UNIVERSIDADE FEDERAL DE PERNAMBUCO
CENTRO ACADÊMICO DE VITÓRIA
PROGRAMA DE PÓS-GRADUAÇÃO EM NUTRIÇÃO, ATIVIDADE FÍSICA E PLASTICIDADE FENOTÍPICA

MÁRCIA MARIA DA SILVA

PLASTICIDADE FENOTÍPICA: efeitos do tratamento neonatal com kaempferol sobre parâmetros morfológicos ósseos em modelo experimental de paralisia cerebral

Vitória de Santo Antão
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Dissertação apresentada a Universidade Federal de Pernambuco, como parte das exigências do Programa de Pós-graduação em Nutrição, Atividade Física e Plasticidade Fenotípica como requisito parcial para obtenção do título de Mestre.

Área de concentração: Bases experimentais e clínicas da plasticidade fenotípica

Orientador: Prof. Dra. Ana Elisa Toscano Meneses da Silva Castro
Coorientador: Prof. Dr. Francisco Carlos Amanajás de Aguiar Júnior

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“Não há, não,
duas folhas iguais em toda a criação.
Ou nervura a menos, ou célula a mais,
não há, de certeza, duas folhas iguais.”
(António Gedeão, 1958)

RESUMO

A paralisia cerebral (PC) compreende um grupo de transtornos do movimento e da postura com limitação da função, relacionados a danos que acometem o sistema nervoso central em desenvolvimento. Existem poucas estratégias de intervenções relacionadas ao manejo e tratamento da paralisia cerebral precoce, ou seja, logo após a lesão. Sendo assim, entre as estratégias nutricionais ou mesmo farmacológicas temos a intervenção com flavonoides, como o kaempferol. Buscamos avaliar o efeito do tratamento neonatal com kaempferol em ratos submetidos à paralisia cerebral experimental sobre parâmetros morfológicos ósseos. Foram utilizados ratos *Wistar* e após o nascimento os filhotes foram ajustados para oito filhotes por ninhada, com peso entre 6-8 gramas no primeiro dia pós-natal, permanecendo com suas respectivas genitoras durante o período de lactação, sendo ainda nesse período distribuídos nos subgrupos: a) Controle Placebo (C+P), b) Controle Kaempferol (C+K), c) Paralisia Cerebral Placebo (PC+P), d) Paralisia Cerebral Kaempferol (PC+K). O modelo de PC consistiu na associação de anoxia perinatal com a restrição sensório motora das patas posteriores. Foi avaliado nos animais em dias específicos, o peso corporal, comprimento naso-anal e comprimento caudal. Após o desmame (P25), os animais foram separados em gaiolas individuais (3-4 animais por gaiola) até os 36° dia de vida pós-natal, quando foram eutanasiados para coleta de tecidos ósseos, (fêmur e tíbia) foi aferido o peso, comprimento e realizada a histomorfometria óssea e análise da densidade radiográfica. Os dados foram analisados por meio do teste *ANOVA two way* ou t*wo way* medidas repetidas, seguidos do pós-teste de *Tukey* ou teste de *Kruskal-Wallis*, seguido do pós-teste de *Dunn's* e o nível de significância considerado foi de $p<0,05$. Os nossos dados sugerem que em P36 a indução de PC experimental promoveu os seguintes resultados: diminuição do peso corporal e comprimento naso-anal, redução do peso absoluto e comprimento da tíbia e fêmur, redução da radiopacidade óssea das regiões analisadas do fêmur e tíbia redução da espessura cortical e área medular, maior densidade de lacunas de osteócitos e menor área lacunas de osteócitos. Sobre os dados dos animais submetidos à PC e que receberam tratamento com kaempferol em comparação com o grupo PC que recebeu placebo sugerem que no dia pós-natal 36 (PC+K vs. PC+P) temos os seguintes resultados: aumento do peso corporal e do comprimento naso-anal e aumento da densidade e área das lacunas de osteócitos. Em resumo, os nossos dados mostram que o modelo de paralisia cerebral reduziu drasticamente o crescimento dos animais no que diz respeito ao peso corporal e do comprimento naso-anal, apresentaram também redução do peso absoluto e do comprimento absoluto da tíbia e do fêmur, reduziu a radiopacidade óssea das regiões analisadas do fêmur e tíbia, redução da espessura cortical e da área medular, bem como redução da área das lacunas de osteócitos e que o tratamento com kaempferol atenuou os efeitos relativos ao peso corporal e comprimento naso-anal, bem como histologicamente pode observar o aumento da área e do número de lacunas de osteócitos. São necessários mais estudos para verificar se esta melhoria no desenvolvimento ósseo pode ser ampliada ao longo do tempo.

Palavras-chave: paralisia cerebral; 3,5,7-Trihydroxy-2-(4-hydroxyphenyl) -4H-chromen-4-one; flavonoide; osso; tíbia; fêmur.

ABSTRACT

Cerebral palsy (CP) comprehends a group of movement and posture disorders with limited function, related to damage that affects the developing central nervous system. There are few intervention strategies related to the management and treatment of early cerebral palsy, soon after the injury. Thus, among nutritional or even pharmacological strategies we have the intervention with flavonoids, such as kaempferol. We sought to evaluate the effect of neonatal kaempferol treatment in rats subject to experimental cerebral palsy on bone morphological parameters. Wistar rats were used and after birth the pups were adjusted to eight pups per litter, with weight between 6-8 grams on the first postnatal day, remaining with their respective genitors during the lactation period, being distributed in the subgroups: a) Placebo Control (C+P), b) Kaempferol Control (C+K), c) Placebo Cerebral Palsy (PC+P), d) Kaempferol Cerebral Palsy (PC+K). The CP model consisted of the association of perinatal anoxia with hind limb sensory motor restriction. Body weight, naso-anal length and caudal length were evaluated in the animals on specific days. After weaning (P25), the animals were separated into individual cages (3-4 animals per cage) until the 36th day of postnatal life, when they were euthanized for bone tissue collection (femur and tibia), weight, length, bone histomorphometry, and radiographic density analysis were performed. The data were analyzed using the two-way ANOVA or two-way repeated measures test, followed by Tukey's post-test or Kruskal-Wallis test, followed by Dunn's post-test, and the significance level considered was $p<0.05$. Our data suggest that in P36 the induction of experimental CP promoted the following results: reduction in body weight and naso-anal length, reduction in absolute weight and length of the tibia and femur, reduction in bone radiopacity of the analyzed regions of the femur and tibia, reduction in cortical thickness and medullary area, greater density of osteocyte lacunae, and smaller area of osteocyte lacuenae. On the data from animals that underwent CP and received kaempferol treatment compared with the PC group that received placebo suggest that on postnatal day 36 (PC+K vs. PC+P) we have the following results: increased body weight and naso-anal length and increased density and area of osteocyte lacunae. In summary, our data show that the cerebral palsy model drastically reduced the growth of the animals in terms of body weight and naso-anal length, that it also reduced the absolute weight and absolute length of the tibia and femur, that it reduced the bone radiopacity of the analyzed regions of the femur and tibia, kaempferol treatment attenuated the effects related to body weight and nasal-anal length, and histologically, an increase in the area and number of osteocyte lacunae could be observed. Further studies are needed to see if this improvement in bone development can be extended over time.

Keywords: Cerebral palsy; 3,5,7-Trihydroxy-2-(4-hydroxyphenyl)-4H-chromen-4-one; flavonoid; bone; tibia; femur.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS E SIGLAS

µM	Micromol
BSP	Sialoproteína de ligação à integrina
C+K	Controle kaempferol
C+P	Controle placebo
DMO	Densidade mineral óssea
DMSO	Dimetilsulfóxido ou sulfóxido de dimetilo
e.p.m.	Erro padrão da média
EDTA	Ácido etilenodiamino tetra-acético
OSX	Osterix ou fator de transcrição Sp7
PC	Paralisia cerebral
PC+K	Paralisia cerebral Kaempferol
PC+P	Paralisia cerebral Placebo
qRT-PCR	PCR de transcrição reversa quantitativa em tempo real
Runx-2	Fator de transcrição 2 relacionado à runt
U-2 OS Line	Células humanas de osteosarcoma

SUMÁRIO

1 APRESENTAÇÃO

A Paralisia Cerebral (PC) compreende um grupo de transtornos do movimento e da postura com limitação funcional que são atribuídos a distúrbios não progressivos do sistema nervoso central fetal ou da criança em desenvolvimento (BAX *et al.*, 2005; BLAIR, 2010). Um grande número dos pacientes com PC exibe aumento dos reflexos, hiper tonicidade, atrofia e fraqueza muscular (KRIGGER, 2006). As crianças com PC são propensas ao desenvolvimento de baixa densidade mineral óssea (DMO) e fraturas de baixo impacto (LEVINE, 2012).

O estado nutricional deficitário em indivíduos com paralisia cerebral está associado aos atrasos do desenvolvimento e na habilidade motora o que pode comprometer ainda mais o desenvolvimento ósseo (DAHLSENG *et al.*, 2012; KUPERMINC; STEVENSON, 2008). Ainda, existem poucas estratégias de intervenções relacionadas ao manejo e tratamento da paralisia cerebral precoce, ou seja, logo após a lesão. Sendo assim, entre as estratégias de intervenção nutricional ou mesmo farmacológica temos a suplementação com flavonoides.

O kaempferol é um composto flavonoide que tradicionalmente tem sido empregado no tratamento de doenças como, hipertensão, dores de cabeça e dores abdominais. (GUO *et al.*, 2012*)*. E em estudos *in vitro*, usando concentrações de 0,1 a 100 µM o kaempferol foi associado a diversas funções biológicas como a promoção, diferenciação e mineralização de osteoblastos e supressão da reabsorção e diferenciação óssea dos osteoclastos (WATTEL *et al.*,2003; MIYAKE *et al.*, 2003; PROUILLET *et al.*, 2004; GUO *et al.*, 2012).

Ainda, em um modelo de osteopenia em ratas ovariectomizadas o kaempferol diminuiu a perda óssea e aumentou as células osteoprogenitoras (TRIVEDI *et al.*, 2008). Entretanto, não encontramos em nossas buscas até o momento, estudos que avaliem o efeito do kaempferol em distúrbios como a paralisia cerebral que promovem importantes modificações ósseas que afetam o desenvolvimento.

Dessa forma, a PC assim como os danos motores e déficits ósseos associados ao quadro desse distúrbio representam temas de estudo relevante; tendo em vista ainda que evidências epidemiológicas apontam que a mesma apresenta uma taxa de prevalência estimada em 2 - 3,5 para cada 1000 crianças nascidas em países desenvolvidos (YEARGIN-ALLSOPP *et al.*, 2008), sendo maior nos países subdesenvolvidos, chegando a 7 para cada 1000 nascidos vivos (ZANINI *et al.*, 2017). Sendo assim, seu estudo é de extrema importância para o desenvolvimento de novas terapias e a compreensão de suas diversidades. Então, o objetivo

desse estudo foi avaliar e elucidar o papel do kaempferol no que diz respeito ao desenvolvimento do tecido ósseo em ratos submetidos a um modelo de paralisia cerebral.

2 REFERENCIAL TEÓRICO

2.1 A paralisia cerebral no contexto da plasticidade fenotípica

A plasticidade fenotípica se refere a capacidade do organismo de se adaptar às diferentes condições ambientais, sem que necessariamente ocorra alguma modificação em nível genético (WEST-EBERHARD, 1986). A variação genética e plasticidade do desenvolvimento são os principais fatores que contribuem para a plasticidade do organismo, ou seja, na habilidade do mesmo de reagir e se adaptar aos desafios ambientais. Este fenômeno pode ser caracterizado ainda, como a relação entre os eventos que ocorrem no útero e sua influência no desenvolvimento da prole, ou seja, a predisposição desse organismo desenvolver doenças em fases mais tardias da vida. Portanto, reporta-se como a habilidade de resposta de um organismo a um *input* do ambiente interno como o útero, ou externo, como, o ambiente de desenvolvimento do indivíduo (WEST-EBERHARD, 1986; BARKER, 2005).

Em conjunto com a plasticidade do desenvolvimento atuam a variabilidade fenotípica, fisiológica e comportamental, e esses fatores podem ser influenciados pelas modificações ambientais, como exemplo temos os efeitos da mãe em sua prole, por exemplo, as pressões ambientais a que as mães foram expostas podem ser refletidas na prole afetando o seu desenvolvimento (WEST-EBERHARD, 2003). Mas a variabilidade de um organismo acontece não somente pela plasticidade fenotípica, ocorrendo também devido aos próprios genes, os organismos podem apresentar ainda padrões distintos de metilações, acetilações, ou ainda, mutações que os diferenciam dos demais organismos e os beneficiam com capacidades responsivas diferentes às variadas pressões ambientais em que está inserido (WEST-EBERHARD, 2005).

A paralisia cerebral (PC), inicialmente reportada por Little em 1861, é um dos distúrbios neurológicos com implicações no desenvolvimento da criança, mais conhecidos mundialmente e foi chamada primeiramente de *"cerebral paresis",* posteriormente seu nome foi modificado para *"cerebral palsy",* pois é um termo mais abrangente e descreve melhor a etiologia e desenvolvimento da pessoa portadora dessa condição (BAX *et al.*, 2005). Em definições mais recentes a PC é reportada como um grupo de transtornos do movimento e da postura com limitação funcional que são atribuídos a distúrbios não progressivos do sistema nervoso central fetal ou da criança em desenvolvimento (BAX *et al.*, 2005; BLAIR, 2010).

A PC apresenta uma taxa de prevalência estimada em 2 - 3,5 para cada 1000 crianças nascidas em países desenvolvidos (STANLEY; BLAIR; ALBERMAN, 2000; YEARGIN-ALLSOPP *et al.*, 2008). Essa prevalência é maior nos países subdesenvolvidos, como é o caso do Brasil, esse número chega a 7 para cada 1000 nascidos vivos, além disso entre 30.000 e 40.000 casos ocorrem a cada ano (ZANINI *et al.*, 2017; MANCINI *et al.*, 2002). A explicação para a diferença na magnitude da prevalência é atribuída às más condições de cuidados pré e pós-natais (MANCINI *et al.*, 2002). Essas evidências epidemiológicas apontam para relevância de estudos clínicos e sobretudo experimentais para o conhecimento dos mecanismos subjacentes à PC e o avanço em sua terapia.

Os fatores de risco para o desenvolvimento da PC podem ocorrer antes, durante, no momento do parto e até posteriormente. Esses fatores incluem infecções, a hipóxia-isquemia, o nascimento prematuro, gravidez múltipla, anomalias congênitas, o retardo no desenvolvimento do feto no útero, a ocorrência de alguns desses fatores culminam em alguma lesão nível cerebral que compromete o desenvolvimento normal da criança (NOVAK *et al.,* 2017).

Sendo então, o resultado de uma má formação ou dano cerebral em crianças em fases iniciais do desenvolvimento. A PC apresenta-se como uma das causas mais comuns de problemas na locomoção e outras debilitações físicas da postura e do movimento, causando distúrbios na função neuromuscular e desenvolvimento inadequado de tecidos musculares esqueléticos (CHRISTENSEN *et al.,* 2014; ROSE *et al.,* 2005; WHITNEY *et al., 2017*).

Crianças diagnosticadas com PC geralmente apresentam na primeira infância dificuldades no desenvolvimento de habilidades motoras grossas e também dificuldades no ato da alimentação, a gravidade do quadro clínico varia com a extensão e região cerebral afetada (BAX *et al.*, 2005). Crianças com paralisia cerebral frequentemente apresentam ainda distúrbios orofaciais (KRICK *et al.*, 1996).

Os danos neurológicos ocasionados pelas lesões cerebrais nos indivíduos acometidos com PC são bem extensos, algumas manifestações são comumente observadas no quadro, como deficiência intelectual, comprometimento à nível motor, quadros de epilepsia e outros, que variam de acordo com a gravidade do dano e regiões nervosas afetadas e também com a idade do indivíduo acometido (BAX *et al.,* 2005).

2.2 Modelos de paralisia cerebral experimental

Vários modelos foram desenvolvidos com animais afim de promover os danos cerebrais que acometem crianças com PC, como o modelo de asfixia que causa lesão no tálamo e tronco cerebral; o acidente vascular cerebral (AVC) perinatal que causa lesão cerebral comum na PC, ou o modelo que infringe lesão na substância branca, simulando basicamente os quadros em que a PC se daria por quadros de hipóxia-isquemia, ou por infecções e até pela combinação dos mecanismos (JOHNSTON, 2005).

Outros estudos simulam os transtornos de migração neuronal, que replicam os danos da PC juntamente com outros quadros clínicos de ordem muitas vezes genéticas e ainda existem outros pesquisadores que combinam os modelos para assim simular de forma mais eficaz o quadro clínico da paralisia cerebral e então montar um panorama completo para facilitar o desenvolvimento de estratégias de tratamento (JOHNSTON, 2005) Dessa forma, os estudos experimentais que simulem a PC em animais são capazes de contribuir de forma significativa nos mecanismos do quadro clínico da PC, dos danos ao desenvolvimento motor e sensorial dos (LACERDA *et al.*, 2017; SILVA *et al.*, 2016).

Os modelos de indução da PC geralmente simulam as manifestações clínicas decorrentes da mesma, modelos experimentais que combinem diferentes insultos parecem ter mais fidedignidade ao representar os déficits dos indivíduos acometidos pela PC (JOHNSTON *et al.*, 2005; COQ *et al.*, 2008). Dessa forma o modelo proposto por Coq e colaboradores que combina a anoxia perinatal com a restrição sensório-motora dos membros posteriores simula os danos a nível de atrasos no desenvolvimento e maturação de reflexos e atividade locomotora, equilíbrio, coordenação, diminuição do peso corporal e até dificuldades nos movimentos mastigatórios (COQ *et al.*, 2008; STRATA *et al.*, 2004; LACERDA *et al.*, 2017).

Várias alterações são descritas na literatura em relação aos indivíduos acometidos pela PC, como por exemplo a diminuição do peso corporal, do desempenho motor e foi observado também em recente revisão, que os distúrbios orofaciais são encontrados em todos os modelos experimentais de paralisia cerebral e em diferentes espécies (SILVA *et al.*, 2016; LACERDA *et al.*, 2017). Os variados aspectos relacionados aos distúrbios que são decorrentes da PC reforçam a importância de ressaltar o fato de que o diagnóstico da mesma deve ser multidisciplinar, assim como seu tratamento, e reforçam ainda, a necessidade de estudos complementares.

2.3 Desenvolvimento do tecido ósseo

O esqueleto humano adulto apresenta 206 ossos que em conjunto promovem ao corpo funções diversas como, de sustentação, movimentação e proteção do organismo, hematopoese e armazenamento de minerais. Cada osso é extensivamente remodelado durante toda a vida do organismo para se adaptar às diferentes mudanças ao longo dela, e para a substituição dos mais velhos por ossos mais novos, mais fortes e resistentes (DATA, *et. al*, 2018; CLARKE, 2008; JUNQUEIRA; CARNEIRO, 2017).

O tecido ósseo é um tipo especializado de tecido conjuntivo composto por algumas células, os osteoblastos que se situam na periferia da matriz e são responsáveis por sintetizá-la, os osteócitos, se encontram no interior de lacunas e têm a função de manter a matriz e por último, os osteoclastos, células multinucleadas e móveis com função de reabsorver a matriz, têm grande importância no processo de remodelação óssea (HENRIKSEN, *et.*, al 2009; JUNQUEIRA; CARNEIRO, 2017; DATA, *et. al*, 2018; ROBLING, *et., al* 2006).

O tecido ósseo apresenta ainda, uma matriz extracelular calcificada e é um dos principais componentes do esqueleto, servindo de suporte para tecidos não calcificados, protegendo os componentes alojados na região craniana e torácica, além de oferecer apoio e participar do processo de movimentação dos músculos esqueléticos, ainda tem o papel de alojar a medula óssea, precursora das células sanguíneas. (OLSEN *et al.*, 2000; JUNQUEIRA; CARNEIRO, 2017).

A matriz mineralizada do tecido ósseo possui componentes orgânicos e inorgânicos; entre os orgânicos se encontra o colágeno do tipo I, formando basicamente 90% dessa matriz, possui ainda, proteínas adesivas e glicosaminoglicanos e proteoglicanos, e fazendo a parte inorgânica apresentam-se os íons fosfato e cálcio que formam os cristais de hidroxiapatita e água. Esses componentes em conjunto atribuem as propriedades do tecido ósseo: rigidez, plasticidade e resistência. (FROST, 2001; JUNQUEIRA; CARNEIRO, 2017; CLARKE, 2008).

Dentre as funções do tecido ósseo estão a sustentação, a proteção, desempenha o papel de importante depósito de alguns minerais, apresenta uma matriz mineralizada, tal matriz confere dureza que permite que o mesmo desempenhe as funções apontadas acima; além dessas, apresenta papéis importantes como o depósito de minerais como cálcio e ferro, liberando-os conforme a necessidade do organismo, mantendo então, uma concentração controlada nos sistemas. Ainda, esse tecido é capaz de absorver e estocar substâncias tóxicas, ou metais

pesados, protegendo assim outros tecidos (FROST, 2001; JUNQUEIRA; CARNEIRO, 2017; CLARKE, 2008).

Já em relação ao desenvolvimento embriológico, os membros superiores e inferiores têm o início do seu desenvolvimento na 4ª semana e vão até a 8ª semana de gestação, compreendendo um período de 5 semanas. O desenvolvimento do tecido ósseo e muscular se inicia na 4ª semana de gestação (SCHOENWOLF *et al.,* 2016). A origem, crescimento e desenvolvimento dos ossos, podem ser de dois tipos, a ossificação endocondral e ossificação intramembranosa (OLSEN *et al.,* 2000; JUNQUEIRA; CARNEIRO, 2017). o processo de remodelação óssea é constante ao longo da vida, mas, é mais constante nos períodos iniciais (HILL, 1998). Assim, é importante observar o período neonatal como uma janela crítica para prevenir problemas que afetem o desenvolvimento ósseo.

A ossificação intramembranosa ocorre em um molde de tecido mesenquimal ou no reparo ósseo, ela acontece quando há a diferenciação de células osteoprogenitoras que sintetizam uma matriz osteóide (não calcificada), os osteoblastos seguem num processo contínuo de formação de matriz, até que ficam presos dentro de lacunas, temos formado então: osteoblastos, matriz óssea e osteócitos; o tecido ósseo segue crescendo enquanto vasos sanguíneos se associam a matriz, esse processo continua até haver a formação do osso propriamente dito, esse tipo de formação óssea é comum em ossos chatos como os do crânio e provém o crescimento em espessura dos ossos longos (OLSEN *et al.,* 2000; JUNQUEIRA; CARNEIRO, 2017; ROSS; PAWLINA, 2016).

A ossificação endocondral, por sua vez ocorre em um molde de cartilagem hialina, onde há a formação de um "colar ósseo" formado por células mesenquimais (esse colar ósseo tem origem intramembranosa, posteriormente se inicia a ossificação endocondral); a cartilagem hialina é gradualmente substituída por matriz óssea, isso ocorre quando os condrócitos da cartilagem hialina entram em apoptose, vasos sanguíneos começam a migrar para essa região e a partir desses vasos há a chegada de células de osteoprogenitoras, em resumo acontece o crescimento do colar ósseo a substituição da cartilagem por tecido ósseo; ocorre nos ossos longos e curtos, proporcionando crescimento em comprimento. (MACKIE. *et al.,* 2008; JUNQUEIRA; CARNEIRO, 2017; ROSS; PAWLINA, 2016).

O tecido ósseo é dividido em dois tipos de acordo com sua fase de maturação: primário (imaturo) e secundário (maduro), sua dinâmica é basicamente a de que o tecido imaturo é

gradativamente substituído pelo maduro, no tecido imaturo os osteócitos e fibras colágenas se organizam de forma aleatória, ao passo que no tecido ósseo maduro as fibras se organizam em lamelas organizadas de forma concêntrica em torno de canais de Harvers, conectados por canais de *Volkmann* (sendo esses longitudinais e transversais, respectivamente). Formando os sistemas de *Harvers* que são essenciais para a nutrição e crescimento do tecido ósseo (HENRIKSEN, *et.,* al 2009; STEVENS; LOWE, 2002; JUNQUEIRA; CARNEIRO, 2017).

Anatomicamente, o fêmur e a tíbia são classificados como ossos longos. Ossos desse tipo possuem uma largura menor em relação ao comprimento e por isso são organizados em uma região intermediária, a diáfise e regiões terminais, alongadas chamadas epífises que promovem a interação com outros ossos, constituindo as articulações (TORTORA; DERRICKSON, 2019).

A osteopenia, por sua vez, é comum em pacientes com PC (FINBRÅTEN *et al.*, 2015). A PC diminui a densidade mineral óssea o que leva a risco elevado de fraturas, comprometendo o desenvolvimento e a reabilitação funcional desses pacientes (MOON *et al.*, 2016). Observa-se então, a importância da realização de estudos em modelo animal para a compreensão dos distúrbios ósseos associados a PC, bem como do desenvolvimento de estratégias intervenção.

2.4 Potencial dos flavonoides

Os polifenóis são uma categoria heterogênica de compostos químicos, podendo ser de dois tipos, os não flavonoides (ácidos fenólicos) e os flavonoides (flavonas, flavonóis, flavanóis, antocianidinas, flavanonas, isoflavonas e calconas), esses compostos são provenientes do metabolismo secundário das plantas. Flavonóides são compostos naturais, sendo um dos metabólitos secundários de plantas mais abundantes do reino vegetal, são solúveis e de baixo peso molecular, constituídos de dois anéis benzênicos e uma cadeia de 3 carbonos (C6-C3-C6) (CIANCIOSI *et al.*, 2018).

Compostos que apresentam polifenóis, são amplamente encontrados na natureza em plantas comestíveis ou não comestíveis e entre seus efeitos mais relatados está a capacidade antioxidante. Dentre as diversas funções que os polifenóis participam estão: assimilação de nutrientes, síntese proteica, atividade enzimática, que participam no processo fotossintético, formação de componentes estruturais da planta e atuam na defesa contra os fatores de risco presentes no ambiente (CHEYNIER; TOMAS-BARBERAN; YOSHIDA, 2015). Por isso, extratos de frutas, cereais, ervas e outros têm sido amplamente estudados pelo seu papel no

retardamento a degradação de lipídios, melhorando a qualidade nutricional do alimento (KÄHKÖNEN *et al.*, 1999).

Os flavonoides, por sua vez, são caracterizados por sua estrutura de difenil propano e por fazer parte dos metabólitos secundários do metabolismo de plantas. São encontrados principalmente em sucos, chás, e nas próprias frutas e vegetais, além de estarem presentes no vinho (VAUZOUR *et al.*, 2008). Flavonoides e outros polifenóis têm sido estudados como importantes preventores em doenças cardíacas e no câncer. Ainda, o estado antioxidante no plasma de humanos tem sido melhorado a partir do consumo de vinho tinto sem álcool ou de compostos polifenólicos extraídos de vinho tinto. (SERAFINI *et al.*, 1998; CARBONNEAU *et al.*, 1998).

O estudo de revisão Calderón-Montaño *et al.* (2011), aponta que o consumo de flavonoides tem sido associado a diminuição do risco de doenças como o câncer e distúrbios cardiovasculares. Em um estudo epidemiológico Hertog *et al.* (1993), avaliaram o perfil de ingestão de flavonoides (quercetina, kaempferol, miricetina, apigenina e luteolina) em 805 homens, entre 65 e 84 anos, em 1985, os homens foram seguidos por 5 anos mostrando que seu consumo é inversamente associado a doenças cardíacas coronárias.

O uso dos mais variados extratos de plantas, bem como de alguns flavonoides têm sido associados à melhora de déficits cognitivos, provavelmente associados incremento na função neuronal, bem como a sua proteção (YOUDIM, 2001). As propriedades neuroprotetoras dos flavonoides têm sido amplamente estudadas buscando elencar a multiplicidade dos efeitos dos mesmos. Dois processos foram indicados como primordialmente alterados pelos flavonoides na sua ação de melhoria das lesões cerebrais, sendo elas, a participação nas cascatas de sinalização de proteínas e quinases lipídicas, e também foram observados ainda efeitos positivos no sistema vascular cerebral (VAUZOUR *et al.*, 2008).

Em revisão realizada por Vauzour *et al.* (2008) apontam que o consumo de flavonoides contribui para a neuroproteção cerebral a partir da sua ação em processos como a inibição da apoptose celular, proteção contra lesão neuronal induzida por neurotoxinas, inibição da neuroinflamação, melhorias no processo de aprendizagem, melhora do desempenho cognitivo e da memória; cabe destacar ainda que os flavonoides são capazes de prevenir as algumas formas de doenças cerebrais.

O potencial dos flavonoides tem sido estudado também no que diz referente ao metabolismo ósseo, *Tridax procumbens* é uma planta comumente usada para tratamento de problemas hepáticos, anemias, artrite, gota, entre outros. Em estudos in vitro, usando os flavonoides isolados do extrato de *Tridax procumbens* foi possível observar suas propriedades em células-tronco mesenquimais (que tem potencial de diferenciação em células osteoblásticas), aumentando sua capacidade osteogênica (AL MAMUN *et al.,* 2017; AL MAMUN *et al.,* 2015).

Achados recentes indicam ainda um importante papel dos flavonoides no que diz referente ao metabolismo do RANKL (ligante do receptor ativador do fator nuclear kappa-B) diminuindo a diferenciação de osteoclastos e a reabsorção óssea e participanndo a diferenciação das células osteoblásticas o que influencia positivamente o processo de formação óssea; o RANKL é uma proteína que está relacionada ao fator mais importante e reconhecido na diferenciação dos osteoclastos. (AL MAMUN *et al.,* 2015).

Em estudos experimentais a densidade mineral óssea e o conteúdo mineral de camundongos tratados com a *Tridax procumbens* aumentou significativamente em comparação com os camundongos do grupo controle, assim como aumentou também os índices dos principais constituintes do metabolismo ósseo. (AL MAMUN *et al.,* 2017). Portanto, o estudo de flavonoides e seus sinônimos tem grande importância na busca por formas alternativas de tratamento e prevenção de doenças e distúrbios.

2.5 Kaempferol e saúde óssea

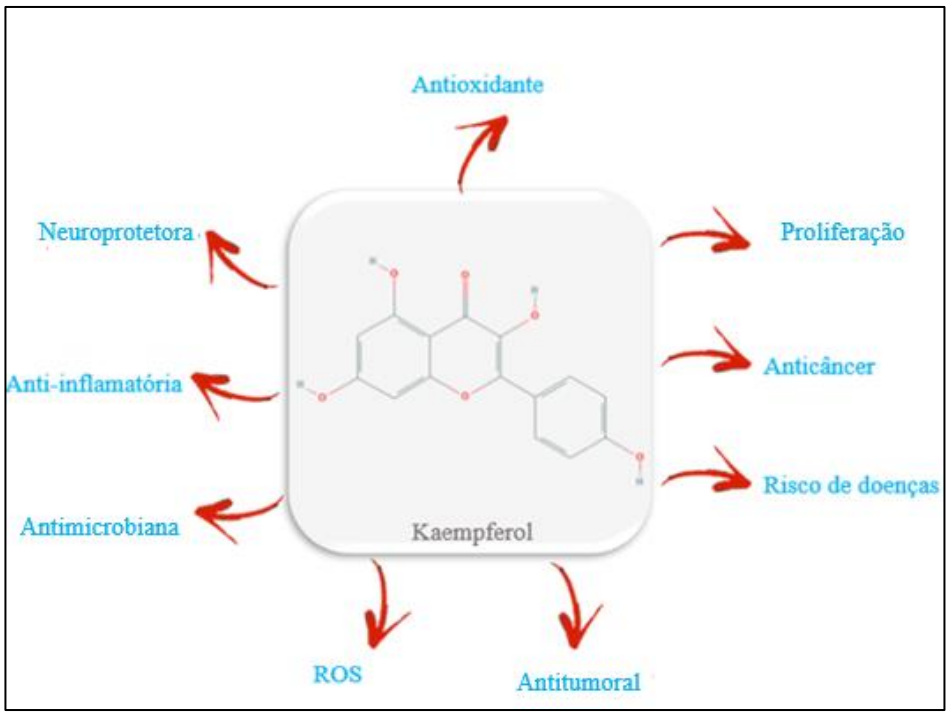
O kaempferol (3,5,7-tri-hidroxi-2- (4-hidroxifenil)-4H-1-benzopiran-4-ona) é um flavoóide conhecido ainda como: kaempferol-flavonol, kaempferol-3, apresenta como fórmula química $C_{15} H_{10} O_{6.}$ o peso molecular 286,23 e seu ponto de fusão está entre 276-278 graus centígrados é um pó amarelo puro translúcido e cristalino, é levemente solúvel em água, mas possui propriedades hidrofóbicas o que acontece por causa de sua estrutura de difenilpropano é altamente solúvel em etanol quente e éter dietílico (CALDERÓN-MONTAÑO *et al.,* 2011; PUBCHEM DATABASE, 2020).

O kaempferol é um flavonol encontrado em diversas partes de plantas, como folhas, flores e sementes; e está presente em alguns vegetais, à saber: o repolho, feijão, morangos, uvas, brócolis, groselha, tomates, frutas cítricas, entre outros. Pode ser encontrado também em plantas usadas na medicina tradicional como o *Ginkgo biloba.* As principais propriedades conhecidas

do kaempferol são: anti-inflamatória, antioxidante, antitumorais, antidiabético, função cardioprotetora, antimicrobiana, neuroproteção, além de apresentar potencial ação anti-carcinogênico (CALDERÓN-MONTAÑO *et al.*, 2011; REN *et al.*, 2019).

Foram desenvolvidos vários estudos procurando elucidar as propriedades do kaempferol na saúde e sua propriedade mais notável e estudada é a de seus efeitos anti-inflamatório e antioxidante, reduzindo o estresse oxidativo, logo após se destacam os estudos relacionados ao combate de diversos tipos de câncer. Em recente revisão Ren e *col*, 2019 desenvolveram um estudo acerca das propriedades do mesmo e viram que o kaempferol pode ainda, atuar na proteção do nervo craniano, contra lesões no fígado, doenças metabólicas, endotélio vascular, manutenção da função cardíaca. Porém, os mecanismos envolvidos nessa proteção ainda não são totalmente compreendidos; assim, mais estudos são necessários (PUBCHEM DATABASE, 2020; REN *et al.*, 2019).

Figura 1 - Resumo das principais funções do Kaempferol



Fonte: A autora.

Em recente revisão Wong, Chin, Ima-Nirwana (2019) fizeram um apanhado de uma série de estudos usando modelos animais e *in vitro* para elencar os efeitos de proteção óssea promovidos pelo kaempferol em ratos recém nascidos, ratas ovariectomizadas com osteoporose pós-menopausa, com osteoporose induzida por glicocorticoides e até modelos de fraturas

ósseas. Os principais mecanismos de proteção óssea de forma geral seriam o de proteção do metabolismo osteoblasto-osteoclasto, supressão da inflamação, auxílio no controle do estresse oxidativo e diminuição da adipogênese.

Além disso outras facetas também foram descobertas nos estudos *in vitro* como a diferenciação e mineralização óssea de células pré-osteoblásticas, aumento da expressão de proteínas relacionadas ao metabolismo ósseo, como Runx-2, OSX e BSP (todos esses genes estão relacionados com o metabolismo ósseo, especialmente aos osteoblastos). Já em estudos em modelo animal foram observados aumento na área e número de osteoblastos, e proteção da morfologia óssea, inibindo assim a osteopenia, dessa forma promovendo a proteção da arquitetura óssea de fêmur e tíbia (WONG; CHIN; IMA-NIRWANA, 2019).

Em estudo realizado com ratas *Sprague-Dawley* ovariectomizadas usando soluções de 0,2 a 5,0 µM, o kaempferol apresentou ação anti-osteoclastogênica, ou seja, impede o desenvolvimento e diferenciação dos osteoclastos. O grupo tratado com o kaempferol obteve ainda o aumento da densidade mineral óssea (DMO) (TRIVEDI *et al.,* 2008), o que pode ser positivo para quadros clínicos em que que o indivíduo apresente osteopenia, como é o caso de indivíduos com PC.

Ainda, em um estudo realizado em células humanas de osteosarcoma (*U-2 OS Line)* o kaempferol inibiu a proliferação de células de osteosarcoma *in vivo* e *in vitro* (Huang *et al.,* 2010). O kaempferol combinado com outro flavonoide a quercetina em concentrações variando de 0,1 a 100 microM apresentaram uma eficiente ação na redução da absorção óssea de maneira dependente tempo e dose *in vitro* (WATTEL *et al.,* 2003).

Pacientes acometidos com algum distúrbio que culmine em uma deficiência motora (como é o caso da PC) apresentam o risco elevado de fraturas em decoerrência disso, têm baixo índice de massa óssea e de DMO, posseum ainda, peso reduzido, e assim, há o comprometimento da força muscular (HENDERSON, 2005). Sendo assim, o estudo de estratégias de intervenção terapêuticas, como o uso do kaempferol são válidas para a compreensão dessas doenças ou quadros clínicos e descoberta de novas formas de tratamento da paralisia cerebral.

3 HIPÓTESE

O Kaempferol administrado no período neonatal diminui a osteopenia de fêmur e tíbia em ratos submetidos à paralisia cerebral experimental.

4 OBJETIVOS

4.1 Objetivo Geral:

Avaliar o efeito do tratamento neonatal com kaempferol em ratos submetidos à paralisia cerebral experimental sobre parâmetros morfológicos ósseos.

4.2 Objetivos específicos:

A. Realizar a análise da caracterização somática: peso corporal, comprimento naso-anal e caudal dos animais;
B. Aferir peso e comprimento absoluto e relativo do fêmur e tíbia;
C. Avaliar a densidade óssea radiográfica através de radiologia digital de regiões do fêmur (cabeça, colo femoral, epífises e diáfise) e tíbia (epífises e diáfise);
D. Analisar a morfologia macroscópica e microscópica (histomorfométrica) do fêmur e da tíbia.

5 MATERIAL E MÉTODOS

5.1 Questões éticas

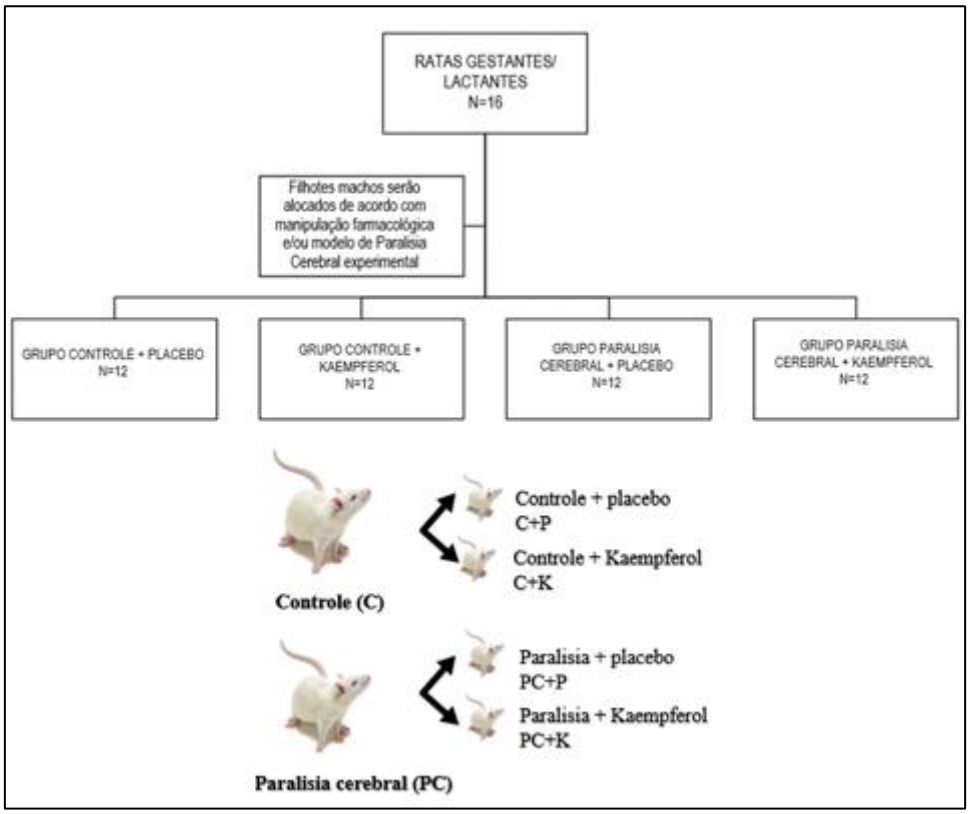
O presente projeto foi submetido a Comissão de Ética em Uso animal (CEUA) da UFPE e aprovado sob o Processo nº 0063/2019 e seguiu as normas do Conselho Nacional de Controle e Experimentação Animal (CONCEA), de acordo com a lei 11.794 de 8 de outubro de 2008, bem como as normas internacionais estabelecidas pelo *National Institute of Health Guide for Care and Use of Laboratory Animals*.

5.2 Animais e Grupos experimentais

Foram utilizados 48 ratos machos da linhagem *Wistar*, provenientes de 20 ratas, mantidos no biotério de experimentação do Departamento de Nutrição da UFPE com temperatura de 22 $\pm$ 2ºC, ciclo claro-escuro de 12/12 horas (luz 20:00h às 08:00h), os animais foram alojados em gaiolas coletivas de polipropileno (49X34X16cm) e livre acesso à água purificada e alimentação com dieta padrão de biotério (*Labina*®). O biotério possui exaustor para redução dos odores de urina e fezes. Após a confirmação da gestação, através da técnica de esfregaço vaginal, as fêmeas foram alojadas em gaiolas individuais.

Após o nascimento os filhotes foram ajustados para oito filhotes por ninhada, com peso entre 6-8 gramas no primeiro dia pós-natal, permanecendo com suas respectivas genitoras durante o período de lactação, sendo ainda nesse período distribuídos de forma aleatória nos subgrupos (figura 2): a) Controle Placebo (CP; n=12), b) Controle Kaempferol (CK; n=12), c) Paralisia Cerebral Placebo (PCP; n=12), d) Paralisia Cerebral Kaempferol (PCK; n=12). Após o desmame (P25), os animais foram separados em gaiolas individuais (3-4 animais por gaiola) até os 36° dia de vida pós-natal, quando foram eutanasiados para coleta de tecidos (ver figura dois para desenho experimental).

Figura 2- Desenho experimental dos grupos e tratamento.



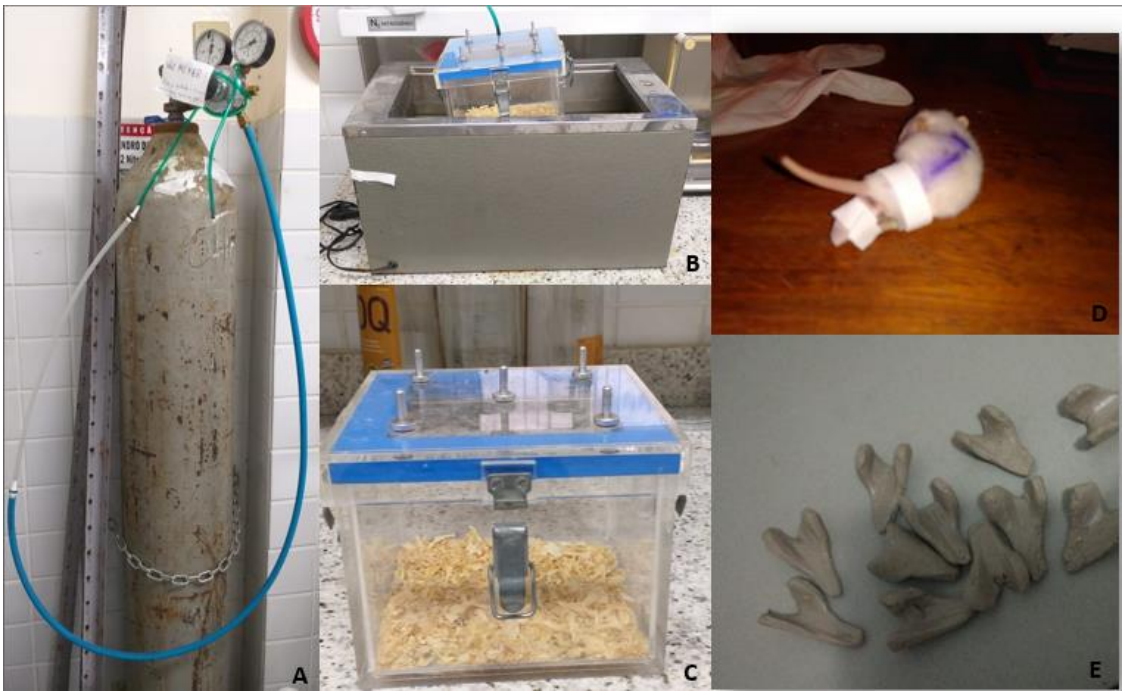
A composição dos grupos está de acordo com intervenção com Kaempferol ou placebo e modelo de paralisia cerebral ou controle. C+P (Controle + Placebo); C+K (Controle + Kaempferol); PC+P (Paralisia Cerebral + Placebo,) e PC+K (Paralisia Cerebral + Kaempferol). Fonte: a autora.

5.3 Paralisia cerebral experimental

Os filhotes dos grupos PC+P e PC+K foram submetidos ao modelo experimental de paralisia cerebral adaptado por Strata *et al.*, 2004 e COQ. *et al., 2008*. Este modelo associou a anoxia perinatal a um modelo de restrição sensório-motora dos membros inferiores semelhante a falta de movimento ocorrida na PC. Ao nascerem, os filhotes foram submetidos a dois episódios de anoxia, no P0 (dia do nascimento) e P1(dia um de vida). O método de anoxia perinatal utiliza uma câmara em banho-maria aos 37°C onde os animais são expostos a uma ventilação de nitrogênio por 12 minutos (N 100%, 9L/min). Do P2 ao P28 foi realizada a restrição sensório-motora das patas posteriores usando fita micropore, esparadrapo e molde feito de epóxi durante 16 horas por dia e nas 8 horas restantes, a livre movimentação do animal foi permitida. O processo de imobilização dos membros é feito de forma que seja permitido apenas movimentos limitados da articulação do quadril,

deixando os membros posteriores estendidos, sem que a eliminação de urina e fezes e os cuidados maternos sejam prejudicados (STRATA *et al.*, 2004; COQ. *et al.*, *2008*; SILVA *et al.*, 2016).

Figura 3 - Materiais utilizados no protocolo de paralisia experimental.



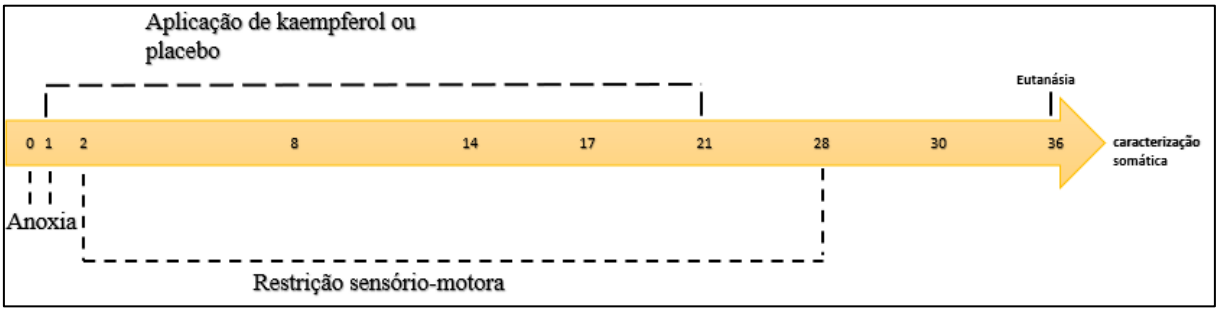
A. Cilindro de Nitrogênio; B. Banho-maria; C. Câmara de anoxia, D. Animal imobilizado. E. Órteses.
Fonte: A autora.

5.4 Tratamento com kaempferol ou placebo

Os filhotes dos grupos CK e PCK receberam, durante os primeiros 21 dias de vida pós-natal, o tratamento com Kaempferol (Cayman Chemical, Ann Arbor, MI, USA) foi aplicado 1mg/Kg, com volume de aplicação de 1 µl/g. Utilizou-se DMSO (dimetilsulfóxido ou sulfóxido de dimetilo) para dissolver o kaempferol e preparar solução estoque obtida pela mistura de 50mg de kaempferol e 5ml de dimetilsulfóxido (DMSO) (m/v), alíquotas de 20µl que foram mantidas à -80°C em tubos *eppendorfs* identificados e protegidos da luz. No dia da aplicação foram acrescidos 1980µl de solução salina comercial à alíquota, obtendo um volume final de 2ml. Desta mistura, foram preparadas seringas com volumes (1ml/100g) de acordo com o peso de cada animal. O kaempferol foi aplicado via intraperitoneal (Lagoa *et al.*, 2009). Os filhotes dos grupos CP e PCP receberam, durante os primeiros 21 dias de vida pós-natal veículo salina

+ DMSO (0,9% NaCl, via intraperitoneal), ver figura 4 para compreensão do delineamento do estudo.

Figura 4 - Resumo delineamento experimental durante o período experimental.



Fonte: A autora.

5.5 Análises experimentais

5.5.1 *Caracterização somática*

5.5.1.1 Peso corporal

O peso corporal dos filhotes foi registrado ao nascimento, e nos dias P1 à 21, P28 e P36 dias de vida com balança de precisão Marte (AD 2000).

5.5.1.2 Comprimento naso-anal

O comprimento naso-anal foi avaliado nos dias 1, 7, 14, 21, 28 e 36 com o auxílio de um paquímetro digital (JOMARCA ®). A distância em milímetros entre o focinho e a base da cauda do animal foi medida. O animal foi comprimido suavemente em uma superfície lisa e plana, então foram feitas marcações com caneta, foi medida a distância em milímetros entre essas marcações.

5.5.1.3 Comprimento caudal:

O comprimento da cauda dos filhotes também foi avaliado nos dias 1, 7, 14, 21, 28 e 36 com o auxílio de um paquímetro digital (JOMARCA ®). Foi realizada a marcação com caneta da base da cauda e da sua extremidade, e a distância em milímetros entre essas marcações foi medida.

5.5.2 Aferição do peso e comprimento do fêmur e tíbia

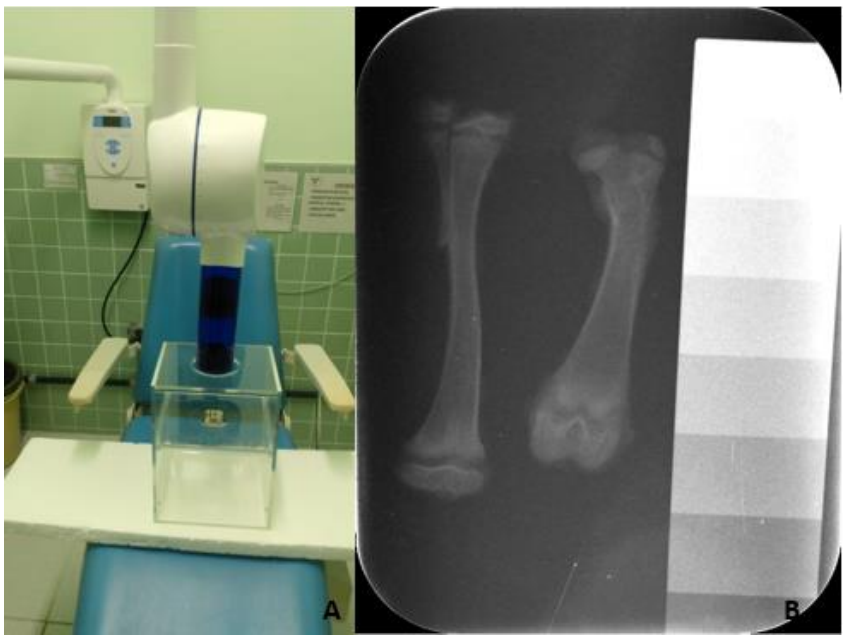
No 36° dia pós-natal (P36) os animais foram eutanasiados por perfusão transcardíaca e os ossos foram coletados. Após a dissecção, os ossos da tíbia e fêmur direitos foram imediatamente pesados em uma balança de precisão de 0,01 g (Marte AD 330). O comprimento total dos ossos da tíbia e do fêmur foram aferidos utilizando um paquímetro digital (Jomarca ®) com uma precisão de 0,01 mm. Sobre superfície lisa com sua face ventral voltada para cima.

5.5.3 Análises radiográficas de fêmur e tíbia

Os fêmures e tíbias direitos foram alinhados na placa de fósforo e radiografados (figura 5). Todas as imagens foram adquiridas utilizando o aparelho de raios X *Heliodent Plus* (Sirona Dental Systems, Bensheim, Alemanha) operando a 70kVp e gráfico 7mA e o sistema digital intraoral Express (Instrumentarium, Tuusula, Finlândia). Placas de fósforo *Digime Optime*® (Soredex, Helsinki, Finlândia) foram utilizadas para a realização de radiografias digitais. O tempo de exposição foi padronizado em 0,10 segundos e a distância utilizada foi padronizada em 30 cm de altura com dispositivo sensor de foco de acrílico para todos os exames radiográficos. Os espécimes, bloco de chumbo e penetrômetro (contendo 8 graus, com intervalo de 1 mm entre eles) foram sempre colocados na placa de fósforo na mesma posição conforme protocolo adaptado de De Marcelos *et al.* (2015).

As imagens foram salvas no formato PNG e exportadas para o programa *ImageJ (U.S. National Institutes of Health, Bethesda, Maryland, EUA)* onde foram medidos os valores de pixel de cada grau do penetrômetro e da área de regiões ósseas de interesse do fêmur (cabeça, colo, epífises e diáfise) e da tíbia (epífises e diáfises). Uma curva foi preparada por meio de um gráfico de dispersão com valores de pixel versus o mmAl correspondente. Assim, obteve-se o valor em mmAl das regiões analisadas. Os dados foram tabulados no programa *Microsoft Office Excel 2016* em uma planilha para cada imagem radiográfica. Essas avaliações radiográficas foram feitas por um único avaliador previamente calibrado.

Figura 5 - A- Equipamento radiográfico. B- Exemplo de radiografia.



Fonte: A autora

5.5.4 Análise histológica e histomorfométrica de fêmur e tíbia

As tíbias e fêmures esquerdos foram dissecados e fixados em solução de formol a 10% por dois dias e depois lavadas em solução tampão de PBS. Foram então incluídos em solução descalcificadora EDTA (ácido etilenodiamino tetra-acético) sendo colocadas em uma mesa agitadora por aproximadamente dois meses, quando constatada a descalcificação, a solução de EDTA foi descartada, os ossos lavados novamente em PBS e armazenados em solução de álcool a 70% até a realização do processamento histológico. Posteriormente, as peças foram desidratadas com soluções crescentes de álcool 70%, diafanizadas em álcool xilol, depois xilol e incluídas em parafina.

Para a análise histomorfométrica foram utilizados cortes transversais da diáfise da tíbia e do fêmur de cada animal. As lâminas foram coradas por Hematoxilina / Eosina (HE) e analisadas por microscopia de luz utilizando uma câmera digital *Tucsen ISH1000* acoplada a um microscópio óptico *Nikon eclipse* E200 sob foco fixo e clareza de campo, utilizando o *software ISCAPTURE* 4.1.3, nesses cortes, a espessura do osso cortical e área medular foi analisada por meio da aquisição de imagens das diáfises com aumento final de 40× Foram efetuadas três e quatro medições em cada corte histológico, respectivamente, na tíbia e no fêmur, priorizando as regiões superior, inferior e lateral de cada corte (adaptado de

CARVALHO *et al.*, 2010). Para determinar a espessura, o osso cortical foi medido desde a superfície periosteal até a superfície endosteal utilizando-se o programa *ImageJ (U.S. National Institutes of Health, Bethesda, Maryland, EUA)* adequadamente calibrado. Quanto a análise histológica, foram feitas aquisição de imagens conforme descrito acima da região da diáfise da tíbia para análise do número de lacunas de osteócitos por campo e área das células, que foram analisadas no aumento final de 400x utilizando-se o programa *ImageJ (U.S. National Institutes of Health, Bethesda, Maryland, EUA)* adequadamente calibrado.

5.6 Análise dos dados

Os resultados foram expressos como média ± e.p.m. (erro padrão da média). A análise de normalidade da amostra foi realizada por meio do teste de *Kolmogorov-Smirnov*. Para os dados considerados paramétricos, a comparação entre os grupos foi realizada por meio do *ANOVA two way* ou *two way* medidas repetidas, seguidos do pós-teste de *Tukey*. Quando foram não paramétricos foi realizado o teste de *Kruskal-Wallis*, seguido do pós-teste de *Dunn's*. As análises foram realizadas no *GraphPad PRISM* 7 ® (GraphPad Software, Inc., La Jolla, CA, USA) e o nível de significância considerado foi de $p<0,05$.

6 RESULTADOS E DISCUSSÃO

Os resultados e a discussão estão apresentados em forma de artigo original, com posterior submissão a Revista BMC Musculoskeletal Disorders / B1 Nutrição – está redigido, portanto, de acordo com as normas previstas pela Revista [Ver Apêndice A].

"**A kaempferol treatment during early life causes increase of body weight, naso-anal length and enlargement of osteocyte lacunae in bone of rats with experimental cerebral palsy"**

7 CONSIDERAÇÕES FINAIS

Em resumo, os nossos dados mostram que o modelo de paralisia cerebral reduziu drasticamente o desenvolvimento dos animais no que diz respeito ao peso corporal, comprimento naso-anal, levou também a alterações nos ossos estudados fêmur e tíbia, como a redução do peso absoluto e comprimento, redução da espessura cortical e da área medular, e apresentou diminuição da radiopacidade óssea e por fim, menor área de lacunas. E o tratamento com kaempferol atenuou os efeitos relativos ao peso corporal e comprimento naso-anal, bem como histologicamente pode observar o aumento da área e do número de lacunas. São necessários mais estudos para verificar se esta melhoria no desenvolvimento ósseo pode ser ampliada ao longo do tempo.

REFERÊNCIAS

AL MAMUN, Md Abdullah *et al.* Flavonoids isolated from Tridax procumbens (TPF) inhibit osteoclasts differentiation and bone resorption. **Biological research**, v. 48, n. 1, p. 51, 2015.

AL MAMUN, Md Abdullah *et al.* Tridax procumbens flavonoids: a prospective bioactive compound increased osteoblast differentiation and trabecular bone formation. **Biological research**, v. 50, n. 1, p. 28, 2017.

BARKER, David JP. The developmental origins of insulin resistance. **Hormone Research in Paediatrics**, v. 64, n. Suppl. 3, p. 2-7, 2005.

BAX, Martin *et al.* Proposed definition and classification of cerebral palsy, April 2005. **Developmental medicine and child neurology**, v. 47, n. 8, p. 571-576, 2005.

Blair E. Epidemiology of cerebral palsies. **Orthop Clin N Am**., v. 41, p. 441-55, 2010.

CARBONNEAU, Marie-Annette *et al.* Improvement in the antioxidant status of plasma and low-density lipoprotein in subjects receiving a red wine phenolics mixture. **Journal of the American Oil Chemists' Society**, v. 75, n. 2, p. 235-240, 1998.

CARVALHO, Ana Carolina Bergmann de *et al.* Bone tissue histomorphometry in castrated rats treated with tibolone. **Jornal Brasileiro de Patologia e Medicina Laboratorial**, v. 46, n. 3, p. 235-243, 2010.

CHEYNIER, Véronique; TOMAS-BARBERAN, Francisco A.; YOSHIDA, Kumi. Polyphenols: from plants to a variety of food and nonfood uses. **Agric. Food Chem**. Aug. 2015.

CHRISTENSEN, Deborah *et al.* Prevalence of cerebral palsy, co-occurring autism spectrum disorders, and motor functioning–Autism and Developmental Disabilities Monitoring Network, USA, 2008. **Developmental Medicine & Child Neurology**, v. 56, n. 1, p. 59-65, 2014.

CIANCIOSI, Danila *et al.* Phenolic compounds in honey and their associated health benefits: A review. **Molecules**, v. 23, n. 9, p. 2322, 2018.

CLARKE, B. Normal Bone Anatomy and Physiology. **Clinical Journal of the American Society of Nephrology**, v. 3, supl. 3, nov. 2008.

COQ, Jacques-Olivier *et al.* Impact of neonatal asphyxia and hind limb immobilization on musculoskeletal tissues and S1 map organization: implications for cerebral palsy. **Experimental Neurology**, v. 210, n. 1, p. 95-108, 2008.

DAHLSENG, Magnus Odin *et al.* Feeding problems, growth and nutritional status in children with cerebral palsy. **Acta paediatrica**, v. 101, n. 1, p. 92-98, 2012.

DATTA, H. K. *et al.* The cell biology of bone metabolism. **Journal of clinical pathology**, v. 61, n. 5, p. 577-587, 2008.

FINBRÅTEN, A.-K. *et al.* Bone mineral density and vitamin D status in ambulatory and non-ambulatory children with cerebral palsy. **Osteoporosis International**, v. 26, n. 1, p. 141-150, 2015.

FROST, Harold M. From Wolff's law to the Utah paradigm: insights about bone physiology and its clinical applications. **The Anatomical Record:** An Official Publication of the American Association of Anatomists, v. 262, n. 4, p. 398-419, 2001.

GUO, Ava Jiangyang *et al.* Kaempferol as a flavonoid induces osteoblastic differentiation via estrogen receptor signaling. **Chinese medicine**, v. 7, n. 1, p. 10, 2012.

HENDERSON, Richard C. *et al.* Longitudinal changes in bone density in children and adolescents with moderate to severe cerebral palsy. **The Journal of pediatrics**, v. 146, n. 6, p. 769-775, 2005.

HENRIKSEN, K. *et al.* Local communication on and within bone controls bone remodeling. **Bone**, v. 44, n. 6, p. 1026-1033, 2009.

HERTOG, Michael GL *et al.* Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. **The lancet**, v. 342, n. 8878, p. 1007-1011, 1993.

HILL, P. A. Bone remodelling. **British journal of orthodontics**, v. 25, n. 2, p. 101-107, 1998.

HUANG, Wen-Wen *et al.* Kaempferol induced apoptosis via endoplasmic reticulum stress and mitochondria-dependent pathway in human osteosarcoma U-2 OS cells. **Molecular nutrition & food research**, v. 54, n. 11, p. 1585-1595, 2010.

JOHNSTON, Michael V. *et al.* Models of cerebral palsy: which ones are best?. **Journal of child neurology**, v. 20, n. 12, p. 984-987, 2005.

JUNQUEIRA, L.C.; CARNEIRO, J. **Histologia Básica** - Texto & Atlas. 13.ed. Rio de Janeiro: Guanabara Koogan, 2017. 568p.

KÄHKÖNEN, Marja P. *et al.* Antioxidant activity of plant extracts containing phenolic compounds. **Journal of agricultural and food chemistry**, v. 47, n. 10, p. 3954-3962, 1999.

KRICK, Jackie *et al.* Pattern of growth in children with cerebral palsy. **Journal of the American Dietetic Association**, v. 96, n. 7, p. 680-685, 1996.

KRIGGER, Karen W. Cerebral palsy: an overview. **American family physician**, v. 73, n. 1, 2006.

KUPERMINC, Michelle N.; STEVENSON, Richard D. Growth and nutrition disorders in children with cerebral palsy. **Developmental disabilities research reviews**, v. 14, n. 2, p. 137-146, 2008.

LACERDA, D. C. *et al.* Oro-facial functions in experimental models of cerebral palsy: a systematic review. **Journal of oral rehabilitation**, v. 44, n. 4, p. 251-260, 2017.

LACERDA, D.C *et al.*, Perinatal undernutrition associated to experimental model of cerebral palsy increases adverse effects on chewing in young rats. **Physiology & Behavior**, v. 173, p. 69–78, 1 Maio 2017.

LAGOA, Ricardo *et al.* Kaempferol protects against rat striatal degeneration induced by 3-nitropropionic acid. **Journal of neurochemistry**, v. 111, n. 2, p. 473-487, 2009.

LEVINE, Michael A. Assessing bone health in children and adolescents. **Indian journal of endocrinology and metabolism**, v. 16, supl 2, p. S205, 2012.

M CALDERON-MONTANO, J. *et al.* A review on the dietary flavonoid kaempferol. **Mini reviews in medicinal chemistry**, v. 11, n. 4, p. 298-344, 2011.

MACKIE, E. *et al.* Endochondral ossification: how cartilage is converted into bone in the developing skeleton. **The international journal of biochemistry & cell biology**, v. 40, n. 1, p. 46-62, 2008.

MANCINI, M. C *et al.* Comparison of functional activity performance in normally developing children and children with cerebral palsy. **Arq Neuropsiquiatr**, 60, 2-B, 446-452, Jun, 2002.

MARCELOS, Priscylla Gonçalves Correia-Leite *et al.* Effects of treatment with fluoxetine on mandibular development: a morphological study in rats. **Acta histochemica**, v. 117, n. 6, p. 582-589, 2015.

MIYAKE, Masaki *et al.* Promoting effect of kaempferol on the differentiation and mineralization of murine pre-osteoblastic cell line MC3T3-E1. **Bioscience, biotechnology, and biochemistry**, v. 67, n. 6, p. 1199-1205, 2003.

MONTEAGUDO, M. D. *et al.* Comparison of the bone robusticity index and bone weight/bone length index with the results of bone densitometry and bone histomorphometry in experimental studies. **Cells Tissues Organs**, v. 160, n. 3, p. 195-199, 1997.

MOON, Sang Young *et al.* Osteopenic features of the hip joint in patients with cerebral palsy: a hospital-based study. **Developmental Medicine & Child Neurology**, v. 58, n. 11, p. 1153-1158, 2016.

NATIONAL CENTER FOR BIOTECHNOLOGY INFORMATION. **PubChem Database**. Kaempferol. CID=5280863. Disponível em: https://pubchem.ncbi.nlm.nih.gov/compound/Kaempferol. Acesso em: 20 abr. 2020.

NOVAK, Iona *et al.* Early, accurate diagnosis and early intervention in cerebral palsy: advances in diagnosis and treatment. **JAMA pediatrics**, v. 171, n. 9, p. 897-907, 2017.

OLSEN, Bjorn R.; REGINATO, Anthony M.; WANG, Wenfang. Bone development. **Annual review of cell and developmental biology,** v. 16, n. 1, p. 191-220, 2000.

PROUILLET, Christophe *et al.* Stimulatory effect of naturally occurring flavonols quercetin and kaempferol on alkaline phosphatase activity in MG-63 human osteoblasts through ERK and estrogen receptor pathway. **Biochemical Pharmacology**, v. 67, n. 7, p. 1307-1313, 2004.

REN, Jie *et al.* Recent progress regarding kaempferol for the treatment of various diseases. **Experimental and therapeutic medicine**, v. 18, n. 4, p. 2759-2776, 2019.

ROBLING, Alexander G.; CASTILLO, Alesha B.; TURNER, Charles H. Biomechanical and molecular regulation of bone remodeling. **Annu. Rev. Biomed. Eng**., v. 8, p. 455-498, 2006.

ROSE, Jessica; MCGILL, Kevin C. Neuromuscular activation and motor-unit firing characteristics in cerebral palsy. **Developmental medicine and child neurology**, v. 47, n. 5, p. 329-336, 2005.

ROSS, Michel H.; PAWLINA, Wojciech. **Ross histologia: texto e atlas**: correlações com biologia celular e molecular. Rio de Janeiro: Guanabara Koogan, 2016.

SCHOENWOLF, Gary C. *et al.* **Larsen embriologia humana**. 5. ed. Rio de Janeiro: Elsevier, 2016.

SERAFINI, Mauro; MAIANI, Giuseppe; FERRO-LUZZI, Anna. Alcohol-free red wine enhances plasma antioxidant capacity in humans. **The Journal of nutrition**, v. 128, n. 6, p. 1003-1007, 1998.

STANLEY, Fiona J.; BLAIR, Eve; ALBERMAN, Eva. **Cerebral palsies: epidemiology and causal pathways**. Cambridge: Cambridge University Press, 2000.

STEVENS, A.; LOWE, J. **Histologia Humana.** 2. ed. São Paulo: Manole, 2002.

STRATA, F. *et al.* Effects of sensorimotor restriction and anoxia on gait and motor cortex organization: implications for a rodent model of cerebral palsy. **Neuroscience**, v. 129, n. 1, p. 141-156, 2004.

TORTORA, G. J.; DERRICKSON, B. **Princípios de anatomia e fisiologia**. 14. edição. Guanabara Koogan. Rio de Janeiro, 2019.

TRIVEDI, Ritu *et al.* Kaempferol has osteogenic effect in ovariectomized adult Sprague–Dawley rats. **Molecular and cellular endocrinology**, v. 289, n. 1-2, p. 85-93, 2008.

VAUZOUR, David *et al.* The neuroprotective potential of flavonoids: a multiplicity of effects. **Genes & nutrition**, v. 3, n. 3, p. 115, 2008.

VINAYAGAM, Ramachandran; XU, Baojun. Antidiabetic properties of dietary flavonoids: a cellular mechanism review. **Nutrition & metabolism**, v. 12, n. 1, p. 60, 2015.

WATTEL, Alice *et al.* Potent inhibitory effect of naturally occurring flavonoids quercetin and kaempferol on in vitro osteoclastic bone resorption. **Biochemical Pharmacology**, v. 65, n. 1, p. 35-42, 2003.

WEST-EBERHARD, Mary Jane. Alternative adaptations, speciation, and phylogeny (a review). **Proceedings of the National Academy of Sciences**, v. 83, n. 5, p. 1388-1392, 1986.

WEST-EBERHARD, Mary Jane. **Developmental plasticity and evolution**. Oxford: Oxford University Press, 2003.

WEST-EBERHARD, Mary Jane. Developmental plasticity and the origin of species differences. **Proceedings of the National Academy of Sciences**, v. 102, n. suppl 1, p. 6543-6549, 2005.

WHITNEY, Daniel G. *et al.* Cortical bone deficit and fat infiltration of bone marrow and skeletal muscle in ambulatory children with mild spastic cerebral palsy. **Bone**, v. 94, p. 90-97, 2017.

WONG, Sok Kuan; CHIN, Kok-Yong; IMA-NIRWANA, Soelaiman. The osteoprotective effects of kaempferol: The evidence from in vivo and in vitro studies. **Drug Design, Development and Therapy**, v. 13, p. 3497, 2019.

YEARGIN-ALLSOPP, Marshalyn *et al.* Prevalence of cerebral palsy in 8-year-old children in three areas of the United States in 2002: a multisite collaboration. **Pediatrics**, v. 121, n. 3, p. 547-554, 2008.

YOUDIM, Kuresh A.; JOSEPH, James A. A possible emerging role of phytochemicals in improving age-related neurological dysfunctions: a multiplicity of effects. **Free Radical Biology and Medicine**, v. 30, n. 6, p. 583-594, 2001.

ZANINI, Graziela; CEMIN, Natália Fernanda; PERALLES, Simone Nique. Paralisia Cerebral: causas e prevalências. **Fisioterapia em Movimento**, v. 22, n. 3, 2017.

APÊNDICE A - ARTIGO ORIGINAL

A kaempferol treatment during early life causes increase of body weight, naso-anal length and enlargement of osteocyte lacunae in bone of rats with experimental cerebral palsy

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ABSTRACT

Background: We sought to evaluate the effect of neonatal treatment with kaempferol in rats submitted to experimental cerebral palsy (CP) on physical characteristics, macroscopic morphometry, bone radiopacity, and microscopic analysis of the cortical bone.

Methods: Male Wistar rats distributed in the subgroups were used: a) Placebo Control (C+P), b) Kaempferol Control (C+K), c) Placebo Cerebral Palsy (CP+P), d) Kaempferol Cerebral Palsy (CP+K). The CP model consisted of the association of perinatal anoxia and sensory motor restriction of the hind legs. After weaning (P25), the animals were separated in cages until the 36th day of postnatal life, when they were euthanized to collect bone, femur and tibia tissues and weight, length and bone histomorphometry and radiographs were performed. Statistical analysis was used considering a 5% significance level.

Results: Our results suggests that in P36 the induction of experimental CP promoted the following results: decreased in body weight and naso-anal length, reduction of absolute weight and size of tibia and femur, reduction of cortical thickness and medullar area, higher density lacunar and lower area of lacunae, reduced the bone radiopacity of the analyzed regions of the femur and tibia. On the data of the animals submitted to CP and that received treatment with kaempferol compared to the CP

group that received placebo suggest that in P36 (CP+K vs. CP+P) we have the following results: increase in body weight and naso-anal length and increase in density of lacunae and area of lacunae. **Conclusion:** In summary our data show that the cerebral palsy model has drastically reduced the development of the animals with respect to decreased in body weight and naso-anal length, reduction of absolute weight and size of tibia and femur, reduction of cortical thickness and medullar area, lower area of lacunae, reduced the bone radiopacity of the analyzed regions of the femur and tibia, and that the treatment with kaempferol has attenuated the effects concerning body weight and naso-anal length, as well as histologically can observe the increase in the area and number of lacunae. More studies are necessary to verify if this improvement in bone development can be extended over time.

Key-words: cerebral palsy; 3.5.7-Trihydroxy-2-(4-hydroxyphenyl)-4H-chromen-4-one; flavonoid; bone; tibia; femur".

BACKGROUND

Cerebral palsy is one of the most common motor disabilities in childhood developmental. Is a group of disorders that culminate in limitations in movement and posture of committed individuals, these disorders are related to damage that affects the developing brain (1–3). Commonly, children with CP are likely to present low-impact fractures and, hypertension, muscle weakness and decreased bone mineral density (4,5). In developed countries CP has an estimated average rate of 3 for every 1,000 live births and in underdeveloped countries this figure can come to 7 for every 1000 live births(2,6,7).

The poor nutritional status of individuals with CP can be associated with retardation in motor development that can further compromise bone tissue development (8,9). There are few early treatment strategies for CP, so we highlight the pharmacological or flavonoid supplementation treatment, that has been extensively studied regarding the maintenance of brain health (10), prevention of neurodegenerative diseases and also osteoporosis (11). Flavonoids have been studied with respect to improvement in bone metabolism (osteogenic capacity), and increase in bone mineral density(12,13).

Kaempferol is a flavonoid compound that presents several functions associated with bone protection and decreased osteopenia. In studies on models of osteoporosis induced by ovariectomy and models of bone fractures it was observed that the use of kaempferol promoted bone protection (14), increased bone mineral density (15). Thus, the study of therapeutic intervention strategies, such as the use of kaempferol are valid for the understanding of the clinical conditions and the discovery of new forms of cerebral palsy treatment.

The being is the result of the interaction of his set of genes with the environment in which he is inserted, as a bioproduct of this operation we have the phenotype. Several changes can occur in the initial periods of an individual's life and lead to changes in the functional and metabolic structure of the individual(16–18). Thus, the individuals have in the perinatal period an optimal critical window for the realization of interventions. Therefore, researchers have been studying extensively the phenotype of phenotypic plasticity, phenotypic plasticity can be defined as a phenomenon in which a genotype exposed to different environmental conditions can come to originate different phenotypes. These adaptive responses are also called "plastic responses"

where this individual can alter his morphology, activity rate and physiological or behavioral state(19,20).

In rodent models of cerebral palsy reduction of body weight, food consumption, reduction of locomotor activity compared to control animals are observed (21,22). In this context our objective was to evaluate if kaempferol administered in the in early period reduces the damages caused by CP experimental in what concerns bone metabolism and physical characteristics of male *Wistar* rats.

METHODS

Ethics statement

All animal experiments were conducted in accordance with guidelines from Animal Research: National Council for Animal Control and Experimentation (CONCEA), in accordance with law 11.794 of 8 October 2008, as well as international standards established by the National Institute of Health Guide for Care and Use of Laboratory Animals and the ethics committee for animal use (CEUA) of Universidade Federal de Pernambuco.

Animals

48 male Wistar rats were used. from 20 rats, kept in a bioterium at the UFPE Department of Nutrition with a temperature of 22 + 2ºC. light-dark cycle from 12/12 hours (light 20: 00h to 08: 00h), the animals were housed in groups polypropylene cages (49X34X16cm) and free access to purified water and feeding with a standard diet (Labina®). The bioterium has an exhaust fan to reduce the odor of urine and feces.

After confirmation of pregnancy the females were housed in individual cages. After birth the pups were adjusted to eight pups per litter, weighing between 6-8 grams

on the first postnatal day, remaining with their respective mothers during the lactation period. In each litter the animals are randomly assigned to the 4 experimental groups according to CP induction and treatment with kaempferol: they have been distributed in the following subgroups: a) Control placebo (C+P; n= 12), b) Control Kaempferol (C+K; n= 12), c) Cerebral palsy placebo (CP+P; n= 12), d) Cerebral Palsy Kaempferol (CP+K; n= 12). After weaning (P25). the animals were separated in individual cages (3-4 animals per cage) until the 36th day of postnatal life (P36) when they were euthanized for tissue collection.

Experimental Model of CP

The pups of the CP+P and CP+K groups were submitted to the experimental model of cerebral palsy adapted by Strata *et al.*, 2004 and COQ. *et al.*, 2008. This model associated perinatal anoxia with a model of sensorimotor restriction of the lower limbs similar to the lack of movement that occurred in CP. At birth, the pups were subjected to two episodes of anoxia. at P0 (day of birth) and P1 (day one of life). The perinatal anoxia method uses a chamber in a water bath at 37°C where the animals are exposed to nitrogen ventilation for 12 minutes (100% N. 9L/min). From P2 to P28, sensorimotor restriction of the hind legs was performed using micropore tape and epoxy mold for 16 hours a day, during the rest of day the free movement was allowed (23,24).

Treatment with kaempferol or placebo

During the first 21 days of postnatal life (P1 to P21) the pups from groups C+K and CP+K received treatment with Kaempferol (Cayman Chemical. Ann Arbor. MI. USA), was applied 1mg/Kg, with an application volume of 1 µ/g. DMSO (dimethyl sulfoxide) was used to dilute the stock solution and preparing kaempferol obtained by

mixing 50mg of kaempferol and 5ml of dimethylsulfoxide (DMSO) (m/v), 20µl aliquots that was kept at -80 ° C in eppendorfs identified and protected from light. On the day of application. 1980µl of commercial saline was added to the aliquot, obtaining a final volume of 2ml. Accordingly, syringes were prepared with volumes (1ml / 100g) according to the weight of each animal. The kaempferol and placebo was administered intraperitoneally (25). The pups of the CP and CP+P groups received. during the first 21 days of postnatal life. saline + DMSO (0.9% NaCl intraperitoneally).

Experimental analysis

Measurement of Body Weight. naso-anal and tail length

The pups body weight was recorded at birth, and on days P1 to 21, P28 and P36 days of life with precision balance Marte (AD 2000). The naso-anal and tail lengths of the pups were also evaluated on days 1, 7, 14, 21, 28 and 36 with Digital calliper (Jomarca ®).

Measurement of the weight and length of the femur and tibia bone's

After dissection. the bone of the right tibia and femur were immediately weighed on a precision balance of 0.01 g (Marte AD 330). The total length of the bones of the tibia and femur were measured using a digital caliper (Jomarca ®) with an accuracy of 0.01 mm on a smooth surface with its ventral face facing upwards.

Radiopacity analysis

The right femur and tibia were aligned and scanned on the phosphorus plate and radiographed. All images were acquired using the Heliodent Plus X-ray machine (Sirona Dental Systems. Bensheim, Germany) operating at 70kVp and 7mA graph and the Express intraoral digital system (Instrumentarium, Tuusula, Finland). Digime

Optime® phosphor plates (Soredex. Helsinki. Finland) were used to perform digital radiographs. The exposure time (ET) was standardized in 0.10 seconds. The distance used was standardized at a height of 30 cm with an acrylic focus-sensor device for all radiographic exams. The specimens, lead block and penetrometer (containing 8 degrees. with an interval of 1 mm between them) were always placed on the phosphor plate in the same position.

The images were saved in PNG format and exported to the ImageJ program (U.S. National Institutes of Health. Bethesda. Maryland. USA, version 1.50i) in which the pixel values of each degree of the penetrometer and the area of bone regions of the femur (head, neck, epiphysis and diaphysis) and tibia (epiphysis and diaphysis) were measured. A curve was prepared using a scatter plot with pixel values versus the corresponding mm AL (aluminum millimeters). Thus, the value in mm AL of the analyzed regions was obtained. The data were tabulated in the Microsoft Office Excel 2016 program in a spreadsheet for each radiographic image. The radiographic evaluations were made by a single, previously calibrated evaluator.

Histological and histomorphometric analysis

The left tibias and femurs were dissected and fixed in 10% formalin solution for two days and then washed in PBS buffer solution. They were then included in an EDTA (ethylenediaminetetraacetic acid) decalcifying solution and placed on a shaking table for approximately two months. When decalcification was verified, the EDTA solution was discarded, the bones were washed again in PBS and stored in a 70% alcohol solution until histological processing. Subsequently, the pieces were dehydrated with increasing solutions of 70% alcohol, diaphanized in xylol alcohol, then xylol and embedded in paraffin.

For histomorphometric analysis, cross sections of the tibial diaphysis and femur of each animal were used. The slides were stained by Hematoxylin/Eosin (HE) and analyzed by light microscopy using a Tucsen ISH1000 digital camera coupled to a Nikon eclipse E200 optical microscope under fixed focus and field clarity, using ISCAPTURE 4.1 software. 3 In these sections, cortical bone thickness and medullary area were analyzed by imaging the diaphyses at a final magnification of 40×. Three and four measurements were taken on each histological section, respectively, of the tibia and femur, prioritizing the upper, lower, and lateral regions of each section. To determine the thickness, the cortical bone was measured from the periosteal surface to the endosteal surface using the appropriately calibrated ImageJ software (U.S. National Institutes of Health, Bethesda, Maryland, USA). As for the histological analysis, images of the region of the tibial diaphysis were acquired as described above to analyze the number of osteocyte lacunae per field, area and perimeter of the cells, which were analyzed at a final magnification of 400x using the ImageJ program (U.S. National Institutes of Health, Bethesda, Maryland, USA) appropriately calibrated.

Statistical Analysis

The results were expressed as mean ± S.E.M. (standard error of the mean). The normality analysis of the sample was performed using the Kolmogorov-Smirnov test. For the data considered parametric, the comparison between the groups was performed using the ANOVA two way or two-way repeated measures, followed by the Tukey post-test; when the data was non-parametric, the Kruskal-Wallis test was performed, followed by the Dunn's post-test. The analyzes were performed in GraphPad 7® (GraphPad Software, Inc., La Jolla, CA, USA) e and the level of significance considered was $p < 0.05$.

RESULTS

Physical characteristics

Body weight

In the evaluation of body weight (Figure 1) there was a statistical difference in the postnatal days 21, 28 and 36 [F (3. 28) = 35.26; p <0.0001]. At 21 days of postnatal life, it was observed that the weight of the CP+P animals was significantly lower when compared to the weight of the animals of the C+P group (CP+P= 23.94±1.611 vs. C+P= 39.59±1.106, p<0.01). At 28 days of postnatal life was again observed that the weight of the CP+P animals was lower compared to C+P (CP+P= 35.07± 3.333 vs. C+P= 67.35 ± 2.339, p<0.0001). At 36 days of postnatal life the weight of the placebo paralysis animals remained lower than the weight of the C+P animals (CP+P= 63.43± 6.197 vs. C+P= 115.5± 8.025, p<0.0001).

It can be seen that the experimental cerebral palsy model significantly reduced the animals' weight. It was also observed an increase in body weight of animals CP+K group compared to CP+P group. (CP+K= 76.40 ± 4.908 vs. CP+P= 63.43± 6.197, p<0.05) which demonstrates that the treatment with kaempferol was able to increase the body weight of rats submitted to cerebral palsy. when compared with the placebo cerebral palsy group.

Naso-anal length

In the assessment of naso-anal length (figure 1) there was a statistical difference in the postnatal days 14. 21. 28 and 36 [F (3. 28) = 39.65; p <0.0001]. At 14 days of postnatal life. the length of the animals in the CP + P group was shorter when compared to the C+P group (CP+P= 74.77 ± 1.835 vs. C+P= 82.84 ± 1.549, p <0.05). At 21 days, the length of the CP+P animals was shorter when compared to the C+P

group (CP+P = 86.00 ± 1.291 vs. C+P = 100.7 ± 1.123, p <0.0001). At 28 and 36 days the length of the animals in the CP+P group remained shorter when compared to the C+P group. postnatal day 28 (CP+P= 104.9 ± 1.578 vs. C+P= 117.2 ± 2.482, p <0.001) postnatal day 36 (CP+P= 124.7 ± 1.687 vs. C+P =144.5 ± 4.066, p <0.0001). These data demonstrate that the cerebral palsy model was able to significantly decrease the length of animals submitted to paralysis.

On day 36. an increase in the length of the animals in the CP+K group compared to the CP+P group was also observed (CP+K = 132.7 ± 2.296 vs. CP+P = 124.7 ± 1.687, p <0.05). It is observed. therefore. that the neonatal treatment with kaempferol was able to increase the naso-anal length of the animals submitted to cerebral palsy when compared with the placebo group.

Tail length

Regarding the tail length of the animals that was evaluated on days 7, 14, 21, 28 and 36. (Figure 1) [F (3. 28) = 6.621; p = 00016]. There was significant difference on the P21, P28 and 36. In P21 the differences are restricted to group CP+P compared to C+K (CP+P 44.65±2.058 vs. C+K= 53.43±2.140). In P28 the differences were between CP+K and C+K (CP+P=53.86±2.001 vs. C+K= 67.99±1.643) and between CP+K and control groups (CP+K= 53.58±2.548 vs. C+P= 62.17±1.765) and (CP+K= 53.58±2.548 vs. C+K= 67.99±1.643). On the P36 the CP+P group shows a shorter tail length than the C+P group (CP+ P = 64.67 ± 3.432 vs. C+P = 75.42 ± 3.428, p <0.01). In relation to the cerebral palsy animals no significant differences were observed in the pups tail length during the experiment, although a tendency to increase was observed in the postnatal day 36.

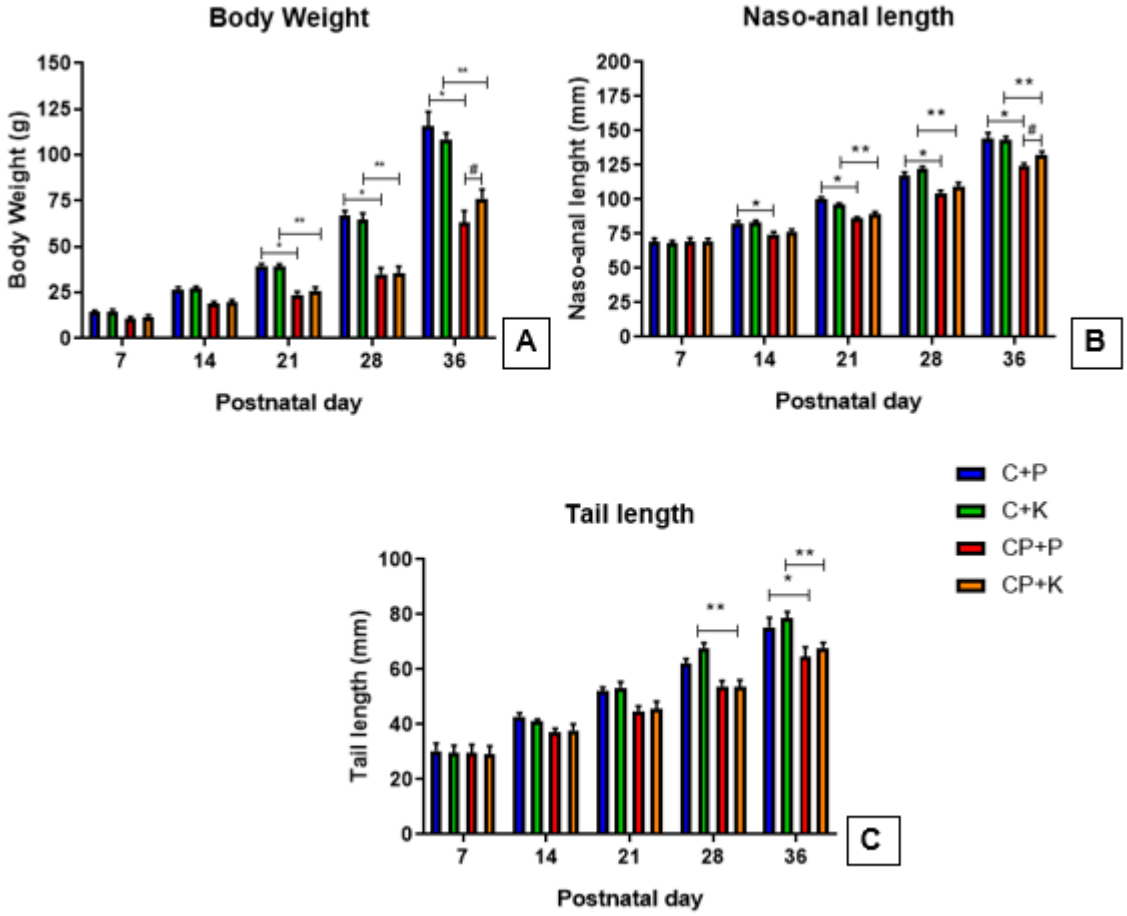

Figure 1: Physical characteristics- Body weight (A), naso-anal length (B) and caudal length (C) of animals in the postnatal days 7, 14, 21, 28 and 36. The composition of the groups is with intervention with Kaempferol or placebo and model of cerebral palsy or control. C+P (Control + Placebo, n=8); C+K (Control + Kaempferol, n= 8); CP+P (Cerebral Palsy + Placebo, n=8); CP+K (Cerebral Palsy + Kaempferol, n=8). The data are presented in mean and standard error of the mean and were analyzed by the two-way ANOVA test repeated measures followed by the post-hoc Tukey test. *= differences between CP+P and C+P; **= differences between CP+K and C+K; #= differences between CP+P and CP+K

Macroscopic morphometry

Femur Weight

In the evaluation of the absolute weight of the femur (figure 2) there was a statistical difference between the groups [F (3. 21)= 28.97, p <0.0001]. The CP+P group had a lower absolute weight when compared to the C+P group (CP+P= 0.2649 ± 0.01490 vs. C+P= 0.4425±0.01057, p <0.0001) The CP+K group had lower absolute weight when compared the C+K group (CP+K= 0.2998 ± 0.02377 vs. C+K= 0.4041 ± 0.02185, p = 0.0006). These data demonstrate that cerebral palsy drastically reduces the absolute femur weight of animals submitted to CP, there is also a tendency to increase the absolute bone weight of the animals of the CP+K group compared to the CP+P group. Regarding the relative weight (figure 2), no significant differences were observed between the groups [F (3. 21) = 1.280. p = 0.3070].

Tibia Weight

In the evaluation of the absolute weight of the tibia (figure 2). there was a statistical difference between the groups [F (3. 21) = 11.18; p= 0.0001]. The CP+P group had lower tibial weight when compared to the C+P group (CP+P = 0.2158 ± 0.01585 vs. C+P = 0.3368 ± 0.01722, p= 0.0005). The CP+K group also had lower absolute tibia weight when compared to C+K (CP+K=0.2443±0.02319 vs. C+K= 0.3223 ± 0.009273, p = 0.0241). Demonstrating that cerebral palsy significantly reduces the absolute tibial weight of the CP animals compared to the animals in the control group. Regarding the absolute tibia weight of the CP groups. we can see a tendency to increase in the kaempferol group when compared to the placebo group. Regarding the relative weight (figure 9). there were no statistical differences between the groups [p = 0.1812].

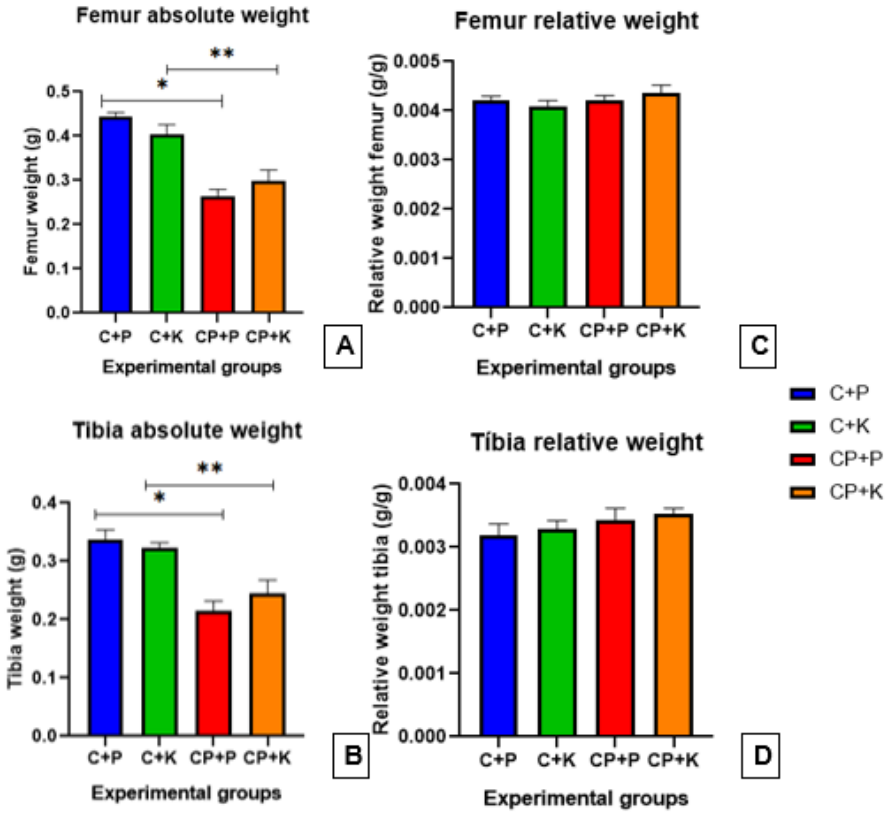

Figure 2: Macroscopic evaluation- Absolute (A, B) and relative weight (C,D) of the femur and tibia bones in P36. The composition of the groups is in agreement with intervention with Kaempferol or placebo and model of cerebral palsy or control. C+P (Control + Placebo, n=8); C+K (Control + Kaempferol, n= 8); CP+P (Cerebral Palsy + Placebo, n=8); CP+K (Cerebral Palsy + Kaempferol, n=8). The data are presented in mean and standard error of the mean and were analyzed by the two-way ANOVA test, followed by the post-hoc Tukey test or by the Kruskal-Wallis test followed by the post-hoc Dunn's test. *= differences between CP+P and C+P; **= differences between CP+K and C+K; #= differences between CP+P and CP+K

Femur Length

In the evaluation of the absolute length of the femur (figure 3). statistical differences were observed between the groups [F (3. 21) = 37.98; p <0.0001]. The CP+P group had a lower absolute length when compared to the C+P group (CP+P =

19.02 ± 0.4555 vs. C+P= 23.32±0.1796, p <0.0001). The CP+K group also had a lower absolute length of the femur when compared as compared to C+K group also had lower absolute length of the femur (CP+K= 19.54±0.5909 vs. C+K= 22.85±0.3755, p<0.0001). The data demonstrate that experimental cerebral palsy decreases the absolute length of the femur. when compared with the control groups. There were no statistical differences between the CP+P and CP+K groups, but a slight improvement trend related to animals treated with kaempferol (CP+K). In relation to the relative length of the femur (figure 2), no statistical differences were observed between the groups p = 0.2464.

Tibia Length

The data related to the absolute length of the tibia (figure 3) demonstrate significant differences between the groups [F (3. 21) = 19.38; p<0.0001]. It is observed that the absolute tibial length of the animal's CP+P was smaller when compared to the group C+P (CP+P= 21.55± 0.7307 vs. C+P= 26.75± 0.4071, p<0.0001). The CP+K group also had the lowest absolute length when compared to the C+K group (CP+K=21.87±0.8632 vs. C+K=26.53±0.4748, p= 0.0003). This demonstrates that cerebral palsy significantly reduces the absolute tibial length of animals compared to control groups. The relative length did not show significant variation between the groups.

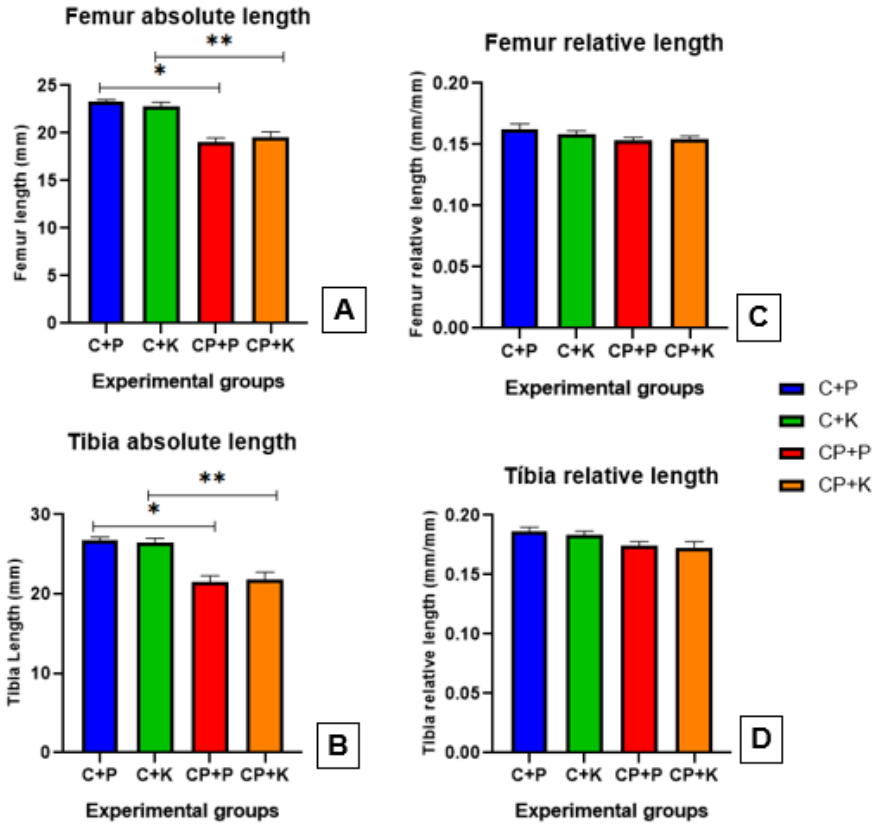

Figure 3: Macroscopic evaluation- Relative absolute length of the femur and tibia bones in P36. The composition of the groups is in agreement with intervention with Kaempferol or placebo and model of cerebral palsy or control. C+P (Control + Placebo, n=8); C+K (Control + Kaempferol, n= 8); CP+P (Cerebral Palsy + Placebo, n=8); CP+K (Cerebral Palsy + Kaempferol, n=8). The data are presented in mean and standard error of the mean and were analyzed by the two-way ANOVA test, followed by the post-hoc Tukey test or by the Kruskal-Wallis test followed by the post-hoc Dunn's test. *= differences between CP+P and C+P; **= differences between CP+K and C+K; #= differences between CP+P and CP+K

Radiopacity results

The data related to radiopacity of the femur regions (head, neck, epiphysis and diaphysis) show differences between the cerebral palsy and control groups (see table 1). The CP+P group shows a decrease in radiopacity in all regions evaluated when

compared to the C+P group in Tukey's multiple comparisons test; head (CP+P vs C+P p: 0.0022), neck (CP+P vs C+P p: 0.0082), proximal epiphysis (CP+P vs C+P p: 0.0031), diaphysis (CP+P vs C+P p: 0.0068) and distal epiphysis (CP+P vs C+P p:0.0005;). Differences are also observed when comparing the CP+K group with the C+K group in the head of femur (CP+K vs C+K p:0.0069), neck (CP+K vs C+K p:0.0086), proximal epiphysis (CP+K vs C+K p:0.0033), diaphysis (CP+K vs C+K p:0.0044) and distal epiphysis (CP+K vs C+K p:0.0022).

Regarding the tibia (see table 2) the CP+P group also presents a decrease in radiopacity in the evaluated regions (proximal epiphysis, diaphysis and distal epiphysis) when compared to the C+P in Tukey's multiple comparisons test; proximal epiphysis (CP+P vs C+P p: 0.0008), diaphysis (CP+P vs C+P p: 0.0063) and distal epiphysis (CP+P vs C+P p: 0.0069;); likewise, the CP+K group presents a decrease when compared to the control groups in the proximal epiphysis (CP+K vs C+K p: 0.0110). Regarding the experimental cerebral palsy groups (CP+P and CP+K), no significant differences were observed between the groups in the analyzed regions. These data show that the cerebral palsy model reduced the bone radiopacity of the analyzed regions of the femur and tibia. likewise, no differences were observed between the control groups.

	GROUPS				
	C+P	**C+K**	**CP+P**	**CP+K**	**F VALUE**
Femur head	0.85±0.058	0.86±0.037	0.54±0.044	0.59±0.068	F (3. 15) = 11.88 P=0.0003
Femur neck	0.84±0.056	0.92±0.059	0.57±0.035	0.65±0.068	F (3. 15) = 10.48 P=0.0006
Proximal Epiphysis	0.84±0.057	0.91±0.057	0.56±0.031	0.63±0.064	F (3. 15) = 12.96 P=0.0002
Diaphysis	0.86±0.054	0.92±0.070	0.59±0.031	0.64±0.064	F (3. 15) = 11.19 P=0.0004
Distal Epiphysis	0.99±0.052	1.0±0.080	0.65±0.028	0.73±0.052	F (3. 15) = 16.15 P<0.0001

Table 1: Evaluation of bone radiopacity of the femoral regions (head, femoral neck, epiphysis and diaphysis). The composition of the groups is with intervention with Kaempferol or placebo and model of cerebral palsy or control. C+P (Control + Placebo, n=6); C+K (Control + Kaempferol, n= 6); CP+P (Cerebral Palsy + Placebo, n=6); CP+K (Cerebral Palsy + Kaempferol, n=6). The data are presented in average and standard error of the mean and were analyzed by the two-way ANOVA test, followed by the post-hoc Tukey test.

	GROUPS				
	C+P	**C+K**	**CP+P**	**CP+K**	**F VALUE**
Proximal Epiphysis	0.85±0.058	0.85±0.042	0.56±0.040	0.63±0.041	F (3. 15) = 13.01 P=0.0002
Diaphysis	0.87±0.063	0.78±0.028	0.58±0.068	0.57±0.061	F (3. 15) = 8.109 P=0.0019
Distal Epiphysis	0.90±0.060	0.77±0.033	0.58±0.086	0.58±0.067	F (3. 15) = 7.208 P=0.0032

Table 2: Evaluation of bone radiopacity of tibia regions (proximal epiphysis, diaphysis and distal epiphysis). The composition of the groups is with intervention with Kaempferol or placebo and model of cerebral palsy or control. C+P (Control + Placebo, n=6); C+K (Control + Kaempferol, n= 6); CP+P (Cerebral Palsy + Placebo, n=6); CP+K (Cerebral Palsy + Kaempferol, n=6). The data are presented in mean and standard error of the mean and were analyzed by the two-way ANOVA test, followed by the post-hoc Tukey test.

Microscopic morphometry

Microscopic results of the cortical bone

Cortical thickness

Regarding the cortical thickness of the femur, there are differences between the groups [F (3, 18) = 8,996; P=0,0007] (see figure 4 and 5). The CP+P group presented lower cortical thickness when compared to the C+P group (CP+P= 263,8 ± 12,18 vs. C+P = 367,5 ±20,72, p= 0,0034). The CP+K group also showed lower cortical thickness when compared to the C+K (CP+K= 274,3 ±16,29 vs. C+K = 354,5 ± 12,91; p= 0,0244). No significant difference is observed between CP groups although there is a graphic tendency (p=0,9749).

Concerning the cortical thickness of the tibia, there are differences between the groups [F (3. 21) = 10.77; P=0.0002] (see figure 4 and 5). The CP+P group showed lower cortical thickness when compared to the C+P group (CP+P= 264.6 ± 16.47 vs. C+P =344.4 ±12.15, p=0.0031). The CP+K group also showed lower cortical thickness when compared to the C+K (CP+K= 285.5 ±13.69 vs. C+K =360.3 ±9.539, p= 0.0056). No significant difference is observed between CP groups although there is a graphical tendency (p=0.7184).

Medullar area

Referring to the medullary area of the femur it is possible to observe differences between the groups [F (3. 18) = 10.43; P=0.0003] (see figure 4 and 5) The CP group presented lower medullary area when compared to the C+P group (CP+P= 693403.8±40668.8 vs. C+P =1236174.3±146620.2, p=0.0083). The CP+K group also showed smaller medullary area when compared to the C+K (CP+K= 716723.4±52492.7 vs. C+K=1329343.2±128968.3, p= 0.0030). No significant difference was observed between the experimental cerebral palsy groups.

On the data of the medullary area of the tibia, it is possible to observe differences between groups [F (3. 21) = 19.92; P<0.0001] (see figure 4 and 5) The

CP+P group presented lower medullary area when compared to the C+P group (CP+P= 398175.2 ± 34840.3 vs. C+P =558913.3 ± 27636.6, p=0.0002). The CP+K group also showed a smaller medullary area when compared to the C+K (CP+K= 457521.3±39110.0 vs. C+K=608797.6±34307.1, p=0.0003). No significant difference was observed between the experimental cerebral palsy groups. although a trend is observed (p=0.2339).

Lacunar density

The data regarding the lacunar density are presented in figure 4, and is illustrated in figure 6 show that there are statistical differences only between the groups [Kruskal-Wallis statistic 16.79; p=0.0008]. CP+K and CP+P (CP+K=33.31± 0.9280 vs. CP+P=28.28±0.8015, p=0.0003); Between CP+P and control groups the difference was not significant, although graphically there is a trend (C+P vs. CP+P, p: 0.7280; C+K vs. CP+P, p: 0.1289), the same occurs between the CP+K group and controls (C+P vs. CP+K, p: 0.0795; C+K vs. CP+K, p: 0.5051).

Lacunar area

The data related to the lacunar area are presented in figure 4, and is illustrated in figure 6 show that there are differences between the groups [Kruskal-Wallis statistic 183.5; P<0.0001]. The CP+P group compared to the C+P control groups showed a lower in the lacunae area (CP+P= 37.65± 0.4377 vs. C+P = 44.77±0.4784, p<0.0001). And the CP+K group also showed reduced lacunar area compared to C+K (CP+K= 39.59±0.4611 vs. C+K=44.70±0.4747, p<0.0001). A significant difference was observed between the experimental cerebral palsy groups (CP+P=37.65± 0.4377 vs. CP+K= 39.59±0.4611. p=0.0110.

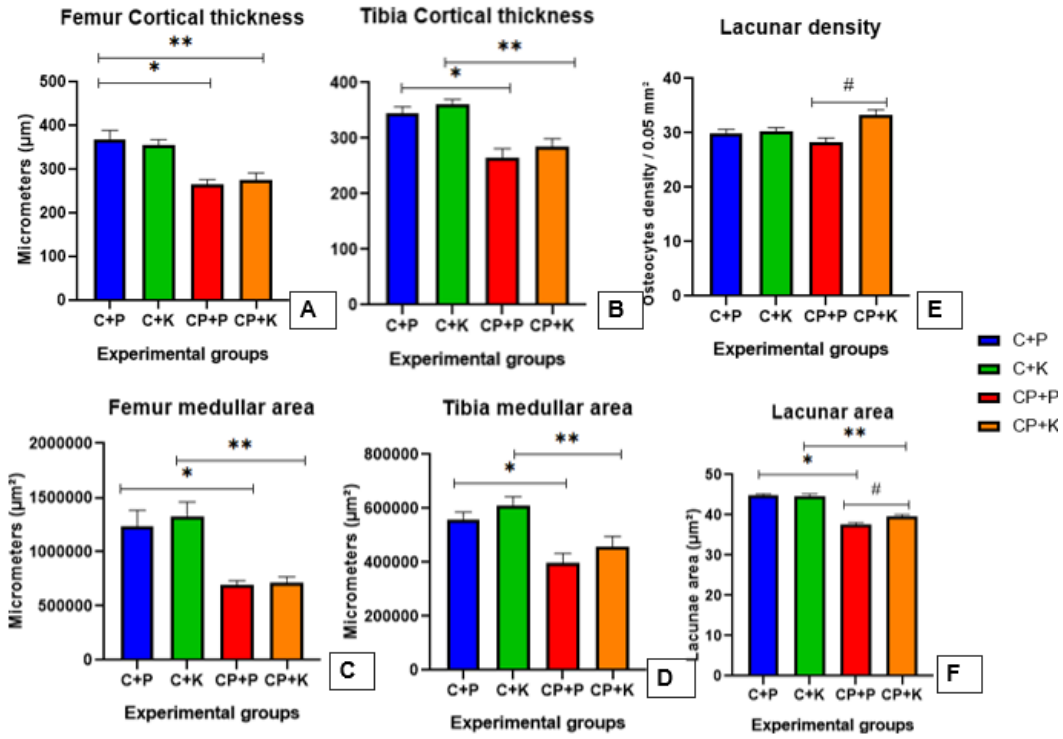

Figure 4: Microscopic morphometry- (A,B)Cortical thickness, (C,D) medullar area, lacunar density(E) and lacunar area(F) of femur and tibia in P36. The composition of the groups is in agreement with intervention with Kaempferol or placebo and model of cerebral palsy or control. Femur cortical thickness and medullar area: C+P (Control + Placebo, n=7); C+K (Control + Kaempferol, n= 7); CP+P (Cerebral Palsy + Placebo, n=7); CP+K (Cerebral Palsy + Kaempferol, n=7). Other data: C+P (Control + Placebo, n=8); C+K (Control + Kaempferol, n= 8); CP+P (Cerebral Palsy + Placebo, n=8); CP+K (Cerebral Palsy + Kaempferol, n=8). The data are presented in mean and standard error of the mean and were analyzed by the two-way ANOVA test, followed by the post-hoc Tukey test or by the Kruskal-Wallis test followed by the post-hoc Dunn's test. *= differences between CP+P and C+P; **= differences between CP+K and C+K; #= differences between CP+P and CP+K.

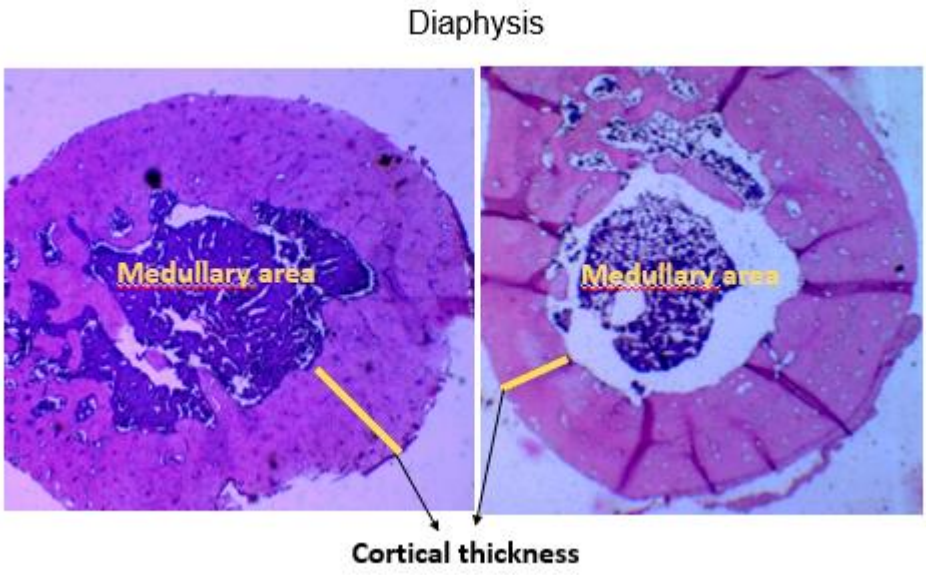

Figure 5- Microscopic morphometry - Photomicrograph showing in cross section evaluated regions of the cortical bone of the femur and tibia: cortical thickness (yellow arrow), medullary area (40x).

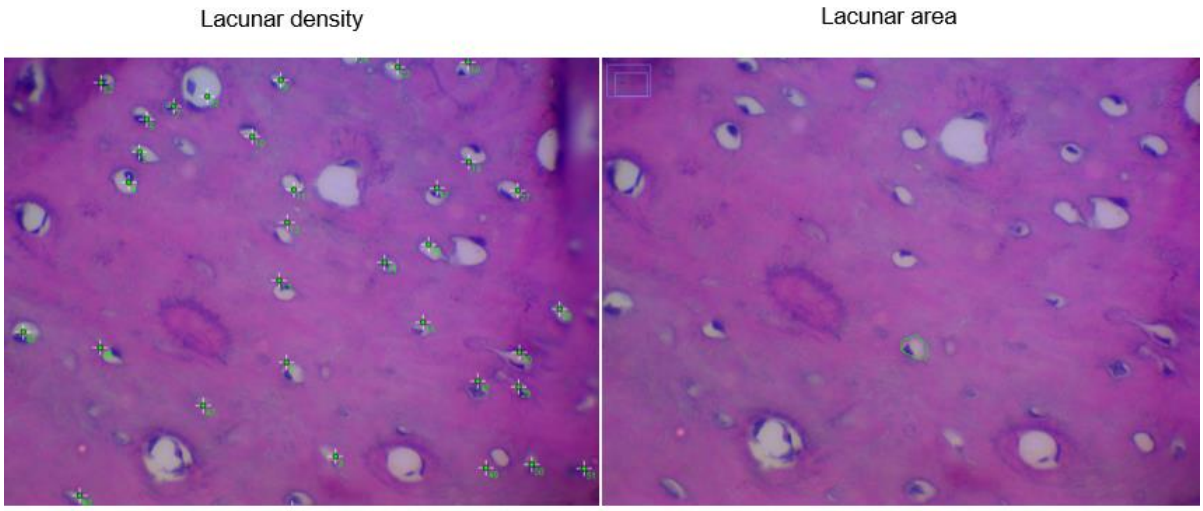

Figure 6- Microscopic morphometry- Photomicrograph demonstrating the parameters evaluated in the lacunar density and lacunar area analysis (400x).

Discussion

As far as we know, this was the first study to evaluate in rats submitted to CP experimental (perinatal anoxia combined with sensory-motor restricted hind paws) the effects of neonatal treatment with kaempferol on body weight, naso-anal and caudal length, bone length and weight, as well as analyses of the radiopacity of areas of the femur and tibia and histomorphometry analyses of the cortical bone region. Our results suggests that in P36 the induction of experimental CP promoted the following results: decreased in body weight and naso-anal length, reduction of absolute weight and size of tibia and femur, reduction of cortical thickness and medullar area, reduced the bone radiopacity of the analyzed regions of the femur and tibia and lower area of lacunae.

On the data of the animals submitted to CP and that received treatment with kaempferol compared to the CP group that received placebo suggest that in P36 (CP+K vs. CP+P) we have the following results: increase in body weight and naso-anal length and increase in density and lacunar area. Regarding the other parameters there are no statistical differences between the groups, although there is a small tendency of improvement of CP+K. These data show that the cerebral palsy model has drastically reduced the growth of the animals and that the treatment with kaempferol has attenuated the effects concerning body weight and naso-anal length, as well as histologically can observe the increase in the area of lacunae.

The experimental model of CP limited the body weight gain of animals, when compared with the control groups, this result corroborates with the results of previous studies using the same model of CP (21,23,26), are also in accordance with the results presented by other studies that evaluated the impact of different models of CP (23,27). This reduction in weight gain can be explained by the process of restriction of the own members of the model that reproduces the CP damages. In addition, animals submitted to CP notably present suction problems and difficulties in masticatory

movements (21). Thus, it is believed that the limitation in weight gain of CP animals occurs by the combination of perinatal anoxia and sensory-motor restriction and the difficulties added to the feeding process of paralytic rats.

After kaempferol administration an attenuation in body weight loss can be observed and increasing the naso-anal length of CP animals, which corroborates with the results of Chaves 2020 in which rats treated with kaempferol showed an increase in body weight after weaning, and with another study (25) in which animals subjected to a model of striatal degeneration by 3-Nitropropionic acid presented several positive changes, such as the prevention of the appearance of injury, decreased mortality and decreased weight loss in animals. In contrast, in other studies kaempferol did not improve the body weight (28).

Children with cerebral palsy commonly have an alimentary deficit, which is associated with a worsening in health and growth (29), which can be reflected in health and bone development. On the absolute weight and length of the femur and tibia bones, it was possible to observe that the CP+P and CP+K groups presented a decrease in the parameters evaluated in comparison to the control group, which was expected due to the induction of experimental CP, this model that combines anoxia with restriction of the hind limbs which can result in a decrease in limb development, a study conducted with the same model of CP presents data that demonstrate that the performance of rats with CP is negatively affected in tests of locomotor activity in comparison to control animals (26).

Also, according to another study, the immobilization process of the limbs is capable of inducing the decrease of bone formation, this is mainly due to the loss of minerals and calcium content, in addition, trabecular bone loss and decrease in bone

length and diameter were demonstrated (30). Until the age assessed P36, treatment with kaempferol was not able to significantly increase the absolute weight and length of the femur and tibia of animals subjected to the cerebral palsy model, although a small improvement is observed, we believe that monitoring the animals to a higher age is necessary for analysis.

The delicate balance between reabsorption and bone formation is influenced by several factors, among them, nutrition and the level of physical activity or immobilization process to which the bone system is exposed. Children with cerebral palsy have several eating disorders and impaired growth which can influence bone development. (31). Bone density in cerebral palsy is extensively impaired. Imaging tests allow a faster and more adequate identification of skeletal damage caused by CP in human patients, as well as help to develop the appropriate treatment strategy (32).

Our data on the radiopacity of areas show that there was a decrease in the bone radiographic density of CP animals in the regions analyzed in comparison with the control groups. Although a small trend is observed, this is in agreement with the data published in human studies, which show the fact that cerebral palsy is associated with osteoporosis and osteopenia (33).

Children with CP generally have inadequate levels of nutrients that are essential for bone health, such as calcium, vitamin D and phosphorus, also associated with a deficient nutritional status that can compromise bone development more severely (8,34) there was no significant improvement with kaempferol treatment in paralytic animals in relation to control animals.

Neurological diseases have a major impact on health and quality of life and can negatively affect bone health (35). Cerebral palsy is usually one of the main causes of

osteopenia in childhood (36). Osteopenia refers to a bone density below normal, but not as low as osteoporosis (37).

Our data suggest that the CP+P and CP+K groups present decreased cortical and medullary thickness of the femur and tibia when compared to the control groups, which is in accordance with other studies in which the immobilization of the hind limbs leads to a decrease in the immobilized limbs proven by changes in bone mass and histology in addition to the diminution of the weight of bone ash compared to the control groups (30,38,39), still in a recent observational study it is possible to relate this result to the clinical picture of CP, in which the affected patients present an increased risk of fractures, due to the low bone mineral density common to the disorder (40). It was not possible to observe any significant difference between the groups CP+P and CP+K in the parameters evaluated.

Regarding histology, it was possible to observe a small decrease in osteocyte density in the CP+P group compared to the controls, but it was not significant at the evaluated age (P36), this can be explained perhaps by the fact that our experiment performs the restriction of hind limbs in the animals until P28, the last week of life (P29-36) of the animals is allowed free movement in the cage. An interesting fact of our study was that the CP+K group showed a significant increase in the number of osteocytes compared to the placebo cerebral palsy group. This indicates an increase in bone differentiation of osteoblasts into ostocytes, an increase in osteocyte density is related to the process of differentiation of osteoblasts into osteocytes which corroborates with other authors presenting kaempferol with osteogenic potential.(41).

The lacunar area of the animals varied significantly among the groups; the animals submitted to experimental paralysis had the smallest lacunar area compared

to the animals of the control group. CP+K animals, which were treated with kaempferol showed a larger lacunar area compared to the paralysis group treated with placebo only, which demonstrates that treatment with kaempferol also stimulated the bone formation process, which is also observed in a study with post-menopausal osteoporosis models (42), the mechanisms by which kaempferol promotes osteogenesis are still unclear, more studies are necessary for this elucidation, but in a recent study Zhao and collaborators (43) using bone marrow mesenchymal stem cells declared that this process can occur through the signaling of the mTOR signaling pathway.

CONCLUSIONS

In summary our data show that the cerebral palsy model has drastically reduced the development of the animals with respect to decreased in body weight and naso-anal length, reduction of absolute weight and size of tibia and femur, reduction of cortical thickness and medullar area, lower area of lacunae, reduced the bone radiopacity of the analyzed regions of the femur and tibia, and that the treatment with kaempferol has attenuated the effects concerning body weight and naso-anal length, as well as histologically can observe the increase in the area and number of lacunae. More studies are necessary to verify if this improvement in bone development can be amplified over time.

List of abbreviations:

µl: microliter

C: Control

CEUA- Ethics Committee on Animal Use

CONCEA: National Council for Animal Control and Experimentation

CP: Cerebral palsy

DMSO: dimethyl sulfoxide

EDTA: ethylenediamine tetra acetic acid

HE Hematoxylin and Eosin

K: Kaempferol

mm AL: Aluminum Millimeters

NaCl: Sodium chloride

P: Postnatal day.

PBS: phosphate buffered saline

S.E.M.: Standard error of the mean

UFPE: Universidade Federal de Pernambuco

Declarations

Availability of data and materials: All data generated or analyzed during this study are included in this published article.

Declaration of interest statement: The authors declare that they have no competing interests.

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REFERENCES:

1. Bax M, Goldstein M, Rosenbaun P, Leviton A, Paneth N, Dan B *et al.* Proposed definition and classification of cerebral palsy, April 2005. Vol. 47, Developmental Medicine and Child Neurology. Cambridge University Press; 2005. p. 571.

2. Stanley F, Blair E, Alberman E. Cerebral Palsies: Epidemiology and Causal Pathways [Internet]. Mac Keith Press; 2000 [cited 2021 Feb 8]. Available from: https://research-repository.uwa.edu.au/en/publications/cerebral-palsies-epidemiology-and-causal-pathways

3. Blair E. Epidemiology of the cerebral palsies [Internet]. Vol. 41, Orthopedic Clinics of North America. Orthop Clin North Am; 2010 [cited 2021 Apr 15]. p. 441–55. Available from: https://pubmed.ncbi.nlm.nih.gov/20868877/

4. Krigger KW. Cerebral Palsy: An Overview [Internet]. Vol. 73, American Family Physician. 2006 Jan [cited 2021 Feb 9]. Available from: www.aafp.org/afp.

5. Levine MA. Assessing bone health in children and adolescents. Indian J Endocrinol Metab [Internet]. 2012 [cited 2020 Sep 28];16(Suppl 2):S205-12. Available from: /pmc/articles/PMC3603027/?report=abstract

6. Yeargin-Allsopp M, Van Braun KN, Doernberg NS, Benedict RE, Kirby RS, Durkin MS. Prevalence of cerebral palsy in 8-year-old children in three areas of the united states in 2002: A multisite collaboration. Pediatrics [Internet]. 2008 Mar [cited 2020 Aug 29];121(3):547–54. Available from: https://pubmed.ncbi.nlm.nih.gov/18310204/

7. Zanini G, Zanini G, Cemin NF, Peralles SN. PARALISIA CEREBRAL: causas e

prevalências. Fisioter em Mov [Internet]. 2017 Sep 4 [cited 2021 Feb 8];22(3). Available from: https://periodicos.pucpr.br/index.php/fisio/article/view/19461

8. Kuperminc MN, Stevenson RD. Growth and nutrition disorders in children with cerebral palsy [Internet]. Vol. 14, Developmental Disabilities Research Reviews. John Wiley and Sons Inc.; 2008 [cited 2020 Sep 28]. p. 137–46. Available from: https://pubmed.ncbi.nlm.nih.gov/18646022/

9. Dahlseng MO, Finbråten AK, Júlíusson PB, Skranes J, Andersen G, Vik T. Feeding problems, growth and nutritional status in children with cerebral palsy. Acta Paediatr Int J Paediatr [Internet]. 2012 Jan [cited 2020 Sep 28];101(1):92–8. Available from: https://pubmed.ncbi.nlm.nih.gov/21767308/

10. Castelli V, Grassi D, Bocale R, D'Angelo M, Antonosante A, Cimini A *et al.* Diet and Brain Health: Which Role for Polyphenols? Curr Pharm Des. 2018;24(2):227–38.

11. Scalbert A, Manach C, Morand C, Rémésy C, Jiménez L. Dietary polyphenols and the prevention of diseases [Internet]. Vol. 45, Critical Reviews in Food Science and Nutrition. Crit Rev Food Sci Nutr; 2005 [cited 2021 Feb 5]. p. 287–306. Available from: https://pubmed.ncbi.nlm.nih.gov/16047496/

12. Al Mamun MA, Islam K, Alam MJ, Khatun A, Alam MM, Al-Bari MAA *et al.* Flavonoids isolated from Tridax procumbens (TPF) inhibit osteoclasts differentiation and bone resorption. Biol Res [Internet]. 2015 Sep 12 [cited 2021 Feb 8];48(1):51. Available from: http://www.biolres.com/content/48/1/51

13. Al Mamun MA, Hosen MJ, Khatun A, Alam MM, Al-Bari MAA. Tridax procumbens flavonoids: A prospective bioactive compound increased osteoblast differentiation and trabecular bone formation. Biol Res [Internet].

2017 Dec 8 [cited 2021 Feb 8];50(1):28. Available from: http://biolres.biomedcentral.com/articles/10.1186/s40659-017-0134-7

14. Wong SK, Chin KY, Ima-Nirwana S. The osteoprotective effects of Kaempferol: The evidence from in vivo and in vitro studies [Internet]. Vol. 13, Drug Design, Development and Therapy. Dove Medical Press Ltd.; 2019 [cited 2021 Feb 8]. p. 3497–514. Available from: /pmc/articles/PMC6789172/?report=abstract

15. Trivedi R, Kumar S, Kumar A, Siddiqui JA, Swarnkar G, Gupta V *et al.* Kaempferol has osteogenic effect in ovariectomized adult Sprague-Dawley rats. Mol Cell Endocrinol [Internet]. 2008 Jul 16 [cited 2021 Feb 8];289(1–2):85–93. Available from: https://pubmed.ncbi.nlm.nih.gov/18400372/

16. West-Eberhard MJ. Alternative adaptations, speciation, and phylogeny. (A review). Proc Natl Acad Sci U S A [Internet]. 1986 [cited 2020 Sep 23];83(5):1388–92. Available from: https://pubmed.ncbi.nlm.nih.gov/16578790/

17. Perng W, Oken E. Programming Long-Term Health: Maternal and Fetal Nutrition and Diet Needs. In: Early Nutrition and Long-Term Health: Mechanisms, Consequences, and Opportunities. Elsevier Inc.; 2017. p. 375–411.

18. Hales CN, Barker DJP. Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis [Internet]. Vol. 35, Diabetologia. Springer-Verlag; 1992 [cited 2020 Sep 7]. p. 595–601. Available from: https://link.springer.com/article/10.1007/BF00400248

19. WEST-EBERHARD MJ. Developmental plasticity and evolution. Oxford Uni. Oxford: Oxford University Press; 2003. 816 p.

20. Pigliucci M. Phenotypic Plasticity: Beyond Nature and Nurture (Syntheses in Ecology and Evolution). Baltimore: Johns Hopkins University Press; 2001. 344 p.

21. Lacerda DC, Ferraz-Pereira KN, Visco DB, Pontes PB, Chaves WF, Guzman-Quevedo O *et al.* Perinatal undernutrition associated to experimental model of cerebral palsy increases adverse effects on chewing in young rats. Physiol Behav. 2017 May 1;173:69–78.

22. Silva KOG da, Pereira S da C, Portovedo M, Milanski M, Galindo LCM, Guzmán-Quevedo O *et al.* Effects of maternal low-protein diet on parameters of locomotor activity in a rat model of cerebral palsy. Int J Dev Neurosci. 2016 Aug 1;52:38–45.

23. Strata F, Coq JO, Byl N, Merzenich MM. Effects of sensorimotor restriction and anoxia on gait and motor cortex organization: Implications for a rodent model of cerebral palsy. Neuroscience. 2004 Jan 1;129(1):141–56.

24. Coq JO, Strata F, Russier M, Safadi FF, Merzenich MM, Byl NN *et al.* Impact of neonatal asphyxia and hind limb immobilization on musculoskeletal tissues and S1 map organization: Implications for cerebral palsy. Exp Neurol [Internet]. 2008 [cited 2020 Sep 28];210(1):95–108. Available from: https://pubmed.ncbi.nlm.nih.gov/18061167/

25. Lagoa R, Lopez-Sanchez C, Samhan-Arias AK, Gañan CM, Garcia-Martinez V, Gutierrez-Merino C. Kaempferol protects against rat striatal degeneration induced by 3-nitropropionic acid. J Neurochem [Internet]. 2009 Oct 1 [cited 2021 Feb 8];111(2):473–87. Available from: http://doi.wiley.com/10.1111/j.1471-4159.2009.06331.x

26. Silva KOG da, Pereira S da C, Portovedo M, Milanski M, Galindo LCM, Guzmán-Quevedo O *et al.* Effects of maternal low-protein diet on parameters of locomotor activity in a rat model of cerebral palsy. Int J Dev Neurosci [Internet]. 2016 Aug 1 [cited 2020 Sep 28];52:38–45. Available from: https://pubmed.ncbi.nlm.nih.gov/27211347/

27. Marcuzzo S, Ferreira Dutra M, Stigger F, Severo do Nascimento P, Ilha J, Kalil-Gaspar PI *et al.* Beneficial effects of treadmill training in a cerebral palsy-like rodent model: Walking pattern and soleus quantitative histology. Brain Res. 2008 Jul 30;1222:129–40.

28. Vishwakarma A, Singh TU, Rungsung S, Kumar T, Kandasamy A, Parida S *et al.* Effect of Kaempferol Pretreatment on Myocardial Injury in Rats. Cardiovasc Toxicol [Internet]. 2018 Aug 1 [cited 2021 Feb 4];18(4):312–28. Available from: https://pubmed.ncbi.nlm.nih.gov/29353381/

29. Fung EB, Samson-Fang L, Stallings VA, Conaway M, Liptak G, Henderson RC *et al.* Feeding dysfunction is associated with poor growth and health status in children with cerebral palsy. J Am Diet Assoc [Internet]. 2002 Mar 1 [cited 2021 Feb 4];102(3):361–73. Available from: http://jandonline.org/article/S0002822302900842/fulltext

30. Tuukkanen J, Wallmark B, Jalovaara P, Takala T, Sjögren S, Väänänen K. Changes induced in growing rat bone by immobilization and remobilization. Bone [Internet]. 1991 [cited 2021 Feb 5];12(2):113–8. Available from: https://pubmed.ncbi.nlm.nih.gov/2064838/

31. Jesus AO, Stevenson RD. Optimizing Nutrition and Bone Health in Children with Cerebral Palsy [Internet]. Vol. 31, Physical Medicine and Rehabilitation

Clinics of North America. W.B. Saunders; 2020 [cited 2021 Feb 19]. p. 25–37. Available from: https://pubmed.ncbi.nlm.nih.gov/31760992/

32. Schroeder KM, Heydemann JA, Beauvais DH. Musculoskeletal Imaging in Cerebral Palsy [Internet]. Vol. 31, Physical Medicine and Rehabilitation Clinics of North America. W.B. Saunders; 2020 [cited 2021 Feb 6]. p. 39–56. Available from: https://pubmed.ncbi.nlm.nih.gov/31760993/

33. Houlihan CM, Stevenson RD. Bone Density in Cerebral Palsy [Internet]. Vol. 20, Physical Medicine and Rehabilitation Clinics of North America. Phys Med Rehabil Clin N Am; 2009 [cited 2021 Feb 6]. p. 493–508. Available from: https://pubmed.ncbi.nlm.nih.gov/19643349/

34. Jesus AO, Stevenson RD. Optimizing Nutrition and Bone Health in Children with Cerebral Palsy [Internet]. Vol. 31, Physical Medicine and Rehabilitation Clinics of North America. W.B. Saunders; 2020 [cited 2021 Feb 6]. p. 25–37. Available from: https://pubmed.ncbi.nlm.nih.gov/31760992/

35. Kelly RR, Sidles SJ, LaRue AC. Effects of Neurological Disorders on Bone Health [Internet]. Vol. 11, Frontiers in Psychology. Frontiers Media S.A.; 2020 [cited 2021 Feb 5]. Available from: https://pubmed.ncbi.nlm.nih.gov/33424724/

36. Paksu MS, Vurucu S, Karaoglu A, Karacalioglu AO, Polat A, Yesilyurt O *et al.* Osteopenia in children with cerebral palsy can be treated with oral alendronate. Child's Nerv Syst [Internet]. 2012 Feb 18 [cited 2021 Feb 5];28(2):283–6. Available from: https://link.springer.com/article/10.1007/s00381-011-1576-9

37. Karaguzel G, Holick MF. Diagnosis and treatment of osteopenia [Internet]. Vol. 11, Reviews in Endocrine and Metabolic Disorders. Rev Endocr Metab Disord; 2010 [cited 2021 Feb 5]. p. 237–51. Available from:

https://pubmed.ncbi.nlm.nih.gov/21234807/

38. Trebacz H, Wójtowicz K. Thermostability of bone tissue after immobilization induced osteopenia in a rat model. Folia Histochem Cytobiol [Internet]. 2008 [cited 2021 Feb 6];46(3):379–82. Available from: https://pubmed.ncbi.nlm.nih.gov/19056544/

39. Weinreb M, Rodan GA, Thompson DD. Osteopenia in the immobilized rat hind limb is associated with increased bone resorption and decreased bone formation. Bone [Internet]. 1989 [cited 2021 Feb 4];10(3):187–94. Available from: https://pubmed.ncbi.nlm.nih.gov/2803854/

40. Martínez de Zabarte Fernández JM, Ros Arnal I, Peña Segura JL, García Romero R, Rodríguez Martínez G. Bone health impairment in patients with cerebral palsy. Arch Osteoporos [Internet]. 2020 Dec 1 [cited 2021 Feb 6];15(1). Available from: https://pubmed.ncbi.nlm.nih.gov/32556612/

41. Prouillet C, Mazière JC, Mazière C, Wattel A, Brazier M, Kamel S. Stimulatory effect of naturally occurring flavonols quercetin and kaempferol on alkaline phosphatase activity in MG-63 human osteoblasts through ERK and estrogen receptor pathway. Biochem Pharmacol [Internet]. 2004 Apr 1 [cited 2021 Feb 6];67(7):1307–13. Available from: https://pubmed.ncbi.nlm.nih.gov/15013846/

42. Nowak B, Matuszewska A, Nikodem A, Filipiak J, Landwójtowicz M, Sadanowicz E *et al.* Oral administration of kaempferol inhibits bone loss in rat model of ovariectomy-induced osteopenia. Pharmacol Reports [Internet]. 2017 Oct 1 [cited 2021 Feb 6];69(5):1113–9. Available from: https://pubmed.ncbi.nlm.nih.gov/29031689/

43. Zhao J, Wu J, Xu B, Yuan Z, Leng Y, Min J *et al.* Kaempferol promotes bone

formation in part via the mTOR signaling pathway. Mol Med Rep [Internet]. 2019 [cited 2021 Feb 8];20(6):5197–207. Available from: https://pubmed.ncbi.nlm.nih.gov/31638215/

ANEXO A - ARTIGO DE COAUTORIA

Early life fluoxetine treatment causes long-term lean phenotype in skeletal muscle of rats exposed to maternal lard-based high-fat diet

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Original article

Early life fluoxetine treatment causes long-term lean phenotype in skeletal muscle of rats exposed to maternal lard-based high-fat diet

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ABSTRACT

There is a concern about early life exposure to Selective Serotonin Reuptake Inhibitors (SSRI) in child development and motor system maturation. Little is known, however, about the interaction of environmental factors, such as maternal nutrition, associated with early exposure to SSRI. The increased maternal consumption of high-fat diets is worrisome and affects serotonin system development with repercussions in body phenotype. This study aimed to assess the short- and long-term effects of neonatal fluoxetine treatment on the body and skeletal muscle phenotype of rats exposed to a maternal lard-based high-fat (H) diet during the perinatal period. A maternal lard-based high-fat diet causes reduced birth weight, a short-term reduction in type IIA fibers in the soleus muscle, and in type IIB fibers in the Extensor Digitorum Longus (EDL) muscle, reducing Lactate Dehydrogenase (LDH) activity in both muscles. In the long-term, the soleus showed reduced muscle weight, smaller area and perimeter of muscle fibers, while the EDL muscle showed reduced Citrate Synthase (CS) activity in offspring from the rats on the maternal lard-based high-fat diet. Early-life exposure to fluoxetine reduced body weight and growth and reduced soleus weight and enzymatic activity in young rats. Exposure to neonatal fluoxetine in adult rats caused a decreased body mass index, less food intake, and reduced muscle weight with reduced CS and LDH activity. Neonatal fluoxetine in young rats exposed to a maternal lard-based high-fat diet caused reduced body weight and growth, reduced soleus weight as well as area and perimeter of type I muscle fibers. In adulthood, there was a reduction in food intake, increased proportion of IIA type fibers, reduced area and perimeter of type IIB, and reduction in levels of CS activity in EDL muscle. Neonatal fluoxetine treatment in rats exposed to a maternal lard-based, high-fat diet induces a reduction in muscle weight, an increase in the proportion of oxidative fibers and greater oxidative enzymatic activity in adulthood.

1. Introduction

The Serotonin (5-HT) system plays a key role in developmental stages such as cell proliferation, migration, differentiation, and morphogenesis, acting in the regulation of neural tissue growth as well as interfering in the development of other systems [1,2]. Influences on 5-HT system have been defined as the main factor contributing to long-lasting changes in physiological and morphological states during the critical period of development [3–5].

There is an increasing number of women using Selective Serotonin Reuptake Inhibitors (SSRIs) during gestation and lactation periods [6]. SSRIs are thought to be effective for treating psychiatric disorders by increasing the synaptic bioavailability of the neurotransmitter serotonin (5-HT) [7]. Epidemiological studies, however, have shown that early life

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exposure to SSRI may have adverse effects on child neurodevelopment [8–10], and some consequences could be aggravated by adverse environmental factors such as inappropriate maternal nutrition [11]. Knowledge of the long-term consequences of perinatal SSRI exposure on neuromotor system maturation is limited [12]. Therefore, special attention is needed to understand the impact of early exposure to these drugs on the development of peripheral tissues.

Early serotoninergic influences of exposure to SSRI play a role in nervous system maturation, impacting body growth, weight gain and feeding behavior [12–15]. Neonatal fluoxetine exposure has been shown, in previous studies with young rats, to modulate serotoninergic neurotransmission, causing hypophagic behavior due to an increased hypothalamic serotonin transporters response [16]. Experimental studies showed the long-term impact of early SSRI exposure on energy balance [17,18], including an induced lean phenotype by increasing the ratio of brown/white adipose tissue [13] and positively affecting mitochondrial bioenergetics in skeletal muscle in the juvenile period [19].

Evidence suggests that serotonin-mediated signaling occurs in skeletal muscle through a 5-HT2A receptor influence on myogenic differentiation and in glucose metabolism [20–22]. Additionally, new evidence has reported that SSRI causes changes in the function, structure and metabolic properties of skeletal muscle [23]. In most mammals, skeletal muscle comprises a large part of body mass. It has an important metabolic function and has the characteristic of adapting to a variety of external stimuli, including substrate availability [24]. A normal mature muscle has both slow type fibers (oxidative) and fast type fibers (glycolytic). Early environmental influences, however, may impact muscle phenotype development [25,26]. Little is known about the impact of early exposure to SSRI on skeletal muscle phenotype, as well as potential interaction of a preexisting insult (e.g. maternal nutrition) that may contribute to impaired development of muscle tissue.

There has been a concern about increased consumption of animal fat in developing countries in the last decades. Women of reproductive age from these regions generally consume more saturated fat than men [27, 28]. Maternal over or undernutrition in the perinatal period is known to impact fetal growth, birth weight, maturation of the nervous system, feeding behavior and to represent a risk for the development of metabolic disorders during a lifetime [29–32]. Maternal intake of high-fat diets affects fetus serotonin system development [33], and has been associated with increased risk of impaired neurodevelopment and mental disorders, such as affective problems, anxiety, and depression in children [33–35].

Studies on animals have shown that during the transition to adulthood there is an increased body weight gain and dysregulation in glucose metabolism, insulin resistance, impaired mitochondrial function and altered structure of skeletal muscle resulting from a maternal lard-based high-fat diet [36–38]. In addition, studies of obesity in animals models have shown that a maternal diet, rich in saturated fats, can lead to important metabolic changes in offspring [39,40].

A significant decrease in nutritional availability during gestation in mammals reduces the formation of secondary myofibers and the ratio of secondary to primary myofibers transition [41]. Therefore, impaired skeletal muscle mass development during the fetal period due to maternal fat intake has long-lasting and negative physiological consequences for the offspring such as altered muscle fiber proportion, reduced fiber cross-section area, and impairment of energy metabolism [42]. Few studies have shown the effects of maternal lard-based, high-fat diet intake in the skeletal muscle phenotype of the offspring, although there is evidence that there exists an impact of a maternal high-fat diet on offspring development [37,42,43].

Considering the higher maternal consumption of saturated fat and the high incidence of early exposure to SSRI, it is opportune to investigate the interaction of maternal lard-based high-fat diet and early serotonin system manipulation on body growth, weight gain, food consumption, and the skeletal muscle development, since there is limited knowledge about this. In one study, authors showed that the manipulation of the serotonin system may prevent metabolic disorder by inducing an increase in the activity of mitochondria and elevation of energy metabolism in the skeletal muscle of mice that consumed a high-fat diet [44]. Another study showed that in overnourished rat pups with impaired metabolism, their chronic exposure to fluoxetine in childhood caused changes in the hypothalamus that resulted in positive effects on their physical well-being and behavior, such as better serum parameters, improvement in adipose profile and positive modulation of hypophagia-related genes [45].

Short and long-term effects of early SSRI exposure on the skeletal muscle phenotype of subjects exposed to a maternal lard-based, high-fat diet are yet to be fully explored. Given this, we hypothesized that the inhibition of serotonin reuptake in early life causes a lean phenotype that involves changes in oxidative skeletal muscle fiber in rats exposed to a lard-based high-fat perinatal maternal diet. In this study, we have evaluated the short-term and long-term effects of neonatal serotonin reuptake inhibition on body weight, growth, food intake, skeletal fiber phenotype and enzymatic activity in the skeletal muscle of rats exposed to or not to a lard-based, high-fat diet during fetal and lactation periods.

2. Material and methods

2.1. Animals and experimental groups

The ethics committee for animal use of the Federal University of Pernambuco (Protocol number 0027/2016) approved all experimental procedures. All experiments and animal use were carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines [46].

A total of twenty (n = 20) female Wistar rats and ten (n = 10) male Wistar rats with 90–120 days of life were obtained from the Department of Nutrition, Federal University of Pernambuco. They were mated in a ratio of two females to one male. Pregnancy was determined by the detection of spermatozoa in vaginal smears. According to dietary manipulation, the female rats were divided randomly into two groups, the Control group, fed on a Normolipidic/Normocaloric diet (N, 354,65 Kcal/100 g, n = 10); and the Experimental group, fed on a lard-based High-fat diet (H, 483,93 Kcal/100 g calories from saturated fats, n = 10) [40,47]. The diets were prepared in the Department of Nutrition of the Federal University of Pernambuco (Brazil). The ingredients and centesimal composition of diets are described in Tables 1 and 2 . At birth, litter size was adjusted to eight pups per dam, and a blind caretaker randomly allocated the pups by weight into two experimental groups. During lactation period (from the first day of postnatal life to the twenty-first day of postnatal life, P1-P21) the male pups received subcutaneously a daily dose (~8:00 a.m.) of either fluoxetine (10 mg/kg, dissolved in saline solution, 10 mL/Kg, bw) or saline (NaCl 0,9 %, 10 mL/Kg, bw) [48]. The pups were assigned into following groups: Normolipidic/Normocaloric diet + saline (NS group, n = 16), Normolipidic/Normocaloric diet + fluoxetine (NF group, n = 16); lard-based High-fat diet + saline (HS, n = 16) and lard-based High-fat diet + fluoxetine (HF, n = 16). After weaning, the pups were fed with commercial laboratory chow (Presence, Brazil) and divided into collective cages, with 4 males per cage, until P30 or P90. Animals were housed under standard conditions at 22 °C and maintained in an inverted cycle of 12/12 h. Water and food were provided *ad libitum*.

Table 1
Ingredients composition of the control diet and experimental diet.

Ingredients (%)	Control Diet	Lard-based High-fat diet
Commercial Chow	100	76
Lard from pork fat	–	24

Table 2
Centesimal Composition of Normolipidic/Normocaloric diet and Lard-Based High-fat diet.

Macronutrients	Normolipidic/Normocaloric Diet (g/100 g)	Lard-Based High-fat Diet (g/100 g)
Proteins	25.51	18.17
Carbohydrates	53.59	40.69
Lipids	4.25	27.61
Ashes	7.6	5.39
Moisture content	9.05	8.14
Kcal/100 g	354.65	483.93

2.2. Measurement of body weight, growth, and food intake

The body weight of the dams was recorded once a week during gestation and lactation period using a MARTE® scale (Model ASF11). Food intake of the dams was evaluated weekly from the first day after pregnancy confirmation until the end of the lactation period. Each week, food intake was determined by the difference between the amount of food (g) provided and the amount of food (g) rejected. The food intake was recorded using the MARTE® scale (Model ASF11).

Body weight for the pups was also recorded using the MARTE® scale (Model ASF11) at P0, P1, P7, P14 and P21 (postnatal day). The body length (distance from nose to anal orifice) was measured by a digital caliper (Jomarca® 0.01 mm) at P30, P60 and P90. The body weight and body length values were used to calculate the Body Mass Index (BMI) – BMI = [body weight (g)/ body length $(cm)^2$] [49]. The percentage of body weight gain was calculated after weaning during the intervals of P22-P30, P30-P60, and P60-P90 by this formula: BWG % = [final body weight (g) x 100/ initial body weight (g)] – 100. To measure food intake the animals were housed in individual cages for five days before the target ages P30, P60, and P90, assessing the daily difference between the amount of food provided at the onset of the dark cycle and the amount of food remaining 24 h later.

2.3. Histochemical analyses of the skeletal muscles

Animals were sacrificed under *ad libitum* feeding conditions by decapitation at P30 and P90 to collect the soleus and *Extensor Digitorum Longus* (EDL) muscles. The muscles were dissected, weighed and immediately immersed in frozen n-hexane. The samples were kept at -80 °C until the analysis of muscle fiber types. The muscle cross-sections (10 μm) were obtained by a cryostat microtome maintained at −30 °C and stained for myofibrillar ATPase (mATPase) [25,50]. Briefly, the cross-sections were preincubated at room temperature for 20 min in a solution containing 140 mM acetic acid and 60 mM sodium acetate (pH adjusted to 4.3 and 4.55). The slides were then washed in distilled water and incubated at 37 °C in a solution containing, 20 mM $CaCl_2$, 2.5 mM ATP salt in 40 mM buffered glycine (pH 9.4). Then the sections were washed in distilled water, followed by soaking in 2% cobalt chloride for 3 min, then they were exposed to 1.5 % ammonium sulfide for 3 min, washed in distilled water, and dehydrated in batches of increasing alcohol content (70–100 %). Finally, they were immersed twice in toluene and placed for drying at room temperature. After drying the slides were covered by coverslips using Entellan resin.

The proportion of each fiber type was determined in each section of the soleus and EDL muscles. Muscle fibers were classified into types I and II based on the presence (type I) or absence (type II) of staining for ATPase after acid pre-incubation at pH 4.3. The muscle fibers were further classified as type I (darkest), type IIa (lightest) or IIb (grey) using differences in staining intensity as criteria for ATPase after acid pre-incubation at pH 4.55 [25,51]. The muscle images were obtained with an optical microscope (Olympus Optical U-CMAD-2, Tokyo, Japan; 40x objective lens). All fibers of soleus and EDL muscles were counted using Mensurim 6 (Jean-François Madre-Amiens) software. The cross-section area (CSA) and perimeter were measured from 500 fibers per animal using Image J (version 1.51p) software.

2.4. Enzymatic activity of citrate synthase and lactate dehydrogenase

Enzymatic activity was determined as described previously [19]. Briefly, for an enzymatic analysis, an individual homogenization of the soleus and EDL muscle was performed. The analysis of citrate synthase activity was carried out with a mixture containing (in mmol/L) 50 Tris·HCl (pH 8.1), 1 $MgCl_2$, 1 EDTA, 0.2 5,5-dithio-bis (2-nitrobenzoic acid) (3 = 13.6 mol/(mL·cm−1), 3 acetyl-CoA, 5 oxaloacetate and 0.3 mg/ml homogenate. The citrate synthase activity was measured by assessing the rate of change in absorbance at 412 nm over an interval of 160 s [19].

Dosage of lactate dehydrogenase, was determined with an assay buffer containing 50 mM potassium phosphate pH 7.4 and 25 °C, 6 mM NADH and 23 mM sodium pyruvate to which was added approximately 200 μg of the sample. The final pH of the assay volume was 2 mL and pH 7.3. The total volume of the assay was 1 mL and the reading was performed over a 60-second interval in a spectrophotometer at 340 nm. The results were expressed as mmol / mg protein / minute [52].

2.5. Statistical analysis

Data normality distribution was assessed by the Kolmogorov-Smirnov test. For the normal distribution, data are expressed as mean ± SEM. The two-way analysis of variance (ANOVA) with repeated measures test followed by Bonferroni's test was applied to body weight, food intake and caloric intake of damns. The Student *t*-test was also used to assess significant differences in birth weight between the groups. Multiple group comparison was performed by two-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. Data from CSA and perimeter of the fiber are expressed as median and 25th and 75th percentile and analyzed by Kruskal-Wallis and Dunn's post-hoc test. The significant level was considered at $p < 0.05$. Statistical analysis was performed using the GraphPad Prism 7.0. ® (GraphPad Software Inc. La Jolla, CA, USA).

3. Results

3.1. Body weight and food intake of dams

In the third week of gestation, pregnant dams exposed to the Lard-Based High-fat diet presented a lower body weight than the control group (Bonferroni's post hoc test: $p < 0.0121$) [$F(3,42) = 248.2$, $p < 0.0001$]. In the second and third weeks of lactation, dams exposed to this diet presented lower body weight than the control group [$F(2,28) = 4.773$, $p = 0.0164$] (Fig. 1A). The weekly food intake evaluated showed lower intake in grams in dams exposed to the Lard-Based High-fat diet compared to control group in gestation [$F(1,18) = 55.2$, $p < 0.0001$] and lactation period [$F(1,18) = 90.68$, $p < 0.0001$] with a significant interaction in lactation period [$F(2,36) = 6.236$, $p = 0.0047$] (Fig. 1B). However, there was no difference and no interaction in caloric intake in dams exposed to the Lard-Based High-fat diet compared to the control group during gestation [$F(2,36) = 0.1118$, $p = 0.8945$] and lactation periods [$F(2,36) = 1.091$, $p = 0.3469$] (Fig. 1C).

3.2. Body weight and growth in offspring

At birth, pups from the pregnant dams exposed to the experimental diet showed reduced birth body weight compared to the control group [$t(62) = 3.102$, $p = 0.002$] (Fig. 2A). In the first week of the lactation period, the HS group presented a reduction in body weight compared to the control group (Tukey's post hoc test: $p = 0.002$) [$F(3,45) = 20.13$, $p < 0.0001$], this remained reduced until P21 (Tukey's post hoc test, $p < 0.0001$) [$F(3,45) = 43.36$, $p < 0.0001$] (Fig. 2B). The nourished pups

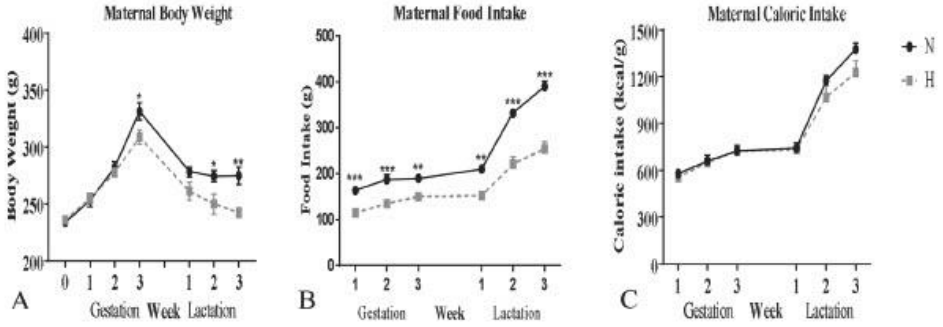

Fig. 1. A) Maternal body weight (grams) during gestation and lactation periods. B) Maternal food intake (grams) during gestation and lactation; C) Maternal caloric intake (Kcal/g) during gestation and lactation. Groups according to maternal diet during gestation and lactation; Normolipidic/Normocaloric diet (N, n = 10), lard-based High-fat diet (H, n = 10). The values were presented as the mean and standard error of the mean. The two-way ANOVA with repeated measures was used to compare the groups over the time period, followed by Bonferroni's post hoc test. *P < 0.05; ** P < 0.01; *** P < 0.001.

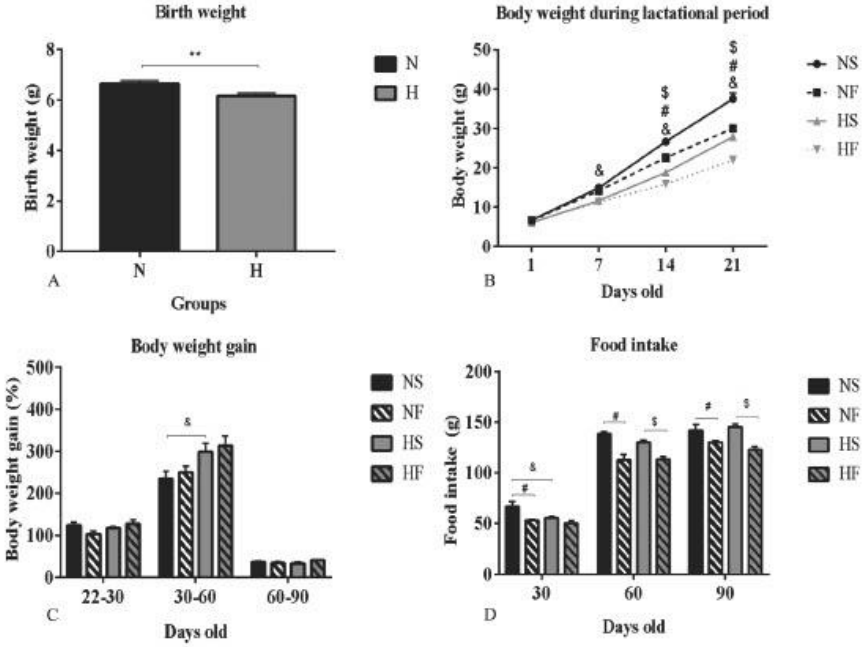

Fig. 2. Parameters of offspring during experiments. A) birth weight: N (pups from mothers exposed to Normolipidic/Normocaloric diet, n = 16); H (pups from mothers exposed to the lard-based High-fat diet, n = 16), B) body weight during lactation. C) Body Weight gain in the post-weaning period. D) Food intake at the postnatal days 30, 60 and 90. Composition of groups according to exposure to maternal diet and pharmacological treatment: NS (control diet + saline, n = 8), NF (control diet + fluoxetine, n = 8), HS (lard-based high-fat diet + Saline, n = 8) HF (lard-based high-fat diet + fluoxetine, n = 8). The values were presented as mean and standard error of the mean. Data were analyzed by two-way ANOVA for repeated measures, followed by the Tukey's *post hoc* test. ** = p < 0.01. & = NS vs. HS, # = NS vs. NF, $ = HS vs. HF. P < 0.05.

treated with fluoxetine presented reduced body weight compared to the control group in the second (Tukey's post hoc test: $p < 0.0001$) to third weeks of the lactation period (Tukey's post hoc test: $p < 0.0001$) [$F(3,45) = 62.7$, $p < 0.0001$] (Fig. 2B). Pups exposed to the lard-based high-fat diet during the perinatal period and treated with fluoxetine during the neonatal period presented reduced body weight in the second week (Tukey's post hoc test: $p < 0.0001$) up to the end of lactation compared to the HS group (Tukey's post hoc test: $p < 0.0001$). A statistical interaction with regard to the body weight of the groups and the lactation period was found [$F(9,180) = 22.59$, $p < 0.0001$] (Fig. 2B).

At 30 days, pups from the HS group presented reduced body weight compared to the control group (Tukey's post hoc test: $p < 0.0001$) (Table 3). Also, neonatal treatment with fluoxetine caused a reduction in body weight of the nourished pups at P30 compared to the control group

Table 3
Somatic and muscle parameters of each experimental group.

AGE	Postnatal day 30				Postnatal day 90			
Group Variable	NS	NF	HS	HF	NS	NF	HS	HF
Body weight (g)	79.74 ± 1.75	**62.89 ± 1.69#******	**60.20 ± 1.64&******	**50.61 ± 1.85 $****	347.10 ± 6.46	**306.2 ± 10.74#****	**307.8 ± 6.74&***	296.91 ± 4.87
Nose-to-anal length (cm)	14.19 ± 0.16	**12.29 ± 0.10#******	**12.15 ± 0.13&******	**11.16 ± 0.18 $*****	22.54 ± 0.17	**20.89 ± 0.20#*****	**21.32 ± 0.34&****	20.73 ± 0.24
Body Mass Index (BMI) g/cm^2)	0.395 ± 0.009	0.416 ± 0.008	0.408 ± 0.011	0.405 ± 0.016	0.683 ± 0.009	**0.603 ± 0.023#***	0.680 ± 0.02434	0.691 ± 0.013
Soleus weight (g)	0.0311 ± 0,001	**0.0207 ± 0.007#******	**0.0227 ± 0.001&*****	**0.0156 ± 0.001$****	0.1710 ± 0.007	**0.1364 ± 0.006#****	**0.1394 ± 0.004&****	0.1239 ± 0.004
Relative weight of soleus muscle (%)	0.0403 ± 0.003	0.0337 ± 0.001	0.03572 ± 0.002	0.03228 ± 0.002	0.04924 ± 0.001	0.04493 ± 0.001	0.04598 ± 0.002	0.04171 ± 0.001
EDL weight (g)	0.0241 ± 0.002	0.0182 ± 0.009	0.0186 ± 0.001	0.0142 ± 0.001	0.1445 ± 0.004	**0.1085 ± 0.009#*****	0.1281 ± 0.001	0.1213 ± 0.002
Relative weight of EDL muscle (%)	0.0306 ± 0.002	0.0281 ± 0.001	0.0292 ± 0.001	0.0272 ± 0.003	0.0417 ± 0.001	0.0353 ± 0.002	0.0422 ± 0.001	0.0407 ± 0.001

Values were showed as mean and standard error of the mean. The data were analyzed by the two-way ANOVA test, followed by Tukey test. & = NS vs. HS, # = NS vs. NF, $ = HS vs. HF. P < 0.05. (control diet + saline, n = 8), NF (control diet + fluoxetine, n = 8), HS (lard-based high-fat diet + saline n = 8) HF (lard-based high-fat diet + fluoxetine n = 8) * p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001.

(Tukey's post hoc test: p < 0.0001) [F (3,45) = 42.63, p < 0.0001] (Table 3). Animals from HS group showed elevated weight gain during the period between 30–60 days (Tukey's post hoc test: p = 0.0080) [F (3,21) = 3.509, p = 0.0332] with significant interaction according to body weight gain during the experimental interval timepoints [F (6,56) = 2.783, p = 0.0193] (Fig. 2C). However, at P90, a lower body weight was observed in the HS group compared to the control group (Tukey's post hoc test: p = 0.0131) and in NF group compared to the control group (Tukey's post hoc test: p < 0.0096) [F (3,21) = 7.431, p = 0.0014] (Table 3). At P90, the HF group did not present reduced body weight compared to their control (HS = 307.8 ± 6.74, n = 8 vs. HF = 296.9 ± 4.87 g, n = 8, p = 0.7833) (Table 3).

At P30, differences in body length was observed in the different groups [F (3,45) = 61.47, p < 0.0001]. Animals from the HS group showed reduced body length compared to the control group (Tukey's post hoc test: p < 0.0001) (Table 3). Neonatal exposure to fluoxetine also reduced the growth of the nourished animals compared to the control group (Tukey's post hoc test: p < 0.0001). Reduced growth was observed in animals exposed to maternal Lard-based high-fat diet during the perinatal period and exposed to fluoxetine in the neonatal period, compared to the HS group (Tukey's post test: p = 0.0005) (Table 3). While at P90, the reduction of body length was continuous in animals from the HS group compared to the control group (Tukey's post hoc test: p = 0.0099) and in animals from the NF group compared to the control group (Tukey's post hoc test: p = 0.0006) [F (3,21) = 11.18, p = 0.0001] (Table 3). At P90, the NF group showed a reduction in BMI compared to the control group (Tukey's post hoc test: p = 0.0136) [F (3,21) = 6.088, p = 0.0038) (Table 3).

3.3. Food intake of offspring

A significant interaction between age and food intake [F (6,42) = 3.031, p = 0.0149] was observed. At P30, animals from the HS group had reduced food intake compared to the control group (Tukey's post hoc test: p = 0.0380) (Fig. 2D). At this age, neonatal exposure to fluoxetine also caused a reduction in food intake of the nourished pups compared to the control group (Tukey's post hoc test: p = 0.0071) [F (3,21) = 19.95, p < 0.0001] (Fig. 2D). This effect was not observed in pups exposed to the experimental maternal diet that had received neonatal exposure to fluoxetine (Tukey's post hoc test: p = 0.5416). At P60, neonatal exposure to fluoxetine cause reduced food intake in nourished pups compared to the control group (Tukey's post hoc test: p < 0.0001). Additionally, while the HF group had shown similar food intake to their control when younger, at P60 they had reduced their food intake, compared to HS group (Tukey's post hoc test: p = 0.0036) [F (3,21) = 18.32, p < 0.0001] (Fig. 2D). At 90 days, the NF group showed reduced food intake compared to the control group (Tukey's post hoc test: p = 0.233) (Fig. 2D). At this age, there was a reduction in food intake between the groups of animals that were both exposed to the experimental maternal diet and treated with fluoxetine during the neonatal period, as compared to HS group (Tukey's post hoc test: p < 0.0001) [F (3,21) = 7.121, p = 0.0017] (Fig. 2D).

3.4. Muscle weight

At P30, there was no statistical difference in EDL weight between groups. While at P90, reduced EDL weight was noted in the nourished group treated with fluoxetine, compared to the control group [F (3,21) = 7.336, p = 0.0015] (Table 3). In the soleus muscle at P30, muscle weight reduction was observed in the group of HS pups compared to their control (Tukey's post hoc test: p < 0.0001) (Table 3). At this age, neonatal administration of fluoxetine resulted in reduced soleus weight in the NF group compared to control (Tukey's post hoc test: p < 0.0001). This was also true for the HF group compared to its respective control (Tukey's post hoc test: p = 0.0032) [F (3,21) = 26.58, p < 0.0001] (Table 3). At P90, the HS group had a reduction in soleus weight compared to control group (Tukey's post hoc test: p < 0.01) (Table 3). Neonatal administration of fluoxetine reduced soleus weight in the nourished group at P90, compared to the control group (Tukey's post hoc test: p = 0.0015) [F (3,21) = 12.6, p < 0.0001]. No differences in relative muscle weight were observed (Table 3).

3.5. Soleus and EDL muscle fibers type composition

At P30, the animals exposed to the experimental diet during perinatal period showed an increased proportion of type I fibers in the soleus muscle (Tukey's post hoc test: p = 0.003) [F (3,12) = 25.32, p < 0.0001 Subsequently, reduction in the IIA fibers was observed in the HS group (NS = 16.77 ± 1.71 VS. HS = 11.59 ± 1.32) (Tukey post hoc test: p < 0.0004) [F (3,12) = 24.85, p < 0.001) (Fig. 3). Neonatal administration of fluoxetine did not cause a change in the proportion of type I fibers in the soleus muscle compared to its control at P30 (NS = 83.22 ± 1.71 %, n = 5 vs. NF = 84.8 ± 1.14 %, n = 3). Animals from HS group that had received neonatal administration of fluoxetine showed similar proportion of type I fibers in the soleus muscle compared to their control (HS = 88.52 ± 1.44 %, n = 5 vs. HF = 90.74 ± 1.89 %, n = 5) (Fig. 3). At P90, no differences were observed in the distribution of soleus muscle fibers between groups. We observed that the adult soleus muscle contained

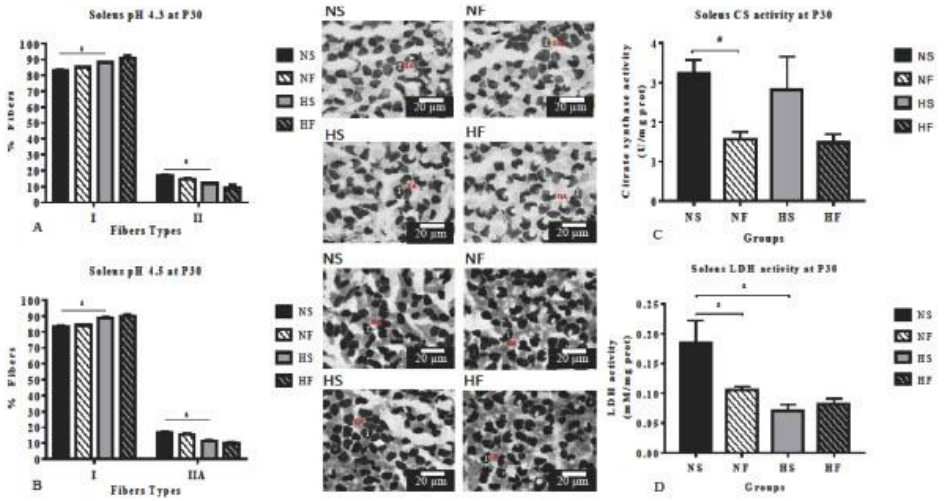

Fig. 3. At 30 days, the proportion of muscle fibers A) at pH 4.3, B) at pH 4.55 and enzymatic activity of C) Citrate Synthase and D) Lactate dehydrogenase of soleus muscle. The composition of the groups according to exposure to maternal diet and pharmacological treatment: NS (Normolipidic/normocaloric diet + saline, n = 5), NF (Normolipidic/normocaloric diet + fluoxetine, n = 5), HS (lard-based high-fat diet + Saline = 5) HF (lard-based high-fat diet + fluoxetine, n = 5). The values were presented as mean and standard error of the mean. The data were analyzed by the ANOVA two-way test, followed by the Tukey's test. & = NS vs. HS, # = NS vs. NF, $ = HS vs. HF. $P < 0.05$.

95.3 ± 1.44 % type I fibers (NS = 95.3 ± 1.44 %, n = 5 vs. NF = 95.42 ± 1.39 %, n = 5, HS = 95.71 ± 0.69 %, n = 5 vs. HF = 95.81 ± 0.42 %, n = 5) (Fig. 4).

In the EDL muscle, animals exposed to an experimental diet presented a reduction in type IIB fibers at P30 (Tukey's post hoc test: p = 0.0086) [F (3,12) = 11.27, p = 0.0008] (Fig. 5). Neonatal administration of fluoxetine caused an increase in the proportion of type I fibers at P30 in the nourished group (Tukey's post hoc test: p = 0.0001) [F (3,12) = 19.83, p < 0.0001] (Fig. 5). The EDL muscle of the nourished animals exposed to fluoxetine in the neonatal period presented an increase in

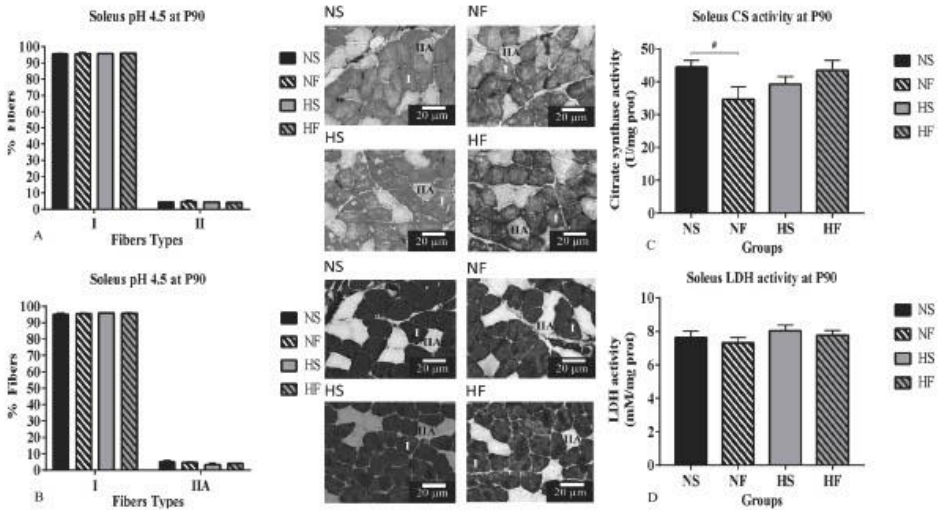

Fig. 4. At 90 days, the proportion of muscle fibers A) at pH 4.3, B) at pH 4.55 and enzymatic activity of C) Citrate Synthase and D) Lactate dehydrogenase of soleus muscle. The composition of the groups according to exposure to maternal diet and pharmacological treatment: NS (Normolipidic/normocaloric diet + saline, n = 5), NF (Normolipidic/normocaloric diet + fluoxetine, n = 5), HS (lard-based high-fat diet + Saline = 5) HF (lard-based high-fat diet + fluoxetine, n = 5). The values were presented as mean and standard error of the mean. The data were analyzed by the ANOVA two-way test, followed by the Tukey's test. & = NS vs. HS, # = NS vs. NF, $ = HS vs. HF. $P < 0.05$.

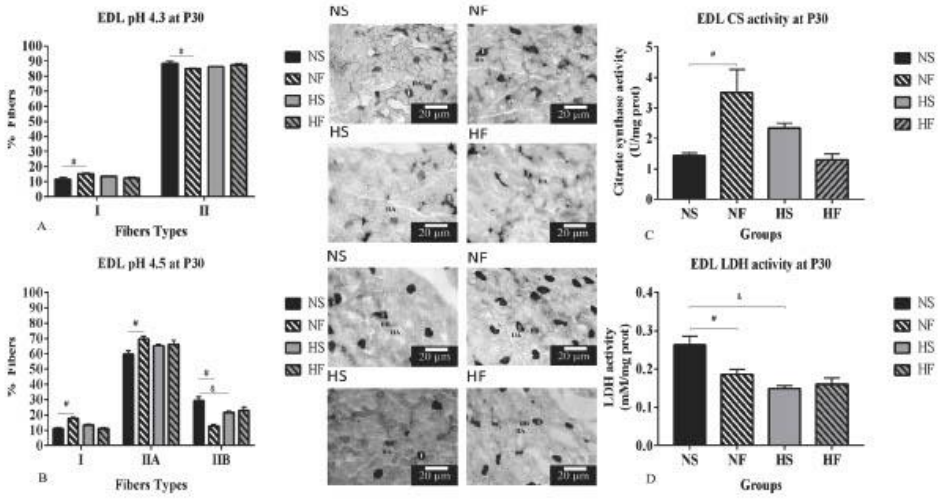

Fig. 5. At 30 days, the proportion of muscle fibers A) at pH 4.3, B) at pH 4.55 and enzymatic activity of C) Citrate Synthase and D) Lactate dehydrogenase of EDL muscle. The composition of the groups according to exposure to maternal diet and pharmacological treatment: NS (Normolipidic/normocaloric diet + saline, n = 5), NF (Normolipidic/normocaloric diet + fluoxetine, n = 5), HS (lard-based high-fat diet + saline = 5) HF (lard-based high-fat diet + fluoxetine, n = 5). The values were presented as mean and standard error of the mean. The data were analyzed by the ANOVA *two*-way test, followed by the Tukey's test & = NS vs. HS, # = NS vs. NF, $ = HS vs. HF. $P < 0.05$.

type IIA fibers compared to their control (Tukey's post hoc test: $p = 0.0004$) [$F(3,12) = 3.975$, $p = 0.0352$], and a reduction of type IIB fibers compared to control group (Tukey's post hoc test: $p = 0.0004$) (NS = 29.21 ± 2.54 vs. NF = 12.65 ± 2.10 %, n = 5, $P < 0.05$) [$F(3,12) = 11.27$, $p = 0.0008$]. These changes were not observed in the HF group, which presented type IIA (HS = 65.33 ± 0.90 %, N = 5 vs. HF = 66.15 ± 2.66 %, n = 5) and type IIB (HS = 21.53 ± 0.72, n = 5 vs. HF = 22.72 ± 2.45 %, n = 5) fibers similar to control at P30 (Fig. 5).

At P90, EDL presented about 5.02 ± 0.25 % of type I fibers in all groups, indicating, at this age, there was no difference between groups in relation to the proportion of type I fibers [$F(3,12) = 0.5091$, $p = 0.6835$]. The group of nourished rats treated with fluoxetine in the neonatal period, at P90, had a higher proportion of type IIB fibers when compared to control group (Tukey's post hoc test: $p = 0.0485$) [$F(3,12)$

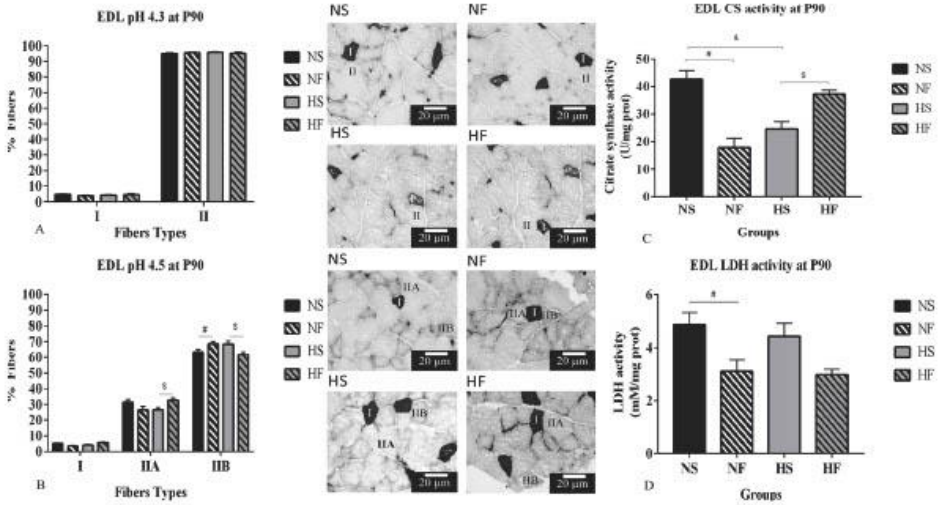

Fig. 6. At 90 days, the proportion of muscle fibers A) at pH 4.3, B) at pH 4.55 and enzymatic activity of C) Citrate Synthase and D) Lactate dehydrogenase of EDL muscle. The composition of the groups according to exposure to maternal diet and pharmacological treatment: NS (control diet + saline, n = 5), NF (control diet + fluoxetine, n = 5), HS (lard-based high-fat diet + saline = 5) HF (lard-based high-fat diet + fluoxetine, n = 5). The values were presented as mean and standard error of the mean. The data were analyzed by the ANOVA two-way test, followed by the Tukey's test & = NS vs. HS, # = NS vs. NF, $ = HS vs. HF. $P < 0.05$.

= 4,298, p = 0.0282] (Fig. 6). Interestingly, at 90d animals form HF group showed an increase in the IIA fiber type (Tukey's post hoc test: p = 0.00138) [F (3,12) = 3.957, p = 0.0357] and a reduction in type IIB fibers compared to HS group (Tukey's post hoc test: p = 0.0047) [F (3,12) = 4.298, p = 0.0282].

3.6. Enzymatic activity of Citrate Synthase (CS) and lactate dehydrogenase (LDH) activity

At P30, the activity of citrate synthase in the soleus muscle of nourished pups that had received neonatal administration of fluoxetine was reduced, compared to their control [F (1,14) = 11.06, p = 0.0050] (Fig. 3). LDH activity was reduced in the soleus muscle of HS group pups compared to control group (Tukey's post hoc test: p < 0.01) (Fig. 3). Neonatal administration of fluoxetine caused a reduction in LDH activity in the soleus muscle of nourished pups, compared to control (Tukey's post hoc test: p < 0.05) [F (1,13) = 4.844, p = 0.0464] (Fig. 3). At 90 days, there was an interaction related to CS activity in the soleus muscle [F (1,17) = 5.205, p = 0.0357]. In soleus muscle CS was reduced in NF group compared to control group [F (1,17) = 5.205, p = 0.0357] (Fig. 4).

In EDL muscle at P30, LDH activity in the HS group was reduced compared to that in the control group (Tukey's post hoc test: p < 0.01) (Fig. 5). Additionally, the neonatal administration of fluoxetine in nourished group promoted a reduction in LDH activity compared to control group (Tukey's post hoc test: p < 0.05) [F (1,16) = 3.708, p = 0.0721] (Fig. 5). In the pups of NF group at P30, the citrate synthase activity was increased in the EDL muscle compared to the control group (Tukey's post hoc test: p < 0.05 [F (1,10) = 20.53, p = 0.0011] (Fig. 5). However, at P30, in the animals exposed to the maternal lard-based diet and treated with fluoxetine in the neonatal period, the enzymatic activity of CS and LDH was unchanged compared to the control group (Fig. 5).

At P90 the CS activity in EDL muscle was reduced in the HS group compared to the control (Tukey's post hoc test: p < 0.01) (Fig. 6). At this age CS presented a reduction in activity in the group of nourished animals treated with fluoxetine in the neonatal period compared to the control group (Tukey's post hoc test: p < 0.0001). Interestingly, pups exposed to the maternal experimental diet and treated with fluoxetine in the neonatal period showed increased CS activity compared to the HS group at 90 days old (Tukey's post hoc test: p < 0.05 [F (1,14) = 4.4774, p = 0.0463] (Fig. 6). LDH activity was reduced in EDL muscle in NF group compared to control group (Tukey's post hoc test: p < 0.05) [F (1,15) = 15.07, p = 0.0015].

3.7. Area and perimeter of muscle fibers

The area of type I fiber of soleus was reduced in animals that received fluoxetine administration during lactation period at P30 compared to animals from NS group (Dunn's post hoc test: p < 0.0001). This area was reduced in the HF group compared to the HS group (Dunn's post hoc test: p < 0.05) (Table 4). The perimeter of type I soleus muscle fiber was reduced in the NF group compared to the control group (Dunn's post hoc test: p < 0.0001). The perimeter of soleus type I fiber was reduced in the HF group compared to HS group (Dunn's post hoc test: p < 0.001) (Table 4). At P90, soleus type IIA fiber was reduced in the area and perimeter of the HS group (Dunn's post hoc test: p < 0.0001) (Table 4).

In the EDL muscle at P30, type I muscle fiber showed a reduction in the area in the HF group (Dunn's post hoc test: p < 0.001) and in fiber in the perimeter of the HF group compared to HS group (Dunn's post hoc test: p < 0.01) (Table 5). At 90 days, the area of type IIB fiber in EDL muscle showed a reduction in the HF group compared to HS group (Dunn's post hoc test: p < 0.0001) and perimeter with type IIB fibers (Dunn's post hoc test: p < 0.0001) (Table 5).

Table 4
Morphometric characteristics of the soleus muscles.

GROUP	NS		NF		HS		HF	
Variable/Age	P30	P90	P30	P90	P30	P90	P30	P90
Area of soleus Type I fiber (μm^2)	595.89 (437.27–781.30)	2627.50 (2159.68–3104.97)	390.97#**** (302.23–544.44)	2498.89 (2057.76–2976.46)	681.63 (514.44–880.55)	1793.25&*** (1502.80–2562.55)	561.38$*(427.09 –743.25)	2123.35 (1721.76-2748.72)
Area of soleus type IIA fiber (μm^2)	525.58 (431.91–770.80)	2372.85 (2033.47 –3210.10)	496.64 (355.28–603.82)	2448.30 (2008.89 –3978.34)	490.05 (342.10–618.97)	2239.74&**** (1524.13–2751.39)	392.26 (268.79–546.59)	2304.69 (1871.27 –2934.35)
Perimeter of soleus type I fiber (μm)	143.41 (122.80–179.01)	242.61 (216.78–257.41)	105.63#**** (83.34–135.78)	247.88 (214.79–270.09)	132.39 (114.17-149.02)	205.70&**** (190.31–241.08)	107.19$*** (92.29–126.16)	212.66 (190.85–242.95)
Perimeter of soleus type IIA fiber (μm)	152.78 (124.81–179.72)	240.19 (212.69–254.23)	124.92 (90.40–161.09)	250.73 (217.14–282.11)	112.89 (93.02–122.97)	194.01&* (187.52–213.53)	124.40 (90.84–181.76)	234.16 (192.99–277.84)

Values were showed as median and interquartil interval (25th and 75th percentile). The data were analyzed by the Kruskal-Wallis test followed by Dunn's post-test. The composition of the groups according to exposure to maternal diet and pharmacological treatment: NS (control diet + saline, n = 5), NF (control diet + fluoxetine, n = 5), HS (lard-based high-fat diet + saline = 5) HF (lard-based high-fat diet + fluoxetine, n = 5). &= NS vs. HS, # = NS vs. NF, $ = HS vs. HF. P < 0.05. * p < 0.05; ** p < 0.01; ***p < 0.001; **** p < 0.0001 .

Table 5
Morphometric parameters of EDL muscle.

GROUP	NS		NF		HS		HF	
Variable/Age	P30	P90	P30	P90	P30	P90	P30	P90
Area of EDL type I fiber (μm^2)	340.32 (282.18–405.66)	1365.90 (1197.34–1676.65)	462.14 (336.90–520.72)	1178.93 (924.40–1703.89)	421.84 (303.52–516.37)	1124.89 (909.78–1364.16)	240.94***$ (213.73–274.58)	1273.51 (1084.79–1363.94)
Area of EDL type IIA fiber (μm^2)	549.32 (473.83–627.42)	1417.99 (1183.40–2009.31)	579.91 (495.27–722.92)	1311.42 (1197.89–1654.64)	547.45 (462.99–656.55)	1494.72 (1304.45–1914.18)	515.52 (374.70–611.07)	1759.46 (1398.59–1975.20)
Area of EDL type IIB fiber (μm^2)	424.53 (375.23–549.16)	2442.85 (1568.73–3750.58)	476.89 (400.04–559.46)	2233.65 (1879.97–2679.08)	549.16 (441.99–586.89)	2322.56 (1840.86–2684.52)	630.18 (496.04–737.77)	1701.502$****(1369.94 – 1983.37)
Perimeter of EDL type I fiber (μm)	92.20 (84.61–105.61)	173.48 (145.37–187.39)	103.29 (87.65–111.37)	167.33 (141.79–193.47)	94.37 (78.08–111.26)	155.22 (149.73–168.54)	75.29**$(69.24 –80.24)	167.409 (153.96–176.37)
Perimeter of EDL type IIA fiber (μm)	102.96 (96.37–115.45)	176.87 (159.08–214.57)	106.92 (98.46–119.63)	169.31 (157.65–181.96)	110.71 (101.82–117.20)	183.86 (167.58–212.56)	103.94 (94.22–118.22)	190.46 (170.35–213.89)
Perimeter of EDL type IIB fiber (μm)	94.53 (86.88–104.65)	231.03 (183.52–272.79)	96.02 (89.15–107.02)	216.54 (194.03–230.14)	99.82 (89.74–118.95)	217.28 (194.76–241.48)	112.06 (98.38–119.31)	195.92 $****(182.42 –213.47)

Values were showed as median and interquartile intervals (25th and 75th percentile). The data were analyzed by the Kruskal-Wallis test followed by Dunn's post-test. The composition of the groups according to exposure to maternal diet and pharmacological treatment: NS (control diet + saline, n = 5), NF(control diet + fluoxetine, n = 5), HS (lard-based high-fat diet + saline = 5) HF (lard-based high-fat diet + fluoxetine, n = 5). & = NS vs. HS, # = NS vs. NF, $ = HS vs. HF. P < 0.05. * p < 0.05; ** p < 0.01; ***p < 0.001; **** p < 0.0001 .

4. Discussion

To our knowledge, this was the first study of neonatal rats treated with fluoxetine and exposed to maternal lard-based high fat diet during the perinatal period showing effects on the skeletal muscle phenotype development. The results showed that neonatal fluoxetine exposure reduces body weight, growth, food intake and skeletal muscle properties in animals exposed to a maternal lard-based high-fat diet during gestation and lactation periods. These changes were observed in both the youth and adulthood of animals. The main finding of this study was to observe muscle weight reduction, alteration in the muscle fiber proportion, and reduction in the morphometric parameters of fibers. Also, we showed altered enzymatic activity of citrate synthase and lactate dehydrogenase in the soleus and EDL muscles. This study confirmed our hypothesis, that neonatal administration of fluoxetine increases the oxidative fibers in skeletal muscle in adult rats exposed to a lard-based high-fat diet.

Pregnant rats that received a lard-based high-fat diet showed a lower body weight at the end of gestation and during lactation, compared to the control group. This was similar to other studies that observed no weight gain in rats during the perinatal period after exposure to a lard-based high-fat diet [53–55]. Our study showed reduced food intake in grams in female rats that received the experimental diet in the perinatal period. However, there were no differences in caloric intake in the same period. Rats that consumed a high-fat diet, reduced their food intake to adjust their calorie intake during the perinatal period [56]. Saturated fat intake seems to cause satiety, and this may be related to increased levels of the peptide hormone YY [57]. Since the gestational and lactation period is characterized by high energy demand, the consumption of a lard-based high-fat diet may have contributed to lower body weight observed in pregnant rats [58]. This fact could be explained by reduced body weight, body length, and placenta size in the fetus during the third week of the gestational period in pregnant rats exposed to a lard-based high-fat diet [55].

Pups whose mothers received the experimental diet showed a reduced birth weight. This result corroborates recent evidence showing that altered maternal nutrition during the gestational period influences the birth weight and impacts on development in early postnatal life [59, 60]. Additionally, epidemiological and animal studies have shown an association between low birth weight and an increase in the risk of metabolic disorders later in life [61–64]. Low birth weight observed in pups from dams exposed to a high-fat diet agrees with the study by de Cunha, Molle, Portella (2015), where the authors observed a similar effect after exposure to a high-fat diet. Restriction of uterine growth, preterm birth, low birth weight and delay in reflex ontogeny are outcomes reported in the literature after the offspring were exposed to a high-fat maternal diet [29,65,66]. The effects of low birth weight are intensified when followed by growth retardation and weight gain in adolescence, agreeing with our results [63]. The imbalance caused by the ingestion of saturated fat can result in permanent changes in appetite control, neuroendocrine functions and fetal energy metabolism affecting the development of offspring [67].

Furthermore, it seems that perinatal exposure to a diet rich in saturated fats impairs the development of the serotonin system in primates [35]. Central serotonin is known to act as developmental signaling that autoregulates serotonin neurons as well as target tissues that may be mediated by specific 5-HT receptor subtypes (e.g. 5-HT2A in skeletal muscle cells) [2,20]. On the other hand, peripheral serotonin plays an important role in weight reduction, in part by accelerating energy consumption in skeletal muscle [44].

The fetal period is crucial to the normal development of skeletal muscle [26]. Studies have suggested, however, that impaired postnatal muscle growth with reduced muscle mass in adulthood may be due to an early life with restricted nutrition [68]. Our study showed that over the short-term, the soleus muscle in animals exposed to a lard-based high-fat diet had the highest proportion of type I fibers and reduced type II at

P30. This change in fiber composition was accompanied by a reduction in muscle weight. The EDL at 30 days had fewer type IIB fibers in pups exposed to a lard-based high-fat maternal diet. Decreased muscle mass reduction results from perturbation of protein degradation induced by a variety of pathophysiological conditions, including reduced substrate availability early in life [26,69]. Additionally, fast-twitch glycolytic fibers are more vulnerable to muscle mass reduction than slow-twitch oxidative fibers [69].

Animals studies have suggested that early exposure to a maternal diet rich in saturated fat may provoke lifelong mitochondrial alterations in the skeletal muscle of offspring [37]. At P30 no differences were observed in citrate synthase activity in either muscle; at P90, however, the EDL muscle showed reduced levels of citrate synthase, possibly indicative of reduced mitochondrial density in skeletal muscle [70]. Other studies have shown that a maternal lard-based high-fat diet causes a lower muscle protein expression of respiratory chain complexes I-V and may downregulate pathways associated with oxidative phosphorylation, electron transport system and ATP synthesis of mitochondrial skeletal muscle of adult rats [37,38,71].

The present study showed the effects of neonatal treatment with fluoxetine on nourished pups or those exposed to a lard-based high fat maternal diet. Reduction of body weight was observed during lactation period and even in the later stage of life in the nourished pups exposed to neonatal treatment with fluoxetine. In a previous study, weight loss and growth retardation had been observed in animals exposed to SSRI in the neonatal period [14,72]. Interestingly, the animals exposed to the maternal lard-based high-fat diet and treated with fluoxetine in the neonatal period showed reduced body weight, although they showed no differences in adulthood, when compared to untreated animals. Early life exposure to fluoxetine seems to impact the energy balance in young and adult rats causing a lean phenotype [18]. Therefore, this occurrence may be related to the important role of the serotonin system in glucose and lipid homeostasis as well in the thermoregulation impact on body weight [73,74].

The main finding of this study was observation of the alteration of muscle phenotype over the short and long-term in rats exposed to a lard-based high-fat maternal diet that had received neonatal administration of fluoxetine. Muscle weight reduction, alteration in the fiber proportion and the enzymatic activity of citrate synthase and lactate dehydrogenase in the soleus and EDL muscle were registered and have been presented here. To our knowledge, this was the first study to show the effects on the proportion of fibers in rats who suffered neonatal treatment with fluoxetine exposed or not to a high fat perinatal maternal diet. We showed that at P30 the nourished and fluoxetine-treated pups presented an increase in the percentage of type I fibers, with an increase in type IIA and reduction in type IIB in the EDL muscle compared to control. At 90 days, there was a higher proportion of type IIB fibers in this same muscle. At 90 days, pups exposed to a high-fat maternal diet and fluoxetine-treated in lactation period showed an increase in type IIA fibers.

Altered serotonin signaling seems to influence skeletal muscle properties and may be a key factor concerning muscle contraction and metabolism [22,75]. Serotonin may also be involved, in skeletal muscle ontogenesis and repair, by causing changes in mRNA that code for proteins involved in myogenic differentiation [20]. Our study showed that early life increased extracellular concentration of serotonin causes reduced glycolytic muscle fibers of EDL muscle in the short-term. Other researchers have shown that increased serotonin levels causes a shift in the profile of muscle fiber type from fast/glycolytic to slow/oxidative in the soleus muscle of mice [44]. This finding suggests that serotonin plays an important role in glucose and lipid metabolism accelerating energy consumption in skeletal muscle [44]. This fact may be related to the elevation of the mRNA expression of the Peroxisome proliferator-activated receptor coactivator 1α (PGC-1α)-b, PGC-1α-c, and genes related to fatty acid oxidation in the skeletal muscle of mice due to serotonin treatment [44]. The coactivator of PGC-1α is the key regulator of mitochondrial biogenesis and fatty acid oxidation in skeletal muscle [76].

Additionally, in agreement with our findings, another study has shown a reduction of muscle weight in the late phase after neonatal treatment with fluoxetine, as well as a reduction in the number of myonuclei in the soleus muscle and a reduction of the cross-sectional area of the muscle at 30 and 90 days of age [77]. In our study, neonatal fluoxetine treatment in pups exposed to a high-fat maternal diet presented at P90 a lower proportion of type IIB fibers in relation to their control, and consequently an increase in type IIA fibers in the EDL muscle. This agrees with another study that showed that the manipulation of serotonin can induce a change from glycolytic to oxidative metabolism in skeletal muscle [38].

Deficiencies in mitochondrial content and function play a role in the postnatal development of metabolic diseases. Recent evidence has shown that certain antidepressants are capable of altering the antioxidant capacity in a number of different systems [19,78–80]. Here we showed different levels of enzymatic activity in citrate synthase and lactate dehydrogenase due to exposure to a high-fat maternal diet in rats treated or untreated with fluoxetine. The activity of citrate synthase is strongly associated with mitochondrial content in the skeletal muscle [70].

We showed in this study that nourished pups treated with fluoxetine underwent an increase in the citrate synthase activity of the EDL muscle. The activity of CS synthase is generally related to the population of type IIA fibers, followed by type I fibers [81]. This finding may be related to the increase in the proportion of type IIA and type I fibers in the EDL muscle of pups nourished and treated with fluoxetine in the lactation period. In another study, even at P60, increased citrate synthase activity was shown in the EDL muscle of rats exposed to fluoxetine during lactation period [19]. Our study showed that at 90 days this effect was not observed. The observed structural and metabolic finds in skeletal muscle might be related to delayed motor development, and motor skills acquisition in children who were early exposed to SSRI drugs [82,83], however, this association needs further investigation.

5. Conclusion

In conclusion, neonatal fluoxetine treatment in rats exposed to a maternal lard-based high-fat diet causes short and long-term changes in structural and enzymatic activity of the skeletal muscle of their offspring. In addition to body weight, growth, and food intake reductions, these influences contributed to a lean phenotype of skeletal muscle, for example in reduced muscle weight, increased proportion of oxidative fibers, and to higher oxidative enzymatic activity in adulthood. Further studies are necessary, however, to explore the cellular and molecular mechanisms involved in skeletal muscle plasticity due to the interaction of a maternal lard-based high-fat diet, and SSRI drug exposure during the critical period of development.

Declaration of Competing Interest

The authors report no declarations of interest.

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References

[1] G.A. Buznikov, W.H. Lambert, J.M. Lauder, Serotonin and serotonin-like substances as regulators of early embryogenesis and morphogenesis, Cell Tissue Res. 305 (2001) 177–186, https://doi.org/10.1007/s004410100408.

[2] P.M. Whitaker-Azmitia, M. Druse, P. Walker, J.M. Lauder, Serotonin as a developmental signal, Behav. Brain Res. 73 (1995) 19–29, https://doi.org/10.1016/0166-4328(96)00071-X.

[3] M. Carolan-olah, M. Duarte-gardea, J. Lechuga, A Critical Review: Early Life Nutrition and Prenatal Programming for Adult Disease, 2015, pp. 3716–3729, https://doi.org/10.1111/jocn.12951.

[4] R. Orozco-Solís, R.J.B. Matos, O. Guzmán-Quevedo, S.L. de Souza, A. Bihouée, R. Houlgatte, R.M. de Castro, F. Bolaños-Jiménez, Nutritional programming in the rat is linked to long-lasting changes in nutrient sensing and energy homeostasis in the hypothalamus, PLoS One 5 (2010), https://doi.org/10.1371/journal.pone.0013537.

[5] S. Frazer, K. Otomo, A. Dayer, Early-life serotonin dysregulation affects the migration and positioning of cortical interneuron subtypes, Transl. Psychiatry 5 (2015), https://doi.org/10.1038/tp.2015.147.

[6] S. Alwan, J. Reefhuis, S.A. Rasmussen, J.M. Friedman, Patterns of antidepressant medication use among pregnant women in a united states population, J. Clin. Pharmacol. 51 (2011) 264–270, https://doi.org/10.1177/0091270010373928.

[7] C.J. Harmer, R.S. Duman, P.J. Cowen, How do antidepressants work? New perspectives for refining future treatment approaches, Lancet Psychiatry 4 (2017) 409–418, https://doi.org/10.1016/S2215-0366(17)30015-9.

[8] S. Corti, P. Pileri, M.I. Mazzocco, C. Mandò, A.F. Moscatiello, D. Cattaneo, S. Cheli, S. Baldelli, L. Pogliani, E. Clementi, I. Cetin, Neonatal outcomes in maternal depression in relation to intrauterine drug exposure, Front. Pediatr. 7 (2019) 1–8, https://doi.org/10.3389/fped.2019.00309.

[9] H. El Marroun, T.J.H. White, N.J.F. Van Der Knaap, J.R. Homberg, G. Fernández, N.K. Schoemaker, V.W.V. Jaddoe, A. Hofman, F.C. Verhulst, J.J. Hudziak, B.H. C. Stricker, H. Tiemeier, Prenatal exposure to selective serotonin reuptake inhibitors and social responsiveness symptoms of autism: population-based study of young children, Br. J. Psychiatry 205 (2014) 95–102, https://doi.org/10.1192/bjp.bp.113.127746.

[10] A.L. Salisbury, K.E. O'Grady, C.L. Battle, K.L. Wisner, G.M. Anderson, L.R. Stroud, C.L. Miller-Loncar, M.E. Young, B.M. Lester, The roles of maternal depression, serotonin reuptake inhibitor treatment, and concomitant benzodiazepine use on infant neurobehavioral functioning over the first postnatal month, Am. J. Psychiatry 173 (2016) 147–157, https://doi.org/10.1176/appi.ajp.2015.14080989.

[11] L. Andersson, I. Sundstro, M. Wulff, M. Åstro, Implications of antenatal depression and anxiety for obstetric outcome, Obstet. Gynecol. 104 (2004) 467–476, https://doi.org/10.1097/01.AOG.0000135277.04565.e9.

[12] M.E. Glover, S.M. Clinton, Of rodents and humans: a comparative review of the neurobehavioral effects of early life SSRI exposure in preclinical and clinical research, Int. J. Dev. Neurosci. 51 (2016) 50–72, https://doi.org/10.1016/j.ijdevneu.2016.04.008.

[13] A.I. da Silva, G.R.P. Braz, A.A. Pedroza, L. Nascimento, C.M. Freitas, D.J. S. Ferreira, R. Manhães de Castro, C.J. Lagranha, Fluoxetine induces lean phenotype in rat by increasing the brown/white adipose tissue ratio and UCP1 expression, J. Bioenerg. Biomembr. 47 (2015) 309–318, https://doi.org/10.1007/s10863-015-9617-9.

[14] T.C.B.J. Deiró, R. Manhães-de-Castro, J.E. Cabral-Filho, S.L. Souza, S.R. Freitas-Silva, L.M.P. Ferreira, R.C.A. Guedes, C.R.V. Câmara, K.M.F.T. Barros, Neonatal administration of citalopram delays somatic maturation in rats, Braz. J. Med. Biol. Res. 37 (2004) 1503–1509, https://doi.org/10.1590/S0100-879X2004001000009.

[15] L.C.M. Galindo, M. da L.D. Barros, I.L. Pinheiro, R.V. de C. Santana, R.J.B. de Matos, C.G. Leandro, S.L. de Souza, R. Manhães de Castro, Neonatal serotonin reuptake inhibition reduces hypercaloric diet effects on fat mass and hypothalamic gene expression in adult rats, Int. J. Dev. Neurosci. 46 (2015) 76–81, https://doi.org/10.1016/j.ijdevneu.2015.07.004.

[16] I.L. Pinheiro, A.I. da Silva, A. Reginato, R.C. da Silva Filho, L.C.M. Galindo, R.J. B. Matos, J.C. de Souza Ferraz, A.E. Toscano Meneses da Silva Castro, M. Milanski Ferreira, R. Manhães de Castro, S.L. de Souza, Neonatal fluoxetine exposure modulates serotonergic neurotransmission and disturb inhibitory action of serotonin on food intake, Behav. Brain Res. (2017), https://doi.org/10.1016/j.bbr.2017.07.038.

[17] G. Scabia, I. Barone, M. Mainardi, G. Ceccarini, M. Scali, E. Buzzigoli, A. Dattilo, P. Vitti, A. Gastaldelli, F. Santini, T. Pizzorusso, L. Maffei, M. Maffei, The antidepressant fluoxetine acts on energy balance and leptin sensitivity via BDNF, Sci. Rep. 8 (2018) 1–12, https://doi.org/10.1038/s41598-018-19886-x.

[18] L.D. Barros, R. Manhães-de-castro, D.T. Alves, O. Guzmán, A. Elisa, A. Bonnin, L. Galindo, Long term effects of neonatal exposure to fluoxetine on energy balance : a systematic review of experimental studies, Eur. J. Pharmacol. 833 (2018) 298–306, https://doi.org/10.1016/j.ejphar.2018.06.013.

[19] A.I. da Silva, G.R.P. Braz, R. Silva-Filho, A.A. Pedroza, D.S. Ferreira, R. Manhães de Castro, C. Lagranha, Effect of fluoxetine treatment on mitochondrial bioenergetics in central and peripheral rat tissues, Appl. Physiol. Nutr. Metab. 40 (2015) 565–574, https://doi.org/10.1139/apnm-2014-0462.

[20] I. Guillet-Deniau, A.-P. Burnol, J. Girard, Identification and localization of a skeletal muscle serotonin 5-HT2A receptor coupled to the Jak/STAT pathway, J. Biol. Chem. 272 (1997) 14825–14829, https://doi.org/10.1074/jbc.272.23.14825.

[21] E. Hajduch, L. Dombrowski, F. Darakhshan, F. Rencurel, A. Marette, H.S. Hundal, Biochemical localization of the 5-HT2A(serotonin) receptor in rat skeletal muscle, Biochem. Biophys. Res. Commun. 257 (1999) 369–372, https://doi.org/10.1006/bbrc.1999.0471.

[22] E. Hajduch, F. Rencurel, A. Balendran, I.H. Batty, C.P. Downes, H.S. Hundal, Serotonin (5-hydroxytryptamine), a novel regulator of glucose transport in rat skeletal muscle, J. Biol. Chem. 274 (1999) 13563–13568, https://doi.org/10.1074/jbc.274.19.13563.

[23] D.B. Visco, R. Manhães-de-Castro, W.F. Chaves, D.C. Lacerda, S. da C. Pereira, K. N. Ferraz-Pereira, A.E. Toscano, Selective serotonin reuptake inhibitors affect structure, function and metabolism of skeletal muscle: a systematic review, Pharmacol. Res. (2018), https://doi.org/10.1016/j.phrs.2018.09.004.

[24] S.A. Bayol, S.J. Farrington, N.C. Stickland, A maternal "junk food" diet in pregnancy and lactation promotes an exacerbated taste for "junk food" and a greater propensity for obesity in rat offspring, Br. J. Nutr. 98 (2007) 843–851, https://doi.org/10.1017/S0007114507812037.

[25] M.H. Brooke, K.K. Kaiser, Muscle fiber types: how many and what kind? Arch. Neurol. 23 (1970) 369–379, https://doi.org/10.1001/archneur.1970.00480280083010.

[26] A.A. Sayer, C. Cooper, Fetal programming of body composition and musculoskeletal development, Early Hum. Deve. 81 (2005) 735–744, https://doi.org/10.1016/j.earlhumdev.2005.07.003.

[27] J. Kearney, Food consumption trends and drivers, Philos. Trans. R. Soc. B Biol. Sci. 365 (2010) 2793–2807, https://doi.org/10.1098/rstb.2010.0149.

[28] R.E. Engell, J. Powles, Global, regional, and national consumption levels of dietary fats and oils in 1990 and 2010: a systematic analysis including 266 country-specific nutrition, BMJ 2272 (2014) 1–20, https://doi.org/10.1136/bmj.g2272.

[29] E.V. Cadena-Burbano, C.C.L. Cavalcanti, A.B. Lago, R. de A.C. Benjamim, T. Rdos P. Oliveira, J.M. Silva, R. Manhães-De-Castro, R. Da Silva Aragão, A maternal high-fat/high-caloric diet delays reflex ontogeny during lactation but enhances locomotor performance during late adolescence in rats, Nutr. Neurosci. 8305 (2017) 1–12, https://doi.org/10.1080/1028415X.2017.1354958.

[30] P.D. Gluckman, M.A. Hanson, C. Pinal, Developmental origins of adult disease, Matern. Child Nutr. 1 (2005) 130–141, https://doi.org/10.1159/000273066.

[31] R. Orozco-Sólis, S. Lopes de Souza, R.J. Barbosa Matos, I. Grit, J. Le Bloch, P. Nguyen, R. Manhães de Castro, F. Bolaños-Jiménez, Perinatal undernutrition-induced obesity is independent of the developmental programming of feeding, Physiol. Behav. 96 (2009) 481–492, https://doi.org/10.1016/j.physbeh.2008.11.016.

[32] A.J. Buckley, B. Keserü, J. Briody, M. Thompson, S.E. Ozanne, C.H. Thompson, Altered body composition and metabolism in the male offspring of high fat-fed rats, Metabolism 54 (2005) 500–507, https://doi.org/10.1016/j.metabol.2004.11.003.

[33] S.D. Bilbo, V. Tsang, Enduring consequences of maternal obesity for brain inflammation and behavior of offspring, FASEB J. 24 (2010) 2104–2115, https://doi.org/10.1096/fj.09-144014.

[34] P. Krakowiak, C.K. Walker, A.A. Bremer, A.S. Baker, R.L. Hansen, I. Hertz-picciotto, Maternal metabolic conditions and risk for autism and other neurodevelopmental disorders, Pediatrics 129 (2012), https://doi.org/10.1542/peds.2011-2583.

[35] J.R. Thompson, J.C. Valleau, A.N. Barling, J.G. Franco, M. DeCapo, J.L. Bagley, E. L. Sullivan, Exposure to a high-fat diet during early development programs behavior and impairs the central serotonergic system in juvenile non-human primates, Front. Endocrinol. 8 (2017), https://doi.org/10.3389/fendo.2017.00164.

[36] R.E.K. MacPherson, L.M. Castelli, P.M. Miotto, S. Prendo-Cumbo, A. Milburn, B. D. Roy, P.J. LeBlanc, W.E. Ward, S.J. Peters, A maternal high fat diet has long-lasting effects on skeletal muscle lipid and PLIN protein content in rat offspring at young adulthood, Lipids 50 (2015) 205–217, https://doi.org/10.1007/s11745-014-3985-5.

[37] C.A. Pileggi, C.P. Hedges, S.A. Segovia, J.F. Markworth, B.R. Durainayagam, C. Gray, X.D. Zhang, M.P.G. Barnett, M.H. Vickers, A.J.R. Hickey, C.M. Reynolds, D. Cameron-Smith, Maternal high fat diet alters skeletal muscle mitochondrial catalytic activity in adult male rat offspring, Front. Physiol. 7 (2016) 1–11, https://doi.org/10.3389/fphys.2016.00546.

[38] A.V. Khamoui, M. Desai, M.G. Ross, H.B. Rossiter, Sex-specific effects of maternal and postweaning high-fat diet on skeletal muscle mitochondrial respiration, J. Dev. Origin Health Dis. 9 (2018) 670–677, https://doi.org/10.1017/S2040174418000594.Sex-Specific.

[39] A.M. Matias, W.M. Estevam, P.M. Coelho, D. Haese, B. Botto, S. Kobi, A.P. Lima-leopoldo, S. Leopoldo, Differential effects of high sugar, high lard or a combination of both on nutritional, hormonal and cardiovascular metabolic profiles of rodents, Nutr. Hosp. 10 (2018) 1–13, https://doi.org/10.3390/nu10081071.

[40] R. Buetter, K.G. Parhofer, M. Woenckhaus, C.E. Wrede, L.A. Kunz-Schughart, J. Scholmerich, L.C. Bollhermel, Defining high-fat-diet rat models: metabolic and molecular effects of different fat types, J. Mol. Endocrinol. 36 (2006) 485–501, https://doi.org/10.1677/jme.1.01909.

[41] T. Zou, D. He, B. Yu, J. Yu, X. Mao, P. Zheng, J. He, Z. Huang, D. Chen, Moderate maternal energy restriction during gestation in pigs attenuates fetal skeletal muscle development through changing myogenic gene expression and myofiber characteristics, Reprod. Sci. 24 (2017) 156–167, https://doi.org/10.1177/1933719116651151.

[42] S.A. Bayol, B.H. Simbi, N.C. Stickland, A maternal cafeteria diet during gestation and lactation promotes adiposity and impairs skeletal muscle development and metabolism in rat offspring at weaning, J. Physiol. 567 (2005) 951–961, https://doi.org/10.1113/jphysiol.2005.089989.

[43] J. Abou Mrad, F. Yakubu, D. Lin, J.C. Peters, J.B. Atkinson, J.O. Hill, Skeletal muscle composition in dietary obesity-susceptible and dietary obesity-resistant

rats, Am. J. Physiol. 262 (1992) 684–688, https://doi.org/10.1152/ajpregu.1992.262.4.R684.
[44] H. Watanabe, T. Nakano, R. Saito, D. Akasaka, K. Saito, H. Ogasawara, T. Minashima, K. Miyazawa, T. Kanaya, I. Takakura, N. Inoue, I. Ikeda, X. Chen, M. Miyake, H. Kitazawa, H. Shirakawa, K. Sato, K. Tahara, Y. Nagasawa, M.T. Rose, S. Ohwada, K. Watanabe, H. Aso, Serotonin improves high fat diet induced obesity in mice, PLoS One 11 (2016) 1–14, https://doi.org/10.1371/journal.pone.0147143.
[45] A.I. da Silva, G.R.F. Braz, S.C. de A. Silva, A.A. da S. Pedroza, N.C. de Lima-Júnior, T.L. de A. Silva, C.J. Lagranha, Body composition, biochemical, behavioral and molecular alterations in overfed rats after chronic exposure to SSRI, Behav. Brain Res. 356 (2019) 62–70, https://doi.org/10.1016/j.bbr.2018.08.007.
[46] C. Kilkenny, W.J. Browne, I.C. Cuthill, M. Emerson, D.G. Altman, Improving bioscience research reporting: the arrive guidelines for reporting animal research, Animals 4 (2013) 35–44, https://doi.org/10.3390/ani4010035.
[47] M.L. Tellechea, M.F. Mensegue, C.J. Pirola, The Association Between High Fat Diet Around Gestation and Metabolic Syndrome-Related Phenotypes in Rats: a Systematic Review and Meta-Analysis, 2017, pp. 1–18, https://doi.org/10.1038/s41598-017-05344-7.
[48] A.E. Toscano, M.A.F. Amorim, E.V. de Carvalho Filho, R. da Silva Aragão, J. E. Cabral-Filho, S.R.A. de Moraes, R. Manhaes-de-Castro, Do malnutrition and fluoxetine neonatal treatment program alterations in heart morphology? Life Sci. 82 (2008) 1131–1136, https://doi.org/10.1016/j.lfs.2008.03.013.
[49] E.L.B. Novelli, Y.S. Diniz, C.M. Galhardi, G.M.X. Ebaid, H.G. Rodrigues, F. Mani, A. A.H. Fernandes, A.C. Cicogna, J.L.V.B. Novelli Filho, Anthropometrical parameters and markers of obesity in rats, Lab. Anim. 41 (2007) 111–119, https://doi.org/10.1258/002367707779399518.
[50] D.C. Lacerda, K.N. Ferraz-Pereira, D.B. Visco, P.B. Pontes, W.F. Chaves, O. Guzman-Quevedo, R. Manhães-de-Castro, A.E. Toscano, Perinatal undernutrition associated to experimental model of cerebral palsy increases adverse effects on chewing in young rats, Physiol. Behav. 173 (2017), https://doi.org/10.1016/j.physbeh.2017.01.043.
[51] R.B. Armstrong, R.O. Phelps, Muscle Fiber type composition of the rat hindlimb, Am. J. Anat. 272 (1984) 259–272, https://doi.org/10.1002/aja.1001710303.
[52] W.J. Reeves, G.M. Fimognari, An improved procedure for the preparation of crystalline lactic dehydrogenase from hog heart, J. Biol. Chem. 238 (1963) 3853–3858.
[53] C. Mendes-da-silva, C.Á. Giriko, L.V. Mennitti, L.F. Hosoume, S. Souto, A. Valotta, Maternal high-fat diet during pregnancy or lactation changes the somatic and neurological development of the offspring, Arq Neuropsiquiat. (2013) 136–144, https://doi.org/10.1590/0004-282X20130220.
[54] P. de Oliveira Andrade, C.C. Fontelles, M.P. Rosim, T.F. de Oliveira, A.P. de Melo Loureiro, J. Mancini-Filho, M.M. Rogero, F.S. Moreno, S. de Assis, L.F. Barbisan, L. Hilakivi-Clarke, T.P. Ong, Exposure to lard-based high-fat diet during fetal and lactation periods modifies breast cancer susceptibility in adulthood in rats, J. Nutr. Biochem. 25 (2014) 613–622, https://doi.org/10.1016/j.jnutbio.2014.02.002.
[55] P.D. Taylor, I.Y. Khan, L. Lakasing, V. Dekou, A.I. Mallet, M.A. Hanson, L. Poston, Uterine artery function in pregnant rats fed a diet supplemented with animal lard, Exp. Physiol. 8 (2003) 389–398, https://doi.org/10.1113/eph8802495.
[56] J.A. Armitage, P.D. Taylor, L. Poston, Experimental models of developmental programming : consequences of exposure to an energy rich diet during development, J. Physiol. 565 (2005) 3–8, https://doi.org/10.1113/jphysiol.2004.079756.
[57] A. Kozimor, H. Chang, J.A. Cooper, Effects of dietary fatty acid composition from a high fat meal on satiety, Appetite 69 (2013) 39–45, https://doi.org/10.1016/j.appet.2013.05.006.
[58] D.J. Naismith, B.L. Morgan, The biphasic nature of protein metabolism during pregnancy in the rat, Br. J. Nutr. 36 (1976) 563, https://doi.org/10.1079/BJN19760109.
[59] L. Englund-Ögge, A.L. Brantsæter, J. Juodakis, M. Haugen, H.M. Meltzer, B. Jacobsson, V. Sengpiel, Associations between maternal dietary patterns and infant birth weight, small and large for gestational age in the Norwegian Mother and Child Cohort Study, Eur. J. Clin. Nutr. 73 (2019) 1270–1282, https://doi.org/10.1038/s41430-018-0356-y.
[60] E.R. Smith, A.H. Shankar, L.S.P. Wu, S. Aboud, S. Adu-Afarwuah, H. Ali, R. Agustina, S. Arifeen, P. Ashorn, Z.A. Bhutta, P. Christian, D. Devakumar, K. G. Dewey, H. Friis, E. Gonso, P. Gupta, P. Kæstel, P. Kolsteren, H. Lanou, K. Maleta, A. Mamadoultaibou, G. Msamanga, D. Osrin, L.Å. Persson, U. Ramakrishnan, J. A. Rivera, A. Rizvi, H.P.S. Sachdev, W. Urassa, K.P. West, N. Zagre, L. Zeng, Z. Zhu, W.W. Fawzi, C.R. Sudfeld, Modifiers of the effect of maternal multiple micronutrient supplementation on stillbirth, birth outcomes, and infant mortality: a meta-analysis of individual patient data from 17 randomised trials in low-income and middle-income countries, Lancet Glob. Heal. 5 (2017) 1090–1100, https://doi.org/10.1016/S2214-109X(17)30371-6.
[61] R.C. Laker, M.E. Wlodek, J.J. Connelly, Z. Yan, Epigenetic origins of metabolic disease: the impact of the maternal condition to the offspring epigenome and later health consequences, Food Sci. Hum. Wellness 2 (2013) 1–11, https://doi.org/10.1016/j.fshw.2013.03.002.
[62] S.C. Langley-Evans, L. Bellinger, D. Sculley, A. Langley-Evans, S. McMullen, Manipulation of the maternal diet in rat pregnancy: different approaches to the demonstration of the programming principle, Adv. Exp. Med. Biol. 573 (2006) 87–102, https://doi.org/10.1007/0-387-32632-4_8.
[63] S.D. Parlee, O.A. MacDougald, Maternal nutrition and risk of obesity in offspring: the Trojan horse of developmental plasticity, Biochim. Biophys. Acta - Mol. Basis Dis. 1842 (2014) 495–506, https://doi.org/10.1016/j.bbadis.2013.07.007.
[64] D. Barker, J. Eriksson, T. Forsen, C. Osmond, Fetal origins of adult disease: strength of effects and biological basis, Int. J. Epidemiol. 31 (2002) 1235–1239, https://doi.org/10.1093/ije/31.6.1235.
[65] S. Cunha, R.D. Molle, A.K. Portella, Both food restriction and high-fat diet during gestation induce low birth weight and altered physical activity in adult rat offspring: the "Similarities in the inequalities" model, PLoS One 10 (2015) 1–18, https://doi.org/10.1371/journal.pone.0118586.
[66] J. Liu, D. Chen, Y. Yao, B. Yu, X. Mao, J. He, Z. Huang, P. Zheng, Intrauterine growth retardation increases the susceptibility of pigs to high-fat diet-induced mitochondrial dysfunction in skeletal muscle, PLoS One 7 (2012) 1–13, https://doi.org/10.1371/journal.pone.0034835.
[67] S. Kabaran, T.T. Besler, Do fatty acids affect fetal programming? J. Heal. Popul. Nutr. 33 (2015) 1–9, https://doi.org/10.1186/s41043-015-0018-9.
[68] S.A. Reed, J.S. Raja, M.L. Hoffman, S.A. Zinn, K.E. Govoni, Poor maternal nutrition inhibits muscle development in ovine offspring, J. Anim. Sci. Biotechnol. 5 (2014) 1–11, https://doi.org/10.1186/2049-1891-5-43.
[69] Y. Wang, J.E. Pessin, Mechanisms for fiber-type specificity of skeletal muscle atrophy, Curr. Opin. Clin. Nutr. Metab. Care 16 (2013) 243–250, https://doi.org/10.1097/MCO.0b013e328360272d.
[70] S. Larsen, J. Nielsen, C.N. Hansen, L.B. Nielsen, F. Wibrand, N. Stride, H. D. Schroder, R. Boushel, J.W. Helge, F. Dela, M. Hey-Mogensen, Biomarkers of mitochondrial content in skeletal muscle of healthy young human subjects, J. Physiol. 590 (2012) 3349–3360, https://doi.org/10.1113/jphysiol.2012.230185.
[71] C. Latouche, S.E. Heywood, S.L. Henry, M. Ziemann, R. Lazarus, A. El-osta, J. A. Armitage, B.A. Kingwell, Maternal overnutrition programs changes in the expression of skeletal muscle genes that are associated with insulin resistance and defects of oxidative phosphorylation in adult, J. Nutr. 144 (2014) 237–244, https://doi.org/10.3945/jn.113.186775.
[72] T.C.B.J. Deiró, R. Manhães-de-Castro, J.E. Cabral-Filho, J.M. Barreto-Medeiros, S. L. Souza, S.M.O.C. Marinho, P.M.M. Castro, A.E. Toscano, R.A. Jesus-Deiró, K.M.F. T. Barros, Sertraline delays the somatic growth and reflex ontogeny in neonate rats, Physiol. Behav. 87 (2006) 338–344, https://doi.org/10.1016/j.physbeh.2005.10.003.
[73] J.M. Mcglashon, M.C. Gorecki, A.E. Kozlowski, K. Caitlin, K.R. Markan, K.L. Leslie, M.E. Kotas, M.J. Potthoff, G.B. Richerson, M.P. Gillum, I. City, I. City, Central serotonergic neurons activate and recruit thermogenic brown and beige fat and regulate glucose and lipid homeostasis, Cell Metab. 21 (2016) 692–705, https://doi.org/10.1016/j.cmet.2015.04.008.
[74] H. Watanabe, R. Saito, T. Nakano, H. Takahashi, Y. Takahashi, K. Sumiyoshi, K. Sato, X. Chen, N. Okada, S. Iwasaki, D.W. Harjanti, N. Sekiguchi, H. Sano, H. Kitazawa, M.T. Rose, S. Ohwada, K. Watanabe, H. Aso, Effect of peripheral 5-HT on glucose and lipid metabolism in wether sheep, PLoS One 9 (2014) 1–12, https://doi.org/10.1371/journal.pone.0088058.
[75] J.J. Kavanagh, A.J. Mcfarland, J.L. Taylor, Enhanced availability of serotonin increases activation of unfatigued muscle but exacerbates central fatigue during prolonged sustained contractions, J. Physiol. 1 (2019) 319–332, https://doi.org/10.1113/JP277148.
[76] E. Supruniuk, A. Miklosz, A. Chabowski, The implication of PGC-1 α on fatty acid transport across plasma and mitochondrial membranes in the insulin sensitive tissues, Front. Physiol. 8 (2017) 1–16, https://doi.org/10.3389/fphys.2017.00923.
[77] V. Caiaffo, A.S.C. Ribeiro, B.D.R. de Oliveira, Y.M.L. de Albuquerque, S.R.A. de Moraes, Histomorphometric evaluation of soleus and lateral gastrocnemius muscles of animals submitted to pharmacological manipulation with fluoxetine, Int. J. Morphol. 32 (2014) 751–755, https://doi.org/10.4067/S0717-95022014000300001.
[78] G.R.P. Braz, C.M. Preitas, L. Nascimento, A.A. Pedroza, A.I. da Silva, C. Lagranha, Neonatal SSRI exposure improves mitochondrial function and antioxidant defense in rat heart, Appl. Physiol. Nutr. Metab. 41 (2016) 362–369, https://doi.org/10.1139/apnm-2015-0494.
[79] C.-C. Chang, C.-T. Lee, T.-H. Lan, P.-C. Ju, Y.-H. Hsieh, T.-J. Lai, Effects of antidepressant treatment on total antioxidant capacity and free radical levels in patients with major depressive disorder, Psychiatry Res. 230 (2015) 575–580, https://doi.org/10.1016/j.psychres.2015.10.006.
[80] T. Liu, S. Zhong, X. Liao, J. Chen, T. He, S. Lai, Y. Jia, A meta-analysis of oxidative stress markers in depression, PLoS One 10 (2015) 1–17, https://doi.org/10.1371/journal.pone.0138904.
[81] M.D. Delp, C. Duan, Composition and size of type I, IIA, IID/X, and IIB fibers and citrate synthase activity of rat muscle, J. Appl. Physiol. 80 (1996) 261–270, https://doi.org/10.1152/jappl.1996.80.1.261.
[82] M. Handal, S. Skurtveit, K. Furu, S. Hernandez-diaz, E. Skovlund, W. Nystad, R. Selmer, Motor development in children prenatally exposed to selective serotonin reuptake inhibitors : a large population-based pregnancy cohort study, BJOG (2015) 1–10, https://doi.org/10.1111/1471-0528.13582.
[83] K. Groove, A.J. Lewis, M. Galbally, Prenatal antidepressant exposure and child motor development: a meta-analysis, Pediatrics 142 (2018), https://doi.org/10.1542/peds.2018-0356.

ANEXO B - PARECER DO COMITÊ DE ÉTICA EM PESQUISA

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Recife, 08 de outubro de 2019

Ofício nº 75/19

Da Comissão de Ética no Uso de Animais (CEUA) da UFPE
Para: **Prof. Ana Elisa Toscano da Silva Castro**
Centro Acadêmico de Vitória
Universidade Federal de Pernambuco
Processo nº 0063/2019

Certificamos que a proposta intitulada "**Plasticidade fenotípica: efeitos do tratamento neonatal com kaempferol sobre parâmetros morfológicos e moleculares ósseos em modelo experimental de paralisia cerebra.**", **registrado com o nº 0063/2019** sob a responsabilidade de **Prof. Ana Elisa Toscano da Silva Castro** o 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 24/09/2019

Finalidade	() Ensino (X) Pesquisa Científica
Vigência da autorização	08/10/2019 a 15/02/2021
Espécie/linhagem/raça	Rato heterogenico
Nº de animais	65
Peso/idade	Adultos(90d ±300g) e Neonatos
Sexo	Macho 53(adultos 5, neonatos 48 Fêmeas nulipares 12 (adultos)
Origem: Biotério de Criação	Bioterio do Departamento de Nutrição da UFPE.
Destino: Biotério de Experimentação	Bioterio de experimentação do Departamento de Nutrição da UFPE.

Atenciosamente,

Prof. Sebastião R. F. Silva
Vice-Presidente CEUA/UFPE
SIAPE 2345681

ANEXO C – PROTOCOLO DO ARTIGO DE REVISÃO

Protocolo do artigo de revisão que está sendo produzida aprovado PROSPERO ID: CRD42020181504

Animal review

To edit the record click *Start an update* below. This will create a new version of the record - the existing version will remain unchanged.

1. * Review title.

Give the working title of the review. This must be in English. The title should have the interventions or exposures being reviewed and the associated health or social problems.

Neuroprotection of flavonoids in early brain injury: a systematic review and meta-analisis of animals studies.

2. Original language title.

For reviews in languages other than English, this field should be used to enter the title in the language of the review. This will be displayed together with the English language title.

None

3. * Anticipated or actual start date.

Give the date when the systematic review commenced, or is expected to commence.

30/05/2020

4. * Anticipated completion date.

Give the date by which the review is expected to be completed.

21/12/2020

5. * Stage of review at time of this submission.

Indicate the stage of progress of the review by ticking the relevant Started and Completed boxes. Additional information may be added in the free text box provided.

Please note: Reviews that have progressed beyond the point of completing data extraction at the time of initial registration are not eligible for inclusion in PROSPERO. Should evidence of incorrect status and/or completion date being supplied at the time of submission come to light, the content of the PROSPERO record will be removed leaving only the title and named contact details and a statement that inaccuracies in the stage of the review date had been identified.

This field should be updated when any amendments are made to a published record and on completion and publication of the review.

The review has not yet started: No

Review stage	Started	Completed
Preliminary searches	Yes	No
Piloting of the study selection process	No	No
Formal screening of search results against eligibility criteria	No	No
Data extraction	No	No
Risk of bias (quality) assessment	No	No
Data analysis	No	No

Provide any other relevant information about the stage of the review here (e.g. Funded proposal, protocol not yet finalised).

6. * Named contact.

The named contact acts as the guarantor for the accuracy of the information presented in the register record.

Márcia Silva

Email salutation (e.g. "Dr Smith" or "Joanne") for correspondence:
Miss Silva

7. * Named contact email.

Enter the electronic mail address of the named contact.

marciasilvamissoes@gmail.com

8. * Named contact address.

PLEASE NOTE this information will be published in the PROSPERO record so please do not enter private information

Enter the full postal address for the named contact.

Universidade Federal de Pernambuco s/n Rua: Alto do Reservatório - Alto José Leal Vitória de Santo Antão - PE 55608-250

9. Named contact phone number

Enter the telephone number for the named contact, including international dialling code.

+5581984160077

10. * Organisational affiliation of the review.

Full title of the organisational affiliations for this review and website address if available. This field may be completed as 'none' if the review is not affiliated to any organisation.

UNIVERSIDADE FEDERAL DE PERNAMBUCO

Organisation web address:
https://www.ufpe.br/

11. * Review team members and their organisational affiliations.

Give the personal details and the organisational affiliations of each member of the review team. Affiliation refers to groups or organisations to which review team members belong. **NOTE: email and country are now mandatory fields for each person.**

Miss Márcia Silva. UNIVERSIDADE FEDERAL DE PERNAMBUCO
Ms Diego Visco. UNIVERSIDADE FEDERAL DE PERNAMBUCO
Dr Ana Elisa Toscano. UNIVERSIDADE FEDERAL DE PERNAMBUCO
Dr Francisco Aguiar Júnior. UNIVERSIDADE FEDERAL DE PERNAMBUCO
Dr Raul Castro. UNIVERSIDADE FEDERAL DE PERNAMBUCO
Dr Omar Quevedo. Instituto Tecnólogico Superior de Tacámbaro

12. * Funding sources/sponsors.

Give details of the individuals, organisations, groups or other legal entities who take responsibility for initiating, managing, sponsoring and/or financing the review. Any unique identification numbers assigned to the review by the individuals or bodies listed should be included.

FACEPE - Fundação de Amparo a Ciência e Tecnologia do Estado de Pernambuco

Grant number(s)

13. * Conflicts of interest.

List any conditions that could lead to actual or perceived undue influence on judgements concerning the main topic investigated in the review.

None

14. Collaborators.

Give the name, affiliation and role of any individuals or organisations who are working on the review but who are not listed as review team members.

15. * Review question.

Give details of the question to be addressed by the review, clearly and precisely.

Do Flavonoids have neuroprotective potential in early brain injuries in an animal model?
In which route of application are flavonoids more effective in the neuroprotective effect in early brain injuries in an animal model?

Context and rationale
Brain injuries largely affect the individual's development, making life difficult in the most common aspects, such as movement and food intake. The use of flavonoids was carried out by several research groups in an attempt to elucidate the characteristics and possibilities of these compounds in protecting the brain. Studies in animal models of brain injuries are important to understand the pathology and to create possible therapies and intervention strategies. Due to the scarcity of randomized clinical trials in humans, evaluating the literature in animals will be important to inform and direct studies on the neuroprotective effects of flavonoids on brain injury in a critical period of development. This systematic review using animal models may be important to understand how flavonoids can help in the treatment of some diseases and clinical conditions resulting from brain injury early in life.

16. * Searches.

Give details of the sources to be searched, and any restrictions (e.g. language or publication period). The full search strategy is not required, but may be supplied as a link or attachment.

The searches will be performed in PubMed, Scopus, Web of Science and Embase without language and publication period restriction.

Search terms will be combined with booleans operator according to MeSH and keywords related to research question as follow:
Early brain injury
Flavonoids
Animals models

17. URL to search strategy.

Give a link to the search strategy or an example of a search strategy for a specific database if available (including the keywords that will be used in the search strategies).

Do not make this file publicly available until the review is complete

18. * Human disease modelled.

Give a short description of the disease, condition or healthcare domain being modelled.

Brain Injury in infants

19. * Animals/population.

Give summary criteria for the animals being studied by the review, e.g. species, sex, details of disease model. Please include details of both inclusion and exclusion criteria.

Inclusion criteria:

Animals of any species and lineage, both sex, exposed to any model of early brain injury.In which the intervention was performed in a pre-injury period (maternal supplementation) or post-injury in the developing rat.

Exclusion criteria:

Genetically modified animals
In vitro study

20. * Intervention(s), exposure(s).

Give full and clear descriptions of the nature of the interventions or the exposures to be reviewed (e.g. dosage, timing, frequency). Please include details of both inclusion and exclusion criteria.

Inclusion criteria:

In vivo studies using animals of any species and lineage, both sex, exposed to an experimental model of early brain injury in the pre, peri or immediately postnatal period.
In vivo studies in which animals are exposed to intervention in the pre-injury period (maternal supplementation) or post-injury in the developing of any animal.
any flavonoids compounds in any dose or via of administration.

Exclusion criteria:

Non Early Brain injury
Exposure occurs later in life.

21. * Comparator(s)/control.

Where relevant, give details of the type(s) of control interventions against which the experimental condition(s) will be compared (e.g. another intervention or a non-exposed control group). Please include details of both inclusion and exclusion criteria.

Inclusion criteria:

A non-exposed control group

Exclusion criteria:

All other control conditions

22. * Study designs to be included.

Give details of the study designs eligible for inclusion in the review. If there are no restrictions on the types of study design eligible for inclusion, or certain study types are excluded, this should be stated. Please include details of both inclusion and exclusion criteria.

Inclusion criteria:

Controlled studies with a separate control group.

Exclusion criteria:

Case studies, cross-over studies, studies without a separate control group.

23. Other selection criteria or limitations applied.

Give details of any other inclusion and exclusion criteria, e.g. publication types (reviews, conference abstracts), publication date, or language restrictions.

all languages, all publication dates.

24. * Outcome measure(s).

Give detail of the outcome measures to be considered for inclusion in the review. Please include details of both inclusion and exclusion criteria.

Inclusion criteria:

Lesion size, brain atrophy, demyelination, ventricular enlargement, loss of neurons, damage to axons and dendrites, oligodendrocyte cell death and alterations of oligodendrocyte development.

Exclusion criteria:
no relevant outcomes reported

25. N/A.

This question does not apply to systematic reviews of animal studies for human health submissions.

26. * Study selection and data extraction.

Procedure for study selection
Phase 1: study titles and / or abstracts will be retrieved using the search strategy and additional sources (for example, reference list of included studies) will be independently tracked by two review authors (SILVA and VISCO).

Phase 2: identify studies that potentially meet the inclusion criteria described above. The full text of these Potentially eligible studies will be retrieved and independently assessed for eligibility by two members, any disagreement between them on the eligibility of specific studies will be resolved through discussion with a third reviewer (TOSCANO).

Prioritise the exclusion criteria
Phase 1: Title/ Abstract screening
1. not an original article
2. not an early brain injury literature
3. not intervention with flavonoids
4. not an animal study

Phase 2: Full-text screening
5. genetically modified animals
6. non control comparison
7. In vitro assessment
8. Exposure occurs later in life
9. Case studies, cross-over studies, studies without a separate control group.
10.No relevant outcomes reported
11. full text not available

Methods for data extraction
Two reviewers will independently extract data from each included article. We first try to extract numerical data from tables, text or figures. If these are not reported, we will extract data from graphs using digital ruler software. In case data are not reported or unclear, we will attempt to contact authors by e-mail (max. 2 attempts). In case an outcome is measured at multiple time points, data from the time point where efficacy is highest will be included.

A pre-piloted and standardized form will be used to extract data from the included studies for assessing the quality of the study and synthesis of evidence. The information extracted will include: characteristics of the animals' baseline, species, lineage, sex, type of model and extent of brain injury, time of insult, details of intervention and control conditions; study methodology; recruitment and completion rates; measurement results and times; suggested mechanisms of exposure action, as well as brain injury markers (lesion extent, number of apoptotic cells, cell death rate); information to assess the risk of bias. Two review authors will extract the data independently, discrepancies will be identified and resolved through discussion (with a third author, when necessary).

Data to be extracted: study design
Experimental groups, control group(s) and number of animals per group.

Data to be extracted: animal model
Species, sex, weight, age, type of experimental brain injury, duration of insult.

Data to be extracted: intervention of interest
Duration of insult exposure, flavonoids name, dose, timing of intervention frequency of intervention, rote of administration.

Data to be extracted: primary outcome(s)

1. brain area (continous, mm2)
2. number of apoptotic cells (continous, n/mm2)
3. rate of cell death (continuous, n/mm2)

Data to be extracted: secondary outcome(s)

1 neurological function score
2 any motor measurement
3. any behavior measurement

Data to be extracted: other

1st author, year of publication, language, journal.

27. * Risk of bias and/or quality assessment.

State whether and how risk of bias and/or study quality will be assessed. Assessment tools specific for pre-clinical animal studies include SYRCLE's risk of bias tool and the CAMARADES checklist for study quality

No risk of bias and/or quality assessment planned

No

By use of SYRCLE's risk of bias tool

Yes

By use of SYRCLE's risk of bias tool adapted as follows:

No

By use of the CAMARADES checklist for study quality

No

By use of the CAMARADES checklist for study quality, adapted as follows:

No

Other criteria, namely

No

Method for risk of bias and/or quality assessment

The risk of bias will be performed by two independent reviewers; discrepancies will be resolved by discussion.

28. * Strategy for data synthesis.

Planned approach

We will provide a narrative synthesis of the findings from the included studies, structured around the type of intervention, animals characteristics, measure outcome and experimental content. We will provide summaries of intervention effects for each study by calculating standardized mean differences for continuous outcomes.

We anticipate that there will be limited scope for meta-analysis because of the range of different outcomes measured across the small number of existing trials. For the accomplishment of the meta-analysis, we stipulated a minimum number of ≥ 3 studies, which include models of brain injury in early periods of life and that there is intervention with flavonoids.

However, where studies have used the same type of intervention and comparator, with the same outcome measure, we will pool the results using a random-effects meta-analysis, with standardised mean differences for continuous outcomes and calculate 95% confidence intervals and two sided P values for each outcome. In studies where the effects of clustering have not been taken into account, we will adjust the standard deviations for the design effect.

Effect measure

Standardized Mean Difference

Effect models

Random-effects

Heterogeneity

I^2 statistic.

Other

If needed, appropriate corrections will be applied.
Corrections will be planned after data extraction and before programming and performing the analyses.

29. * Analysis of subgroups or subsets.

Subgroup analyses

1. model type
2. flavonoids type
3. age of assessment

Sensitivity

None planned

Publication bias

Publication bias will be assessed with a funnel plot, Egger regression and trim and fill.

30. * Review type.

Type of review

Animal model review	Yes
Experimental animal exposure review	No
Pre-clinical animal intervention review	Yes

31. Language.

Select each country individually to add it to the list below, use the bin icon to remove any added in error.

English

There is not an English language summary

32. * Country.

Select the country in which the review is being carried out from the drop down list. For multi-national collaborations select all the countries involved.

Brazil

Mexico

33. Other registration details.

List other places where the systematic review protocol is registered. The name of the organisation and any unique identification number assigned to the review by that organisation should be included.

34. Reference and/or URL for published protocol.

Give the citation and link for the published protocol, if there is one.

No I do not make this file publicly available until the review is complete

35. Dissemination plans.

Give brief details of plans for communicating essential messages from the review to the appropriate audiences.

Do you intend to publish the review on completion?

Yes

36. * Keywords.

Give words or phrases that best describe the review. Separate keywords with a semicolon or new line.

early brain injury;
neuroprotection;
flavonoids;
animal study.

37. Details of any existing review of the same topic by the same authors.

Give details of earlier versions of the systematic review if an update of an existing review is being registered, including full bibliographic reference if possible.

38. * Current review status.

Review status should be updated when the review is completed and when it is published.

Review_Ongoing

39. Any additional information.

Provide any further information the review team consider relevant to the registration of the review.

40. Details of final report/publication(s) or preprints if available.

This field should be left empty until details of the completed review are available OR you have a link to a preprint. Give the full citation for the preprint or final report or publication of the systematic review.

ANEXO D - NORMAS DA REVISTA "BMC MUSCULOSKELETAL DISORDERS"

Research article

Criteria

Research articles should report on original primary research, but may report on systematic reviews of published research provided they adhere to the appropriate reporting guidelines which are detailed in our editorial policies. Please note that non-commissioned pooled analyses of selected published research will not be considered. Studies reporting descriptive results from a single institution will only be considered if analogous data have not been previously published in a peer reviewed journal and the conclusions provide distinct insights that are of relevance to a regional or international audience.

BMC Musculoskeletal Disorders strongly encourages that all datasets on which the conclusions of the paper rely should be available to readers. We encourage authors to ensure that their datasets are either deposited in publicly available repositories (where available and appropriate) or presented in the main manuscript or additional supporting files whenever possible. Please see Springer Nature's information on recommended repositories. Where a widely established research community expectation for data archiving in public repositories exists, submission to a community-endorsed, public repository is mandatory. A list of data where deposition is required, with the appropriate repositories, can be found on the Editorial Policies Page.

Cropped gels and blots can be included in the main text if it improves the clarity and conciseness of the presentation. In such cases, the cropping of the blot must be clearly evident and must be mentioned in the figure legend. Corresponding uncropped full-length gels and blot should be included in the supplementary information. These uncropped images should indicate where they were cropped, be labelled as in the main text and placed in a single supplementary figure. The manuscript's figure legends should state that 'Full-length blots/gels are presented in Supplementary Figure X'. Further information can be found under 'Digital image integrity' which are detailed on our Standards of Reporting page.

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figshare. Checks are carried out as part of a submission screening process to ensure that researchers who should use a specific community-endorsed repository are advised of the best option for sharing and archiving their data. Use of Research Data Support is optional and does not imply or guarantee that a manuscript will be accepted.

Preparing your manuscript

The information below details the section headings that you should include in your manuscript and what information should be within each section.

Please note that your manuscript must include a 'Declarations' section including all of the subheadings (please see below for more information).

Title page

The title page should:

- present a title that includes, if appropriate, the study design e.g.:
    - "A versus B in the treatment of C: a randomized controlled trial", "X is a risk factor for Y: a case control study", "What is the impact of factor X on subject Y: A systematic review"
    - or for non-clinical or non-research studies a description of what the article reports
- list the full names and institutional addresses for all authors
    - if a collaboration group should be listed as an author, please list the Group name as an author. If you would like the names of the individual members of the Group to be searchable through their individual PubMed records, please include this information in the “Acknowledgements” section in accordance with the instructions below
- indicate the corresponding author

Abstract

The Abstract should not exceed 350 words. Please minimize the use of abbreviations and do not cite references in the abstract. Reports of randomized controlled trials should follow the CONSORT extension for abstracts. The abstract must include the following separate sections:

- **Background:** the context and purpose of the study
- **Methods:** how the study was performed and statistical tests used
- **Results:** the main findings
- **Conclusions:** brief summary and potential implications

- **Trial registration:** If your article reports the results of a health care intervention on human participants, it must be registered in an appropriate registry and the registration number and date of registration should be in stated in this section. If it was not registered prospectively (before enrollment of the first participant), you should include the words 'retrospectively registered'. See our editorial policies for more information on trial registration

Keywords

Three to ten keywords representing the main content of the article.

Background

The Background section should explain the background to the study, its aims, a summary of the existing literature and why this study was necessary or its contribution to the field.

Methods

The methods section should include:

- the aim, design and setting of the study
- the characteristics of participants or description of materials
- a clear description of all processes, interventions and comparisons. Generic drug names should generally be used. When proprietary brands are used in research, include the brand names in parentheses
- the type of statistical analysis used, including a power calculation if appropriate

Results

This should include the findings of the study including, if appropriate, results of statistical analysis which must be included either in the text or as tables and figures.

Discussion

This section should discuss the implications of the findings in context of existing research and highlight limitations of the study.

Conclusions

This should state clearly the main conclusions and provide an explanation of the importance and relevance of the study reported.

List of abbreviations

If abbreviations are used in the text they should be defined in the text at first use, and a list of abbreviations should be provided.

Declarations

All manuscripts must contain the following sections under the heading 'Declarations':

- Ethics approval and consent to participate

- Consent for publication
- Availability of data and materials
- Competing interests
- Funding
- Authors' contributions
- Acknowledgements
- Authors' information (optional)

Please see below for details on the information to be included in these sections.

If any of the sections are not relevant to your manuscript, please include the heading and write 'Not applicable' for that section.

Ethics approval and consent to participate

Manuscripts reporting studies involving human participants, human data or human tissue must:

- include a statement on ethics approval and consent (even where the need for approval was waived)
- include the name of the ethics committee that approved the study and the committee's reference number if appropriate

Studies involving animals must include a statement on ethics approval and for experimental studies involving client-owned animals, authors must also include a statement on informed consent from the client or owner.

See our editorial policies for more information.

If your manuscript does not report on or involve the use of any animal or human data or tissue, please state "Not applicable" in this section.

Consent for publication

If your manuscript contains any individual person's data in any form (including any individual details, images or videos), consent for publication must be obtained from that person, or in the case of children, their parent or legal guardian. All presentations of case reports must have consent for publication.

You can use your institutional consent form or our consent form if you prefer. You should not send the form to us on submission, but we may request to see a copy at any stage (including after publication).

See our editorial policies for more information on consent for publication.

If your manuscript does not contain data from any individual person, please state "Not applicable" in this section.

Availability of data and materials

All manuscripts must include an ‘Availability of data and materials’ statement. Data availability statements should include information on where data supporting the results reported in the article can be found including, where applicable, hyperlinks to publicly archived datasets analysed or generated during the study. By data we mean the minimal dataset that would be necessary to interpret, replicate and build upon the findings reported in the article. We recognise it is not always possible to share research data publicly, for instance when individual privacy could be compromised, and in such instances data availability should still be stated in the manuscript along with any conditions for access.

Data availability statements can take one of the following forms (or a combination of more than one if required for multiple datasets):

- The datasets generated and/or analysed during the current study are available in the [NAME] repository, [PERSISTENT WEB LINK TO DATASETS]
- The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.
- All data generated or analysed during this study are included in this published article [and its supplementary information files].
- The datasets generated and/or analysed during the current study are not publicly available due [REASON WHY DATA ARE NOT PUBLIC] but are available from the corresponding author on reasonable request.
- Data sharing is not applicable to this article as no datasets were generated or analysed during the current study.
- The data that support the findings of this study are available from [third party name] but restrictions apply to the availability of these data, which were used under license for the current study, and so are not publicly available. Data are however available from the authors upon reasonable request and with permission of [third party name].
- Not applicable. If your manuscript does not contain any data, please state 'Not applicable' in this section.

More examples of template data availability statements, which include examples of openly available and restricted access datasets, are available here.

BioMed Central also requires that authors cite any publicly available data on which the conclusions of the paper rely in the manuscript. Data citations should include a persistent identifier (such as a DOI) and should ideally be included in the reference list. Citations of datasets, when they appear in the reference list, should include the minimum information

recommended by DataCite and follow journal style. Dataset identifiers including DOIs should be expressed as full URLs. For example:

Hao Z, AghaKouchak A, Nakhjiri N, Farahmand A. Global integrated drought monitoring and prediction system (GIDMaPS) data sets. figshare. 2014. http://dx.doi.org/10.6084/m9.figshare.853801

With the corresponding text in the Availability of data and materials statement:

The datasets generated during and/or analysed during the current study are available in the [NAME] repository, [PERSISTENT WEB LINK TO DATASETS]. [Reference number]

If you wish to co-submit a data note describing your data to be published in *BMC Research Notes*, you can do so by visiting our submission portal. Data notes support open data and help authors to comply with funder policies on data sharing. Co-published data notes will be linked to the research article the data support (example).

Competing interests

All financial and non-financial competing interests must be declared in this section.

See our editorial policies for a full explanation of competing interests. If you are unsure whether you or any of your co-authors have a competing interest please contact the editorial office.

Please use the authors initials to refer to each authors' competing interests in this section.

If you do not have any competing interests, please state "The authors declare that they have no competing interests" in this section.

Funding

All sources of funding for the research reported should be declared. The role of the funding body in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript should be declared.

Authors' contributions

The individual contributions of authors to the manuscript should be specified in this section. Guidance and criteria for authorship can be found in our editorial policies.

Please use initials to refer to each author's contribution in this section, for example: "FC analyzed and interpreted the patient data regarding the hematological disease and the transplant. RH performed the histological examination of the kidney, and was a major contributor in writing the manuscript. All authors read and approved the final manuscript."

Acknowledgements

Please acknowledge anyone who contributed towards the article who does not meet the criteria for authorship including anyone who provided professional writing services or materials.

Authors should obtain permission to acknowledge from all those mentioned in the Acknowledgements section.

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If you do not have anyone to acknowledge, please write "Not applicable" in this section.

Group authorship (for manuscripts involving a collaboration group): if you would like the names of the individual members of a collaboration Group to be searchable through their individual PubMed records, please ensure that the title of the collaboration Group is included on the title page and in the submission system and also include collaborating author names as the last paragraph of the “Acknowledgements” section. Please add authors in the format First Name, Middle initial(s) (optional), Last Name. You can add institution or country information for each author if you wish, but this should be consistent across all authors.

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Footnotes can be used to give additional information, which may include the citation of a reference included in the reference list. They should not consist solely of a reference citation, and they should never include the bibliographic details of a reference. They should also not contain any figures or tables.

Footnotes to the text are numbered consecutively; those to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data). Footnotes to the title or the authors of the article are not given reference symbols.

Always use footnotes instead of endnotes.

References

Examples of the Vancouver reference style are shown below.

See our editorial policies for author guidance on good citation practice

Web links and URLs: All web links and URLs, including links to the authors' own websites, should be given a reference number and included in the reference list rather than within the text of the manuscript. They should be provided in full, including both the title of the site and the URL, as well as the date the site was accessed, in the following format: The Mouse Tumor Biology Database. http://tumor.informatics.jax.org/mtbwi/index.do. Accessed 20 May 2013. If an author or group of authors can clearly be associated with a web link, such as for weblogs, then they should be included in the reference.

Example reference style:

Article within a journal

Smith JJ. The world of science. Am J Sci. 1999;36:234-5.

Article within a journal (no page numbers)

Rohrmann S, Overvad K, Bueno-de-Mesquita HB, Jakobsen MU, Egeberg R, Tjønneland A *et al.* Meat consumption and mortality - results from the European Prospective Investigation into Cancer and Nutrition. BMC Medicine. 2013;11:63.

Article within a journal by DOI

Slifka MK, Whitton JL. Clinical implications of dysregulated cytokine production. Dig J Mol Med. 2000; doi:10.1007/s801090000086.

Article within a journal supplement

Frumin AM, Nussbaum J, Esposito M. Functional asplenia: demonstration of splenic activity by bone marrow scan. Blood 1979;59 Suppl 1:26-32.

Book chapter, or an article within a book

Wyllie AH, Kerr JFR, Currie AR. Cell death: the significance of apoptosis. In: Bourne GH, Danielli JF, Jeon KW, editors. International review of cytology. London: Academic; 1980. p. 251-306.

OnlineFirst chapter in a series (without a volume designation but with a DOI)

Saito Y, Hyuga H. Rate equation approaches to amplification of enantiomeric excess and chiral symmetry breaking. Top Curr Chem. 2007. doi:10.1007/128_2006_108.

Complete book, authored

Blenkinsopp A, Paxton P. Symptoms in the pharmacy: a guide to the management of common illness. 3rd ed. Oxford: Blackwell Science; 1998.

Online document

Doe J. Title of subordinate document. In: The dictionary of substances and their effects. Royal Society of Chemistry. 1999. http://www.rsc.org/dose/title of subordinate document. Accessed 15 Jan 1999.

Online database

Healthwise Knowledgebase. US Pharmacopeia, Rockville. 1998. http://www.healthwise.org. Accessed 21 Sept 1998.

Supplementary material/private homepage

Doe J. Title of supplementary material. 2000. http://www.privatehomepage.com. Accessed 22 Feb 2000.

University site

Doe, J: Title of preprint. http://www.uni-heidelberg.de/mydata.html (1999). Accessed 25 Dec 1999.

FTP site

Doe, J: Trivial HTTP, RFC2169. ftp://ftp.isi.edu/in-notes/rfc2169.txt (1999). Accessed 12 Nov 1999.

Organization site

ISSN International Centre: The ISSN register. http://www.issn.org (2006). Accessed 20 Feb 2007.

Dataset with persistent identifier

Zheng L-Y, Guo X-S, He B, Sun L-J, Peng Y, Dong S-S *et al.* Genome data from sweet and grain sorghum (Sorghum bicolor). GigaScience Database. 2011. http://dx.doi.org/10.5524/100012.

Figures, tables and additional files

See General formatting guidelines for information on how to format figures, tables and additional files.