UNIVERSIDADE FEDERAL DE PERNAMBUCO
CENTRO DE CIÊNCIAS DA SAÚDE
DEPARTAMENTO DE FISIOTERAPIA
PROGRAMA DE PÓS-GRADUAÇÃO EM FISIOTERAPIA

SABRINA DA CONCEIÇÃO PEREIRA

EFEITOS DO TRATAMENTO COM O FATOR DE CRESCIMENTO DE FIBROBLASTOS 19 (FGF-19) SOBRE A ATROFIA MUSCULAR, MORFOMETRIA ÓSSEA E DESEMPENHO DAS FUNÇÕES MOTORAS EM RATOS SUBMETIDOS À PARALISIA CEREBRAL EXPERIMENTAL

Recife
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Dissertação apresentada ao Programa de Pós-Graduação em Fisioterapia da Universidade Federal de Pernambuco, como requisito parcial para a obtenção do título de mestra em Fisioterapia.

Área de concentração: Fisioterapia na atenção à saúde.

Orientadora: Profª. Drª. Ana Elisa Toscano Menezes da Silva Castro
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Recife
2019

Catalogação na fonte:
Bibliotecário: Elaine Freitas, CRB4:1790

P439e Pereira, Sabrina da Conceição.
Efeitos do tratamento com o fator de crescimento de fibroblastos 19 (FGF-19) sobre a atrofia muscular, morfometria óssea e desempenho das funções motoras em ratos submetidos à paralisia cerebral experimental/ Sabrina da Conceição Pereira. - Recife: o autor, 2019.
91 f.; il.

Orientadora: Ana Elisa Toscano Menezes da Silva Castro.
Dissertação (mestrado) – Universidade Federal de Pernambuco. Centro de Ciências da Saúde. Programa de pós-graduação em Fisioterapia.
Inclui referências, apêndices e anexo.

1. Modelos animais. 2. Paralisia cerebral. 3. Músculo esquelético. 4. Habilidades motoras. I. Castro, Ana Elisa Toscano Menezes da Silva (orientadora). II. Título.

615.8 CDD (23.ed.) UFPE (CCS 2019 - 121)

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Aprovada em: 28/02/2019

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Dedico esse trabalho a força que me impulsiona a buscar sempre o melhor, meus pais.

AGRADECIMENTOS

Quero agradecer primeiramente à Deus por todas as oportunidades e desafios vencidos até agora que me fizeram crescer como pessoa e profissionalmente. Pelos anjos que colocou em meu caminho e só me ajudaram nessa trajetória e agora dividem comigo a alegria de dever cumprido.

A minha mãe, Lindinalva Conceição, minha maior inspiração de força, por toda dedicação em me educar da melhor forma possível e por estar comigo em todos os momentos. Ao meu pai, Ediberto Pereira, por todo incentivo e apoio nas minhas escolhas.

A minha orientadora, de hoje e sempre, prof Ana Elisa Toscano, por todo o suporte e esforço, sempre ajudando a tornar realidade esse sonho de ser pesquisadora e agora mestre e me mostrando o melhor caminho a seguir. Ao meu co-orientador, prof Raul Manhães de Castro, pelo aprendizado e conselhos.

Aos colegas de laboratório do LENIB e do CAV, fundamentais em minha jornada, por partilhar o conhecimento e experiências. Em especial ao prof Francisco Amananjás, pelo suporte na condução dos procedimentos experimentais das tíbias e por disponibilizar o laboratório de Biotecnologia e Fármaco do CAV para esta finalidade.

À minha querida estagiária Vanessa Souza, por sua eficiência e responsabilidade em me ajudar nos experimentos. À Debora Vieira e Manuela Amaral que também contribuíram de forma significativa nos experimentos.

À todos os professores do departamento de fisioterapia que contribuíram para a construção da pessoa que sou hoje. E a grande facilitadora, que resolve todos os problemas com o sorriso no rosto Niege.

À todos meu amigos e familiares, que estejam onde estiver, estão torcendo e rezando por mim.

À Propesq/UFPE.

RESUMO

O objetivo do presente estudo foi avaliar os efeitos do tratamento com o fator de crescimento de fibroblastos 19 (FGF-19) sobre a atrofia muscular, morfometria óssea e o desempenho das funções motoras em ratos submetidos à paralisia cerebral experimental. Foram utilizados 46 ratos machos Wistar, distribuídos nos grupos experimentais: Veículo (n=11), receberam solução tampão fosfato-salino com albumina de soro bovino (PBS/BSA a 0,1%), Paralisia Cerebral(PC)+Veículo (n=12), submetidos a PC experimental e solução de PBS/BSA a 0,1%; Veículo+FGF19 (n=10), receberam FGF19 em solução veículo (0,1 mg/kg de FGF-19 em solução de PBS/BSA a 0,1%) e PC+FGF19 (n=13), submetidos PC experimental e tratados com FGF19 (0,1 mg/kg de FGF-19 em solução de PBS/BSA a 0,1%). O modelo de PC consistiu na associação de ánoxia perinatal e restrição sensóriomotora das patas posteriores. A administração farmacológica foi via subcutânea do 22º a 28º dia de vida pós-natal. Foi avaliada a evolução ponderal e o desempenho das funções motoras, sendo a atividade locomotora, coordenação motora e força muscular. Aos 29 dias foram coletados os músculos sóleo e extensor longo dos dedos (EDL) para análise do peso, tipos de fibras, área e perímetro das fibras musculares, e a tíbia para o peso, comprimento e morfometria óssea. Os resultados foram analisados utilizando o *ANOVA Two Way* e o *Two Way Medidas Repetidas* seguido do pós-teste de *Tukey*, considerando $p<0,05$. Os animais submetidos à PC experimental em comparação ao veículo apresentaram menor peso corporal, redução da locomoção e coordenação motora, menor peso muscular relativo e menor área e perímetro das fibras musculares do sóleo e EDL, além de menor espessura cortical, área medular e volume trabecular e aumento da porosidade da tíbia. O tratamento com FGF-19 nos animais PC manteve o peso corporal dos animais, aumentou a atividade locomotora, o peso relativo do EDL e a área e perímetro das fibras musculares do sóleo e EDL, e aumentou a espessura cortical, o volume trabecular e reduziu a porosidade da tíbia comparado ao grupo PCV. Concluímos que o tratamento com FGF-19 na PC experimental melhora a atividade locomotora e reduz os efeitos da PC sobre a atrofia muscular e perda de massa óssea em ratos.

Palavras-chave: Modelos animais. Paralisia cerebral. Músculo esquelético. Habilidades motoras.

ABSTRACT

The aim of the present study was to evaluate the effects of treatment with fibroblast growth factor 19 (FGF-19) on muscle atrophy, bone morphometry and the performance of motor functions in rats submitted to experimental cerebral palsy. A total of 46 male Wistar rats, distributed in the experimental groups: Vehicle (n = 11), received phosphate-saline buffer solution with bovine serum albumin (PBS / 0.1% BSA), Cerebral Palsy (PC) + Vehicle = 12), submitted to experimental PC and 0.1% PBS / BSA solution; Vehicle + FGF19 (n = 10), received FGF19 in vehicle solution (0.1 mg / kg FGF-19 in 0.1% PBS / BSA solution) and PC + FGF19 (n = 13) and treated with FGF19 (0.1 mg / kg FGF-19 in PBS / 0.1% BSA solution). The CP model consisted of the association of perinatal anoxia and sensory-motor restraint of the hind legs. Pharmacological administration was subcutaneous from the 22nd to the 28th day of postnatal life. The weight evolution and the performance of the motor functions were evaluated, being the locomotor activity, motor coordination and muscle strength. At 29 days, the soleus and extensor digitorum longus (SDL) muscles were collected to analyze the weight, fiber types, area and perimeter of muscle fibers, and the tibia for bone weight, length and morphometry. The results were analyzed using ANOVA Two Way and Two Way Repeated Measures followed by Tukey's post-test, considering $p < 0.05$. The animals submitted to the experimental CP in comparison to the vehicle presented lower body weight, reduced locomotion and motor coordination, lower relative muscle weight and smaller area and perimeter of soleus muscle fibers and EDL, as well as lower cortical thickness, medullary area and trabecular volume and increased porosity of the tibia. FGF-19 treatment in PC animals maintained the animals' body weight, increased locomotor activity, relative EDL weight and the area and perimeter of soleus muscle fibers and EDL, and increased cortical thickness, trabecular volume and reduced the porosity of the tibia compared to the PCV group. We conclude that treatment with FGF-19 in the experimental PC improves locomotor activity and reduces the effects of CP on muscle atrophy and loss of bone mass in rats.

Keywords: Models animal. Cerebral palsy. Muscle skeletal. Motor skills.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS E SIGLAS

EDL	Extensor longo dos dedos
EDTA	ácido etilenediaminetetracético
EVA	Etil Vinil Acetato
FGF-19	Fator de crescimento de fibroblastos 19
HE	Hematoxilina-eosina
MyHC	Miosina de cadeia pesada
PBS/BSA	Solução tampão fosfato-salino com albumina de soro bovino
PC	Paralisia Cerebral
Por	Porosidade
SN	Sistema Nervoso
SNC	Sistema Nervoso Central
VTO	Volume trabecular ósseo

SUMÁRIO

1 INTRODUÇÃO

O fenótipo de um indivíduo é resultado da interação do seu genótipo e do ambiente (WEST-EBERHARD, 1986). De acordo com a teoria da plasticidade fenotípica, um genótipo poderia expressar-se de modo diverso a depender da interação com o meio ambiente, revelando a capacidade de um organismo reagir aos desafios impostos pelo ambiente, com modificações de sua forma, estado, movimento ou padrão de atividade (WEST-EBERHARD, 1986). Neste sentido, estudos epidemiológicos e experimentais têm demonstrado que variações ambientais na vida precoce podem levar às mudanças fenotípicas com repercussões permanentes nos sistemas fisiológicos (BARKER, 1995; TOSCANO et al., 2010).

O sistema musculoesquelético exibe um elevado potencial adaptativo (FLUCK; HOPPELER, 2003; PETTE, 2002), em que as fibras musculares e o tecido ósseo têm a habilidade de ajustar suas propriedades metabólicas, moleculares e funcionais a fim de acomodar demandas específicas (AMMANN, 2009; BASSEL-DUBY; OLSON, 2006; HOPPELER; FLÜCK, 2002; PETTE, 2002). Diversos estímulos influenciam o fenótipo musculoesquelético como a atividade contrátil, suprimento de substrato e fatores ambientais, como a hipóxia (FLUCK; HOPPELER, 2003; PETTE, 2002). Sendo que doenças neurológicas afetam esse sistema, assim como ocorre na paralisia cerebral (PC) (NOBLE et al., 2018).

A PC é descrita como um grupo heterogênio de síndromes clínicas que resultam em desordens permanentes no movimento e na postura devido à agressão ocorrida durante o desenvolvimento cerebral (GULATI; SONDHI, 2017; KRIGGER, 2006; STAVSKY et al., 2017). Está entre as maiores categorias de deficiências do desenvolvimento neuromotor (PATEL et al., 2010) que incide de 2-3 por 1000 nascidos vivos no mundo (STAVSKY et al., 2017). As crianças acometidas pela PC são propensas a apresentar fragilidade óssea e sarcopenia prematura (PETERSON; GORDON; HURVITZ, 2013; WARD et al., 2006), e também caracterizam-se por atraso na aquisição de habilidades motoras (MOCKFORD; CAULTON, 2010), alterações de tônus muscular, incapacidade de controlar as funções motoras, além de atrofia e fraqueza muscular (KRIGGER, 2006).

A fim de reproduzir os danos neurofuncionais e esclarecer os mecanismos subjacentes à esta patologia, foram propostos modelos experimentais de PC em animais (LACERDA et al., 2017a). Dentre os modelos destaca-se o que associa

anóxia perinatal e restrição sensóriomotora das patas posteriores de roedores, em virtude de ocasionar alterações duradouras e semelhantes às ocorridas em crianças com PC, tais como aumento do tônus muscular (STRATA et al., 2004), redução da densidade de sarcômeros (STIGGER et al., 2011), desorganização na representação cortical dos membros inferiores no córtex somatossensorial (S1) (COQ et al., 2008), redução na atividade locomotora (SILVA et al., 2016), prejuízos na mastigação (LACERDA et al., 2017b), além de prejuízos no desempenho motor (MARCUZZO et al., 2010). Por esta razão, este modelo torna-se uma ferramenta relevante para elucidação da patogênese da PC e evidencia a importância de estudos que se propõem a agregar novas perspectivas de tratamento para esta patologia.

Apesar das conhecidas repercussões clínicas da PC, atualmente o tratamento desses pacientes é de assistência às sequelas no desenvolvimento motor, sendo incapaz de reverter os danos no sistema musculoesquelético. Recentemente, BENOIT et al. (2017) estudaram um membro da família dos fatores de crescimento de fibroblastos, o FGF-19. Este fator de crescimento promoveu efeito muscular hipertrófico em camundongos submetidos à modelos de atrofia muscular, demonstrando que há presença de receptores de FGF19 e seu co-receptor βKlotho em vários músculos, incluindo sóleo, tibial anterior e gastrocnêmio, bem como em miofibras isoladas e em cultura primária de miotubos humanos (BENOIT et al., 2017). Isto indica que o sistema musculoesquelético é alvo direto para o FGF19 e que esse papel regulador no crescimento musculoesquelético propõe que o FGF19 possui potencial terapêutico para melhorar a estrutura muscular e poderia também ser ampliado para outros modelos experimentais (BENOIT et al., 2017) inclusive modelos de doenças neurológicas que afetam o sistema musculoesquelético.

Assim, diante do exposto, postulamos que o tratamento com FGF19 melhora a atrofia muscular decorrente da PC experimental, com consequências benéficas para a morfometria óssea e o desempenho das funções motoras afetadas pela doença. Portanto, o objetivo desse estudo é avaliar os efeitos do tratamento com FGF19 sobre a atrofia muscular, morfometria óssea e o desempenho das funções motoras em ratos submetidos à paralisia cerebral experimental.

2 REFERENCIAL TEÓRICO

2.1 Paralisia Cerebral

A paralisia cerebral (PC) é descrita como um grupo heterogênio de síndromes clínicas que resultam em desordens permanentes no movimento e na postura (GULATI; SONDHI, 2017; STAVSKY et al., 2017). É uma condição neurológica estática, não progressiva, consequente à agressão ocorrida durante o desenvolvimento cerebral (KRIGGER, 2006). A etiologia da PC é diversificada e multifatorial (GULATI; SONDHI, 2017) e está relacionada às condições maternas antes da concepção, à gravidez, ao trabalho de parto e/ou ao período neonatal (PATEL et al., 2010; STAVSKY et al., 2017).

A prevalência global de PC é estimada em 2 por 1000 nascidos vivos, variando de acordo com a localização geográfica com base no desenvolvimento do cuidado obstétrico e neonatal (GULATI; SONDHI, 2017). Incluem-se como fatores de risco malformações congênitas, restrição do crescimento fetal, gestações múltiplas, infecção durante o período fetal e neonatal, asfixia ao nascimento, extremo baixo peso ao nascer (OSKOUI et al., 2013), hemorragia intracraniana e trauma (KRIGGER, 2006). A prematuridade extrema é o principal fator de risco para o desenvolvimento da PC (JONES et al., 2007; KESAR et al., 2012; PATEL et al., 2010; STAVSKY et al., 2017) devido à maior fragilidade dos vasos sanguíneos cerebrais e propensão à amplas oscilações no fluxo sanguíneo cerebral que podem resultar em hemorragia intraventricular, leucomalácia periventricular ou leucoencefalopatia cerebral (JOHNSTON et al., 2009; JOHNSTON; HOON, 2006). O grau e a área afetada irão refletir diretamente as incapacidades resultantes, sendo algumas áreas cerebrais mais susceptíveis que outras (JONES et al., 2007; KESAR et al., 2012).

Embora seja uma desordem não progressiva, a expressão clínica altera-se ao logo do tempo com a maturação cerebral (GULATI; SONDHI, 2017; STAVSKY et al., 2017). As crianças caracterizam-se por atraso na aquisição de habilidades motoras (MOCKFORD; CAULTON, 2010) e por distúrbios do processamento sensorial (PAVÃO; ROCHA, 2017) e de percepção (QIU; YANG; LI, 2017). Além de que comorbidades podem estar associadas aos prejuízos motores, como desordens

comportamentais, epilepsia, desordens visuais, dificuldades na fala e deglutição, falha no crescimento e desordens ortopédicas (GULATI; SONDHI, 2017).

A deterioração funcional, especialmente da mobilidade, é geralmente atribuída à perda de força, equilíbrio e diminuição da capacidade física (PETERSON; GORDON; HURVITZ, 2013). Tais alterações estão relacionados à sarcopenia prematura que é a perda de massa muscular com o aumento da vulnerabilidade à fraqueza, deficiência, comorbidez e autonomia diminuída (PETERSON; GORDON; HURVITZ, 2013). Além disso, nesses indivíduos há incapacidade de recrutar a musculatura alvo durante a atividade voluntária, ou seja, a inefetividade neural, e um recrutamento e coativação da musculatura antagonista (PETERSON; GORDON; HURVITZ, 2013; TUGUI et al., 2005). Essas deficiências de controle motor reduzem drasticamente a eficiência da marcha e o movimento, e aumentam o gasto de energia e a fadiga durante as atividades (PETERSON; GORDON; HURVITZ, 2013).

Além disso, crianças com PC apresentam baixa densidade mineral óssea (HENDERSON et al., 2002; HOULIHAN, 2014), sendo descrita como “osteopenia fisiológica”. Esta é resultante principalmente da musculatura insuficiente e anormalmente funcional e da imobilidade, ambas as quais reduzem a carga para o esqueleto em desenvolvimento e prejudica o desenvolvimento saudável dos ossos (WARD et al., 2006). Assim, são propensas a fraturas por fragilidade e, consequentemente, a uma qualidade de vida muito reduzida (HOULIHAN; STEVENSON, 2010; WARD et al., 2006).

Vários fatores são preditores para os desfechos das crianças com PC (LIPTAK et al., 2004), inclusive a sobrevivência está relacionada à severidade da incapacidade, com o número de graves deficiências que tenham o maior efeito (GULATI; SONDHI, 2017) e com a deficiência intelectual (LIPTAK et al., 2004). Fatores clínicos como complicações secundárias, fatores familiares como o ambiente social e recursos financeiros, e fatores relacionados ao tratamento também interferem diretamente nas atividades, participação e bem-estar dessas crianças (LIPTAK et al., 2004).

A fim de reproduzir os danos neurofuncionais e esclarecer os mecanismos subjacentes à esta patologia, foram propostos modelos experimentais de PC em animais (LACERDA et al., 2017a), os quais se baseiam no insulto precoce, durante o período crítico de desenvolvimento, como a hipóxico-isquemia pré-natal (TAN et al., 2005), infecção materna (POGGI et al., 2005; ROUSSET et al., 2006; TOSO et al.,

2005) ou acidente vascular encefálico por hipoperfusão cerebral (UEHARA et al., 1999). No entanto, destaca-se o modelo que associa anóxia perinatal e restrição sensóriomotora dos membros posteriores de roedores, em virtude de ocasionar alterações duradouras com características típicas da PC (COQ et al., 2008; MARCUZZO et al., 2010; STRATA et al., 2004).

A asfixia perinatal foi um dos primeiros mecanismos a ser apresentado como modelo de PC em animais (JOHNSTON et al., 2005). É capaz de reproduzir os danos no sistema nervoso central como a lesão de substância branca periventricular e decréscimo da densidade neuronal, resultantes do evento isquêmico no cérebro imaturo e consequente estresse oxidativo (BLOMGREN; HAGBERG, 2006; COQ et al., 2016). Mas este modelo, por si só, apresenta uma limitação considerável, por não ocasionar incapacidade crônica característica da PC (JOHNSTON et al., 2005), sendo observadas apenas alterações discretas no tônus muscular e nas habilidades motoras em ratos submetidos apenas à anóxia perinatal (STRATA et al., 2004). Dessa forma, a associação da anóxia com a restrição sensóriomotora dos membros posteriores no modelo proposto por COQ et al., 2008 e STRATA et al., 2004 reforça a degradação na performance motora e altos níveis de distorção na representação cortical, com evidente aumento no tônus muscular, rigidez articular e limitação de movimento interferindo no padrão de marcha e nas habilidades motoras que não são vistos somente com a indução da anóxia.

Estudos utilizando este modelo de PC que associa anóxia e restrição sensóriomotora dos membros posteriores mostram que os animais apresentam alterações no tônus muscular (STRATA et al., 2004), desorganização na representação cortical dos membros inferiores no córtex somatossensorial (S1) (COQ et al., 2008), redução na atividade locomotora (SILVA et al., 2016), prejuízos na mastigação (LACERDA et al., 2017b), além de habilidades motoras prejudicadas semelhantes às ocorridas em crianças com PC (MARCUZZO et al., 2010). Por esta razão, este modelo torna-se uma ferramenta relevante para elucidação da patogênese da PC, incluindo como esta afeta o desenvolvimento das crianças principalmente no sistema músculoesquelético, e evidencia a importância de estudos que se propõem a agregar novas perspectivas de tratamento para esta patologia.

2.2 Desenvolvimento do sistema musculoesquelético

O sistema musculoesquelético constitui aproximadamente 40% ou mais do peso corporal humano adulto e compreende ossos, músculos esqueléticos, tendões e ligamentos (BASKIN et al., 2016; ENDO, 2015). Origina-se durante a gastrulação no período embrionário, a partir de uma das camadas germinativas, o mesoderma (MOORE; PEARSADE; TORCHIA, 2016).

No período embrionário, o mesoderma paraxial segmenta-se originando os somitos, em ambos os lados do tubo neural (MOORE; PEARSADE; TORCHIA, 2016; WIGMORE; EVANST, 2002). Cada somito se diferencia em duas porções: a porção ventral é o esclerótomo, suas células formam as vértebras e costelas; e a porção dorsal é o dermomiótomo, em que as células provenientes do miótomo formam mioblastos (células musculares primordiais) e as provenientes do dermátomo formam a derme (ENDO, 2015; MOORE; PEARSADE; TORCHIA, 2016; WIGMORE; EVANST, 2002). Assim, desde a fase embrionária o desenvolvimento e a funcionalidade dos ossos, cartilagem e articulações requerem a integração precisa com outros componentes do sistema, e está relacionado com o desenvolvimento concomitante dos músculos esqueléticos (BERENDSEN; OLSEN, 2015).

As células que formarão as fibras musculares dos membros são derivadas de somitos adjacentes ao broto do membro em desenvolvimento que se desprendem da borda lateral do dermomiótomo e migram para a localização da formação muscular (GRETA; WIGMORE, 1998; WIGMORE; EVANST, 2002). Inicialmente, são originadas as fibras da musculatura axial e, posteriormente, a musculatura apendicular nos vertebrados superiores (STOCKDALE, 1992). De forma semelhante, a esqueletogênese inicia com migração das células mesenquimais para os locais dos futuros ossos, em que os somitos são a fonte celular do esqueleto axial, enquanto as células mesodérmicas da placa lateral formam o esqueleto apendicular (BERENDSEN; OLSEN, 2015).

Na formação óssea, há a estruturação de condensações de alta densidade celular que delineia a forma e o tamanho dos futuros ossos, ou seja, a identidade anatômica do elemento esquelético é pré-determinado (BERENDSEN; OLSEN, 2015). Dentro das condensações, até final do período embrionário de mamíferos, as células mesenquimais se diferenciam em condrócitos e formam os moldes de

cartilagem dos futuros ossos (formação óssea endocondral) ou diferenciam-se em osteoblastos para formar diretamente osso (formação óssea intramembranosa) (BERENDSEN; OLSEN, 2015; MOORE; PEARSADE; TORCHIA, 2016).

Formação óssea endocondral ocorre na base do crânio e na parte posterior do crânio, no esqueleto axial e no esqueleto apendicular. Formação óssea intramembranosa ocorre no neuro e viscerocrânio membranoso e em parte da clavícula (BERENDSEN; OLSEN, 2015). Enquanto o feto cresce, por volta da 12ª semana de gestação, aparecem os centros de ossificação primária que posteriormente se expandem em centros de ossificação secundária, a partir da 32ª semana de gestação (BERENDSEN; OLSEN, 2015; MOORE; PEARSADE; TORCHIA, 2016). Isso resulta no desenvolvimento da placa de crescimento epifisária, responsável pelo crescimento longitudinal dos ossos no período pós-natal (BERENDSEN; OLSEN, 2015).

Dentro das placas de crescimento os condrócitos são organizados em zonas estruturais e funcionais, cada uma com padrões distintos de expressão gênica, em que fatores locais e sistêmicos, como os fatores de crescimento, regulam a proliferação, diferenciação e apoptose de condrócitos desta região (BERENDSEN; OLSEN, 2015). Com a idade, as placas de crescimento tornam-se delgadas e são eventualmente substituídas por osso em vários momentos após a puberdade até a idade adulta em humanos (BERENDSEN; OLSEN, 2015).

Quanto à formação das fibras musculares, todas as fibras são produzidas pela fusão de células miogênicas, os mioblastos, formando os miotubos, estimulados por fatores reguladores miogênicos (MOORE; PEARSADE; TORCHIA, 2016; WIGMORE; EVANST, 2002). Sendo a formação de fibras musculares em mamíferos descrita como bifásica e dividida em uma onda inicial e tardia de produção, formando as fibras primárias e secundárias (WIGMORE; EVANST, 2002).

As fibras primárias se formam durante os estágios iniciais da miogênese aumentando rapidamente em diâmetro e desempenham um papel significativo na geração de fibras (GRETA; WIGMORE, 1998). As fibras secundárias são caracterizadas por serem menores e se formarem posteriormente, usando a superfície das fibras primárias como ponto para a fixação e fusão de células miogênicas (GRETA; WIGMORE, 1998). No rato, as fibras primárias se formam

entre o 14º e 16º dia embrionário, enquanto as fibras secundárias se formam entre o 17º dia gestacional e o período neonatal, sendo que ambos os tipos continuam a crescer e aumentar por fusão celular no período pós-natal (GRETA; WIGMORE, 1998; WIRSEN; LARSSON, 1964).

Na fase final da gestação até aproximadamente o 1º dia pós-natal no rato, ocorre o fim da miogênese sendo este marcado pela cessação da formação da fibra e diminuição do diâmetro das fibras primárias (GRETA; WIGMORE, 1998; WIRSEN; LARSSON, 1964). Como resultado, a diferença de tamanho entre as duas gerações de fibras desaparecem e elas não são mais distinguíveis pela morfologia (GRETA; WIGMORE, 1998). No entanto, essas fibras podem ser ainda classificadas em diferentes tipos, caracterizadas por especificidades no metabolismo e velocidade de contração (BASKIN et al., 2016; WIGMORE; EVANST, 2002; WIRSEN; LARSSON, 1964).

Durante o desenvolvimento fetal, as fibras são normalmente classificadas apenas como rápida ou lenta reconhecidas desde a sua formação pela expressão de isoformas de miosina de cadeia pesada (MyHC) que predizem seu tipo final de fibra (GRETA; WIGMORE, 1998). Todas as fibras primárias são inicialmente lentas, mas uma pequena proporção posteriormente é convertida em rápida. Em contraste, as fibras secundárias são todas inicialmente rápidas, mas uma proporção se converte em lenta, sendo que as isoformas de MyHC apresentam expressão sequencial nesse período estabelecendo o padrão de tipos de fibras rápidas e lentas dentro dos músculos individuais (GRETA; WIGMORE, 1998).

Todas as fibras primárias expressam MyHC-Embrionário (MyHC-Emb) ao longo da miogênese, e em seguida começam a expressar MyHC-β que é predominante nas fibras lentas adultas e também é encontrado no músculo cardíaco. Pouco antes do final da formação das fibras primárias, algumas começam a expressar MyHC-Perinatal (MyHC-Peri) nas regiões superficiais de músculos como tibial anterior e gastrocnemio, assim como as fibras secundárias. Durante o período pós-natal, essas regiões superficiais das fibras primárias expressarão a isoforma MyHC-IIb de fibras rápidas, enquanto aquelas em regiões mais profundas manterão a expressão do MyHC-β lenta. Assim, a distribuição de MyHC-β e MyHC-Peri prefigura a distribuição de fibras rápidas e lentas no músculo adulto (WIGMORE; EVANST, 2002).

Dessa forma, diferentes subtipos de miofibras são detectados durante a vida embrionária, mas a padronização dos tipos de fibras dentro dos principais grupos musculares é estabelecida no período pós-natal (BASSEL-DUBY; OLSON, 2006). Ocorre maior refinamento do padrão de fibras rápidas e lentas, não intrínseco ao músculo, mas dependente de sinais neurais ou de fatores de crescimento (GRETA; WIGMORE, 1998), em que no rato somente por volta do 7º dia pós-natal a musculatura alcança a aparência adulta (WIRSEN; LARSSON, 1964).

No músculo adulto, quatro tipos de fibras distinguem-se normalmente pelo seu metabolismo e isoformas de MyHC, que são uma única fibra lenta (tipo I) e 3 tipos de fibras rápidas (tipo IIA, IIB e IIX) (GRETA; WIGMORE, 1998). E continuam a mudar em resposta às exigências impostas a eles, convertendo-se para a próxima forma mais rápida ou mais lenta ao longo da sequência I <-> IIA <-> IIX <-> IIB (WIGMORE; EVANST, 2002).

Fibras de contração lenta apresentam baixa fatigabilidade, alta capacidade oxidativa e preferência por ácidos graxos como substrato para a produção de ATP (BASKIN et al., 2016), sendo identificados como músculos vermelhos (SCHIAFFINO; REGGIANI, 2011). As fibras de contração rápida possuem maior força de contração, menor capacidade oxidativa e preferência pela glicose como substrato para a produção de ATP através da glicólise anaeróbica, apresentando maior fatigabilidade (BASKIN et al., 2016) e identificados como músculos brancos (SCHIAFFINO; REGGIANI, 2011). Assim, os tipos de fibras musculares irão determinar a função muscular como a estabilização e manutenção da postura pelas fibras lentas, ou produção de movimento pelas fibras rápidas (GRETA; WIGMORE, 1998).

Embora eventos ambientais sejam determinantes no processo de desenvolvimento das fibras musculares (FLUCK; HOPPELER, 2003; PETTE, 2002), a proporção relativa de qualquer tipo de fibra pode variar de acordo com a espécie e sítio anatômico (SCHIAFFINO; REGGIANI, 2011). Além disso, a heterogeneidade das fibras musculares está relacionada com neurônios motores com padrão de descarga específico organizado em unidades motoras (SCHIAFFINO; REGGIANI, 2011). O que demostra que a capacidade dos músculos esqueléticos de gerar força e movimento é explorada pelo sistema nervoso para alcançar uma variedade de tarefas motoras (SCHIAFFINO; REGGIANI, 2011).

Por exemplo, nos músculos das pernas, os músculos mais estudados do corpo, as fibras lentas tipo I são mais abundantes no compartimento posterior, onde se localiza o músculo sóleo que é lento típico, devido ao maior papel deste músculo na manutenção postural (SCHIAFFINO; REGGIANI, 2011). Sabe-se que em ratos na idade adulta o sóleo é composto por aproximadamente 90% de fibras tipo I, sendo complementado por uma pequena proporção de fibras tipo IIa (SOUKUP; ZACHAŘOVÁ; SMERDU, 2002). Enquanto no extensor longo dos dedos (EDL), músculo do compartimento anterior da perna, ocorre o inverso, este é composto por maior proporção de fibras do tipo IIb, seguida de IIa e tipo I (SOUKUP; ZACHAŘOVÁ; SMERDU, 2002), sendo assim um músculo de contração rápida. Neste sentido, a diversidade dos tipos de fibras musculares é fundamental para o desempenho das atividades funcionais e conhecer esse sistema e suas possíveis alterações na presença de patologias é importante para estudos que buscam estratégias de intervenção no sistema musculoesquelético.

2.3 Fator de Crescimento de Fibroblastos

A família de fatores de crescimento de fibroblastos (FGFs) é um grupo composto de 22 proteínas que estão envolvidas em diversas funções biológicas como no desenvolvimento participando da organogênese e metabolismo (BENOIT et al., 2017; FERNANDES-FREITAS; OWEN, 2015). São nomeados sequencialmente (FGF 1 – 23), sendo que o FGF15 e 19 são proteínas ortólogas, ou seja, proteínas com sequências homólogas e função similar, sendo o FGF15 encontrado em ratos e o FGF19 em humanos, assim sendo referidos como FGF15/19 (FERNANDES-FREITAS; OWEN, 2015; MARKAN; POTTHOFF, 2016).

A maioria dos membros da família FGF age de forma autócrina ou parácrina e medeia seus efeitos através da ligação a receptores na superfície celular, que são receptores tirosina quinases codificadas por quatro genes (FGFR1–4) (POTTHOFF; KLIEWER; MANGELSDORF, 2012). Geralmente, esses FGFs requerem uma interação com glicosaminoglicanos heparan sulfato na matriz extracelular para ativar seus receptores (POTTHOFF; KLIEWER; MANGELSDORF, 2012). Contudo, três FGFs endócrinos (FGF15/19, 21 e 23) podem ser liberados na corrente sanguínea para agir em todo o corpo (FERNANDES-FREITAS; OWEN, 2015; POTTHOFF;

KLIEWER; MANGELSDORF, 2012). Para compensar sua incapacidade de interagir com heparan sulfato, os FGFs endócrinos requerem membros da família de proteínas Klotho para ligação ao receptor de alta afinidade (POTTHOFF; KLIEWER; MANGELSDORF, 2012).

O grupo de proteínas transmembrana Klotho é formada pelo αKlotho, βKlotho, e lactase like (ZHANG et al., 2015). Estas interagem com os receptores de FGF para permitir a ligação seletiva dos três FGF endócrinos, sendo que αKlotho serve como o co-receptor para FGF23, e βKlotho serve como co-receptor para FGF15/19 e FGF21, e o FGF15/19 também pode sinalizar através de complexos lactase like (POTTHOFF; KLIEWER; MANGELSDORF, 2012). Embora os FGFRs tenham distribuições de tecidos muito amplas, a expressão das proteínas Klotho é mais restrita. Assim, os locais de ação para o FGFs endócrinos são largamente ditados pela presença ou ausência das proteínas Klotho (POTTHOFF; KLIEWER; MANGELSDORF, 2012).

Durante o desenvolvimento embrionário e fetal, o FGF15/19 é expresso no sistema nervoso central (SNC) onde desempenha um papel importante suprimindo a proliferação e promovendo a diferenciação de precursores neurais (POTTHOFF; KLIEWER; MANGELSDORF, 2012; ZHANG et al., 2015). Nos adultos, entretanto, o FGF15/19 não é detectado no SNC, sendo expresso no intestino delgado e cólon, e seu papel é evidenciado na regulação da homeostase metabólica (FERNANDES-FREITAS; OWEN, 2015; POTTHOFF; KLIEWER; MANGELSDORF, 2012; ZHANG et al., 2015). Dessa forma, o FGF15/19 não atravessa a barreira hematoencefálica o que restringe sua ação nos órgãos periféricos (FERNANDES-FREITAS; OWEN, 2015; HSUCHOU; PAN; KASTIN, 2013).

Uma função primária do FGF15/19 no adulto é regular a homeostase dos ácidos biliares, sendo um regulador negativo de síntese e transporte do ácido biliar (POTTHOFF; KLIEWER; MANGELSDORF, 2012; ZHANG et al., 2015). O FGF15/19 entra na circulação porta e atua no fígado através do receptor FGF-4 (FGFR4) em complexo com a proteína transmembrana essencial βKlotho (FERNANDES-FREITAS; OWEN, 2015). Além dos efeitos sobre a homeostase dos ácidos biliares, há efeitos metabólicos pós-prandiais mais amplos no fígado (FERNANDES-FREITAS; OWEN, 2015). Semelhante à insulina, estimula a síntese de proteína e de glicogênio e inibe a gliconeogênese (FERNANDES-FREITAS; OWEN, 2015). No entanto, existem diferenças importantes. Os níveis de pico pós-prandiais de

FGF15/19 ocorrem no sangue substancialmente mais tarde, e duram mais tempo, que os da insulina (FERNANDES-FREITAS; OWEN, 2015) e não estimula a lipogênese (POTTHOFF; KLIEWER; MANGELSDORF, 2012).

Recentemente, um estudo indicou que o sistema musculoesquelético é um alvo direto para o FGF19 (BENOIT et al., 2017). Onde foi observado efeito muscular hipertrófico do tratamento com FGF19 humano recombinante em camundongos adultos e jovens submetidos à modelos de atrofia muscular induzida por dexametasona, atrofia relacionada ao envelhecimento e modelo de obesidade (BENOIT et al., 2017). Foram encontrados níveis detectáveis de receptores de FGF e proteína βKlotho em vários músculos de camundongo, incluindo sóleo, tibial anterior e gastrocnêmio, em miofibras isoladas e em cultura primária de miotubos humanos (BENOIT et al., 2017). Ademais, utilizando células musculares humanas primárias, o FGF19 não afetou a proliferação celular ou a expressão de fatores miogênicos, mas aumentou substancialmente a área dos miotubos durante o processo de diferenciação dos mioblastos aos miotubos, representando assim, os mecanismos de ação por trás dessa hipertrofia muscular resultante do tratamento com FGF19 (BENOIT et al., 2017).

Esse papel regulador no crescimento musculoesquelético propõe que o FGF19 possui potencial terapêutico para melhorar a estrutura muscular e poderia também ser ampliado para outros modelos experimentais. Outras condições patológicas inclusive modelos de doenças neurológicas poderiam ser investigadas já que o mecanismo de lesão central difere do periférico estudado anteriormente, sendo que o tratamento farmacológico com o FGF19 poderia atuar através da plasticidade do sistema musculoesquelético.

2.4 Plasticidade fenotípica e sistema musculoesquelético

Os organismos vivos possuem uma notável capacidade de responder a insultos ambientais com mudanças na forma e função. Ajustes fenotípicos podem ocorrer em diferentes períodos, duráveis e às vezes mudanças irreversíveis (GIUDICE, 2015). Dependendo de como a palavra “fenótipo” é definida (por exemplo, evento desenvolvimental, ajuste fisiológico, mudança de comportamento, expressão gênica dependente do ambiente, etc.) todos processos biológicos são, de alguma forma, influenciados pelo ambiente e, consequentemente, qualquer

modificação resultante pode ser categorizada como plasticidade fenotípica (KELLY; PANHUIS; STOEHR, 2012).

Dessa forma, a plasticidade fenotípica pode ser amplamente definida como a capacidade de um genótipo de produzir diferentes fenótipos em resposta as condições ambientais levando à alterações na forma, estado, movimento ou taxa de atividade (KELLY; PANHUIS; STOEHR, 2012; TURCOTTE; LEVINE, 2016; WEST-EBERHARD, 1986). Pode ser reversível, chamada de flexibilidade fenotípica, ou irreversível, como a plasticidade desenvolvimental (TURCOTTE; LEVINE, 2016). Dependendo dos fatores que induzem a plasticidade fenotípica e do tempo de indução, pode afetar múltiplas gerações através de efeitos maternos ou de fatores epigenéticos (TURCOTTE; LEVINE, 2016).

Vários fatores bióticos e abióticos podem induzir a plasticidade fenotípica como fatores ambientais locais, alterações químicas, sociais e hormonais (TURCOTTE; LEVINE, 2016). Mas a plasticidade é, na verdade, uma variação intraespecífica (WEST-EBERHARD, 1998), em que alguns indivíduos são mais suscetíveis a esses fatores. Isso pode ser atribuído às diferenças nos mecanismos precoces envolvidos na coleta de informações do meio ambiente e traduzindo-o em efeitos fenotípicos, que são mediadores da plasticidade no nível precoce (GIUDICE, 2015). Estudos mostram que o período perinatal corresponde ao período de maior plasticidade e representa a janela crítica de desenvolvimento (HÜBENER; BONHOEFFER, 2014; JENSEN, 2002) em que insultos precoces podem levar à repercussões permanentes nos sistemas fisiológicos (BARKER, 1995; TOSCANO et al., 2010).

O sistema musculoesquelético exibe um elevado potencial adaptativo (FLUCK; HOPPELER, 2003; PETTE, 2002). Esse potencial é resultante da habilidade das fibras musculares em ajustar suas propriedades metabólicas, moleculares e funcionais a fim de acomodar demandas específicas (BASSEL-DUBY; OLSON, 2006; HOPPELER; FLÜCK, 2002; PETTE, 2002). Assim, o fenótipo definitivo de uma fibra muscular esquelética adulta é o resultado de eventos que começam no embrião e são continuamente modulados e refinados ao longo da vida do organismo (STOCKDALE, 1992).

Diversos estímulos influenciam o fenótipo musculoesquelético como a atividade contrátil (exercícios de endurance, eletroestimulação, denervação), condições de carga (treinamento de resistência, microgravidade), suprimento de substrato

(intervenções nutricionais) ou fatores ambientais (hipóxia) (FLUCK; HOPPELER, 2003; PETTE, 2002). Ademais, estudos experimentais são capazes de demonstrar em diferentes modelos animais, as alterações resultantes no fenótipo muscular, incluindo alterações nas propriedades biomecânicas do músculo (TOSCANO et al., 2010) e na proporção dos tipos de fibras (LACERDA; MORAIS, 2017).

Os quatro tipos principais de fibras musculares (uma lenta e três tipos de fibras rápidas) tem sido até agora a principal referência para estudar a heterogeneidade e a plasticidade muscular de mamíferos que é representativo dos músculos dos membros, que geralmente são considerados o paradigma para estudos de diversidade muscular (SCHIAFFINO; REGGIANI, 2011). A transição dos tipos de fibras exibem características da relação dose-resposta e ocorrem em uma ordem sequencial dentro do espectro das fibras que se estende desde o fenótipo das rápidas (Tipo IIB) para as lentas (Tipo I) que depende da qualidade, intensidade e duração do estímulo (PETTE, 2002). Isso também ocorre a nível de propriedades metabólicas, em que um aumento na atividade neuromuscular implica em aumento no conteúdo mitocondrial e potencial aeróbico oxidativo, ou seja, transição de uma fibra rápida para lenta, enquanto alterações na direção oposta ocorrem quando a atividade neuromuscular é reduzida (PETTE, 2002).

Além disso, o músculo esquelético exibe notável plasticidade em suas respostas metabólicas à disponibilidade calórica e à atividade física (BASKIN et al., 2016). Sendo que a composição dos tipos de fibras do músculo esquelético afeta profundamente o consumo de energia sistêmica quanto à homeostase da glicose e dos lipídios (BASKIN et al., 2016; STOCKDALE, 1992). Por exemplo, o exercício aumenta o número de fibras de contração lenta, aumentando assim a utilização de ácidos graxos, enquanto a obesidade aumenta as fibras de contração rápida e faz com que as fibras de contração lenta se tornem resistentes à insulina (BASKIN et al., 2016).

Outro componente importante do sistema musculoesquelético que está relacionado a adaptações fenotípicas é o tecido ósseo. Este pode suportar traumas de baixa energia e estímulos repetidos, como caminhar e correr, até certo ponto (AMMANN, 2009). A capacidade do osso de suportar o estresse fisiológico é governada tanto por seus determinantes, incluindo massa, geometria e microarquitetura, mas também pela qualidade do tecido ósseo intrínseco (AMMANN,

2009). Esta qualidade intrínseca refere-se ao grau de mineralização e características da matriz, como a orientação das fibras de colágeno e estrutura química, ambas ajudando a caracterizar a ultraestrutura óssea (AMMANN, 2009).

Esses determinantes relacionados a ultraestrutura do osso são afetados pela remodelação óssea e, portanto, são considerados como alvos de estudos de intervenção e atividade nutricional, hormonal e terapêutica (AMMANN, 2009). Assim, modelos animais são de grande importância na compreensão dos mecanismos fisiopatológicos na alteração da força óssea e como este se adapta a uma perda de massa óssea (AMMANN, 2009). Diversas patologias podem provocar alterações adaptativas no osso levando à fragilidade, inclusive doenças neurológicas principalmente pela falta de carga normal da musculatura, além da imobilização prolongada que aumenta a reabsorção óssea (COHEN et al., 2009; WARD et al., 2006).

Dessa forma, a plasticidade fenotípica é um fenômeno comum (TURCOTTE; LEVINE, 2016) em que no sistema musculoesquelético diversos fatores podem favorecer, desde insultos ambientais ocorridos precocemente durante o desenvolvimento (LACERDA et al., 2017b; SILVA et al., 2016b) até eventos tardios, como o exercício, fatores endócrinos e patologias (BASKIN et al., 2016; WARD et al., 2006). E estudos de experimentais são fundamentais para a compreensão dos mecanismos associados a plasticidade do sistema musculoesquelético, principalmente com intervenções nas patologias que acometem esse sistema.

3 JUSTIFICATIVA

A paralisia cerebral (PC) é descrita como um grupo heterogênio de síndromes clínicas que resultam em desordens permanentes no movimento e na postura devido à agressão ocorrida durante o desenvolvimento cerebral (GULATI; SONDHI, 2017; KRIGGER, 2006; STAVSKY et al., 2017). Está entre as maiores categorias de deficiências do desenvolvimento neuromotor. As crianças acometidas apresentam diversas alterações do sistema musculoesquelético que resultam na deterioração funcional, em que a sobrevivência está relacionada à severidade da incapacidade. Os tratamentos atuais visam minimizar a limitação da funcionalidade da criança e melhorar as comorbidades associadas à PC. Estudos que investiguem tratamentos para os distúrbios primários, como a atrofia muscular, são escassos.

Recentemente, um estudo demonstrou que o musculoesquelético é um alvo direto para o fator de crescimento de fibroblastos 19 (FGF19), em que foi observado efeito muscular hipertrófico do tratamento com FGF19 em camundongos submetidos à modelos de atrofia muscular induzida, sendo detectada a presença de receptores de FGF e proteína co-receptora βKlotho em vários músculos. Este achado promissor encoraja estudos na busca de incorporar uma alternativa de tratamento com enfoque sobre os danos no sistema musculoesquelético resultantes da PC. Neste contexto, estudos experimentais de PC são ferramentas para elucidar o mecanismo subjacente a esta patologia e investigar potenciais terapias, como a manipulação farmacológica com FGF19.

Os dados provenientes desse estudo irão contribuir para o esclarecimento do potencial papel do FGF19 sobre os prejuízos no sistema músculoesquelético resultantes de doença neurológica, a PC experimental, como a atrofia muscular, e consequentemente déficit nas funções motoras e perda de massa óssea. Além disso, irá auxiliar na compreensão do impacto da PC experimental sobre o sistema musculoesquelético e consequentemente no desenvolvimento motor. Os conhecimentos que foram agregados, quanto à esses efeitos, possibilitará também novas janelas de atuação terapêutica, visto que a utilização de modelo experimental de PC, obedecendo às normas éticas, permite extrapolar informações relevantes com os devidos cuidados para a criança acometida por essa doença, que afeta milhares no mundo e que é tão comum no dia-a-dia do fisioterapeuta e dos outros profissionais da área da saúde.

4 HIPÓTESE

O tratamento com FGF19 melhora a atrofia muscular decorrente da PC experimental, com consequências benéficas para a morfometria óssea e o desempenho das funções motoras afetadas pela condição neurológica.

5 OBJETIVOS

5.1. Objetivo Geral

Avaliar os efeitos do tratamento com FGF19 sobre a atrofia muscular, morfometria óssea e desempenho das funções motoras relacionadas a locomoção na paralisia cerebral experimental.

5.2. Objetivos Específicos

Avaliar em ratos submetidos à paralisia cerebral expostos ou não ao tratamento com FGF19:

a) a evolução ponderal;
b) a atividade locomotora;
c) a coordenação motora;
d) a força muscular;
e) o peso dos músculos sóleo e extensor longo dos dedos (EDL);
f) a proporção dos tipos de fibras, a área e perímetro dos músculos sóleo e EDL;
g) a antropometria óssea da tíbia, quanto ao peso e comprimento;
h) a morfometria da tíbia.

6 MATERIAIS E MÉTODOS

Trata-se de um estudo experimental com animais que foi realizado nas dependências do Laboratório de Estudos em Nutrição e Instrumentação Biomédica (LENIB) do Centro de Ciências da Saúde UFPE – Recife e nos laboratórios do CAV (Centro Acadêmico de Vitória) UFPE – Vitória de Santo Antão.

6.1 Animais e condições de biotério

Foram utilizados 46 ratos machos *Rattus Norvegicus Albinus* da linhagem *Wistar* provenientes do biotério de criação do Departamento de Nutrição da UFPE, mantidos no biotério de manutenção do Departamento de Nutrição da UFPE com temperatura de 22 ± 2 ° C, ciclo claro-escuro invertido de 12/12 horas, alojados em gaiolas de polipropileno com acesso livre a água e dieta. O projeto foi aprovado pelo comitê de ética em experimentação animal do Centro de Ciências Biológicas da Universidade Federal de Pernambuco (número do protocolo CEUA: 0011/2017) (Anexo 1). Todos os procedimentos foram realizados em conformidade com as diretrizes do Conselho Nacional de Controle de Experimentação Animal (CONCEA) e com as normas internacionais do *National Institute of Heath Guide for Care and Use of Laboratory Animals* (8ª ed).

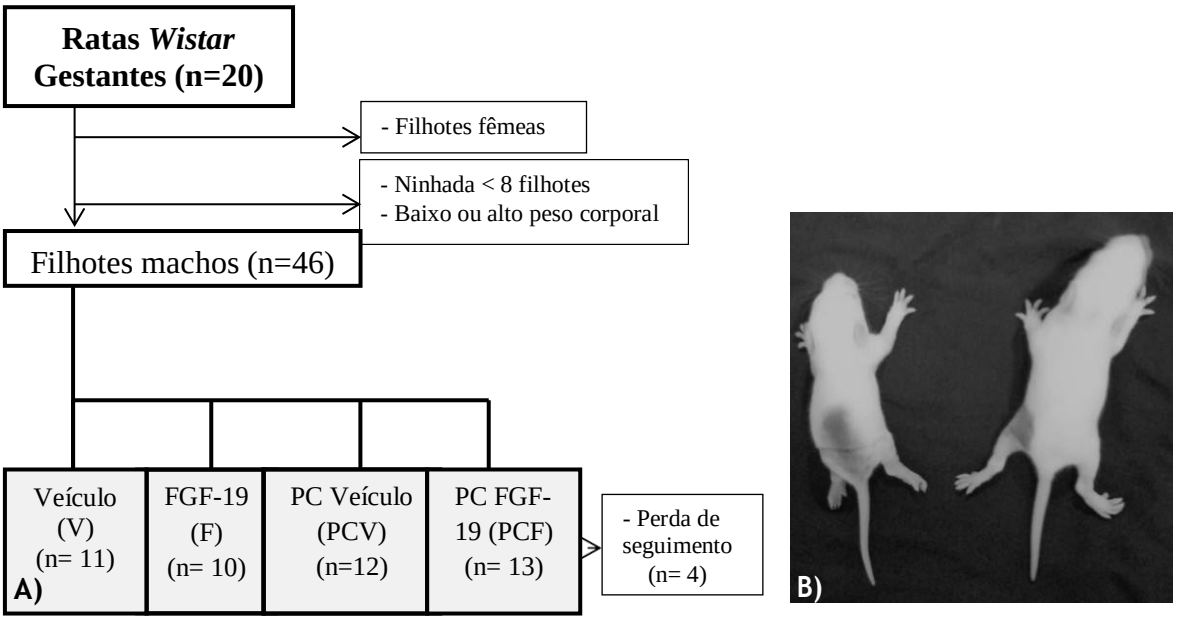
6.2 Grupos experimentais

20 ratas da espécie *Rattus Norvegicus Albinus* da linhagem *Wistar* provenientes do biotério de criação do Departamento de Nutrição da UFPE foram utilizadas neste estudo para obtenção dos filhotes. Foram colocadas para acasalar com machos reprodutores na proporção de duas fêmeas para um macho, sendo utilizadas fêmeas primíparas, com idade de 90 a 120 dias, pesando entre 220 e 250g. Após a confirmação da prenhez através de esfregaço vaginal, as gestantes foram distribuídas em gaiolas individuais e acompanhadas neste período. No dia do nascimento foi realizada a distrubuição dos filhotes machos nos grupos experimentais de forma aleatória, sendo incluidos filhotes machos saudáveis com um peso corporal ideal (6 a 8 gramas), em que cada ninhada fosse composta dos 4 grupos experimentais. As filhotes fêmeas foram utilizadas apenas para completar a ninhada de 8 filhotes até o dia do desmame, além de que foram excluídos os filhotes

machos que não finalizaram a pesquisa devido ao protocolo de indução da PC que levou a perda de alguns animais (n=4).

Os quatro grupos experimentais, foram formados com base na indução da paralisia cerebral e na manipulação farmacológica administrada aos animais: 1-Veículo (V, n=11); 2-FGF19 (F, n=10); 3- Paralisia Cerebral+Veículo (PCV, n=12); 4-Paralisia Cerebral+FGF19 (PCF, n=13). O desmame ocorreu no 25º dia de vida pós-natal em que os filhotes machos foram colocados em gaiolas individuais até a eutanasia por decaptação no 29º dia pós-natal (Figura 1).

Figura 1 **-** Formação dos grupos experimentais. A) Fluxograma de captação da amostra. B) Ratos aos 8 dias de vida, o animal marcado no dorso representa os grupos submetidos à PC (PCV e PCF) e o animal marcado no membro posterior esquerdo representa os grupos controles (V e F).

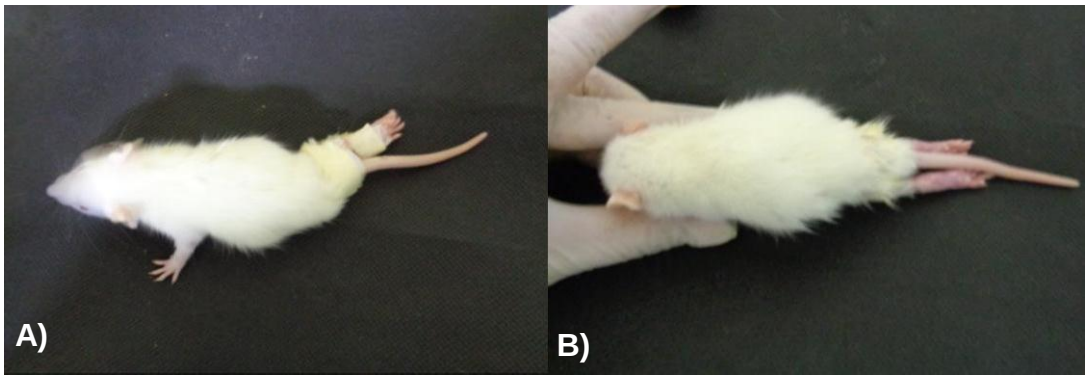


6.3 Modelo de paralisia cerebral experimental

O modelo experimental de PC foi baseado nos experimentos que associam a anóxia perinatal e restrição sensório-motora das patas posteriores (COQ et al., 2008; LACERDA et al., 2017b; SILVA et al., 2016b; STRATA et al., 2004). Os filhotes dos grupos PC foram submetidos a dois episódios de anóxia pós-natal, no dia do nascimento e no primeiro dia de vida (P0 e P1). Os filhotes foram colocados dentro de uma câmara de acrílico parcialmente imersa em água a 37 graus e expostos a

nitrogênio (100%) a 9L/min durante 12 minutos, e em seguida, recuperados em ar e temperatura ambiente e devolvidos as suas respectivas mães. Do 2º ao 28º dia de vida (P2 ao P28) foi feita a restrição sensóriomotora das patas posteriores durante 16 horas por dia, sendo permitida nas 8 horas restantes, a livre movimentação do animal. Para a restrição sensóriomotora foi utilizada uma órtese feita com molde de epóxi, deixando as patas posteriores estendidas, sem que a eliminação de urina e fezes e os cuidados maternos fossem prejudicados (STRATA et al., 2004) (Figura 2).

Figura 2 **-** Modelo experimental de paralisia cerebral. A) Rato utilizando órtese durante a restrição sensóriomotora; B: Animal em período de livre movimentação sem órtese.



6.4 Manipulação Farmacológica

A manipulação farmacológica foi realizada diariamente, entre o 22º e 28º dia de vida, por via subcutânea no dorso do animal. A administração foi realizada as 8h da manhã após a transição do ciclo claro para o escuro, pois este momento coincide com os níveis de pico pós-prandiais de FGF15 nos animais (FERNANDES-FREITAS; OWEN, 2015). De acordo com os grupos experimentais, os animais receberam solução Veículo, que corresponde a uma solução tampão fosfato-salino com albumina de soro bovino (PBS/BSA a 0,1%), ou receberam a solução FGF19 humano recombinante a uma concentração de 0,1 mg/kg na solução Veículo (FGF19 + PBS/BSA a 0,1%) (BENOIT et al., 2017).

6.5 Coleta dos dados

6.5.1 Evolução ponderal

Para monitorar o crescimento dos animais, o peso corporal dos filhotes foi verificado no dia do nascimento, no 8^{o}, 14^{o}, 17^{o} e do 22^{o} ao 29^{o} dia de vida pós-natal. A pesagem dos animais foi obtida através de uma balança digital eletrônica (Marte, S-1000 modelo, a capacidade de 1 kg e 0,1 g de sensibilidade).

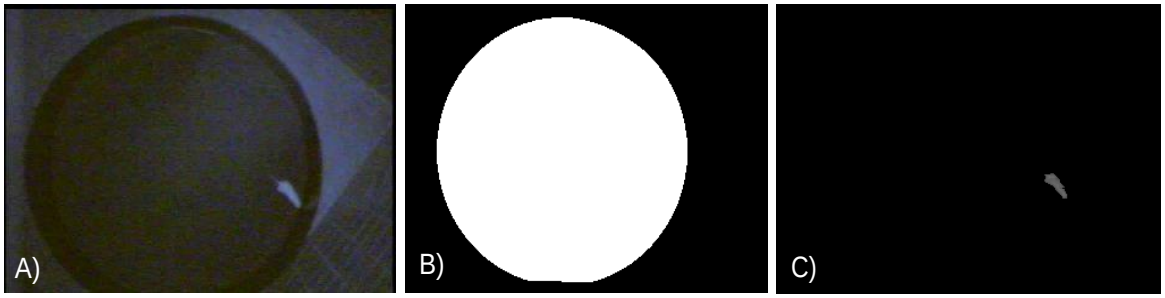
6.5.2 Avaliação da atividade locomotora

O estudo da atividade locomotora foi realizado no 22º e no 28º dia de vida pós-natal, que correspondem ao período pré e pós tratamento farmacológico. Um sistema de monitorização em um campo aberto foi utilizado (ARAGÃO et al., 2011; SILVA et al., 2016b), em que o teste ocorreu em sala escura anexa ao biotério de manutenção, durante o ciclo escuro, em que o animal está naturalmente no estado de vigília após três horas de aplicação do fármaco deivido ao estresse provocado. Os animais foram colocados no centro do campo aberto e gravados em vídeo durante um período de 5 minutos cada (Figura 3). O campo foi limpo com hipoclorito 3% e o EVA trocado para que o odor do animal anterior não influenciasse no teste do animal seguinte.

Os parâmetros analisados foram:

- Distância percorrida (m);
- Velocidade média (m/s);
- Tempo que o animal permaneceu parado(s);
- Número total de paradas feitas pelo animal;
- Proporção entre o tempo parado/número de paradas(s);

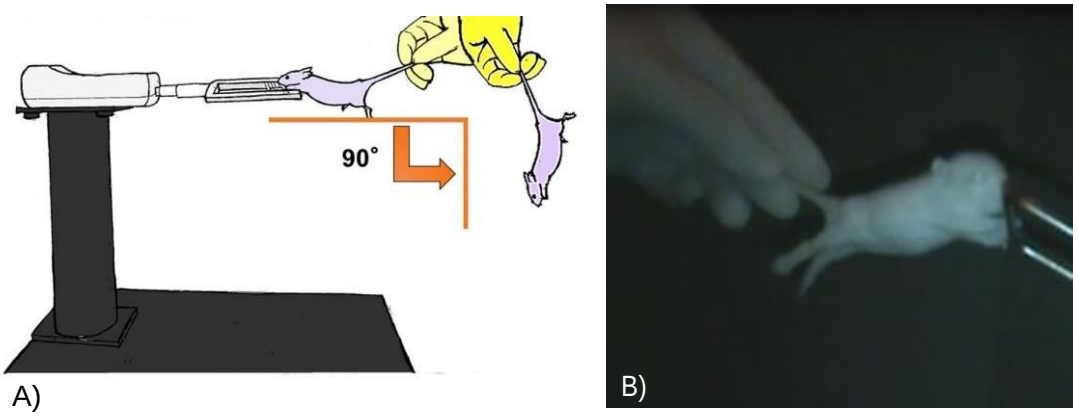
Figura 3 **-** Análise da atividade locomotora em campo aberto. A: Filmagem do animal em atividade espontânea; B: Máscara usada para isolar a imagem do campo com o animal, C: Imagem final (figura B sobre a figura A) utilizada no programa Matlab® para calcular os parâmetros avaliados.



6.5.3 Avaliação da força muscular

Nas idades de 14, 17, 22 e 28 dias de vida pós-natal foi realizada a análise da força muscular através da filmagem do teste de suspensão (*forelimb grip test*). Foi realizado uma hora após ao teste da atividade locomotora, em que um animal por vez foi posicionado em um cabo de aço revestido (3 mm de diâmetro), distante 1 metro do chão, tendo que manter-se agarrado pelas patas anteriores por um tempo limite de 60 segundos enquanto suspensos pela cauda. Posteriormente os vídeos foram analisados por um avaliador cego através do programa *Windows Movie Maker* em que foi registrada a latência de queda em segundos (adaptado de TEO; MORRIS; JONES, 2017) (Figura 4).

Figura 4 **-** Avaliação da força muscular através do teste de suspensão. A: Representação esquemática do teste; B: Filmagem do teste em execução.

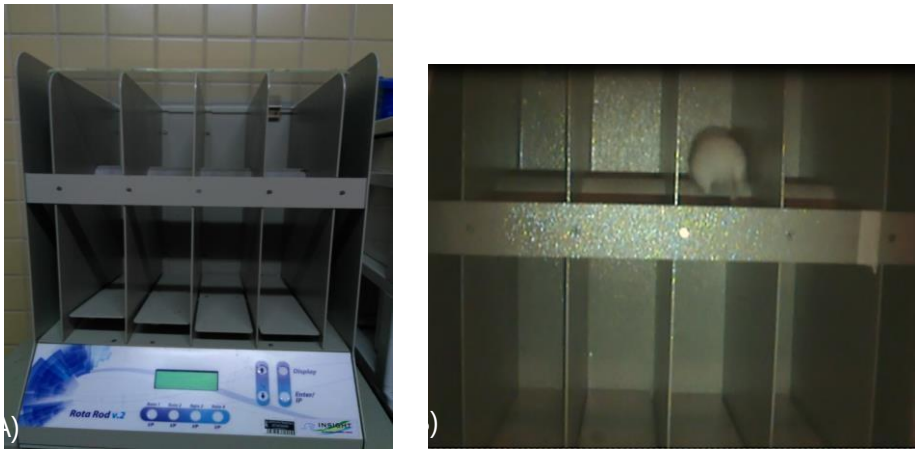


Fonte (Figura 4 A): Takeshita et al, 2017.

6.5.4 Avaliação da Coordenação Motora

Foi realizado o teste de perfomance em rotarod aos 29 dias de vida pós-natal por um avaliador cego quanto à manipulação farmacológica. Um animal por vez foi colocado no equipamento rotarod sobre uma haste de 60 mm de diâmetro e 75 mm de comprimento em rotação. Inicialmente, os animais passaram por um período de adaptação no equipamento por 2 minutos a uma velocidade de 16rpm. Após aguardar o período de descanso de 2 min, os animais foram colocados individualmente no rotarod em 5 tentativas, respeitando o intervalo de 2 min para descanso, com uma rotação a uma velocidade de 25 rpm por no máximo de 3 minutos para que a latência de queda fosse registrada (adaptado de STRIGGER, 2011) (Figura 5). A análise da latência de quedas foi sintetizada como média das 5 tentativas de acordo com os grupos experimentais.

Figura 5 **-** Avaliação da coordenação motora atravéz do teste de perfomance em rotarod. A: Equipamento rotarod; B: Execução do teste em rotarod.



A) B)

6.5.5 Coleta e aferição do peso dos músculos sóleo e extensor longo dos dedos

Os animais, aos 29 dias de vida pós-natal, foram eutanasiados por decapitação. Em seguida, os músculos sóleo e extensor longo dos dedos foram dissecados, pesados, congelados em n-hexano (dióxido de carbono pré-arrefecida solidifica -78,5 °C) e armazenados a -80 °C para posterior procedimento histoquímico e análise histomorfométrica. Para pesar os músculos foi utilizada uma balança de precisão (modelo Marte AUW220, capacidade de 220g e 0,1 mg de

sensibilidade), onde foi obtido o peso absoluto de ambos os músculos e seu peso relativo, normalizando o peso de cada músculo através do peso corporal.

6.5.6 Análise histomorfométrica dos músculos sóleo e extensor longo dos dedos

Foram realizadas secções transversais (8um), através de criostato a -30°C, dos músculos sóleo e extensor longo dos dedos, coletados no 29º dia de vida. As secções foram fixadas em lâminas e o procedimento de coloração pela técnica de ATPase miofibrilar começou depois de atingir a temperatura ambiente (BROOKE; KAISER, 1970; LACERDA et al., 2017b). As fibras musculares foram coradas em relação aos três tipos de fibras principais (I, IIa, IIb), de acordo com as diferenças na intensidade da coloração ATPase após pré-incubação de ácido (pH 4.3 e 4.55). Na pré-incubação com pH 4.3 as fibras foram coradas em tipo I (mais escura) e tipo II (mais clara) e no pH 4,55 as fibras são coradas em tipo I (mais escura), tipo IIa (pálida) e tipo IIb (cinza). Os cortes foram analisados com um microscópio óptico (Olympus BX-41, 100x) conectado a um computador com software de captura de imagem *Analysis Get It*. Todas as fibras musculares foram contadas em cada secção histológica, sendo os valores apresentados para os diferentes tipos de fibras como uma percentagem do número total. Também foi feita a análise da área e perímetro de cada fibra muscular, defindo-se a área como a medida da superfície de cada fibra e o perímetro como a soma das medidas de todos os lados de cada fibra. Para contagem e análise da área e perímetro das fibras foi utilizado o software *ImageJ* (versão 1.51p).

6.5.7 Antropometria e morfometria óssea da tíbia

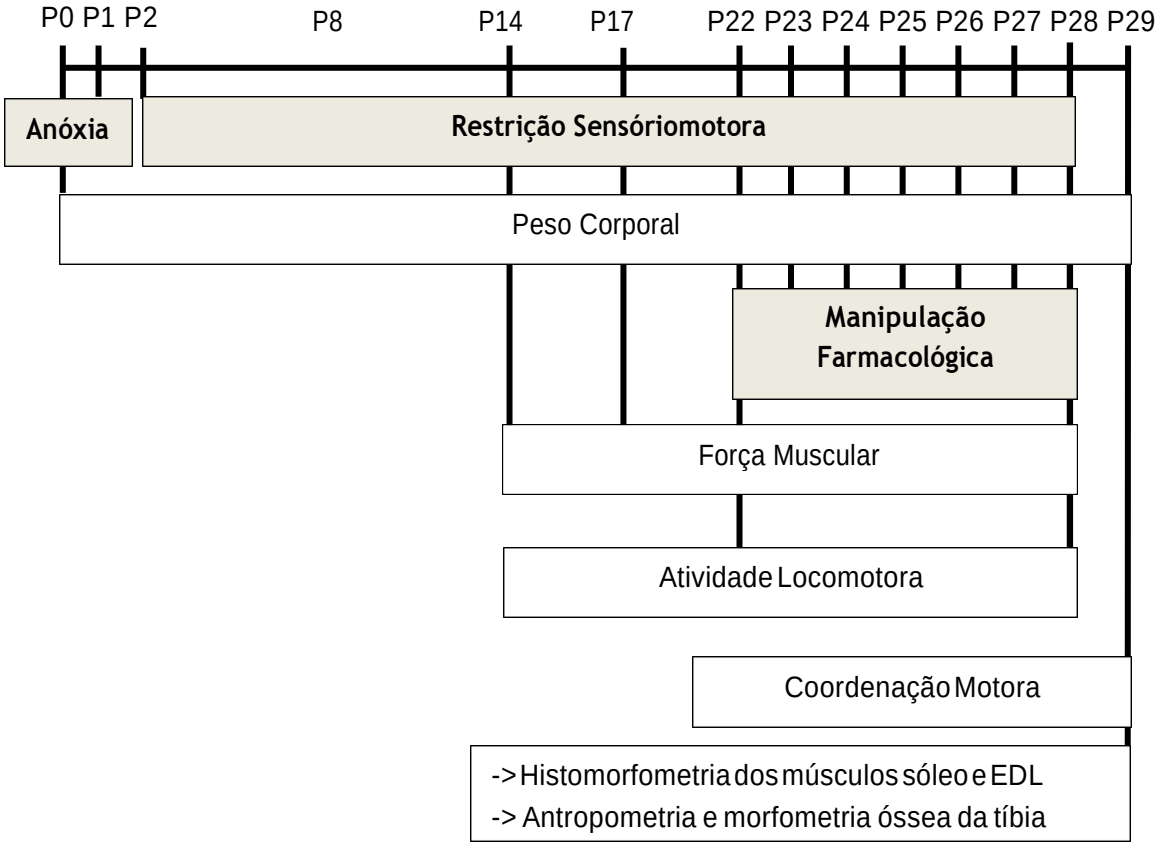
As tíbia de ambas as patas posteriores foram dissecadas e pesadas em balança de precisão (modelo Marte AUW220, capacidade de 220g e 0,1 mg de sensibilidade), sendo obtido seu peso absoluto e relativo no P29. Além disso, foi mensurado o comprimento longitudinal da tíbia utilizando um paquímetro universal analógico (Digimess, 0,02mm), em que foi feita a média de duas medidas. O comprimento das tíbias também foi normalizado pelo comprimento longitudinal do animal, também chamado de comprimento nasoanal, para obter a medida de comprimento relativo. Após a dissecação e pesagem, as tíbias foram fixadas em

formol tamponado 10% por 48h para iniciar o processo de descalcificação em solução quelante ácido etilenediaminetetracético (EDTA). Quando a descalcificação foi concluída o tecido foi pós fixado com sacarose 15% e 30% por 24h cada, em seguida as tíbias foram congeladas em n-hexano e armazenadas em freezer -80º. As tíbias foram cortadas em sentido transversal na diáfise e em sentido coronal na epífise proximal, sendo esses fragmentos incluídos em parafina para obtenção dos cortes histológicos em micrótomo (*Leica Biosystems*) a 4µm, corados com hematoxilina-eosina (HE) e fotografados em microscópio óptico (Olympus BX-41, aumento de 100x). A morfometria consistiu na avaliação da espessura do osso cortical, desde a superfície periostal até a superfície endostal, avaliação da espessura do periósteo e da área medular da diáfise da tíbia com aumento de 100x. Para análise de cada parâmetro foi realizada a média de 3 medidas (Figura 1) (adaptado de CARVALHO et al., 2010). No corte longitudinal foi avaliada a região que compreende de 0,75 a 1,76mm abaixo da placa de crescimento, sendo os parâmetros morfométricos o volume trabecular ósseo (VTO) e a porosidade (Por) com aumento de 40x (adaptado de IWAMOTO; SEKI; SATO, 2014; RAFFI; MÉNDEZ; RIET-CORREA, 1997), de acordo com as seguintes fórmulas:

$$VTO = \frac{\text{nº de intersecções que incidem sobre o osso trabecular x100}}{\text{nº total de intersecções}}$$

$$Por = \frac{\text{nº de intersecções que incidem sobre as cavidades x100}}{\text{nº total de intersecções}}$$

Figura 6 - Organograma de atividades experimentais.



7 DEFINIÇÃO E OPERACIONALIZAÇÃO DE VARIÁVEIS

7.1 Variáveis independentes:

De interesse: Paralisia cerebral experimental e tratamento farmacológico com FGF19.

De Controle: Sexo dos filhotes, idade e peso corporal no dia do nascimento.

7.2 Variáveis dependentes:

Desfechos primários: Peso dos músculos sóleo e EDL, área, perímetro e proporção dos tipos de fibras musculares.
Desfechos secundários: Peso corporal, atividade locomotora, coordenação motora, força muscular, antropometria e morfometria óssea da tíbia.

7.3 Variáveis Quantitativas Contínuas

a) Peso Corporal (g)
b) Atividade locomotora através dos parâmetros: distância percorrida (m), velocidade média (m/s), potência média (mW), tempo parado(s); proporção entre o tempo parado/número de paradas(s)
c) Coordenação motora (latência de queda em rotarod (s))
d) Força muscular (latência de queda no teste de suspensão (s))
e) Peso dos músculos sóleo e EDL (g)
f) Peso da tíbia (g)
g) Comprimento da tíbia (mm)
h) Área e perímetro das fibras musculares (μm^2 e µm)
i) Morfometria óssea da diáfise da tíbia (espessura cortical e do periósteo (µm) e área medular (μm^2).

7.4 Variável Quantitativa discreta

a) número de paradas (n) na atividade locomotora

7.5 Variáveis Qualitativas Nominais

a) Proporção dos tipos de fibras (Tipo I, IIa e IIb) nos músculos sóleo e EDL
b) Volume trabecular e porosidade da tíbia (%)

8 ANÁLISE ESTATÍSTICA

A análise estatística dos resultados foi realizada através do software estatístico GraphPad Prism 6.0, sendo as variáveis quantitativas contínuas expressas em média e erro padrão de média. Foi realizado o teste de normalidade de *Kolmogorov Smirnov*. Sendo a distribuição normal, para comparação intergrupos foi utilizada o teste paramétrico *ANOVA Two Way* com os fatores paralisia cerebral experimental e manipulação farmacológica, seguido do pós-teste de *Tukey.* Para as variáveis em que a análise foi feita em várias idades foi utilizado o teste *ANOVA Two Way Medidas Repetidas* seguido do pós-teste de *Tukey*. Foi atribuído um nível de significância de 95%.

9 RESULTADOS

Os resultados deste estudo foram apresentados em forma de artigo original.

Artigo Original – FIBROBLAST GROWTH FACTOR 19 IMPROVES LOCOMOTION AND MUSCULOSKELETIC SYSTEM IMPAIRMENT ON EXPERIMENTAL CEREBRAL PALSY MODEL

Artigo original elaborado a partir dos resultados do presente estudo, seguindo as normas da revista a qual será submetido: *THE FASEB JOURNAL* (Qualis A1 na área 21 da CAPES, fator de impacto 5,595) (APÊNDICE A).

10 CONSIDERAÇÕES FINAIS

A paralisia cerebral experimental promoveu prejuizos nos animais com relação à evolução ponderal, nas funções motoras e no sistema musculoesquelético. Dessa forma, foi observada a redução no ganho de peso corporal a partir do 14º dia de vida, redução da locomoção aos 28 dias, menor latência de quedas no teste de coordenação motora em rotarod aos 29 dias, menor peso muscular relativo e menor área e perímetro das fibras musculares do sóleo e EDL, além de menor espessura cortical, área medular e volume trabecular da tíbia com aumento na porosidade neste osso.

O tratamento com FGF19 nos animais submetidos á PC manteve o peso corporal dos animais, e foi capaz de melhorar os aspectos da locomoção aos 28 dias de vida com efeitos benéficos para o sistema musculoesquelético. Houve o aumento do peso relativo do EDL, aumento da área e perímetro das fibras musculares do sóleo e EDL, aumento da espessura cortical e do volume trabecular da tíbia e redução na porosidade, resultados que sugerem a atenuação da atrofia muscular por desuso e perda de massa óssea decorrente da PC experimental.

Nos animais controle, a administração de FGF19 também manteve o peso corporal dos animais e promoveu repercussões no músculo, aumentando o peso relativo do sóleo e aumento a área e perímetro do EDL.

Quanto às limitações do estudo, podemos incluir a perda de animais devido ao modelo de indução da PC que torna os animais frágeis e com maior risco de mortalidade. Além disso, a escolha do teste para análise da força muscular pode não ter sido adequado pois foi observado algumas vezes que alguns animais, inclusive controles, não executavam a tarefa e alguns dos PCs executavam com muita dificuldade pelo tempo máximo.

11 PERSPECTIVAS

Os resultados apresentados nesta dissertação abrem novas possibilidades para estudos que se proponham a investigar intervenções para a PC e para estudos que busquem entender os efeitos do FGF19 no sistema músculoesquelético, que ainda precisa ser melhor elucidado.

Assim, propusemos de forma sintetizada alguns aspectos relevantes para investigações futuras:

- Avaliar os efeitos do FGF19 no metabolismo muscular, abrangendo de forma integrada com seus efeitos sobre o fígado.
- Avaliar a presença de receptores e co-receptores para o FGF-19 no osso e sua forma de atuação incluindo diferentes idades de animais.
- Aprofundar a análise dos músculos sóleo e EDL do ponto de vista molecular.
- Avaliar a histomorfometria das articulações dos membros posteriores dos animais submetidos à PC experimental associada com o tratamento com FGF-19
- Avaliar a atividade locomotora e alterações musculoesqueléticas após o tratamento com FGF-19 a longo prazo.
- Avaliar o controle postural dos animais submetidos à PC desde o período neonatal até a vida adulta.
- Utilizar outras formas de avaliação da força muscular nos animais submetidos à PC.

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APENDICE A — FIBROBLAST GROWTH FACTOR 19 MINIMIZE DAMAGES IN LOCOMOTION, MUSCULAR ATTROPHY AND BONE MASS LOSS IN AN EXPERIMENTAL MODEL OF CEREBRAL PALSY

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ABBREVIATIONS: CP, Cerebral Palsy; FGF-19, fibroblast growth factor 19; PBS / BSA, phosphate-buffered saline solution with bovine serum albumin; SN, Nervous System; CNS, Central Nervous System, EDL, extensor digitorum longus; EVA, ethyl vinyl acetate; EDTA, ethylenediaminetetraacetic acid; HE, hematoxylin-eosin.

ABSTRACT

The aim of the present study was to evaluate the effects of FGF19 treatment on muscle atrophy, bone morphometry and motor function performance in rats submitted to experimental cerebral palsy (CP). Male Wistar rats, distributed in the experimental groups: Vehicle (n = 11), received 0.1% PBS / BSA solution, Vehicle + FGF19 (n = 10) received FGF19 (0.1 mg / kg in PBS / 0.1% BSA); Cerebral Palsy (PC) + Vehicle (n = 12); and PC + FGF19 (n = 13). The weight evolution and histomorphometry, as well as the morphometry of the diaphysis and the proximal tibial epiphysis, were evaluated. The weight evolution and histomorphometry were evaluated in animals, locomotion, motor coordination, muscle strength and soleus and extensor digitorum muscles. The animals submitted to the CP compared to controls had a reduction in body weight, locomotion and motor coordination, lower muscle weight, lower area and perimeter of the soleus muscle fibers and EDL, as well as lower cortical thickness and trabecular volume of the tibia. Treatment with FGF-19 on CP maintained the animals' body weight, increased locomotor activity, relative EDL weight, area and perimeter of soleus muscle fibers and EDL, cortical thickness and trabecular volume of the tibia. We conclude that FGF-19 treatment improves locomotor activity and reduces the effects on muscle atrophy and bone loss in the experimental CP.

Keywords: Animal models, cerebral palsy, musculoskeletal fibers, motor skills.

INTRODUCTION

The musculoskeletal system exhibits a high adaptive potential (1,2) in which muscle fibers and bone tissue have the ability to adjust their metabolic, molecular and functional properties in order to accommodate specific demands (2-5). Several stimuli influence the musculoskeletal phenotype, such as contractile activity, substrate supply and environmental factors, such as hypoxia (1,2). Even neurological diseases, such as cerebral palsy (CP), also affect this system (6).

PC is present in every 2-3 per 1,000 live births worldwide (7) and is described as a heterogeneous group of clinical syndromes that result in permanent movement and posture disorders due to aggression during brain development (7-9). Children with PC experience premature sarcopenia, which is loss of muscle mass with increased vulnerability to weakness and disability. In addition to the inability to recruit the target musculature during voluntary activity, that is, neural ineffectiveness, and a

recruitment and coactivation of the antagonist musculature (10,11). These motor control deficits drastically reduce walking efficiency and movement, and increase energy expenditure and fatigue during activities (10). The low bone mineral density (12,13), described as "physiological osteopenia" may also be present. This is mainly due to insufficient and abnormally functional musculature and immobility, both of which reduce the load on the developing skeleton and impair the healthy development of bones (14). Thus, they are prone to fragility fractures and, consequently, to a reduced quality of life (14,15).

In order to reproduce the neurofunctional damages and clarify the mechanisms underlying this pathology, experimental models of CP in animals have been proposed (16). Among them, we highlight what associates perinatal anoxia and sensory-motor restraint of the hind paws of rodents, due to the fact that they cause lasting changes similar to those in children with CP, such as increased muscle tone (17), reduction of sarcomere densities (18), disorganization in the cortical representation of the lower limbs in the somatosensory cortex (S1) (19), reduction in locomotor activity (SILVA et al., 2016), impairment in mastication (21), and impaired motor skills. For this reason, this model becomes a relevant tool to elucidate the PC pathogenesis and highlights the importance of studies that propose to add new perspectives of treatment for this pathology.

Despite the known clinical repercussions of CP, currently the pharmacological and non-pharmacological treatment approaches are to assist the sequels and associated comorbidities, being unable to reverse the damages in the musculoskeletal system. In this context, one study demonstrated that a member of the fibroblast growth factor family (FGF), FGF19, acts directly on the muscle promoting hypertrophic effect in models of induced muscular atrophy (23). This indicates that the musculoskeletal system is a direct target for FGF19 and that this regulatory role in musculoskeletal growth proposes that this factor has therapeutic potential to preserve and / or improve muscle structure and could also be extended to other experimental models (23) including models of neurological diseases that affect the musculoskeletal system.

Thus, in view of the above, we postulate that FGF19 treatment improves muscle atrophy due to the experimental PC, with beneficial consequences for bone morphometry and the performance of the motor functions affected by the disease. Therefore, the aim of this study is to evaluate the effects of FGF19 treatment on

muscle atrophy, bone morphometry and the performance of motor functions related to locomotion in rats submitted to experimental cerebral palsy.

MATERIALS AND METHODS

Animals and experimental groups

Twenty Wistar rats were used from the breeding room of the Department of Nutrition of UFPE. The animals were kept in the maintenance room of the Department of Nutrition of the UFPE with temperature of 22 ± 2 ° C, reversed light-dark cycle of 12/12 hours housed in polypropylene cages with free access to water and diet. The project was approved by the animal experiment ethics committee of the Biological Sciences Center of the Federal University of Pernambuco (CEUA: 0011/2017). All procedures were performed in accordance with the guidelines of the National Council for the Control of Animal Experimentation (CONCEA) and the International Standards of the National Institute of Heath Guide for Care and Use of Laboratory Animals (8th ed). On the day of birth, the male cubs (weight 6 to 8 grams) were randomly distributed in the experimental groups. The female pups were only used to complete the litter of 8 pups until the day of weaning. The four experimental groups were formed based on the induction of cerebral palsy and the pharmacological manipulation administered to the animals: Vehicle (n = 11); Cerebral Palsy (PC) + Vehicle (n = 12); Vehicle + FGF19 (n = 10); PC + FGF19 (n = 13). Weaning occurred on the 25th day postnatal life in which male cubs were placed in individual cages until euthanasia by decapitation on the 29th postnatal day.

The experimental model of adopted CP consisted of the association of perinatal anoxia and sensory-motor restriction of the hind paws (17,19,21,24). The offspring of the PC groups were submitted to two episodes of postnatal anoxia on the day of birth and on the first day of life (P0 and P1) being exposed to nitrogen (100%) at 9L / min for 12 minutes. From the 2nd to the 28th day of life (P2 to P28) the sensory-motor restraint of the hind paws was made for 16 hours per day, allowing the free movement of the animal in the remaining 8 hours. Pharmacological manipulation was performed daily between the 22nd and 28th day of life, subcutaneously on the animal's back. According to the experimental groups, the animals received vehicle solution, which corresponds to a phosphate-buffered saline solution with bovine

serum albumin (PBS / 0.1% BSA), or received the FGF19 solution at a concentration of 0.1 mg / kg in Vehicle solution (FGF19 + PBS / 0.1% BSA) (23).

Weight evolution

To monitor the growth rate of the animals, the pups' body weight was verified on the day of birth, on the 8th, 14th, 17th and 22nd to 29th days of postnatal life. The animals were weighed using a digital electronic scale (Mars, S-1000 model, 1 kg capacity and 0.1 g sensitivity).

Locomotor activity

The study of locomotor activity was performed on the 22nd and 28th days of postnatal life, which correspond to the period before and after the pharmacological treatment. An open field monitoring system was used (24,25), in which the test was performed in a dark room attached to the maintenance room during the dark cycle, in which the animal is naturally in the waking state. The animals were placed in the center of the open field and recorded in video during a period of 5 minutes each, being obtained the parameters: Distance traveled (m); Average speed (m / s); Time the animal remained stationary; Total number of stops made by the animal; Proportion between the stopped time / number of stops (s).

Muscular Strength

At the ages of 14, 17, 22 and 28 days of postnatal life the analysis of muscle strength was performed through the filming of the forelimb grip test. One animal at a time was positioned on a coated steel cable (3 mm in diameter), 1 meter from the ground, having to hold the front legs for a time limit of 60 seconds while suspended by the tail. Later, the videos were analyzed by a blind evaluator through the Windows Movie Maker program in which the latency of the fall was recorded in seconds (adapted from TEO; MORRIS; JONES, 2017) (Figure 4).

Motor Coordination

A rotarod perfomance test was performed at 29 days postnatal life by a blind evaluator regarding pharmacological manipulation. One animal at a time was placed in the rotarod equipment on a rod 60 mm in diameter and 75 mm in length. Initially, the animals undergo an adaptation period in the equipment for 2 minutes at a speed

of 16rpm. After waiting for the rest period of 2 min, the animals were placed individually in the rotarod in 5 trials, respecting the interval of 2 min for rest, at a speed of 25 rpm for a maximum of 3 minutes for the drop latency to be recorded (adapted from STRIGGER, 2011). The latency analysis of falls was synthesized as mean of the 5 trials according to the experimental groups.

Analysis of soleus and extensor digitorum longus muscles

The animals, at 29 days postnatal life, were euthanized by decapitation. Then, the soleus and extensor digitorum longus muscles were dissected for absolute and relative weight, frozen in n-hexane (pre-cooled carbon dioxide solidifies -78.5 ° C) and stored at -80 ° C for posterior histochemical procedure and histomorphometric analysis. Cross sections (8 µm) were performed through a cryostat at -30 ° C of both muscles. Sections were fixed on slides and the staining procedure by myofibrillar ATPase technique started after reaching room temperature (21, 27). The muscle fibers were stained with respect to the three main fiber types (I, IIa, IIb), according to the differences in the intensity of ATPase staining after acid preincubation (pH 4.3 and 4.55). In pre-incubation at pH 4.3 the fibers are stained in type I (darker) and type II (lighter) and at pH 4.55 the fibers are stained in type I (darker), type IIa (pale) and type IIb (gray). The sections were photographed under an optical microscope (Olympus BX-41, 100x) connected to a computer with Analysis Get It image capture software. All muscle fibers were counted in each histological section, the values presented for the different types of fibers as a percentage of the total number. We also analyzed the area and perimeter of each muscle fiber, defining the area as the measurement of the surface of each fiber and the perimeter as the sum of the measurements on all sides of each fiber. For counting and analysis of the area and perimeter of the fibers, the software Image J.

Anthropometry and bone morphometry of the tibia

The tibia of both hind paws were dissected, weighed in a precision scale (model Mars AUW220, capacity of 220g and 0.1 mg of sensitivity), obtaining its absolute and relative tibial weight in P29. In addition, the longitudinal length of the tibia was measured using a universal analog caliper (Digimess, 0.02mm), which averaged two measurements. The length of the tibia was also normalized by the longitudinal length of the animal, also called the nasoanal length, to obtain the

measure of relative length. After dissection and weighing, the tibias were fixed in 10% buffered formalin for 48h to initiate the decalcification process in ethylenediaminetetraacetic acid (EDTA) chelating solution. When decalcification was completed the tissue was post-fixed with 15% sucrose and 30% for 24h each, then the tibias were frozen in n-hexane and stored in a freezer at -80º. The tibias were cut transversely in the diaphysis and coronally in the proximal epiphysis, and these fragments were included in paraffin for histological sections in microtome (Leica Biosystems) at 4µm, stained with hematoxylin-eosin (HE) and photographed under an optical microscope (Olympus BX-41, increase of 100x). Morphometry consisted of evaluation of the thickness of the cortical bone, from the periostal surface to the endostal surface, evaluation of the thickness of the periosteum and the medullary area of the tibial shaft with an increase of 100x. For analysis of each parameter, the average of 3 measurements was performed (Figure 1) (adapted from CARVALHO et al., 2010). In the longitudinal section, the region comprised of 0.75 to 1.76 mm below the growth plate was evaluated, the morphometric parameters being the trabecular bone volume (VTO) and the porosity (Por) with a 40x magnification (adapted from IWAMOTO, SEKI , SATO, 2014, RAFFI, MÉNDEZ; RIET-CORREA, 1997), according to the following formulas:

STATISTICAL ANALYSIS

The results were analyzed through the statistical software GraphPad Prism 6.0, with continuous quantitative variables being expressed as mean and standard error of mean. The Kolmogorov Smirnov normality test was performed. As the normal distribution, for intergroup comparison, the ANOVA Two Way parametric test was used with experimental cerebral palsy and pharmacological manipulation, followed by the Tukey post-test. For the variables in which the analysis was performed at several ages, the ANOVA Two Way Repeated Measure followed by the Tukey post-test was used. A significance level of 95% was assigned.

RESULTS

Weight evolution

The animals submitted to cerebral palsy had a lower body weight compared to the control group from the 14th day of life to the last day of the experiment, 29th day of

life (14th day: PCV: 19.38 ± 3.24g / V: 25 , 60 ± 3.40 g p <0.05, 29 th day: PCV: 44.66 ± 7.09 g / V: 67.60 ± 5.74 g p <0.05). The animals of the PCF group presented higher body weight from the 22nd day compared to the PCV group (22nd day: PCF: 36.09 ± 3.61g / PCV: 28.70 ± 5.04g p <0.05; 29th day: PCF: 54.32 ± 5.09g / PCV: 44.66 ± 7.09g p <0.05), as well as the F group compared to V (22nd day: F: 49.22 ± 5.06g / V : 41.95 ± 5.10g p <0.05; 29th day: F: 79.53 ± 7.51g / V: 67.06 ± 5.74g p <0.05) (Figure 2).

Locomotor activity

At 22 days, there was no difference between the experimental groups. At 28 days, the animals submitted to CP, compared to the vehicle group, had the lowest actual distance traveled (PCV: 13.86 ± 5.72 / V: 22.29 ± 4.93 p <0.05), lower average speed (PCV: 0.1233 ± 0.02 / V: 0.1630 ± 0.02 p <0.05) and longer standing time (PCV: 196.55 ± 31.01 / V: 158.26 ± 35.46 p <0.05). As the PC associated with FGF-19 manipulation presented a greater distance traveled (PCF: 29.63 ± 4.23 / PCV: 13.86 ± 5.72 p <0.05) and the mean velocity (PCF: 0, 1613 ± 0.02 / PCV: 0.1233 ± 0.02 p <0.05) and reduced the standing time of these animals at 28 days of age (PCF: 141.79 ± 45.71 / PCV: 196.55 ± 31.01 p <0.05). There was no difference between the groups in the number of stops and in the proportion between the stopped time / number of stops between the experimental groups (Figure 3).

Motor Coordination

The animals in the experimental group presented lower latency of the motor coordination test in rotarod compared to the control group (PCV: 2.3 ± 1.2 s / V: 23.61 ± 13.29 s, p <0.05). Administration of FGF-19 in animals submitted to CP was not able to improve this aspect significantly (PCF: 7.77 ± 6.01s / PCV: 2.3 ± 1.2s) (Figure 4).

Muscular Strength

There was no difference between the experimental groups regarding the analysis of muscle strength in the suspension test (Table 1).

Table 1: Muscle strength through the sleep test (latency of seconds) in the experimental groups at the ages of 14, 17, 22 and 28 days postnatal life. Data are

expressed as mean and standard deviation, p <0.05. (PCVxCVα; CVxCFβ; PCVxPCF *)

Latência de queda (s)	Veículo (V) (n=10)	FGF-19 (F) (n=10)	Paralisia Cerebral + Veículo (PCV) (n=10)	Paralisia Cerebral + FGF-19 (PCF) (n= 10)	p valor
P14	23,98 ±12,05	34,98 ±17,37	25,98 ±23,41	28,09 ±16,14	p>0,05
P17	40,67 ±19,95	35,26 ±20,34	35,05 ±17, 58	30,92 ±14,89	p>0,05
P22	48,88 ±20,75	44,01 ±20,23	35,90 ±21,21	39,28 ±21,14	p>0,05
P28	59,53 ±1,00	52,70 ±12,04	44,55 ±17,50	60,00 ±0,00	p>0,05

P: postnatal day; n: number of animals per group. Data are expressed as mean and standard deviation. Two-way ANOVA repeated measures were used followed by the Tukey post-test, p <0.05.

Soleus muscle weight

The experimental group presented lower absolute weight (PCV: 0.0125 ± 0.003g / V: 0.0258 ± 0.003gp <0.05) and relative (PCV: 0.0003 ± 0.0001g / V: 0, 0004 ± 0.0000g p <0.05) of the soleus muscle at 29 days compared to the control group. The animals submitted to the FGF-19 treated PC presented higher absolute weight of this muscle (PCF: 0.0175 ± 0.003g / PCV: 0.0125 ± 0.003g p <0.05). There was a significant difference between the controls, in which the animals that received FGF-19 presented higher absolute weight of the soleus (F: 0.0364 ± 0.005g / V: 0.0258 ± 0.003gp <0.05), as well as observed in the relative weight (F: 0.0005 ± 0.0001g / V: 0.0004 ± 0.0000g p <0.05) (Figure 5).

EDL muscle weight

The PCV group presented lower absolute weight (PCV: 0.0146 ± 0.0032g / V: 0.0254 ± 0.0025g p <0.05) and relative (PCV: 0.0003 ± 0.0000g / V: 0, (PF 0.0201 ± 0.0028g / PCV: 0, p <0.05). The results showed that FGF- 0146 ± 0.0032g p <0.05) and relative (PCF: 0.0004 ± 0.0001g / PCV: 0.0003 ± 0.0000g p <0.05) of this muscle compared to the PC group that was submitted to vehicle administration. FGF-19 also

showed a higher absolute EDL weight compared to vehicle group (F: 0.0290 ± 0.0021g / V: 0.0254 ± 0.0025g p <0.05) (Figure 6).

Soleus and EDL muscle histomorphometry

The animals of the CP group presented a smaller area and perimeter of the muscle fibers in the soleus muscle compared to the control group submitted to vehicle administration only (Area: PCV: 287.68 ± 121.24 / V: 952.38 ± 289 , 23; Perimeter: PCV: 75.23 ± 14.06 / 179.84 ± 54.98, p <0.05). Similarly, this group had a smaller area and perimeter of muscle fibers in the EDL muscle (Area: PCV: 272.27 ± 128.2 / V: 644.98 ± 300.93; Perimeter: PCV: 99.82 ± 43, 97 / V: 172.67 ± 77.6, p <0.05). FGF-19 administration in the animals submitted to PC promoted an increase in the area and perimeter of the muscle fibers of the soleus (Area: PCF: 362.97 ± 130.46 / PCV: 287.68 ± 121.24; Perimeter: PCF: 126.49 ± 32.03 / PCV: 75.23 ± 14.06, p <0.05) and EDL (Area: PCF: 408.25 ± 189.48 / PCV: 272.27 ± 128.2; Perimeter: PCF: 123.64 ± 51.88 / PCV: 99.82 ± 43.97, p <0.05). In addition, administration of FGF-19 in the control animals also increased the area and perimeter of muscle fibers in the EDL (Area: F: 762.89 ± 307.6 / V: 644.98 ± 300.93; 192.51 ± 84.03 / V: 172.67 ± 77.6, p <0.05) (Figure 7).

As for the proportion of fiber types in the soleus muscle there was no difference between groups at pH 4.3 in relation to type I fibers (V: 82.3 ± 4.05% / F: 84.1 ± 4.14% / PCV: 84.1 ± 4.17% / PCF: 82.2 ± 4.11%) or type II (V: 17.0 ± 4.05% / F: 16.5 ± 4.14% / PCV: 6 ± 4.17% / PCF: 17.5 ± 4.11%). At pH 4.55, the animals of the PCV group had a higher proportion of type I fibers compared to group V (PCV: 83.2 ± 1.74 / V: 75.8 ± 1.75; p <0.05). Moreover, at this pH there was no difference between groups in relation to type IIa fibers (V: 5.6 ± 1.64% / F: 3.2 ± 1.99% / PCV: 1.9 ± 0.57% / PCF: 4.7 ± 6.76%) and type IIb (V: 18.6 ± 3.07% / F: 23.1 ± 10.32% / PCV: 14.9 ± 1.66% / PCF : 18.4 ± 10.35%) (Figure 8 and 9).

In the EDL muscle, there was a difference in the proportion of fiber types in PH 4.3 between the control animals, in which the FGF-19 group presented a reduction of type I fibers (F: 33.5 ± 6.46% / V: 55 , P <0.05) and increased type II fibers (F: 66.2 ± 6.46% / V: 45.0 ± 12.33%, p <0.05). At pH 4.55, the increase in the proportion of type II fibers in group F compared to group V was confirmed, being significant for type IIa fibers (F: 36.2 ± 2.78% / V: 25.7 ± 9 , 49%, p <0.05). The animals of the CP group had a reduction of type IIa fibers (PCV: 15.9 ± 1.52% / V: 25.7 ± 9.49%, p <0.05),

and an increase in type IIb fibrosis (PCV: 58.9 ± 2.21% / V: 54.1 ± 4.72%, p <0.05) at pH 4.55. FGF-19 administration in the experimental PC at this pH promoted the opposite effect, increasing type IIa fibers (PCF: 25.1 ± 9.69% / PCV: 15.9 ± 1.52%, p <0.05) and reducing type IIb fibers (PCF: 50.6 ± 5.93% / PCV: 58.9 ± 2.21%, p <0.05) (Figure 10 and 11).

Anthropometry and bone morphometry of the tibia

The anthropometric analysis of the tibia at 29 days of life showed a reduction in the absolute weight of the tibia of the animals submitted to CP in comparison to the vehicle group (PCV: 0.0792 ± 0.015g / V: 0.1278 ± 0.010g; , 05). FGF-19 administration in PC animals promoted the increase of this weight (PCF: 0.0936 ± 0.015g / PCV: 0.0792 ± 0.015g, p <0.05). There were no changes between the groups when the weight of the tibia was normalized by body weight. The tibia length was lower in the animals of the PCV group compared to the V group (PCV: 16.63 ± 1.25 mm / V: 20.25 ± 0.75 mm, p <0.05) (Figure 12). In relation to tibial diaphysis morphometry, the animals of the PCV group, compared to the V group, presented lower cortical thickness (PCV: 160.4 ± 20.2µm / V: 202.9 ± 60.4 µm, p <0, 05) and medullary area (PCV: 616.099.402 ± 39.795.262µm / V: 906.210.111 ± 212.467.276µm, p <0.05). Treatment with FGF-19 on the PC increased the cortical thickness of the tibia (PCF: 215.2 ± 27.3µm / PCV: 160.4 ± 20.2µm, p <0.05). There was no difference between the groups in the analysis of periosteal thickness. In the analysis of the proximal epiphysis of the tibia, the reduction of the trabecular volume (PCV: 57.04 ± 4.22% / V: 75.44 ± 6.31%, p <0.05) and increase in porosity : 42.96 ± 4.22% / 24.56 ± 6.31%, p <0.05) in the PCV group compared to the group V. The animals submitted to FGF-19 associated PC presented increased trabecular volume (PCF: 71.85 ± 7.99 / PCV: 57.04 ± 4.22%, p <0.05) and reduction of porosity (PCF: 28.15 ± 7.99 / PCV: 42.96 ± 4 , 22%, p <0.05) compared to the PCV group (Figure 13).

DISCUSSION

The present study evaluated the effects of FGF-19 treatment on changes in the musculoskeletal system, and on the impairment of motor functions due to experimental CP in rats. We observed that the experimental PC causes impairment in locomotion and motor coordination and negatively interferes in the physical

development of the animals with reduction in body weight, lower area and perimeter of muscle fibers, and reduction of bone mass of the tibia. The administration of FGF-19 in animals submitted to CP improved locomotion and musculoskeletal parameters such as the area and perimeter of muscle fibers, as well as bone mass of the tibia. Thus, the findings of this study show the musculoskeletal and functional changes of CP in rats and is the pioneer to investigate the effects of FGF-19 treatment, in face of this neurological disease, on the locomotor activity and bone morphometry of rats.

The reduction in body weight from the 14th day of life observed in the animals submitted to the experimental CP confirms that CP affects the somatic development of the animals. This result corroborates previous studies that used the same model of CP, which associates perinatal anoxia and sensorimotor restriction of the hind limbs (17,20,22). It is known that aggressions suffered from the early stages of life such as anoxia associated with sensory-motor restraint promote long-lasting effects such as reduction in body growth rate (17). In a study using the same CP model, the animals presented a reduction in the masseter muscle mass, the main masticatory muscle, with functional consequences for chewing as a lower frequency of masticatory cycles and lower food intake over a period of time (21). Simple activities such as feeding can be difficult on the PC resulting in food dysfunction due to movement disorders and changes in postural control (13). In children with CP, deficiencies in oral feeding are the main cause of inadequate nutrition that can lead to low growth, sub-optimal body fat reserves and poor general health status (13,31). The main problems are related to motor incapacity such as swallowing difficulties and airway protection problems, positioning difficulties requiring feeding assistance and prolonged feeding time (31). In addition, muscle atrophy, demonstrated in the present study by the reduction of muscle weight and the area and perimeter of muscle fibers, may also have contributed to the reduction of body weight in the animals of the PC group.

The administration of FGF-19 in the experimental PC, as well as in the control animals, maintained the body weight gain with the progression of the age of the animals, from the 22nd to the 29th day of life. This result is different from previous studies using this drug, which demonstrated the reduction in body weight in mice using the same route of application (23,32). It is known that FGF-19 participates in glucose and lipid homeostasis in the liver (32,33), which may be related to the preservation of energy reserves and maintenance of body weight. The role of FGF-19 includes the increase of protein and glycogen synthesis in the liver with functions

to suppress gluconeogenesis being similar to insulin (32,34,35). Although, in mammals, the highest concentration of glycogen is found in the liver, a large amount is stored in muscle tissue due to its wide distribution (36). In this context, the effects of FGF-19 on glucose metabolism could influence concomitantly in the musculoskeletal system, since this is also one of the largest glycogen stores, and is intimately related to the liver (36,37). Additionally, it is known that the muscle is a direct target for FGF-19 (23) in that its role could also be to act on muscle metabolism in addition to regulating its growth. Thus, it is possible that the administration of FGF-19 may have contributed to the improvement of muscle glycogen metabolism thus maintaining the energy reserves to be used according to the needs of the organism.

The PC also caused losses in the locomotor activity of the animals, as observed by the reduction in the distance traveled and average speed and increase of the stopped time during the test in the open field. This reduction in locomotion is consistent with previous studies that demonstrated that the CP model promotes physical changes that interfere with gait performance (17,18). Animals submitted to PC models present a delay in the transition from crawling to walking, an increase in the angle of the hind limb foot that is correlated with gait instability (38), and injury of white and gray matter in the brain resulting in deficiencies in motor skills (39). Since sensorimotor restraint alone leads to an increase in knee and ankle resistance and stiffness to passive elongation, and limited range of motion (17) aggravating the reduction in locomotion and exploratory ability in spontaneous activity.

Locomotion involves moving the center of mass of their bodies against gravity using their limbs, which depends on the degrees of freedom of the joints and muscular synergism, as well as the generation of motor commands for locomotion from the higher centers such as the cerebral cortex, integrating information visual, vestibular and somatosensory (40). Although the development of the gait pattern occurs in the rat around P15-P16 due to qualitative changes in postural control (41), the experimental CP impairs the motor development milestones interfering with the gait pattern in the medium and long term (24,42,43). In our results, we observed changes at 28 days, which corroborates with studies that used the same model of PC that causes lasting losses in locomotion, similar to what happens with children affected by the disease.

The damages in locomotor activity due to experimental CP were improved with FGF-19 treatment, since there was an increase in the distance covered and average speed, and a reduction in the time stopped at 28 days of life. The finding that the musculoskeletal is a direct target of FGF-19 is recent and its mechanism of action still needs to be better elucidated. It is known that the musculoskeletal has receptors and the co-receptor βKloto so that it can act to regulate the growth of muscle fiber (23). In this sense, the improvement of the musculoskeletal is fundamental for locomotion in which the intrinsic force, together with its architecture and metabolism, play important roles in controlling movement, facilitating adjustments to alter the motor demands (44,45). In addition to the action of FGF-19 on muscle atrophy, we have shown that this leads to an increase in the proportion of type IIa fibers and a decrease in type IIb fibers in the EDL muscle in animals submitted to CP. The type IIb fibers are characterized by being of intermediate (oxidative-glycolytic) metabolism (45), suggesting that there was greater resistance to fatigue during locomotion. Thus, it is assumed that FGF-19 acts with its possible role on muscle metabolism and attenuation of atrophy in the muscles of the hind limbs affected by CP, reaching locomotion-related motor function, improving motor performance.

In addition to the influence of muscle metabolism, motor coordination also interferes in locomotion, in which we demonstrated that the experimental CP promoted a poor coordination of the animals in the rotarod performance test. This result corroborates previous studies that used the same CP model (18) and studies that used other models that observed impairment in the motor performance of the animals (46). Coordination is related to movement control, including muscle synergy, in which the neural control activates the co-contraction of specific muscles resulting in force generation and movement in space including gait (47). However, it is established in the literature that motor control mechanisms are deficient in the PC, in which, for example, children with spastic CP exhibit desynchronization in the somatosensory cortex during motor planning and execution (39). In the rat the postural stabilization of the trunk is an important factor for the development of the coordinated movements between the trunk and limbs in order to reach the adult locomotion, which happens from the P15 in which the electromyographic activity in the long muscles of the back modulates more or less consistently with the cycle of steps (41). Thus, early changes in the control of movements associated with musculoskeletal changes characteristic of CP may have favored motor coordination

deficit. FGF-19 was able to improve locomotion and muscular parameters, but it was not enough to improve the motor coordination in the animals submitted to CP. Possibly, because of the FGF-19's performance, which is restricted to the musculature and peripheral organs failing to cross the blood-brain barrier (35,48) and consequently did not act in a central way that would be essential for motor coordination.

Regarding the analysis of the muscular strength of the anterior paws in the suspension test, no significant difference was observed between the groups. This result is in agreement with previous studies that used the same test in a CP model and in an intervention study analyzing the effects on neurodevelopment (26,49). Although this test set the analytical goal, no changes resulting from the experimental PC were observed at any of the ages in the present study. The application of the suspension test requires other aspects besides the strength of anterior limbs such as horizontal positional control, and gravity (50). It is also important to consider which adaptive mechanisms may have contributed to this result, since the anterior paws may have been hyperstimulated to compensate for the immobility and sensory deprivation of the hind paws.

In addition to the motor repercussions of the experimental PC, muscular alterations resulting from this were also evaluated in the present study. Consistent with previous studies, we observed the reduction of relative muscle weight, the area and perimeter of the soleus muscle fibers and EDL in the animals submitted to CP. The PC model causes damage to the muscles of the hind limbs of animals including this decline in muscle mass, previously demonstrated in studies with the same model (18,19,24). The sensorimotor restriction is the main factor that induces the reduction in the cross-sectional area of the muscle due to disuse atrophy, since it simulates the immobility induced by spasticity (18). The cross-sectional area of the muscle fibers during activity interferes in the generation of force in which muscles with longer fibers require a greater volume of muscle to perform their function (44), which may explain the reduction of locomotion and motor coordination observed in the present study .

Another important aspect for muscle function is the constitution of the types of fibers (51). In our study, we observed that PC animals had a higher proportion of type I fibers in the soleus muscle. Type I fibers are known to be slow-contracting and act in postural maintenance, being found widely in the soleus muscle because of its role in standing posture (51,52). Experimental PC may have further increased the

proportion of these fibers in the soleus because of the positioning of the hind limbs of the animals in extension during the period of sensory-motor restraint. In addition, the probable presence of spasticity, characteristic of the PC model used (17), may have contributed to stimulate the activation of the soleus in the extension of the hind limbs. In the EDL muscle, we confirmed that it is constituted in greater proportion by type II fibers at 29 days. Stigger et al using the same PC model also observed a higher proportion of type II fibers when analyzing the anterior tibial muscle (18). In the present study, CP promoted the increase of type IIb fibers and reduction of type IIa fibers. Studies that specifically investigate the proportion between type II fibers are scarce and their mechanism of action are not known, which limits the understanding of these data. This change in fiber types may suggest the repercussions of CP on the maturation of EDL muscle fibers, increasing the proportion of fast fibers with glycolytic metabolism and with greater potential for fatigue (45). And FGF-19 treatment in CP may have influenced positively in this aspect in which we observed opposite effect, increasing Type IIa fibers that are more resistant to fatigue than type IIb (45), which consistently favors the increase of locomotion.

Regarding the analysis of the tibia, the experimental CP caused a reduction in the absolute weight and in the absolute and relative length of the tibia, which is consistent with the reduction in body weight of the animals observed in this study. In addition, there was reduction of the cortical thickness, the medullary area in the tibial diaphysis, and reduction of the trabecular volume and increase of porosity in the proximal epiphysis, thus, suggestive of loss of bone mass. The reduction in the thickness of the cortical bone may reflect the decrease in the bone strength in the PC (53). Several factors are involved in the reduction of bone mineral density including low body weight, changes in motor skills and prolonged immobilization (53), these factors being demonstrated in the present study. The musculoskeletal also influences this process, since the mechanical forces directed by the muscles adjacent to the bone are important for the postnatal bone development (14). Thus, the reduction of body weight, reduction of locomotion and muscle alterations of the hind limbs, observed in the present study, may have contributed to this result. FGF-19 treatment promoted increased cortical thickness and trabecular bone volume, and reduced porosity suggesting that this factor may act on the bone indirectly through the muscle with its effect on the growth of muscle fibers (23). But this result raises the assumption that FGF-19 could also participate in the regulation of bone growth

directly. The literature is scarce in relation to the effects of FGF-19 on the musculoskeletal system, and its sites and mechanisms of action still need to be better elucidated. It is known that a member of the same family of growth factors, FGF-23 has actions on mineral metabolism that in excess causes hypophosphatemia, resulting in diseases such as osteomalacia, in which there is a decrease in mineralization of the cortical and trabecular bone (54 , 55). Therefore, as a proposal we could assume that FGF-19 could be one of the mechanisms for the homeostasis of this process acting contrary to FGF-23 in the PC.

In summary, we studied the effects of FGF-19 on the musculoskeletal and functional alterations of the experimental PC. From our results, we confirm our hypothesis of the beneficial effects of FGF-19 on musculoskeletal performance, with beneficial consequences for locomotion and bone structure, being important to arouse the interest of studies that propose to add new perspectives for the treatment of neuromusculoskeletal diseases , so that in future the quality of life of these children can be improved. We suggest new studies evaluating the effects of FGF-19 on muscle metabolism, investigations on the mechanism of action of FGF-19 in bone tissue and possibly in the joints. In addition, our results complement the knowledge of the repercussions of CP on locomotion, motor coordination and on the musculoskeletal system, using the animal model that is similar to what happens in children affected by CP.

ACKNOWLEDGEMENT

The authors thank Prof. Francisco Amanajás for his support in conducting the processing and analysis of the tibias and Propesq / UFPE.

CONTRIBUTION OF AUTHORS

A.E. Toscano, R. Manhães-de-Castro and H. Vidal designed the research and gave the intellectual assumption and orientation; S.C. Pereira and V.S. Souza performed the experiments and analyzed the data. S.C. Pereira and A. E. Toscano wrote the manuscript; All the authors involved reviewed and approved the final manuscript.

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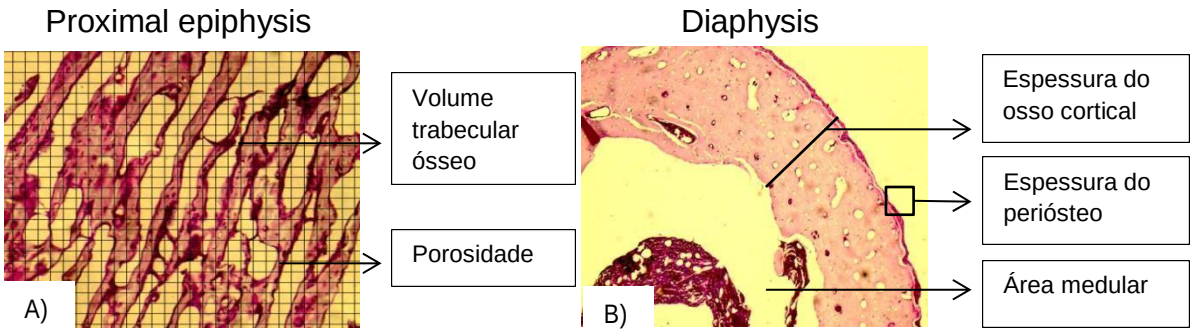


Figure 1: Morphometric analysis of the tibia. A: Photomicrography demonstrating the longitudinal cut of the proximal tibia epiphysis demonstrating the intersections that affect the trabecular bone (in pink) and on the cavities (in yellow) (40x); B: Photomicrography of the transverse section of the tibial shaft and its parameters analyzed cortical thickness, periosteal thickness and medullary area (100x).

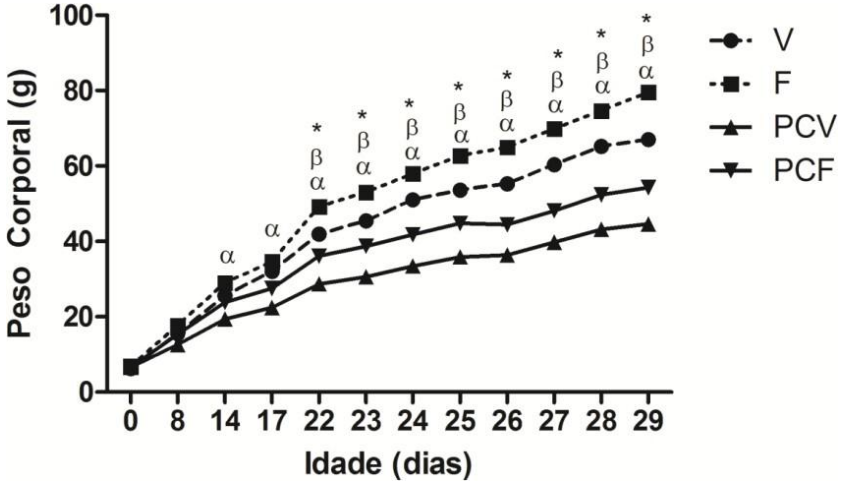


Figure 2: Weight evolution at the ages of 0, 8, 14, 17, 22-29 days postnatal life according to the experimental groups: V: Vehicle (n = 11); F: FGF-19 (n = 10); PCV: PC + Vehicle (n = 12); PCF: PC + FGF-19 (n = 13). Data are expressed as mean and standard error of mean, p <0.05. Two-way ANOVA repeated measures were used followed by the Tukey post-test, p <0.05. (PCV x CVα; CV xCFβ; PCV x PCF *)

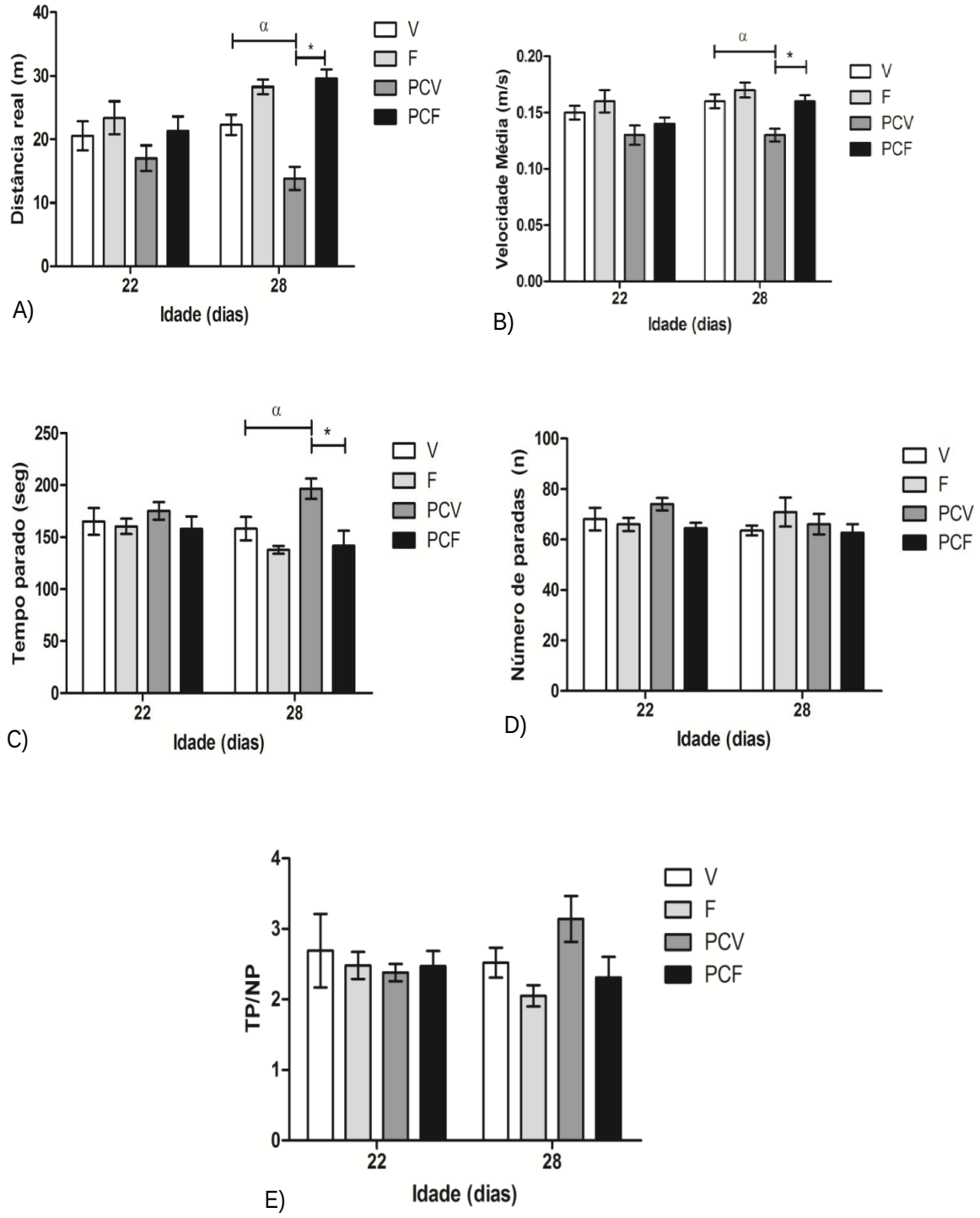


Figure 3: Locomotor activity at the 22nd and 28th day postnatal life according to the experimental groups: V: Vehicle (n = 10); F: FGF-19 (n = 10); PCV: PC + Vehicle (n = 10); PCF: PC + FGF-19 (n = 10). A) Actual distance traveled (m); B) Average speed (m / s); C) Stopped time (s); D) Number of stops (n); E) Time relationship stopped by number of stops (T / P) Data are expressed as mean and standard error of mean.

Two-way ANOVA repeated measures were used followed by the Tukey post-test, p <0.05. (PCVxCVα; CVxCFβ; PCVxPCF *)

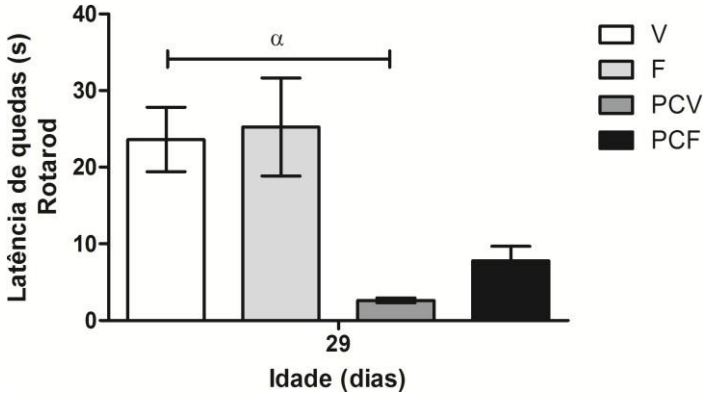


Figure 4: Motor coordination through the performance test in rotarod at 29 days of life according to the experimental groups: V: Vehicle (n = 10); F: FGF-19 (n = 10); PCV: PC + Vehicle (n = 10); PCF: PC + FGF-19 (n = 10). Data are expressed as mean and standard error of mean. Two-way ANOVA followed by Tukey post-test was used, p <0.05. (PCVxCVα; CVxCFβ; PCVxPCF *)

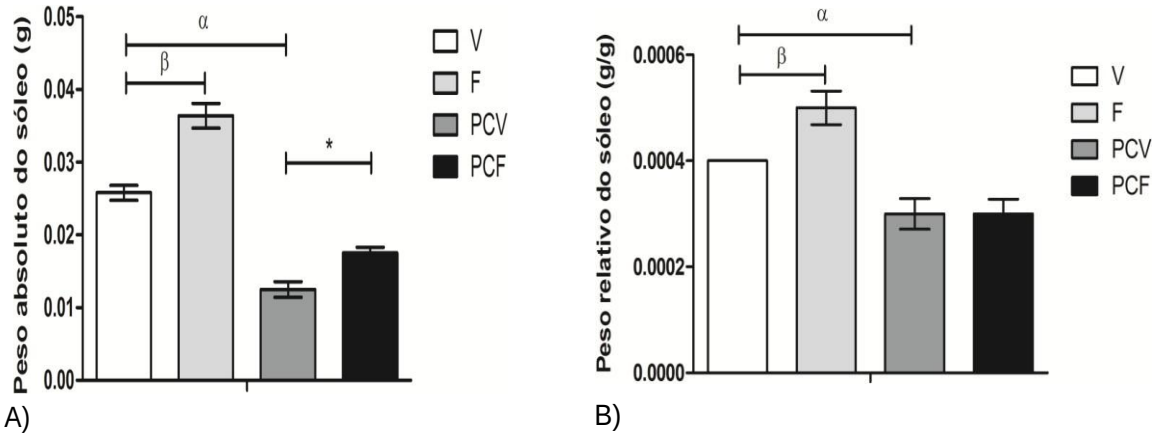


Figure 5: Soleus muscle weight at the 29th postnatal day according to the experimental groups: V: Vehicle (n = 11); F: FGF-19 (n = 10); PCV: PC + Vehicle (n = 12); PCF: PC + FGF-19 (n = 13). A) Absolute weight (g); B) Relative weight (g / g). Data are expressed as mean and standard error of mean, p <0.05. Two-way ANOVA followed by Tukey post-test was used, p <0.05. (PCVxCVα; CVxCFβ; PCVxPCF *)

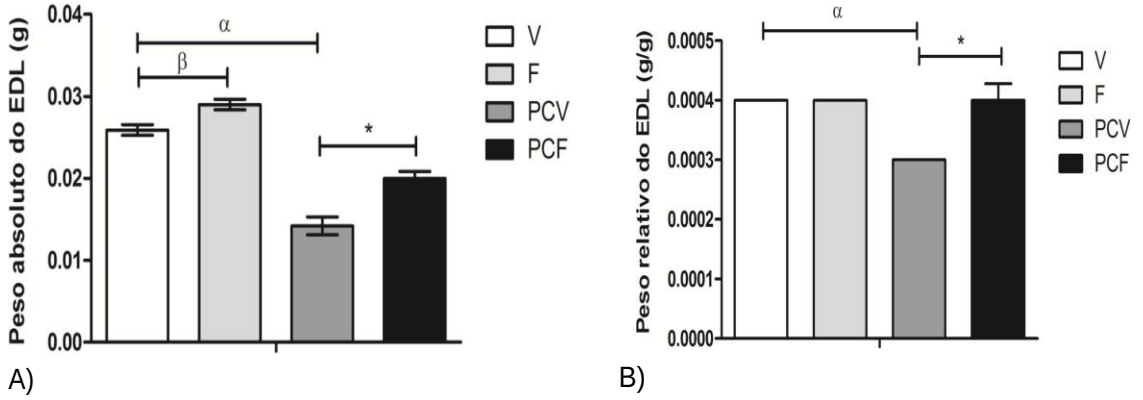


Figure 6: Weight of the EDL muscle at the 29th postnatal day according to the experimental groups: V: Vehicle (n = 11); F: FGF-19 (n = 10); PCV: PC + Vehicle (n = 12); PCF: PC + FGF-19 (n = 13). A) Absolute weight (g); B) Relative weight (g / g). Data are expressed as mean and standard deviation, p <0.05. (PCVxCVα; CVxCFβ; PCVxPCF *)

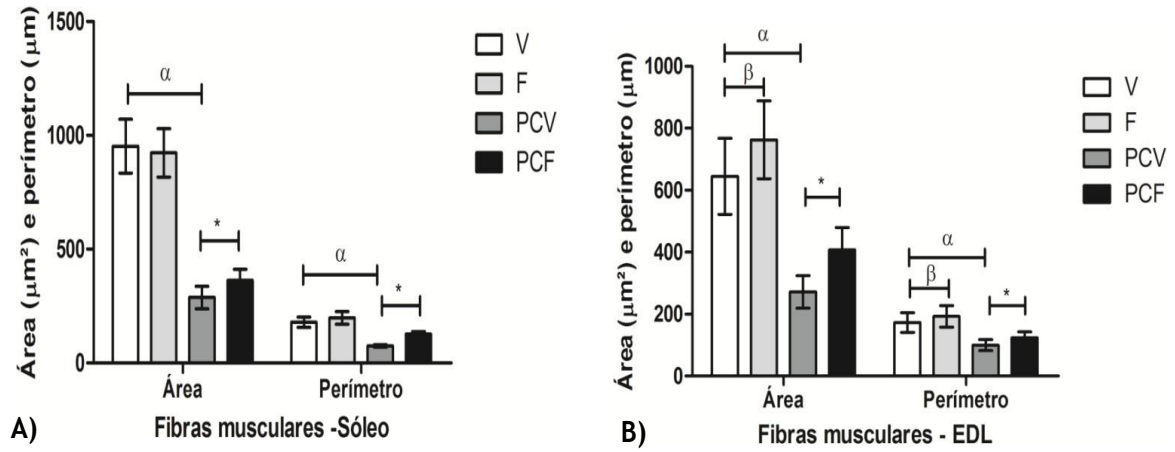


Figure 7: Area (µm²) and perimeter (µm) of muscle fibers at 29 days of life according to the experimental groups: V: Vehicle (n = 6); F: FGF-19 (n = 6); PCV: PC + vehicle (n = 6); PCF: PC + FGF-19 (n = 6). A) Soleus muscle; (B) EDL muscle. Data are expressed as mean and standard error of mean. Two-way ANOVA followed by Tukey post-test was used, p <0.05. (PCVxCVα; CVxCFβ; PCVxPCF *)

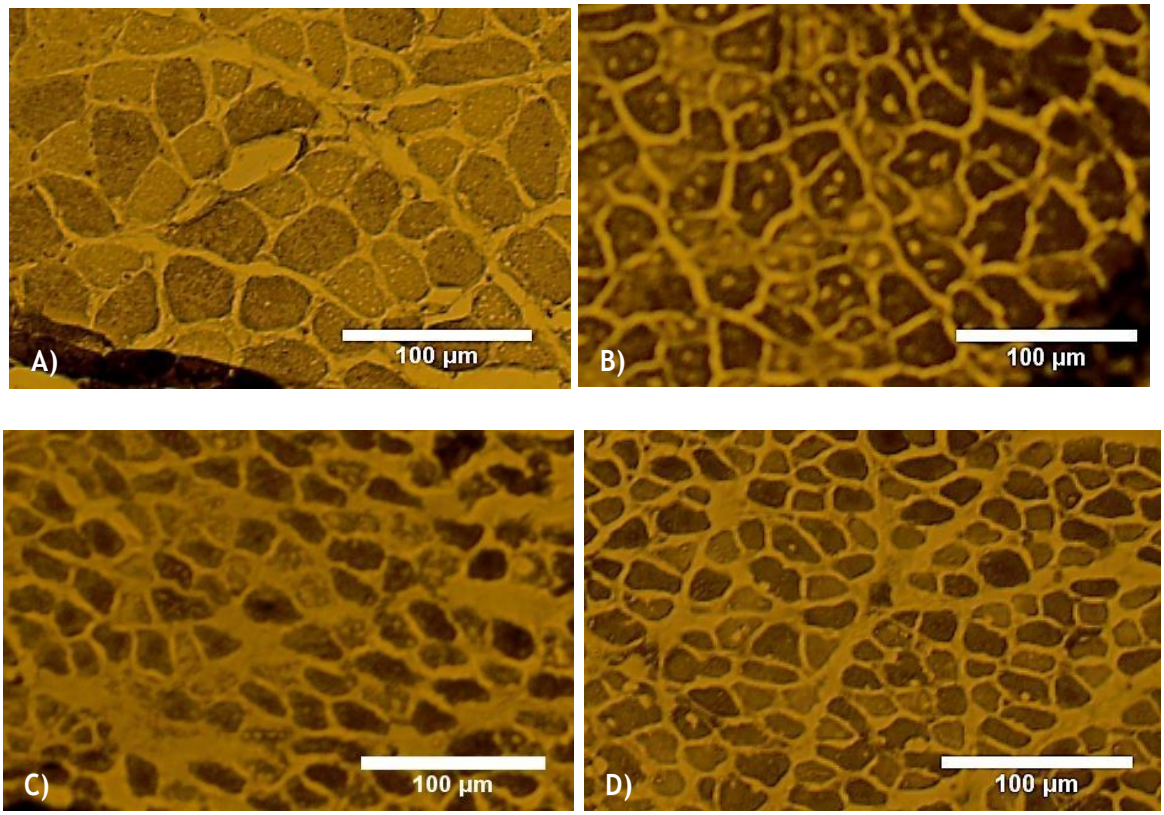


Figure 8: Photomicrography of the soleus muscle at 29 days, according to the experimental groups: A) V: Vehicle; B) F: FGF-19; C) PCV: PC + Vehicle; D) PCF: PC + FGF-19. Prepunched ATPase staining at pH 4.3, showing type I (darker) and type II (lightest) fibers.

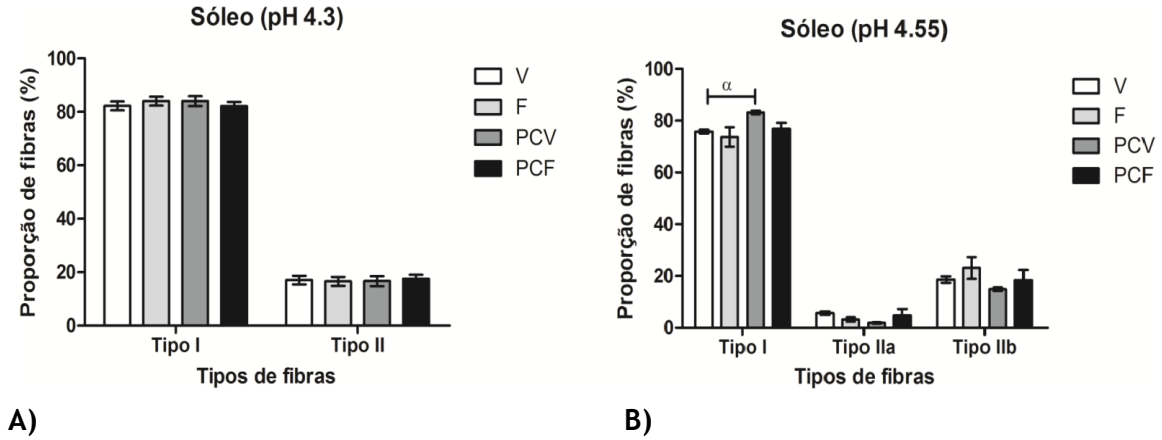


Figure 9: Proportion of muscle fiber types (%) in the soleus muscle at pH 4.3 (A) and pH 4.55 (B) at 29 days of life according to the experimental groups: V: Vehicle (n = 6); F: FGF-19 (n = 6); PCV: PC + vehicle (n = 6); PCF: PC + FGF-19 (n = 6). Data are expressed as mean and standard error of mean. Two-way ANOVA followed by Tukey post-test was used, $p < 0.05$. (PCVxCVα; CVxCFβ; PCVxPCF *)

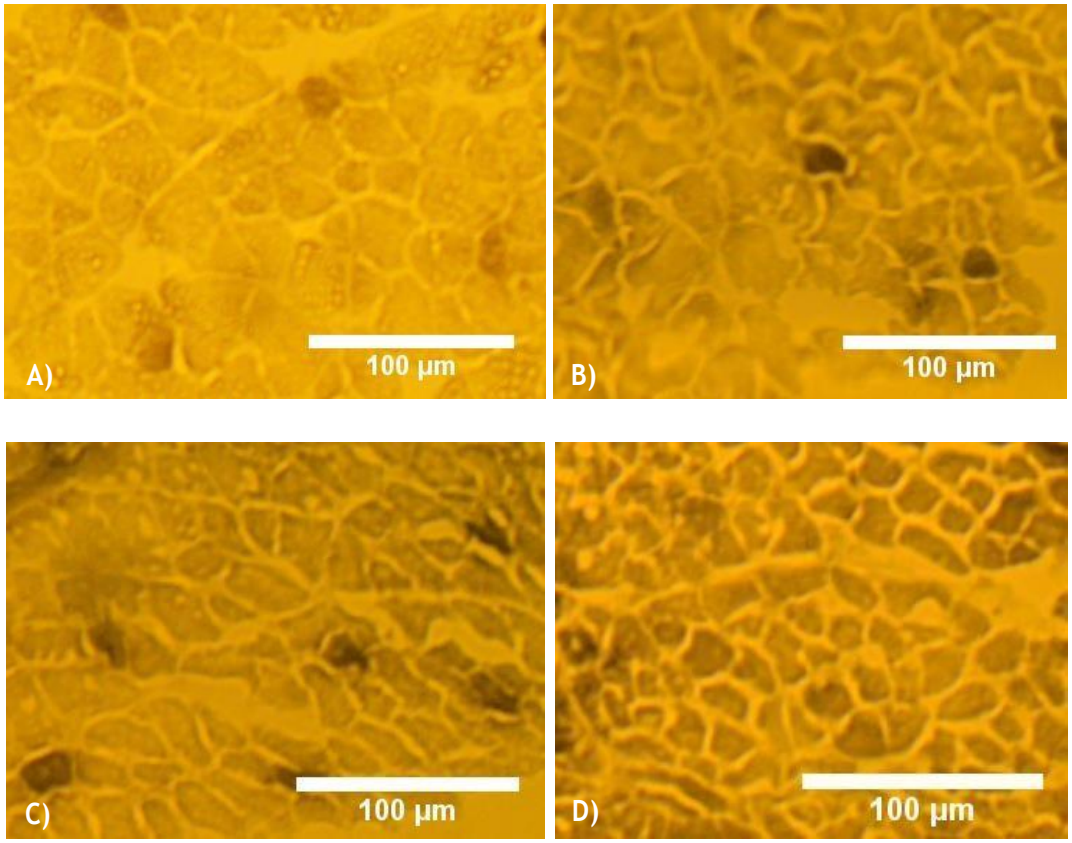


Figure 10: Photomicrography of the EDL muscle at 29 days, according to the experimental groups: A) V: Vehicle; B) F: FGF-19; C) PCV: PC + Vehicle; D) PCF: PC + FGF-19. ATPase staining in pre-incubation at pH 4.3, evidencing type I (darker) and type II (lightest) fibers.

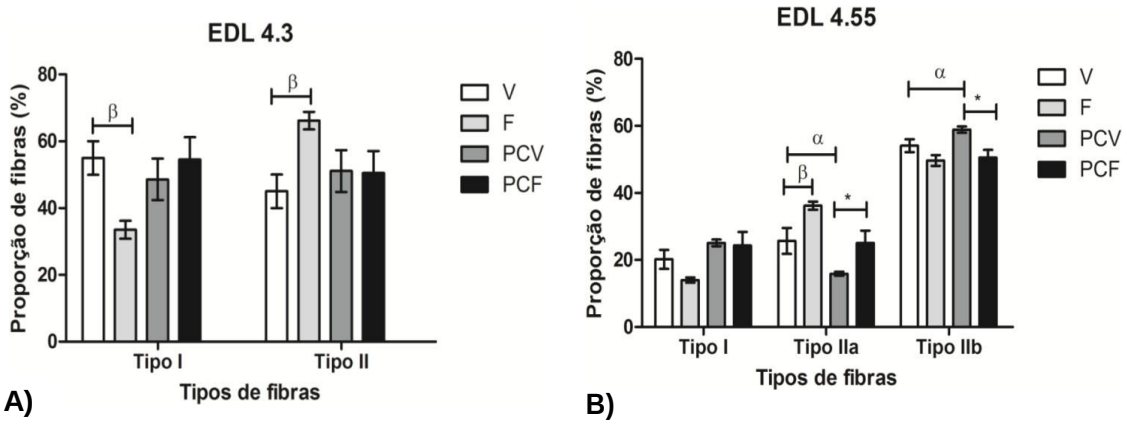


Figure 11: Proportion of muscle fiber types (%) in the EDL muscle at pH 4.3 (A) and at pH 4.55 (B) at 29 days of life according to the experimental groups: V: Vehicle (n = 6); F: FGF-19 (n = 6); PCV: PC + vehicle (n = 6); PCF: PC + FGF-19 (n = 6). Data are expressed as mean and standard error of mean. Two-way ANOVA followed by Tukey post-test was used, $p < 0.05$. (PCVxCVα; CVxCFβ; PCVxPCF *)

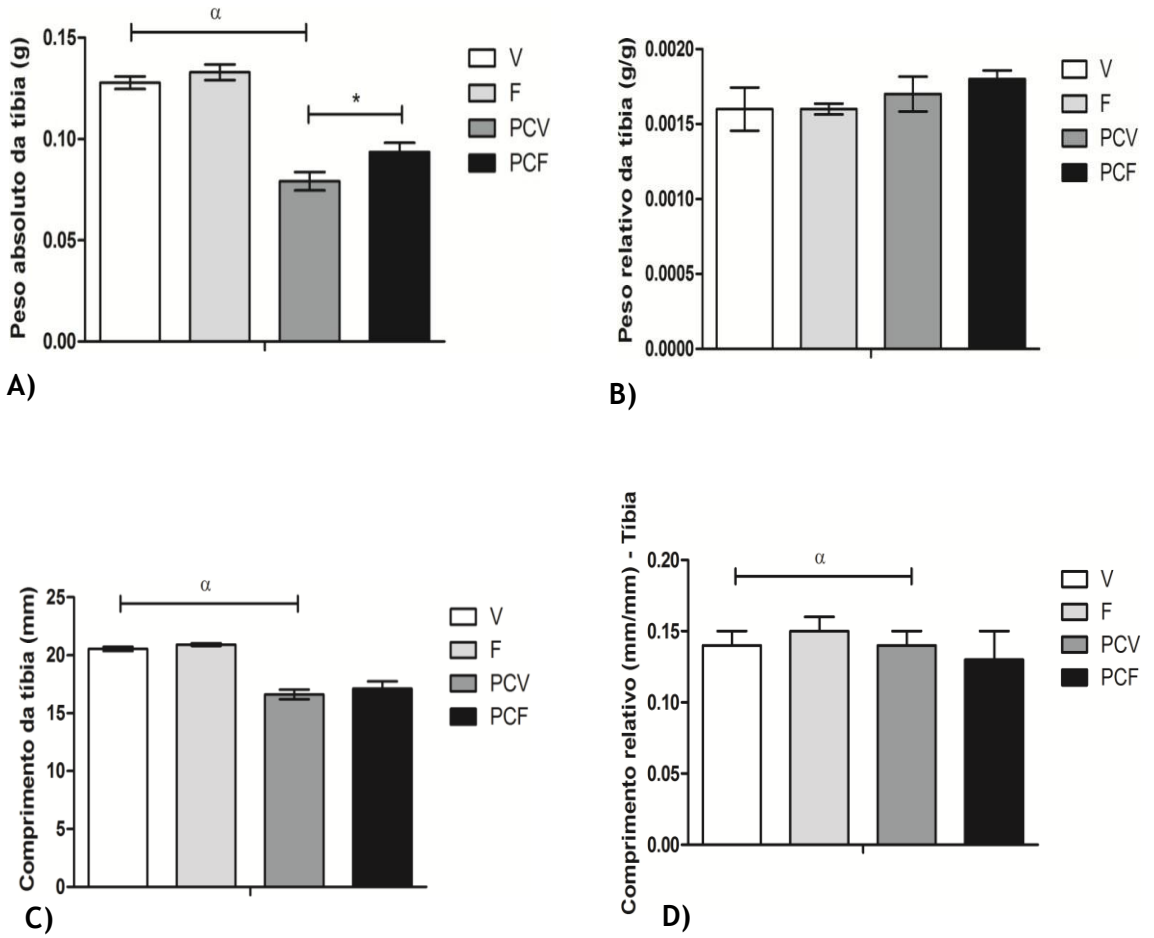
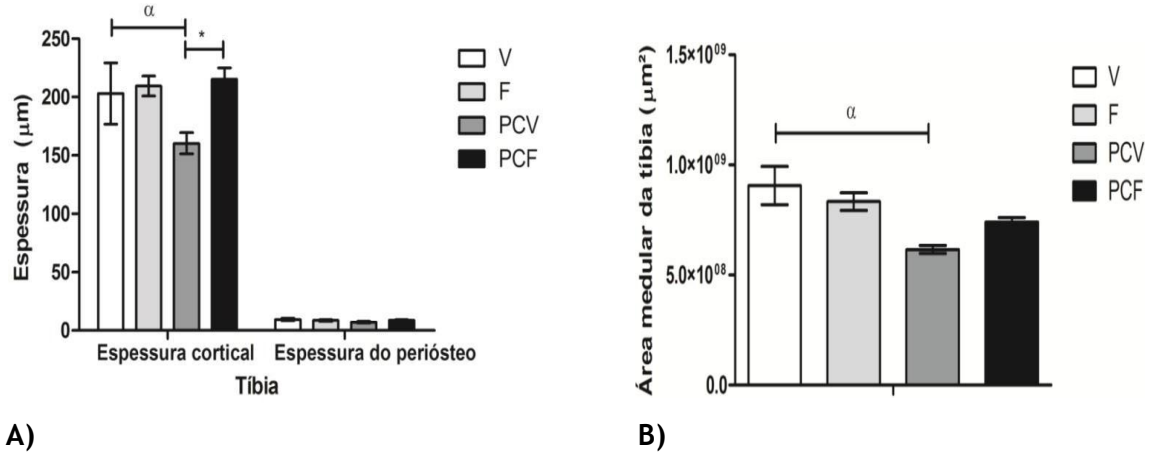


Figure 12: Anthropometry of the tibia at the 29th day postnatal life according to the experimental groups: V: Vehicle (n = 11); F: FGF-19 (n = 10); PCV: PC + Vehicle (n = 12); PCF: PC + FGF-19 (n = 13). A) Absolute tibia weight (g); B) Relative weight of the tibia (g / g); C) Length of the tibia (mm); D) Relative length of the tibia (mm / mm). Data are expressed as mean and standard deviation. Two-way ANOVA followed by Tukey's post-test, p <0.05 (PCVxCVα; CVxCFβ; PCVxPCF *) was used.



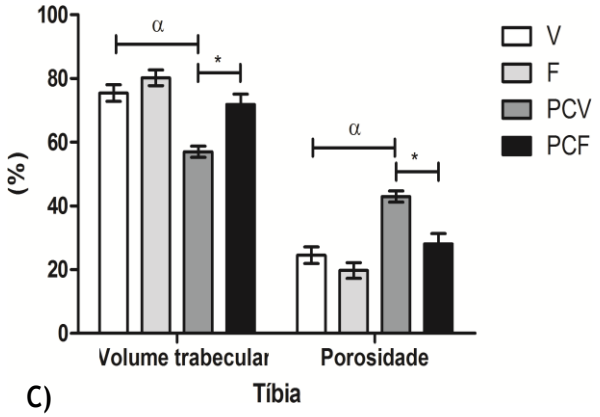


Figure 13: Morphometry of the tibia at the 29th day postnatal life according to the experimental groups: V: Vehicle (n = 6); F: FGF-19 (n = 7); PCV: PC + Vehicle (n = 5); PCF: PC + FGF-19 (n = 8). A) Cortical thickness and periosteal thickness (μm); B) Medullary area (μm); C) Trabecular volume and bone porosity (%). Data are expressed as mean and standard deviation. Two-way ANOVA followed by Tukey's post-test, p <0.05 (PCVxCVα; CVxCFβ; PCVxPCF *) was used.

APENDICE B — SELECTIVE SEROTONIN REUPTAKE INHIBITORS AFFECT STRUCTURE, FUNCTION AND METABOLISM OF SKELETAL MUSCLE: A SYSTEMATIC REVIEW

Pharmacological Research 136 (2018) 194–204

Contents lists available at ScienceDirect

Pharmacological Research

journal homepage: www.elsevier.com/locate/yphrs

Selective serotonin reuptake inhibitors affect structure, function and metabolism of skeletal muscle: A systematic review

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ARTICLEINFO

Chemical compounds studied in this article:
Serotonin (Pubchem CID: 5202)
Fluoxetine (Pubchem CID: 3386)
Citalopram (Pubchem CID: 2771)
Escitalopram (Pubchem CID:146570)
Fluvoxamine (Pubchem CID: 5324346)
Dexamethasone (Pubchem CID: 5743)
Insulin (Pubchem CID:70678557)

Keywords:
Serotonin uptake inhibitors
Serotonin
Skeletal muscle
Systematic review

ABSTRACT

Selective Serotonin Reuptake Inhibitors (SSRIs) may have side eff ects, such as stiff ness, tremors and altered tonic activity, as well as an increased risk of developing insulin resistance and diabetes mellitus. However, little is known about the structural, functional and metabolic changes of skeletal muscle after administration of SSRIs. The aim of this systematic review was to explore and discuss the eff ects of SSRIs on skeletal muscle properties described in human and rodent studies. A systematic search of PUBMED, SCOPUS, and WEB OF SCIENCE was performed. The inclusion criteria were intervention studies in humans and rodents that analysed the eff ects of SSRIs on skeletal muscle properties. The research found a total of six human studies, including two randomized controlled trials, one non-randomized controlled trial, one uncontrolled before-after study and two case reports, and six preclinical studies in rodents. Overall, the studies in humans and rodents showed altered electrical activity in skeletal muscle function, assessed through electromyography (EMG) and needle EMG in response to chronic treatment or local injection with SSRIs. In addition, rodent studies reported that SSRIs may exert eff ects on muscle weight, the number of myocytes and the cross-sectional area of skeletal muscle fibre. The results showed eff ects in energy metabolism associated with chronic SSRI use, reporting altered levels of glycogen synthase activity, acetyl-CoA carboxylase phosphorylation, citrate synthase activity, and protein kinase B Ser phosphorylation. Moreover, changes in insulin signalling and glucose uptake were documented. In this context, we concluded based on human and rodent studies that SSRIs aff ect electrical muscle activity, structural prop- erties and energy metabolism in skeletal muscle tissue. However, these changes varied according to pre-existing metabolic and functional conditions in the rodents and humans.

1. Introduction

Antidepressant drug use has increased over recent decades [1]. Specifically, selective serotonin reuptake inhibitors (SSRIs) are widely prescribed for a range of behavioural and psychiatric problems, such as depressive disorder treatment in teens, adults and particularly women during perinatal and postpartum [1,2]. This class of medication increases the extracellular level of serotonin by blocking serotonin

Abbreviations: SSRIs, selective serotonin reuptake inhibitors; EMG, electromyography; SERT, serotonin transporter; 5-HT, serotonin; 5-HT2A, serotonin receptor 2A; PRISMA, preferred reporting items for systematic review and meta-analyses; CAMARADES, collaborative approach to meta-analysis and review of animal data from experimental studies; PROSPERO, international prospective register of systematic reviews; MeSH, medical subject heading; SYRCLE, systematic review center for laboratory animal experimentation; CI, confidence interval; ROB, risk of bias; RCT, randomized clinical trial; GS, glycogen synthase CSP, cutaneous silent period; ARV, averaged rectified value; iSCI, incomplete spinal cord injury; CaPICs, calcium-mediated persistent inward currents; pACC, phosphorylated acetyl-CoA carboxylase; pPP2A, phosphorylated protein phosphatase 2A; NREM, non-rapid eye movement ROS, reactive oxygen species, CS, citrate synthase; EDL, extensor digitorum longus; PKB, protein kinase B; GLUT4, glucose transporter 4; RG, red gastrocnemius; Px, pancreactomized; SK channels, small conductance calcium-activated potassium channels; BDNF, brain-derived neurotrophic factor

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https://doi.org/10.1016/j.phrs.2018.09.004
Received 13 June 2018; Received in revised form 1 September 2018; Accepted 4 September 2018
Available online 05 September 2018

transporters (SERTs), resulting in an increase in the availability of serotonin in the synaptic cleft and may exert effects centrally and peripherally [3,4]. Biological substrates, receptors and pathways for serotonin are candidates that mediate not only the therapeutic actions of SSRIs but also their side effects [3].

SSRI exposure has been associated with side effects such as movement problems, rigidity [6], tremor [7] and altered tonic activity [8]; these are indications of serotonergic system involvement in motor activity [9]. Alternatively, there is concern about the alteration of the functionality of the immune system [10], as well as structural and metabolic abnormalities in various organs related to treatment with SSRIs [11]. Furthermore, some conditions, such as appetite disturbance, impaired weight gain and energy imbalance, may be related to structural changes in the hypothalamus after SSRI administration [5].

The serotonin system is suggested to play a role in a modulatory manner at the cellular and behaviour levels [6]. Evidence has shown that early exposure to drugs that change serotonin levels could alter serotonin circuitry, neurotransmission and behaviour-mediated 5-HT signalling, even later in life [7–9]. However, peripheral serotonin plays an important role in the regulation of glucose and lipid homeostasis [10]. In recent years, several studies have reported an association between the use of SSRIs and metabolic abnormalities [11–13] Moreover, studies have concluded that some serotonergic antidepressants may reduce hyperglycaemia, normalize glucose homeostasis and increase insulin sensitivity [14,15]. Conversely, impaired glucose tolerance is commonly observed in depressed patients treated with SSRIs, increasing the risk of developing insulin resistance and diabetes mellitus [16–18]. Nevertheless, there is a critical unanswered question concerning the responsiveness of skeletal muscle in these conditions, as well as which skeletal muscle properties are altered by SSRI administration.

Serotonin-mediated signalling in target cells occurs through specific receptors [19,20]. Evidence suggests that the functional serotonin 5-HT2 A receptor is expressed in rat myoblasts, activating intracellular phosphorylation, on the plasma membrane and at the level of T-tubules in contracting myotubes [21]. Conversely, it is believed that this receptor is located exclusively in the plasma membrane and is thus unlikely to be related to the muscle excitation-contraction process [22]. In response to altered 5-HT signalling, this receptor seems to influence myogenic differentiation and muscle glucose utilization [21]. Thus, the pharmacological manipulation by SSRIs might modify these important regulatory functions; however, the mechanisms are unexplored.

Therefore, it is necessary to know the different responses to antidepressant treatment in humans and rodents [23]. While studies on skeletal muscle plasticity have often used rodents as models, the results are often extrapolated to humans [24,25]. Furthermore, human and rodent studies have shown the impact of SSRI exposure on central structures, such as the hippocampus and hypothalamus [26] and peripheral organs as well as heart morphology [27], pulmonary haemodynamics [28] and bone homeostasis [29]. However, little is discussed about the functional, structural and metabolic changes in skeletal muscle following SSRI administration. In this context, the following focused question of this systematic review is "What are the effects of SSRIs on skeletal muscle properties of humans and rodents?". The aim of this systematic review was to explore and discuss the literature examining the effects of SSRIs on skeletal muscle properties in humans and rodents.

2. Methods

The author reports of this systematic review were performed based on the Preferred Reporting Items for Systematic Review and Meta-analyses PRISMA Statement [30]. Our review was carried out using a protocol published on CAMARADES and the PROSPERO database (registration number CRD42018081792).

2.1. Search strategy

Relevant articles were identified by searching the PUBMED (1966 - Jan 2018), SCOPUS (1950 - Jan 2018) and WEB OF SCIENCE (1900 - Jan 2018) databases. Searches were also performed by two independent reviewers hand searching for articles. Discrepancies that could not be resolved by discussion were resolved by an additional investigator. The search was conducted between November 2017 and January 2018. The list of references of the included articles was also assessed. Electronic databases were searched based on MeSH terms (Medical Subject Headings) and keywords: "serotonin reuptake inhibitors" or "serotonin uptake inhibitors" or "SSRI"; "skeletal muscle" or "muscle, skeletal" or "muscle striated". The initial phase of screening was based on the title and abstract in relation to the research question in studies using rodents or humans. The full-text screening was based on inclusion/exclusion criteria.

2.2. Inclusion criteria

The inclusion criteria of the included studies were primary studies, preclinical and controlled experiments that used rodent models with any sex and age at testing, exposure to SSRIs during pregnancy or prenatal, perinatal or postnatal exposure. In humans, intervention studies that measured skeletal muscle outcomes after treatment with SSRIs were included. Any method to assess the muscle skeletal properties after SSRI administration was included. The search has no language and publication date restriction. We excluded studies that did not report skeletal muscle outcomes or treatment with non-SSRIs (Table 1).

2.3. Data extraction

The data were extracted through reading titles, abstracts and full text in accordance with the inclusion and exclusion criteria by two independent researchers. The characteristics extracted from each included article were the name of the first author, year, title, human or animal model characteristics (strain, sex, age at testing), sample size, number of groups, type of intervention, administration method, dosage and measurements of muscle outcomes.

2.4. Assessment of methodology quality

Bias assessment was performed in rodent studies using the SYRCLE risk of bias (RoB) tool. This tool is specific to improving critical appraisal of evidence in animal studies [31]. Every study was assessed, and the judgement of each item was low, unclear or high risk. Random sequence generation, baseline characteristics, allocation concealment, random housing, blinding of participants and personnel, random

Table 1
Population-Intervention-Comparator-Outcome (PICO) statement.

Inclusion criteria	Exclusion criteria
Participants/Population (intervention studies) • Humans. • Rodents. Intervention • Exposure to SSRIs during foetal life or postnatal life. Comparators • Vehicle-only treatment control. Outcomes • Measurements of skeletal muscle properties. Publication Parameters • Original data. • Full-text available.	• All non-human and non-rodent animals and organisms, including wildlife and aquatic species. Genetically modified animals. • Exposure to non-SSRIs or SSRIs in combination with another drug. • No determination of outcomes with respect to skeletal muscle tissue. • No original data, e.g., editorials, reviews.

outcome assessment, blinding of outcome assessment, incomplete outcome data, selective reporting, and other sources of bias were the items judged. For human studies, the Cochrane risk bias tool [32] was applied in studies that presented at least a control group and described the risk (low, unclear or high risk) for selection bias, performance bias, detection bias, attrition bias, reporting bias, and other sources of bias. The Kappa statistics were performed to evaluate the inter-observer agreement for items of the risk of bias tool [33]. We synthesized the information in summary tables, in a figure and in a descriptive summary, enabling comparison of results. The Review Manager Software Package 5.3 was used to create figures of risk bias summary and the PRISMA flow chart diagram.

3. Results

A total of 2536 articles were identified through electronic database searching. A total of 1704 duplicates were removed. A total of 832 records were screened for title and abstract, 154 full texts were assessed for eligibility, and 142 were excluded because of the exclusion criteria. Twelve articles (six human studies and six rodent studies) were included in the final analysis. All of the studies were published in English. The search steps are shown in Fig. 1.

3.1. Assessment of quality of studies

The risk of bias was carried out using the SYRCLE RoB tool for rodent studies and the Cochrane ROB tool for human studies by two independent researchers. The results of the Kappa tests revealed substantial agreement, Kappa = 0.697, 95% CI (0.28–1.0) for the items of the tools. In the included human studies, the risk of bias was assessed in two randomized clinical trials (RCTs) [34,35] and only one non-randomized experimental study [36]. In these studies, only one reported a random sequence generation [35], while each one clearly described the allocation concealment. On the other hand, all studies reported that participants were blinded, even though blinding of the outcome assessment was not reported. In one study, it was unclear whether the author used the complete outcome data [34]. Each author clearly described selective reporting bias; however, these studies did not show other biases that warrants discussion (Fig. 2). The risk of bias in other human studies was not analysed since they are not clinical trials. The

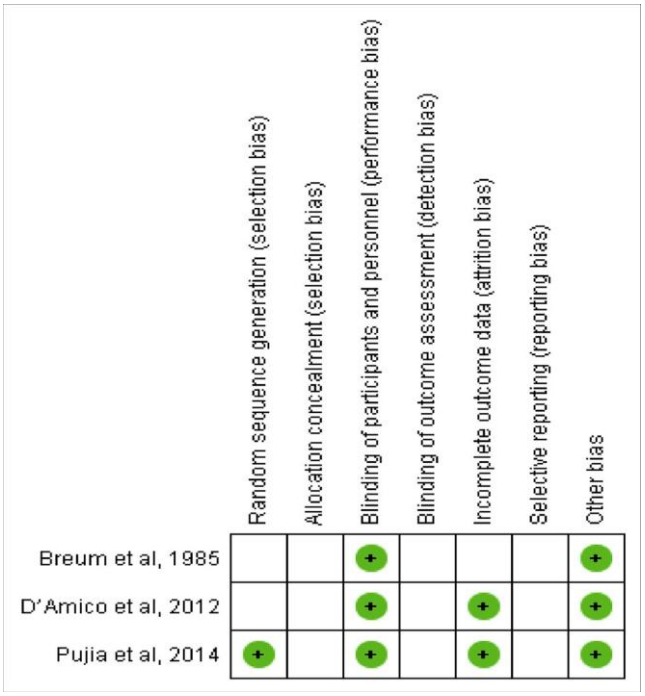

Fig. 2. Risk of bias summary of human studies: review authors' judgements about each risk of bias item for each included article. + (green) low risk of bias; - (red) high risk of bias; (uncoloured) unclear risk of bias. The Cochrane Risk bias tool was applied for studies that included at least a control group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

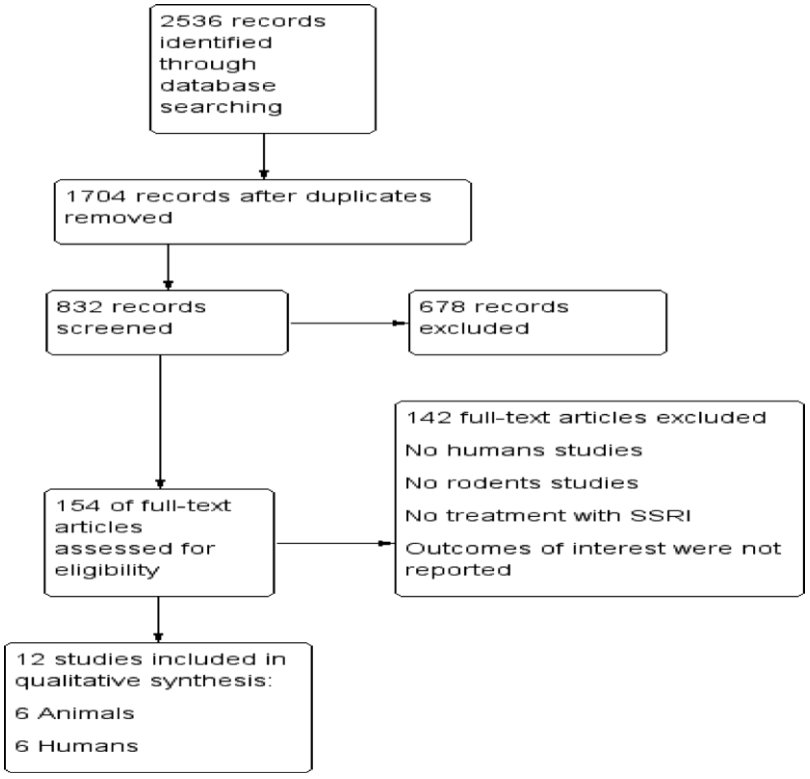

Fig. 1. Flow diagram of the study selection process. Selection of studies occurred in four phases: 1) Identification: potential studies were gathered from databases; 2) Screening: studies were selected for full-text assessment using titles and abstracts, based on PICO statement; 3) Eligibility: studies were selected for inclusion using *a priori* criteria; 4) Inclusion: studies were selected for qualitative analysis.

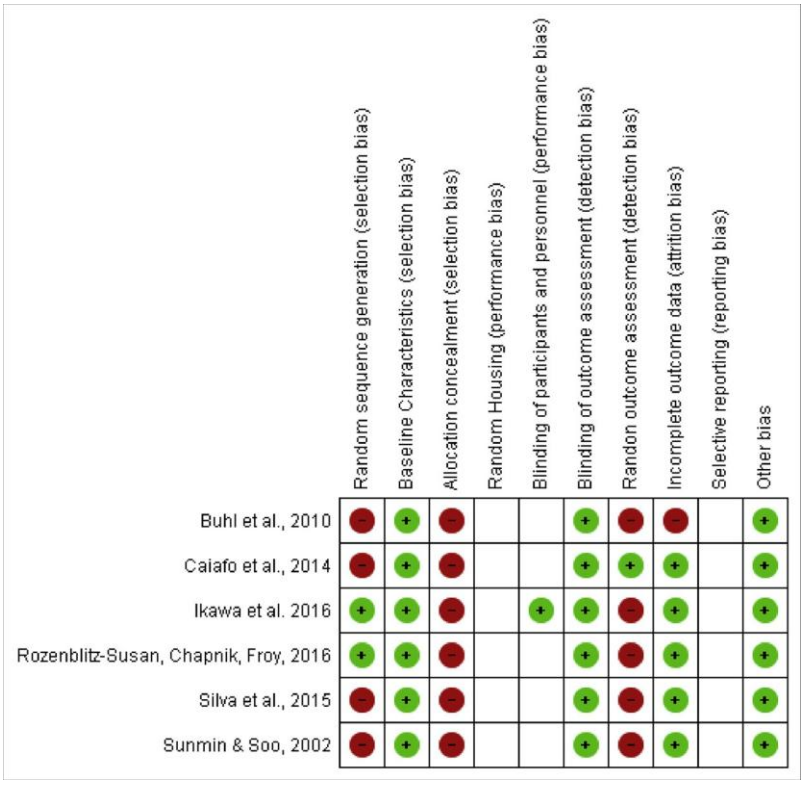

Fig. 3. Risk of bias summary of rodent studies: review authors' judgements about each risk of bias item for each included article. + (green) low risk of bias; - (red) high risk of bias; (uncoloured) unclear risk of bias. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

study of Chisari et al. (2009) is a study devoid of a control group (i.e., it is considered a before and after study design). Regarding the studies of Colomer (2006) [38] and Tarlaci (2004) [39], because they are case reports, it is not feasible to analyse the risk of bias. Most of the articles including rodents did not report adequate sequence generation and allocation concealment; there were two studies that performed a random sequence generation [40,41], however, the baseline characteristics of the included rodent studies were similar. One article selected the sample by random to assess the outcome [42]. One article reported the exclusion of animals because of assessment parameters [43]. All studies did not report random housing or blinding of the researcher or caregivers. Fig. 3 summarizes the risk of bias analysis in rodents (Fig. 3).

3.2. Clinical and methodological characteristics

Two human studies were randomized clinical trials [34,35], one was a non-randomized controlled trial [36], one was an uncontrolled before-after study [37], and two were case reports [38,39]. One RCT enrolled 40 obese subjects with non-insulin-dependent diabetes mellitus or with impaired glucose tolerance [34]. Another RCT enrolled 23 healthy subjects to elucidate the role of monoaminergic pathways in mediating a transient period of electromyographic (EMG) activity [35]. In one study [37], authors used as a sample, 3 patients with myotonic dystrophy type 1 to perform a needle and surface EMG. Another study [36] observed outcomes in 4 subjects with spinal cord injury (complete or incomplete) compared to 9 participants with no known neurologic injury or impairment to assess the constitutive monoamine receptor activity in the development of spasticity in human spinal cord injury [36]. The cases reported included a 58-year-old woman with jaw tremor [39], while another case report described a patient with congenital myasthenic syndrome [38] (Table 2). The rodent studies included animals at different ages, all were male animals, and all were compared with at least one control group. Two studies used young and young adult Wistar rats [42,44]. Two studies used 40- and 120-day-old Sprague-Dawley rats [43,45]. Finally, two studies used young adult C57BL/6 male mice [40,41] (Table 3).

3.3. Intervention

The human studies used different doses and routes of administration. Three studies used fluoxetine with daily doses between 40–60 mg/day [34,37,38]. Two studies used citalopram at a dose of 20 mg/day [36,39]. One study used escitalopram at a dose of 20 mg/day [35]. Oral dosing was used in 5 human studies, and only one study used a local injection dose of an SSRI [37] (Table 2). In rodents, the doses used were 1.4 mg/ml and between 10–100 mg/day. Three studies used fluoxetine [42,44,45], one study used escitalopram [43], one study used citalopram [40], and one study used fluvoxamine [41]. Two studies used the drug dissolved in water [41,45], three studies injected the drug subcutaneously [40,42,44], and one study applied the drug intraperitoneally [43] (Table 3).

3.4. Outcomes in skeletal muscle function

The RCT conducted by Pujia et al. (2014) showed in healthy subjects (n = 23; male/female) that a single oral dose of escitalopram (20 mg) induces an increase in the duration of cutaneous silent period (CSP) after 3 h ($p = 0.004$) as recorded by electromyography (EMG) after isometric contraction of the first dorsal interosseous muscle [35] (Table 2). Alternatively, Chisari et al. (2009) conducted a study to verify the effects of a muscular injection of fluoxetine on both needle EMG "myotonic runs" and on the surface EMG pattern in 3 patients affected by myotonic dystrophy type 1 [37]. The recordings were performed on the tibialis anterior. The resting electrical activity and the myotonic discharge were detected before and after the local injection of 100 μL of fluoxetine. Assessments from needle EMG showed that the injection of fluoxetine induced a clear reduction of the basal electrical activity and made it impossible to evoke "myotonic runs" in the patients tested [37]. Surface EMG was recorded after 300 μL of fluoxetine and showed that patients presented a clear and complete recovery of the normal increase of the typical averaged rectified value (ARV)

Table 2
Characteristics of the included human studies.

Author (Year)	Sample	Age assessment	Study design	Intervention/ Dose	Muscle outcomes
Pujia et al., 2014.	Healthy subjects	~ 32 years old	Randomized controlled trial	Single oral dose of escitalopram 20 mg	Increase in cutaneous silent period after an isometric contraction in the first dorsal interosseous muscle in the EMG assessment.
D'Amico et al., 2012.	4 subjects with incomplete spinal cord injury	Adults 23-61 years old	Non-randomized controlled trial	Oral dose of citalopram 20 mg	Long-lasting reflex responses (spasm) were facilitated on tibialis anterior and soleus muscle recorded by EMG
Chisari et al., 2009.	3 subjects with myotonic dystrophy type 1	25-38 years old.	Uncontrolled before-after study	Local injected solution of fluoxetine, 100 and 300 μL at a final concentration of 10 μM.	Reduction of basal electrical activity of tibialis anterior assessed by needle EMG. Normalized sarcolemma excitability recorded by surface EMG.
Colomer et al., 2006.	Patient with congenital myasthenic syndrome	15 years old.	Case report	Fluoxetine 40 mg/day gradually increased to 60 mg/day over two months.	Increased amplitude of muscle action potential, decreased response in abductor pollicis brevis, improvement of strength and endurance and activities of daily living.
Tarlaci, 2004.	Woman with jaw tremor	58 years old	Case report	Citalopram 20 mg/day	No activity other than the rhythmic activity associated with the tremor obtained in masseter, digastric and orbicularis oris muscles examined by needle EMG
Breum et al., 1985.	40 obese (M/F) participants with non-insulin-dependent diabetes mellitus.	43.6 years old	Randomized controlled trial	Fluoxetine (60 mg/ day) or placebo	Total glycogen synthase activity increased of vastus lateralis muscle.

Table 3
Characteristics of included rodent studies.

Author (Year)	Strain	Age assessment	Groups	Intervention/ Dose	Muscle outcomes
Rozenblitz-Susan, Chapnik, Froy, 2016.	C57BL/6 male mice	84 days	Experimental and control	Fluvoxamine (9 mg/kg/day) dissolved in water or placebo (water) for three weeks	Increased levels of phosphorylated acetyl CoA carboxylase on C2C12 myotubes. Reduced fatty acid synthesis as a result of increased b-oxidation. No overall change in lipid content in muscle cells.
Ikawa et al., 2016.	C57BL/6 male mice	49-70 days	3 groups: Experimental (Cit10), Experimental (Cit100), and Control	Subcutaneous osmotic minipump with citalopram (10 mg/kg/day), citalopram (100 mg/kg/day) or placebo (saline, NaCl 0.9%) for 6 days	Higher dose increased the time engaged in masseter EMG activity in the second half of the dark period and the first half of the light period during non-rapid eye movement (NREM) sleep.
Silva et al., 2015.	Male Wistar rats	60 days	Experimental and control during weaning period.	Subcutaneous injection of fluoxetine (10 mg/kg) or placebo(saline, NaCl 0.9%), during suckling period.	Increased citrate synthase activity, increased mitochondrial respiration and decreased reactive oxygen species of extensor digitorum longus muscle.
Caiafo et al., 2014.	Male Wistar rats	30 and 90 days	Experimental and control, during weaning period	Subcutaneous injection of fluoxetine (10 mg/kg) or placebo (saline, NaCl 0.9%), during suckling period.	Reduction of the weight, number of myonuclei in the muscle fibre and reduction of cross sectional area in soleus and lateral gastrocnemius.
Buhl et al., 2010.	Pregnant and male Sprague-Dawley rats	40 days	Mother treated with dexamethasone or saline 3 groups: control, low birthweight saline, LBW-ESC	Injected intraperitoneally twice a day with either escitalopram 1.4 mg/ml or placebo (saline)	Rates of insulin-stimulated glucose uptake in red gastrocnemius muscle were recovered by escitalopram exposure. Increased protein kinase B Ser phosphorylation and improvement in insulin signalling in muscle of low birthweight rats.
Sunmin & Soo, 2002.	Male Sprague-Dawley rats	120 days	Pancreatectomized (Px) or sham surgery. Both of the Px and sham rats were divided 2 groups: FXTN or placebo	Orally fluoxetine 5 mg/kg. Placebo: mint-flavoured water over 8 weeks	Increased glycogen deposits, decreased triglyceride content and increased glucose uptake of the soleus muscle.

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curve after injection [37]. The authors concluded that the local application of fluoxetine produces functional modifications in myotonic dystrophy type 1 muscle electrical properties, encouraging further therapeutic approaches in this disease [37] (Table 2). D'amico et al. (2012) used incomplete spinal cord injury (iSCI) and uninjured (as a control) subjects and administered a 20 mg oral dose of citalopram. The authors suggested that in incomplete spinal cord injury, citalopram facilitated muscle spasm, mediated by calcium-mediated persistent inward currents (CaPICs) and assessed through the surface EMG [36]. In the control group, which had intact descending 5-HT axons, citalopram was effective in increasing the PIC-mediated spasm. If subjects with iSCI have some residual descending monoaminergic axons, a 20 mg dose of citalopram should be effective in increasing the PIC-mediated long- lasting reflex (spasm) [36] (Table 2). Finally, two case reports were included: Tarlaci (2004) showed a case of jaw tremor after 20 mg/day of citalopram. A needle EMG investigation was carried out to examine the masseter, digastric and orbicularis oris muscle. The authors report no activity other than rhythmic, harmonic and continuous activity as- sociated with the tremor in these muscles. This activity was compared to other muscles of the region (i.e., deltoid, trapezius and sternoclei- domastoid muscle) that did not display such rhythmic activity and were all silent at rest. This was the first report in the literature of a transient jaw tremor associated with citalopram use, even though authors be- lieved that this occurrence can be explained by an indirect inhibitory effect on central dopaminergic activity [39] (Table 2). In a case of a 15- year-old patient, who was diagnosed with congenital myasthenic syn- drome early in life, fluoxetine (40–60 mg/day) therapy was started and gradually increased over two months, and the subject showed an in- creased amplitude of the compound of muscle action potential, a de- creased response in abductor pollicis brevis, and improvement in muscle strength and endurance, as well as better performance in daily activities (Table 2). However, the authors believe that the increase in strength observed in the patient may have resulted from a direct effect of fluoxetine at the endplate of the patient [38] (Table 2). Ikawa et al. (2016) [45] investigated the effects of chronic administration of cita- lopram on sleep/wake cycle and masseter muscle electromyogram ac- tivity over a 24-hour period in C57BL/6 J mice. Through a sub- cutaneous minipump, citalopram (10 mg/kg/day or 100 mg/kg/day) or saline was administered in different groups [45]. The authors con- cluded that citalopram did not modify mean masseter EMG activity during any of the vigilance states and did not affect temporal changes related to the shifts between dark/light periods; however, citalopram increased the time engaged in masseter EMG activation during non- rapid eye movement (NREM) sleep in the second halves of the dark and light periods [40] (Table 3).

3.5. Outcomes in skeletal muscle structure

Caiafo et al. (2014) [42] observed a reduction in the number of myonuclei in the soleus and lateral gastrocnemius at 30 and 90 days old after chronic neonatal treatment with 10 mg/kg/day fluoxetine. The muscle cross-sectional area was also reduced in animals treated with fluoxetine both early and later phases of development [42] (Table 3).

3.6. Outcomes in skeletal muscle metabolism

The RCT conducted on obese subjects (male and female) with non-insulin-dependent diabetes mellitus with a mean age of 43.6 years showed that after 12 months, patients who received 60 mg/day of fluoxetine, compared to the placebo group, had total skeletal muscle glycogen synthase (GS) activity increased by 31% ($p < 0.01$) in biopsies of vastus lateralis muscle. Even after adjustment for fasting glucose, insulin, weight loss and diabetic state, a positive effect of fluoxetine remained on the total GS activity. Thus, the authors concluded that fluoxetine seemed to improve insulin sensitivity beyond the effects mediated through weight loss by possible effects on GS activity in skeletal muscle tissue [34] (Table 2). Rozenblit-Susan et al. (2016) [46] studied the effect of fluvoxamine on cell differentiation to myotubes and metabolic genes and protein expression in mouse muscle. At 60 days of age, enzymatic calorimetric tests, extraction of RNA from muscle, lipid content quantification, quantitative real-time PCR and Western blot analysis were performed [46]. There was an increased level of phosphorylated acetyl CoA carboxylase (pACC) on C2C12 myotubes in mice after fluvoxamine (9 mg/kg/day; dissolved in water) administration, which could indicate reduced fatty acid synthesis as a result of increased β-oxidation [41]. These results showed that there was no overall change in lipid content in muscle cells, probably due to the high pACC on the one hand and low phosphorylated protein phosphatase 2 A (pPP2 A) on the other hand [41] (Table 3). Silva et al. (2015) [44] studied the effects of chronic treatment with fluoxetine (10 mg/kg) injected subcutaneously in male Wistar pups during the suckling period on the mitochondrial bioenergetics of the skeletal muscle of young rats. The enzymatic activity, mitochondrial protein concentration, mitochondrial oxygen consumption, mitochondrial reactive oxygen species (ROS) production, mitochondrial pore opening, and oxidative stress evaluation were determined in muscle samples [49]. The results indicated that increasing serotonin at synaptic terminals early in life resulted in increased citrate synthase (CS) activity, increased mitochondrial respiration and decreased ROS production in extensor digitorum longus (EDL) muscle in 60-day-old male rats after fluoxetine treatment [49]. Neonatal fluoxetine treatment caused higher mitochondrial respiration rates and decreased ROS levels in the EDL muscle of young adult male rats [49]. Associated with this occurrence, the EDL muscle showed more resistance to calcium-induced mitochondrial pore opening [49] (Table 3). Buhl et al. (2010) [43] studied the effects of escitalopram (1.4 mg/ml) injected intraperitoneally to restore insulin sensitivity in low birthweight rats. These authors evaluated estimated rates of insulin-stimulated glucose transport activity in muscle tissue, glycogen levels, protein kinase B (PBK) Ser phosphorylation expression levels and total crude membrane GLUT4 contents in skeletal muscle at 40 days old [43]. The results showed that an SSRI could change metabolic processes and signalling of insulin [43]. Insulin caused an increase in red gastrocnemius (RG) PKB Ser phosphorylation, suggesting that insulin resistance can be reversed by treatment with escitalopram [43]. Rates of insulin-stimulated glucose uptake in RG were normalized with escitalopram treatment in low birthweight rats, but this was not observed in white gastrocnemius muscle tissue [43]. Glycogen content and GLUT4 protein were similar in all groups of the study, with RG muscle tissue showing the lowest glycogen content and the highest GLUT4 protein concentration compared to white gastrocnemius [43]. These results suggested that escitalopram administration leads to a tissue-specific improvement in insulin signalling in RG muscle of low birthweight Sprague-Dawley rats [43] (Table 3). Insulin and glucose uptake was evaluated after oral fluoxetine (5 mg/kg) treatment in the study of Sunmin & Soo (2002) [46]. Muscle tissue was used to determine glucose uptake and glycogen and triglyceride contents [46]. This study showed that glycogen deposits in the soleus muscle were affected by fluoxetine administration and diabetes status in pancreatectomized (Px) rats or after sham surgery in male Sprague-Dawley rats at 120 days old [46]. Fluoxetine treatment increased glycogen deposits more than placebo in the soleus muscle of sham and Px rats [46]. Triglyceride content decreased with fluoxetine administration in sham and Px rats compared to placebo [46]. Glucose uptake was higher with fluoxetine administration than placebo in sham and Px rats [46] (Table 3).

4. Discussion

4.1. Main findings

In this paper, we aimed to summarize clinical and preclinical evidence of skeletal muscle property changes due to SSRI exposure. To our

knowledge, this systematic review is the first to summarize and appraise the current evidence on the effects of SSRI administration on skeletal muscle properties in humans and rodents. Based on the included articles, this review showed that skeletal muscle is responsive to the influences of SSRI administration, with alterations in functional, structural and metabolic properties. Nevertheless, our conclusion is based on low numbers and levels of evidence in publications from the human and rodent literature. Here, all available literature up to January 2018 was systematically searched.

Overall, the included articles that used humans as a sample showed alterations in electrical activity produced by skeletal muscle after SSRI administration. The activity of serotonin neurons is positively correlated with the level of behavioural arousal, which, in turn, appears to be related to the level of tonic motor activity (muscle tone), especially in muscle groups associated with gross motor function [47]. Interestingly, in humans, these drug effects are primarily restricted to motor signs (e.g., myoclonus and tremor) [48]. In addition, it appears that the 5-HT projections to the spinal cord increase its activity with improvements in motor response [49]. Furthermore, 5-HT has an important regulatory role on the excitability of spinal motoneurons influencing spinal reflexes and the input-output gain mechanisms in motoneurons, allowing better activation of motor units and, consequently, motor gain control [50–52].

Therefore, in healthy subjects, the increase in the cutaneous silent period can be explained by the inhibition of descending corticospinal tracts on the excitatory inputs of the motoneurons that maintain voluntary muscle contraction [35]. It may be a benefit in cases of incomplete spinal cord injury that subjects present a reduction in the duration of the CSP [53]. In cases of spinal cord injury, for example, muscle spasm is mediated by the activation of 5-HT2 receptor [54]. So drugs that selectively block these constitutively active monoamine receptors may provide better control of spasticity in skeletal muscle, especially in motor complete spinal cord injury where reducing motoneuron excitability is the primary goal [54]. In contrast, in cases of iSCI, an oral dose of citalopram increased the muscle spasm, assessed in tibial anterior muscle, but without statistical significance [36]. However, further research is needed to understand the influence of SSRIs on CSP in cases of iSCI.

Inhibiting the reuptake of serotonin in the central nervous system can also affect the peripheral tissues [4]. Although, the peripheral effects of direct application of these drugs in muscle tissue are largely unexplored. Therefore, to date, the use of intramuscular administration of fluoxetine has been examined in only one study, in which fluoxetine transiently modified the sarcolemmal excitation [37]. It is believed that fluoxetine's action on human SK channels (small conductance calcium-activated potassium channels) is responsible for this occurrence because fluoxetine is reported to affect different ion channels in different tissues and cells [55,56]. In this sense, the reduction of persistent contractions induced by myotonia could decrease muscle fibre dys- function through the lower calcium influx observed in tibialis anterior muscle after intramuscular injection of fluoxetine [37]. Even though fluoxetine is reported to affect the function of vascular tissue in skeletal muscle [57], there is no consensus on the contractile influence of these vessels on the rat muscle, but it is believed that there is a role in the sensitivity changes of Ca2+ [57]. It may be that the effects of SSRIs could normalize the calcium influx in cases of myotonia and reduce fibre dysfunction, presenting a potential objective of further research mainly in subjects with myotonic dystrophy type I [58].

In the same line, fluoxetine may reduce the pathological characteristics of slow-channel congenital myasthenic syndrome, which is associated with a cationic overload of the endplate, subsequent degeneration of the neuromuscular junction, and eventually, endplate myopathy [59]. Due to the diverse clinical presentation of the human subjects, different doses, routes of administration, and reduced quality of evidence, in addition to the different muscle outcomes presented in the included studies, the mechanisms by which SSRIs influence skeletal muscle function in humans seems to be discrepant; however, in regard to skeletal muscle function, the magnitude of the problem or benefit should be based on and interpreted according to the clinical presentation of subjects. Meanwhile, these mechanisms need to be further investigated.

However, structural changes in muscle may be associated with reduced weight gain observed after chronic SSRI exposure [60]. The impact on body weight caused by chronic fluoxetine exposure is associated with higher energy expenditure, increased sensitivity to the acute leptin system and increased hypothalamic brain-derived neurotrophic factor (BDNF) expression. Additionally, the reduction in white adipose tissue caused by fluoxetine exposure was related to changes in peripheral organs [60]. Reduction of the number of nuclei of myocyte cells and cross-sectional area of muscle fibre can be linked with the lean phenotype observed in young and adult animals after neonatal exposure to 10 mg/kg of fluoxetine [42,61]. In addition, a possible hypophagic response may have a direct consequence on the nutritional supply required for muscle development resulting in lower muscle weight even in adult rats [61]. The lean phenotype caused by chronic exposure to fluoxetine may be related to positive modulation of uncoupling protein and mitochondrial bioenergetics of brown adipose tissue [63]. It is possible that this occurs due to the increase of hypothalamic brain-derived neurotrophic factor (BDNF) caused by fluoxetine administration [60].

The involvement of serotonin in the muscle maturation process was evidenced in an *in vitro* study. The authors noted that serotonin may promote muscle growth due to the inhibition of myostatin, which is negatively related to the regulation of muscle mass [64]. Faced with this evidence, fluoxetine may cause muscle mass decrease. If this is the case, SSRI chronic exposure may cause a negative effect on skeletal muscle structural properties in subjects without functional comorbidities, leading to reduced muscle mass [42]. In addition, studies in rodents and humans utilizing SSRIs demonstrated structural changes in other organs such as brain, lung and heart [27,65,66]. Neonatal treatment with fluoxetine decreased heart weight, the cross-sectional area of the heart, and the cross-sectional area and perimeter of the cardiac cells [27]. It is interesting that the differences in cardiac morphology are associated with lower body weight [27]. Finally, as peripheral serotonin may play an important role in the relief of obesity, shifting the profile of muscle fibre type from fast to slow and accelerating energy consumption in skeletal muscle [67], the influence of SSRIs on skeletal muscle phenotype may present new possibilities for the development of interventions strategies for metabolic diseases such as obesity.

Although there is a concern about the metabolic effects of long-term antidepressant exposure, contradictory findings regarding the association between SSRI and metabolic state are presented in the literature [11]. Skeletal muscle is recognized as the primary site for glucose uptake and storage and is often involved in various metabolic diseases [68,69]. However, little is known about what occurs in this tissue in individuals with metabolic comorbidities who use SSRIs. This systematic review revealed an article in which chronic fluoxetine administration (60 mg/kg/day) resulted in total glycogen synthase activity in vastus lateralis muscle [34]. Fluoxetine administration seemed to improve insulin sensitivity and glucose uptake, beyond the effect mediated through weight loss, by a possible effect on GS activity of skeletal muscle tissue in obese subjects with non-insulin dependent diabetes [34]. It is increasingly recognized that treatment with SSRIs improves glucose homeostasis in nondiabetic depressed patients in the short term [70].

Serotonin may be involved in the control of glucose uptake and reduced circulating levels of blood glucose via the 5HT2 A receptor, [71]. Corroborating this idea, one included rodent study showed increased glycogen deposits and higher glucose uptake with chronic fluoxetine (5 mg/kg) administration compared to placebo administration in sham and Px rats [46]. The long-term effects must be investigated in clinical trials, as well as identifying the involvement of

skeletal muscle tissue in these phenomena; therefore, in this context, SSRI effects on skeletal muscle may result in positive metabolic effects, and these interactions are relevant as an area of research.

The serotonergic system may be involved in mechanisms that alter factors of myogenic differentiation and intracellular phosphorylation through 5-HT2 A receptors in myoblasts and at the level of T-tubules in rat myotubes [72]. Although it is believed that this receptor is located exclusively in the plasma membrane, thus being unlikely to be related to the excitation-contraction process [22]. The environmental influences during the critical period of development and skeletal muscle capacity to respond to these challenges has been an increasing area of research over recent decades [73,74].

In this context, it was observed that during the early stage of development, chronic exposure to SSRIs showed persistent effects on skeletal muscle including increased citrate synthase activity and mitochondrial respiration, and reduced oxidative stress [44]. In previous studies, authors have shown that fluoxetine administered during the critical period of development may positively affect the mitochondrial bioenergetics and antioxidant defence in the cardiac tissue and in brain areas, such as the hypothalamus and the hippocampus [75,76]. In contrast to the negative effects observed on skeletal muscle structure due to neonatal treatment with fluoxetine [42], here, neonatal exposure to fluoxetine (10 mg/kg) seemed to have a persistent beneficial effect in young adult rats, resulting in a positive modulation of skeletal muscle mitochondrial respiration [44].

In addition, chronic exposure to SSRIs influenced phosphorylation activity in muscle cells and increased uptake of glucose by muscle [43]. While the occurrences seen in the included articles are described as increased glycogen stores, a decrease in triglyceride content associated with a reduction in fatty acid synthesis from the administration of SSRIs may suggest a positive impact of this drug on skeletal muscle energy metabolism [41,46]. From the metabolic point of view, it is important to maintain the glycogen stores and the cellular oxidative capacity to avoid compromising the energy status in demanding metabolic conditions such as during physical exercise or disorders such as obesity and diabetes [10,77]. In addition, obese subjects may present oxidative damage in skeletal muscle [78] and therefore improved muscle oxidative capacity in these cases may also mean, in the long-term, a beneficial effect of the use of SSRI. That is, SSRI administration in cases of obesity may present a potential object of study due to skeletal muscle metabolic implications discussed here.

It is believed that the therapeutic effects of SSRIs are mediated through their action at SERTs; however, SSRIs have interactions with other biogenic amine neurotransmitter transporters, such as the norepinephrine transporter (NET) and the dopamine transporter (DAT), that can lead to severe side effects. For example, an included article showed a citalopram-induced jaw tremor with rhythmic activity of the masseter by an indirect inhibitory effect on central dopaminergic activity [39]. However, the authors described normal activity with normal motor unit action potentials and no spontaneous activity other than the rhythmic activity associated with citalopram-induced jaw tremor [39].

SSRIs possess halogen atoms at specific positions that are key determinants for the drug specificity for SERTs [79]. Therefore, the side effects of SSRIs on skeletal muscle could be predicted by receptor selectivity and site of action [79]. However, the SSRI subtypes used in the included papers in this review (e.g., fluoxetine, escitalopram, citalopram and fluvoxamine) are described as relatively selective for SERT blockade [79,80] with reduced potency at the norepinephrine and dopamine transporters [81].

Minimal therapeutic doses of citalopram or fluoxetine had a significant effect on striatal SERT binding potential and increased levels of basal serotonin [23,82]. In addition, binding studies have demonstrated that SSRIs are more selective for SERT over NET and generally have low affinities for DAT [80,81,83]. It is important to consider the concentration of the drug at the site of action relative to its affinity (which is the concentration of drug that binds to 50% of the receptors) for this concentration is what determines how much of the drug will be bound to its target [81]. Knowledge of these effects in varying conditions of health or disorder can help to elucidate the potential and the most appropriate choice of SSRI for a specific condition. Further research will help to maximize the effectiveness and tolerability of an intervention and help develop highly selective and potent drugs.

The evidence presented in this systematic review revealed aspects of the influence of SSRIs on skeletal muscle. However, the mechanisms require further research to elucidate the skeletal muscle response under these conditions. Studies investigating the mechanism underlying the changes in skeletal muscle in some conditions such as exercise, models of obesity and metabolic syndrome should be performed to clarify the role of serotonin in those metabolic conditions associated with SSRI exposure.

The findings discussed in this systematic review have important implications for the knowledge of the involvement of the serotoninergic system on motor activity and even in the control of muscle activation in subjects with spinal cord injury. Additionally, in cases of congenital myasthenic syndrome and myotonic dystrophy type I, these findings may help guide the development of pharmacological interventions to provide a better response to the skeletal muscle dysfunction in these disorders. In terms of muscle structure, it is intriguing to observe alterations in skeletal muscle fibre associated with body weight reduction related to chronic SSRI use. Finally, due to implications for skeletal muscle metabolism with SSRI use, it may have potential for guiding the development of interventions directed to subjects with metabolic disorders such as obesity and diabetes.

4.2. Quality of evidence and potential biases

In the present study, we found heterogeneities between the included articles. Even with the reduced number of included articles, the human and rodent studies presented had low levels of quality of evidence. However, measurements performed in rodents were robust. Therefore, the studies used different doses, routes of administration, periods of administration, age at testing and especially the outcome assessed.

The results of the risk of bias tool suggested a reduced level of evidence, which could be associated with the lack of the establishment of a random sequence and lack of random housing for the animals used as samples [31]. The blinding of the researcher and caregivers was not reported by any author, making the process of bias analysis difficult. It should be noted that the baseline characteristics were similar in all rodents used as samples. However, we elucidate the importance of improving the internal validity of animal studies. Many entries in the RoB tool were judged as “unclear” risk of bias, which revealed that reporting experimental and human studies details on sample characteristics, methods and selective reporting are very poor [31,84]. The RCTs in humans had cited the use of randomization and blinding of the participants. While the housing conditions and ethical procedures in rodent models were adequate to assess the outcomes of interest in each study, the possible adverse effects were not reported for any included article. These conditions illustrate the necessity of accuracy and additional safety investigations on the role of SSRIs in the functional, structural and metabolic properties of skeletal muscle. Moreover, a systematic review can contribute to improving the translation of animal research to human studies. It is important to further improve animal research to maximize its contribution to evidence-based translational research [85].

4.3. Strengths and weaknesses of this review

To our knowledge, no systematic review has been conducted in human and rodent models addressing the effects of SSRI administration on skeletal muscle outcomes, such as the functional, structural, and metabolic changes described here. Major limitations of this review are

related to the weakness and reduced number of studies included. For example, it was not feasible to conduct a meta-analysis due to clinical and methodological heterogeneity across studies. Furthermore, in reviewing the current evidence, preclinical studies using rodents to explore SSRI use and skeletal muscle properties are more robust compared to clinical research, mainly because these studies showed similar baseline characteristics of the sample compared to the human studies. The human studies also lack generalizability, as they were conducted in patients with different clinical presentations (e.g., obesity, spinal cord injury, myotonic dystrophy, and congenital myasthenic syndrome compared with healthy subjects). Moreover, the human literature presented in this review had limited sample sizes. It is necessary to develop RCTs that show more robust effects on the influence of SSRI administration on skeletal muscle tissue, which may be a potential object of study in cases of metabolic disorders such as obesity and diabetes, where there is an alteration of some properties of muscle tissue.

5. Conclusions

The evidence presented in this systematic review revealed aspects of the side effects due to exposure to SSRIs on skeletal muscle properties. Human and rodent studies demonstrated that SSRIs modified electrical muscle activity, skeletal muscle structure, and energy metabolism, al- though these changes varied according to pre-existing metabolic and functional conditions in rodents and humans. High-quality studies are needed to elucidate the skeletal muscle response mechanisms following SSRI administration.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

The authors thank the Coordination for the Improvement of Higher Education Personnel (CAPES) and Foundation to Support Science and Research in Pernambuco State – Brazil (FACEPE) for their financial support.

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ANEXO A - PARECER DA COMISSÃO DE ÉTICA E USO DE ANIMAIS

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Recife, 22 de agosto de 2017.

Ofício nº 80/17

Da Comissão de Ética no Uso de Animais (CEUA) da UFPE
Para: **Prof. Raul Manhães de Castro**
Departamento de Nutrição
Centro de Ciências da Saúde
Universidade Federal de Pernambuco
Processo nº **0011/2017**

Certificamos que a proposta intitulada "**Efeitos do tratamento com o fator de crescimento de fibroblastos 19 sobre o músculo esquelético de ratos submetidos a paralisia cerebral experimental**", registrada com o **nº 0011/2017** sob a responsabilidade de **Prof. Raul Manhães de Castro** que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (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 nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo CONSELHO NACIONAL DE CONTROLE DE EXPERIMENTAÇÃO ANIMAL (CONCEA), e foi aprovada pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) DA UNIVERSIDADE FEDERAL DE PERNAMBUCO (UFPE), em reunião de 02/08/2017.

Finalidade	( ) Ensino (X) Pesquisa Científica
Vigência da autorização	11 meses
Espécie/ linhagem/raça	Ratos heterogênicos/Wistar
Nº de animais	176
Peso/Idade	220-250g / 90-120 dias
Sexo	136 machos e 40 fêmeas
Origem	Bioterio do Departamento de Nutrição - CCS/UFPE

Atenciosamente,

Prof. Sebastião R. F. Silva
Vice-Presidente CEUA/UFPE
SIAPE 2345991