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ANÁLISES COMPORTAMENTAL E MORFOLÓGICA DA ASSOCIAÇÃO DE EXPOSIÇÃO PRÉ-NATAL À LIPOPOLISSACARÍDEO E ASFIXIA INTRAUTERINA

Dissertação de Mestrado

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Aos meus amores
Federico D'Aurizio, Rodrigo Raio Timmen Raimundo
À memória de Rodrigo Raimundo

“A vida não é a que a gente viveu e sim a que a gente recorda, e como recorda para contá-la.”

Gabriel García Marques

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RESUMO

A exposição do encéfalo imaturo a uma infecção maternal e asfixia perinatal podem predispor ao desenvolvimento da paralisia cerebral (PC) em humanos. A indução desses eventos lesivos isoladamente em roedores não reproduz os déficits motores, cognitivos e morfológicos observados em crianças com PC. Assim, o presente estudo teve como objetivo verificar se a associação desses eventos, inflamação pré-natal por lipopolissacarídeo (LPS) e asfixia intrauterina, produz um fenótipo mais semelhante ao da PC em ratos. Injeções de LPS ou de solução salina estéril foram administradas por via intraperitoneal em ratas prenhas entre o décimo sétimo e vigésimo primeiro dia gestacional. No vigésimo segundo dia de gestação, o procedimento de asfixia intrauterina foi realizado em parte dos animais. Assim, os filhotes foram divididos em quatro grupos: Controle (CT), Asfixia (A), LPS e LPS-Asfixia (LA). A partir do terceiro dia pós-natal (P3) até o P14, os filhotes foram avaliados quanto ao desenvolvimento neuromotorneonatal utilizando a avaliação da aquisição de marcos do desenvolvimento. No P29, a função motora e cognitiva desses animais foi observada no *rotarod,* no *grip test* e no teste de reconhecimento do objeto reposicionado. Os filhotes foram submetidos à eutanásia no P30 para a obtenção de amostras encefálicas. A imunorreatividade para proteína básica de mielina no estriado, e para caspase-3 e sinaptofisina na região hipocampal do giro denteado foi analisada. Os animais dos grupos LPS e A apresentaram menor ganho de peso corporal quando comparados ao controle. O fator LPS sozinho ou em associação com a asfixia retardou o desenvolvimento da resposta auditiva de sobressalto e proprioceptiva de colocação do membro posterior, mas acelerou a resposta de preensão do membro anterior. Nenhum dos grupos apresentou déficits nos testes motores e no teste de reconhecimento do objeto reposicionado. Não houve diferença significativa na imunorreatividade para proteína básica da mielina no estriado em nenhum dos grupos estudados. Nos grupos LPS e LA houve uma redução na expressão de sinaptofisina no giro denteado,a qual foi associada a um aumento na expressão de caspase-3 nesta mesma região no grupo LA. Apesar de ter causado danos e uma redução na atividade sináptica hipocampal, o modelo experimental avaliado neste estudo não alterou o desenvolvimento neuromotorneonatal dos animais e não afetou as funções motoras e cognitivas avaliadas. Desta forma, a associação da inflamação pré-natal e asfixia intra-uterina

utilizadas nesse estudo, não foram capazes de reproduzir características comportamentais e/ou deficitis funcionais encontrados em modelos de PC em ratos.

ABSTRACT

The exposure of the developing brain to injuries such as maternal infections and perinatal asphyxia can induces permanent neurological deficits which may be potentially similar to those observed in cerebral palsy (CP). Since motor deficits observed in patients with CP are poorly reproduced in experimental models, the aim of this study was to examine if the combination of prenatal inflammation caused by lipopolysaccharide (LPS) and intrauterine asphyxia reproduces in rats functional and morphological changes similar to those found in patients with CP. Pups were divided in 4 groups: Control (CT), Asphyxia (A), LPS-injected (LPS) and LPS-injected plus asphyxia (LA). Intraperitoneal LPS (in LPS and LA groups) or sterile saline (in CT and A groups) injections was performed in pregnant rats between the 17th and 21st gestational days. At the 22nd gestational day, the asphyxia procedure was carried out in A and LA groups. All animals were weighed daily during the first 14 postnatal days (P14) and at P30. The developmental milestones were assessed between the P3 to P14. Animal's motor skills were evaluated on the rotarod and grip test at P29. The repositioned object test, used to evaluate spatial memory, was also performed at P29. In the following day, the animals were euthanized and brain samples were collected. The caspase-3 and synaptophysin immunoreactivity in the dentate gyrus (DG) region of the hippocampus and myelin basic protein immunoreactivity in the striatum were analyzed. LPS and A groups showed less weight gain compared to control animals. LPS factor alone or in association with asphyxia delayed the acquisition of audio startle response and hindlimb proprioceptive placement, but accelerated the appearance of the forelimb grasp. No motor deficits were observed on rotarod and grip test. Deficits in spatial memory were also not verified. The DG morphological analysis showed that the LPS and LA group had a reduction in synaptophysin expression. Caspase-3 expression increased in DG of LA group compared to the control one. In striatum, no differences in mielin basic protein immunoreactivity was observed between the groups. Although the experimental model used increased cell death and decreased the synaptic activity in hippocampus, only subtle changes were observed in motor behavior abilities of the animals. Besides, the experimental approach used did not affect the myelin in the striatum, an

important region for motor control. Thus, the association of prenatal inflammation and intrauterine asphyxia, in association or alone, not shown to be capable of reproducing a PC model in rats.

SUMÁRIO

LISTA DE ABREVIATURAS

DO	Densitometria Óptica
E	Dia Embrionário
P	Dia Pós-natal
CRF	Fator de Liberação de Corticotrofina
TNF	Fator de Necrose Tumoral
GD	Giro Denteado
GDs	Glicocorticóides
IL	Interleucina
LPS	Lipopolissacarídeo
LPV	Leucomácia Periventricular
NPY	Neuropeptídeo Y
OL	Oligodendrócitos
PC	Paralisia Cerebral
POMC	Pro-opiomelanocortina
PG	Prostaglandina
GFAP	Proteína Ácida Fibrilar Glial
MBP	Proteína Básica de Mielina
TLR	Receptor Tipo Toll
SYP	Sinaptofisina
SNC	Sistema Nervoso Central

LISTA DE FIGURAS

1 INTRODUÇÃO

A paralisia cerebral (PC) pode ser conceituada como um grupo de desordens da postura e do movimento, atribuídas a lesões persistentes e não progressivas que comprometem o desenvolvimento encefálico normal (Rosenbaum, 2007; Bax et al., 2005). Esta patologia afeta de 1 a 3 crianças a cada 1000 nascidos vivos (Nelson, 2002; Grether, Cummins e Nelson, 1992). O dano ao sistema nervoso pode ocorrer durante o período gestacional, no momento do parto ou nos dois primeiros anos de vida (Koman, Paterson e Shilt, 2004).

Alterações no padrão de maturação do encéfalo podem afetar as funções sensoriais, cognitivas e comportamentais, prejudicando principalmente o controle motor. Alterações musculoesqueléticas também podem ocorrer como consequência dos déficits no controle motor (Bax et al., 2005). O grau de comprometimento motor observado em indivíduos com PC tende a ser proporcional à gravidade das lesões encefálicas. Os substratos neurobiológicos encontrados mais frequentemente na PC incluem danos na substância branca, como a leucomalácia periventricular (LPV) (Volpe, 2000), hemorragia intraventricular e lesões no córtex, nos núcleos da base, no cerebelo e no tálamo (Folkberth, 2005; Kadhim et al., 2005). Acredita-se que a patogênese da LPV é baseada na interação de três fatores: asfixia perinatal (Yager e Ashwal, 2009; Younkin, 1992; Hill, 1991; Hill e Volpe, 1981), inflamação-infecção sistêmica (Yoon et al., 2003) e a vulnerabilidade intrínseca das células precursoras de oligodendrócitos que mielinizam o sistema nervoso central (SNC; Volpe, 2001).

A sinaptogênese é o substrato para comunicação celular neuronal. Em humanos, esta etapa do desenvolvimento do SNC tem início nos primeiros meses de gestação, atinge o pico entre 1-2 anos de idade e segue até a adolescência (Huttenlocher, 1987). Estudos mostram que disfunções sinápticas são importantes indicadores de declínio cognitivo (Coleman et al., 2004; Lacor et al., 2007). A sinaptofisina (SYP) é uma glicoproteína integral de membrana presente nas vesículas pré-sinapticas de neurônios. O aumento da imunorreatividade para esta glicoproteína é indicativo de integridade sináptica, fundamental para a neuroplasticidade (Ding et al., 2002).

De acordo com o tipo de disfunção motora, a PC pode ser classificada como espástica, discinética, atáxica, hipotônica e mista (Olney et al., 1995). Conforme a

distribuição topográfica de envolvimento dos membros, pacientes com PC podem ser hemiplégicos, diplégicos ou quadriplégicos (Peterson et al., 1998; Olney et al., 1995). A diplegia é o quadro clínico encontrado mais frequentemente, sendo associada a lesões em áreas periventriculares e subcorticais da substância branca (Romeo e col. 2008; Voorman et al., 2007; Ostenjo et al., 2004). A complexa etiologia desse distúrbio pode explicar a variabilidade das manifestações clínicas na PC.

Em mais de 30% dos casos de PC os fatores de risco que levaram ao desenvolvimento dessa condição são desconhecidos (Rosenbaum, 2003; Taft, 1999). Entretanto, a asfixia intraparto é tradicionalmente considerada uma importante causa da PC (Yoon et al., 2013). O feto pode sofrer episódios de asfixia severa próximo do período do nascimento devido a vários fatores que incluem a compressão do cordão umbilical, danos à placenta que comprometem o fluxo sanguíneo útero-placentário, contrações uterinas anormais, parto prematuro ou prolongado e incapacidade do neonato de iniciar a ventilação (Haan et al., 2006).

Bjelke e colaboradores (1991) desenvolveram um modelo em roedores de asfixia perinatal induzida por meio de uma cesárea seguida pelo clampeamento de um corno uterino contendo os filhotes. Essa estrutura é mantida a uma temperatura semelhante à corporal por determinados períodos de tempo. Este método é relativamente não invasivo e tem sido amplamente aplicado com algumas modificações (Morales et al., 2010; Saraceno et al., 2010; Strackx et al., 2010; Capani et al., 1997, 2001, 2003, 2009; Cebral et al., 2006; Weitzdoerfer et al., 2004; Boksa e El-Khodor, 2003; Chen et al., 1995), uma vez que mimetiza a ocorrência de uma asfixia no momento do parto.

Em neonatos, poucos minutos após o insulto isquêmico inicia-se uma resposta imune inata (Algra et al., 2013). São verificadas duas fases de morte neuronal em avaliações clínicas e modelos animais (Penrice et al., 1996; Gluckman e Williams, 1992): - a fase primária ocorre no momento do insulto, e; - a fase secundária após um período de latência de no mínimo 6 horas (Inder e Volpe, 2000). Na fase primária, a saturação de oxigênio no sangue é reduzida, alterando a via metabólica aeróbica para anaeróbica, com acúmulo de lactato nos compartimentos extracelulares, acidose e morte celular (Wyss et al., 2011). Em insultos severos, a perda neuronal na fase secundária é significativa e os mecanismos envolvidos incluem excitoxicidade, ativação microglial e apoptose (Yenari et al., 2010; Inder e

Volpe, 2000; Figura 2). Nessas condições, a permeabilidade da membrana mitocondrial é aumentada, favorecendo a liberação de fatores pró-apoptóticos que ativam a caspase-3, resultando em apoptose neural (Pacher, Beckman, Liaudet, 2007; Chen et al., 2005; Daval et al., 2004). As estruturas encefálicas mais comprometidas pela asfixia são: o hipocampo, o córtex cerebral e os núcleos da base (sobretudo o corpo estriado; Loidl et al., 2000). Essas regiões estão envolvidas no processamento de informações motoras e emocionais, além do aprendizado e memória (Galeano et al., 2011).

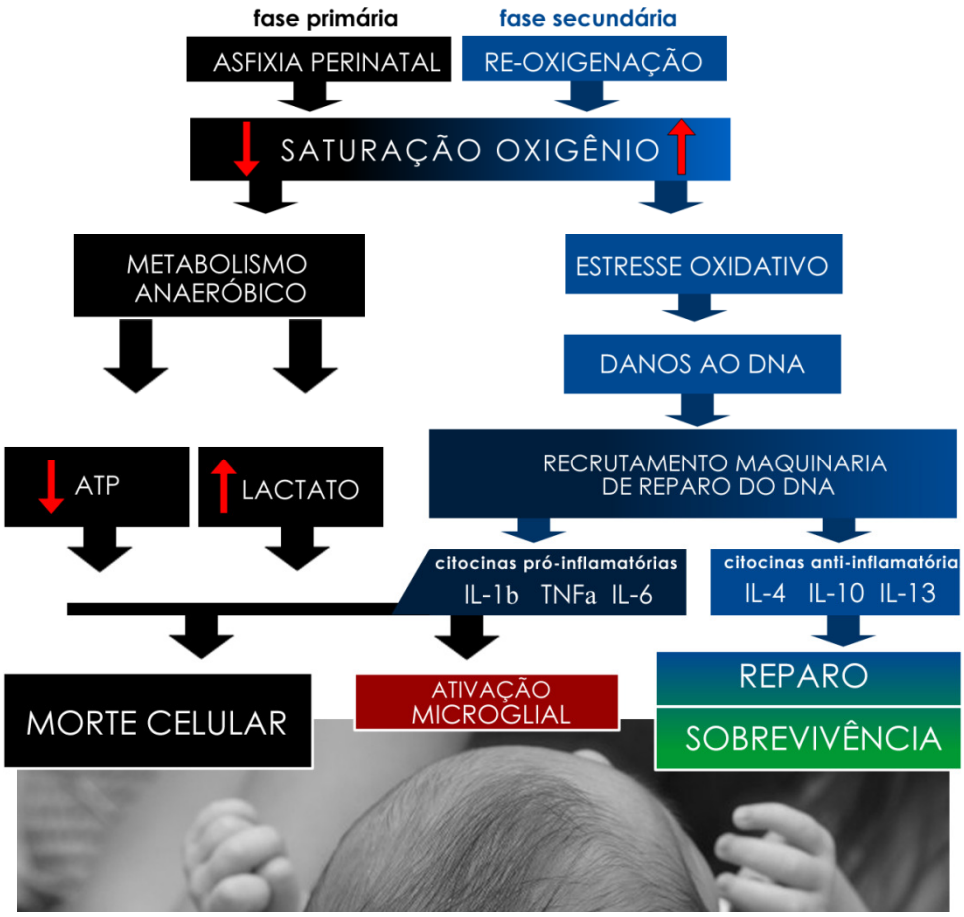

Figura 1. Representação esquemática das fases primária e secundária de morte neuronal após uma asfixia perinatal e os mecanismos de reparo subsequentemente ativados. Adaptado de Herrera-Marschitz e colaboradores (2014)

Estudos mais atuais indicam que a asfixia está relacionada apenas com uma fração dos casos de PC, revelando que outros fatores como infecções/inflamações intrauterinas também podem desempenhar um importante

papel na gênese desta desordem (Yoon et al., 2013). Uma resposta inflamatória fetal desencadeada por infecções maternais, como a corioamnionite, pode provocar danos ao encéfalo fetal durante o período perinatal (Leviton et al., 2010; Nelson e Chang, 2008; Bracci e Buonocore, 2003). Além disso, casos de corioamnionite podem se agravar, uma vez que o início dessa doença é frequentemente subclínico e sem sinais aparentes de infecção placentária (Leviton et al., 2010; Russel, 1979). Os efeitos de uma infecção materna sobre o SNC do feto têm sido reproduzidos experimentalmente em roedores usando abordagens imunogênicas com a administração de lipopolissacárideo (LPS), vírus influenza ou ácido policitidilico (poli IC). Esses agentes são aplicados em diferentes estágios da gestação e por meio de vias de inoculação variadas (Boksa, 2010). O LPS é uma endotoxina presente em bactérias Gram-negativas que estimula fortemente a resposta imunológica inata de animais sadios. Quando administrado por via sistêmica, o LPS prejudica o desenvolvimento normal do encéfalo fetal de maneira indireta a partir da sua ligação a receptores tipo Toll-4 (TLR-4) em células imunes (Ashdown et al., 2006; Goto et al., 1994). Esta ativação desencadeia uma cascata de sinalização que inclui a síntese e liberação de mediadores pró-inflamatórios, como as citocinas (Aderem e Ulevitch, 2000; Luheshi, 1998). Estas citocinas interagem com alvos específicos no encéfalo materno, provocando febre, anorexia e aumento na liberação de glicocorticóides (GCs; Roth et al., 2009). Os mediadores inflamatórios maternos causam disfunções na placenta e apoptose do trofoblasto (Haider and Knöfler, 2009; Kakinuma et al., 1997). Além disso, estes mediadores também prejudicam o desenvolvimento normal do encéfalo fetal (Liverman et al., 2006; Paintlia e col., 2004; Gayle et al., 2004; Cai et al., 2000), podendo resultar em déficits comportamentais na prole no período pós-natal (Figura 2).

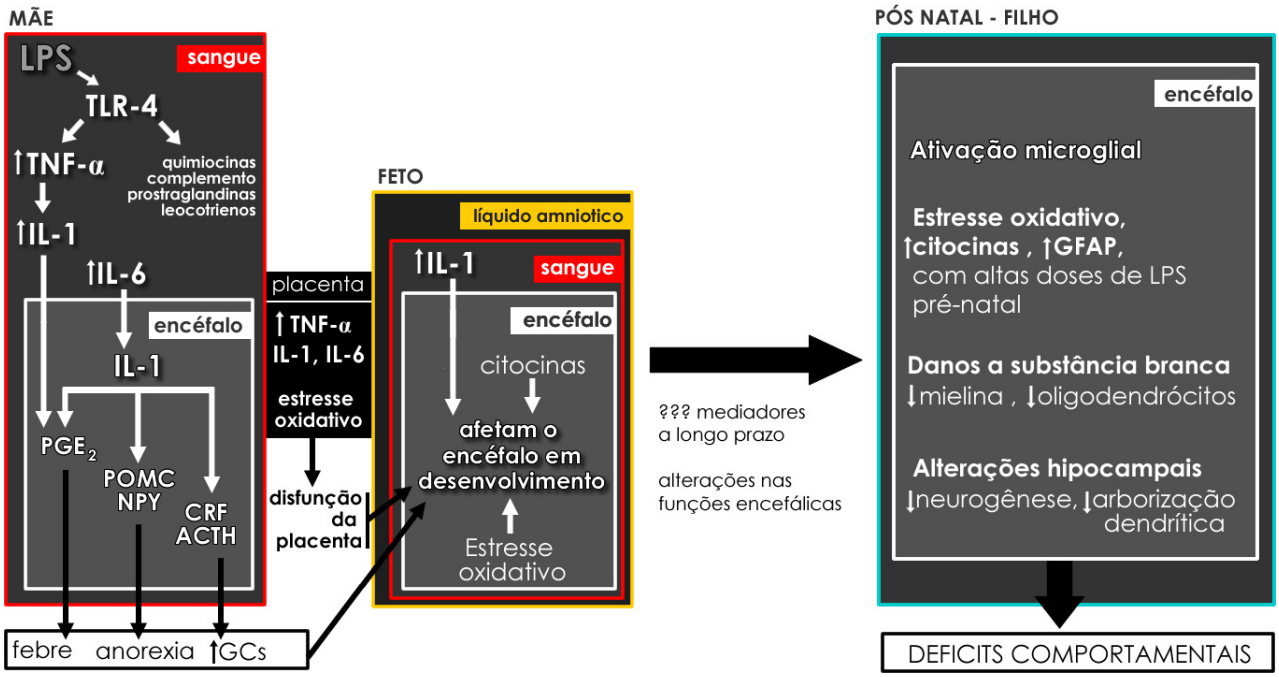

Figura 2. Possível mecanismo mediador dos efeitos de uma infecção pré-natal sobre as funções encefálicas fetais. CRF, fator de liberação de corticotrofina; GCs, glicocorticóides; GFAP, proteína glial fibrilar ácida; IL, interleucina; LPS, lipopolissacarídeo; NPY, neuropeptídeo Y; PGE, prostaglandina E; POMC, pro-opiomelanocortina; TLR, receptor toll-like; TNF, fator de necrose tumoral. Adaptado de Boksa (2010)

1.1 Modelos experimentais de paralisia cerebral

Diversos modelos experimentais, baseados na exposição de animais a fatores de risco associados à PC, têm sido desenvolvidos com o intuito de reproduzir as lesões encefálicas e os déficits funcionais encontrados em pessoas com esta patologia. Por exemplo, Toso e colaboradores (2005) induziram uma inflamação pré-natal pela administração intrauterina de LPS, simulando uma infecção do trato urogenital durante a gestação, e verificaram um atraso de alguns parâmetros do desenvolvimento sensório-motor dos filhotes. Porém, na idade adulta, os animais não apresentaram déficits motores (Toso et al., 2005). Outro modelo experimental expôs os animais à privação de oxigênio nas primeiras horas de vida, por meio de uma anóxia perinatal com 100% de N_2, durante 25 minutos, em uma câmara fechada (Dell Anna et al., 2000). Os animais submetidos a esse procedimento apresentaram hiperatividade motora transitória, embora o desenvolvimento motor não tenha sido

alterado. Todavia, esses animais apresentaram déficits de memória espacial, quando avaliados aos 45 dias de vida.

Como a maioria desses estudos reproduz somente aspectos sutis da PC, a associação de fatores de risco também tem sido uma estratégia para tentar aproximar os modelos animais do quadro clínico visto em humanos. A indução de uma resposta inflamatória pela exposição de ratas prenhas a injeções intraperitoneais de LPS no período embrionário combinada a uma hipóxia-isquemia induzida pela ligadura permanente da artéria carótida comum 24 horas após o parto, causou déficits motores e lesões corticais e subcorticais mais extensas quando comparadas àquelas produzidas pelos procedimentos isoladamente (Girard et al., 2009). Usando um modelo similar de PC, Hu e colaboradores (2013) também mostraram que esses procedimentos causam prejuízos motores, alterações cognitivas e anormalidades no desenvolvimento do sistema nervoso.

Entretanto, uma limitação desses modelos experimentais que utilizam vários fatores de risco associados à PC tem sido a falta de distúrbios motores permanentes como os observados em humanos. Stigger e colaboradores (2011) propuseram um modelo em ratos associando dois fatores de risco da PC (infecção materna por LPS e anóxia perinatal) associados à restrição sensório-motora (RSM). Essa restrição baseia-se na contenção dos membros posteriores dos animais entre o segundo e vigésimo oitavo dia pós-natal. A avaliação da repercussão individual destes fatores (LPS, anóxia perinatal e RSM), mostrou que esse último é imprescindível para o estabelecimento de alterações motoras de longo prazo para um modelo experimental de PC.

A variedade dos modelos descritos até o presente momento representa a própria heterogeneidade desta patologia. Porém, os déficits comportamentais e neurobiológicos descritos até o momento pelos modelos de PC atuais não têm reproduzido de forma significativa a multiplicidade de déficits observados nessa patologia. Assim, o estudo da associação de uma inflamação pré-natal e asfixia intrauterina pode contribuir para a criação de um modelo de PC com fenótipo mais semelhante ao observado em humanos com essa condição clínica.

2 Objetivos

2.1 Objetivo Geral

Investigar as repercussões da exposição de filhotes de ratos *Wistar* a inflamação pré-natal e asfixia perinatal, isoladamente ou em combinação, sobre parâmetros motores, cognitivos e morfológicos.

2.2 Objetivos Específicos

a) Avaliar o desenvolvimento neuromotor de filhotes de ratos submetidos a inflamação pré-natal e asfixia perinatal, utilizando como parâmetro de avaliação a aquisição de marcos do desenvolvimento;
b) Verificar o equilíbrio e a coordenação de filhotes de ratos submetidos a inflamação pré-natal e asfixia perinatal utilizando o *rotarod*;
c) Analisar os efeitos da inflamação pré-natal e asfixia perinatal sobre a destreza dos membros anteriores de filhotes de ratos por meio do *grip test*;
d) Avaliar a memória espacial de filhotes de ratos submetidos a inflamação pré-natal e asfixia perinatal utilizando o teste de reconhecimento do objeto reposicionado;
e) Avaliar os efeitos da inflamação pré-natal e da asfixia perinatal sobre a expressão de um marcador de morte celular (através da imunomarcação para caspase-3) e sobre a plasticidade sináptica (através da imunomarcação para sinaptofisina) no hipocampo de filhote de ratos;
f) Avaliar a mielina no corpo estriado de filhotes de ratos submetidos a inflamação pré-natal e asfixia perinatal por meio de imunoistoquímica para proteína básica de mielina.

3 RESULTADOS

3.1 Artigo - **Prenatal exposure to LPS and intrauterine asphyxia does not change the neonatal development and cognitive function nor myelination in the striatum, but causes cell death and impaired synaptic plasticity in the hippocampal DG in rats.** *Maria Augusta Timmen Raimundo, Ethiane Segabinazi, Samir Khal de Souza, Silvia Barbosa, Otávio Américo Augustin, Marilia Rossato Marques, Francele Valente Piazza, Matilde Achaval, Simone Marcuzzo.*

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Prenatal exposure to LPS and intrauterine asphyxia does not change the neonatal development and cognitive function nor myelination in the striatum, but causes cell death and impaired synaptic plasticity in the hippocampal DG in rats.

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Keywords: Cerebral palsy; Lipopolysaccharide; Maternal infection; Intrauterine asphyxia

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Abstract

Introdution: Aggressive events that occur during the prenatal, perinatal and postnatal periods, are the main risk factors that the complex multifactorial etiology of CP imples.

However, certain functional changes commonly observed in patients with CP are poorly mimicked in experimental models of this disease in rats.

Objectives: The aim of the present study was examine if the combination of prenatal inflammation by lipopolysaccharide (LPS) and intrauterine asphyxia plays in rats some aspects of the CP patients's fenotype.

Methods: This study was approved by the Ethics Committee of UFRGS (18450). LPS or saline was administered i.p. in pregnant rats between the 17th and 21st gestational day and on the 22nd intrauterine asphyxia was performed. The rat pups was divided in four experimental groups: Control (CT), Asphyxia (A), LPS and LPS-Asphyxia (LA). The pups were weighed daily between the 1st and 14th postnatal day (P1 to P14) and sensorimotor development was assessed by the Developmental Milestones between the P3 to P14. On the P29, the animals were subjected to evaluations (of balance on the rotarod), (muscle strength in the grip test) and and cognition (on the novel object-placement recognition task - NOPR). The expression of caspase-3 and synaptophysin in the dentate gyrus region of the hippocampus and myelin basic protein in the striatum were analyzed by optical densitometry in software Image Pro Plus 6.0 by using images from immunohistochemistry.

Results: Animals exposed to LPS and A treatments had less weight gain in the first two weeks of life compared to controls: LPS ($p < 0.001$) and A ($p = 0.004$, respectively). At 30 days old, only LPS factor reduced the body weight of animals ($p < 0.001$).The LPS factor alone or in association with asphyxia delayed the development of startle response ($p < 0.01$) and hindlimb proprioceptive placement ($p = 0.03$), but accelerated the appearance of the forelimb grasping ($p = 0.004$). No deficits were observed in LPS, A and LA groups in the rotarod, grip test and NOPR. About the morphological analyses, LPS and LA groups howed a reduction in the it synaptophysin expression in the dentate gyrus ($p = 0.0001$). In the LA group an increase in the caspase-3 expression the dentate gyrus was observed ($p < 0.001$)

compared to the others groups (CT, A, LPS). There was no difference in the mielin basic protein immunoreactivity among the groups.

Conclusion: Although have caused cell death and impairment in the synaptogenesis, combined with intrauterine asphyxia caused only subtle changes in the development, not being useful as a model of CP.

INTRODUCTION

Cerebral palsy (CP) is the most common motor disability in childhood. In developed countries, it is estimated that 1.5 newborns per 1.000 will be affected by the moderated form of CP, 2.5 the severe one. In developing countries, that number raises to 7 cases to each 1.000 newborns (Stanley et al., 2000; Winter et al., 2002). This disorder has no cure, and despite not being progressive, the motor deficit limits individual activities and induces to sedentary habits that end up by increasingly the damaging motor function and quality of life (Marques et al., 2014; Damiano et al., 2006).

By definition, the term Cerebral Palsy (CP) describes a group of movement and posture disorders that causes activity limitation and are attributed to non progressive lesions in the developing fetal or infant brain (Bax et al, 2005; Rosenbaum, 2007). The damage can occur in utero, during childbirth, or in the first 2 years of life (Koman, Paterson, Shilt, 2004).

Due to its multifatorial etiology, different types of injuries can cause CP and sometimes those injuries can be associated together during the prenatal, perinatal and postnatal periods. The prenatal period seems to be the most critical period to the development of brain injuries, since 70% up to 80% of the registred cases of CP had a history of exposure to risk factors during this time of neurological development (Johnston & Hoom, 2006; Krigger, 2006).

The neuropathological substrate of CP includes periventricular leukomalacia, intraventricular hemorrhage, and damage to cortex, basal ganglia, cerebellum and thalamus (Folkberth, 2005; Kadhim et al., 2005). Among the many events that predisposes to CP and affect the development of these structures are the maternal inflammation, perinatal asphyxia, trauma and meningitis (Johnston & Hoom, 2006; Krigger, 2006, Blair & Watson, 2005).

The prenatal inflammation can lead to a fetal inflammatory syndrome, which can inflict damage on the white matter, and that is related to motor and cognitive deficits (Burd et al, 2012). Experimentally, gestational inflammation can be induced by exposition to lipopolysaccharides (LPS), a structural constituent of most gram-negative bacteria. LPS activates the innate immune system through interaction with toll-like receptors (TLRs) which leads to 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; Stigger et al., 2011). The increase of pro-inflammatory cytokines, the microglial and astroglial activations and the lack of oligodendrocytes maturation are some of the consequences derivated from prenatal inflammation, which culminates in the hypomyelination and increase of non-mature brain vulnerability to others perinatal injuries, such as hypoxia-ischemia (Volpe et al., 2011; Coumans et al., 2003). Infections on gestational period are associated to perinatal asphyxia, and as a matter of fact, chances of these children develop CP increases on 70% (Coumans et al., 2003).

Perinatal asphyxia is also another important factor that causes neonatal deaths and irreversible damage to the brain (de Haan et al., 2006; Morales et al., 2011). The severe asphyxia can lead to severe disorders such as CP, dystonia and epilepsy, while episodes of moderated asphyxia can lead to cognitive and attention disfunctions (Calamandrei et al., 2004). Among brain structures that are compromised by asphyxia are the hippocampus, the cerebral cortex and basal ganglia, especially the striate nucleus (Loidl et al., 2000). Those parts are involved in motor, emotional processes, memory and learning (Galeano et al., 2011).

The animal models of CP that have been used are very heterogeneous, just as much as the patology itself (Johnston & Hoom, 2006), and there is also variations between the kind of intervention and the animal species used (Stigger et al., 2011). According with these protocols, distinct characteristics are reproduced in each model, which indicates that the animal models are complementary. However, the studies that used inflammation during gestational period combined, or not, with perinatal anoxia or neonatal hypoxia-ischemia as animal model in rats, produced only slight and transitory motor alterations (Lubics et al., 2005; Poggi et al., 2005, Toso et al., 2005; Girard et al., 2009).

Seen it, it is necessary to establish an animal model of CP that covers a wider range of characteristics observed on clinical cases, once the lack of an animal model that induce severe functional deficits represents an obstacle on the development of prophylactic and therapeutic strategies (Derrick et al., 2007; Marcuzzo et al., 2010).

A large number of studies had been searching for an association between aggressors in order to cause synergistic deleterious effects and, with that, produce a phenotype that resembles CP (Strata et al., 2004; Girard et al., 2009). Studies about the effects of association of LPS exposure during embryonic period and perinatal intrauterine asphyxia in rats were not found. On this context, the objective of this study was provide an analysis if the prenatal inflammation induced by LPS associated to intrauterine asphyxia produces in rats the typical phenotype of CP. For this, evaluated the development of pups, using as the parameter the acquisition of developmental milestones; the balance and coordination, using the rota rod; dexterity of the forelimbs, using the *grip test*; the spatial memory, through the repositioned object recognition test. Using immunohistochemistry, analyzed the expression of the cell death marker (caspase-3) and the synaptic plasticity (synaptophysin) in the hippocampal GD and the myelin in the striatum (myelin basic protein).

MATERIALS AND METHODS

CP Animal Model Induction

All procedures performed on this study were approved by the Universidade Federal do Rio Grande do Sul (UFRGS) Ethical Committee (number 18450). The animals, 12 males and 12 females Wistar rats with 60 days of age, were provided by a local breeding colony. Animals were maintained in a controlled temperature ambient (20 ± 2 ºC), with a dark/light cicle of 12 hours and food and water *ad libitum*, according to the Law nº 6638 of 8/5/79 that regulates the use of animals for teaching and scientific practice. All efforts were done to minimize animal suffering as well as to reduce the number of animals. Prior to experiments, the animals were left undisturbed for 7 days and from there, they were mated to obtain offspring.

The prenatal inflammation was induced according to Girard et al., (2009). Thus, from the 17th to the 21th day of gestational period (E17 a E21) half of the pregnant rats (n = 3) received LPS injections i.p. (200 µg/kg diluted in 100 µl of saline, Sigma, USA), while the other female rats (n = 3) received only the vehicle (100 µl of saline) every 12 hours.

In the last day of gestational period (E22), the pregnant rats were submitted to euthanized by cervical dislocation and their uterine horns were separated through caesarean (Bjelke et al. 1991; Yang et al. 2011; Souza et al., 2013). One of the uterine horns containing the puppies was clamped on its ends and transferred to a saline solution at 37ºC, where it remained for 15 minutes (asphyxia procedure). The other uterine horn was immediately opened in order to obtain the control group of neonates (Figure 1). The day of birth was considered P0.

After hysterectomy, all the puppies were removed, cleaned, stimulated to breath and had their umbilical cords clamped, They remained at room temperature for 60 minutes seeking recovery from the procedure before they were delivered to a surrogate mother. They stayed with this surrogate mother until P21, when they were all weaned.

The 6 litters used on this study had its size limited to a number between 5 and 8 puppies each litter, and also were composed by both sexes. A total of 45 animals (21 females and 24 males) were used on this study. Therefore, experimental groups are: rats submitted to saline injection (CT, n = 8); rats submitted to LPS injection (LPS, n = 7); rats submitted to saline injection and intrauterine asphyxia (A, n = 14); rats submitted to LPS injection and intrauterine asphyxia (LA, n = 13).

Functional analysis

The animals were weighed daily since P1 to P14 (CT = 9, A = 15, LPS = 8 and LA = 14) and again at P30 (CT = 9, A = 14, LPS = 6 and LA = 8). Examinations of developmental milestones were performed between the days P3-14. In the P29, the grip test, rotarod and the novel object-placement recognition task (NOPR) test were performed.

Physical and Sensorimotor Development

The assessment of developmental milestones were performed daily, since P3 to P14 as previously described (Marcuzzo et al., 2010; Roberson et al., 2006; Toso et

al., 2005; Lubics et al., 2004). The signs and neurological reflexes evaluated were: (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 of pups to remain suspended for 10 s after grasping thin rod with their forepaws; (5) hindlimb placing - pups had the head and trunk supported, the hindlimbs were suspended near to the edge of a platform and the test was considered positive when touching the paw's dorsal surface was followed by simultaneous hip and knee extensions and ankle-plantar flexion; (6) open field activity - time to move off a circle of 13 cm diameter. Each developmental test response was considered positive based on its first appearance. All measurements were time-limited to a maximum of 30 s.

Motor skills assessments

Grip Test

The grip test was used to evaluate the forelimb coordination and muscle strength (CT = 9; A = 16; LPS = 8; LA = 14 pups). To this, animals were suspended by both their forepaws on a horizontal nylon rope (4-mm diameter) stretched horizontally and suspended 1 m from the ground. The latency to the animals fell off the rope was recorded (maximum 60 seconds; Bona, Johansson, Hagberg, 1997).

Rotarod

The motor balance and coordination were evaluated using a rotarod (Insight, Brazil; CT = 6; A = 10; LPS = 5; LA = 10 pups). The equipment have *a* 60 mm diameter textured rod with 75 mm in length, that is attached to a rotating motor. The test was performed at a speed of 30 rpm. In this test, pups were placed on the rotating drum and the total time the animal remains on the rotarod was measured (maximum 3 minutes). The time spent by the animal on the rotarod was considered as the latency to fall (Girard et al., 2009).

Spatial Memory

The novel object-placement recognition task (NOPR) was chosen to evaluate hippocampal-dependent spatial memory and was adapted from Revsin et al. (2009). The test was performed in a open field (40x 34x 25cm), divided in 9 squares with the same area (CT = 7, A = 7, LPS = 7 and LA = 10). The evaluation consists of two steps, a sample trial and a test trial. On the sample trial, the animal is positioned in open field on the opposite side containing two bottles of water (one placed in the northwest zone and another in the southwest zone). The rat stays in the open field for 5 minutes exploring freely the objects, and then returned to its house box. After a 50 minutes interval, the rat is again positioned on the open field for more 5 minutes to the test trial. However, the southwest object was moved to the east zone, in order to analyze the exploration of the relocated object. The day before the test, the animals were placed individually on the open field with no objects during a period of 3 minutes, in order to adapt the animal to this new environment. The field-boxes and the objects were sanitized with ethanol 70% on every trial to remove possible odors. Both trials were recorded with a camera (DCR-SR47, Sony, Japan) and the time used to explore the objects was analyzed by two evaluators. Exploration behavior was considered when the animal makes physical contact with the object by the nose, forepaws or vibrissae (as long as the head was directioned towards the object). The physiological response to this test is characterized by the bigger exploration between the two objects or the equal exploration of both objects on the test trial (Revsin et al., 2009). The calculation of the exploration percentage was performed by the ratio exploration of the repositioned object / exploration of both objects X100 (Revsin et al, 2009).

Morphological analysis

At P30, 3 animals from each group were deeply anesthetized with sodium thiopental (50 mg/kg, i.p.; Cristália, Brazil), injected with 1000 IU heparin (Cristália, Brazil) and submitted to transcardiac perfusion with 200 mL of saline solution and 250 mL of 4% paraformaldehyde (Reagen, Brazil) diluted in 0.1 M phosphate buffer (PB; pH 7.4) using a peristaltic pump (Milan, Brazil, 30 mL/min).

The brains were dissected, post-fixed in the same fixative solution at room temperature for 4 h and cryoprotected by immersion in 15 % and 30 % sucrose solution (Synth, Brazil) in PB at 4 °C until they sank. The brains were then quickly frozen in isopentane (Merck, Germany), cooled in liquid nitrogen and kept in a freezer (–80 °C) for further analyses.

Coronal sections (40 µm thick) of the dorsal hippocampus and corpus striatum were obtained using a cryostat (CM1850, Leica, Germany) at –20 °C. Slices were serially collected on gelatin coated slides. The point of origin for dorsal hipocampus was located approximately 2.30 to 4.52 mm posterior to the bregma (Paxinos and Watson 1982). For the corpus striatum, the point of origin was located approximately 1.92 to -0.60 mm posterior to the bregma (Paxinos and Watson 1982). Every sixth section (240 µm apart) was processed for immunostaining (described below) (Malberg et al. 2000).

Myelin basic protein (MBP), **Caspase-3 and Synaptophysin (SYP), immunohistochemistry**

The free-floating brain sections were washed in saline phosphate buffer (PBS), pre-treated with 3% hydrogen peroxide for 30 minutes, washed in PBS and in PBS containing 0.4% Triton X-100 (PBS-Tx) for 15 minutes and treated with 2% bovine serum albumin (BSA; Sigma Aldrich, USA) in PBS-Tx for 30 minutes. Then, the sections were incubated with primary polyclonal rabbit antibody to myelin basic protein (BMP; 1:500; Sigma Aldrich, USA), or caspase-3 (1:500; Sigma Aldrich, USA) or synaptophysin (1:500; Chemicon-Millipore, USA) for 48 h at 4ºC. After this, the sections were washed in PBS-Tx and incubated in the secondary anti-rabbit IgG-peroxidase antibody (1:500; Sigma Aldrich, USA) during 2 hours at room temperature. The immunohistochemical reaction was revealed using a solution of 0.06 % of 3,3-diaminobenzidine (DAB; Sigma Aldrich, USA) and 10 % of hydrogen peroxide for 5 minutes. Finally, the sections were washed in PBS, dehydrated in ethanol, cleared with xylene, covered with balsam (Merck, Germany) and cover slips. Control sections were prepared omitting the primary antibody.

Optical Densitometry (OD)

Images of SYP, caspase-3 and MBP immunoreactivity in diaminobenzidine stained brain sections were evaluated by means of regional semi-quantitative optical

densitometry (OD; Xavier et al. 2005). Images from the DG of the dorsal hippocampus and corpus striatum were obtained using an Optiphot-2 microscope (200×, Nikon, Japan) coupled to a CMOS camera (518CU, Micrometrics, USA) and Image Pro Plus Software 6.0 (Media Cybernetics, USA). All lighting conditions and magnifications were held constant. From each animal, 6 images were analyzed (3 sections per animal, both left and right sides), 3 animals per group. For analysis, images were converted to an 8-bit gray scale (0–255 gray levels). Picture elements (pixels) employed to measure optical density were obtained from the mean of 3 squares, each measuring 0.0051279 μm2, (area of interest, AOI), overlaid in the hilus of the DG of the hippocampus (Ferreira et al. 2011). Background correction was performed according to Xavier and cols. (2005). The following formula was used for OD: $OD(x,y) = -\log[(INT(x,y) - BL)]/(INC - BL)$.Where “OD” is the optical density; “INT (x,y)” or intensity is the intensity at pixel (x,y), “BL” or black is the intensity generated when no light goes through the material and “INC” is the intensity of the incidental light.

Statistical analysis

Male and female pups of all experimental groups were evaluated in all tests. Firstly, the data obtained were submitted to multifactorial ANOVA analysis followed by Tukey *post hoc* test, to evaluate if there was a difference between the responses of the genders (significance level of $p<0.05$). As there was no difference on the comparison between the genders in all tests, male and female pups composed the four experimental groups.

The weight monitoring between P1 and P14 was submitted to ANOVA for repeated measures. Other evaluations were analyzed by two-way ANOVA. Both tests were followed by post-hoc of Tukey test, when necessary. All data were expressed as average ± S.E.M. Differences between groups were considerate significant when $p< 0.05$. The data were analyzed by the Statistica software, version 6.0.

RESULTS

Neonatal Development

The animals were weighed daily, from P1 to P14, in order to evaluate their gain of weight. The two-way ANOVA for repeated measures revealed significant effect between the following factors: *LPS* ($F(1, 42) = 29.97$; $p < 0.001$); *asphyxia* ($F(1, 42) = 9.23$; $p = 0.004$); *time* ($F(12, 504) = 3194.78$; $p < 0.001$); however, the interaction *LPS* x asphyxia x *time* was no significant ($F(12, 504) = 0.97$; $p > 0.05$; Figure 3). The three experimental groups (LPS, A e LA) had smaller weight when compared to the control group (CT) however this difference was not significant statistically. However, the association of injuries (LA) did not exercises synergistic effect compared to the effects caused by isolated factors (LPS and A) during the first two weeks of lifetime.

On the 30 days of life, only the LPS factor continued to interfere on the weight gain in the animals. The two-way ANOVA showed effects of the *LPS* factor ($F(1, 33) = 15.437$; $p < 0.001$); but the *asphyxia* factor was not significant ($F(1, 33) = 0,356$; $p > 0,05$); such as the interaction *LPS* x *asphyxia* ($F(1, 33) = 2.087$; $p > 0.05$; Figure 3). On this evaluation, the groups exposed to LPS (LPS and LA) had less weight in comparison to control and asphyxia groups (CT and A), but they did not differ between themselves.

Regarding the sensorimotor development, two-way ANOVA showed that most part of the development milestones (open field activity, eye opening, cliff aversion, surface righting e stability on an inclined surface) were acquired without any differences between the groups. However, the animals exposed to LPS (LPS e LA) showed delay on the hindlimb proprioceptive placing (2 days, Tabela 1, $p = 0.03$) and audio startle (1 day, $p < 0.01$), and had an increase on the development of forelimb grasp (1 day, $p = 0.004$) in comparison to other groups (control and asphyxia).

Motor and cognitive evaluations

The performance of animals in the grip test and rotarod did not show significant differences between the experimental groups (Figure 4), indicating no motor and coordination impairments. In addition, no significant alterations in the novel object-placement recognition test (NOPR) were observed, indicating no spatial memory deficits (Figure 5).

MBP, Caspase-3 and SYP immunoreactivity

The results of densitometric measurements of immunoreactivity of MBP in the striatum region analyzed by two-way ANOVA were similar between groups (Figure 6). Caspase-3 immunoreactivity in the hippocampal DG showed a significant effect of *LPS* factor ($F_{(1,8)} = 38.09$, $p < 0.001$), *asphyxia* factor ($F_{(1,8)} = 58.48$, $p < 0.001$) and of *LPS X asphyxia interaction* ($F_{(1,8)} = 34.02$, $p < 0.001$). The *post hoc* analysis revealed that the LA group showed an increase in the caspase-3 OD compared to the others groups ($p < 0.001$). LPS and A groups showed no significant difference when compared to the control group (Figure 7). Two-way ANOVA revealed that the groups exposed to LPS (LPS and LA) have lower expression of synaptophysin in the DG hilus of hippocampus in comparison with control (CT) and asfixia (A) groups [$F(1,8) = 45.2$, $p < 0.001$]. The *post hoc* test showed that the LPS and LA groups had a lower synaptophysin immunoreactivity when compared to CT and A groups ($p < 0.01$) (Figure 8).

DISCUSSION

The present study is the first, in our knowledge, to examine the effects of the association of LPS and intrauterine asphyxia in order to see if this interventions would be able to reproduce the findings of CP in rats. Although have caused cell death and impairment in the synaptic activity, this model caused only few subtle and transitory changes in the development, not generating late motor and cognitive deficits. Therefore, this experimental model proposed no reproduces the phenotypic characteristics of CP, not being viable to study biological mechanisms of this pathology and therapeutic proposals. However, interesting results were obtained in the hippocampus evaluation, indicating that LPS and asphyxia promote brain changes, although not severe enough to promote functional deficits.

Children with moderate or severe CP frequently experience poor nutritional status that compromises the development. Consequently, occurs an increase health care utilization and a decrease in the children participation in normal activities (Samson et al, 2002). This study demonstrated that animals exposed to the prenatal inflammation induced by LPS and intrauterine asphyxia had lower weight gain

compared to the control animals in the first two weeks of life, the period in which feed only breast milk. In P30, the only animals subjected to LPS (LPS and LA) continued to lower body weight and the only animals subjected to asphyxia seem to have recovered since they were not different from control animals. In experimental studies, adequate body weight is considerate an indicative of well-being and normal growth (Rousset et al., 2013). Similar data were found in other studies (Chen et al, 2011; Strackx et al, 2010; Poggi et al, 2004; Van de Berg et al, 2003), demonstrating that the inflammation by LPS seems to be the essential factor to produce a reduction in the body weight gain at the first weeks of the life. Gestational inflammation induced by LPS triggers the production of pro-inflammatory cytokines by the mother and fetus, such as TNF-α (Burd et al., 2012). TNF-α may play a key role in the modulation of lipid metabolism by stimulation of lipolysis and leptin production, and inhibition of lipogenesis (Sethi & Hotamisligil, 1999). In turn, the decrease in oxygen supply potentiates the inflammatory response by TNF-α in both white and brown adipocytes and downregulates the transcription factors involved in adipocyte function (Bhattacharya et al., 2015). Furthermore, it was shown that LPS is capable of causing anorexia even in mice that were deficient in leptin or its receptor (Faggioni et al., 1997). These data suggest that LPS can have a longer lasting effect on body weight via TNF-α production, decreased appetite and food intake.

One of the diagnostic criteria for the CP is the delay in the acquisition of developmental milestones (Dodge, 2008). Only animals exposed to LPS had their sensorimotor development altered. LPS and LA groups delayed the development of startle response and hindlimb proprioceptive placement, but accelerated the appearance of the forelimb grasp. It is possible that these changes in sensorimotor development of these animals was relate to its low weight, since malnourished mice have impaired their motor development (Gramsbergen & Westerga, 1992).

There are studies that corroborate our findings, in which the LPS affected the development of some developmental milestones (Poggi et al., 2004; Toso et al., 2005; Rousset et al., 2013), but intrauterine asphyxia did not alter (Hoeger et al., 2000). In addition, others, who at variance showed that intrauterine asphyxia, perinatal anoxia and LPS combined anoxia interfere with the acquisition of several developmental milestones (Kiss et al., 2009; Stigger et al., 2013). The effect of maternal inflammation has on motor development is not well understood, since there is a divergence of literature data on the types of abnormal reflexes and its

manifestation is accelerated or delayed, but it indicates that inflammation certainly disturb motor development system.

However, at P29, neither experimental group showed change in motor tasks in the Rotarod and the Grip test, showing that their balance and motor coordination and musculoskeletal system are normal. The motor deficits caused by maternal inflammation may disappear over time (Toso et al., 2005). At P7 there is a hypomyelination in the brain exposed to LPS, but when analyzed in adulthood has normal myelination. There is a hypothesis that suggests that myelination of the brain in early development is not altered by oligodendrocyte death induced by exposure to LPS, but is just delayed by the insult (Rousset et al., 2013). The absence of late alterations in the white matter of the striatum, an area thought to be highly hypoxia-sensitive (Rademakers et al., 2005), suggests that changes may be transient due to repair mechanisms in the rat immature nervous system (Kohlhauser et al., 2000), so the initial deficits motors are offset by such mechanisms. This is also in agreement with our results of densitometry MBP in striatum, showing a normal myelination structure in all groups.

With regard to asphyxia and perinatal anoxia protocols, the motor disability are also subtle or transient (Kiss et al., 2009; Strata et al., 2004). Studies examining the effects of neonatal hypoxia-ischemia on motor function of rats, also noted that the motor deficit, especially in their development, tend to be compensated with age (Lubics et al., 2005; Zuravin et al., 2004). This motor recovery can be explained by several factors. The neonatal brain is in maturing process and may occur mechanisms to adapt to injuries in that period. Several injuries in neonatal rodents were recovered by plastic mechanisms, for example, development of compensatory projections corticospinal tract neonatal rat with extensive lesions (Castro, 1975; Hoeger et al., 2000). According to previous studies, the brain of newborn rodents are less susceptible to oxygen deprivation, and the time of asphyxia as well the degree of brain maturity at the time of injury are determining factors for the evolution of injury in the CNS (Davis, 1979; Vanucci et al., 1980). Furthermore, asphyxiated pups issue more ultravocalizations than a normoxemic puppy (Veronesi et al., 2006). The ultravocalizations are a potent stimulant of maternal care, so the increased demand for maternal care for the animals asphyxiated may partly explain the non installation of motor deficits, as possibly the mothers stimulated more the puppies, and this

increased sensory and proprioceptive stimulation may have offsetting the motor deficits. This hypothesis should be studied in future experiments in this animal model.

The hippocampus has a central role in learning and memory, being particularly vulnerable to these inflammatory insults (Lynch et al, 2004; Cunningham and Souza, 1993). For this reason, injuries in early life stage permanently alters the function of the hippocampus (Herguido et al, 1999; Bauman et al, 1997). We investigated the cellular death by apoptosis in DG of hippocampus and synaptic plasticity 30 days after the induction of the experimental model. The group exposed to insults in combination (LA) showed the higher Caspase-3 expression in relation to the other groups. This suggests that the combination of insults enhances their separate effects on apoptosis in the hippocampus DG. In other studies, the maternal infection and the asphyxia singly were able to produce cell death in the DG of hippocampus in young rats (Morales et al., 2008; Golam et al., 2005). In addition to increased cell death by apoptosis, we found a reduction in the synaptic activity, indicating an impairment in the neuronal connectivity of hippocampus. Huang et al (2015) also found that prenatal exposure to LPS reduces the synaptophysin content in the hippocampus of offspring. Synaptophysin is a marker of synaptic plasticity modifications, located in the presynaptic vesicle membranes (Wiedenmann et al., 1986). Synaptic plasticity is fundamental in neurology for learning and memory ability. The reduction of neuronal synaptic plasticity and functions do harms to the ability of learning and memory (Meng et al., 2013) and the alteration in synaptophysin is a result normally observed after injuries (Prechtl, 1997).

However, cell death and damage to hippocampal plasticity seen in our study, were unable to cause memory deficit in the novel object-placement recognition task (NOPR). One possible explanation for this is that this test assesses spatial memory and intrauterine asphyxia, regardless of severity; it seems to have specific deleterious effects on non-spatial memory (Van de Berg et. al., 2000; Hoeger et al., 2000; Morales et al., 2009). Although interconnected, neural circuits responsible for spatial memory processing are different from that process the non-spatial memory. Spatial memory is processed by a pathway involving cortical association areas, the medial thalamus, and other subcortical areas; while the space depends on the circuitry that connects the hippocampus, pre limbic cortex and parietal cortex (Steckler et al., 1998, Simola et al., 2008). Although both pathways are damaged by intrauterine asphyxia (Bjelke et al., 1991; Kohlhauser et al., 1999, Simola et al.,

2008), probably the pathway involved with spatial memory may be less sensitive to injury caused by asphyxia or compensatory mechanisms may justify this pathway functionality after insult (Simola et al., 2007).

There are few studies that describe the effect of perinatal inflammation by LPS on memory, however, Chen et al. (2011) found that the LPS administration in late pregnancy CD-1 mice had no deleterious effect on the spatial and non-spatial memory of the puppies at 35 days of life, effect manifested itself only when the animals had 400 days. Wang et al. (2009) also studied the effects of prenatal inflammation by LPS and found spatial memory deficit from 400 days in CD-1 mice. Therefore, prenatal LPS inflammation causes no immediate damage on the spatial and non-spatial memory.

CONCLUSION

The difficulty of playing in rats, motors and cognitive impairment found in CP patients is due to the fact that this disease does not exist naturally for this specie and after the insults, the animals tend to die or recover (Wright & Rang, 1990). The recovery of rodents after these insults can be explained by the fact that their brains are more primitive, less complex than human and therefore more plastic (Rousset et al., 2013). In addition, the quadruped position, the organization and function of the corticospinal tract of rodents differ from humans, which may justify the fact that brain injuries do not reflect functional deficits (Eyre, 2007). However, it is necessary to keep testing different insults in order to establish a CP animal model in rats because this specie is easy to handle, it reproduces and mature very quickly (Rooney et al., 1997, Rice & Barone, 2000), and it is possible to evaluate different types of behavior that would be unworkable in other species.

Although cell death and impairments in the hippocampal synaptic activity were observed, this model caused only subtle changes in the development without late motor and cognitive deficits, contrary to what occurs in the clinic, where motor deficits are permanent. Sensorimotor restriction seems to be necessary to produce motor deficits in rats (Stigger et al., 2011; Marcuzzo et al., 2010; Marcuzzo et al., 2008; Coq et al., 2008; Strata et al., 2004) and the risk factors used in our approach may have not been aggressive enough to mimic the long-term motor deficits of PC, not being useful as an animal model for this disease. To this end, the risk factors for CP have

been associated to the sensorimotor restriction, and seems to be necessary to produce the late motor deficits (Stigger et al., 2011; Marcuzzo et al., 2010; Marcuzzo et al., 2008; Coq et al., 2008; Strata et al., 2004).

LEGENDS

Figure 1. Schematic representation of intrauterine asphyxia procedure. (A) Anatomy of uterus of pregnant rat. (B) Uterine horn containing neonates with clamped extremities dipped in saline solution at 37°C for 15 minutes. Illustrated by Maria Augusta Timmen Raimundo.

Figure 2. Time line of the experimental procedures. G – gestational day; P – postnatal day.

Figure 3. (A) Body weight gain of animals since postnatal day 1 (P1) to P14. CT – control group (n = 9), A – asphyxia group (n = 15), LPS - Lipopolysaccharide group (n = 8) and LA - Lipopolysaccharide and asphyxia group (n = 14). Values are expressed as mean ± S.E.M. ANOVA for repeated measures: * $p < 0.05$ (A, LPS and LA vs. CT). (B) Body weight at P30. CT – control group (n=9), A – asphyxia group (n=14), LPS - Lipopolysaccharide group (n=6) e LA - Lipopolysaccharide and asphyxia group (n=8). Values are expressed as mean ± S.E.M). Two-way ANOVA: * $p < 0.001$ (LPS and LA vs. CT and A).

Figure 4 (A) Latency to fall in the grip test. CT – control group (n = 9), A – asphyxia group (n = 16), LPS - Lipopolysaccharide group (n = 8) and LA - Lipopolysaccharide and asphyxia group (n = 14). (B) Latency to fall on rotarod test. CT – control group (n = 6), A – asphyxia group (n = 10), LPS - Lipopolysaccharide group (n = 5) and LA - Lipopolysaccharide and asphyxia group (n = 10). Values are expressed as mean ± SEM. Two-way ANOVA. No significant differences were found between the experimental groups.

Figure 5. Percentage of preference for the novel object the during the test trial in the novel object-placement recognition task (NOPR). CT - control group (n = 7), A - asphyxia group (n = 7), LPS - Lipopolysaccharide group (n = 7) and LA - Lipopolysaccharide and asphyxia group (n = 10). Values are expressed as mean ± S.E.M. Two-way ANOVA. No significant differences were found between the experimental groups.

Figure 6. Myelin basic protein (MBP) in striatum. (A) Digital images of representative coronal sections of the striatum stained for MBP. Magnification of 200x. Scale bar =

20 µm. (B) Optical Density (OD) measurement of MBP immunoreactivity in the striatum. CT – control group (n = 3), A – asphyxia group (n = 3), LPS – Lipopolysaccharide group (n = 3) and LA – Lipopolysaccharide and asphyxia group (n = 3). Values are expressed as mean ± SEM. Two-way ANOVA. No significant differences were found between the groups.

Figure 7. Caspase-3 in dentate gyrus (DG) of hippocampus. (A) Digital images of representative coronal sections of the DG stained for Caspase-3. Magnification of 200x. Scale bar = 20 µm. (B) Optical Density (OD) measurement of Caspase-3 immunoreactivity in DG of hippocampus. Values are expressed as mean ± SEM. CT – control group (n = 3), A – asphyxia group (n = 3), LPS – Lipopolysaccharide group (n = 3) and LA – Lipopolysaccharide and asphyxia group (n = 3). Values are expressed as mean ± SEM. for groups. * Tukey *pos hoc.*: $p < 0.001$ (LA vs. CT, A and LPS).

Figure 8. Synaptophysin (SYP) in dentate gyrus (DG) of hippocampus. (A) Digital images of coronal sections of the DG stained for synaptophysin (SYP). Magnification of 200x. Scale bar = 20 µm. B) Optical Density (OD) measurement of synaptophysin immunoreactivity in the hilus of DG. CT – control group (n = 3), A – asphyxia group (n = 3), LPS – Lipopolysaccharide group (n = 3) and LA – Lipopolysaccharide and asphyxia group (n = 3). Values are expressed as mean ± SEM. for groups. * Tukey *pos hoc.*: $p < 0.01$ (LPS and LA vs. CT and A).

Table 1. Latency to the acquisition of developmental milestones in control (CT), asphyxia (A), Lipopolysaccharide (LPS) and Lipopolysaccharide and asphyxia groups (LA). Data were expressed in mean ± SEM.

ASSESSMENT	DAY OF RESPONSE PRESENTATION (IN AVERAGE)			
	CT (n=9)	A (n=16)	LPS (n=8)	LA (n=12)
Gait	8,778 ± 1,382	10,813 ± 0,867	9,875 ± 1,217	9,333 ± 1,082
Eye opening	14,778 ± 0,147	14,813 ± 0,101	15,000 ± 0	14,917 ± 0,083
Limb placing	4,889 ± 0,564	5,313 ± 0,313	7,375 ± 1,224	6,250 ± 0,906
Forelimb grasp	5,000 ± 0,441	5,125 ± 0,287	4,000 ± 0,327*	4,083 ± 0,260*
Audio startle	13,444 ± 0,242	13,500 ± 0,183	14,000 ± 0,327*	14,250 ± 0,218*
Cliff aversion	5,222 ± 0,324	4,750 ± 0,371	4,500 ± 0,267*	4,583 ± 0,149*
Righting reflex	3,111 ± 0,111	3,250 ± 0,144	3,375 ± 0,183	3,583 ± 0,499
Negative geotaxis	9,667 ± 0,500	8,313 ± 0,711	9,125 ± 0,766	8,333 ± 0,689

Two-way ANOVA revealed significant effect of the fator LPS on proprioceptive placing [$F(1, 41) = 5{,}2766$; $p = 0.03$], auditory startle [$F(1, 41) = 7.48$; $p < 0.01$] and forelimb grasp [$F(1, 41) = 9{,}1944$; $p = 0.004$]. * $p < 0.05$ (LPS and LA vs. CT and A).

Figure 1.

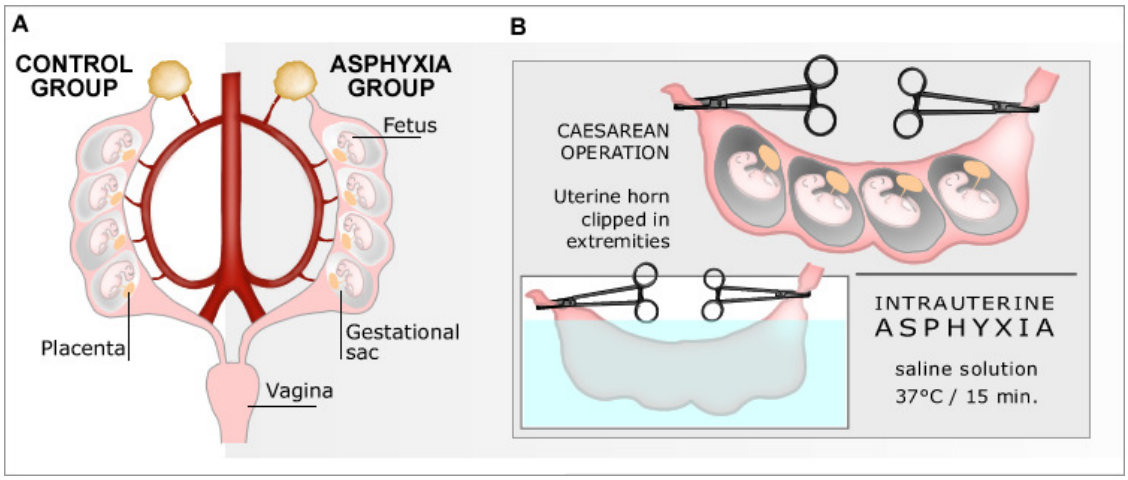


Figure 2.

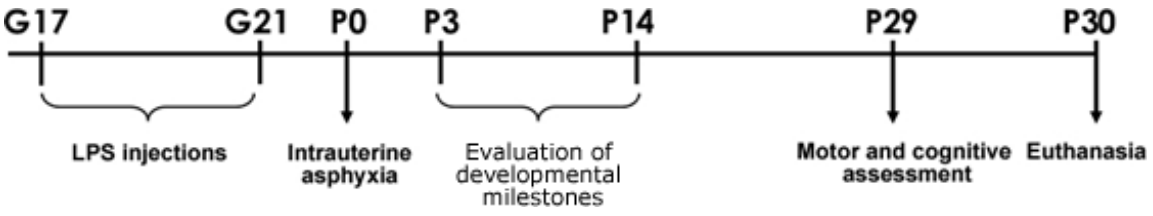
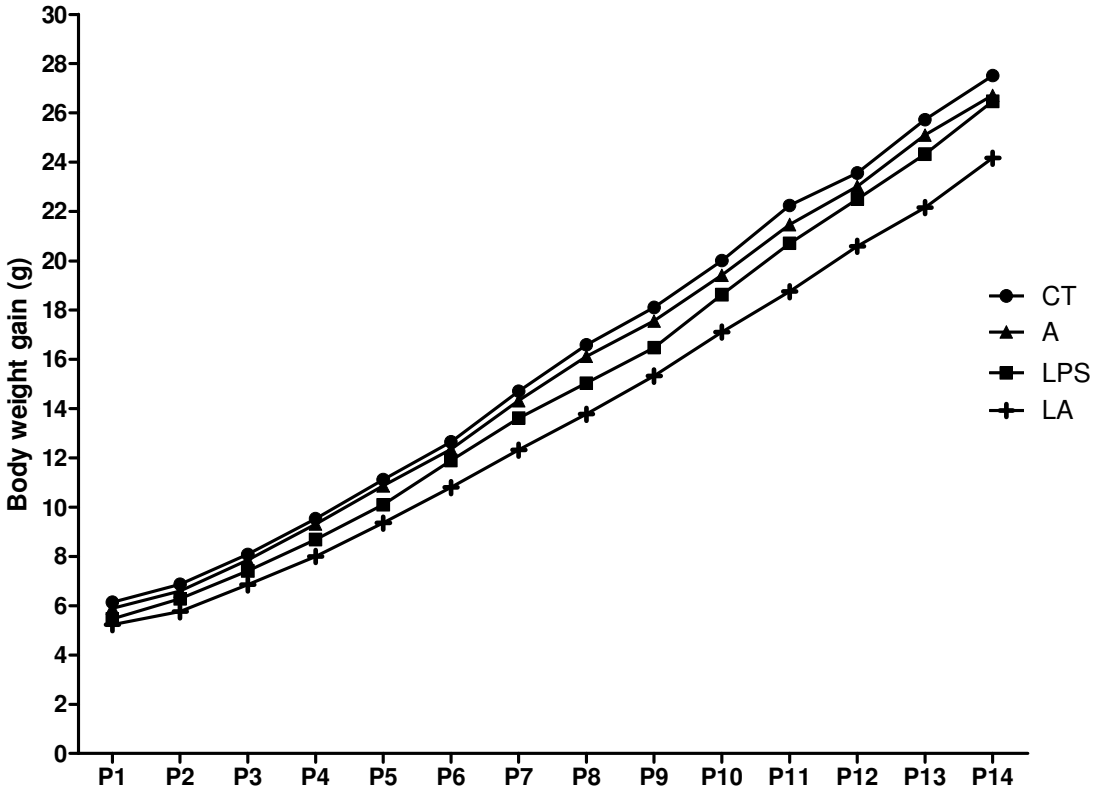


Figure 3.

A


Body weight gain (g)
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
P1
P2
P3
P4
P5
P6
P7
P8
P9
P10
P11
P12
P13
P14
CT
A
LPS
LA

B

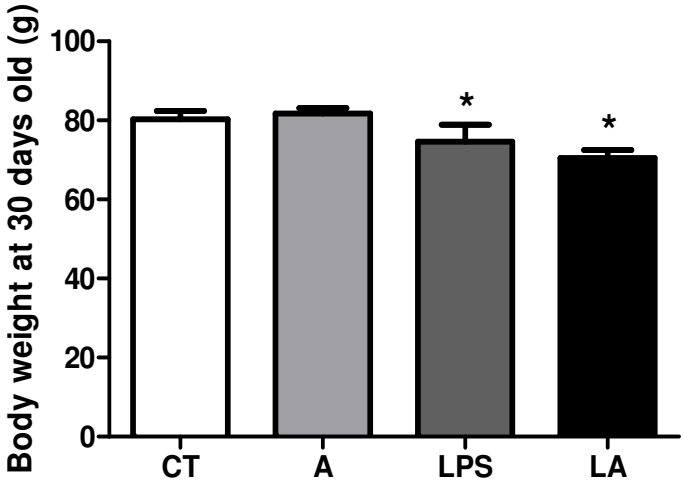
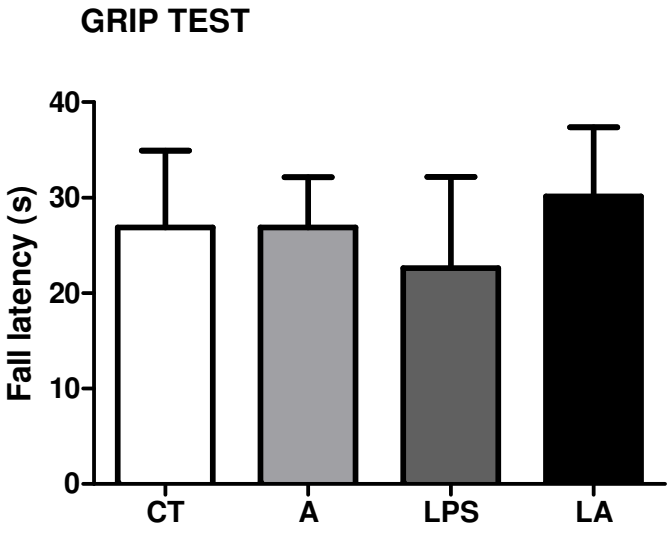

Body weight at 30 days old (g)
100
80
60
40
20
0
*
*
CT
A
LPS
LA

Figure 4

A


GRIP TEST
Fall latency (s)
40
30
20
10
0
CT
A
LPS
LA

B

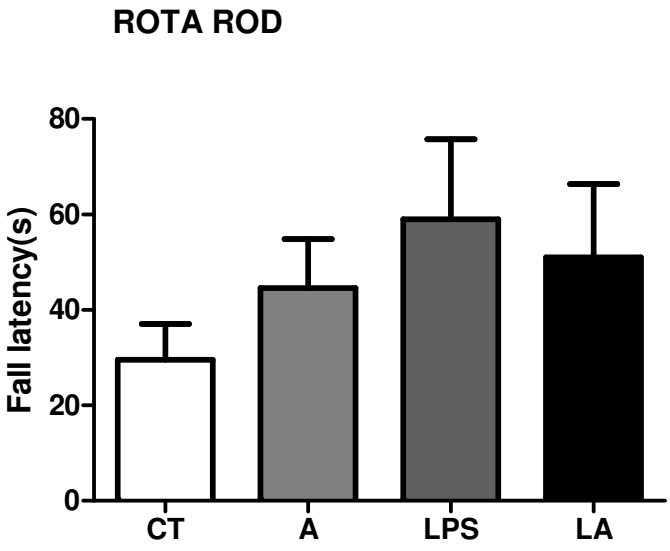

ROTA ROD
Fall latency(s)
80
60
40
20
0
CT
A
LPS
LA

Figure 5

A

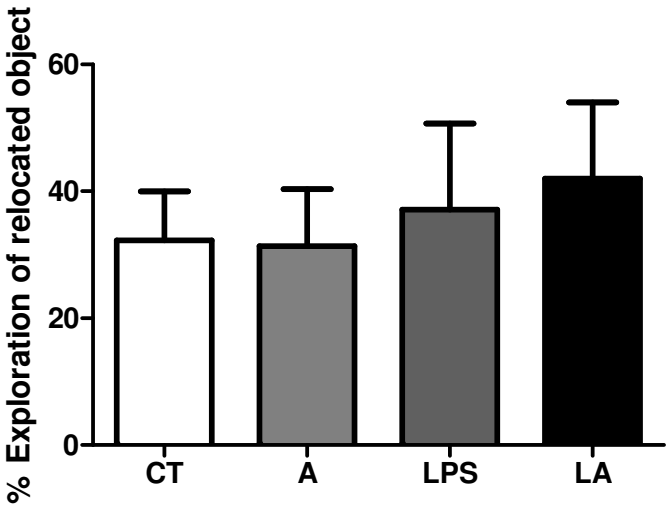
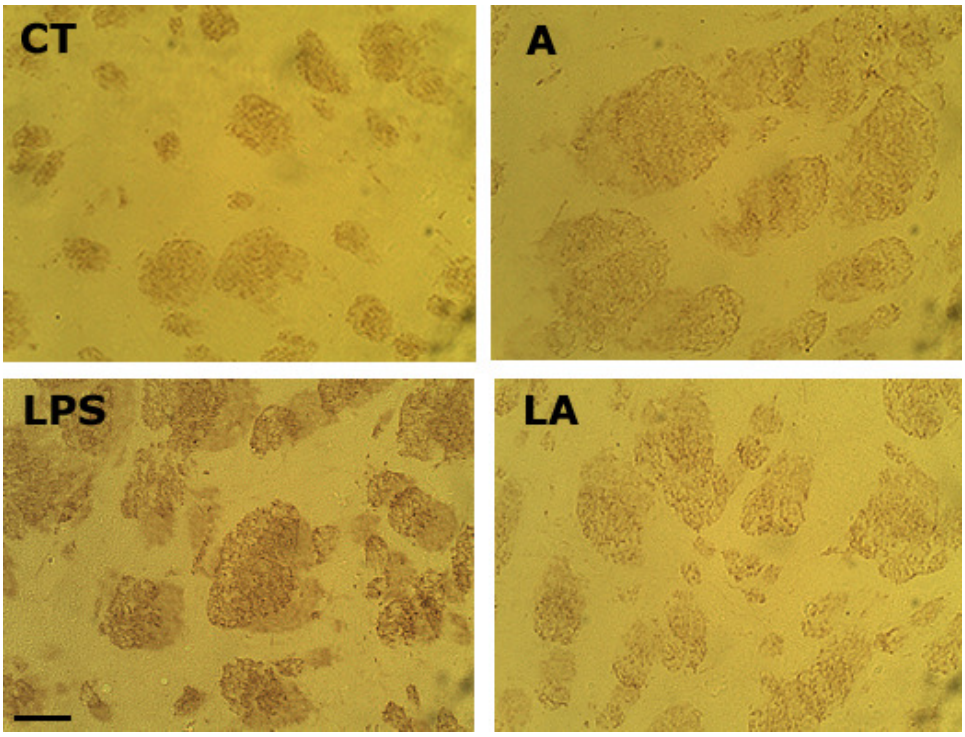


Figure 6

A



B

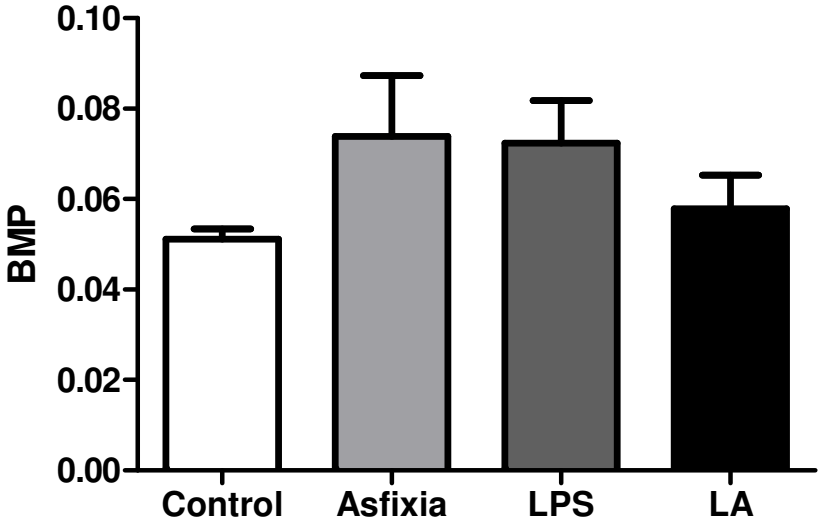
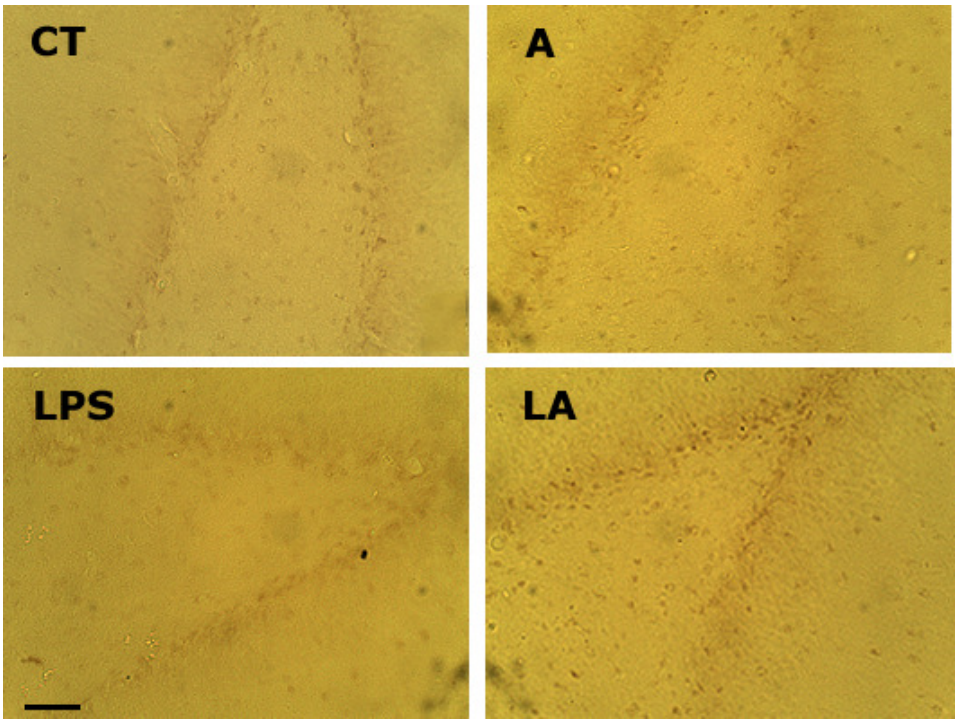


Figure 7

A



B

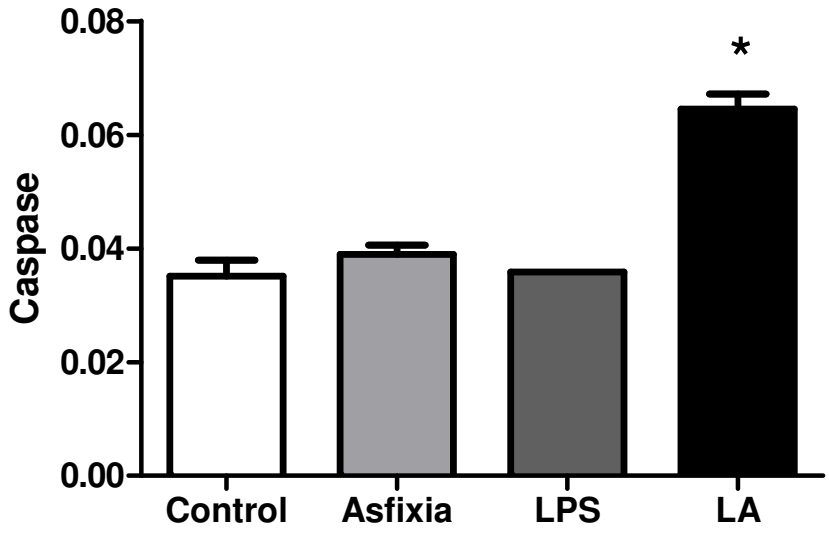
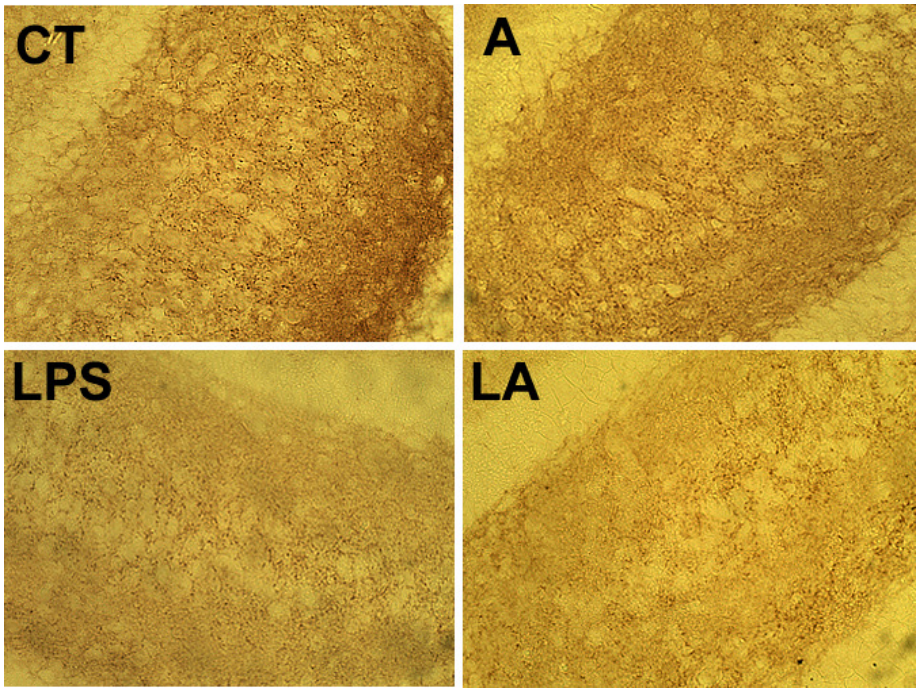
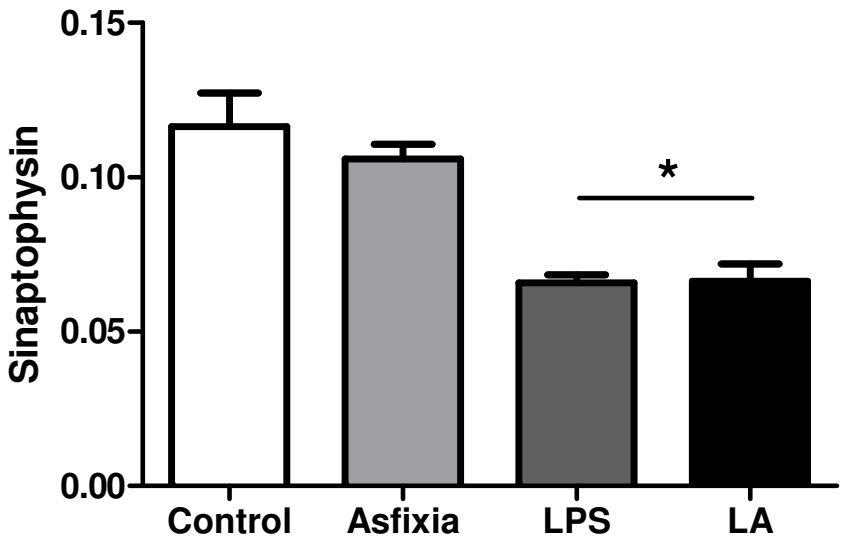


Figura 8

A


CT
A
LPS
LA

B


0.15
0.10
0.05
0.00
Sinaptophysin
*
Control
Asfixia
LPS
LA

Acknowledgments

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4 CONCLUSÕES

Os resultados obtidos nessa dissertação nos permitem concluir que:

- Nas duas primeiras semanas de vida, os animais expostos tanto a resposta inflamatória fetal como a asfixia intrauterina, ou a combinação de ambas intervenções, tiveram menor ganho de peso;
- No P30, apenas os animais submetidos ao fator LPS, isolado ou associado à anóxia, mantiveram um peso corporal menor;
- Os animais expostos ao fator LPS, isolado ou associado à anóxia, apresentaram alterações na avaliação dos marcos do desenvolvimento(as respostas de sobressalto e colocação do membro posterior foram retardadas e a preensão palmar adiantada) em relação aos grupos CT e A;
- Nenhum dos grupos experimentais demonstraram déficits nas funções motoras avaliadas pelo *Rotarod* e teste de *suspensão na barra*, além disso não apresentaram déficit de memória espacial;
- Não houve diferença na mielinização entre os grupos experimentais, avaliada pela expressão da BMP no estriado;
- A combinação dos fatores LPS e asfixia aumentou a expressão de caspase-3 no giro denteado do hipocampo, um marcador de morte celular por apoptose, ;
- A expressão da glicoproteína sinaptofisina, avaliada no hipocampo, foi significativamente reduzida nos grupos submetidos a inflamação fetal, LPS e LA.

Esse estudo demonstrou que a inflamação gestacional combinada ou não a asfixia intrauterina causa apenas alterações motoras sutis e transitórias, ao contrário do que ocorre na clínica, onde os déficits motores nos pacientes são debilitantes e permanentes. Desta forma, a associação de uma inflamação pré-natal e asfixia intra-uterina utilizadas nesse estudo não foi capaz de reproduzir um modelo de PC em ratos. O estabelecimento de um modelo experimental de PC com um maior número de características da patologia pode contribuir para a elucidação da fisiopatologia dessa condição clínica e para o desenvolvimento de estratégias de tratamento mais eficazes.

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