, based on the association of PA and early hind-limb disuse. Our results are in agreement with previous results that show that this model produces long-lasting effects on the locomotor system, both functionally and at a histopathological level. In our study, chronic sensorimotor restriction caused the majority of gait deficits, but anoxia may have contributed to a more complex phenotype that still requires further characterization in this model. Sustained activity, such as low-intensity treadmill training, improved some, but not all, functional and histological parameters of possible importance in human cerebral palsy. Thereby, this model has proved to be a useful strategy to understand not only the disease but also some possible mechanisms of improvement after physical intervention.

4. Experimental procedures

4.1. *Animals*

Pregnant female Wistar rats from a local breeding colony (ICBS, Universidade Federal do Rio Grande do Sul, Brazil) were housed in standard plexiglass boxes, under 12:12 h light/dark cycle, in a temperature-controlled environment (20±1 °C), with food and water available *ad libitum*. Approximately 5 days before delivery, they were housed individually and the presence of pups was checked daily. The day of birth was postnatal day 0 (P0). The number of pups was culled to 8 per dam by removing essentially the females (or males if necessary). After weaning (postnatal day 21), the females were removed from the boxes and discarded from the study. All the procedures were approved by the Ethical Committee at the Federal University of Rio Grande do Sul. All the animals were cared for in accordance with Brazilian law and the recommendations of the Brazilian Society for Neurosciences, Review Committee of the School of Veterinary Surgery, University of Buenos Aires and the 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).

4.2. *Experimental groups*

The male pups from 13 litters were randomly assigned to four different groups: control (CT; $n=14$), anoxic (PA; $n=16$), sensorimotor-restricted (SR; $n=14$) and anoxic and sensorimotor-restricted (PA-SR; $n=12$).

4.3. *Anoxia and sensorimotor restriction procedures*

PA and SR were performed according to Strata et al. (2004). Briefly, the two anoxic groups (PA and PA-SR) were exposed to two episodes of anoxia, at P0 and P1. The pups were placed into a glass chamber partially immersed in 37 °C water and exposed to nitrogen (100%) at 9 L/min for 12 min each epoch. The pups were then resuscitated in room air, and returned to their dams. The SR procedure was performed from P2 to P28 (Fig. 1). Rats' hind limbs were bound together with paper tape and placed in an extended position maintained with an epoxy cast for 16 h/day (see Strata et al., 2004 Fig. 1). This procedure was well tolerated by the pups.

4.4. *Training procedure*

After the end of the restriction period (P28), the rats were assigned to *untrained*: control (CT, $n=7$); perinatal anoxia (PA, $n=8$) sensorimotor restriction (SR, $n=7$); perinatal anoxia plus sensorimotor restriction (PA-SR, $n=7$) and *trained*: trained control (TrCT, $n=7$); trained perinatal anoxic (TrPA, $n=8$), trained sensorimotor-restricted (TrSR, $n=7$), trained perinatal anoxic and sensorimotor-restricted (TrPA-SR, $n=5$) groups. The training consisted of walking on a treadmill for three weeks from P31 (once a day, 5 sessions/week, leaving one day for rest and another for functional tests). The initial speed was determined by observing the best walking pattern developed by restricted rats (because they had the worst walking pattern). In the first week, the speed was 5 m/min and the duration of training started with 10 min on the first day and progressed gradually until 15 min on the fifth day. In the next two weeks, each training session included a warm-up period of 5 min running at 5 m/min, 6–15 min (progressed gradually) running at 6 m/min and 7 m/min (respectively in second and third weeks) and 5 min recovery at 5 m/min.

4.5. *Gait testing*

Walking pattern evaluation consisted of two spatiotemporal parameters: hind paw stride length and foot angle measurements on P31, P38, P45 and P52. For stride length, 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 (see Fig. 1, adapted from Kunkel-Bagden et al., 1993). For the foot angles, each rat walked once along a 100-cm-long corridor, while being filmed from the lateral view with a camera at 30 frames per second. The video sequences were digitized and examined with VirtualDub software (available at http://www.virtualdub.org). Three frames from different step cycles (each one from the beginning of the stance, that is the first frame with the foot in contact with the ground) were selected from each rat. The images were then

analyzed with the UTHSCSA ImageTool 2.0 software (University of Texas, San Antonio, TX, USA, http://ddsdx.uthscsa.edu/dig/). The foot angle was obtained by the intersection between the line connecting the lateral malleolus with metatarsus–fifth toe junction and the line parallel to ground was measured (see Fig. 2, adapted from Varejão et al., 2002), and the lowest of the three value was taken for the analysis.

4.6. *Histological and morphometric analysis*

After treadmill training (on P52) the animals (n=5 for all the groups, n=4 for TrPA-SR) were anesthetized with sodium thiopental (50 mg/kg, i.p.; Cristália, Brazil), injected with 1000 IU heparin (Cristália, Brazil) and were transcardially perfused with 150 ml of saline solution, followed by 0.5% glutaraldehyde (Sigma, USA) and 4% paraformaldehyde (Reagen, Brazil) in 0.1 M phosphate buffer (PB, pH 7.4) at room temperature. The left soleus muscles were carefully dissected free from surrounding tissue. Small samples (2×1 mm) of the central part of each soleus muscle were selected and postfixed in the same fixative solution until processed. The soleus muscle samples were washed in PB and postfixed in 1% OsO_4 (Sigma, USA) in PB for 1 h. The samples were then washed with PB and dehydrated in a graded series of alcohol and propylene oxide (Electron Microscopy Sciences, USA), embedded in resin blocks (Durcupan, ACM-Fluka, Switzerland), maintained in vacuum for 24 h, and, afterwards, polymerized for 48 h at 60 °C. Transverse-semithin sections (1 μm) were obtained using an ultramicrotome (MT 6000-XL, RMC, Tucson, USA) and stained with 1% toluidine blue (Merck, Germany) in 1% sodium tetraborate (Ecibra, Brazil).

Afterwards, images of the soleus muscles were captured and digitalized (initially 20× and further amplified 200% for analysis) using a Nikon Eclipse E-600 microscope (Japan) coupled to a Pro-Series High Performance CCD camera and Image Pro Plus Software 4.1 (Media Cybernetics, USA). For morphometric evaluation, a set of 5 images was chosen using random sampling of one slice. The total area examined of each soleus muscle was the sum of the 5 randomly selected areas (0.429 mm^2 in total). The average number of analyzed fibers was 239.

Morphometric measurements included (1) the estimation of the mean muscle fiber area (μm^2) and (2) muscle fiber density (number of fibers/mm^2). Individual soleus fibers were counted and the soleus fiber density was determined by examining the ratio of the soleus fibers/total analyzed area. The soleus fiber areas were estimated with a point-counting technique (Hermel et al., 2006), using grids with point density of one point per 62.32 μm^2 and the equation: $\hat{A}=\sum p.a/p$. Where $\hat{A}$ is area, $\sum p$ is the total of counted areas/point and a/p is the area/point value (62.32 μm^2).

4.7. *Statistical analysis*

Body weight, stride length and foot angle evaluations were analyzed using three-way repeated measures analysis of variance (ANOVA) with *anoxia*, *restriction* and *treadmill training* as the independent variables and *time* (evaluations days) as repeated measure (n=5–8 per group). Soleus muscle cross-sectional fiber density and mean area, and area–frequency histograms were analyzed using three-way ANOVA (n=4–5 per group). All analyses were followed by *post hoc* Fisher's Least Significant Difference (LSD). Data were expressed as means±SEM. Probability values less than 5% were considered significant. Statistical analysis was performed using the Statistica software package.

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4.3. Resultados do segundo artigo: "Different effects of anoxia and hind-limb immobilization on sensorimotor development and cell numbers in the somatosensory cortex in rats". Submetido.

Nesse segundo estudo foram avaliados a aquisição de marcos do desenvolvimento e o desempenho em dois testes de habilidades motoras. Adicionalmente, foi feita uma série de medidas morfométricas: espessura de S1 (na área de representação dos membros inferiores); espessura do corpo caloso e espessura do córtex cerebelar. Foi realizado também contagem de células neuronais e gliais na camada V do córtex S1, bem como analisamos a área dos somas neuronais em S1. A área dos membros inferiores de S1 foi escolhida para estudo já que há uma grande sobreposição dos mapas motores e sensoriais nessa região (DONOGHUE, 1995). Além disso, modificações no padrão de movimentação dos membros inferiores causam diversas modificações nessa região encefálica (LANGLET et al., 1999; DeFELIPE et al., 2002; DUPONT et al., 2005).

Novamente, a PA, sozinha, não afetou o ganho de peso ou causou déficits sensório-motores. Por outro lado, a SR, unicamente ou associada à PA, diminuiu o ganho de peso, causou criptorquidismo em metade dos animais, prejudicou a aquisição de marcos do desenvolvimento (atraso na mobilidade em plano inclinado e acelerou o aparecimento de reflexo de colocação, ou "placing") e causou déficits nos testes de habilidades motoras. As espessuras de S1, corpo caloso e córtex cerebelar não foram alteradas significativamente, embora haja diminuição da espessura do corpo caloso no grupo SR e do córtex cerebelar no grupo PA-SR. Além disso, a PA, associada ou não à SR, aumentou o número de células gliais em S1, enquanto o número de neurônios em S1 foi reduzido pela SR, somente. Finalmente, a combinação de PA e SR aumentou o tamanho de somas neuronais na camada

V de S1. Há poucos trabalhos que tenham mostrado um aumento de tamanho celular neuronal (ROY et al., 2005). Porém, mecanismos compensatórios podem estar envolvidos com tal aumento do soma neuronal, como o aumento de conexões sinápticas entre neurônios sobreviventes, como já demonstrado em outro modelo de asfixia perinatal, onde houve perda neuronal, porém houve um aumento compensatório no número de botões pré-sinápticos no córtex parietal e no estriado (VAN DE BERG et al., 2000).

Nesse trabalho mostramos alguns novos achados nesse modelo, tais como: o criptorquidismo, o comprometimento periférico causado pela SR que pode alterar a aquisição de marcos do desenvolvimento precocemente, porém somente aqueles dependentes do desempenho motor dos membros inferiores (mobilidade em plano inclinado e o reflexo de colocação). Adicionamos dados ao prejuízo na habilidade motora dos membros inferiores mostrados por Strata e cols (2004). E, por fim, mostramos que além da desorganização do mapa cortical dos membros inferiores em S1, há modificações em nível celular, como redução de neurônios, aumento de células gliais e aumento do soma neuronal. Além de uma tendência na diminuição da espessura do corpo caloso e do córtex cerebelar em animais submetidos à SR e PA-SR, respectivamente.

4.4. SEGUNDO ARTIGO

DIFFERENT EFFECTS OF ANOXIA AND HIND-LIMB IMMOBILIZATION ON SENSORIMOTOR DEVELOPMENT AND CELL NUMBERS IN THE SOMATOSENSORY CORTEX IN RATS

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Abstract

Cerebral palsy (CP) is a group of movement and posture disorders attributed to insults in the developing brain. In rats, CP-like motor deficits can be induced by early hind limb sensorimotor restriction (SR; from postnatal days P2 to P28), associated or otherwise with perinatal anoxia (PA; on P0 and P1). In this study, we address the question of whether PA, early SR or a combination of both produces alterations to sensorimotor development. Developmental milestones (surface righting, cliff aversion, stability on an inclined surface, proprioceptive placing, auditory startle, eye opening) were assessed daily from P3 to P14. Motor skills (horizontal ladder and beam walking) were evaluated weekly (from P31 to P52). In addition, on P52, the thickness of the somatosensory (S1) and cerebellar cortices, and corpus callosum were measured, and the neuronal and glial cell numbers in S1 were counted. SR (with or without PA) significantly delayed the stability on an inclined surface and hastened the appearance of the placing reflex and impaired motor skills. No significant differences were found in the thickness measurements between the groups. Quantitative histology of S1 showed that PA, either alone or associated with SR, increased the number of glial cells, while SR alone reduced neuronal cell numbers. Finally, the combination of PA and SR increased the size of neuronal somata. We conclude that SR impairs the achievement of developmental milestones and motor skills. Moreover, both SR and PA induce histological alterations in the S1 cortex, which may contribute to sensorimotor deficits.

Keywords: Perinatal asphyxia; Developmental disuse; Cerebral palsy; Developmental milestones; Motor skills; Somatosensory cortex plasticity

1. Introduction

Cerebral palsy is primarily a disorder of movement and posture [1]. Etiologies are very diverse and multifactorial, and lead to the brain's inability to control motor functions and consequently affect global development [2]. The common initial clues to the diagnosis of CP are delayed achievement of motor milestones and abnormal muscle tone and posture [3]. The paucity of perinatal animal models manifesting neurobehavioral deficits has been an obstacle to the development of therapies for CP [4]. In rodents, perinatal hypoxic-ischemic insults induce only subtle and transient motor alterations [5-7]. Animal disuse models, however, have shown to produce more degraded motor functions [8-10].

Strata and cols. [10] proposed hind-limb immobilization (named sensorimotor restriction, SR) as a strategy to impair the locomotor development, and compared it to perinatal anoxia (PA) or the association of both interventions. The SR produced long-lasting deficits such as reduced body growth rate, increased muscular tone, abnormal gait patterns and primary motor cortex disorganization. Later, Coq and cols. [11] showed that this CP-like behavior is associated to muscle fiber atrophy, ankle and knee joint degeneration, and distorted hind-limb representation in the primary somatosensory cortex (S1). On the other hand, PA only caused mild alterations in muscle tonus, motor performance and cortical organization.

The association of PA and SR has been shown to be a simple, inexpensive and easily reproducible model of a CP-like motor phenotype. Moreover, the altered motor behavior shown in restrained rats underlines the crucial role played by the motor experience during this period, and the importance of early intervention to prevent degradation of motor control and voluntary movements [10,12,13]. However, other complex features of CP, such

as the delayed achievement of developmental milestones and motor skills, as well as the altered brain histology still require evaluation in this model.

In order to describe further alterations in this CP-like model, developmental milestones (surface righting, cliff aversion, stability on an inclined surface, proprioceptive placing, auditory startle, eye opening) were assessed daily from P3 to P14, and motor skills (horizontal ladder and beam walking tests) were evaluated weekly, after the end of the immobilization period. Also, the thickness of theS1 and cerebellar cortices, and corpus callosum were measured on P52, and the neuronal and glial cell numbers in the S1 were counted.

2. Experimental Procedures

2. 1. Animals

Pregnant female Wistar rats from a local breeding colony were housed in standard plexiglass boxes, under a 12:12 h light/dark cycle, in a temperature-controlled environment (20 ±1 °C), with food and water available *ad libitum*. Approximately 5 days before delivery, they were housed individually and the presence of pups was checked daily. The day of birth was postnatal day 0 (P0). The number of pups was culled to 8 per dam (males and females; 4 males at least per dam). After weaning (postnatal day 21), the female pups were removed from the boxes and discarded from this study and the remaining 56 male pups were used. All the procedures were approved by the Ethics Committee of the Federal University of Rio Grande do Sul (2006631). All the animals were cared for in accordance with Brazilian law and the recommendations of the Brazilian Society for Neurosciences, Review Committee of the School of Veterinary Surgery, University of Buenos Aires and the 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).

2. 2. Experimental groups

Male pups from 13 litters were randomly assigned to four different groups: control (CT; n = 14), anoxic (PA; n = 16), sensorimotor restricted (SR; n = 14) and anoxic and sensorimotor restricted (PA-SR; n = 12).

2. 3. Anoxia and sensorimotor restriction procedures

PA and SR were performed according to Strata and cols. [10]. Briefly, the two anoxic groups (PA and PA-SR) were exposed to two episodes of anoxia, at P0 and P1. The pups were placed into a glass chamber partially immersed in water at 37 °C and exposed to nitrogen (100%) at 9 L/min for 12 min each epoch. The pups were then resuscitated in room air, and returned to their dams. The SR procedure was performed from P2 to P28. The rats' hind limbs were bound together with paper tape and placed in an extended position maintained with an epoxy cast daily from 6 p.m. to 10 a.m (see Strata and cols. [10]; Figure 1). This procedure was well tolerated by the pups. No eating difficulties were observed in any of the groups.

2. 4. Neonatal developmental tests

The developmental milestones were evaluated daily by paired observers, blinded to treatment groups, from P3 to P14, always at 4 p.m. (period without restriction), and assessed as following (based on Poggi and cols. [14]): (1) surface righting: pups were placed in a supine position, and positive response was obtained when the animal returned to

prone position, with all paws on the ground; (2) cliff aversion: pups were positioned with forepaws and snout over the edge of a shelf, a positive response consisted of turning and crawling away from the edge; (3) stability on an inclined surface (formerly referred to as negative geotaxis) [15]: pups were placed head down on a 45°-inclined surface, and the positive response consisted of a 180^{o} turn with upward crawling; (4) hind-limb proprioceptive placing: pups had the head and trunk supported while the hind limbs were pendant near the edge of a platform: the test was considered positive when touching the paw's dorsal surface was followed by simultaneous hip and knee extensions and ankle-plantar flexion; (5) audio startle: pups responded with a quick involuntary jump after a small metal object was dropped on a lab bench 10 cm away from the pup; (6) eye opening: the day the pups opened their eyes was noted. Each developmental test response was considered positive based on its first appearance. All measurements were time-limited to a maximum of 30 seconds.

2. 5. Motor skills evaluation

The animals' motor skills (n = 7 for all the groups, n = 8 for PA) were evaluated weekly after the immobilization period (on P31, P38, P45 and P52). The number of animals used in motor skills' evaluations was reduced in comparison to those assessed for developmental milestones because half the pups were assigned to another study (data not shown). A horizontal ladder and the beam walking test were used to examine hind-limb sensorimotor function. For each test, the animals were filmed 3 times (30 frames per second) from the lateral view. The ladder apparatus was a 100 cm- long, 5 cm- wide, with horizontal parallel metal rungs (initially, 1 cm apart) with a little shelter at the end. The gaps between the rungs were randomly modified each week to increase task difficulty. The

number of hind limb step errors was counted by two blinded observers. The beam walking test consisted of crossing a 100 cm- long, 2 cm- wide flat surface beam elevated 30 cm above the floor with a little shelter at one end. The task difficulty was increased weekly by reducing the beam's width. A scoring system was used to assess the ability of animals to cross the beam [16]: 0 was counted as complete inability to walk on the beam (animals fell down immediately), 0.5 was scored if the animals was able to traverse half of the beam, 1 point was given for crossing the whole length, 1.5 points when stepping with the hind limbs was partially possible, and 2 points were given for normal weight support and accurate foot placement.

2. 6. Histological and morphometric analysis

On P52 the animals (n = 5 for all the groups, n = 4 for CT) were anesthetized with sodium thiopental (50 mg/kg, i.p.), injected with 1000 IU heparin and transcardially perfused with 150 mL of saline solution, followed by 0.5% glutaraldehyde (Sigma, USA) and 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4; 150 mL) at room temperature. Brains and cerebellum were removed and postfixed in the same fixative solution until processed. Brain coronal sections (20 μm) were obtained using a vibratome and every second section was collected and stained using the Nissl method. For the cerebellum, parasagittal sections from the left cerebellar hemisphere (30 μm) were obtained using a vibratome, each second section was colleted and stained using the Nissl method. Afterwards, images of the hind-limb representation area in the S1, identified using stereotaxic coordinates (-2,30 to -3,30 mm of bregma) [17] were captured and digitalized (in 2X for measurement of cortical and corpus callosum thickness and in 20X for cell counting in layer V) using a Nikon Eclipse E-600 microscope (Japan) coupled to a digital

camera and Image Pro Plus Software 6.0 (Media Cybernetics, USA). For each rat, 5 coronal slices (both sides) of hind-limb S1 were used to measure thickness of cortical layers I–VI and corpus callosum. Within each slice, 3 measures of S1 were taken every 0.28 mm starting at 1.8 mm lateral from midline and perpendicular to the white matter (adapted from Anderson and cols. [18]). In each corpus callosum two measures were performed adjacent to midline. No attempt was made to correct tissue shrinkage, which was expected to be equivalent across specimens. Neurons and glial cells were counted per unit area (6,692.52 μm^2). Neurons were identified by the presence of a cresyl violet–stained cytoplasm and their generally larger size and nonspherical outline. Glial cells were identified by the absence of stained cytoplasm and spherical aspect. Counts were made in 10 fields in each section (five in the left hemisphere and five in the right) [19]. The glial-neuronal index, calculated as the ratio of glial cells per neuron in each field, was used in order to better analyze the results. Data of S1 and corpus callosum thickness, neurons, glial cells, and the indexes were totaled for all sections, and means were determined for each animal. The mean neuronal area (μm^2) was estimated using a pointcounting technique [20], using grids with a point density of one point per 27.70 μm^2. For measurement of cerebellar cortical thickness, images of six sections of whole cerebellum were captured and digitalized (in 1X) and three cerebellar lobules (simple, crus 1 and crus 2) [17] were randomly selected. The apices of three gyri were measured and a mean value obtained.

2. 7. Statistical analysis

Body weight, number of errors on the horizontal ladder and the scores from the beam walking test were analyzed using two-way repeated measures analysis of variance (ANOVA) with *anoxia* and *restriction* as the independent variables and *time* as the repeated

measure. Developmental milestones, S1, corpus callosum and cerebellar thickness, numbers of neurons and glial cells, glial-neuron index and neuron cross-sectional areas were analyzed using two-way ANOVA. All analyses were followed by *post-hoc* Fisher's Least Significant Difference (LSD). Data were expressed as means ± SEM. Probability values less than 5% were considered significant. Statistical analysis was performed using the Statistica software package.

3. Results

3. 1. Physical examination

In accordance with Strata and cols. [10], PA did not produce abnormal physical signs, compared to CT group, but SR, with or without PA, led to significantly less body weight gain in all evaluations, mainly in the post-weaning period (P31; $P<.001$, Figure 1).

Qualitative observations in the SR and PA-SR animals were also in accordance with previous studies, including hind-limb muscle atrophy, abnormal extension of the limb, and grossly altered gait patterns [10, 21]. It is noteworthy that, in our study, half the SR and PA-SR animals also presented long-lasting uni- or bilateral cryptorchidism after the immobilization period. In contrast to humans, in rabbits and rats the inguinal canals remain open throughout life, so the testes are held in the scrotum by a combination of gravity and intra-abdominal pressure, except when retracted by contraction of the cremaster muscle [22]. In our study, we think that the forced adduction and extension of the hips during hind limb immobilization decreased space for the testes kept the cremaster muscle in a shortened position contributing to the failure of the testes to descend.

3. 2. Neonatal developmental tests

The appearance of certain neurological reflexes can be influenced by various factors such as neonatal hypoxia-ischemia [6] and chronic lipopolysaccharide exposure [23]. At birth the rat is capable of some specific activities, but its movements are uncoordinated and seemingly random, its tactile sensitivity is not fully developed, and its ear canals and eyes remain closed until several days after birth [24]. Most of the developmental milestones (surface righting, cliff aversion, auditory startle and eye opening) were successfully achieved without differences between groups (Table 1). However, SR-exposed animals, with or without PA, presented stability on an inclined surface significantly later when compared to CT and PA (approximately 3 days later, Table 1, $P<.001$). Proprioceptive placing was achieved earlier by SR and PA-SR groups than CT and PA animals (2 days before, Table 1, $P<.001$).

3. 3. Motor skills

In a previous study Strata and cols. [10], motor performances in the rotor rod and suspended bar were significantly affected in SR and PA-SR rats compared to control and PA rats. In our study we used a horizontal ladder test (observing the ability of animals to walk on an irregular surface, counting the hind-limb step errors) and a beam walking test (scoring full weight support and normal plantar paw placing while the animals were crossing the beam). These tasks are easily performed in intact rats, and require fine sensorimotor control and precise foot placing, and therefore rely partly on an intact corticospinal tract [16, 25]. The CT and PA groups presented equally good performances in the horizontal ladder and beam tests. On the other hand, similarly to Strata and cols. [10], the overall performances of the SR and PA-SR animals in both tests were significantly

affected by hind-limb joint rigidity in the extended position, compromising fluency and movement coordination. The gross alterations seen in these groups gradually reduced, but the differences between the SR and PA-SR groups on the one hand and the CT and PA groups on the other remained. Regarding the horizontal ladder, SR and PA-SR groups showed significant impairment when compared to the CT and PA groups during the whole period of the evaluation (Fig. 2A). Regarding the beam walking, the SR and PA-SR groups obtained significantly lower scores in the first three evaluations ($P<.001$), and the PA-SR group values remained different from controls in the last evaluation ($P<.001$), despite moderate improvement (Fig. 2B).

3. 4. Histological and morphometrical analysis

Microscopic examinations of Nissl stained brain and cerebellum sections did not reveal areas of infarcts or large alterations. Statistical analysis failed to show significant thickness alterations in the S1, cerebellar cortex and corpus callosum. However, there was a tendency toward a reduction of the corpus callosum in the SR group, in the cerebellar cortex in the PA-SR group and an increase in S1 thickness in the PA-SR group (Table 2 in bold).

The neurons and glial cells in the S1 were counted per unit area (6,692.52 μm^2). Neurons were identified by the presence of Nissl granules in the cytoplasm and their generally larger and triangular shape and a pale nucleus with 1 or 2 nucleoli. Glial cells were identified by the absence of stained cytoplasm, their spherical aspect and smaller size. Figure 3 shows representative photomicrographs of the neurons in layer V of the S1 cortices from the CT, PA, SR and PA-SR groups.

The number of neurons was significantly reduced in the SR group (6.32±0.33) compared to the CT and PA groups (7.56±0.30 and 8.32±0.37; respectively, $P<.05$); whereas the PA-SR group (7.30±0.28) was not different from the CT and PA group (Fig. 4A). The number of glial cells was significantly increased in the PA (7.00±0.66) and PA-SR (7.10±0.26) groups compared to the CT group (5.00±0.34, $P<.01$), whereas the SR group (6.08±0.32) did not differ from the CT and PA groups (Fig. 4B). Glial-neuronal index data, calculated as the ratio of glial cells per neuron in each field, showed that the SR and PA-SR groups (0.96±0.11 and 0.96±0.10) had significantly greater numbers of glial cells per neuron per unit area than the CT group (0.67±0.17; $P<.01$; Fig 4C). The mean cross sectional areas of neuronal somata in the S1 were 169.45±5.58; 173.94±13.26; 189.3±10.94; 215.42±12.74, respectively in the CT, PA, SR and PA-SR groups. The neuronal cross-sectional area in the PA-SR group was 27.12% larger than that of the CT group ($P<.01$; Fig. 5A). Additionally, the size distributions of the neuronal cross-sectional areas (frequency histogram, Fig. 5B) showed that the PA-SR group had fewer neurons within the 0-100 μm² and 100-200 μm² range and more in the 200-300, 300-400 and 400-500 μm² ranges.

4. Discussion

Similarly to previous results [10], we found that SR, with or without PA, led to significantly less body weight gain in the post-weaning period when compared to CT group. It is possible that muscle atrophy or lower bone density due SR, as well as changes in food intake may have contributed to this result. Martí and cols. [26] showed that handling of adult male rats did not cause anorexia, but restraint slightly reduced food intake and immobilization drastically reduced it. Also, undernourished rats have delayed

locomotor development [27]. Impaired growth and failure to thrive are frequent and well known characteristics of children with CP, reinforcing deficits in weight gain as an important outcome in this model.

In our study, half of the SR and PA-SR animals presented uni- or bilateral cryptorchidism. There is a higher incidence of cryptorchidism in children with CP, as well as delayed testicular descent, possibly due to spasticity of cremaster muscle in CP during early childhood, which would either cause the testis to retract out of the scrotum or prevent elongation of the spermatic cord with growth [28]. In our study, we think that the position of the hind-limbs and a shortened cremaster muscle may have contributed to this finding. Hence, it suggests that restriction of movements may be a risk factor to failure in testicular descent in CP patients.

Stability on an inclined surface, formerly called negative geotaxis, has long been considered a measure of vestibular and postural reflex [29], but recently its accuracy when assessing vestibular and sensory function only has been debated (see a discussion in Motz and Alberts) [15]. It is considered a form of compensatory response or even an emergency reaction to postural instability. In our study, both SR and PA-SR groups seemed to spend more time and effort trying to rotate 180^{o} upwards because of difficulties in moving hind limbs and an excessive dependence on the forelimbs. Animals often rolled down the sloping surface, especially in the first evaluations, suggesting that some peripheral aspects of limb immobilization, like joint stiffness and muscle atrophy may have sensitized the test.

Accordingly, we observed abnormal signs in proprioceptive placing test when performed by SR and PA-SR animals. Although it depends on intact spinal circuits [30] we suggest that SR and PA-SR heightened limb reflex responses could be due to the spasticity itself. Muscle stretching caused by limb extension during application of the test could lead

to earlier or exaggerated contraction responses. Since both stability on inclined surface and placing were less specific for central nervous system testing, we found these tests not reliable to assess pure neuronal function in mixed models of asphyxia and disuse. On the other hand, a phenotype comprising both neuronal and peripheral impairments should make this model suitable to explore other features of spastic hypertonia.

SR, with or without PA, significantly affected the overall performance of the rats in the horizontal ladder and beam walking tests. Despite moderate improvements, visible alterations persisted throughout the experimental period. In rodents, long-lasting motor deficits are difficult to achieve by the use of early-life neurological insults. In rats, unilateral common carotid ligation followed by hypoxia at P7 produces delays in several neurological reflexes, but the subjects generally achieve normal performances by 5 weeks of age [6]. Similarly, intra-uterine perinatal asphyxia on the last day of gestation also fails to produce abnormal results in neurological reflexes, maze and open field tests [5]. The same limitations apply to intra-uterine or postnatal insults using lipopolysaccharide injections that, despite inflammatory response and white matter damage, do not cause alterations to developmental milestones or open-field and rotarod tests [14, 31]. In the present study, we found persistent motor deficits that were in accordance with previous research, reinforcing this model as suitable to study skill and gait impairments.

The hind-limb representation area in S1 was chosen for cell counting because there is intense overlapping of motor and sensory maps in this region [32]. Moreover, alterations of the hind-limb movement pattern cause several alterations in this area [11, 33, 34]. We found a neuronal cell reduction in S1 cortical layer V in the SR group, an increase in the number of glial cells in the PA and PA-SR groups, an increased glial-neuronal index (increase in glial cells per neurons) in the SR and PA-SR groups and an increase in

neuronal somata area in PA-SR group. In addition, there was a slight increase in S1 cortical thickness (not significant, Table 2 in bold) in PA-SR group. These findings are in accordance with worse pathological findings in the presence of SR, but reveal a role for PA in sensorimotor alterations. However, further studies are needed to affirm whether or not these results could be related to alterations in cortical somatotopy.

We also found that the number of glial cells was significantly increased in the PA and PA-SR groups when compared to the CT group. However, the glial-neuronal index showed that there was a significant increase in glial cells per neuron in the SR and PA-SR groups, thus reaffirming the worsened pathological findings in the presence of SR. On the other hand, the results clearly show glial cell proliferation after the anoxic procedures. Whether gliosis may play a neuroprotective role or contribute to functional impairments in this animal model is not yet known. Two episodes of anoxia could be neuroprotective, because brief episodes of sublethal ischemia were shown to protect against damage from subsequent ischemia in brain [35]. Specific protective pathways have been identified by which glial cells can protect or even help to regenerate brain tissue after acute insults. This could explain the lesser decrease in neuronal cell numbers when PA was associated to SR. Nevertheless, the existence of the glial scarring, which forms around damaged brain tissue, is a key process in regeneration failure [36]. A previous study showed transient prenatal systemic hypoxic-ischemic insult on the 18th embryonic day produced a three-fold increase in astrocytes and neuronal cell loss in the cortex [7]. In humans, a prenatal insult results in gliosis, and oligodendrocyte and neuronal loss [37]. In our study, we think that, although PA-induced gliosis may be neuroprotective to neuronal cell numbers, it may impair synaptogenesis in the PA-SR rats, thus contributing to receptive field degradation, which was not seen in PA alone.

Finally, the combination of PA and SR produced an increase in neuronal area. There are a few reports showing an increase in cell size [38]. However, compensatory mechanisms may be involved, such as the increase in the number of synaptic connections between survivor neurons. in another model of perinatal asphyxia, neuronal death was shown to be compensated by an increase in presynaptic boutons in the parietal cortex and striatum [39].

These results present new data on the pathophysiology of this model and its implications for motor disorders like CP, where movement deprivation and altered experience may themselves worsen motor development and performance. Although the brain pathology in CP is static, motor aspects worsen with time [40], leading to increased disability [12]. Therefore, our results reaffirm that therapeutic interventions in CP should start as early as possible, in order to prevent further motor impairments.

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Legends

Fig. 1. Body weight. PA alone did not affect body weight gain, but SR, with or without PA, led to significantly less body weight gain, mainly in the post-weaning period (P31) compared to CT and PA groups. Data are expressed as means and SEM. *P<.001. Two-way repeated measures ANOVA revealed significant effects of the factors restriction ($F(1,52)=58.304$; P<.001) and time ($F(2,104)=2118.268$; P<.001) on body weights, with a significant restriction × time interaction ($F(2,104)=47.657$; P<.001).

Fig. 2. Motor skills. (A) Horizontal ladder walking: SR and PA-SR groups had significant impairments compared to the CT and PA groups during the whole period of the evaluation. (B) Beam walking: the SR and PA-SR groups obtained significantly lower scores in the first three evaluations, and the PA-SR group values remained different from controls also in the last evaluation, despite moderate improvement. Data are expressed as means and SEM. *SR significantly different from CT and PA (P<.01). ** PA-SR significantly different from CT and PA (P<.01). Regarding the horizontal ladder test, repeated measures two-way ANOVA revealed significant effects of the factors restriction ($F(1,25)=53.76$; P<.001) and time ($F(3,75)=11.85$; P<.001) with a significant restriction × time interaction ($F(3,75)=3.22$; P<.001). Regarding the beam walking test, repeated measures two-way ANOVA revealed significant effects of the factors anoxia ($F(1,25)=7.74$; P<.01), restriction ($F(1,25)=455,05$; P<.001) and time ($F(3,75)=47.50$; P<.001) with a significant restriction × anoxia interaction ($F(1,25)=7.74$; P<.01).

Fig. 3. Representative digitalized images of Nissl-stained sections showing the layer V of the hind-limb representation area in the somatosensory cortex of CT, PA, SR and PA-SR

groups. Neurons (arrows) were identified by the presence of a cresyl violet–stained cytoplasm and their generally larger shape and nonspherical outline. Glial cells (arrowheads) were identified by the absence of stained cytoplasm. Note the uniform distribution of neurons including minimal size variations in CT and PA groups. The PA group displays an increase in glial cell numbers (free and satellite glial cells). In SR there was a decrease in neuron numbers. PA-SR shows an increase in mean cross-sectional areas of neuronal somata and increased glial cell numbers. Scale bar = 40 μm.

Fig. 4. Measurements of neurons and glial cells per unit area: (A) S1 neurons (B) S1 glial cells (C) S1 glial-neuronal index (rate of glial cells per neuron). Data are expressed as means and SEM. * significantly different of CT ($P<.05$). Area = 6,692.52 μm^2. Two-way ANOVA revealed significant effect of the factor restriction ($F(1,14)=7.58$; $P<.05$) on neuronal numbers; and the factor anoxia ($F(1,14)=13.68$; $P<.01$) on glial cell numbers; of the factor anoxia ($F(1,14)=14.35$; $P<.01$) on the glial-neuronal index.

Fig. 5. (A) Cross-sectional mean areas of neuronal somata and (B) cross-sectional areas distribution of neurons. Note fewer neurons within the 0-100 μm² and 100-200 μm² range and more in the 200-300, 300-400 and 400-500 μm² ranges in the PA-SR group. Data are expressed as means. Two-way ANOVA revealed significant effect of the factor restriction ($F (1,14)=7.49$; $P<.05$) on cross-sectional mean areas.

Table 1
Average day ± S.E.M of appearance of neonatal behavioral tests

Behavior	**CT**	**PA**	**SR**	**PA-SR**
Cliff aversion	4.85 ± 0.28	4.37 ± 0.26	4.50 ± 0.28	4.75 ± 0.30
Negative geotaxis	5.71 ± 0.35	5.87 ± 0.32	8.64 ± 0.35*	8.33 ± 0.38*
Proprioceptive placing	7.28 ± 0.27	7.06 ± 0.25	5.00 ± 0.27*	5.28 ± 0.29*
Auditory startle	12.14 ± 0.11	12.12 ± 0.10	11.78 ± 0.11	11.83 ± 0.12
Eye opening	13.71 ± 0.15	13.68 ±0.14	13.78 ± 0.15	14.00 ± 0.16

Two-way ANOVA revealed significant effect of the factor restriction on negative geotaxis ($F(1,52)=57.76$; $P<.001$) and on proprioceptive placing ($F(1,52)=46.66$; $P<.001$). *$P < 0.001$

Table 2 – Thickness measurements

Thickness	**CT**	**PA**	**SR**	**PA-SR**
S1 Cortex	1498.90 ± 54.80	1541.47± 45.68	1508.94 ± 38.60	**1613.75 ± 65.35**
Corpus Callosum	437.24 ± 18.45	405.52 ± 17.79	**381.13 ± 13.91**	406.81 ± 9.65
Cerebellar Cortex	273.93 ± 24.90	261.82 ± 9.77	257.50 ± 19.61	**245.86 ± 19.50**

Statistical analysis did not show significant differences in S1, corpus callosum and cerebealar cortex thickness between the groups.

Figure 1

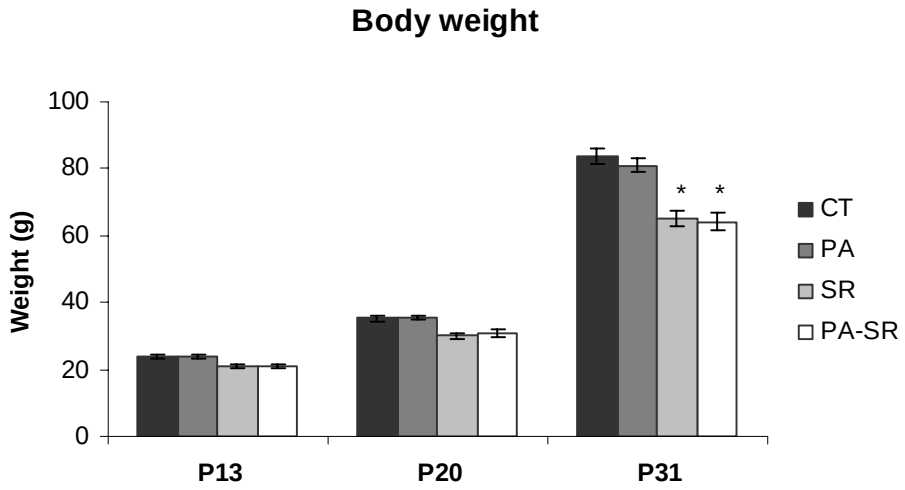


Figure 2

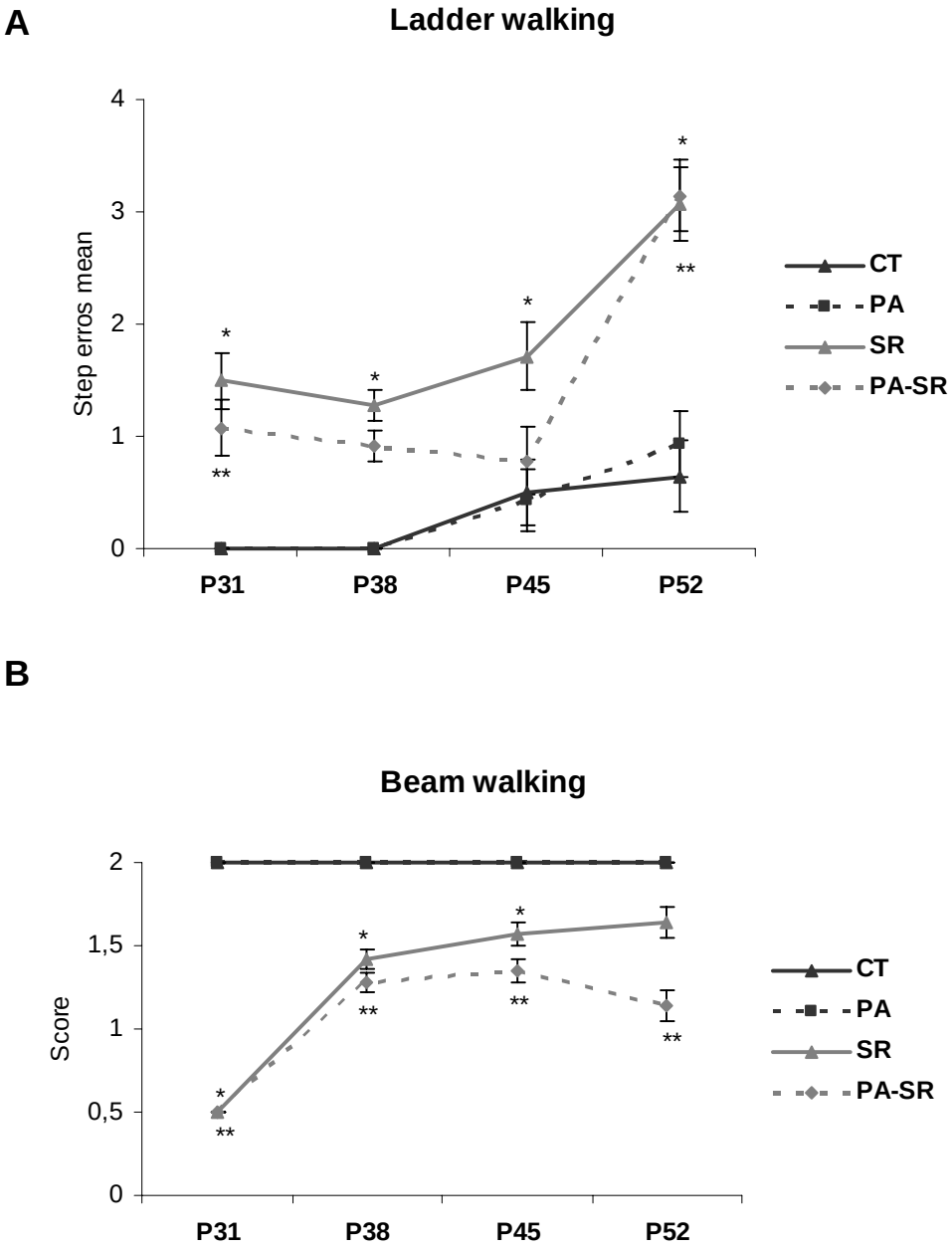

A
Ladder walking
Step erros mean
4
3
2
1
0
P31
P38
P45
P52
CT
PA
SR
PA-SR
B
Beam walking
Score
2
1,5
1
0,5
0
P31
P38
P45
P52
CT
PA
SR
PA-SR

Figure 3

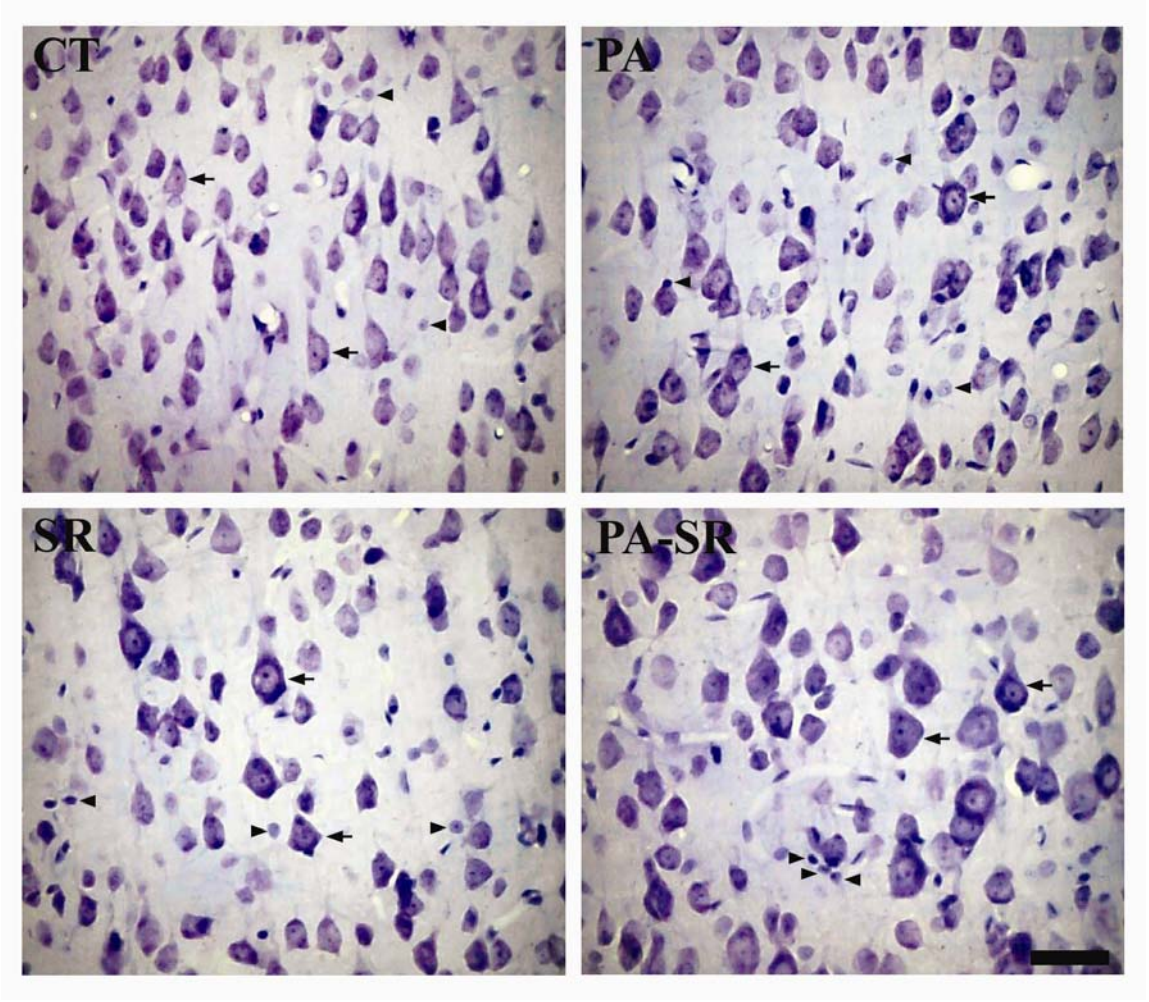


Figure 4

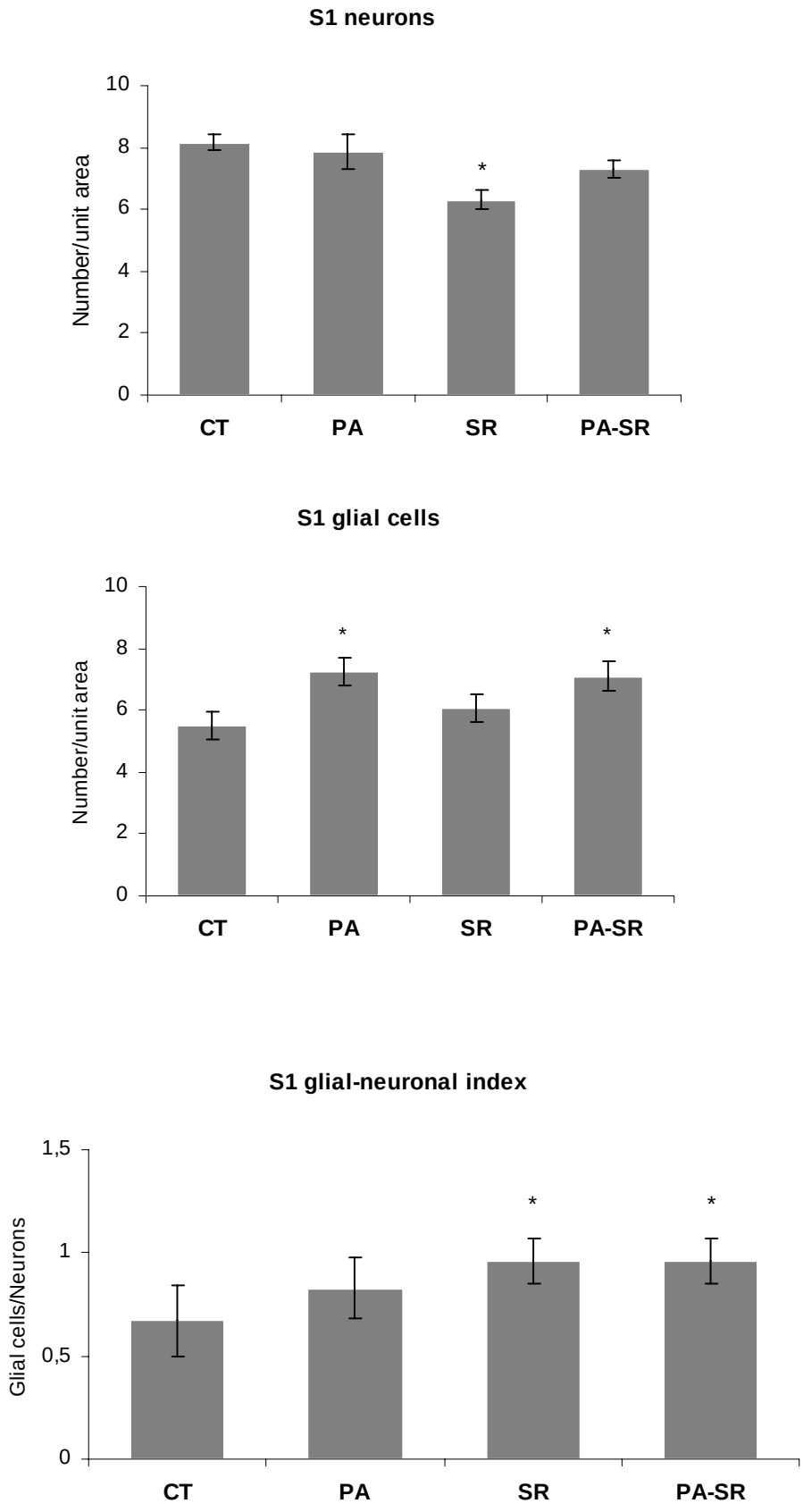
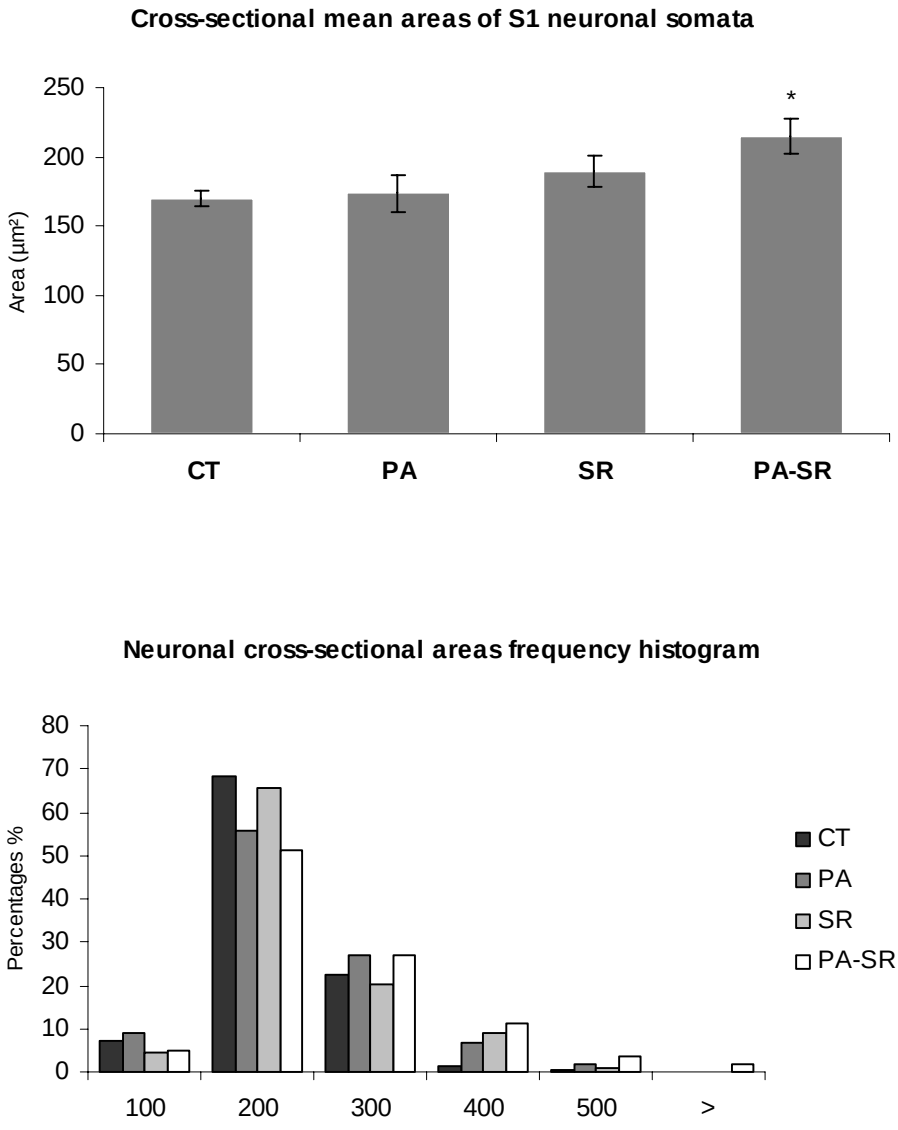

S1 neurons
Number/unit area
10
8
6
4
2
0
*
CT
PA
SR
PA-SR
S1 glial cells
Number/unit area
10
8
6
4
2
0
*
*
CT
PA
SR
PA-SR
S1 glial-neuronal index
Glial cells/Neurons
1,5
1
0,5
0
*
*
CT
PA
SR
PA-SR

Figure 5


Cross-sectional mean areas of S1 neuronal somata
Area (µm²)
250
200
150
100
50
0
*
CT
PA
SR
PA-SR
Neuronal cross-sectional areas frequency histogram
Percentages %
80
70
60
50
40
30
20
10
0
100
200
300
400
500
>
CT
PA
SR
PA-SR

5. CONCLUSÕES E PERSPECTIVAS

5. Conclusões e perspectivas

O desenvolvimento de um modelo animal adequado propicia um melhor entendimento dos mecanismos fisiopatogênicos da PC e o desenvolvimento de estratégias terapêuticas efetivas. A combinação da anoxia perinatal e da restrição sensório-motora, baseada em trabalho seminal de Strata e cols. (2004), mostrou-se simples, acessível, facilmente reprodutível e produziu um fenótipo motor duradouro semelhante àquele encontrado na doença. Somando-se aos resultados obtidos originalmente, demonstramos que a imobilização periférica (restrição sensório-motora) é capaz de causar déficits motores mensuráveis (alteração da aquisição de marcos do desenvolvimento, diminuição do tamanho da passada, aumento do ângulo do pé, prejuízos de habilidades motoras), alterações musculares (redução da área de fibras do músculo sóleo e aumento da densidade de fibras por área estudada) e modificações encefálicas (redução de neurônios no córtex somatossensorial). Por sua vez, o protocolo de anoxia perinatal utilizado nesse trabalho não produziu déficits motores aparentes, porém causou aumento do número de células gliais em S1. Quando os dois procedimentos foram combinados (anoxia combinada à restrição sensório-motora), os aspectos funcionais foram semelhantes àqueles obtidos com a restrição sensório-motora apenas, porém houve aumento do tamanho dos neurônios no córtex somatossensorial, e prejuízo da recuperação morfológica de fibras do sóleo após treinamento físico. Concluímos que, a experiência motora normal durante o desenvolvimento pós-natal precoce é imprescindível para o desenvolvimento normal do sistema locomotor em ratos. Além disso, a atividade física após as alterações motoras já estarem estabelecidas também mostrou benefícios funcionais neste modelo, o que pode reforçar seu potencial terapêutico na paralisia cerebral.

Esse trabalho soma-se a outros nessa linha de pesquisa que procura esclarecer o papel prejudicial da falta de movimentação, ou, a existência de movimentação anormal no início da vida, e suas repercussões na patofisiologia da paralisia cerebral. Essa doença não é naturalmente existente em ratos, porém induzida artificialmente, fica evidenciado nessa espécie a necessidade de restrição de movimentos para indução de alterações motoras duradouras. E que a restrição de movimentos é capaz de gerar dano central. Em humanos o dano central é que é responsável pela gênese da patologia. Porém, danos adicionais podem advir da falta de movimentos e é nessa premissa que deve se basear a fisioterapia precoce nessa patologia.

Esse trabalho é o primeiro nessa linha de pesquisa realizado no Laboratório de Histofisiologia Comparada do Departamento de Ciências Morfológicas – ICBS - UFRGS. A partir desses dados encontram-se em desenvolvimento outros modelos de insultos perinatais, como a modificação do protocolo de anoxia e associação da restrição sensório-motora à indução de reação inflamatória perinatal por exposição a lipopolissacarídeo, a fim de abranger outros aspectos histopatológicos da paralisia cerebral.

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