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

ESTUDO FUNCIONAL E MORFOLÓGICO DA ASSOCIAÇÃO DE LIPOPOLISSACARÍDEO, ANÓXIA E RESTRIÇÃO SENSÓRIO-MOTORA EM RATOS: IMPLICAÇÕES PARA UM MODELO ANIMAL DE PARALISIA CEREBRAL

Dissertação de Mestrado

Felipe de Souza Stigger

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**“The choices we make, not the chances we take,
determine our destiny”**

Autor desconhecido

RESUMO

A paralisia cerebral (PC) é uma complexa desordem da locomoção, postura e do movimento que pode ser causada por uma lesão pré- peri- ou pós-natal ao encéfalo em desenvolvimento. Em ratos, déficits motores semelhantes à PC podem ser induzidos por imobilização dos membros posteriores (restrição sensório-motora; SR), associada ou não à anóxia peri-natal (PA). Além disso, estudos prévios têm mostrado que a exposição pré-natal à endotoxina bacteriana (lipopolissacarídeo; LPS) pode contribuir como um fator patogênico para as características da PC. No presente estudo, investigamos os efeitos à longo prazo da exposição pré-natal ao LPS, da anóxia peri-natal e da imobilização dos membros posteriores durante o desenvolvimento em testes de habilidades motoras e na morfologia do sóleo e tibial anterior. LPS, PA e SR sozinhos ou em combinação mostraram-se capazes de induzir déficits motores no Rotarod. Todos os grupos submetidos à SR, associada ou não à outros agressores, apresentaram prejuízos motores, avaliados pela contagem de erros das patas posteriores nos testes da escada horizontal e da barra estreita suspensa. A imobilização, sozinha ou em combinação, levou à atrofia do sóleo, um aumento no comprimento de seus sarcômeros e uma diminuição de sua densidade. A área de secção transversal (AST) das fibras musculares do tibial anterior, comprimento e densidade dos sarcômeros não foram afetadas pela imobilização, mas interessantemente, uma diminuição da AST foi induzida pela associação com LPS e PA. Foi observada a transição do tipo de fibras musculares no sentido lento para rápido tanto no sóleo como no tibial em todos os grupos restritos.

Estes dados sustentam achados prévios e sugerem que uma experiência sensório-motor aberrante durante a maturação pode reproduzir desordens no movimento e alterações musculares correlacionadas com as observadas em crianças com PC.

ABSTRACT

Cerebral palsy (CP) is a complex disorder of locomotion, posture and movements that can be caused by pre-, peri- or postnatal damage to the developing brain. CP-like movement deficits were more reliably reproduced in rats by hind limb sensorimotor restriction (SR) during development than perinatal asphyxia (PA). Additionally, previous studies showed that prenatal exposure to bacterial endotoxin (lipopolisacharide; LPS) contribute as a critical pathogenic factor underlying CP characteristics. In the present study, we investigated the long-term effects of prenatal LPS exposure, perinatal anoxia and hind-limb immobilization during development in motor skills tests and soleus and anterior tibialis muscles' morphophysiology. LPS, PA and SR alone or in combination showed to induce motor deficits on Rotarod. All goups submitted to SR, associated or not to other treatment exhibited motor impairments, measurable by hind-limb errors counted on horizontal ladder and suspended bar tests. Immobilization alone or in combination induced soleus atrophy, an increase of its sarcomere length and a decrease density. Tibialis anterior cross-section area (CSA), sarcomere length and density were not affected by the immobilization alone, but interestingly, a decrease in CSA was induced by the association with LPS and PA. A slow-to-fast fiber type transition phenomenon was observed on soleus and tibialis anterior only in restricted groups.

These data support previous findings and suggest that aberrant sensorimotor experience during maturation can reliably reproduce the disabling movement disorders and muscular alterations correlated to the observed in children with CP.

SUMÁRIO

ABREVIATURAS

AST	Área De Secção Tranversal
H/I	Hipóxia - Isquemia
LPS	Lipopolissacarídeo
LPV	Leucomalácia Periventricular
MN	Motoneurônio
P	Dia Pós-Natal
PA	Anoxia Perinatal *(Perinatal Anoxia)*
PC	Paralisia Cerebral
RNAm	Ácido Ribonucleico Mensageiro
S1	Córtex Somatossensorial
SN	Sistema Nervoso
SR	Restrição Sensório-Motora

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1. INTRODUÇÃO

1.1 Paralisia Cerebral

Paralisia cerebral (PC) é a causa mais comum de deficiência motora na infância (Himmelmann et al., 2005; Zarrinkalam et al., 2010), com uma prevalência de 2-2,5/1000 nascidos vivos (Stanley et al., 2000; SCPE, 2002; Winter et al., 2002). A PC dispõe de um grupo de distúrbios permanentes do movimento e da postura, causando limitação da atividade, que são atribuídos a danos não progressivos que ocorrem no encéfalo (Rosenbaum et al., 2007; Bax et al., 2005). O dano pode ocorrer ainda no útero, durante o nascimento ou nos primeiros dois anos de vida, caracterizando uma lesão no sistema nervoso (SN) em desenvolvimento (Koman et al., 2004).

Embora uma ampla variedade de fatores de risco pré-natais e perinatais foram definidas para a PC (Kraus e Acheen, 1999; Wu; Colford, 2000), em muitos casos, a etiologia pode ser difícil de se estabelecer (Stanley et al., 2000). A maioria dos casos, 70 a 80%, são adquiridos no período pré-natal. As complicações intra-parto como a asfixia, infecções e traumas envolvem de 10 a 20% dos fatores de risco pré-natais (Johnston; Hoom, 2006). Condições peri-natais 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, 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).

Os substratos neuropatológicos da PC incluem lesão da substância branca, conhecida como leucomalácia periventricular (LPV); hemorragia com extensão

intraventricular, e lesão do córtex cerebral, dos núcleos da base, do tálamo e do cerebelo (Folkerth, 2005; Kadhim et al., 2005). A LPV, identificada como a lesão mais prevalente, resulta da vulnerabilidade dos oligodendrócitos imaturos antes da 32ª semana de gestação (Johnston; Hoon, 2006). Alguns investigadores acreditam que a asfixia seja o principal fator patogênico para a LPV devido a uma peculiar vulnerabilidade das células precursoras dos oligodendrócitos à isquemia (Volpe, 2001). Embora a morte de oligodendrócitos parecer representar um importante papel na patofisiologia da LPV e PC, isso pode não ocorrer como um mecanismo independente. Evidências clínicas, epidemiológicas e experimentais sugerem que respostas inflamatórias maternais/neonatais infecciosas ou não, podem contribuir para o dano encefálico perinatal (Leviton; Paneth, 1990; Zupan et al., 1996; Nelson et al., 1998; Nelson; Chang, 2008), sugerindo que possa existir uma interação entre infecções sistêmicas e a asfixia perinatal (Nelson; Grether, 1998). O componente mais nocivo da resposta inflamatória fetal é a ativação de células do sistema imunológico que atravessam a barreira hematoencefálica causando dano, tanto diretamente, como ativando células locais tais como a microglia e os astrócitos (Damman et al., 2001).

A PC incorpora uma ampla variedade de sinais sendo seu diagnóstico primeiramente clínico. Observações no atraso do desenvolvimento motor, a presença de tônus muscular anormal e alterações posturais são pistas iniciais importantes para o diagnóstico (Krigger, 2006; Dodge, 2008). Os achados motores da PC são comumente acompanhados por epilepsia, problemas musculoesqueléticos secundários e distúrbios sensoriais, perceptivos, cognitivos e comunicativos (Zarrinkalam et al., 2010).

Uma das características mais surpreendentes da PC é a variabilidade de suas manifestações clínicas (Stanley et al 2000, Graham; Selber 2003). A natureza ampla dessa variabilidade causa um problema para classificá-la. Métodos tradicionais de classificação

têm focado a distribuição topográfica, a severidade e o tipo de desordem associada (Dodge, 2008). A distribuição topográfica (Tabela 1) classifica as crianças com base no envolvimento dos membros e sua distribuição pelo corpo. Os principais termos utilizados relacionados à distribuição são hemiplegia, diplegia e quadriplegia, entretanto, termos como monoplegia e triplegia também são utilizados (Delgado; Albright 2003). A severidade dos sintomas normalmente requer a classificação das limitações motoras baseada no grau de limitação, utilizando de termos como: leve, moderado e severo (Oeffinger et al, 2004).

Tabela 1. Classificação Topográfica da Paralisia Cerebral *(Adaptado de Jones, 2006)*

Hemiplegia	Envolvimento de um hemicorpo
Monoplegia	Um membro afetado
Diplegia	Envolvimento maior das extremidades inferiores
Triplegia	Três membros afetados
Quadriplegia	Todas os membros afetados

A classificação da PC pelo tipo de desordem motora inclui: espasticidade (85%), discinesia (7%), ataxia (5%), hipotonia (0,5%) e mista (2,5%) (Stanley et al., 2000). A PC espástica é o tipo mais comum caracterizada por um desequilíbrio do sistema sensório-motor associado à hiperreflexia e ao aumento da resposta muscular ao alongamento passivo, correlacionado com a taxa de alongamento e dependente da velocidade (Lance,

1990; Stanley et al., 2000). A discinesia é caracterizada pela presença de movimentos involuntários e tônus flutuante (Delgado; Albright 2003). As desordens mistas freqüentemente envolvem a espasticidade e a discinesia (Gorter et al., 2004) enquanto as ataxias e hipotonias são relativamente raras (Reddihough; Collins 2003).

1.2 Alterações Musculares na Paralisia Cerebral

Embora os músculos não sejam lesados inicialmente, a doença normalmente evolui com mudanças morfológicas e bioquímicas no sistema neuromuscular (Goldstein, 2004). A musculatura esquelética possui um grande potencial adaptativo em resposta às alterações nas demandas funcionais como mudanças na atividade neuromuscular (Lieber, 1986). Tendo em vista que as crianças com PC possuem o estilo de vida mais sedentário entre as incapacidades pediátricas (Moreau et al., 2009), é razoável assumir que a musculatura dessas crianças apresentará características semelhantes às observadas decorrentes da inatividade porém, não existe um consenso se a espasticidade representa um modelo de desuso ou sobrecarga (Gough, 2009). Em crianças com PC, os músculos esqueléticos não relaxam durante a atividade, devido à espasticidade. Tanto a espasticidade como o espasmo muscular resistem ao alongamento e tendem a manter o músculo em uma posição encurtada, favorecendo o encurtamento muscular (O'Dwye et al., 1989) Além disso, o ciclo de inatividade e incapacidade nas crianças e adolescentes com PC estão freqüentemente associados ao desenvolvimento progressivo de contraturas e fraqueza muscular (Moreau et al., 2009). A incapacidade motora piora o condicionamento físico que, por sua vez, agrava a incapacidade (Graham; Selber, 2003; Damiano, 2006). Em termos motores, a PC resulta em uma lesão do motoneurômio (MN) superior que causa uma série de aspectos neurais e

mecânicos que interagem reproduzindo a patologia músculo-esquelética (Graham e Selber, 2003 – Figura 1).

Figura 1. *Diagrama mostrando a patologia músculo-esquelética na PC. Em termos motores, a PC resulta em uma lesão do motoneurônio (MN) superior que causa uma série de aspectos que interagem (mecanismos neurais somam-se a mecanismos mecânicos) para produzir a patologia músculo-esquelética (adaptado de Graham; Selber, 2003).*

A fraqueza muscular na PC é multifatorial e esta relacionada à mudanças no tipo de fibra muscular, ao recrutamento patológico das unidades motoras, à co-contracção de agonistas e antagonistas, à redução do controle seletivo, à redução do volume e

comprimento da fibra muscular e ao aumento da deposição de colágeno entre outras causas (Damiano et al., 1995; Rose; McGill, 1998).

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), atrofia das fibras (Marbini et al., 2002) e depósitos de fibras de colágeno (Booth et al., 2001). 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 das extremidades podem apresentar alterações de comprimento (Novacheck; Gage, 2007).

1.3 Modelos Animais de Paralisia Cerebral

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;Raju, 1997; Rice; Barone, 2000). Alguns investigadores acreditam que a hipóxia-isquemia (H/I) seja o principal fator patogênico para a LPV devido a uma peculiar vulnerabilidade das células precursoras dos oligodendrócitos à isquemia (Volpe, 2001). Tendo isso em vista, modelos de H/I perinatal têm sido bastante utilizados 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 H/I 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 clínicas, epidemiológicas e experimentais sugerem que respostas inflamatórias maternais/neonatais infecciosas ou não, podem contribuir para o dano encefálico perinatal (Leviton; Paneth, 1990; Zupan et al., 1996; Nelson et al., 1998; Nelson; Chang, 2008), sugerindo que possa existir uma interação entre infecções sistêmicas e a asfixia perinatal (Nelson; Grether, 1998).

Na última década foram elucidados os mecanismos específicos da infecção, à partir da descoberta de que lipopolissacarídeos (LPS) ativam o sistema imunológico através da interação com receptores *toll-like* conduzindo a iniciação da resposta adaptativa imune pela produção de citocinas inflamatórias tais como a interleucina 1-beta e o fator de necrose tumoral-alfa (FNT-α) (Kopp; Medzhitov, 1999). A produção dessas citocinas inflamatórias pode induzir a apoptose de oligodendrócitos e a degeneração da mielina (Damman et al., 2001).

Insultos aplicados nos períodos intra-uterino e pós-natal, usando injeção de LPS, induzem resposta inflamatória e causam dano da substância branca encefálica, porém não causam atrasos no desenvolvimento motor ou alterações em testes motores (Poggi et al., 2007; Roberson et al., 2006). Estudos experimentais mostraram que a pré-exposição ao LPS aumenta a vulnerabilidade do encéfalo imaturo à H/I (Eklind et al., 2001; Rousset et al., 2008). A indução de uma resposta inflamatória pela exposição de ratas prenhas à injeção intraperitonial de LPS ao 17º dia até o final da gestação, combinada a H/I, 24 h

após o parto causou déficits motores e lesões corticais e subcorticais extensas (Girard et al., 2008).

No entanto, em roedores, apesar da H/I, associada ou não ao LPS, causar atrofia de regiões encefálicas, como o estriado, o córtex sensório-motor e o hipocampo dorsal no lado ipsilateral à lesão, nenhum desses modelos reproduz todos os aspectos da PC, como por exemplo, alterações específicas da marcha, transição de fenótipo das fibras musculares principalmente em membros inferiores, assim como mudanças nas propriedades mecânicas desses músculos.

Desta forma, tendo em vista que estas alterações são claramente observadas em modelos de desuso (Basso et al., 1996; Anderson et al., 1999), Strata e colaboradores (2004), desenvolveram um modelo animal de PC, que consiste na combinação de anóxia nos dois primeiros dias pós-natais (P0 e P1) adicional à contenção dos membros posteriores, chamada restrição sensório-motora (do P2 ao P28, por 16 horas por dia – Figura 2), assim causando o desuso dos membros semelhante à falta de movimento que ocorre na PC. Nesse modelo, obtiveram-se alterações de longo prazo típicas da PC, tais como, redução do crescimento corporal, aumento do tônus muscular dos membros posteriores, padrão de marcha anormal e uma desorganização intensa no córtex motor primário, assim como alterações específicas da marcha, como redução do tamanho da passada, alterações de ângulos articulares, atrofia muscular (Strata et al., 2004; Coq et al., 2008; Marcuzzo et al., 2008). Este trabalho mostrou que a imobilização precoce dos membros posteriores, combinada ou não à 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

alteração na representação dos membros posteriores nos córtices motor e somatossensorial, que seriam semelhantes àquelas alterações encontradas em crianças com PC.

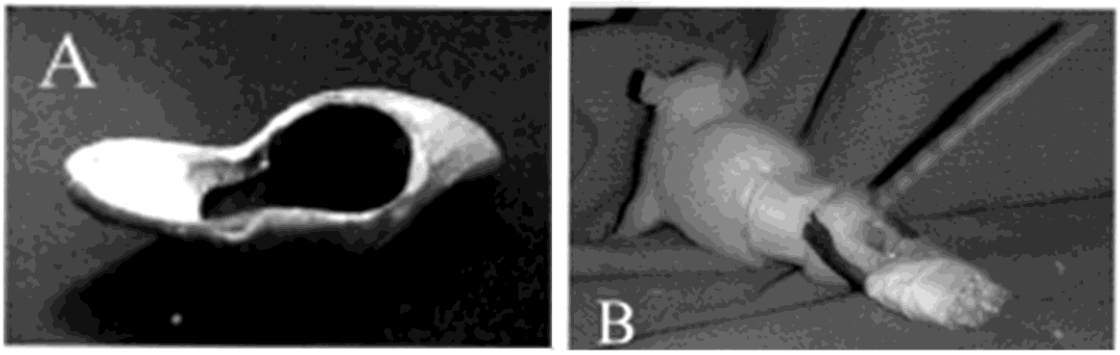

Figura 2. A) Moldura de epóxi utilizada para a restrição sensório-motora dos membros posteriores. B) Filhotes com os membros imobilizados em posição estendida com utilização de fitas adesivas e a moldura (adaptado de Strata et al., 2004).

1.4 Alterações periféricas e centrais do desuso no período de desenvolvimento

Tendo em vista que os 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, doenças que afetam a motricidade no início da vida produzem efeitos deletérios sobre a maturação do sistema motor (Eyre, 2007; Martin et al., 2007). Na PC, à lesão encefálica inicial, soma-se inatividade no início da vida, que pode agravar os déficits motores. Apesar da lesão encefálica não ser progressiva, o agravamento das desordens motoras normalmente é (Graham; Selber, 2003), causando mais inabilidade (Damiano, 2006).

Lesões durante o período de maturação do SN em crianças acarretam em uma mudança na mobilidade espontânea (Ferrari et al., 1990), porém, somente mais tarde são

observados os sinais neurológicos característicos da PC (Eyre, 2007). 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, assim como 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 a 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 a partir do 15º dia de vida o padrão de marcha adulto é estabelecido (Eyre, 2007; Clowry, 2007). Estudos em que a atividade muscular foi reduzida na primeira e na segunda semana pós-natal causam modificações periféricas e centrais de longo prazo, tais como, retardo 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), retardo na maturação de MN (Pastor et al., 2003), aumento no número de neurônios córtico-espinhais (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).

Utilizando o modelo de asfixia e desuso, Coq e colaboradores (2008) demonstraram que a PA não produziu alterações dos mapas corticais, porém a imobilização dos membros posteriores causou uma desorganização topográfica da representação dos membros posteriores em S1. Ratos imobilizados apresentaram mapas podais degenerados, com

campos receptivos maiores e uma aumentada responsividade cortical à estimulação tátil. Esta desorganização aumentou quando o desuso e a PA foram associados, e a combinação de ambos os procedimentos causou diminuição das áreas corticais envolvidas com o 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, além de não serem descritas as diversas alterações musculares presentes na PC, este modelo não apresenta os aspectos neuropatológicos encefálicos presentes na doença (Marcuzzo et al., 2009).

2. JUSTIFICATIVA

A PC é uma doença de características motoras com alta prevalência em nosso meio. Ela causa prejuízos motores importantes, levando a um ciclo de inatividade e incapacidade que pioram o condicionamento físico e, por sua vez, limitam as atividades de vida diária levando à dependência funcional (Stanley et al., 2000; SCPE, 2002; Winter et al., 2002).

Os modelos animais freqüentemente estudados que procuram mimetizar esta desordem não conseguem reproduzir um fenótipo semelhante à PC em humanos. Enquanto modelos de H/I e exposição à LPS não causam as alterações motoras típicas da PC, o modelo que associa a anoxia perinatal e a restrição sensório-motora se aproxima mais do fenótipo motor, no entanto, este modelo não mostrou as alterações encefálicas características da doença.

Portanto, cada tipo de procedimento: injeção de LPS no período embrionário, anóxia perinatal e restrição sensório-motora durante o desenvolvimento da locomoção influenciam diferentes e complementares aspectos presentes na patogênese da PC, podendo juntos resultar modelo animal mais semelhante à PC em humanos.

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

3.1 Objetivo Geral

Delinear um novo modelo animal de PC, a partir da combinação de três tratamentos relacionados a gênese da PC, capaz de reproduzir tanto os déficits anatômicos quanto funcionais e motores, a fim de que se obtenha um fenótipo comportamental e neuropatológico mais semelhante à PC.

3.2 Objetivos Específicos

Analisar o efeito de cada tratamento (injeções de LPS, anóxia e restrição sensório-motora) e suas combinações no comportamento motor e em aspectos morfológicos musculares.

Avaliar o equilíbrio e a coordenação, utilizando testes sensório-motores (Rotarod, escada horizontal e barra estreita suspensa);

Analisar as variáveis histomorfométricas e histoquímicas de amostras musculares do sóleo e do tibial anterior.

4. RESULTADOS

4.1 *Artigo – Felipe de S. Stigger, Arthur L. de S. Felizzola, Glaucia A. Kronbauer, Gabriela K.Couto, Matilde. Achaval, Simone Marcuzzo.* ***Effects of Fetal Exposure to Lipopolysacharide, Anoxia and Sensorimotor Restriction on Motor Skills and Musculoskeletal tissue: Implications for an Animal Model of Cerebral Palsy***

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Effects of Fetal Exposure to Lipopolysaccharide, Anoxia and Sensorimotor Restriction on Motor Skills and Musculoskeletal tissue: Implications for an Animal Model of Cerebral Palsy

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Keywords: Lipopolysacharide; Perinatal asphyxia; Developmental disuse; Motor behavior; Muscle atrophy; Muscular fiber type; Cerebral palsy

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Abstract

Cerebral palsy (CP) is a complex disorder of locomotion, posture and movement that can be originated by pre, peri or postnatal damage during brain development. CP-like movement deficits were more reliably reproduced in rats by hind limb sensorimotor restriction (SR) during development than perinatal asphyxia (PA). Additionally, previous studies have shown that prenatal exposure to bacterial endotoxin (lipopolissacharide; LPS) contributes as a critical pathogenic factor underlying CP characteristics.

In the present study, we investigated the long-term effects of prenatal LPS exposure, perinatal anoxia and hind-limb immobilization during development on motor behavior and soleus and anterior tibialis muscle histology. LPS, PA and SR alone or in combination were shown to induce motor deficits in Rotarod tests. All rats submitted to SR, associated or not to other treatments (LPS and/or PA) exhibited motor impairments, measurable by hind-limb errors counted on horizontal ladder and suspended bar tests. Immobilization alone or in combination with LPS and/or PA induced soleus atrophy and an increase in sarcomere length and density. In the tibialis anterior, the cross-section area (CSA), sarcomere length and density were not affected by the immobilization alone, but interestingly, a decrease in CSA was induced by the association of LPS+PA+SR. A slow-to-fast fiber type transition phenomenon was only observed in soleus and tibialis anterior in restricted rats. These data support previous findings and suggest that aberrant sensorimotor experience during maturation can reliably reproduce the disabling movement disorders and muscular alterations correlated to those observed in children with CP. However, when combined, they induced the worst impairments on motor function and histological changes in the soleus and tibialis anterior muscles. The mechanisms by which these procedures contribute

to these pathophysiological aspects are still unclear and more studies must be done to support this hypothesis.

Introduction

Cerebral palsy is primarily a motor and posture disorder (Krigger, 2006), which is attributed to very diverse and multifactorial etiologies, leading to the brain's inability to control motor functions, consequently affecting global development which limit activity (Jones et al., 2007). Muscle function frequently becomes progressively more compromised in children with spastic CP contribution to the development of contractures and weakness (Moreau et al, 2009). Histological analyses of muscle biopsies from patients with CP have shown changes in muscle fiber type, size and collagen accumulation (Rose et al., 1994; Ito et al., 1996; Booth et al., 2001; Marbini et al., 2002).

Oxygen deprivation such as asphyxia, before, during or after delivery, and hypoxic/ischemic (H/I) events are still considered the main mechanisms promoting brain damage (Kadhim et al., 2005; Nelson, 2003). However, in rodents, perinatal hypoxic–ischemic (H/I) insults induce only subtle and transient motor alterations (Hoeger et al. 2000; Lubics et al. 2005; Robinson et al. 2005).

Additionally, several clinical findings support the hypothesis that maternal inflammatory process could contribute as a pathogenic factor (Nelson et al., 1998; Zupan et al., 1996; Leviton; Paneth 1990; Nelson; Chang, 2008), suggesting the existence of an interaction between systemic infections and perinatal asphyxia (Nelson; Grether, 1998). In addition, other studies (Eklind et al.; 2001; Rousset et al.; 2008) have shown that embryonic exposure to bacterial endotoxin (LPS) dramatically increased brain vulnerability to subsequent injury in rats. Girard and cols. (2009) have shown that immune activation of rats on the 17^{th} day of pregnancy, combined with H/I, caused several motor deficits and cortical lesions.

Disuse animal models, however, have been shown to produce more degraded motor functions (Westerga; Gramsbergen, 1993; Strata et al., 2004). As a strategy to impair the locomotor development, hind-limb immobilization, also known as sensorimotor restriction (SR), alone or in association with perinatal anoxia (PA), presented good results in mimicking the motor functions of a CP patient (Strata et al., 2004; Marcuzzo et al., 2009). The SR produced long-lasting deficits such as reduced body growth rate, increased muscular tone, abnormal gait patterns and primary motor cortex disorganization. Another study showed that this CP-like behavior is associated to muscle fiber atrophy, ankle and knee joint degeneration, as well as brain disorganization (Coq et al., 2008). On the other hand, anoxia alone caused mild alterations in muscle tonus and motor performance (Strata et al., 2004). The association of PA and SR has been shown to be a simple, inexpensive and easily reproducible model of a CP-like motor phenotype (Marcuzzo, et al., 2009).

Consequently, considering that gait alterations are observed in disuse models (Basso et al. 1996; Strata et al., 2004), and that LPS-injection in the embryonic period and perinatal anoxia are related to pathogenesis of CP lesions (Eklind et al.; 2001; Rousset et al.; 2008; Girard et al., 2008), the aim of the present study is to investigate the combination of these three treatments and its possible implications for an animal cerebral palsy model. Therefore, the specific objective of this study is to analyze the effects of LPS, PA and SR, combined or not, on motor skill tests such as the suspended bar and horizontal ladder and Rotarod as well as the morphological and histochemical aspects of the soleus and tibialis anterior muscles in order to gather more information that may help in the development of a cerebral palsy model in rodents.

Materials and methods

Animals

For this study, we used 20 female and 10 male Wistar rats aged 75 days from the colony of the Federal University of Rio Grande do Sul (Porto Alegre, Brazil). Animals were housed in standard plexiglass boxes, under a 12:12 h light/dark cycle in a temperature-controlled environment (20 ± 1 ºC) with food and water available *ad libitum*, according to the Brazilian law that regulates animal use for didactic-scientific practice. All procedures were approved by the Ethical Committee at the Federal University of Rio Grande do Sul (n^{o} 2008189) and animals care followed the recommendations of the Brazilian Society for Neuroscience, 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).

Estrous phase was determined under microscope examination of daily vaginal smears starting the 75th day of age. Sexually receptive females were housed overnight with a sexually experienced male. Successful mating was confirmed next morning by the presence of spermatozoids in the vaginal smear and that day was designated as gestation day (GD) 1.

Experimental groups

After delivery, male pups were randomly assigned to eight different groups (n = 6 - 8): rats submitted to saline injection during the embryonic period (CT); LPS injection during the embryonic period (LPS); saline injection and anoxia (PA); LPS injection and anoxia (LPS+PA); saline injection and sensorimotor restriction (SR); LPS injection and

sensorimotor restriction (LPS+SR); saline injection, anoxia and sensorimotor restriction (PA+SR); LPS, anoxia and sensorimotor restriction; (LPS+PA+SR).

LPS, Anoxia and Sensorimotor Restriction procedures

Figure 1 shows the time line of the experimental procedures. Female rats were observed until GD17. At this period, they were divided into 2 groups according to Girard and col. (2008): Group 1 (n = 10), LPS injected rats (200 µg/kg diluted in 100 µL of sterile saline; Sigma, USA) and Group 2 (n = 10), vehicle injected rats (100 µL of sterile saline). The pregnant rats were injected with LPS or saline at 12-hour intervals until the end of gestation. After delivery pups were submitted to anoxia for 20 min on the day of birth (P0). For this procedure, rat pups were put in a temperature controlled chamber (37º C ± 1º C) with a flow of 9 L/min of 100% N_2 (White Martins, Brazil). Rats were submitted to sensorimotor restriction (16 h/day) from P2 to P28. The pup's hind limbs were immobilized together, in an extended position, by a support made of epoxy resin and tape (Henkel, Brazil). This procedure was well tolerated by the pups, and it does not prejudice the elimination of urine and feces, or interfere with maternal care (Strata et al., 2004; Marcuzzo et al., 2008; 2009).

Motor Skills Assessment

Rotarod

Motor balance and coordination were evaluated on P29 using a Rotarod (Hugo Basile, Italy). The animals were placed on a 60 mm diameter textured rod, 75 mm in length, rotating at a speed of 30 rpm. The time spent by the animal on the rotating rod was

considered as the latency to fall. Each animal was tested 5 times with a 2 minute interval between each trial. The maximum duration of the test was 3 minutes.

Horizontal ladder and narrow suspended bar

On P29 and P45, the animals were submitted to the horizontal ladder and the narrow suspended bar tests, which were used to assess the hindlimb sensorimotor function. During each test, the animals were filmed 3 times (30fps), with a digital camera (Sony; DCR-SR47, USA).

The suspended bar is a 100 x 2.5 cm rectangular bar positioned 30 cm from the floor. The horizontal ladder has the same length, but is 5 cm in width, with parallel metal rungs (2 cm apart). Motor skills were assessed based on the ability to walk on these apparatus. During the course of the tests the number of hindlimb step errors was counted by two blinded observers (adapted from Marcuzzo et al., 2009)

Histological, morphometric and histochemical analysis

At P45, after being tested, rats were anesthetized with sodium thiopental (50 mg/kg, i.p.; Cristália, Brazil), injected with 1000 IU heparin (Cristália, Brazil) and transcardially perfused with 100 ml of saline solution, followed by 150 ml of a solution containing 0.5 glutaraldehyde (Sigma, USA) and 4% paraformaldehyde (Reagen, Brazil) diluted in 0.1 M phosphate buffer (PB; pH 7.4) at room temperature. An additional experiment was conducted to obtain samples from the soleus and tibialis anterior muscles for histochemical analysis.

Soleus and Tibialis Anterior sarcomere length and density calculation

During the saline perfusion, left non-fixated soleus and tibialis anterior muscles were dissected free of their attachments (sodium thiopental - 50 mg/kg, i.p.; Cristália, Brazil). After this procedure the soleus and tibialis anterior muscles were immersed in a 0.1M phosphate buffer solution (pH 7.4) containing 4% paraformaldehyde for 3h. Subsequently, each muscle was placed in a separate dish containing 30% nitric acid for 48 h to partially digest the connective tissue. The muscle tissue was then transferred to a 50% glycerol solution. Six fibers from each muscle were carefully teased out and placed on glass slides. Three regions from each fiber were obtained, using a Nikon Eclipse E-600 microscope (Japan) coupled camera and an imaging software (Image Pro Plus Software 6.1, Media Cybernetics, USA) was used to calculate sarcomere length and density. For each image was considered a rectangular region of interest (ROI). The ROI length was calculated and then, sarcomere length was determined by dividing the ROI length for the number of sarcomeres counted in this ROI. The density, which represents the number of sarcomeres in each mm, was obtained by dividing the number of sarcomeres for the ROI length (adapted from Butterfield et al., 2005).

Soleus and Tibialis Anterior cross-section area

After perfusion, right soleus and tibialis anterior were carefully dissected free from surrounding connective tissue. Small samples (2x1 mm) of the central part of the muscle were selected and postfixed in the same fixative solution until processed. Muscle samples were washed in PB and postfixed in 1% OsO_4 (Sigma, USA) in PB for 1 h and then newly washed with PB and dehydrated in a graded series of alcohol and propylene oxide (Eletron Microscopy Sciences, USA), embedded in resin (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 (RMC; PT-x, USA) and stained with 1% toluidine blue (Merck, Germany) in 1% sodium tetraborate (Ecibra, Brazil).

Afterwards, images of soleus and tibialis anterior muscles were captured and digitalized (initially 20x and further amplified 200% for analysis) using a Nikon Eclipse E-600 microscope (Japan) coupled to a imaging software (Image Pro Plus Software 6.1, Media Cybernetics, USA). For morphometric evaluations, a set of 6 images was chosen using random sampling of one slice. We examined 20 muscle fibers of each one of the 6 randomly selected areas.

Morphometric measurement was made by the estimation of the mean muscle fiber area; CSA (μm^2). The soleus and tibialis anterior fiber areas were estimated with a point-counting technique as described by Marcuzzo et al. (2008).

Soleus and Tibialis Anterior ATP-ase histochemical analysis

Soleus and tibialis anterior non fixed muscles were dissected and quickly frozen. Serial sections (25 mm) were obtained using a cryostat (Leica; CM1850, Germany) at -20°C. The myosin adenosine triphosphatase (ATPase) activity of these muscle sections were determined using a modification of the procedure of Brooke and Kaiser (1970). Sections were incubated at room temperature for 11 min in a 0.1M glycine/NaCl buffer with 0.75M $Cacl_2$ and 5mg of ATP adjusted to pH 9.6. After washing sections were immersed in a 2% $COCl_2$ solution for 5 min. Sections were newly washed and then immersed in 1:10 ammonium sulphide solution for 30 seconds after this procedure sections were well rinsed in tap water. The microscopic observations of the sections were analyzed

using a Nikon Eclipse E-600microscope (Japan) and an imaging software (Image Pro Plus Software 6.1, Media Cybernetics, USA) was used to count the number of positively (black) and negatively (white) stained fibers, respectively classified as type II (fast-twitch) fibers and type I (slow-twitch). Were classified more than 200 fibers per section (total number of muscle fiber) that were used to classify the composition pattern of the muscle accordingly to the proportion of each muscle fibers type.

Statistical analysis

Data were analyzed using three-way analysis of variance (ANOVA) with LPS, anoxia and sensorimotor restriction as the independent variable. All analyses were followed by *post hoc Duncan test*. Data were expressed as means ± SEM. Probability values less than 5% were considered significant. Statistical analysis was performed using the Statistica software package.

Results

Functional assessment

To evaluate the functional consequences of the exposure of LPS, PA and SR, combined or not, pups were submitted at two different postnatal periods to functional tests. In a first set of experiments (P29), motor balance and coordination were evaluated using a Rotarod. Rats were placed on the rotting rod and the latency to fall was registered for each rat. As shown in Figure 2, CT rats performed significantly better than any other group, whereas, any restricted rat, independently of the combination of treatments, showed the worst performances. SR, LPS+SR, PA+SR, and LPS+PA+SR rats remained a shorter time period on the Rotarod when compared to CT ($P<0.001$). LPS, PA and LPS+PA rats

presented similar performances between them and significantly better than the all restricted rat ($P<0.05$) but worst than CT ($P<0.05$). As already described, restricted rats in our study used a different strategy to walk on the rotting rod, they did not move their hind limbs until it were total extended and they use their forelimbs to pull up the whole body trough the rod (Strata et al., 2004).

At the same postnatal period a horizontal ladder and a narrow suspended bar were used to examine hind limb sensory motor function (counting the hind limb step errors). As shown in Figure 3, these tasks were easily performed by non restricted rats. The CT, PA, LPS and LPS+PA groups presented equally good performances. Therefore, similarly to Strata et al. (2004) and Marcuzzo et al. (2009), the performances of all SR group were significantly worst due to the lack of fluency and movement coordination caused by the hind limb immobilization ($P<0.001$). Interestingly, the combination of the three treatments, was seem to cause the worst performances on these tests. LPS+PA+SR rats were significantly different than CT rats ($P<0.001$) and than any other SR rat independently of the combination of treatments ($P<0.05$ - $P<0.001$).

At P45, rats were submitted to a second set of evaluations using the same horizontal ladder and a narrow suspended bar used on P29. Although, the number of step errors was reduced in all groups, compared to P29, similar differences between groups were observed at this time. All SR groups showed significantly impairments when compared to non restricted rats ($P<0.001$), but at this period the LPS+PA+SR was similar to SR group but worst than LPS+SR and PA+SR ($P<0.05$).

Histological, morphometric and histochemical analysis

The results sarcomere length and density of soleus and tibialis anterior are shown in Table 1. Neither tibialis anterior sarcomere density nor sarcomere length showed significant differences between groups. However, immobilization, alone or in combination with other treatments, induced an increase on sarcomere length, as well as, decreased sarcomere density on soleus muscle compared to control ($P<0.05$).

Myofiber CSA of both muscles were differentially affected by the all experimental treatments (Table 1). Soleus fibers of CT rats showed a mean CSA of 998.5 ± 71.6 μm^2. Whereas no difference on soleus's CSA was observed in LPS, PA and LPS+PA groups (1178.6 ± 37.6, 1097.4 ± 38.0 and 1154.5 ± 89.9, respectively) when compared to CT, soleus muscle atrophy was confirmed in SR, LPS+SR, PA+SR and LPS+PA+SR groups (741.6 ± 59.7 ($P<0.05$); 720.0 ± 70.9 ($P<0.01$); 677.8 ± 37.6 ($P<0.01$); 549.8 ± 97.8 ($P<0.001$), respectively. Atrophy of tibialis anterior was only detected in LPS+PA+SR rats (635 ± 41.4). A significantly decrease on CSA was observed in this group when compared to CT (978.0 ± 79.0; $P<0.05$), LPS (947.8 ± 53.1; $P<0.05$), PA (952.5 ± 60.2; $P<0.05$), SR (1192.2 ± 153.5; $P<0.001$), LPS+SR (1014.5 ± 104.1; $P<0.05$) and PA+SR (938.1 ± 97.2; $P<0.05$) group (Figure 4, Table 1).

Soleus atrophy was accompanied by a fiber-type transition phenomenon reveled by the ATP-ase activity. The proportion of type I fibers to the total fiber number displayed significant differences between groups. Table 1 shows the percentage of soleus and tibialis anterior fibers type in each group. The percentage of soleus type I fibers was 79.1 ± 1.8 % for the CT group and 60.4 ± 2.0 % for the LPS+PA+SR group. The percentage of type II soleus fibers was 20.9 ± 1.8 % for the CT group and 39.6 ± 2.0 % for the LPS+PA+SR group. Thus, in the LPS+PA+SR group, the soleus muscle had a significantly higher

proportion of type II than type I fibers compared with the CT group ($P<0.001$). Similar results were detected in SR, LPS+SR, PA+SR groups ($P<0.001$). PA, LPS and LPS+PA groups had similar results to the CT group. The same pattern of fibre-type transition was observed on anterior tibialis (Figure 4, Table 1).

Discussion

The etiology of CP is very diverse and data from different animal models need to be combined in order to reproduce a complete picture of CP pathogenesis. A better understanding of the physiopathogenic mechanisms underlying CP development could be facilitated by generating an animal model that could mimic the human CP condition with a demonstrable phenotype (Girard et al., 2008). For the first time, the present study has demonstrated the effects of prenatal exposure to LPS injection, neonatal asphyxia and chronic hind-limb disuse on motor behavior, as well as, on the morphologic aspect of the soleus and tibialis anterior muscles in male Wistar rats. Our main findings can be summarized as follow: First, all SR rats, whatever the combined treatment, displayed severely impaired motor performance. Interestingly, the LPS+PA+SR group demonstrated the worst results in the three performed tests. Second, soleus muscles were found to have a decrease in sarcomere density and an increase in its length. Third, soleus fiber atrophy was only detected in rats submitted to SR (associated or not with LPS and/or PA) and only the association of the three treatments led to a CSA loss in the tibialis anterior muscle. Fourth, soleus and tibialis anterior muscles from all the SR rats presented the classical effects of disuse that result in a fiber type transition phenomenon in which the proportion of fast fibers increases.

In the present study, the LPS, PA and LPS+PA groups presented similar results in functional evaluations. Although Rotarod was sufficiently sensitive to detect motor impairments, the horizontal ladder and the narrow suspended bar did not show any motor function deficit. McQuillen and col. (2003) reported that hypoxic animals at P2 performed significantly worse in motor-function tests such as Rotarod, beam walking and stair climbing. Additionally, neither, Strata and col. (2004) nor Marcuzzo and col. (2009), found impairments when PA rats performed the narrow beam test. Actually, Strata and col. (2004) found PA rats presented significantly longer time spent on the Rotarod than CT. Differences in PA procedure account for the discrepancy in our findings. Several perinatal hypoxic/anoxic models have been used to induce motor dysfunctions that match the symptoms of CP (Brake et al., 2000; Derrick et al., 2004) but the impairments caused by PA did not completely succeed in reproducing the long term characteristic disabilities of this disease (Hoeger et al., 2000; Lubics et al., 2005).

There are few experiments showing the effects of LPS in association or not with other treatments on motor capacities. For example, the induction of an inflammatory response by the exposure of pregnant rats to LPS intracervically on the gestational period showed a delay in the achievement of some developmental milestones and a trend towards decreasing mean locomotion speed, but no sign of motor impairments was shown using the Rotarod (Toso et al. 2005). In another study, rats exposed to postnatal LPS injections did not display any significant motor impairment when assessed using the Rotarod and narrow beam tests (Roberson et al., 2006). On the other hand, Girard and col. (2008), employing the same LPS protocol used in the present study, found impairments to motor function and coordination in rats exposed to LPS alone or additionally to H/I. It seems that LPS sensitizes the rat brain, making it more vulnerable to a hypoxic event (Girard et al., 2008;

Rouseet et al., 2008). Although, these studies demonstrated motor impairments, they did not result in the severe motor deficits and muscular alterations that are typical of PC, such as an abnormal walking pattern, chronic spasticity, muscle atrophy or mechanical and phenotype transition (Ito et al., 1996; Marbini et al., 2002; Givon, 2009).

Rats submitted to hind-limb SR displayed alterations in gait such as decreased stride length, wider foot angle, posteriorly extended hind limbs, reduced joint movements and elevated hindquarters (Strata et al., 2004; Marcuzzo et al.,2008). In accordance with previous studies (Strata et al., 2004; Marcuzzo et al., 2009), SR animals displayed significant motor performance impairments when assessed using the Rotarod, horizontal ladder and narrow suspended bar tests. On P29, the combination of LPS+PA+SR caused the worst performance on the horizontal ladder and the narrow suspended bar tests, showing that the SR together with LPS+PA might, in some way, contribute to the impairments found during the motor skill tests. Asphyxia for 20 min seems to induce neuronal loss by apoptosis and a depletion of neurotransmitters within the rat striatum (Loidl et al., 1994; Van derBerg et al., 2002) and, since, striatum plays an important role in motor control and sensorimotor integration (Calabresi et al., 1997) these previus data may be related to our findings. Additionally, LPS has been shown to induce an inflammatory response in the brain leading to decreased myelinization (Toso et al., 2005) and also to enhance the vulnerability of the immature brain to excitotoxic insults (Rousset et al., 2008).

Rat spinal cord undergoes a significant continuous transformation during perinatal development (Vinay, et al., 2000) and the first postnatal week is a critical period for the development of postural reactions in the hindlimbs (Brocard et al., 1999). Motor activity and proprioceptive input seems to play an important role in motoneuronal development (Inglis et al., 2000) and locomotion in the rat (Westerga and Gramsbergen, 1993).

Unilateral hindlimb immobilization from P1 to P20 caused abnormalities in the timing of the EMG activation patterns in the gastrocnemius and the tibialis anterior muscles (Westerga and Gramsbergen, 1993). Our study confirms the importance of voluntary movements during the maturation of the central and peripheral nervous system and we also showed that abnormal proprioceptive input during the first four postnatal weeks can contribute to some motor behaviors that are compatible to those observed in children with CP. Disuse does not simply reduce proprioceptive feedback from the hind limbs, it also leads to a remodeling within the somatosensory cortex (Langlet, Canu, & Falempin, 1999; Coq et al., 2007), motor cortex (Strata et al., 2004) and a change in neuronal activation (Dupont, Canu, & Falempin, 2003) that could contribute to the abnormal motor-skill performance seen in these animals.

Previous studies have shown that mammalian skeletal muscle fibers display a great adaptive potential in response to altered functional demands, such as changes in neuromuscular activity seen in disuse (Lieber, 1986). These adaptations can alter muscle fiber CSA and the expression of myofibrillar and other protein isoforms resulting in muscle fiber type transitions (Zhang, Chen and Fan, 2007; Urso, 2009). Cerebral palsy is described as a disabling condition that is permanent during the life of the affected children (Krägeloh-Mann & Cans, 2009), often associated with spasticity (Ito et al., 1996) and muscle contractures (O'Dwye et al., 1989). It is reasonable to assume that muscles of children with CP would become atrophic compared with normal muscles since such children are less mobile than children without disabilities (Rose; McGill, 1998), but there is no agreement as to whether spasticity represents a disuse or an overuse model (Foran et al., 2005). Histological analyses of muscle biopsies from patients with CP showed abnormal muscle fiber size, fiber I predominance and fiber atrophy (Rose et al., 1994; Ito et al., 1996;

Marbini et al., 2002). However, varying patterns of myofiber alterations have been reported in patients with CP and spasticity depending on the patient's clinical status and the muscle assessed (Sjöström et al., 1980; Scelsi et al., 1984; Rose et al., 1994).

In this study, several musculoskeletal changes were detected in rats that underwent SR alone or in combination with LPS and/or PA. SR induced alterations to structure, trophism and fiber type composition in the soleus muscle that represent important parameters for determining the functional properties of muscles (Bodine et al., 1982). SR induced a decrease in soleus CSA. Combining LPS, PA and SR treatments apparently enhances the musculoskeletal pathological changes observed in SR alone. The soleus CSA tends to decrease, though not significantly, while the tibialis anterior CSA was only affected by this combination of treatments. Scelsi and col. (1984) reported muscle fiber atrophy following anoxia, however, altered fiber size is more often seen with the persistent decrease of activity imposed by disuse (Huckstorf et al., 2000; Ohira et al., 2001; Coq et al., 2007; Marcuzzo et al., 2008). In developing animals, the mechanical activity imposed on the muscle fiber could play an important role in the maturation of innervation (Greensmith et al., 1998), thus, disruption of motor activity during the developmental period seems to produce modification to the neuromuscular system. Immobilization for different periods of times promotes atrophy ranging from 15 to 70%, depending on the type of animal and the evaluated muscle fibers (Qin et al., 1997). Additionally, Kannus and col. (1998) showed a reduction in the fiber area of the soleus immobilized for 3 weeks. In accordance with our findings, using the same SR protocol, Coq and col. (2008) and Marcuzzo and col, (2008) found different atrophy levels in hind limb muscles.

This study also confirmed, for soleus and tibialis muscles, the classical slow-to-fast phenotypic transition caused by disuse in all restricted groups. Previous studies

investigating muscle pathology in cerebral palsy demonstrated type I fiber predominance and type I and type II fiber atrophy as an almost constant finding (Rose et al., 1994; Ito et al., 1996). Type I fiber predominance may come about as a consequence of continuous muscle activation due to spasticity since this type of fiber transition occured in animals subjected prolonged high frequency stimulation (Sreter et al., 1982). However, there is no agreement that spasticity represents an increased or decreased use model (Foram et al., 2005) and reduced proportions of type I fibers were observed in spastic muscles (Olsson et al., 2006).

In the present study, we also found an increase in sarcomere length and a decrease in sarcomere density in all the SR groups when compared to control. These findings are in accordance with previous reports showing that following immobilization, in a shortened position, the muscle loses sarcomeres, thereby shortening its fiber length to suit its new functional length. Consequently, in order to obtain an optimum overlap of myosin and actin filaments and hence enable the muscle to generate maximum tension in the immobilized position, there is an increase in the length of the remaining sarcomeres (Tabary et al., 1972; Williams; Goldspink, 1978). According to O'Dwye (1989), the development of muscle contracture in cerebral palsy can best be understood from this perspective: spasticity resists stretching and tends to maintain the muscle in a shortened position. Thus, this change in muscle arrangement caused by SR, which simulates the decreased excursion induced by spasticity, may have implications for the muscle contracture found in CP patients.

Our goal was to design an animal model that could mimic the CP-like characteristics found in humans. The present study highlights the importance of SR, which mimics the immobility induced by the spasticity, on the morphological changes to the soleus and tibialis anterior muscles and in the lack of motor ability. The immobilization led

to both morphological and functional abnormalities, associated or not with the other treatments. In addition, our findings suggest that the combination of LPS, PA and SR may play a role in the observed motor skills impairments. When combined, they induced the worst motor function impairments and greatest histological changes in the soleus and tibialis anterior muscles. The mechanisms by which these procedures contribute to these pathophysiologic aspects remain unclear and therefore further studies are required.

Although, there is evidence that the SR procedure is crucial for, and most definitely succeeded in, inducing the motor behavior and muscular aspects of cerebral palsy in rodents, LPS and PA, in some way, contributed to these data showing that this combination might lead not only to muscular alterations but also to the brain damage found in CP. Additional experiments are necessary to corroborate this hypothesis.

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Legends

Fig. 1. Time line of the experimental procedures. The pregnant rats (GD17) were injected with LPS or saline with 12-hour interval until the end of gestation. After delivery (P0) pups were submitted to anoxia (100% nitrogen at 9 L/min for 20 min). The sensorimotor restriction procedures was performed daily (16 h / day) from P2 to P28. Motor skill assessments were performed on P29 and P45.

Fig. 2. Performance of control and experimental rats in the Rotarod test. Differences in mean motor performance between all groups were determined at P29. All experimental rats showed a decrease in their latency to fall when compared to control animals. Three-way ANOVA revealed significant effects of the factor LPS ($F(1,49) = 6.9486$; $P=0.01$), restriction ($F(1,49) = 53.7686$; $P<0.001$) and LPS X restriction interaction ($F(1,49) = 4.5714$; $P=0.03$).

a Significantly different from CT, $P < 0.05$.

b Significantly different from CT, $P < 0.001$.

c Significantly different from all SR groups, $P < 0.05$.

Fig. 3. Motor skill in control and experimental rats attained with the horizontal ladder (A, B) and the narrow suspended bar (C, D) tests. At P29 (A, C) and P45 (B, D) all rats submitted to SR had significant impairments compared to non restricted rats. Regarding the horizontal ladder test on P29, three-way ANOVA revealed significant effects of the factor LPS ($F(1,49) = 16.3653$; $P=0.01$), SR ($F(1,49) = 164.0935$; $P<0.0001$) and LPS X SR interaction ($F(1,49) = 13.0475$; $P<0.001$).

Regarding the narrow suspended bar test on P45, three-way ANOVA revealed significant effects of the factor SR ($F(1,49) = 88.3364$; $P<0.001$) and LPS X PA interaction ($F(1,49) = 10.4659$; $P=0.002$).

Regarding the narrow suspended bar test on P29, three-way ANOVA revealed significant effects of the factor LPS ($F(1,49) = 23.1943$; $P=0.01$), SR ($F(1,49) = 141.2905$; $P<0.001$), LPS X PA interaction ($F(1,49) = 5.4960$; $P=0.02$), LPS X SR interaction ($F(1,49) = 12.0260$; $P=0.001$) and LPS X PA X SR interaction ($F(1,49) = 4.6855$; $P=0.03$).

Regarding the narrow suspended bar test on P45, three-way ANOVA revealed significant effects of the factor SR ($F(1,49) = 76.4873$; $P<0.001$) and PA X SR interaction ($F(1,49) = 4.4817$; $P=0.03$).

a Significantly different from CT, $P < 0.05$.

b Significantly different from CT, $P < 0.001$.

c Significantly different from LPS+PA+SR, $P < 0.05$.

d Significantly different from LPS+PA+SR, $P < 0.001$.

Fig. 4. Digitalized images showing histological, histochemical and morphometric analysis. Left panel (A, C, E, G, I, K) corresponds to CT rats. Right panel (B ,D, F, H, J, L) corresponds to LPS+PA+SR rats. (A, B, C, D) Digitalized images of CT and LPS+PA+SR fiber segment of a soleus muscle at rest under a 400 x magnification showing the cross-striation pattern used to calculate the sarcomere number inside the ROI and then estimate its length and density. (E, F, G, H) Digitalized images of ATP-ase histochemical analyses of soleus (E, F) and tibialis anterior (G, H) muscles used to count the number of positively (black) and negatively (white) stained fibers, respectively classified as type II and type I. (I,

J, K, L) Digitized images of transverse-semithin sections (1 μm) obtained from soleus (I, J) and tibialis anterior (K, L) muscles used to estimate the CSA.

Table 1. Cross-Sectional Area (CSA), Fiber Type Distribution, Sarcomere Length, and Sarcomere Density of Soleus and Tibialis Anterior Muscles of all experimental groups

	Soleus muscle					Tibialis anterior muscle				
Group	CSA (μm^2, M±SD)	Fiber-type distribution (%)		Sarcomere		CSA (μm^2, M±SD)	Fiber-type distribution (%)		Sarcomere	
		Type I	Type II	Length (µm)	Density (n^o/mm)		Type I	Type II	Length (µm)	Density (n^o/mm)
CT	998.5 ± 71.6	79.1	20.9	2.32	4.37	978.0 ± 79.0	3.7	96.3	2.22	4,54
LPS	1178.6 ± 37.6	75.9	24.1	2.42	4.18	974.8 ± 53.1	3.0	97.0	2.24	4,46
PA	1097.4 ± 38.0	77.9	22.1	2.55	3.95	952.5 ± 60.2	2.9	97.1	2.26	4,45
LPS+PA	1154.5 ± 89.9	73.8	26.2	2.52	3.98	720.8 ± 48.9	1.8[a]	98.2[a]	2.32	4,34
SR	741.6 ± 59.7[a]	61.5[b]	38.5[b]	2.81[a]	3.30[a]	1192.2 ± 153.5	1.1[b]	98.9[b]	2.21	4,64
LPS+SR	720.0 ± 70.9[a]	65.1[b]	34.9[b]	2.73[a]	3.67[a]	1014.5 ± 104.1	1.2[b]	98.8[b]	2.37	4,24
PA+SR	677.8 ± 37.6[a]	62.9[b]	37.1[b]	2.82[a]	3.56[a]	938.1 ± 97.2	1.4[b]	98.5[b]	2.42	4,17
LPS+PA+SR	549.8 ± 97.8[b]	60.4[b]	39.6[b]	2.84[a]	3.53[a]	635.7 ± 41.4[a]	0.3[b]	99.7[b]	2.33	4,30

Three-way ANOVA revealed significant effect of the factor SR (F (1.32) = 84.811; P<0.001) and LPS x SR interaction (F (1.32) = 4.193; P<0.05) on soleus CSA; LPS (F (1.32) = 9.0691; P<0.01) and PA (F (1.32) = 12.9130; P=0.001) on tibialis anterior CSA; SR (F (1.32) = 65.166; P<.001) on soleus fiber type distribution; LPS (F (1.32) = 5.5213; P<0.05), PA (F (1.32) = 4.4999; P<0.05) and SR (F (1.32) = 36.4521; P<0.05) on tibialis anterior fiber type distribution; SR (F (1.32) = 15.632; P<0.001) on sarcomere length and SR (F (1.32) = 12.679; P=0.001) on sarcomere density of soleus muscle.

a Significantly different from CT, $P < 0.05$.

b Significantly different from CT, $P < 0.001$.

Figures

Figure 1.

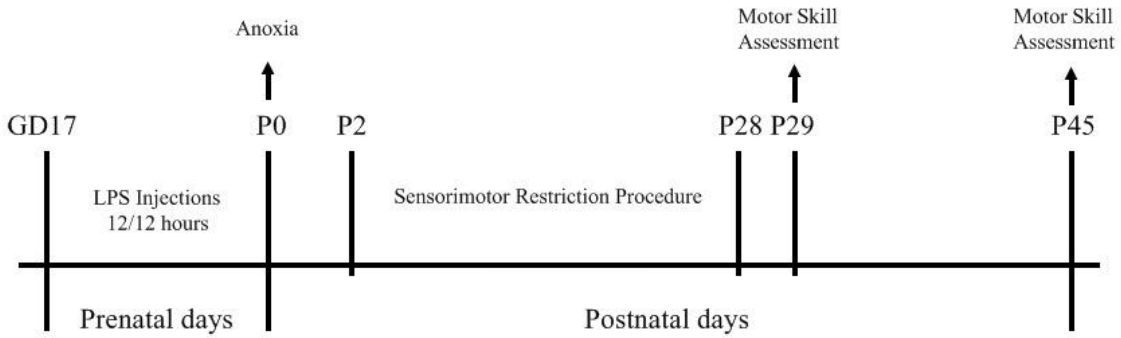


Figure 2.

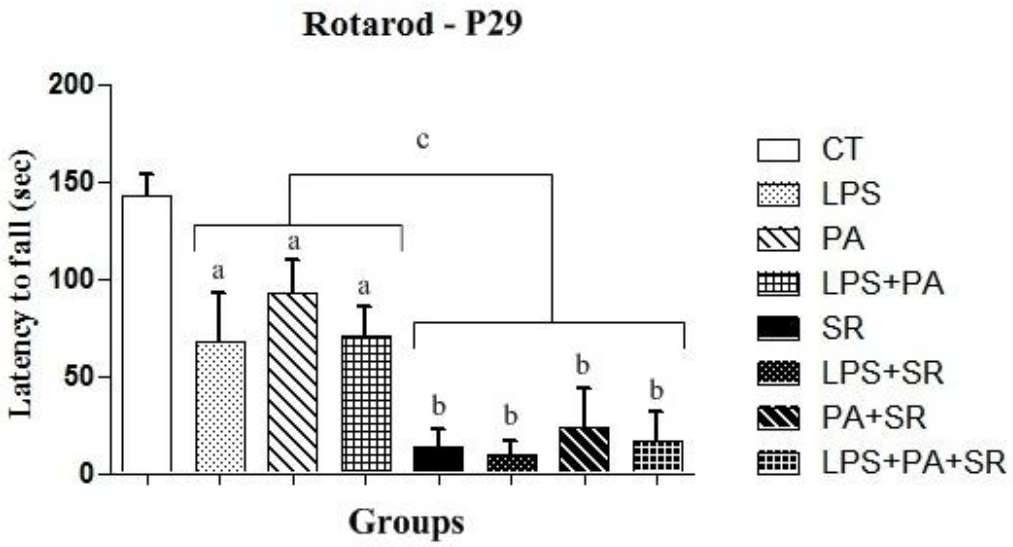

Rotarod - P29
Latency to fall (sec)
200
150
100
50
0
c
a
a
a
b
b
b
b
Groups
CT
LPS
PA
LPS+PA
SR
LPS+SR
PA+SR
LPS+PA+SR

Figure 3.

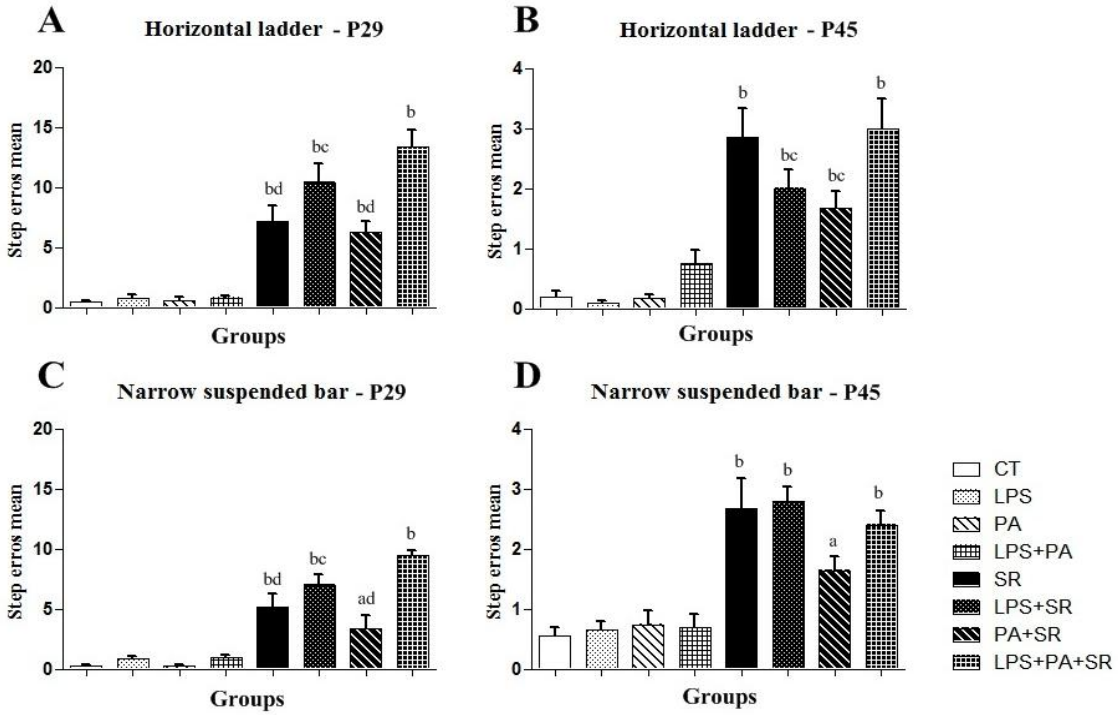
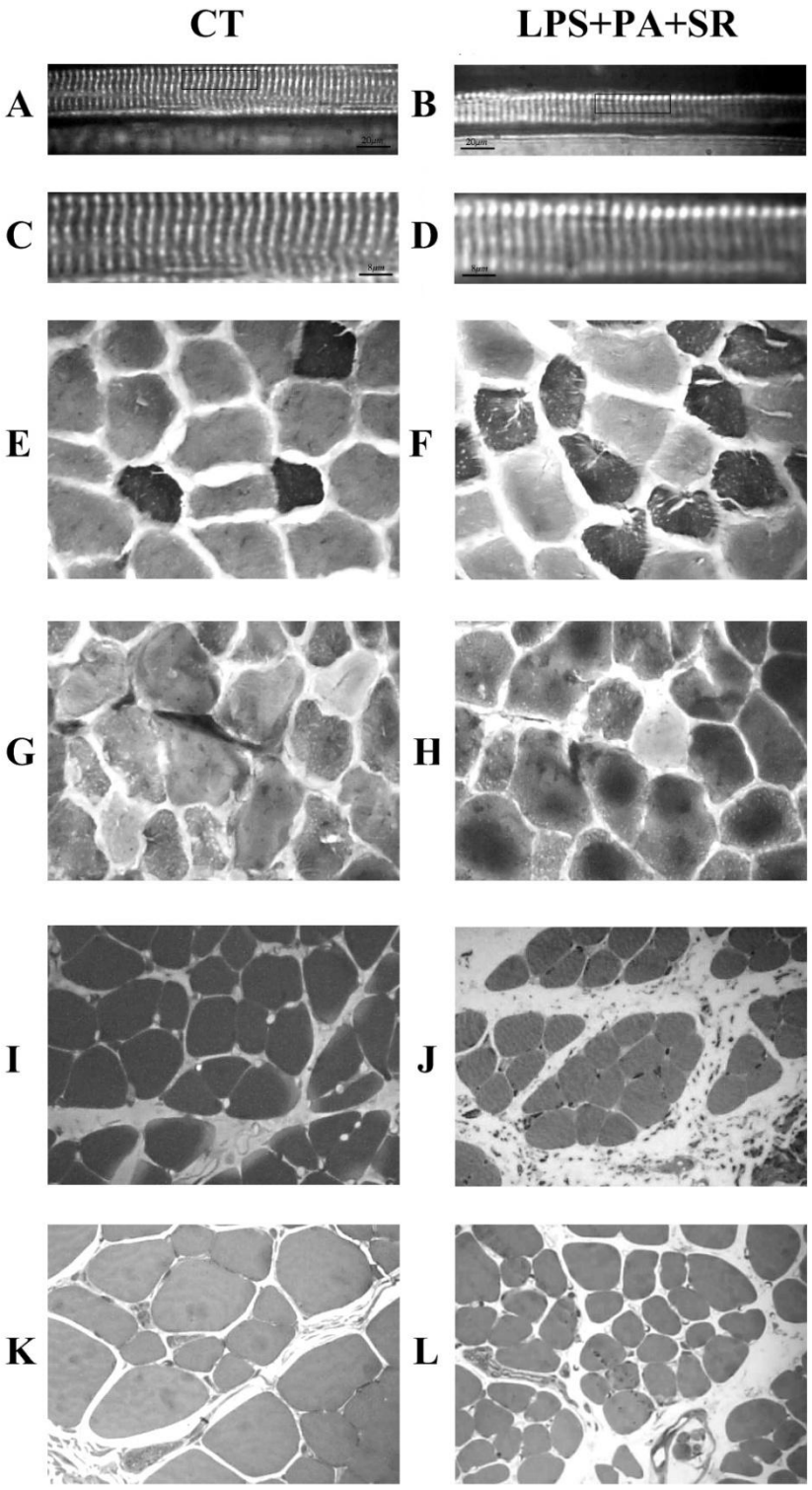

A
Horizontal ladder - P29
Step erros mean
Groups
bd
bc
bd
b
B
Horizontal ladder - P45
Step erros mean
Groups
b
bc
bc
b
C
Narrow suspended bar - P29
Step erros mean
Groups
bd
bc
ad
b
D
Narrow suspended bar - P45
Step erros mean
Groups
b
b
a
b
CT
LPS
PA
LPS+PA
SR
LPS+SR
PA+SR
LPS+PA+SR

Figure 4.



5. CONCLUSÕES E PERSPECTIVAS

O presente estudo mostrou a importância da experiência motora normal durante o desenvolvimento do sistema locomotor em ratos. Concluímos que a SR, simulando a imobilidade relacionada à espasticidade, é um fator importante em mimetizar as características motoras e musculares de crianças com paralisia cerebral. A imobilização levou a alterações morfológicas e funcionais associada ou não aos outros tratamentos.

Nossos dados sugerem também que a combinação do LPS, PA e SR pode estar envolvida com os prejuízos motores observados. A combinação dos três insultos – LPS, anóxia e restrição sensório-motora – foi o tratamento mais agressivo, resultando nos piores prejuízos motores. Já em relação à histomorfologia, podemos perceber evidências de que a restrição é crucial em reproduzir as alterações musculares encontradas. A imobilização levou a um fenômeno de transição de fibras no sentido lento para rápido tanto no sóleo como no tibial anterior. No músculo sóleo essa transição foi associado a uma diminuição na área das fibras e ao aumento no comprimento com diminuição do número de sarcômeros. Já no tibial anterior, a área das fibras somente foi afetada pela combinação dos três tratamentos não havendo alterações na densidade nem no comprimento dos sarcomeros.

Embora podemos perceber o protagonismo da SR na indução deste modelo, o LPS e a PA, contribuíram, de alguma forma, tanto para as prejuízos motores como para as alterações musculares observadas. Os mecanismos pelo qual o LPS e a PA e sua associação possam contribuir para a atrofia muscular ainda não estão bem estabelecidos. Coq e colaboradores (2007) mostraram alterações variadas na trofismo muscular dependendo da musculatura avaliada, e não existem estudos mostrando a alterações musculares após submeter ratos ao LPS. Finalmente, as perspectivas desse estudo estão relacionadas em analisar regiões encefálicas ligadas à motricidade, como o córtex

sensório-motor, o estriado e o cerebelo, além da porção lombar da medula espinhal assim tentando esclarecer os mecanismos responsáveis pelos distúrbios motores encontrados.

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