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INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO NEUROCIÊNCIAS

INFLAMAÇÃO PRÉ-NATAL, ASFIXIA PERINATAL E RESTRIÇÃO SENSÓRIO-MOTORA: IMPLICAÇÕES PARA UM MODELO DE PARALISIA CEREBRAL EM RATOS

Tese de Doutorado

Felipe de Souza Stigger

Porto Alegre

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You see things; and you say "Why?" But I dream things that never were; and I say "Why not?"

George Bernard Shaw

RESUMO

O objetivo desta tese foi avaliar o efeito de distintos eventos agressivos, pertinentes ao contexto fisiopatológico da Paralisia Cerebral, na gênese de um modelo animal capaz de reproduzir as alterações anatômicas, bioquímicas e funcionais a fim de que se obtenha um fenótipo comportamental e neuropatológico mais semelhante à doença. Para isso, foram realizados três experimentos. No primeiro, foram examinados os efeitos de baixas doses da endotoxina bacteriana (lipopolissacarídeo, LPS), associada ou não com a anóxia perinatal (AP), nos parâmetros inflamatórios e de estresse oxidativo no córtex cerebral de filhotes de ratos Wistar machos recém nascidos. Foram avaliadas os níveis de TNF-α, IL-1, IL-4, SOD, CAT e DCF. Outros animais foram submetidos à avaliação dos marcos do desenvolvimento do dia 1º ao 21º dia de vida (P1 - P21). O comportamento motor também foi avaliado no P29 utilizando-se o campo aberto e o Rotarod. O LPS e a AP sozinhos obtiveram impactos diferentes na atividade de SOD e nos níveis de IL-1, TNF-α e radicais livres acompanhados com leve impacto no desenvolvimento e desempenho motor. Quando o LPS e a AP foram combinados, as mudanças nos parâmetros inflamatórios e de estresse oxidativo foram maiores. Adicionalmente, na combinação dos eventos, maiores limitações no desenvolvimento motor e na coordenação foram observadas. No segundo e terceiro experimentos, investigamos parâmetros de plasticidade morfológica na medula espinal de ratos submetidos à restrição sensório-motora (RS), associada ou não à estimulação locomotora. Ratos Wistar machos foram expostos à RS do P2 ao P28. Ratos controle e experimentais foram submetidos ao treinamento de estimulação locomotora em esteira com duração de três semanas (do P31 até P52). Foram determinadas a área de secção transversal (AST) de motoneurônios ao nível da lombar (L5), assim como de fibras nervosas mielinizadas do nervo isquiático (segundo experimento). Também, foram avaliadas a imunorreatividade à sinaptofisina e à caspase-3 no corno ventral da medula espinal (L5; terceiro experimento). Após a RS, a área média do soma dos motoneurônios foi reduzida, acompanhada pela redução da AST média das fibras nervosas do nervo isquiático.

Adicionalmente, houve redução na imunorreatividade à sinaptofisina, associada a um aumento na expressão de caspase-3. Essas alterações foram revertidas, alcançando níveis do grupo controle, quando os animais submetidos à RS foram expostos à estimulação locomotora. Nossos resultados sugerem que, em ratos, infecções/inflamação, asfixia perinatal e a inatividade no início da vida pós-natal podem criar substratos para reproduzir um fenótipo clínico relevante e similar aos observados na PC.

ABSTRACT

The aim of this thesis was to evaluate the effect of different aggressive events, relevant to pathophysiological context involved in Cerebral Palsy (CP), in the genesis of an animal model able to reproduce the anatomical, biochemical and functional alterations in order to obtain a behavioral and neuropathological phenotype more similar to CP. For this, we made three experiments. In the first one, the effects of maternal exposure to low doses of bacterial endotoxin (lipopolysaccharide, LPS) associated or not with perinatal anoxia (PA) on oxidative stress and inflammatory parameters which were examined in cerebral cortices of newborns Wistar male pups. Concentrations of TNF-α, IL-1, IL-4, SOD, CAT and DCF were measured. Other newborn rats were assessed for neonatal developmental milestones from day 1 to 21 (P1 – P21). Motor behavior was also tested at P29 using open-field and Rotarod. LPS and PA alone had different impacts on SOD activity and IL-1, TNF-α and free radicals levels accompanied with slight impact on development and motor performance. When LPS and PA were combined, changes in inflammatory and oxidative stress parameters were greater. In addition, greater motor development and coordination impairments were observed. In the second and third experiments, we investigated morphologic parameters of spinal cord plasticity in rats that undergone through sensorimotor restriction (SR), associated or not to locomotor stimulation. Male Wistar rats were exposed to SR from P2 to P28. Control and experimental rats underwent locomotor stimulation training in a treadmill for three weeks (from P31 to P52). The cross-sectional area (CSA) of spinal motoneurons at lumbar level (L5) as well of myelinated fibers from ischiatic were determined. Also, the intensity of the synaptophysin and caspase-3 immunoreaction was assessed within ventral horn of spinal cord (L5; third experiment). After SR, the mean motoneuron soma size was reduced accompanied by a reduction in the mean fiber and axon CSA of ischiatic nerve. In addition, there was a synaptophysin immunoreactivity reduction accompanied by an increased caspase-3 immunoreactivity. Those alterations were reversed and reached the control levels when animals submitted to SR were exposed to locomotor stimulation. Our results suggest that, in rodents, infections/inflammation, perinatal anoxia and inactivity during early postnatal life could play an important role by creating substrates for the pathological behavior thus, contributing to reproduce clinically relevant phenotype similar to those observed in CP.

SUMÁRIO

ABREVIATURAS

AP	Asfixia perinatal
HI	Hipóxia-isquemia
L-1β	Interleucina 1-beta
LPS	Lipopolissacarídeo
LPV	Leucomalácia periventricular
PC	Paralisia cerebral
RS	Restrição sensório-motora
SNC	Sistema nervoso central
TLR	Receptores *toll-like* (do inglês *toll-like receptors*)
TNF-α	Fator de necrose tumoral-alfa (do inglês *tumor necrosis factor-α*)

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1.INTRODUÇÃO

1.1. Paralisia Cerebral

Paralisia Cerebral (PC) é considerada a causa mais comum de deficiência na função motora na infância, abrangendo uma ampla condição clínica relacionada a alterações neurológicas que afetam o movimento (HIMMELMANN et al., 2005; ZARRINKALAM et al., 2010). A definição clássica da PC foi descrita, pela primeira vez, em 1964 como um "distúrbio permanente, embora não imutável, do movimento e da postura, devido a defeito ou lesão não progressiva do encéfalo no começo da vida" (BAX, 1964; LEITE & PRADO, 2004). Essa definição inicial foi modificada em 1992 para atender a heterogeneidade das desordens associadas ao termo PC. Assim sua definição passou a ser: "um termo guarda chuva que envolve um grupo de síndromes de limitação motora, não progressivas, porém, frequentemente mutáveis decorrentes de lesões ou anormalidades no encéfalo em estágios iniciais do desenvolvimento" (MUTCHet al 1992). Em 2006, a definição foi revisada e atualmente, o termo mais amplo e aceitável descreve um "grupo de desordens no desenvolvimento do movimento e da postura, causando limitação da atividade, que são atribuídos a distúrbios não progressivos que ocorrem no encéfalo em desenvolvimento" (ROSENBAUM et al., 2007).

Diferentes estudos epidemiológicos realizados em países desenvolvidos têm relatado a incidência de PC em 2,0 e 2,5 por 1.000 nascidos vivos (STANLEY, BLAIR, ALBERMAN, 2000; HAGBERG et al., 2001; PHAROAH, COOKE & ROSEN BLOOM, 1989). Em contrapartida, quando comparados aos índices de países subdesenvolvidos sua incidência é maior, aproximando-se de 7 por cada 1.000 nascidos vivos sendo que no Brasil, os dados estimam cerca de 30.000 a 40.000 novos casos por ano (MANCINI et al., 2002). Atualmente a prevalência parece estar aumentando em

decorrência aos declínios na mortalidade infantil principalmente relacionado aos de recém-nascidos pré-termos (MUTCH et al 1992; CANS, 2000).

A PC é uma condição clínica de etiologia complexa, por vezes múltipla, e, apesar de uma ampla variedade de fatores de risco já ter sido demonstrada (KRAUS & ACHEEN, 1999; WU & COLFORD, 2000; JONES et al., 2007) (Tabela 1), em muitos casos é difícil de ser estabelecida (STANLEY et al., 2000). De fato, mais de 30% dos casos são de etiologia desconhecida (ROSENBAUM, 2003). Os fatores de risco foram estabelecidos em decorrência do período do desenvolvimento encefálico em que a lesão tem origem. O dano pode ocorrer, portanto, no período pré-natal (durante o período de gestação até a iniciação do parto), perinatal (desde a iniciação do parto até o nascimento propriamente dito) ou pós-natal (a partir do nascimento até aproximadamente 2 anos de idade) (CANS, 2000; KOMAN, SMITH & SHILT, 2004). A grande maioria dos casos, 70 a 80%, são desenvolvidos no período pré-natal (KUBAN &, LEVITON, 1994), sendo que complicações como a asfixia, infecções ou traumas envolvem aproximadamente 10 a 20% dos fatores de risco neste período (JOHNSTON & HOOM, 2006). Ainda entre os fatores de risco pré-natais estão incluídos também gestações múltiplas e condições maternas, tais como retardo mental e hipertireoidismo (SANKAR & MUNDKUR, 2005). A idade gestacional é um dos fatores perinatais que aumenta dramaticamente o risco de desenvolvimento da PC e, de fato, a prematuridade representa atualmente 25% de todos os casos de PC (HAGBERG & HAGBERG, 1984; HAGBERG et al., 2001; ANCEL et al., 2006). Além disso, 4-12% das crianças com baixo peso ao nascimento têm maior probabilidade de ter prejuízos neurológicos e desenvolver PC (DERRICK et al., 2004). As infecções, os acidentes cerebrovasculares e os traumatismos são causas comuns que ocorrem após o nascimento (REID, LANIGAN

& REDDIHOUGH, 2006) compreendendo entre 10 a 20% dos casos de PC (KRIGGER, 2006).

Tabela 1. Fatores de risco associados à Paralisia Cerebral

Pré-natal	Perinatal	Pós-natal
▪ Hipóxia	▪ Asfixia	▪ Asfixia
▪ Desordens genéticas	▪ Prematuridade	▪ Crise convulsiva
▪ Desordens metabólicas	▪ Incompatibilidade sanguínea	▪ Infarto cerebral
▪ Gestações múltiplas	▪ Infecções	▪ Hiperbilirrubinemia
▪ Infecções uterinas	▪ Parto instrumental	▪ Meningite
▪ Exposição a agentes teratogênicos	▪ Descolamento placentário	▪ Trauma
▪ Corioamnionite		▪ Hemorragia intraventricular
▪ Febre Maternal		
▪ Exposição a toxinas		
▪ Malformação congênita		
▪ Insultos vasculares		
▪ Trauma abdominal		
▪ Restrição de crescimento intra-uterino		

Adaptado de Jones et al., 2007

Ao longo dos anos, os substratos neuropatológicos habitualmente encontrados em lactentes prematuros foram a leucomalácia periventricular (LPV) e o infarto hemorrágico periventricular (associadoà hemorragia na matriz germinal, com ou semhemorragia intraventricular) (KADHIM, 2005; VOLPE, 2008). Tendo em vista que a incidência de infarto hemorrágico periventricular diminuiu ao longo da última década, a LPV assumiu o papel de destaque como lesão patológica encefálica mais prevalente identificada na PC (FOLKERTH, 2005). Esse tipo de lesão é resultante da vulnerabilidade dos oligodendrócitos imaturos antes da 32ª semana de gestação (JOHNSTON & HOON, 2006). Estudos epidemiológicos têm demonstrado uma

associação entre infecções, como a corioamnionite, com a LPV, sugerindo que a resposta fetal inflamatória desencadeada pelo aumento de citocinas pró-inflamatórias, contribuiria para a lesão na substância branca (WU & COLFORD, 2000). Já as lesões encontradas em substância cinzenta englobam lesão no córtex, nos núcleos da base, no tálamo e no cerebelo (FOLKERTH,2005). Essas lesões, em contraste à LPV e às hemorragias periventriculares, são mais encontradas em nascidos a termo (VOLPE, 2008).

A lesão no encéfalo em desenvolvimento originada por diferentes causas acaba por afetar o desenvolvimento motor, e, apesar do dano encefálico ser estático e não progressivo, o desempenho motor piora com o tempo, acarretando déficits significativamente maiores no sistema motor (GRAHAM & SELBER, 2003). As características clínicas usualmente encontradas na PC são: tônus muscular anormal, persistência de reflexos e problemas relacionados à manutenção da postura e execução de movimentos (DAMIANO, 2006). Enquanto crianças normais dispõem de um diversificado repertório de movimentos espontâneos, crianças com PC têm movimentos escassos e com padrões estereotipados, com ausência na variação de padrão, de complexidade e de fluência do movimento (HADDERS-ALGRA, 2004; EINSPIELER &PRECHTL, 2005).

O diagnóstico da PC é basicamente realizado a partir da observação clínica do paciente, sendo que o atraso do desenvolvimento motor, alterações no tônus muscular e nos reflexos profundos são uns dos principais achados em crianças com PC (DODGE et al., 2008). Apesar da PC ser caracterizada principalmente pela disfunção motora, contudo, o quadro pode ser acompanhado de outras desordens, como afecções sensoriais, cognitivas, comunicativas, perceptivas e/ou comportamentais, assim como epilepsia (KENT, 2013).

Tendo em vista a ampla variabilidade das manifestações clínicas envolvidas na PC, torna-se difícil a sua classificação (STANLEY et al 2000, GRAHAM & SELBER 2003). Com esse intuito, no início da década de 50, foi desenvolvida uma série de modelos de classificações para PC (PAKULA, VAN NAARDEN BRAUN, & YEARGIN-ALLSOPP, 2009). As diretrizes de classificação foram estabelecidas de acordo com as diferentes desordens do movimento, ou seja, nos comportamentos posturais relacionados ao tônus e às características dos gestos motores em associação com sua distribuição topográfica (BOBATH & BOBATH, 1989, COLVER & SETHUMADHAVEN, 2003). A área do encéfalo afetada está diretamente relacionada com as limitações resultantes (JONES et al, 2007). Desta forma, de acordo com os aspectos fisiológicos, a PC pode ser dividida em dois principais grupos: piramidal e extrapiramidal (PAKULA, VAN NAARDEN BRAUN, & YEARGIN-ALLSOPP, 2009). A PC piramidal é resultante de lesão nas vias córtico-espinhais, também descrita como lesão dos neurônios motores superiores, enquanto a PC extrapiramidal é decorrente principalmente de lesões envolvendo o cerebelo ou os núcleos da base (JONES et al., 2007).

Outra forma de classificação refere-se às desordens do movimento associadas à PC, como a espasticidade, as discinesias e as ataxias. A espasticidade, referida como um desequilíbrio sensório-motor caracterizado pelo aumento da resposta muscular ao alongamento passivo com caráter velocidade-dependente e hiperreflexia (LANCE, 1990), é encontrada em aproximadamente 76% dos casos, sendo as formas discinéticas e hipotônicas menos comuns (STANLEY et al., 2000; GRAHAM & SELBER, 2003; COLVER & SETHUMADHAVEN, 2003). Ainda podemos classificar a PC de acordo com a distribuição topográfica com base no padrão de envolvimento dos membros, assim como a sua distribuição pelo corpo. Dentre os termos mais utilizados em relação à

distribuição topográfica encontram-se a hemiplegia (comprometimento de um hemicorpo), diplegia (afetando membros inferiores) e quadriplegia (comprometimento envolvendo tanto membros superiores como inferiores) (DELGADO & ALBRIGHT 2003; SINNO, CHARAFEDDINE & MIKATI, 2013 – Figura 1). Já em relação à severidade dos comprometimentos da PC, sua classificação utiliza termos como: leve, moderado e severo (OEFFINGER et al, 2004) e normalmente está associada às limitações dasatividades e à presença de comorbidades (BAX et al., 2005).

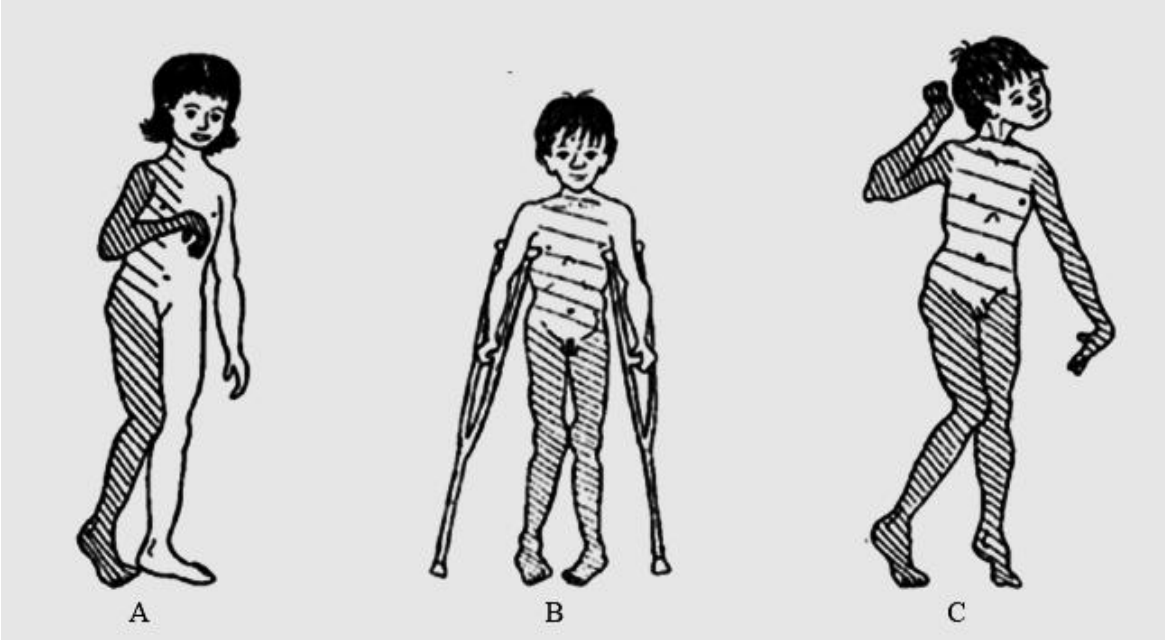

Figura 1. Classificação topográfica da Paralisia Cerebral. A. Hemiplegia à direita; B. Diplegia; C. Quadriplegia. (Adaptado de Sinno, Charafeddine & Mikati, 2013)

1.2. Alterações Secundárias no Sistema Nervoso Central

Como descrito anteriormente, diferentes eventos patológicos no período de desenvolvimento do sistema nervoso central (SNC) podem causar danos encefálicos. Esses danos podem alterar a transmissão adequada do trato córtico-espinal e, consequentemente, causar prejuízos relacionados à habilidade motora e aos movimentos espontâneos (JONES et al., 2007). Embora em humanos as vias córtico-espinhais

alcancem a medula cervical aproximadamente na 17º semana de gestação, e, a lombar na 29º semana (HUMPHREY, 1960), aparentemente, o padrão adulto das projeções sinápticas aos motoneurônios medulares não é presente até o nascimento, ocorrendo durante a primeira semana pós-natal em paralelo com o aparecimento de movimentos independentes e supressão dos reflexos primitivos (LAWRENCE & HOPKINS, 1976; PALMER et al, 1983). Neste período, a maturação do sistema sensório motor é caracterizada pelo estabelecimento dos mapas corticais topográficos, o surgimento de conexões de longo alcance e acentuada plasticidade (KILLACKEY, RHOADES& BENNETTCLARKE, 1995; LOPEZ-BENDITO, G. & MOLNAR, 2003; JAIN et al., 2003). Estes processos são dependentes da atividade, assim, a experiência motora e os movimentos espontâneos são essencialmente necessários para o desenvolvimento motor normal (KHAZIPOV et al., 2004). Se uma lesão ocorrer durante este período, o padrão de conectividade destas vias, assim como seus *inputs* pré-sinápticos, podem se tornar alterados, levando ao desenvolvimento de comportamentos anormais (BROUWER&ASHBY, 1991). De fato, a presença de anormalidades na experiência sensório motora, evidenciada em crianças com PC, (PRECHTL, 1997; HADDERS-ALGRA, 2004; EINSPIELER & PRECHTL, 2005; HADDERS-ALGRA, et al., 2010) demonstrou impactos profundos na reorganização cortical, tanto em humanos (CLAYTON, FLEMING & COPLEY, 2003; WINGERT et al., 2008; ANDIMAN et al., 2010) como em animais submetidos à restrição de movimentos (STRATA et al., 2004; COQ, et al., 2008) e, consequentemente limitações funcionais foram observadas.

Tendo em vista que tanto estímulos descendentes, provenientes do córtex motor, como os ascendentes, oriundos de receptores periféricos, possuem como alvo a mesma região da medula espinal (GIBSON, ARNOTT & CLOWRY, 2000), as alterações funcionais na PC podem não ser exclusivamente devido à disfunção cerebral, mas,

também em decorrência de anormalidades na circuitaria medular (DE LOUWet al., 2002). Durante o período do desenvolvimento, essas duas entradas de informação na medula espinal interagem, a partir de um mecanismo dependente de atividade, com o objetivo de produzir padrões maduros de inervação e comportamentos motores (GIBSON, ARNOTT & CLOWRY, 2000). Dessa forma, associada à perda de controle motor descendente, os movimentos escassos e anormais, oriundos da inatividade, podem resultar em impulsos anômalos à medula espinal contribuindo para o aumento da eliminação sináptica e o desenvolvimento de reflexos aberrantes (CLOWRY, 2007). Adicionalmente, modelos experimentais de desuso de membros têm demonstrado o retardo na maturação (PASTOR et al., 2003) e a redução no número de motoneurônios medulares (GREENSMITH & VRBOVÁ, 1992).

1.3. Fisioterapia na Paralisia Cerebral

Diferentes estudos mostram que crianças com PC possuem diminuição na capacidade aeróbica (KUSANO et al., 2001), na força e na resistência muscular (DAMIANO, KELLY, & VAUGHN, 1995; PARKERet al., 1992), que, consequentemente, limitam sua atividade motora. Como citado anteriormente, apesar do dano encefálico ser estático, as disfunções motoras são progressivas e pioram com o crescimento da criança, resultando em alterações no desempenho como a dificuldade de deambulação. De fato, crianças com PC apresentam diminuição na velocidade da marcha, no comprimento dos passos e na cadência (EAGLETON et al., 2004). Neste contexto a fisioterapia possui um importante papel na PC pelo treinamento específico de atos motores como: levantar-se, dar passos ou caminhar, além de exercícios destinados a aumentar a força e resistência muscular e melhorar o controle sobre os movimentos, objetivando a funcionalidade (Leite & Prado, 2004). Um dos recursos utilizados pela

fisioterapia em crianças com PC é o treino orientado à tarefa onde são focadas habilidades motoras específicas como, por exemplo, a marcha (Salem & Godwin, 2009). Dessa forma, a estimulação locomotora em esteira, por estimular o sistema de controle da locomoção praticando comportamentos específicos relacionados à marcha, pode desempenhar um papel importante tanto na prevenção como na diminuição dos prejuízos secundários observados (DAMIANO, 2006; FOWLER et al., 2007). Adicionalmente, levando em consideração a relação entre o padrão locomotor e a execução de atividades de vida diária, desenvolver a deambulação é um importante objetivo para crianças com PC (LEPAGE, NOREAU & BERNARD, 1998). Com esse intuito, nos últimos anos a estimulação locomotora em esteira ergométrica ganhou popularidade entre os métodos de intervenção na PC (FRANKI et al., 2012) (Figura 2). A prática da locomoção com auxílio de esteira ergométrica tem se mostrado eficiente no tratamento da PC auxiliando na melhora da postura, do equilíbrio, da força e a adequação do tônus muscular (JENG et al., 2013; VERSCHUREN et al., 2013), melhorando consequentemente a função motora de crianças com diagnóstico de PC e a concomitante limitação da marcha (BRYANT et al., 2013). Mudanças espaço-temporais também foram observadas em crianças com PC em decorrência da estimulação locomotora. Dois estudos relataram o aumento significativo na velocidade da marcha em crianças entre 6 e 14 anos, com limitações leves e severas (PROVOST et al., 2007; DODD & FOLEY, 2007). Ainda, associado ao aumento na velocidade na marcha, também foram demonstradas melhoras no comprimento da passada (PALISANO et al., 1997) e cadência (JOHNSTONet al., 2011) de crianças com PC.

Figura 2. Fotografia do treino de marcha em esteira em crianças com PC utilizando suporte de peso corporal (Adaptado de JOHNSTON et al., 2011)

O padrão de atividade desenvolvido no treinamento em esteira necessário para facilitar a reorganização e recuperação do SN, assim como os mecanismos envolvidos neste processo, ainda não foi devidamente esclarecido. No entanto, a ativação de geradores de padrão centrais, existentes na medula espinal parece ser um dos mecanismos sugeridos (SCHINDLet al., 2000).

1.4. Modelos Animais de Paralisia Cerebral

Muito dos conhecimentos atuais em relação à patofisiologia envolvida nos distúrbios neurológicos pré, peri e pós natais são decorrentes de modelos animais. Como citado anteriormente, a PC possui uma etiologia diversa e multifatorial sendo atribuída adiferentes fatores de risco pré-natais, perinatais e/ou pós-natais (NELSON,

2003; KADHIM et al., 2005). Dessa forma, diferentes modelos animais, incluindo primatas e espécies não primatas como coelhos e roedores, foram delineados com o intuito de estudar o efeito de diferentes fatores etiológicos no desenvolvimento de distúrbios neurológicos.

Embora alguns estudos epidemiológicos indiquem que a asfixia em nascidos a termo não seja a causa mais comum da PC, ela é um dos mecanismos bastante utilizados em modelos animais envolvendo essa condição clínica. A asfixia perinatal (AP) tem sido reproduzida em macacos (RANCK & WINDLE, 1959), porcos (HERPINet al., 1996), ovelhas (MALLARDet al., 2003) e coelhos (LAWSON& THACH, 1977). Nesses modelos, a asfixia produz lesões e alterações funcionais similares ao padrão produzido pela asfixia intraparto em neonatos a termo (BARKOVICH et al., 1995; MALLER et al., 1998). Porém, em ratos, a AP induz somente alterações motoras sutis e transitórias, limitadando-se em reproduzir uma das principais características da PC: a inatividade crônica (HOEGER et al., 2000; LUBICS et al., 2005;ROBINSON et al., 2005).

Outro tipo de mecanismo de lesão, a hipóxia-isquemia (HI) encefálica, é considerado um importante fator desencadeante da PC (BERGER&GARNIER,1999; LEE et al., 2005). Em decorrência disto, modelos de HI têm sido bastante utilizados no intuito de causar lesões no encéfalo imaturo e assim se estudar suas repercussões (JANSEN & LOW, 1996; HOEGER et al., 2000; ZHURAVIN, DUBROVSKAYA &TUMANOVA, 2004; LUBICS et al., 2005; ROBINSON et al., 2005). Atualmente, o modelo mais utilizado para o estudo da HI neonatal em roedores é o introduzido pelo grupo de Vannucci há mais de 20 anos (HAGBERG, PEEBLES &MALLARD, 2002). Este modelo consiste na associação de uma isquemia unilateral, realizada pela oclusão da artéria carótida comum de um dos lados, seguida por um evento hipóxico sistêmico a

partir da inalação de uma mistura gasosa contendo apenas 8% de oxigênio por um determinado período de tempo (VANNUCCI & VANNUCCI, 2005). Embora neste modelo sejam encontradas alterações metabólicas e no fluxo sanguíneo ocasionando danos em córtex, estriado, hipocampo e tálamo, similares aos observados na lesão cerebral perinatal humana, o dano é geralmente limitado a um hemisfério cerebral, ipsilateral à oclusão, e as alterações motoras resultantes, apesar de mais severas, continuam sendo transitórias, não sendo observadas na idade adulta (JANSEN & LOW, 1996; HOEGER et al., 2000; ZHURAVIN, DUBROVSKAYA &TUMANOVA, 2004; LUBICS et al., 2005; ROBINSON et al., 2005).

Grande parte dos estudos envolvendo AP ou HI não leva em consideração a possível contribuição das infecções intrauterinas ou maternais que também aumentam o risco de desenvolvimento de PC (JOHNSTON et al. 2005). No entanto, diferentes estudos vêm demonstrando uma associação entre PC e infecções intrauterinas durante o período gestacional (MURPHY et al., 1995;NELSON et al., 1998; COLFORD, 2000), sendo estas, responsáveis pelo aumento na incidência de lesões encefálicas como a LPV (YOON, PARK&CHAIWORAPONGSA, 2003). Os mecanismos específicos pelos quais as infecções poderiam levar à lesão encefálica foram esclarecidos a partir da descoberta de que o lipopolissacarídeo (LPS), constituinte da membrana externa das bactérias gram-negativas, é capaz de ativar o sistema imunológico por meio de sua interação com receptores *toll-like* (TLR). O LPS facilita a ativação do TLR-4,em astrócitos e microglia que, por sua vez, inicia uma cascata de eventos intracelulares resultando na produção de citocinas inflamatórias, tais como a interleucina 1-beta (L-1β) e o fator de necrose tumoral-alfa (TNF-α) (ADEREM &ULEVITCH, 2000). Além da acentuada produção de agentes inflamatórios, que, por si só são lesivos ao encéfalo (GILLES, AVERILL & KERR, 1977; YOUNG, YAGEL & TOWFIGHI, 1983), o LPS

pode induzir hipoperfusão, hipoglicemia e acidose láctica, além de estimular a produção de radicais livres, que também podem possuir papéis distintos e importantes no dano encefálico (YOUNG, HERNANDEZ& YAGEL, 1982; YOUNG, YAGEL & TOWFIGHI, 1983, GILLES, AVERILL & KERR, 1977, BLASIG et al., 2001).

Diferentes modelos animais usando o LPS como agente patogênico distinguem-se quanto à janela temporal na qual o animal é submetido à endotoxina, variando entre os períodos intra-uterino e pós-natal (TOSO et al., 2005; ROUSSET et al., 2006; ROBERSON et al., 2006). Estudos demonstraram que o LPS, quando administrado tanto no período pré-natal, como em um período pós-natal precoce, causa uma redução nos marcadores para oligodendrócitos e mielina, assim como danos em substância branca (CAI et al., 2000; PAINTLIA et al., 2004; POGGI et al., 2005; ROBERSON et al., 2006). Porém, a administração de LPS não é capaz de produzir lesões encefálicas macroscópicas, nem déficits motores similares aqueles observados no contexto humano (POGGI et al. 2005; ROBERSON et al., 2006). Um fato importante abordado em estudos recentes é relacionado à possível sensibilização do SNC em resposta ao LPS. A exposição prévia ao LPS parece aumentar dramaticamente a vulnerabilidade do encéfalo a um subsequente evento agressivo (EKLIND et al., 2001; ROUSSET et al., 2008; GIRARD et al., 2008). De fato, Eklind et al (2001) demonstrou que o LPS, quando administrado 4 h antes de um episódio de HI, é capaz de sensibilizar o encéfalo à lesão, causando danos maiores do que os induzidos pelo LPS isolado. Neste experimento, ratos com 7 dias de vida submetidos à exposição ao LPS, seguidos por 20 minutos de HI, apresentaram lesões mais extensas, quando comparados aos animais nos quais somente foi administrado salina. Adicionalmente, Girard et al., (2008) demonstraram que a indução de uma resposta inflamatória, pela exposição de ratas prenhas à injeção intraperitonial de LPS, no período embrionário, combinada à HI, realizada 24 h após o

parto (Figura 3), foi capaz de causar lesões corticais e subcorticais extensas e, consequentemente, ocasionar alterações no comportamento motor dos animais. Estes estudos sugerem a existência de uma potencial interação entre as infecções sistêmicas e a asfixia perinatal na patogênese da PC (GRETHER & NELSON, 1997; NELSON & GRETHER, 1998). Porém, apesar da HI, associada ou não ao LPS, causar lesões em diferentes regiões encefálicas, nenhum desses modelos é capaz de reproduzir os principais aspectos observados em indivíduos com PC, como o atraso na aquisição de marcos do desenvolvimento motor, as alterações específicas da marcha e a espasticidade.

Tendo em vista a ausência de déficits motores característicos da PC nesses modelos em ratos foi desenvolvido um modelo de PC a partir da associação de dois episódios de anóxia seguidos por 26 dias de restrição sensório-motora (RS; Figura 4) dos membros posteriores (STRATA et al., 2004). Este modelo é uma estratégia para mimetizar a diminuição da atividade motora no período crítico do desenvolvimento motor vivenciada pelas crianças com PC. De fato, a escassez de movimentos e/ou movimentos repetitivos anormais, acarretam em informações errôneas ao córtex motor e sensorial, e foram capazes de produzir uma reorganização cortical em ambos os córtices (STRATA, et al., 2004; COQ et al., 2008). O desuso prolongado também contribuiu para induzir alterações patológicas nos tecidos musculares e articulares (COQ et al., 2008; MARCUZZO et al., 2008). A combinação das alterações corticais e musculares foi associada à redução do crescimento corporal, ao aumento do tônus muscular dos membros posteriores, ao atraso na aquisição de algumas habilidades motoras, à degradação da função motora e ao aparecimento de um padrão de marcha patológico que se assemelham ao fenótipo encontrado em crianças com PC (STRATA et al., 2004; MARCUZZO et al., 2008; MARCUZZO et al., 2010). Esses estudos, diferentemente

dos modelos animais abordados anteriormente, demonstraram a importância da inatividade precoce dos membros posteriores, combinada ou não à anóxia perinatal, em produzir déficits motores duradouros em ratos.

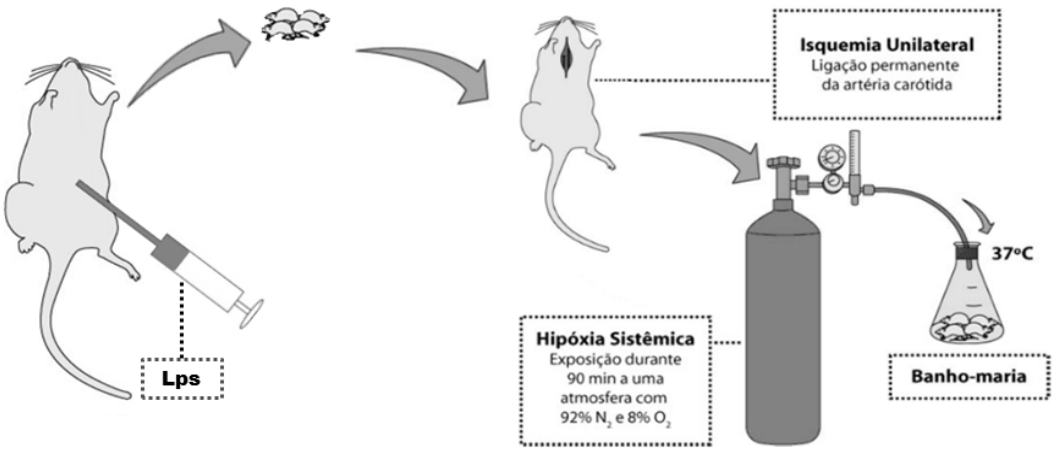

Figura 3. Modelo animal descrito por Girard et al., (2008). Exposição ao LPS (injeções i.p. de 200 µg/kg de LPS realizadas de 12 em 12 h do 17º de gestação até o dia do nascimento). Ratos recém-nascidos foram submetidos ao procedimento combinação de hipóxia e isquemia.

No entanto, apesar das consideráveis alterações motoras, este modelo não foi capaz de mostrar as alterações encefálicas características da PC (MARCUZZO et al., 2010). Com isso, associado ao modelo sugerido por Strata et al., (2004) e, tendo em vista a possível sensibilização do encéfalo à inflamação pré-natal (GIRARD et al., 2008), o LPS no período gestacional foi associado à AP e RS (STIGGER et al., 2011a). Nesse estudo, a combinação dos três insultos resultou em pior quadro motor associado a alterações musculares mais evidentes, como a transição no padrão de tipo de fibras musculares e diminuição na área de secção transversa das fibras musculares, assim como no aumento do comprimento dos sarcômeros. Embora este estudo tenha demonstrado que a RS é crucial na indução de um modelo com fenótipo similar ao de

PC em humanos, o LPS e a PA, isoladamente foram capazes de, alguma forma, contribuir para os prejuízos motores, demonstrados pela diminuição do tempo de permanência no Rotarod. Ainda, animais expostos à combinação de ambos os procedimentos apresentaram alterações musculares, mostrando uma maior porcentagem de fibras do tipo II, similares à combinação dos três agressores. Estes resultados indicam que cada agressor possui um papel na criação de substratos neuropatológicos envolvidos na gênese da PC, sugerindo a importância da combinação de insultos para o desenvolvimento de um modelo animal que mimetize o quadro motor da PC.

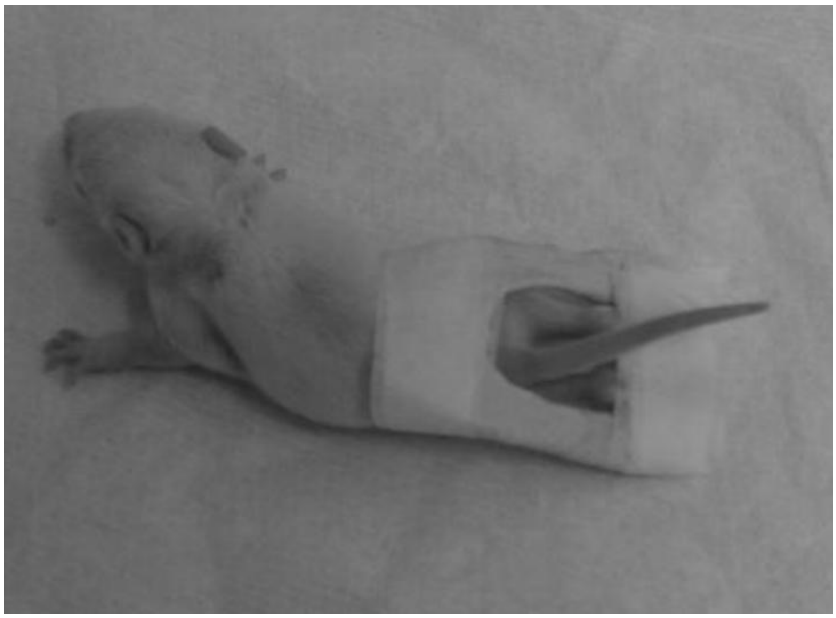

Figura 4. Procedimento de restrição sensório motora (RS) realizado do P2 ao P28 por 16 h por dia. Os membros posteriores são contidos juntos com uma fita adesiva e colocados em uma posição estendida mantida com uma moldura feita de um material moldável. Esse procedimento é bem tolerado pelos filhotes não prejudicando a eliminação de urina e fezes, nem os cuidados maternos (Adaptado de COQ et al., 2011).

2.JUSTIFICATIVA

A PC é considerada a causa mais comum de deficiência motora na infância sendo uma condição clínica com características motoras altamente debilitantes. Os modelos animais de PC mais comumente estudados não conseguem mimetizar este fenótipo motor. Foi desenvolvido em nosso laboratório um modelo animal, a partir da associação da exposição fetal à LPS, anóxia perinatal e restrição sensório-motora, que se aproxima mais aos achados motores envolvidos nessa condição clínica (STIGGER et al., 2011a). Nesse estudo foi demonstrada a importância da experiência motora normal no desenvolvimento do sistema locomotor em ratos, concluindo que a RS é uma estratégia importante para mimetizar as características motoras e musculares de crianças com PC. Embora tenha sido possível perceber o protagonismo da RS na indução de um modelo com fenótipo motor similar ao observado em crianças com PC, o LPS e a AP, foram capazes de contribuírem para os prejuízos motores e para as alterações musculares observadas.

Claramente, cada procedimento influenciou diferentes e complementares aspectos presentes na patogênese da PC, porém, os mecanismos pelos quais cada um deles contribuiu para tais achados não foram estudados. O entendimento dos mecanismos patológicos envolvidos nessa enfermidade pode auxiliar no desenvolvimento de estratégias terapêuticas mais eficazes. Com este intuito, baseado em hipóteses envolvendo modificações no perfil inflamatório e oxidativo, na primeira parte desta tese foi estudado o envolvimento de citocinas inflamatórias e a produção de radicais livres envolvidas na anóxia perinatal, ou no processo inflamatório pré-natal, assim como no contexto de um insulto duplo combinando ambos LPS e PA. Os resultados desta primeira hipótese serão apresentados na forma de artigo (Capítulo 1).

Na segunda etapa deste estudo, considerando possíveis alterações medulares e do sistema nervoso periférico em decorrência do desuso, foram investigados os aspectos

morfológicos, tanto dos motoneurônios e dos nervos que inervam os músculos dos membros posteriores de ratos após o protocolo de RS. Ainda, como parte do esforço em entender o papel do desuso precoce no desenvolvimento deste quadro motor, na terceira etapa desta tese objetivou-se analisar se a limitação da atividade, induzida pela RS, afetaria a expressão de marcadores de plasticidade sináptica e morte celular no corno ventral da medula espinal lombar. Nessas duas últimas etapas ainda testou-se a hipótese de que a estimulação locomotora em esteira ergométrica, aplicada aos animais previamente submetidos à RS, poderia auxiliar na modulação da plasticidade neuronal, associada a benefícios funcionais. Os resultados da segunda e terceira etapa também serão apresentados em formato de artigos (Capítulo 2 e 3, respectivamente).

3.OBJETIVOS

3.1. Objetivo Geral

O objetivo geral desta tese foi avaliar o efeito de distintos eventos agressivos, pertinentes ao contexto fisiopatológico envolvidos na paralisia cerebral, na gênese de um modelo animal em ratos capaz de reproduzir as alterações anatômicas, bioquímicas e funcionais dessa condição clínica: (1) infecção maternal - administração pré-natal de lipopolissacarídeo; (2) anóxia perinatal e (3) inatividade – restrição sensório-motora.

3.2. Objetivos Específicos

3.2.1. Analisar o desenvolvimento motor, as capacidades motoras e a expressão de marcadores inflamatórios - conteúdo de Interleucina 1 (IL-1), Interleucina 4 (IL-4) e Fator de Necrose Tumoral Alfa (TNF-α) e de estresse oxidativo - conteúdo de 2', 7' diclorofluoresceína (DCF) e atividade das enzimas antioxidantes - catalase (CAT), superóxido dismutase (SOD) no córtex de filhotes expostos à combinação da administração pré-natal de lipopolissacarídeo e anóxia perinatal.

3.2.2. Estudar parâmetrosmorfológicos na medula espinal lombar e no nervo ciático em filhotes submetidos à restrição sensório-motora e à estimulação locomotora.

3.2.3. Avaliar o desenvolvimento motor e a expressão de Sinaptofisina e de Caspase-3 no corno ventral da medula espinal de filhotes submetidos à restrição sensório-motora e à estimulação locomotora.

4.RESULTADOS

Os resultados desta tese serão apresentados na forma de artigos que relatam os experimentos relacionados aos objetivos específicos descritos anteriormente.

4.1. **CAPÍTULO 1** (Objetivo 3.2.1)

"Inflammatory response and oxidative stress in developing rat brain and its consequences on motor behavior following maternal administration of LPS and perinatal anoxia" - Felipe Stigger, Gisele Lovatel, Marília Marques, Karine Bertoldi, Felipe Moysés, Viviane Elsner, Ionara Rodrigues Siqueira, Matilde Achaval, Simone Marcuzzo.

Artigo aceito para publicação na revista "International Journal of Developmental Neuroscience" no dia 5 de outubro de 2013.

4.2. **CAPÍTULO 2** (Objetivo 3.2.2)

"Treadmill Training Induces Plasticity in Spinal Motoneurons and Sciatic Nerve After Sensorimotor Restriction During Early Postnatal Period: New Insights into the Clinical Approach for Children with Cerebral Palsy" - Felipe Stigger, Patrícia S. do Nascimento, Márcio F. Dutra, Gabriela K.Couto, Jocemar Ilha, Matilde Achaval, Simone Marcuzzo.

Artigo publicado em dezembro de 2011 na revista "International Journal of Developmental Neuroscience", Volume 29, Páginas 833–838.

4.3. **CAPÍTULO 3** (Objetivo 3.2.3)

"Expression of Synaptophysin and Caspase-3 on Lumbar Segments of Spinal Cord After Sensorimotor Restriction During Early Postnatal Period and Treadmill Training" - Felipe Stigger, Silvia Barbosa, Marília Marquesa, Ethiane Segabinazi, Bruno Santos Campos Gomes, Otávio Américo Augustin, Matilde Achavala, Simone Marcuzzo.

Artigo a ser submetido.

CAPÍTULO 1

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Inflammatory response and oxidative stress in developing rat brain and its consequences on motor behavior following maternal administration of LPS and perinatal anoxia

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ABSTRACT

Cerebral palsy (CP) is a disorder of locomotion, posture and movement that can be caused by prenatal, perinatal or postnatal insults during brain development. An increased incidence of CP has been correlated to perinatal asphyxia and maternal infections during gestation. The effects of maternal exposure to low doses of bacterial endotoxin (lipopolysaccharide, LPS) associated or not with perinatal anoxia (PA) in oxidative and inflammatory parameters were examined in cerebral cortices of newborns pups. Concentrations of TNF-α, IL-1, IL-4, SOD, CAT and DCF were measured by the ELISA method. Other newborn rats were assessed for neonatal developmental milestones from day 1 to 21. Motor behavior was also tested at P29 using open-field and Rotarod. PA alone only increased IL-1 expression in cerebral cortex with no changes in oxidative measures. PA also induced a slight impact on development and motor performance. LPS alone was not able to delay motor development but resulted in changes in motor activity and coordination with increased levels of IL-1 and TNF-α expression associated with a high production of free radicals and elevated SOD activity. When LPS and PA were combined, changes on inflammatory and oxidative stress parameters were greater. In addition, greater motor development and coordination impairments were observed. Prenatal exposure of pups to LPS appeared to sensitize the developing brain to effects of a subsequent anoxia insult resulting in an increased expression of pro-inflammatory cytokines and increased free radical levels in the cerebral cortex. These outcomes suggest that oxidative and inflammatory parameters in the cerebral cortex are implicated in motor deficits following maternal infection and perinatal anoxia by acting in a synergistic manner during a critical period of development of the nervous system.

1. Introduction

Human newborns, particularly preterm, are at high risk of brain injury (Brochu et al., 2011). Considering the variety of encephalopathy that severely limits motor function in young children, cerebral palsy (CP) is the most prevalent (O'Shea, 2008). The CP is caused by non-progressive brain damage that arises early in life, and is characterized by chronic disorders of movement or posture. This condition is frequently accompanied by seizure disorders, sensory impairment, and cognitive limitation (Nelson, 2003). The CP etiology is complex and many causal pathways have been suggested to operate antenatally and to interact with intrapartal and postnatal factors (Eklind et al., 2005). Much of the current CP epidemiological research has focused on two potential mechanisms of brain damage: one mechanism involves insufficient cerebral perfusion; the other, cytokine-mediated damage, potentially triggered by events such as maternal infections (O'Shea, 2002).

In fact, studies with CP patients have shown increase in inflammatory cytokine and oxidative imbalance in the first 48 h of life in neonates who have suffered perinatal hypoxic–ischemic brain injury, correlated with deficits neurodevelopment subsequent,

Abbreviations: CP, cerebral palsy; LPS, lipopolysaccharide; PA, perinatal anoxia; IL-1β, interleukin-1beta; TNF-α, tumor necrosis factor-alpha; H/I, hypoxic–ischemia; G17, gestational day 17; P0, day of birth; CT, control group; IL-4, interleukin 4; PMSF, phenylmethylsulfonyl fluoride; DCFH-DA, 2′-7′-dichlorofluorescein diacetate; SOD, superoxide dismutase; CAT, Catalase; P1, postnatal day 1; P15, postnatal day 15; P21, postnatal day 21.

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assessed at 12 months (Vasiljevic et al., 2011). Interestingly, it was also found increase in inflammatory responses in preterm children with CP at 7.2 mean age (Lin et al., 2010).

Maternal infections also seem to promote an inflammatory reaction involving oxygen free radicals and the synthesis of proinflammatory cytokines such as interleukin-1beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) (Kopp and Medzhitov, 1999). Experimentally, administration of LPS, a cell wall component from Gram negative bacteria, is a well-characterized and widely accepted model of bacterial infection, and pre-exposure to the LPS potentiates hypoxic–ischemic (H/I) brain injury in newborn animals triggered by unilateral common carotid artery ligation followed by systemic hypoxia by inhalation of 8% oxygen-balance nitrogen (Girard et al., 2009; Cai et al., 2000).

However, a critical issue in animal models of perinatal brain injury is to adapt the pertinent pathophysiological scenarios in order to induce neuropathological and behavioral characteristics suggestive to perinatal CP (Girard et al., 2009). Across laboratories, studies vary regarding the type, dose and timing of immunogen administration during gestation, species used, postnatal age examined and specific outcome measure quantified. However, with regard to mechanisms, evidence for roles for several acute mediators of effects of prenatal immune activation have emerged, including circulating interleukin-6 and oxidative stress in the fetal brain (Boksa, 2010).

A previous study showed that exposure to LPS during the prenatal period (intraperitoneally, twice a day, from gestational day 17 until the end of the gestation) and perinatal anoxia (PA; on the day of birth, during 20 min in a 100% N2 chamber), alone or in combination, caused various degrees of consequences to motor behavior (Stigger et al., 2011). Given several evidences linking cytokines and oxidative stress to infection and anoxia, we suggest that cytokines could influence neurodevelopment by acting in a period of the rat's gestation that is roughly correspondent to the late second trimester in human CNS development. Also, at this period, fetus is particularly vulnerable to squeals such as periventricular leucomalacia (Berger and Garnier, 1999). As understanding the mechanisms of perinatal brain injury is essential to the design of effective prevention and neuroprotective interventions, this present study was designed to evaluate the inflammatory responses and oxidative stress on total cerebral cortices after a LPS and PA protocol used by our lab (Stigger et al., 2011). Our main goal was to understand how these procedures operate alone and in combination, and related this with subsequent neurodevelopment, evaluating the acquisition of development milestones, skill and motricity.

2. Materials and methods

2.1. Gestational LPS and perinatal anoxia: experimental design

All procedures were approved by the Ethical Committee at the Federal University of Rio Grande do Sul (no. 2008189) and animal 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). Animals were bred under conventional conditions, housed in plexiglass boxes at 20 ± 1 °C, and allowed free access to food and water; the light/dark cycle was set at 12 h according to the Brazilian law that regulates animal use for didactic-scientific practice. For gestational LPS treatment, timed pregnant Wistar rats were divided into 2 main groups: Group 1 (n = 10), LPS injected rats (200 μg/kg diluted in 100 μL of sterile saline; from *Escherichia coli*, 0127:B8; Sigma, USA) and Group 2 (n = 10), vehicle injected rats (100 μL of sterile saline). The pregnant rats received intraperitoneal injections of either LPS or saline every 12 h starting from gestational day 17 (G17) until the end of the gestation. On the day of birth (P0), half of the pups born from both the LPS-treated mothers and saline treated mothers were subjected to anoxia. For this procedure, rat pups were placed in a temperature controlled chamber (37 ± 1 °C) with a flow of 9 L/min of 100% N_2 (White Martins, Brazil) for 20 min (Stigger et al., 2011). The number of pups/litter was culled to eight by removing primarily the females (or males if necessary). After weaning (P21), the females were removed from the boxes and discarded from the study. In total, 54 male pups were used in this study. Pups were randomly assigned to four different groups: (1) rats receiving saline injection during the embryonic period (CT, n = 13); (2) LPS injection (LPS, n = 14); (3) saline injection and anoxia (PA, n = 14); (4) LPS injection and anoxia (LPS + PA, n = 13).

2.2. Preparation of samples

At P0, 30 min after the anoxia procedure, neonatal pups were decapitated, brain was removed, cortices were dissected out on ice, immediately frozen in liquid nitrogen and stored at −80 °C. Cerebral cortex, which has been previously shown to be altered after PA or LPS procedure (Eklind et al., 2001; Strata et al., 2004; Coq et al., 2008; Girard et al., 2009; Marcuzzo et al., 2010), was chosen due its relation to motor behavior. According to Schieber and Fuglevand (2006) several areas of the cerebral cortex are directly involved in deciding which movements to make and in executing the selected movements (posterior parietal, dorsolateral prefrontal, secondary motor, and primary motor cortex). The samples were used to evaluate inflammatory and oxidative parameters (n = 5–6).

2.2.1. Inflammatory parameters

The levels of TNF-α, IL-1β, IL-4 were measured by the ELISA Assay kits (colorimetric detection, catalog number 88-7340; 88-6010 eBioscience; 555198, BD OptEIA EUA, respectively) according to the manufacturer's instructions. Briefly, 96 well plates were coated with specific monoclonal antibody. The plates were then blocked for non-specific binding using assay diluents. The cortices were homogenized with specific kit lyses buffer. Lysates were centrifuges and the supernatant and standards curve were incubated with the capture antibody followed by detection antibody. The plates were incubated with detection enzyme avidin-HRP and the absorbance was measured on a microplate reader (450 nm). Protein concentration of each sample was measured by Lowry method (Peterson, 1977). The cytokines levels were expressed as pg/mL.

2.2.2. Oxidative parameters

For the analysis of oxidative parameters, brain tissue was homogenized in ice-cold phosphate buffer (0.02 M, pH 7.4) containing EDTA (0.002 M) and phenylmethylsulfonyl fluoride (PMSF, 0.1 M) in a Teflon-glass homogenizer. After that the homogenate was centrifuged at 1000 × g for 10 min at 4 °C and the supernatant was used for the assays. To assess the free radicals content we used 2′-7′-dichlorofluorescein diacetate (DCFH-DA) as a probe (Lebel et al., 1990). The supernatant was incubated with DCFH-DA (100 mM) at 37 °C for 30 min. The formation of the oxidized fluorescent derivative (DCF) was monitored at excitation (488 nm) and emission (525 nm) wavelengths using a fluorescence spectrophotometer. All procedures were performed in the dark and blanks containing DCFH-DA (no homogenate) were processed (Driver et al., 2000 and Sriram et al., 1997). The free radicals content was quantified using a DCF standard curve and results were expressed as pmol of DCF formed/mg protein. Superoxide dismutase (SOD) activity was determined using a RANSOD kit (Randox Labs., USA). This method employs xanthine and xanthine oxidase to generate O_2^- that react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to form a red formazan dye which is assayed spectrophotometrically at 505 nm at 37 °C. The inhibition on production of the chromogen is proportional to the activity of SOD present in the sample. The SOD activity was expressed as percentage of control and the control group was considered 100% of activity. Catalase (CAT) activity was measured by the method described by Aebi (1984) with samples incubated in ethanol (10%) and triton (10%). The activity was assayed at 25 °C by determining the rate of degradation of H_2O_2 at 240 nm in 10 mM potassium phosphate buffer (pH 7.0). The CAT activity was expressed as percentage of control and the control group was considered 100% of activity. The protein content of the tissue homogenates was measured by the Comassie blue method using bovine serum albumin as standard (Bradford, 1976).

2.3. Neonatal developmental tests

The neonatal developmental milestones were evaluated daily by a blinded observer, from P1 to P15, always at the same time (10 a.m.). Newborn rats were (n = 6–8) assessed for neonatal developmental milestones as following (based on Poggi et al., 2005): (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) negative geotaxis (pups were placed head down on a 45°-inclined surface, and the positive response consisted of a 180 turn with upward crawling), (3) 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), (4) forelimb grasp (the ability to pups remain suspended for 10 s after grasping thin rod with their forepaws), (5) hind limb 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), and (6) open field activity (time to move off a circle of 13 cm diameter). The behaviors measured all occur at differing stages throughout the first 15 days corresponding to the development throughout the neonatal period. Each developmental test

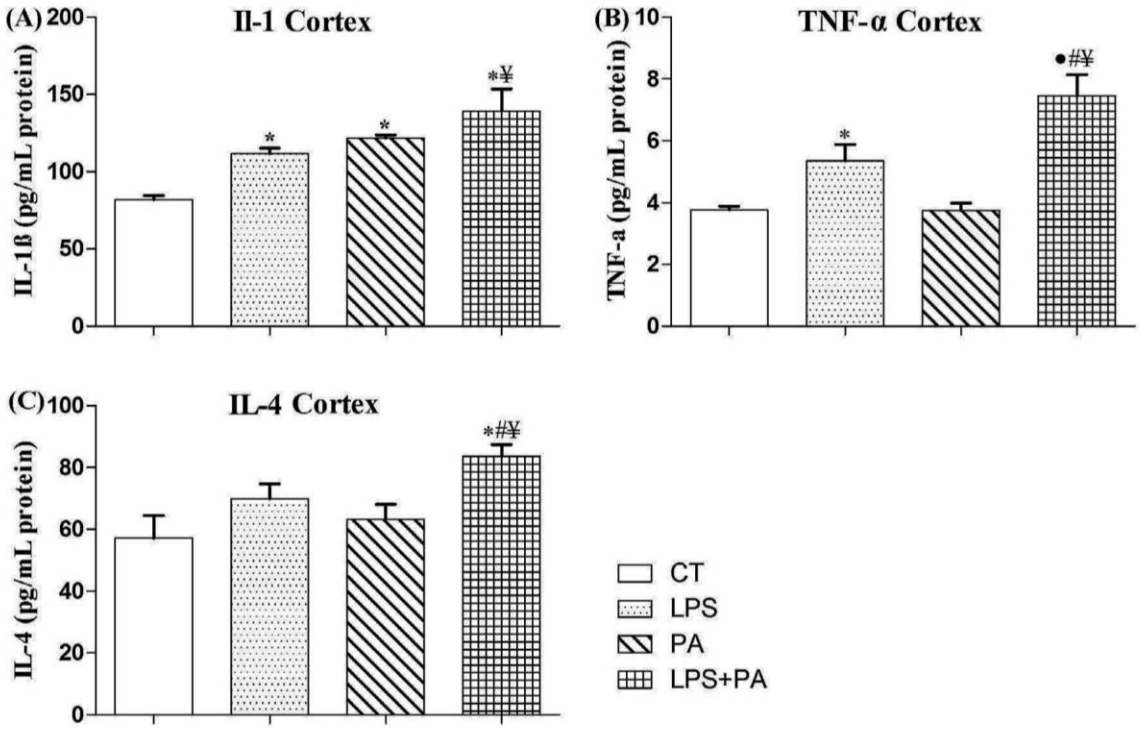

Fig. 1. Interleukin-1 (A), tumor necrosis factor-alpha (B) and interleukin-4 expression in cerebral cortex of control and experimental rats were calculated for each group and plotted. Two-way ANOVA followed by Duncan test. Columns represent means ± SEM. * Different from CT, $P < 0.05$, ● Different from CT, $P < 0.001$, # Different from PA, $P < 0.001$, ¥ Different from LPS, $P < 0.01$

response was considered positive based on its first appearance. All measurements were time-limited to a maximum of 30 s.

2.4. Motor skills evaluation

Offspring ($n = 6$–8) were also tested for motor skills and locomotor activity at P29 using a Rotarod and an open field apparatus. For motor coordination and balance pups were tested on the 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 Rotarod was considered as the latency to fall. A blinded observer tested each animal 5 times with a 2 min interval between each trial. The maximum duration of the test was 3 min.

Spontaneous locomotor activity was assessed using open-field apparatus, consisting in a 60 cm × 30 cm × 40 cm Plexiglas enclosure. Rat pups were always placed facing the same direction, in the upper left corner of the apparatus and pups displacements were all recorded for 5 min with a high quality digital camera placed directly over the arena. The testing began as soon as the animal was placed in the open field and the total distance traveled was registered by a blinded observer.

2.5. Statistical analysis

Neonatal behavior, motor skills evaluations, brain cytokine and oxidative status were analyzed using two-way analysis of variance (ANOVA), with LPS and Anoxia as independent variables. All analyses were followed by post hoc Duncan's test for multiple comparisons, whenever indicated. Data are expressed as means ± SEM. Probability values less than 5% were considered significant. All statistical analysis was performed using the Statistica® software package.

3. Results

3.1. Inflammatory parameters

Two-way ANOVA results examining IL-1 concentrations on cerebral cortex revealed significant effect of the factors LPS ($P < 0.05$) and PA ($P = 0.05$). The post hoc analyses from IL-1 of all experimental groups are shown in Fig. 1A. As observed, the IL-1 expression in cerebral cortex is significantly increased in LPS (111.59 ± 3.53 pg/mL), PA (121.72 ± 1.97 pg/mL) and LPS + PA (139.28 ± 14.19 pg/mL) animals compared to CT (81.93 ± 2.43 pg/mL). It was also observed that the IL-1 expression is higher in LPA + PA when compared to LPS animals.

Considering the TNF-α concentrations in cerebral cortex, two-way ANOVA revealed significant effect of the factors LPS ($P < 0.0001$), PA ($P < 0.05$) and a LPS × PA interaction ($P < 0.05$). The post hoc analyses of all experimental groups are shown in Fig. 1B. As shown in Fig. 1B, there was no difference between CT (3.76 ± 0.12 pg/mL) and PA (3.75 ± 0.24 pg/mL) groups. TNF-α expression in cerebral cortex was increased in rats exposed to LPS (5.35 ± 0.53 pg/mL) and LPS + PA (7.45 ± 0.68 pg/mL) animals). Both LPS and LPS + PA pups were significantly different from CT and PA groups. When prenatal administration LPS was associated with PA TNF-α expression was even higher. LPS + PA group was significantly different from all experimental groups.

The IL4 levels are illustrated in Fig. 1C. Two-way ANOVA showed no differences between PA (63.17 ± 4.95 pg/mL) and LPS (69.95 ± 4.79 pg/mL) animals compared to CT (57.12 ± 7.34 pg/mL). An increased IL-4 expression was observed in LPS + PA group (83.63 ± 3.79 pg/mL) when compared to all experimental groups.

3.2. Oxidative parameters

The free radical levels and SOD and CAT activity are summarized in Fig. 2. Two-way ANOVA revealed that rats exposed to LPS (158.57 ± 6.62 pg/mL) and LPS + PA (152.05 ± 4.71 pg/mL) had higher levels of free radicals compared to CT (100.22 ± 7.94 pg/mL) and PA (100.68 ± 8.2 pg/mL) rats (Fig. 2A). However, there was no significant difference on DCF levels of PA rats when compared to CT rats.

Two-way ANOVA also showed that SOD activity was higher in the LPS and LPS + PA groups than in CT group (about 7% and 11% respectively; Fig. 2B). There were no differences between SOD activity comparing CT and PA groups. In addition, an increase in SOD activity was observed in LPS + PA when compared to PA rats.

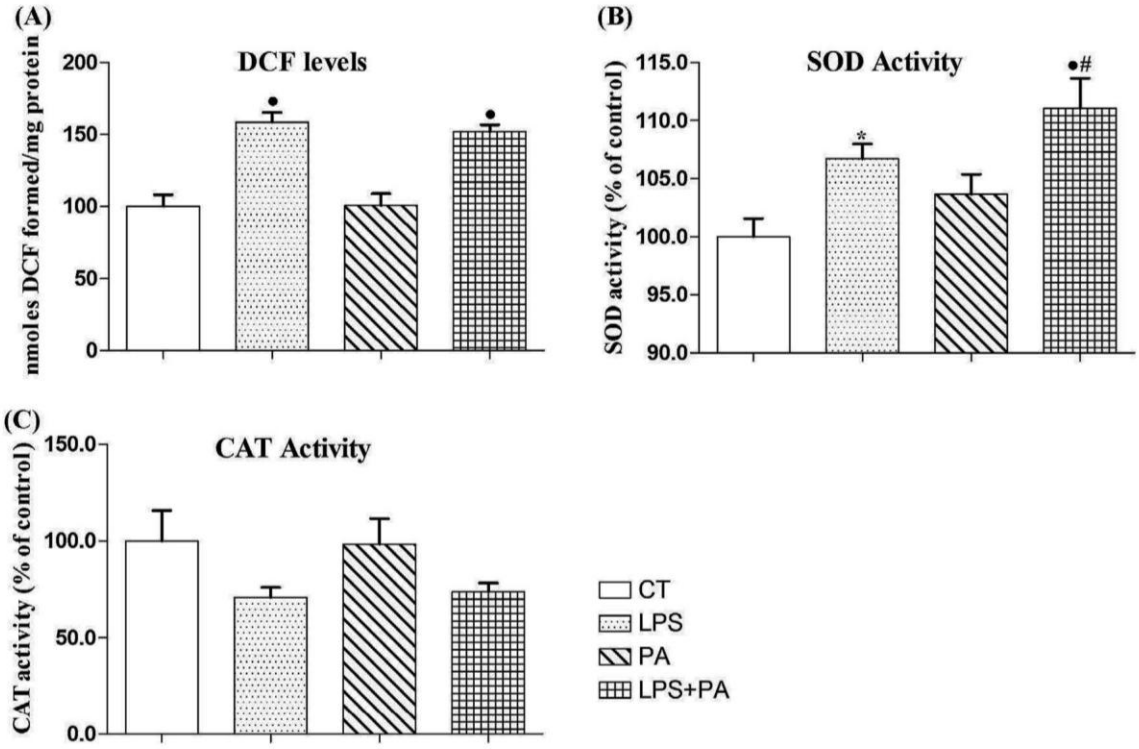

Fig. 2. Levels of free radical evaluated by DCF (A) and the expression of antioxidant enzymes, Superoxide dismutase (B) and Catalase (C) in cerebral cortex of control and experimental rats were calculated for each group and plotted. Two-way ANOVA followed by Duncan test. Columns represent means ± SEM. * Different from CT, $P < 0.05$, ● Different from CT, $P < 0.001$, # Different from PA, $P < 0.001$, ¥ Different from LPS, $P < 0.01$

The CAT activity was not altered in any of the experimental groups compared to CT (Fig. 2C).

3.3. Neonatal developmental tests

As shown in Tables 1 and 2, surprisingly, neonatal LPS rats showed a significantly earlier first day of performance in achieving forelimb grasp ($P < 0.05$) compared to CT. PA-exposed pups performed cliff aversion ($P < 0.01$), and motor activity ($P = 0.01$) latter than CT animals. LPS + PA pups were delayed achieving cliff aversion ($P < 0.01$), hind limb placement ($P < 0.01$) and motor activity ($P < 0.01$). There was a trend toward delaying motor activity in LPS and delaying negative geotaxis LPS + PA groups compared to CT ($P < 0.075$).

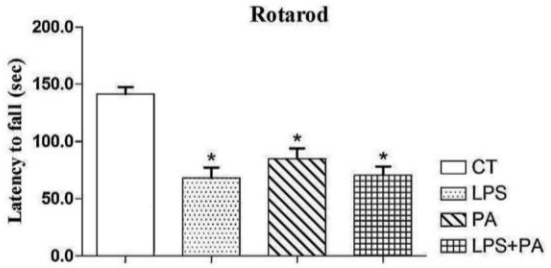

Fig. 3. Rotarod performance in control and experimental rats. Differences in mean motor performance between all groups were determined at P29 and plotted. Columns represent means ± SEM. Two-way ANOVA followed by Duncan test. * Different from CT, $P < 0.01$

3.4. Motor skills evaluation

Two-way ANOVA of Rotarod test revealed only significant effects of the factor LPS ($P = 0.01$). Fig. 3 shows the results of the post hoc analyses. CT rats performed significantly better than any other group. Rats exposed to LPS (68.01 ± 8.71 s), PA (85.14 ± 6.48 s) alone or in combination (LPS + PA, 70.58 ± 5.70 s) showed deficits in balance and coordination tested on the Rotarod when compared to CT (142.72 ± 4.18 s). Two-way ANOVA of analysis of total traveled distance revealed significant effects of the factor LPS ($P = 0.01$). Post hoc results of locomotor activity are shown in Fig. 4. LPS-exposed animals (135.20 ± 22.74 cm) had significantly less spontaneous locomotor activity in the open field in comparison to CT (902.57 ± 68.72 cm), PA (689.0 ± 98.92 cm) and LPS + PA

Table 1
Day of first performance of neonatal developmental sensory-motor behaviors.

Behavior	CT ($n = 7$)	LPS ($n = 8$)	PA ($n = 6$)	LPS + PA ($n = 7$)
Surface righting	0.43 ± 0.20	0.75 ± 0.41	0.66 ± 0.33	1.00 ± 0.30
Cliff aversion†	3.14 ± 0.70	4.50 ± 0.26	6.33 ± 0.55†	5.28 ± 0.56†
Negative geotaxis	6.85 ± 0.45	7.62 ± 0.7	6.83 ± 0.70	7.14 ± 0.70
Hind limb placing†	5.00 ± 0.84	6.25 ± 0.49	6.66 ± 0.91	10.00 ± 0.53†
Forelimb grasp*	5.28 ± 0.71	8.62 ± 0.80*	6.83 ± 0.83	8.42 ± 0.68
Activity†	7.14 ± 0.59	9.50 ± 1.12	11.16 ± 0.90†	11.00 ± 1.38†

Values represented as means ± SEM.
* Mean significantly different from CT, $P < 0.05$.
† Mean significantly different from CT, $P \leq 0.01$.

Table 2
Days of observation for neonatal behavioral tests.

Behavior	Day of observation															
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Surface righting	CT; LPS LPS + PA	PA	●	●												
Cliff aversion†	●	●	●	●	CT	LPS	LPS + PA †	PA†	●	●	●					
Negative geotaxis	●	●	●	●	●	●	●	CT	PA; LPS	LPS + PA	●	●				
Hind limb placing†	●	●	●	●	●	●	CT; PA	●	LPS	●	●	LPS + PA†	●	●		
Forelimb grasp*	●	●	●	●	●	●	LPS* LPS + PA	CT; PA	●	●	●	●	●	●		
Activity†	●	●	●	●	●	●	●	●	●	CT	●	LPS	PA†	LPS + PA†	●	●

CT, first day in which >50% of pups receiving saline performed behavior; PA, first day in which >50% of pups exposed to perinatal anoxia performed behavior; LPS, first day in which >50% of pups receiving LPS performed behavior; LPS + PA, first day in which >50% of pups exposed to combined LPS and PA performed behavior. ● Days in which the variable was tested.

* Mean significantly different from CT, $P < 0.05$.

† Mean significantly different from CT, $P \leq 0.01$.

(741.0 ± 78.54 cm) animals. PA and LPS + PA group did not show any difference regarding CT of traveled distance.

4. Discussion

Both the expression of pro-inflammatory cytokines and oxidative stress have been implicated to brain damage and consequently poor motor performance. In the current study, we explored the involvement of inflammatory and oxidative process under "pure" perinatal anoxia, or sole endotoxic conditions and in the context of a "double insult" combining both endotoxin and PA. To our knowledge, this is the first report characterizing the pattern of inflammatory and oxidative response involving these interrelated components, related to the physiopathology of CP, in the context of motor development and performance.

The main findings of the present study can be summarized as follow: First, we found that PA alone only increases IL-1 expression in cerebral cortex with no changes in oxidative measures. PA also induced a poor motor performance with a slight impact on motor development. Second, although LPS alone was not able to delay motor development, it resulted in changes on motor activity and coordination with increased levels of IL-1 and TNF-α expression associated to a high production of free radicals and elevated SOD activity. Third, changes on inflammatory and oxidative stress parameters were even greater when LPS and PA were combined. Additionally, coordination impairments were also observed with the combination of these procedures and motor development delay was more prominent in this group.

4.1. Impact of LPS, PA alone or in combination on inflammatory responses

There have been several trials on animal models to test the effects of LPS and PA on motor abilities. Although most of these studies reported impairments on motor performance, they were, however, not reminiscent of those observed in human CP and the adult offspring were able to compensate to the damage occurred (Boksa et al., 1995; Poggi et al., 2005; Strata et al., 2004; Toso et al., 2005; Ujházy et al., 2006; Roberson et al., 2006; Rousset et al., 2013). Using an animal model that mimics a chronic maternal inflammation in association to a perinatal model of anoxia which resulted in increased levels of IL-1, TNF-α (pro-inflammatory cytokines), and DCF (a marker for oxidative stress) we have demonstrated a motor phenotype that is relevant to mimic the human CP condition.

As previously described, both maternal infections and perinatal asphyxia seem to promote an inflammatory reaction linked to the synthesis of pro-inflammatory cytokines and oxygen free radicals in human newborns (Sävman et al., 1998; Kopp and Medzhitov, 1999; Lin et al., 2010; Vasiljevic et al., 2011). In fact, human neuropathological studies involving children with perinatal brain damage had described an overexpression of intracerebral pro-inflammatory cytokines (Kadhim et al., 2003, 2006) demonstrating higher levels of IL-1β in the cord blood, amniotic fluid and neonatal blood of children with CP as compared to non-CP children (Kadhim et al., 2005).

Our results demonstrate that, within perinatal cerebral cortex, LPS, PA, or its association, upregulate IL-1 synthesis. Several experiments suggested the possible involvement of IL-1β, in the cascade leading to perinatal brain damage and subsequent development of CP. Studies using animal models have reported expression of IL-1β in perinatal brain injuries induced either by H/I, an model widely used to investigate the mechanisms involved in PA where P7 rats are exposed to unilateral carotid artery occlusion, followed by a period of systemic hypoxia (Szaflarski et al., 1995; Zhai et al., 1997; Bona et al., 1999) or LPS exposures during different time-points (Cai et al., 2000; Rousset et al., 2006).

Concerning the LPS mechanism involved in brain damage, Kohmura et al. (2000), using LPS as an inflammatory agent, have

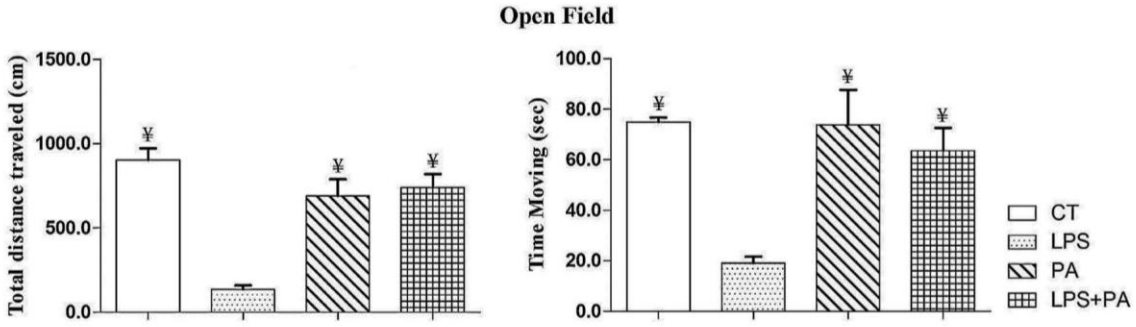

Fig. 4. Motor activity analyzed by the total distance traveled (A) and mean time moving (B) with the open field test in control and experimental rats. Differences in mean motor performance between all groups were determined at P29 and plotted. Columns represent means ± SEM. Two-way ANOVA followed by Duncan test. ¥ Different from CT, $P < 0.01$

previously suggested that LPS could pass through placenta to fetuses and generate an inflammatory response in the fetus and also in the fetal brain leading to an expression of both IL-1β and TNF-α. In fact, LPS treated rats showed a concomitant increase of IL-1 and TNF-α. These findings corroborates to a previous human study that found high levels of cytokine in infants in whom there was evidence of infection and concomitant CP (Kadhim et al., 2003). Several changes in brain environment have been associated with the inflammatory response in fetal brain triggered following antenatal LPS administration. Such changes are related to microglial activation (Paintlia et al., 2004a,b), reactive astrogliosis (Cai et al., 2000; Yu et al., 2003a), apoptosis of oligodendrocyte precursors (Paintlia et al., 2004a,b; Rousset et al., 2006), hypomyelination (Toso et al., 2005; Rousset et al., 2006), loss of dopaminergic neurons within the striatum (Ling et al., 2004) and programmed cell death in the gray matter (Rousset et al., 2006).

The inflammatory response was not only observed in LPS pups, mild changes on inflammatory parameters were also observed in rats that experienced only PA at birth. Higher expression of IL-1 and TNF-α mRNA in the CNS was previous observed after hypoxia-ischemia in newborn rats (Szaflarski et al., 1995). Although we could observe an increase in IL-1 expression, we were not able to correlate this with an overexpression of TNF-α. Since this cytokine is not normally expressed in a constitutive manner, cells that produce TNF-α require the presence of signaling molecules such as IL-1 (Bethea et al., 1992). In an in vitro experiment, an ischemic incubation induced the expression of IL-1 during the early period and the peak level of TNF-α expression was not reached until 2 h after ischemia. These results, in addition to the fact that IL-1, during some pathological states, is a potent inducer of TNF-α (Bethea et al., 1992) indicate that one of the possible reasons we could not observe any increase in the expression of TNF-α in anoxic pups is related to this cascade and, the time-frame between PA procedure and pups euthanasia could justify the absence of changes on TNF-α levels.

Summarizing, both LPS and PA were related to an increase of IL-1β and TNF-α levels. Previous studies have related cytokine levels to brain damage and consequently CP. Thus, the combination of LPS and PA in animal models that aim to reproduce the features of CP seems appropriated. IL-1β is produced by microglia and activates other proinflammatory cytokines such TNF-α (Vilcek and Le, 1994). IL-1 receptor antagonist has been shown to reduce excitotoxic and ischemic brain injuries (Martin et al., 1994; Relton and Rothwell, 1992), and the overexpression of IL-1 receptor antagonist was found to attenuate ischemic injuries suggesting that IL-1 is involved in the processes leading to brain injury (Betz et al., 1995).

Another finding in the current study is that IL-4 expression was increased in LPS + PA animals compared to all experimental groups. Several studies have demonstrated the neuroprotective effect of IL-4 on pathological states of the CNS (Chao et al., 1993; Furlan et al., 2001). It has been previously shown that IL-4 has the ability to suppress the production of free radical (Zhao et al., 2006) and down-regulate TNF-α expression (Butovsky et al., 2005). In accordance with this, it seems that the expression of IL-4 was up-regulated in animals exposed to LPS and PA in response to the overexpression of pro-inflammatory cytokines and oxygen free radicals observed in this group. Based on that, we can hypothize that, although failed, this response could be in attempt to inhibit the production of free radical and pro-inflammatory cytokines, and thus, protect brain from oxidative and cytokine toxicity.

4.2. Effects of LPS, PA alone or in combination on oxidative stress parameters

Another important finding that emerged from this study is that the inflammatory response can significantly contribute to the high levels of free radicals. The oxidative stress, an imbalance between the activity of free radicals generation and scavenging systems, has been also implicated in brain damage. In fact, LPS has been shown to stimulate reactive oxygen and nitrogen species production in the rat brain (Blasig et al., 2001) and to induce activation of brain microglia, which generate ROS, including superoxide and nitric oxide (NO), that subsequently contribute to neurodegeneration (Mayer, 1998). The brain tissue is sensitive to oxidative damage (Cechetti et al., 2012) and ROS have been associated to second messengers that trigger mitogen-activated protein kinase cascades (Griendling et al., 2000; Torres, 2003). Additionally, LPS-induced neuronal apoptotic cell death has been shown to be linked to ROS production from cortical neurons (Kim et al., 2002).

Perinatal asphyxia also has been reported to induce oxidative imbalance (Capani et al., 2001; Kumar et al., 2008). Indeed, there are several evidences suggesting the role of oxidative stress in neuronal damage both in animal models of neonatal hypoxia (Palmer, 1997; Barth et al., 1998) and in human newborns suffering from birth asphyxia (Ray et al., 1998; Yu et al., 2003b). Also, previous studies have already demonstrated that PA leads to an increased release of glutamate into the extracellular space which, resulting in excitotoxicity (Kohlhauser et al., 1999), delayed neuronal death (Dell'Anna et al., 1997a,b; Van de Berg et al., 2002), loss of GABAergic projection neurons and interneurons (Van De Berg et al., 2003) and a depletion of neurotransmitters (Loidl et al., 1994).

Although, we failed to demonstrate an increase of free radical production in PA animals, one of the main reasons could be explained by the time point that animals were euthanized. In the present study the pups were euthanized following approximately 30 min of reoxygenation. It has been previously shown that the more consistent ROS release occurs in the early reperfusion phase reaching a peak at 20 min of reoxygenation (Kontos, 1989) and then returning to CT levels (Capani et al., 2001).

4.3. Consequences of LPS, PA alone or in combination on motor development and motor skills

Based on our results, the association of LPS and PA seems to promote a more prominent response leading to an increased inflammatory and oxidative imbalance that could be involved in a more drastic brain injury and consequent disturbance in motor abilities. In fact, using a CP animal model protocol based in association of LPS administration and hypoxic/ischemia (H/I), Girard et al. (2009) observed that LPS sensitizes the developing brain to the effects of an eventually upcoming H/I. It seems that the synergy of both procedures could create a more severe histopathological damage, involving both gray and white cerebral matters, which correlated to a more limitating disability and thus, remindful of the human context (Girard et al., 2009).

Our results corroborate the hypothesis that LPS increases the vulnerability to motor impairments in animals exposed to a subsequent anoxic insult. Through the animal model proposed in our experiment, which is based in interventions during the pre and perinatal period, we were able to identify developmental motor sequels in the neonatal period that ranged to the adolescent period. Animals exposed to both LPS and PA showed delay in cliff aversion, hind limb placing and motor activity with a trend in delaying negative geotaxis which, in terms, represents signs of sensorimotor impairment (Toso et al., 2005).

Concomitantly with the phenotypic indication of developing delay, 29-days-old animals exposed to LPS in association with PA, showed remarkable motor impairment in Rotarod. The long lasting motor deficits observed here corroborate previous findings of our laboratory showing that impairments on coordination and balance of LPS + PA animals last until P29 (Stigger et al., 2011). Despite the developmental and coordination deficits, LPS + PA animals did not show any striking motor impairment in open field showed by

total distance traveled. Even though LPS animals presented lower distance traveled in the open field test, it seems that when combined with PA there was a loss of this disabilitating condition. It has been already reported motor hyperactivity in animals exposed to hypoxia with a greater distance traveled and time moving on open field (Nyakas et al., 1991; Dell'Anna et al., 1997a,b), contrasting with the principal impairments observed in CP which are mostly characterized by loss of motor abilities and accompanied to reduced movements.

5. Concluding remarks

According to previous study in our laboratory, using a CP rat model using both LPS and PA in association to a sensorimotor restriction, it seems that LPS and PA played an important physiopathological role inducing motor impairments. Based on inflammatory and oxidative hypothesis our main goal was to understand how the combination of these aggressors could contribute reproducing this complex phenotype. Our data support the idea that changes of inflammatory cytokines and oxidative stress, caused to the LPS + PA, may be involved to a more severe motor disability, creating a neurophatological substrate to cellular damage. In conclusion, prenatal exposure of pups to LPS seems to sensitize the developing brain to the effects of an eventually upcoming perinatal anoxia that resulted in an increased expression of pro-inflammatory cytokines and free radicals levels in cerebral cortex. These neuropathological alterations associated to the motor behavioral observed provide strong evidences, pertinent to the human context, that would help to understand the disease mechanisms and, more prominently, to analyze the efficiency of new neuroprotective strategies designed to reduce the increased disability encountered in human CP.

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CAPÍTULO 2

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Treadmill training induces plasticity in spinal motoneurons and sciatic nerve after sensorimotor restriction during early postnatal period: New insights into the clinical approach for children with cerebral palsy

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ABSTRACT

The aim of the present study was to investigate whether locomotor stimulation training could have beneficial effects on the morphometric alterations of spinal cord and sciatic nerve consequent to sensorimotor restriction (SR). Male Wistar rats were exposed to SR from postnatal day 2 (P2) to P28. Control and experimental rats underwent locomotor stimulation training in a treadmill for three weeks (from P31 to P52). The cross-sectional area (CSA) of spinal motoneurons innervating hind limb muscles was determined. Both fiber and axonal CSA of myelinated fibers were also assessed. The growth-related increase in CSA of motoneurons in the SR group was less than controls. After SR, the mean motoneuron soma size was reduced with an increase in the proportion of motoneurons with a soma size of between 0 and 800 μm^2. The changes in soma size of motoneurons were accompanied by a reduction in the mean fiber and axon CSA of sciatic nerve. The soma size of motoneurons was reestablished at the end of the training period reaching controls level. Our results suggest that SR during early postnatal life retards the growth-related increase in the cell body size of motoneurons in spinal cord and the development of sciatic nerve. Additionally, three weeks of locomotor stimulation using a treadmill seems to have a beneficial effect on motoneurons' soma size.

1. Introduction

Different studies have shown that the neuromuscular system display great adaptive potential in response to decreased neuromuscular activity (Lieber, 1986a,b; Marcuzzo et al., 2008; Stigger et al., 2011; Ilha et al., 2011). Most of these studies were focused on skeletal muscle but there is also convincing evidence that disuse produces neural adaptations (Canu et al., 2009). At least, in early stages of development, the mechanical activity imposed on the muscle fiber seems to play an important role in the maturation of the innervations (Greensmith et al., 1998). In fact, Nagatomo et al. (2009), using a model of hind limb unloading, showed that the increase in soma size of alpha motoneurons during development is regulated by motor activity and could be inhibited by a decrease in such activity. Additionally, during development, the neural impulse activity can affect myelinization (Fields, 2005; Zalc and Fields, 2000).

Cerebral palsy (CP) is considered to be a motor disorder resulting from a primary lesion in central nervous system (CNS) leading to impaired motor control, neuromuscular disorder and inactivity (Graham and Selber, 2003; Foran et al., 2005). Patients with CP exhibit both nerve and dorsal rootlet demyelization (Chen, 2000; Fukuhara et al., 2010) and although there is a lack of studies, evidence shows that the muscle condition found in those patients is secondary to a pathological change in peripheral nerve (Chen, 2000). In order to enhance motor skills and muscle strength, a child with CP usually begins treatment soon after diagnosis (Damiano, 2006). Several studies using animal models of CP have attempted to clarify the mechanisms involved in functional recovery. However, the major problem in most of these models is that they do not present the characteristic motor deficits seen in CP.

Abbreviations: BDNF, brain-derived neurotrophic factor; CNS, central nervous system; CP, cerebral palsy; CSA, cross-sectional area; CT, control; IGF-I, insulin growth factor I; NT-3, neurotrophim 3; P2, postnatal day 2; P14, postnatal day 14; P21, postnatal day 21; P28, postnatal day 28; P31, postnatal day 31; P52, postnatal day 52; PB, phosphate buffer; ROI, region of interest; SR, sensorimotor restriction; TrCT, trained control; TrSR, trained sensorimotor restriction.

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In order to mimic the motor deficits observed in children with CP, Strata et al. (2004) designed a rodent model based on perinatal asphyxia and chronic sensorimotor restriction (SR). The clinical relevance of the SR is based on the immobility imposed by pathological motor condition in CP. A series of studies using the SR procedure showed that the lack of movement and the abnormal proprioceptive input during early stages of development seemed to contribute the most to the abnormal pattern of movement observed (Strata et al., 2004; Coq et al., 2008; Marcuzzo et al., 2008, 2010; Stigger et al., 2011). A more in-depth characterization of neural changes due to the SR paradigm used in this animal model is needed and would help to clarify the mechanisms that underlie the motor disturbance following a primary central lesion as seen in children with CP. Thus, the aim of the present study is to investigate the changes in morphologic properties of both the motoneurons and nerves that innervate the hind limb muscles following sensorimotor restriction. Most importantly, since activity-based programs such as treadmill training have been used as treatment strategy for CP patients (Damiano, 2006), the effects of locomotor stimulation on a treadmill will be assessed in an attempt to obtain new insights into the clinical approach adopted in this pathological condition.

2. Materials and methods

All procedures were approved by the Ethical Committee at the Federal University of Rio Grande do Sul (2006631). All 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). All efforts were done to minimize animal suffering as well as to reduce the number of animals.

2.1. Experimental animals

Pregnant Wistar rats (5) were obtained from a local breeding colony (Institute of Basic Health Sciences, at the Universidade Federal do Rio Grande do Sul, Brazil). The day of birth was considered day 0. Litters were culled to a maximum of eight pups per litter. Animals were maintained in a 12/12 h light/dark cycle in an air-conditioned constant temperature room (20 ± 1 °C), with food and water available *ad libitum*. After weaning (postnatal day 21), the females were removed from the boxes and discarded from the study.

At postnatal day 2 (P2), pups were assigned randomly to: control group (CT, $n = 10$) or sensorimotor restriction group (SR, $n = 10$). The SR procedure was performed from P2 until P28 by bounding together both hind limbs with paper tape and maintained in an extended position with an epoxy cast for 16 h per day (Strata et al., 2004; Coq et al., 2008; Marcuzzo et al., 2008, 2010; Stigger et al., 2011). After the end of the SR period (P28), half of the animals of each group were submitted to a locomotor stimulation by a treadmill training: untrained: control (CT, $n = 5$); sensorimotor restriction (SR, $n = 5$) and trained: trained control (TrCT, $n = 5$); trained sensorimotor restricted (TrSR, $n = 5$). The training consisted of a locomotor stimulation by walking on a treadmill, with low speed, for three weeks from P31 (once a day, 5 sessions per week). The initial speed was determined by observing the best walking pattern developed by restricted rats. 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. For details see Marcuzzo et al. (2008).

2.2. Histological and morphometric analysis

After treadmill training (on P52) animals were deeply 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 spinal cord segments at L4-5 level were removed after cautious laminectomy and left sciatic nerves were carefully dissected free from surrounding tissue.

2.2.1. Spinal cord analyses

Transversal sections of the post fixed lumbar segment (200 μm) were cut using a vibratome (Leica, Germany). Four samples were embedded in resin blocks (Durcupan, ACM-Fluka, Switzerland), maintained in vacuum for 24 h, and, afterwards, polymerized for 48 h at 60 °C. One of the samples was randomly selected and transverse-semithin sections (1 μm) were obtained using an ultramicrotome (MT 6000-XL, RMC, Tucson, USA). Every 10 μm, one section was collected and stained with 1% toluidine blue (Merck, Germany) in 1% sodium tetraborate (Ecibra, Brazil). Images of the spinal cord were captured (initially 20× and further amplified 200% for analysis) using a Nikon Eclipse E-600 microscope (Japan) coupled to a digital camera and Image Pro Plus Software 6.0 (Media Cybernetics, USA). Digital images from left ventral horn were taken and the cross-sectional areas of the motoneurons in which the nucleolus was visible were estimated. The area of each individual motoneuron was estimated by the point-counting technique (Hermel et al., 2006) using grids with a point density of one point per 26.29 μm^2 and the equation: $\hat{A} = \Sigma p \cdot a/p$. Where $\hat{A}$ is area, Σp is the total of counted areas/point and a/p is the area/point value (26.29 μm^2). This procedure was performed by a blinded examiner. The average of the cross-sectional areas of each individual rat was based on the mean of the motoneuron areas measured per animal.

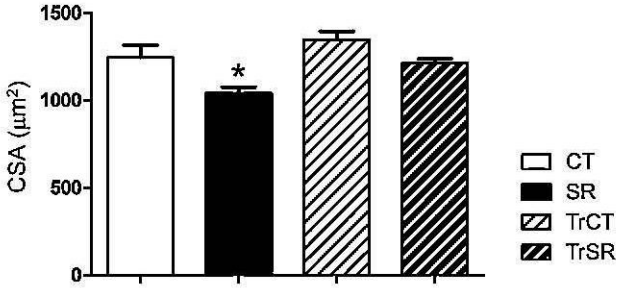

Fig. 1. Cross-sectional areas (CSA) of motoneurons in control (CT), sensorimotor-restricted (SR), trained (TrCT) and sensorimotor-restricted trained (TrSR) rats. Values are expressed as means ± SEM. * Significantly different from CT, $P < 0.05$.

2.2.2. Sciatic nerve analyses

For nerve analysis, small samples of the sciatic nerve (≈3 mm) were postfixed in the same fixative solution described for spinal cord segment. The samples were also embedded in resin blocks, maintained in vacuum, and polymerized. Transverse-semithin sections (1 μm) were obtained using the same ultramicrotome and stained with 1% toluidine blue (Merck, Germany) in 1% sodium tetraborate (Ecibra, Brazil). Afterwards, images of the sciatic nerve were captured and digitalized (initially 100× and further amplified 200× for analysis). For morphological evaluation, a set of 6 images was obtained from each nerve, 3 random images from the periphery and 3 random images from the center of the nerve. The morphometric measurements were calculated in both large and small myelinated fibers (both sensory and motor fibers) that were located inside an area of interest (823.72 μm^2). Morphometric measurements included the (1) average myelinated fiber area (μm^2); (2) average axon area of the myelinated fiber (μm^2); (3) average myelin sheath thickness (μm); (4) *g* ratio (the quotient axon diameter/fiber diameter, a measurement of the degree of myelination) and were performed by a blinded examiner. The measurements of areas were estimated using the point-counting technique already described (point density of 1 point per 1.06 μm^2). The average myelin sheath thickness was estimated using the measurement tools of the Image Pro Plus software.

2.3. Statistical analysis

The data were analyzed using two-way analysis of variance (ANOVA) with *restriction* and *treadmill training* as the independent variables. All analyses were followed by *post hoc* Duncan's test. 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. Motoneuron morphometry

A total of 1008 motoneurons were analyzed (CT, $n = 336$; SR, $n = 188$; TrCT, $n = 228$; TrSR, $n = 256$). The mean soma sizes of the motoneurons are shown in Fig. 1. The mean CSA of the motoneurons soma was 1246.7 μm^2 for the CT and 1037.7 μm^2 for SR groups. The distribution histograms of motoneuron soma sizes are illustrated in Fig. 2. For the CT group, the proportions of the total number of counted motoneuron soma sizes were 25.1% between 0 and 800 μm^2, 43% between 800 and 1600 μm^2 and 31.9% between 1600 and 2400 μm^2. For the SR group, 38.6% of motoneuron soma sizes were between 0 and 800 μm^2, 45.4% between 800 and 1600 μm^2 and 16% between 1600 and 2400 μm^2. A decrease in the proportions of motoneurons with soma sizes located between 1600 and

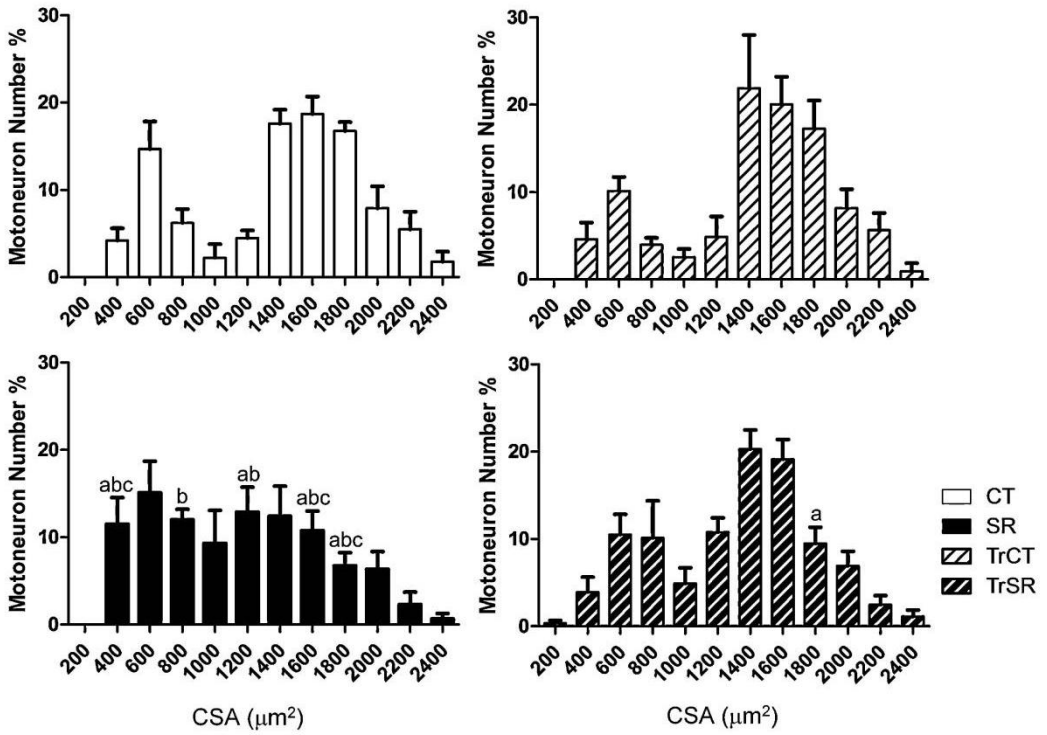

Fig. 2. Frequency distributions of percentage of motoneurons in the ventral horn of the spinal cord in control (CT), sensorimotor-restricted (SR), trained (TrCT) and sensorimotor-restricted trained (TrSR) rats. Values are expressed as means ± SEM ($n = 5$). a, Significantly different from CT, $P < 0.05$; b, significantly different from TrCT, $P < 0.05$ and c, significantly different from TrSR, $P < 0.05$.

2400 μm^2 in association with an increased proportion of motoneuron soma sizes between 0 and 800 μm^2 was observed after the SR procedure. These results suggest that the mean motoneuron soma size diminished after the SR procedure (Figs. 3 and 4).

After training, the mean motoneuron soma size returned to normal CT levels. In the TrSR group 24.9% of motoneuron soma sizes were between 0 and 800 μm^2, 55.1% between 800 and 1600 μm^2 and 20% between 1600 and 2400 μm^2. The mean CSA of the motoneuron soma sizes was 1216.2 μm^2. No significant differences were found between the CT and TrCT groups nor between the TrCT and TrSR groups.

3.2. Nerve morphometry

Table 1 shows the mean diameter of myelinated fibers from CT, SR, TrCT and TrSR rats. The mean fiber diameter of SR and TrSR rats decreased when compared to CT ($P \leq 0.05$).

The axon diameter of SR and TrSR also presented a decrease when compared to CT ($P < 0.05$). No significant differences were found when comparing the mean myelin sheath thickness and g ratios in all experimental groups.

4. Discussion

It is well established that changes in neuromuscular activity can induce alterations in skeletal muscle (Lieber, 1986a,b). Disuse induces muscle fiber atrophy and alters the expression of myofibrillar and other protein isoforms resulting in muscle fiber type transitions from slow to fast (Zhang et al., 2007; Urso, 2009). In fact, Stigger et al. (2011), using the same 26-day SR procedure used in this study, found atrophy and a slow-to-fast fiber type transition in soleus and tibialis anterior muscles. Since skeletal muscles are innervated by motoneurons from the ventral horn of the spinal cord (Nicolopoulos-Stournaras and Iles, 1983; Peyronnard et al., 1986) and morphological and metabolic features of these motoneurons correspond with those from the innervated muscle fibers (Ishihara et al., 1995, 1997), it is reasonable to suggest that motoneurons innervating skeletal muscles would also demonstrate some changes in their properties after a period of disuse.

The present study showed that after SR the motoneuron soma size was diminished. Our results suggest that the restriction of movement during postnatal growth altered the development of these motoneurons causing an inhibition of the growth-related increase in soma size. Our study also showed that the SR-induced reduction in mean motoneuron soma size was reversed and normal control levels were reestablished after a period of locomotor stimulation on a treadmill. The increase in soma size was not observed in TrCT rats. This could be attributable to the training protocol used in this study. The velocity and time of training sessions were established based in an optimal gait movement of SR group (Marcuzzo et al., 2008) thus, considering that a therapeutic intervention targeting a cellular adaptations or function must provide an overload to the system (Mueller and Maluf, 2002), probably the intensity of training was not a challenge to TrCT rats and therefore, not able to promote a motoneuron adaptation.

The change in soma size in the motoneurons after SR was accompanied by a reduction in the fiber and axon area. These results suggest that the mechanical and neural activity of hind limb muscles can also influence the axonal characteristics of these motoneurons. One explanation for our results is based on activity-dependent modulation of neurotrophic factors. In fact, four weeks of immobilization, has already been shown to decrease the insulin-like growth factor-I peptide (IGF-I) levels in spinal cord (Suliman et al., 2001). IGFs are muscle derived trophic factors that have been detected in developing skeletal muscles (Girbau et al., 1992; Neff et al., 1993) and are found to play an important role in the growth and branching of motoneuron axons in the developing neuromuscular system (D'Costa et al., 1998). This hypothesis could also explain the normalization of growth-related changes of motoneurons' soma after the training. Exercise may affect not only the

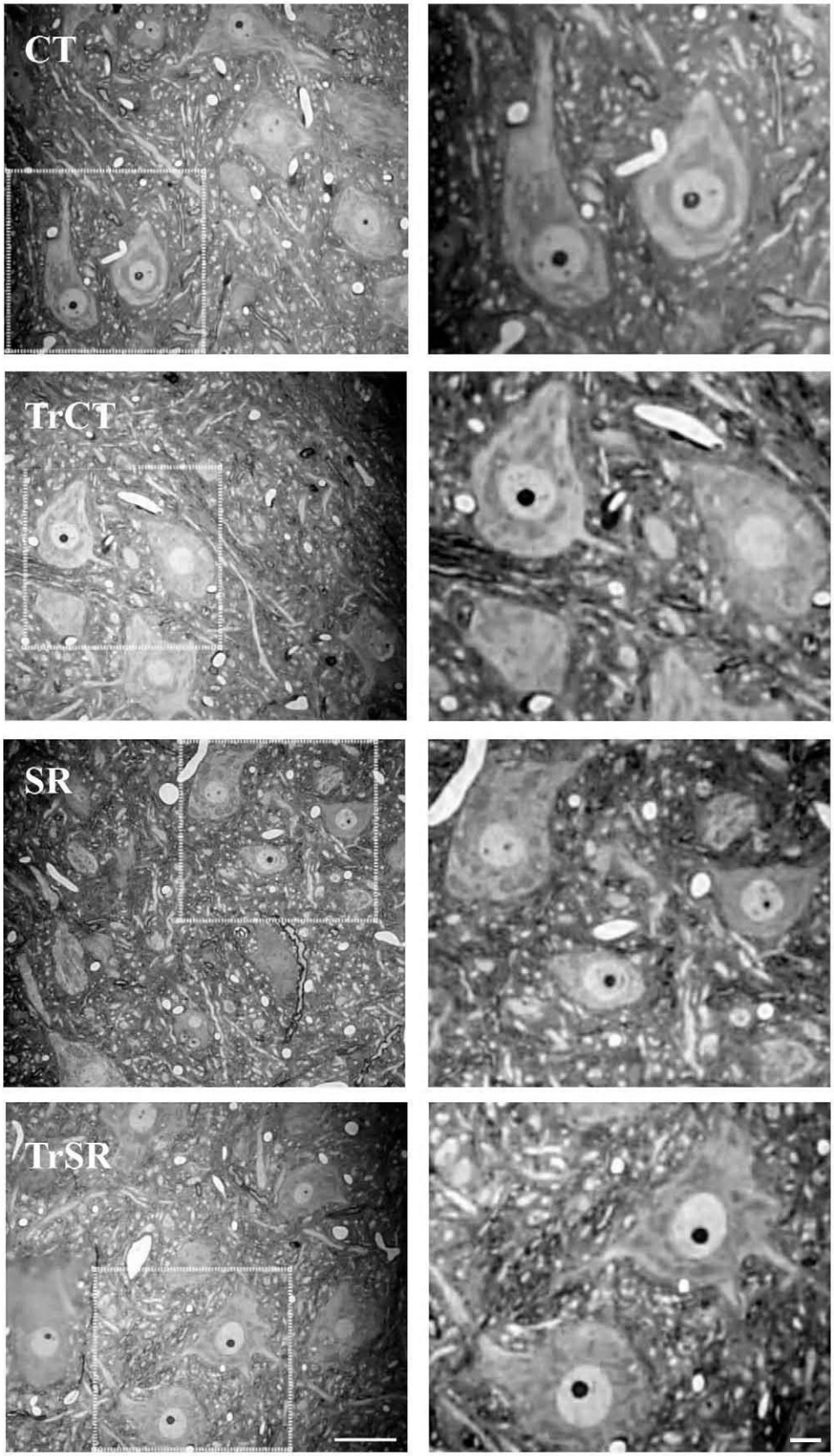

Fig. 3. Representative photomicrographs of motoneurons from left ventral horn in sections stained with 1% toluidine blue of all experimental groups (captured at 20× – left and amplified 200% – right). Bar indicates 20 μm.

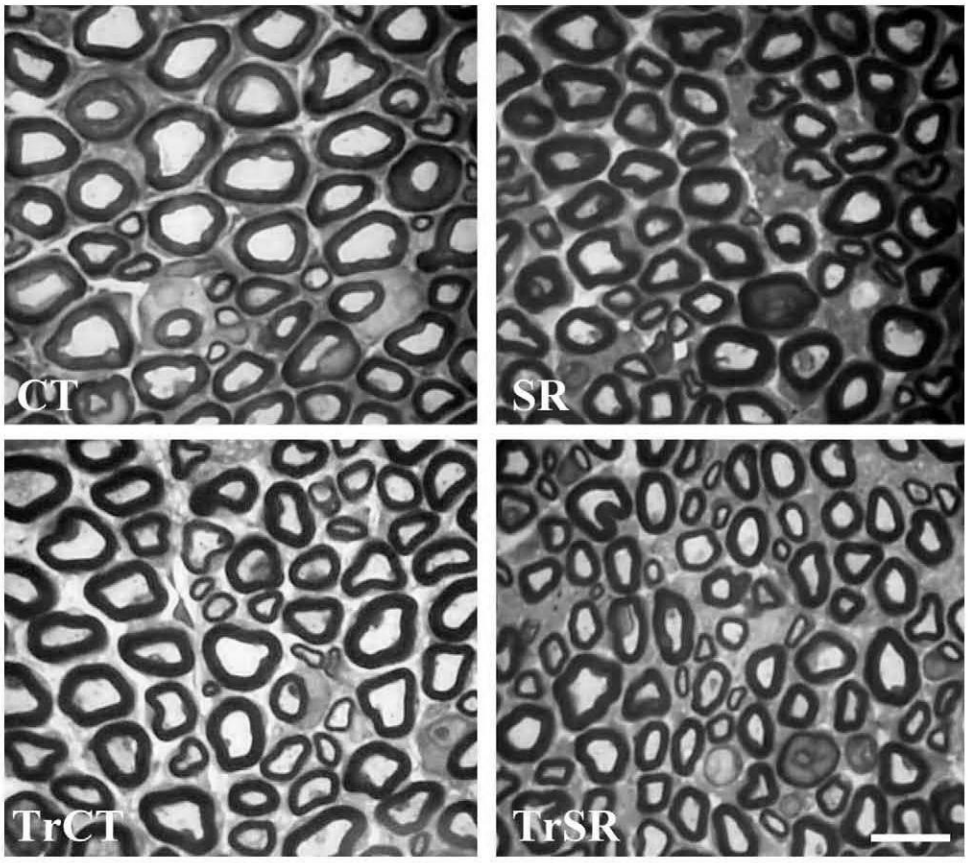

Fig. 4. Digitized images of transverse-semithin sections (1 μm) obtained from sciatic nerves of CT, SR, TrCT and TrSR rats (captured at 100×). Reduction of axonal area was apparent comparing SR with CT. Semithin sections were stained with 1% toluidine blue. Bar indicates 10 μm.

expression of IGF-I (Eliakim et al., 1997; Heo et al., 2001), but also of other neurotrophic factors, such as brain-derived neurotrophic factor (BDNF) and neurotrophin 3 (NT-3) in soleus muscle and lumbar spinal cord (Gómez-Pinilla et al., 2001; Ilha et al., 2011).

IGF-I not only appears to enhance the survival of spinal motor neurons in the spinal cord (Neff et al., 1993), but evidence also suggests a central role for IGF-I in corticospinal motoneuron outgrowth and/or survival (Özdinler and Macklis, 2006). In a series of experiments, Kalb and Hockfield (1990, 1988) suggested that the coordinated input from upper neurons and proprioceptive afferents during early postnatal life is required for the normal development of motoneurons. A twenty-six-day SR procedure does not simply reduce proprioceptive input from the hind limbs; it also leads to remodeling within the somatosensory and motor cortex (Strata et al., 2004; Coq et al., 2008). It is known that the distribution of neurons that send axons to spinal cord varies between infant and adult rats, and an adult pattern is reached between P14 and P21 (Stanfield et al., 1982; Stanfield and O'Leary, 1985). Remodeling within the motor cortex could contribute to abnormal projections from the motor cortex to spinal motoneurons and consequently produce the motor-skill impairments experienced by these rats in a previous study (Marcuzzo et al., 2008, 2010). It is postulated that the projections from the motor cortex to spinal motoneurons are altered in CP (Brouwer and Ashby, 1991).

One implication of our results is the existence of a critical period of motor system development during which the cellular and molecular aspects could be finely sculpted and consolidated. In this period, motoneurons seem to have a morphological and functional adaptability in response to changed demands such as those seen either after injury to the motor system in CP or with training. In addition, spinal cord seems to respond positively to locomotor stimulation, indicating that it could serve as a substrate for the therapeutic approach to this pathological condition. An element involved in this plasticity might reside in the fact that during locomotor training both sensory and motor activity are stimulated, as well as a neural circuit within the lumbar spinal cord capable to generate organized and repetitive motor patterns, such as locomotion, the central pattern generator (Ichiyama et al., 2008; Edgerton et al., 2008; Edgerton and Roy, 2009).

CP children are less mobile than children without disabilities, a child's experience in early postnatal life such as walking, running or jumping might provide a pattern of neuronal activity (primary

Table 1
Two-way ANOVA revealed significant effect of the factor SR. Values are expressed as means ± SEM.

Groups	Fiber area (μm²)	Axonal area (μm²)	Myelin sheath thickness (μm)	*g* ratio
CT	45.44 ± 2.82	24.73 ± 1.53	1.00 ± 0.03	0.74 ± 0.003
SR	40.16 ± 1.34*	21.11 ± 0.85*	0.98 ± 0.01	0.72 ± 0.004
TrCT	44.16 ± 3.64	23.29 ± 1.95	1.02 ± 0.04	0.73 ± 0.003
TrSR	39.03 ± 1.98*	20.86 ± 1.35*	0.95 ± 0.02	0.73 ± 0.007

Morphometric parameters of sciatic nerve of all experimental groups.
* Significantly different from CT, $P < 0.05$.

afferent and descending inputs on motor neurons) that could lead to normal motoneuron differentiation and consequently optimal neuromuscular performance in adulthood.

This animal model could help to enhance our understanding of the role played by the lack of voluntary movement in early postnatal life in CP, where movement deprivation and altered experience may themselves worsen motor development and performance. A description of the morphological background linked to the motor deficits or the protective effect of training in this model allows a better comprehension of this phenomenon and provides useful suggestions regarding efficacious lifestyles and rehabilitation strategies for patients with this condition. Once again, we highlight the importance of early interventions, such as those found in physiotherapy programs, for CP patients in order to prevent the disability found in this population.

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CAPÍTULO 3

Expression of Synaptophysin and Caspase-3 on Lumbar Segments of Spinal Cord After Sensorimotor Restriction During Early Postnatal Period and Treadmill Training

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Keywords: Sensorimotor restriction; Developmental disuse; Rat spinal cord; Apoptosis; Spinal cord plasticity; Treadmill training

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Abstract

The aim of the present study was to investigate whether locomotor stimulation training could have beneficial effects on spinal cord plasticity and development consequent to sensorimotor restriction (SR). Male Wistar rats were exposed to SR from postnatal day 2 (P2) to P28. Control and experimental rats underwent locomotor stimulation training in a treadmill for three weeks (from P31 to P52). The intensity of the synaptophysin and caspase-3 immunoreaction was determined on ventral horn of spinal cord. The synaptophysin immunoreactivity was lower in the ventral horn of sensorimotor restricted rats compared to controls animals. The changes in synaptophysin immunoreactivity were accompanied by a an increased caspase-3 immunoreactivity. Those alterations were reversed and reached the control level at the end of the training period. Our results suggest that immobility affects the normal developmental process that spinal cord undergoes in early postnatal life influencing both pro-apoptotic and synapse marker. Also, we demonstrated that this phenomena was reversed by three weeks of locomotor stimulation using a treadmill.

1. Introduction

Patients confined to bed/wheelchair or simple subjected to physical hypoactivity due to neurological disorders suffer from functional impairment in motor tasks. One of the most common causes of motor disability in children is Cerebral palsy (CP) (Himmelmann et al., 2005). CP is considered to be a heterogeneous clinical condition resulting from a primary lesion in central nervous system (CNS) that leads to impaired motor control, neuromuscular disorder and inactivity (Graham and Selber, 2003; Foran et al., 2005). Although the primary lesion involved in the CP's brain pathology is static, it is proposed that motor performance could be worsened with time, due to the privation of voluntary movement imposed by spasticity and impaired motor control (Graham and Selber, 2003).

There are several animal models, mainly focused in maternal infections or perinatal asphyxia and hypoxic–ischemic injuries, trying to reproduce the developmental and motor deficits that would be typical of human CP condition. Although most of these studies reported impairments on motor performance, they were, however, not reminiscent of those observed in human CP and, with time, offspring are able to compensate to the damage occurred (Boksa et al., 1995; Poggi et al., 2005; Strata et al., 2004; Toso et al., 2005; Ujházy et al., 2006; Roberson et al., 2006, Rousset et al., 2013). A motor phenotype similar to those described in CP patients were more reliably reproduced in rats by motor restriction during the early stages of development (Strata et al., 2004; Coq et al., 2008;Marcuzzo et al., 2008; Marcuzzo et al., 2010, Stigger et al., 2011a). This confirms the significance of voluntary movements during the maturation of the central and peripheral nervous system to the development of posture and locomotion. Sensorimotor restriction (SR), used in rats to mimic the immobility imposed by the pathological motor condition in CP, is a valuable model which allows

gaining new insights into the underlying mechanisms of neural adaptations that occurs in response to immobility. A series of studies using the SR procedure showed that SR reduce proprioceptive feedback from the hind limbs and leads to remodeling within the somatosensory cortex (Coq et al., 2008) and motor cortex (Strata et al., 2004) with a diminished number of neurons within somatosensory cortex (Marcuzzo et al., 2009). Although evident the central effects of disuse, the functional impairment in motor tasks could be as a result of the combination of both central and peripheral factors. In fact, it has been previously proposed that the locomotor abnormalities of CP may not be solely derived from cerebral dysfunction but could also partly because d by dysfunction of the spinal cord neural network (de Louw et al., 2002).

It is well established that the rat spinal cord undergoes a significant continuous plasticity during peri/postnatal development (Vinay et al., 2000). The first postnatal week is a critical period for the development of postural reactions in the hind limbs (Brocard et al., 1999). Through this period, 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). In fact, our recent experiment (Stigger et al., 2011b) showed that a 26-day period of sensorimotor restriction altered the development of motoneurons in the ventral horn of the spinal cord causing an inhibition of the growth-related increase in soma size.

Based in our recent finding indicating that there is a period of activity-dependent plasticity in the developing CS system and that morphological aspects of motoneurons could be changed by disuse, the aim of the present study is investigate whether diminished sensorimotor stimulation, induced by SR, affects spinal cord plasticity and development by analyzing the expression of synaptophysin, an intrinsic synaptic vesicle membrane protein related to the activity-dependent synapse formation, and caspase-3,

an pro-apoptotic molecules involved in programed cell death (PCD) during development, in the ventral horn of lumbar segments of spinal cord.

Additionaly, since a child with CP usually begins treatment soon after diagnosis in order to enhance motor skills and muscle strength (Damiano, 2006) and treadmill training have been successfully used to prevent motor and morphological alterations on neuromuscular system after SR (Marcuzzo et al., 2008; Stigger et al., 2011b), the effects of locomotor stimulation on a treadmill will be assessed in an attempt to obtain new insights into the clinical approach on pathological conditions comprising developmental disuse.

2. Materials and methods

All procedures were approved by the Ethical Committee at the Federal University of Rio Grande do Sul (2006631). All 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). All efforts were done to minimize animal suffering as well as to reduce the number of animals.

2.1.Experimental animals

Pregnant Wistar rats (5) were obtained from a local breeding colony (Institute of Basic Health Sciences, at the Universidade Federal do Rio Grande do Sul, Brazil). The day of birth was considered day 0. Litters were culled to a maximum of eight pups per litter. Animals were maintained in a 12/12 h light/dark cycle in an air-conditioned constant temperature room (20±1 ◦C), with food and water available ad libitum. After weaning

(postnatal day 21), the females were removed from the boxes and discarded from the study.

At postnatal day 2 (P2), pups were assigned randomly to: control group (CT, n = 10) or sensorimotor restriction group (SR, n = 10). The SR procedure was performed from P2 until P28 by bounding together both hind limbs with paper tape and maintained in an extended position with an epoxy cast for 16 h per day (Strata et al., 2004; Coq et al., 2008; Marcuzzo et al., 2008; 2010; Stigger et al., 2011a; Stigger et al., 2011b). After the end of the SR period (P28), half of the animals of each group were submitted to a locomotor stimulation by a treadmill training: untrained: control (CT, n = 5); sensorimotor restriction (SR, n = 5) and trained: trained control (TrCT, n = 5); trained sensorimotor restricted (TrSR, n = 5). The training consisted of a locomotor stimulation by walking on a treadmill, with low speed, for three weeks from P31 (once a day, 5 sessions per week). 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. For details see Marcuzzo et al., 2008.

2.2.Immunoistochemical procedure

After treadmill training (on P52) animals were deeply 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 spinal cord segments at L4-

5 level were removed after cautious laminectomy. The L5 level of spinal cord was post-fixed in a solution containing 4% paraformaldehyde in 0,1M PB for 4h and cryoprotected by immersion in a 15% and 30% sucrose (Synth, Brazil) solution in PB at 4ºC until they sank. After, the segments of spinal cord were quickly frozen in isopentane (Merck, Germany) cooled in liquid nitrogen and kept in a freezer (-70ºC) for further analyses. Transversal sections (40µm) were cut using a cryostat (CM1850, Leica, Germany) at -20ºC and collected in a PB saline (PBS), pH 7,4. The free-floating sections were washed in PBS, pre-treated with 3% hydrogen peroxide for 30 min, washed again in PBS and after in PBS containing 0,4% Triton X-100 (PBS-Tx) for 15 min, treated with 2% bovine serum albumin (In lab, Brazil) in PBS-Tx for 30 min an incubated with the primary antibody Monoclonal Anti-Synaptophysin or Caspase-3 (Sigma Chemical Co, USA) diluted 1:200 in PBS-Tx for 48h at 4ºC. The sections were again washed in PBS-Tx and incubated in the secondary antibody Anti-Mouse IgG-Peroxidase (Sigma Chemical Co, USA) diluted 1:500 for 2h at room temperature. The reaction was revealed in a medium containing 0,06% 3,3-diaminobenzidine (DAB, Sigma Chemical Co, USA) dissolved in PBS for 10 min and after in 0,06% DAB with en 2µL of 3% hydrogen peroxide for 10 min. Finally, the sections were washed in PBS, dehydrated in ethanol, cleared with xylene and covered with Entellan (Merck, Germany) and coverslips. Control sections were prepared omitting the primary antibody by replacing it with PBS.

2.3.Optical densitometry

To measure the intensity of the synaptophysin and caspase-3 immunoreaction was used a semi-quantitative densitometric analysis. Digitalized images of the dorsal horn of the spinal cords were obtained with a Nikon Optiphot-2 microscope (200x, Tokyo, Japan) coupled to a Micrometrics camera (Accu Scope, Commack, NY, USA). The digitized

images obtained from the selected areas were converted to an 8-bit gray scale (0–255 gray levels) with the Image Pro Plus 6.0 software for further analysis. Picture elements (pixels) employed to measure optical density were obtained from tree areas of interest (AOI) measuring 3775μm2 each overlaid on the gray scale image. All of the lighting conditions and magnifications were held constant. Both the left and right dorsal horn of spinal cord were used. For each rat, 30 measures were taken and the results shown were the total mean value from the three studied AOIs. Background staining subtraction and correction were done in accordance with our previous published protocol to calculate the optical density (Xavier et al., 2005).

2.4.Neonatal developmental tests

The neonatal developmental milestones were evaluated daily by a blinded observer, from P1 to P15, always at the same time (10 a.m.). Newborn rats were assessed for neonatal developmental milestones as following (based on Poggi et al., 2005): (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) negative geotaxis (pups were placed head down on a 45°-inclined surface, and the positive response consisted of a 180 turn with upward crawling), (3) 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), (4) forelimb grasp (the ability to pups remain suspended for 10 seconds after grasping thin rod with their forepaws), (5) hind limb 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), and (6) open field activity (time to move off a circle of 13cm diameter). The behaviors measured all occur at differing stages throughout the first 15 days

corresponding to the development throughout the neonatal period. Each developmental test response was considered positive based on its first appearance. All measurements were time-limited to a maximum of 30s.

2.5.Statistical analysis

The data for each neonatal developmental tests were analyzed using unpaired Mann-Whitney statistics. Synaptophysin and caspase-3 expressionresults were analyzed using two-way analysis of variance (ANOVA) with restriction and treadmill training as the independent variables followed by post-hoc Duncan's test. 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.Optical densitometry

As observed in Figure 1, the OD analysis of the lumbar segment showed that the Synaptophysin immunoreactivity (synaptophysin-ir) was lower in the ventral horn of from the SR group (0,182±0.002) when compared to either CT (0,193±0.002), CTTr (0,192±0.002) or SRTr groups (0,197±0.003) ($P<0.05$). There were no differences between the CT and CTTr groups, or between the CT and SRTr groups. Interestingly, the decreased synaptophysin-ir observed in the SR group was accompanied with a increased caspase-3 immunoreactivity (caspase3-ir). Caspase3-ir was increased within the ventral horn of SR animals (0,181±0.001) when compared to CT (0,170±0.001), CTTr (0,169±0.001) or SRTr groups (0,174±0.001)($P<0.05$). Again, no differences were observed between the CT and CTTr groups, or between the CT and SRTr groups (Figure 2).

3.2.Neonatal developmental tests

As showed in Table 1, sensorimotor restricted pups performed cliff aversion ($P < 0.01$), negative geotaxis ($P < 0.01$), hind limb placement ($P < 0.01$) and motor activity ($P = 0.05$) latter than CT animals. No differences were observed on surface righting and forelimb grasp.

4. Discussion

The present study provides evidence for a change in the activity-dependent plasticity in lumbar spinal cord in response to a 26-day period of sensorimotor restriction and locomotor stimulation in rats at early stages of development. As results, first we found that the synaptophysin expression in the ventral horn of the lumbar segment of spinal cord was lower in SR animals comparing to all experimental groups, this results were accompanied with an increased expression of caspase-3. Second, the locomotor stimulation program was able to reverse the alterations in synaptophysin and a lower caspase-3 expression induced by SR. Also, SR induced changes in motor development. Taken together, these results suggest that motor activity is essential to the normal process of development, that includes synaptogenesis and programed cell death, and thus, these process could participate as substrates for the motor deficits observed in developmental disuse conditions such CP. Additionally, the present data encourage early locomotor stimulation in developmental disuse conditions considering that spinal cord plasticity alterations are reversible in response to treatment.

The rat spinal cord undergoes a significant continuous transformation in the course of pre/perinatal and early postnatal development. An initial phase of differentiation of motoneurons including fiber outgrowth and synapses formation initiates at the last week before birth (Altman and Bayer, 1984) and, following the next 3 weeks of postnatal life, motoneurons undergoes a rapid period of development. This period is associated with the maturation of both their afferent inputs and efferent connections with their target muscle fibres and establishment of mature and functional synapses. Through this process, the storage and release of neurotransmitters from synaptic vesicles seems to be essential (Bergmann et al., 1991). Synaptophysin have been suggested to participate with an important role in regulating activity-dependent synapse formation (Tarsa and Goda, 2002). In fact, Bergmann et al., (1991) have correlated transcription and translation of synaptophysin within the neurons of the spinal cord with proliferation, migration, fiber outgrowth and the formation of transient and/or permanent synapses, demonstrating that synaptophysin is a marker for fiber outgrowth and synapse formation.

One of our results is the reduced expression of synaptophysin within the ventral horn of spinal cord in response to immobilization. In the rats' spinal cord, synaptophysin expression has been shown to start at embryonic day (ED) 12, reaching a constant level, which is kept until birth, after ED 14. It' expression is followed by a postnatal rise to reach the adult pattern (Bergmann et al., 1991). In present study we demonstrate that the synaptophysin expression could be modulated for either decreased or increased motor activity. Actually, the synaptophysin expression seems to occur in an activity-dependent manner modulated by neurotrophic factors such as brain-derived neurotrophic factor (BDNF). At the time of nerve–muscle contact BDNF, as well other neurotrophins, such as neurotrophin-3 (NT-3), are expressed by embryonic muscle cells

(Schecterson and Bothwell, 1992; Henderson et al., 1993; Trupp et al., 1995) and can contribute to formation and maturation of neuronal synapses (Wang, Liao and Li, 2010).

Not only, BDNF expression could be involved in changes on spinal circuitry. Further than peripheral stimulus, descending inputs that occur during practice also seems to have a crucial role in development of muscle afferents (Wolpaw and Tennissen, 2001). Following developmental disuse, remodeling within the somatosensory cortex (Coq et al., 2008) and motor cortex (Strata et al., 2004) occurs. Remodeling within central areas could contribute to abnormal projections from the motor cortex to spinal motoneurons and consequently impaired development of motoneurons (Stigger et al., 2011b). The activity arising from corticospinal input initiates a critical period in the development that is essential in guiding the development of spinal cord segmental circuitry, refining muscle afferent connectivity and modulating synaptic networks between spinal interneurons (Clowry, 2007). Thus, changes in synaptophysin expression could be due peripheral mechanisms, but, also have supraspinal contributions since motor areas could be affected by disuse.

Our study also showed that the SR-induced decrease in synaptophysin expression was accompanied to a delay in achieving some developmental milestones and locomotion. Synaptogenesis in rats' spinal cord seems to coincide with the acquisition of postural and locomotor functions (Kerai et al. 1995). The rats' hind limbs are not motile at birth (Geisler et al., 1993; Jamon and Clarac, 1998). This, at least in some rodents, corresponds to a period when only few synapses are present in the gray matter of spinal cord (Gingras and Cabana, 1998). Actually, the synaptogenesis of spinal motoneurons with axons originated within the brain occurs mostly postnatally (Kamiyama et al., 2006, Eyre, 2007; Clowry, 2007) and only at the end of the first postnatal week that animals become able to lift their trunk from the floor and to walk spontaneously

(Geisler et al., 1993; Jamon and Clarac, 1998). Rat CS axons enter the spinal cord at postnatal day 0 (P0) (Porter and Lemon 1993; Stanfield 1992), reach the lower cervical cord by P3, and extend to the end of the spinal cord by the end of the second postnatal week (Donatelle 1977; Jones et al. 1982; Stanfield et al., 1982). Consequently, the disturbance in activity level within early postnatal period due SR procedure could be responsible to alter normal development and acquisition of a mature pattern of motor behavior.

Besides synaptogenesis, cell death during normal development has been also described in nearly all neuronal cell types in both central and peripheral nervous systems (Oppenheim, 1991; Spreafico et al., 1995). In approximately all neuronal populations, the early phase of neuron's generation is followed by a phase of programmed cell death (PCD) that results in the elimination of the initial number of cells (Oppenheim, 1991). In fact, during development, a wave of apoptosis in the gray matter in both embryonic and postnatal spinal cord of rats was reported (Lawson et al., 1997; Yamamoto et al., 1999; Lowrie and Lawson, 2000). Cysteine proteases, including the caspase family, are considered to be among the most highly conserved pro-apoptotic molecules involved in PCD during development (Oppenheim et al., 2001). Our study demonstrated that SR increases the expression of caspase-3 compared to on normal developmental rats within the ventral horn of the lumbar spinal cord, what could be related to motoneurons and/or interneurons death. During development, neurons within spinal cord seem to die by apoptosis following nerve injury (Lawson and Lowrie, 1998). To our knowledge there are only few trials examining spinal cord apoptosis following non traumatic disuse such as limb immobilization or hind limb unloading. Although Islamov, et al (2011) did not report any sign of apoptosis within hind limb motoneurons during 35-day antiorthostatic hind limb suspension, their results indicated the increased expression of anti-apoptotic

factors. It seems that this resistance of motoneurons to a decreased motor activity is not found in neonatal rats since we found an overexpression of caspase-3 in motoneurons within ventral horn. In fact, previous studies from our laboratory showed that SR during development is able to alter motoneurons maturation (Stigger et al., 2011b) inducing reduction in mean soma size. In the same way that occurred to synaptophysin expression, both central and peripheral diminished/abnormal inputs could be related to changes in caspase-3 expression. One point that should be noted is that, although this results could possibly be relate to amplified cell death, it seems that it is not permanent since locomotor stimulus could normalize this parameter.

Since neurotropic factors are involved not only in synaptic plasticity of the central and peripheral nervous system but also in neuronal survival (Constandil et al., 2011), a neurotrophic hypothesis could explain both the increased caspase-3 and decreased synaptophysin expression during SR procedure and also explain the normalization of its expression after the training. Neurotrophic factors are also known to be activity dependent, with increases in their expression resulting from neural activity (Neeper et al., 1996). The reduction of synaptophysin expression was reversed after the SR rats participated in treadmill exercise. Therefore, although the locomotor stimulation used in our study is considered to be a low intensity exercise, the demand that these animals were exposed during our training protocol was sufficient to normalize their activity to levels comparable to those of CT rats. Exercise affects the expression of several neurotrophic factors such as insulin-like growth factor-I peptide (IGF-I) (Eliakim et al., 1997; Heo et al., 2001), BDNF and NT-3 (Gómez-Pinilla et al., 2001; Ilha et al, 2011). Also, BDNF has the ability to inhibit caspase-3 activation and subsequent apoptosis protecting against neuronal injury (Han et al., 2000). Adictionally, IGF-I, that has been previously revealed decreased in spinal cord after immobilization (Suliman et al., 2001),

has been found to exert neuroprotective functions, reducing programmed death of motoneurons during development, after axotomy and spinal cord transection (Lewis et al., 1993; Neff et al., 1993; Li et al., 1994).

5. Concluding remarks

To conclude, the present study demonstrates that immobility affects the normal developmental process that spinal cord undergoes in early postnatal life. Our data extend these results and demonstrate that locomotor stimulation may also take advantage in the developmental process related to spinal cord. Considering our results here and in our previous study, an altered plasticity in spinal cord may participate to the alteration of the motor performance reported in rats submitted to developmental disuse. This empathizes that rehabilitation strategies will be more efficient by taking into account the potential of spinal cord plasticity. A better knowledge of spinal cord plasticity occurring during disuse, such those observed in children with CP and other developmental conditions that induces inactivity, will enable suitable intervention strategies to promote functional recovery and/or to prevent disability to be developed. Further studies comprising the specific sites of plasticity that could include synaptic connections made by descendent fibers, interneurons interposed between descendent inputs and motoneurons, synaptic connections on motoneurons, and the motoneurons themselves should be addressed.

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Legends

Figure 1. Synaptophysin immunoreactivity in ventral horn of spinal cord were measured for each group and plotted. Control (CT), sensorimotor-restricted (SR), trained (TrCT) and sensorimotor-restricted trained (TrSR). Two-way ANOVA followed by Duncan test. Columns representmeans ± SEM.

* Different from CT, $P < 0.05$

Figure 2. Caspase-3 immunoreactivity in ventral horn of spinal cord were measured for each group and plotted. Control (CT), sensorimotor-restricted (SR), trained (TrCT) and sensorimotor-restricted trained (TrSR). Two-way ANOVA followed by Duncan test. Columns representmeans ± SEM.

* Different from CT, $P < 0.05$

Table 1. Day of first performance of neonatal developmental sensory-motor behaviors in control (CT), sensorimotor-restricted (SR) animals.

Values represented as means ± SEM.

∗ Mean significantly different from CT, $P < .05$.

† Mean significantly different from CT, $P \leq .01$.

Table 1.

Table 1. Day of first performance of neonatal developmental sensory-motor behaviors

Behavior	CT (n=7)	SR (n=8)
Surface righting	3.00±0.00	3.00±0.00
Cliff aversion†	4.71±0.70	9.25±0.67†
Negative geotaxis†	7.57±0.48	10.25±0.52†
Hind limb placing†	4.42±0.61	8.25±0.75†
Forelimb grasp	3.00±0.00	3.25±0.25
Activity*	7.14±1.16	11.12±0.54*

Values represented as means ± SEM

∗ Mean significantly different from CT, $P < .05$.

† Mean significantly different from CT, $P \leq .01$.

Figure 1.

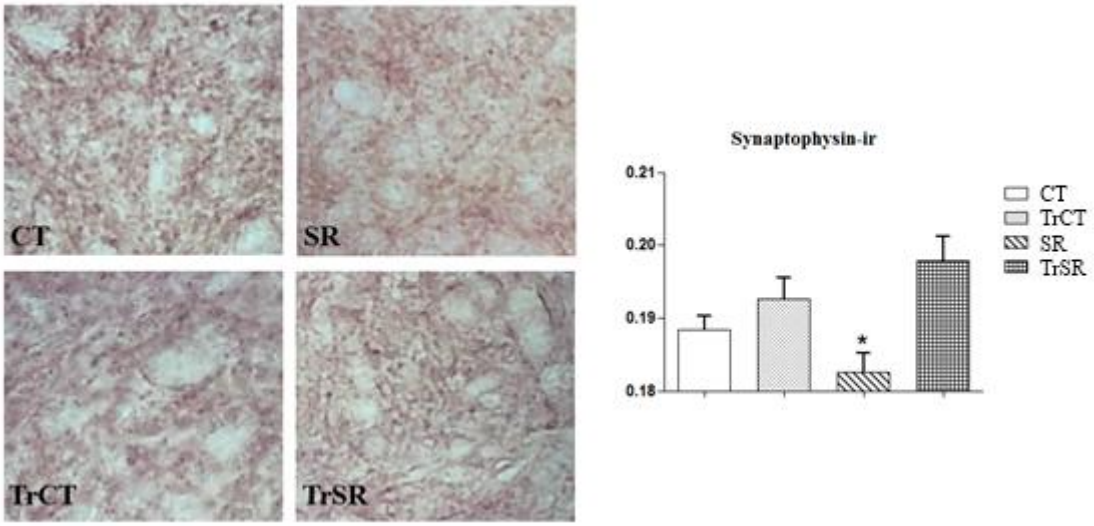
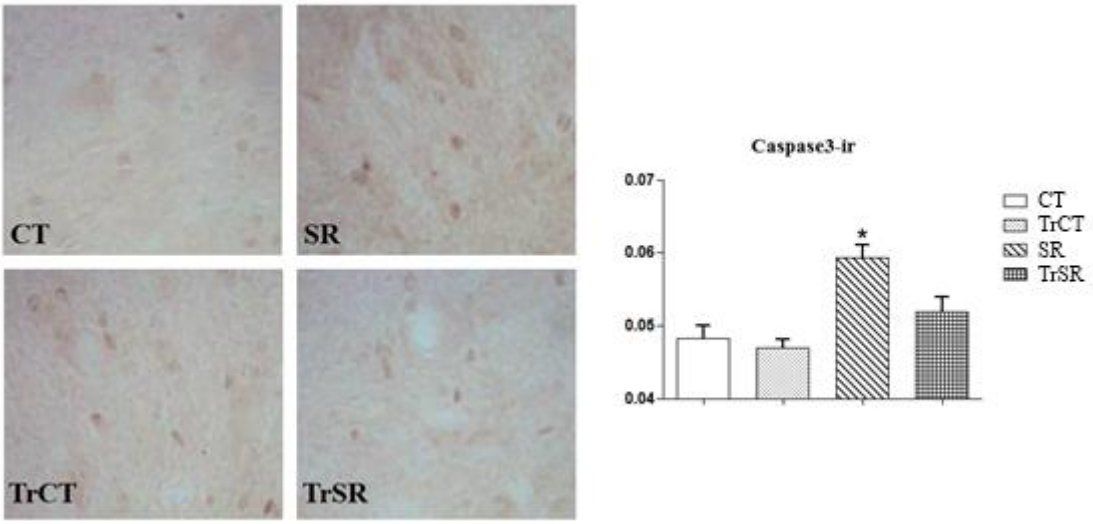

CT
SR
TrCT
TrSR
Synaptophysin-ir
0.21
0.20
0.19
0.18
*
CT
TrCT
SR
TrSR

Figure 2.



5. DISCUSSÃO GERAL

O delineamento de um modelo animal que inclua as características motoras da PC auxilia na melhor compreensão dos mecanismos fisiopatogênicos envolvidos na evolução da doença e os seus desfechos clínicos, além de fornecer uma possibilidade de testar e de desenvolver diferentes estratégias terapêuticas. Até então, os modelos animais com o intuito de mimetizar a PC não abrangem os principais achados clínicos e morfofisiológicos observados em humanos (JANSEN & LOW, 1996; HOEGER et al., 2000; ZHURAVIN, DUBROVSKAYA & TUMANOVA, 2004; LUBICS et al., 2005; POGGI et al. 2005; ROBINSON et al., 2005; ROBERSON et al., 2006). Tanto as infecções maternais, como a depleção na oferta de oxigênio e também o processo de desuso dos membros têm sido relacionados a alterações no SNC e subsequente PC. A combinação desses fatores causou o pior quadro clínico quando comparada às repercussões dos eventos isolados, vistos em um estudo anterior (STIGGER et al., 2011). Essa função motora deteriorada evidencia que cada evento tem um papel complementar na criação de substratos neuropatológicos envolvidos na gênese da PC. Diferentes modelos animais sugerem que a combinação de múltiplos insultos no período de desenvolvimento do SN é importante na elaboração de modelos animais que mimetizem achados mais condizentes com o contexto clínico (GIRARD et al., 2008; STRATA et al., 2004; COQ et al., 2008; MARCUZZO et al., 2008; EKLIN et al., 2001). Desta forma, esta tese teve como objetivo geral avaliar o efeito de distintos eventos agressivos (LPS, AP, RS), pertinentes ao contexto fisiopatológicoda PC, na gênese de um modelo animal capaz de reproduzir as alterações anatômicas, bioquímicas e funcionais semelhante à PC.

No primeiro capítulo apresentado nesta tese, foi explorado o envolvimento dos processos inflamatórios e a formação de radicais livres relacionados à inflamação pré-natal e à anóxia perinatal, assim como a repercussão nesses parâmetros da combinação

de ambos os procedimentos. O principal achado deste estudo foi o aumento de citocinas pró-inflamatórias e de radicais livres na combinação do LPS e AP, associadoa alterações na coordenação motora e ao atraso na aquisição de marcos de desenvolvimento motor. Embora os efeitos do LPS e AP isoladamente no comportamento motor não tenham sido tão evidentes, juntos eles parecem exercer um importante mecanismo fisiopatológico nos resultados encontrados. De fato, tanto as infecções maternais, como a asfixia perinatal, tem sido relacionadas com a síntese de citocinas pró-inflamatórias e/ou expressão de moléculas oxidativas em recém-nascidos (SÄVMAN et al., 1998, KOPP & MEDZHITOV, 1999; LIN et al., 2010; VASILJEVIC, 2011).

A expressão de citocinas inflamatórias e radicais livres tem sido investigada em diferentes modelos animais (SZAFLARSKI et al., 1995; CAPANI et al., 2001; ROUSSET et al 2006). Corroborando com outros estudos (SZAFLARSKI et al., 1995; CAPANI et al., 2001; ROUSSET et al 2006), nossos resultados mostraram que tanto a administração pré-natal de LPS como o episódio de anóxia, isoladamente ou em conjunto, resultam na liberação de citocinas pró-inflamatórias no córtex de ratos neonatos. Na presença de infecções ou qualquer estímulo inflamatório, a microglia e os astrócitos podem potencializar a expressão de citocinas pró-inflamatórias podendo induzir lesão cerebral (LEE et al., 1993; LIEBERMAN et al., 1989). Adicionalmente, nossos resultados ainda demonstram que, associado ao aumento na expressão de moléculas inflamatórias, a administração pré-natal de LPS foi capaz de desencadear a produção de moléculas de radicais livres que, tendo em vista a sensibilidade do tecido nervoso ao processo oxidativo, poderiam contribuir para lesão cerebral (MAYER, 1998).

Apesar de não ser abordada a análise de aspectos histológicos neste estudo, inúmeros experimentos clínicos sugerem o envolvimento destes agentes na lesão encefálica e o consequente desenvolvimento de PC (SÄVMAN et al., 1998, KOPP &MEDZHITOV, 1999; LIN et al., 2010; VASILJEVIC, 2011). De fato, os diferentes modelos animais envolvendo LPS e AP têm evidenciado alterações no SNC como a ativação microglial (PAINTLIA et al., 2004) e a astrogliose (CAI et al, 2000; YU et al., 2003a). Ademais, tanto o LPS como a AP causam lesão em substância branca (LIU et al., 2002; TOSO et al., 2005; ROUSSET et al., 2006) e morte neuronal em diferentes regiões encefálicas (DELL'ANNA et al., 1997; VAN DER BERG et al., 2003; VAN DER BERG et al., 2003; LING et al., 2004; ROUSSET et al., 2006) que poderiam explicar as alterações motoras encontradas neste estudo.

Diferente de outros modelos, baseados tanto em protocolos de administração de LPS no período embrionário como em asfixia perinatal, o modelo utilizado neste estudo causou atraso na aquisição de habilidades motoras e alterações envolvendo a coordenação que perduraram até o período correspondente a adolescência. Porém, embora claramente observadas disfunções relacionadas ao controle motor, animais expostos à combinação de LPS e AP não demonstraram inatividade, contrastando com a principal limitação observada na PC, fundamentalmente caracterizada pela perda das habilidades motoras associada à redução dos movimentos (JONES et al., 2007). Em humanos o dano encefálico que causa a PC desempenha um papel importante na limitação do movimento, seja pela alteração na via córtico-espinal, como na perda da inibição descendente (JONES et al., 2007). Em ratos, o possível dano encefálico resultante das alterações inflamatórias e de estresse oxidativo produzidas pelo LPS e/ou AP não foi suficiente para induzir tal comprometimento, tornando-se evidente a necessidade da restrição de movimento, induzida artificialmente, para o modelo

abranger características motoras mais consistentes com a PC (STRATA et al., 2004; MARCUZZO et al., 2008; STIGGER et al., 2011a).

Na segunda e terceira partes dessa tese, tendo em vista que a piora progressiva do quadro motor observado na PC (DAMIANO et al., 2006) possam não ser exclusivamente em decorrência de uma disfunção cerebral e sim, em parte, causada por modificações na circuitaria medular (DE LOUW et al., 2002), foi abordado o envolvimento da RS em criar alterações na medula espinal. O modelo de restrição utilizado no presente estudo possibilitou expandir o conhecimento em relação ao papel da inatividade no período pós-natal imediato no desenvolvimento da medula espinal. Como principal resultado destes estudos foi possível observar que 26 dias de restrição sensório motora limitou o desenvolvimento dos motoneurônios lombares, com redução do calibre do nervo ciático e alterações na expressão de sinaptofisina e caspase -3.

Durante o período de desenvolvimento, a medula espinal passa por um processo de transformação contínuo envolvendo tanto a maturação dos motoneurônios como formação de sinapses funcionais e a eliminação do número inicial de neurônios e sinapses não utilizadas (ALTMAN& BAYER, 1986; OPPENHEIM, 1991). Os motoneurônios são a "via final" na comunicação entre o sistema nervoso central e o músculo esquelético (TAKAZAWA et al. 2012). Durante seu desenvolvimento o motoneurônio se torna morfologicamente mais complexo, ocorrendo um aumento no tamanho do corpo celular com extensas ramificações dentríticas (ALTMAN & BAYER, 2001; CARRASCAL et al., 2005; LI, BURKE & ASCOLI, 2005). A diferenciação e maturação desses neurônios parecem ocorrer em decorrência de um processo dependente de atividade mediada por fatores neurotróficos como o fator neurotrófico derivado do cérebro (BDNF) e a neurotrofina 3 (NT-3; LOEB & FISCHBACH, 1997; D'COSTA et al., 1998). De fato, tanto o BDNF como a NT-3 são expressas nos

músculos e parecem transportados retrogradamente para o corpo celular de motoneurônios onde parecem exercer um papel importante no seu crescimento, ramificação e sobrevivência (LOEB & FISCHBACH, 1997). O período de maturação do sistema locomotor parece responder a diferentes demandas de atividade, sendo a atividade motora espontânea e os *inputs* proprioceptivos imprescindíveis para a maturação dos motoneurônios e desenvolvimento da locomoção em ratos (INGLIS et al., 2000; WESTERGA & GRAMSBERGEN, 1993). Nossos resultados indicam que a atividade muscular dos membros inferiores pode influenciar as características dos motoneurônios que os inervam, assim como o padrão da expressão de sinaptofisina na medula, uma proteína vesicular pré-sináptica encontrada em terminais nervosos e indicativa de plasticidade neuronal (WALAAS; BROWNING; GREENGARD, 1988).

Modelos animais de desuso sugerem que a inatividade não somente diminui o *feedback* proprioceptivo, ela também altera a expressão de neurotrofinas (SULIMAN et al., 2001) e fatores pró-apoptóticos (LAWSON & LOWRIE, 1998) que poderiam contribuir para alterações deletérias no processo de maturação dos motoneurônios assim como na expressão de sinaptofisina encontradas neste estudo. De fato, Suliman et al. (2001) demostraram a diminuição nos níveis de neurotrofinas na medula espinal após o período de quatro semanas de imobilização. Adicionalmente, a expressão de sinaptofisina também parece ser regulada de forma atividade-dependente induzida pela liberação de BDNF (TARSA & GODA, 2002; WANG, LIAO & LI, 2010) o que poderia estar relacionada a alteração na atividade sináptica na região abordada no estudo.

Como citado anteriormente, é normal o processo de apoptose durante o desenvolvimento da na medula espinal. Cisteína-proteases, incluindo a família das caspases, são consideradas as principais moléculas envolvidas neste processo (KUAN,

et al., 2000). Associada à diminuição na expressão de sinaptofisina, nosso estudo também demonstrou que a inatividade no período de maturação medular é capaz de induzir um aumento na expressão de caspase-3. O aumento do processo apoptótico é aumentando em modelos de desuso. Lawson & Lowrie (1998), a partir de uma lesão no nervo isquiático no segundo dia de vida pós-natal demostrou um aumento na morte celular na medula espinal. Já Qin-Wei et al., (1994) ao examinarem a sobrevivência celular de neurônios lombares em desenvolvimento, em decorrência da transecção torácica, observou uma perda de 25% do número total de neurônios. Adicionalmente, podemos inferir a participação neurotrofinas no aumento da morte celular. A expressão de fatores neurotróficos, como o BDNF e a NT-3, não está somente relacionada à maturação e desenvolvimento funcional das sinapses, tanto o BDNF como a NT-3 também apresentam um papel importante na sobrevivência celular (WANG, XEI & LU, 1995). O BDNF tem se mostrado eficaz em inibir a liberação de caspase-3, diminuindo o processo apoptótico e protegendo o SNC de possíveis lesões (HAN et al., 2000). Sabe-se que durante o processo de desenvolvimento normal, a diminuição de neurotrofinas parece relacionar-se com a fase de eliminação sináptica (FUNAKOSHI et al., 1995). O que podemos hipotetizar é que associado a este processo, a imobilização pode potencializar a depleção de neurotofinas já existente neste período, o que poderia indicar um aumento na morte celular em decorrência ao desuso do período pós-natal.

Somando-se aos estímulos ascendentes, oriundos de receptores periféricos, as informações descendentes, provenientes do córtex motor possuem importante função na maturação dos motoneurônios medulares (GIBSON, ARNOTT & CLOWRY, 2000). No período de desenvolvimento pós-natal, a maturação do sistema sensório motor é caracterizada pelo estabelecimento dos mapas corticais topográficos, o surgimento de conexões de longo alcance, além de acentuada plasticidade (KILLACKEY, RHOADES

& BENNETTCLARKE, 1995; LOPEZ-BENDITO& MOLNAR, 2003; JAIN et al., 2003). Em ratos, as vias córtico-espinais chegam à medula cervical apenas no terceiro dia pós-natal, e, somente ao final da segunda semana alcançam o nível lombar (DONATELLE 1977; JONES et al. 1982; STANFIELD et al., 1992; STANFIELD & O'LEARY, 1985). Tendo em vista que a atividade motora é imprescindível para refinar as conexões e estabelecer o padrão futuro de especificidade topográfica e de conexões do sistema motor (EYRE, 2007), doenças que afetem a motricidade no início da vida podem produzir efeitos deletérios sobre a maturação do sistema motor. De fato, a restrição dos movimentos espontâneos, no início do período pós-natal, pode contribuir para gerar impulsos sensoriais anormais ao SNC, resultando em informações sensoriais aberrantes e repetitivas e, dessa forma, contribuir para uma reorganização deletéria dos córtices motor e somatosensorial, e consequentemente piora no desempenho motor (COQ et al., 2008).

Com relação ao supracitado, torna-se coerente hipotetizar que a restrição sensório-motora, por induzir a inatividade em um período crucial de maturação do SN, interfere na expressão de neurotrofinas e fatores pró-apoptóticos, levando ao desenvolvimento anormal dos motoneurônios, bem como alteração no estabelecimento de sinapses funcionais. Desta forma, as alterações motoras da PC, inicialmente impostas pelas alterações de tônus muscular, não parecem ser exclusivamente devido à disfunção cerebral, mas sim, ser um processo progressivo, decorrente de um conjunto de alterações neurais, tanto descendentes, como ascendentes e que envolvem anormalidades na circuitaria medular (DE LOUW et al., 2002).

Outro resultado importante desta tese foi que o padrão de alteração medular relacionado à RS é modificável com o incremento da atividade motora. Esses dados indicam que a estimulação locomotora pode influenciar a maturação dos motoneurônios

e aumentar a atividade sináptica, o que pode estar relacionado a uma plasticidade neuronal mais funcional e eficaz. As hipóteses relacionadas à expressão de fatores neurotróficos também podem ser aplicadas às mudanças observadas em decorrência da estimulação locomotora. O exercício aumenta a expressão de diferentes fatores neurotróficos, como o fator de crescimento semelhante à insulina tipo 1 (IGF-1; ELIAKIM et al., 1997; HEO et al., 2001), BDNF e a NT-3 (GÓMEZ-PINILLA et al., 2001; ILHA et al, 2011), ambos relacionados à formação sináptica e sobrevivência celular (HAN et al., 2000; LEWIS et al., 1993; NEFF et al., 1993; LI et al., 1994) além de possivelmente inibir a expressão de caspases (HAN et al., 2000). Nossos resultados sugerem a possibilidade que estratégias de reabilitação, como a estimulação locomotora, têm a capacidade de modular o potencial plástico da medula espinal de crianças com PC e de inibir os efeitos deletérios causados pelo desuso. Desta forma, possibilitando discutir seu envolvimento na melhora do quadro motor observado utilizando-se a estimulação locomotora na prática clínica na PC a partir do aumento da formação sináptica e depleção dos níveis de apoptose encontrados no presente estudo.

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6. CONCLUSÕES E PERSPECTIVAS

Os resultados apresentados nesta tese nos permitem concluir que:

- A inflamação no período gestacional e a anóxia perinatal possuem papéis distintos e sinérgicos no processo inflamatório e de estresse oxidativo cortical.
- O aumento de citocinas inflamatórias e espécies reativas de oxigênio podem estar relacionados ao atraso na aquisição de habilidades motoras e no comportamento motor aberrante, observado no período da adolescência quando os eventos de inflamação gestacional e de anóxia perinatal são combinados.
- A inatividade no início do período pós-natal retarda o processo de maturação dos motoneurônios medulares e do nervo ciático.
- O aumento da caspase-3 e diminuição de sinaptofisina no corno ventral da medula espinal induzidos pelo desuso corroboram os achados de alteração na maturação dos motoneurônios medulares também vistos nesse procedimento.
- A estimulação locomotora em esteira ergométrica é capaz de reverter as alterações encontradas na medula espinal induzidas pelo período de inatividade.

Por fim, as perspectivas desse estudo estão relacionadas a analisar regiões encefálicas ligadas ao controle motor, como o córtex motor, córtex somatossensorial, o estriado desta forma tentando esclarecer os mecanismos responsáveis pelos distúrbios motores encontrados no modelo composto pela associação do LPS, AP e RS a nível encefálico.

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