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PÂMELA BURATTI

AVALIAÇÃO FUNCIONAL E MORFOLÓGICA DO MÚSCULO ESTRIADO ESQUELÉTICO E JUNÇÕES NEUROMUSCULARES EM RATOS SUBMETIDOS A UM MODELO DE PARALISIA CEREBRAL

CASCAVEL-PR
Maio/2017

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RESUMO GERAL

A paralisia cerebral (PC) corresponde a uma desordem motora, ocasionada por lesão não progressiva no cérebro imaturo, por conseguinte, acarreta alterações no tônus muscular, movimento e postura. Este estudo objetivou investigar as características morfofuncionais do músculo plantar em um modelo animal de PC. Na obtenção das ninhadas, ratos *Wistar* adultos foram utilizados para o pareamento (11 fêmeas e 06 machos) e as fêmeas prenhas foram agrupadas em: ratas injetadas intraperitonealmente com veículo (100 µL de solução salina estéril); e ratas injetadas intraperitonealmente com lipopolissacarídeo (LPS; 200 µg/kg de LPS em 100 µL de solução salina estéril), a cada 12 horas, a partir do 17º dia gestacional até o final da gestação (21º dia gestacional). Os filhotes machos foram separados em: Grupo controle (GC, *n*= 8), filhotes de mães injetadas com solução salina durante a gestação; e Grupo PC (GPC, *n* = 8), filhotes de mães injetadas com LPS na gestação, submetidos à anóxia perinatal e restrição sensório-motora. Para a anóxia perinatal, os filhotes foram colocados em uma câmara fechada, parcialmente imersa em água a 37°C±1, com fluxo de 9 L/min de nitrogênio (100%) durante 20 minutos no dia do nascimento (dia pós-natal 0, P0). Do 1º ao 30º dia pós-natal (P1 até P30), os animais do GPC foram submetidos à restrição sensório-motora durante 16 horas/dia. E, aos 29 e 45 dias de vida, os animais foram avaliados no teste de campo aberto, coletados os dados de tempo de deslocamento, cruzamento, frequências de erguidas (*rearing*) e de autolimpeza (*grooming*). No 48º dia pós-natal, os animais foram pesados e foi realizada a coleta, além das mensurações do peso e do comprimento muscular. As fibras musculares foram avaliadas em secções histológicas submetidas às técnicas de Hematoxilina-Eosina (HE), reação da NADH-TR (Nicotinamida Adenina Dinucleotídeo – Tetrazolium Redutase) e microscopia eletrônica de transmissão. O colágeno intramuscular foi estudado com a coloração de Tricrômico de Masson e as junções neuromusculares (JNMs) foram averiguadas pela reação Esterase Inespecífica. Os dados foram avaliados estatisticamente com o teste paramétrico *t de Student* e teste não paramétrico *Mann-Whitney*, de acordo com o teste de normalidade *Kolmogorov-Smirnov*. Verificou-se que, quando comparado ao GC, o GPC apresentou: redução significativa no tempo de deslocamento, no número de cruzamentos e no *rearing* aos 29 dias de idade; no tempo de deslocamento e no *rearing* aos 45 dias de idade; nos parâmetros corporais (peso corporal, peso e comprimento do músculo sóleo); nas relações núcleo/fibra e capilar/fibra; aumento na porcentagem de colágeno; redução da área de secção transversal das fibras musculares tipo I; aumento na contagem de fibras do tipo I; no número de desorganização miofibrilar, da linha Z e dissolução da linha Z; e redução na área, diâmetros maior e menor das JNMs. Conclui-se, portanto, que o modelo de PC utilizado alterou as características morfológicas do músculo estriado esquelético e causou déficits na atividade locomotora dos animas, comprovado pelos resultados da avaliação em campo aberto.

Palavras-chave: lipopolissacarídeo; anóxia perinatal; restrição sensório-motora; morfologia; morfometria.

GENERAL ABSTRACT

FUNCTIONAL AND MORPHOLOGICAL EVALUATION OF SKELETAL MUSCLE AND NEUROMUSCULAR JUNCTIONS IN RATS SUBMITTED TO A MODEL OF CEREBRAL PALSY

Cerebral palsy (CP) corresponds to a motor disorder, caused by non-progressive injury in the immature brain, resulting in changes in muscle tone, movement and postureThis study aimed to investigate the morphofunctional characteristics of the plantar muscle in an animal model of CP. To obtain the litters were used for paired adult *Wistar* rats (11 females and 06 males). The pregnant females were separated in: rats injected intraperitoneally with vehicle (100 µL of sterile saline) and rats injected intraperitoneally with lipopolysaccharide (LPS; 200 µg / kg LPS in 100 µL sterile saline). The injections were performed every 12 hours, from the 17th gestational day until the end of gestation (21st gestational Day). The male offspring were separated in: Control group (CG, n = 8) - pups of rats injected with saline solution during pregnancy, and CP group (CPG, n = 8) - pups of rats injected with LPS during pregnancy, submitted to perinatal anoxia and sensorimotor restriction. For perinatal anoxia, the pups were placed in a closed chamber, partially immersed in water at 37 °C ± 1, with a flow of 9 L/min of nitrogen (100%) for 20 minutes on the day of birth (postnatal day 0, P0). From the 1st to the 30th postnatal day (P1 to P30), the CPG animals were submitted to sensorimotor restriction for 16 hours/day. At 29 and 45 days of age, the animals were evaluated in the open-field test, being collected time of displacement, crossing, rearing and grooming frequency. At the 48th postnatal day the animals were weighed and the collection, measurement of weight and muscle length were performed. The muscle fibers were evaluated in histological sections submitted to Hematoxylin-Eosin (HE), NADH-TR reaction (Nicotinamide Adenine Dinucleotide - Tetrazolium Redutase) and transmission electron microscopy; intramuscular collagen was studied with Masson's trichrome staining; and the neuromuscular junctions (NMJs) ascertained by the Nonspecific Esterase reaction. The data were statistically evaluated using the parametric Student t-test and the non-parametric Mann-Whitney test, according to the Kolmogorov-Smirnov normality test.It was verified that when compared to CG the CPG presented: reduction in time of displacement, number of crosses and rearing at 29 days of age; in the time of displacement and rearing at 45 days of age; of body parameters (body weight, weight and length soleus muscle); of the nuclei/fiber and capillary/fiber relations; increased percentage of collagen; reduction of cross-sectional area of muscle fibers type I; increased counts of fibers type I; in the number of myofibrillar disorganization, of the Z line and dissolution of the Z line; and reduction in area, larger and smaller diameters of NMJs. It is concluded that the PC model used altered the morphological characteristics of the skeletal striated muscle, causing deficits in the locomotor activity of the animals, as evidenced by the results of the evaluation in the open field.

Keywords: lipopolysaccharide; perinatal anoxia; sensorimotor restriction; morphology; morphometry.

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LISTA DE ABREVIATURAS

PC	Paralisia cerebral
LPS	Lipopolissacarídeo
JNMs	Junções neuromusculares
HIV	Vírus da Imunodeficiência Humana
LPV	Leucomalácia periventricular
HE	Hematoxilina-Eosina
mATPase	ATPase miofibrilar
ACh	Acetilcolina
IL-1β	Interleucina 1β
TNF-α	Fator de necrose tumoral- α
G	Dia gestacional
P	Dia pós-natal
GC	Grupo controle
GPC	Grupo paralisia cerebral
NADH-TR	Nicotinamida Adenina Dinucleotídeo Tetrazolium Redutase

INTRODUÇÃO GERAL

A paralisia cerebral (PC), conhecida também pelo termo encefalopatia crônica nãoprogressiva da infância, é um grupo de desordens motoras, ocasionadas por lesão não progressiva durante o desenvolvimento neurológico (COLVER; FAIRHURST; PHAROAH, 2014; ROSENBAUM *et al.,* 2007). Esse distúrbio acarreta alterações do tônus muscular, movimento e postura e pode estar associado à epilepsia, transtorno do desenvolvimento intelectual, transtornos sensoriais, cognitivos, de comunicação, percepção e comportamento (BAX *et al.,* 2005; GOMES; ARAÚJO; MACIEL, 2014; LIMA *et al*., 2014; MIURA; PETEAN, 2012). Embora a PC seja considerada lesão cerebral não progressiva, as alterações motoras ocasionadas por ela podem se agravar com o passar do tempo e permanecerão longo da vida útil do indivíduo (BAX *et al.,* 2005; GRAHAM; SELBER, 2003).

Crianças portadoras de PC enfrentam uma série de problemas nas atividades diárias, que vão além dos problemas físicos, como a interação com os colegas e no ambiente escolar, pois muitas instituições não estão preparadas com suporte adequado (LIMA *et al*., 2014). Há também implicações para os familiares e em especial a mãe, já que as necessidades físicas e emocionais exigem esforço, dedicação e tempo de seus cuidadores, as quais podem trazer também desgastes financeiro, emocional e social (GUYARD *et al*., 2011; MIURA; PETEAN, 2012; SÁ; RABINOVICH, 2006; WIJESINGUE; FONSEKA; HEWAGE, 2013).

É uma das desordens motoras mais comuns (SOUZA *et al.*, 2013). No Brasil não existem estudos atuais a respeito de incidência de PC (MIURA; PETEAN, 2012; ROTTA, 2002). No entanto, em países subdesenvolvidos, o número de casos

pode atingir sete em cada 1.000 nascidos vivos (BRASIL, 2013; PIOVESANA *et al.*, 2002; ROCHA; AFONSO; MORAIS, 2008).

Devido ao impacto do distúrbio, modelos animais têm sido realizados a fim de que se reproduzam as características observadas em pacientes com PC, visando ao melhor entendimento sobre o tema. Para isso, insultos como infecção maternal, anóxia perinatal, e restrição sensório-motora foram utilizados. Na indução da infecção pré-natal, o lipopolissacarídeo (LPS), constituinte estrutural da maioria das bactérias gram-negativas, tem sido empregado (DAMMANN; DURUM; LEVITON, 2001; STIGGER *et al.*, 2011). Contudo, observou-se que ele sozinho não reproduz as alterações motoras observadas em pacientes com PC (POGGI *et al.*, 2005). Outros insultos utilizados são a anóxia perinatal, caracterizada pela diminuição de trocas gasosas (MACLENNAN, 1999; VAN HANDEL *et al.,* 2007) e também a restrição sensório-motora, empregada a fim de se mimetizar o desuso observado em pacientes com PC (STRATA *et al.*, 2004). A associação da anóxia perinatal e restrição sensório-motora ocasionaalterações histológicas no córtex S1, o que pode contribuir para déficits sensório-motores (MARCUZZO *et al.,* 2010). Quando utilizados em conjunto, o LPS, a anóxia perinatal e a restrição sensório-motora causam déficits de equilíbrio e coordenação além de alterações na morfologia dos músculos sóleo e tibial anterior, redução na área de secção transversal das fibras musculares, aumento do comprimento e diminuição da densidade do sarcômero (STIGGER *et al.*, 2011).

O conhecimento das alterações que ocorrem na musculatura é essencial para a compreensão do comprometimento da capacidade funcional dos pacientes com PC (DIAS *et al.*, 2013). No entanto, estudos que avaliem a ultraestrutura das fibras musculares e as junções neuromusculares (JNMs) em modelos animais de PC ainda não foram realizados. Desta forma, questiona-se sobre as implicações da associação da exposição pré-natal ao LPS, anóxia perinatal e restrição sensório-motora sobre a musculatura estriada esquelética em ratos. Com base na literatura consultada, hipotetiza-se que tais insultos associados podem ocasionar alterações nas características morfofuncionais musculares e nas JNMs, com comprometimento da funcionalidade.

Os fatores relacionados às lesões provocadas por essa associação ainda não foram completamente esclarecidos, então acredita-se que uma melhor compreensão das adaptações musculares e das JNMs possa contribuir para a

melhoria dos tratamentos e até mesmo para o desenvolvimento de estratégias terapêuticas relacionadas a pacientes com PC. Com isso, este trabalho objetivou analisar os efeitos de um modelo animal de PC nas fibras musculares, JNMs no músculo estriado esquelético e na funcionalidade de rato.

REVISÃO GERAL DE LITERATURA

Paralisia cerebral

A PC, também denominada de encefalopatia crônica não progressiva da infância, está entre as principais causas de deficiências motoras infantis (SOUZA *et al*., 2013). É conceituada como um grupo de desordens permanentes do desenvolvimento e da postura que causam limitação de atividade, atribuídas a distúrbios não progressivos que ocorrem no encéfalo em desenvolvimento (BAX *et al.,* 2005; LIMA *et al.,* 2014; ROSENBAUM *et al.,* 2007).

Apesar do desenvolvimento tecnológico e do aumento da assistência materno-infantil nas últimas décadas, a incidência manteve-se entre dois e três a cada 1.000 nascidos vivos (PIN; ELMASRY; LEWIS, 2013; ROCHA; AFONSO; MORAIS, 2008; SOUZA *et al.*, 2013; VICENTE, 2014). Em países subdesenvolvidos, como o Brasil, a incidência é maior e pode estar presente em sete a cada 1.000 nascidos vivos (PIOVESANA *et al.*, 2002). Sua elevada frequência no País é decorrente dos poucos cuidados com as gestantes e com os recém-nascidos (BONOMO *et al.*, 2007; BRASIL, 2013; SOUZA *et al.*, 2013). Além disso, apenas 20% das crianças recebem atendimento especializado (CACCIA-BAVA, 2001).

Existem muitos fatores de risco associados ao desenvolvimento de PC, os quais podem ocorrer nos períodos pré, peri e pós-natal. A maioria das ocorrências é resultante de interferências no desenvolvimento do cérebro ainda no útero materno, período pré-natal, quando cerca de 70 a 80% dos casos são adquiridos (COLVER; FAIRHURST; PHAROAH, 2014; HIMMELMANN; UVEBRANT, 2014; JOHNSTON; HOON, 2006; KRIGGER, 2006). Complicações intraparto como asfixia, infecções e traumas envolvem de 10 a 20% dos fatores de risco pré-

natais (JOHNSTON; HOON, 2006). Dentre as infecções, destacam-se toxoplasmose, citomegalovírus, herpes, rubéola, sífilis e Vírus da Imunodeficiência Humana (HIV) (ROTTA, 2002; DODGE, 2008; HIMMELMANN; UVEBRANT, 2014). No período perinatal, a ocorrência de PC é menor e representa cerca de 6% dos casos, quando nascimento com menos de 32 semanas de gestação, peso menor que 2.500 gramas, retardo no crescimento intrauterino, hemorragia intracraniana e trauma podem causar PC (JONES; MORGAN; SHELTON, 2007; KRIGGER, 2006). Já no pós-natal, a PC pode ser consequente de meningite bacteriana, encefalite viral, traumatismos e hiperbilirrubinemia, com estimativa de 10 a 20% dos casos nesse período (DODGE, 2008; KRIGGER, 2006).

Dentre os fatores que levam ao acometimento deste distúrbio podem-se citar a leucomalácia periventricular (LPV), hemorragia intraventricular e lesão no córtex cerebral, núcleos da base, cerebelo e tálamo (FOLKERTH, 2005; JONES; MORGAN; SHELTON, 2007). A lesão mais comum é a LPV, caracterizada pela lesão da substância branca presente ao redor dos ventrículos. Está presente em até 75% dos casos e resulta da vulnerabilidade dos oligodendrócitos imaturos durante o desenvolvimento cerebral, entre 24 e 32 semanas de gestação (VOLPE *et al.*, 2011; ZHU; JIANG, 2006). Acredita-se que a asfixia perinatal seja o principal fator patogênico para o desenvolvimento da LPV, devido à sensibilidade das células precursoras de oligodendrócitos à isquemia (VOLPE *et al.*, 2011). Além disso, respostas inflamatórias maternas também podem estar relacionadas a danos encefálicos, devido à ativação de células do sistema imunológico que atravessam a barreira hematoencefálica causando danos de forma direta ou ativando células como a microglia e os astrócitos (DAMMANN; DURUM; LEVITON, 2001).

Sabendo-se da complexidade da PC, classificações padronizadas são essenciais para a investigação e estudo desta síndrome (COLVER; FAIRHURST; PHAROAH, 2014). A distribuição do comprometimento motor pode ser classificada como espástica, discinética (distônica e coreoatetoide) ou atáxica (BAX *et al.,* 2005). O tipo espásticopode ainda ser dividido em hemiplegia, diplegia e quadriplegia, de acordo com o envolvimento topográfico (KOMAN; SMITH; SHILT*,* 2004). A hemiplegia acomete um lado dos membros superiores e inferiores, enquanto a diplegia é a mais comum e atinge os membros inferiores com leve envolvimento dos membros superiores, e a quadriplegia gera consequências de mesma gravidade nos membros superiores e inferiores (OLIVEIRA *et al.,* 2013).

Pacientes espásticos apresentam aumento dos reflexos profundos do tendão muscular, tremores, hipertonia e fraqueza. O indivíduo com PC discinética possui movimentos atípicos das mãos, pés, membros superiores e/ou inferiores, os quais são acentuados durante o estresse e ausentes durante o sono. Já a forma atáxica afeta o equilíbrio e a coordenação (BRASIL, 2013; KRIGGER, 2006). As manifestações clínicas da PC podem estar relacionadas à região lesionada do sistema nervoso central. Desta forma, problemas neurológicos podem ou não estar presentes e incluem convulsões, dificuldades visuais, auditivas e na fala, deficiência sensorial dos membros superiores, transtorno do desenvolvimento intelectual, hidrocefalia, disfunção autonômica, problemas alimentares, respiratórios e dificuldade na aprendizagem (ABPC, 2014; KOMAN; SMITH; SHILT, 2004). Contudo, o sintoma comum entre os indivíduos com PC é o distúrbio motor (ABPC, 2014).

Músculo estriado esquelético

O músculo estriado esquelético é formado por células denominadas de fibras musculares, que apresentam formas alongada e cilíndrica, e também são multinucleadas, cujos núcleos se localizam na periferia celular (DAL PAI-SILVA; CARVALHO, 2007) (Figura 1). A ultraestrutura das fibras musculares mostra que são compostas por proteínas contráteis e elásticas que realizam contração e se organizam para gerar um padrão de bandas claras e escuras alternadas, as estriações transversais, e formar os sarcômeros (SILVERTHORN, 2010).

As faixas escuras são conhecidas como bandas A e as faixas claras como bandas I. Na banda A, pode-se observar uma fina faixa mais clara, enquanto a banda H é composta por filamentos espessos. Na região central da banda H, observa-se a banda M, formada por um arranjo de proteínas que unem os filamentos espessos. A banda I, constituída por filamentos finos, é dividida por uma linha escura, o disco Z. A região da miofibrila localizada entre dois discos Z forma um sarcômero (Figura 2). Os sarcômeros se unem para formar as miofibrilas, que se organizam em feixes, os fascículos musculares (DAL PAI-SILVA; CARVALHO, 2007; MATHEWSON; LIEBER, 2015; SILVERTHORN, 2010).

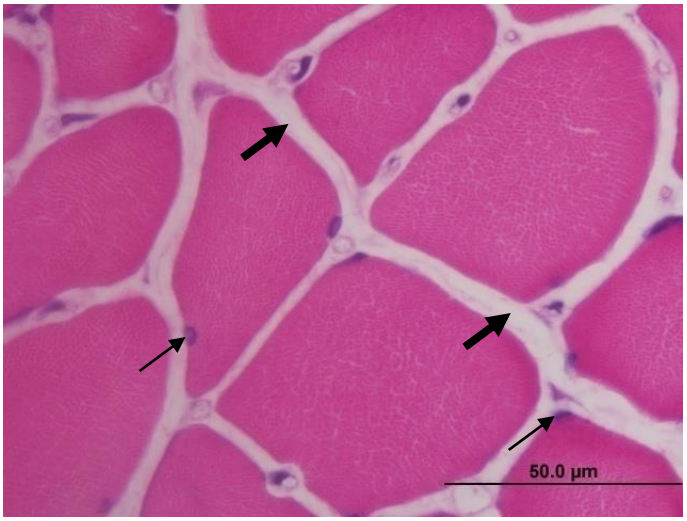

Figura 1 - Fotomicrografia da estrutura do músculo estriado esquelético. Secção transversal. Hematoxilina-Eosina (HE). Fibras multinucleadas com núcleos em posição periférica (seta fina) e endomísio (seta espessa).Fonte: autor.

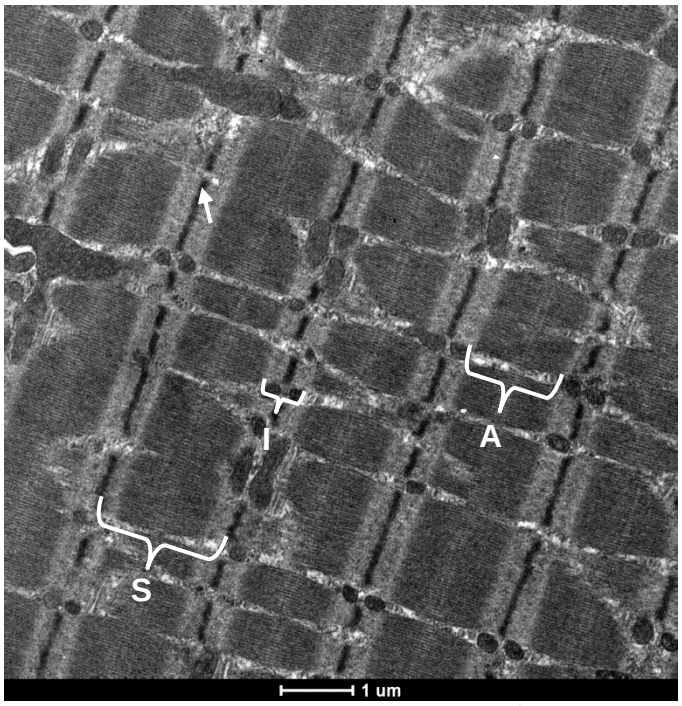

Figura 2 - Ultraestrutura da fibra muscular estriada esquelética. Miofibrilas organizadas em sarcômeros (S) com bandas A, I e linha Z (seta). Fonte: autor.

A musculatura esquelética é constituída por diferentes tipos de fibras que apresentam características morfológicas e funcionais distintas (MINAMOTO, 2005). Diferentes terminologias foram adotadas para classificar as fibras musculares como resultado de uma grande variedade de procedimentos (MINAMOTO, 2005). Dentre elas, a classificação mais utilizada foi proposta com basena atividade da enzima ATPase miofibrilar (mATPase) em meio de incubação em pH 9.4, precedido de pré-incubação em meio ácido (pH 4.3 - 4.6) e alcalino (pH 10.4 - 10.6) (BROOKE;

KAISER, 1970). As fibras musculares foram classificadas em tipos I, IIA e IIB. As fibras tipo I reagem fortemente com pré-incubação ácida e são de contração lenta; as fibras tipo IIB reagem fortemente com pré-incubação alcalina e são 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 (BROOKE; KAISER, 1970).

Os músculos são constituídos por diferentes tipos de fibras e podem apresentar predomínio de um tipo específico. O músculo plantar é considerado um músculo de contração rápida (FULLER *et al.,* 2006), composto predominantemente por fibras tipo II (TERENA *et al.,* 2017). Desempenha função como coadjuvante nos movimentos fásicos rápidos e rítmicos de flexão plantar durante a marcha (CORNACHIONE, 2011). Em situações normais, o músculo plantar não é tonicamente ativo na manutenção da postura, porém é o extensor primário do tornozelo de ratos na corrida (JASMIN; GARDINER, 1987).

O tecido conjuntivo presente nos músculos está organizado no epimísio, perimísio e endomísio. O músculo é envolto externamente pelo epimísio, internamente em torno dos feixes de fibras encontra-se o perimísio, enquanto cada fibra muscular é circundada pelo endomísio (Figura 1) (DAL PAI-SILVA; CARVALHO, 2007; GAO *et al*., 2008; JÄRVINEN *et al.,* 2002; JUNQUEIRA; CARNEIRO, 2013). A composição e disposição destas estruturas são importantes para a função muscular e podem sofrer alterações quando ocorrem desordens musculares (MATHEWSON; LIEBER, 2015).

A atividade muscular é controlada por estímulos provenientes do sistema nervoso e participando do mecanismo de controle encontram-se as JNMs, que são regiões sinápticas localizadas entre neurônios motores e fibras musculares esqueléticas (BLOCH-GALLEGO, 2015; WU; XIONG; MEI, 2010). Estas estruturas são responsáveis pela transmissão dos impulsos nervosos (FAGERLUND; ERIKSSON, 2009; SANES; LICHTMAN, 1999; WU; XIONG; MEI, 2010).

Os impulsos nervosos chegam até a fibra muscular por meio de um neurônio motor, o qual possui o corpo celular no corno anterior da medula espinal e se prolonga para a periferia por um axônio mielinizado, de forma que se ramifica e entra em contato com várias células musculares para formar uma unidade motora. O terminal nervoso não possui bainha de mielina como o restante do axônio, mas é coberto pelas células de Schwann (MARTYN; FAGERLUND; ERIKSSON, 2009). O

terminal nervoso é separado da superfície do músculo por uma fenda sináptica (MARTYN; FAGERLUND; ERIKSSON, 2009; NETO *et al.*, 2015). Quando o impulso nervoso atinge o terminal sináptico ocorre a liberação de neurotransmissores tais como a acetilcolina (ACh), das vesículas para a fenda sináptica. Quando a ACh atinge a membrana pós-sináptica localizada na superfície da fibra muscular se inicia um potencial de ação, levando ao estímulo ou à inibição da contração muscular (MARTYN; FAGERLUND; ERIKSSON, 2009; WALTON, 1988).

Basicamente a JNM é formada por um terminal axônico contendo ACh; células de Schwann e seus prolongamentos citoplasmáticos que envolvem o terminal axonal (exceto na membrana pré-sináptica); uma fenda, contendo a enzima acetilcolinesterase e revestida por lâmina basal, chamada goteira sináptica primária; uma membrana pós-sináptica, correspondente a uma região especializada da membrana sarcoplasmática, que contém receptores para ACh e, por fim, um sarcoplasma juncional, que suporta estrutural e metabolicamente a região pós-sináptica (FAGERLUND; ERIKSSON, 2009; OGATA, 1988; SCHIAFFINO; REGGIANE, 2011) (Figura 3). A forma e o tamanho do terminal axônico, assim como a complexidade das membranas pré e pós-sinápticas, variam de acordo com os diferentes tipos de fibras musculares (OGATA, 1988).

Para que ocorra a transmissão de forma adequada, as JNMs apresentam adaptações que variam conforme o tipo de fibra a que estão associadas (SCHIAFFINO; REGGIANE, 2011). As fibras musculares tipo I possuem JNMs com dimensões pequenas, forma arredondada ou ligeiramente elíptica, ramificação axonal grosseira com botões terminais dilatados, goteira sináptica rasa e dobras juncionais pouco profundas e simples. As fibras musculares tipo IIB apresentam JNMs com dimensões maiores, forma elíptica, terminais axônicos finos, longos, ramificados e com botões terminais delicados, goteira sináptica profunda e dobras juncionais profundas e complexas. As fibras musculares tipo IIA exibem JNMs com morfologia característica, evidenciando aspectos estruturais que ficam entre aqueles apresentados pelas fibras tipos I e IIB (OGATA, 1988).

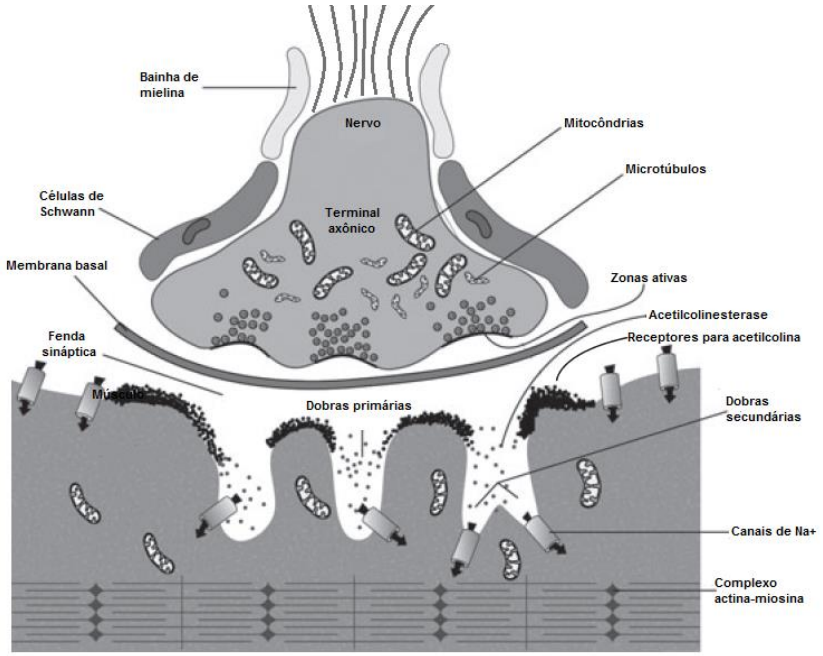

Figura 3 - Estrutura de uma junção neuromuscular e seus principais constituintes. Adaptado de Martyn, Fagerlund e Eriksson (2009).

Efeitos da PC no tecido muscular

Inicialmente, a lesão associada à PC ocorre no cérebro em desenvolvimento, no entanto, os sintomas são comumente tratados em nível muscular (MATHEWSON; LIEBER, 2015). A musculatura torna-se comprometida em pacientes espásticos, o que ocasiona redução da mobilidade (BOOTH; CORTINA-BORJA; THEOLOGIS, 2001). Com base nestas informações, estudos foram realizados com o objetivo de investigar os diferentes tipos de alterações que ocorrem na estrutura do músculo espástico em humanos. Essas alterações podem ocorrer tanto em nível macroscópico quanto microscópico (DIAS *et al.*, 2013).

Avaliações macroscópicas mostraram que músculos de pacientes com PC espástica apresentam redução do volume, da espessura e de comprimento (FRY; GOUGH; SHORTLAND, 2004; MALAIYA *et al.*, 2007; MOHAGHEGHI *et al.*, 2007; MOREAU; TEEFEY; DAMIANO, 2009). Consequentemente, indivíduos com PC possuem déficit mecânico que gera fraqueza na musculatura e pode ocasionar perda do controle motor e falta de equilíbrio (DIAS *et al.*, 2013; GRAHAM; SELBER, 2003). Além disso, observou-se a redução de 37% da área em secção transversa do músculo gastrocnêmio medial (BARBER; BARRETT; LICHTWARK, 2011). Resultados semelhantes a esse foram encontrados em outro estudo, quando foram avaliados os músculos reto femoral e vasto lateral de pacientes com PC, os quais

sugerem que as alterações observadas podem acarretar em diminuição da capacidade para geração de força, velocidade e amplitude de movimento (MOREAU; TEEFEY; DAMIANO, 2009).

Foi observado aumento na densidade de colágeno tipo I de acordo com a gravidade em relação à análise microscópica, com o objetivo de avaliar o papel que o tecido conjuntivo muscular desempenha na PC. Assim, quanto mais grave é o estado do distúrbio, maior é o acúmulo de colágeno no músculo (BOOTH; CORTINA-BORJA; THEOLOGIS, 2001; SMITH *et al.*, 2011). A quantidade de tecido conjuntivo é importante para seu desenvolvimento, reparação após lesão e na manutenção da fisiologia do organismo, portanto, quando presente em grande quantidade, pode trazer consequências. Desta forma, acredita-se que o excesso de colágeno pode estar envolvido no aumento da rigidez muscular observada na espasticidade e consequentemente relacionado à fibrose muscular em indivíduos com PC (BOOTH; CORTINA-BORJA; THEOLOGIS, 2001; SMITH *et al.*, 2011).

As características das fibras musculares também podem ser afetadas em pacientes com PC e apresentar aumento da área das fibras e do espaço entre fibras, assim como formato arredondado (BOOTH; CORTINA-BORJA; THEOLOGIS, 2001; MARBINI *et al.*, 2002). Ao se analisar a predominância dos diferentes tipos de fibra muscular, estudos verificaram predominância das fibras tipo I e deficiência das fibras tipo II (ITO *et al.*, 1996; MARBINI *et al.*, 2002; ROSE *et al.*, 1994). Foi também detectada variação no diâmetro da fibra muscular, especialmente nas fibras tipo I, em pacientes idosos com PC espástica. Estes achados podem estar relacionados com a gravidade e duração do estado espástico (ITO *et al.*, 1996).

Além das alterações nas fibras musculares, a organização das JNMs também pode se alterar na PC. Verificou-se em microscopia eletrônica de transmissão que houve redução das mitocôndrias pré-sinápticas, modificação na conformação das dobras pós-sinápticas e alteração na localização da acetilcolinesterase e laminina β2, componentes da lâmina basal essenciais para organização e eficácia da neurotransmissão das JNMs, em crianças com escoliose e PC espástica (ROBINSON *et al.*, 2013).

Modelos experimentais de PC

Modelos animais têm sido utilizados na tentativa de que se reproduzam as características observadas em pacientes com PC. Para isso, levam-se em

consideração os fatores pré e pós-natais, responsáveis pelo desenvolvimento da síndrome.

O LPS, quando presente no organismo, se liga a receptores do tipo Toll e ativa o sistema imunológico ocasionando uma resposta adaptativa imune a partir da produção de citocinas inflamatórias como Interleucina 1β (IL-1β) e o Fator de Necrose Tumoral α (TNF-α) (KOPP; MEDZHITOV, 1999). A produção dessas citocinas pode ocasionar apoptose de oligodendrócitos, causar danos à substância branca encefálica devido à inflamação e a degeneração da mielina (DAMMANN; DURUM; LEVITON, 2001; KOPP; MEDZHITOV, 1999). Modelos animais usando infecção por LPS mostraram que, apesar de suas consequências sobre o sistema nervoso, não são geradas alterações no desenvolvimento motor, comumente observadas na PC (POGGI *et al*., 2005; ROBERSON *et al.*, 2006; STIGGER *et al.*, 2013). No entanto, o LPS afeta a atividade e coordenação motora com o aumento dos níveis de IL-1β e TNF-α associada a um elevado índice na fabricação de radicais livres, que ocasiona uma mielinização deficiente (STIGGER *et al.*, 2013; SVEDIN *et al.,* 2005).

Assim como o LPS a anóxia perinatal também é utilizada em estudos que envolvem a PC. A indução de hipóxi-isquemia no período embrionário leva à redução de peso e ao atraso no desenvolvimento fisiológico (ZHURAVIN; DUBROVSKAYA; TUMANOVA, 2004). Não foram observadas alterações na marcha, no peso nem na histologia do músculo quando foram avaliadas as consequências desse insulto sozinho (MARCUZZO *et al.*, 2008). No entanto, observou-se que a expressão de IL-1 no córtex cerebral pode aumentar, mas as medidas oxidativas não são alteradas e também induzir a um leve impacto no desenvolvimento motor (STIGGER *et al.*, 2013). Alterações motoras foram encontradas em outro estudo, ocasionadas pela atrofia cerebral, porém, a qualidade motora melhorou ao longo do período experimental. Além disso, verificou-se a redução do peso nos animais do grupo experimental (LUBICS *et al.*, 2005).

Quando os testes funcionais foram avaliados, verificou-se a redução do cruzamento e do comprimento médio da passada, sugerindo-se que insultos hipóxi-isquêmicos pré-natal induzem a um déficit funcional que persiste nos animais afetados (ROBINSON *et al.*, 2005). Injeções de LPS (a partir do 17º dia gestacional até o final da gestação, que compreende o 21º dia gestacional) associadas à anóxia perinatal (induzida pela ligação da artéria carótida comum direita no 1º dia pós-natal)

ocasionaram alterações comportamentais e motoras mais intensas no grupo em que foram associados os dois insultos (GIRARD *et al.*, 2009). Quando LPS e anóxia perinatal foram combinados, houve ainda maior deficiência no desenvolvimento e coordenação motora (STIGGER *et al.*, 2013).

A anóxia perinatal quando associada à restrição sensório-motora pós-natal provoca redução do peso e alteração na marcha, que compreende redução no comprimento da passada e maior ângulo de pé, além de alterações na histologia do músculo sóleo como atrofia muscular. No grupo submetido à restrição sensório-motora com posterior treinamento locomotor, verificou-se melhora nestes fatores (MARCUZZO *et al.*, 2008). A restrição sensório-motora pós-natal, associada ou não à anóxia perinatal, pode reduzir a taxa de crescimento corporal, aumentar o tônus muscular, ocasionar comportamento do andar anormal, atrofia das fibras musculares, degeneração articular e desorganização do córtex motor primário (STRATA *et al.,* 2004). A longo prazo, a combinação de asfixia perinatal com desuso dos membros pélvicos ocasiona atrofia das fibras musculares, alterações da matriz extracelular no músculo e degeneração articular, bem como a desorganização do córtex somatossensorial primário. Tal desorganização é maior quando são associados o desuso e a asfixia perinatal (COQ *et al.*, 2008).

Stigger *et al.*(2011) avaliaram as consequências da exposição pré-natal ao LPS, anóxia perinatal e restrição sensório-motora de forma isolada e também as possíveis combinações entre elas. E demonstraram que ratos expostos ao LPS, à anóxia perinatal e à restrição sensório-motora isoladamente ou combinados apresentaram redução do equilíbrio e coordenação no teste de Rotarod. Os animais submetidos à restrição sensório-motora (imobilização dos membros pélvicos) tiveram alterações motoras mais graves. A análise do músculo sóleo mostrou redução na área da seção transversal das fibras musculares, aumento do comprimento do sarcômero e diminuição na densidade do sarcômero no grupo restrito. Foi observada também redução na área de secção transversal das fibras no músculo tibial anterior quando os três insultos foram associados. Além disso, redução no número de fibras tipo I e aumento das fibras tipos II nos músculos sóleo e tibial anterior nos grupos com restrição sensório-motora. Com isso, sugeriu-se que os insultos isolados ou combinados possuem efeitos e diferentes gravidades sobre a atividade motora e as características musculares (STIGGER *et al.,* 2011).

Verifica-se que a combinação mais eficaz para o estudo desta desordem é a associação da infecção materna pelo LPS, anóxia perinatal e restrição sensório-motora com base na literatura consultada e nos modelos experimentais envolvendo PC. Como as características da musculatura estriada esquelética ainda não foram bem estabelecidas neste modelo, a abordagem mais completa das alterações musculares é relevante para futuras pesquisas relacionadas à PC.

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ARTIGO CIENTÍFICO

MORPHOFUNCTIONAL CHARACTERISTICS OF SKELETAL MUSCLE IN RATS WITH CEREBRAL PALSY

Morphofunctional characteristics of skeletal muscle in rats with cerebral palsy

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Abbreviations: CP –cerebral palsy; LPS – lipopolysaccharide; P – postnatal day; CG – control group; CPG – cerebral palsy group; HE – hematoxylin-eosin; NADH-TR – nicotinamide adenine dinucleotide-tetrazolium reductase.

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ABSTRACT

Knowledge of the adaptations that occur in skeletal muscle is important for the understanding of the functional deficits found in cerebral palsy (CP). Therefore, the objective of this study was to investigate the morphofunctional characteristics of striated muscle in an animal model of CP. For induction of CP, pregnant *Wistar* rats were injected intraperitoneally with saline or lipopolysaccharide (LPS) over the last 5 days of pregnancy (gestational days 17 to 21). The control group (n = 8) consisted of male pups born to females injected with saline. The CP group (n = 8) consisted of male pups born to females injected with LPS, which were submitted to perinatal anoxia (day of birth, postnatal day 0 - P0) and sensorimotor restriction (P1 to P30). The open-field test was undertaken on P29 and P45. On P48, the animals were weighed and the plantaris muscle was collected and its weight and length were measured. Muscle fiber morphology was analyzed by light microscopy after staining with hematoxylin-eosin and NADH-TR and by transmission electron microscopy. Intramuscular collagen was evaluated by staining with Masson's trichrome and neuromuscular junctions by the nonspecific esterase reaction. The parametric Student *t*-test and nonparametric Mann-Whitney test were used for statistical analysis. In the CP group, reductions were observed in mobility time, number of crossings and rearing frequency, as well as in body weight, muscle weight and length, and nucleus-to-fiber and capillary-to-fiber ratios. In addition, there was an increase in collagen percentage; a reduction in the area and an increase in the number of type I muscle fibers; an increase in myofibrillar disorganization and Z-line disorganization and dissolution, and a reduction in the area and largest and smallest diameters of neuromuscular junctions. In conclusion, the animal model of CP produced morphofunctional alterations in striated muscle, causing motor deficits as demonstrated by the results of the open-field test.

Keywords: lipopolysaccharide; perinatal anoxia; sensorimotor restriction; ultrastructure; neuromuscular junction

1. INTRODUCTION

Cerebral palsy (CP) describes a group of permanent disorders affecting movement and posture, which are attributed to nonprogressive disturbances that occur in the developing fetal or infant brain (Rosenbaum et al., 2007). The estimated incidence of CP in developed countries is 2 to 3 per 1,000 live births (Pin et al., 2013; Souza et al., 2013). However, in underdeveloped or developing countries, this incidence can reach 7 per 1,000 live births (Piovesana et al., 2002). The high incidence of CP observed in Brazil might be related to inadequate prenatal and neonatal care (Brasil, 2013).

The pathophysiology of CP is not fully understood, but might be related to events that occur during neural development triggered by pre-, peri- or postnatal factors (Agarwal and Verma, 2012). The main prenatal factor in the etiology of CP are infections (Jacobsson and Hagberg, 2004; Dodge, 2008). Preterm birth and intrapartum asphyxia increase the risk of developing CP during the perinatal period (Jacobsson and Hagberg, 2004). Postnatal causes include infections, traumas, and anoxic injury (Dodge, 2008; Hugues et al., 2012). Considering these risk factors, experimental models have been proposed to standardize a protocol of CP using insults of prenatal exposure to lipopolysaccharide (LPS; the structural component of most Gram-negative bacteria), perinatal anoxia, and sensorimotor restriction (Strata et al., 2004; Marcuzzo et al., 2008; Stigger et al., 2011a). These insults alone or in combination exert different effects and severity on motor activity and muscle characteristics. Their combination appears to better represent the alterations observed in humans (Stigger et al., 2011a).

Patients with CP exhibit a series of disorders of movement, posture and coordination that can vary according to type, severity and etiology (McCullough et al., 2013). In this respect, knowledge of the changes that occur in skeletal muscle is essential to understand the impairment of functional capacity in patients with CP (Dias et al., 2013). Therefore, the objective of the present study was to investigate the morphofunctional characteristics of striated muscle in an animal model of CP.

2. MATERIAL AND METHODS

2.1. Animals and experimental induction of CP

All animal procedures of this study were approved by the Ethics Committee on Animal Use of Unioeste (Approval No. 24/16; Appendix A). Litters were obtained using 3-month-old *Wistar* rats (11 females and 6 males). These animals were housed in polycarbonate boxes (27 x 26 x 31 cm) covered with shavings and kept under controlled conditions of temperature (20 ± 2ºC) and light (12-h light/dark cycle), with water and standard chow available *ad libitum*.

First, the females were submitted to colpocytological examination for the verification of estrous cycle. When in the receptive phase (proestrus), the females were transferred to individual cages (19 x 11 x 10 cm) with one male for mating. Once pregnancy was confirmed by colpocytological examination, the pregnant females were injected intraperitoneally with sterile saline (100 μL) or LPS (200 μg/kg in 100 μL sterile saline). These injections were administered at intervals of 12 h from day 17 to day 21 of gestation (end of pregnancy) (Stigger et al., 2011a).

On the day of birth (postnatal day 0, P0), the litters were standardized. Male pups derived from at least four litters were divided into two groups: control group (CG, n = 8)

consisting of pups born to females injected with saline during pregnancy; CP group (CPG, *n* = 8) consisting of pups born to females injected with LPS during pregnancy and submitted to perinatal anoxia and sensorimotor restriction.

Perinatal anoxia was induced on P0 by placing pups of CPG in a closed chamber partially immersed in water at 37 ± 1°C, under a nitrogen (100%) flow of 9 L/min for 20 min. Next, the pups were rapidly removed, kept under normal atmospheric conditions, and observed until return to their normal breathing pattern (Stigger et al., 2011a). Pups of CG were submitted to a similar procedure and maintained for the same period of time in the chamber, which remained open and with normal flow of atmospheric air.

From P1 to P30, animals of CPG were submitted to sensorimotor restriction for 16 hours/day. The pelvic limbs of the animals were bound together with microporous adhesive tape and the hip, knee and ankle were held in an extended position with a properly positioned epoxy mold adjusted to the size of the animal. This type of restriction permits the animal to perform limited movements of the hip and slight ankle movements become possible over time (Strata et al., 2004). This intervention appears to be well tolerated by pups and does not restrict miction, defecation, suckling or other maternal care (Marcuzzo et al., 2008). The pelvic limbs of CG animals were manipulated for approximately 2 min, a period similar to that necessary to place the immobilization device in CPG (Strata et al., 2004).

2.2. Evaluation of motor activity in the open-field test

Locomotor activity was evaluated on P29 and P45 using the open-field test. The test uses a box (40 x 100 x 100 cm) whose floor is subdivided into 12 squares (33.3 x 25 cm). The behavior of the animals was filmed by positioning a video camera (height of 150 cm) in such a way that it would encompass the whole field. The animals were first placed in the northeast corner of the box and filmed for 5 min. The recordings were used to collect the following

data: number of crossings (number of squares crossed by the animal during the test); rearing frequency (number of times the animal stands only on its pelvic limbs, keeping its thoracic limbs elevated), and grooming frequency (raising the thoracic limbs to the nose) (Stigger et al., 2013).

2.3. Collection of the plantaris muscle

At 48 days of age, the animals were weighed on an analytical scale (Shimadzu UX620H, São Paulo, Brazil) and anesthetized by intraperitoneal injection of ketamine hydrochloride (50 mg/kg; Cristália, Brazil) and xylazine hydrochloride (10 mg/kg; Cristália, Brazil). For collection of the plantaris muscle, the skin of the pelvic limbs was elevated and the gastrocnemius and soleus muscles were removed for exposure and bilateral dissection of the plantaris muscle. The length (mm) of the right muscle antimere (muscle belly) was then measured with a digital caliper (Digimess®, São Paulo, Brazil) and the specimen was weighed on an analytical scale (Shimadzu UX620H, São Paulo, Brazil). The right and left muscle antimeres were cut transversely into smaller fragments with a stainless-steel blade for histological and histoenzymological analysis of muscle fibers and histochemical study of neuromuscular junctions (NMJs).

2.4. Study of muscle fibers and intramuscular collagen

For the study of muscle fibers and intramuscular collagen, the right antimere of the plantaris muscle was removed and kept at room temperature for 30-40 min (Khan, 1977). After this period, the material was covered with neutral talc for tissue preservation (Moline and Glenner, 1964), frozen in liquid nitrogen for 2 min, transferred to cryotubes, and stored in a biofreezer at -80ºC for subsequent processing. The muscle segments thus frozen were transferred to a cryostat chamber (LUPETEC CM 2850 Cryostat Microtome) at -20°C and

maintained for 30 min for temperature stabilization. Next, the ends of these segments were glued to a metal support with Jung Tissue Freezing Medium (Leica, Germany). The muscle specimens were cut into 7-µm semi-serial sections (in which the five sections in between were discarded) and submitted to the staining technique and enzymatic reaction described below.

2.4.1. Histological study

Cross-sections of the plantaris muscle were stained with hematoxylin-eosin (HE) (Junqueira and Junqueira, 1983). These HE-stained slides were used for the quantification of nuclei, muscle fibers and capillary-to-fiber ratio in 10 microscopic fields (40X objective) per animal. The number of nuclei and muscle fibers was used to determine the nucleus-to-fiber ratio. To obtain the capillary-to-fiber ratio, muscle fibers covering the upper right margins were included in the count, while those found in the lower left margins were excluded from the analysis in order to minimize errors, since capillaries present in the microscopic fields belonged to the vascularization of all fibers, including those that were not complete in the image. Quantification was performed individually by two trained evaluators and the mean of the two values was used for analysis (Fernandes et al., 2012).

The presence of collagen in connective tissue was also evaluated. Cross-sections of the plantaris muscle were stained with Masson's trichrome (Bancroft and Stevens, 1990) and the percentage of intramuscular collagen was obtained by analyzing three microscopic images per animal (20X objective).

2.4.2. Histoenzymological and morphometric study

Cross-sections of the muscle were also submitted to the nicotinamide adenine dinucleotide-tetrazolium reductase (NADH-TR) reaction as described by Pearse (1972) and modified by Dubowitz and Brooke (1973). The material obtained was used for the analysis of

oxidative and glycolytic metabolism of the three types of muscle fibers. Morphometric analysis of approximately 200 muscle fibers was performed by measuring their respective areas in cross-sections of the plantaris muscle. The three types of muscle fibers were also quantified. Three microscopic fields per animal were randomly chosen (20X objective).

2.5. Ultrastructural study of muscle fibers

For the ultrastructural study of muscle fibers, the left antimere of the plantaris muscle was removed and fixed in Karnovsky fixative (Karnovsky, 1965). The specimens were cut into approximately 1-mm wide longitudinal fragments for processing. These fragments were washed in 0.1 M phosphate buffer, pH 7.3 (15 min), and postfixed in 1% osmium tetroxide in the same buffer (2 hours). The specimens were then washed in distilled water (3 times of 5 min each), incubated in 0.5% uranyl acetate in aqueous solution (2 hours), dehydrated in an increasing acetone series, and immersed in a mixture of resin and 100% acetone (12 hours) for subsequent embedding in resin. Fields showing the largest amount of adequate material for analysis were selected in semi-thin sections (0.5 µm thick), and ultrathin (90 nm thick) sections were then obtained with an ultramicrotome (Ultracut UCT, Leica®, Germany). The ultrathin sections were stained with a saturated solution of uranyl acetate (20 min) and lead citrate (10 min) for subsequent analysis.

2.6. Histochemical and morphometric study of NMJs

For analysis of NMJs, a fragment of the left antimere of the plantaris muscle was removed and fixed in Karnovsky fixative (Karnovsky, 1965). This fragment was cut longitudinally into several slices with a stainless-steel blade and the sections obtained were submitted to the nonspecific esterase reaction (Lehrer and Ornstein, 1959). For morphometry,

the area and largest and smallest diameters of 100 NMJs were measured in microscopic images (20X objective).

2.7. Image analysis

The morphology and morphometry of muscle fibers and NMJs were analyzed in images captured with an Olympus Bx60® microscope equipped with an Olympus DP71 camera (Tokyo, Japan) using the DP Controller 3.2.1 276 software. This material was analyzed using the Image Pro Plus 6.0® program (Media Cybernetics, Maryland, USA). For ultrastructural analysis, the material was examined and photographed under a transmission electron microscope (CM100, Philips®, The Netherlands).

2.8. Statistical analysis

The data were analyzed with the GraphPad Prism® program (La Jolla, USA), considering the results of the Kolmogorov-Smirnov test of normality. Normally distributed data were analyzed by the Student *t*-test, while the Mann-Whitney test was used for nonparametric analysis. A p value < 0.05 was considered significant.

3. RESULTS

3.1. Motor activity

Evaluation of the locomotor parameters showed reductions of 23% in mobility time ($p = 0.0012$), of 42% in the number of crossings ($p = 0.0003$), and of 57% in rearing frequency ($p = 0.0008$) in CPG animals at 29 days of age when compared to CG. At 45 days, animals of CPG exhibited a reduction of 32% in mobility time ($p = 0.0344$) and of 41% in rearing

frequency (p = 0.0310) compared to CG. The other parameters evaluated at 29 and 45 days of age were similar in the two groups studied (Table 1).

3.2. Macroscopic parameters

Macroscopic inspection was performed on the day of euthanasia, at 48 days of age. There was a body weight reduction of 18% in CPG animals compared to CG (p = 0.0009). With respect to muscle characteristics, a reduction in the weight (50%; p = 0.0009) and length (24%; p = 0.0007) of the plantaris muscle was observed in CPG when compared to CG. Muscle weight in relation to body weight was reduced by 36% in CPG animals (p = 0.009), demonstrating a significant loss of muscle tissue (Table 2).

3.3. Morphology and morphometry of muscle fibers and intramuscular collagen

The general architecture of skeletal muscle fibers was preserved. These fibers had a polygonal shape, were multinucleated with peripheral nuclei, and were organized into fascicles. Blood capillaries were found interspersed among muscle fibers in the connective tissue (Figure 1A and 1B).

There was no significant difference in the area of muscle fibers stained with HE between the groups studied (p = 0.073). Analysis of fiber count showed a reduction of 29% in CPG compared to CG (p = 0.023). No significant difference in the number of peripheral nuclei was found between the two groups (p = 0.178). The nucleus-to-fiber ratio was reduced by 33% in CPG compared to CG (p = 0.002) (Table 2). Evaluation of the capillary-to-fiber ratio showed a 36% reduction in CPG compared to CG (p = 0.002) (Figure 1E).

Collagen was predominant in the perimysium that surrounds the muscle fiber bundles (Figure 1C and 1D). There was an increase of 27% in the percentage of intramuscular collagen in CPG compared to CG (p = 0.038) (Figure 1F).

The muscle fibers were classified as proposed by Brooke and Kaiser (1970). The NADH-TR reaction demonstrated the presence of type I (small diameter and intense oxidative activity), IIA (intermediate diameter and moderate oxidative activity), and IIB (large diameter and weak oxidative activity) fibers in the two groups studied (Figure 2A and 2B). The mean area of type I fibers was reduced by 20% in CPG compared to CG ($p = 0.024$), while no significant difference was observed for type IIA ($p = 0.292$) or IIB fibers ($p = 0.732$) (Figure 2C). With respect to muscle fiber count, the number of type I fibers was 35% higher in CPG compared to CG ($p = 0.034$), while type IIA or IIB fiber count did not differ between groups (Figure 2D).

Ultrastructural analysis by transmission electron microscopy showed a well-defined morphology of the muscle fibers in CG, which were organized in sarcomeres following the striated pattern of light and dark bands (I and A bands, respectively) (Figure 3A). Ultrastructural alterations characterized by disorganization of the myofibrillar pattern and Z- and M-line were observed in CPG (Figure 3B). Statistical analysis revealed a significant increase in Z-line disorganization (333.3%; $p = 0.034$; Figure 3C), myofibrillar disorganization (142.8%; $p = 0.0026$; Figure 3D), and Z-line dissolution (184.2%; $p = 0.0011$; Figure 3E) in CPG when compared to CG.

3.4. Morphology and morphometry of NMJs

The NMJs had an oval, round and elliptical shape in CG and CPG (Figure 4A and 4B). Morphometric analysis revealed a reduction in the area (25%; $p = 0.004$) and in the largest (11%; $p = 0.048$) and smallest diameter (11%; $p = 0.031$) of NMJs in CPG compared to CG (Figure 4C, 4D and 4E).

4. DISCUSSION

The use of animal models for the investigation of human diseases is only relevant if cellular responses occur in both the human and the animal organism (Feather-Schussler and Ferguson, 2016). Regarding CP, no well-established experimental model exists for the study of encephalopathy. Stigger et al. (2011a) proposed that the combination of prenatal exposure to LPS, perinatal anoxia and sensorimotor restriction is more effective in reproducing the characteristics observed in patients with CP. However, studies evaluating in detail the striated muscle in this experimental model are sparse. The present study provides additional results regarding the effects of the model proposed by Stigger et al. (2011a) on the motor function and morphology of muscle fibers and NMJs of the plantaris muscle.

The evolution of locomotor parameters was evaluated in the open-field test at 29 and 45 days of age. The results showed a reduction in mobility time, number of crossings and rearing frequency at 29 days of age in CPG animals compared to CG. With the removal of sensorimotor restriction at 30 days of age, the mobility time and rearing frequency continued to be altered in CPG at 45 days of age. Reduced activity in the open-field test has been reported by other authors in models of hypoxia-ischemia (Schuch et al., 2016) and CP (Girard et al., 2009; Silva et al., 2016). These alterations might be due to a decrease in exploratory activity or an increase in anxiety (Lubics et al., 2005; Carletti et al., 2012). Furthermore, studies have shown that prematurity and hypoxic events affect the striatum (Jamon, 2006; Durán-Carabali et al., 2017), which is responsible for the initiation and control of movement (Piek et al., 2008). Thus, we believe that the present results are related to damage to the striated body that leads to the motor deficits observed.

The evaluation of body parameters showed a reduction in body weight and in the weight and length of the plantaris muscle in CPG compared to CG. Other studies also

demonstrated a weight reduction in animals with CP (Marcuzzo et al., 2010; Silva et al., 2016). This finding could be attributed to muscle atrophy or lower bone density caused by sensorimotor restriction (Marcuzzo et al., 2010). The ratio between body weight and muscle weight showed marked impairment of muscle weight when compared to the total body weight of CPG animals. Reduced muscle weight and length are common features observed in patients with CP that can cause mechanical deficits, resulting in muscle weakness, loss of motor control and lack of balance (Graham and Selber, 2003; Dias et al., 2013). These changes are suggested to be due to the limitation of movement caused by immobilization, compromising muscle development (Marques et al., 2014) that occurs between the $4^{th}$and 6^{th} postnatal week in rats (Dayanidhi and Lieber, 2014).

The remodeling capacity of skeletal muscle permits its adaptation according to functional demands (Mathewson and Lieber, 2015; Baehr et al., 2016). In the present study, a reduction was observed in the number of nuclei and, consequently, in the nucleus-to-fiber ratio in CPG. According to the myonuclear domain hypothesis, each nucleus is responsible for a specific amount of sarcoplasm and for the necessary protein synthesis in the respective domain (Mitchell and Pavlath, 2004). The myonuclear domain is not fixed and varies according to muscle fiber type, being inversely related to the oxidative capacity of the muscle fiber (Van Der Meer et al., 2011). Because of their high oxidative activity, type I muscle fibers have a smaller myonuclear domain than type IIA and IIB fibers (Jaspers et al., 2006). In this respect, the number of nuclei per fiber is related to cell volume and muscle fiber atrophy is therefore associated with the loss of nuclei (Van Der Meer et al., 2011; Delhaas et al., 2013). The reduction in cell volume requires less protein turnover and consequently a smaller number of nuclei for production of the necessary protein volume (Van Wessel et al., 2010). Thus, the reduction in the nucleus-to-fiber ratio found might be related to the atrophy of type I muscle fibers observed in this study.

Since the number of capillaries is directly proportional to muscle mass (Kano et al., 2000), reduced capillarity may be associated with the reduction in type I muscle fiber area observed in this study. Decreased muscle activity and immobilization reduce the capillary network and consequent oxygen supply (Pontén and Stal, 2007). This affects muscle metabolism, increasing fatigability due to impaired supply of energy substrates and oxygen, factors that are important for maintaining essential metabolic characteristics of the muscle (Degens and Always, 2006; Schiaffino and Reggiane, 2011). Another factor is the accumulation of connective tissue, which was also found in the present study, impairing intramuscular blood circulation by increasing the connective tissue barrier between the capillary and muscle fiber (Järvinen et al., 2002).

Connective tissue is important for providing mechanical support to muscles (Gagliano et al., 2013). Its composition and arrangement interfere with muscle function and are altered in the presence of muscle disorders (Mathewson and Lieber, 2015). Immobilization causes an increase in intramuscular connective tissue that results in the loss of extensibility and movement limitation (Järvinen et al., 2002; Pucciarelli et al., 2016). An increase in intramuscular collagen has been observed in patients with CP, which is associated with the installation of stiffness observed in disused muscles (Booth et al., 2001; Smith et al., 2011). In this study, a higher percentage of collagen in connective tissue was found in CPG. Since the same was observed by other authors who combined perinatal anoxia and sensorimotor restriction in an experimental model of CP (Coq et al., 2008), we believe that these insults were responsible for the increase in collagen percentage.

Studies using the same model of CP as employed here have shown muscle fiber atrophy in soleus (Marques et al., 2014) and tibialis anterior muscle (Stigger et al., 2011a). Muscle atrophy is a feature seen in patients with CP (Marbini et al., 2002) and type I fibers are more susceptible to the alterations resulting from inactivity in these patients (Walden et

al., 2012; Wang and Pessin, 2013). In the present study, a reduction in the cross-sectional area of type I fibers was observed in CPG, in agreement with the literature, while no changes were found for type IIA or IIB fibers. This result may be explained by the metabolism of each muscle fiber type. Type I fibers require high protein turnover and therefore depend on a higher rate of protein synthesis and degradation than type IIA and IIB fibers (Appel, 1990). Given this knowledge and since muscle vascularization is impaired in this model, we believe that the metabolic substrates are not sufficient to maintain protein synthesis in CPG animals. Since type I fibers are dependent on a higher protein supply, it is suggested that they are more affected, with the observation of significant differences compared to type IIA and IIB fibers that were able to maintain their metabolism. Thus, muscle fiber atrophy is an adaptive response to reduce metabolic demands in order to protect cell viability and the contractile function of the muscle (Clanton and Klawitter, 2001). In addition, muscle fiber atrophy has been associated with fiber adaptation to motor disabilities caused by brain injury and loss of sarcomeres (Mohaguegui et al., 2007). There was also an increase in the number of type I fibers, which is associated with a reduction in fiber area, resulting in a larger number of fibers per microscopic field analyzed.

The ultrastructure of muscle fibers is little studied in CP. To our knowledge, this is the first study to evaluate skeletal muscle ultrastructure in an animal model of CP. The changes observed, including Z-line disorganization, myofibrillar disorganization and Z-line dissolution, were focal, corroborating the results obtained for patients with CP. In humans, Marbini et al. (2002) demonstrated various degrees of myofibrillar disorganization or disorientation, with focal loss of the striated pattern which was always accompanied by changes in the Z-line. The Z-line provides transverse support to the sarcomere, ensuring transmission of contraction force in the myofibril (Schiaffino and Reggiane, 2011). Desmin filaments are linked to the Z-line through crystallin and plectin, forming a network among

myofibrils in the sarcoplasm and the sarcolemma which protects against the mechanical stress during muscle contraction (Clark et al., 2002). It is important to note that the contractile apparatus is bound to the basement membrane through association with the Z-line and M-line. The contractile force is thus transmitted to the basement membrane where it is transmitted laterally to the muscle end (Clark et al., 2002). Taken together, these findings reinforce that the loss of structural organization observed, even if focal, compromises the contractile action of the whole muscle.

The structural integrity of NMJs influences neuromuscular transmission and consequently muscle function (Pratt et al., 2015). In spastic children, transmission electron microscopy analysis showed a reduction in presynaptic mitochondria, modifications in postsynaptic folds, and changes in structures that are essential for the organization and efficiency of neurotransmission in NMJs (Robinson et al., 2013). The interruption of motor activity during development results in modifications in the peripheral nervous system (Marcuzzo et al., 2008), since the mechanical activity applied to the muscle fiber is important for the maturation of innervation (Greensmith et al., 1998). Animals submitted to sensorimotor restriction exhibit a reduced cross-sectional area of motoneurons (Stigger et al., 2011b). The morphology of NMJs adapts to the functional demands of different motor units (Sieck et al., 2012) and depends on the muscle fibers with which they are associated (Schiaffino and Reggiane, 2011). Few studies have investigated the changes that occur in NMJs in animal models of CP. In the present study, reductions were observed in the area and largest and smallest diameters of the NMJs analyzed. We suggest these results to be related to the atrophy of type I muscle fibers discussed above and that sensorimotor restriction was the determinant factor of these changes. Since these structures are affected in patients with CP, this animal model appears to reproduce some of the characteristics observed in studies on humans.

5. CONCLUSION

The animal model of CP that combines prenatal exposure to LPS, perinatal anoxia and sensorimotor restriction caused the accumulation of intramuscular collagen, muscle fiber atrophy, and ultrastructural alterations in muscle fibers and NMJs. These adaptations culminated in motor deficits demonstrated by the open-field test. This model showed the relationships between structural alterations in striated muscle and could be used in animal studies investigating CP, thereby contributing to the development of new therapeutic strategies designed to improve the quality of life of patients with this disorder.

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Table 1. Mobility time, number of crossings and rearing and grooming frequency of control animals and animals with cerebral palsy at 29 and 45 days of age.

Parameter (n = 8)	**CG P29**	**CPG P29**	**CG P45**	**CPG P45**
Mobility time (s)	165.4 ± 22.7	126.3 ± 15.1**	99.9 ± 20.3	67.2 ± 33.7*
Crossings	119.8 ± 15.1	69.4 ± 26.0***	89.5 ± 26.1	58.5 ± 35.2
Rearing	46.2 ± 11.2	19.9 ± 13.5***	29.9 ± 11.3	17.7 ± 8.7*
Grooming	4.4 ± 1.6	5.0 ± 2.6	2.6 ± 1.6	4.4 ± 1.8

CG: control group; CPG: cerebral palsy group; P29: 29 days of age; P45: 45 days of age.

Values are expressed as the mean ± standard deviation.*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$(Student *t*-test).

Table 2. Macroscopic and microscopic parameters of control rats and rats with cerebral palsy at 48 days of age.

Parameter	**CG**	**CPG**
Body weight (g)[a]	198.5 ± 5.3	162 ± 16.2***
Plantaris muscle weight (g)[a]	0.2 ± 0.05	0.1 ± 0.03***
Muscle length (mm)[b]	26.2 ± 3	19.9 ± 2.8***
Muscle weight/body weight ratio[a]	0.11 ± 0.02	0.07 ± 0.02***
Muscle fiber area (μm^2)[b]	1461 ± 120	1214 ± 238.5
Number of muscle fibers[b]	361.8 ± 38.1	467.8 ± 77.1*
Number of peripheral nuclei[b]	458.6 ± 47.4	397.6 ± 79.1
Nucleus-to-fiber ratio[b]	1.3 ± 0.2	0.8 ± 0.1**

CG: control group; CPG: cerebral palsy group.

Values are expressed as the mean ± standard deviation. Macroscopic parameters: $n = 8$. Microscopic parameters: $n = 5$. [a]Mann-Whitney test; [b]Student *t*-test. *p < 0.05; **p < 0.01; ***p < 0.001.

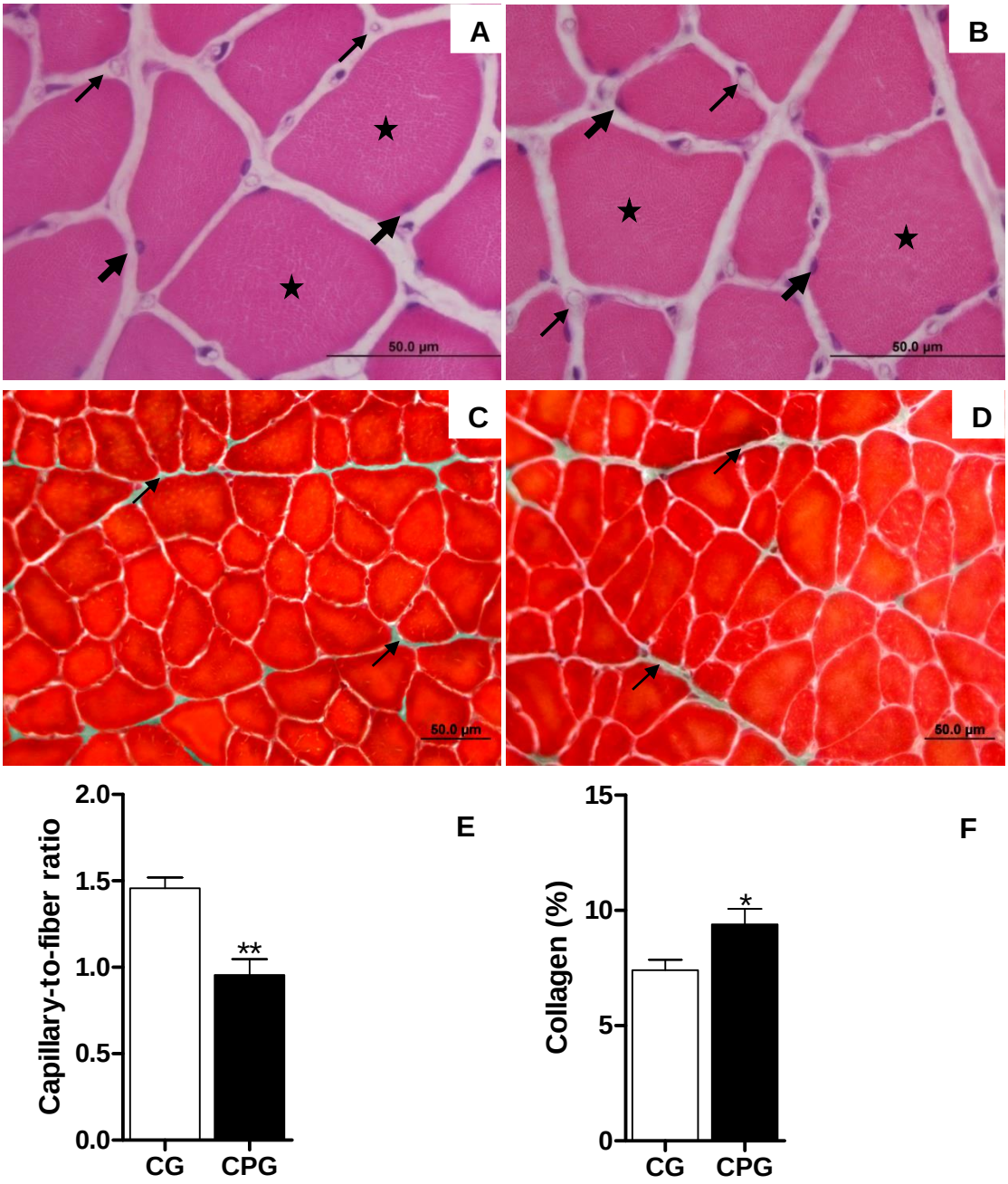

Figure 1. Photomicrographs of cross-sections of the plantaris muscle obtained from48-day-old *Wistar* rats. **A and B:** Muscle fibers (star), peripheral nuclei (thick arrow) and capillaries (thin arrow) in the control (CG) and cerebral palsy group (CPG), respectively. HE. **C and D:** Perimysium (arrow) in CG and CPG, respectively. Masson's trichrome. **E:** Capillary-to-fiber ratio in CG and CPG. **F:** Percentage of intramuscular collagen in CG and CPG. Values are expressed as the mean ± standard deviation. *p < 0.05; **p < 0.01 (Student *t*-test).

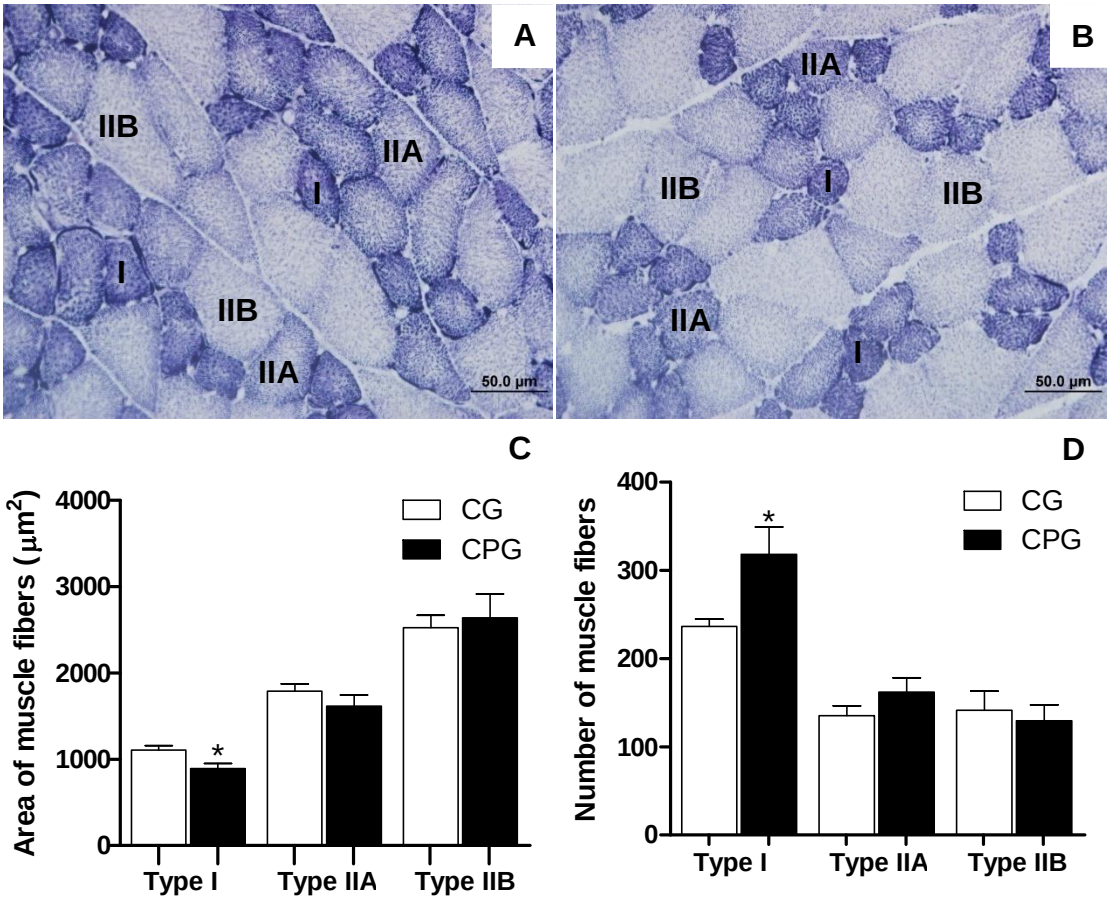

Figure 2. Photomicrographs of cross-sections of the plantaris muscle obtained from 48-day-old *Wistar* rats and submitted to the NADH-TR reaction. **A and B:** Type I, IIA and IIB muscle fibers in the control (CG) and cerebral palsy group (CPG), respectively. **C and D:** Comparison of area and number of the different muscle fiber types between animals of CG and CPG. Values are expressed as the mean ± standard deviation. *$p < 0.05$ (Student *t*-test).

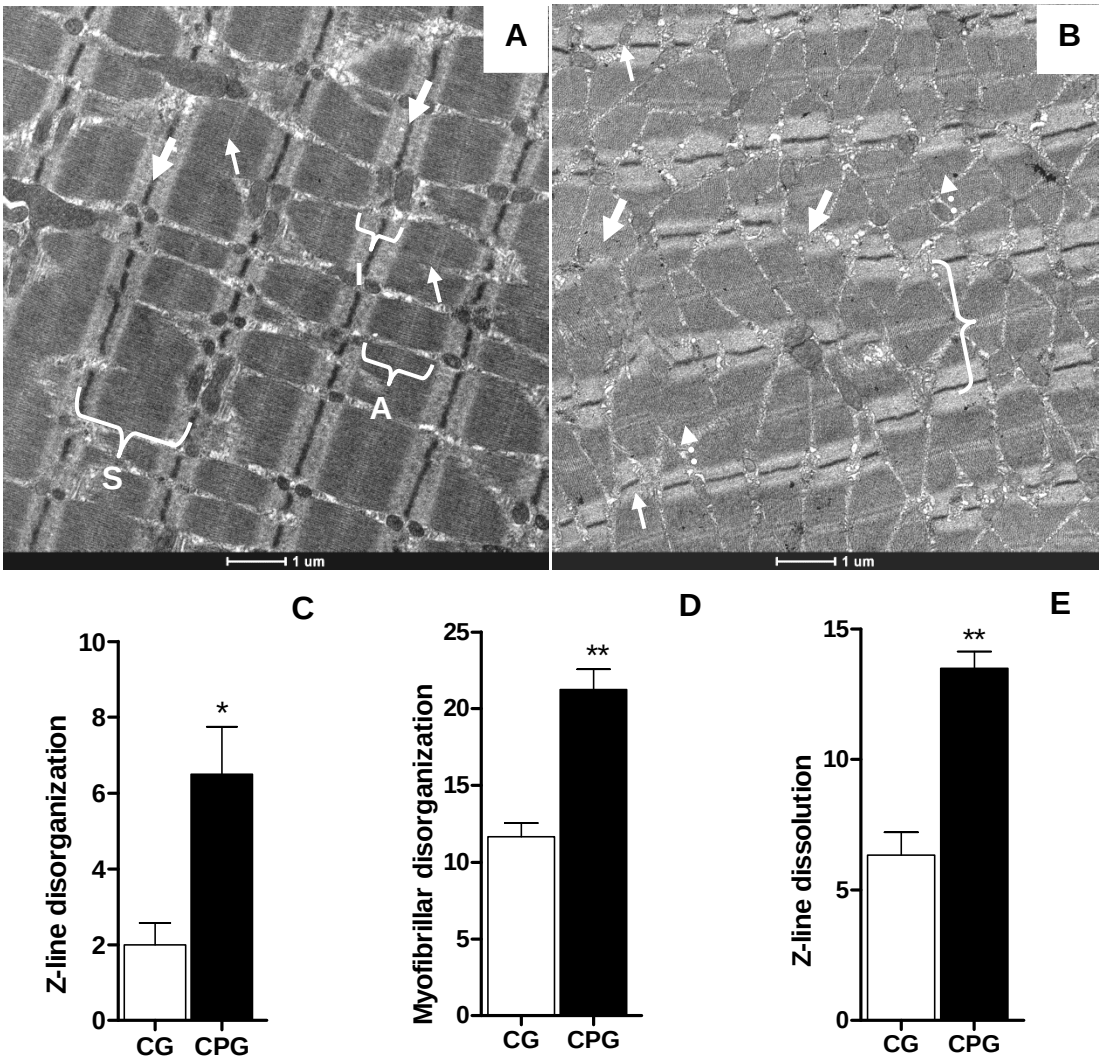

Figure 3. Electromicrographs of longitudinal sections of the plantaris muscle obtained from 48-day-old *Wistar* rats. **A:** Preserved muscle fiber showing the organized sarcomere (S), A-band (A), I-band (I), Z-line (thick arrow), and M-line (thin arrow) in the control group (CG). **B:** Muscle fiber showing a disorganized Z-line (thin arrow), disorganized M-line (dotted arrow), myofibrillar disorganization (brace), and Z-line dissolution (thick arrow) in the cerebral palsy group (CPG). **C, D and E:** Comparison of Z-line disorganization, myofibrillar disorganization and Z-line dissolution between CG and CPG. Values are expressed as the mean ± standard deviation. *$p < 0.05$; **$p < 0.01$ (Student *t*-test).

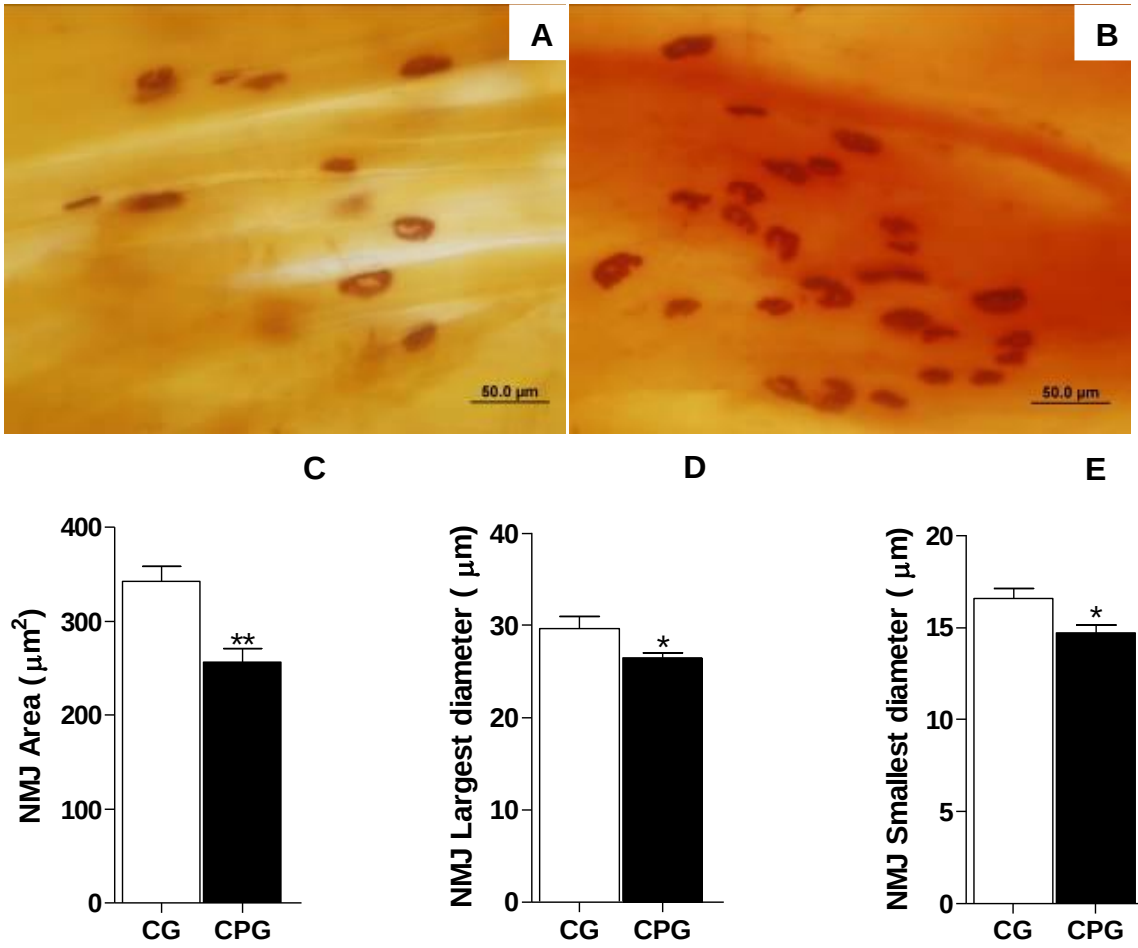

Figure 4. Photomicrographs of longitudinal sections of neuromuscular junctions (NMJs) in the plantaris muscle obtained from 48-day-old *Wistar* rats and submitted to the nonspecific esterase reaction. **A and B:** Observe the morphological characteristics of the NMJs in the control (CG) and cerebral palsy group (CPG), respectively. **C, D and E:** Comparison of area and largest and smallest diameters of NMJs between animals of CG and CPG. Values are expressed as the mean ± standard deviation. *$p < 0.05$; **$p < 0.01$ (Student *t*-test).

ANEXO A:

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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 "Lígia 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
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Espécie/linhagem/raça	Rato/Wistar
Nº de animais	34 Machos e 32 Femêas
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 da revista científica – MICRON: The International Research and Review Journal for Microscopy

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