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AVALIAÇÃO DE UM MODELO ANIMAL DE PARALISIA CEREBRAL SOBRE A MORFOLOGIA DO MÚSCULO EXTENSOR LONGO DOS DEDOS

CASCAVEL - PR

(Junho/2016)

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Dissertação apresentada ao Programa de Pós-graduação *Stricto Sensu* em Biociências e Saúde - Nível Mestrado, do Centro de Ciências Biológicas e da Saúde, da Universidade Estadual do Oeste do Paraná, como requisito parcial para obtenção do título Mestre em Biociências e Saúde.

Área de concentração: Fatores que influenciam a morfofisiologia orgânica.

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RESUMO GERAL

A paralisia cerebral (PC) caracteriza-se por desordens do movimento e da postura, por isso causam limitação na atividade do indivíduo, as quais são atribuídas por distúrbios não progressivos que ocorreram no desenvolvimento cerebral fetal ou infantil. Modelos animais têm sido utilizados na tentativa de reproduzir as lesões e características da PC. Porém, ainda não existe uma intervenção capaz de reproduzir as características desta patologia no âmbito experimental. Assim, o objetivo deste estudo foi verificar os efeitos de um modelo de PC que associa exposição pré-natal ao lipopolissacarídeo (LPS), anóxia perinatal e restrição sensório-motora sobre as fibras musculares e junções neuromusculares (JNMs) do músculo extensor longo dos dedos (EDL) de ratos. Filhotes *Wistar* machos foram separados em dois grupos: Grupo controle (CTL - n = 10) - filhotes de mães injetadas com solução salina durante a gestação e Grupo Paralisia Cerebral (PC - n = 10) - filhotes de mães injetadas com LPS durante a gestação, submetidos à anóxia perinatal e à restrição sensório-motora. No dia do nascimento, os filhotes do grupo PC foram colocados em câmara fechada com fluxo de nitrogênio (100%) para a realização da anóxia perinatal. A partir do primeiro dia pós-natal (P1) até o P30, esses filhotes também foram submetidos à restrição sensório-motora por imobilização das patas pélvicas. Avaliações do desempenho motor foram realizadas em um campo aberto no P29 e P45 para os dois grupos. Após a eutanásia, amostras do músculo EDL foram processadas para análise morfológica e morfométrica das fibras musculares e JNMs. Quanto à performance motora, o tempo de locomoção e o número de erguidas (*rearing*) foi significativamente menor no grupo PC em comparação ao CTL aos 29 dias de idade ($p< 0,001$ e $p< 0,01$, respectivamente). Aos 45 dias de idade, o tempo de locomoção dos animais do grupo PC também foi menor em relação ao grupo CTL ($p < 0,05$). O peso corporal, peso e comprimento do músculo EDL foram 18%, 17% e 15% menores, respectivamente, no grupo PC em relação ao CTL. Os animais do grupo PC apresentaram hipertrofia das fibras do tipo IIB, todavia, não houve diferença entre os grupos nas demais fibras. Não teve diferenças entre os grupos estudados quanto ao número de fibras musculares dos tipos I, IIA e IIB. A relação núcleo/fibra, e a relação capilar/fibra, foi significativamente maior no grupo PC (21% e 18%, respectivamente). Quanto às fibras intrafusais, os animais do grupo PC apresentaram atrofia de 26% na área de secção transversal e redução de 26% na área do fuso muscular. O colágeno intramuscular aumentou 34% nos animais do grupo PC. O estudo ultraestrutural do músculo EDL do grupo PC mostrou desarranjo miofibrilar, desorganização e dissolução da linha Z. As JNMs do grupo PC apresentaram aumento de 22% na área e de 11% no diâmetro maior quando comparado ao grupo CTL. Em conclusão, o modelo animal de PC que utiliza injeções de LPS, anóxia perinatal e restrição sensório-motora produz déficits motores que também são observados em crianças com PC.

Palavras-chave: modelo animal de paralisia cerebral; lipopolissacarídeo; anóxia perinatal; restrição sensório-motora; morfologia muscular.

GENERAL ABSTRACT

Cerebral palsy (CP) is characterized by movement and postural disorders that limit the individual's activity, and is attributed to the occurrence of non-progressive disturbances during the development of the fetal or infant brain. Animal models have been used in an attempt to reproduce the lesions and characteristics of CP. However, there is still no intervention capable of reproducing the characteristics of this pathology in the experimental area. Thus, the objective of this study was to verify the effects of a CP model that combines prenatal exposure to lipopolysaccharide (LPS), perinatal anoxia and sensorimotor restriction on the muscle fibers and neuromuscular junctions (NMJs) of the *extensor digitorum longus* (EDL) of rats. Male Wistar pups were separated into two groups: Control group (CTL - n = 10) - pups of mothers injected with saline during pregnancy and Cerebral Palsy Group (CP - n = 10) - pups of mothers injected with LPS during pregnancy. submitted to perinatal anoxia and sensorimotor restriction. On the day of birth, the offspring of the CP group were placed in a closed chamber with nitrogen flow (100%) to perform the perinatal anoxia. Additionally, from the first postnatal day (P1) to P30, those pups were also submitted to sensorimotor restriction by immobilizing the hind limbs. Motor performance was assessed in both groups during open field tests on P29 and P45. After euthanasia, EDL muscle samples were processed for morphological and morphometric analysis of the muscle fibers and NMJs. Regarding motor performance, the time of locomotion and the number of rearings were significantly lower in the CP group compared to CTL group at 29 days of age ($p < 0.001$ and $p < 0.01$, respectively). At 45 days of age, the time of locomotion of the animals in the CP group was also lower in relation to the CTL group ($p < 0.05$). The total body weight, weight and length of the EDL muscle were 18%, 17% and 15% lower, respectively, in the CP group in relation to the CTL. The animals in the CP group presented hypertrophy of the type IIB fibers. However, regarding the other fibers there was no difference between groups. There were no differences between groups in the number of muscle fiber types I, IIA and IIB. The nuclei/fiber ratio, and the capillary/fiber ratio, were significantly higher in the CP group (21% and 18%, respectively). Regarding the intrafusal fibers, the animals from the CP group presented atrophy in 26% of the cross-sectional area and a reduction of 26% in the muscle spindle area. Intramuscular collagen increased by 34% in the animals from the CP group. The ultrastructural study of the EDL muscle in the CP group showed myofibrillar disruption and Z-line disorganization and dissolution. The NMJs in the CP group presented an increase of 22% in area and 11% in diameter when compared to the CTL group. In conclusion, the CP animal model that uses injections of LPS, perinatal anoxia and sensorimotor restraint produces motor deficits that are also observed in children with CP.

Keywords: animal model of cerebral palsy; Lipopolysaccharide; Perinatal anoxia; Sensorimotor restriction; Muscular morphology.

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LISTA DE ABREVIATURAS

ACh	Acetilcolina
CEUA	Comitê de Ética no Uso de Animais
COBEA	Colégio Brasileiro de Experimentação Animal
CTL	Controle
EDL	Extensor longo dos dedos
G	Gramas
G	Dia gestacional
HE	Hematoxilina-eosina
HI	Hipóxico-isquêmico
IL-1b	Interleucina – 1b
JNM	Junção neuromuscular
JNMs	Junções neuromusculares
LABEM	Laboratório Experimental de Morfologia
LPS	Lipopolissacarídeo
LPV	Leucomalácia periventricular
mm	Milímetros
mATPase	ATPase miofibrilar
µm	Micrômetros
N	Número amostral
N_2	Nitrogênio
NADH-TR	Nicotinamida adenina dinucleotídeo - tetrazolium reductase
O_2	Oxigênio
TF	Tampão fosfato
P	Dia pós-natal
PC	Paralisia cerebral
SNC	Sistema nervoso central

INTRODUÇÃO GERAL

Conceitua-se paralisia cerebral (PC) como uma encefalopatia crônica da infância, que causa distúrbios motores em diferentes graus de severidade (ROTTA, 2002). De acordo com o Ministério da Saúde (BRASIL, 2013), a incidência de PC é de sete a cada 1000 nascidos vivos em países subdesenvolvidos, portanto é a causa mais comum de deficiência física. As desordens encefálicas frequentemente observadas nesta patologia são as lesões de substância branca, do córtex cerebral, da medula espinal e do tronco encefálico (KOMAN; SMITH; SHILT, 2004). Crianças com PC apresentam padrões de movimentos escassos, monótonos e estereotipados e não possuem complexidade, variação e fluência (HADDERS-ALGRA, 2004). As alterações musculoesqueléticas observadas nesses pacientes incluem atrofia ou hipertrofia das fibras musculares, aumento do tecido conjuntivo na musculatura, malformação óssea secundária e degeneração da cartilagem, os quais são devido aos déficits na execução de movimentos espontâneos (COQ *et al.*, 2008; GRAHAM; SELBER, 2003). Assim, apesar de ser considerada uma lesão cerebral não progressiva, as alterações motoras na PC tendem a se agravar com o passar do tempo (GRAHAM; SELBER, 2003).

Familiares de pacientes com PC relatam dificuldades em relação a transportes especiais, preconceito da sociedade e sensação de desamparo em relação ao suporte profissional (SIMÕES *et al.*, 2013). A qualidade de vida dos cuidadores primários de crianças com PC também é prejudicada, principalmente pelas visitas frequentes ao médico, uso de medicações e hospitalizações (PRUDENTE; BARBOSA; PORTO, 2010). Foram aplicados questionários aos pacientes adultos portadores da doença e, verificou-se que poucos possuíam

índices de ensino superior, enquanto apenas 24% dos pacientes com PC em graus leves da doença tinham atividades laborais em tempo integral (ANDERSSON; MATTSON, 2001).

Tendo em vista o impacto dessa condição clínica sobre a vida do indivíduo com PC e de sua família, estudos com modelos animais estão sendo desenvolvidos para reproduzir os sinais, sintomas e os achados encefálicos e musculoesqueléticos encontrados em pacientes com PC (COQ *et al.*, 2008; DELCOUR *et al.*, 2011; LUBICS *et al.*, 2005; MARCUZZO *et al.*, 2010; STRATA *et al.,* 2004).

Um estudo prévio utilizando anóxia perinatal e restrição sensório-motora para a indução da PC registrou aumento do tônus muscular, alterações na marcha e redução na taxa de crescimento dos animais (STRATA *et al.,* 2004). Em outro estudo utilizando o mesmo modelo de lesão, a restrição sensório-motora atrasou a aquisição dos marcos do desenvolvimento e produziu alterações histológicas no córtex somatossensorial primário, o que pode contribuir para o desenvolvimento de déficits sensório-motores (MARCUZZO *et al.*, 2010). A exposição pré-natal de filhotes de ratos à endotoxina bacteriana através de injeções de lipopolissacarídeo (LPS) também pode contribuir para a reprodução das características da PC em animais experimentais. Estudos anteriores revelam que a administração de LPS durante o período gestacional pode produzir lesões na substância branca, semelhantes às observadas em pacientes com a mesma patologia (ROUSSET *et al.*, 2013).

Quanto ao sistema musculoesquelético, a indução de um modelo animal de PC que associa a exposição pré-natal de filhotes de ratos ao LPS, anóxia perinatal e restrição sensório-motora produziu atrofia muscular nos músculos tibial anterior e sóleo, além de transição do tipo de fibra lenta para rápida com repercussões negativas sobre as habilidades motoras desses animais (STIGGER *et al.*, 2011). Entretanto, a ampla caracterização dos déficits produzidos pelo modelo animal de PC, que associa essas três intervenções, ainda não foi realizada. Assim, o objetivo deste estudo foi verificar os efeitos da associação de injeções pré-natais de LPS, anóxia perinatal e restrição sensório-motora sobre as fibras musculares e JNMs do músculo extensor longo dos dedos (do inglês, EDL) em filhotes de ratos, visando à reprodução de achados patológicos observados em pacientes com PC. A investigação detalhada das possíveis alterações que este modelo animal causa sobre diferentes músculos e junções neuromusculares (JNMs) pode nortear o

desenvolvimento de novas terapias para a recuperação funcional de pacientes com PC.

REVISÃO DE LITERATURA

Incidência, fisiopatologia, classificação e diagnóstico da PC

A PC refere-se a um conjunto de desordens do movimento e da postura, atribuídas a lesões não progressivas que ocorreram durante o desenvolvimento cerebral (ROSENBAUM *et al.*, 2005). A incidência mundial da PC é estimada em cerca de 2 a cada 1000 nascidos vivos, porém, pode variar devido à escassez de registros formais (PANETH; HONG; KORZENIEWSKI, 2006). Em países subdesenvolvidos, tal patologia pode acometer sete em cada 1000 nascidos vivos, e ser considerada a causa mais comum de deficiência física (MANCINI *et al*, 2002). No Brasil, sabe-se que existe ocorrência maior dessa patologia no sexo masculino, sendo o tipo espástico o mais frequente (BRASIL, 2013).

Fatores pré-natais que ocorrem desde a concepção até antes do nascimento como infecções e parasitoses (rubéola, toxoplasmose, citomegalovírus, HIV), exposição a radiações diagnósticas ou terapêuticas, intoxicações por drogas, álcool, tabaco, pesticidas e medicações, diabetes mellitus, hipertensão, anemia grave, desnutrição, idade avançada, traumatismos e alterações anatomopatológicas (nó no cordão umbilical, cordão umbilical curto, malformações do cordão umbilical) são condições descritas em cerca de 70-80% dos casos de PC (KRIGGER, 2006; DODGE, 2008; JOHNSTON; HOON JUNIOR, 2006; ROTTA, 2002; McINTYRE *et al.*, 2013).

O período perinatal estende-se desde o início do trabalho de parto até 6 horas após o nascimento (ABPC, 2014). Eventos ocorridos nessa fase, tais como

asfixias, desproporção cefálico-pélvica e prematuridade, representam risco de 6% para o desenvolvimento de PC (KRIGGER, 2006; ROTTA, 2002). As asfixias pré e perinatal causam extenso comprometimento cerebral do recém-nascido, e são consideradas as principais causas de morbidade neurológica que levam à PC (COLVER; FAIRHURST; PHAROAH, 2014; ROTTA, 2002).

Fatores de risco pós-natais acontecem durante a infância e são associados a aproximadamente 10-20% dos casos de PC (JOHNSTON; HOON JÚNIOR, 2006; KRIGGER, 2006). Nessa fase, os danos cerebrais podem ser causados por infecções, hiperbilirrubinemia, traumas, distúrbios metabólicos como hipocalcemia, hipoglicemia, hipomagnesemia e desnutrição (DODGE, 2008; JOHNSTON; HOON JUNIOR, 2006; McINTYRE *et al.*, 2013; ROTTA, 2002).

As lesões encefálicas mais comumente observadas na PC são a leucomalácia periventricular (LPV), hemorragias intraventriculares e isquemia transitória ou irreversível (com necrose celular secundária) (KOMAN; SMITH; SHILT, 2004). A LPV caracteriza-se por necrose focal e gliose difusa da substância branca. Esse tipo de lesão também provoca perturbação da maturação de células da linhagem oligodendroglial, levando à hipomielinização e à formação de cistos e cicatrizes na substância branca (FOLKERTH, 2005). Sugere-se que infecções maternas aumentam o risco do desenvolvimento fetal de LPV, uma vez que a resposta inflamatória sistêmica fetal contribui para a ocorrência de lesões da substância branca (WU; COLFORD, 2000).

Bebês nascidos prematuros, como é o caso de parte dos pacientes com PC, apresentam fragilidade dos vasos sanguíneos intracerebrais e oscilações no fluxo sanguíneo cerebral. Assim, esses bebês são mais vulneráveis ao desenvolvimento de hemorragias intraventriculares (JOHNSTON; HOON JUNIOR, 2006). Além disso, a autorregulação do fluxo sanguíneo cerebral é um mecanismo de proteção que mantém a velocidade do fluxo sanguíneo cerebral estável em crianças normais, independentemente de variações da pressão arterial sistêmica. Tal controle ocorre por modificações do tônus arteriolar cerebral, induzidas por secreção de alguns fatores humorais. Tais mecanismos são funcionalmente imaturos em crianças prematuras. Devido a esse fato, a hipertensão sistêmica pode causar fenômenos hemorrágicos, enquanto a hipotensão sistêmica pode causar fenômenos isquêmicos (DISTEFANO; PRATICÒ, 2010). Os eventos hipóxico-isquêmicos (HI) promovem apoptose e necrose neuronal. Neurônios imaturos de recém-nascidos são mais

susceptíveis à morte apoptótica do que neurônios maduros de adultos. Por este motivo, a HI predomina como a principal causa de PC (McLEAN; FERRIERO, 2004).

As manifestações clínicas apresentadas pelos pacientes com PC dependem da distribuição topográfica, gravidade e extensão da lesão encefálica (ABPC, 2014; MY CHILD ^{TM}, 2014). Os principais sinais e sintomas observados nesses pacientes incluem comprometimentos do tônus muscular, das funções motoras grossas e finas, do equilíbrio dos reflexos e da postura (MY CHILD^{TM}, 2014). Déficits auditivos e visuais, convulsões, dificuldades de aprendizagem e transtornos de linguagem também são comumente observados em pacientes com PC (DODGE, 2008; KRIGGER, 2006; ROTTA, 2002).

A PC pode ser classificada em espástica, discinética e atáxica (de acordo com o comprometimento motor observado). A PC espástica é a mais comum, além de ser caracterizada por padrões anormais de postura e/ou movimento, aumento do tônus muscular e reflexos patológicos (hiperreflexia e/ou sinais de liberação piramidal). A forma discinética está presente em 16% dos casos de PC e pode ser subdivida em formas distônica (onde ocorre hipocinesia associada à hipertonia) e coreoatetoide (caracterizada por uma hipercinesia e hipotonia). A forma mais rara é a atáxica, a qual perfaz 5% dos casos de PC, e ocorre principalmente em crianças nascidas a termo. Nessa última, ocorre perda de coordenação, alteração de força, do ritmo e da metria do movimento (CANS, 2000; HIMPENS *et al.*, 2008; KRIGGER, 2006).

Mesmo após avanços tecnológicos significativos, o diagnóstico da PC continua sendo clínico (DODGE, 2008). Atrasos no desenvolvimento motor normal, tônus muscular anormal, postura incomum e persistência de reflexos primitivos são alertas iniciais para o diagnóstico de PC (KRIGGER, 2006). Outras ferramentas usadas para avaliar a extensão da lesão no SNC são exames de imagem, como a ressonância magnética, tomografia computadorizada e ultrassonografia de crânio (KOMAN; SMITH; SHILT, 2004). Preconiza-se que o tratamento não seja adiado pela espera diagnóstica ou avaliação etiológica, mas deve ser iniciado precocemente (DODGE, 2008).

Músculo Estriado Esquelético e Alterações na Paralisia Cerebral

Características do músculo estriado esquelético e das JNMs

As fibras musculares adultas são células alongadas e cilíndricas que possuem capacidade contrátil. Tais fibras são multinucleadas e os núcleos se localizam principalmente na periferia da célula (DAL PAI-SILVA; CARVALHO, 2007). O citoplasma das fibras musculares é denominado sarcoplasma e é constituído por miofibrilas. As proteínas contráteis dentro de uma miofibrila estão dispostas em unidades idênticas chamadas de sarcômeros. A organização dos sarcômeros em série confere a aparência estriada ao músculo (DAL PAI-SILVA; CARVALHO, 2007).

Em um músculo, as fibras estão organizadas em feixes chamados de fascículos, os quais são unidos por tecido conjuntivo. A camada de tecido conjuntivo que recobre o músculo inteiro é chamada de epimísio. No interior do músculo, com a separação dos fascículos, encontram-se finos septos de tecido conjuntivo, o perimísio. Mais internamente, encontra-se o endomísio, o qual envolve cada fibra muscular individualmente (JUNQUEIRA; CARNEIRO, 2013).

Os músculos são ainda constituídos por fusos musculares, localizados profundamente ao ventre muscular e são formados por fibras intrafusais especializadas. As fibras intrafusais são inervadas por neurônios sensoriais, monitoram as mudanças na extensão e taxa de alongamento dos fascículos musculares e transmitem a informação para o SNC, o qual responde, imediatamente, com ajustes corretivos à tonicidade dos grupos musculares (ZVARITCH; MACLENNAN, 2015).

A musculatura esquelética é constituída por tipos de fibras musculares que apresentam características morfológicas e funcionais distintas (MINAMOTO, 2005). Brooke e Kaiser (1970), com base na ATPase miofibrilar (mATPase) em meio de incubação em pH 9,4, precedido de pré-incubação em meios ácido (pH 4,3 - 4,6) e alcalino (pH 10,4 - 10,6), classificaram as fibras em tipos I, IIA e IIB. As fibras do tipo I reagem fortemente com a pré-incubação ácida, de contração lenta; as fibras do tipo IIB reagem fortemente com pré-incubação alcalina, de contração rápida. As fibras tipo IIA respondem, de forma variada, à reação pela mATPase. Geralmente, a

reação é moderada após pré-incubação em pH alcalino e fraca após pré-incubação em pH ácido.

O músculo EDL, alvo deste estudo, contém principalmente fibras do tipo II, as quais possuem pouca quantidade de mitocôndrias, linha Z delgada e retículo sarcoplasmático altamente desenvolvido (SCHIAFFINO; HANZLÍKOVÁ; PIEROBON, 1970). O EDL origina-se no epicôndilo lateral do fêmur e divide-se em quatro tendões, inserindo-se no terceiro, quarto e quinto dedo do membro pélvico de ratos, cuja função é a extensão dos dedos (HEBEL; STROMBERG, 1976). Quando o rato está apoiado somente pelos membros pélvicos, o músculo EDL não é ativado e seu sinal na eletromiografia é quase plano. Quando o rato está andando, a atividade eletromiográfica caracteriza-se por rajadas rítmicas, que correspondem à fase de balanço do ciclo da marcha (SLAWIRISKA *et al.*, 1996).

Os músculos estriados esqueléticos de ratos são imaturos ao nascimento e mudanças importantes no tipo de fibra podem ocorrer durante as fases iniciais de desenvolvimento pós-natal (SCHIAFFINO; REGGIANI, 2011). A contração muscular espontânea produz a ativação dos fusos musculares de forma somatotópica. A interação entre sinais de retroalimentação sensorial disparados pelos movimentos e oscilações do fuso moldam a formação de conexões corticais necessárias para a coordenação sensório-motora (KHAZIPOV *et al.*, 2004). Além disso, a contração muscular é um fator importante para o desenvolvimento muscular normal e maturação das JNMs, promove o crescimento miofibrilar correto para a estabilização do receptor de acetilcolina nas JNMs (SCHIAFFINO; REGGIANI, 2011).

As JNMs são sinapses especializadas encontradas entre os motoneurônios e as fibras musculares esqueléticas (WU; XIONG; MEI, 2010). Mais especificamente, os neurônios motores inferiores que fazem parte dessas junções possuem seus corpos celulares no corno anterior da medula espinal e projetam-se até a periferia através de axônios mielinizados. Esses terminais axonais possuem acetilcolina (ACh) e ramificam-se amplamente ao se aproximarem do músculo-alvo. Devido a essa ramificação, o axônio entra contato com várias fibras musculares para formar grupos funcionais conhecidos como unidades motoras. Logo antes de atingir a fibra muscular, o axônio perde a bainha de mielina e passa a ser coberto apenas pelas células de Schwann (MARTYN; FAGERLUND; ERIKSSON, 2009).

Existe uma fenda chamada de goteira sináptica primária entre o axônio e a fibra muscular, a qual é revestida por lâmina basal e contém a enzima

acetilcolinesterase. A parte do músculo oposta ao neurônio é chamada de membrana pós-sináptica, contendo receptores para ACh e o sarcoplasma juncional, o qual suporta estrutural e metabolicamente essa região (Figura 1). Com o amadurecimento da JNM, a membrana pós-sináptica se invagina e forma as dobras juncionais. Os receptores para ACh estão concentrados apenas nas cristas destas dobras juncionais (WU; XIONG; MEI, 2010).

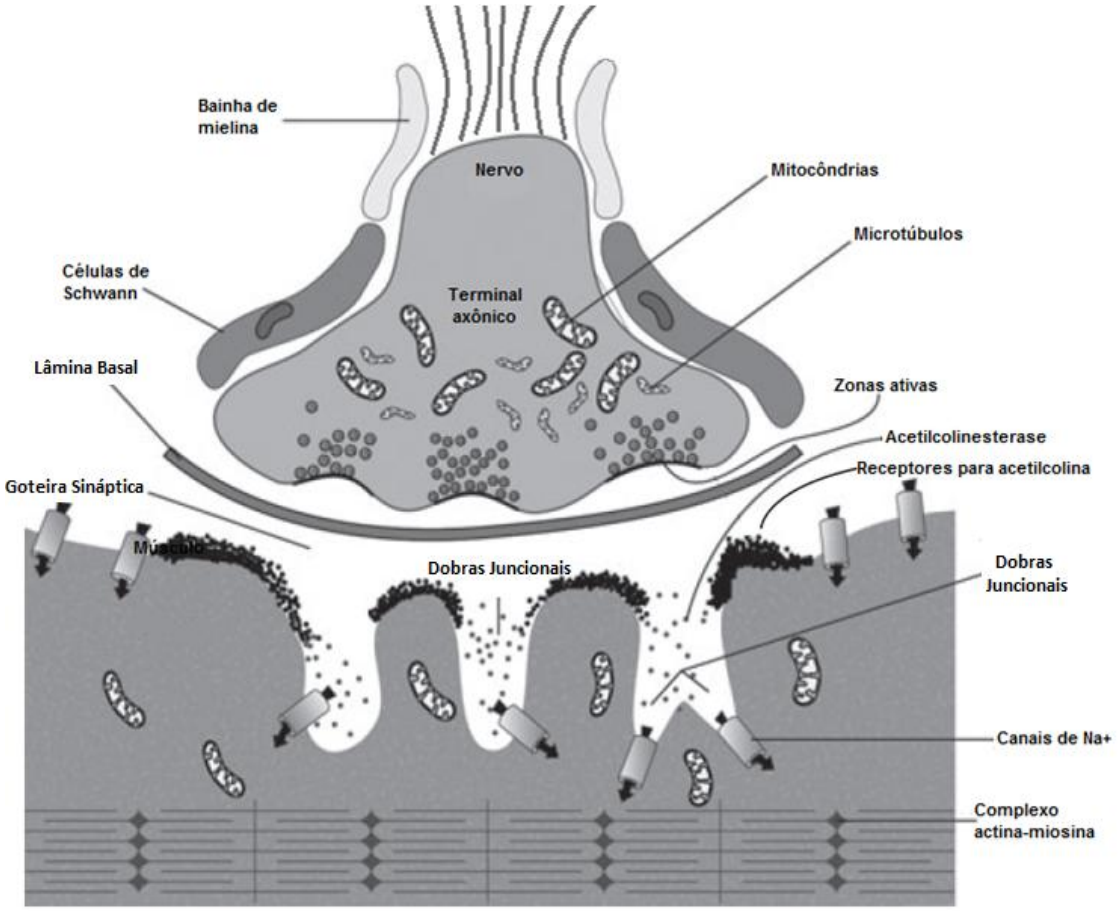

Figura 1 - Esquema da estrutura de uma JNM e seus principais constituintes. Adaptado de Martyn; Fagerlund; Eriksson, 2009

De acordo com os diferentes tipos de fibras musculares, podem existir variações na forma e tamanho do terminal axônico e também na complexidade da membrana pós-sináptica. As fibras musculares do tipo I possuem JNMs com dimensões pequenas, forma arredondada ou ligeiramente elíptica, ramificação axonal grosseira com terminais axônicos dilatados, goteira sináptica rasa e dobras juncionais pouco profundas e simples. Em contrapartida, as fibras musculares do tipo IIb apresentam junções com dimensões maiores, forma elíptica, terminais axônicos finos, longos, ramificados e delicados, goteira sináptica profunda e dobras

juncionais profundas e complexas. As fibras do tipo IIA exibem junções com aspectos estruturais que ficam entre aqueles apresentados pelas fibras dos tipos I e IIB (OGATA, 1988).

As JNMs são funcionais ao nascimento, mas sofrem modificações no período pós-natal. Os terminais pré e pós-sinápticos tornam-se mais interdependentes e ocorre melhor comunicação entre os terminais nervosos e os receptores de ACh. Quando atingem a maturação, essas estruturas mantêm-se de forma estável, mas são suscetíveis à remodelação. As JNMs possuem ainda capacidade de regeneração após lesão do nervo periférico ou músculo (SANES; LICHTMAN, 1999). Em estudo realizado por Robinson *et al.* (2013), a organização das JNMs foi avaliada em crianças com PC do tipo espástica e que apresentavam escoliose. Redução das mitocôndrias nas terminações nervosas pré-sinápticas e diferenças na conformação das dobras juncionais foram observadas no músculo espinal desses pacientes. Todavia, descrições detalhadas sobre as JNMs em humanos e em modelos animais ainda são escassas.

Estudos em Humanos com PC

Para que o crescimento muscular ocorra adequadamente são necessários alongamentos musculares regulares (GRAHAM; SELBER, 2003). Na maioria dos casos de PC, os músculos não relaxam devido à presença da espasticidade (MARBINI *et al.*, 2002). Assim, pode ocorrer uma redução do volume do ventre associado ao encurtamento muscular nesses pacientes (MALAIYA *et al.*, 2007). Tais alterações musculares podem causar uma torção dos ossos longos, instabilidade articular e alterações degenerativas prematuras em articulações (GRAHAM; SELBER, 2003). Consequentemente, crianças com PC apresentam redução da atividade motora devido à fraqueza, à dor e à falta de equilíbrio (GRAHAM; SELBER, 2003).

As crianças com PC do tipo espástico podem apresentar aumento na quantidade de colágeno muscular, o que pode contribuir para o aparecimento de rigidez (BOOTH; CORTINA-BORJA; THEOLOGIS, 2001). Análises ultraestruturais de biópsias realizadas no músculo adutor longo e tríceps sural de crianças com PC também mostram desorganização ou desorientação de miofibrilas, alterações mitocondriais (degeneração, agregação, polimorfismo e hiperplasia) e alteração

nuclear (núcleos picnóticos) (MARBINI *et al.*, 2002). Músculos espásticos apresentam ainda redução no comprimento do sarcômero em repouso (FORAN *et al.*, 2005). E, apesar de não ter qualquer anormalidade muscular específica desta patologia, mudanças na distribuição do tipo de fibra (predominância de fibras do tipo I) e variações no tamanho da fibra muscular (hipertrofia das fibras do tipo II) são alterações vistas em biópsias do músculo gastrocnêmio de pacientes jovens (ITO *et al*., 1996).

Modelos animais de PC

A PC é resultado da interação de múltiplos fatores de risco, que podem agir em diferentes momentos do desenvolvimento do SNC (ROSENBAUM *et al.*, 2005). Por tais motivos, as alterações anatomopatológicas observadas nessa encefalopatia crônica são variáveis (ROTTA, 2002). Assim como em humanos, ao considerarmos modelos animais de PC, o momento e a natureza da lesão podem produzir diferentes fenótipos (CLOWRY, 2014).

Em filhotes de ratos, a infecção induzida através de injeções de LPS no período pré-natal pode causar lesões na substância branca, possivelmente devido à produção de citocinas inflamatórias (DAMMANN; DURUM; LEVITON, 2001; WANG *et al.*, 2006). A anóxia perinatal leva ao aumento do tônus muscular, alterações na marcha e desorganização no córtex motor primário (STRATA *et al.*, 2004). Além disso, a restrição sensório-motora no período pós-natal produz redução do peso corporal e da densidade óssea e prejuízos no desempenho motor em testes de caminhada e em escadas (MARCUZZO *et al.*, 2010).

Filhotes de ratos expostos à infecção por LPS associada com anóxia perinatal apresentam déficits na coordenação motora e diminuição dos movimentos espontâneos mais graves do que filhotes expostos à LPS ou anóxia perinatal isoladamente (GIRARD *et al.*, 2009). Quando foi realizada a combinação de anóxia perinatal e a restrição sensório-motora, houve aumento do colágeno e alterações na matriz do tecido conjuntivo nos músculos glúteo máximo, quadríceps femoral e tríceps sural (COQ *et al.*, 2008). Esse segundo tipo de associação causou também atrofia no músculo sóleo e diminuição do comprimento da passada (MARCUZZO *et al.*, 2008).

Stigger *et al.* (2011) realizaram estudo com injeções de LPS, anóxia perinatal e restrição sensório-motora em associação ou isoladamente. Nos testes motores, todos os grupos submetidos à restrição sensório-motora apresentaram resultados significativamente inferiores devido à falta de fluência e coordenação. Porém, a associação dos três insultos produziu os piores desempenhos nos testes motores realizados. Além disso, a atrofia muscular do tibial anterior somente foi observada no grupo onde houve tal associação. Acredita-se que a restrição sensório-motora imita a imobilidade induzida pela espasticidade, enquanto a associação com LPS e anóxia perinatal desempenham importante papel na reprodução de um fenótipo mais complexo de PC. Sendo assim, o presente estudo utilizou a associação de injeções de LPS, anóxia perinatal e restrição sensório-motora para identificar e caracterizar as alterações musculares causadas neste modelo animal de PC.

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ARTIGO CIENTÍFICO

AN EVALUATION OF AN ANIMAL MODEL OF CEREBRAL PALSY: THE EFFECTS ON THE MORPHOLOGY OF THE *EXTENSOR DIGITORUM LONGUS* MUSCLE

AN EVALUATION OF AN ANIMAL MODEL OF CEREBRAL PALSY: THE EFFECTS ON THE MORPHOLOGY OF THE *EXTENSOR DIGITORUM LONGUS* MUSCLE

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ABSTRACT

Background: Cerebral palsy (CP) refers to a chronic childhood encephalopathy that causes movement disorders, which is the main cause of physical disability.
Objective: Animal models have been used in attempting to reproduce the clinical characteristics of the CP. However, as yet, there is no model capable of faithfully reproducing the findings of this clinical condition in the experimental setting. Thus, the objective of this study was to verify the effects of a CP model that combines prenatal exposure to lipopolysaccharide (LPS), perinatal anoxia and sensorimotor restriction on muscle fibers and neuromuscular junctions (NMJs) of the *extensor digitorum longus* muscle (EDL) in rats.
Study design: Male Wistar rat pups were separated into two groups: a) Control (CTL - n = 10) - pups of mothers injected with saline during pregnancy and b) Cerebral Palsy (CP - n = 10) - pups of mothers injected with LPS during pregnancy, and submitted to perinatal anoxia and sensorimotor restriction. Motor performance was assessed in an open field on postnatal day 29 (P29) and P45 in both groups. After euthanasia, EDL muscle samples were processed for morphological and morphometric analysis of muscle fibers and NMJs.
Results: Motor performance, locomotion time and number of rearings were significantly lower in the CP compared to the CTL group at 29 days of age ($p < 0.001$ and $p < 0.01$, respectively). At 45 days, the locomotion time of the animals from the CP group was also shorter in relation to the CTL group ($p < 0.05$). The body weight, weight and length of the EDL muscles were 16%, 16% and 15% lower, respectively, in the CP group in relation to the CTL. The animals in the CP group presented hypertrophy in the type IIB fibers, but there were no differences between the studied groups regarding the type I, IIA and IIB muscle fiber counts. The nuclei/fiber and capillary/fiber ratios were significantly higher in the CP group than in the CTL group. Regarding the intrafusal fibers, the animals form the CP group presented 26% atrophy in the cross-sectional area of these structures and 26% reduction in the muscle spindle area. Intramuscular collagen volume was 34% higher in the animals from the CP group. In the animals from the CP group, the EDL muscle showed myofibrillar disruption and Z-line disorganization and dissolution. The NMJs from the CP group presented increases of 22% in area and 11% in diameter when compared to the CTL group.
Conclusion: The animal model of CP using injections of LPS, perinatal anoxia and sensorimotor restraint, produces motor deficits and macro and microscopic alterations and in the ultrastructure of the EDL muscle that are also observed in patients with CP.

Keywords: Cerebral Palsy animal model; Lipopolysaccharide; Perinatal anoxia; Sensorimotor Restriction; *Extensor Digitorum Longus* Muscle.

INTRODUCTION

Cerebral palsy (CP) is defined as a chronic encephalopathy that causes sensorimotor disorders at different degrees of severity[1], and is considered the most common cause of physical deficiency in childhood[2]. CP is believed to result from the interaction of multiple pre, peri, or postnatal risk factors[3]. The musculoskeletal changes observed in these patients are mainly due to deficits in the execution of spontaneous movements and include atrophy or hypertrophy of the muscular fibers, increased volumes of connective tissue in the musculature, bone malformation and degeneration of the articular cartilage[4,5].

An earlier study also shows that children with spastic CP may also present alterations in the organization of the neuromuscular junctions (NMJs), with reduced mitochondria in the presynaptic nerve endings and differences in the conformation of the junctional folds of the spinal muscle[6]. NMJs are elements of the nervous system that play a key role in transmitting signals between motor neurons and muscle fibers[7]. Spontaneous muscle contraction is known to be an important factor for the maturation of NMJs and normal muscle development[8].

During muscle contraction, the muscle spindles are activated. These structures are located deep within the muscular belly and are formed of specialized intrafusal fibers[9]. Each muscle spindle is comprised of 2-10 intrafusal fibers, which are surrounded by a capsule of connective tissue. Intrafusal fibers can be classified into three types: dynamic nuclear bag fibers; static nuclear bag fibers and nuclear chain fibers[10]. The intrafusal fibers monitor changes in the length of the muscular fascicles and send information to the central nervous system, which responds by adjusting the tone of the muscular groups[9]. The sensory feedback signals triggered by the movements and oscillations of the muscle spindle lead to the formation of the cortical connections necessary for sensory-motor coordination[11].

Animal models are being developed with the purpose of reproducing the signs, symptoms and brain and musculoskeletal characteristics found in CP patients[4,12-15]. Inducing an animal CP model that combines prenatal exposure of rats to lipopolysaccharide (LPS), perinatal anoxia and post-natal sensorimotor restriction produced muscular atrophy in the anterior *tibialis* and *soleus* muscles, the transition from type I to type II fibers and changes in the motor skills of the animals[16]. In this experimental model, sensorimotor restriction is believed to mimic spasticity-induced

immobility, while LPS and perinatal anoxia may mimic neuropathological findings in CP. However, a broad characterization of the muscular deficits produced by this animal model of CP in muscles in which type II fibers predominate, such as the EDL, has not yet been conducted. Therefore, the objective of this study was to investigate the effects of the combination of prenatal injections of LPS, perinatal anoxia and post-natal sensorimotor restriction on muscle fibers and NMJs in the EDL muscle in rat pups.

METHODOLOGY

Place, animals and induction of the animal model of CP

This research was approved by the Ethics in the Use of Animals Committee (*CEUA*) of UNIOESTE. During all stages of the experiment, the ethical principles recommended by the Brazilian College of Animal Experimentation (COBEA) were followed.

At the beginning of the experimental protocol, adult Wistar rats (42 females and 10 males), approximately three months old, from the UNIOESTE Central Bioterium (Animal Facility) were used. These animals were housed in polycarbonate boxes (27 x 26 x 31cm) covered wood shavings, in an environment with a 12h light/dark cycle, controlled temperature (20 ± 2ºC), with water and food *ad libitum*.

In order to verify the estrous cycle, the rats were submitted to daily colpocytological examination, based on the analysis of vaginal smear samples. When receptive (the proestrus phase), the females were placed with a male for mating. Pregnancy was confirmed the following morning, through the presence of spermatozoa in a second colpocytological examination. Then, some of the females were injected intraperitoneally with vehicle (n=15; 100 µl of sterile saline); and the others with LPS (n=27; approximately 200 µg/kg LPS in 100 µl of sterile saline). Both injections were performed every 12 hours (07:30 and 19:30 h), as from the 17th gestational day (G17) until the end of gestation (G21).

The day of birth of the offspring was considered the postnatal day 0 (P0), when the pups were separated into two groups: 1) the Control group - pups of rats injected with saline during pregnancy (CTL, n = 10) and 2) the CP group - pups of rats injected with LPS during pregnancy, submitted to perinatal anoxia and

sensorimotor restriction (CP, n=10). Only male offspring were used in this study, as females were excluded due to the influence of sex hormones on brain development. In the first days after birth, the litters were standardized into 8 offspring (with preferential removal of females).

The offspring from the CP group underwent perinatal anoxia on P0. For this intervention, the pups were placed in a closed chamber, partially immersed in water at 37° C, with a flow of 9 L/min of nitrogen (100%) for 20 minutes. Following this procedure, the animals were quickly removed, kept under normal atmospheric conditions and observed until their normal respiratory pattern returned[16]. The male offspring from the CTL group were also placed in the same chamber for 20 minutes, which remained open and with normal atmospheric airflow. To avoid rejection by the mother following this intervention, direct contact by the experimenter was prevented by ensuring the pups were wrapped in wood shavings from the animal box during handling. Whenever possible, the mothers remained close to the pups, but in their housing boxes.

Finally, the pups from the CP group underwent sensorimotor restriction 16 hours/day, from the first postnatal day (P1) to P30. Restriction was achieved with the aid of adhesive microporous tape and an epoxy mold (adjusted according to the size of the animal), which allowed the hind limbs to be immobilized in an extended position. The animals from the CTL group received manipulation in the hind limbs similar to that performed on the restricted animals to prevent possible differences in brood development.

Evaluating the displacement time in the open field

The locomotor activity and exploratory behavior of the animals were evaluated on P29 and P45. The test apparatus, called the open field, consisted of an arena (40 x 100 x 100 cm) with the floor subdivided into 12 squares. For each test, the animals were initially positioned in the northeast corner of the arena and removed after a period of five minutes. Each evaluation was filmed from above for later evaluation of the following parameters: number of line quadrants crossed, number of rearings, grooming frequency, total time immobile and total time mobile.

Sample collection

At 48 days of age, the animals from both groups were weighed and anesthetized with an overdose of ketamine hydrochloride (50 mg/kg, ip; *Cristália*, Brazil) and xylazine hydrochloride (10 mg/kg, ip; *Cristália*, Brazil). The skin of the hind limb was folded back and the anterior tibial muscle was removed for bilateral dissection of the EDL muscle. The EDL muscle from the right antimere was then weighed and its length (muscular belly) measured using a digital caliper (Digimess®, São Paulo, Brazil). The muscles were sectioned using a stainless steel blade and prepared for histological, histoenzymological and histochemical examination.

The histological and histoenzymological study of the muscle fibers and quantification of intramuscular collagen

After dissection, the proximal portions of the right antimere EDL muscle were maintained at room temperature for 30 to 40 minutes. After which, the samples were covered with neutral talc (JOHNSON'S®, São Paulo, Brazil) and frozen in liquid nitrogen for storage in a Biofreezer at -80ºC. Subsequently, cross sections (7µm thick) of these samples were obtained using a cryostat (LUPETEC CM 2850 Cryostat Microtome).

Some of the sections obtained were stained using the Hematoxylin-Eosin (HE) technique[17], and the slides were mounted with Permount (Fisher Scientific®, New Jersey, USA). To quantify the nuclei, muscle fibers and capillary/fiber ratio, 10 images of randomized visual fields were used per animal (magnification of 400X). When measuring the capillary/fiber ratio, muscle fibers overlapping the upper and right margins were included in the count, while those overlapping the lower and left margin were excluded. Two trained evaluators individually performed the quantification and the mean values obtained were used[18]. For the muscle spindle measurements, images (magnification of 1000X) of the same muscle spindle were captured in 10 different cuts per animal. Standardized measurements were made of the area and diameter of the muscle spindle, mean cross-sectional area of intrafusal fibers, number of intrafusal fibers and thickness of the spindle capsule in the muscle spindle with the largest diameter.

Some of the other the histological sections of the EDL muscle was submitted to NADH-TR (Nicotinamide Adenine Dinucleotide - Tetrazolium Reductase) reaction

to analyze the oxidative and glycolytic metabolism of the muscle fibers (Pearse's technique[19], modified by Dubowitz and Brooke[20]). The slides were dehydrated, diaphanized and mounted using Permount (Fisher Scientific, New Jersey, USA). Images of two visual fields (200x magnification) were used for the morphometric analyzes (cross-sectional area and counts of the three types of muscle fibers) of approximately 300 fibers per animal.

To quantify the percentage of intramuscular collagen, the remainder of the histological sections of the EDL muscle was stained with Masson's Trichrome. Three microscopic images from each animal were used in the analysis (magnification 200X). Once the images were captured, the percentage of collagen/total area was calculated.

Transmission electron microscopy (T.E.M.) study

To visualize the ultrastructure of the muscle fibers from the EDL, the right antimere was removed and reduced to longitudinal fragments (approximately one mm wide) and immersed in glutaraldehyde (2.5%) for fixation. Subsequently, the samples were washed in 0.1M phosphate buffer, pH 7.3 (15 minutes) and post-fixed in 1% osmium tetroxide for two hours. After which, they were washed in distilled water, incubated in 0.5% uranyl acetate for 2 hours, dehydrated in acetone and soaked in a mixture of resin and 100% acetone (12 hours) for the subsequent formation of blocks. The desired fields were selected from semi-thin sections and ultra-thin sections were obtained using an ultramicrotome (Ultracut UCT, Leica®, Germany). The ultrathin sections were stained with a saturated solution of uranyl acetate and lead citrate. The material obtained was examined and photographed in a transmission electron microscope (CM100, Philips®, The Netherlands).

Evaluation of the NMJs

For the study of the NMJs, the proximal part of the left antimere of the EDL muscle was removed and immersed in Karnovsky[21] at room temperature. Subsequently, the muscle was sectioned longitudinally into three or four slices with stainless steel blades for the nonspecific esterase reaction[22]. The sections were dehydrated, diaphanized and mounted with Permount (Fisher Scientific®, New Jersey, USA). Images of the NMJs (magnification of 200x) were obtained and

measurements of the area and largest and smallest diameters of 100 NMJs were taken for each animal.

Image analysis

Images of the histological sections stained with HE, Masson's Trichrome staining and photo-documentation of the NMJs were taken using an Olympus Bx60 microscope coupled to an Olympus DP17 camera (Tokyo, Japan) with the aid of the DP Controller 3.2.1 276 program. Morphometric analyzes were performed using Image Pro Plus 6.0 software (Media Cybernetics, Maryland, USA). The analysis of the cross-sectional area and counts of the three types of muscle fibers (NADH-TR reaction) was conducted on a Primo Star, Zeiss (Oberkochen, Germany) microscope coupled to a camera (AxiocamERc5s) and a computer, measurements were performed with the aid of Axiovision Rel. 4.8 software (Carl Zeiss Microimaging Inc., Germany).

Statistical analysis

The data obtained were statistically analyzed considering the results of the normality tests. Student's t-test was used when the data distribution was normal. In cases where the data distribution was not normal, the non-parametric Mann-Whitney statistical test was used. For the NADH-TR analysis, the two-way ANOVA test was used using the factors CP induction of and muscle fiber type, followed by Bonferroni's post-hoc test. Values of $p < 0.05$ were considered significant. The statistical analysis was performed with the aid of GraphPad Prism 5.0 software (La Jolla, USA).

RESULTS

Locomotor performance

The animals from the CP group spent less time in locomotion compared to the CTL group ($p < 0.001$) at 29 days of age, and at 45 days of age the difference between the groups persisted ($p < 0.05$). No differences were observed between the CP and CTL groups regarding the number of quadrants crossed in the open field at 29 and 45 days of age (Table 1).

Regarding exploratory behavior, the frequency of rearing in the CP group was only lower when compared to the CTL group at 29 days of age ($p < 0.01$). There were no differences between the experimental groups regarding the frequency of grooming behavior at either of the two evaluations.

Anthropometric variables

At 48 days of age, the body weight of the animals in the CP group was 16% lower in relation to the CTL group ($p < 0.001$). The animals in the CP group also presented a 16% reduction in weight ($p < 0.01$) and a 15% reduction in EDL muscle length ($p < 0.01$) when compared to the CTL group (Table 2).

Morphological and morphometric analysis of the muscle fibers

The muscle fibers of the EDL muscle from the two experimental groups had a normal morphological aspect, regular diameter, a polygonal shape with rounded angles and peripheral nuclei in the subsarcolemmal position (Figures 1A and 1B). The fibers were arranged in fascicles surrounded by the perimysium, with each fiber being surrounded by endomysium (Figures 2A and 2B). Vessels and nerves were present in the connective tissue of the muscle. The muscle spindles in the EDL muscle were observed in the two studied groups and were found to have a normal organizational pattern, that is, smaller and thinner intrafusal fibers, surrounded by a connective tissue capsule (Figures 1C and 1D).

Regarding morphometry, there was an increase in the number of peripheral nuclei in the CP group compared to the CTL group ($p = 0.034$), while the number of muscle fibers was similar between the studied groups (Figures 1A and 1B; Table 3). Thus, the core/fiber ratio was 27% higher in the CP group compared to the CTL group ($p < 0.01$). There was also an increase of 23% in the capillary/fiber ratio (Table 3). In the CP group, the muscle spindle area and the mean cross-sectional area of the intrafusal fibers were 26% smaller when compared to the CTL group (Figures 1C and 1D; Table 3). However, there no significant difference between the groups in terms of the largest diameter of the muscle spindle, number of intrafusal fibers, or muscle spindle capsule thickness. Intramuscular collagen showed a 34% increase in the CP group compared to the CTL group ($p = 0.009$; Figure 2C).

The histoenzymological analysis permitted the muscle fibers from the CP and CTL groups to be characterized as types I, IIA and IIB[10] (Figures 3A and 3B). There was a significant increase in the cross-sectional area of the type IIB fibers from the CP group compared to CTL (two-way ANOVA showed significant interaction effect (($F_{(2.42)}$ = 3.35; p = 0.04), for the group ($F_{(1.42)}$ = 6.88; p = 0.01) and for the fibers ($F_{(2.42)}$ = 187; p < 0.001). Regarding the other fiber types, there were no differences between the groups (Figure 3C). Similarly, there was no significant difference between the groups in terms of count of the three identified muscle fiber types (Two-way ANOVA showed a significant effect for the fibers ($F_{(2.42)}$ = 13.1; p < 0.001), whereas no effect was observed for the interaction ($F_{(2.42)}$ = 1.88; p = 0.16) and for the group ($F_{(1.42)}$ = 0.11; p = 0.74) (Figure 3D).

Ultrastructural analysis of the EDL muscle

The morphology of the EDL muscle from the CTL group exhibited sarcoplasm with the presence of well-defined myofibrils and an organized Z-line (Figure 4A). In the animals from the CP group, there was dissolution and disorganization of the Z-line in several regions (p <0.05) and rarefied or loosely arranged myofibrils (p <0.001, Figures 4B and 4C) were observed.

Morphological and morphometric analysis of NMJs

The NMJs from the CP and CTL groups were polymorphic, round, oval and elliptical in shape (Figures 5A and 5B). The morphometric analysis showed an increase in the area (22%, p <0.001, Figure 5C) and in the larger diameter (11%, p <0.05) of the NMJs from the CP group compared to the CTL group (Figure 5D). There was no significant difference between the groups studied in relation to the smallest diameter of the NMJs (Figure 5E).

DISCUSSION

In the present study, the animals from the CP group spent less time locomotion during the open field test when compared to the CTL group at 29 and 45 days of age. There was no improvement in this parameter even after the removal of the sensorimotor restriction, which took place at 30 days of age. Regarding the

number of quadrants crossed by the animals during that test, the CP and CTL groups were similar at 29 and 45 days of age. These results show the animals in the CP group moved for less time, but appeared to have traveled a distance similar to that of the CTL animals. Sensorimotor restraint causes intrinsic muscle disruption due to disuse[4] and animals submitted to this procedure have difficulty discharging the plantar weight during the support phase, which may compromise gait and other motor skills[15]. However, the result might be related to the fact the animals from the CP group compensated for their deficits by greater use of the thoracic limbs. When using LPS injections combined with perinatal anoxia, Stigger *et al*. [23], also found no difference in the distance covered in the open field test at 29 days of age. Grooming frequency was also similar between the CP and CTL groups at both evaluations. Although LPS injections and perinatal anoxia are known to be able to cause damage to the white matter and disorganization of M1[15,24,25], these two insults may not have affected thoracic limb motor control in the CP group. As for the number of rearings, a behavior that requires strength and control of the pelvic limbs, there was a reduction in the CP group when compared to the CTL group at 29 days. Thus, sensorimotor restriction would seem to play a fundamental role in inducing motor alterations in the hind limbs. Spontaneous motor activity is known to be essential for the development of the corticospinal tract[26]. Motor experiments reinforce and refine synaptic connectivity, favor myelination of the corticospinal tract and maturation of the motor unit and reflex circuits. Interventions that impair the performance of movements during the development of locomotion, such as sensorimotor restraint, may disturb the establishment of connections within the motor circuit [26], leading to functional impairment.

The body weight of the animals from the CP group was significantly lower than that of the CTL group. In humans, low birth weight is one of the main risk factors associated with CP[27,28]. Marques *et al*. [29], using LPS injections, perinatal anoxia and sensorimotor restriction to induce CP, also observed reduced body weight in the animals and attributed the finding to a decrease in bone mineral density due to lack of weight discharge. In this study, a reduction in the length of the EDL muscle was also observed in the CP group. Longitudinal muscle growth occurs in the first four to six postnatal weeks in rats. Radial muscle growth occurs simultaneously and lasts a further 10 weeks[30]. The lack of longitudinal growth of skeletal muscle in patients with CP leads to muscle contractures, long bone twitches and early joint degeneration[5].

Again, this animal model of CP reproduced one of the characteristic clinical findings of this pathology, muscular shortening. To the best of our knowledge, this is the first description of these parameters using such an experimental CP model.

Striated skeletal muscle is a highly dynamic tissue that can alter its morphological, metabolic and functional characteristics in response to changes in the quantity and/or pattern of neuromuscular activity[31]. In the present study, there was an increase in the number of peripheral nuclei and in the core/fiber ratio in the CP group. Muscle fibers that have a greater number of nuclei generally have a larger cross-sectional area, but in certain circumstances, these parameters may change independently of one another[32]. The CP group was also found to have a higher capillary/muscle fiber ratio associated with hypertrophy of type IIB fibers in the EDL muscle. Increased capillary density, resistance, and hypertrophy of muscle fibers are some of the changes seen in target muscles of continuous muscular activation due to spasticity[33,34]. Although the sensorimotor restriction model used in this study is considered of disuse, the characteristics found in the EDL muscle of the CP group are compatible with those observed in spastic muscles.

In this study, an increase in intramuscular collagen was observed. Neuronal activity regulates the synthesis of collagen and in spastic muscles the neuronal stimulation is intensified[35]. Gagliano *et.al* (2013) [36] observed that patients with spastic CP also have increased collagen in the tendons. These muscular alterations lead to muscle stiffness and reduced function[4]. Spasticity-induced fibrosis directly limits muscle length growth[35], which together forms a mechanical barrier to muscle regeneration[37]. Additional factors that may contribute to muscle rigidity are the organization of collagen, the distribution of collagen types and the proteoglycan content[38].

This is the first time the characteristics of muscle with a predominance of type II fibers following the induction of this animal model of CP have been observed. In an earlier study, Marcuzzo *et al*. [39] showed that the sensorimotor restriction alone or associated with perinatal anoxia causes atrophy of the soleus muscle of rats, which was improved after treadmill training. Stigger *et al*. [39] noted a reduction in the cross-sectional area of the *soleus* and *anterior tibial* muscle fibers using the same CP model. The predominance of fibers within a muscle will cause it to have different biochemical and structural properties[40], which promote varied responses to certain insults[41].

Regarding the muscle spindles, the absence of weight discharge is known to be able to reduce the cross-section area of the intrafusal fibers, decrease the diameter of the equatorial region and fragment the nerve endings[42]. In the present study, there was a decrease in the muscle spindle area and the cross-sectional area of the intrafusal fibers in the CP group. Apparently, the period between removal of the sensorimotor restraint and the euthanasia of the animals was insufficient to repair the muscle spindle structure, although the intrafusal fibers presented high concentrations of satellite cells and a smaller myonuclear domain (amount of cytoplasm supported by a single nucleus), which favor growth, regeneration and repair[42].

The ultrastructural study of the EDL muscle showed alterations such as myofibrillar disruption and Z-line disorganization and dissolution in the animals from the CP group. Studies using electron microscopy in animal models of CP are scarce and are generally conducted through biopsies of individuals with this pathology. Patients with myofibrillar myopathies exhibit ultrastructural alterations that are typically focal. Some regions contain normal sarcomeres alternating with areas of pronounced myofibrillar disruption[42]. This animal model reproduced focal ultrastructural changes similar to those found in patients with CP. According to Marbini *et al*. (2002)[34], myofibrillar disruption is found in all cases, which is associated with Z-line disorganization and a consequent loss of the striation that is characteristic of the skeletal muscle.

The animals from the CP group presented an increase in the area and in the largest diameter of the NMJs when compared to the CTL group. NMJs are structures that can remodel themselves according to the functional demands[45]. Conditions such as disuse, neuromuscular disorders and aging lead to muscle weakness, causing NMJs to lose functionality in the most affected regions[46]. In this study, the disuse caused by the sensorimotor restriction may have caused the longitudinal lengthening of the nerve terminals in order to reinforce synaptic transmission and maintain muscle function[47].

CONCLUSION

The association of LPS injections, perinatal anoxia and sensorimotor restraint produced lasting effects on the function and structure of the muscular system, as

shown by the reduction in rearing frequency and in muscle length. There were increases in the nuclei/fiber ratio, capillary/fiber ratio, type IIB fiber muscle hypertrophy and intramuscular collagen, which are findings commonly found in patients with spastic CP. The morphological alterations found in the muscle spindle may affect posture maintenance and movement coordination. The ultrastructural study of the EDL muscle showed alterations similar to those found in biopsies of patients with CP. However, the alterations to the NMJs caused by the animal model of CP constitute new discoveries, which require further investigation.

This animal model proved to be a useful means to understand the pathophysiology of this clinical condition. It is suggested that new studies be performed with evaluations at different periods, that is, during sensorimotor restriction and soon after its removal. The need for such studies became evident sue to the fact that immobilization in the developmental period of locomotion aggravated the repercussions of the clinical condition. Given this, this experimental model could be an important tool to check the efficacy of early-start rehabilitation programs.

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Table 1. Locomotor variables of the animals from the CTL and CP groups evaluated in the open field at 29 and 45 days of age.

Variables **(n = 10)**	**CTL** **P29**	**CP** **P29**	**CTL** **P45**	**CP** **P45**
Locomotion time (seconds)	94.1 ± 15.8	55.9 ± 24.2***	67.3 ± 16.8	49.2 ± 21.6*
Number of quadrant crossings	96.1 ± 22.1	85.2 ± 35.9	72.2 ± 30.6	69.9 ± 39.2
Grooming frequency	2.5 ± 1.0	2.7 ± 0.8	2.3 ± 0.9	3.1 ± 1.3
Number of rearings	37.0 ± 7.6	21.0 ± 12.5**	21.3 ± 12.6	22.3 ± 12.3

Values expressed as mean ± standard deviation. Grooming frequency: Mann Whitney test. Other parameters: Student's *t*-test. * Represents p <0.05, ** Represents p <0.01, *** Represents p <0.001.

Table 2. Body weight and EDL muscle weight and length in rats at 48 days of age.

Variables (n = 10)	CTL	CP
Body weight (g)	197 ± 6.8	165 ± 17.2***
Muscle weight (g)	0.08 ± 0.01	0.07 ± 0.01**
Muscle length (mm)	21.4 ± 3.1	18.2 ± 2.1**

Values expressed as mean ± standard deviation. Animal weight: Mann Whitney test. Other parameters: Student's *t*-test. ** Represents p <0.01, *** Represents p <0.001.

Table 3. Morphometry of the EDL muscle and muscle spindles from the CTL and CP groups at 48 days of age.

Variables (n = 8)	CTL	CP
Number of peripheral nuclei	869.3 ± 82.6	1060 ± 214.7*
Number of muscular fibers	492.4 ± 60.4	472.9 ± 79.8
Nuclei/fiber	1.8 ± 0.3	2.2 ± 0.3**
Capillary/fiber	2.2 ± 0.2	2.7 ± 0.3**
Largest diameter of muscle spindles (µm)	74.5 ± 16.1	82.5 ± 40.3
Cross-sectional area of muscle spindles (μm^2)	3443 ± 1264	2541 ± 1603*
Number of intrafusal fibers	4.1 ± 0.4	4.3 ± 0.7
Cross-sectional area of intrafusal fibers (μm^2)	182.4 ± 56.3	135.3 ± 35.2*
Muscle spindle capsule thickness (µm)	20.3 ± 13.4	19.4 ± 15.2

Values expressed as mean ± standard deviation. * Represents $p < 0.05$. ** Represents $p < 0.01$. Number of peripheral nuclei, Number of muscle fibers, Nuclei/fiber, Capillary/fiber and Largest diameter of muscle spindles: Student *t*-test. Other parameters: Mann Whitney test.

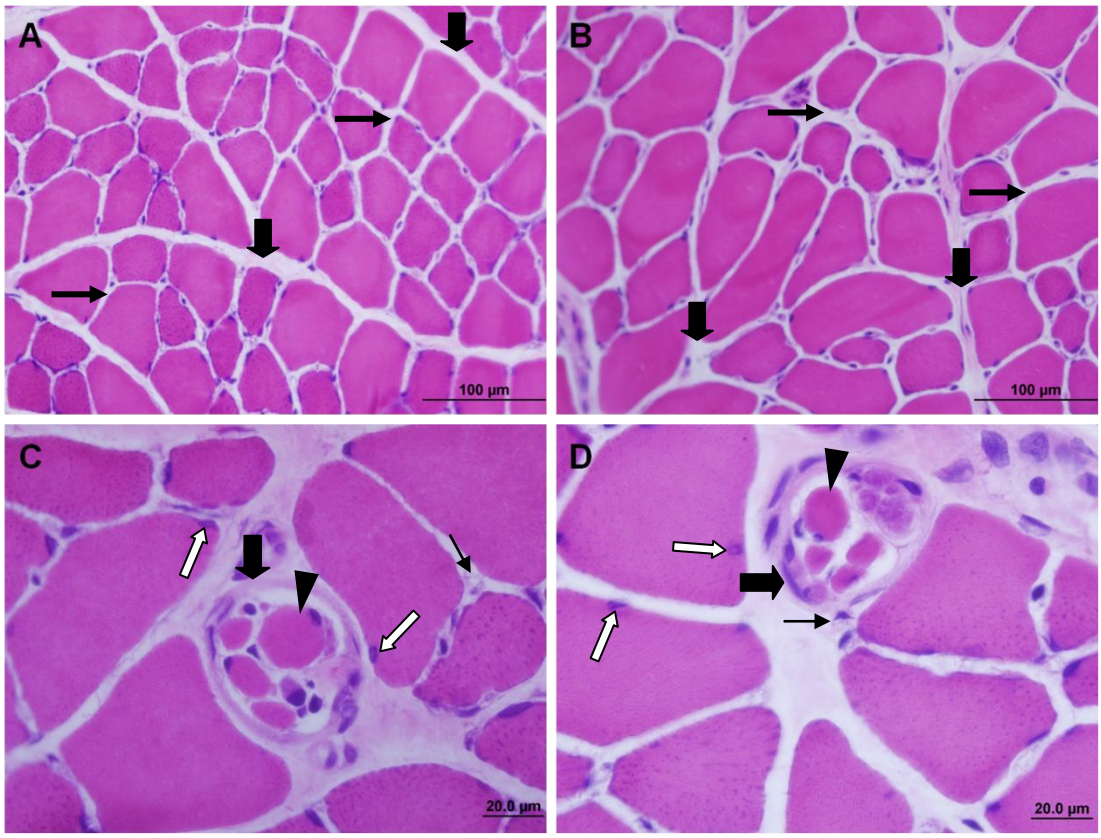


Figure 1 - Photomicrographs of EDL muscle fibers from Wistar rats at 48 days of age. Cross section, HE staining. CTL (**A** and **C**) and CP (**B** and **D**) groups. **A** and **B**: Note the perimysium (thick arrows), endomysium (long arrows). **C** and **D**: Note the peripheral nuclei (white arrows), muscle spindle capsule (thick arrows), intrafusal fibers (arrow heads) and capillaries (thin arrows).

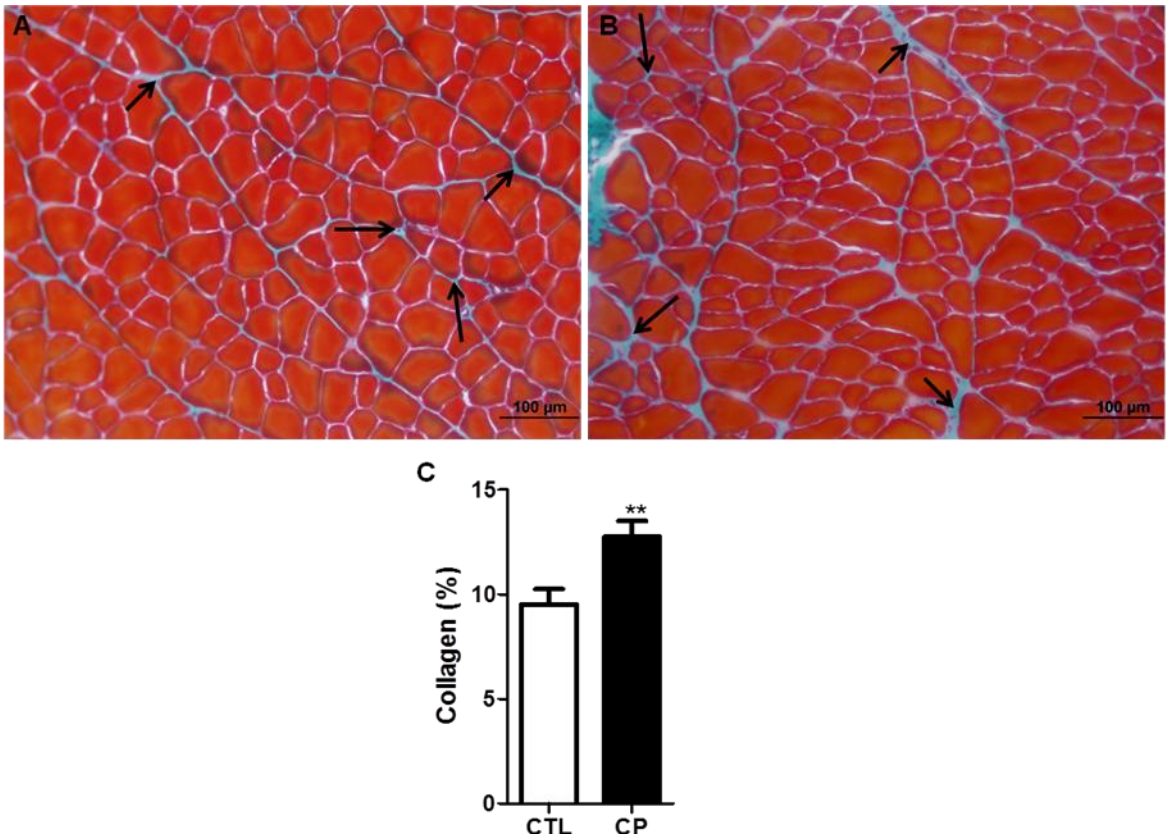


Figure 2 - Photomicrographs of the collagen quantification in the EDL muscle of Wistar rats at 48 days of age. Longitudinal section, Masson trichrome. **A** and **B**: Note the perimysium (short arrow) and endomysium (long arrow) in the animals from the CTL and CP groups, respectively. **C**: Percentage of the intramuscular collagen of the animals from the CTL and CP groups. Values expressed as mean ± standard deviation. **Represents $p < 0.01$; Student's *t*-test.

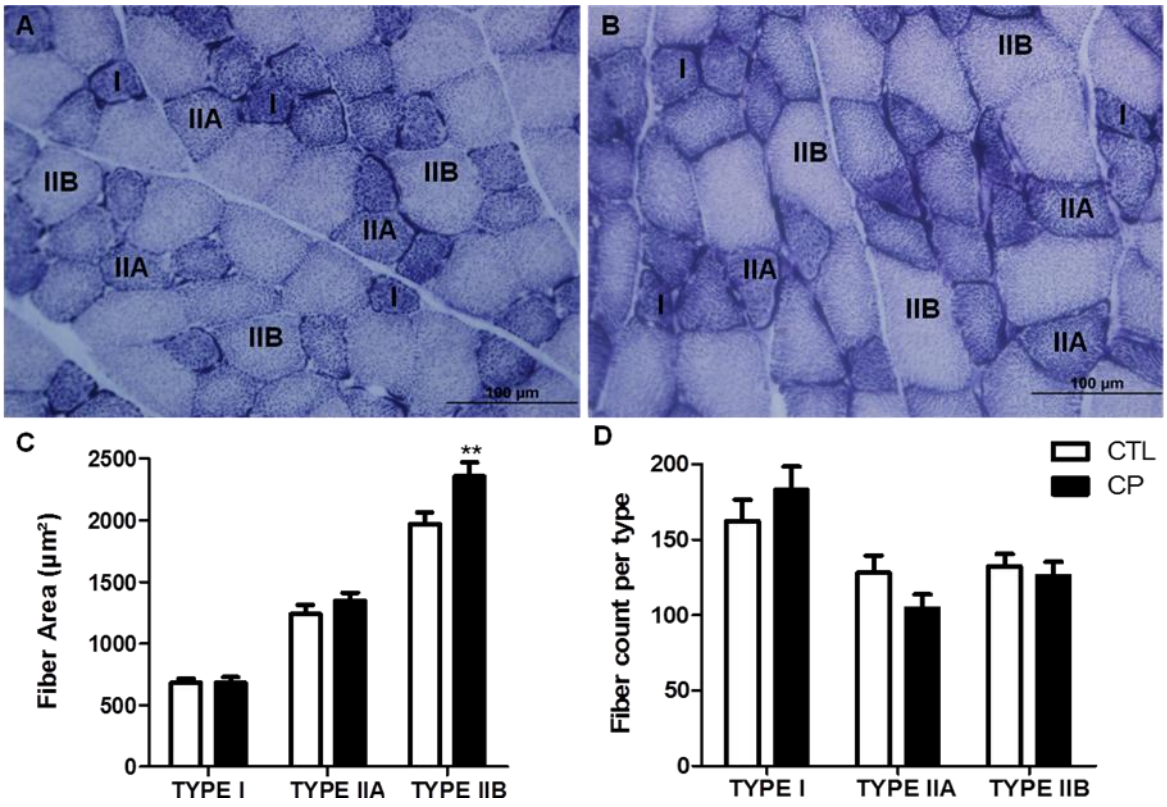
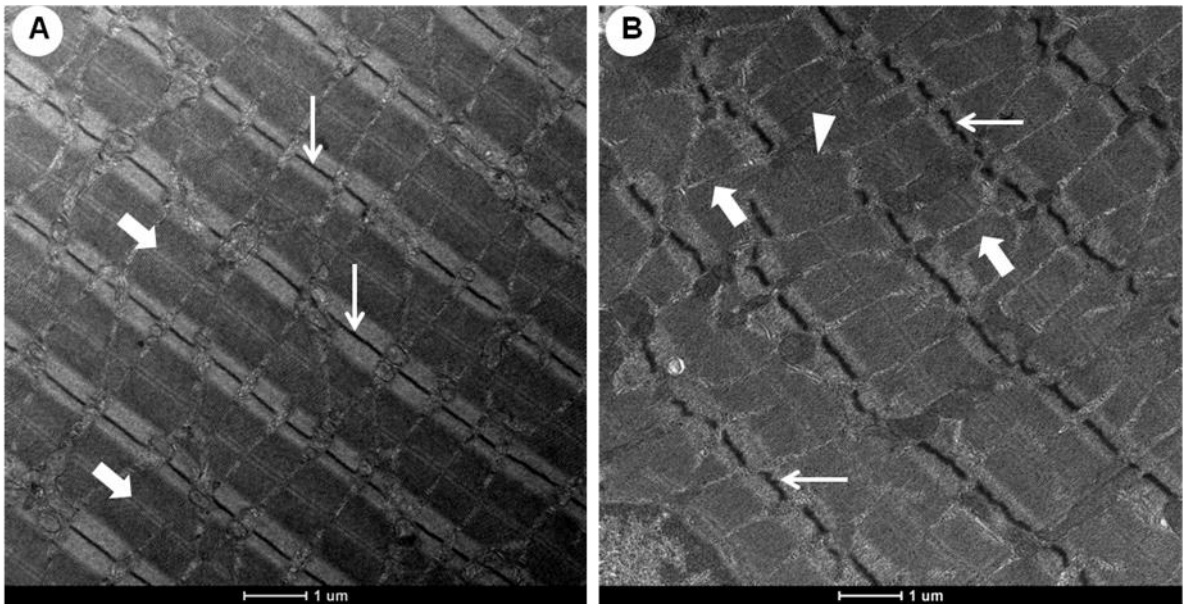


Figure 3 - Photomicrographs of the NADH-TR reaction in the EDL muscle fibers of Wistar rats at 48 days of age. Cross section. **A** and **B**: Morphology of the different types of muscle fibers in the CTL and CP groups, respectively. Fibers with small diameter (I), intermediate diameter (IIA) and large diameter (IIB). **C**: Cross-sectional area of the different types of muscle fibers observed in the EDL muscle in rats from the CP and CTL groups. **D**: Number of muscle fibers of types I, IIA and IIB observed in the EDL muscle of rats from the CP and CTL groups. Values expressed as mean ± standard deviation. Two-way ANOVA, *post-hoc* Bonferroni test.



C

Observations	CTL Group	CP Group
Z Line disorganization (%)	18,3 ± 10,4	67,25 ± 8,3***
Z line dissolution (%)	5,0 ± 5,0	23,8 ± 14,7*
Myofibrillar disruption (%)	9,0 ± 9,6	25,8 ± 8,5*

Figure 4 - Transmission electro-micrographs of 48-day-old rat EDL muscle in longitudinal section. **A**: CTL Group - Organized myofibrils (short arrow) and alignment of the Z line (long arrow). **B**: CP Group - Myofibrils, sparse or loosely arranged (short arrow), disorganization (long arrow) and dissolution of the Z line (arrowhead). Bar = 1µm. **C**: Values expressed as mean ± standard deviation. Z-line dissolution: Mann Whitney test. Other parameters: Student's *t*-test.

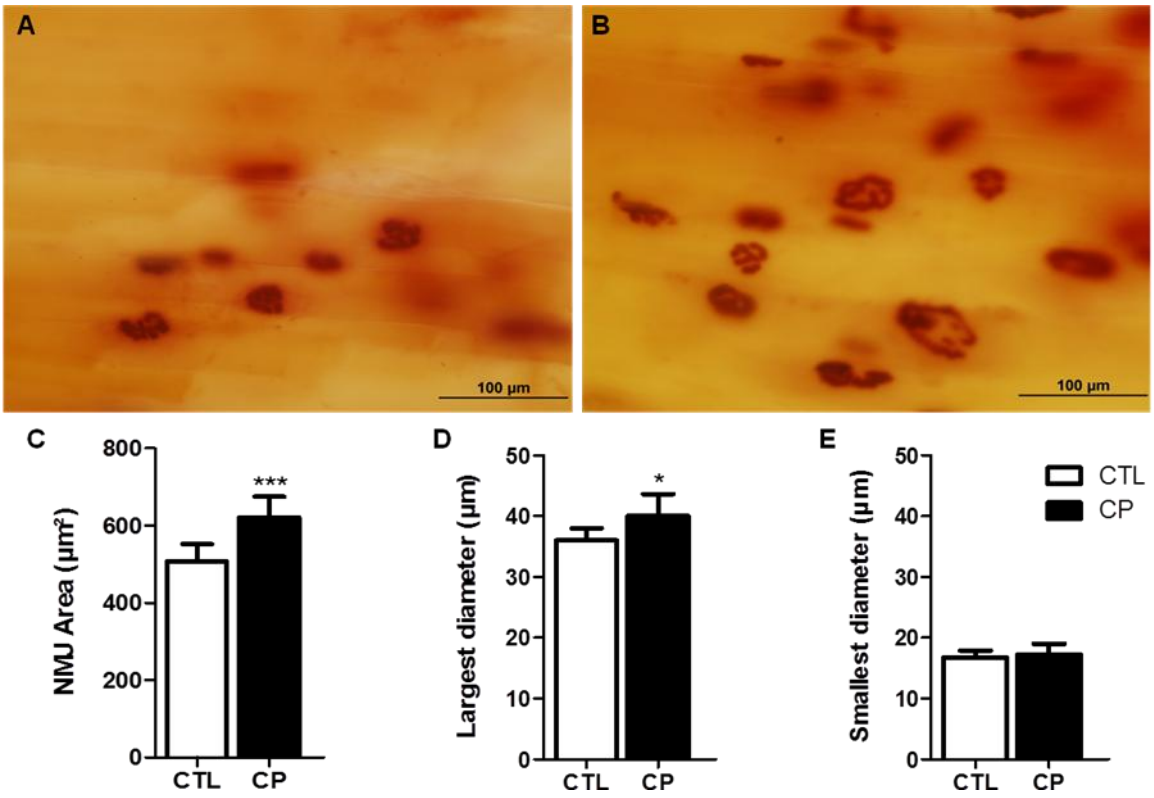


Figure 5 - Photomicrographs of the NMJs observed in the EDL muscle of Wistar rats at 48 days of age. Longitudinal section, Non-specific esterase reaction. **A** and **B**: Morphological characteristics of the NMJs of the animals from the CTL and CP groups. **C**, **D** and **E**: Area, largest diameter and smallest diameter of the NMJs of the animals from the CTL and CP groups, respectively. Values expressed as mean ± standard deviation. ***Represents $p < 0.001$; *Represents $p < 0.05$. Smallest diameter of NMJs: Mann Whitney test. Other parameters: Student's *t*-test.

ANEXO A:

unioeste
Universidade Estadual do Oeste do Paraná
PRO-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
COMITÊ DE ÉTICA EM PESQUISA - PRPPG

Certificado Experimental no Uso de Animais em Pesquisa Nº 24/16 - CEUA

Certificamos que a proposta intitulada "Implicações de um modelo de paralisa cerebral sobre a função motora, cognitiva e morfologia do sistema nervoso e muscular", registrada com o número "02/14", sob a responsabilidade de "Ligia A. Centenaro", que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vetebrata (exceto humanos), para fins de pesquisa científica (ou ensino) – encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do decreto nº6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovado pelo COMITÊ DE ÉTICA NO USO DE ANIMAIS (CEUA) DA UNIVERSIDADE ESTADUAL DO OESTE DO PARANÁ (UNIOESTE), em reunião de 13/05/2016.

Finalidade	Pesquisa Científica
Vigência da autorização	15/09/2014 – 31/08/2014
Espécie/linhagem/raça	Rato/Wistar
Nº de animais	34 Machos e 32 Fêmeas
Peso/Idade	42=250g/60 dias 24=5g/Neonatos
Sexo	Masculino/Feminino
Origem	Biotério Central da Unioeste

Prof. Dirceu Baumgartner
Coordenadora Suplente do CEUA UNIOESTE
Portaria n°3730/2016

ANEXO B:

Normas das revistas científicas

American Journal of Obstetrics & Gynecology

Article Types

- Every submission must include a title page with a disclosure statement and a signed statement of authorship form. This requirement applies to ALL article types listed in the following section; including letters, replies, mixed media, etc.
- The editors encourage the supplementary use of multimedia components such as PowerPoint, additional images, or video clips. Color figures and images are free.

Original Research

Manuscripts are limited to 3000 words of main text (not counting the title page, abstract, condensation, acknowledgements, references, tables, figures, and legends).

All authors must meet authorship criteria (see Named Authors and Contributors).

Systematic review and metaanalysis studies: please refer to Systematic Reviews.

Research articles must be accompanied by a structured abstract between 250 and 500 words, accompanied by as many alphabetized key words or short phrases as needed for indexing.

Structured Abstract - applies to Original Research and Reports of Major Impact

Between 250 and 500 words with the following required headings:

1. **Background:** an explanation of the basis of the study (NOTE: This is a NEW heading)
2. **Objective(s):** the purpose of the study (hypothesis being tested)
3. **Study Design:** the setting for the study, subjects (number and type), treatment or intervention, and type(s) of statistical analysis used
4. **Results:** the outcome(s) of the study and, if appropriate, their statistical significance
5. **Conclusion(s):** overall significance of the results

The type(s) of non-human animals or other species used in an investigation must be named in the Title, Abstract, and Materials and Methods sections of the manuscript.

Each original research article is published in 2 formats: 1) in the printed journal: in an

abbreviated form, that includes the abstract and 1 figure or table, and 2) in full on the Journal website (ajog.org), where the abstract also appears. The full length article is the official version and is linked to search engines. (Note: Expert Reviews, Systematic Reviews, Clinical Opinion, Call to Action, Viewpoint, and Point/Counterpoint are published in full in boththe print and online versions.)

The full-length article becomes available online for citation before the print issue containing the abstract and accompanying material. Impact Factors and other citation indices are based on the full-length online article.

Translational Science

Translational science is typically presented in the form of an original research manuscript; however, the only type of non-clinical research considered must be translational in nature and contain biological implications for obstetrics and gynecology. Basic science without direct clinical relevance will not be considered; please see Editorial Policies for examples.

Reports of Major Impact

Authors who believe their original research article has the potential for affecting clinical practice in a major way or is otherwise of urgent importance may submit the manuscript under this category. The editors, in consultation with experts in the area addressed in the article, will assess the likely impact of the article and notify the authors whether it is being considered for this category or as a regular original research manuscript.

Articles accepted as Reports of Major Impact are reviewed and published as rapidly as possible. The authors are to follow the format for original research articles. As with any article, concern about any potential conflict arising from the timing of publication relative to a presentation at a scientific meeting may be communicated to the editors, who will, at the authors' request, delay publication until the paper has been presented.

Reviews

Expert Reviews

These invited articles provide concise reviews on a topic in which the author has expertise. The manuscript should be comprehensive and balanced, but not exhaustive. Expert Reviews must be evidence based but may include some expert opinion and recommendations. The goal is to provide a concise update on the state of the art and guidelines for clinical care.

Expert Reviews are limited to 3500 words of main text (not counting the title page, abstract, condensation, acknowledgments, references, tables, legends, and figures). An unstructured abstract (1 paragraph, no categories) of no more than 350 words and as many alphabetized key words or short phrases as needed for indexing must be provided.

Subheadings to separate and identify sections of text should be unique to the topic; the 4 prescribed subheadings required for research articles do not apply. To prevent such subheadings from occupying many lines on a page, they should be as short as possible, not exceeding approximately 6 words, and preferably 1 to 4 words.

The full-length article appears both in print and on the Journal website.

Systematic Reviews

Each article in this category provides a comprehensive and exhaustive systematic review of the literature related to the topic, collating all relevant evidence meeting pre-specified eligibility criteria. Systematic reviews may not be combined with other manuscript types.

Systematic reviews must include a clearly stated set of objectives with reproducible methodology, a systematic search, eligibility criteria for selecting studies, assessment of study quality (risk of bias), an assessment of the validity of the findings and systematic synthesis of these findings. Metaanalysis, the use of statistical techniques to combine and summarize results across studies, may or may not be contained within a systematic review.

Authors must adhere to the PRISMA and MOOSE guidelines (for guidance see Editorial Policies).

Systematic Reviews are limited to 5000 words of main text (not counting the title page, abstract, condensation, acknowledgments, references, tables, legends, and figures). Include a structured abstract containing no more than 350 words and as many alphabetized key words or short phrases as needed for indexing.

Title: The title should identify the report as systematic review or metaanalysis.
Abstract: Include a structured summary according to PRISMA guidelines with the following headings:

- Objective
- Data sources (including years searched)
- Study eligibility criteria (study design, populations, and interventions [if applicable])
- Study appraisal and synthesis methods
- Results
- Conclusions

Main text: Headings and subheadings in the main text should include the following; note that subheadings may be modified to best represent the specific report.

- Introduction (rationale, explain impetus for Review)
- Objective(s)
- Methods

  Eligibility criteria, information sources, search strategy
  Study selection
  Data extraction
  Assessment of risk of bias
  Data synthesis

- Results

  Study selection
  Study characteristics
  Risk of bias of included studies
  Synthesis of results

- Comment

  Main findings
  Strengths and limitations
  Comparison with existing literature
  Conclusions and Implications

The full-length article appears both in print and on the Journal website.

Clinical Opinion

A Clinical Opinion paper presents a balanced, evidence-based discussion of a clinical issue pertinent to obstetricians and gynecologists. The paper may address an emerging or controversial topic or bring attention to a topic of increasing clinical significance. Opinions rendered must be based on an interpretation of available evidence.

Not appropriate for this category: 1) a review of an extensively researched subject. Submit as a Systematic Review. 2) an essay about issues for which minimal data exist, such as certain clinical, ethical, educational, practice, and research issues. Submit as a Viewpoint paper.

A Clinical Opinion paper is limited to 3000 words of main text (not counting the title page, abstract, condensation, acknowledgments, references, tables, legends, and figures). An unstructured abstract (1 paragraph; no headings) of no more than 350 words and as many alphabetized key words or short phrases as needed for indexing must be provided.

Subheadings to separate and identify sections of the text should be unique to the topic; the 4 prescribed categories required for research articles do not apply. To prevent such subheadings from occupying many lines on a page, they should be as short as possible, not exceeding approximately 6 words and preferably 1 to 4 words.

The full-length article appears both in print and on the Journal website.

Special Report

A Special Report is released by a consensus committee, working group, task force, or similar group, or summarizes the findings of an important meeting.

Include a condensation, an unstructured abstract (1 paragraph, no subheadings) of no more than 350 words, and as many alphabetized key words or short phrases as needed for indexing.

Subheadings to separate and identify sections of the text should be unique to the topic; the 4 prescribed categories required for research articles do not apply. To prevent such subheadings from occupying too many lines on a page, they should be as short as possible, not exceeding approximately 6 words and preferably 1 to 4 words.

The full-length article appears both in print and on the Journal website.

Viewpoint

Viewpoint articles are well-founded, scholarly pieces that address a scientific, ethical, academic, or practice-related topic in women's health. The article should be balanced and based on a critical analysis of the literature. Viewpoint articles are intended to stimulate discussion.

Viewpoint articles are limited to 1500 words of main text (not counting the title page, condensation, abstract, acknowledgments, references, tables, legends, and figures).

Include a condensation, an unstructured abstract (1 paragraph, no subheadings) of no more than 350 words, and as many alphabetized key words or short phrases as needed for indexing.

The full-length article appears both in print and on the Journal website.

Point/Counterpoint

Point/Counterpoint presents 2 essays of differing views about a controversial issue of interest to AJOG's readers. These articles are generally solicited by the editors, but readers are encouraged to suggest topics for this section.

Each essay is limited to 1500 words of main text (not counting the title page, condensation, abstract, acknowledgments, references, tables, legends, and figures).

Include a condensation, an unstructured abstract (1 paragraph, no subheadings) of no more than 350 words, and as many alphabetized key words or short phrases as needed for indexing.

Subheadings to separate and identify sections of the text should be unique to the topic; the 4 prescribed categories required for research articles do not apply. To prevent such subheadings from occupying too many lines on a page, they should be as short as possible, not exceeding approximately 6 words, and preferably 1 to 4 words.

The essays appear in full, both in print and on the Journal website.

Call to Action

Call to Action is a topical piece highlighting a problem related to a clinical, research, social, ethical, political, or economic issue pertinent to obstetricians and gynecologists and a suggested solution to that problem. Accordingly, the author must include a suggested corrective action; describing the problem alone is not sufficient.

Call to Action articles are limited to 2000 words of text (not counting the title page, condensation, abstract, acknowledgements, references, tables, legends, and figures).

Include a condensation, an unstructured abstract (1 paragraph, no subheadings) of no more than 350 words, and as many alphabetized key words or short phrases as needed for indexing. The main text must include: 1) “The Problem:,” a one-sentence statement of the problem being presented; 2) “A Solution:,” a one-sentence summary of the proposed solution; and 3) the presentation.

The full-length article appears both in print and on the Journal website.

Case Report

The Journal no longer publishes Case Reports.

Editorials

Editorials are written or solicited by the editors. Spontaneous submissions are not considered for publication.

Images in Obstetrics; Images in Gynecology

An Interesting image of clinical significance, such as a clinical photograph or evidence of a diagnostic test (ultrasound scan, MRI film, slide, photomicrograph,

DNA blot, or similar), is accompanied by a case description of no more than 350 words (not counting the title page, acknowledgements, references, tables, legends, and figures). The manuscript must include:

1. Condensation: a 1-sentence condensation of the paper, consisting of no more than 25 words, to be placed in the Table of Contents.
2. Case Notes: a brief case presentation (under the heading "Case Notes") with introduction of relevant image(s) accompanied either in text or a figure legend by a short description of each
3. Comment: a discussion of the clinical relevance of the figure (under the heading "Comment". (Together, the case notes and comment should not exceed 350 words.)
4. Figure and (if applicable) video legends.
5. 7 or fewer references.

The full text of the article appears both in print and online. The print version generally includes 1 image. Up to 5 images may be submitted for the online version. If the paper is accepted, the editors will work with the author to choose which image to display in the print. In addition to images, we encourage (for use online) the inclusion of multimedia components such as PowerPoint, additional images, or video clips.

Surgeon's Corner

This content provides high-quality instruction or an application of a procedure or part of a procedure, designed to aid the practicing obstetrician or gynecologist in improving care. Surgeon's Corner is published in full online; the abstract, manuscript, and a photo or graphic are published in the print journal.

The manuscript must include all of the following:

1. Condensation: a 1-sentence condensation of the paper, consisting of no more than 25 words, to be placed in the Table of Contents.
2. An unstructured abstract of no more than 300 words that summarizes the clinical situation and surgical solution, explains the figure used in the print edition (see item 4), and refers to the video.
3. A description of the clinical situation or problem (under the heading: "Problem") followed by your surgical solution (under the heading: "Our solution"), in 600 words or less (not counting the title page, acknowledgement, references, tables, legends, and figures). Lists and bullet points may be used as appropriate. The text should refer to the figures/photos and video (see items 4 and 5).
4. At least one high-quality photograph (300+ dpi; not taken from a website or cell phone), graphic, or figure, to be published in the print edition; this, plus up to 5 additional photos/figures may be included for the online version.

5. A video clip or computer graphic not longer than 5 minutes, or a **maximum of 50MBs** or less per clip, to be published in the online version.
6. Figure and video legends.
7. 7 or fewer references.

Sketches

These articles describe interesting aspects of medical careers, work life, professional or personal development, or moments of insight, transformation, or inspiration related to professional experiences. Sketches are limited to 1000 words of main text, 7 or fewer references, and require a condensation.

The full-length article appears both in print and on the Journal website.

Mixed Media

Mixed Media may include photos, graphic art, poems, animation, video, interviews, or other forms of creative expression that portray historical or contemporary topics of interest to obstetricians and gynecologists. Typically, Mixed Media articles are published online only; however, applicable portions may appear in the print edition of the Journal. Provide a title suitable for the table of contents.

Authors are encouraged to supply with their manuscripts, for publication, a professional “head shot” of the lead author. This must be a high-resolution digital photograph of at least 300 dpi and not taken from a website or cell phone. Photographs are optional.

Letters to the Editors, Replies and Replies

Every Letter to the Editors, Reply, and Research Letter must include a title page, conflict of interest disclosure, and a Statement of Authorship signed by all authors. These submissions are subject to minor editorial alterations, may be shortened without the authors' approval, and published both in print and on the Journal website.

Per ACOG/SMFM standard practice, letters related to these joint society guidelines are not published. As ACOG and SMFM are interested in feedback, AJOG will forward letters related to guideline articles to the committee and they will reply personally. Please see Clinical Opinion as a venue for presenting a scholarly, evidence-based point of view about controversial issues in OB/GYN.

Letters to the Editor and Replies

Selected Letters to the Editors that cite at least 1 article published in the *American Journal of Obstetrics & Gynecology* within the previous 4 issues are considered for publication.

Letters to the Editors are limited to 3 authors, 400 words (not counting the title page

or references), and 1 to 4 references. At least one of the references must cite the related Journal article(s). All data presented must be fully citable and cited in the supporting reference list.

The editors routinely invite the author(s) of the related article to respond in writing. Published letters are accompanied by either a reply from the original authors or the statement "Reply declined."

Research Letters

Research Letters, not linked to items published in AJOG, briefly summarize the results of original data. Each Research Letter is considered a scientific publication; authors must meet all requirements regarding responsible conduct of research (eg, appropriate IRB approval, data integrity, data retention). Most undergo external peer review. Reviews, case reports, and opinion pieces are not considered for publication under this category.

Research Letters are limited to 7 authors, 500 words (not counting the title page, references, or legend), 5 references and may include either 1 table or 1 figure. Online supplementary materials are not permitted. Research Letters should be formatted similar to the structured abstract guidelines for original research and divided into 4 sections: Objective, Study Design, Results, and Conclusion. Research Letters do not include an abstract or condensation.

Editorial Policies

Queries about submission requirements may be addressed to either of the managing editors:

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Ethics of the editorial process

A policy of the Journal entitled "Specific Inappropriate Acts in the Publication Process" describes the Journal's policies regarding ethical practices, which apply to all submitted articles, whether accepted for publication or not. **Authors must review this document prior to submission.**

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