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DIEGO BULCÃO VISCO

EFEITOS DO TRATAMENTO NEONATAL COM KAEMPFEROL SOBRE O DESENVOLVIMENTO NEURO-MÚSCULO-ESQUELÉTICO EM MODELO DE PARALISIA CEREBRAL EXPERIMENTAL

Recife
2022

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Tese apresentada ao Programa de Pós-Graduação em Nutrição da Universidade Federal de Pernambuco, como requisito parcial para obtenção do título de Doutor em Nutrição. Área de concentração: Bases experimentais da Nutrição.

Orientador (a): Dr. Raul Manhães de Castro

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RESUMO

A Paralisia Cerebral (PC) é caracterizada por uma lesão cerebral em período crítico de desenvolvimento do sistema nervoso central, e em consequência déficits motores, comportamentais e de aprendizado são observados nos indivíduos acometidos. Dentre as estratégias de intervenção nutricionais ou mesmo farmacológicas que estão sendo estudadas atualmente, os flavonoides, como o Kaempferol tem se destacado por suas propriedades anti-inflamatórias e neuroprotetoras. Nesse contexto, o objetivo desse estudo foi avaliar os efeitos do tratamento neonatal com kaempferol sobre o desenvolvimento neuro-músculo-esquelético de ratos submetidos à PC. Este estudo foi aprovado pela Comissão de Ética no Uso de Animais (nº 0058/2018) da Universidade Federal de Pernambuco. Para a composição dos grupos experimentais ratos *Wistar* machos foram alocados de forma randomizada após o nascimento em quatro grupos de acordo com o modelo de PC ou controle e tratamento neonatal com kaempferol ou veículo, sendo: C = controle – veículo; K = controle – Kaempferol; PC = Paralisia Cerebral – veículo; PCK = Paralisia Cerebral – Kaempferol. O modelo de paralisia cerebral consistiu em anóxia perinatal associada à restrição sensório-motora das patas posteriores durante a infância (P2-P28). O tratamento com Kampeferol (1 mg / kg) foi realizado por via intraperitoneal durante o período neonatal (P2-P21). Na prole foram avaliados a caracterização somática, parâmetros de maturação física, a ontogênese dos reflexos, o desenvolvimento da atividade locomotora, a coordenação motora, a força muscular, e parâmetros cinéticos, temporais e espaciais da marcha. Ao 36º dia de vida pós-natal os animais foram divididos para realização das análises experimentais do cérebro e do músculo esquelético. Após perfusão intracardíaca o encéfalo foi dissecado e submetido aos procedimentos de imuno-histoquímica para análise de proliferação celular no giro denteado do hipocampo, densidade e perfil de micróglias e proliferação de micróglias no giro denteado do hipocampo. Para análises do músculo esquelético os animais foram eutanasiados por decaptação para dissecação dos músculos sóleo. Os músculos foram processados através da técnica de ATPase miofibrilar para tipagem de fibras musculares e para quantificação de proteínas miofibrilares através de *western blot*. O tratamento neonatal com kaempferol atenuou os déficits sobre o desenvolvimento da atividade locomotora, da maturação das características físicas, da ontogenia dos reflexos e da coordenação motora ratos com PC. Também o

tratamento neonatal com kaempferol preveniu o impacto sobre a proliferação de células progenitoras, no aumento do perfil de micróglias ativadas e proliferação microglial no giro denteado do hipocampo em animais com PC. Além disso, observou-se redução dos efeitos deletérios da PC sobre o fenótipo corporal, atenuando também déficits na força muscular e na marcha. Adicionalmente, o tratamento mitigou o impacto sobre o fenótipo muscular ao prevenir a redução da proporção de fibras oxidativas e de medidas histomorfométricas do músculo sóleo em ratos com PC. Os resultados demonstram que o tratamento neonatal com kaempferol atenuou os déficits de desenvolvimento locomotor e da maturação do sistema neuro-músculo-esquelético em ratos submetidos a PC.
Palavras-chave: paralisia cerebral; flavonoides; modelos animais; plasticidade celular.

ABSTRACT

Cerebral palsy (CP) is characterized by brain damage during the critical period of development of the central nervous system, and as a result, motor, behavioral, and learning deficits are observed in affected subjects. Among the nutritional or even pharmacological intervention strategies that are currently being studied, flavonoids such as Kaempferol have the potential due to their anti-inflammatory and neuroprotective properties. In this context, the aim of this study was to assess the effects of neonatal treatment with kaempferol on the neuromusculoskeletal development of rats submitted to CP. This study was approved by the Ethics Committee on the Use of Animals (nº 0058/2018) of the Federal University of Pernambuco. For the composition of experimental groups, male Wistar rats were randomly allocated after birth into four groups according to the CP model or control and neonatal treatment with kaempferol or vehicle, as follows: C = control – vehicle; K = control – Kaempferol; CP = Cerebral Palsy – vehicle; PCK = Cerebral Palsy – Kaempferol. The model of cerebral palsy consisted of perinatal anoxia associated with sensorimotor restriction of hind limbs during infancy (P2-P28). Treatment with Kaempferol (1 mg/kg) was performed intraperitoneally during the neonatal period (P2-P21). In the offspring were evaluated: somatic features, physical maturation, reflex ontogenesis, development of locomotor activity, motor coordination, muscle strength, and gait kinetic, temporal and spatial parameters. On the 36th day of postnatal life, the animals were divided to carry out experimental analyzes of the brain and skeletal muscle. After intracardiac perfusion, the brain was dissected and submitted to immunohistochemical procedures for analysis of cell proliferation in the dentate gyrus of the hippocampus, density and profile of microglia and proliferation of microglia in the dentate gyrus of the hippocampus. For skeletal muscle analysis, the animals were euthanized by decapitation for soleus muscle dissection. The muscles were processed using the myofibrillar ATPase technique for muscle fiber typing and for quantification of myofibrillar proteins through western blot. Neonatal treatment with kaempferol attenuated deficits in the development of locomotor activity, the maturation of physical characteristics, the ontogeny of reflexes, and motor coordination in rats with CP. Neonatal treatment with kaempferol also prevented the impact on the proliferation of progenitor cells, increased profile of activated microglia, and microglial proliferation in the dentate gyrus of the hippocampus in animals with CP. In addition, a reduction in

the deleterious effects of CP on the body phenotype was observed, also attenuating deficits in muscle strength and gait. Also, the treatment mitigated the impact on muscle phenotype by preventing the reduction in the proportion of oxidative fibers and histomorphometric measurements of the soleus muscle in rats with CP. The results demonstrate that neonatal treatment with kaempferol attenuated deficits in locomotor development and neuromusculoskeletal system maturation in rats submitted to CP.
Keywords: cerebral palsy; flavonoids; animal models; cell plasticity.

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1 INTRODUÇÃO

A Paralisia Cerebral (PC) é um distúrbio do neurodesenvolvimento associado ao comprometimento do movimento, mudanças de comportamento e disfunção cognitiva, como memória espacial e déficits de aprendizagem (BUNNEY et al., 2017; HADDERS-ALGRA, 2018; LEE et al., 2017; LYALL et al., 2015; WEE et al., 2017). Esta desordem é considerada a causa mais comum de deficiência motora na infância sendo definida como um distúrbio não progressivo da postura, movimento e tônus, que resulta de dano cerebral estático durante um período crítico do cérebro em desenvolvimento (GRAHAM et al., 2016; ROSENBAUM et al., 2007).

Os mecanismos que levam ao desenvolvimento da PC podem ser atribuídos a qualquer condição que afete o cérebro em desenvolvimento devido a condições intrauterinas (malformações congênitas, distúrbios vasculares da placenta, infecção / inflamação), complicações intraparto (descolamento prematuro da placenta, corioamnionite e asfixia ao nascimento) e / ou insulto pós-natal (acidente vascular cerebral neonatal, hemorragia intraventricular, leucomalácia periventricular, sepse) (MOR et al., 2016; STAVSKY et al., 2017a).

O período fetal e neonatal representam uma janela crítica de maturação do Sistema Nervoso Central (SNC) (BAX et al., 2006; VASUNG et al., 2019). Estudos de neuroimagem e estudos pós-morte em animais revelaram uma variedade de combinações de lesões no córtex cerebral, substância branca, gânglios da base e cerebelo em indivíduos com PC (DOS SANTOS et al., 2017; KORZENIEWSKI et al., 2008; VOLPE, 2009). Portanto, dependendo do período, do tipo de lesão cerebral, da localização da lesão cerebral e da resposta difusa dos tecidos, a PC pode estar associada a uma variedade de deficiências motoras, sensoriais, cognitivas e comportamentais (GRAHAM et al., 2016; MARRET; VANHULLE; LAQUERRIERE, 2013).

Nos estágios iniciais do desenvolvimento do cérebro, estudos revelam que a inflamação cerebral é um fator importante para a fisiopatologia da PC (KUYPERS et al., 2012). A micróglia, que representa de 5 a 15% das células cerebrais, participa do início e da progressão da resposta inflamatória do SNC após a lesão (GALLUZZI et al., 2016). Estudos experimentais com mamíferos neonatais mostraram que modelos de lesão cerebral precoce causam aumento da expressão de marcadores para micróglia e macrófagos ao redor das lesões nas primeiras 24 horas após o insulto inicial ou até mesmo meses após o dano (BURD; BALAKRISHNAN; KANNAN, 2012;

MALLARD; TREMBLAY; VEXLER, 2019; SAVIGNON et al., 2012). Além disso, a ativação da micróglia e consequente indução de citocinas pró-inflamatórias e outros mediadores inflamatórios está estabelecida como uma característica de lesão cerebral perinatal em vários modelos animais (MALLARD; TREMBLAY; VEXLER, 2019). Entretanto, a proliferação de micróglia e seu papel na neuroplasticidade após lesão cerebral em período perinatal tem sido pouco explorada (VISCO et al., 2021a).

Uma vez estabelecido, o insulto cerebral ou problemas estruturais não parecem progredir com o tempo, mas os indivíduos com PC estão sujeitos a uma série de condições secundárias que podem interferir em aspectos importantes da qualidade de vida, como independência funcional e participação social (STAVSKY et al., 2017b). A PC está associada a comportamentos sedentários exagerados e a uma progressão acelerada de patologias musculares em comparação com crianças e adultos com desenvolvimento típico (PETERSON et al., 2012). Fatores como excesso de deposição de tecido adiposo, problemas de captação de glicose, resistência à insulina e inflamação crônica podem aumentar a gravidade da patologia muscular durante a vida adulta e levar ao risco de doença cardiometabólica e / ou mortalidade precoce (PETERSON et al., 2012). Em conjunção com esses fatores, a musculatura esquelética em quadros de PC sofre importantes impactos como, redução da massa muscular; fraqueza; inabilidade de recrutar a musculatura alvo para determinado movimento; e co-ativação da musculatura antagonista, que contribuem para os déficits de controle motor para realização da marcha e atividades de vida diária (CHEN et al., 2015; SHORTLAND, 2009; STACKHOUSE et al., 2005).

Devido à estreita janela de tempo terapêutica após a lesão cerebral, as opções para o tratamento da PC em fase precoce permanecem muito limitadas e poucos tratamentos neuroprotetores foram desenvolvidos com sucesso para prevenir danos cerebrais e atraso no neurodesenvolvimento (JUUL; FERRIERO, 2014). Dentre as intervenções nutricionais, ou mesmo farmacológicas, muito se tem discutido sobre potencial neuroproteror e anti-inflamatório dos compostos polifenólicos como os flavonoides (HUANG et al., 2012; PANDEY; RIZVI, 2009; S.S. et al., 2013). Os flavonoides, uma classe de metabólitos vegetais secundários, têm sido relatados por promover a regeneração neuronal, restaurar a funcionalidade dos neurônios e mediar os efeitos neuroprotetores contra o estresse oxidativo, inflamação e atividade persistente da micróglia (SCHROETER et al., 2001; ZHENG et al., 2008).

Dentre os flavonoides, o Kaempferol é considerado por apresentar potencial neuroprotetor mais evidente na literatura científica, através dos mecanismos subjacentes às propriedades anti-inflamatórias e efeitos antioxidantes destes compostos (LI et al., 2019a; LY et al., 2015; PANDIMA et al., 2015). **Além disso, o kaempferol tem sido relatado por proporcionar** benefícios comportamentais e cognitivos em modelos animais de lesão cerebral (DAS et al., 2018; LI et al., 2019a).

Em modelos de isquemia cerebral, o kaempferol atenuou a neuro-inflamação e reduziu os déficits neurológicos (YU et al., 2013). Nesse estudo com ratos, os autores relataram que o tratamento com kaempferol após isquemia cerebral, inibe mecanismos pró-inflamatórios no cérebro, com redução da inflamação e diminuição da micróglia com morfologia ativada (YU et al., 2013). Os autores observaram que o potencial antioxidante do kaempferol protege a apoptose celular no hipocampo e atenua os déficits de memória (YU et al., 2013; EL-KOTT et al., 2020). Além disso, o tratamento com kaempferol reduziu os danos aos neurônios e axônios em comparação com os controles tratados com veículo (YU et al., 2013). Mesmo com essas evidências, não existem estudos que investigaram o potencial efeito benéfico do kaempferol ou mesmo de outros polifenóis, em modelos de lesão cerebral perinatal como a paralisia cerebral (VISCO et al., 2021b).

Nesse contexto, nosso objetivo foi avaliar os efeitos do tratamento neonatal com kaempferol sobre o desenvolvimento neuro-músculo-esquelético em modelo de paralisia cerebral. Com isso, hipotetizamos que a exposição neonatal ao kaempferol promove melhor proliferação celular e menor ativação de micróglia no hipocampo, beneficiando o desenvolvimento dos sistemas nervoso e muscular e consequente repercussões sobre a motricidade em ratos submetidos ao modelo de PC.

2 REVISÃO DA LITERATURA

2.1 O PERÍODO CRÍTICO DE DESENVOLVIMENTO E A PARALISIA CEREBRAL

O Sistema Nervoso Central (SNC) é caracterizado por apresentar um período crítico e um período sensitivo durante o seu desenvolvimento em fase precoce da vida. O período crítico é o tempo durante o qual a ação de uma influência específica interno ou externa é necessária (crítica) para o progresso normal de desenvolvimento (BHUTTA; ANAND, 2002). Em contraste, o período sensitivo, caracteriza-se por ser o tempo durante o qual o SNC é altamente suscetível aos efeitos de condições internas ou externas prejudiciais ou deletérias (BHUTTA; ANAND, 2002). Portanto, um período crítico ocorre quando certas condições são necessárias para o SNC para desenvolver normalmente; um período sensível é o tempo em que o dano ao SNC pode levar a alterações, reorganização e potenciais aberrações do sistema (BHUTTA; ANAND, 2002; DOBBING; SMART, 1974).

A origem e maturação do SNC pode ser dividida em uma série de eventos. As primeiras semanas do período pré-natal é caracterizada pela organogênese, neurogênese e a ocorrência de migração neuronal, em seguida ocorrem mecanismos de crescimento e maturação neuronal (SOUSA et al., 2017; STILES; JERNIGAN, 2010). A organização do desenvolvimento do SNC ocorre de forma precisa e sequencial através de mecanismos bioquímicos e moleculares complexos. De forma simples os eventos que caracterizam a maturação do SNC são: 1) indução dorsal, (2) indução ventral, (3) proliferação neuronal, (4) migração neuronal, (5) organização / diferenciação (crescimento axonal e dendrítico, sinaptogênese, morte celular / axonal) e (6) mielinização (BHUTTA e ANAND, 2002; DARNELL e GILBERT, 2017; HALLETT AND PROCTOR, 1996). Entretanto sabe-se que o neurodesenvolvimento depende de influências genéticas e epigenéticas (VOLPE, 1992; ZAHIR; BROWN, 2011).

Como a formação do cérebro é um processo complexo, este está susceptível a influências ambientais em qualquer estágio do seu desenvolvimento (STAUDT, 2010). Acredita-se que momento de ocorrência de um insulto pode ser mais importante do que a natureza do próprio insulto na determinação do padrão de malformação ou funcionamento cerebral (VOLPE, 1992). Em outras palavras, o espectro de distúrbios do SNC após insultos perinatais é determinado não só pela natureza e a gravidade do insulto, mas também pelo estágio de maturação do cérebro no momento de insulto (MARÍN, 2016).

Desordens cerebrais nos primeiros anos de vida podem ser causadas por uma variedade de fatores incluindo: distúrbios genéticos, trauma, hemorragia, acidente vascular cerebral, infecção, metabolismo alterado, falta de oxigênio e exposição a toxinas (HAGBERG; DAVID EDWARDS; GROENENDAAL, 2016). Cada influência desta, pode destruir, de forma direta ou indireta, alguma parte do cérebro, ou afetando o desenvolvimento normal e maturação do SNC (BERGER; GARNIER, 2000). Em outras palavras, uma lesão em período perinatal pode então afetar toda a sequência de desenvolvimento que se segue (BHUTTA; ANAND, 2002). Isso, certamente se deve aos distúrbios de formação de sinapses, problemas de conexões neuronais com o córtex cerebral e mielinização pós-natal (BERGER e GARNIER, 2000; HAGBERG et al., 2016).

Dentre as desordens de neurodesenvolvimento, causadas por lesão cerebral em período perinatal, destaca-se a Paralisia Cerebral. A Paralisia Cerebral (PC) é considerada a causa mais comum de deficiência motora na infância (GRAHAM et al., 2016; ROSENBAUM et al., 2007). A PC é definida como um distúrbio não progressivo da postura, movimento e tônus, que resulta de dano cerebral estático durante um período crítico do cérebro em desenvolvimento (GRAHAM et al., 2016; ROSENBAUM et al., 2007). Com o início precoce de deficiências motoras, a PC está associada a incapacidade e dependência funcional ao longo da vida (PATEL et al., 2020). Os distúrbios motores da PC são frequentemente acompanhados por deficiências sensoriais, problemas cognitivos e comportamentais, representando uma importante demanda aos serviços de saúde (GRAHAM et al., 2016).

Mesmo com os avanços na assistência perinatal, a taxa total de PC está relativamente estável nas últimas décadas em todo o mundo (STAVSKY et al., 2017b). Os estudos de base populacional relatam uma faixa de 1,5 a 4 por 1.000 na estimativa de prevalência desta desordem (MAENNER et al., 2016; OSKOUI et al., 2013; VAN NAARDEN BRAUN et al., 2016). Na Europa, 10.756 casos foram notificados durante o período de 1980 a 2009 (SELLIER et al., 2020). Um estudo populacional que avaliou diferenças de gênero revelou que a incidência da PC em homens é maior do que em mulheres (CHOUNTI et al., 2013).

Os mecanismos que levam ao desenvolvimento de PC podem ser atribuídos a qualquer condição que afete o cérebro em desenvolvimento devido a condições intrauterinas (malformações congênitas, distúrbios vasculares da placenta, infecção / inflamação), complicações intraparto (descolamento prematuro da placenta,

corioamnionite e asfixia ao nascimento), e / ou insulto pós-natal (acidente vascular cerebral neonatal, hemorragia intraventricular, leucomalácia periventricular, sepse) (O'SHEA et al., 2009; STAVSKY et al., 2017a). Além disso, o nascimento prematuro, baixo peso ao nascer e privação de oxigênio em período perinatal são os principais fatores de risco para o desenvolvimento de PC (O'SHEA et al., 2009; STAVSKY et al., 2017a).

Adicionalmente, vários estudos associam a condição socioeconômica como fator de risco para ocorrência de PC em países em desenvolvimento e desenvolvidos (DOLK et al., 2010; HJERN; THORNGREN-JERNECK, 2008; OSKOUI et al., 2016). A exposição crônica a estressores socioeconômicos como, nutrição parental inadequada, nível reduzido de atividade física, falta de recursos de saúde e condições de moradia inadequadas estão associadas a alta prevalência de PC, incluindo apresentação de fenótipos clínicos mais graves (FORTHUN et al., 2018; GALEA et al., 2019; MCINTYRE et al., 2013; OSKOUI et al., 2016). Atualmente, há uma preocupação com o atual contexto pandêmico sobre essas condições socioeconômicas e como isso pode influenciar a prevalência de PC no futuro.

O período fetal e neonatal representa uma janela crítica de maturação do SNC (VASUNG et al., 2019). O estágio de maturação do cérebro durante o dano cerebral define o tipo, o local e a resposta do tecido à lesão (GRAHAM et al., 2016). O processo destrutivo que lesa o tecido cerebral saudável representa a maioria dos casos de ocorrência de PC, em comparação com as anormalidades de má formação do cérebro (BAX; TYDEMAN; FLODMARK, 2006). Estudos de neuroimagem e estudos pós-morte em animais revelaram uma variedade de combinações de lesões no córtex cerebral, substância branca, gânglios da base e cerebelo em indivíduos afetados pela PC (DOS SANTOS et al., 2017; KORZENIEWSKI et al., 2008; VOLPE, 2009). Logo, dependendo do período de tempo e do tipo de lesão cerebral, da localização da lesão cerebral e da resposta difusa dos tecidos, a PC pode estar associada a uma variedade de deficiências motoras, sensoriais, cognitivas e comportamentais (GRAHAM et al., 2016; MARRET; VANHULLE; LAQUERRIERE, 2013).

A PC é geralmente classificada em subtipos baseados na topografia (hemiplegia, tetraplegia, diplegia ou distúrbios extrapiramidais), que geralmente resulta de danos a diferentes áreas do cérebro que ocorrem no útero, durante o parto, ou após o nascimento (GRAHAM et al., 2016; PATEL et al., 2020). Com base na apresentação clínica de comprometimento neurológico do sistema motor, a PC é

geralmente caracterizada por espasticidade, discinesia, hipotonia e ataxia. Além disso, a apresentação mista pode ser comumente observada em crianças afetadas (GRAHAM et al., 2016; PATEL et al., 2020).

O fenótipo clínico mais comum da PC é a diplegia espástica, quando as vias corticospinais e talamocorticais motoras são afetadas devido a danos aos oligodendrócitos imaturos no útero (COLVER et al., 2013; GRAHAM et al., 2016; MARRET; VANHULLE; LAQUERRIERE, 2013). O nascimento prematuro é geralmente associado à tetraplegia espástica (20% das crianças com PC) e este fenótipo clínico é devido à leucomalácia periventricular e encefalomalácia cortical multicística (COLVER et al., 2013; GRAHAM et al., 2016; MARRET; VANHULLE; LAQUERRIERE, 2013). O prognóstico para crianças com tetraplegia espástica está relacionado à limitação funcional significativa, déficits cognitivos e de aprendizagem com outras condições associadas. Enquanto que, a hemiplegia espástica é comum em crianças que nascem a termo que sofreram um acidente vascular cerebral intrauterino ou perinatal (COLVER et al., 2013; GRAHAM et al., 2016; MARRET; VANHULLE; LAQUERRIERE, 2013). O fenótipo de menor incidência da PC é o extrapiramidal, com fenótipo clínico associado à forma coreoatetótica, distônica ou discinética (Colver et al., 2013; Marret et al., 2013). A maioria desses casos é observada em crianças nascidas a termo que sofrem de encefalopatia hipóxico-isquêmica, kernicterus, distúrbio neurometabólico ou neurogenético, com pior prognóstico relacionado a déficits cognitivos, problemas comportamentais, convulsões, distúrbios do sono, deficiências visuais e auditivas e dificuldades de alimentação (COLVER et al., 2013; GRAHAM et al., 2016; MARRET; VANHULLE; LAQUERRIERE, 2013).

Diversos modelos animais têm sido desenvolvidos para entender os mecanismos de lesão cerebral subjacentes à PC (CAVARSAN; GORASSINI; QUINLAN, 2020; HAGBERG; PEEBLES; MALLARD, 2002). Tais modelos, portanto, fornecem um meio prático de obter informações fundamentais sobre a resposta do SNC, com ênfase especial no papel da patogênese, mecanismos fisiopatológicos e eventos espaço-temporais no neurodesenvolvimento (CAVARSAN; GORASSINI; QUINLAN, 2020; CLOWRY; BASUODAN; CHAN, 2014b). Estes também são essenciais para o desenvolvimento de intervenções eficazes para aumentar a capacidade regenerativa endógena do cérebro imaturo ou mesmo desenvolvimento de estratégias de prevenção ao tecido nervoso (CAVARSAN; GORASSINI; QUINLAN,

2020; CLOWRY; BASUODAN; CHAN, 2014b). Bem como fornecem informações sobre danos cerebrais e comprometimento neurocomportamental (CAVARSAN; GORASSINI; QUINLAN, 2020; CLOWRY; BASUODAN; CHAN, 2014b; DA CONCEIÇÃO PEREIRA et al., 2021; FRAGOPOULOU et al., 2019; GOUVEIA et al., 2020; HAGBERG; PEEBLES; MALLARD, 2002; LACERDA et al., 2017a; SILVA et al., 2016). Nesse sentido, estudos pre-clínicos apresentam potencial para o entendimento de como a lesão cerebral perinatal favorece o desenvolvimento da paralisia cerebral bem como favorecem o estudo dos comprometimentos motores, sensoriais, comportamentais e cognitivos.

2.2 PLASTICIDADE NEURAL NO HIPOCAMPO DURANTE O DESENVOLVIMENTO E APÓS LESÃO CEREBRAL PERINATAL

O hipocampo é uma estrutura do lobo temporal medial importante para uma série de processos cognitivos críticos, incluindo, mas não se limitando a memória episódica, regulação do estresse, navegação espacial e aprendizado (EICHENBAUM; COHEN, 2014). Esta região cerebral, compreende um número considerável de subestruturas complexas, incluindo o giro dentado, o Cornu Ammonis, o subículo e os tratos de substância branca (INSAUSTI; CEBADA-SÁNCHEZ; MARCOS, 2010). Durante o desenvolvimento em humanos, o hipocampo surge primeiramente na parte dorsal da lâmina terminal e pode ser identificado histologicamente já na 9ª semana gestacional (HUMPHREY, 1967; INSAUSTI; CEBADA-SÁNCHEZ; MARCOS, 2010). Durante o segundo semestre do período gestacional, importantes mudanças ocorrem na estrutura do hipocampo. O giro denteado e o Cornu Ammonis dobram-se progressivamente no lobo temporal, juntamente com o sulco hipocampal que se fecha gradualmente, formando a fissura hipocampal (INSAUSTI; CEBADA-SÁNCHEZ; MARCOS, 2010). Entre a 18ª e 20ª semana gestacional, a estrutura do hipocampo já possui características que são observadas na fase adulta (INSAUSTI; CEBADA-SÁNCHEZ; MARCOS, 2010). Uma trajetória linear de aumento do volume do hipocampo foi encontrada em fetos saudáveis e bebês prematuros e a termo (JACOB et al., 2011). Em roedores, os neurônios do hipocampo são produzidos a partir do neuroepitélio dentado no dia Embrionário 13 (E13) (URBÁN; GUILLEMOT, 2014). A formação hipocampal é amplamente desenvolvida por E20 em roedores e por volta de 20 semanas de gestação em humanos. No entanto, um desenvolvimento

volumétrico significativo persiste até P21 em roedores e até 2 anos em humanos (SEMPLE et al., 2013).

O hipocampo se comunica com regiões disseminadas do córtex por meio de um grupo de regiões cerebrais altamente interconectadas no lobo temporal medial. Estas regiões são geralmente referidas como sistema hipocampal (WIBLE, 2013). O sistema hipocampal consiste em Giro Denteado (DG), campos de Cornu Ammonis (CA) e subículo. O giro dentado é uma região de entrada, que recebe entrada do córtex entorrinal. Os campos Cornu Ammonis (CA) do hipocampo consistem em células piramidais e geralmente são subdivididos em quatro regiões (CA1-CA4) (WIBLE, 2013). A fisiologia do hipocampo é única e confere à região um alto nível de plasticidade, que é importante para o aprendizado e a memória; esta propriedade também tem implicações importantes para lesões cerebrais em período perinatal (KOZAREVA; CRYAN; NOLAN, 2019; WIBLE, 2013).

O hipocampo é uma região do cérebro dos mamíferos que apresenta uma capacidade impressionante de reorganização estrutural sendo fundamental para ocorrência de neuroplasticidade cerebral (KOZAREVA; CRYAN; NOLAN, 2019). Os circuitos neurais preexistentes sofrem modificações na complexidade dendrítica e no número de sinapses, e conexões neurais inteiramente novas são formadas por meio do processo de neurogênese (CAYRE; CANOLL; GOLDMAN, 2009; SANCHEZ; RIBAK; SHAPIRO, 2012). Antigamente, pensava-se que esses tipos de mudança estrutural se restringiam ao desenvolvimento. No entanto, agora é geralmente aceito que o hipocampo permanece estruturalmente plástico ao longo da vida (KOZAREVA; CRYAN; NOLAN, 2019).

Embora os neurônios piramidais das regiões CA3 e CA1 sejam gerados exclusivamente durante o desenvolvimento embrionário, a população de células granulares do giro denteado é produzida durante um período prolongado que começa durante a gestação e continua após o nascimento (SERESS, 2007). Em roedores, a camada de células granulares apresenta um pico na gênese celular durante a primeira semana de vida pós-natal (SERESS, 2007). Em período pós-natal a Zona Subgranular (ZSG) do GD do hipocampo é uma das poucas áreas cerebrais que mantém a capacidade proliferativa das células troncos neurais em produzir células gliais e neurônios (LAJUD; TORNER, 2015).

O SNC se desenvolve a partir de um pequeno número de células altamente plásticas que proliferam, adquirem identidades regionais e produzem diferentes tipos

de células (DARNELL; GILBERT, 2017). Essas células foram definidas como células-tronco neurais, com base em seu potencial para gerar múltiplos tipos de células (por exemplo, neurônios e glia) e sua capacidade de auto renovação (CHEN et al., 2002). O período neonatal é importante para a maturação do SNC (DARNELL; GILBERT, 2017). Enquanto a maioria das células granulares do hipocampo de roedores são gerados até o dia pós-natal (P) 10 (ALTMAN; BAYER, 1990). As células tronco neurais permanecem abundantes no desenvolvimento do cérebro até o P14 quando elas começam a se diferenciar em células progenitoras neurais (ALTMAN; BAYER, 1990). Isso é seguido pela transformação em neuroblastos e finalmente em células granulares excitatórias que se integram no circuito por volta do P21 (KRIEGSTEIN; ALVAREZ-BUYLLA, 2009).

O surgimento de novos neurônios pode, portanto, diferir em sua resposta a influências ambientais e fatores modificadores da doença em vários momentos durante a vida, o que também tem implicações funcionais (KOZAREVA; CRYAN; NOLAN, 2019). Assim, a neurogênese hipocampal e sua relevância funcional têm sido o tema de intensa pesquisa nas últimas décadas, e recentemente no contexto da PC (VISCO et al., 2021a). A neurogênese é definida como um processo de geração de novos neurônios a partir células-tronco neurais ou células neurais progenitoras (RAMASAMY et al., 2013).

Nas regiões neurogênicas, existem microambientes (nichos) únicos e altamente especializados que regulam rigidamente o desenvolvimento neuronal das células-tronco neurais adultas (CAYRE; CANOLL; GOLDMAN, 2009). Evidências sugerem que a glia, participa no controle de várias etapas da neurogênese adulta dentro dos nichos, desde a proliferação e especificação do destino dos progenitores neurais até a migração e integração da progênie neuronal em circuitos neuronais pré-existentes no cérebro adulto (MA; MING; SONG, 2005).

Nesse contexto, sabe-se que um importante regulador do desenvolvimento do SNC é a micróglia, células imunes inatas do SNC (DENG, 2010). A micróglia tem uma ampla gama de funções ao longo da vida e em diferentes regiões do SNC (BOCHE; PERRY; NICOLL, 2013). Durante o desenvolvimento, a micróglia no SNC contribui para a formação de circuitos neuronais e promovem sua sobrevivência por meio da liberação de neurotrofinas, fatores de crescimento e citocinas (NAYAK; ROTH; MCGAVERN, 2014). Além disso, foi mostrado que a micróglia poda neurônios redundantes, iniciando programas de morte celular seguido por fagocitose ou mesmo

limpando restos celulares após apoptose (NAYAK; ROTH; MCGAVERN, 2014). Elas também promovem a sobrevivência de células neuronais precursoras no SNC em desenvolvimento, englobando sinapses intactas menos ativas e regulando a remodelação sináptica (REEMST et al., 2016). Em período neonatal, a micróglia desempenha um papel importante na mielinização e neurogênese do cérebro em desenvolvimento (WLODARCZYK et al., 2017).

Evidências sobre a origem da micróglia demonstraram que essas células são derivadas de progenitores mieloides aproximadamente no dia embrionário 7.5 (E7.5) em camundongos e se infiltram no cérebro através dos vasos sanguíneos entre E8.5 e E9.5 (GINHOUX et al., 2010; SCHLEGELMILCH; HENKE; PERI, 2011). Esse processo ocorre imediatamente antes da formação dos primeiros progenitores radiais para neurônios e glia, que ocorre em E10.5 (KAUR; RATHNASAMY; LING, 2017).

Em humanos, aproximadamente 5% das células cerebrais são micróglia (PELVIG et al., 2008). Originalmente, a micróglia destaca-se por determinarem os níveis de inflamação no ambiente celular, o que subsequentemente determina se os neurônios recém-gerados sobrevivem (TONG; VIDYADARAN, 2016). No entanto, evidências têm demonstrado que a micróglia desempenha papéis diversos na neurogênese, tanto nos estágios embrionários quanto nos estágios adultos pós-natal (LEGENDRE; LE CORRONC, 2014).

No hipocampo em período pós-natal, dois tipos principais de células microgliais foram reconhecidas, micróglia tipo ameboide (ativada) e a micróglia ramificada (inativada), e para cada tipo de célula, diferentes subtipos podem ser distinguidos (DALMAU et al., 1998). Além dessas duas células, células semelhantes ao tipo de micróglia reativa também foram reconhecidos (DALMAU et al., 1998). Ao nascimento, o giro dentado possui muito menos micróglias do que a área CA (REZNIKOV, 1991). Durante a primeira semana pós-natal, células ameboides são progressivamente transformadas em células microgliais ramificadas primitivas (Reznikov, 1991; Dalmau et al., 1998). A partir da segunda semana pós-natal, o número de células micróglias ramificadas primitivas aumentam, gradualmente em quase todas as sub-regiões dentadas, e progressivamente transformam-se em células microgliais inativadas (DALMAU et al., 1998).

Basicamente, ao nascimento as micróglias são confinadas na região hilar do hipocampo. A maioria das micróglias apresentam perfil ameboide, embora seja possível reconhecer as ramificadas próximas a camada de células granulares

(Dalmau et al., 1998; Matcovitch-Natan et al., 2016). Em P3, a parte externa molecular do estrato hipocampal é invadido por processos celulares da micróglia distribuída no estrato lacunoso da área CA1 (DALMAU et al., 1998). Em P6, os corpos celulares de micróglias ramificadas primitivas foram encontradas pela primeira vez na camada molecular dentada. Finalmente, através de análise celular transcriptômica, autores concluíram que a micróglia passa por três estágios de desenvolvimento: micróglia 'precoce' (E10-14), 'pré-micróglia' [E14 – P9] e micróglia 'adulta' (P28 em diante) (MATCOVITCH-NATAN et al., 2016). Entretanto, durante o desenvolvimento pós-natal uma séria de fatores intrínsecos e extrínsecos podem afetar a densidade e o fenótipo de células microgliais. Logo, essas células desempenham papéis importantes no estabelecimento da rede de conectividade do cérebro durante o desenvolvimento do cérebro pós-natal (MALLARD; TREMBLAY; VEXLER, 2019).

Como as micróglias são consideradas as células residentes do sistema imune no SNC, estas células são importantes no contexto da lesão cerebral em período perinatal. A lesão da substância branca causada por inflamação, hipóxia e ou isquemia-hipóxia, é uma característica comum das lesões cerebrais no início da vida (MCNAMARA; MIRON, 2020). Em recém-nascidos pré-termo, foi relatado em estudo *post-mortem* maior densidade de micróglias, com fenótipo ativado em região periventricular do cérebro (VERNEY et al., 2012). Além disso, em caso de lesão cerebral perinatal, as micróglias mostram expressão aumentada de marcadores pró-inflamatórios IL-1β e fator de necrose tumoral alfa (TNF-α) (DAMMANN; O'SHEA, 2008)(DAMMANN; O'SHEA, 2008). Evidências mostram que micróglia tem papel na lesão da substância branca e defeitos de mielinização associadas em neonatos (ZAGHLOUL; PATEL; AHMED, 2017).

A ativação microglial, tem sido relatada como uma característica da lesão cerebral perinatal (MALLARD; TREMBLAY; VEXLER, 2019). Em modelos de inflamação neonatal, houve um aumento transitório no número absoluto e densidade celular da micróglia Iba1-positiva, bem como uma alteração persistente no estado inflamatório hipocampal na micróglia (SMITH et al., 2014). No cérebro neonatal, as células microgliais sofrem transformação morfológica após modelos de isquemia-hipóxia e acidente vascular arterial focal no cérebro (MALLARD; TREMBLAY; VEXLER, 2019). Acredita-se que micróglia / macrófagos ativados contribuem para o quadro pós-lesão em modelo de isquemia-hipóxia (IVACKO et al., 1997; IVACKO; SUN; SILVERSTEIN, 1996) e lesões excito-tóxicas (DOMMERGUES et al., 2003a).

Em momentos de lesão, a micróglia residente pode promover a capacidade regenerativa endógena e têm a capacidade de promover o reparo na substância branca, mas a microgliose persistente pode causar uma extensão dessa lesão por ativação inadequada ou patológica de vias citotóxicas. Apesar do papel crítico da ativação microglial em muitas outras doenças do SNC, os mecanismos moleculares de ativação microglial no hipocampo, ainda permanecem mal definidos. Além disso, pouco se sabe como os níveis de proliferação da micróglia pode afetar o desenvolvimento e estabelecimento de disfunções no tecido cerebral após desordens do desenvolvimento como a PC.

2.3 REPERCUSSÕES DOS MODELOS ANIMAIS DE PARALISIA CEREBRAL SOBRE OS SISTEMAS NERVOSO E MÚSCULO ESQUELÉTICO

Modelos animais têm sido desenvolvidos nas últimas décadas com o objetivo de contribuir para o conhecimento dos distúrbios neurológicos. Os modelos que envolvem danos cerebrais durante o período perinatal (pré, peri e / ou pós-natal) são essencialmente relevantes para a compreensão dos distúrbios do neurodesenvolvimento. Vários modelos de distúrbios cerebrais perinatais mostraram comprometimento do desenvolvimento cerebral e da função locomotora (DA CONCEIÇÃO PEREIRA et al., 2021; VISCO et al., 2021a). Esses modelos são baseados em dano cerebral perinatal, expondo os animais a diversas condições, aplicadas isoladamente ou em combinação, como inflamação pré-natal, privação de oxigênio no útero, asfixia perinatal, isquemia neonatal, hipóxia, hemorragia intraventricular e restrição sensório-motora dos membros posteriores em animais (COQ et al., 2016; DELCOUR et al., 2018; LACERDA et al., 2017a; P. et al., 2018; PEREIRA et al., 2021; SILVA et al., 2016).

A PC é frequentemente classificada de acordo ao comprometimento topográfico do corpo. Entre eles, a PC diplégica é um dos tipos mais incidentes nas clínicas (STAVSKY et al., 2017b). Esta condição é caracterizada pela fraqueza e rigidez das pernas com comprometimento significativo da marcha funcional (ROSENBAUM et al., 2007). Nesse sentido, para reproduzir esse fenótipo, o dano cerebral perinatal também é associado aos procedimentos de restrição sensório motora dos membros posteriores durante a infância em roedores (LACERDA et al., 2017b, 2019; PEREIRA et al., 2021; SILVA et al., 2016). O estabelecimento do fenótipo da PC em diversas espécies e linhagens animais contribui para o

entendimento das janelas críticas de maturação do sistema nervoso e favorece a tradução dos achados, tornando-os relevantes para a clínica (CLANCY; DARLINGTON; FINLAY, 2001). Nesse sentido, o conceito de impor o SNC animal a um ambiente perinatal adverso, como por episódios de privação cerebral de suprimento de oxigênio e redução da experiência sensório-motora no início da vida pós-natal representa as características observadas em indivíduos com PC (CLOWRY; BASUODAN; CHAN, 2014a).

A asfixia perinatal é uma condição caracterizada por fluxo sanguíneo reduzido ou troca gasosa de ou para o feto no período imediatamente antes, durante ou após o parto (BOOG, 2011). Quando a troca gasosa placentária (pré-natal) ou pulmonar (pós-natal imediata) está comprometida ou cessa completamente, há uma falta parcial (hipóxia) ou completa (anóxia) de oxigênio para os órgãos vitais (POPESCU et al., 2020). Asfixia perinatal e a resultante encefalopatia hipóxico-isquêmica são uma das causas identificáveis mais comuns de PC em recém-nascidos a termo (ZHANG et al., 2020).

A asfixia perinatal pode resultar em efeitos sistêmicos, incluindo lesão neurológica, dificuldade respiratória e hipertensão pulmonar e disfunção hepática, miocárdica e renal (POPESCU et al., 2020). Dependendo da gravidade e do momento da anóxia, um recém-nascido com encefalopatia hipóxico-isquêmica devido à asfixia perinatal pode apresentar uma variedade de achados neurológicos. A lesão cerebral hipóxica neonatal vem sendo implementada em animais para avaliar os mecanismos celulares e moleculares, o potencial para novas estratégias terapêuticas e para caracterizar os correlatos funcionais e comportamentais associados (HAMDY et al., 2020).

Estudos mostram que ratos submetidos a restrição sensório-motora, associada ou não a anóxia perinatal, tinham representação reduzida dos membros posteriores no córtex motor primário (STRATA et al., 2004). A restrição sensório=-motora em ratos jovens causa desorganização topográfica da representação do membro posterior cortical somatossensorial (COQ et al., 2008). Entretanto essa desorganização cortical se agrava quando o desuso imposto pela restrição sensório-motora estava associada a anóxia perinatal (COQ, et al., 2008). Na mesma linha, a restrição sensório-motora pós-natal altera as áreas do mapa somatossensorial e motor dedicadas à representação dos membros posteriores (DELCOUR ET AL., 2018). Além disso, foi sugerido que, mesmo sem dano cerebral, a restrição sensório-

motora pós-natal leva à degradação secundária dos mapas dos membros posteriores no córtex somatossensorial e córtex motor primário, com propriedades de resposta cortical alteradas e hiperexcitabilidade cortical (COQ et al., 2020). A asfixia perinatal e restrição sensório-motora causam um aumento no número de células gliais no córtex somatossensorial, enquanto a restrição sozinha reduz o número de células neuronais (MARCUZZO et al., 2010).

Recentemente, vários estudos têm mostrado os efeitos periféricos dos modelos animais de PC. Estudos mostram que músculo esquelético é um dos principais tecidos afetados pela PC. Em estudos anteriores, foi constatado que em músculos de ratos submetidos à PC, ocorre alterações nos níveis de expressão de vários genes relacionados ao desenvolvimento muscular (PEREIRA et al., 2021). Outro estudo mostrou que a asfixia perinatal associada à restrição sensório motora pós-natal reduz a massa muscular do sóleo, afetando o índice de massa muscular / peso corporal, além de modificar proteínas da via de degradação musculares como MuRF-1 no músculo sóleo (SILVA et al., 2016).

No estudo de Stigger et al. (2011), foi observado redução da área de secção transversa das fibras musculares do músculo tibial anterior e do sóleo de ratos submetidos ao modelo de PC. Além disso, uma transição do tipo de fibra lenta para rápida foi observada no músculo tibial anterior e no músculo sóleo em ratos com PC (STIGGER et al., 2011). Quando a restrição sensório-motora foi aplicada em ratos, uma redução significativa da densidade do sarcômero foi observada no músculo sóleo de ratos jovens (STIGGER et al., 2011). Este procedimento também causou um aumento significativo no comprimento do sarcômero no músculo sóleo de ratos submetidos a restrição sensório-motora (STIGGER et al., 2011).

O estudo de Marcuzzo et al. (2008) foi constatado que os animais submetidos a restrição sensório-motora, com ou sem anóxia perinatal, apresentaram atrofia das fibras do sóleo devido à redução da área de seção transversal e densidade das fibras. Foi observado em um estudo anterior que não apenas os músculos locomotores foram afetados pela asfixia perinatal associada com restrição sensório-motora, mas também um comprometimento global do fenótipo do músculo esquelético. Foi observada redução da massa muscular do músculo masseter e digástrico em ratos submetidos ao modelo PC (LACERDA et al., 2017). Além disso, foi observado um impacto significativo na proporção das fibras musculares (LACERDA et al., 2017). No músculo digástrico foi relatada uma redução na fibra muscular tipo I, associada a um aumento

nas fibras tipo IIA, enquanto no grupo masseter foi observada uma redução significativa nas fibras musculares tipo IIB (LACERDA et al., 2019; 2017).

A PC está associada a uma diversidade de alterações comportamentais e cognitivas que afetam principalmente os níveis de funcionalidade dos indivíduos acometidos (STAVSKY et al., 2017b)s. As deficiências do comportamento motor em roedores, geralmente são avaliadas na literatura científica através de instrumentos com que envolvem diferentes testes motores (PEREIRA et al., 2021). Em estudo prévio foi mostrado que em relação aos modelos mais eficazes para causar danos na locomoção, a combinação de restrição sensório-motora associada a lesão cerebral perinatal, potencializa o dano na locomoção e coordenação motora a curto e longo prazo (PEREIRA et al., 2021). Além disso, a restrição sensório-motora associada ao modelo de asfixia perinatal replica os problemas de mastigação observados pela redução da quantidade de movimentos mastigatórios necessários da função oral (LACERDA et al., 2017). Além disso, modelos experimentais de PC induzidos por inflamação materna ou hipóxia-isquemia, levam a outros déficits orofaciais comportamentais, incluindo incoordenação de sucção e deglutição; redução dos movimentos da cabeça durante a alimentação; danos a aquisição de alimentos; e resposta aversiva olfativa prejudicada (LACERDA et al., 2017).

Em relação às deficiências comportamentais sensoriais, a restrição sensório-motora combinada com o modelo de asfixia perinatal diminuiu a sensibilidade intraoral em ratos (LACERDA et al., 2017). Os animais submetidos a esse modelo de PC foram menos responsivos ao recebimento do estímulo hedônico oferecido pela administração de uma solução contendo sacarose (LACERDA et al., 2017). No entanto, diferentes avaliações sensoriais comportamentais precisam ser investigadas em estudos futuros, para se obter mais informações sobre a eficácia dos modelos de PC em mimetizar o dano às habilidades sensoriais.

Cerca de 40% das crianças prematuras nascidas com PC desenvolvem deficiências cognitivas moderadas a graves, que limitam a interação social e levam à dependência funcional (COQ et al., 2020). Modelos experimentais de PC podem induzir os distúrbios cognitivos comumente encontrados em crianças com PC (COQ et al., 2018). Entre os modelos de PC, a hipóxia isquemia uterina realizada por estenose das artérias uterinas no dia embrionário 17, induziu déficits na codificação de informação e déficits, em curto e longo prazo, em tarefas de memória (COQ et al., 2020). Os déficits em tarefas de memória foram verificados pela dificuldade dos

animais com PC em codificar configurações espaciais e características do objeto (COQ et al., 2020). Curiosamente, quanto maior o grau de retardo de crescimento em animais com PC, maior foi déficit cognitivo avaliado pela tarefa de memória (COQ et al., 2020). No entanto, faltam informações na literatura sobre a eficácia de outros modelos experimentais de PC em replicar déficits cognitivos. Além disso, é pertinente explorar outras habilidades cognitivas diferentes, incluindo aprendizagem espacial e memória de trabalho. Em resumo, esses déficits comportamentais e cognitivos induzidos por modelos de PC parecem recapitular alguns sintomas comumente encontrados em crianças com PC.

Mesmo havendo progresso nas últimas décadas em relação ao entendimento da fisiopatologia das desordens cerebrais perinatais, não existem estudos que avaliaram o desenvolvimento neuro-musculo-esquelético em modelos animais de PC. Nesse sentido, se faz necessário investigar os efeitos da anóxia perinatal e restrição sensório-motora sobre parâmetros de desenvolvimento e maturação do sistema nervoso, sobre o desenvolvimento e histologia do hipocampo, bem como quais as repercussões desse modelo em parâmetros da marcha e seu impacto no fenótipo corporal e muscular.

2.4 OS POLIFENÓIS COMO AGENTES TERAPÊUTICOS EM DESORDENS NEUROLÓGICAS

Os polifenóis são um grupo de compostos bioativos essenciais para uma variedade de funções nas plantas. Esses compostos naturais são comumente encontrados na dieta humana, principalmente em frutas, vegetais, cereais, café e bebidas (SILVA; POGAČNIK, 2020). Nas últimas décadas, eles têm sido estudados devido às suas propriedades antioxidantes e anti-inflamatórias (SILVA; POGAČNIK, 2020). Atualmente, há uma crescente atenção ao potencial dos compostos polifenólicos sob várias condições de saúde. Em um estudo anterior, destacamos e discutimos os benefícios metabólicos e neurológicos dos polifenóis em modelos de doenças crônicas não transmissíveis como derrame cerebral, doenças neurodegenerativas e desordens neurocognitivas (LACERDA et al., 2021). Outros estudos também mostraram a ação neuroprotetora dos polifenóis em distúrbios neurológicos (BELLONE et al., 2019; BEN YOUSSEF et al., 2021; CHEN et al., 2020; MACHADO et al., 2019; VARGA et al., 2020). Este compostos também vêm apresentando apresentando benefícios na cognição e no comportamento (ROSLI et

al., 2021; YANG et al., 2021). Na literatura científica, entretanto, existem poucos estudos explorando o potencial terapêutico dos compostos polifenólicos após lesão cerebral durante um período crítico de desenvolvimento (VISCO et al., 2021b).

Evidências recentes destacam as propriedades neuroprotetoras dos compostos polifenólicos e seus benefícios cognitivos e comportamentais. Como os polifenóis apresentam inúmeras atividades biológicas, devido às suas propriedades antioxidantes e anti-inflamatórias, estes compostos apresentam-se como um potencial agente terapêutico no tratamento de doenças neurológicas (LACERDA et al., 2021).

Acredita-se que os polifenóis causam suas ações neuroprotetoras por meio de sua capacidade de proteger os neurônios contra danos induzidos por estresse oxidativo e neuroinflamação. Esses processos atuam para manter a função cerebral e desempenham papéis importantes na plasticidade neuronal (VAUZOUR, 2012). Assim, se faz necessária investigação dos efeitos terapêuticos dos polifenóis na neuroinflamação, uma característica dos distúrbios cerebrais perinatais, após lesão cerebral precoce. A literatura é escassa, mas algumas evidências experimentais indicam que, quando usados como agente profilático, os polifenóis podem prevenir danos morfológicos, estresse oxidativo e neuroinflamação em modelos de encefalopatia neonatal (ARTEAGA et al., 2015; GAO et al., 2018; ISAC et al., 2017; WEST; ATZEVA; HOLTZMAN, 2007a).

Recentemente, estudos em modelos de hipóxia-isquemia neonatal mostraram efeitos neuroprotetores dos polifenóis na redução da inflamação cortical e apoptose celular, diminuição do volume do infarto cerebral (WU; LIU; GUO, 2019), redução do edema cerebral, e da ruptura da barreira hematoencefálica e consequente redução de morte celular neuronal (MIN et al., 2015). Além disso, há relatos de aumento da proliferação de células progenitoras de oligodendrócitos com melhorias na mielinização (QU et al., 2014). Esses efeitos foram associados a melhora no desempenho cognitivo e desfechos neurocomportamentais (MIN et al., 2015; QU et al., 2014).

Dentre os compostos polifenóicos que tem se destacado na literatura, os flavonoides vêm chamando atenção devido aos seus efeitos benéficos para a saúde. Os flavonoides representam um grupo diverso de compostos naturais que são biossintetizados a partir da fenilalanina e são abundantes em pigmentos verdes no reino vegetal (HAVSTEEN, 2002). Os flavonoides são metabólitos vegetais secundários comumente encontrados em frutas e vegetais. mais de 7.000 flavonoides

foram relatados de fontes naturais, incluindo plantas medicinais, vegetais, frutas e vinhos (ISHIGE; SCHUBERT; SAGARA, 2001).

Os flavonoides apresentam uma estrutura comum que consiste em dois anéis aromáticos (A e B), que são unidos por três átomos de carbono, formando um heterociclo oxigenado (anel C) (DWIVEDI; MALIK; CHHOKAR, 2017). Com base nas variações na saturação do sistema de anel de flavan básico, sua alquilação e / ou glicosilação e o padrão de hidroxilação das moléculas, os flavonoides podem ser divididos em sete subclasses: flavonóis, flavonas, flavanonas, antocianidinas, isoflavonoides e flavanois (WANG et al., 2017a). As principais diferenças entre os grupos individuais residem no padrão de hidroxilação da estrutura do anel, o grau de saturação do anel C e a substituição na posição 3 (WANG et al., 2017a). Por exemplo, as flavonas como apigenina, luteolina (encontrados na salsa, cebolinha, alcachofra e aipo), quando sofrem a hidroxilação na posição 3 da estrutura da flavona, dá origem às 3-hidroxiflavonas, sendo conhecidas como flavonóis (kaempferol, quercetin), que são encontrados em cebolas, alho-poró e brócolis (MANACH et al., 2004).

Estes compostos têm a capacidade de se ligar a várias proteínas do corpo e modificar os transportadores, enzimas, hormônios, DNA, quelação de metais pesados e eliminar os radicais livres; portanto, possuem fortes propriedades antioxidantes (MASTROIACOVO et al., 2015). Os efeitos benéficos de alimentos ricos em flavonoides como cacau, chá verde e baga azul podem ser atribuídos às interações dos flavonoides e seus metabólitos com vários alvos celulares e moleculares (MASTROIACOVO et al., 2015).

Os flavonoides sofrem extensa biotransformação e conjugação que ocorre durante sua absorção no trato gastrointestinal, no fígado e finalmente nas células (SPENCER et al., 2001). No intestino delgado e no fígado, os flavonoides (e outros polifenóis) são metabolizados em glucuronídeos, sulfatos e O-metilados derivados (SPENCER et al., 1999). Também, foi relatado que o metabolismo ocorre no cólon, onde as enzimas da microflora intestinal induzem a quebra dos flavonoides em ácidos fenólicos simples que podem então sofrer absorção e ser metabolizados no fígado (XIAO; HOGGER, 2013). Os mecanismos metabólicos intracelulares estão relacionados a 3 vias: 1- metabolismo oxidativo; 2- metabolismo relacionado a P450; 3- Conjugação com tióis, particularmente a glutationa (SPENCER et al., 2003).

Para que os flavonoides acessem o cérebro, eles devem primeiro cruzar a barreira hematoencefálica, que controla a entrada de xenobióticos no cérebro

(YOUDIM et al., 2003). Seu grau de penetração de BBB é dependente da lipofilicidade do composto, o que significa que metabólitos O-metilados menos polares podem ser capazes de maior captação pelo cérebro do que os flavonoides glicuronídeos mais polares (YOUDIM et al., 2003). Após exposição a alimento ricos em flavonoides, estudos relataram acumulo desses compostos em regiões do cérebro como hipocampo e córtex (ABD EL MOHSEN et al., 2002; KALT et al., 2008; PASSAMONTI et al., 2005).

No sistema nervoso, estes compostos apresentam importantes propriedades anti-inflamatórias, antioxidativas e neuroprotetoras (VAUZOUR et al., 2008). Sua ação antioxidante é devido as propriedades estruturais desses compostos (DWIVEDI; MALIK; CHHOKAR, 2017). Os grupos hidroxila são essenciais pois eliminam os radicais livres e espécies reativas de oxigênio, pela doação de um próton (JUSTINO et al., 2009). Ao promover ação antioxidantes, consequentemente ocorrem vários benefícios relacionados neuroproteção celular (VAUZOUR et al., 2008). A forma mais eficaz de neuroproteção por flavonoides é prevenir a formação de radicais livres pela modulação das vias de sinalização celular (SPENCER, 2007). Por fim, três mecanismos distintos de proteção pelos flavonoides podem ser identificados na literatura: a alteração do metabolismo da Glutationa (GSH), extinção de ROS e a inibição do influxo de Ca^{2+} que sinaliza a última etapa da cascata de morte celular induzida por glutamato (ISHIGE; SCHUBERT; SAGARA, 2001). Além disso, os flavonoides protegem as células neuronais da lesão oxidativa causada por ácido homocisteico (HCA), privação de cistina, butionina sulfoximina (BSO), hipoglicemia e peróxido de hidrogênio (H_2O_2) (ISHIGE; SCHUBERT; SAGARA, 2001).

Na mesma linha, foi demonstrado que os flavonoides naturais exercem propriedades neuroprotetoras ao exercer um efeito anti-inflamatório por meio da interferência no desenvolvimento de mediadores inflamatórios, como IL-6, TNF-α e IL-1β em várias linhagens celulares. Nesse sentido, uma série de evidências tem mostrado que esses compostos naturais exercem inibição sobre micróglias (JANG; KELLEY; JOHNSON, 2008). Fatores de transcrição como NF-kβ, AP-1, junto com ERK1 / 2, p38 e c-Jun-N-terminal quinase (JNK) regulam a expressão do gene inflamatório durante a resposta inflamatória (JANG; KELLEY; JOHNSON, 2008). Os flavonoides retardam significativamente a ativação de NF- kβ e AP-1, bem como vias MAPK na micróglia ativada, inibindo a produção de moléculas inflamatórias (CHEN et al., 2005; JANG; KELLEY; JOHNSON, 2008). Assim, ao modular o transcriptoma

microglial e inibir a produção de IL-1β, TNFα, oxido nítrico e prostaglandina E2, efeitos antioxidantes e neuroprotetores são obtidos através dos flavonoides (DIRSCHERL et al., 2010). Estudos mostraram que os flavonoides reduziram a quantidade de inflamação e estresse oxidativo no ambiente celular por vários meios (ALTHALI; HASSAN; ABDEL-WAHHAB, 2019; LIN et al., 2006; WEST; ATZEVA; HOLTZMAN, 2007b). Portanto, compostos flavonoides eliminam diretamente os radicais livres e espécies reativas de oxigênio, aumentam a concentração intracelular de GSH e moléculas antioxidantes, modulam as vias de sinalização do NF-κβ reduzindo a apoptose celular (GINWALA et al., 2019).

O interessante é que a exposição a esses compostos pode também prevenir a ocorrência de diversas doenças crônicas como AVC, Alzheimer e Parkinson (SPAGNUOLO; MOCCIA; RUSSO, 2018). Adicionalmente, o consumo de comida ricas em flavonoides pode reduzir o risco de mortalidade (BONDONNO et al., 2019). A partir dessas propriedades, diversos estudos investigaram o potencial benefícios desses compostos em modelos de doenças neurológias (GROSSO et al., 2013). Entretanto, poucas são as evidências que abordaram os benefícios dos flavonoides em casos de insulto cerebral perinatal.

Apesar de não haver estudos em doenças perinatais e de desordens de desenvolvimento como a paralisia cerebral, diversos estudos pré-clínicos investigaram os efeitos desses compostos em casos de infecção e ou inflamação fetal, modelos de lesão cerebral traumática, modelos de hipóxia-isquemia e de doenças degenerativas logo importantes considerações podem ser feitas a partir desses outros modelos (J.-W. et al., 2017;WANG; LIU, 2018). Em um estudo em ratos, foi constatado que quercetina alivia lesões cerebrais fetais induzidas por exposição materna ao LPS em ratos, tornando-o um potencial terapêutico para suprimir danos cerebrais infantis como resultado de infecção materna (WANG; LIU, 2018). Neste estudo, a quercetina suprimiu a produção de TGF-1β, reduzi a expressão da Bax (pró-apoptotica)e aumento a expressão da Bcl-2 (anti-apoptótica)(WANG; LIU, 2018). Um estudo realizado em leitões recém-nascidos, mostrou que o uso de nanossomos intravenosos de quercetina melhoram a função cerebral e a instabilidade hemodinâmica após hipóxia grave (BLASINA et al., 2015).

O flavonóide c-glicosilado, vitexin (5, 7, 4-trihidroxiflavona-8-glucosídeo) é um composto natural encontrado em muitas plantas medicinais, que tem atividade inibidora do HIF-1α (CHOI et al., 2006). A administração intraperitoneal de vitexin

imediatamente (5 min) após o insulto HI em ratos perinatais atenuou o aumento de HIF-1α e fator de crescimento endotelial vascular (VEGF), redução do tamanho do infarto cerebral, melhora do edema cerebral, da ruptura da barreira hematoencefálica e da morte celular neuronal, e melhora os resultados neurocomportamentais (CHOI et al., 2006; LUO et al., 2018). O pré-tratamento com vitexin antes de HI mostrou os mesmos resultados, diminuindo a via de sinalização pró-apoptótica ao inibir a fosforilação da proteína quinase dependente de Ca2 + / Calmodulina II e aumentando a razão BCL-2 / proteína BAX 24 horas após a lesão (MIN et al., 2017).

O pré-tratamento com hesperidina reduziu significativamente a perda de tecido cerebral induzida por isquemia-hipóxica e melhorou os desfechos neurológicos e comportamentais (RONG et al., 2013). Os efeitos neuroprotetores da hesperidina são resultantes da prevenção de um aumento nas espécies reativas de oxigênio intracelular e nos níveis de peróxido lipídico (RONG et al., 2013). O tratamento com hesperidina também ativou uma quinase de sinalização de sobrevivência chave, Akt, e suprimiu o nível de P-FoxO3 (RONG et al., 2013). Logo, os autores concluíram que o pré-tratamento com hesperidina protegeu o tecido cerebral em ratos neonatais após encefalopatia hipóxico-isquêmica reduzindo os radicais livres e ativando a quinase sinalizadora Akt fosforilada (RONG et al., 2013). Em outro estudo de modelo neonatal de hipóxia-isquemia a baicalina, um flavonoide, pode proteger cérebros de ratos neonatais contra lesão hipóxico-isquêmica ao regular positivamente o transportador de glutamato 1 por meio da via de sinalização PI3K / Akt (ZHOU et al., 2017).

Dentre os flavonoides, o kaempferol tem demonstrado maior potencial em lesões cerebrais. O kaempferol é considerado um dos flavonoides mais potentes (PANCHE; DIWAN; CHANDRA, 2016). Por exemplo, em modelo de isquemia/reperfusão em ratos o tratamento com kaempferol protegeu contra o aumento de marcadores de morte celular, como atividade da caspase-9 e poli (ADP-ribose) polimerase e de marcadores de morte celular (TUNEL) (LÓPEZ-SÁNCHEZ et al., 2007). Além disso reduziu o dano no hipocampo em 40-50% (LÓPEZ-SÁNCHEZ et al., 2007). Em estudo recente, os autores mostraram que o kaempferol reduziu o efeitos neurocomportamentais através da redução do estresse oxidativo ao reduzir os níveis de oxido nítrico, e concentrações de malondialdeído (MDA) e aumentar a atividade de glutationa peroxidase (GPx), catalase e superóxido dismutase (SOD) que foram avaliadas no hipocampo (AKEFE; AYO; SINKALU, 2020).

Em um estudo anterior, foi demonstrado que as concentrações micromolares de kaempferol proporcionavam proteção contra a apoptose de neurônios granulares cerebelares de rato em cultura induzida por privação de K^+ no meio extracelular (SAMHAN-ARIAS; MARTÍN-ROMERO; GUTIÉRREZ-MERINO, 2004). Em modelo de traumatismo craniano em ratos jovens (P31) um aumento significativo na conectividade neural foi observado após o tratamento com kaempferol (PARENT et al., 2020). Através de ressonância magnética funcional e imagem por tensor de difusão foi relatado melhor integridade sináptica e axonal com maior mudanças nos córtices frontal e parietal e no hipocampo pós-traumatismo craniano em ratos jovens (PARENT et al., 2020). Além disso, o tratamento com kaempferol também melhorou parâmetros estruturais do corpo caloso, indicando melhora mensurável na conectividade inter-hemisfério (PARENT et al., 2020). Finalmente, descobertas sugerem que o tratamento pós-isquêmico com kaempferol previne lesão cerebral isquêmica e neuroinflamação pela inibição de STAT3 e ativação de NF-κB, tendo potencial terapêutico para doenças relacionadas à neuroinflamação, como acidente vascular cerebral isquêmico (YU et al., 2013).

Recentemente, vimos que a exposição crônica com o kaempferol reduz a densidade microglial, bem como favorece a redução do perfil de micróglia ativada no hipotálamo de camundongos obesos (ROMERO-JUÁREZ et al., 2021). Apesar de evidências mostrarem grande potencial do tratamento com flavonoides, em especial o kaempferol em desordens neurológicas, pouco se sabe do impacto da exposição em período precoce a esses compostos em desordens cerebrais perinatais como a paralisia cerebral. Nesse sentido se faz necessário o desenvolvimento de estudos que avaliem os efeitos benéficos do kaempferol em modelos de desordens cerebrais perinatais como a paralisia cerebral.

3 HIPÓTESE

O tratamento neonatal com kaempferol melhora o desenvolvimento neuro-músculo-esquelético de ratos submetidos ao modelo de paralisia cerebral experimental.

4 OBJETIVOS

4.1 OBJETIVO GERAL

Avaliar as repercussões do tratamento neonatal com Kaempferol sobre o desenvolvimento neuro-músculo-esquelético em ratos submetidos à paralisia cerebral experimental.

4.2 OBJETIVOS ESPECÍFICOS

Na prole, em ratos jovens submetidos à Paralisia cerebral experimental que receberam o tratamento neonatal com Kaempferol foi avaliado:

- Experimento 1:

a) as medidas corporais e características somáticas;

b) a maturação física

c) a ontogênese dos reflexos;

d) o desenvolvimento da atividade locomotora;

e) a coordenação motora;

f) a proliferação celular no hipocampo;

g) o perfil de micróglias no hipocampo.

h) a proliferação de micróglias no hipocampo

- Experimento 2:

a) o peso corporal e comprimento corporal durante a infância;

b) a força muscular;

c) as características estáticas e dinâmicas da marcha.

d) a tipagem de fibras musculares;

e) a expressão de proteínas miofibrilares.

5 MÉTODOS

5.1 CONSIDERAÇÕES ÉTICAS

Este projeto de pesquisa 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, e com as normas internacionais estabelecidas pelo *National Institute of Health Guide for Care and Use of Laboratory* Animals. Este estudo teve início após aprovação pela Comissão de Ética em Uso animal (CEUA) da Universidade Federal de Pernambuco (UFPE) (nº processo 0058/2018) (ANEXO A).

5.2 ANIMAIS

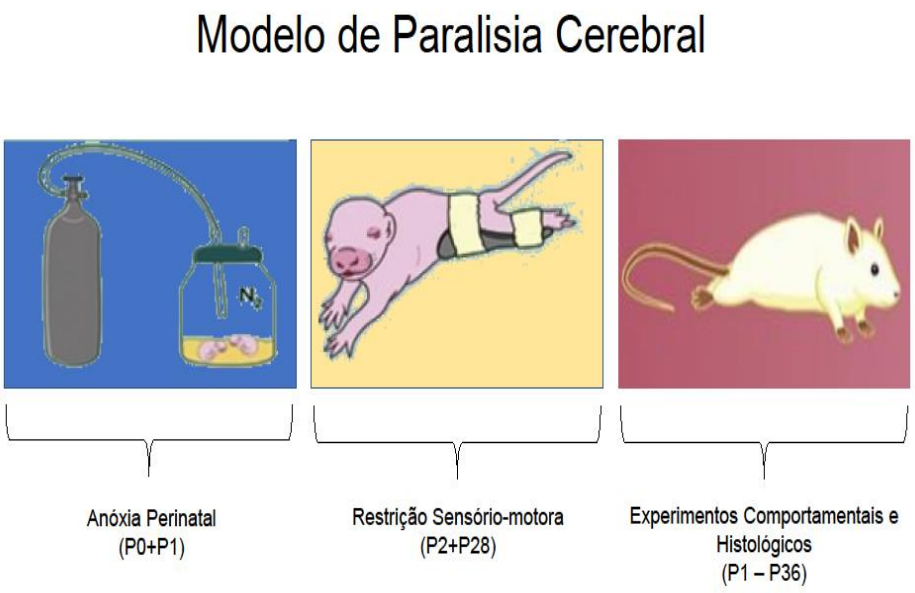
Foram utilizados ratos da linhagem *Wistar* provenientes da colônia do Departamento de Nutrição da UFPE. Todos os animais foram mantidos em biotério padrão com ciclo invertido de luz (20:00 às 8:00) e escuridão (08:00 às 20:00), temperatura 22 ± 2°C e com livre acesso à água e ração, alojados em gaiolas de polipropileno (46cmx34cmx20cm) coberta com maravalha estéril. Para obtenção das ninhadas, foram acasalados animais machos (n=18) e fêmeas (n=36) (proporção 1:2). Para acasalamento as ratas nulíparas tinham idade entre 90 - 120 dias e peso entre 220 - 250 gramas.

5.3 MODELO DE PARALISIA CEREBRAL

O modelo experimental de paralisia cerebral foi baseado nos experimentos de Coq et al. (2008), Strata et al. (2004) e em experimentos prévios (LACERDA et al., 2017c, 2019; PEREIRA et al., 2021; SILVA et al., 2016). Este modelo associa a anóxia perinatal a um modelo de restrição sensório-motora das patas posteriores, semelhante ao fenótipico clínico dediplégico observado em crianças com PC (COQ et al., 2008; STRATA et al., 2004). Após o nascimento, os filhotes machos foram submetidos a dois episódios de anóxia, o primeiro ocorreu no dia do nascimento, considerado o P0 e o segundo, no segundo dia pós-natal, considerado o P1. Para realização da Anóxia Perinatal (AP), os filhotes foram colocados dentro de uma câmara de acrílico parcialmente imersa em água a 37°C e expostos a nitrogênio (100%) a 9L/min por 12 minutos em cada episódio. Em seguida, os filhotes foram mantidos em temperatura ambiente até reestabelecimento da coloração e respiração e devolvidos as suas respectivas nutrizes após tal procedimento (COQ et al., 2008; STRATA et al., 2004). Do P2 ao P28 foi realizada a restrição sensório-motora durante 16 horas por dia (das

4 p.m. às 8 a.m do dia seguinte), nas 8 horas restantes foi permitida a livre movimentação do animal (COQ et al., 2008; STRATA et al., 2004). Para a restrição sensório-motora um molde de epóxi foi usado de forma que apenas movimentos limitados da articulação do quadril foram permitidos, deixando os membros posteriores estendidos com o auxílio de fita adesiva complementar ao molde, sem que a eliminação de urina e fezes, bem como os cuidados maternos fossem prejudicados (COQ et al., 2008; STRATA et al., 2004) (Figura 1).

Figura 1 – Modelo de Paralisia Cerebral em ratos.



Fonte: adaptado de Dos SANTOS et al. (2017). O modelo de paralisia cerebral que mimetiza o fenótipo da PC diplégica em ratos consiste em episódios de privação do oxigênio imediatamente após o nascimento seguido de restrição sensório-motora do membro posterior durante a infância em ratos.

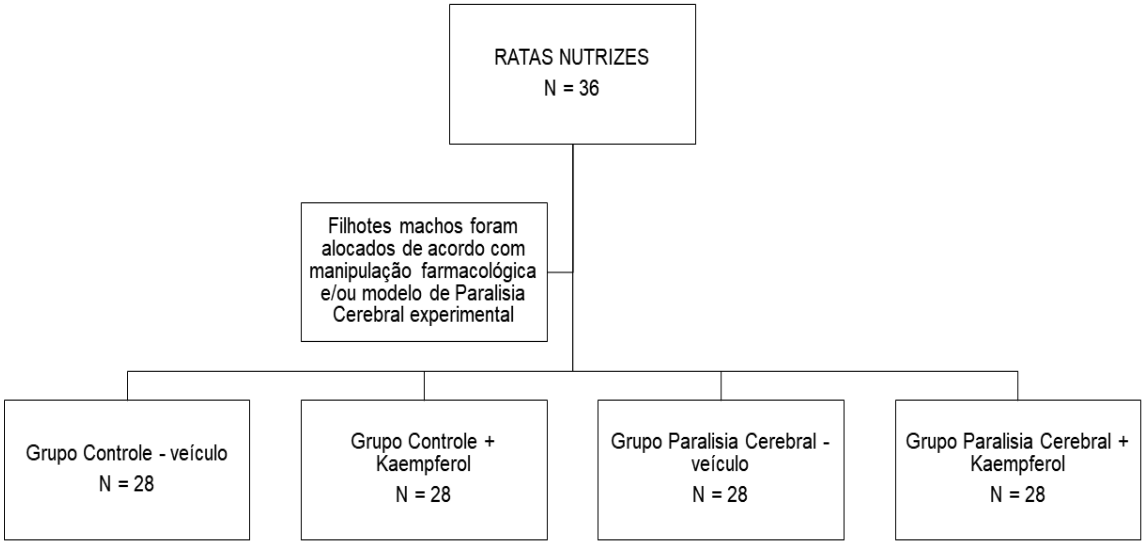
5.4 TRATAMENTO NEONATAL COM KAEMPFEROL

Do P2 ao P21, os filhotes machos receberam uma injeção intraperitoneal (i.p) diária de Kaempferol ou veículo. A solução estoque Kaempferol (Cayman Chemical, Ann Arbor, MI, EUA) foi preparada com solvente Dimetil Sulfóxido (DMSO) puro e armazenada a -80 °C. A injeção diária de kaempferol foi aplicada através da suspensão de uma solução fresca de solução veículo (0.1% v/v DMSO em solução salina normal) na dose de 1 mg / kg (PARENT et al., 2021, CHAVES et al., 2021). Os animais de controle receberam a solução de veículo sem composto de kaempferol. As aplicações diárias ocorreram no horário entre 9 – 10 a.m.

5.5 GRUPOS EXPERIMENTAIS

Após o nascimento dos filhotes, as ninhadas foram ajustadas para conter 8 filhotes por ninhada. As fêmeas foram utilizadas apenas para compor a ninhada. Para composição dos grupos experimentais os filhotes machos foram alocados de forma randomizada nos grupos de acordo com o modelo de paralisia cerebral ou controle e ao tratamento neonatal com kaemperol ou veículo. Dessa forma, os grupos experimentais foram divididos em: Controle - veículo (C), n = 28; Controle - Kaempferol (K), n = 28; Paralisia Cerebral - veículo (PC), n = 28; Paralisia Cerebral - Kaempferol (PCK), n = 28 (Figura 2).

Figura 2 – Grupos Experimentais.



Fonte: o autor (2022). Após o nascimento os animais foram alocados de forma randomizada para a composição dos grupos experimentais de acordo com intervenção com Kaempferol ou veículo e modelo de paralisia cerebral ou controle. C (Controle - veículo, n=28); K (Controle - Kaempferol, n=28); PC (Paralisia Cerebral - veículo, n=28); PC (Paralisia Cerebral - Kaempferol, n=28).

5.6 ANÁLISES EXPERIMENTAIS

5.6.1 Experimento 1

5.6.1.1 Análises das medidas corporais

O peso corporal dos filhotes foi avaliado diariamente de P1 a P21 e no P28, P33 e P36. O peso corporal foi avaliado com uma balança digital (Marte®, modelo S-1000, 0,1 g). Para a análise das medidas corporais um paquímetro digital (Jomarca®

0,01 mm) foi utilizado para medir as seguintes medidas corporais: a distância da ponta anterior do osso nasal à borda posterior do osso occipital (Eixo Craniano Anteroposterior - ECAP), e a distância da direita para a esquerda da linha temporal do osso parietal (Eixo Craniano Latero-Lateral -ECLL) e o comprimento do corpo (distância nasal a anal) foram medidos com um paquímetro nas idades alvos (GOUVEIA et al., 2019; SANTANA MUNIZ et al., 2014). As medidas foram realizadas entre 9-11 horas da manhã.

5.6.1.2 Avaliação da maturação física e ontogenia dos reflexos

Para a análise da maturação das características físicas, o dia em que cada característica física foi observada pela primeira vez, foi considerado como a idade de maturação (dia pós-natal). As características físicas avaliadas foram abertura dos olhos, exposição dos incisivos superiores e exposição dos incisivos inferiores (CADENA-BURBANO et al., 2017; GOUVEIA et al., 2020).

A ontogênese reflexa foi avaliada conforme descrito por Feather-Schussler (2016), Fox (1965), Dobbing and Smart (1974) e em estudo prévio (GOUVEIA et al., 2020). A resposta reflexa foi avaliada diariamente (entre 9-11h) durante o período neonatal, pelo mesmo avaliador cego, até o dia da maturação. O primeiro de uma série de três dias consecutivos em que a resposta esperada apareceu ou desapareceu completamente, foi considerado o dia de maturação do reflexo. Os reflexos testados foram preensão palmar (testado em P3-P9), endireitamento (testado em P2-P8), colocação de vibrissas (testado em P4-P11), aversão ao precipício (testado em P3-P8), endireitamento em queda livre (testado em P9-P17) e geotaxia negativa (ângulo 45º) (testado em P13-P21). As descrições das avaliações dos reflexos estão apresentadas na Tabela 1.

Tabela 1 – Descrição dos parâmetros para avaliação dos reflexos em ratos.

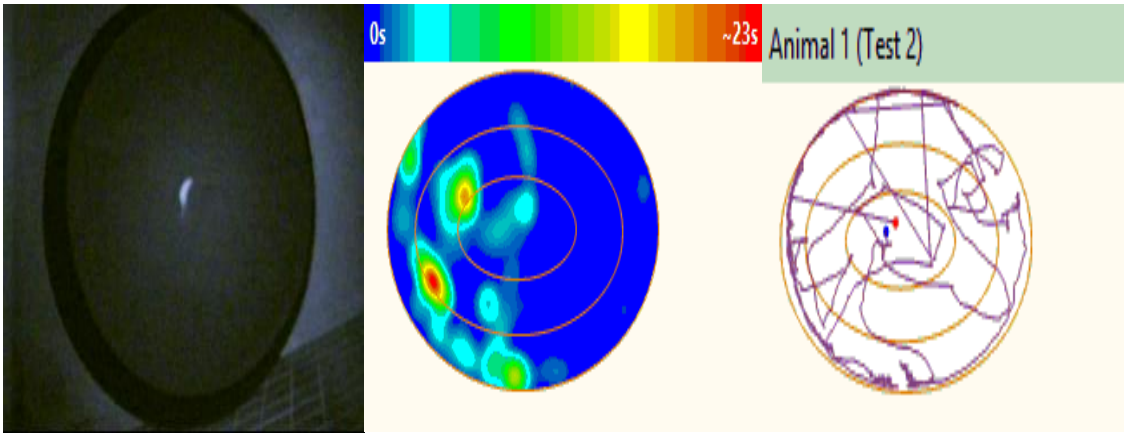
Reflexos	Período de avaliação	Resposta
Endireitamento	P2-P8	Rotação corporal em decúbito dorsal para decúbito ventral em 10 segundos.
Preensão palmar	P3-P9	Falha em realizar flexão rápida dos dedos após leves percussões na palma da pata dianteira com um clipe metálico.
Aversão ao precipício	P3-P8	Deslocamento angular de 45 ° do animal em até 10 segundos, quando colocado com as patas dianteiras na margem de uma superfície plana e alta.
Colocação pelas vibrissas	P4-11	Colocação das patas dianteiras na mesa, tentando caminhar quando suspenso pela cauda em até 10 segundos.
Resposta ao susto	P6-P18	A retração simultânea e rápida com imobilização involuntária após percussão em objeto metálico.
Endireitamento em queda livre	P9-P17	Quando segurado pelas quatro patas em decúbito dorsal e deixado cair de uma altura de 30 cm; na queda gira totalmente o corpo pousando sobre as quatro patas em uma superfície com algodão.
Geotaxia negativa	P13-P21	Rotaciona o corpo em 10 segundos e posiciona a cabeça para cima quando colocada no centro de uma rampa inclinada de 45º, posicionada com a cabeça para baixo.

Fonte: o autor (2022), adaptado de Gouveia et al. (2020).

5.6.1.3 Avaliação do Desenvolvimento da atividade locomotora

O desenvolvimento da atividade locomotora foi avaliado por meio de um campo aberto circular (1 metro de diâmetro) e uma câmera digital infravermelha (VTR® 6638 - Sistema CCTV) posicionada verticalmente do centro do campo aberto (2,4 m de distância do chão do campo). As gravações foram realizadas em uma sala sem iluminação anexa ao biotério, durante uma fase escura do ciclo circadiano (2:00 – 4:00 p.m.). Os animais foram filmados em uma sessão de 5 minutos de locomoção livre em P8, P14, P17, P21 e P28 conforme estudo prévio (SILVA et al., 2016). O software de rastreamento comportamental ANY-maze (Figure 3) foi usado para analisar automaticamente a locomoção durante as idades de interesse. As medidas avaliadas foram distância percorrida (m), velocidade média (m / s), velocidade máxima (m / s), tempo imóvel (s) e tempo despendido (s) nas zonas (central, intermediária e periférica) (Figure 3).

Figura 3 – Avaliação do desenvolvimento da atividade locomotora.



Fonte: o autor (2022). Avaliação do desenvolvimento da atividade locomotora em campo aberto. A imagem da esquerda representa uma amostra de imagem das gravações feita utilizando campo aberto circular. As análises foram feitas pelo Software Any-maze para mensuração dos parâmetros de distância percorrida (m), velocidade média (m / s), velocidade máxima (m / s), tempo imóvel (s) e tempo despendido (s) nas zonas (central, intermediária e periférica). A imagem central representa um *heat-map* do tempo despendido nas zonas do campo aberto. A imagem da direita representa um *track plot* de um rato no P14.

5.6.1.4 Avaliação da coordenação e equilíbrio

A coordenação motora e o equilíbrio foram testados usando o Teste do Rotarod em P33. Para a realização do teste utilizou-se o equipamento Rota Rod v.2 (Insight Ltda.) (Figura 4). O teste foi realizado colocando o rato em uma haste rotativa para medir o tempo que o animal era capaz de manter o equilíbrio na haste (BOHLEN et al., 2009). Um treinamento de pré-teste de habituação foi realizado em dois dias consecutivos antes do dia do teste usando 10 rpm por 2 minutos cada dia. Os testes foram realizados com uma velocidade de corrida em aceleração de até 37 rpm alcançada em 5 minutos. O tempo médio gasto na haste durante três ensaios consecutivos foi obtido para cada animal. O intervalo entre os ensaios para cada animal foi de 5 min.

Figura 4 – Equipamento Rota Rod para avaliação da coordenação e equilíbrio.

Fonte: insightlitda.com.br (2022). O Rota Rod é um equipamento que mede atividade motora demonstrando alterações de equilíbrio e coordenação em animais de laboratório. As dimensões do equipamento são (A x L x C) mm: 530 x 400 x 410. Peso do equipamento 15kg. Possui motor de 12v acoplado para rotação da haste. Equipamento confeccionado em alumínio com pintura eletrostática , motorizado, com configurações de rampa de aceleração.

5.6.1.5 Histologia do cérebro

No P36, os ratos foram anestesiados com Ketamina (100 mg / kg) e Xilazina (12 mg / kg) e perfundidos intracardialmente com 300 ml de solução salina (NaCl 0,9%) e 200ml de Paraformaldeído (PFA) 4% em tampão Fosfato 0,1 M (PB). Os cérebros perfundidos (n = 7 por grupo), foram dissecados, pesados (com uma balança digital (Marte®, precisão 0,001 mg), armazenados durante a noite com 4% de PFA, em uma solução de sacarose a 30% e, em seguida, congelados. Seções coronais de 30 µm (270 µm uma da outra) (Bregma -2.80 à -4.15 mm) foram cortadas em um criostato (-30 °C; Leica) e mantidos em solução anti-congelante até processamento por imuno-histoquímica.

5.6.1.6 Proliferação celular no hipocampo

No P5 e P6 os animais foram submetidos a injeção intraperitoneal de 40 mg/kg de Bromodeoxiuridina (5-bromo-2'-deoxiuridina – BrdU). A BrdU, é uma pirimidina análoga da timidina e é seletivamente incorporada ao DNA celular (durante a fase S do ciclo celular), sendo comumente utilizada como marcador de proliferação celular

(HARRIS; ZALUCKI; PIPER, 2018). Para avaliar a proliferação de células neurais precursoras através de imuno-histoquímica, seções encefálicas (30 µm) flutuantes foram enxaguadas em PB e incubadas em PBT (PB + Triton X-100 0,3%, St. Louis, MO, EUA) contendo 10% de peróxido de hidrogênio. Posteriormente, as seções foram incubadas em metanol absoluto, lavadas em PB e incubadas em formamida (50% SSC, Sigma-Aldrich) a 65 ° C. Após lavagem em solução SSC, a desnaturação do DNA foi realizada em HCl (1N) a 37 ° C seguida, realizou-se incubação em uma solução tampão de borato (pH 8,4) (DIAZ-CHÁVEZ et al., 2020; LAJUD et al., 2013). Após o pré-tratamento, as secções foram incubadas *overnight* (4 ° C) em solução com anticorpo primário anti-BrdU (anti-BrdU em camundongo, 1: 30.000 Roche Molecular). Em seguida, as seções foram incubadas em solução de anticorpo secundário biotinilado (anti-camundongo, 1: 750, Vector Laboratories) e revelada em complexo avidina-biotina (kit Elite ABC, Vector Laboratories) e Diaminobenzidina (kit de coloração DAB, Vector Laboratories) (CAJERO et al., 2012). Após secagem as secções foram montadas com Citosol em lâminas gelatinizadas. As imagens foram capturadas usando um microscópio óptico com a lente objetiva de 20x em 4-5 seções por animal. A contagem do número células $BrdU^+$ no hipocampo foi avaliado por um pesquisador cego. Os limites da zona granular e subgranular do Giro Denteado foram delineados digitalmente usando o software ImageJ. A proporção de células / área foi então calculada ($BrdU^+$ /mm^2).

5.6.1.7 Perfil de micróglia no hipocampo

Para a coloração da micróglia, as seções do cérebro foram incubadas em 10% de H_2O_2 em metanol e 10% em tampão de fosfato (0,1 M, pH 7,4), contendo 3% de PBT. Posteriormente, os cortes foram incubados a 4 ºC por 48h, em anti-corpo primário para molécula adaptadora de ligação de cálcio ionizada 1 (IBA1) (anti-Iba1 / IAF1 em coelho, 1: 3000, Wako), que foi diluído em 5% de soro de cavalo em PBT (DIAZ-CHÁVEZ et al., 2020).

As seções foram então incubada em anti-corpo secundário biotinilato secundário (IgG em coelho da Sigma-Aldrich), ambos os anticorpos foram diluídos a 1: 750 em PBT e as secções foram incubados por 2 h a 4 °C. Posteriormente, as seções foram incubadas com soluções do complexo avidina-biotina peroxidase (ABC Elite Kit; Vector Laboratories, Burlingame, CA, EUA) e uma solução de kit de coloração com diaminobenzidina (DAB Kit; Vector Laboratories) para marcação das micróglias

(DIAZ-CHÁVEZ et al., 2020). As seções de cada grupo foram executadas em paralelo para evitar efeitos de coloração inespecíficos. Os cortes foram então montados em lâminas de vidro revestidas com gelatina 1% e lamínulas com Cytoseal (Thermo Scientific, EUA).

Para avaliação de células Iba1^{+}, 3 campos por seção em um total de 4-6 seções por cérebro (n = 7 por grupo) no hipocampo dorsal (-2,80 a -4,15 mm de bregma) foram selecionados aleatoriamente para avaliar a contagem de células da micróglia (DIAZ-CHÁVEZ et al., 2020). Os campos selecionados foram analisados com um microscópico óptico em uma ampliação de 20x. Um pesquisador cego usando o software ImageJ realizou a análise do número de células / área e classificou o perfil da micróglia acordo com descrições anteriores (DIAZ-CHÁVEZ et al., 2020). As células microgliais com um pequeno soma e poucos a numerosos processos foram consideradas micróglia ramificada (tipos I-III), enquanto aquelas com um grande soma ou corpo amebóide e processos mais espessos e curtos foram consideradas micróglia ativada (tipos IV-V) (SAAVEDRA; NAVARRO; TORNER, 2018). A proporção de células Iba1^{+} ativadas foi estimada conforme descrito anteriormente (ROQUE; OCHOA-ZARZOSA; TORNER, 2016).

5.6.1.8 Colocalização de BrdU/Microglia

Para identificar micróglia proliferativa no hipocampo através da colocalização de células Brdu^{+} e Iba1^{+}, foi realizada imunohistoquímica fluorescente. Resumidamente, após o pré-tratamento, as secções foram incubadas durante 24h (4 ° C) na solução de anticorpo primário anti-BrdU (anti-BrdU em rato, 1: 400; Wako). Posteriormente, as secções foram incubadas em solução de anticorpo secundário biotinilado (anti-camundongo, 1: 250, Vector Laboratories) e visualizadas com o kit de substrato avidina-FITC fluorescente (Vector Laboratories). Após a coloração com BrdU, as secções foram incubadas no anticorpo primário IBA1 (1: 500) durante 48h a 4 °C. As seções foram então visualizadas com o anticorpo secundário em coelho Alexa Fluor 594 IgG (1: 500, Jackson Imuno Research), em seguida foram montados com Vectashield em lâminas gelatinizadas. A avaliação da colocalização de núcleos BrdU^{+} totais com Iba1+ foi feita com um microscópio confocal Olympus Fluoview FV1000. Uma série de microfotografias com 1 µm de distância do eixo z das secções foram obtidas para gerar uma vista ortogonal. As imagens foram processadas com o

software Fluoview FV10-ASW 2.0 e apenas núcleos $BrdU^+$ que apresentaram colocalização no eixo z foram considerados para quantificação.

5.6.2 Experimento 2

5.6.2.1 Análises das medidas corporais

O peso corporal dos filhotes foi avaliado diariamente de P1 a P21 e no P28, P33 e P36. O peso corporal foi avaliado com uma balança digital (Marte®, modelo S-1000, 0,1 g). Para a análise do comprimento corporal um paquímetro digital (Jomarca® 0,01 mm) foi utilizado para medir distância naso-anal no P7, P14, P21, P28 e P36 (GOUVEIA et al., 2019; SANTANA MUNIZ et al., 2014). As medidas foram realizadas entre 9-11 horas da manhã.

5.6.2.2 Teste de Força

O teste de força de preensão manual é comumente usado na área biomédica, como um indicador da força muscular geral (FRAGOPOULOU et al., 2019). O teste de força de preensão da pata é semelhante ao teste de preensão da mão para pessoas, pois avalia a capacidade de segurar um dispositivo com a pata, é não invasivo e fácil de executar e fornece informações reproduzíveis (MEYER et al., 1979). Os ratos em cada grupo de idade foram testados em P33 para determinar o pico de força de preensão da pata. Para medição, os ratos foram posicionados horizontalmente a partir de uma barra de apoio Grip Strength System (San Diego Instruments, San Diego, CA, EUA) e puxados para trás de forma lenta e continua até soltarem (Figura 5). Isso foi repetido três vezes, e o pico de força para as patas dianteiras foi medido. Os ratos foram pesados na data do teste de força de preensão para que a força de preensão fosse normalizada pelo peso corporal, de modo que o pico de força fosse expresso em relação ao peso corporal.

Figura 5 – Instrumento utilizado para avaliação da força de preensão manual.

Fonte: sandiegoinstruments.com (2022). Grip Strength System (San Diego Instruments, San Diego, CA, EUA). O aparelho foi projetado para mensurar a força de preensão das patas de ratos ou camundongos. Pode-se utilizar um ou dois medidores de força. O aparato consiste em uma plataforma em acrílico com suporte para os medidores de força que possuem alças com sensores digitais. O sistema ainda conta com um software que permite a análise acurada dos dados.

5.6.2.3 Análise dos parâmetros da Marcha

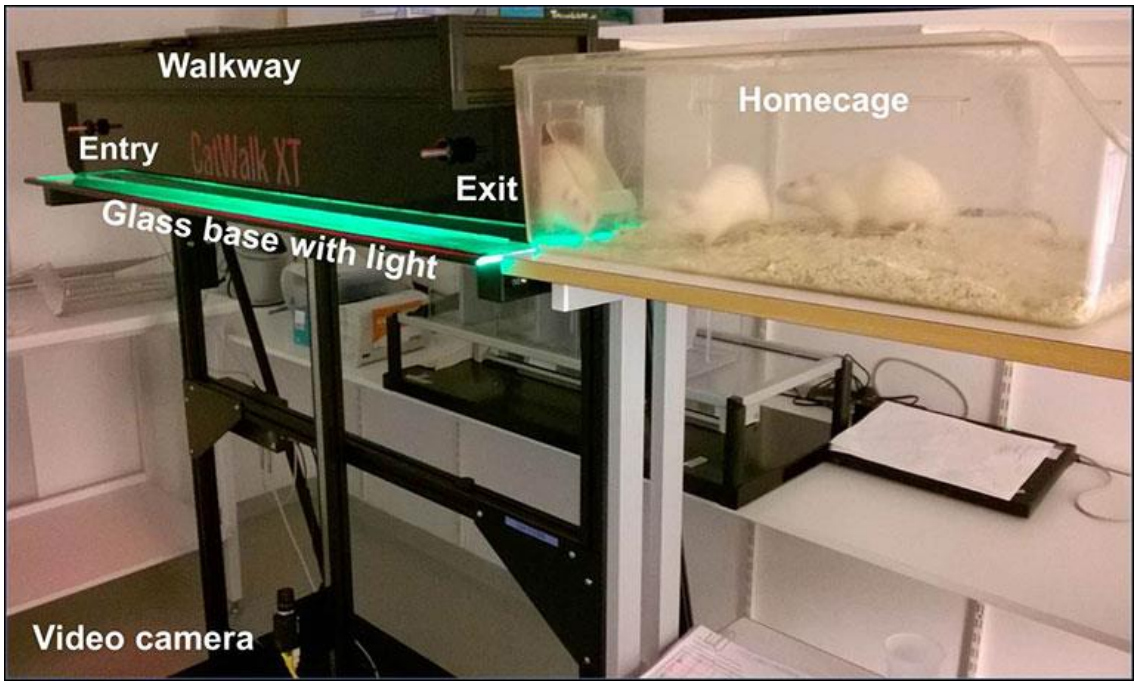
A análise da marcha dos ratos de cada grupo foi conduzida usando o sistema de análise quantitativa automatizado assistido por vídeo CatWalk XT (Noldus Information Technology, Holanda) no P33. Para a realização dos testes os animais foram habituados à sala de teste (sala escura e silenciosa). Também, antes da gravação, os animais puderam se habituar e cruzar a passarela livremente.

Em resumo, o CatWalk System consiste em uma passarela fechada (placa de vidro) que é iluminada por luz fluorescente (HEROLD et al., 2016). Além disso, o sistema é equipado com uma câmera colorida de alta velocidade conectada a um computador com o software de detecção adequado (CatwalkXT9.1) (Figura 6). O software é capaz de detectar vários parâmetros dinâmicos e estáticos durante o andar de um rato (HEROLD et al., 2016; KAPPOS et al., 2017). Para a detecção de todos

os parâmetros usados nos experimentos, o ganho da câmera foi definido em 20 e o limiar de detecção em 0,1 (HEROLD et al., 2016; KAPPOS et al., 2017). As corridas deveriam ter duração entre 0,50 e 5,00 s e a variação de velocidade máxima permitida de 60%, para que fossem consideradas corridas bem-sucedidas. Para cada animal, 3 execuções compatíveis foram adquiridas na avaliação. Após cada execução, o aparelho foi cuidadosamente limpo com papel toalha umedecido com solução de etanol a 75%. As corridas foram classificadas para todos os membros e analisadas estatisticamente (HEROLD et al., 2016; KAPPOS et al., 2017). O sistema CatWalk identificou automaticamente cada impressão da pata e gerou uma série de parâmetros de interesse, incluindo: cadência (passos / segundo), índice de regularidade da sequência de passos (%), velocidade média (cm/s), base de apoio (cm); e as estatísticas das patas traseiras, incluindo: tempo em fase de apoio (s), tempo em de balanço (s), velocidade de balanço (cm/s), área máxima de contato (cm^2), comprimento (cm) e largura (cm) de impressão da pata.

A cadência é definida pelo número de passos por segundo durante a locomoção na plataforma (HEROLD et al., 2016; KAPPOS et al., 2017).O índice de regularidade da sequência de passos, é definido como o uso exclusivo de padrões de sequência de passos normais durante a locomoção ininterrupta, mostrando o grau de coordenação entre os membros (KOOPMANS et al., 2005). O cruzamento da plataforma com um índice de 100%, é considerado uma corrida totalmente coordenada. A velocidade média mede a velocidade de cada corrida sendo representativa para status geral de locomoção (HEROLD et al., 2016; KAPPOS et al., 2017). A Base de suporte (BS) (cm) é calculada pela média da largura no eixo y entre as patas anteriores direitas / esquerda (PAD / PAE) ou posteriores direitas / esquerda (PPD / PPE) (patas anteriores BS = yPAD - yPAE; patas posteriores de BS = yPPD - yPPE) (ZHENG et al., 2021). A base de apoio reduzida representa uma maior estabilidade do tronco (ZHENG et al., 2021). As impressões das patas permitem mensurar os parâmetros estáticos envolvidos na locomoção como as medidas de área máxima de contato (cm^2), comprimento e largura da impressão da pata. Em geral, os aumentos dessas medidas representam uma melhor função motora fina e melhor estabilidade do tronco (PITZER; KURPIERS; ELTOKHI, 2021; ZHENG et al., 2021).

Figura 6 – Sistema de análise quantitativa da marcha Catwalk.



Fonte: noldus.com. Imagem com a representação dos componentes do sistema de análise da marcha quantitativa CatWalk XT. O sistema consiste em: um corredor que permite o movimento livre do animal em linha reta para facilitar o aprendizado e melhorar a reprodutibilidade; uma passarela com base de vidro que permite a tecnologia *"Illuminated Footprints"* do CatWalk XT; uma câmera colorida de alta velocidade para resolução espacial e temporal extremamente precisa; um software CatWalk XT para o registro e análise automatizada da capacidade locomotora de roedores.

5.6.2.4 Histologia do músculo esquelético

Para análise histológica do músculo esquelético, os animais foram sacrificados em condições de alimentação *ad libitum* por decapitação em P36 para coleta do músculo sóleo. Os músculos foram dissecados, pesados e imediatamente imerso em n-hexano resfriado. As amostras foram mantidas em -80 ◦C até o processamento. As seções transversais do músculo (10 µm) foram obtidas por um micrótomo criostato mantido a - 30 ◦C e corado através da técnica ATPase miofibrilar (mATPase) (BROOKE; KAISER, 1970). Resumidamente, as seções transversais foram pré-incubadas em temperatura ambiente por 20 min em uma solução contendo ácido acético 140 mM e acetato de sódio 60 mM (pH ajustado para 4,3 e 4,55) (BROOKE; KAISER, 1970). As lâminas foram então lavadas em água destilada e incubadas a 37 ◦C em uma solução contendo CaCl2 20 mM, 2,5 mM, Sal de ATP em glicina tamponada 40 mM (pH 9,4) (BROOKE; KAISER, 1970). Então as seções foram lavadas em água destilada, seguido de imersão em cloreto de cobalto a 2% por 3 min, então elas foram expostas a sulfeto de amônio 1,5% por 3 min, lavadas em água destilada e desidratadas em lotes de aumento teor de álcool (70–100%) (BROOKE;

KAISER, 1970). Finalmente, elas foram imersas duas vezes em tolueno e colocadas para secar em temperatura ambiente. Depois de secar as lâminas foram cobertas por lamínulas com resina Entellan (VISCO et al., 2020).

A proporção de cada tipo de fibra foi determinada em cada seção do músculo sóleo. As fibras musculares do músculo sóleo foram classificadas em tipos I e II com base na presença (tipo I) ou ausência (tipo II) de coloração para ATPase após pré-incubação ácida em pH 4.3 (BROOKE; KAISER, 1970). As imagens musculares foram obtidas com um microscópio óptico (Olympus Optical U-CMAD-2, Tóquio, Japão; lentes objetivas 40X). Todas as fibras dos músculos sóleo foram contadas usando Software Mensurim 6 (Jean-François Madre-Amiens). A área de seção transversal área e perímetro das fibras foram medidas a partir de 500 fibras por animal usando o software Image J (versão 1.51p) (VISCO et al., 2020).

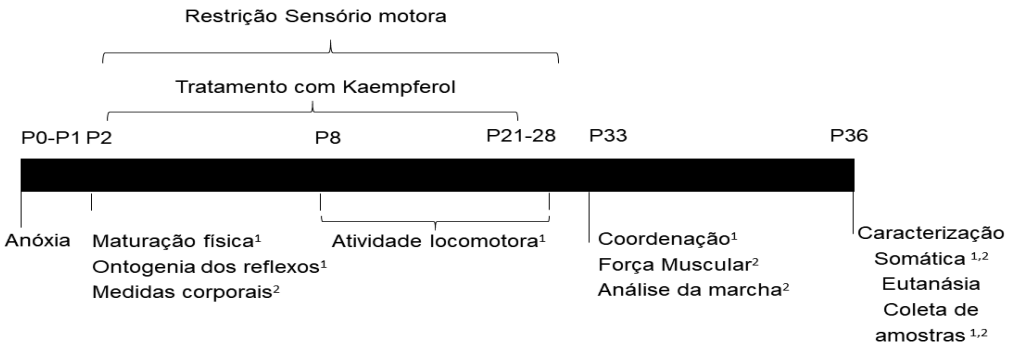
5.6.2.5 Análise da expressão de proteínas no músculo esquelético

Após eutanásia dos animais por decaptação, o músculo foi imediatamente dissecado, congelado e mantido em -80 ºC até a realização dos experimentos. O músculo sóleo da pata direita foi homogeneizado em tampão de extração (pH 7,5; 10mM de EDTA, Trisma base 100mM, Pirofosfato de Na 10mM, Fluoreto de Na 100mM, PMSF 2mM, Ortovanadato de Na 10 mM Aprotinina 0,01mg/ml – os dois últimos reagentes sempre foram adicionados no momento da homogeneização). Triton X-100 1% foi acrescentado ao homogenato e em seguida foi centrifugado a 15000RPM (Rotações Por Minuto) durante 20min em temperatura de 4°C (MARZUCA-NASSR et al., 2019). Após centrifugação, todo o sobrenadante foi separado e utilizamos apenas 2uL para a dosagem do conteúdo total de proteínas utilizando o método proposto por Bradford (1976). A solução de Bradford foi preparada usando Azul de Coomassie G250 (0,01%), etanol (4,75%) e ácido fosfórico (8,5%). O corante se liga à cadeia proteica, gerando um complexo de cor azul. A reação é colorimétrica e a absorbância foi determinada a 595 nm (MARZUCA-NASSR et al., 2019). Os resultados de absorbância foram utilizados no cálculo da concentração de proteínas baseado em uma equação de reta de uma curva padrão de albumina sérica bovina. Para elaboração da curva padrão de proteína utilizamos as seguintes concentrações de albumina: 1,0 mg/mL; 0,5 mg/mL; 0,25 mg/mL; 0,125 mg/mL; 0,0625 mg/mL (MARZUCA-NASSR et al., 2019)

A determinação da quantidade das proteínas relacionadas à via de síntese proteica miofibrilar conforme a técnica de *Western Blotting* foi feita com anticorpo para proteína total e fosforilada. Após avaliação do conteúdo de proteínas de cada amostra, 20µg de proteínas totais por *slot* do gel foram separadas por eletroforese de acordo com seu peso molecular, utilizando gel poliacrilamida 12% (SDS-PAGE). Feita essa separação, as proteínas do gel foram transferidas para uma membrana de nitrocelulose (BIORAD®) a 80 volts por 1 hora e 40 minutos. Ligações inespecíficas dos anticorpos foram inibidas incubando a membrana por 18 horas à temperatura ambiente em solução de bloqueio (pH 7,5, 10 mM Tris–HCl, 150 mM NaCl, 0,05% Tween-20; T–TBS) acrescida de 5% albumina bovina sérica (BSA), sob agitação constante. Posteriormente as membranas foram incubadas overnight (12 horas) a 4°C, sob agitação constante, e foram adicionados os anticorpos primários (Rabbit Cell Signaling: S6 Ribosomal Protein [5G10] #2217; Akt #9272; Phospho-S6 Ribosomal Protein [Ser240/244] #2215; Phospho-Akt [Ser473] #9271; IRS-1 [59G8] #2390) diluído na solução de bloqueio na proporção de 1:1000. Ao final desse período de incubação, as membranas foram lavadas em T–TBS 3 vezes, por 10 minutos cada, e incubadas com anticorpo secundário (Anti-rabbit IgG HPR-linked Antibody #7074, Cell Signaling) na proporção de 1:5000 durante 2 horas. Após o término do período de incubação, as membranas foram novamente lavadas em T–TBS 3 vezes, por 10 minutos cada, e então incubadas com a solução de revelação contendo peroxidase (luminol; SuperSignal West Pico Chemiluminescent Substrate System – Pierce Biotechnology) por 1 minuto e imediatamente seguido de leitura através do fotodocumentador (ChemiDoc XRS+). O filme foi revelado e as intensidades das bandas foram quantificadas com o auxílio do software ImageLab (5.2.1 BIO-RAD). As densidades das bandas foram normalizadas após a revelação e quantificação das bandas da membrana corada com Ponceau (MARZUCA-NASSR et al., 2019).

As análises e procedimentos experimentais estão resumidas em linha do tempo na figura 7.

Figura 7 – Linha do tempo das análises e procedimentos experimentais.



Fonte: o autor (2022). Descrição temporal dos procedimentos para exposição ao modelo de paralisia cerebral e do tratamento neonatal com kaempferol em ratos. As análises experimentais estão indicadas conforme período ou idade de realização. [1] = análises experimentais realizadas no experimento 1. [2] = análises experimentais realizadas no experimento 2.

5.7 ANÁLISE ESTATÍSTICA

As análises estatísticas foram realizadas com o GraphPad Prism 8.0 (software GraphPad, EUA). A distribuição da normalidade dos dados foi avaliada pelo teste de Kolmogorov-Smirnov. Os dados de maturação física e ontogenia reflexa são expressos como mediana e 25º e 75º percentis e analisados pelo teste post-hoc de Kruskal-Wallis e pelo teste *post-hoc* de Dunn. Para outras variáveis, a comparação múltipla entre os grupos foi realizada pela Análise de Variância (ANOVA) *two-way* e pelo teste *post-hoc* de Tukey ou Bonferroni. Os resultados são expressos como a média ± erro padrão da média (S.E.M.) ou pelo valor de F. Os resultados foram considerados significativos quando $p<0.05$.

6 RESULTADOS

6.1 RESULTADOS EXPERIMENTO 1

6.1.1 Maturação de características físicas, ontogenia dos reflexos e caracterização somática

Durante o período neonatal, o modelo de paralisia cerebral causou um impacto significativo na maturação das características físicas. Foi observado um atraso da erupção dos incisivos superiores (C vs. PC, p = 0,0002) e inferiores (C vs. PC, p = 0,0001) em animais com paralisia cerebral não tratados em comparação com o controle grupo (C vs. PC, p = 0,0001) (Tabela 2). Além disso, a abertura dos olhos nos animais com PC não tratados foi atrasada em comparação com o grupo controle (C vs. PC, p = 0,0003). Em animais com PC, o tratamento neonatal com Kaempferol atenua o impacto do modelo na maturação dessas características físicas sem diferença estatística em relação aos animais controle (C vs. PCK, p>0.05) (Tabela 2).

Foi observado atraso no desaparecimento do reflexo de preensão do membro anterior no grupo com PC não tratado em comparação com o grupo controle (C vs. PC, p = 0,0228) (Tabela 2). A aquisição do endireitamento nos animais com PC não tratados foi atrasada em comparação com o grupo de controle (C vs. PC, p = 0,001) (Tabela 2). A resposta de aversão ao precipício apresentou atraso nos animais com PC não tratados em relação ao grupo controle (C vs. PC, p = 0,0032) (Tabela 2). A aquisição da colocação pelas vibrissas também apresentou atraso nos animais com PC não tratado em relação ao grupo controle (C vs. PC, p = 0,0004) (Tabela 2). Em comparação com o grupo de controle, a aquisição da habilidade motora como, o endireitamento em queda livre (C vs. PC, p <0,0001) e a geotaxia negativa (C vs. PC, p <0,0001) foi retardado em animais com PC não tratados (Tabela 2). Não houve diferença na resposta ao susto entre os grupos. O tratamento neonatal com kaempferol atenuou o impacto do modelo de paralisia cerebral na aversão ao precipício, colocação pelas vibrissas, quando comparados com as mensurações do grupo controle em relação aos animais controle (C vs. PCK, p>0.05) (Tabela 2).

No P36 foram observadas mudanças significativas em relação as medidas corporais dos animais dos animais. O modelo de paralisia cerebral causou redução significativa no peso corporal e comprimento corporal em comparação com os animais do grupo controle. O eixo craniano látero-lateral dos animais do grupo PC apresentou redução quando comparado aos animais controle. Adicionalmente, o peso do encéfalo apresentou redução nos animais do grupo PC em comparação com os animais

controle. O tratamento neonatal com kaempferol atenuou essas mudanças nos animais com paralisia cerebral (Tabela 2).

No P36 foram observadas mudanças significativas em relação a caracterização somática dos animais através das análises de medidas corporais. O modelo de PC causou uma redução significativa no peso corporal [$F (3, 42) = 21,82$, $p < 0,0001$] e comprimento corporal em comparação com os animais do grupo de controle [$F (3, 42) = 32,54$, $p < 0,0001$] (Tabela 2). O eixo craniano látero-lateral dos animais do grupo PC apresentou redução quando comparado aos animais controle [$F (3, 42) = 11,62$, $p < 0,0001$]. Além disso, o peso encefálico apresentou redução nos animais do grupo PC em relação aos animais controle [$F (1, 28) = 25,34$, $p < 0,0001$]. O tratamento neonatal com Kaempferol mitigou essas alterações em animais expostos ao modelo de PC, quando comparado com as medidas dos animais controle (Tabela 2).

Tabela 2 – Maturação física, ontogenia dos reflexos e caracterização somática.

Parâmetro	GRUPOS			
	C	**K**	**PC**	**PCK**
Maturação das características físicas				
Erupção dos Incisivos Superiores	10 (9-10)	10 (9-11)	**12 (11-12)*** **	11 (10-11.5)
Erupção dos Incisivos Inferiores	11 (10-11)	10 (10-11)	**13 (13-14)*** **	12 (11-12)
Abertura dos olhos	13 (13-14)	14 (13-14.25)	**15 (14.75-15.25)*** **	14 (13.75-15)
Ontogenia dos reflexos				
Endireitamento	3.5 (3-4)	3 (3-4)	**5 (4.75-5.25)*** **	5 (4-5)
Preensão Palmar	6 (5-6)	6 (5-6.25)	**7 (6-8)***	7 (7-8)
Aversão ao precipício	6 (5-6)	6 (5-6)	**7 (6.25-8)****	7 (6-7)
Colocação pelas vibrissas	7 (6-7)	7 (6-7)	**9 (8-11)*** **	9 (7-9)
Resposta ao susto	12 (11 - 13.25)	12 (11-13)	13 (12-13)	12 (11.75-13)
Queda livre	15 (14-15)	15 (14-15.5)	**18 (17-19)*****	16 (16-17.25)
Geotaxia negativa	15 (15-15)	15 (14.75-16)	**21 (21-22)*****	20.5 (19-21.25)
Caracterização somática				
Peso corporal (g) no P36	107.8 ± 5.704	100.4 ± 3.543	**64.27 ± 3.998*****	79.31 ± 3.85-
Comprimento corporal (mm) no P36	147.0 ± 2.432	144.1 ± 1.875	**124.5 ± 1.765*** **	126.3 ± 2.947
ECAP (mm) no P36	37.62 ± 0.462	38.03 ± 0.676	35.39 ± 0.593	35.78 ± 0.519
ECLL (mm) no P36	23.06 ± 0.921	21.54 ± 0.240	**19.08 ± 0.210*****	20.0 ± 0.325
Peso encefálico (g) no P36	1.51 ± 0.041	1.437 ± 0.042	**1.203 ± 0.052*** **	1.324 ± 0.024

Fonte: O Autor (2022). Idade (dia pós-natal) de aparecimento ou maturação de características físicas e da ontogenia dos reflexos em período neonatal. Para caracterização somática ao final do experimento foram avaliadas as medidas corporais e peso do encéfalo. Grupos de acordo com o tratamento com kaempferol e/ou exposição ao modelo de Paralisia Cerebral. C, n = 15; K, n = 15; PC, n = 15; PCK, n = 15. Dados de maturação física e ontogenia dos reflexos estão apresentados em mediana e intervalo interquartil. Para essas variáveis a comparação estatística feita através do Teste de Kruskall-Wallis com pós teste de Dunn's. Os dados de medidas corporais e peso encefálico estão apresentados em média e erro padrão da média e foram analisados pelo teste ANOVA *two-way* seguido do *post-hoc* teste de Tukey. * = C vs. PC; # = PC vs. PCK. * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$; **** = $p<0.0001$.

6.1.2 Desenvolvimento da atividade locomotora

A análise do desenvolvimento da atividade locomotora mostrou que os animais controles apresentaram aumento progressivo na capacidade de locomoção no período entre de P8 a P28. A análise da diferença intergrupos mostrou diferenças significativas na distância percorrida em P17 [F (3, 33) = 5,512, p = 0,0035] (Figura 8A). As comparações múltiplas revelaram, nesta idade, que os animais do grupo PC apresentaram redução significativa da distância percorrida em relação aos animais do grupo controle (C = 19,35 ± 2,536 vs. PC = 7,981 ± 1,641, p <0,0,002) (Figura 8A). Uma redução significativa da distância percorrida no grupo PC em comparação com os animais controle também foi observada no P21 (C = 22,95 ± 2,155 vs. PC = 13,16 ± 2,255, p <0,0010) [F (3, 33) = 4,06, p = 0,0146] e em P28 (C = 26,66 ± 1,157 vs. PC = 18,35 ± 2,28, p <0,0005) [F (3, 33) = 5,88, p = 0,0025] (Figura 7A). No P17 os animais do PCK apresentaram aumento da distância percorrida em relação ao grupo PC (PC = 7,981 ± 1,641 vs. PCK = 14,67 ± 2,123, p = 0,0486). Aumento na distância percorrida do grupo PCK também foi observado em P21 em comparação ao grupo PC (PC = 13,16 ± 2,255 vs. PCK = 20,43 ± 1,763, p = 0,0252) (Figura 8A).

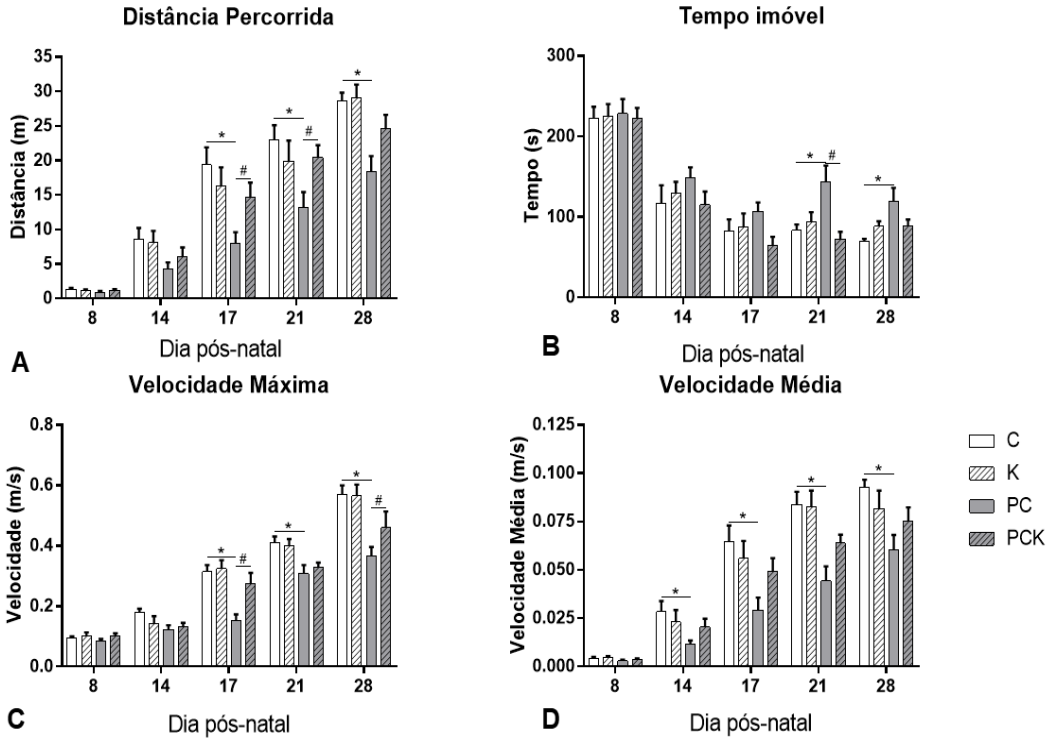
A comparação do tempo imóvel revelou mudanças significativas entre os grupos [F (3, 44) = 5,829; p <0,0019). Em comparação ao grupo controle, os animais do grupo PC apresentaram aumento do tempo imóvel no P21 (C = 83,44 ± 7,226 vs. PC = 143,4 ± 20,59, p = 0,0021) e no P28 (C = 69,33 ± 3,566 vs. PC = 119,4 ± 16,66, p = 0,0060) (Figura 8B). O grupo PCK apresentou tempo imóvel reduzido em comparação com PC no P21 (PC = 143,4 ± 20,59 vs. PCK = 72,53 ± 9,042, p = 0,0021) (Figura 8B).

Diferença significativa da velocidade máxima foi observada entre os grupos [F (3, 44) = 17,35; p <0,0001)] em diferentes momentos. O parâmetro de velocidade máxima apresentou redução nos animais do grupo PC em comparação com o grupo controle em P17 [F (3, 33) = 8,711; p = 0,0002], em P21 [F (3, 33) = 5,098; p = 0,0052] e em P28 [F (3, 33) = 6,238; p = 0,0018] (Figura 8C). Comparado ao grupo de paralisia cerebral não tratado, o grupo PCK apresentou maior velocidade máxima em P17 (PC = 0,1533 ± 0,0193 vs. PCK = 0,2752 ± 0,0349, p = 0,0139) e em P28 (PC = 0,3676 ± 0,0287 vs. PCK = 0,4598 ± 0,0536, p = 0,0443) (Figura 8C).

A análise da velocidade média revelou mudanças significativas entre os grupos [F (3, 44) = 12,44; p <0,0001)]. Os animais do grupo PC apresentaram

velocidade média reduzida no P14 [F (3, 33) = 2,947; p = 0,0471] (C = 0,0282 ± 0,023 vs. PC = 0,0113 ± 0,020; p = 0,0316), em P17 [F (3, 33) = 4,45; p = 0,0099] (C = 0,0645 ± 0,008 vs. PC = 0,0290 ± 0,006; p = 0,0070), em P21 [F (3, 33) = 7,72; p = 0,005] (C = 0,0837 ± 0,006 vs. PC = 0,0443 ± 0,007; p = 0,0011) e em P28 [F (3, 33) = 3,643; p = 0,0225] (C = 0,0928 ± 0,003 vs. PC = 0,0604 ± 0,007; p = 0,0142) (Figura 8D).

Figura 8 – Desenvolvimento da atividade locomotora.

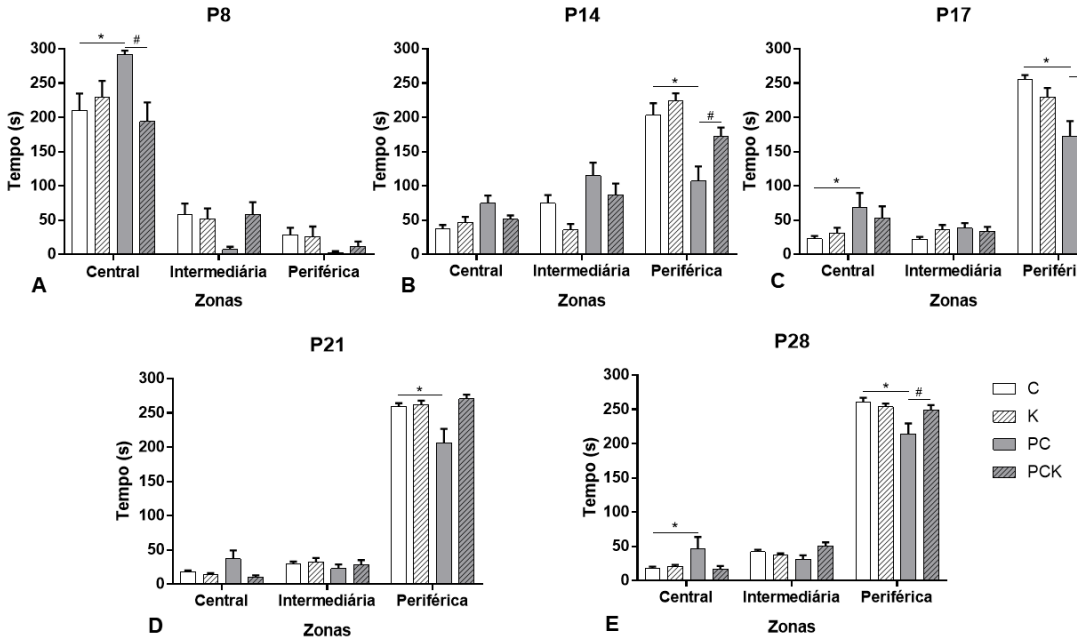


Fonte: o autor (2022). Avaliação do desenvolvimento da atividade locomotora nas idades alvos de P8, P14, P17, P21 e P28. A – Distância percorrida; B - Tempo imóvel; C – Velocidade máxima; D – Velocidade média. A composição dos grupos está de acordo com intervenção com Kaempferol ou veículo e modelo de paralisia cerebral ou controle. C (Controle - veículo, n=12); K (Controle - Kaempferol, n=12); PC (Paralisia Cerebral + veículo, n=12); PCK (Paralisia Cerebral - Kaempferol, n=12). Os dados estão apresentados em média e erro padrão da média e foram analisados pelo teste ANOVA *two-way* medidas repetidas seguido do *post-hoc* teste de Tukey. * = C vs. PC; # = PC vs. PCK; p<0.05.

A avaliação do tempo gasto nas zonas do campo aberto, mostrou diferenças no comportamento entre os grupos em diferentes momentos. Uma interação significativa foi observada em P8 entre a zona do campo aberto e os grupos experimentais [F (6, 132) = 4,606; p <0,0003)]. No P8 os animais do grupo PC apresentaram aumento do tempo na zona central em comparação ao grupo controle

(C = 209,7 ± 25,35 vs. PC = 291,8 ± 5,89, p = 0,0031) (Figura 9A). No P8 os animais do PCK apresentaram tempo reduzido na zona central em comparação ao grupo PC (PC = 291,8 ± 5,89 vs. PCK = 193,9 ± 28,2, p = 0,003) (Figura 9A). No P14, os animais do grupo PC apresentaram redução do tempo gasto na zona periférica em relação ao grupo controle (C = 203,4 ± 17,59 vs. PC = 107,1 ± 21,42, P <0,0001). No P14 foi observada uma interação significativa entre as zonas do campo e grupos experimentais [F (6, 132) = 10,74; p <0,0001). No P14, os animais do PCK apresentaram aumento do tempo gasto na zona periférica em comparação ao grupo PC (PC = 107,1 ± 21,42 vs. PCK = 172,3 ± 13,06, p = 0,0047) (Figura 9B). Em P17 foi observada uma interação significativa entre as zonas e grupos [F (6, 132) = 5,493; p <0,0001). No P17 os animais do PC apresentaram maior tempo de permanência na zona central em relação ao grupo controle (C = 22,81 ± 4,302 vs. PC = 68,98 ± 20,79, p = 0,0461). No P17, os animais do PC apresentaram redução do tempo gasto na zona periférica em relação ao grupo controle (C = 255,4 ± 6,532 vs. PC = 172,5 ± 22,26, p <0,0001). No P17, os animais do grupo PCK apresentaram maior tempo gasto na zona periférica em comparação ao grupo PC (PC = 172,5 ± 22,26 vs. PCK = 242,5 ± 12,0, p = 0,0006) (Figura 9C). No P21 foi observada uma interação significativa entre as zonas e grupos [F (6, 132) = 6,416; p <0,0001). No P21, os animais do grupo PC apresentaram redução do tempo gasto na zona periférica em comparação ao grupo controle (C = 259,6 ± 4,859 vs. PC = 206,6 ± 20,2, p <0,0001). No P21, os animais do grupo PCK apresentaram aumento do tempo gasto na zona periférica em comparação ao grupo PC (CP + P = 206,6 ± 20,2 vs, CP + K = 270,8 ± 6,159, p <0,0001) (Figura 9D). Em P28 foi observada uma interação significativa entre as zonas e grupos [F (6, 132) = 5,269; p <0,0001). No P28 os animais do grupo PC apresentaram aumento do tempo gasto na zona central em relação ao grupo controle (C = 17,77 ± 2,737 vs. PC = 47,13 ± 16,87, p = 0,0464), com redução do tempo gasto na zona periférica em comparação para o grupo controle (C = 260,8 ± 6,365 vs. PC = 214,3 ± 15,42, p = 0,0003). Em P28 os animais do grupo PCK apresentaram redução do tempo na zona central em comparação ao grupo PC (PC = 47,13 ± 16,87 vs. PCK = 16,83 ± 4,8, p = 0,0371), com aumento do tempo na zona periférica em relação ao grupo PC (PC = 214,3 ± 15,42 vs. PCK= 249,5 ± 6,849, p = 0,0105) (Figura 9E).

Figura 9 – Tempo gasto por zona durante o desenvolvimento da atividade locomotora.

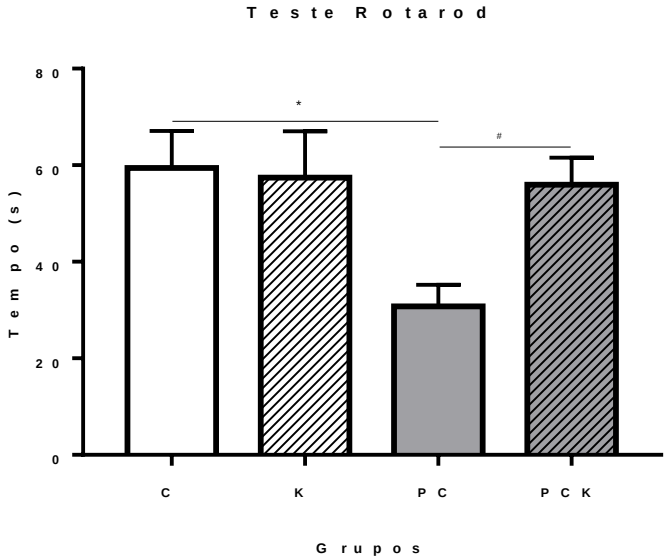


Fonte: o autor. Tempo gasto nas zonas do campo aberto. Zonas: central, intermediária e periférica. A - Tempo gasto nas zonas no P8; B - Tempo gasto nas zonas no P14; C - Tempo gasto nas zonas no P17; D - Tempo gasto nas zonas no P21; E - Tempo gasto nas zonas no P28. A composição dos grupos está de acordo com intervenção com Kaempferol ou veículo e modelo de paralisia cerebral ou controle. C (Controle - veículo, n=12); K (Controle - Kaempferol, n=12); PC (Paralisia Cerebral + veículo, n=12); PCK (Paralisia Cerebral - Kaempferol, n=12). Os dados estão apresentados em média e erro padrão da média e foram analisados pelo teste ANOVA *two-way* medidas repetidas seguido do *post-hoc* teste de Tukey. * = C vs. PC; # = PC vs. PCK; $p<0.05$.

6.1.3 Coordenação motora

Importantes diferenças foram observadas na avaliação da coordenação motora entre os grupos [$F(3, 33) = 4,755$; $p = 0,0073$). O teste do Rotarod revelou que os animais do grupo PC tiveram desempenho reduzido no teste em relação ao grupo controle (C = 59,46 ± 7,660 vs. PC = 30,74 ± 4,523, $p = 0,0114$). Os animais do grupo PCK apresentaram maior tempo no aparelho em comparação com os animais do grupo PC (CP = 30,74 ± 4,523 vs. CP = 55,95 ± 5,624, $p = 0,0309$) (Figura 10).

Figura 10 - Desempenho durante o teste de coordenação e equilíbrio.

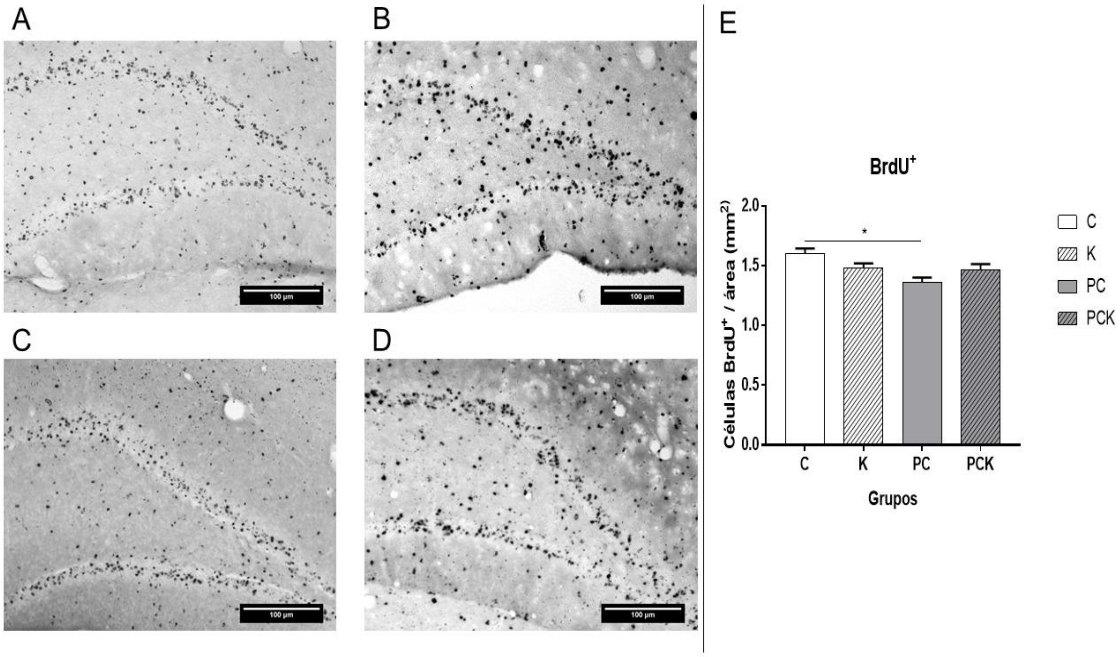


Fonte: o autor (2022). Desempenho dos animais durante o teste de coordenação e equilíbrio no aparato Rotarod. A composição dos grupos está de acordo com intervenção com Kaempferol ou veículo e modelo de paralisia cerebral ou controle. C (Controle - veículo, n=12); K (Controle - Kaempferol, n=12); PC (Paralisia Cerebral + veículo, n=12); PCK (Paralisia Cerebral - Kaempferol, n=12). Os dados estão apresentados em média e erro padrão da média e foram analisados pelo teste ANOVA *two-way* medidas repetidas seguido do *post-hoc* teste de Tukey. * = C vs. PC; # = PC vs. PCK; $p<0.05$.

6.1.4 Proliferação celular no hipocampo

Na zona subgranular (ZSG) e camada de células granulares (CCG) do hipocampo, uma diferença significativa no número de células $BrdU^+$ que proliferaram em período pós-natal precoce foi observada entre os grupos [$F(3, 216) = 5,235$; $p = 0,0017$]. Uma redução significativa na razão do número de células proliferativas por área foi observada no grupo PC em comparação com o grupo controle (C = 1,599 ± 0,0452 vs PC = 1,359 ± 0,0413, p = 0,0006) (Figura 11).

Figura 11 - Proliferação celular no hipocampo.

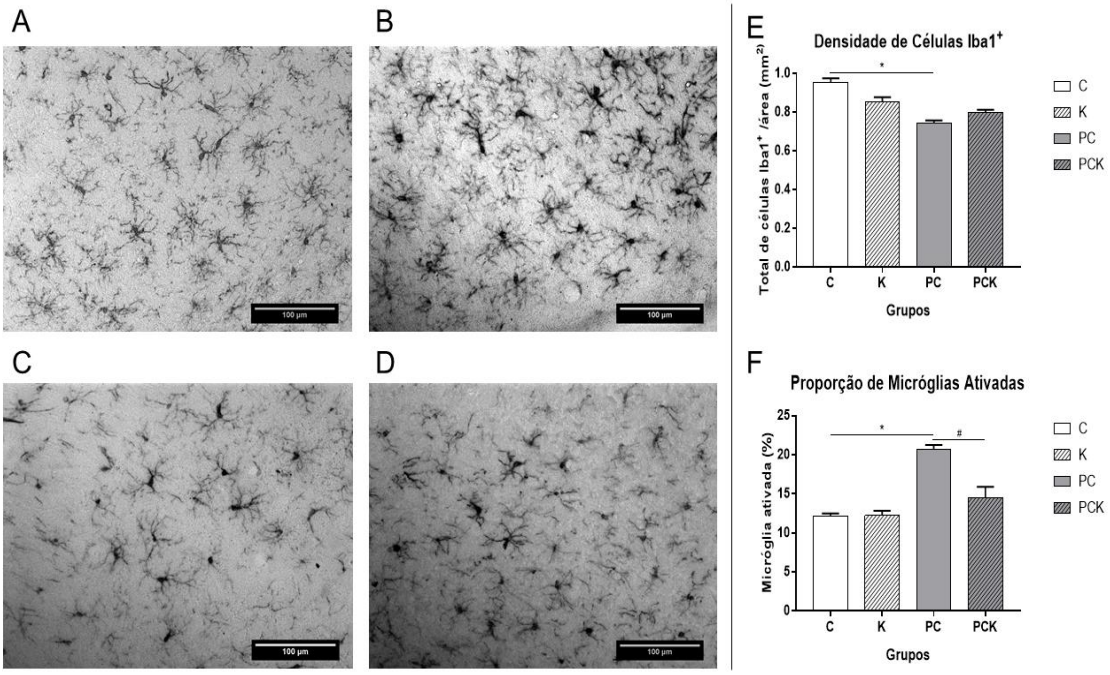


Fonte: o autor (2022). Imagens representativas da imuno-histoquímica para células $BrdU^+$ na camada de células granulares e zona subgranular do giro denteado do hipocampo A-D; A- Grupo controle - veículo; B- Grupo controle - Kaempferol; C- Grupo Paralisia Cerebral – veículo; D- Grupo Paralisia Cerebral - Kaempferol. E – Número de células $BrdU^+$ / área. A composição dos grupos está de acordo com intervenção com Kaempferol ou veículo e modelo de paralisia cerebral ou controle. C (Controle - veículo, n=7); K (Controle - Kaempferol, n=7); PC (Paralisia Cerebral + veículo, n=7); PCK (Paralisia Cerebral - Kaempferol, n=7). Os dados estão apresentados em média e erro padrão da média e foram analisados pelo teste ANOVA *two-way* medidas repetidas seguido do *post-hoc* teste de Tukey. * = C vs. PC; # = PC vs. PCK; $p<0.05$.

6.1.5 Análise morfológica da micróglia hipocampal

O modelo experimental de Paralisia Cerebral induziu uma redução significativa da densidade da micróglia no hilo do hipocampo em comparação com o grupo controle [F (3, 165) = 25,65; p <0,0001]. Além disso, um efeito significativo no perfil ativado da micróglia foi observado no grupo PC em comparação com o grupo controle [F (3, 165) = 22,89; p <0,0001 (Figura 12). A comparação múltipla revelou um aumento da proporção de micróglia ativada no hilo de do grupo PC em comparação com o grupo de controle (C = 12,11 ± 0,3736 vs. PC = 20,65 ± 0,5982, p <0,0001). O tratamento neonatal com Kaempferol induziu uma redução significativa na porcentagem de micróglia ativada no hilo de animais com paralisia cerebral em comparação com animais com paralisia cerebral não tratados (PC= 20,65 ± 0,5982 vs. PCK= 14,46 ± 1,432, p <0,0001) (Figura 12).

Figura 12 – Perfil e número de micróglias no giro denteado do hipocampo.



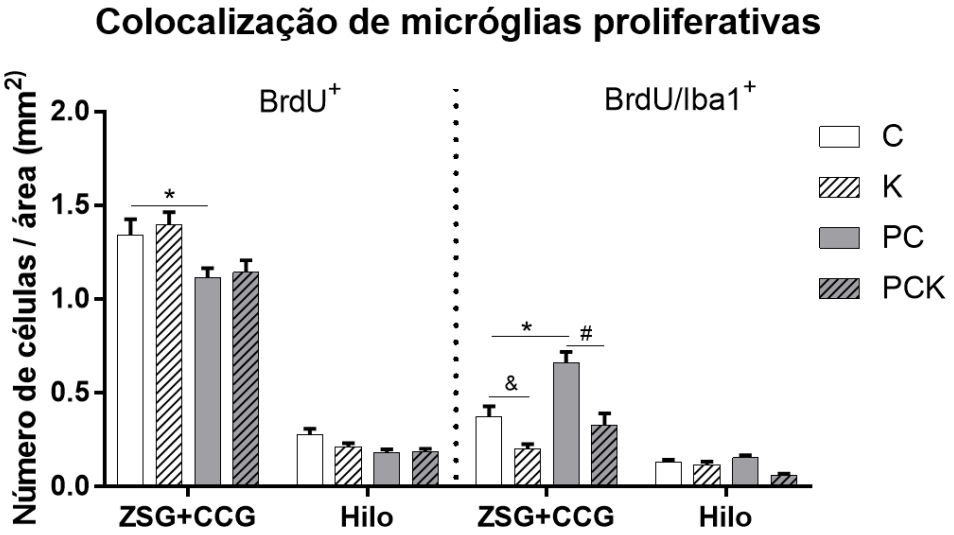
Fonte: o autor (2022). Imagens representativas da imuno-histoquímica para células Iba1⁺ A-D; A-Grupo controle - veículo; B- Grupo controle - Kaempferol; C- Grupo Paralisia Cerebral – veículo; D-Grupo Paralisia Cerebral - Kaempferol. E – Número de células BrdU⁺ / área. A composição dos grupos está de acordo com intervenção com Kaempferol ou veículo e modelo de paralisia cerebral ou controle. C (Controle - veículo, n=7); K (Controle - Kaempferol, n=7); PC (Paralisia Cerebral + veículo, n=7); PCK (Paralisia Cerebral - Kaempferol, n=7). Os dados estão apresentados em média e erro padrão da média e foram analisados pelo teste ANOVA *two-way* medidas repetidas seguido do *post-hoc* teste de Tukey. * = C vs. PC; # = PC vs. PCK; $p<0.05$.

6.1.6 Proliferação de micróglias no hipocampo

A análise de imunofluorescência através de microscópio confocal revelou um efeito significativo entre os grupos na contagem de células BrdU⁺ [$F(3, 198) = 4,806$; $p = 0,0030$]. A comparação múltipla entre os grupos mostrou que o modelo de paralisia cerebral causou uma diminuição significativa nas células indiferenciadas marcadas com BrdU⁺ nas regiões do hipocampo ZSG + CCG [$F(3, 96) = 6,594$; $p = 0,0004$] (C = 1,343 ± 0,082 vs. PC = 1,127 ± 0,035, $p < 0,0001$) (Figura 13). A colocalização de BrdU / Iba1⁺ apresentou um efeito significativo entre os grupos [$F(3, 102) = 15,08$; $p < 0,0001$] (Figura 13). As comparações múltiplas mostraram que o tratamento neonatal kaempferol em animais controle reduziu a colocalização das células microgliais no ZSG + CCG do hipocampo (C = 0,372 ± 0,055 vs. K = 0,203 ± 0,024, $p = 0.0289$) (Figura 13). O modelo de paralisia cerebral causou um aumento significativo na proliferação de células microgliais no ZSG + CCG em comparação com os animais controle (C = 0,372 ± 0,055 vs. PC = 0,651 ± 0,055, $p < 0,0001$) (Figura 13). O

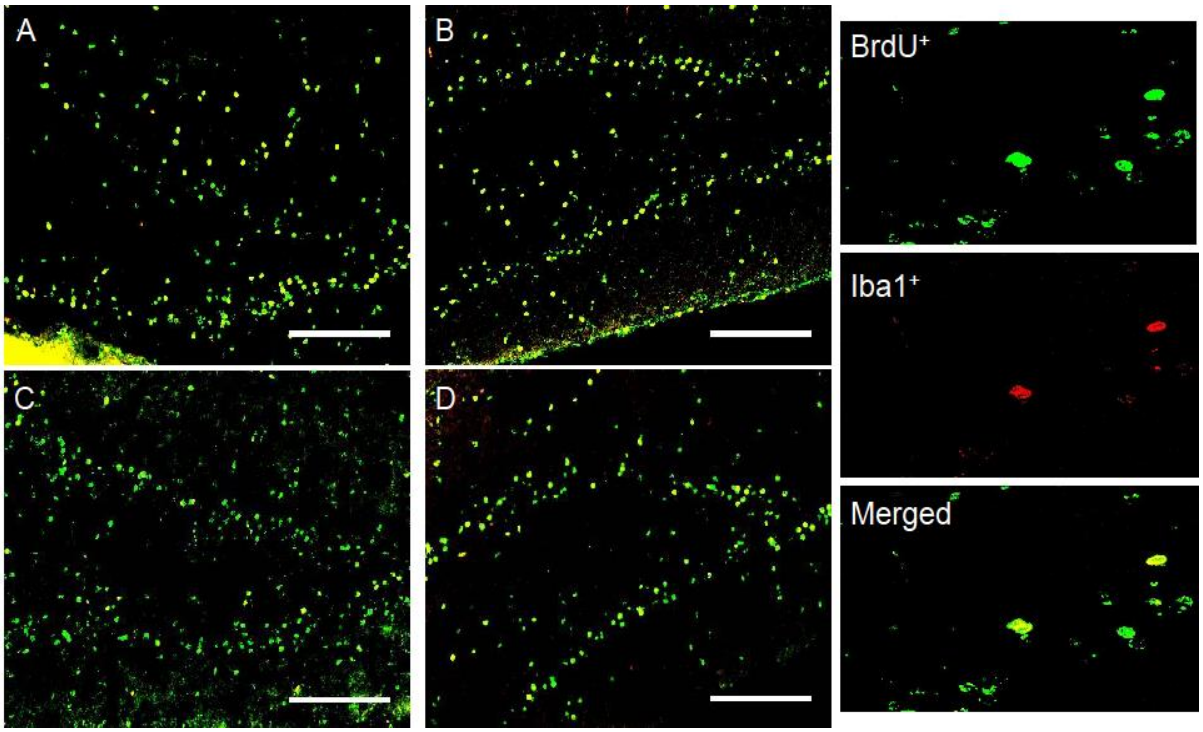
tratamento neonatal de kaempferol em animais com paralisia cerebral causou uma redução significativa da proliferação de células microgliais na ZSG + CCG em comparação com animais do grupo PCK (PC = 0,651 ± 0,055 vs. PCK = 0,293 ± 0,052, p <0,0001) (Figura 14).

Figura 13 – Proliferação de micróglias no hipocampo.



Fonte: o autor (2022). Colocalização de micróglias proliferativas na Zona Subgranular (ZSG) e Camada de Células Granulares (CCG) e no hilo do giro denteado do hipocampo. A composição dos grupos está de acordo com intervenção com Kaempferol ou veículo e modelo de paralisia cerebral ou controle. C (Controle - veículo, n=7); K (Controle - Kaempferol, n=7); PC (Paralisia Cerebral + veículo, n=7); PCK (Paralisia Cerebral - Kaempferol, n=7). Os dados estão apresentados em média e erro padrão da média e foram analisados pelo teste ANOVA two-way medidas repetidas seguido do post-hoc teste de Tukey. * = C vs. PC; & = C vs. K; # = PC vs. PCK; p<0.05.

Figura 14 – Imagens representativas da colocalização de micróglias proliferativas no hipocampo.



Fonte: o autor (2022). Imagens representativas de imunofluorescência avaliando a colocalização de micróglias proliferativas. Em verde células BrdU⁺. Em vermelho micróglias. Em amarelo micróglias colocalizadas com BrdU⁺. A- Grupo controle - veículo; B- Grupo Paralisia cerebral - veículo; C – grupo controle - Kaempferol; D – grupo Paralisia Cerebral - Kaempferol.

6.2 RESULTADOS EXPERIMENTO 2

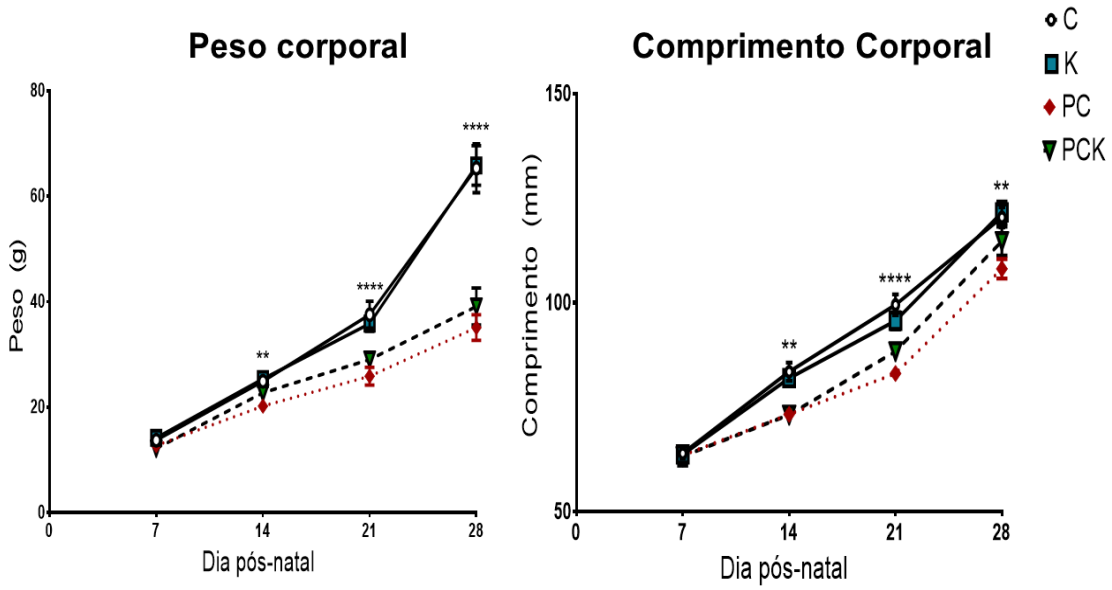
6.2.1 Peso corporal e crescimento corporal em período neonatal

Durante o período neonatal, um efeito significativo para o peso corporal [F (9, 132) = 12,48, p <0,0001] e o crescimento corporal [F (9, 132) = 2,58, p = 0,009] foi observado entre os grupos. O peso corporal foi reduzido no grupo PC em comparação com os animais controle no P14 (C = 24,93 ± 0,99 vs. PC = 20,22 ± 1,10, p <0,0011) [F (3, 33) = 8,79, p = 0,0002), no P21 (C = 37,56 ± 2,54 vs. PC = 25,88 ± 1,69, p <0,0001) [F (3, 33) = 11,9, p <0,0001), e no P28 (C = 65,31 ± 4,63 vs. PC = 35,09 ± 2,43, p <0,001) [F (3, 33) = 18,43, p <0,0001) (Figura 15). O tratamento neonatal de Kaempferol reduziu o impacto do modelo de PC sobre o fenótipo corporal, atenuando os efeitos sobre o peso corporal e comprimento corporal no P21 (Figura 15).

Foi observado um comprimento corporal reduzido do grupo PC quando comparados a animais controle no P14 (C = 83,48 ± 2,18 vs. PC = 73,26 ± 1,27, p = 0,0018) [F (3, 33) = 9,36, p = 0,0001], no P21 (C = 99,48 ± 2,48 vs. PC = 83.04 ± 1.79

p <0,0001) [F (3, 33) = 13,35, p <0,0001], e no P28 (C = 120,40 ± 2,39 vs. PC = 108,1 ± 2,31, p = 0,0092) [F (3, 33) = 5,81, p <0,0026] (Figura 15). O tratamento neonatal com kaempferol não afetou o comprimento corporal dos animais controle durante os experimentos (Figura 15). Em animais expostos ao modelo de PC, no entanto, o kaempferol atenuou o impacto sobre o comprimento do corporal na terceira semana. Após as quatro semanas pós-natais, não foram observadas diferenças entre o grupo PCK e os animais controle (Figura 15).

Figura 15 – Análise do peso e do comprimento corporal.



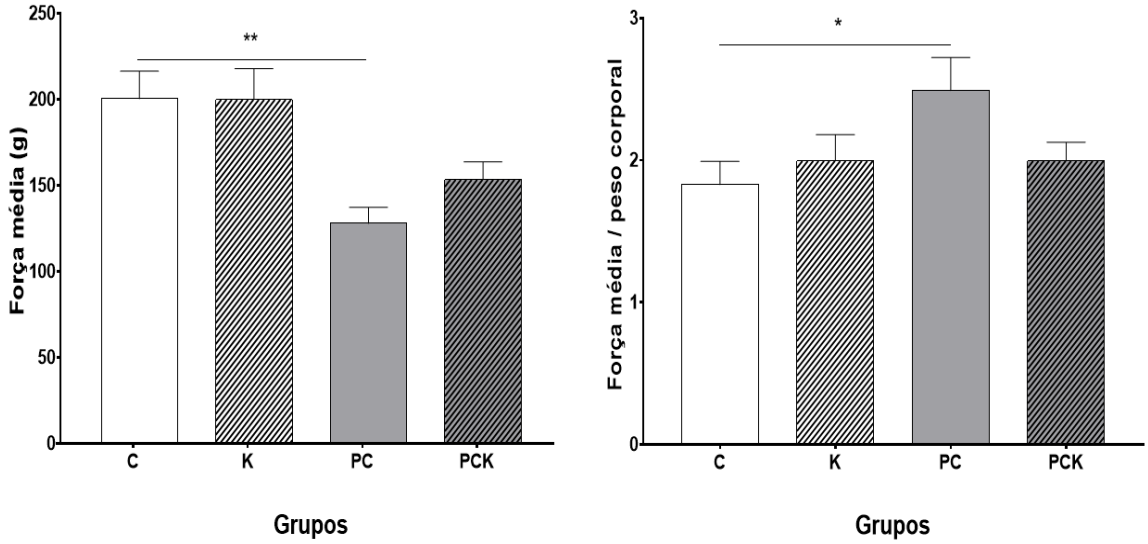
Fonte: o autor (2022). Peso corporal e comprimento corporal dos ratos nas idades P7, P14, P21 E P28. A composição dos grupos está de acordo com intervenção com Kaempferol ou veículo e modelo de paralisia cerebral ou controle. C (Controle - veículo, n=12); K (Controle - Kaempferol, n=12); PC (Paralisia Cerebral + veículo, n=12); PCK (Paralisia Cerebral - Kaempferol, n=12). Os dados estão apresentados em média e erro padrão da média e foram analisados pelo teste ANOVA *two-way* medidas repetidas seguido do *post-hoc* teste de Tukey. * = C vs. PC; & = C vs. K; # = PC vs. PCK; p<0.05.

6.2.2 Análise da força de preensão

Um efeito significativo entre os grupos foi observado na análise da força de preensão [F (3, 33) = 7,24, p = 0,0007]. As análises de comparações múltiplas revelaram que o grupo PC apresentou redução da força média das patas dianteiras avaliada pelo *GripStrenght* (C = 200,4 ± 16,05 vs. PC = 127,90 ± 9,31, p = 0,0028)

(Figura 16). O tratamento neonatal com kaempferol não causou aumentos significativos na força média nos animais expostos ao modelo de PC, porém, observou-se uma redução do impacto do modelo sobre a força (C = 200,4 ± 16,05 vs CPK = 153,3 ± 10,39, p> 0,05). Os dados foram normalizados em relação ao peso corporal e revelaram um aumento na taxa de força média / peso corporal nos animais do grupo PC (C = 1,82 ± 0,16 vs. PC = 2,49 ± 0,23, p = 0,0424) (Figura 16).

Figura 16 – Análise da força de muscular através do Grip Strenght.



Fonte: o autor (2022). A composição dos grupos está de acordo com intervenção com Kaempferol ou veículo e modelo de paralisia cerebral ou controle. C (Controle - veículo, n=12); K (Controle - Kaempferol, n=12); PC (Paralisia Cerebral + veículo, n=12); PCK (Paralisia Cerebral - Kaempferol, n=12). Os dados estão apresentados em média e erro padrão da média e foram analisados pelo teste ANOVA *two-way* medidas repetidas seguido do *post-hoc* teste de Tukey. * = C vs. PC; & = C vs. K; # = PC vs. PCK; p<0.05.

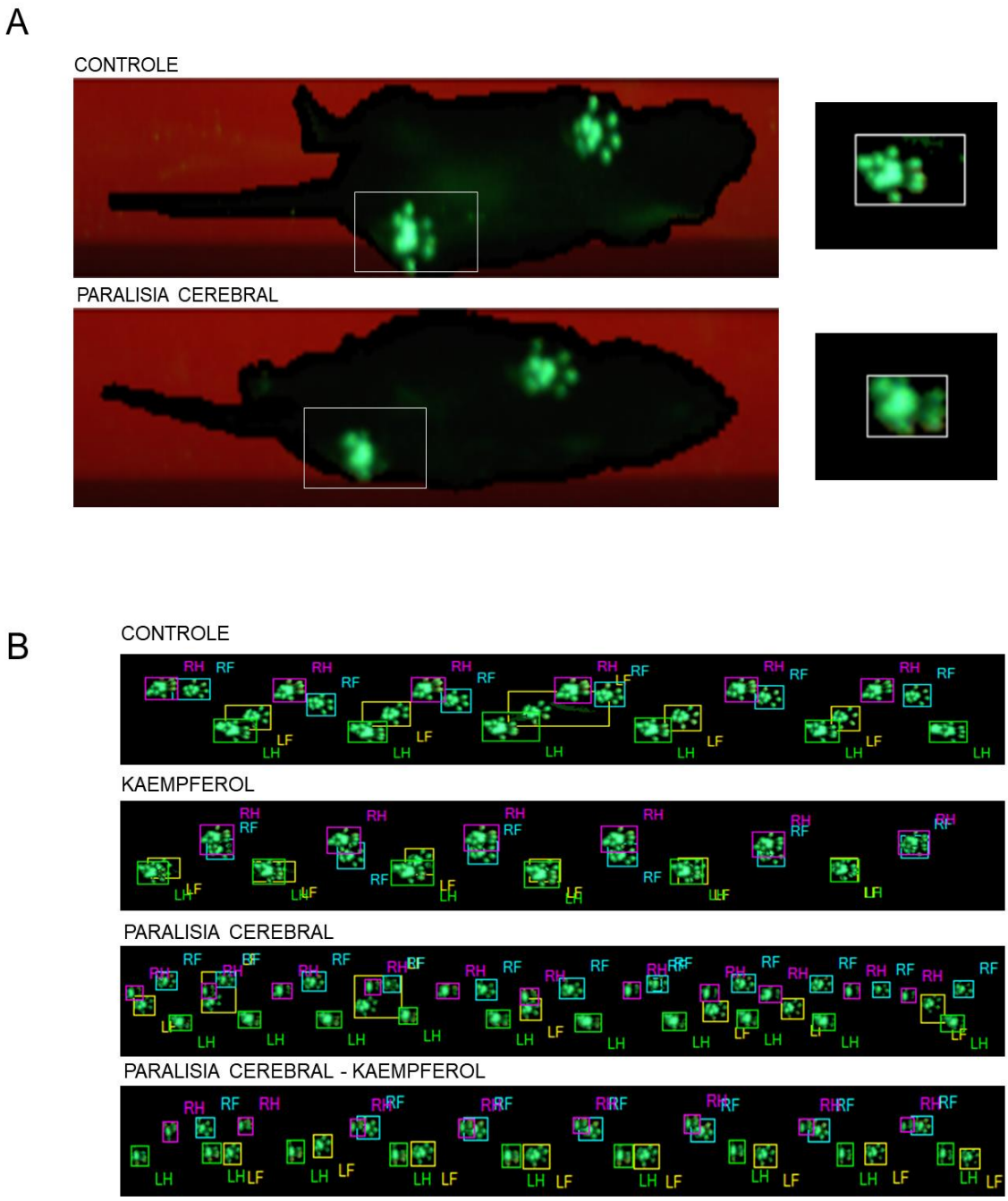
6.2.3 Análise dos parâmetros estáticos e dinâmicos da marcha

Para avaliar o comportamento motor após o tratamento neonatal com Kaempferol em ratos submetidos ao modelo de PC, os parâmetros da marcha e as estatísticas das patas foram quantificados pelo sistema de passarela (Figura 17). Uma vez que a restrição sensório-motora dos membros posteriores em ratos mimetiza a

PC do tipo diplégica, foi observado um efeito do modelo sobre a cadência da marcha [F (3, 27) = 3,683, p = 0,0241].

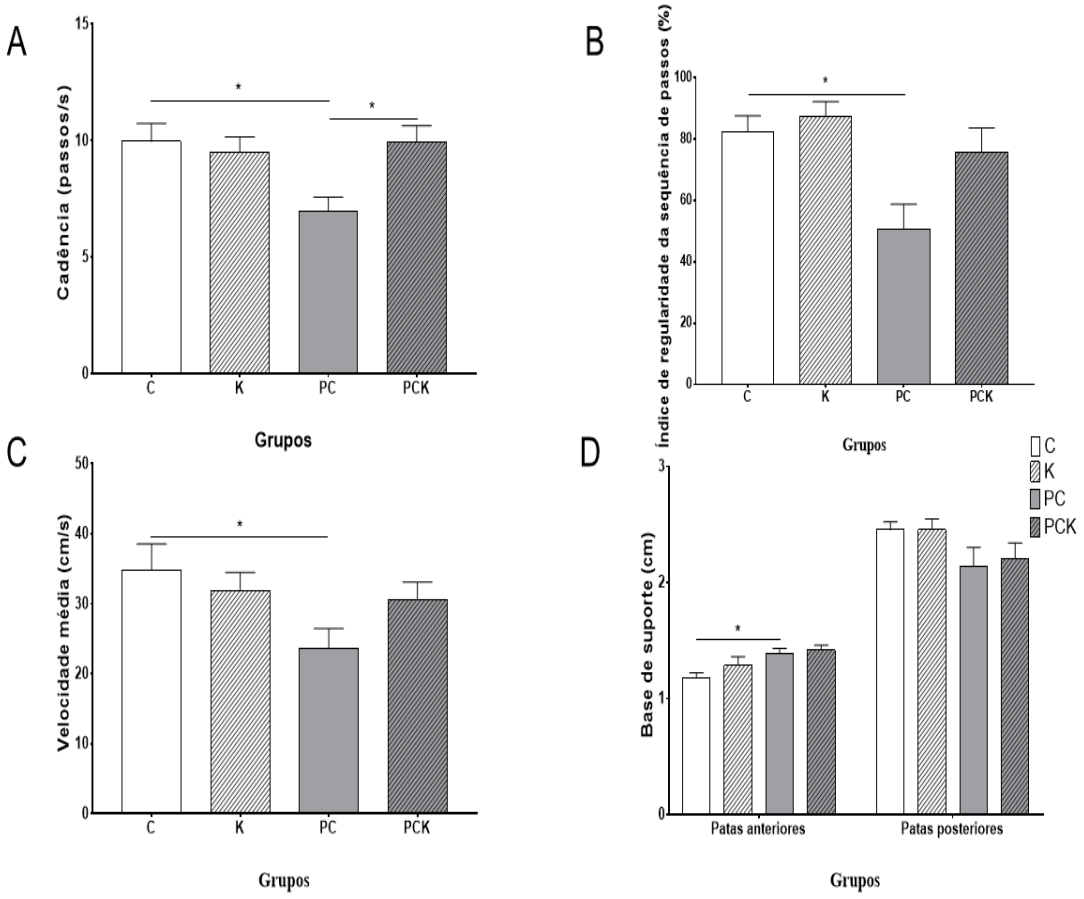
As comparações múltiplas revelaram que o grupo com PC apresentou número reduzido de passos por segundo ao longo de sua caminhada na plataforma (C = 9,965 ± 0,775 vs. PC = 6,978 ± 0,603, p = 0,04) (Figura 17A). O tratamento neonatal com kaempferol atenuou o comprometimento da cadência da marcha em animais com PC (PC = 6,978 ± 0,603 vs. PCK = 9,933, p = 0,042). O índice de regularidade da sequência de passos (% para o grau de coordenação entre os membros durante a marcha) mostrou um efeito significativo do modelo de PC [F (3, 27) = 5,569, p = 0,0042]. Observou-se notável impacto do modelo PC na coordenação inter-membros durante a marcha (C = 82,56 ± 5,10 vs. PC = 50,63 ± 8,11, p = 0,014) (Figura 17B), enquanto o grupo PCK não apresentou diferenças significativas em relação ao controle grupo (C = 82,56 ± 5,10 vs. PCK = 75,69 ± 8,01, p> 0,05) (Figura 18B), Como mostrado na Figura 17C, um efeito significativo foi observado na velocidade média entre os grupos com PC [F (3, 27) = 4,129, p = 0,0156]. Os animais submetidos ao modelo PC apresentaram velocidade média (cm / s) reduzida durante os experimentos em relação ao grupo controle (C = 34,85 ± 3,691 vs. PC = 23,61 ± 2,83, p = 0,0109). A velocidade média reduzida foi atenuada em animais com PC que receberam tratamento neonatal com Kaempferol (C = 34,85 ± 3,691 vs; PCK = 30,64 ± 2,46, p> 0,05) (Figura 18C). Ainda, foi observado que modelo de PC afeta a base de apoio (cm) durante a marcha. Um efeito significativo foi observado para base de apoio das patas dianteiras entre os grupos [F (3, 27) = 5,679, p = 0,0038]. Em comparação com o grupo controle, os animais com PC apresentaram maior base de apoio das patas dianteiras (C = 1,18 ± 0,042 vs. PC = 1,391 ± 0,040, p = 0,0144), (C = 1,18 ± 0,042 vs. PCK = 1,41 ± 0,042, p = 0,0053) (Figura 18D).

Figura 17 – Avaliação dos parâmetros da marcha com o sistema Catwalk.



Fonte: o autor (2022). Avaliação dos parâmetros estáticos e dinâmicos da marcha através do sistema *Catwalk* durante a locomoção do animal pela plataforma. A- Comparação qualitativa entre um animal do grupo controle com um animal submetido ao modelo de paralisia cerebral. A direita observa-se impressões da pata posterior dos animais controle e com PC. B- Imagens representativas das impressões das patas durante a locomoção ativa dos animais pela plataforma. RH- *right hind* (pata posterior direita); LH- *left hind* (pata posterior esquerda); RF- *right front* (pata anterior direita); LF- *left front* (pata anterior esquerda). A composição dos grupos está de acordo com intervenção com Kaempferol ou veículo e modelo de paralisia cerebral ou controle.

Figura 18 – Avaliação dos parâmetros cinéticos da marcha através do CatWalk.



Fonte: o autor (2022). Comparações dos parâmetros de marcha entre os grupos no P33. A- Cadência da marcha (passos por segundo); B- Índice de regularidade da sequência de passos (uso de padrões normais de passos de forma ininterrupta durante a locomoção, demonstrando o grau de coordenação entre os membros); C- Velocidade média (velocidade média durante a locomoção ativa na plataforma); D- Base de suporte (largura entre as patas dianteiras ou traseiras na fase de apoio durante a locomoção). A composição dos grupos está de acordo com intervenção com Kaempferol ou veículo e modelo de paralisia cerebral ou controle. C (Controle - veículo, n=10); K (Controle - Kaempferol, n=10); PC (Paralisia Cerebral + veículo, n=10); PCK (Paralisia Cerebral - Kaempferol, n=10). Os dados estão apresentados em média e erro padrão da média e foram analisados pelo teste ANOVA *two-way* medidas repetidas seguido do *post-hoc* teste de Tukey. * = C vs. PC; & = C vs. K; # = PC vs. PCK; $p<0.05$.

Além dos parâmetros da marcha, a análise das estatísticas das patas mostrou alterações importantes. Um efeito significativo entre os grupos foi observado para o tempo da fase de balanço das patas traseiras [F (3, 72) = 5,905, p = 0,0012]. A análise mostrou que o grupo PC teve um tempo maior de fase de balanço do membro posterior direito em comparação ao grupo controle (C = 0,124 ± 0,005 vs. PC = 0,1877 ± 0,014, p = 0,0113) [F (3, 27) = 5,947, p = 0,0030] (Figura 19B). A velocidade de balanço das patas traseiras apresenta uma redução nas comparações dos grupos [F (3, 72) =

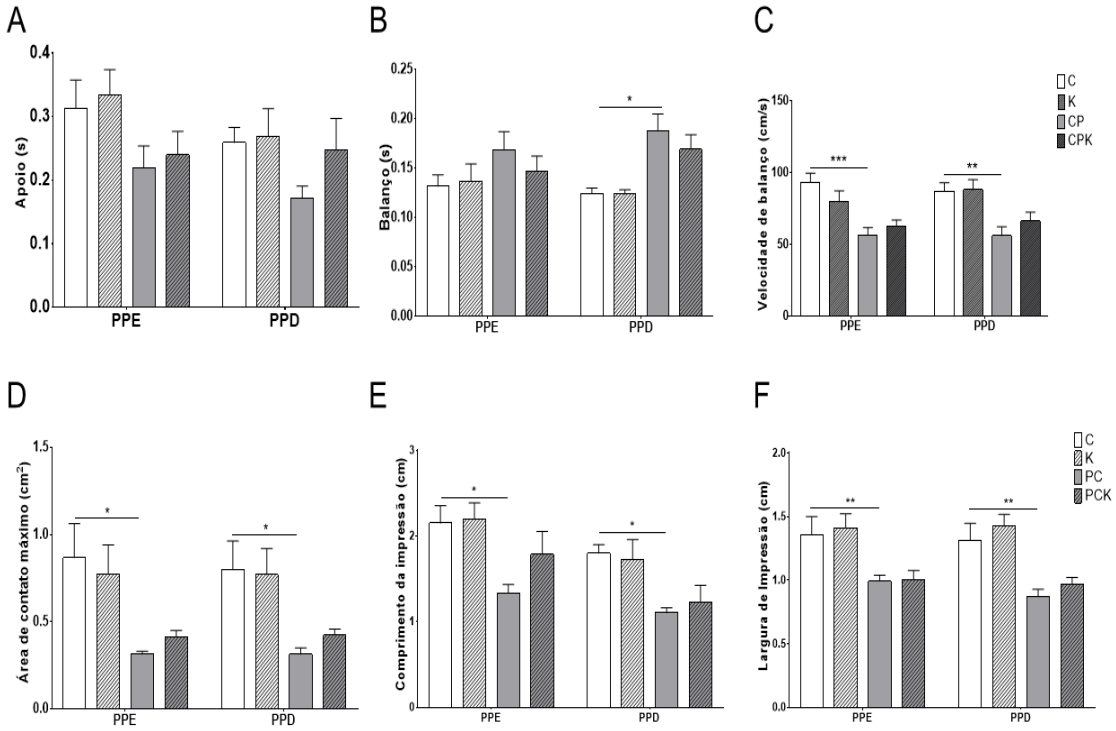
13,59, p <0,0001. Em comparação com o grupo controle, os animais do grupo PC apresentaram velocidade de balanço reduzida da pata traseira esquerda (C = 93,21 ± 6,442 vs. PC = 56,22 ± 5,5, p = 0,0004) da pata traseira direita (C = 87,15 ± 5,76 vs. PC = 56,0 ± 6,223 p = 0,0039) (Figura 19C).

A análise da área de contato máxima das patas posteriores mostrou efeito significativo entre as comparações dos grupos [F (7, 32) = 8,812, p <0,0001]. Os animais do grupo PC mostraram área de contato máxima reduzida da pata posterior esquerda em comparação com o grupo controle (C = 0,866 ± 0,195 vs. PC = 0,3125 ± 0,016, p = 0,0107) [F (7, 32) = 3,649, p = 0,0249] (Figura 19D). Além disso, a área de contato máxima da pata posterior direita foi reduzida em animais do grupo PC (C = 0,797 ± 0,166 vs. PC = 0,3131 ± 0,036, p = 0,0310) [F (7, 32) = 4,423, p = 0,0118] (Figura 19D). Os animais com PC que receberam o tratamento neonatal com Kaempferol mostraram um efeito menor do modelo na área máxima de contato de ambas as patas posteriores em comparação com o controle (PPE: C = 0,866 ± 0,195 vs. PCK = 0,4119 ± 0,0368, p = 0,0501); (PPD C = 0,797 ± 0,166 vs. PCK = 0,4234 ± 0,034, p = 0,1281) (Figura 19D).

Um efeito significativo foi observado entre os grupos para a análise do comprimento das impressões das patas posteriores [F (3, 72) = 8,264, p <0,0001]. Em comparação com o grupo de controle, os animais do grupo PC tiveram o comprimento da impressão reduzida na pata posterior esquerda (PPE: C = 2,151 ± 0,205 vs. PC = 1,335 ± 0,099, p = 0,0114) e na pata posterior direita (PPD: C = 1,801 ± 0,994 vs PC = 1,104 ± 0,058, p = 0,0402) (Figura 19E). Enquanto, não foram observadas diferenças em animais com PC que receberam o tratamento neonatal com kaempferol em comparação com o grupo controle (PPE: C = 2,151 ± 0,205 vs. PCK = 1,786 ± 0,267, p = 0,490) (PPD: C = 1,801 ± 0,994 vs PCK = 1,232 ± 1,191, p = 0,1282) (Figura 19E).

A análise da largura da impressão das patas posteriores revelou um efeito significativo entre os grupos [F (3,72) = 13,12, p <0,0001]. Em comparação com o grupo controle, os animais do grupo PC tiveram largura de impressão reduzida da pata posterior esquerda (PPE: C = 1,361 ± 0,139 vs. PC = 0,994 ± 0,070, p = 0,039) [F (3,27) = 4,816, p = 0,0082] e da pata posterior direita (PPD: C = 1,313. ± 0,135 vs. PC = 0,872 ± 0,056, p = 0,087) [F (3,27) = 7,676, p = 0,0007] (Figura 19F). O grupo de animais que recebeu o tratamento neonatal com Kaempferol não apresentou largura de impressão reduzida das patas traseiras em comparação com o grupo controle (Figura 19F).

Figura 19 – Avaliação dos parâmetros temporais e espaciais da marcha através do CatWalk.



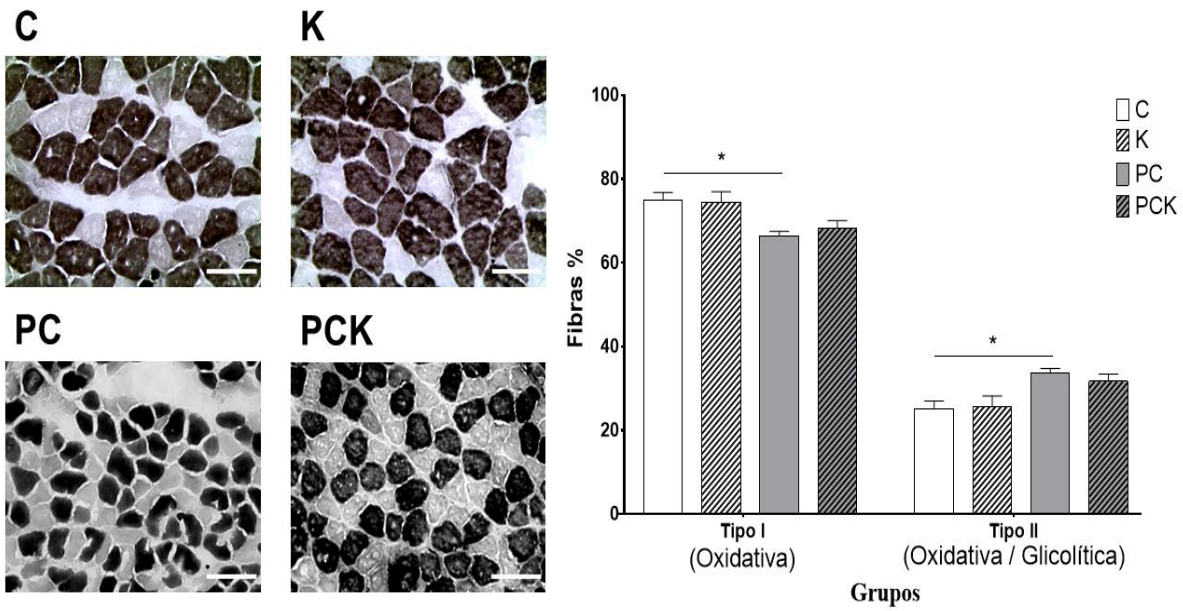
Fonte: o autor (2022). Comparações dos parâmetros temporais e espaciais das patas posteriores dos animais no P33. A- Tempo de apoio das patas posteriores durante a locomoção. B- Tempo durante a fase de balanço nas patas posteriores. C- Velocidade das patas posteriores durante a fase de balanço. D- Medida da área das patas posteriores em contato com a plataforma durante a locomoção. E- Comprimento de impressão das patas posteriores durante a locomoção. F- Largura de impressão das patas posteriores durante a locomoção. A composição dos grupos está de acordo com intervenção com Kaempferol ou veículo e modelo de paralisia cerebral ou controle. C (Controle - veículo, n=10); K (Controle - Kaempferol, n=10); PC (Paralisia Cerebral + veículo, n=10); PCK (Paralisia Cerebral - Kaempferol, n=10). Os dados estão apresentados em média e erro padrão da média e foram analisados pelo teste ANOVA *two-way* medidas repetidas seguido do *post-hoc* teste de Tukey. * = C vs. PC; & = C vs. K; # = PC vs. PCK; p<0.05.

6.2.4 Análise do fenótipo corporal e muscular

No P36, foi observado um efeito significativo para as comparações de peso corporal e comprimento corporal entre os grupos. Os animais do grupo PC apresentaram peso corporal reduzido em comparação ao grupo controle (C vs. PC, p <0,0001) [F (3,33) = 16,86, p <0,0001] (Tabela 3). Além disso, o comprimento corporal foi reduzido no grupo PC em relação ao controle no P36 (C vs. PC, p <0,0001) [F (3,33) = 12,34, p <0,0001] (Tabela 3). O peso absoluto do músculo sóleo apresentou redução no grupo PC em relação ao grupo controle (C vs. PC, p <0,0001) [F (3,15) = 15,99, p = 0,0116] (Tabela 3).

A análise histológica da composição dos tipos de fibra no músculo sóleo revelou uma interação significativa entre os grupos [F (3, 32) = 10,19, p <0,0001]. As comparações múltiplas mostraram que os animais submetidos ao modelo de PC apresentavam uma proporção reduzida de fibra tipo I no músculo sóleo em comparação ao grupo controle no P36 (C = 74,91 ± 1,86 vs. PC = 66,41 ± 1,29, p = 0,0201) e consequente maior proporção de fibra tipo II (C = 25,08 ± 1,86 vs. PC = 33,61 ± 1,13, p = 0,0197) (Figura 20). O tratamento neonatal com kaempferol reduziu o impacto do modelo sobre a proporção das fibras do músculo sóleo (Tipo I: C = 74,91 ± 1,86 vs. PCK = 68,32 ± 1,73, p = 0,1123) (Tipo II: C = 25,08 ± 1,86 vs. PCK = 31,67 ± 1,735, p = 0,1125) (Figura 20).

Figura 20 – Tipagem de Fibras Musculares no músculo Sóleo.



Fonte: o autor (2022). Imagens representativas de secções do músculo sóleo de animais em cada grupo experimental. O gráfico mostra as comparações da proporção das fibras musculares no músculo sóleo no P36. A composição dos grupos é de acordo com a intervenção com Kaempferol ou veículo e modelo ou controle de paralisia cerebral. C (Controle - veículo, n = 5); K (Controle - Kaempferol, n = 5); CP (paralisia cerebral + veículo, n = 5); PCK (Paralisia Cerebral - Kaempferol, n = 5). Barra de escala 25 µm. Os dados são apresentados como a média e o erro padrão da média e analisados usando o teste ANOVA *two-way* seguido pelo teste post-hoc de Tukey. * = C vs. CP; & = C vs. K; # = PC vs. PCK; p <0,05.

As análises morfométricas das fibras do músculo sóleo revelaram efeito significativo entre os grupos para área e perímetro das fibras do tipo I e do tipo II. O grupo PC apresentou área reduzida das fibras do tipo I em comparação com o grupo

controle (C vs. PC, p = 0,0157) [F (3,12) = 9,159, p = 0,0020] (Tabela 3). Além disso, foi observada redução do perímetro das fibras do tipo I do grupo PC em relação ao grupo controle (C vs. PC, p = 0,0101) [F (3,12) = 16,28, p = 0,002] (Tabela 3). Além disso, a área das fibras musculares do tipo II nos músculos sóleo foi reduzida no grupo PC em comparação com o grupo controle (C vs. PC, p = 0,04) [F (3,12) = 6,754, p = 0,0064] (Tabela 3). O perímetro das fibras tipo II no músculo sóleo apresentaram redução no grupo PC em relação ao grupo controle (C vs. PC, p = 0,0046) [F (3,12) = 23,80, p <0,0001] (Tabela 3).

Tabela 3 – Comparação do fenótipo corporal e muscular no P36.

Variáveis	Grupos			
	C	K	PC	PCK
Peso corporal (g)	108.5 ± 4.842	99.31 ± 4.05	**74.25 ± 4.283**$^{\&***}$	76.65 ± 3.224
Comprimento corporal (mm)	148.1 ± 3.677	143.9 ± 2.129	**128.00 ±3.596**$^{\&****}$	131.2 ± 2.569
Peso absoluto do sóleo (g)	0.0853 ± 0.003	0.0831 ± 0.008	**0.043 ± 0.003**$^{\&***}$	0.0606 ± 0.004
Peso relativo do sóleo (%)	0.0828 ± 0.0044	0.0854 ± 0.0097	0.0701 ± 0.0128	0.0798 ± 0.0061
Área das fibras tipo I do sóleo (μm^2)	318.80 ± 26.49	321.30 ± 11.25	**208.20 ± 10.23**$^{\&*}$	211.70 ± 20.55
Perímetro das fibras tipo I do sóleo (µm)	71.42 ± 2.869	74.78 ± 0.795	**59.36 ± 0.952**$^{\&*}$	56.44 ± 2.204
Área das fibras tipo II do sóleo (μm^2)	246.60 ± 24.55	250.40 ± 14.5	**168.50 ± 9.18**$^{\&*}$	161.50 ± 14.16
Perímetro das fibras tipo II do sóleo (µm)	65.65 ± 2.55	68.00 ± 1.01	**54.48 ± 0.639**$^{\&**}$	49.42 ± 1.56

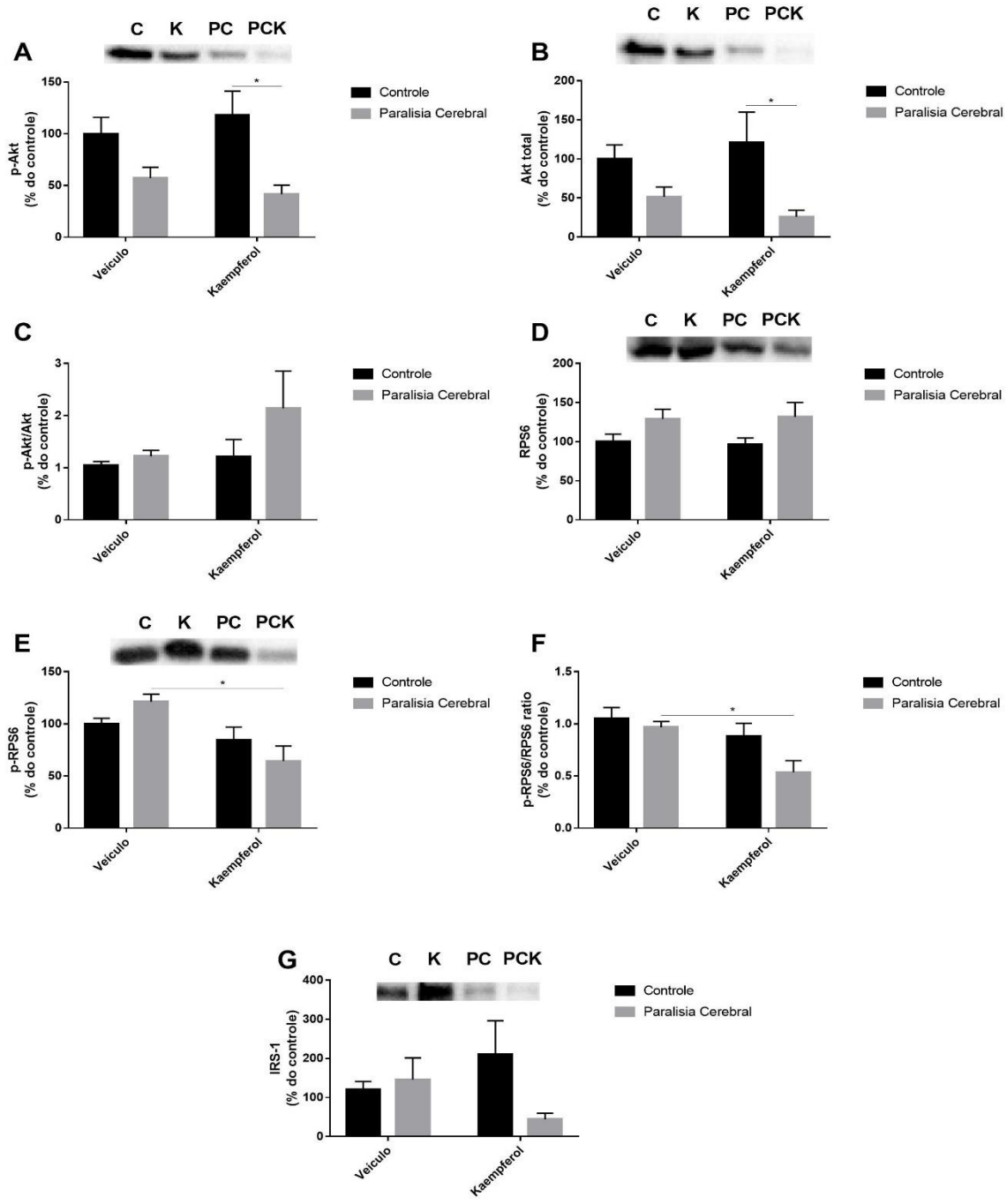
Fonte: o autor (2022). Comparações dos fenótipos corporal e muscular dos ratos que receberam tratamento neonatal com kaempferol ou veículo, e que foram submetidos ou não ao modelo de PC no P36. Os valores estão apresentados como média e erro padrão da média. Os dados foram analisados pelo teste ANOVA *two-way*, seguido do teste de Tukey. & = C vs. PC, # = C vs. K, $ = PC vs. CPK. C (controle - veículo, n = 5), K (controle - kaempferol, n = 5), PC (paralisia cerebral - veículo n = 5) PCK (paralisia cerebral - kaempferol, n = 5) * p <0,05; ** p <0,01; *** p <0,001; **** p <0,0001.

6.2.5 Expressão de proteínas miofibrilares no músculo sóleo

Um efeito significativo entre os grupos foi observado para a análise da p-Akt [F (1, 21) = 16,83, p = 0,0005] e Akt total [F (1, 21) = 16,83, p = 0,0005]. O grupo PCK apresentou redução na expressão de p-Akt em relação aos respectivos animais controle (K = 118,20 ± 23,06 vs. CPK = 41,67 ± 8,82, p = 0,0121) (Figura 21A). Além disso, a Akt total foi reduzida no grupo PCK em comparação com os animais de controle tratados com kaempferol (K = 121,00 ± 38,93 vs. PCK = 25,96 ± 8,447, p = 0,0271) [F (1, 21) = 12,85, p = 0,0017] (Figura 21B).

O tratamento neonatal de kaempferol reduziu a expressão sóleo p-S6 em ratos PCK em comparação com o grupo PC que não receberam o tratamento (PC = 121,40 ± 7,206 vs. PCK = 64,29 ± 14,44, p = 0,0022) [F (1, 21) = 12,82, p = 0,0002] (Figura 21E). Além disso, no grupo PCK, a razão p-S6 / S6 total no músculo sóleo foi reduzida em comparação com o grupo PC não tratado (PC = 0,996 ± 0,057 vs. PCK = 0,5367 ± 0,110, p = 0,0199) [F (1, 21) = 9,35, p = 0,006] (Figura 21F).

Figura 21 – Expressão de proteínas miofibrilares no músculo sóleo.



Fonte: o autor (2022). Efeitos do tratamento neonatal com kaempferol nas vias de sinalização associada à síntese de proteínas no músculo sóleo de ratos com PC. Os conteúdos de: (A) Akt fosforilado; (B)- Akt total; (C)- p-Akt / Akt total; (D)- RPS6; (E)- p-RPS6; (F)- p-RPS6 / RPS6; e (G)- IRS-1 nos músculos sóleo foram determinados por Western blotting. Os resultados são apresentados como média ± S.E.M. com base na carga de proteína total indicada pelas medições Ponceau S, n = 5-8 animais por grupo. Os resultados foram comparados usando ANOVA de dois fatores e teste post hoc de Bonferroni.

7 DISCUSSÃO

Este é o primeiro estudo a avaliar o efeito do tratamento neonatal com Kaempferol no desenvolvimento motor e proliferação da micróglia no hipocampo de ratos expostos ao modelo de PC. Descrevemos o impacto do modelo nos parâmetros de maturação do sistema nervoso, desenvolvimento da atividade locomotora e coordenação usando um modelo de anóxia perinatal e restrição sensório-motora de membros posteriores. Além disso, observamos uma redução na proliferação de células precursoras neurais, um aumento no perfil da micróglia ativada e um aumento na proliferação da micróglia em SGZ e GCL do hipocampo de ratos jovens submetidos ao modelo. A exposição neonatal ao kaempferol atenuou os efeitos do modelo sobre parâmetros de características físicas, maturação do sistema nervoso e desenvolvimento da locomoção. Além disso, evitou o impacto na redução da proliferação de células precursoras neurais no período crítico de desenvolvimento; no aumento do perfil da microglia ativada; e proliferação microglial no giro denteado do hipocampo. Esses resultados sugerem que o kaempferol mitiga o impacto do modelo de paralisia cerebral no desenvolvimento neuromotor, com uma influência significativa no perfil da micróglia e na proliferação de precursores neurais pós-natais.

Na literatura, vários estudos demonstram desenvolvimento motor alterado durante o período neonatal usando modelos de lesão cerebral perinatal em animais. O reflexo do neurodesenvolvimento alterado é uma característica comum da paralisia cerebral e sua ocorrência está relacionada ao início precoce de danos cerebrais (CARVALHO et al., 2019) bem como, os testes de reflexo são úteis na avaliação do grau de maturação neural em mamíferos em desenvolvimento (NGUYEN; ARMSTRONG; YAGER, 2017), e são indicadores confiáveis de desenvolvimento normal (FOX, 1965). Uma característica da paralisia cerebral é uma persistência de reflexos primitivos e atraso no desenvolvimento motor (FEATHER-SCHUSSLER; FERGUSON, 2016). Aqui, mostramos que a anóxia perinatal e a restrição sensório-motora do membro posterior causa persistência na preensão do membro anterior, aquisição tardia de endireitamento, aversão precipício e colocação de vibrissas, bem como em endireitamento em queda livre e geotaxia negativa. Além disso, o modelo utilizado atrasou a maturação das características físicas. Até o momento, nenhum estudo avaliou o efeito do kaempferol nos reflexos do neurodesenvolvimento em modelos de distúrbios neurológicos. Vimos que a exposição precoce ao kaempferol

em ratos submetidos ao modelo PC, evitou o atraso da maturação física e a persistência dos reflexos primitivos em ratos com paralisia cerebral. A razão pela qual o kaempferol previne o reflexo alterado pode estar relacionada às propriedades antioxidantes e anti-inflamatórias do kaempferol na proteção do tecido nervoso e, consequentemente, favorecendo o desenvolvimento do cérebro (WANG et al., 2020).

Neste estudo, observamos que o retardo na aquisição da resposta motora ativa foi associado a déficits no desenvolvimento da atividade locomotora observados em ratos com paralisia cerebral durante o período neonatal. De acordo com estudos anteriores (SILVA et al., 2016), na segunda semana de vida pós-natal em ratos, foi observado um efeito negativo na locomoção e no padrão exploratório em animais com paralisia cerebral. Porém, observamos em ratos com PC que receberam o tratamento neonatal com Kaempferol, maior distância percorrida, melhor velocidade média, menor tempo de imobilidade e exploração periférica em campo aberto em comparação aos não tratados. Além disso, os ratos com PC tratados com kaempferol realizaram melhores testes de equilíbrio e coordenação motora. Esses resultados estão de acordo com um estudo com um modelo de degeneração estriatal em ratos que mostrou que uma injeção intraperitoneal diária de kaempferol atenuou os déficits neurológicos e motores (LAGOA et al., 2009). Esses resultados têm implicações importantes para o efeito do kaempferol no desenvolvimento locomotor em indivíduos com paralisia cerebral, pois existem poucos estudos sobre os benefícios dos flavonoides nos distúrbios do neurodesenvolvimento. Além disso, são necessários estudos que avaliem o perfil de segurança e o desenvolvimento farmacológico para a exposição precoce ao kaempferol em crianças.

Os dados sugerem que polifenóis, como os flavonoides, têm importante implicações metabólica e neurológica em modelos de doenças crônicas não transmissíveis (LACERDA et al., 2021). Estudos com suplementação dietética com alimentos ricos em flavonoides, como mirtilo, chá verde e Ginkgo biloba, levam a resultados significativos na memória espacial e no aprendizado. Além disso, estudos sugerem que os mecanismos que sustentam sua capacidade de induzir melhorias na memória estão ligados ao potencial de flavonoides absorvidos e seus metabólitos para interagir e modular vias de sinalização críticas, fatores de transcrição e expressão de genes e / ou proteínas que controlam a memória e processos de aprendizagem no hipocampo; a estrutura do cérebro onde ocorre a aprendizagem espacial (RENDEIRO et al., 2012; VAUZOUR et al., 2008).

Um estudo mostrou que a suplementação materna com óleo e polpa de abacate (rico em flavonoides) antecipa a maturação reflexa e o desenvolvimento somático pós-natal (MELO et al., 2019), e melhora a memória durante as fases adolescente e adulta (MELO et al., 2019). Além disso, efeitos positivos nas atividades locomotoras e nos reflexos sensoriais motores, bem como no aprendizado e memória da prole exposta a altas doses de extrato de chá verde, no período neonatal foram observados (AJAREM et al., 2017). Em outro modelo, descobriu-se que os flavonoides influenciam positivamente a atividade locomotora (BECKMANN et al., 2014; KE et al., 2016).

Recentemente, destacamos e discutimos a proliferação, migração e diferenciação celular em nichos neurogênicos em modelos de lesão cerebral perinatal (VISCO et al., 2021a). Dependendo do período de tempo da lesão cerebral (pré-natal vs. pós-natal) e do tipo de lesão cerebral, a neurogênese responde de forma diferente ao insulto frente ao desafio do desenvolvimento em uma fase crítica da vida. Danos cerebrais em cérebros muito imaturos afetam a neurogênese, resultando em uma diminuição na geração de células-tronco neurais (VISCO et al., 2021a). Isso contrasta com os modelos em que o dano cerebral foi produzido na primeira semana após o nascimento em roedores, nos quais houve um aumento da proliferação de células precursoras neurais (VISCO et al., 2021a).

Aqui, mostramos pela primeira vez a proliferação de células precursoras neurais no giro denteado do hipocampo usando um modelo de asfixia perinatal e restrição sensório-motora de membro posterior em ratos jovens. No geral, os resultados mostraram células proliferativas reduzidas que sobrevivem na zona subgranular do giro dentado em ratos jovens expostos ao modelo CP. No estudo de Takada et al., (2016) um episódio de anoxia em P1 causou redução do número de neurônios recém-gerados no DG durante a adolescência. Fagel et al., (2006) mostraram que a privação de oxigênio neonatal crônica reduz no período neonatal o número de células precursoras neurais proliferativas em SVZ e hipocampo. A redução da proliferação de células precursoras neurais, aqui observada, pode estar associada ao retardo do desenvolvimento motor em ratos do grupo PC e à redução da massa encefálica. Interessante que Marcuzzo et al., (2010), relataram que a restrição sensório-motora sozinha causa uma redução na célula neuronal no córtex somatossensorial primário. No entanto, poucos estudos destacaram o mecanismo e o padrão de migração celular em modelos de lesão cerebral perinatal.

Os flavonoides podem proteger o cérebro por sua capacidade de modular os sinais intracelulares, promovendo a sobrevivência celular (DAJAS et al., 2003). Como as primeiras semanas do período neonatal são cruciais para o desenvolvimento e maturação do cérebro, a intervenção proposta para a paralisia cerebral deve ser aplicada de forma aguda (STAVSKY et al., 2017a). Nossos resultados sugerem que o tratamento neonatal com kaempferol atenua o número reduzido de células precursoras neurais na zona subgranular e camada de células granulares da DG do hipocampo em ratos expostos à anóxia perinatal e restrição sensório-motora dos membros posteriores. Evidências revelaram que os flavonoides promovem a diferenciação de células precursoras neurais em neurônios e causam uma redução da morte neuronal; o aumento da neurogênese e da plasticidade sináptica na idade adulta, levando ao aumento da função sináptica (MATIAS et al., 2016). Recentemente, um estudo mostrou que o Kaempferol atenua o déficit cognitivo por meio da regulação de antioxidantes e da neuroinflamação (KOUHESTANI; JAFARI; BABAEI, 2018), promovendo retenção de memória e densidade de neurônios no CA1 do hipocampo (DARBANDI et al., 2016).

Nas primeiras semanas após o nascimento, a célula da micróglia tem um papel importante para o desenvolvimento e maturação do cérebro (ANDERSON; VETTER, 2018; SCHLEGELMILCH; HENKE; PERI, 2011, 2011). Além disso, após a lesão cerebral perinatal, a proliferação da micróglia pode, portanto, ser um fator que contribui para a recuperação neurológica (GIRARD et al., 2009). A ativação inicial da micróglia é benéfica para proteger os neurônios, mas o excesso de citocinas inflamatórias pode levar à superativação da micróglia, causando uma cascata de citocinas inflamatórias e inflamação descontrolada (BACHILLER et al., 2018). Nossos resultados mostraram que um modelo de asfixia perinatal e restrição sensório-motora em ratos causou redução da densidade de micróglia no GD, além do aumento da proporção de micróglia ativada em ratos PC. Um estudo com estresse precoce e desafio imunológico mostrou densidade reduzida de micróglia e aumento da proporção de micróglia ativada na região CA3 e no hilo do hipocampo (SAAVEDRA; NAVARRO; TORNER, 2018). Além disso, a ativação da micróglia foi estabelecida como uma característica de lesão cerebral perinatal em vários modelos (MCRAE et al., 1995; TAHRAOUI et al., 2001; TREMBLAY et al., 2017). No dano cerebral perinatal, a micróglia mostra expressão aumentada de marcadores pró-inflamatórios como IL-1β e fator de necrose tumoral alfa (TNF-α). Evidências mostram que a

micróglia na lesão da substância branca promove defeitos de mielinização em neonatos (DOMMERGUES et al., 2003b; ERKENSTAM et al., 2016; YOON et al., 1997).

Nossos resultados mostraram que na ZSG e na CCG do hipocampo, os animais com PC não tratados apresentaram aumento da proliferação de micróglia. Este resultado corrobora com um estudo com modelo de isquemia-hipóxia neonatal em camundongos, onde os autores relataram aumento do número de células BrdU / Iba1^{+} no hipocampo (QIU et al., 2007). O aumento da proliferação da micróglia, pode ser devido à modulação da resposta inflamatória cerebral (LALANCETTE-HEBERT et al., 2007). No entanto, na literatura, poucos estudos avaliam a proliferação da micróglia pós-natal em modelos de paralisia cerebral e como isso contribui afetando a neurogênese (VISCO et al., 2021a). Nesse sentido, a redução na geração de novos neurônios e oligodendrócitos, pode ser associado ao aumento da taxa de proliferação da micróglia no GD.

Evidências experimentais sugerem que a micróglia superativada pode ser extremamente prejudicial após distúrbios neurais agudos. Um estudo mostrou que a ativação microglial prejudica a neurogênese hipocampal adulta após dano tecidual induzido por LPS pré-natal (EKDAHL et al., 2003). Além disso, as evidências sugerem que a micróglia pró-inflamatória está associada à hipomielinização na lesão cerebral perinatal (MCNAMARA; MIRON, 2020). Para isso, os autores sugerem que existem drogas anti-inflamatórias que podem atenuar as respostas microgliais prejudiciais (MCNAMARA; MIRON, 2020).

Diversas evidências mostraram que o bloqueio da ativação da micróglia causa uma resposta de neuroproteção em diferentes modelos de lesão do SNC (COLELLA et al., 2018; MCNAMARA; MIRON, 2020; MONJE, 2003). Após a ablação microglial, houve um aumento no número de células apoptóticas concomitante com o aumento da molécula pró-inflamatórias (LALANCETTE-HEBERT et al., 2007). Devido às propriedades anti-inflamatórias e antioxidantes, os flavonoides têm sido propostos como uma potencial intervenção para doenças neuroinflamatórias (LAN et al., 2017; WANG et al., 2017b). Os flavonoides exercem uma série de ações neuroprotetoras no cérebro, incluindo um potencial para proteger os neurônios contra lesões induzidas por neurotoxinas, uma capacidade de suprimir a neuroinflamação e o potencial de promover a memória, o aprendizado e a função cognitiva (VAUZOUR et al., 2008). Além disso, induzem efeitos benéficos no sistema vascular, levando a alterações no

fluxo sanguíneo cerebrovascular. Estes compostos são capazes de causar angiogênese, neurogênese e alterações na morfologia neuronal (VAUZOUR et al., 2008). Nossos resultados mostraram que o tratamento neonatal de Kaempferol previne a redução da densidade da micróglia em animais com PC e restaura o perfil de repouso da micróglia.

Recentemente, um estudo relatou que o tratamento com kaempferol em um modelo de isquemia / reperfusão atenua a inflamação do cérebro isquêmico por meio da inibição da ativação microglial (LI et al., 2019b)(LI et al., 2019b; PARK et al., 2011). Em modelos de isquemia cerebral, o kaempferol atenuou a neuroinflamação e reduziu os déficits neurológicos (YU et al., 2013). Neste estudo com ratos, os autores relataram que o tratamento com kaempferol após isquemia cerebral, inibe a cadeia pró-inflamatória cerebral, com redução da neuroinflamação e diminuição da micróglia com morfologia ativada (YU et al., 2013). O tratamento com Kaempferol reduziu os danos aos neurônios e axônios em comparação com os controles tratados com veículo. Os autores observaram que o potencial antioxidante do kaempferol protege a apoptose celular no hipocampo e atenua os déficits de memória (EL-KOTT et al., 2020; YU et al., 2013). Além disso, em outro estudo o tratamento com kaempferol reduziu os danos aos neurônios e axônios em comparação com os controles tratados com veículo (YU et al., 2013).

Lan et al (2017) mostraram que a injeção intravenosa de 5 mg / kg de Pinocembrina protege o cérebro do modelo de hemorragia intracerebral em camundongos (LAN et al., 2017). Os autores encontraram redução do volume da lesão, déficits neurológicos de edema cerebral e ativação da microglia suprimida com redução das citocinas pró-inflamatórias. Em outro estudo com um modelo de lesão cerebral traumática em ratos, o pós-tratamento com Pinocembrina diminui a ativação da micróglia de forma aguda, proporcionando proteção cerebral (WANG et al., 2017b).

Mostramos com este estudo que a anóxia perinatal associada à restrição sensório-motora dos membros posteriores durante a infância impacta significativamente o fenótipo corporal de ratos, reduzindo o peso corporal e o comprimento corporal durante o período neonatal. Em estudos anteriores, essas repercussões na antropometria também foram observadas em ratos durante a exposição ao modelo de CP (SILVA et al., 2016, LACERDA et al., 2017, LACERDA et al., 2019; LACERDA et al., 2021). Lacerda et al., (2019) sugerem que o impacto do modelo sobre esses parâmetros pode estar relacionado aos déficits motores que

prejudicam o acesso a lactação e ao alimento. Além disso o modelo compromete a sucção nos primeiros dias de vida pós-natal e a funcionalidade da mastigação após o desmame (LACERDA et al. 2017; 2019). Observamos que o tratamento neonatal com Kaempferol previne a redução do peso corporal e do comprimento corporal principalmente após o término do período de tratamento. Esses efeitos podem estar relacionados aos efeitos benéficos do kaempferol na prevenção do atraso da função motora ativa, favorecendo o consumo da ingestão alimentar (Visco et al. 2022, dados não publicados). Além disso, um estudo mostrou que a administração neonatal de kaempferol aumenta o peso corporal e o crescimento somático na prole exposta a uma dieta materna rica em gordura (CHAVES et al., 2020).

Reforçando o impacto negativo do modelo de CP no fenótipo corporal, relatamos pela primeira vez que a anóxia perinatal e a restrição sensório-motora do membro posterior durante a infância contribuem para déficits globais de força em ratos jovens. Os resultados aqui apresentados mostraram a redução da força média da análise da força de preensão das patas dianteiras em ratos do grupo PC. Fragopoulou et al. (2019), relatam que a inflamação sistêmica neonatal combinada com hipóxia afeta a força de preensão das patas anteriores de ratas adolescentes em comparação com o grupo de controle.

Nossos resultados demonstraram pela primeira vez uma caracterização do padrão de marcha do modelo que combina a anóxia perinatal e a restrição sensório-motora em ratos. A análise pelo Catwalk mostrou que em ratos submetidos ao modelo de PC, a cadência da marcha é afetada, causando descoordenação entre os membros e aumento da base de apoio das patas dianteiras durante a locomoção ativa. Além disso, ratos com PC apresentaram aumento do tempo de balanço das patas e redução da velocidade de balanço das patas posteriores durante a marcha. Além disso, foi observado reduzido contato das patas posteriores durante a fase de apoio na marcha. Akefe, Ayo, Sinalu, et al., (2020) sugerem que o mecanismo pelo qual o kaempferol mitigou a alteração nos parâmetros comportamentais pode ser devido ao seu potencial antioxidante.

Para avaliar as repercussões da anóxia perinatal e da restrição sensório-motora dos membros posteriores durante a infância em ratos, foi realizada a análise histomorfométrica e a avaliação das vias de sinalização associadas à síntese protéica no músculo sóleo de ratos com PC. Observamos redução da massa muscular, redução da proporção de fibras oxidativas e menor área de secção transversal e

perímetro do músculo sóleo de ratos com PC. Este achado é sugestivo de disfunção e retardo da maturação do músculo sóleo que se caracteriza como um músculo oxidativo (em P36 ~ 75% do tipo I) (HOWARD; HERZOG, 2021). Em concordância com nossos resultados, Buratti et al., (2019) observaram em ratos PC redução do peso corporal, peso muscular e comprimento e redução na área e aumento no número de fibras musculares tipo I no músculo plantar (BURATTI et al., 2019). O mesmo modelo de PC encontrou massa muscular reduzida no músculo sóleo e tibial anterior (MARQUES et al., 2014; STIGGER et al., 2011). No presente estudo, encontramos uma redução da área da secção transversal e do perímetro de ambos os tipos de fibras musculares do músculo sóleo. A atrofia muscular é uma característica observada em pacientes com PC, e as fibras do tipo I são mais suscetíveis às alterações decorrentes da inatividade nesses pacientes (MARBINI et al., 2002; WANG; PESSIN, 2013).

O tratamento neonatal com kaempferol reduziu o impacto do modelo CP no peso do sóleo e na área de secção transversal das fibras musculares do tipo I do sóleo. No entanto, surpreendentemente, não observamos redução das vias de sinalização de expressão associadas à síntese de proteínas no músculo sóleo de ratos CP. Por outro lado, os ratos com PC que receberam o tratamento neonatal de Kaempferol apresentaram redução de p-Akt e AKT total em comparação com os animais de controle tratados com Kaempferol. Além disso, em comparação com ratos CP não tratados, foi observada expressão reduzida de p-RPS6 em ratos CP que receberam tratamento neonatal com kaempferol. Em um estudo com camundongos, foi observado que o kaempferol aumentou a atividade de fosforilação no fígado, no entanto, nem as proteínas nem suas atividades foram alteradas nos tecidos musculares esqueléticos (ALKHALIDY et al., 2018).

Apesar de numerosos estudos in vitro e in vivo terem relatado os polifenóis como moléculas bioativas eficazes em atenuar a atrofia muscular e aumentar a saúde muscular, nossos resultados não confirmaram o potencial do kaempferol no aumento da expressão das vias de sinalização associadas à síntese de proteínas no músculo sóleo de ratos com PC.

8 CONSIDERAÇÕES FINAIS

O tratamento neonatal com kaempferol atenuou os déficits de desenvolvimento e maturação do sistema neuromotor, além de prevenir o impacto sobre proliferação de células precursoras neurais, no aumento do perfil de micróglias ativadas e proliferação microglial no giro denteado do hipocampo de ratos jovens submetidos ao modelo de paralisia cerebral. Esses resultados sugerem que o kaempferol previne o impacto do modelo de PC sobre o desenvolvimento neuromotor ao inibir a proliferação desregulada e o estado persistente de ativação da micróglia no giro denteado do hipocampo. Adicionalmente os resultados sugerem que o tratamento neonatal com kaempferol em ratos com PC atenua o impacto do modelo sobre o fenótipo corporal, prevenindo redução significativa do peso e crescimento corporal em ratos jovens. Ainda, foram observados efeitos benéficos da intervenção ao reduzir os déficits de força, e o impacto sobre os parâmetros cinéticos, temporais e espaciais da marcha. Adicionalmente, o tratamento neonatal com kaempferol preveniu a redução de fibras oxidativas no músculo sóleo, bem como diminui o impacto do modelo sobre as medidas histomorfométricas das fibras musculares. O tratamento com kaempferol apresenta potencial como agente neuroprotetor para desordens cerebrais perinatais. Sugere-se que mais estudos sejam realizados para elucidar os mecanismos celulares, moleculares e bioquímicos nos quais o kaempferol afeta de forma benéfica os sistemas nervoso e músculo esquelético.

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APÊNDICE A – "A SYSTEMATIC REVIEW OF NEUROGENESIS IN ANIMAL MODELS OF EARLY BRAIN DAMAGE: IMPLICATIONS FOR CEREBRAL PALSY".

A systematic review of neurogenesis in animal models of early brain damage: implications for cerebral palsy

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ABSTRACT

Brain damage during early life is the main factor in the development of cerebral palsy (CP), which is one of the leading neurodevelopmental disorders in childhood. Few studies, however, have focused on the mechanisms of cell proliferation, migration, and differentiation in the brain of individuals with CP. We thus conducted a systematic review of preclinical evidence of structural neurogenesis in early brain damage and the underlying mechanisms involved in the pathogenesis of CP. Studies were obtained from Embase, Pubmed, Scopus, and Web of Science. After screening 2329 studies, 29 studies, covering a total of 751 animals, were included. Prenatal models based on oxygen deprivation, inflammatory response and infection, postnatal models based on oxygen deprivation or hypoxic-ischemia, and intraventricular hemorrhage models showed varying neurogenesis responses according to the nature of the brain damage, the time period during which the brain injury occurred, proliferative capacity, pattern of migration, and differentiation profile in neurogenic niches. Results mainly derived from rodent studies suggest, that prenatal brain damage impacts neurogenesis and curbs generation of neural stem cells, while postnatal models show increased proliferation of neural precursor cells, improper migration, and reduced survival of new neurons.

Keywords: Neurogenesis. Cerebral Palsy. Animal models.

Introduction

Brain damage during the critical period of development has been associated with movement disorders, behavior deficits and cognitive impairments related to spatial memory and learning (Bunney et al., 2017; Hadders-Algra, 2018; Lyall et al., 2015; Seung et al., 2017; Wee et al., 2017). Early brain damage is related to cerebral palsy (CP), which is a major cause of disability in childhood and results from nonprogressive brain injury before, during or after birth (Shevell, 2019; Velde et al., 2019). Oxygen deprivation and related inflammation are the most common causes of CP, accounting for 23% - 35% of neonatal death worldwide (Lawn et al., 2014; Moshiro et al., 2019). Despite advances in perinatal care, interventions to prevent brain damage and support the recovery process are of limited effectiveness (Hadders-Algra et al., 2017).

The human brain undergoes a number of critical stages of development early in life (Bjornsson et al., 2015). Neural stem cells (NSCs) emerge during the embryonic period and the first few weeks after birth and remain active throughout life (Martínez-Cerdeño et al., 2006). This critical window is the period during which neurons are born and migrate to their final locations and networks are formed (Silbereis et al., 2016). Early postnatal development is also characterized by massive outgrowth of dendrites and axons, followed by synaptogenesis, glial proliferation, and myelination (Silbereis et al., 2016).

In mammals, postnatal neurogenesis persists during development in specific brain areas, known as neurogenic niches which include the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus (DG) of the hippocampus (Lajud and Torner, 2015; Paridaen and Huttner, 2014; Urbán and Guillemot, 2014). The subsequent maturation of neurogenic niches and proper migration of newly generated neurons and their postmitotic differentiation leads to the formation of structures and rapid expansion of the central nervous system (CNS) early in life (Silbereis et al., 2016). The functional importance of this

phenomenon also means that it has implications for behavioral and cognitive development throughout life (Kozareva et al., 2019).

The regenerative capacity of the newborn brain has important implications for future approaches to the treatment of early brain damage that involve regeneration (Donega et al., 2013). However, assessing the capacity of neural stem precursor (NSP) cell proliferation, migration, and differentiation into neurons and glial cells in humans poses great challenges. Furthermore, developmental neurogenesis in individuals with CP has not been fully elucidated and requires further investigation and better characterization of the critical neuroimmunological mechanisms involved in the response of neural tissue to brain development following CP.

Animal models are able to shed important light on the brain injury mechanisms underlying CP (Cavarsan et al., 2019; Hagberg et al., 2002). Such models therefore provide a practical means to obtain fundamental information on central nervous system response, with a special emphasis on the role of pathogenesis, pathophysiological mechanisms, and spatiotemporal events in neurodevelopment (Cavarsan et al., 2019; Clowry et al., 2014). These are also essential for the development of effective interventions to enhance the endogenous regenerative capacity of the immature brain (Cavarsan et al., 2019; Clowry et al., 2014). Given the wide range of risk factors for CP, various animal models, involving oxygen deprivation in utero, intrauterine inflammation/infection, birth asphyxia, intraventricular hemorrhage (IVH) or hypoxic-ischemia (HI) have been developed in a variety of different species as a way of providing information on brain damage and neurobehavioral impairment (Cavarsan et al., 2019; Clowry et al., 2014; Fragopoulou et al., 2019; Hagberg et al., 2002; Lacerda et al., 2017; Lacerda et al., 2019; Silva et al., 2016; Wilson, 2015).

In the immature murine brain, a transition occurs from proliferative and multipotent NSPs to fully differentiated neurons and glias, including astrocytes and oligodendrocytes (Urbán and Guillemot, 2014). There has, however, been little discussion of the mechanisms

underlying cell proliferation, migration, and differentiation in individuals with CP. While the processes leading to recovery are still not fully understood, one possible mechanism may involve the brain's innate ability to continuously generate new neurons in postnatal neurogenic niches (Jin, 2016).

Postnatal neurogenesis may be able to mitigate the damage caused by the injury, although the new brain cells that are produced in response to injury are not capable of fully replacing the damaged neurons (Niimi and Levison, 2018). Furthermore, they do not promote long-term survival of these newly generated neurons (Niimi and Levison, 2018). Depending on the time period during which perinatal brain injury occurred, different neurogenesis responses may be related to the generation of new NSP cells, migration, or differentiation (Amrein et al., 2004; Taylor et al., 2013).

In view of differences in the pathogenesis of CP and the diversity of animal models of brain damage prior to birth, during birth or during the neonatal period, we conducted a systematic assessment of all literature related to neurogenesis mechanisms in CP models as a way of obtaining comprehensive knowledge of the responses of the neurogenic niche to cell proliferation, the pattern of migration, and the eventual fate of cells. Evidence gathered from a systematic review of animal models is also valuable for achieving improved extrapolation to humans. This may in future serve as a guide for the development of interventions to bring about "replacement", "reduction" and "refinement" in animal experiments and to improve the accuracy of preclinical research.

METHODS

Systematic Review Report and Protocol Registration

The present systematic review followed the recommendations of the PRISMA statement for transparent reporting of systematic reviews and meta-analyses (Liberati et al.,

2009). The protocol adopted for this systematic review has been registered on the International Prospective Register of Systematic Review (PROSPERO) (Registration nº CRD42019121645) and can be consulted in full (Visco et al., 2020a).

Search strategy

Studies in animals that reported structural neurogenesis after experimental model of CP (Leenaars et al., 2012) were taken from Embase (1947-2020), PubMed (1966-2020), Scopus (1969-2020) and Web of Science (1900-2020). All searches were performed up to August 2020. The search strategy was performed using Mesh, keywords, and synonyms appropriate for each database using the following terms: cerebral palsy AND neurogenesis AND animals (Table 1). An 'animal studies" search filter was applied in Pubmed. The lists of references of included studies were also consulted to find additional reports. The full search strategy for each database can be found in the dataset (Visco et al., 2020b).

Table 1. Standard terms used in search strategy.

Search Strategy	
Component	Terms / Boolean operators
CP Brain Injury Models	(cerebral palsy) OR (cerebral palsy, spastic, diplegic) OR (cerebral palsy, spastic quadriplegic) OR (spastic diplegia) OR (spastic cerebral palsy) OR (palsy, cerebral) OR (brain palsy) OR (brain paralysis) OR (central palsy) OR (encephalopathia infantilis) OR (perinatal asphyxia) OR (perinatal asphyxia encephalopathy) OR (anoxia) OR (fetal hypoxia) OR (hypoxic-ischemic encephalopathy) OR ((hypoxia, brain AND neonatal)) OR ((hypoxia-ischemia, brain AND fetal)) OR (neonatal stroke) OR (leukomalacia, periventricular) OR ((white matter damage AND infant)) OR (inflammatory-mediated white matter damage) OR (lipopolysaccharide exposure) OR (cerebral intraventricular hemorrhage) OR (inflammatory-mediated white matter damage) OR (infant white matter damage) OR (fetal infection OR (hindlimb immobilization AND anoxia) OR (sensorimotor restriction)
	AND
Neurogenesis	(neurogenesis) OR (cell differentiation) OR (neuron) OR (neural regeneration) OR (neurotization) OR (neural stem cell) OR (nervous system development) OR (nerve regeneration) OR (neural progenitor cell) OR (neural/stem progenitor cell) OR (neural recovery)
	AND
Animals	Laboratory animals Search Filters and terms (de Vries et al., 2011; Hooijmans et al., 2010; Leenaars et al., 2012)

Note: the terms used varied according to the requirements of the database.

Elegibility

We included all studies of neurogenesis identified in an experimental model of CP, using inclusion and exclusion criteria in phase 1 (title and abstract) and phase 2 (full-text screening) as follows: phase 1 – Criteria for exclusion, 1- Not an original article; 2- Not a cerebral palsy article; 3- Not an animal study. In phase 2, the exclusion criteria applied during the screening were: 4- Genetically modified animal; 5- Non-controlled group comparison; 6- Outcome not related to neurogenesis reported; 7- *In vitro* assessment; 8- Treatment exposure of any kind; 9- Full text not available (Table 2).

Table 2. Inclusion and exclusion criteria.

Inclusion Criteria	Exclusion Criteria
Participants: o Animals.	Participants: o Humans. o Genetically modified animals.
Exposure: o Models of Cerebral Palsy.	Exposure: o Brain injury after perinatal/neonatal period. o Treatment exposure of any kind
Control: o Sham.	Control: o Studies without control group.
Outcomes: o Assessment of neurogenesis.	Outcomes o *In vitro* assessment of neurogenesis.
Study type: o Original data. o Full-text available.	Study type: o Not original data (e.g reviews, editorial).

For inclusion as an assessment of neurogenesis, the study was required to report quantified structural markers measuring cell proliferation, migration, or differentiation. The characteristics of each marker used by the studies included are described in Table 3. (Bernal and Arranz, 2018; Dehmelt and Halpain, 2004; Eng, 1985; Gleeson et al., 1999; Harris et al., 2018; Kim et al., 2017; Korzhevskii and Kirik, 2016; Kucharova, Karolina and Stallcup, 2011; Lang et al., 2013; Lee, 1997; Lind et al., 2005; Lo et al., 1991; Ross and Hall, 1995; Seki and Arai, 1999; Strzalka and Ziemienowicz, 2011; Zhang and Jiao, 2015). Discrepancies were resolved after discussion between the two authors (VISCO and ROMERO) or were referred to a third author (TOSCANO).

Table 3. Markers used to measure cell proliferation, migration and differentiation.

MARKER	DESCRIPTION
Bromodeoxyuridine (BrdU)	A synthetic thymidine analog that incorporates DNA of dividing cells during the S-phase of the cell cycle
5-ethynyl-2′-deoxyuridine (EdU)	A thymidine analog incorporated into the DNA of dividing cells
Nestin	A class VI intermediate filament protein known as an NSP cell marker during development of the central nervous system
Antigen Ki-67 (Ki-67)	A DNA-binding protein expressed in all active phases of the cell cycle
Phosphohistone H3 (pHH3)	A nuclear core histone protein of DNA chromatin, which plays an important role in chromosome condensation and cell-cycle progression during mitosis and meiosis
Proliferating Cell Nuclear Antigen (PCNA)	A DNA clamp that acts as a processivity factor for DNA polymerase δ in eukaryotic cells and is essential for replication
Polysialylated form of the Neural Cell Adhesion Molecule (PSA-NCAM)	Participates in neural plasticity and neurogenesis and is involved in cell migration
Doublecortin (Dcx)	A microtubule-associated protein, expressed extensively by migrating neurons, exclusively in postmitotic neurons during periods of migration
Neuronal nuclear antigen (NeuN)	A DNA-binding protein that is abundantly and exclusively expressed in neurons
Neurogenic Differentiation 1 (Neuro D)	A transcription factor of the NeuroD-type found to regulate multiple genes involved in cell cycle progression, cell-fate determination and cellular differentiation
Neuron-specific enolase (NSE)	A cell-specific isoenzyme of the glycolytic enzyme enolase, a highly specific neuron marker
Glial Fibrillary Acidic Protein (GFAP)	an intermediate-filament protein expressed uniquely in astrocytes
Tubulin beta III (TuJ1)	A tubulin thought to be involved specifically during differentiation of immature neuronal cell types
Achaete-scute homolog 1 (MASH1)	A protein that plays a role in neuronal commitment and differentiation
Microtubule-associated protein 2 (MAP-2)	A neuron-specific protein that stabilizes microtubules in the dendrites of postmitotic neurons
Calcium binding protein β (*S100β*)	A protein detected in a subgroup of specific postmitotic astrocytes
Chondroitin sulfate proteoglycan (NG2)	A marker for oligodentrocyte progenitors cells
Tumor suppressor Adenomatous Polyposis Coli (APC)	A protein that regulates oligodendrocyte differentiation
Ionized calcium binding adaptor molecule 1 (IBA1)	A *microglia/*macrophage-specific calcium-binding protein

Data extraction

Data extraction was performed by two researchers using a piloted form (Visco et al., 2020b). The following details were extracted from each study: 1- publication year and name of first author; 2- characteristics of animals used (species, sex, number of animals per group); 3- characteristics of cerebral palsy model (period and type of injury); 4- region of brain assessed; 5- outcome measurement using information on proliferation, migration, and differentiation (the specific marker of structural neurogenesis assessed, the timing of outcome assessments and the corresponding p-values); 6- mean and standard deviation for each control and treatment group; 7- statistical method used and 8- descriptive results. If data were missing, we tried to contact the author to obtain more specific information.

Assessment of methodological quality

Two authors independently assessed the methodological quality of the articles included, using Syrcle's Risk of Bias (RoB) tool (Hooijmans et al., 2014). This tool is based on Cochrane's RoB tool and has been adjusted for aspects of bias that play a specific role in animal studies (Hooijmans et al., 2014). Syrcle's RoB tool for animal studies contains 10 items. These items concern selection bias (1- sequence generation; 2- baseline characteristics, and 3- allocation concealment), performance bias (4- random allocation housing of animals and 5- blinding of animal caregivers and researchers), detection bias (6-random outcome assessment and 7- blinding of outcome assessor), attrition bias (8-incomplete outcome data), reporting bias (9- selective outcome reporting) and 10- other sources of biases (Hooijmans et al., 2014). A "yes" judgment indicates a low risk of bias; a "no" judgment indicates a high risk of bias; an "unclear" judgment indicates that insufficient details have been reported to provide a proper assessment of the risk of bias (Hooijmans et al., 2014). Discrepancies were resolved by discussion between the two authors (VISCO and ROMERO) or were referred to a third author

(TOSCANO). RevMan v.5.3 software was used to create the 'risk of bias summary' figure and 'risk of bias' graph.

Statistical analysis

The Kappa statistic for the interobserver agreement of inclusion criteria was calculated using GraphPad QuikCalcs.

RESULTS

Study Selection

We identified 2329 potentially relevant records from four main databases (Embase= 516; Pubmed=283; Scopus = 914; Web of Science = 616), of which 2014 articles remained after removal of duplicates. After examination of the title and abstract, 1273 further articles were excluded for at least one of the following reasons: (1) not an original article, n=344; (2) not cerebral palsy article, n= 534; or (3) not an animal study, n= 395. Finally, 741 articles underwent full-text analysis and a total of 712 were excluded for the following reasons: (1) used genetically modified animals, n = 44; (2) no comparisons with control animals, n =7; (3) no neurogenesis measurement, n = 530; (4) in vitro assessment, n = 42; (5) treatment exposure of any kind, n = 60; (6) full text not available, n = 29. Ultimately, 29 studies were included in this systematic review for qualitative assessment (Figure 1). The authors' judgments regarding inclusion criteria showed substantial agreement according to the Kappa Statistic (Kappa = 0.687, 95% CI [0.509-0.865]).

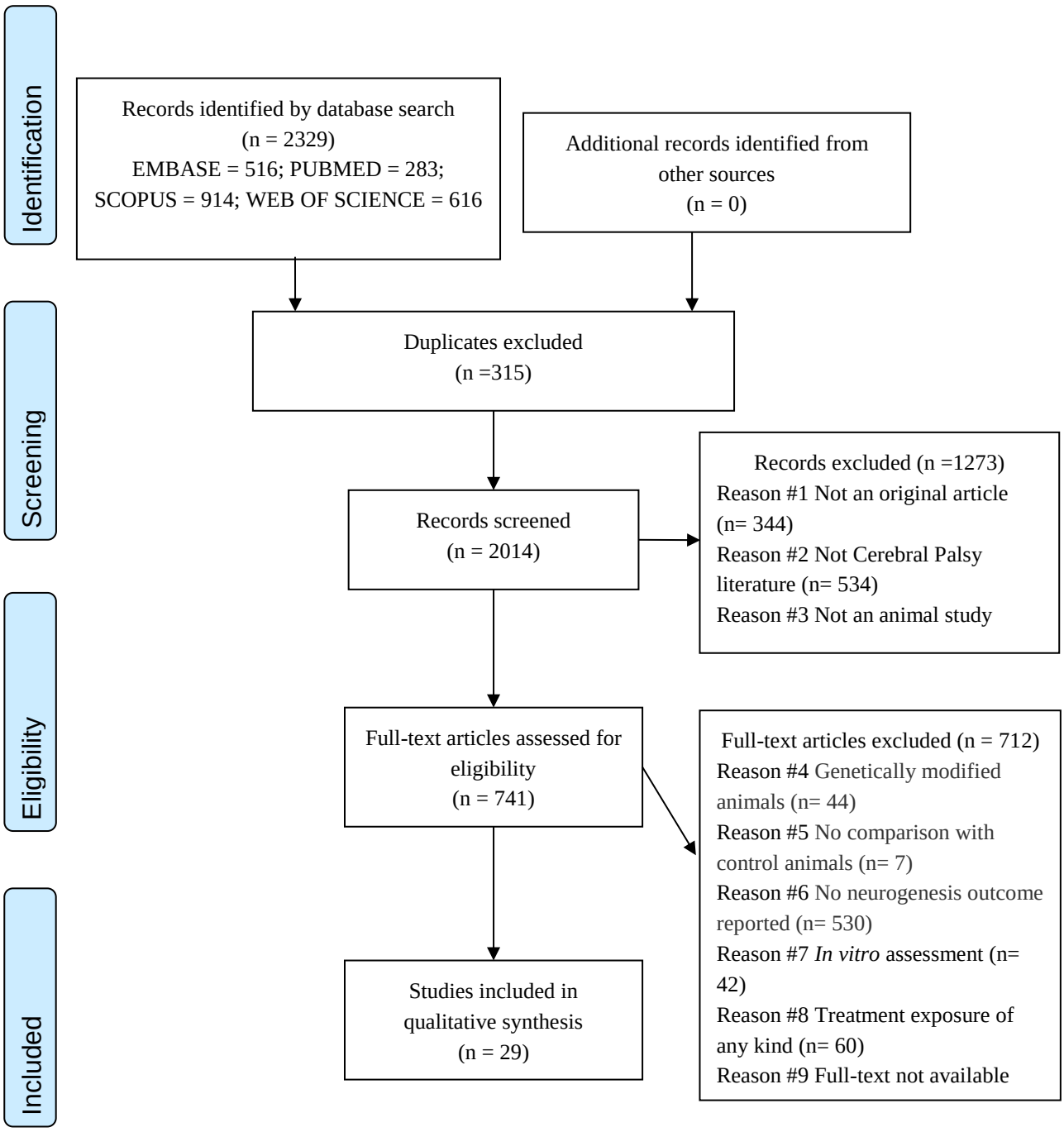

Figure 1. PRISMA flow diagram of study selection process.

Study characteristics

The 29 studies included covered a total of about 751 animals. The vast majority of the studies were performed in rodents. Six studies used Wistar rats (Felling et al., 2006; Graciarena et al., 2010; Hayashi et al., 2005; Ikeda et al., 2005), four studies C57BL/6 mice (Al Mamun et al., 2018; Dawes et al., 2017; Qiu et al., 2007; Schneider et al., 2012), seven used Sprague-Dawley (SD) rats (Chung et al., 2019; Daval et al., 2004; Lin and Wang, 2014; Ong et al., 2005; Pang et al., 2016; Schmitt et al., 2013; Yin et al., 2014), four CD-1 mice (Kadam et al., 2008; Plane et al., 2004; Segado-Arenas et al., 2018; Xue et al., 2003), and one was conducted using Spiny mice (Fleiss et al., 2011). Three studies did not specify the strain of rodent used (Jiang et al., 2012; Sanchez et al., 2012; Waddell et al., 2016) and one study was performed using guinea pigs (Chung et al., 2015). One study used piglets (Ara and De montpellier, 2013) and another ewes (Gussenhoven et al., 2018).

Early brain damage in animals was replicated by creating different types of brain lesions in prenatal, perinatal, or postnatal models. The majority of studies used neonatal rodent models to assess the outcome of interest after brain damage. The specific models of early brain damage used in the literature were: oxygen deprivation in utero in three studies (Chung et al., 2015; Fleiss et al., 2011; Morales et al., 2008); an inflammation- or infection-based model in five studies (Graciarena et al., 2010; Gussenhoven et al., 2018; Jiang et al., 2012; Lin and Wang, 2014; Pang et al. 2016); neonatal oxygen deprivation in seven (Ara and De montpellier, 2013; Daval et al., 2004; Fagel et al., 2006; Sanchez et al., 2012; Schmitt et al., 2013; Schneider et al., 2012; Takada et al., 2016); intraventricular haemorrhage in three (Dawes et al., 2017; Segado-Arenas et al., 2018; Xue et al., 2003); and postnatal ischemia or hypoxia-ischemia in eleven (Al Mamun et al., 2018; Chung et al., 2019; Felling et al., 2006; Hayashi et al., 2005; Ikeda et al., 2005; Kadam et al., 2008; Ong et al., 2005; Plane et al., 2004; Qiu et al., 2007; Waddell et al., 2016; Yin et al., 2014). All of the 29 studies included assessed at least one type

of cell proliferation marker, one assessed an outcome related to specific cell migration markers (Felling et al., 2006), while 19 studies assessed outcomes related to cell differentiation markers (Al Mamun et al., 2018; Ara and De montpellier, 2013; Daval et al., 2004; Dawes et al., 2017; Fagel et al., 2006; Felling et al., 2006; Graciarena et al., 2010; Hayashi et al., 2005; Ikeda et al., 2005; Jiang et al., 2012; Kadam et al., 2008; Lin and Wang, 2014; Morales et al., 2008; Ong et al., 2005; Qiu et al., 2007; Sanchez et al., 2012; Schneider et al., 2012; Segado-Arenas et al., 2018; Takada et al., 2016). Figure 2 presents a schematic representation of NSP cell proliferation and neuron differentiation and gives the time period during which brain damage occurred for each model used in the studies included.

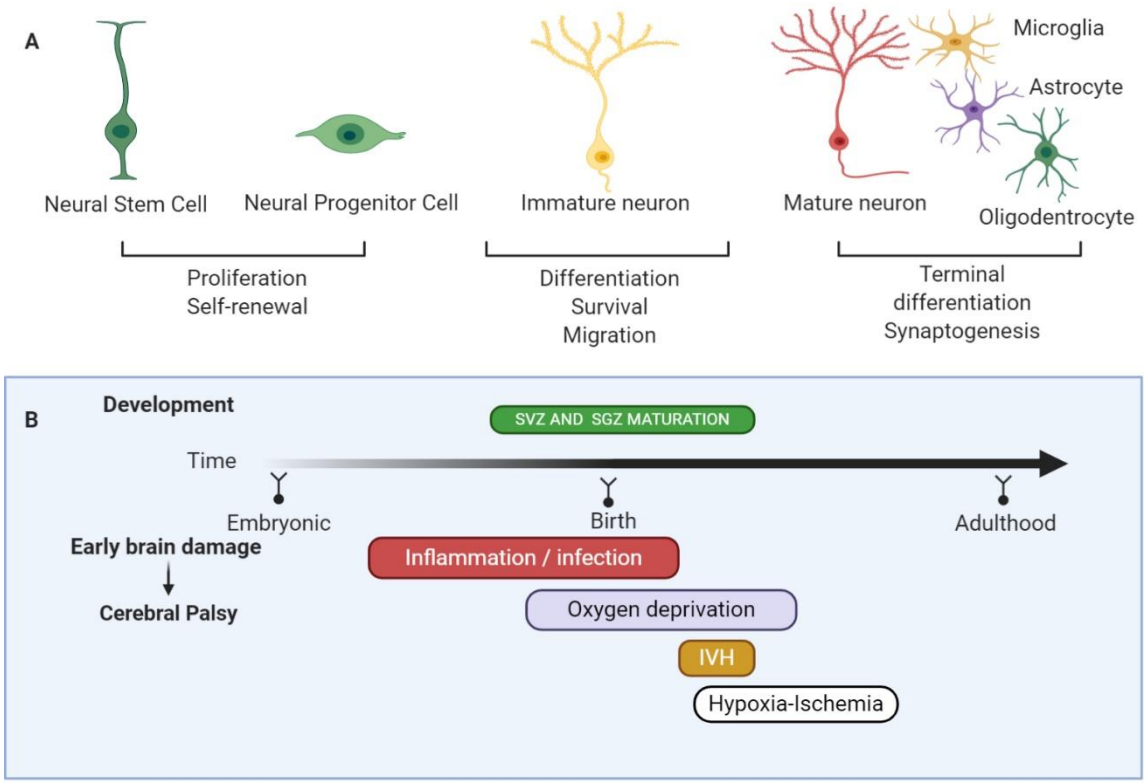

Figure 2. A) Schematic representation of neural stem/progenitor cell proliferation and self-renewal for fully mature neuron differentiation, survival, proper migration, terminal differentiation, and synaptogenesis with the contribution of astrocytes, oligodentrocytes and microglia. B) The critical window for SVZ and SGZ maturation during the embryonic period and postnatal life, and the time periods of brain damage in each animal model in studies included; SVZ: Subventricular Zone; SGZ: Subgranular Zone; IVH: Intraventricular Hemorrhage.

Neurogenesis outcomes in studies included

Prenatal oxygen deprivation models

Oxygen deprivation in utero represents one of the various mechanisms originating cerebral palsy (Dong et al., 2012; Gunn and Bennet, 2009; Perlman, 2006). Cell proliferation in the hippocampus and in the SVZ have been shown to be unaltered in preterm guinea pigs after being submitted to the chronic placental insufficiency model (Chung et al., 2015). In mice, uterine hypoxia has been shown to cause hippocampal functional deficits but without structural changes related to cell proliferation ($PCNA^+$) (Fleiss et al., 2011). Term asphyxia in utero, on the other hand, has been shown to cause increased cell proliferation ($BrdU^+$) in the DG of neonatal (P7) and young (P30) Wistar rats (Morales et al., 2008). Furthermore, significant increases in BrdU/MAP-2^+ cells have been found in the CA1, suprapyramidal band, infrapyramidal bands, and in the hilus of the dentate gyrus of perinatal asphyxia-exposed rats compared to the control group in P7 (Morales et al., 2008) (Table 4).

Early-life inflammation- or infection-based models

Intrauterine infections and early-life inflammation have been associated with the development of cerebral palsy (Girard et al., 2009; Jiang et al., 2018; Minagawa et al., 2002). Intra-amniotic exposure to LPS causes a significant reduction in the mitotic cells of the hippocampus ($pHH3^+$) two days before birth in ewes (Gussenhoven et al., 2018). After chronic LPS during the fetal period, a reduced hippocampal proliferative capacity has been found by assessment of $BrdU^+$ cells in young and adult rats (Graciarena et al., 2010). A single intraperitoneal injection of LPS during the second week of gestation has been found to cause reduced cell proliferation in infant and adult SD rats (Lin and Wang, 2014), while, an intrauterine infection model was found to cause increased BrdU/$nestin^+$ in the DG of the hippocampus in rats in the neonatal period (P3, P7, and P14) (Jiang et al., 2012). Furthermore,

LPS exposure in newborn SD rats has been shown to increase cell proliferation (Ki-67$^+$) in the SVZ and DG of the hippocampus in P21 (Pang et al., 2016) (Table 4).

Maternal LPS exposure during gestation causes a reduction in immature neurons (Dcx$^+$) in the SGZ and granular layers of the hippocampus in both P21 and P90 compared to control SD rats (Lin and Wang, 2014), while no differences in BrdU/Dcx$^+$ were observed in the same period (Lin and Wang, 2014). In P67, the number of Dcx$^+$ cells in the SGZ was lower compared to the control group after chronic maternal LPS exposure during gestation in Wistar rats (Graciarena et al., 2010). Likewise, the number of BrdU/Dcx$^+$ cells was significantly lower in exposed rats compared to the control group in the same period (Graciarena et al., 2010). This same study reported reduced numbers of BrdU/NeuN$^+$ cells in the SGZ of rats during adulthood (Graciarena et al., 2010). In another study, no differences in the proliferation of neurons (BrdU/NeuN$^+$) and astrocytes (BrdU/GFAP$^+$) were found in the rat hippocampus in P28 in the LPS group compared to the control (Jiang et al., 2012) (Table 4).

Neonatal oxygen deprivation models

Oxygen deprivation in newborns is a leading cause of infant brain damage, causing long-term neurodevelopmental deficit and representing an important cause of cerebral palsy (Ellenberg and Nelson, 2013; Phelan et al., 2005). Hypoxia occurs when a newborn receives inadequate oxygen leading to brain injury (Fatemi et al., 2009). Acute hypoxia in newborn SD rats (P0) has been found to cause an increase in cell proliferation (BrdU$^+$) in the hippocampus and in the SVZ in P20 (Daval et al., 2004). However, a model involving chronic hypoxia after birth (P4-P8) found no increases in cell proliferation (BrdU$^+$) in the DG of the hippocampus nor in the SVZ in P13 and P39 in SD rats (Schmitt et al., 2013). In C57/B1 mice, chronic hypoxia (P3-P11) caused a reduction in BrdU$^+$ cells at P11, but an increase in the same cells in P18 in the DG and the SVZ (Fagel, et al., 2006) (Table 4).

One study in piglets found that, one week after recovery (P7) from birth hypoxia, there were an increased number of Dcx+ cells with a migrating neuroblast profile in the striatum of animals undergoing preconditioning (PC) hypoxia and associated with HI (PC+HI) compared to those submitted to normoxic conditions (Ara and De montpellier, 2013). Furthermore, the proportion of BrdU/Dcx^{+} cells was significantly higher in PC and in PC+HI compared to normoxic animals (Ara and De montpellier, 2013). In this same study, an increased number of proliferative neuronal stem/progenitor cells (BrdU/nestin^{+}) and astrocytes (BrdU/GFAP^{+}) was reported in P7 in the SVZ (Ara and De montpellier, 2013) (Table 4).

In neonatal C57/Bl6 mice, the number of immature neurons (Dcx^{+}) increased in the hippocampus in both acute or chronic models of hypoxia, with a different response following periods of reoxygenation (Schneider et al., 2012). Another study showed that, during childhood (P40), there were an increased number of Dcx^{+} cells in the dentate gyrus after a single hypoxia episode in P10 in rats (Sanchez et al., 2012). However, in P60, rats showed a reduced number of BrdU/NeuN^{+} cells in the dentate gyrus after hypoxia damage in P1 in Wistar rats (Takada et al. 2016) (Table 4).

Intraventricular hemorrhage models

Intraventricular hemorrhage (IVH) is a common complication of preterm birth that may lead to a prognosis of cerebral palsy (Gotardo et al., 2019; Radic et al., 2015). In models involving autologous injection of blood into the periventricular tissue of mice, a reduced number of proliferative cells (Ki-67^{+}) has been found in the SVZ 8h and 7d after the procedure (Xue et al., 2003). By contrast, a few days after intracranial injection of autologous blood in P0, an increased number of proliferative marker EdU+ cells was found in the SVZ in P4 (Dawes et al., 2017). This increase is accompanied by greater proliferation of EdU/MASH1^{+} and astrocytes (Edu+/GFAP^{+}) in the SVZ in P4 in C57BL/6 mice (Dawes et al., 2017). In another study, intracerebroventricular injection of collagenase VII in CD-1 mice in P7 induced a long-

term reduction in the number of proliferative cells ($BrdU^+$) and Dcx^+ cells in the ipsi- and contralateral hippocampus compared to control animals (Segado-Arenas et al., 2018). There was also a reduction in Dcx^+ cells in the ipsilateral SVZ compared to the control group (Segado-Arenas et al., 2018). Likewise, the ipsilateral DG and SVZ showed a reduction in BrdU/Dcx^+ cells in P70 (Segado-Arenas et al., 2018) (Table 4).

Postnatal ischemia or hypoxia-ischemia models

Hypoxia-ischemia during the critical period of development is an important cause of cerebral palsy and associated disabilities in children (Evans et al., 2001; Kurinczuk et al., 2010; Martinez-Biarge et al., 2011). The studies covered here included hypoxic-ischemic models generated between P6 and P12 in models involving rats and mice, and one study tested the model involving HI in P21. Increased cell proliferation was reported at different time points during the infancy period. The number of $BrdU^+$ cells in Wistar rats has been found to be increased a few days after lesions in P8 (Felling et al., 2006), and P12 in the SVZ (Waddell et al., 2016). Increased cell proliferation ($BrdU^+$) has been found in SVZ in P14 in Wistar rats (Hayashi et al., 2005; Ikeda et al., 2005). Likewise, seven days after HI, an increase in the number of hippocampal $BrdU^+$ cells has been reported in P17 in rats (Waddell et al., 2016). After the neonatal period, the quantity of BrdU+ cells in the SVZ was found to have increased by P21 and P28 in rats (Hayashi et al., 2005; Ikeda et al., 2005; Ong et al., 2005) and by P24 in mice (Plane et al., 2004). Even after a chronic period of recovery, increased cell proliferation has been found in the SVZ in P30 in C57BL/6 mice (Al Mamun et al., 2018) and in P42 in rats (Ikeda et al., 2005). However, in a model using only the permanent ligation of the right common carotid artery, the numbers of $BrdU^+$ cells in the hippocampus declined, but had increased in the SVZ by P40 in mice (Kadam et al., 2008). Later in life, in P45 and P57, a reduction in the number of Brdu+ cells in the hippocampus has been found after HI was performed in C57/BL6 mice in P9 or P21 respectively (Qiu et al., 2007) (Table 4).

Nestin, a marker of neuronal precursor cell proliferation, was found in increased quantities in the SVZ and in the hippocampus after HI in P10 (Yin et al., 2014), P14 (Chung et al., 2019), and in P18, P21 and P28 in rats (Yin et al., 2014). PCNA/nestin^{+} cells and nestin/PSA-NCAM/Ki-67^{+} were also found in increased numbers in the SVZ of rats (Felling et al., 2006). After several days of hypoxic-ischemia, the contralateral SVZ has been found to present an abundance of cells expressing PSA-NCAM, while a lack of PSA-NCAM^{+} cells was found in the ipsilateral hemisphere, in P8 (Felling et al., 2006). In the same study, an increased number of Dcx^{+} cells with a migrated neuroblast profile were found to have migrated from the SVZ, three weeks after recovery from HI, in the ipsilateral striatum, in rats (Felling et al., 2006). Another study showed that, seven days after HI, the newly produced cells migrated to the damaged area (Hayashi et al., 2005). Dcx^{+} cells were distributed in the peri-infarcted area, mainly in the striatum close to the lateral ventricle (Hayashi et al., 2005). Likewise, BrdU/Dcx^{+} cells were found in the peri-infarcted area in the brains of Wistar rats (Hayashi et al., 2005). Increased numbers of Dcx^{+} cells were found in the SVZ of SD rats in response to HI in P14, P21, and P28 (Ong et al., 2005) (Table 4).

Studies have reported and quantified a number of cell differentiation parameters. Even after a longterm period of recovery (5 weeks) subsequent to HI, surviving BrdU/NeuN^{+} cells have been found in the ipsilateral striatum of Wistar rats (Felling et al., 2006). Conversely, cell staining with BrdU/NeuN^{+} and -GFAP^{+} has revealed sparse cell labelling only 14 days after HI in the ipsilateral cortex, but not at 28 and 42 days after HI. NeuN and GFAP account for 1% and 4.6% of BrdU^{+} cells , respectively, after 14 days of HI in rats (Ikeda et al., 2005). An increased number of BrdU/GFAP^{+} cells has been reported in P21 in SD rats (Ong et al., 2005) and in P40 in CD-1 mice (Kadam et al., 2008). Furthermore, BrdU/NG2^{+} cells have been found to be increased in P14 and P21 in the SVZ of Wistar rats (Hayashi et al., 2005). Larger numbers of BrdU/NG2^{+} cells have been found in P40 in the SGZ and SVZ of CD-1 mice (Kadam et al.,

2008). An investigation of the differences between males and females for HI outcomes have revealed a larger number of BrdU/NeuN^{+} cells in the ipsilateral lesion in females compared to male mice, indicating more active neurogenesis in females in response to brain HI in P30 (Al Mamun et al., 2018). Increased proliferative microglias were found in the SVZ in P7 in post-HI injury acute response in Wistar rats (Hayashi et al., 2005). However, after a long-term period subsequent to HI, the number of BrdU/IBA1^{+} cells was found to be increased in the DG of C57/BL6 mice (Qiu et al., 2007). At this age, increased numbers of BrdU/ACP^{+} and BRDU/ S100β^{+} cells have also been reported in the hippocampus of C57/BL6 mice (Qiu et al., 2007) (Table 4).

Table 4. Characteristics and main findings of the studies included.

Study (year)	Species (sex, n per groups)	Model characteristics	Region of interest	Neurogenesis	Outcome Index	Intergroup differences (time)
Prenatal oxygen deprivation models						
Chung et al. (2015)	Dunkin–Hartley guinea pigs (sex NS; Control or experimental at GD 50 or 60; n= 8 per group).	At GD 30–32, maternal blood vessels of the uterine horn were ligated at the cervical end of the arterial cascade	SGZ and SVZ	Proliferation	1. ↔$PCNA^+$	1.p>0.05 (GD 50 and 60).
Fleiss et al. (2011)	Spiny mice (male and female; Control or birth asphyxia, n=6 per group)	At GD 37, the fetuses became hypoxic and acidemic within the excised uterus for 7.5 min	Hippocampus (DG)	Proliferation	1. ↔$PCNA^+$	1.p>0.05 (P5)
Morales et al. (2008)	Wistar rats (male; control or perinatal asphyxia, n = 6 per group)	At GD 22, the fetuses were asphyxiated by immersing the uterine horns in a water bath at 37º C for 20 min	Hippocampus (DG; CA1)	Proliferation Differentiation	1. ↑$BrdU^+$ 2. ↑BrdU/$MAP\text{-}2^+$	1. p<0.005 (P7, P30) 2. p<0.005 (P7)
Early-life Inflammation-- or infection-based models						
Graciarena et al. (2010)	Wistar rats (male; Control, or LPS, n=5-6 per group)	Maternal sc injection of LPS (0.5 mg/kg) or saline on GD 14, 16, 18, and 20.	DG (SGZ)	Proliferation Differentiation	1. ↓$BrdU^+$ 2. ↓Dcx^+ 3. ↓BrdU/Dcx^+ 4. ↓BrdU/$NeuN^+$	1.p<0.001 (P67); p<0.05 (P90) 2.p<0.05(p67) 3.p<0.01 (P67) 4.p<0.01 (P67); p<0.05 (P90)
Gussenhoven et al. (2018)	Ewes (male and female; control or maternal LPS, n=6-7 per group)	Intra-amniotic injection of 10mg Escherichia coli-derived LPS at 5, 12, or 24 h or 2, 4, 8, or 15 days before pre-term delivery	Hippocampus	Proliferation	1. ↓ $pHH3^+$	1. 0.05<p<0.100 (2 days after LPS)
Jiang et al. (2012)	Rats (sex and strain NS; control group n =55, LPS n=55)	At GD 15, maternal endocervical injection of 0.4 ml of E. coli suspension.	Hippocampus (DG)	Proliferation Differentiation	1. ↑$BrdU^+$ 2. ↑BrdU/$Nestin^+$ 3. ↔BrdU/$NeuN^+$ 4. ↔BrdU/$GFAP^+$	1. p<0.05 (P3, 7, 14) 2. p<0.01 (P7) 3. p>0.05 (P28) 4. p>0.05 (P28)
Lin et al. (2014)	Sprague-Dawley rats (male; control or LPS, n=24 per group)	Pregnant rats receive a single intraperitoneal (i.p.) injection of LPS at gestational day 10	Hippocampus (SGZ and DG granular layer)	Proliferation Differentiation	1. ↓ $BrdU^+$ 2. ↓Dcx^+ 3. ↔BrdU/Dcx^+	1.p<0.01 (P21, 90) 2.p<0.01 (P21, 90) 3.p>0.05 (P21, P90)
Pang et al. (2016)	Sprague-Dawley (male	At P3, i.p. injection of LPS (E. coli,	SVZ and DG	Proliferation	1. ↑ $Ki\text{-}67^+$	1. p<0.01 (P21)

	and female; control and LPS, n=8 per group)	serotype O55:B05) at 1 mg/kg				

Neonatal oxygen deprivation in neonatal period

Ara and De Montpellier (2013)	Piglets (female; control normoxic, hypoxic preconditioned, hypoxic–ischemic, hypoxic preconditioned + hypoxic–ischemic; n=4-5 per group)	At P1 female piglets were placed in a plexiglass chamber and exposed to hypoxia (8% O_2/92% N_2) for 3 h. For hypoxic-ischemia, animals were subjected to 5% FiO_2 for 40 min with 10 min hypotension	SVZ	Proliferation Differentatio n	1. ↑Dcx^+ 2. ↑$BrdU/Dcx^+$ 3. ↑$BrdU/GFAP^+$ 4. ↑$BrdU/Nestin^+$	1. p<0.05 (P7) 2. p<0.05 (P7) 3. P<0.05 (P7) 4. p<0.01 (P7)
Daval et al. (2014)	Sprague-Dawley rats (sex NS; control or hypoxia; n = 5 per group)	Neonates were placed in a chamber for 20 min with 100% N_2 at P0	SVZ	Proliferation Differentiatio n	1. ↑$BrdU^+$	1. p<0.01 (P20)
Fagel et al. (2006)	C57/B1 mice (sex NS, control and hypoxia, n= 7 per group)	Chronic hypoxia (P3-11) in an airtight Plexiglas chamber, maintained at a 9.5–10.5% O_2 concentration	SVZ and Hippoca mpus (DG)	Proliferation Differentiatio n	1. ↓$BrdU^+$ 2. ↑ $BrdU^+$ 3. ↔$BrdU/NeuN^+$	1. p<0.05 (P11) 2. p< 0.01 (P18) 3.p>0.05
Sanchez et al. (2012)	Rats (strain and sex NS; Control, n=6, hypoxic n=7, hypoxic-seizure, n=7)	In P10, rats were placed individually in a small cage within an airtight chamber, at 33–34°C, and exposed to 100% N_2	Hippoca mpus (DG)	Differentiatio n	1. ↑Dcx^+	1. p<0.05 (P40)
Schaeffer et al. (2013)	Sprague–Dawley rats (male; control or hypoxia, n = 20 per group)	Chronic, repeated hypoxia (11 % O_2, 89 % N_2) was imposed from P4 to P8 by placing animals in an air-tight plastic chamber for 6 h per day	Hippoca mpus (DG) and SVZ	Proliferation	1. ↔$BrdU^+$	1. p>0.05 (P13; P39)
Schneider et al. (2012)	C57/Bl6 wild-type mice (sex NS; control, acute hypoxia, chronic hypoxia, n = 5-6 per group)	Acute hypoxia: P0 exposure to acute systemic hypoxia with FiO_2 of 8% O_2 for 6h following períod of reoxygenation (24h, 72h, 7d) Chronic hypoxia: pregnant and neonatal mice (P7)	Hippoca mpus (DG and CA3)	Differentiatio n	1. ↑Dcx^+ acute Dcx acute (p0+7d reoxygenation) 2. ↑Dcx+ chronic (GD 14-20+72 reoxygenation)	1.p<0.05 (P7 CA3) 2. p<0.05 (P3 DG)

		were kept under continuous hypoxia using FiO_2 of 10% O_2 for 7 d starting on GD 14 (intrauterine hypoxia) or P7 respectively				
Takada et al. (2016)	Wistar rats (male; control, n=5 or anoxia, n=5)	P1 exposure to continuous flow (3 L/min) of 100% N_2 for 25 min at 37ºC in non-hermetic chamber	Hippocampus (DG)	Proliferation Differentiation	1. ↓BrdU/NeuN$^+$	2.p<0.001(P60)
Intraventricular hemorrhage models						
Dawes et al. (2017)	C57BL/6 mice (sex NS; control n= 4 or intraventricular haemorrhage n= 5)	P0 stereotactic intracranial injection of autologous blood	SVZ	Proliferation Differentiation	1. ↑EdU$^+$ 2. ↑EdU/MASH1$^+$ 3. ↑EdU/GFAP$^+$	1. p<0.001 (P4) 2. p<0.05 (P4) 3. p<0.05 (P4)
Segado-Arenas et al. (2017)	CD-1 mice (male and female, control and experimental group Col 0.1 and experimetal group Col 0.3), n = NS)	In P7, offspring subjected to lesion by collagenase VII (Col) intracerebroventricular (icv) infusion	SVZ and SGZ	Proliferation Differenatiation	1. ↓BrdU$^+$ 2. ↓Dcx$^+$ 3. ↓BrdU/Dcx$^+$ in DG	1. p<0.05 (P70) 2. p<0.05 (P14) 3.p<0.01 (P14); p<0.05 (P70)
Xue et al., (2003)	CD-1 mice (sex NS; control, sham control, experimental, n=4-5 per group at each time point)	In P1, injection of autologous blood into the periventricular tissue.	SVZ	Proliferation	1. ↓Ki-67$^+$	1. p<0.05 (8h to 7d)
Postnatal ischemia or hypoxia-ischemiaModels						
Al Mamun et al., (2018)	Wide-type C57BL/6 mice (male and female; control and HI, n= 6 per group)	In P10, right common carotid artery ligation + exposure to 90% N_2 for 60 min.	SVZ	Proliferation Differentiation	1. ↑BrdU$^+$ 2. ↑BrdU/NeuN$^+$	1. p<0.05 (P30) 2. p<0.05 (P30)
Chung et al. (2019)	Sprague-Dawley rats (sex NS; control and HI, n = 18 per group)	In P7, right carotid artery + hypoxic (8% O_2 and 92% N_2 for 2 h.	SVZ	Proliferation	1. ↑ Nestin$^+$	1. p<0.05 (P14)
Felling et al. (2006)	Wistar rats (sex NS, control or HI, n = 6 per group)	In P6, permanent right common carotid ligation + exposure to of 8%	SVZ	Proliferation Migration Differentiation	1. ↑BrdU$^+$ 2. ↑PCNA$^+$ 3. ↔PCNA/Nestin$^+$ 4. ↔BrdU/GFAP$^+$	1. p<0.05 (P8) 2.p<0.001 (P8) 3.p<0.002 (P8)

		O_2 / 92% N_2 for 90 min			5. ↔BrdU/S100$^+$ 6. ↑Nestin/PSA-NCAM/Ki67$^+$ 7. ↑BrdU/Dcx$^+$ 8. ↑BrdU/NeuN$^+$	4 and 5. p>0.05 (P8) 6.p>0.05 (P8) 7.p<0.05 (3w) 8.P<0.05 (5w)
Hayashi et al. (2005)	Wistar rats (sex NS; Control, n = 3 and HI, n = 4-5)	In P7, left common carotid artery sectioned + exposure to hypoxia (8% O_2 and 92% N_2) for 2h	SVZ	Proliferation Differentiation	1. ↑BrdU$^+$ 2. ↑BrdU/Dcx$^+$ 3. ↔BrdU/GFAP$^+$ 4. ↑BrdU/NG2$^+$ 5. ↑BrdU/IBA1$^+$	1. p<0.01 (P14,21,28) 2. p<0.01 (P14,21) 3. p>0.01 4. p<0.01 (P14,21) 5. p<0.01 (P7)
Ikeda et al. (2005)	Wistar rats (sex NS; control and HI. n=6 per group)	In P7, left common carotid artery sectioned + hypoxia (8% O_2, 92% N_2) for 2h	SVZ	Proliferation Differentiation	1.↑BrdU$^+$ 2. ↔BrdU/NeuN$^+$ 3. ↔BrdU/GFAP$^+$	1. p<0.01 (P14, 28, 42) 2. p>0.05 3. p>0.05
Kadam et al (2008)	CD-1 mice (male and female; Control n= 10; experimental, n=11)	In P12, permanent right common carotid artery	SGZ; (CA3; CA1, DG); SVZ	Proliferation, Migration, Differentiation	1. ↓BrdU$^+$ (DG) 2. ↑BrdU$^+$ (SVZ) 3. ↔BrdU/NeuN 4. ↑BrdU/GFAP 5. ↑BrdU/NG2	1. p<0.01 (P40) 2. p<0.01 (P40) 3. p>0.05 (P40) 4. p<0.01 (P40) 5. p>0.05 (P40)
Ong et al. (2005)	Sprague-Dawley rats (male and female; control n=5-6, hypoxia, n=5-6, ischemia-hypoxia, n=11-12)	In P7, right common carotid artery ligation + 8% O_2/balanced N_2 for 90 min.	SVZ	Proliferation Differentiation	1. ↑ BrdU$^+$ 2. ↑ Dcx$^+$ 3. ↔BrdU/NeuN$^+$ 4. ↑ BrdU/GFAP$^+$	1. p<0.02 (P21) 2. p<0.05 (P14; 21;28) 3. p>0.05 4. p<0.05 (P21)
Plane et al. (2004)	CD-1 mice (sex NS; Control, n=8; hypoxia, n=9; hypoxia-ischemia, n=13)	In P10, right common carotid artery ligation + exposure to 10% O_2 balanced N_2 for 45 min.	SVZ	Proliferation	1. ↑ BrdU$^+$	1. p<0.01 (P24)
Qiu et al. (2007)	C57/BL6 mice (male; control and HI, n=13 per group)	In P9 or P21, left common carotid artery ligation + exposure to 10% O_2 in N_2 for ~30 min in chamber at 36ºC	Hippocampus (CA1-4 and DG)	Proliferation Differentiation	1. ↑ BrdU$^+$ 2. ↑ BrdU/NeuN$^+$ 3. ↑ BrdU/IBA1$^+$ 4. ↑ BrdU/APC$^+$ 5. ↑BrdU/ S100β$^+$	1. p<0.001 (P45; P57) 2. p<0.05 (P57) 3. p<0.001 (P57) 4. p<0.001 (P57) 5. p<0.01 (P45; P57)
Waddell et al. (2016)	Rats (strain NS; male and female; control,	In P10, ligation of right carotid artery + exposure to 8% O_2-92% N_2 in water	Hippocampus (CA1,	Proliferation	1. ↑ BrdU$^+$	1. p<0.05 (P12; P17)

	n=28 and HI, n = 26)	bath (37° C) for 60 min	CA3 and DG)			
Yin et al. (2014)	Sprague-Dawley rats (sex NS; control, hypoxic and hypoxic-isquemic groups; n=3-4 per group).	In P7, double ligation of the left carotid artery + exposure to airflow mixture of 8% O_2 and 92% N_2 for 2.5h	Hippocampus and SVZ	Proliferation	1. ↑ $Nestin^+$	1. $p<0.05$ (P10; P14;P21;P28)

N, number of animals; NS, not specified; P, postnatal day; GD, gestational day; DG, dentate gyrus, CA, Cornu Ammonis; FiO_2, inspired oxygen fraction; w, week; ↑, increased; ↓, reduced; ↔, no change.

Study Quality Assessment

The methodological quality of studies included was assessed using Syrcle's RoB tool (Hooijmans et al., 2014) (Figure 3). Six of the 29 studies presented low risk of bias for random sequence generation (Graciarena et al., 2010; Gussenhoven et al., 2018; Jiang et al., 2012; Takada et al., 2016; Waddell et al., 2016; Yin et al., 2014). Eighteen studies presented low risk for the baseline charactistics of the animals (Daval et al., 2004; Fagel et al., 2006; Fleiss et al., 2011; Graciarena et al., 2010; Gussenhoven et al., 2018; Jiang et al., 2012; Kadam et al., 2008; Lin and Wang, 2014; Morales et al., 2008; Ong et al., 2005; Pang et al., 2016; Plane et al., 2004; Qiu et al., 2007; Schneider et al., 2012; Segado-Arenas et al., 2018; Takada et al., 2016; Xue et al., 2003; X. Yin et al., 2014). Fourteen studies presented low risk of bias for allocation concealment (Ara and De montpellier, 2013; Chung et al., 2015; Daval et al., 2004; Dawes et al., 2017; Fleiss et al., 2011; Graciarena et al., 2010; Gussenhoven et al., 2018; Lin and Wang, 2014; Morales et al., 2008; Schmitt et al., 2013; Takada et al., 2016; Waddell et al., 2016; Yin et al., 2014). No included studies reported random housing allocation. Three studies presented low risk for blinded caregivers and researchers (Al Mamun et al., 2018; Chung et al., 2015; Jiang et al., 2012). Eleven studies presented low risk of bias for random outcome assessment (Daval et al., 2004; Fagel et al., 2006; Felling et al., 2006; Graciarena et al., 2010; Ikeda et al., 2005; Kadam et al., 2008; Ong et al., 2005; Plane et al., 2004; Sanchez et al., 2012; Schaeffer et al., 2013; Yin et al., 2014). Thirteen studies presented low risk for blinding of outcome

assessessment (Al Mamun et al., 2018; Chung et al., 2015; Fagel et al., 2006; Fleiss et al., 2011; Graciarena et al., 2010; Gussenhoven et al., 2018; Jiang et al., 2012; Kadam et al., 2008; Ong et al., 2005; Plane et al., 2004; Sanchez et al., 2012; Schneider et al., 2012; Xue et al., 2003). One study presented high risk for attrition bias (Daval et al., 2004) and, finally, all the studies were found to be free of bias for incomplete outcome assessment and other sources of bias. A 'risk of bias' percentage graph covering all studies included is presented in Figure 3A. The 'risk of bias' summaries for individual studies are presented in Figure 3B.

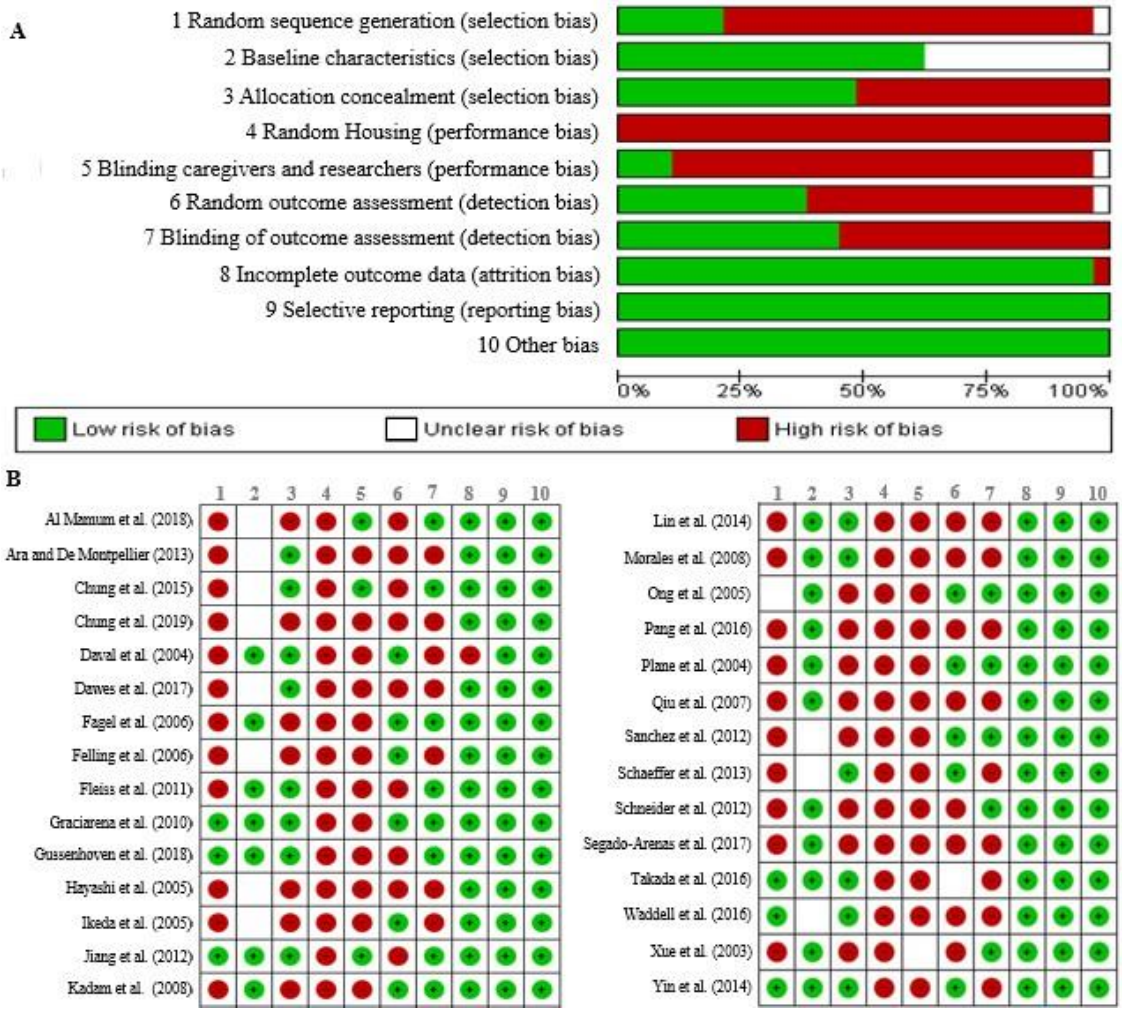

Figure 3. Quality assessment of studies using Syrcle Risk of Bias tool. A) 'Risk of bias' graph: review authors' judgements regarding each 'risk of bias' item presented as percentages for all included studies as a whole. B) 'Risk of bias' summary: review authors' judgements regarding each 'risk of bias' item for each study included.

Discussion

This is the first study to systematically review and summarize the current findings on the structural neurogenesis response utilizing models involving animals with early brain damage related to the etiology of cerebral palsy. We have described the temporal structural changes relating to markers of cell proliferation, migration, and differentiation subsequent to brain damage occurring during the critical period of development. The studies selected revealed a different neurogenesis response in models that were potentially relevant to a CP-like phenotype throughout life.

Overall, the evidence presented here indicates that prenatal brain damage impacts neurogenesis, resulting in a decrease in generation of neural stem cells. This contrasts with models in which brain damage was produced in the early postnatal period, in which there was an increased proliferation of neural precursor cells. This may be related to the compensatory mechanism of the neurogenic niche, which is capable of restoring brain structure at the same neuronal rate. However, such mechanisms are insufficiently effective in the SVZ and in the hippocampus, and, in the long-term, the greater proliferation of neuronal precursor cells does not result in more extensive survival of neuron cells, diminishing the capacity of the immature brain's repair mechanism.

Models of oxygen deprivation in the pre-, peri-, or postnatal period show an altered cell proliferation response during infancy. In HI models in the neonatal period, however, the response involving cell proliferation and neuronal and glial differentiation was substantially improved in neurogenic niches during infancy and adulthood. Meanwhile, models involving inflammation, infection and ventricular hemorrhage, in general, present discrepant results in relation to cell proliferation and migration, and neuronal and glial differentiation, even in adulthood. The pattern of cell migration after brain damage in the neurogenic niches in these models requires further more detailed investigation. Nevertheless, the various approaches

adopted in terms of model and period of brain damage mean that interpretation of these results can shed important light on understanding of the mechanism of neurogenesis involved in the pathophysiology of cerebral palsy.

The perinatal period represents a critical window for neurogenesis and brain development. Over the years, animal models of early brain damage have been developed to simulate the various aspects of neurodevelopmental disorders in humans, as a way of providing better understanding of the underlying pathophysiology and exploring potential treatments (Cavarsan et al., 2019; Clowry et al., 2014). Interpretation of the results for neurogenesis require consideration of variables such as the type of brain lesion, the areas affected, the period of brain damage, in addition to differences between species, strains and sexes, all of which are important factors (Cavarsan et al., 2019; Netto et al., 2017). The majority of studies included in the present review were performed in rodents, with only two involving the use of large mammals. Furthermore, the relation between sex and differences in neurogenesis was poorly explored in this group of studies.

Systematic reviews of preclinical evidence have been reported to facilitate extrapolation of animal research to humans (Hooijmans and Ritskes-Hoitinga, 2013; Ritskes-Hoitinga et al., 2014). While brain maturation landmarks in rodents and large mammals do show differences, various studies have indicated that maturational states are broadly equivalent across animal species, which could greatly facilitate the provision of greater understanding of the related mechanisms (Finlay and Darlington, 1995; Knoth et al., 2010; Workman et al., 2013). Studies have shown that the E18 and E21 rat brain corresponds to that of the human embryo in weeks 8-9 and weeks 15-16 after fertilization respectively (Pressler and Auvin, 2013). In rodents, hippocampal neurons are produced from the dentate neuroepithelium from E13.5 onwards (Urbán and Guillemot, 2014). The formation of the hippocampus is mostly developed by E20 in rodents and by around 20 weeks of gestation in humans (Bayer, 1980;

Gómez and Edgin, 2016). However, significant volumetric development continues to occur up to P21 in rodents and for up to two years in humans (Hevner, 2016).

Furthermore, a similar pattern has been found when comparing adult hippocampal neurogenesis in humans and rodents (Knoth et al., 2010). Models of brain damage in the embryonic and neonatal periods impact brain development at a critical period that performs a specific function in terms of neural plasticity (Ciric et al., 2019). The models used to simulate CP and perinatal brain damage successfully reproduce the brain lesion mechanisms, the pattern of cell death in white and gray matter, and behavioral impairment (Lacerda et al., 2017; Morales et al., 2008; Rumajogee et al., 2016; Silva et al., 2016; Stavsky et al., 2017).

At present, there is increasing evidence regarding a sex-dependent response to brain damage during infancy. Clinical and experimental findings have indicated that males are more susceptible to cerebral palsy and other neurodevelopmental disorders than females (Chounti et al., 2013; Johnston and Hagberg, 2006). Studies have also noted that there are sex differences in behavior and cellular mechanism after brain injury during the perinatal period (Arteni et al., 2010; Netto et al., 2017). It has been reported that neonatal males exhibit a different pattern of cell proliferation in neurogenic niches from that of females (Bowers et al., 2010; Zhang et al., 2008).

These sex-related differences in neurogenesis early in life may have implications for the understanding of cerebral palsy throughout life and for the development of intervention strategies. However, only one study included here investigated the influence of sex differences on neurogenesis after early brain damage, making it difficult to draw any general conclusions in this regard. There is a need for further investigation of the influence of sex on neurogenesis in models of perinatal brain damage. Examination of the advantages and disadvantages observed in each sex during intervention strategies may also pave the way for the development of more specific treatments.

As cerebral palsy is a multifactorial disorder, with various pathophysiological mechanisms (Stavsky et al., 2017; Velde et al., 2019), it is difficult to establish one single representative animal model for CP. However, animal models of early brain damage, such as perinatal hypoxia, inflammation, infection, intraventricular hemorrhage, ischemia, in isolation or in combination, have greatly contributed to our understanding of cerebral palsy and continue to play an important role in preclinical testing of new treatments (Clowry et al., 2014; Hagberg et al., 2002; Rojas et al., 2013).

The immature brain can reorganize its structure to recover from or compensate for injury during the critical period of development (Donega et al., 2013; Kitagawa et al., 2001; Urbán and Guillemot, 2014). The brain's response to injury during early life development maintains a balance between the activation of neurodestructive components and endogenous protective mechanisms (Bossenmeyer-Pourié et al., 2002; Walton et al., 1999). In early-life brain damage, there is a decrease in dendritic spine density in the hippocampus, reduced arbor maturation, and disturbance of synapse formation in the cortical neurons (Back et al., 2001; McClendon et al., 2014; Rojas et al., 2013). This also leads, in the long-term, to inhibition of regeneration, predisposing the brain to additional injuries, such as myelin deficits, thereby contributing further to abnormal brain development (Fleiss and Gressens, 2012). The correct functioning of cell proliferation, migration, and subsequent differentiation mechanisms in the neurogenic niches is essential for normal brain development and cognitive function after injury (Pramparo et al., 2010; Wynshaw-Boris et al., 2010).

After an injury, the behavior of NSP cells is strictly regulated in both time and space in order to achieve normal brain development, (Fuentealba et al., 2012). This is achieved by establishing a balance between intrinsic cell mechanisms and microenvironmental factors (Fuentealba et al., 2012). Increased cell loss, increased oxidative stress and disrupted expression of growth and differentiation factors in the neurovascular niche as a consequence of brain

lesions may explain repair of the impaired tissue (Pfisterer and Khodosevich, 2017; Wang et al., 1998). However, the neurogenesis that occurs as a result of neural stem/precursor cells and the survival of newly differentiated cells contributes to self-repair after neuron loss and functional recovery (Jin et al., 2003; Nakatomi et al., 2002).

Furthermore, cell migration and network formation are both characteristics of the neonatal brain and important factors in brain regeneration after injury (Teixeira et al., 2012), It would appear that most of the progenitors that migrate postnatally from neurogenic niches in response to injuries in most cases either become interneurons or glial cells or do not survive in the long term (Ong et al., 2005; Plane et al., 2004; Teramoto et al., 2003; Yang et al., 2007). It is also possible that the vasculature might not only serve as a guide for migration but also provide support for survival, differentiation, and thereby long-term neurogenesis in non-neurogenic structures (Palmer et al., 2000; Teixeira et al., 2012).

Yang and Levison (2007) found that that ~ 1/3 of the newly produced cells migrating out of the SVZ after HI differentiated into neurons, 1/3 differentiated into astrocytes, and 1/3 became oligodendrocytes. Hayashi et al. (2005), however, suggested that some of the newly produced cells might migrate to the damaged area and attempted to differentiate into neuron cells. Likewise, some cells proliferating in the first week appear to migrate from the SVZ to the damaged striatum to differentiate into phenotypically mature striatal neurons (Arvidsson et al., 2003; Felling et al., 2006; Parent et al., 2002).

The SVZ contains a population of stem cells and more mature progenitors of neurons, astrocytes, and oligodendrocytes (Goldman, 1995; Lois and Alvarez-Buylla, 1993). These cells do not express the proteins that distinguish mature neurons or glias. Normally, neural progenitors from the SVZ migrate along the rostral migratory stream into the olfactory bulb, where they differentiate into interneurons (Lois and Alvarez-Buylla, 1993; Luskin, 1993). However, after injury, some of these cells migrate toward damaged areas and differentiate into

both neurons and glias (Arvidsson et al., 2003; Ong et al., 2005; Zhang et al., 2001).The SVZ of rats is known to expand in size during acute recovery from neonatal HI with an increase in tripotential neural progenitors, followed later by an increase in the production of new neuroblasts, neurons, astrocytes, oligodendrocyte precursors, and oligodendrocytes (Alagappan et al., 2013; Back et al., 2001; Bain et al., 2010; Dizon et al., 2010; Felling et al., 2006; Ong et al., 2005; Plane et al., 2004; Yang and Levison, 2007; Zaidi et al., 2004).

Inflammation also plays an important neuroregulatory role during early development (Belarbi and Rosi, 2013; Musaelyan et al., 2014) Evidence suggests that neurogenesis is affected by aspects of the immune response such as microglia activation and cytokine release (Musaelyan et al., 2014; Sierra et al., 2014). Microglia may thus be a significant contributing factor for neurological recovery (Girard et al., 2009). Furthermore, cytokines released by the microglia are involved in the control of many crucial aspects of cell life, from development to differentiation to cell death (Girard et al., 2009). After early brain injury, it is known that the microglias enhance neural stem cell proliferation and subsequent migration to the injured area by secretion of diffusible factors (Aarum et al., 2003). Microglia can also have a positive or negative effect on the proliferation, survival or differentiation of newborn cells (Sierra et al., 2014). Finally, after early brain damage, there is an increase in neuron precursor cells, astroglias, oligodendroglia progenitors and microglias, which demonstrates the important role these cells play in the brain repair mechanism (Hayashi et al., 2005).

Strengths and limitations

This is the first systematic review that addresses neurogenesis in animal models of brain damage related to the pathophysiology of cerebral palsy and provides information on proliferation, migration, and cellular differentiation in different periods following early brain damage. Rigorous methodological criteria were used throughout this review, from publication and multiple database search protocols, to data extraction and the reporting of results. We also

provided a quality assessment of the literature included, indicating the risk of bias in the execution of experimental studies. This systematic review may also help to improve the extrapolation of outcomes in animals to humans, since it is difficult to obtain clinical information on neurogenesis in children with cerebral palsy.

This systematic review has a number of limitations. It was not possible to conduct a meta-analysis, owing to the methodological heterogeneity of the studies. The great variety of results, models, and time-points for measuring neurogenesis impaired the quantitative comparison of findings. Likewise, the diversity of protocols used for BrdU or EdU cell markers precluded quantitative analysis. Furthermore, with regard to risk of bias, only a few of the studies involved random sequence generation, blinding of researchers, or random outcome assessment. Finally, the studies included here did not make a comparison between male and female animals, making it difficult to draw any conclusions regarding sex-dependent differences in neurogenesis in these models.

Implications for future investigation

The findings from these investigations, taken as a whole, improve our understanding of cell proliferation, migration, and differentiation in the brains of subjects with cerebral palsy and can be used as a guide for future studies. This systematic review may also pave the way to the replacement, refinement, and reduction of animal experimentation in this field. The mechanisms underlying neurogenesis are still poorly understood and this requires further investigation in a context involving early brain damage such as cerebral palsy. Future research exploring the potential therapies and methodological issues discussed here will undoubtedly contribute to better extrapolation to humans. The potential use of environmental enrichment for neurogenesis needs to be investigated in models of early brain damage (Rojas et al., 2013), and the efficacy of various intervention strategies needs to be assessed through further systematic reviews and meta-analyses.

The molecular and cellular composition of the neurogenic niches that favor regeneration in the immature brain need therefore to be defined more precisely and connected to the behavioral and cognitive changes observed in such subjects. One of the most challenging issues to be solved in this area of research is elucidation of the cell migration mechanism. This was not covered by many of the studies included here. The outlook for future studies should combine approaches involving both neuromotor behavior and cognitive function associated with neurogenesis. Furthermore, the influence of sex on the neurogenesis response after these models requires further investigation in primary studies. This would facilitate the extrapolation of animal findings to the field of human health.

Conclusion

Animal models of cerebral palsy help us to understand the neurogenesis mechanisms related to abnormal development in children afflicted by this syndrome and its secondary complications. Important insights derived from such models include the divergent responses of the neurogenic niches to the specific insults depending on the age of the animal at time the brain damage occurs, the location, and severity of the injury. Prenatal brain damage impacts neurogenesis, reducing the generation of neural stem cells, while postnatal models show increased proliferation of neural precursor cells, improper migration, and reduced survival of newly generated neurons. In both neurogenic niches, increased differentiation of neuroblasts, astrocytes, and oligodendrocytes were reported in response to the brain damage in perinatal period. In the long term, however, the neurogenesis response during brain development in these early brain damage models proved insufficient. These findings were observed in preclinical studies in models of brain damage mostly performed during the neonatal period in rodents and in preterm larger mammals. Future research is needed to identify the behavioral changes associated with the neurogenesis response in CP subjects.

Disclosure

The authors declare that they have no conflicts of interest.

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Appendix A. supplementary data.

The supplementary dataset for this article can be consulted on-line at doi: 10.17632/mdhjfmkdpg.1

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APÊNDICE B – "COULD POLYPHENOLS BE USED AS A NEUROPROTECTOR THERAPEUTIC AGENT IN PERINATAL BRAIN DISORDERS?".

COULD POLYPHENOLS BE USED AS A NEUROPROTECTOR THERAPEUTIC AGENT IN PERINATAL BRAIN DISORDERS?

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Polyphenols are a group of bioactive compounds, essential for a variety of functions in plants. These natural compounds are commonly found in the human diet mainly in fruits, vegetables, cereals, coffee, and beverages [1]. In the last decades, they have been studied due to their free radical scavenging and metal chelating properties [1]. Currently, there is growing attention to the potential of polyphenolic compounds under various health conditions. In a previous study, we highlighted and discussed the metabolic and neurologic benefits of polyphenols in models of non-communicable disease [2]. Other studies have shown the neuroprotective action of polyphenols in several neurologic disorders [3–7] with benefits in cognition and behavior [8,9]. In the scientific literature, however, there are few studies exploring the therapeutic potential of polyphenolic compounds after brain damage during a critical period of development.

Despite advances in perinatal care, brain damage early in life remains an important public health problem. Because there are fewer preventive and therapeutic strategies available, perinatal brain damage may cause acute mortality, especially in newborns, and a high rate of morbidity in infancy. During early life, perinatal brain injury might cause altered development and maturation of the central nervous system [10]. As a result of this non-progressive brain injury, neurologic, motor, sensory and cognitive disabilities have been reported during early development [11].

Perinatal brain injury can affect infant development at any gestational age or early in postnatal life [12,13]. Epidemiological data indicates that approximately 3 out of 1000 live births result in neonatal encephalopathy [14]. Associated with this, there is a growing concern about the increase in the prevalence of developmental disabilities [15]. Few neuroprotective strategies, however, are aimed at reducing the risk and severity of perinatal brain injury.

Recent evidence has supported the neuroprotective properties of polyphenolic compounds and their cognitive and behavioral benefits. Polyphenols display numerous biological activities, due to their antioxidant and anti-inflammatory properties, making them a potential therapeutic agent in the treatment of neurological disorders. Polyphenols have been studied in clinical and experimental evidence in neurodegenerative diseases, such as Alzheimer's and Parkinson's diseases, and neurological disorders such as stroke, and hypoxic-ischemic injury. No studies have been found concerning the benefits of polyphenols to immature brains after an injury.

It’s believed that polyphenols cause their neuroprotective actions through their capacity to protect neurons against damage induced by oxidative stress and neuroinflammation. These processes act to maintain brain function and play important roles in neuronal plasticity [16]. Investigation is necessary of the therapeutic effects of polyphenols on neuroinflammation, a characteristic of perinatal brain disorders, after early-life brain injury. In the literature, some experimental evidence indicates that when used as a prophylactic agent, polyphenols can prevent morphological damage, oxidative stress, and neuroinflammation in models of neonatal encephalopathy [17–20].

In the clinical practice, to use these compounds for preventive purposes, there must be the screening of potential risk factors (e.g.: preconceptional factors, genetic disorders, infections, exposure to toxins, maternal chronic illness, maternal nutritional deficiencies) and the identification of clinical conditions during pregnancy that might result in fetal brain damage (e.g.: pregnant-related complications, perinatal infections, multi-fetal pregnancy, uterine abnormalities, placental bleeding, prematurity, and low birth weight). Studies on these need to be developed to assess the safety and efficacy of dietary polyphenolic intervention, both during pregnancy and the suckling period. In

addition, due to overall health benefits, increasing the consumption of polyphenols by people of reproductive age could be stimulated.

Recently, studies in models of neonatal hypoxia-ischemia showed neuroprotective effects of polyphenols in reducing cortical inflammation and cell apoptosis, decreased brain infarct volume [21], ameliorated brain edema, blood brain barrier disruption, and neuronal cell death [22]. In addition, there are reports of increased proliferation of oligodendrocytes progenitor cells with improvements in myelination [23]. These effects were accompanied by improvements in cognition performance and neurobehavioral outcomes [22,23].

As little is known about neuroprotective mechanisms of polyphenols in early life, various cellular, molecular and behavioral parameters need to be assessed in preclinical models, such as intrauterine growth restriction, in-utero oxygen deprivation, perinatal asphyxia, neonatal stroke, neonatal intraventricular hemorrhage, and also in models of infant traumatic brain injury. Furthermore, the cognitive and behavioral benefits of these compounds have been reported in the literature may be favorable in neurodevelopmental disorders, such as cerebral palsy, autism spectrum disorders, Attention Deficit Hyperactivity Disorder (ADHD), and learning disabilities. Also, these investigations could favor translational purposes to introduce this knowledge to clinical perspectives.

Finally, given the need for the development of novel interventions in perinatal brain disorders, and the neuroprotective and anti-inflammatory potential of polyphenolic compounds, further investigation is urged. Studies assessing the short and long-term brain response, cognitive and behavioral adaptations after exposure to polyphenolic compounds, are required, not only in the context of prevention but also

as a therapeutic agent for neuroprotection during early development. Also, the investigations should address safety and tolerability issues for human health, considering the type of brain disorder (time of injury, lesion extension, clinical condition), the intervention characteristic (polyphenols formulation, dose, route, frequency, bioavailability), and outcomes of interest (brain structure and function, motor symptoms, behavioral and cognitive aspects).

In the literature, the majority of studies used polyphenols as an extract or as pure compounds, followed by polyphenol-rich foods [24]. For future investigation, there is an urgent need to understanding the effects of polyphenols during early life, including potential benefits of maternal consumption of polyphenol-rich foods during pregnancy and breastfeeding, mainly for women with adverse clinical conditions. Further, studies are required to assess the effects of maternal supplementation of polyphenols (dietary programs, supplementation with extract or pure compounds). Also, intervention strategies might be developed using this approach in the neonatal and pediatric population through supplementation of polyphenols in infant formulae, foods, and beverages. In addition, the inclusion of polyphenol-rich food in the dietary program of children with developmental disabilities could be beneficial for nervous system function and maturation.

Conflict of interest

The authors declare no conflicts of interest.

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APÊNDICE C – "NEONATAL EXPOSURE TO KAEMPFEROL ATTENUATES THE IMPAIRED NEUROMOTOR DEVELOPMENT, HIPPOCAMPAL CELL PROLIFERATION AND MICROGLIA ACTIVATION IN A RAT MODEL OF CEREBRAL PALSY".

Neonatal exposure to kaempferol attenuates the impaired neuromotor development, hippocampal cell proliferation and microglia activation in a rat model of cerebral palsy

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ABSTRACT

Neuroinflammation is a characteristic of the pathophysiology of perinatal brain damage. Kaempferol, a natural flavonoid, has been reported as a neuroprotector agent, due to its anti-inflammatory and antioxidant properties. Nevertheless, the potential benefits of kaempferol in neurodevelopmental disorders, like cerebral palsy, remain unexplored. The presented study aims to assess the neonatal treatment of kaempferol on neuromotor development, proliferation of neural precursors cells, and microglia profile in the dentate gyrus of the hippocampus. A rat model of Cerebral Palsy was established by perinatal anoxia and sensorimotor restriction of hindlimbs during infancy. Kaempferol (1 mg/ kg) was intraperitoneally administered during the neonatal period. Kaempferol neonatal treatment reduces the impact of the CP model in reflexes ontogeny, physical features maturation. The impairment of locomotor activity development and motor coordination was attenuated by kaempferol treatment during the neonatal period in CP exposed rats. In the dentate gyrus (DG) of the hippocampus, the neonatal treatment of kaempferol in cerebral palsy rats prevents the reduction of neural precursors cells, the activated profile of microglia, and the increased proliferation of microglia in the subgranular zone and cell granular layer of DG. In conclusion, kaempferol attenuates the impact of cerebral palsy on neuromotor behavior development and hippocampal microglia activation and the decreased cell proliferation in a neurogenic niche.

Keywords: Cerebral Palsy. Flavonoids. Locomotion. Microglia. Hippocampus.

1. Introduction

Cerebral Palsy (CP) is a neurodevelopmental disorder associated with impaired movement, behavioral changes, and cognitive dysfunction, such as spatial memory and learning deficits [1–5]. This disorder, considered the most common cause of motor disability in infancy, is defined as a group of permanent disorders of the development of movement and posture, which results from static brain damage during a critical period of the developing brain [6,7].

Several brain regions could be affected by CP, depending on the time period of injury, local and extension of the lesion, as well as the type of insult to the brain [7]. Among them, the hippocampus is a brain structure that plays a critical role during early development and after brain injury [8,9]. This structure is a site of new neural cells proliferation in postnatal life, considered a key mechanism for the maturation and repair of the brain [9,10].

Due to the interaction of the motor system, the hippocampus may reflect the role of memory in motor learning [11] and subsequent motor behavior [12]. The dentate gyrus (DG) of the hippocampus is a structure that contributes to the formation of new memories, spatial exploration, and motor skills acquisition during development [13,14]. Then, its hippocampal impairment also is associated with the motor, behavioral and cognitive problems observed in CP [15].

Perinatal brain injury, with pathophysiology related to CP, impacts the hippocampal neurogenesis during early development [16]. The hippocampal response may vary according to the nature of the brain damage, the time period during which the brain injury occurred, proliferative capacity, pattern of migration, and differentiation profile in the neurogenic niches [16]. However, the mechanisms that change

hippocampal neural cell proliferation, and its impact on neuromotor development and spatial exploration in CP subjects, remain unexplored [16].

In the early stages of brain development, studies have shown that brain inflammation is an important component in the pathophysiology of perinatal brain disorders [17–22]. Brain inflammation early in life affects the number of proliferating and differentiating neural progenitors cells in the hippocampus, causing altered development and behavior even in adulthood [23,24]. Microglia, the permanent resident immune cell of the central nervous system (CNS) [25,26], are essential for brain development and homeostasis via neuronal–microglial interactions [26,27]. Further, this cells participate in the onset and progression of the inflammatory response in the CNS after the neonatal brain injury performing an essential role in plasticity [20,27,28].

Studies showed that perinatal brain damage cause increased expression of markers for activated microglia and macrophages around the lesions in response of the initial insult, that may persists even months after the damage [20,26,29,30]. In addition, significant activation of microglia and induction of pro-inflammatory cytokines and other inflammatory mediators, has been established as a hallmark of perinatal brain injury in several models [31–34]. These microglia alterations are associated with neurodevelopmental disorders [35,36]. However, the hippocampal microglia profile, as well, survival and differentiation of newly generated cells in models of perinatal brain damage with implication of CP has been little explored [37].

Recently there is increased attention for the development of intervention strategies based on polyphenolic compounds, especially the flavonoids in neurological disorders [38–42]. Kaempferol (3, 4, 5, 7, -tetrahydroxyflavone, KAE), a natural

flavonoid found in a variety of plants has been increasingly investigated as a neuroprotective agent, due to its well-established anti-inflammatory and antioxidant properties [43–46].

Experimental evidence has demonstrated that kaempferol prevents neurological deficits, cognitive and motor impairments in models of neurological disorders [46,47]. Further, kaempferol attenuates brain inflammation by the inhibition of microglia activation [48,49]. A study that uses a model of brain damage in adolescent rats, found that prognosis was significantly altered at adolescence by early Kaempferol treatment with improved neural functionality, microstructural integrity [44] and sensorimotor behavior [50]. Even with this evidence, there are no studies that have investigated the potential beneficial effect of kaempferol in models of perinatal brain damage such as cerebral palsy [39].

In this context, we assess the neonatal treatment of kaempferol on neuromotor development, microglial activation profile, and proliferation of newly generated cells in the dentate gyrus of the hippocampus. Thus, we hypothesized that neonatal exposure to kaempferol attenuates the impact of cerebral palsy on neuromotor behavior development and hippocampal microglia activation, preventing the decreased proliferation in a neurogenic niche.

2**. Methods**

2.1 Ethical Considerations

This research project followed the standards of the National Council for Animal Control and Experimentation (CONCEA-Brazil) and began after the authorization by the Ethics Committee on Animal Use (CEUA) of the Federal University of Pernambuco (UFPE) (process number 0058/2018). All animal experiments comply with the

guideline of the "National Institute of Health Guide for Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978)". The research reports followed the recommendations of the "Animal Research: Reporting In Vivo Experiments (ARRIVE) guideline 2.0" [51]

2.2 Animals

A randomized, controlled, preclinical study was conducted using Wistar rats from the colony of the Department of Nutrition at UFPE. All animals were kept in standard conditions with an inverted light cycle (lights on 8:00 p.m. to 8:00 a.m.), controlled temperature 22 ± 2 °C, free access to water and food, housed in polypropylene cages (46cmx34cmx20cm) covered with sterile wood shavings. In order to obtain the litters, male (n = 18) and female (n = 36) animals (1: 2 ratio) were mated. For mating, the nulliparous rats were aged between 90 - 120 days and weighed between 220 - 250 grams.

2.3 Experimental groups

After the rat pups were born, the litters were adjusted to contain 8 animals per litter. For the composition of the experimental groups, newborn male rat pups were randomly allocated to the groups (using a computer-based random order generator), according to the model of cerebral palsy or control and to neonatal treatment with kaempferol or vehicle. Thus, the experimental groups were divided in: Control vehicle (C), n = 15; Control Kaempferol (CK), n = 15; Cerebral Palsy vehicle (CP), n = 15; Cerebral Palsy Kaempferol (CPK), n = 15. All efforts were made to minimize the number of animals used. The estimation of sample size was calculated using power analysis. To this, the standard deviation of behavioral parameters of locomotor activity

development in earlier studies and the presumed differences between the means groups was used.

2.4 Experimental Model of Cerebral Palsy

The experimental model of cerebral palsy was based on the experiments by Coq et al., (2008), Strata et al., (2004) and on previous experiments [54–56]. The model was reproduced only in males rats due to the higher biological vulnerability to develop CP in males. [57,58]. This model associates the perinatal anoxia with sensorimotor restriction of the hindlimbs, similar to the clinical phenotype of spastic diplegia [52,53]. After birth, male rat pups underwent two episodes of anoxia, the first occurred on the day of birth, considered as P0, and the second, on the next postnatal day (P), considered as P1. To reproduce Perinatal Anoxia (PA), newborn rats were placed in a plexiglass airtight chamber, partially immersed in water (controlled temperature ~37 ° C) and exposed to a flow of Nitrogen (100 % N_2) (9 L / min) for 12 minutes. After each episode of anoxia, rat pups were removed from the chamber to be kept in normal atmospheric conditions until they recovered their breathing, skin color, and postural reflexes. They were then returned to their mother [52,53].

From P2 to P28, the sensorimotor restriction of the hindlimbs was performed for 16 hours [52,53]. For the sensorimotor restriction, the rat's pups' feet were gently bounded together with medical tape and their hindlimbs were restrained in an extended position with a cast of epoxy allowing only limited movements of the hip joint. For 8h per day, the restrained rats could move freely without the casts [52,53]. These procedures did not impair the pup's elimination of urine and stools and receiving maternal care.

2.5 Neonatal treatment with Kaempferol

To assess the effect of early intervention after the brain injury and to obtain neuroprotection during exposure to the CP model, the male rat pups received a daily intraperitoneal (i.p) injection of Kaempferol or vehicle from P2 to P21. The Kaempferol stock solution (Cayman Chemical, Ann Arbor, MI, USA) was prepared with pure Dimethyl Sulfoxide (DMSO) solvent and stored at -80 °C. The daily injection of kaempferol was applied to rat pups as a suspension freshly dissolved in-vehicle solution (0.1% v/v DMSO in saline) at a dose of 1 mg/kg. Control animals received the vehicle solution without kaempferol compound. Daily applications occurred between 9 - 10 a.m. The dose was established according to a previous study of the effects of kaempferol in somatic growth [59].

2.6 Analysis of body measurements

For the analysis of somatic measures a digital caliper (Jomarca® 0.01 mm) was used to perform the following body measurements at P36: the distance from the anterior tip of the nasal bone to the posterior edge of the occipital bone (Anteroposterior Axis of Skull - APCA), and the distance from right to left of the temporal line of the parietal bone (Latero-Lateral Axis of Skull - LLAS) and body length (nasal to anal distance) [60,61]. The body weight was assessed at P36 with a digital scale (Marte®, model S-1000, 0.1 g). Measurements were taken between 9-11 am.

2.7 Evaluation of physical maturation and ontogeny of reflexes

For the evaluation of the maturation of the physical features, the day when each physical feature was first observed was considered as the age of maturation. The physical features evaluated were eyes opening, the eruption of the upper dental incisors, and of the lower dental incisors [62]. Reflex ontogeny was assessed as described by Feather-Schussler (2016) [63], Fox (1965), Dobbing and Smart (1974)

and in a previous studies [62,66]. The reflex response was assessed daily (between 9-11 am) during the neonatal period. The first in a series of three consecutive days in which the expected response appeared or disappeared completely, was considered the reflex maturation day by an observer blind to the experimental design. The reflexes tested were righting (tested in P2-P8), forelimb grasp (tested in P3-P9), cliff aversion (tested in P3-P8), vibrissae placing (tested in P4-P11), auditory startle (tested in P6-P18), free-fall righting (tested in P9-P17) and negative geotaxis (45º angle) (tested in P13-P21). The reflexes assessments are described in Table 1.

2.8 Evaluation of the development of locomotor activity

The development of locomotor activity was assessed using a circular open field (1 meter in diameter) and an infrared digital camera (VTR® 6638 - CCTV system) positioned vertically from the center of the open field (2.4 m away from the ground). The records were made in a room with no lighting, attached to the vivarium, during a dark phase of the circadian cycle (2:00 - 4:00 p.m.). The animals were placed in the center of the open field and filmed in a 5-minute free locomotion session at P8, P14, P17, P21 and P28 according to a previous study [55]. The ANY-maze behavioral tracking software was used to perform the analysis of locomotion at target ages. The measures evaluated were distance traveled (m), average speed (m / s), maximum speed (m / s), immobile time (s) and time spent in the zones (central, intermediary and peripheral).

2.9 Assessment of coordination and balance

The motor coordination and balance were tested using the Rotarod performance test at P33. This test was performed by placing the rat on a rotating cylinder (rod) to measure the time of locomotor activity that the animal was able to

maintain on the rod [67]. The acclimatization pretest training was performed two consecutive days before the test day using 10 rpm for 2 minutes each day. The tests were performed with an accelerated running speed of up to 37 rpm progressively reached in 5 minutes. The mean time spent on the apparatus during three consecutive tests was measured for each animal by a researcher blind to the experimental design. The interval between tests for each animal was 5 min.

2.10 Histology of the brain

At P36, the rats were anesthetized with Ketamine (100 mg / kg) and Xylazine (12 mg / kg) and transcardially perfused with 300 ml of saline (0.9% NaCl) and 200 ml of 4% Paraformaldehyde (PFA) in buffer 0.1 M phosphate (PB). The perfused brains (n = 7 per group) were dissected, weighed (with a digital scale (Marte®, precision 0.001 mg), stored overnight with 4% PFA, in a 30% sucrose solution. Frozen coronal sections of 30 µm (270 µm from each other) were cut in a cryostat (-30 °C; Leica) and kept in antifreeze solution until immunohistochemical processing.

2.10.1 Cellular proliferation in the hippocampus

At P5 and P6 the animals received an intraperitoneal injection of Bromodeoxyuridine (5-bromo-2'-deoxyuridine – BrdU, Sigma-Aldrich Co. LLC) (40 mg / Kg). BrdU, a pyrimidine analog of thymidine that is selectively incorporated into cell DNA during the S phase of the cell cycle, is commonly used as a marker of cell proliferation [68]. To assess the proliferation of neural precursors cells through immunohistochemistry, floating brain sections (30 µm) were rinsed in PB and incubated in PBT (PB + Triton X-100 0.3%, St. Louis, MO, USA) containing 10% hydrogen peroxide. Subsequently, the sections were incubated in absolute methanol, washed in PB and incubated in formamide (50% in saline sodium citrate SSC, Sigma-

Aldrich) at 65 °C. After washing in SSC solution, DNA denaturation was performed in HCl (1N) at 37 °C followed by incubation in a borate buffer solution (pH 8.4) [31,69]. After pre-treatment, the sections were incubated overnight (4 °C) in solution with primary anti-BrdU antibody (mouse anti-BrdU, 1: 30,000 Roche Molecular). Then, the sections were incubated in a solution containing anti-mouse biotinylated secondary antibody (1: 750, Vector Laboratories) and developed in avidin-biotin complex (Elite ABC kit, Vector Laboratories) and Diaminobenzidine (DAB staining kit, Vector Laboratories) [70]. Brain sections were then washed and mounted on gelatinized slides with Cytoseal after drying. The images were captured using an optical microscope with a 20x objective lens in 4-5 sections per animal. The count of the number of $BrdU^+$ cells in the hippocampus was assessed by a researcher blinded to the experimental design. The boundaries of the granular cell layer (GCL) and subgranular zone (SGZ) of the Dentate Gyrus were digitally outlined using the ImageJ software. The proportion of cells / area was then calculated ($BrdU^+$ / mm^2).

2.10.2 Microglia profile in the hippocampus

For staining the microglia, sections of the brain were incubated in 10% H_2O_2 in methanol and 10% in phosphate buffer (0.1 M, pH 7.4), containing 3% Triton X-100. Subsequently, the sections were incubated at 4 ºC for 48h, in primary antibody for an ionized calcium binding adapter molecule 1 (Iba1) (rabbit anti-Iba1 / IAF1, 1: 30,000, Wako), which was diluted with 5% of horse serum in PBT [33]. The sections were then incubated for 2h at 4 °C in secondary biotinylated anti-rabbit antibody (1:750, Sigma-Aldrich). Subsequently, the sections were incubated with solutions of the avidin-biotin peroxidase complex (ABC Elite Kit; Vector Laboratories, Burlingame, CA, USA) and a diaminobenzidine staining kit solution (DAB Kit; Vector Laboratories) to mark microglia [33]. The sections of each group were performed in parallel to avoid unspecific staining

effects. The sections were then mounted on glass slides coated with 1% gelatin and coverslips with Cytoseal (Thermo Scientific, USA). For evaluation of $Iba1^+$ microglial cells, 3 fields per section for a total of 4-6 sections per brain (n = 7 per group) in the hippocampus dentate gyrus (DG) (-2.80 to -4.15 mm bregma) were randomly selected for evaluate the microglia cell count [33]. The selected fields were analyzed with an optical microscope at 20x magnification. A blinded researcher using the ImageJ software performed the analysis of the number of cells / area and classified the microglia profile according to previous descriptions [33]. Microglial cells with a small sum and few to numerous processes were considered branched microglia (types I-III), while those with a large sum or amoeboid body and thicker and shorter processes were considered activated microglia (types IV-V) (Saavedra; Fenton-Navarro; Torner, 2018). The proportion of activated microglial cells was estimated as previously described (Roque; Ochoa-Zarzosa; Torner, 2016).

2.10.3 Hippocampal Colocalization of BrdU / Microglia

To identify proliferative microglia in the hippocampus through the colocalization of $BrdU^+$ and $Iba1^+$ cells, fluorescent immunohistochemistry was performed. Briefly, after pre-treatment, the sections were incubated for 24h (4° C) in a solution with primary mouse anti-BrdU antibody (1: 400; Roche Molecular). Subsequently, the sections were incubated in a solution containing the anti-mouse biotinylated secondary antibody (1: 250, Vector Laboratories) and visualized with the fluorescent avidin-FITC substrate kit (Vector Laboratories). After staining with BrdU, the sections were incubated with the primary antibody anti-Iba1 (1: 500, rabbit, Wako) for 48 h at 4° C. The sections were then visualized with the secondary antibody Alexa Fluor 594 IgG (1: 500, anti-rabbit, Jackson Imuno Research), then they were mounted with Vectashield on gelatinized slides. The evaluation of the colocalization of total

$BrdU^+$ nuclei with $Iba1^+$ was performed with an Olympus Fluoview FV1000 confocal microscope. A series of microphotographs 1 µm away from the z axis of the sections were obtained to generate an orthogonal view. The images were processed with the Fluoview FV10-ASW 2.0 software and only $BrdU^+$ cores that showed colocalization on the z axis were considered for quantification.

2.11 Statistics

Statistical analyses were performed using GraphPad Prism 8.0 (GraphPad Software Inc. La Jolla, CA, USA). Data normality distribution was assessed by the Kolmogorov-Smirnov test. The physical maturation and reflex ontogeny data are expressed as median and 25th and 75th percentiles and analyzed using the Kruskal-Wallis test and Dunn's post hoc test. For other variables, multiple comparisons between the groups were performed using the two-way Analysis of Variance (ANOVA) and the Tukey post hoc test. The results are expressed as the mean ± standard error of the mean (S.E.M.) or by the value of F. The level of significance was 5% ($p < 0.05$).

3. Results

3.1 Physical features maturation, reflex ontogeny and somatic measurement

During the neonatal period, the model of CP impacts the development and maturation of the physical features. There was observed a delay in the eruption of upper dental incisors (C vs. CP, $p = 0.0002$) and lower dental incisors (C vs. CP, $p = 0.0001$) in untreated cerebral palsy animals compared to the control group (Table 2). In addition, the eyes opening in the untreated CP animals was delayed compared to the control group (C vs. CP, $p = 0.0003$). In CP animals the neonatal treatment with kaempferol attenuates the impact of the model on these physical features maturation with no statistic difference compared to control group (C vs. CPK, $p>0.05$) (Table 2).

The disappearance of forelimb grasp reflex was delayed in the untreated CP animal compared to the control group (C vs. CP, p = 0.0228) (Table 2). The righting reflex acquisition in the untreated CP animals was delayed compared to the control group (C vs. CP, p = 0.001) (Table 2). The cliff aversion presented a delay in the untreated CP animals compared to control group (C vs. CP, p = 0.0032) (Table 2). The vibrissae placing acquisition was also delayed in CP animals compared to the control group (C vs. CP, p = 0.0004). Compared to the control group, the motor ability acquisition as, the free-fall righting (C vs. CP, p<0.0001) and the negative geotaxis (C vs. CP, p<0.0001) was delayed in untreated CP animals (Table 2). There was no difference in auditory startle response between groups. The neonatal treatment of kaempferol attenuated the impact of the CP model in all reflexes tested compared to control animals (Table 2).

At P36, significant changes were observed in relation to the somatic measures of the animals. The CP model caused a significant reduction in body weight [$F_{(3, 42)}$ = 21.82, p < 0.0001] and body length compared to animals in the control group [$F_{(3, 42)}$ = 32.54, p < 0.0001] (Table 2). The laterolateral cranial axis of the animals in the CP group showed a reduction when compared to the control animals [$F_{(3, 42)}$ = 11.62 , p < 0.0001]. Additionally, the encephalic weight showed a reduction in the animals of the CP group compared to the control animals [$F_{(1, 28)}$ = 25.34, p < 0.0001]. Neonatal kaempferol treatment mitigated these changes in animals exposed to the CP model (Table 2).

3.2 Locomotor activity development.

The analysis of locomotor activity development showed that control animals presented a progressive increase in distance traveled from P8 to P28. The intergroups difference analysis showed significant differences on distance traveled on P17 [$F_{(3, 33)}$ = 5.512, p = 0.0035]. The multiple comparisons revealed in this age, that animals from CP showed significant reduction of distance traveled compared to Control animals (C = 19.35 ± 2.536 vs. CP = 7.981 ± 1.641, p<0.002). A significant reduction of distance traveled in CP group compared to control animals was also observed on P21 (C = 22.95 ± 2.155 vs. CP = 13.16 ± 2.255, p<0.0010) [$F_{(3, 33)}$ = 4.06, p = 0.0146] and on P28 (C = 26.66 ± 1.157 vs. CP = 18.35 ± 2.28, p<0.0005) [$F_{(3, 33)}$ = 5.88, p = 0.0025] (Fig. 1A). On the P17 animals from CPK showed an increase of distance traveled compared to the CP (CP = 7.981 ± 1.641 vs. CPK = 14.67 ± 2.123, p = 0.0486). Increase in distance traveled of CPK group was also observed on P21 compared to CP group (CP = 13.16 ± 2.255 vs. CP = 20.43 ± 1.763, p = 0.0252) (Fig. 1A).

The immobile time comparison revealed significant changes between groups [$F_{(3, 44)}$ = 5.829; p<0.0019). In comparison to control group, animals from CP showed increased immobile time on P21 (C = 83.44 ± 7.226 vs. CP = 143.4 ± 20.59, p = 0.0021) and on P28 (C = 69.33 ± 3.566 vs. CP = 119.4 ± 16.66, p = 0.0060) (Fig. 1B). The CPK group showed reduced immobile time compared to CP on P21 (CP = 143.4 ± 20.59 vs. CPK = 72.53 ± 9.042, p = 0.0021) (Fig. 1B).

A significant difference of max speed was observed between groups [$F_{(3, 44)}$ = 17.35; p<0.0001)] in different time points. Reduced max speed parameter was observed in untreated CP animals compared to the control group on P17 [$F_{(3, 33)}$ = 8.711; p=0.0002], on P21 [$F_{(3, 33)}$ = 5.098; p=0.0052] and on P28 [$F_{(3, 33)}$ = 6.238; p=0.0018] (Fig. 1C). Compared to untreated CP group, the CPK group showed higher

max speed on P17 (CP = 0.1533 ± 0.0193 vs. CPK = 0.2752 ± 0.0349, p=0.0139) and on P28 (CP = 0.3676 ± 0.0287 vs. CPK = 0.4598 ± 0.0536, p=0.0443) (Fig. 1C).

The mean speed analysis revealed significant changes between groups [$F_{(3, 44)}$ = 12.44; p<0.0001)]. Animals from CP group presented reduced mean speed on P14 [$F_{(3, 33)}$ = 2.947; p=0.0471] (C = 0.0282 ± 0.023 vs. CP = 0.0113 ± 0.020; p=0.0316), on P17 [$F_{(3, 33)}$ = 4.45; P=0.0099] (C = 0.0645 ± 0.008 vs. CP = 0.0290 ± 0.006; p=0.0070), on P21 [$F_{(3, 33)}$ = 7.72; P=0.005] (C = 0.0837 ± 0.006 vs. CP = 0.0443 ± 0.007; p=0.0011) and on P28 [$F_{(3, 33)}$ = 3.643; P=0.0225] (C = 0.0928 ± 0.003 vs. CP = 0.0604 ± 0.007; p=0.0142) (Fig. 1D).

The assessment of time spent in zone showed differences on the behavior between groups in different timepoints. A significant interaction was observed on P8 between zone and groups [$F_{(6, 132)}$ = 4.606; p < 0.0003)]. On P8 animals from CP showed increased time in central zone compared to control group (C = 209.7 ± 25.35 vs. CP = 291.8 ± 5.89, p = 0.0031) (Fig. 2A). On P8 animals from CPK showed reduced time in central zone compared to the CP group (CP = 291.8 ± 5.89 vs. CPK = 193.9 ± 28.2, p = 0.003) (Fig. 2A). On P14 animals from CP group showed reduced time spent in peripheral zone compared to the control group (C = 203.4 ± 17.59 vs. CP = 107.1 ± 21.42, P<0.0001) (Fig. 2B). On P14 a significant interaction of zones and groups was observed [$F_{(6, 132)}$ = 10.74; p<0.0001). On P14 animals from CPK showed increased time spent in peripheral zone compared to the CP group (CP = 107.1 ± 21.42 vs. CPK = 172.3 ± 13.06, p = 0.0047) (Fig. 2B). On P17 a significant interaction of zone and groups was observed [$F_{(6, 132)}$ = 5.493; p<0.0001) (Fig. 2). On P17 animals from CP showed increased time spent in central zone compared to the control group (C = 22.81 ± 4.302 vs. CP = 68.98 ± 20.79, p = 0.0461) (Fig. 2C). On P17 animals from CP showed reduced time spent in peripheral zone compared to the control group (C = 255.4 ±

6.532 vs. CP = 172.5 ± 22.26, p < 0.0001) (Fig. 2C). On P17 animals from CPK group showed increased time spent in peripheral zone compared to the CP group (CP = 172.5 ± 22.26 vs. CPK = 242.5 ± 12.0, p = 0.0006) (Fig. 2C). On P21 a significant interaction of zone and groups was observed [$F_{(6, 132)}$ = 6.416; p < 0.0001). On P21 animals from CP group showed reduced time spent in peripheral zone compared to the control group (C = 259.6 ± 4.859 vs. CP = 206.6 ± 20.2, p < 0.0001) (Fig. 2D). On P21 animals from CPK group showed increased time spent in peripheral zone compared to the CP group (CP = 206.6 ± 20.2 vs, CPK = 270.8 ± 6.159, p <0.0001) (Fig. 2D). On P28 a significant interaction of zone and groups was observed [$F_{(6, 132)}$ = 5.269; p < 0. 0001). On P28 animals from CP group showed increased time spent in central zone compared to the control group (C = 17.77 ± 2.737 vs. CP= 47.13 ± 16.87, p = 0.0464, with reduced time spend in peripheral zone compared to the control group (C = 260.8 ± 6.365 vs. CP = 214.3 ± 15.42, p = 0.0003) (Fig. 2E). On P28 animals from CPK group showed reduced time in central zone compared to the CP group (CP = 47.13 ± 16.87 vs. CPK = 16.83 ± 4.8, p = 0.0371), with increased time in peripheral zone compared to the CP group (CP = 214.3 ± 15.42 vs. CPK = 249.5 ± 6.849, p = 0.0105) (Fig. 2E).

3.3 Motor coordination

Significant change on motor coordination assessment was observed between groups [$F_{(3, 33)}$ = 4.755; p = 0.0073). Rotarod test revealed that animals from CP group had reduced performance on the apparatus compared to the control group (C = 59.46 ± 7.660 vs. CP = 30.74 ± 4.523, p = 0.0114) (Fig. 3). Animals from CPK group showed increased time on apparatus compared to the animals from CP group (CP = 30.74 ± 4.523 vs. CPK = 55.95 ± 5.624, p = 0.0309) (Fig. 3).

3.4 Cell proliferation in the hippocampus.

In the subgranular zone and granular cell layer (GCL) of hippocampus a significant difference in the number of $BrdU^+$ cells that proliferated and survived was observed between groups [$F_{(3, 216)} = 5.235$; $p = 0.0017$]. A significant reduction in the ratio of number of proliferative cell per area was observed in the CP group compared to the control group (C = 1.599 ± 0.0452 vs CP = 1.359 ± 0.0413, p = 0.0006) (Fig. 4E).

3.5 Morphological analysis of hippocampal microglia

The experimental model of CP induced a reduction of microglia density in the hilus of the hippocampus compared to the control group [$F_{(3, 165)} = 25.65$; $p < 0.0001$] (C = 0.9537 ± 0.2051 vs CP = 0.7442 ± 0.0129, p < 0.0001) (Fig. 5E). In addition, a significant effect on microglia activated profile was observed in CP group compared to control group [$F_{(3, 165)} = 22.89$; $p < 0.0001$]. Multiple comparison revealed an increase percentage of activated microglia in the hilus of CP compared to the control group (C = 12.11 ± 0.3736 vs. CP = 20.65 ± 0.5982, p < 0.0001) (Fig. 5F). Kaempferol induce a significant reduction in the percentage of activated microglia in the hilus of CP animals compared to untreated CP animals (CP = 20.65 ± 0.5982 vs. CPK = 14.46 ± 1.432, p < 0.0001) (Fig. 5F).

3.6 Colocalization of proliferative microglia in the hippocampus.

The confocal analysis revealed a significant main effect between groups in the number of $BrdU^+$ cells [$F_{(3, 198)} = 4.806$; $p = 0.0030$]. Multiple comparison showed that cerebral palsy model causes a significant decrease in $BrdU^+$ labeled undifferentiated cells in the SGZ+GCL [$F_{(3, 96)} = 6.594$; $p = 0.0004$] (C = 1.343 ± 0.082 vs. CP = 1.127 ± 0.035, p < 0.0001) (Fig. 6). Colocalization of BrdU/$Iba1^+$ presented a significant main effect between groups [$F_{(3, 102)} = 15.08$; $p < 0.0001$] (Fig. 6). Multiple comparison showed that kaempferol in control animals reduced the colocalization of microglial cells

in the SGZ+GCL of the hippocampus (C = 0.372 ± 0.055 vs. CK = 0.203 ± 0.024). The cerebral palsy model causes a significant increase in the proliferation of microglial cells in the SGZ+GCL compared to control animals (C = 0.372 ± 0.055 vs. CP = 0.651 ± 0.055, $p < 0.0001$) (Fig. 6). The neonatal treatment of kaempferol in cerebral palsy animals cause a significant reduction of proliferation of microglial cells in the SGZ+GCL compared to untreated cerebral palsy animals (CP = 0.651 ± 0.055 vs. CPK = 0.293 ± 0.052, $p < 0.0001$). There were no differences of $BrdU^+$ and $BrdU/Iba1^+$ in the hilus of hippocampus between groups (Fig. 6).

4. Discussion

To our knowledge, this is the first study to evaluate the effect of neonatal treatment with kaempferol on motor development and proliferation of microglia in the hippocampus of rats exposed to a perinatal brain disorder. The new findings presented here describes the impact of perinatal anoxia and sensorimotor restriction on parameters of maturation of the nervous system, development of the locomotor activity, and coordination. Also, we observed a reduction in the proliferation of neural precursors cells, an increase in the activated microglia profile and an increase in microglial proliferation in SGZ and GCL of the hippocampus of young rats submitted to the model. Neonatal exposure to kaempferol attenuated the effects of the model on parameters of physical characteristics, nervous system maturation and development of locomotion. Additionally, it prevented the impact on the reduced proliferation of neural precursors cells in the critical period of development; in the increase of the profile of activated microglia; and microglial proliferation in the dentate gyrus of the hippocampus. These results suggest that kaempferol mitigates the impact of the cerebral palsy model on neuromotor development with an important influence on microglia profile and postnatal neural precursors proliferation.

In the literature, several studies demonstrate altered motor development during neonatal period using models of perinatal brain damage in animals [71]. Altered neurodevelopmental reflex is a common characteristic of cerebral palsy and its occurrence is related to the early onset of brain damage [72] as well as, reflex tests are useful in assessing the degree of neural maturation in the developing mammals [73], and are reliable indicators of normal development [64]. A characteristic of cerebral palsy is the persistence of primitive reflexes and delay in motor development [74]. In neonatal model of cerebral palsy a significant motor deficits with persistent grasping reflex were observed in mice [63]. Here, we show that perinatal anoxia and sensorimotor restriction of hindlimb causes persistence on forelimb grasp, delayed acquisition of righting, cliff aversion and vibrissae placing, as well as in free-fall righting and negative geotaxis. Also, the model used delayed the maturation of the physical features. To date, no studies assess the kaempferol effect on neurodevelopment reflexes in models of neurological disorders. Here, it seems that early exposure to kaempferol in rats submitted to the CP model, prevented the delay of physical maturation and the persistence of primitive reflexes in cerebral palsy rats. The reason on which kaempferol prevent altered neurodevelopmental reflex may be related to antioxidant and anti-inflammatory properties of kaempferol in protecting the nervous tissue and consequently favoring the development of the brain [75].

In this study, we observed that the delayed acquisition of active motor response was associated with deficits in the development of locomotor activity observed in rats with cerebral palsy during neonatal period. In agreement with previous studies [55], in the second week of postnatal life on rats, a negative effect on locomotion and exploratory pattern in animals with cerebral palsy was observed. However, we observed in CP rats that received the neonatal kaempferol treatment,

greater distance traveled, better average speed, less immobility time, with a higher peripheral exploration in the open field compared to untreated. Also, the CP rats treated with kaempferol performed better balance and motor coordination tests. These results are in agreement with a study with a model of striatal degeneration in rats that showed a daily intraperitoneal injection of kaempferol attenuate neurological and motor deficits (Lagoa et al., 2009). These results have important implications for the effect of kaempferol on locomotor development in subjects with CP, because there are few studies of the benefits of flavonoids in neurodevelopmental disorders. Further, studies assessing the safety profile and pharmacology development for the early exposure to kaempferol in children are required

Data suggests that polyphenols compounds, as the flavonoids, has important metabolic and neurological benefits in models of non-communicable disease [41]. Studies with dietary supplementation with flavonoid-rich foods, such as blueberry, green tea and Ginkgo biloba, lead to improvement on spatial memory and learning [77,78]. Furthermore, animal and cellular studies suggest that the mechanisms underpinning their ability to induce improvements in memory are linked to the potential of absorbed flavonoids and their metabolites to interact with and modulate critical signaling pathways, transcription factors and gene and/or protein expression which control memory and learning processes in the hippocampus; the brain structure where spatial learning occurs [77,78].

One study showed maternal supplementation with avocado oil (rich in flavonoids) and pulp anticipates reflex maturation and somatic postnatal development [79], and improves memory during the adolescent and adult phases [79]. Also, positive effects were observed on locomotor activities and sensory motor reflexes, as well as learning and memory of the offspring exposed to high dose green tea extract, better

response of sensory motor reflex in the neonatal period [80]. In another models it was found that flavonoids positively influences locomotor activity [81,82].

Recently we highlight and discuss the cell proliferation, migration and differentiation in neurogenic niches in models of perinatal brain damage [37]. Depending of the time period of brain injury (pre-natal vs. postnatal) and the type of brain lesion, the neurogenesis responds differently to the insult in face to the challenge of development in a critical phase of life. Brain damage in very immature brain impacts neurogenesis, resulting in a decrease in generation of neural stem cells [37]. This contrasts with models in which brain damage was produced in the first week after birth in rodents, in which there was an increased proliferation of neural precursor cells [37].

Here, we show for the first time the neural precursors cell proliferation in the dentate gyrus of hippocampus using a model of perinatal asphyxia and sensorimotor restriction of hindlimb in young rats. Overall the results showed reduced proliferative cells that survive in sub granular zone of dentate gyrus in young rats exposed to the CP model. In the study of Takada et al., (2016) an episode of anoxia at P1 cause reduced number of newly generated neurons in DG during adolescence. Fagel et al., (2006) showed that chronic neonatal oxygen deprivation reduces in the neonatal period the number of proliferative neural precursors' cell in SVZ and hippocampus. The reduction in neural precursors cell proliferation observed here, may be associated to the delayed motor development in rats of CP group and reduced brain mass. Interesting that Marcuzzo et al., (2010), reported that sensorimotor restriction alone cause a reduction in neuronal cell in the primary somatosensory cortex. However, few studies have highlight the cell migration mechanism and pattern in models of perinatal brain damage.

Flavonoids can protect the brain by their ability to modulate intracellular signals promoting cellular survival [86]. As the firsts weeks of neonatal period are crucial for brain development and maturation, the intervention proposed for cerebral palsy must be acutely applied [87]. Our results suggest that neonatal treatment with kaempferol attenuates the reduced neural precursor cells number in the sub granular zone and granular cell layer of DG of hippocampus in rats exposed to perinatal anoxia and sensorimotor restriction of the hindlimbs. Evidences revealed that flavonoids promote the differentiation of neural precursors cells into neurons and cause a reduction of neuronal death; together with an increase of neurogenesis and synaptic plasticity in the adulthood, leading to increased synaptic function [88]; Recently a study showed that Kaempferol attenuates cognitive deficit through regulating antioxidants and neuro-inflammation [47], promotes memory retention and density of hippocampal CA1 neurons [89].

In the first weeks after birth, microglial cells has an important role for brain development and maturation (Anderson and Vetter, 2018; Schlegelmilch et al., 2011). Also, after perinatal brain injury, the proliferation of microglia can therefore be a contributing factor for neurological recovery [92]. Initial activation of microglia is beneficial to protect neurons, but excessive inflammatory cytokines led to over-activation of microglia which caused a cascade of inflammatory cytokines and uncontrolled inflammation [93]. Our results showed that a model of perinatal asphyxia and sensorimotor restriction in rats caused reduced density of microglia in the DG, in addition the proportion of activated microglia in CP rats were increased. A study with early life stress and immune challenge showed reduced density of microglia and increased proportion of activated microglia in CA3 region and in the hilus of hippocampus [94]. Activation of microglia has been established as a hallmark of

perinatal brain injury in various models [95–97]. Additionally, in perinatal brain damage, microglia show enhanced expression of pro-inflammatory markers as Interleukin 1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α) and emerging evidence implicates microglia in white matter injury and associated myelination defects in neonates [98–100].

Our results showed that in the SGZ and GCL of hippocampus, untreated CP animals, presented increased proliferation of microglia. A study with a neonatal HI in mice, authors reported increased number of BrdU / $Iba1^{+}$ cell in hippocampus [101]. The increase of microglia proliferation, could be due to the modulation of brain inflammatory response [102]. However, in the literature, few studies assess post-natal microglia proliferation in models of cerebral palsy, and how this contributes to impaired neurogenesis [37]. In response to injury, increased proliferation and activation of microglia may result in increased death of newly generated neurons or reduced survival of young neurons in DG. Corroborating, we observed an increased proportion of activated microglia with reduced neural precursors cell proliferation in DG.

Experimental evidence suggesting that inflammatory response may be extremely detrimental for neurogenesis. A study showed that hippocampal neurogenesis is impaired after brain insult associated with tissue damage and inflammation [103]. Also, evidence suggests that pro-inflammatory microglia are associated with hypomyelination in perinatal injury [104]. To this, authors suggest that the use of anti-inflammatory drugs may dampen damaging microglial responses existent [104]

Several evidence showed that the blockage of microglia activation cause a neuroprotection response in different models of CNS injury [104–106]. After microglial ablation, there was an increase in the number of apoptotic cells concomitant with

increased proinflammatory molecules [102]. Due to their anti-inflammatory and antioxidant properties, the flavonoids, have been proposed as potential intervention for neuroinflammatory conditions [107,108]. Flavonoids exert a multitude of neuroprotective actions in the brain, including a potential to protect neurons against neurotoxin-induced injuries, an ability to suppress neuroinflammation and the potential to promote memory, learning and cognitive function [78]. In addition, they induce beneficial effects on the vascular system, leading to changes in cerebrovascular blood flow, capable of causing angiogenesis, neurogenesis and changes in neuronal morphology [78]. Our results showed that neonatal treatment of kaempferol prevents the reduction of microglia density in CP animals and restore the microglia to resting profile.

Recently a study reported that kaempferol treatment in a model of ischemia/reperfusion attenuates the inflammation of ischemic brain by inhibiting the microglial activation (Li et al., 2019). In cerebral ischemia models, kaempferol attenuated neuroinflammation and reduced neurological deficits [111]. In this study with rats, the authors reported that treatment with kaempferol after cerebral ischemia, inhibits the cerebral proinflammatory chain, with reduced neuroinflammation and decreased microglia with activated morphology [111]. Kaempferol treatment reduced damage to neurons and axons compared to vehicle-treated controls. The authors noted that kaempferol's antioxidant potential protects cellular apoptosis in the hippocampus and mitigates memory deficits (Yu et al., 2013; El-kott et al., 2020). In addition, kaempferol treatment reduced damage to neurons and axons compared to vehicle-treated controls [111].

Further, evidences showed that the flavonoid Pinocembrin protects the brain tissue in models of brain disorders. Lan et al (2017) showed that intravenous injection of 5 mg/kg of Pinocembrin protects brain from intracerebral hemorrhage model in mice

[108]. Authors found reduced lesion volume, brain edema neurological deficits, and suppressed microglia activation with reduction in pro-inflammatory cytokines. In another study with a model of Traumatic brain injury in rats, post-treatment with Pinocembrin decrease microglia activation acutely providing cerebral protection [107].

5. Conclusion

The cerebral palsy model impairs the maturation of the central nervous system, the development of locomotor activity and coordination in rats. Also, reduce the proliferation of neural precursors cells, increase the activated microglia profile and microglial proliferation in the dentate gyrus of the hippocampus of young rats. Neonatal treatment with kaempferol attenuated the development and maturation deficits of the neuromotor system. Additionally, the kaempferol prevented the impact on the proliferation of neural precursors cells, reducing the profile of activated microglia and microglial proliferation in the dentate gyrus of the hippocampus of young rats submitted to a cerebral palsy model. These results suggest that kaempferol mitigates the consequences of the cerebral palsy model on neuromotor development by inhibiting unregulated proliferation and the persistent state of microglial activation in the dentate gyrus of the hippocampus. Further studies are needed to demonstrate the benefits of flavonoids in perinatal brain disorders.

Conflict of interest

The authors declare no conflicts of interest.

Data availability statement

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

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TABLES AND FIGURES

Table 1. Description of reflexes assessment.

Reflex	Period of assessment	Response
Righting	P2-P8	Body rotation in dorsal decubitus for ventral decubitus within 10 seconds.
Forelimb grasp	P3-P9	Failure to perform rapid flexion of the fingers after two slight percussions in the forepaw palm.
Cliff aversion	P3-P8	Angular displacement of 45° of the animal in up to 10 seconds, when placed with forepaws on the margin of a flat and high surface.
Vibrissae Placing	P4-11	Placement of forepaws on the table, trying to walk when suspended by the tail in up to 10 seconds.
Auditory Startle	P6-P18	The simultaneous and rapid retraction with involuntary immobilization after acute percussion on metallic object.
Free-fall Righting	P9-P17	When held by all four paws in dorsal decubitus and let fall 30 cm, full turn the body landing on all four legs on a cotton bed.
Negative Geotaxis	P13-P21	Rotation within 10 seconds and positions the head upwards when placed in the center of a 45° incline ramp, positioned with the head downwards.

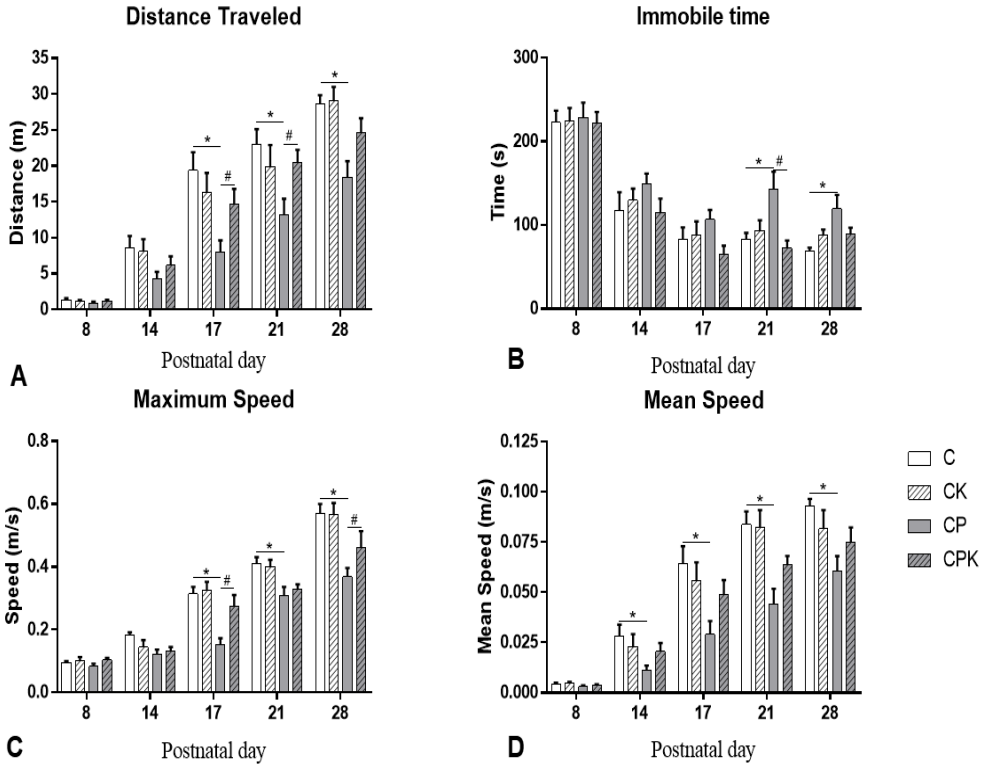
Note: According previous studies Campos et al. (2021) and Gouveia et al. (2020).

Table 2. Maturation of physical features, reflexes ontogeny and somatic measures.

MEASURE	GROUPS			
	C	**CK**	**CP**	**CPK**
Physical Features				
Upper Incisors Eruption	10 (9-10)	10 (9-11)	**12 (11-12)^{a***}**	11 (10-11.5)
Lower incisors Eruption	11 (10-11)	10 (10-11)	**13 (13-14)^{a***}**	12 (11-12)
Eyes Opening	13 (13-14)	14 (13-14.25)	**15 (14.75-15.25)^{a***}**	14 (13.75-15)
Reflexes Ontogeny				
Righting	3.5 (3-4)	3 (3-4)	**5 (4.75-5.25)^{a***}**	5 (4-5)
Forelimb grasp	6 (5-6)	6 (5-6.25)	**7 (6-8)^{a*}**	7 (7-8)
Cliff aversion	6 (5-6)	6 (5-6)	**7 (6.25-8)^{a**}**	7 (6-7)
Vibrissae Placing	7 (6-7)	7 (6-7)	**9 (8-11)^{a***}**	9 (7-9)
Auditory Startle	12 (11-13.25)	12 (11-13)	13 (12-13)	12 (11.75-13)
Free-fall Righting	15 (14-15)	15 (14-15.5)	**18 (17-19)^{a****}**	16 (16-17.25)
Negative Geotaxis	15 (15-15)	15 (14.75-16)	**21 (21-22)^{a****}**	20.5 (19-21.25)
Somatic Measures				
Body weight (g) at P36	107.8 ± 5.704	100.4 ± 3.543	**64.27 ± 3.998 ^{a****}**	79.31 ± 3.85
Body Length (mm) at P36	147.0 ± 2.432	144.1 ± 1.875	**124.5 ± 1.765 ^{a***}**	126.3 ± 2.947
APAS (mm) at P36	37.62 ± 0.462	38.03 ± 0.676	35.39 ± 0.593	35.78 ± 0.519
LLAS (mm) at P36	23.06 ± 0.921	21.54 ± 0.240	**19.08 ± 0.210^{a****}**	20.0 ± 0.325
Encephalic Weight (mg) at P36	1.51 ± 0.041	1.437 ± 0.042	**1.203 ± 0.052^{a***}**	1.324 ± 0.024

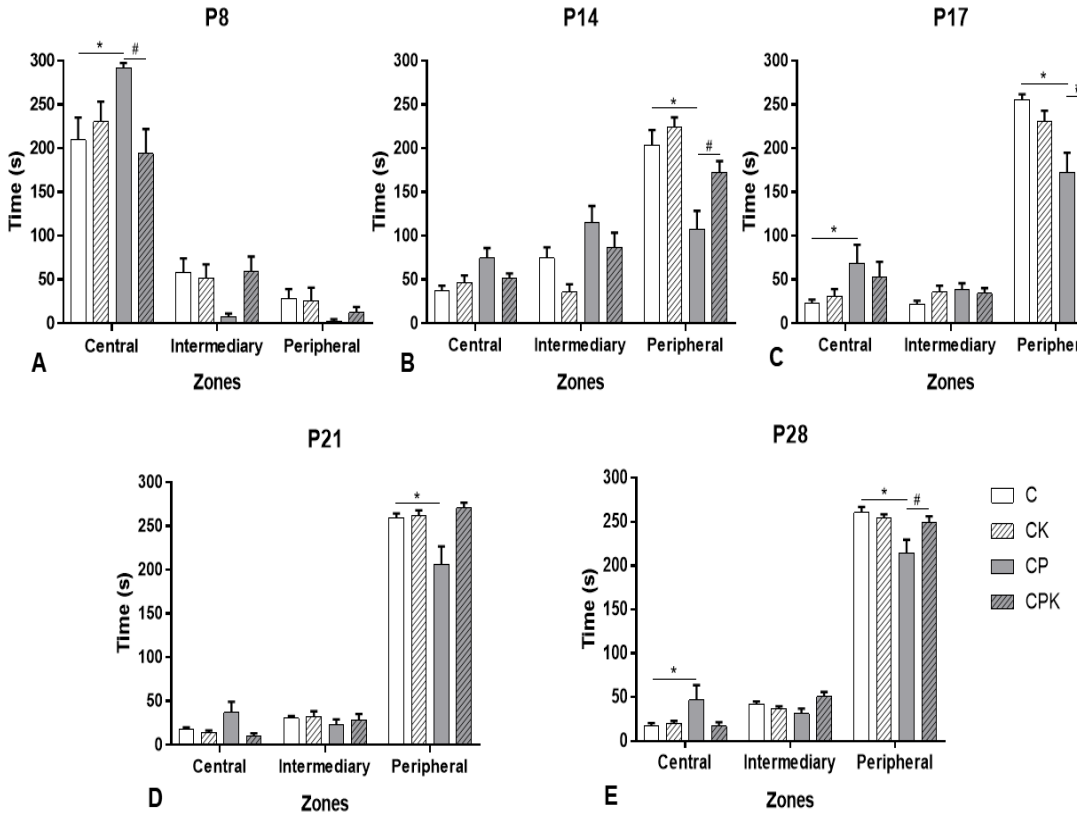
Age of appearance or maturation of physical characteristics and the ontogeny of reflexes. The composition of the groups is according to the intervention with Kaempferol or vehicle and model of cerebral palsy or control. C (Control vehicle, n = 15); CK (Control Kaempferol, n = 15); CP (Cerebral Palsy vehicle, n = 15); CPK (Cerebral Palsy Kaempferol, n = 15). Data presented as median and interquartile range. Statistical comparison made using the Kruskal-Wallis test with Dunn's post-test. Somatic measures at P36 expressed as mean and standard error of mean. Multiple comparisons performed by ANOVA two-way with Tukey's post-test. ^{a} = C vs. CP; * = p <0.05; ** = p <0.01; *** = p <0.001; **** = p <0.0001.

Figure 1. Development of locomotor activity.



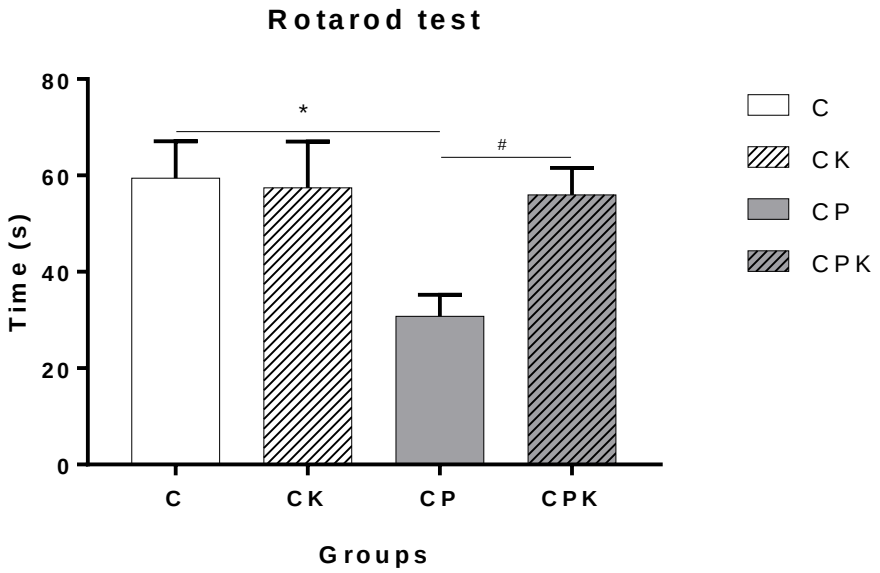
Evaluation of the development of locomotor activity at the target ages P8, P14, P17, P21 and P28. A - Distance traveled; B - Immobile time; C - Maximum speed; D - Mean speed. The composition of the groups is according to the intervention with Kaempferol or vehicle and model of cerebral palsy or control. C (Control vehicle, n = 12); CK (Control Kaempferol, n = 12); CP (Cerebral Palsy vehicle, n = 12); CPK (Cerebral Palsy Kaempferol, n = 12). The data are presented as mean and standard error of the mean and were analyzed using the ANOVA two-way repeated measures test followed by the post-hoc Tukey test. * = C vs. CP ; # = CP vs. CPK; $p < 0.05$.

Figure 2. Time spent in zones during the development of locomotor activity.



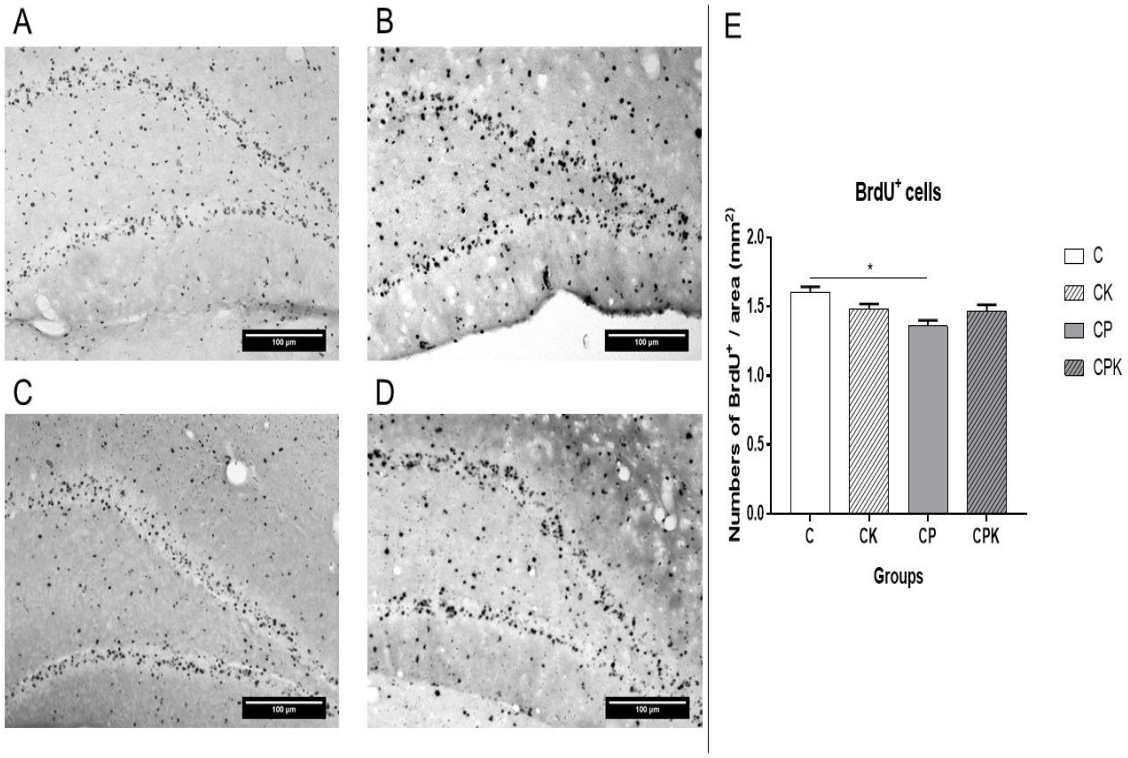
Time spent in open field zones in each postnatal day. Zones: central, intermediary and peripheral. A - Time spent in the zones at P8; B - Time spent in the zones at P14; C - Time spent in the zones at P17; D - Time spent in the zones at P21; E - Time spent in the zones at P28. The composition of the groups is according to the intervention with Kaempferol or vehicle and the model of cerebral palsy or control. C (Control vehicle, n = 12); CK (Control Kaempferol, n = 12); CP (Cerebral Palsy vehicle, n = 12); CPK (Cerebral Palsy Kaempferol, n = 12. The data are presented as mean and standard error of the mean and were analyzed using the ANOVA two-way test followed by the post-hoc Tukey test. * = C vs. CP; # = CP vs. CPK; $p < 0.05$.

Figure 3. Performance during the coordination and balance test.



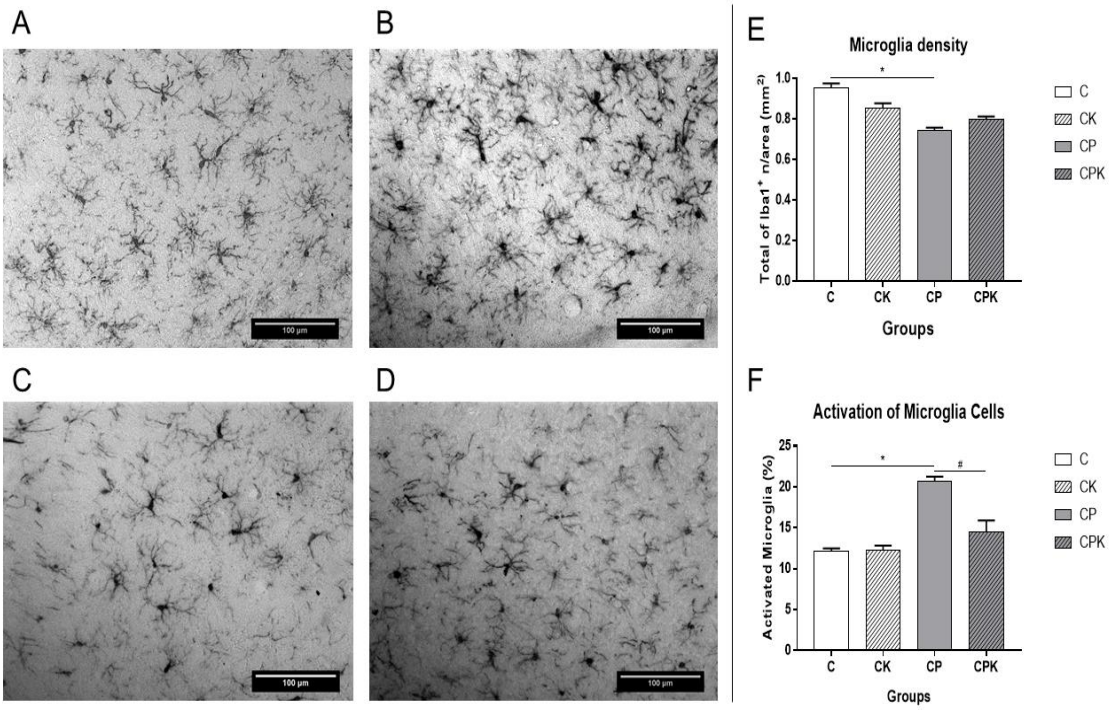
Animal performance during the coordination and balance test in the Rotarod apparatus. The composition of the groups is according to the intervention with Kaempferol or vehicle and the model of cerebral palsy or control. C (Control vehicle, n = 12); CK (Control Kaempferol, n = 12); CP (Cerebral Palsy vehicle, n = 12); CPK (Cerebral Palsy Kaempferol, n = 12). The data are presented as mean and standard error of the mean and were analyzed using the ANOVA two-way test followed by the post-hoc Tukey test. * = C vs. CP; # = CP vs. CPK; $p < 0.05$.

Figure 4. Cellular proliferation in the hippocampus.



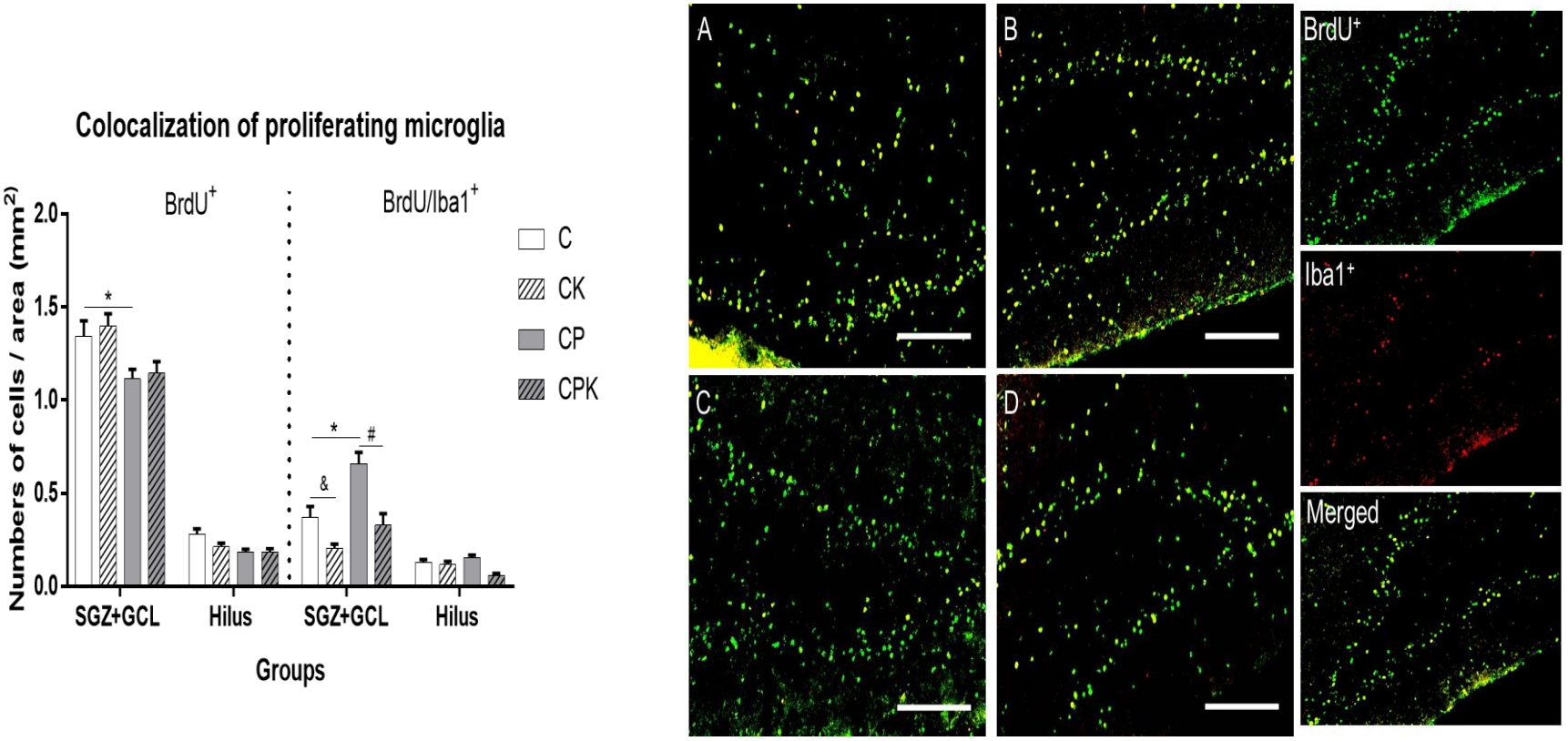
Representative images of immunohistochemistry for $BrdU^+$ cells in the granular cell layer and subgranular zone of the DG of the hippocampus A-D; A- Control vehicle; B- Control Kaempferol; C- Cerebral palsy vehicle; D - Cerebral Palsy Kaempferol. E - Number of $BrdU^+$ proliferative cells / area mm^2. The composition of the groups is according to the intervention with Kaempferol or vehicle and the model of cerebral palsy or control. C (Control vehicle, n = 7); CK (Control Kaempferol, n = 7); CP (Cerebral Palsy vehicle, n = 7); CPK (Cerebral Palsy Kaempferol, n = 7). The data are presented as mean and standard error of the mean and were analyzed using the ANOVA two-way test followed by the post-hoc Tukey test. * = C vs. CP; # = CP vs. CPK; $p < 0.05$.

Figure 5. Profile and number of microglia in the dentate gyrus of the hippocampus.



Images of immunohistochemistry for Iba1^{+} cells. A-D; A - Control vehicle; B - Control Kaempferol; C - Cerebral palsy vehicle; D - Cerebral Palsy Kaempferol. E - Iba1^{+} cell density; F - proportion of microglia with an activated profile in the DG of the hippocampus. The composition of the groups is according to the intervention with Kaempferol or vehicle and the model of cerebral palsy or control. C (Control vehicle, n = 7); CK (Control Kaempferol, n = 7); CP (Cerebral Palsy vehicle, n = 7); CPK (Cerebral Palsy Kaempferol, n = 7). Scale bar 100 µm. The data are presented as mean and standard error of the mean and were analyzed using the ANOVA two-way test followed by the post-hoc Tukey test. * = C vs. CP; # = CP vs. CPK, $p < 0.05$.

Figure 6. Proliferation of microglia in the hippocampus.



Colocalization of proliferative microglia in the subgranular zone (SGZ) and granular cell layer (GCL) and in the hilus of the DG of the hippocampus (left figure / graph). The composition of the groups is according to the intervention with Kaempferol or vehicle and the model of cerebral palsy or control. C (Control vehicle, n = 7); CK (Control Kaempferol, n = 7); CP (Cerebral Palsy vehicle, n = 7); CPK (Cerebral Palsy Kaempferol, n = 7). The data are presented as mean and standard error of the mean and were analyzed using the ANOVA two-way test followed by the post-hoc Tukey test. * = C vs. CP; # = CP vs. CPK; $p < 0.05$. Immunofluorescence images evaluating the colocalization of proliferative microglia (right figures) in the hippocampal DG in a sample from CP group. In green cells $BrdU^+$. In red $Iba1^+$ cells. In yellow $Iba1^+$ colocalized with $BrdU^+$ (orthogonal view). A- Control vehicle; B- Cerebral palsy vehicle; C - Control Kaempferol; D - Cerebral Palsy Kaempferol. Scale bars 100 µm.

APÊNDICE D – "NEONATAL KAMPFEROL EXPOSURE ATTENUATES GAIT AND STRENGTH DEFICITS AND PREVENTS THE ALTERED MUSCLE PHENOTYPE IN A RAT MODEL OF CEREBRAL PALSY"

NEONATAL KAMPFEROL EXPOSURE ATTENUATES GAIT AND STRENGHT DEFICITS AND PREVENTS THE ALTERED MUSCLE PHENOTYPE IN A RAT MODEL OF CEREBRAL PALSY

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ABSTRACT

Cerebral palsy (CP) is characterized by brain damage at a critical period of development of the central nervous system, and as a result, motor, behavioral, and learning deficits are observed in those affected. Among the nutritional or even pharmacological interventions, that are currently being studied, flavonoids such as Kaempferol have demonstrated potential given their anti-inflammatory and neuroprotective properties. In this context, the aim of this study was to assess the effects of neonatal treatment with kaempferol on the neuro-musculoskeletal development of CP rats. This study was approved by the Ethics Committee on the Use of Animals (nº 0058/2018) of the Federal University of Pernambuco. For the composition of experimental groups, male Wistar rats were randomly allocated after birth into four groups according to the CP model or control and neonatal treatment with kaempferol or vehicle, as follows: C = control – vehicle; K = control – Kaempferol; CP = Cerebral Palsy – vehicle; CPK = Cerebral Palsy – Kaempferol. The model of cerebral palsy consisted of perinatal anoxia associated with sensorimotor restriction of hind paws during childhood (P2-P28). The treatment with Kaempferol (1 mg/kg) was performed intraperitoneally during the neonatal period (P2-P21). In the offspring, the body weight and body length, muscle strength, and gait kinetic, temporal, and spatial parameters were evaluated. On the 36th day of postnatal life, the animals were euthanized by decapitation for soleus muscle dissection. The muscles were processed using the myofibrillar ATPase technique for muscle fiber phenotype and for quantification of myofibrillar proteins by western blot. A reduction in the impact of CP on the body phenotype was observed, also attenuating deficits in muscle strength and gait. Additionally, the treatment mitigated the impact on muscle phenotype by preventing the reduction in the proportion of oxidative fibers and histomorphometric measurements of the soleus muscle in rats with CP. The results demonstrate that neonatal treatment with kaempferol attenuated gait deficits, impaired muscle strength and skeletal muscle maturation in rats submitted to CP.

Keywords: Cerebral Palsy. Flavonoids. Animal Models. Skeletal Muscle.

1 Introduction

Cerebral Palsy (CP) is a neurodevelopmental disorder associated with movement deficits, behavioral changes, and cognitive dysfunction, such as spatial memory and learning deficits (BUNNEY et al., 2017; HADDERS-ALGRA, 2018; LEE et al., 2017; LYALL et al., 2015; WEE et al., 2017). This disorder is considered the most common cause of motor disability in childhood and is defined as group of permanent disorders of the development of movement and posture, which results from static brain damage during a critical period of the developing brain (GRAHAM et al., 2016; ROSENBAUM et al., 2007).

The mechanisms leading to the development of CP can be attributed to any condition that affects the developing brain due to intrauterine conditions, intrapartum complications, and/or postnatal insult. The variety of motor, sensory, cognitive and behavioral disabilities depend on the period and type of brain injury, the location of the brain injury and the diffuse response of nervous tissue (GRAHAM et al., 2016; ROSENBAUM et al., 2007; VISCO et al., 2021a). The prematurity and oxygen deprivation early in life is almost related with diplegic CP, one of the most incidents in the clinics (STAVSKY et al., 2017). This condition is characterized by the weakness and stiffness of legs with significant impairment of the functional gait (ROSENBAUM et al., 2007). Also, the morbidity of subjects of diplegic CP could be affected by sedentary behavior.

The muscle weakness is considered to be a major component of CP pathology. Caused by multiple etiologies including variations in the muscle fiber type, pathologic motor unit function, spasticity, and muscle size, weakness interferes with function and leads to limited function and participation (HOWARD; HERZOG, 2021). These impairments contribute to the abnormal development of locomotion, affecting gait in

subjects with CP (GAGE; NOVACHECK, 2001; GIVON, 2009). Overall muscle volume and length have been found to be decreased in CP, likely secondary Factors such as muscle atrophy, excess adipose tissue deposition, insulin resistance, and chronic inflammation may increase the severity of muscle pathology throughout adulthood and lead to cardiometabolic disease risk and/or early mortality (HOWARD; HERZOG, 2021; PETERSON et al., 2012).

Animal models have been developed in the last decades aiming to contribute to the knowledge of neurologic disorders and also to favor the development of intervention strategies. Models that involve brain damage during the perinatal period (pre, peri, and /or postnatal life) are essentially relevant to understanding neurological impairments in CP. Several models of perinatal brain disorders showed impaired early brain neurogenesis and maturation locomotion function (DA CONCEIÇÃO PEREIRA et al., 2021; VISCO et al., 2021a). In rats, the association of perinatal anoxia and sensorimotor restriction of hindlimbs affects the development of locomotion with a significant impact on skeletal muscle phenotype, presenting reduced muscle mass and lower cross-section area of muscle fibers (LACERDA et al., 2017a, 2019b; PEREIRA et al., 2021).

Recently, in the preclinical research there is increased attention for the development of intervention strategies based on polyphenolic compounds, especially the flavonoids in neurological disorders (DA SILVA et al., 2020; LACERDA et al., 2021; LAN et al., 2017; NORRIS et al., 2016; SCHEFF; ANSARI; ROBERTS, 2013; STAGNI et al., 2017; VISCO et al., 2021b). Kaempferol (3, 4, 5, 7, -tetrahydroxyflavone, KAE), a natural flavonoid found in a variety of plants, has been increasingly investigated as a neuroprotective agent, due to its established anti-inflammatory and antioxidant properties (CHITTURI; SANTHAKUMAR; KANNURPATTI, 2019; LAGOA et al., 2009;

PARENT et al., 2020; YU et al., 2013). Experimental evidence has demonstrated that kaempferol provides general health benefits, including effects on energy metabolism and hypothalamic inflammation (CHEN; CHEN, 2013; ROMERO-JUÁREZ et al., 2021). A study found that Treatment with KPF leads to an increase in skeletal myocyte oxygen consumption (DA- SILVA et al., 2007).

Recently, a study found beneficial effects of kaempferol after traumatic brain injury (TBI) in young rats. The author's reported that intraperitoneally injection of kaempferol (1 mg/kg) causes positive effects on metabolic profile with improvements in sensorimotor behavior after TBI during infancy in rats (PARENT et al., 2020). In addition, it was found that kampferol increase glucose uptake as well as glycogen content in rat soleus muscle (CAZAROLLI et al., 2009). However, there is no evidence of the effects of kaempferol on skeletal muscle development and maturation. In addition, there are no studies that have investigated the potential beneficial effect of kaempferol in models of perinatal brain damage such as cerebral palsy.

In this context, we assess the neonatal treatment of kaempferol on the body phenotype, strength and gait performance as well skeletal muscle phenotype of soleus muscle in rats exposed to a model o CP. Thus, we hypothesized that neonatal exposure to kaempferol attenuates the impact of cerebral palsy on gait, strength and body and skeletal muscle phenotype.

2 Methods

2.1 Ethical Considerations

This research project followed the standards of the National Council for Animal Control and Experimentation (CONCEA-Brazil) and began after the authorization by the Ethics Committee on Animal Use (CEUA) of the Federal University of Pernambuco

(UFPE) (process number 0058/2018). All animal experiments comply with the guideline of the "National Institute of Health Guide for Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978)". The research reports followed the recommendations of the "Animal Research: Reporting In Vivo Experiments (ARRIVE) guideline 2.0" (PERCIE DU SERT et al., 2020).

2.2 Animals

A randomized, controlled, preclinical study was conducted using Wistar rats from the colony of the Department of Nutrition at UFPE. All animals were kept in standard conditions with an inverted light cycle (lights on 8:00 p.m. to 8:00 a.m.), controlled temperature 22 ± 2 °C, free access to water and food, housed in polypropylene cages (46cmx34cmx20cm) covered with sterile wood shavings. In order to obtain the litters, male (n = 18) and female (n = 36) animals (1: 2 ratio) were mated. For mating, the nulliparous rats were aged between 90 - 120 days and weighed between 220 - 250 grams.

2.3 Experimental groups

After the rat pups were born, the litters were adjusted to contain 8 animals per litter. For the composition of the experimental groups, newborn male rat pups were randomly allocated to the groups (using a computer-based random order generator), according to the model of cerebral palsy or control and to neonatal treatment with kaempferol or vehicle. Thus, the experimental groups were divided in: Control vehicle (C), n = 12; Control Kaempferol (CK), n = 12; Cerebral Palsy vehicle (CP), n = 12; Cerebral Palsy Kaempferol (CPK), n = 12. All efforts were made to minimize the number of animals used. The estimation of sample size was calculated using power analysis. To this, the standard deviation of behavioral parameters of locomotor activity

development in previous studies and the presumed differences between the means groups was used.

2.4 Experimental Model of Cerebral Palsy

The experimental model of cerebral palsy was based on the experiments by Coq et al., (2008), Strata et al., (2004) and in previous studies (LACERDA et al., 2017b, 2019a; SILVA et al., 2016). The model was reproduced only in males rats due to the higher biological vulnerability to develop CP in males (CHOUNTI et al., 2013; JOHNSTON; HAGBERG, 2007). This model associates the perinatal anoxia with sensorimotor restriction of the hindlimbs, similar to the clinical phenotype of spastic diplegia (COQ et al., 2008; STRATA et al., 2004). After birth, male rat pups underwent two episodes of anoxia, the first occurred on the day of birth, considered as P0, and the second, on the next postnatal day (P), considered as P1. To reproduce Perinatal Anoxia (PA), newborn rats were placed in a plexiglass airtight chamber, partially immersed in water (controlled temperature ~37 ° C) and exposed to a flow of Nitrogen (100 % N_2) (9 L / min) for 12 minutes. After each episode of anoxia, rat pups were removed from the chamber to be kept in normal atmospheric conditions until they recovered their breathing, skin color, and postural reflexes. They were then returned to their mother (COQ et al., 2008; STRATA et al., 2004).

From P2 to P28, the sensorimotor restriction of the hindlimbs was performed for 16 hours (COQ et al., 2008; STRATA et al., 2004). For the sensorimotor restriction, the rat's pups' paws were gently bounded together with medical tape and their hindlimbs were restrained in an extended position with a cast of epoxy allowing only limited movements of the hip joint. For 8h per day, the restrained rats could move freely without the casts (COQ et al., 2008; STRATA et al., 2004). These procedures did not impair the pup's elimination of urine and stools and to receiving maternal care.

2.5 Neonatal treatment with Kaempferol

To assess the effect of early intervention after the brain injury and to obtain neuroprotection during exposure to the CP model, the male rat pups received a daily intraperitoneal (i.p) injection of Kaempferol or vehicle from P2 to P21. The Kaempferol stock solution (Cayman Chemical, Ann Arbor, MI, USA) was prepared with pure Dimethyl Sulfoxide (DMSO) solvent and stored at -80 °C. The daily injection of kaempferol was applied to rat pups as a suspension freshly dissolved in-vehicle solution (0.1% v/v DMSO in saline) at a dose of 1 mg/kg. Control animals received the vehicle solution without kaempferol compound. Daily applications occurred between 9 - 10 a.m. The dose was established according to a previous study of the effects of kaempferol in somatic growth (CHAVES et al., 2020).

2.6 Analysis of body measurements

During the neonatal period (P7, P14, P21) and on the target ages (P28 and P36) the body weight was assessed at with a digital scale (Marte®, model S-1000, 0.1 g). In the same period, for the analysis of body length a digital caliper (Jomarca® 0.01 mm) was used to measure the distance from the anterior tip of the nasal bone to the base of the tail (GOUVEIA et al., 2019; SANTANA MUNIZ et al., 2014). Measurements were taken between 9-11 am.

2.7 Grip Strength analysis

The grip strength test is commonly used in the biomedical field as an indicator of general muscle strength. The paw grip strength test is similar to the hand grip test for people in that it assesses the ability to hold a device with the paw, is non-invasive and easy to perform, and provides reproducible information (MEYER et al., 1979). Rats in each group were tested at p33 to determine peak paw grip strength (g). For measurement, rats were positioned horizontally from a Grip Strength System handrail

(San Diego Instruments, San Diego, CA, USA) and pulled back slowly and continuously until released. This was repeated three times, and the peak force for the forepaws was measured. The grip strength was also normalized with animal body weight.

2.8 CatWalk-assisted gait analysis

Gait analysis of rats in each group was conducted using the CatWalk XT video-assisted automated quantitative analysis system (Noldus Information Technology, Netherlands) on P33. To carry out the tests, the animals were habituated to the test room (dark and silent room). Also, before recording, the animals were able to habituate to the apparatus to it and cross the runway freely.

In summary, the CatWalk System consists of an enclosed walkway (glass plate) that is illuminated by fluorescent light (HEROLD et al., 2016). Furthermore, the system is equipped with a high-speed color camera connected to a computer with the appropriate detection software (CatwalkXT9.1). The software detects various dynamic and static parameters while walking a rat (HEROLD et al., 2016; KAPPOS et al., 2017). For the detection of all parameters used in the experiments, the camera gain was set to 20 and the detection threshold to 0.1 (HEROLD et al., 2016; KAPPOS et al., 2017).The runs had to last between 0.50 and 5.00 s and the maximum allowed speed variation of 60%, to be considered successful runs. For each animal, 3 compatible runs were acquired in the evaluation. After each run, the instrument was carefully cleaned with a paper embembed with 75% ethanol solution. The runs were rated for all paws and statistically analyzed (HEROLD et al., 2016; KAPPOS et al., 2017). The CatWalk system automatically identified each paw print and generated a number of parameters of interest, including cadence, step sequence regularity index, average speed, the

base of support; and hind paw statistics including stand time, swing time, swing speed, maximum contact area, print length, and print width.

2.9 Histochemical analyses of the skeletal muscles

Animals were sacrificed under *ad libitum* feeding conditions by decapitation at P36 to collect the soleus muscle. The muscles were dissected, weighed and immediately immersed in frozen n-hexane. The samples were kept at -80ºC 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) (BROOKE; KAISER, 1970; LACERDA et al., 2017b). Briefly, the cross-sections were preincubated at room temperature for 20 minutes in a solution containing 140mM acetic acid and 60mM sodium acetate with pH adjusted to 4.3. 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 minutes, then they were exposed to 1.5% ammonium sulfide for 3 minutes, 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.

Muscle fibers in the soleus muscle 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 (ARMSTRONG; PHELPS, 1984; BROOKE; KAISER, 1970). The muscle images were obtained with an optical microscope (Olympus Optical U-CMAD-2, Tokyo, Japan; 40x objective lens). All fibers of soleus 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 rat (VISCO et al., 2020)

2.10 Western blotting

The soleus muscle of the right paw was homogenized in extraction buffer (pH 7.5; 10mM EDTA, 100mM Trisma base, 10mM Na pyrophosphate, 100mM Na fluoride, 2mM PMSF, 10mM Na Orthovanadate Aprotinin 0.01mg/ml – the last two reagents were always added at the time of homogenization). 1% Triton X-100 was added to the homogenate and then centrifuged at 15000RPM (Rotations Per Minute) for 20min at 4°C (MARZUCA-NASSR et al., 2019). After centrifugation, all the supernatant was separated and we used only 2uL to measure the total protein content using the method proposed by Bradford (1976).

Bradford's solution was prepared using Coomassie Blue G250 (0.01%), ethanol (4.75%), and phosphoric acid (8.5%). The dye binds to the protein chain, generating a blue-colored complex. The reaction is colorimetric and the absorbance was determined at 595 nm. The absorbance results were used to calculate the protein concentration based on a straight-line equation of a standard curve for bovine serum albumin. For the elaboration of the protein standard curve, we used the following albumin concentrations: 1.0 mg/mL; 0.5 mg/ml; 0.25mg/ml; 0.125 mg/ml; 0.0625 mg/ml (MARZUCA-NASSR et al., 2019).

The determination of the amount of proteins related to the myofibrillar protein synthesis pathway according to the Western Blotting technique was performed with antibodies to the total and phosphorylated protein. After evaluating the protein content of each sample, 20μg of total proteins per gel slot were separated by electrophoresis according to their molecular weight, using 12% polyacrylamide gel (SDS-PAGE). After

this separation, the gel proteins were transferred to a nitrocellulose membrane (BIORAD®) at 80 volts for 1 hour and 40 minutes. Nonspecific antibody binding was inhibited by incubating the membrane for 18 hours at room temperature in blocking solution (pH 7.5, 10 mM Tris–HCl, 150 mM NaCl, 0.05% Tween-20; T–TBS) plus 5 % bovine serum albumin (BSA), under constant agitation.

Subsequently, the membranes were incubated overnight (12 hours) at 4°C, under constant agitation, and the primary antibodies were added (Rabbit Cell Signaling: S6 Ribosomal Protein [5G10] #2217; Akt #9272; Phospho-S6 Ribosomal Protein [Ser240] /244] #2215; Phospho-Akt [Ser473] #9271; IRS-1 [59G8] #2390) diluted in the blocking solution 1:1000. At the end of this incubation period, the membranes were washed in T-TBS 3 times, for 10 minutes each, and incubated with secondary antibody (Anti-rabbit IgG HPR-linked Antibody #7074, Cell Signaling) at a ratio of 1:5000 during 2 hours. After the end of the incubation period, the membranes were again washed in T-TBS 3 times, for 10 minutes each, and then incubated with the developing solution containing peroxidase (luminol; SuperSignal West Pico Chemiluminescent Substrate System – Pierce Biotechnology) for 1 minute and immediately followed by reading through the photodocumentator (ChemiDoc XRS+). The film was developed and the band intensities were quantified with the help of ImageLab software (5.2.1 BIO-RAD). Band densities were normalized after developing and quantifying the membrane bands stained with Ponceau.

2.11 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

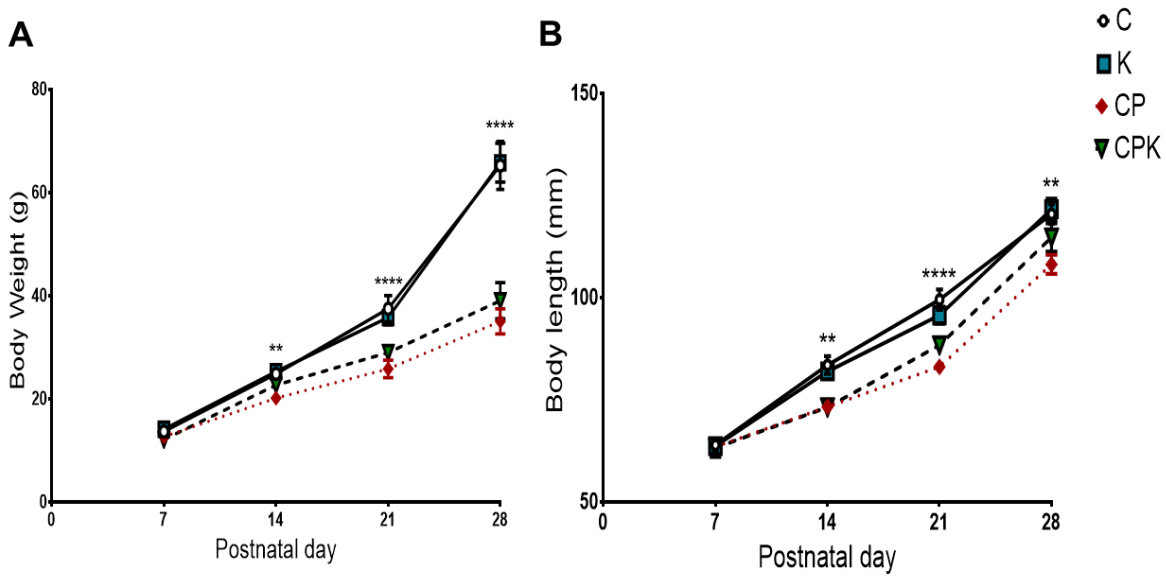
applied to body weight, and body length. Multiple group comparison was performed by two-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. The significant level was considered at $p<0.05$. Statistical analysis was performed using the GraphPad Prism 8.0. ® (GraphPad Software Inc. La Jolla, CA, USA).

3 Results

3.1 Body weight and growth during neonatal period

During the neonatal period a significant effect for body weight [$F_{(9, 132)} = 12.48$, $p < 0.0001$] and body growth [$F_{(9, 132)} = 2.58$, $p = 0.009$] was observed among groups. The body weight was reduced in the CP group compared to control animals at P14 (C = 24.93 ± 0.99 vs. CP= 20.22 ± 1.10, $p < 0.0011$) [$F_{(3, 33)} = 8.79$, $p = 0.0002$), at P21 (C = 37.56 ± 2.54 vs. CP = 25.88 ± 1.69, $p < 0.0001$) [$F_{(3, 33)} = 11.9$, $p < 0.0001$), and at P28 (C = 65.31 ± 4.63 vs. CP = 35.09 ± 2.43, $p < 0.001$) [$F_{(3, 33)} = 18.43$, $p < 0.0001$) (Figure 1). The neonatal treatment of kaempferol didn't cause significant changes on body weight in control animals, however, reduced the impact of the CP model on this parameter. It was observed a reduced body length of CP group on P14 (C = 83.48 ± 2.18 vs. CP = 73.26 ± 1.27, $p = 0.0018$) [$F_{(3, 33)} = 9.36$, $p = 0.0001$], at P21 (C = 99.48 ± 2.48, $p < 0.0001$) [$F_{(3, 33)} = 13.35$, $p < 0.0001$], and at P28 (C = 120.40 ± 2.39 vs. CP = 108.1 ± 2.31, $p = 0.0092$) [$F_{(3, 33)} = 5.81$, $p < 0.0026$]. The neonatal treatment with kaempferol did not affect the body length of control animals during the experiments (Figure 1). In animals exposed to the PC model, however, kaempferol attenuated the impact on body length in the third week. After the four postnatal weeks, no differences were observed between the PCK group and the control animals (Figure 1).

Figure 1. Analysis of body weight and body length.



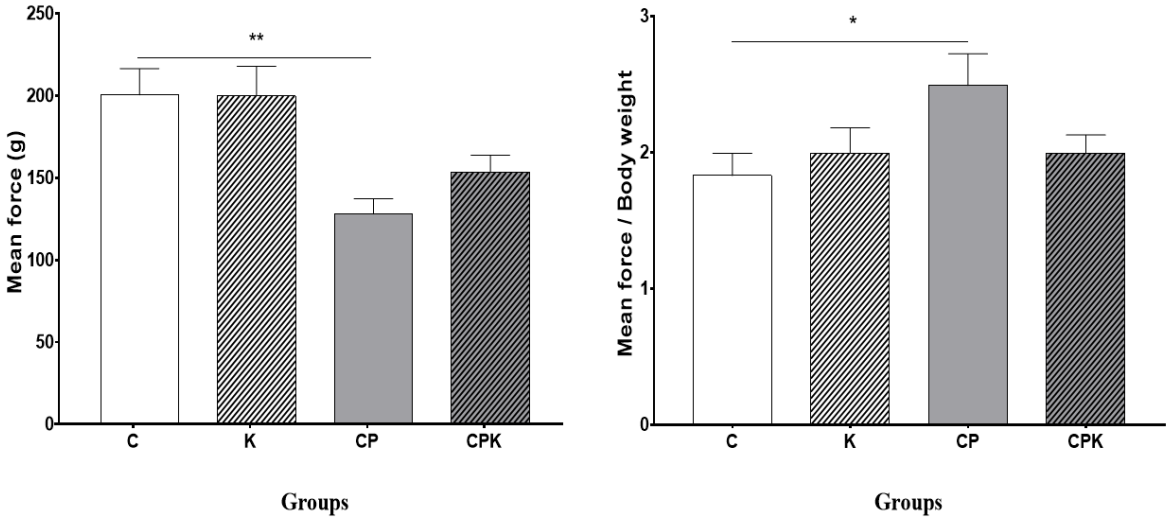
Body weight and body length of rats at ages P7, P14, P21 and P28. The composition of the groups is according to intervention with Kaempferol or vehicle and cerebral palsy model or control. C (Control - vehicle, n=12); K (Control - Kaempferol, n=12); CP (Cerebral Palsy + vehicle, n=12); PCK (Cerebral Palsy - Kaempferol, n=12). Data are presented as mean and standard error of the mean and were analyzed using the two-way repeated measures ANOVA test followed by Tukey's post-hoc test. * = C vs. CP; & = C vs. K; # = PC vs. PCK; $p<0.05$.

3.2 Grip strength analysis

A significant effect among groups was observed in the grip strength analysis [$F_{(3, 33)} = 7.24$, $p = 0.0007$]. Multiple comparisons revealed that the CP group showed the reduced mean force of the front paws assessed by the Grip strength apparatus (C = 200.4 ± 16.05 vs. CP = 127.90 ± 9.31, $p = 0.0028$) (Figure 2). The neonatal treatment of kaempferol didn't cause significant increases in the mean force in animals exposed to the CP model, however, it was observed a reduced effect of the model on the mean force (C = 200.4 ± 16.05 vs CPK = 153.3 ± 10.39, $p > 0.05$). The data was normalized against body weight and revealed an increased on the rate of Mean force / body weight

in animals of the CP group (C = 1.82 ± 0.16 vs. CP = 2.49 ± 0.23, p = 0.0424) (Figure 2).

Figure 2. Grip Strength analysis



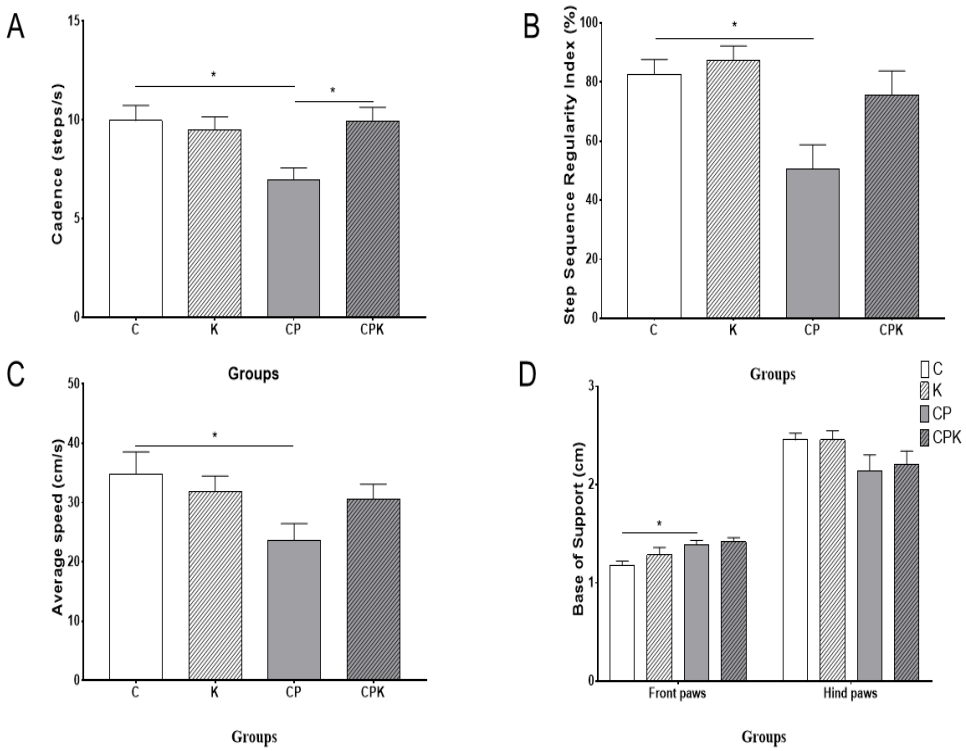
The composition of the groups is according to intervention with Kaempferol or vehicle and cerebral palsy model or control. C (Control - vehicle, n=10); K (Control - Kaempferol, n=10); CP (Cerebral Palsy + vehicle, n=10); PCK (Cerebral Palsy - Kaempferol, n=10). Data are presented as mean and standard error of the mean and were analyzed using the two-way repeated measures ANOVA test followed by Tukey's post-hoc test. * = C vs. CP; & = C vs. K; # = PC vs. PCK; $p<0.05$.

3.3 Catwalk system analysis

To assess the motor behavior after the neonatal treatment of kaempferol in rats submitted to the CP model, the gait parameters and paws statistics were quantified by the catwalk system. Since the sensorimotor restriction of hindlimbs mimitizes diplegic cp, we found a main group effects on the gait cadence [$F_{(3,\ 27)}$=3.683, p =0.0241]. Multiple comparisons two-way ANOVA revealed that the CP group showed reduced number of steps per second along its walking path (C = 9.965 ± 0.775 vs. CP = 6.978 ± 0.603, p =0.04) (Figure 3A). The neonatal treatment of kaempferol

attenuated the impairment on the cadence of the gait in animals with CP (CP = 6.978 ± 0.603 vs. CPK = 9.933, p = 0.042). The step sequence regularity index (an % index for the degree of interlimb coordination during gait) showed a significant main group effect [$F_{(3, 27)}$ = 5.569, p = 0.0042]. It was observed a notable impact of the CP model on interlimb coordination during gait (C = 82.56 ± 5.10 vs. CP = 50.63 ± 8.11, p = 0.014) (Figure 3B), whereas the CPK group showed no significant differences compared to the control group (C = 82.56 ± 5.10 vs. CPK = 75.69 ± 8.01, p > 0.05). As shown in Fig 3C a significant main group effect was observed in the average speed among groups [$F_{(3, 27)}$ = 4.129, p = 0.0156]. Animals that underwent the CP model presented reduced average speed (cm/s) during experiments compared to the control group (C = 34.85 ± 3.691 vs. CP = 23.61 ± 2.83, p = 0.0109). The reduced average speed is attenuated in animals with CP that received neonatal treatment of kaempferol (C = 34.85 ± 3.691 vs; CPK = 30.64 ± 2.46, p >0.05). The model of CP affects the base of support (cm) during the gait. A main group effect was observed for BOS of front paws [$F_{(3, 27)}$ = 5.679, p = 0.0038]. Compared to the control group, animals with CP had higher BOS of the front paws (C = 1,18 ± 0.042 vs. CP = 1.391 ± 0.040, p = 0.0144), (C = 1,18 ± 0.042 vs. CPK = 1.41 ± 0.042, p = 0.0053). Despite reduced BOS of the hindlimb no statistical differences were observed in animals with CP (Figure 3D).

Figure 3. Gait parameters evaluation with CatWalk.



The composition of the groups is according to intervention with Kaempferol or vehicle and cerebral palsy model or control. C (Control - vehicle, n=10); K (Control - Kaempferol, n=10); CP (Cerebral Palsy + vehicle, n=10); PCK (Cerebral Palsy - Kaempferol, n=10). Data are presented as mean and standard error of the mean and were analyzed using the two-way repeated measures ANOVA test followed by Tukey's post-hoc test. * = C vs. CP; & = C vs. K; # = PC vs. PCK; $p<0.05$.

Additionally, to the gait parameters, the analysis of paws statistics showed important changes. There was a reduction in the standing time of hind paws of CP animals, no differences were observed among groups (Fig. 4A). A main group effect was observed for time of swing phase of hind paws [$F (3, 72) = 5.905$, $p = 0.0012$]. The analysis shown that CP group had a longer time of swing phase of the right hindlimb compared to control group (C = 0.124 ± 0.005 vs. CP = 0.1877 ± 0.014, $p = 0.0113$) [$F (3, 27) = 5,947$, $p = 0.0030$] (Fig. 4B). The swing speed of hind paws present a reduction in group comparisons [$F (3, 72) = 13.59$, $p < 0.0001$. Compared to control

group animals from CP group showed reduced swing speed of the left hind paw (C = 93.21 ± 6.442 vs. CP = 56.22 ± 5.5, p = 0.0004) and the right hind paw (C = 87.15 ± 5.76 vs. CP = 56.0 ± 6.223 p = 0.0039) (Fig. 4C).

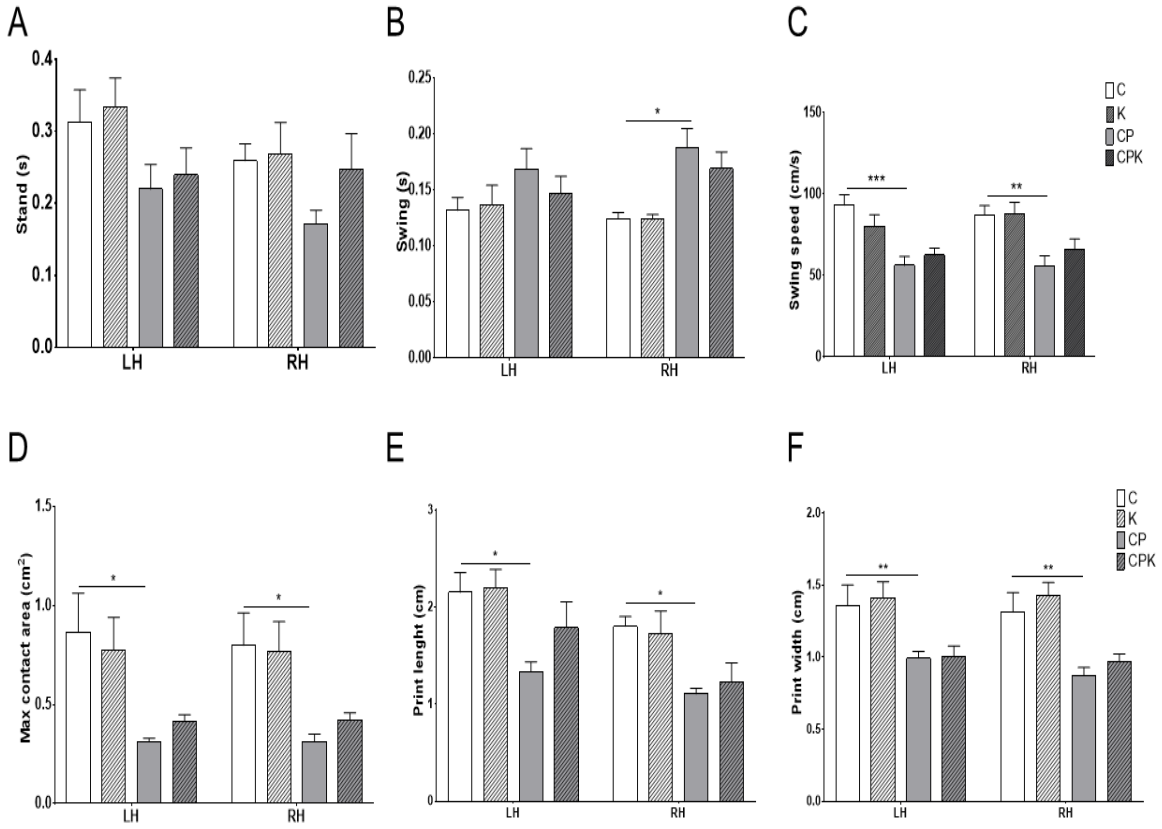
The paws max contact area analysis showed significant effect among group comparisons [$F_{(7, 32)} = 8.812, p < 0.0001$]. Animals from CP group showed reduced max contact area left hind paw compared to the control group (C = 0.866 ± 0.195 vs. CP = 0.3125 ± 0.016, p = 0.0107) [$F_{(7, 32)} = 3.649, p = 0.0249$]. Also, the max contact area of the right hind paw were reduced in animals from CP group (C = 0.797 ± 0.166 vs. CP = 0.3131 ± 0.036, p = 0.0310) [$F_{(7, 32)} = 4.423, p = 0.0118$]. The animals with CP that receive the neonatal treatment of kaempferol showed a minor effect of the model in the max contact area of both hind paws compared to the control (LH paw: C = 0.866 ± 0.195 vs. CPK = 0.4119 ± 0.0368, p = 0.0501) ;(RH paw C = 0.797 ± 0.166 vs. CPK = 0.4234 ± 0.034, p = 0.1281) (Figure 4E).

A significant effect was observed among groups for the analysis of print length of the hind paws [$F_{(3, 72)} = 8.264, p < 0.0001$]. Compared to the control group animals from the CP group had reduced print length in left hind paw (LH: C = 2.151 ± 0.205 vs. CP = 1.335 ± 0.099, p = 0.0114) and right hind paw (RH: C = 1.801 ± 0.994 vs CP = 1.104 ± 0.058, p = 0.0402) (Figure 4D). While, no differences were observed in animals with CP that receive the neonatal treatment with kaempferol compared to the control group (LH: paw (LH: C = 2.151 ± 0.205 vs. CPK = 1.786 ± 0.267, p = 0.490) (RH: C = 1.801 ± 0.994 vs CPK = 1.232 ± 1.191, p = 0.1282) (Figure 4D).

The print width analysis of the hind paws revealed a main group effect [$F_{(3,72)} = 13.12, p < 0.0001$]. Compared to the control group, animals from CP group had reduced print width of the left hind paw (LH: C = 1.361 ± 0.139 vs. CP = 0.994 ± 0.070,

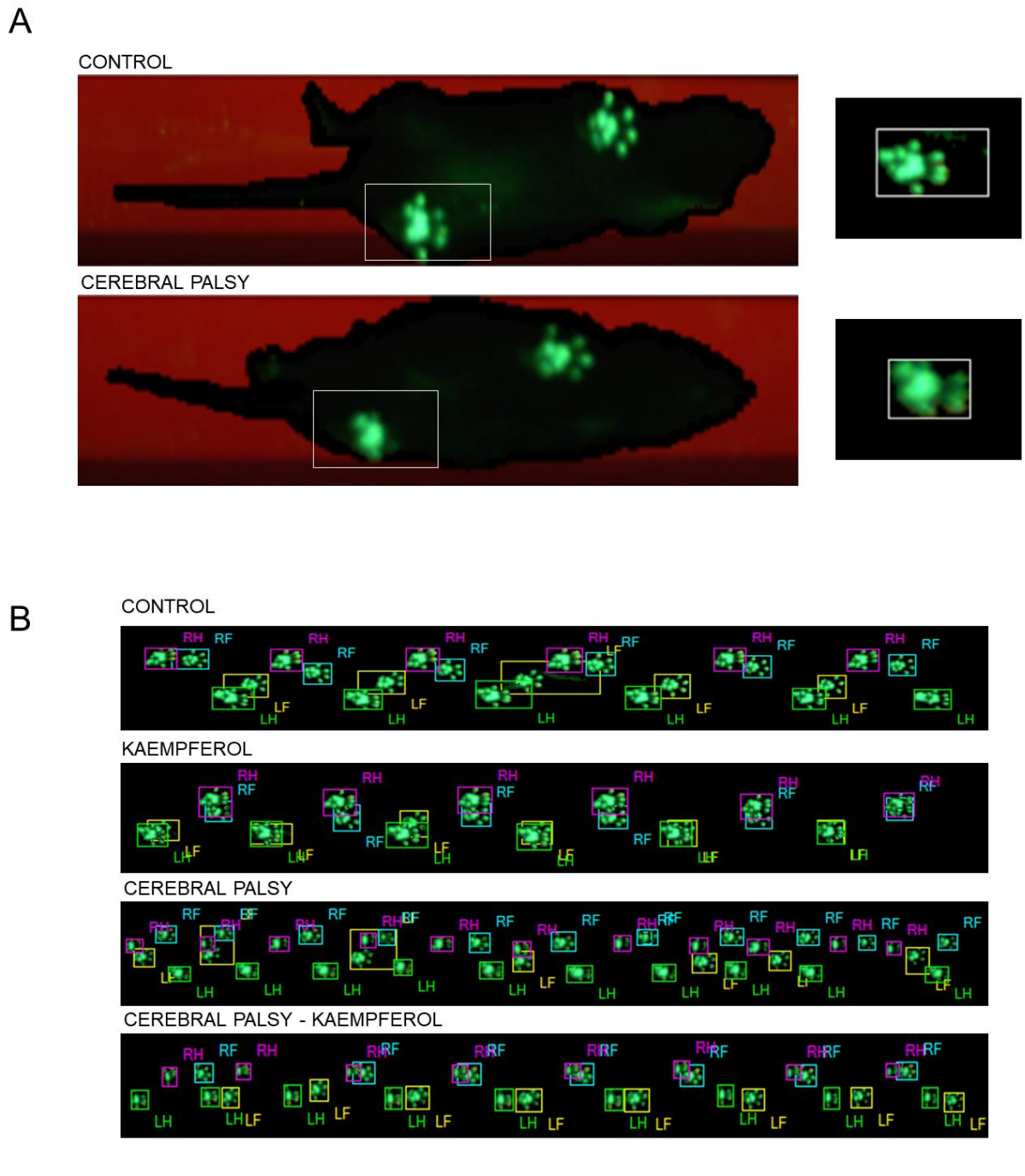
p = 0.039) [$F_{(3,27)}$ = 4.816, p = 0.0082] and of the right hind paw (RH: C = 1.313. ± 0.135 vs. CP = 0.872 ± 0.056, p = 0.087) [$F_{(3,27)}$ = 7.676, p = 0.0007] (Figure 4E). The group of animals that received the neonatal treatment of kaempferol did not showed reduced print width of the hind paws compared to the control group (Figure 4F).

Figure 4. Hind paws statistics.



The composition of the groups is according to intervention with Kaempferol or vehicle and cerebral palsy model or control. C (Control - vehicle, n=10); K (Control - Kaempferol, n=10); CP (Cerebral Palsy + vehicle, n=10); PCK (Cerebral Palsy - Kaempferol, n=10). Data are presented as mean and standard error of the mean and were analyzed using the two-way repeated measures ANOVA test followed by Tukey's post-hoc test. * = C vs. CP; & = C vs. K; # = PC vs. PCK; p<0.05.

Figure 5 – Gait assessment with Catwalk.



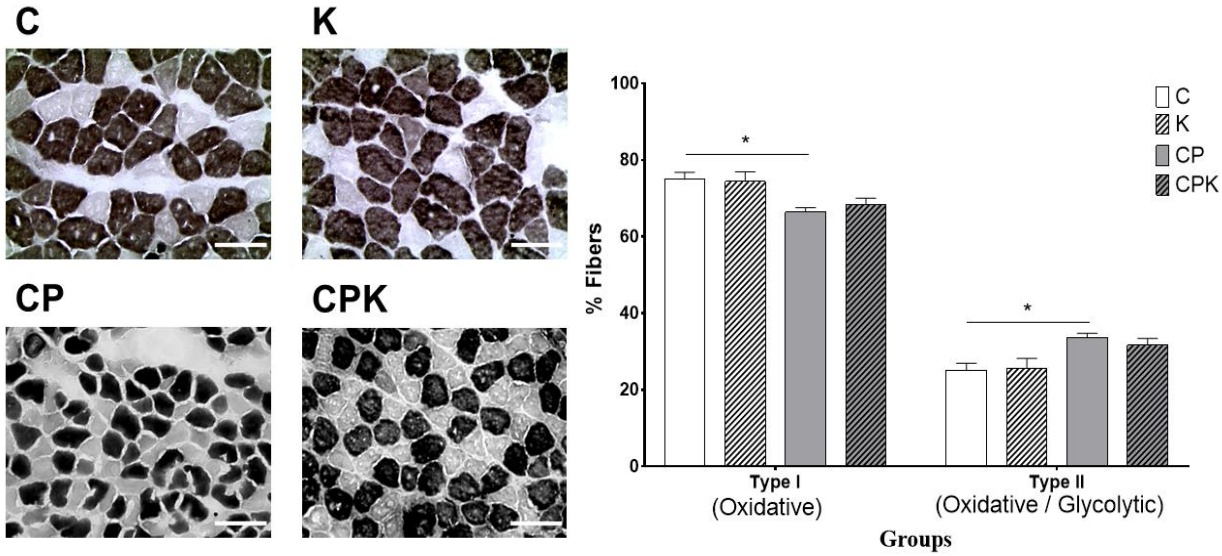
Evaluation of static and dynamic gait parameters by the Catwalk system during the animal's locomotion through the platform. A- Qualitative comparison between an animal of the control group and an animal submitted to the cerebral palsy model. On the right, hind paw impressions of control and CP animals. B- Representative images of paw prints during active locomotion of the animals on the platform. RH- right hind paw; LH- left hind paw; RF- right front paw; LF- left front paw. The composition of the groups is according to intervention with Kaempferol or vehicle and cerebral palsy model or control.

3.4 Body and skeletal muscle phenotype

At the P36 a main group effect was observed for body weight and body length comparisons. Animals from CP group showed reduced body weight compared to the control group (C vs. CP, $p < 0.0001$) [$F_{(3,33)} = 16.86$, $p < 0.0001$] (Table 1). Also, the body length was reduced in the CP group compared to control (C vs. CP, $p < 0.0001$) [$F_{(3,33)} = 12.34$, $p < 0.0001$] (Table 1). The absolute weight of the soleus muscle are reduced in the CP group compared to the control group (C vs. CP, $p < 0.0001$) [$F_{(3,15)} = 15.99$, $p = 0.0116$] (Table 3).

The histological analysis of fiber type composition in the soleus muscle revealed a significant interaction among groups [$F_{(3, 32)} = 10.19$, $p < 0.0001$]. The multiple comparisons revealed that animals submitted to the CP model presented a reduced proportion of type I fiber in the soleus muscle compared to the control group at P36 (C = 74.91 ± 1.86 vs. CP = 66.41 ± 1.29, $p = 0.0201$) and the consequent increases of the proportion of II fiber type (C = 25.08 ± 1.86 vs. CP = 33.61 ± 1.13, $p = 0.0197$) (Figure 6). The neonatal treatment of kaempferol prevented the significant changes on soleus muscle fiber proportion of animals submitted to the CP model (Type I: C = 74.91 ± 1.86 vs. CPK = 68.32 ± 1.73, $p = 0.1123$) (Type II: C = 25.08 ± 1.86 vs. CPK = 31.67 ± 1.735, $p = 0.1125$) (Figure 6).

Figure 6. Proportion of muscle fibers in soleus muscle.



At p36, the representative images of soleus section of each group and the comparisons of proportion of muscle fibers type in soleus muscle. The composition of the groups is according to intervention with Kaempferol or vehicle and cerebral palsy model or control. C (Control - vehicle, n=5); K (Control - Kaempferol, n=5); CP (Cerebral Palsy + vehicle, n=5); PCK (Cerebral Palsy - Kaempferol, n=5). Data are presented as mean and standard error of the mean and were analyzed using the two-way ANOVA test followed by Tukey's post-hoc test. * = C vs. CP; & = C vs. K; # = PC vs. PCK; $p<0.05$.

The morphometric analysis of soleus muscle fibers revealed significant main group effect of type I and type II fibers The CP group showed reduced area of type I fibers compared to the control group (C vs. CP, $p = 0.0157$) [$F_{(3,12)} = 9.159$, $p = 0.0020$] (Table 1). Also, it was observed a reduction of the perimeter of type I fibers of CP group compared to the control group (C vs. CP, $p = 0.0101$) [$F_{(3,12)} = 16.28$, $p = 0.002$] (Table 1). Additionally, the area of the type II muscle fibers in soleus muscles was reduced in the CP group compared to the control group (C vs. CP, $p = 0.04$) [$F_{(3,12)} = 6.754$, $p = 0.0064$] (Table 1). The perimeter of the type II soleus muscle are reduced in the CP group compared to the control group (C vs. CP, $p = 0.0046$) [$F_{(3,12)} = 23,80$, $p < 0.0001$] (Table 1).

Table 1. Body and soleus muscle phenotype at P36.

Variables	Groups			
	C	K	CP	CPK
Body weight (g)	108.5 ± 4.842	99.31 ± 4.05	**74.25 ± 4.283**$^{\&***}$	76.65 ± 3.224
Body Lenght (mm)	148.1 ± 3.677	143.9 ± 2.129	**128.0 ±3.596**$^{\&****}$	131.2 ± 2.569
Soleus weight (g)	0.0853 ± 0.003	0.0831 ± 0.008	**0.043 ± 0.003**$^{\&***}$	0.0606 ± 0.004
Relative weight of soleus (%)	0.0828 ± 0.0044	0.0854 ± 0.0097	0.0701 ± 0.0128	0.0798 ± 0.0061
Area of soleus Type I fiber (μm^2)	318.80 ± 26.49	321.30 ± 11.25	**208.20 ± 10.23**$^{\&*}$	211.70 ± 20.55
Perimeter of soleus Type I fiber (µm)	71.42 ± 2.869	74.78 ± 0.795	**59.36 ± 0.952**$^{\&*}$	56.44 ± 2.204
Area of soleus Type IIA fiber (μm^2)	246.60 ± 24.55	250.40 ± 14.5	**168.50 ± 9.18**$^{\&*}$	161.50 ± 14.16
Perimeter of soleus Type IIA fiber (µm)	65.65 ± 2.55	68.00 ± 1.01	**54.48 ± 0.639**$^{\&**}$	49.42 ± 1.56

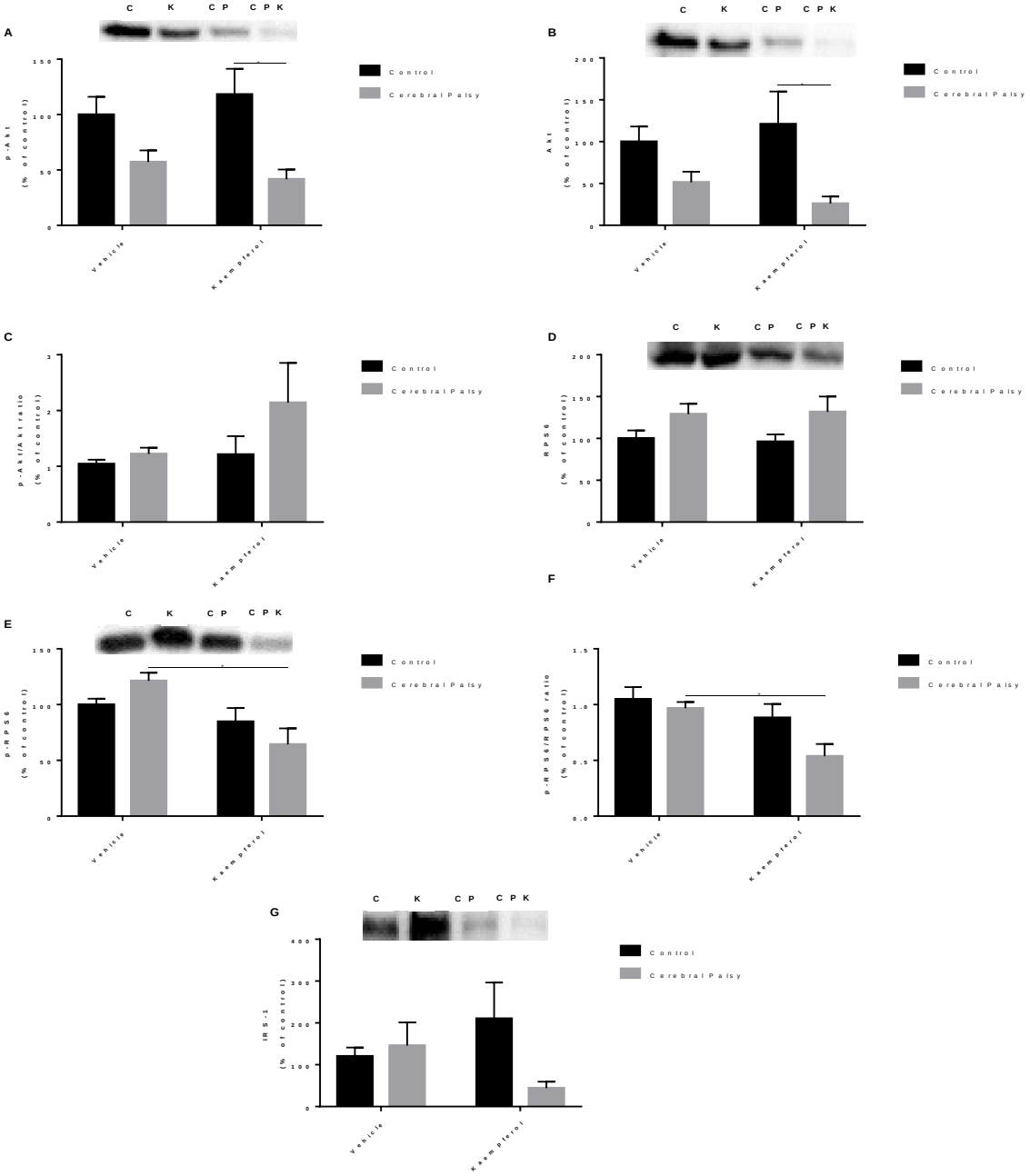
Values were showed as mean and standard error of the mean. The data were analyzed by the two-way ANOVA test, followed by Tukey's test. & = C vs. CP, # = C vs. K, $ = CP vs. CPK. C (control - vehicle, n= 5), K (control - kaempferol, n = 5), CP (Cerebral Palsy - vehicle n = 5) CPK (Cerebral palsy – kaempferol, n = 5) * p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001.

3.5 Muscle protein expression

A main group effect of p-Akt [$F_{(1, 21)} = 16.83$, $p = 0.0005$] and total Akt [$F_{(1, 21)} = 16.83$, $p = 0.0005$] was observed in CP animals. The CPK group showed reduction in the p-Akt expression compared to the respective control animals (K = 118.20 ± 23.06 vs. CPK = 41.67 ± 8.82, p = 0.0121) (Figure 7A). Also, the total Akt was reduced in the CPK group compared to control animals treated with kaempferol (K = 121.00 ± 38.93 vs. CPK = 25.96 ± 8.447, p = 0.0271) [$F_{(1, 21)} = 12.85$, $p = 0.0017$] (Figure 7B).

The neonatal treatment of kaempferol reduces the soleus p-S6 expression in CPK rats compared to the CP control group (CP = 121.40 ± 7.206 vs. CPK = 64.29 ± 14.44, p = 0.0022) [$F_{(1, 21)} = 12.82$, $p = 0.0002$] (Figure 7E). In addition, in the CPK group the p-S6 / total S6 ratio in the soleus muscle was reduced compared to the untreated CP group (CP = 0.996 ± 0.057 vs. CPK = 0.5367 ± 0.110, p = 0.0199) [$F_{(1, 21)} = 9.35$, $p = 0.006$] (Figure 7F).

Figure 7. Signaling pathways associated with protein synthesis in the soleus muscle.



Effects of neonatal kaempferol treatment on signaling pathways associated with protein synthesis in the soleus muscle of CP rats. The contents of: (A) Phosphorylated Akt; (B) - Total Akt; (C) - p-Akt / total Akt; (D) - RPS6; (E) - p-RPS6; (F) - p-RPS6 / RPS6; and (G) - IRS-1 in the soleus muscles were determined by Western blotting. Results are presented as mean ± S.E.M. on the basis of total protein loading as indicated by the Ponceau S measurement, n = 5-8 animals per group. Results were compared using two-way ANOVA and Bonferroni's post hoc test.

4 Discussion

To our knowledge, this is the first study to evaluate the effect of neonatal treatment with kaempferol on body phenotype, gait performance, muscle strength and skeletal muscle fiber type composition, and protein synthesis expression in a rat model of CP. The new finds presented here describe the impact of perinatal anoxia and sensorimotor restriction on body weight and body length during infancy; on gait kinetic, temporal, and spatial parameters; and muscle strength. Also, we found a great impact of the CP model on muscle fiber type distribution and fiber cross-section area and perimeter. The neonatal treatment of kaempferol reduces the impact of anoxia and sensorimotor restriction of hindlimbs in rats, preventing the altered body phenotype, the gait performance, and maturation of skeletal muscle fiber in soleus muscle. However, kaempferol affects the signaling pathways associated with protein synthesis in the soleus muscle of CP rats.

We showed that perinatal anoxia associated with sensorimotor restriction of the hindlimbs during infancy significantly impacts the body phenotype of rats, reducing the body weight and body length during the neonatal period. In previous studies, these repercussions in anthropometrics were also observed in rats during exposure to the CP model (LACERDA et al., 2017a, 2019b; SILVA et al., 2016). Lacerda et al. (2019) suggest that the model's impact on these parameters may be related to motor deficits that impair access to lactation and food. In addition, the model compromises suction in the first days of postnatal life and the functionality of chewing after weaning (LACERDA et al., 2017a, 2019b). We observed that the neonatal treatment of kaempferol prevents the reduction of the body weight and body length mainly after the end of the treatment period. These effects could be related to the beneficial effects of kaempferol in preventing the delay in the active motor function, favoring the food intake

consumption (Visco et al. 2022, non-published data). In addition, a study showed that neonatal administration of kaempferol increases the body weight and somatic growth in the offspring exposed to a maternal high-fat diet (CHAVES et al., 2020).

Reinforcing the negative impact of the CP model on the body phenotype, we report for the first time that perinatal anoxia and sensorimotor restriction of the hindlimb during infancy contributes to global strength deficits in young rats. The results here presented showed the reduced mean force of the grip strength analysis of the forepaws in rats from the CP group. Fragopoulou et al. (2019), report that neonatal systemic inflammation combined with hypoxia affects the grip strength of adolescent female rats compared to the control group (FRAGOPOULOU et al., 2019). Grip requires significant sustained strength, rather than dexterity or linear force, mainly in the digits and paws. In a study with mice submitted to a CP model, it was found reduced grip strength during the neonatal period (FEATHER-SCHUSSLER; FERGUSON, 2016). The reduction in the grip strength in CP animals could be related to the delay in the maturation of the grasping reflex and consequently acquisition of voluntary motricity (FEATHER-SCHUSSLER; FERGUSON, 2016). The neonatal treatment of kaempferol seems to reduce the impact of the CP model on the grip strength performance in rats. However, the mechanism needs to be further investigated. This occurrence could be associated with better body weight and body length in animals from the CPK group. However, a body of preclinical evidence suggests that polyphenolic compounds improve muscle strength by promoting better mitochondrial function, cellular energy metabolism and affecting the muscle fibers proportion (ABEER F. MOSTAFA, M.D., SHEREEN M. SAMIR, 2019; CHEN et al., 2019; OMMATI et al., 2020)

Our results demonstrated for the first time a characterization of the gait pattern of the model that combines the perinatal anoxia and sensorimotor restriction in rats.

Catwalk analysis showed in rats submitted to the CP model altered cadence of the gait, with interlimb incoordination, and increasing the base of support of the forepaws during active locomotion. In addition, rats with CP presented increased time of paw swing, reduced swing velocity of hind paws during gait. Also, it was observed reduced contact of the hindpaws during the stance phase. Another model of neurological disorders in rodents have been assessed with the catwalk system. In agreement with our results, the study of Delcour et al., (2018) assesses the effects of sensorimotor alone in the gait parameters in rats. It was found the maximal foot contact area during weight-bearing (ie, footprint surface) in sensorimotor restricted rats was smaller than that of controls at P30 (DELCOUR et al., 2018). Here, it was demonstrated that the neonatal treatment of kaempferol prevents the impact of CP in rats' gait, increasing the number of steps, and the coordination of the hindlimb during active locomotion. Also, CP rats that received kaempferol during the neonatal period showed a reduced effect on swing paws time and velocity during gait. Akefe, Ayo and Sinkalu (2020) suggest that the mechanism by which kaempferol mitigated the alteration in the behavioral parameters may be due to its anti-oxidative potential.

To assess the repercussions of the perinatal anoxia and sensorimotor restriction of hindlimbs during infancy in rats it was performed the histomorphometric analysis and the assessment of the signaling pathways associated with protein synthesis in the soleus muscle of CP rats. We observed reduced muscle mass, reduced proportion of oxidative fibers, and smaller cross-section area and perimeter in soleus muscle of CP rats. This finding is suggestive of dysfunction and delay of the maturation of soleus muscle that is characterized as an oxidative muscle (at P36 ~75% of type I).

In agreement with our results, Buratti et al., (2019) observed in CP rats reduced body weight, muscle weight, and length and reduction in the area and increase in the number of types I muscle fibers in the plantaris muscle (BURATTI et al., 2019). Studies that use the same CP model found reduced muscle mass in soleus and tibialis anterior muscle (MARQUES et al., 2014; STIGGER et al., 2011). In the present study, we found a reduction in the cross-section area and perimeter of both types of muscle fibers in soleus muscle. Muscle atrophy is a feature seen in patients with CP,and type I fibers are more susceptible to the alterations resulting from inactivity in these patients (MARBINI et al., 2002; WANG; PESSIN, 2013). The CP model does not affect only skeletal muscle tissue related to locomotion. Lacerda et al. (2017;2019) in previous studies showed that muscle involved in the chewing movement also presents reduced muscle mass with reduced oxidative fibers in the masseter muscle in CP rats.

The neonatal treatment of kaempferol reduced the impact of the CP model on the soleus weight and in the cross-section area of the type I muscle fibers in the soleus. However, surprisingly we do not observe reduced expression signaling pathways associated with protein synthesis in the soleus muscle of CP rats. Conversely, the rats with CP that receive the neonatal treatment of kaempferol showed reduced p-Akt and total AKT compared to control animals treated with the kaempferol. In addition, in comparison to untreated CP rats, reduced expression of p-RPS6 was observed in CP rats that receive the neonatal treatment with kaempferol. Despite numerous in vitro and in vivo studies that have reported polyphenols as strongly effective bioactive molecules that attenuate muscle atrophy and enhance muscle health, our results did not confirm the potential of kaempferol to increase the signaling pathways associated with protein synthesis in the soleus muscle of CP rats. Alklady et al, found that

kaempferol increased phosphorylation activity in the liver, however, neither proteins nor their activities were changed in skeletal muscle tissues (ALKHALIDY et al., 2018).

5 Conclusion

The neonatal treatment with kaempferol in rats with CP attenuates the impact of the model on the body phenotype, preventing weight reduction and body growth in young rats. Furthermore, beneficial effects of the intervention were observed by reducing strength deficits, and the impact on the kinetic, temporal and spatial parameters of gait. In addition, neonatal treatment with kaempferol mitigates the reduction of oxidative fibers in the soleus muscle, as well as the impact of the model on the histomorphometric measurements of muscle fibers. Kaempferol treatment has the potential as a neuroprotective agent for perinatal brain disorders as well as presenting beneficial effects for skeletal muscle tissue. It is suggested that further studies be carried out to elucidate the cellular, molecular and biochemical mechanisms in which kaempferol beneficially affects the nervous systems and skeletal muscle.

Conflict of interest

The authors declare no conflicts of interest.

Data availability statement

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

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APÊNDICE E - "METABOLIC AND NEUROLOGICAL CONSEQUENCES OF THE TREATMENT WITH POLYPHENOLS: A SYSTEMATIC REVIEW IN RODENT MODELS OF NONCOMMUNICABLE DISEASES"

METABOLIC AND NEUROLOGICAL CONSEQUENCES OF THE TREATMENT WITH POLYPHENOLS: A SYSTEMATIC REVIEW IN RODENT MODELS OF NONCOMMUNICABLE DISEASES

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Abstract

Noncommunicable diseases (NCDs) are the main cause of death worldwide. In most cases, NCDs lead to drastic metabolic alterations with associated energy balance and body weight changes, two related physiological processes regulated by the brain. Polyphenol-based treatments for NCDs have emerged as a promising therapy, which seems to involve energy balance modulation. However, it remains unclear what the most effective polyphenols-based treatment is to attenuate adverse effects in energy balance of NCDs. This systematic review aimed to evaluate the literature on the metabolic and neurological effects of polyphenols-based treatment in rodent models of NCDs. To select the articles evaluated in this systematic review, we carried out a literature search in the following databases: CINAHL, Medline/PubMed, SCOPUS, and Web of Science. For title and abstract screening, original papers with polyphenols exposure in rodents were selected. For full-text screening, studies with models of NCDs that reported metabolic and neurological outcomes when treated with polyphenols were selected for inclusion in this review. The assessment of methodological quality was performed according to the adapted points highlighted by the analysis tool risk of bias proposed by Hooijams *et al* (2014). The level of agreement between reviewers evaluating studies for data extraction and assessments study quality (risk of bias) was assessed by Kappa Statistics. 23 articles, using individual compound (11 articles) or polyphenols extracts (12 articles), were included in this review: 5 articles using tea polyphenols, 12 articles using grape-derived polyphenols, 3 articles using the polyphenol quercetin, and 3 articles using other polyphenol sources. All the studies evaluated effects of different sources of polyphenols in rodents subjected to NCDs including stroke, depression, anxiety and stress disorder, neurocognitive and neurodegenerative disorders, diabetes, and obesity. Most results agree on the beneficial effect of polyphenols in attenuating alterations in energy balance and body weight in animals subjected to different NCDs. Interestingly, such effects were associated with neuroprotective responses in different brain areas including hippocampus and hypothalamus, a key regulator of energy balance. Moreover, studies using resveratrol or quercetin showed higher internal validity according to analysis of risk of bias. In conclusion, this review shows that the treatment with polyphenols, especially resveratrol or quercetin, attenuates adverse effects of NCDs on energy balance and are associated with neuroprotective effects.

Keywords: Energy balance; Neuroprotection; Noncommunicable disease; Polyphenols; Rodents; Systematic review.

1. Introduction

NCDs, also known as chronic diseases, are the main cause of death worldwide according to WHO data of 2015. NCDs, including cardiovascular disease and diabetes, kill 19.5 million people annually, both closely related to obesity and metabolic syndrome. WHO recommends their opportune detection, screening and, especially, treatment as key actions of the response to NCDs [1]. WHO recommends their opportune detection, screening and, especially, treatment as key actions of the response to NCDs [1].

The etiological component of these NCDs is, in part, a sustained positive energy balance (EB), which results in the accumulation of adipose tissue and the subsequent development of overweight and obesity, an important risk factor to develop other medical conditions including type 2 diabetes, cardiovascular diseases, fatty liver, and some types of cancer [2]. Body weight (BW) and body composition are determined by a long-term balance between energy intake (calories from food) and energy expenditure (EE), which mainly consists of energy spent during physical activity, basal metabolism and adaptive thermogenesis [3,4]. EB is considered as positive if calorie intake is higher than calorie expenditure [5]. Body composition, on the other hand, is the amount and distribution of fat and fat-free tissues (muscle and bone) [6].

EB is finely regulated by a complex range of signals of metabolic, mechanical, endocrine, and neural nature, which ultimately induces appetite and energy storage or satiety and EE [7]. The most important signals are leptin and insulin, two hormones produced by adipose tissue and the pancreas, respectively. This, so-called “adiposity signal”, passes through the blood-brain barrier (BBB) and lets the central nervous system (CNS) sense the levels of energy stored in the body as body fat mass, which are proportional to the aforementioned hormone circulation [8,9]. Other types of signals are the circulating levels of cholesterol, triglycerides, glucose, several neuroendocrine stimuli (cholecystokinin, glucagon-like peptide1 and peptide YY), and increase of vagal nerve activity, which are activated after food intake (FI) and send the information to stop eating and feel satiety [10].

The current treatments for overweight and obesity are based in lifestyle modifications and drug consumption, which have reduced long-term success and limited effectiveness [11]. Since overweight and obesity are complex medical conditions, and there are no effective long-term therapies so far, the interest in alternative treatment is growing. Thus, special focus has been given to natural compounds, because several plant extracts possess the ability to decrease glucose levels and to improve EB regulation, as polyphenols do [12].

Polyphenols are a wide group of more than 8,000 molecules produced as secondary metabolism by plants; they include flavonoid (e.g. quercetin, kaempferol, apigenin, catechins) and stilbene (e.g. resveratrol) compounds. Studies from the past three decades involving polyphenols have shown beneficial effects on health due to their capacity to act as antioxidants and exert other health-related functions, like anti-diabetic, anti-cancerogenous and as anti-inflammatory agents [13]. With regard to the management of BW and metabolic parameters, polyphenols have the potential in lowering FI, decreasing adiposity, activating sympathetic thermogenesis, and attenuating inflammation and oxidative stress [14]. Polyphenols modulate different genes, enzymes and signaling cascades in peripheral organs like liver, muscle, adipose and digestive tissues, but mainly, and most importantly, they are able to modulate signaling pathways within the CNS [15,16].

The CNS is a key factor in energy homeostasis maintenance due to fuel-sensing neurons located in the hypothalamus that respond to energy signals from the periphery and make the adjustments (by acting on second order neuro-circuits) to promote an equilibrium between EE/energy intake and metabolic parameters [17]. More specifically, in the arcuate nucleus (ARC), two neuron populations are excited or inhibited by nutritional signals including leptin, glucose and fatty acids. One cell population NPY/AGRP (Neuropeptide Y/agouti related peptide) has orexigenic effects, while on the other hand, POMC/CART (pro-opiomelanocortin/cocaine and amphetamine-regulated transcript) neurons have anorexigenic outcomes [18]. Obesity is closely related to hypothalamic inflammation, which is characterized by over-activated microglia and rises in pro-inflammatory cytokines [19,20]. These are shown to be important targets of natural compounds like polyphenols. Some flavonoids, like anthocyanins have neuroprotective, antidiabetic and cardioprotective effects [21]. Also, recent studies have probed strong evidence of polyphenol effect over activation of nuclear factor erythroid 2-related factor 2 (Nrf2) and its downstream pathway enzymes (heme oxygenase-1) to play a beneficial role in metabolic diseases [22].

Although there is substantial evidence of the beneficial effects of polyphenols in brain areas regulating EB, the neurological mechanisms involved in such effects are still unclear. Its knowledge will allow the development of other types of studies (e.g. clinical) and suitable strategies according to health condition, dosage and way of administration, and the comprehension of other possible targets and natural molecule derivatives to treat this kind of metabolic disturbance.

2. Methods

2.1. Systematic review reporting and protocol registration

This systematic review complies with Preferred Report Items for Systematic Reviews and Meta-Analysis (PRISMA) [23]. In addition, its protocol was published in the Collaborative Approach to Meta-Analysis and Review of Animal Data for Experimental Studies (CAMARADES) [24].

2.2. Search strategy

The literature search was conducted from January to March 2020 in the following databases: Medline/PubMed (1966-2020), SCOPUS (1969-2020), CINAHL (1937-2020), and Web of Science (1900-2020). It was carried out in electronic databases by two independent reviewers (Lacerda DC and Urquiza-Martínez MV) based on a predefined protocol. The search strategy was composed of terms relating to describing the population, intervention, comparison and outcomes. The guiding question was composed of the points of PICO, which consist of **Population**: rodent models (rats or mice) reproducing any NCDs, **Intervention**: treatment with polyphenols, **Comparison**: rodents subjected to any NCDs which have not been treated with polyphenols, and **Outcomes**: metabolic and neurological parameters. The primary outcomes were neurological-dependent effects including BW and EB, which depends on FI and EE (thermogenesis and/or physical activity-based EE). The secondary outcomes comprised metabolic parameters such as plasma levels of glucose, lipids (cholesterol and triglycerides), hormones (insulin, insulin-like growth factor, glucagon, leptin, cholecystokinin, ghrelin) as well as glucose tolerance and adiposity. Combination of MeSH descriptors or DECS descriptors and keywords were included in the search: rats, mice, NCDs, neoplasms, cardiovascular diseases, respiration disorders, diabetes mellitus, depressive disorder, neurodegenerative diseases, anxiety disorders, physiological stress, obesity, polyphenols, eating, energy metabolism, feeding behavior, biomarkers, and nervous system.

To assess the level of agreement among reviewers in the screening phase, the Kappa index was calculated using the Statistical Package for the Social Sciences – SPSS version 20 for Windows (IBM SPSS Software, Armonk, NY, USA). A third reviewer (Manhães-de-Castro R) was consulted when needed as a mediator for the definition of inclusion or exclusion of items when there was no agreement between reviewers.

2.3. Inclusion criteria

To select the articles for this systematic review, the screening of studies was performed in two phases. In phase 1, screening of studies was performed by reading the title and abstract of original papers. Then, in phase 2, the full-texts of the studies were considered. The following inclusion criteria were applied: studies that evaluated rodents subjected to NCDs and treated by polyphenols, studies that have control group in the study design, and studies that analysed metabolic and neurological outcomes were considered. Additionally, the following exclusion criteria were adopted: articles that evaluated animals subjected to a method of treatment not suitable for replication and articles that evaluated treatment with polyphenols coupled with other interventions. For search and selection of articles there was no year of publication restrictions, as well as no restriction of the articles' language.

2.4. Data extraction

Data extraction was performed by two researchers using a piloted form. The following details were extracted from each study: article name, authors, year of publication, and journal of publication. The polyphenol or main active polyphenol present in the treatment was extracted. The characteristics of animals used in the studies were extracted: type of rodent, animal age and the animal's sex. The following information regarding the characteristics of the intervention was addressed: type of polyphenol; route of administration, dose/concentration used, time of day of treatment, and duration of treatment. Finally, we analyzed the effects of the treatment with polyphenols on neurological-related effects including BW, EB variables, and metabolic parameters. To evaluate the results of the included articles, the values of mean (M), standard deviation (SD) or standard error of mean (SEM) were analyzed.

2.5. Analysis of risk of bias

The analysis of the risk of bias of the included studies were performed according to the adapted points highlighted by the analysis tool risk of bias proposed by Hooijmans *et al*. (2014) [25]: randomization of the sample, animal care (room temperature, water and diet), blinding of the intervention and evaluation and presence of ethical code register. The level of agreement between reviewers evaluating the studies for data extraction and assessments study quality (risk of bias) was assessed by Kappa Statistics using Statistical Package for the Social Sciences – SPSS version 20 for Windows (IBM SPSS Software, Armonk, NY, USA).

3. Results

3.1. Study selection

Initial database searching resulted in 6107 articles. The number of articles identified in each database was as follows: 3453 articles in Medline/Pubmed, 1052 articles in Scopus, 759 articles in CINAHL and 843 articles in Web of Sciences. After the analysis of the title and abstract, 5985 articles were excluded either because it did not meet the PICOS points or because it did not meet the defined inclusion criteria. Finally, after evaluating the full-text of the 122 remaining articles, 99 articles were excluded due to disagreement with the PICO points as well as the eligibility criteria. Therefore, a total of 23 articles were included in this review; a flow diagram of the article selection process is shown in **Figure 1**.

3.2. Study characteristics

The characteristics of the studies included in this review are summarized in **Table 1-4**, according to the source and type of the polyphenol administered. According with the source of the polyphenols we found 6 articles using tea polyphenols (**Table 1**) and 11 articles using grape derived polyphenols (**Table 2**). Quercetin was individually used in 3 articles (**Table 3**) and 3 articles used other polyphenol sources (**Table 4**). Interestingly, 11 articles (44% of included articles) used polyphenols individually. The single compound mentioned in these studies were: resveratrol (6 articles), trans-resveratrol (1 article), quercetin (3 articles), and paeonol (1 article). The chemical structure of the main polyphenols employed in the studies is illustrated in **Figure 2**. Of the 23 articles included, most studies used male animals, except Chan *et al*. (2006) [26] (**Table 1**) and Wang *et al*. (2013) [27] (**Table 2**) who used females. Also, Chang *et al*. (2018) [28] and Ferreira *et al*. (2018) [29] did not inform the sex of the animals.

Regarding the rodent type, most studies used rats (Wistar or Sprague-Dawley); however, some models of mice (C56Bl/6j, ICR, B6/SJL and SMP8) were used as well (**Table 1-4**). Also, a wide heterogeneity was observed regarding the route of administration and dose of polyphenols used (**Table 1-4**). Indeed, 9 articles used oral administration, 4 used oral gavage, 4 did intragastric gavage, 4 used intraperitoneal administration, and 2 articles did intravenous injection. According to the dose used, we found variations in a range between 30 and 500 mg/kg when oral administration was done, 2 and 500 mg/kg by oral gavage, 5 and 80 mg/kg by intragastric gavage, 20 and 100 mg/kg when intraperitoneally administration was done, and 0.1 and 200 mg/kg by intravenous injection (**Table 1-4**). Furthermore, a large variation in terms of treatment duration was also observed, from a single dose to a daily treatment for 1, 3, 4, 7, 8, 10, 12, 14, 16 or 24 weeks (**Table 1-4**). All the studies included in this systematic review evaluated effects of different sources and types of polyphenols in rodents subjected to NCDs

including stroke, depression, anxiety and stress disorder, neurocognitive and neurodegenerative disorders, diabetes, and obesity (**Table 5**).

3.3. Main findings

The results related to metabolic and neurological outcomes of the articles included in this review are summarized in **Table 5**. Of the 23 articles included, 9 analyzed BW, 2 measured FI, 11 assessed parameters of EE, 10 evaluated metabolic parameters including serum levels of glucose and lipids. In general, the treatment with polyphenols was more effective to reduce metabolic and neural damage in obesity, diabetes, and anxiety/disorder models. However, mild effects of the treatment with polyphenols were found in rodents subjected to cognitive decline and stroke disease. Among active polyphenols, resveratrol and quercetin induced higher effects, reducing metabolic and neural damage in animals subjected to NCDs.

Regarding the EB outcomes, two studies evaluated the effect of polyphenols treatment on FI; however, only one reported the results obtained. Ibars *et al*. (2018) found that oral administration of grape derivate polyphenols (100 mg/kg, per day for 7 weeks) reduced FI in obese rats [30]. The authors propose that this result was the consequence of hypothalamic action of the treatment including up-regulation of proopiomelanocortin (POMC) and increased leptin sensitivity through the modulation of the hypothalamic leptin signal pathway [30] (**Table 5**). On the other hand, Phyu *et al*. (2016) did not inform FI results [31]. Concerning EE analysis, studies showed heterogeneous results. Among 11 studies that evaluated EE, 4 found an increase in EE after polyphenols therapy (**Table 5)**. Ibars *et al*. (2018) showed that oral administration of grape derivate polyphenols (100 mg/kg, per day for 7 weeks), increased EE due to lipid use and elevation of respiratory quotient in obese animals [30] (**Table 5**). The neural mechanisms underlying these findings were an elevation of expression of anorexigenic neuropeptides in the hypothalamus [30]. In addition, Chang *et al*. (2018) verified that intraperitoneal administration of resveratrol (20 mg/kg, per day for 1 week) increased EE by enhancing locomotor activity in animals subjected to stroke disease [28]. The neural mechanisms associated with these findings were reduced neuronal loss, reduced expression of inflammatory cytokines and decreased oxidative stress (**Table 5)** [28]. Besides, Kosari-Nasab *et al*. (2019) found that oral supplementation with quercetin (50 mg/kg, per day for 2 weeks) increases locomotor activity in animals submitted to anxiety/stress disorder [32]. Elevation of EE involved a reduction in activity of the HPA axis (**Table 5)** [32]. Also, Chen *et al*. (2009) found a comparable increase in locomotor activity after treatment with green tea, during 3 weeks [33]. This result was related to antioxidant activity and increased serum levels of norepinephrine and dopamine [33]. Finally,

among 10 studies that analyzed metabolic parameters, 7 articles showed that different sources of polyphenols were able to reduce serum levels of glucose in rodent models of stroke, diabetes and obesity. In addition, the treatment with polyphenols was able to reduce lipid accumulation on peripheral cells [34], decrease cellular content of lipids in adipocytes [34], and reduce serum levels of cholesterol (**Table 5)** [27].

In different models of NCDs it was observed that polyphenols, independently of the source, normalize the alterations of BW induced by the experimental disease. Abd El-Fattah *et al*. (2018) observed that oral treatment with resveratrol (80 mg/kg, per day for 8 weeks) attenuated BW loss in rats subjected to a model of depression [35]. This result was associated with a reduction in the activation of the hypothalamic–pituitary–adrenal axis (HPA), increased hippocampal serotonin levels, inhibition of monoamine oxidase (MAO) activities and increased expression level of Brain-derived neurotrophic factor (BDNF) in hippocampus (**Table 5)** [35]. Moreover, four studies demonstrated that treatment with polyphenols reduced BW loss of animals subjected to experimental diabetes (**Table 5)** [29,36–38]. Control of BW in diabetic animals was linked to a reduction of oxidative and nitrosative stress in different segments of the peripheral nervous system, including dorsal root ganglia, sciatic nerve and myenteric plexus [29,36–38]. Moreover, supplementation with polyphenols decreased BW in obese animals [27,39]. According to Macedo *et al.* (2019), oral administration of green tea (500 mg/kg, 5 days per week for 12 weeks) decreased inflammation in the cerebral cortex, brain stem and cerebellum by increasing some of the antioxidant enzymes. Also, Wang *et al*. (2013) found that control of BW was associated with neuroprotection of hippocampus [27]. However, Phyu *et al*. (2016) only observed transitory reduction of BW after a low dose of resveratrol (2 mg/kg, per day for 8 weeks) (**Table 5)** [31]. Madhavadas *et al*. (2016), on the other hand, did not inform about BW [40].

3.4. Quality assessment of included studies

The analysis of risk of bias of the articles included was performed according to the adapted SYRCLE (Systematic Review Centre for Laboratory animal Experimentation) tool; it consists of 5 entries related to 4 types of bias: selection bias, performance bias, detection bias, and other biases. Results were summarized in **Table 6**. After analyzing the studies, we observed methodological deficits in all included articles, except Kosari-Nasab *et al*. (2019) [32], which described clearly all topics of risk of bias tool used. Of the 23 included studies, 17 referred to randomization of sample; however they did not inform the method applied. Most articles have adequately described the conditions of animal care (room temperature, water and diet).

However, Ferreira *et al* (2018) [29] did not make it clear whether proper care was taken with animal care. Only Kosari-Nasab *et al*. (2019) [32] performed blinding of intervention. However, it is important to highlight that blinding animal interventions are almost unfeasible due to induction of NCDs, which limit keeping unknown animal identity. Besides, the phenotype of animals submitted to NCDs may be a signal for evaluators about animal identity, which limits blinding methods. In addition, of all included studies, Akinrinmade *et al* (2017) [41], Chang *et al*. (2018) [28], Demir *et al*. (2016) [37] and Kosari-Nasab *et al*. (2019) [32] performed blinding of evaluation method, which may increase the accuracy and reliance of extracted results. Finally, only Chen *et al*. (2009) [33] did not inform about ethic code register. The reviewers presented substantial agreement for screening and in the risk of bias analysis (Kappa = 0.752).

4. Discussion

This systematic review provided all information available of studies involving the physiological effects of polyphenols on metabolic and neurological parameters in rodent models of NCDs. The main findings of included articles show that treatment with polyphenols leads to: 1) reduced serum levels of glucose and lipids, 2) increased EE, 3) reduced FI, and 4) and maintenance of BW under normal conditions, avoiding excessive gain or losses. These benefits were associated with some neural responses, including: reduced oxidative stress and neuro-inflammation on different brain segments, this includes cerebral cortex, brain stem, cerebellum, hippocampus regions (CA1 and CA3), and hypothalamus. Also, treatment with polyphenols inhibited the HPA axis activity. Moreover, treatment with polyphenols decreased neural cell death, reduced apoptosis markers and increased expression levels of BDNF mainly in the hippocampus. In addition, the therapy with polyphenols up-regulated the expression of hypothalamic anorexigenic neuropeptides. Thus, neuroprotective effects induced by polyphenols seem to be more relevant in the hypothalamus and hippocampus and reproducible between individual polyphenol or extract treatments. This information will allow the development of studies employing other sources of polyphenols, analyzing its intervention on specific parts of the neurological pathways involved on EB and metabolic parameters in NCDs, or even, design future studies with humans.

Food intake

The treatment with polyphenols was effective on reducing FI in rats subjected to obesity. According to Ibars *et al*. (2018) [30], oral administration of grape derivate polyphenols (100 mg/kg, per day for 7 weeks), reduced FI in obese rats. Furthermore, the decrease in FI was associated with elevation of leptin sensitivity through the modulation of the hypothalamic leptin

signal pathway, and up-regulation of gene expression of POMC in the hypothalamus [30]. The CNS regulates EB through modulation of FI and expenditure and energy supply. In addition, control of EB occurs mainly in the ARC of the hypothalamus. This area of the hypothalamus has neurons that express POMC and CART neuropeptides, which inhibit appetite, and has another population of neurons that express NPY and AgRP with opposite effect [42]. Thus, up-regulation of gene expression of POMC, verified after oral administration of grape derivate polyphenols (100 mg/kg, 10 weeks), may have contributed to the decrease FI in obese rats [42]. In addition, it is important to highlight the role of leptin in modulation of EB. Leptin is an important sign of energy status, interacting with hypothalamic receptors to maintain BW homeostasis and EB. In CNS, leptin acts mainly on neurons in the ARC in order to reduce FI and increase EE [43]. The central action of leptin occurs by inhibiting orexigenic hypothalamic neurons (NPY/AgRP) and stimulation of the anorexigenic ones (POMC/CART). Thus, treatment with grape derivate polyphenols led to an improvement of leptin sensitivity in ARC, which may explain the reduction of FI in obese animals [30]. In summary, it seems that supplementation with grape derivate polyphenols, may be used as a clinic strategy to control BW in obesity.

Energy expenditure

To complete the evaluation of EB modulation, we searched articles that analyzed the effects of the treatment with polyphenols on EE outcomes. Based on this, different methods of treatment with polyphenols were able to increase EE in rodents subjected to obesity [30], stroke [28] and anxiety/stress disorder [32,33]. The main neural mechanisms underlying these findings were the following: an elevation of expression of anorexigenic neuropeptides in hypothalamus, decreased activity of the HPA axis, increased serum levels of norepinephrine and dopamine and reduction of oxidative stress and neuronal loss. Therefore, treatment with grape derivate polyphenols induced up-regulation of hypothalamic neurons that express POMC and may contribute to enhance EE [30]. In addition, the elevation of serum levels of norepinephrine, induced by green tea treatment may also contribute to increase EE [33]. Neurotransmitters such as epinephrine, norepinephrine and serotonin promote activation of anorexigenic pathways, especially in the ARC [44].

Body weight

Coupled with analysis of EB variables, we searched articles that investigate the role of polyphenols on modulation of BW in different models of NCDs. NCDs, including depression and diabetes led to a drastic reduction of BW [29,35–37]. Conversely, polyphenols therapy

attenuated weight loss, which contributes to keeping BW into normal range, taking control animals as reference [35–37]. Increased BW in depressive rats was associated with multiple neural responses, including: reduced activaty of the HPA axis, increased hippocampal serotonin levels, inhibition of MAO activities and increased expression level of BDNF in hippocampus [35]. Studies report that the hormone CRH is a potent anorexigenic marker [42,45]. Thus, it is suggested that the reduction in the activity of the HPA axis may explain the increase in BW in depressive rats treated with resveratrol. Under challenge, a release of glucocorticoids (corticosterone in rodents or cortisol in humans) is activated by the stress system. This system is composed by corticotropin-releasing hormone (CRH) and locus coeruleus-norepinephrine (LC/NE)-autonomic systems along with their peripheral effectors (the pituitary-adrenal axis and the limbs of the autonomic system) [46]. Among their functions, glucocorticoids are able to modify the energy metabolism by stimulating hepatic gluconeogenesis, inhibiting insulin action on skeletal muscle and potentiating the storege in adipose tissue promoting BW gain, dyslipidemia, visceral adiposity and metabolic syndrome [47,48]. The effect of the polyphenols on the HPA axis is to suppresses its hyperactivity by reducing serum corticosterone and ACTH (adrenocorticotropic hormone) levels, leading to its homeostasis [49].

Concerning the increase of BW in diabetic rats, the authors found a reduction of oxidative and nitrosative stress in different segments of peripheral nervous system, including dorsal root ganglia, sciatic nerve and myenteric plexus [29,36,37]. Moreover, supplementation with polyphenols decreased BW in obese animals [27,39]. Among neural mechanisms, the authors highlight that polyphenols decrease the inflammation in the cerebral cortex, brain stem, and cerebellum [39] and exert neuroprotective actions in the hippocampus [27].

Antioxidant

The results obtained show a clear antioxidant effect against oxidative damage by its dihydroxy and trihydroxy structure (see **Figure 2**), which reduce reactive oxygen and nitrogen species production and/or promote the expression of endogenous antioxidant genes [31,33]. This activity prevents the damage of molecules like lipids, which are part of the cell membrane's phospholipids, in a type of harm called lipoperoxidation, which alters the fluidity and the selectivity function of the membrane, compromising the integrity of the cell [26]. CNS proper composition, which is high in easily oxidizable polyunsaturated fatty acids (PUFA) plus reduced levels of antioxidant enzymes and a high quantity of redox active transition metal ions (e.g. iron), makes it a very susceptible tissue to oxidative damage [50]. Proteins and nucleic acids are potentially attacked by oxidation, causing protein aggregation and apoptosis. Such

damage can alter and cause malfunction in several brain areas besides triggering inflammation [51].

Oxidative stress is related to increases in lipid hydroperoxides (LOOH) and nitric oxide (NO) levels, decrease in glutathione (GSH) levels and reduction in enzyme activity of Superoxide dismutase (SOD) and Glutathione S-transferases (GST), intensifying tissue damage. Several studies showed the beneficial effect of polyphenols reverting oxidative damage. Such is the case of resveratrol, which was able to restore the activity of several antioxidant enzymes via the nuclear factor Nrf-2-dependent mechanisms [35]. Also, studies in a diabetes model, showed that resveratrol or *Cornus mas* L. extracts were able to increase endogenous levels of antioxidants (e. g. SOD, GSH and MCH) and neutralize active forms of oxygen and inhibit lipid peroxidation through increasing catalytic activity and expression of SIRT1 [29,36].

Anti-inflammatory

It is important to highlight that different sources of polyphenols therapy reduced neuro-inflammation. Astrocytes and microglia are responsible for the response in the face of any insult on brain tissue. They produce many pro-inflammatory cytokines like Tumor Necrosis Factor alpha (TNFα), interleukins like one-beta and six (Il-1β and IL-6), the production of NO, and the translocation of Nuclear Factor kappa-B (NF-κB) [52]. Results from this review show that polyphenols (e. g. quercetin) have the ability to suppress the expression of pro-inflammatory cytokines (TNFα and NF-κB). They promote the expression of a heme oxygenase 1 (HO-1), whose function is suppressing TNFα signaling by diminishing NF-κB activation, thus reducing the microglia-mediated inflammatory response, which is central part of neuro-inflammation and neurodegenerative disorders [53,54].

Pre-treatment with tea polyphenols was able to reduce brain edema and ameliorate BBB elevated permeability on ischemic injury due to attenuation of oxidative damage and reduction in the expression of the tight junction proteins claudin-5, occluding and ZO-1 [41]. Other studies showed that administration of resveratrol or quercetin induced inhibition of NF-κB, TNF-α, Il-6, and Il-1β expression in the hippocampus of rats with depression-like behavior via Akt/GSK3β signaling pathway [35,37,55]. These results are consistent with Macedo *et al*. (2019) [39], which evaluate the effect of green tea extracts, rich in catechins and caffeine, over an obese model probing that overnutrition can lead to chronic inflammation and oxidative stress attenuated by polyphenol treatment.

Gene expression

Regarding genetic parameters, polyphenols can modulate gene expression of stress-related proteins. Indeed, it has been reported that polyphenols affect the expression of metallothioneins, heat shock proteins, interleukin-2 (enhancer of humoral immunity) [33], and insulin-like growth factor 1 (IGF-1) which acts as a promoter of brain activity and reaches the hypothalamus leading to peripheral metabolic effects [56,57]. Moreover, they may exert an effect on neurogenesis and neuroplasticity by promoting expression of glucocorticoid receptors and proteins involved in the maintenance of neuronal caliber and axon formation [49]. Also, IGF-1 protects against oxidative stress in energy metabolism [56]. Also, polyphenols from red wine were probed to increase expression of nitric oxide synthase (eNOS) and prevent tissue damage and apoptosis in cerebral ischemia [58,59]. In a depression model, resveratrol increased the expression level of BDNF in the hippocampus via CREB pathway, attributed to an increase in serotonin levels and resulting in an antidepressant-like behavioral outcome [35]. In an anxiety model, the flavonoid quercetin exerted a therapeutic effect by down-modulating expression of hypothalamic CRF and reducing the activation of the HPA axis. This translates into a reduction of the ACTH secretion and, in consequence, corticosterone in the serum [32]. In addition, grape and cherry (rich phenolic fruits) consumption led to an overexpression of both POMC and the leptin receptor Obrb in the hypothalamus [30]. This increase was associated with a down-regulation of AgRP and tyrosine-protein phosphatase non-receptor type 1 (Ptb 1b), exlplaining lower FI (anorexigenic effect) as part of the leptin signaling in a photoperiod-dependent manner [30].

Neurogenesis, neuroplasticity and cell survival

There is a well established relationship between obesity, metabolic syndrome, inflammation and cognitive declination [60], associated with neurophysiological and structural changes in the brain such as hypometabolism, microvascular dysfunction and reduced neuroplasticity [61]. Polyphenol treatment shows an increase on neurogenesis and neuronal signaling (neuroplasticity) associated with a rise in IGF-1 receptor signaling through phosphatidylinositol-3 kinase (PI3K) and its downstream partner mammalian target of rapamycin (mTOR) regulation [27,40,49,57]. Also, studies had shown an important anti-apoptotic effect associated to its antioxidant capacity [31,62]. The use of polyphenols in an ischemia/reperfusion model, probed the rescue of CA1 pyramidal neurons, and showed that they contribute to the disposal of ROS and promote chaperoning, which leads to neuroprotection via JAK/ERK/STAT signaling pathway [28]. ERK pathway is an intracellular signaling cascade involved in differentiation, survival, and functional as well as structural

plasticity in neurons, stimulated also by BDNF induced by resveratrol [35,63]. Furthermore, also chronic stress can cause synaptic remodeling, atrophy, and dendritic spine loss in the hippocampus (CA1 pyramidal neurons and DG granular neurons), as well as neurobehavioral alterations which may be attenuated by phenolic compounds like Paeonol [64].

5. Conclusion

This systematic review clearly demonstrates the capacity of polyphenols to regulate metabolic and neurological parameters in different models of NCDs. Particular effects were observed with resveratrol and quercetin, which were able to regulate different parameters of EB and BW and induce neuroprotective effects at the brain level. Most of the included articles showed that polyphenols promote effects on BW, EB, and inflammation, which is highly related to obesity, suggesting that these natural compounds might be managed to treat obesity and obesity-related diseases. The treatment with polyphenols in some diseases including cognitive decline and stroke showed, however, mild effects on EB parameters. In this systematic review, it was observed that treatments with individual polyphenols reproduce the metabolic effects induced by extracts. This fact is relevant for development of potential therapies to combat metabolic diseases.

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Disclosure of Interests

The authors declare no competing interests.

Data Availability statement

This systematic review was performed by a global screening and presentation of data published in the following original articles:

Article	Data Availability Statement
M.-F. Ritz, P. Ratajczak, Y. Curin, E. Cam, A. Mendelowitsch, F. Pinet, R. Andriantsitohaina, **Chronic treatment with red wine polyphenol compounds mediates neuroprotection in a rat model of ischemic cerebral stroke**., J. Nutr. 138 (2008) 519–525.	The data that support the findings of this study are openly available in the website of the Journal at https://academic.oup.com/jn/article/138/3/519/4670240 doi:10.1093/jn/138.3.519.
M.-F. Ritz, Y. Curin, A. Mendelowitsch, R. Andriantsitohaina, **Acute treatment with red wine polyphenols protects from ischemia-induced excitotoxicity, energy failure and oxidative stress in rats**., Brain Res. 1239 (2008) 226–234.	The data that support the findings of this study are available, with access fees, in the website of the Journal at https://www.sciencedirect.com/science/article/abs/pii/S0006899308020969?via%3Dihub doi:10.1016/j.brainres.2008.08.073.
O. Akinrinmade, S. Omoruyi, D. Dietrich, O. Ekpo, **Long-term consumption of fermented rooibos herbal tea offers neuroprotection against ischemic brain injury in rats**., Acta Neurobiol. Exp. (Wars). 77 (2017) 94–105.	The data that support the findings of this study are openly available in the website of the Journal at at https://www.ane.pl/cookie?page=linkout.php?pii=7709 doi:10.21307/ane-2017-040.
C. Chang, Y. Zhao, G. Song, K. She, **Resveratrol protects hippocampal neurons against cerebral ischemia-reperfusion injury via modulating JAK/ERK/STAT signaling pathway in rats**., J. Neuroimmunol. 315 (2018) 9–14.	The data that support the findings of this study are available, with access fees, in the website of the Journal at https://www.sciencedirect.com/science/article/abs/pii/S0165572817304721 doi:10.1016/j.jneuroim.2017.11.015.
A.A. Abd El-Fattah, A.T. Fahim, N.A.H. Sadik, B.M. Ali, **Resveratrol and dimethyl fumarate ameliorate depression-like behaviour in a rat model of chronic unpredictable mild stress**., Brain Res. 1701 (2018) 227–236.	The data that support the findings of this study are available, with access fees, in the website of the Journal at https://www.sciencedirect.com/science/article/abs/pii/S0006899318304918 doi:10.1016/j.brainres.2018.09.027.
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X.-L. Zhu, J.-J. Chen, F. Han, C. Pan, T.-T. Zhuang, Y.-F. Cai, Y.-P. Lu, **Novel antidepressant effects of Paeonol alleviate neuronal injury with concomitant alterations in BDNF, Rac1 and RhoA levels in chronic unpredictable mild stress rats.,** Psychopharmacology (Berl). 235 (2018) 2177–2191.	The data that support the findings of this study are available, with access fees, in the website of the Journal at https://link.springer.com/article/10.1007/s00213-018-4915-7 doi:10.1007/s00213-018-4915-7.
R. Xue, G. Wu, X. Wei, J. Lv, R. Fu, X. Lei, Z. Zhang, W. Li, J. He, H. Zhao, J. Zhao, X. Ding, **Tea polyphenols may attenuate the neurocognitive impairment caused by global cerebral ischemia/reperfusion injury via anti-apoptosis**., Nutr. Neurosci. 19 (2016) 63–69.	The data that support the findings of this study are available, with access fees, in the website of the Journal at https://www.tandfonline.com/doi/full/10.1179/1476830514Y.0000000160 doi:10.1179/1476830514Y.0000000160.
W.-L. Zhu, H.-S. Shi, Y.-M. Wei, S.-J. Wang, C.-Y. Sun, Z.-B. Ding, L. Lu, **Green tea polyphenols produce antidepressant-like effects in adult mice.,** Pharmacol. Res. 65 (2012) 74–80.	The data that support the findings of this study are available, with access fees, in the website of the Journal at https://www.sciencedirect.com/science/article/abs/pii/S1043661811002611 doi:10.1016/j.phrs.2011.09.007.
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Figure and Table Captions

Figure 1. Flow diagram of the article selection process. Selection of studies occurred in four phases: 1) Identification: potential studies were gathered from databases; 2) Screening: studies were selected for full-text evaluation using titles and abstracts, based on PICO statement; 3) Eligibility: studies were selected for inclusion using eligibility criteria; 4) Inclusion: studies were selected for risk of bias analysis.

Figure 2. Chemical structure of the main polyphenol used in the articles included in this review. Colored circles illustrate the number of studies using the polyphenols located around it. Grape and wine polyphenols (red circle, 11 articles) include resveratrol, hydroxycinnamic acids, gallic acid, myricetin, anthocyanins, catechin, and quercetin. Tea polyphenols (brown-yellow circle, 6 articles) include catechin, quercetin, epigallocatechin 3-gallate, epicatechin 3-gallate, and epigallocatechin. 3 articles (gray circle) used isolated quercetin. Other sources of polyphenols (3 articles) used anthocyanins, phenolic acids, and flavonols.

Table 1. Description of the sample found in studies that administered tea polyphenols.

Table 2. Description of the sample found in studies that administered grape derivate polyphenols.

Table 3. Description of the sample found in studies that administered quercetin.

Table 4. Description of the sample found in studies that administered other sources of polyphenols.

Table 5. Summary of the main results regarding metabolic and neurological outcomes found in all included studies.

Table 6. Risk of bias summary of the included studies: review authors' judgments about each risk of bias item for each included article, evaluated by the items highlighted by Hooijmans *et al* (Yes) low risk of bias; (Unclear) when the articles did not inform methodological details; (No) high risk of bias.

Figures And tables

Graphical abstract

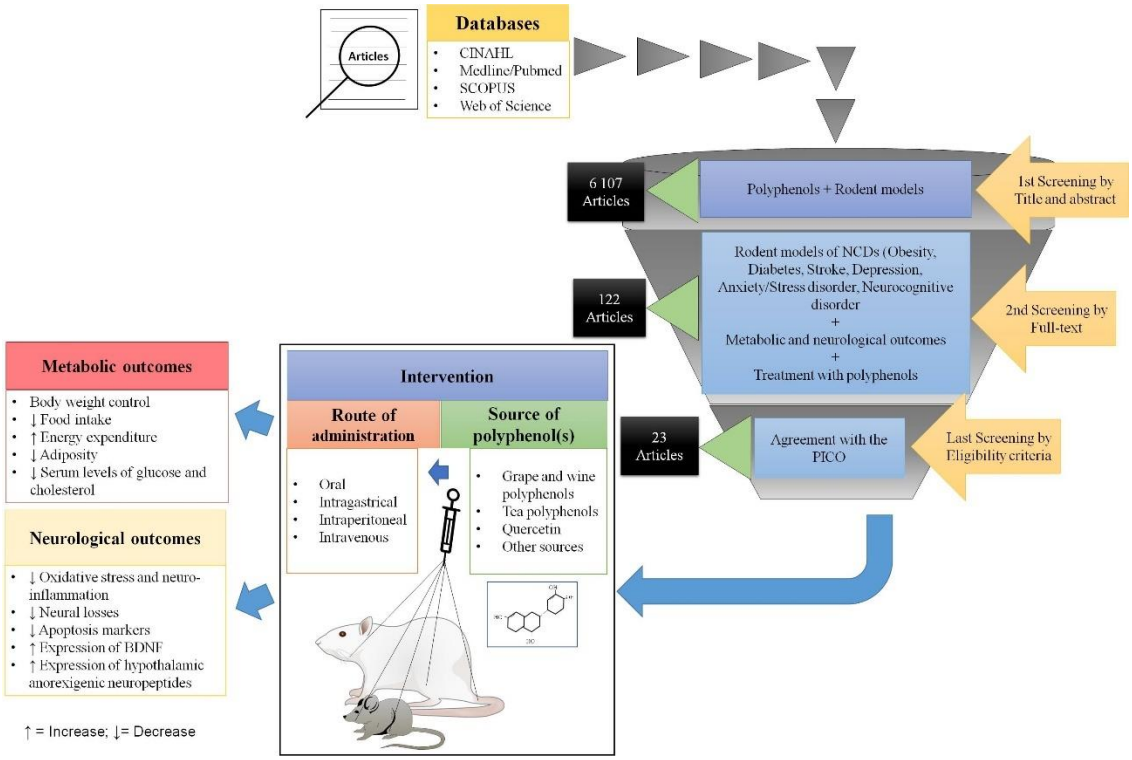

Figure 1.

a

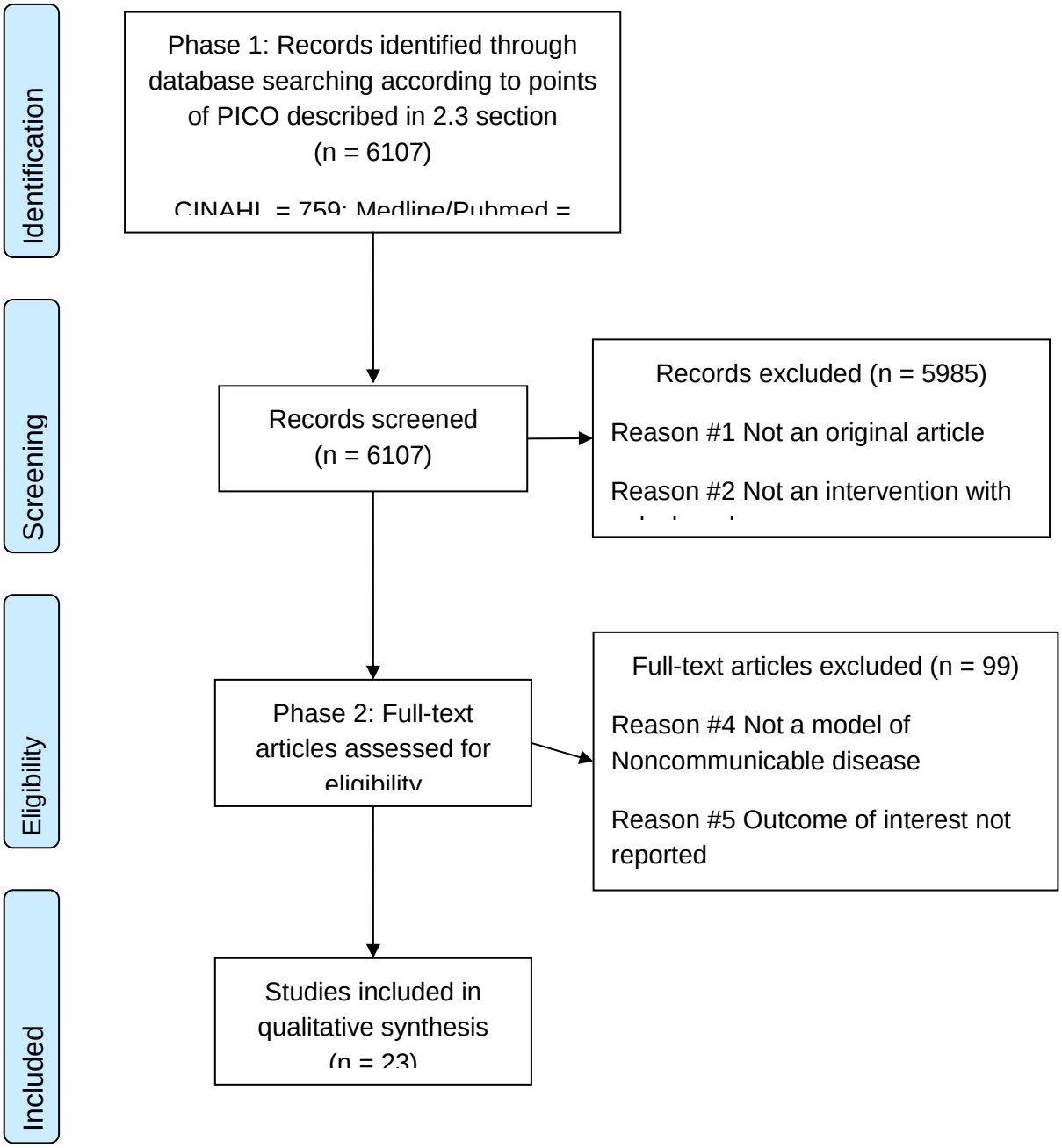
Identification
Screening
Eligibility
Included
Phase 1: Records identified through database searching according to points of PICO described in 2.3 section (n = 6107)
CINAHL = 759; Medline/Pubmed =
Records screened (n = 6107)
Records excluded (n = 5985)
Reason #1 Not an original article
Reason #2 Not an intervention with
Phase 2: Full-text articles assessed for eligibility
Full-text articles excluded (n = 99)
Reason #4 Not a model of Noncommunicable disease
Reason #5 Outcome of interest not reported
Studies included in qualitative synthesis (n = 23)

Figure 2.

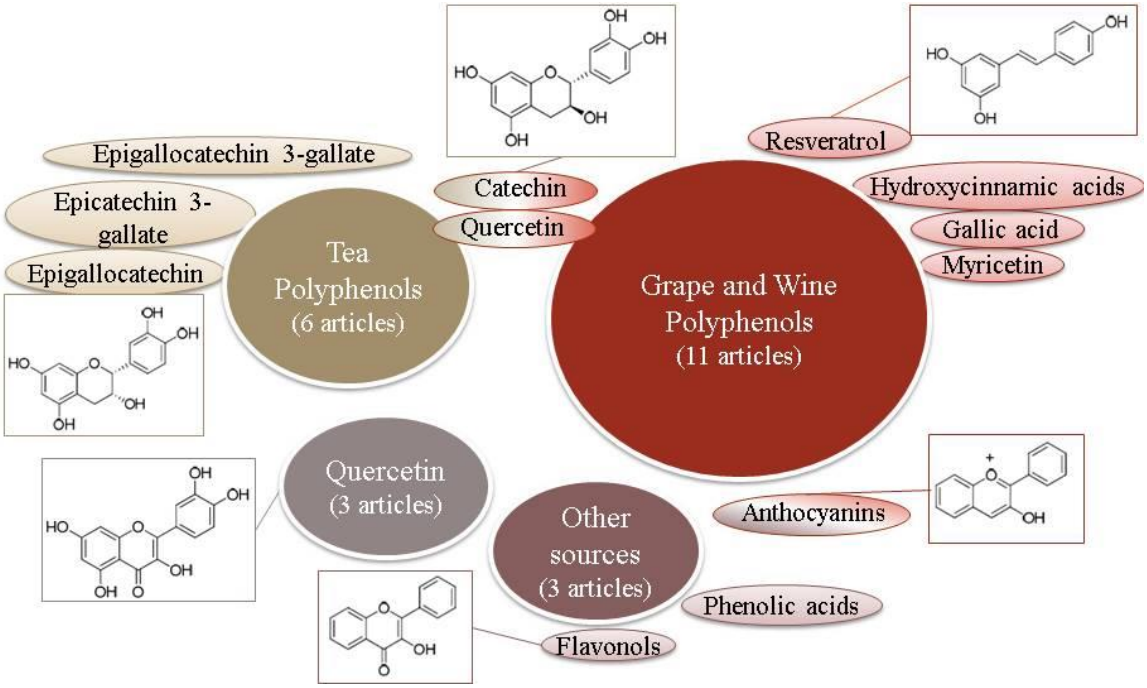
Epigallocatechin 3-gallate
Epicatechin 3-gallate
Epigallocatechin
Tea Polyphenols (6 articles)
Catechin
Quercetin
Grape and Wine Polyphenols (11 articles)
Resveratrol
Hydroxycinnamic acids
Gallic acid
Myricetin
Quercetin (3 articles)
Other sources (3 articles)
Anthocyanins
Phenolic acids
Flavonols

Table 1.

Source of polyphenol(s)	Main active polyphenol(s)	Model	Route of administration	Dose	Duration of treatment	Reference
Rooibos tea Supplier: Rooibos Ltd, Clanwilliam, South Africa	Aspalathin, chrysoeriol, isoorientin, isoquercitrin, isovitexin, luteolin, nothofagin, orientin, quercetin, rutin and vitexin	Male Wistar rats	Orally (drinking)	2 g/100 mL *ad libitum*	7 weeks	Akinrinmade *et al*., 2017 [41]
Green Tea Powder Supplier: Sigma Chemical Company, St. Louis, MO, USA	Catechins and quercetin	Male Wistar rats	Oral gavage	500 mg/kg	12 weeks	Macedo *et al*., 2019 [39]
Tea Polyphenols Supplier: Sigma Chemical Company, St. Louis, MO, USA	Epicatechin	Male Sprague-Dawley rats	Intravenous (tail vain)	200 mg/kg	Single application	Xue *et al*., 2016 [62]
Green Tea Polyphenols (*Camelia sinensis*) Supplier: Ao-Jing Science and Technology Development Co., Ltd., China	Epicatechin, epigallocatechin-3-gallate, epigallocatechin, and epicatechin-3-gallate	Male ICR mice	Intragastric gavage	5, 10 and 20 mg/kg	1 week	Zhu *et al.*, 2012 [49]

Green Tea Polyphenols Supplier: ZheJiang Yixin Pharmaceutical Co. Ltd., China	Catechin and epigallocatechin gallate	Male Sprague-Dawley rats	Orally	Low (0.1%), Medium (0.5%) or High: (1%)	3 weeks	Chen *et al.*, 2009 [33]
Oolong Tea or Green Tea Polyphenols Supplier: Suntory Co., Ltd., Osaka, Japan	Epicatechin gallate and epigallocatechin gallate	Male and female SMP8 mice	Orally	1%	16 weeks	Chan *et al.*, 2006 [26]

Table 2.

Source of polyphenol(s)	Main active polyphenol(s)	Model	Route of administration	Dose	Duration of treatment	Reference
Resveratrol Supplier: Sigma Chemical Company, St. Louis, MO, USA	Resveratrol	Male Wistar rats	Orally	80 mg/kg	8 weeks	Abd El-Fattah *et al.*, 2018 [35]
Trans-Resveratrol Supplier: Sigma Chemical Company, St. Louis, MO, USA	Trans-resveratrol	Sprague-Dawley rats (did not inform sex of animals)	Intraperitoneally	20 mg/kg	1 week	Chang *et al*., 2018 [28]
Resveratrol Supplier: did not inform	Resveratrol	Male Wistar rats	Oral gavage	10 mg/kg	16 weeks	Ferreira *et al.*, 2018 [29]
Royal Down Sweet Cherries (*Prunus avium L*.) or Black Grapes (*Vitis vinifera L*.) Supplier: Barcelona and Tarragona, Spain	Anthocyanins (Cyanidin 3-O-rutinoside, Cyanidin 3-O-glucoside), Flavanols (Epicatechin, Catechin), Phenolic acids (3-Caffeoylquinic acid, 3-p-Coumaroylquinic acid)	Male Fischer rats	Orally	100 mg/kg	7 weeks	Ibars *et al.*, 2018 [30]
Resveratrol Supplier: Solarbio Life Science Company, Beijing, China	Resveratrol	Male Sprague Dawley rats	Intraperitoneally	40 or 80 mg/kg	4 weeks	Shen *et al.*, 2019 [55]

Resveratrol Supplier: Sigma-Aldrich, Merck KGaA, Darmstadt, Germany	Resveratrol	Male Wistar rats	Intraperitoneally	80 mg/kg	3 weeks	Zhang *et al.*, 2017 [63]
Standardized Grape Polyphenol (Resveratrol, Grape Seed and Concord Grape Juice) Supplier: ChromaDex, Los Angeles, USA	Resveratrol, anthocynains, proanthocyanidin oligomers, phenolic acids, catechin, epicatechin, quercetin-3-O-glucoside, quercetin-3-O-glucuronide and quercetin	Female C56Bl/6j mice.	Intragastric gavage	Resveratrol (400 mg/kg), Grape Seed (200 mg/kg), and Concord Grape Juice (183 mg/kg)	24 weeks	Wang *et al.*, 2013 [27]
Red Wine Cabernet Sauvignon Supplier: The National Institute Wine, Yalta, Russia	Anthocyanins, resveratrol, galic acid, catechin, myricetin and quercetin	Male Wistar rats.	Orally	300 ml/70 kg	2 weeks	Drel *et al.*, 2010 [38]
Red Wine Polyphenol Compounds Supplier: Provinols, SFD Vallont Pont D'Arc, France	Proanthocyanidins, anthocyanins, catequin, hydroxycinnamic acids, flavonols and polymeric tannis	Male Wistar rats	Intravenous (femoral vein)	0.1 mg/kg	Single Dose	Ritz *et al.*, 2008 [59]

Red Wine Polyphenol Compounds Supplier: Provinols, SFD Vallont Pont D'Arc, France	Proanthocyanidins, anthocyanins, catequin, hydroxycinnamic acids, flavonols and polymeric tannis	Male Wistar rats	Orally	30 mg/kg	1 week	Ritz *et al*., 2008 [58]
Resveratrol Supplier: Sigma Chemical Company, St. Louis, MO, USA	Resveratrol	Male Wistar rats	Oral gavage	2 mg/kg	8 weeks	Phyu *et al*., 2016 [31]

Table 3.

Source of polyphenol(s)	Main active polyphenol(s)	Model	Route of administration	Dose	Duration of treatment	Reference
Quercetin Supplier: Sigma Chemical Company, St. Louis, MO, USA	Quercetin	Male mice	Oral gavage	50 mg/kg	2 weeks	Kosari-Nasab *et al.*, 2019 [32]
Quercetin Supplier: Jena Bioscience, Jena, Germany.	Quercetin	Male B6/SJL mice	Orally	50 mg/kg	8 weeks	Yang *et al*., 2017 [34]
Quercetin Supplier: did not inform	Quercetin	Male Wistar rats	Intraperitoneally	50 mg/kg or 100 mg/kg	3 weeks	Demir *et al.,* 2016 [37]

Table 4.

Source of polyphenol(s)	Main active polyphenol(s)	Model	Route of administration	Dose	Duration of treatment	Reference
Cornelian Cherry Extracts (*Cornus mas L*) Supplier: Bolestraszyce, Poland	Anthocyanins, phenolic acids, and flavonols	Male Wistar rats	Intragastrically	20 mg/kg	2 weeks	Dzydzan *et al.*, 2019 [36]
Paeonol Supplier: Xuancheng Pharmaceutical Co.,Ltd., Xuancheng, China	Phenolic acid	Male Sprague-Dawley rats	Intragastrically	25 mg/kg or 80 mg/kg	4 weeks	Zhu *et al*, 2018 [64]
Dark Chocolate Cocoa Solids Supplier: Nature Products, Bangalore, India	Epicatechin	Male Sprague-Dawley rats	Orally	500 mg/kg	12 weeks	Madhavadas *et al*., 2016 [40]

Table 5.

Reference	NCD	Metabolic outcomes	Neurological outcomes
Ritz *et al*., 2008 [59]	Stroke	Lower basal concentrations of lactate in the brain parenchyma.	Prevented the burst of excitatory amino acids (glutamate, aspartate, taurine). Also promoted vasodilatation and consequent enhanced the residual cerebral blood flow.
Ritz *et al*., 2008 [58]	Stroke	Lower basal concentrations of energy metabolites, including glucose and lactate in the brain parenchyma.	Reduced brain infarct volumes, prevented the burst of excitatory amino acids. Also promoted vasodilatation and consequent enhanced the residual cerebral blood flow.
Akinrinmade *et al.*, 2017 [41]	Stroke	No effects on energy expenditure evaluated by locomotor activity (open field test).	Reduced brain edema and neuronal apoptosis (lower neuronal death), reduced lipid peroxidation levels and increased total antioxidant capacity
Chang *et al.*, 2018 [28]	Stroke	Increased energy expenditure by enhance of locomotor activity (open field test).	Reduced neuronal loss, reduced expression of inflammatory cytokines, decreased oxidative stress and alleviated neuroinflammation.
Abd El-fattah *et al.*, 2018 [35]	Depression	Increased body weight.	Decreased activation of the hypothalamic–pituitary–adrenal axis (HPA), increased hippocampal serotonin levels, inhibition of monoamine oxidase (MAO) activities, increased expression level of Brain-derived neurotrophic factor (BDNF) in

			hippocampus, neuroprotection due to its antioxidant, anti-inflammatory and anti-apoptotic.
Shen *et al.*, 2019 [55]	Depression	No effects on energy expenditure evaluated by locomotor activity (open field test).	Negative modulation of proinflammatory related (TNF-α, IL-6, and IL-1β) and reduced apoptosis markers (Bax and Bcl-2) in hippocampus and prefrontal cortex.
Zhu *et al.*, 2018 [64]	Depression	No effects on energy expenditure evaluated by locomotor activity (open field test).	Dendritic length and complexity and the density of dendritic spines markedly increased in the hippocampal CA1 and the dentate gyrus (DG).
Zhang *et al.*, 2017 [63]	Depression	No effects on energy expenditure evaluated by locomotor activity (open field test).	Upregulation o hippocampal BDNF expression.
Zhu *et al.*, 2012 [49]	Depression	No effects on energy expenditure evaluated by locomotor activity (open field test).	Inhibition of the hypothalamic–pituitary–adrenal axis (HPA), reducing circulating glucocorticoids.
Kosari-Nasab *et al.*, 2019 [32]	Anxiety/Stress disorder	Increased energy expenditure by enhancing locomotor activity (open field test).	Attenuation of anxiety-related behaviors and HPA axis hyperreactivity by the suppression of the CRF expression or its receptors.
Chen *et al.*, 2009 [33]	Anxiety/Stress disorder	Increased energy expenditure by enhance of locomotor activity (open field test).	Antioxidant activity and increased serum levels of norepinephrine and dopamine.

Xue *et al*., 2016 [62]	Neurocognitive disorder	No effects on energy expenditure evaluated by locomotor activity (open field test).	Reduced neuronal apoptosis in the hippocampal CA1 region.
Chan *et al*. 2006 [26]	Neurocognitive disorder	No effects on energy expenditure evaluated by locomotor activity (open field test).	Significantly less spongy degeneration and lower percentages of lipofuscin in the hippocampus.
Madhavadas *et al*., 2016 [40]	Neurodegenerative disorder	Significantly lowered the glucose levels, no difference of cholesterol levels. The authors did not report body weight results.	Enhanced cholinergic activity in the hippocampus by reducing acetylcholinesterase activity and increase in cell volume in the hippocampal CA3 region.
Phyu *et al*., 2016 [31]	Obesity and diabetes	Transitory decrease in body mass. No significant effect on blood glucose. They do not report food intake results.	Antioxidant effects and improved neural function by reducing tactile allodynia.
Wang *et al*., 2013 [27]	Obesity and diabetes	Significantly reduced the body weight gain, reduced levels of glucose and cholesterol.	Prevention or rescue of the synaptic abnormality (increased metabolic signaling) and improvement of the long term potentiation at the CA1 hippocampal region.
Ibars *et al.*, 2018 [30]	Obesity	Reduced food intake, increased energy expenditure due to lipid use and elevation of respiratory quotient.	Increase leptin sensitivity through the modulation of the hypothalamic leptin signal pathway. Hypothalamic gene expression of pro-opiomelanocortin was significantly up-regulated.

Macedo *et al.*, 2019 [39]	Obesity	Reduction of body weight, body weight gain, adiposity index, reduction of serum levels of glucose.	Reducing inflammation in cerebral cortex, brain stem and cerebellum by increasing some of the antioxidant enzymes.
Yang *et al.*, 2017 [34]	Obesity	Reduced accumulation of lipids in peripheral cells, and decrease cellular content of triglyceride.	Reduced the levels of inflammatory cytokines and microglia activation markers in the hypothalamus.
Drel *et al*., 2010 [38]	Diabetes	Contributed to normalize body weight (increase of body weight).	Reduced the 3-nitrotyrosine (NT) content in sciatic nerve, dorsal root ganglia (DRG) neurons and spinal cord.
Demir *et al.*, 2016 [37]	Diabetes	Contributed to normalize body weight (increase of body weight), reduced serum levels of glucose.	Not alter HPA axis related parameters.
Dzydzan *et al.*, 2019 [36]	Diabetes	Contributed to normalize body weight (increase of body weight), lowered blood glucose and improved glucose tolerance.	Reduced oxidative stress.
Ferreira *et al.*, 2018 [29]	Diabetes	Contributed to normalize body weight (increase of body weight), reduced serum levels of glucose.	Attenuated oxidative and nitrosative stress, and prevented neuronal loss in myenteric plexus.

Table 6.

Reference	Randomization of sample	Animal care	Blinding of the intervention	Blinding of evaluation	Ethical code register
Ritz *et al*., 2008 [59]	No	Yes	No	No	Yes
Ritz *et al*., 2008 [58]	No	Yes	No	No	Yes
Akinrinmade *et al.*, 2017 [41]	Yes	Yes	No	Yes	Yes
Chang *et al.*, 2018 [28]	Yes	Yes	No	Yes	Yes
Abd El-fattah *et al.*, 2018 [35]	Yes	Yes	No	No	Yes
Shen *et al.*, 2019 [55]	Yes	Yes	No	No	Yes
Zhu *et al.*, 2018 [64]	Yes	Yes	No	No	Yes
Zhang *et al.*, 2017 [63]	No	Yes	No	No	Yes
Zhu *et al*., 2012 [49]	No	Yes	No	Yes	Yes
Kosari-Nasab *et al.*, 2019 [32]	Yes	Yes	Yes	Yes	Yes
Chen *et al*., 2009 [33]	No	Yes	No	No	No
Xue *et al*., 2016 [62]	No	Yes	No	No	Yes
Chan *et al*. 2006 [26]	No	Yes	No	No	Yes
Madhavadas *et al*., 2016 [40]	No	Yes	No	No	Yes
Phyu *et al*., 2016 [31]	Yes	Yes	No	No	Yes
Wang *et al*., 2013 [27]	Yes	Yes	No	No	Yes

Ibars *et al.*, 2018 [30]	No	Yes	No	No	Yes
Macedo *et al.*, 2019 [39]	No	Yes	No	No	Yes
Yang *et al.*, 2017 [34]	Yes	Yes	No	No	Yes
Drel *et al*., 2010 [38]	No	Yes	No	Yes	Yes
Demir *et al.*, 2016 [37]	Yes	Yes	No	Yes	Yes
Dzydzan *et al.*, 2019 [36]	Yes	Yes	No	No	Yes
Ferreira *et al.*, 2018 [29]	Yes	No	No	No	Yes

APÊNDICE F – "DIETARY FLAVONOID KAEMPFEROL REDUCES OBESITY-ASSOCIATED HYPOTHALAMIC MICROGLIA ACTIVATION AND PROMOTES BODY WEIGHT LOSS IN MICE WITH OBESITY"

Dietary flavonoid kaempferol reduces obesity-associated hypothalamic microglia activation and promotes body weight loss in mice with obesity

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[6]Laboratório de Nutrição Experimental, Departamento de Nutrição, Universidade Federal da Paraíba, João Pessoa, Brazil;

[7]Departmento de Enfermagem, Universidade Federal de Pernambuco, Vitória de Santo Antão, Pernambuco, Brasil;

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[#]These authors contributed equally

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Abstract

Background: Obesity results from an unbalance in the ingested and burned calories. Energy balance is critically regulated by the hypothalamic arcuate nucleus by promoting appetite or anorectic actions. Hypothalamic inflammation, driven by high activation of the microglia, has been reported as a key mechanism involved in the development of diet-induced obesity. Kaempferol, a flavonoid-type polyphenol present in a large number of fruits and vegetables, was shown to regulate both energy metabolism and inflammation. **Objectives:** In this work we studied the effects of both the central and peripheral treatment with kaempferol on hypothalamic inflammation and energy balance regulation in mice with obesity. **Methods:** Obese adult mice were chronically (40 days) treated with kaempferol (0.5 mg/kg/day, intraperitoneally). During the treatment, body weight, food intake, feed efficiency, glucose tolerance, and insulin sensitivity were determined. Analysis of microglia activation in the arcuate nucleus of the hypothalamus at the end of the treatment was also performed. Body weight, food intake, and feed efficiency changes were also evaluated in response to 5µg kaempferol, centrally administrated. **Results:** Chronic administration of kaempferol decreased ~43% of the density, and ~30% of the ratio, of activated microglia in the arcuate nucleus. These changes were accompanied by body weight loss, decreased feed efficiency, reduced fasting blood glucose, and a tendency to improve insulin sensitivity. Finally, acute central administration of kaempferol reproduced the effects on energy balance triggered by peripheral administration. **Conclusion:** These findings suggest that kaempferol might fight obesity by

regulating central processes related to energy balance regulation and hypothalamic inflammation.

Keywords: kaempferol, obesity, energy balance, hypothalamic inflammation, microglia.

Abbreviations: ARC, arcuate nucleus; BW, body weight; EB, energy balance; EE, energy expenditure; FE, feed efficiency; FI, food intake; GTT, glucose tolerance test; HFD, high-fat diet; icv, intracerebroventricular; IL-1β, interleukin-1 β; IL-6, inleukin-6; ip, intraperitoneal; ITT, insulin tolerance test; KF, kaempferol; LPS, lipopolysaccharide; POMC, proopiomelanocortin; SFA, saturated fatty acid; T2D, type 2 diabetes; TLR4, toll-like receptor 4; TNF-α, tumor necrosis factor α.

1. Introduction

Obesity and overweight are affecting over one third of the world's population nowadays. Defined as an abnormal or excessive fat accumulation that affects health, obesity is closely related to high cardiometabolic risk, some types of cancer, poor mental health, but mainly type 2 diabetes (T2D) [1,2]. Body weight (BW) and T2D are closely related as 65% of patients with T2D have obesity or overweight [3].

BW is critically regulated by the hypothalamus, which controls energy balance (EB) (intake/expenditure of energy) involving central and peripheral nutritional signals such as hormones, nutrients and signals from the gastrointestinal nervous system [4]. In the arcuate nucleus (ARC) of the hypothalamus metabolic signals from the periphery are detected and adaptive neuroendocrine responses involving the melanocortin system are triggered to maintain energy balance [4]. The melanocortin system is composed of proopiomelanocortin (POMC) and agouti-related peptide (AgRP) neurons, which have opposing functions; POMC neurons

reduce food intake (FI) and increase energy expenditure (EE), while AgRP neurons increase FI and reduce EE [5]. Hormonal actions of insulin (pancreas derived) and leptin (fat derived) on POMC and AgRP neurons in the ARC are known to regulate glycemia and EB, promoting reduced FI and increased EE [6].

Excessive consumption of high-fat diets (HFD) associated with low consumption of vegetables has been widely recognized as one of the main factors for the development of obesity [7,8]. Recent studies have strongly linked HFD-induced obesity and hypothalamic inflammation evidenced by an increase in density and activation of the microglia (bigger soma size) [9–13]. By secreting pro-inflammatory cytokines including TNF-α, IL-1β, and IL-6 (tumor necrosis factor α, interleukin-1 β, and inleukin-6, respectively), in response to a HFD, activated microglia in the ARC impairs the melanocortin system function and EB regulation leading to the development of obesity [9–13]. Blocking microglia activation and the toll-like receptor 4 (TLR4) actions reduces cytokine production, protecting against the development of obesity, indicating that these two mechanisms are necessary for the development of HFD-induced obesity [9,11].

Kaempferol (KF), or 3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one, is a flavonoid-type polyphenol present in a large number of dietary sources (e.g. onion, spinach, broccoli, grapefruit, blackberries, and olive oil) and plants used in traditional medicine (e.g. tea, *Aloe vera*, *Ginkgo biloba*, and *Moringa oleifera*) [14,15]. Around 5.38 mg of KF are ingested daily from diet and beverages [16] and growing evidence from *in vitro* and *in vivo* studies highlight healthy effects of this flavonoid on metabolism disorders and inflammation [17,18]. Studies in mice have shown that KF decreases BW, adiposity, hyperglycemia, glucose intolerance, and insulin resistance reduction [19,20]. Anti-neuroinflammatory effects of KF in both mice and cultured microglia, challenged with lipopolysaccharides (LPS), have also been reported [21,22].

In this context, the aim of the present study was to test whether the chronic treatment with kaempferol reduces hypothalamic inflammation and improves the EB regulation on mice with obesity. We also investigated whether KF directly modulates EB within the brain.

2. Materials and Methods

All the experiments were performed in C57Bl/6J adult male mice (n=65), which were purchased from the Institute of Neurobiology of the "Universidad Nacional Autónoma de México" (UNAM, Juriquilla, Queretaro, Mexico) and habituated for a week in our facilities before any intervention. The mice were housed individually and kept in light/dark cycles of 12h (lights on-off at 7 a.m.-7 p.m., respectively) and at a temperature of 22 ± 2ºC. They had free access to food (Nutricubos, Agribrands Purina Mexico) and water, unless otherwise indicated. Experiments were carried out strictly following the guidelines of the Mexican (NOM-062-ZOO-1999) and international (National Institutes of Health, USA) regulations for the care and use of laboratory animals, emphasizing on minimizing any suffering and number of mice used. All procedures were approved by the Ethical Committee of the "Mexican Institute of Social Security" (IMSS) (R-2015-785-103). Experimental reporting is in accordance with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines 2.0 [23].

2.1 Selection of the dose of kaempferol

In order to establish the dose of KF required to perform the chronic treatment, a preliminary acute test was conducted in non-obese mice fed on standard diet (SD) (see **Table 1** for diet composition). 8-weeks old mice, fasted during 24-h, were randomly assigned into the following 3 groups: Vehicle, KF 0.5 mg/kg, and KF 1 mg/kg (n=6/group) in accordance with the treatment. Vehicle and KF mice (Tocris Bioscience, USA) were injected via intraperitoneal (ip) (volume: 1 µL/g of BW) using Dimethyl sulfoxide (DMSO)/saline (25%/75%) as solvent, the ip administration of DMSO/saline without KF constituted the Vehicle group. This route of administration was chosen to increase the bioavailability of the compound, which is marginal

when administered orally [24]. In this way, the bioavailability for the hypothalamus, the tissue of interest in this study, is increased. Although intravenous administration can lead to a higher concentration of KF in plasma, ip administration was chosen as it is more suitable for chronic treatment. Drug administration was done just before (6 p.m.) the start of the dark phase (the period of greatest physical activity and metabolism of rodents) using an Ultra-Fine Insulin Syringe 0.3 mL (Becton-Dickinson, Mexico). After injection the food was provided in their cages and the effect of KF on 24-h BW changes and FI were determined using a digital scale (Sartorius, Göttingen, Germany) [25]. BW change was determined by subtracting BW at injection to BW after 24-h (BW change=BW_{final}-$BW_{initial}$). FI was recorded 1, 2, 4, and 24-h after the administration injection. Also, feed efficiency (FE), expressing the capability of the organism to transform calories ingested in BW, was calculated as following: FE=BW gain/cal ingested (g/cal). Furthermore, 24-h FI normalized to the BW was calculated, by using the following formula FI= $FI_{(g)}/BW_{(g)}$*100. Based on previous work [26,27], the mice in this experiment were subjected to a 24-h fasting period to induce hunger and thus, be able to assess a potential anorectic effect of KF. FI and BW changes were evaluated in a period of 24-h to cover the different phases of the circadian cycle. The experimental design of this experiment is summarized in **Figure 1A**. The dose modulating any parameter of EB was selected to the chronic treatment.

2.2 Effect of chronic administration of kaempferol on diet-induced obesity

2.2.1 Obesity induction

In this study the widely studied model of diet-induced obesity was used [28]. 8-weeks old mice were exposed for 12 weeks to a HFD (see **Table 1** for diet composition) elaborated following the recommendation established by the "American Institute of Nutrition" (AIN-93) for rodent diets. Fresh diet was added twice a week. The correct progress of obesity induction was

evaluated by monitoring BW weekly. Alterations in fasting blood glucose, commonly observed in obesity, were also evaluated after 12 weeks of HFD exposure.

2.2.2 Chronic administration of kaempferol

To evaluate the effect of chronic treatment with KF on diet-induced obesity, 20-week old obese mice were randomly assigned into 2 groups according to the treatment received: Vehicle group (n=7) and KF group (n=6). Before the treatment, mice underwent a period of adaptation, during 7 consecutive days, to handling and restraint (without injection). This was carried out to reduce BW loss related to stressful manipulation. Afterwards, mice (21-weeks old) received a daily dose of 0.5 mg/kg or vehicle solution (like in Section 2.1) throughout 40 days. To avoid, as much as possible, the stress associated with the experiment, the injections were made using an Ultra-Fine Insulin Syringe (like in ***Section 2.1***) and alternating both sides of the peritoneum. BW and FI were recorded during the treatment. FE was also calculated using BW change and 40-days cumulative FI. Both injections and measurements were always made at 6 p.m. The experimental design of this experiment is summarized in **Figure 1B**. During the treatment with KF the animals remained on HFD.

2.2.3 Glucose and insulin tolerance tests

To verify whether the chronic treatment with KF improved glucose control alterations associated with obesity, we performed the glucose tolerance test (GTT) and the insulin tolerance test (ITT) at day 32 and 38 of the study, respectively (**Figure 1B**). After 6-h of fasting (started at 7 a.m.) basal glucose levels (0 min) were determined for both groups (n=6/group) using a glucometer (One Touch® Ultra® 2, LifeScan, USA) with blood samples from the tail vein. Afterwards, a glucose solution (Meyer, Mexico) (2 g/kg ip) or insulin (AMSA, Mexico) (1 U/kg ip) was injected and blood glucose levels were measured at 15, 30, 45, 60, and 120 min (GTT) or 15, 30, 60, 90, and 120 min (ITT), after injection. The glucose and insulin doses were based

on a previous report of the literature [10]. The time of fasting was selected following the considerations and guidelines for mouse metabolic phenotyping in diabetes research [29].

2.3 Immunohistochemistry and microglia activation analysis

On the 40th day of treatment, mice (n=6/group) were euthanized (**Figure 1B**) with sodium pentobarbital (Sedalphorte, Mexico) (200 mg/kg, ip) and perfused transcardially with cold PBS (0.1 M), followed by 4% paraformaldehyde (PFA)/PBS. Mice were decapitated and brains were collected and post fixed at 4°C overnight in 4% PFA/PBS [9]. Then, brains were incubated into 30% sucrose solution at 4°C. Afterwards, 30 µm-thick coronal sections containing the hypothalamus were cut using a cryostat (Leica, Germany). Free-floating brain sections were stored in anti-freezing medium containing 30% ethylene glycol and 30% glycerol in PBS at −20°C, until immuno-processing.

Free-floating brain sections were washed (3x10 min) with PBS and incubated at room temperature (RT) in 3% H_2O_2/PBS before blocking with 5% normal goat serum (Sigma) and 0.3% Triton X-100/PBS for 1 h at RT. Afterwards, brain sections were incubated for 48-h at 4°C with a rabbit primary anti-Iba1 antibody (1:15000, FUJIFILM Wako Chemicals, USA). 3x10 min washes with PBS were made followed by the incubation with a secondary anti-rabbit biotinylated antibody (1:750, Sigma, USA) in 1% normal goat serum/PBS for 2-h at RT. After a new washing with PBS, incubation in a solution containing a peroxidase-conjugated avidin-biotin complex (VECTASTAIN Elite ABC System, Vector Laboratories, USA) was then performed. A repetition of washes was performed and microglia staining was visualized using a DAB Kit (Vector Laboratories, USA). Brain slices were washed again, mounted on slides, and coverslipped.

Microglial cell counts and morphological analyses were performed on digital images (10X) captured using a light microscope (Zeiss, Germany) containing the ARC (from bregma −1.22

to bregma −2.70 mm) by a blind observer. Microglia counts and phenotype were examined with ImageJ Software on right- and left-side on the ARC, using a counting frame (0.167 mm^2) with uniform area placed into the ARC. Both experimental groups were analyzed for the same ARC region investigated. The density of Iba1-immuno-reactive (IR) cells (number cells/mm^2) was determined for 3 hypothalamic sections per brain. Microglia activation levels were determined by the morphology exhibited according to the criteria established by Diz-Chaves et al. (2012) [30]. Briefly, microglia was considered in a resting state (inactivated) when it exhibited a small cell body with thin process (phases I, II, and III), while activated microglia exhibited larger soma with thicker process (phases IV and V) [30]. In order to demonstrate that obese mice in this study had the typical microglia activation in the ARC [9,12,13], brain sections from mice fed a HFD (n=6) or SD (n=6) were also analyzed during 12 weeks.

2.4 Effect of intracerebroventricular administration of kaempferol on energy balance

Obese mice with 12 weeks of HFD exposure were stereotaxically implantated with a stainless steel cannula into the cerebral lateral ventricle (coordinates relative to Bregma: A-P -0.5; M-L -1.2; D-V -2.1) [31]. Surgery was performed under isoflurane anesthesia with previous injection subcutaneously of buprenorphine (0.1 mg/kg) and lidocaine (0.1 mL of a 0.5% solution). After surgery, mice received analgesic in the water (paracetamol 2 mg/100 mL) for up to 2 days. After two weeks of post-operative recovery, 24-h fasted animals (n=6) were injected icv with 5 µg KF in 1 µL DMSO right before dark phase. Vehicle group (n=6) received 1 µL DMSO without drug. After injection, the food was put back into their cages and 24-h BW changes, FI, and FE were determined (**Figure 1C**) as in ***Section 2.1***. The amount of KF injected was established from previous tests of our laboratory showing regulatory effects of 5 µg of other compounds on EB regulation. The correct implantation of the cannula was verified post mortem by histological analysis.

2.5 Effect of intracerebroventricular administration of kaempferol on cytokine expression

Cannula implantation was conducted as in ***Section 2.4***, in obese mice that were also exposed to HFD for 12 weeks. After two weeks of postoperative recovery, mice (n=5) were injected icv with 5µg KF in 1 µL DMSO just before the onset of the dark phase. Vehicle group (n=5) received 1 µL DMSO without drug. Dose was defined from the result observed regarding the effect of 5µg KF on energy balance regulation.

2.5.1 Tissue collection

2-h after KF injection, animals were euthanized by decapitation. Then, hypothalami were quickly dissected out and frozen on dry ice. Samples were kept at -80°C for further processing. The time between injection and euthanasia was established from previous studies in our laboratory. The correct implantation of the cannula was verified as in ***Section 2.4***.

2.5.2 Quantitative real-time PCR analysis

Hypothalami were homogenized in Trizol (Invitrogen, USA) and total RNA was isolated using a standard chloroform/isopropanol protocol. Then, 1µg of total RNA was reverse transcribed using M-MLV RT 200 U (Invitrogen, USA) and primed with oligo(dT) 12-18 primers (Invitrogen, USA). Real-time PCR was carried out on a CFX96 Real Time PCR Detection System (Bio-Rad, USA) using VeriQuest Fast SYBR Green qPCR Master Mix (USB Affymetrix, USA). The genes analyzed and their respective primers were as followed: Tnf-α (NM_013693), forward (5'-3') GCCTCTTCTCATTCCTGCTT, reverse (5'-3') TGGGAACTTCTCATCCCTTT; Il-1β (NM_008361), forward (5'-3') GAAGAAGAGCCCATCCTCTG, reverse (5'-3') TCATCTCGGAGCCTGTAGTG. The relative expression of both genes was normalized respect to the housekeeping control gene Nono (GNM_023144), forward (5'-3') CTGTCTGGTGCATTCCTGAACTAT, reverse (5'-3') AGCTCTGAGTTCATTTTCCCATG. The relative expression was calculated using the comparative $2^{-\Delta\Delta CT}$ method. mRNA expression levels are expressed as the fold changes from the reference group.

2.6 Statistical analysis

Data were presented as means ± SEM and analyzed using the following tests: 1) Student t for the comparison of means between two groups, 2) one-way analysis of variance (ANOVA) to compare means between 3 groups, 3) two-way ANOVA to compare groups considering treatment and time as factors. When a significant difference was found between groups the Bonferroni post-hoc test was applied for multiple comparisons. Values of $p<0.05$ were considered significant. All analyses were performed using the statistical program GraphPad Prism6 (La Jolla, California, USA).

3. Results

3.1 Establishing the dose of kaempferol capable of modulating energy balance

In order to determine the dose of KF necessary to modulate EB, mice fed on SD (normal-weight) were administered via ip 0.5 or 1 mg/kg KF and the acute effect on BW changes, FI and FE was evaluated. The results showed that the administration of both doses led to a tendency of reducing 24-h BW gain compared to the vehicle group (**Fig. 2A**). This effect was unrelated to FI changes, since there is no difference in absolute FI (**Fig. 2B**) nor in BW-normalized FI was observed (**Fig. 2C**). It could also be verified that the treatment did not present a transitory effect on FI, given the fact that all groups consumed the same amount of food at 1, 2, and 4-h (**Fig. 2B**). Interestingly, the animals treated with KF exhibited a decrease in FE, which was significant for the group treated with 0.5 mg/kg KF (**Fig. 2D**). The results show that acute KF ip promotes a negative EB.

3.2 Effect of chronic treatment with kaempferol on diet-induced obesity

The typical BW gain (**Fig. S1A**), hyperglycemia (**Fig. S1B**), and microglia activation (**Fig. S2**) were observed after 12 weeks of HFD intake, indicating that the model of obesity was correctly established. Given that 0.5 mg/kg KF promoted a negative EB, we hypothesized that a chronic treatment with this amount of KF improves the state of diet-induced obesity. Thus, the effects

of a single dose of 0.5 mg/kg KF, administered daily to obese mice, on BW, FI, FE, glucose control, and microglia activation were evaluated.

3.2.1 Chronic treatment with kaempferol promotes a negative energy balance and body weight loss

Treatment with KF started after a period of habituation to handling and restraint where mice showed a slight tendency to reduce BW during the 7 days of handling, but the change was not significant (**Fig. S3**) indicating a tolerance to the experiment-associated stress. As expected, the treatment showed that KF led to a reduction in BW of obese mice compared to the obese mice receiving the vehicle (**Fig. 3A and Table S1**) from day 35 up to the end of treatment. As in lean mice, fed on SD, KF had no effect on FI (**Fig. 3B** and **3C**). However, total FI tended to decrease (**Fig. 3B**) and when calculating the average of FI normalized to BW no effect was observed (**Fig. 3C**), suggesting it was due to the loss of BW. In the same line, FE was significantly reduced by KF (**Fig. 3D**) reproducing the effect observed in lean mice.

3.2.2 Chronic treatment with kaempferol improves glucose control

High levels of fasting blood glucose and reduced insulin sensitivity are a hallmark of obesity. In this sense, we evaluated whether the chronic treatment with KF is able to improve these parameters. At day 32 of treatment both vehicle- and KF-treated mice were challenged with exogenous glucose (2 g/kg BW) exhibiting no differences in glucose tolerance between groups (**Fig. 4A** and **4B**). At this time point, KF treatment also did not alter fasting blood glucose (**Fig. 4C**). When ITT was carried out (day 38 of treatment), we observed that the treatment with KF led to a clear tendency ($p=0.067$) to improve insulin sensitivity (**Fig. 4D** and **4E**). Interestingly, at this time point of treatment with KF fasting blood glucose was found significantly reduced in the KF group compared to the Vehicle group (**Fig. 4F**).

3.2.3 *Chronic treatment with kaempferol decreases obesity-associated hypothalamic microglia activation*

Before evaluating the effect of KF treatment, we assessed the prevalence of hypothalamic inflammation, widely reported in the literature. To do this, we analyzed the density and activation levels of microglia in the ARC of lean (SD) and HFD-induced obese mice. This analysis revealed the presence of typical increase in density and activation levels of microglia in the ARC observed in HFD-exposed obese mice (**Fig. S2**), validating our model. Further analysis revealed that the inflammatory phenotype (**Fig. 5A**) is improved by chronic treatment with KF (**Fig. 5B**). Indeed, KF led to a decrease in both Iba1-IR cell with activated phenotype (**Fig. 5D**) and percentage of activation (**Fig. 5E**). Regarding the total number of microglia, it was found that the group treated with KF tended to decrease the density of IR-Iba1 cells (**Fig. 5C**).

3.4 Acute intracerebroventricular administration of kaempferol regulates energy balance

Given the results obtained in the previous analysis, we investigated whether this compound is able to modulate EB by acting in the brain of obese mice. We found that the icv administration of KF reproduced the effect observed in obese mice injected ip (**Fig. 6**). Actually, 24-h fasted mice injected with KF exhibited a reduced 24-h BW gain compared to the Vehicle group (**Fig. 6A**). Also, no changes in absolute (**Fig. 6B**) or relative (**Fig. 6C**) FI were observed. Moreover, KF reduced around 50% FE (**Fig. 6D**).

3.5 Intracerebroventricular administration of kaempferol does not affect hypothalamic expression of pro-inflammatory cytokine

After the anti-inflammatory effect of KF observed with chronic treatment, we evaluated whether its acute administration via icv modulates the expression of pro-inflammatory cytokines in obese mice. Analysis of mRNA expression showed that KF did not affect the expression of neither Tnf-α (Fig. 7A) nor Il-1β (Fig. 7B).

4. Discussion

Obesity development has been long associated with high consumption of HFDs and poor consumption of vegetables [32]. Due to the limited success of the pharmacological therapies developed against obesity, the scientific community has intensified its research on the use of diets rich in bioactive compounds for its treatment [33–35]. Dietary antioxidants, particularly polyphenols, have the ability to regulate both metabolism and neuroinflammation [36]. However, their mechanisms of action are poorly understood, especially at a brain level where EB and BW are finely regulated.

From a previous study, conducted *in vitro* using muscle cells, da-Silva et al., (2007) showed that KF promotes a strong increase in EE throughout type-2-iodothyronine deiodinase (D2)-depending thyroid hormone (T3) production [37]. In this logic, we evaluated the effects of the acute treatment with 0.5 or 1 mg/kg KF on the EB of mice with normal BW. Both doses of KF showed a tendency to decrease BW without changes in FI. However, FE was significantly decreased with the lower dose, suggesting an induction of EE by the treatment.

Next, 0.5 mg/kg KF was chronically administrated to HFD-induced obese mice for a time of 40 days. We observed that mice subjected to chronic KF treatment exhibited an improvement in parameters critically associated with obesity. Indeed, mice treated with KF had BW loss, improved glucose control, and reduced hypothalamic microglia activation. In a previous report, Wang et al., (2020) evaluated the 8-weeks anti-obesity effect of 200 mg/kg KF, in the diet [19]. Consistent with our result, in such study the authors observed that KF blocked BW gain of mice exposed to a HFD, demonstrating the ability of KF to modulate EB and BW. However, discrepant data reporting the effect of KF on BW have also been reported. Using the same obesity model, mice receiving 50 mg/kg/day KF via gavage for 6 weeks did not show changes in BW [20]. Such discrepancy can be explained by the different amount of KF applied in both studies. Consistent with that, another study showed that 250 mg/kg KF, but not 50, added to the

diet blocked BW gain [38]. Thus, low doses (50 mg/kg) could fail to reach effective bioavailability of KF, since marginal absorption of this polyphenol is associated with oral administration [24]. Surprisingly, the dose used in this study is much lower than the doses shown to have an effect on EE. We attribute this discrepancy to the different administration routes that are used. The high doses were given orally to animals, as mentioned above, this route leads to marginal absorption. Thus, ip administration could avoid reduced intestinal absorption and the transformation by the microbiota.

In contrast to the effect observed on BW, we did not observe the effect of the chronic treatment with KF on FI. No changes in FI were reported in the above referred papers as well, regardless of the use of low [20] or high doses of kaempferol [38], even for long periods of treatment. BW loss associated with a stable FI indicates a reduction in FE, that is, a reduced capability to transform the calories ingested into BW. As in the acute treatment in lean mice, KF administrated chronically reduced FE in obese mice, indicating that this flavonoid promotes a negative EB. Negative EB indicates that the body burns more calories than ingested; thus, we can conclude that KF is triggering EE of obese mice.

EE expenditure depends, in part, on thermogenesis and physical activity. Works showing the ability of KF to modulate both parameters are scarce. Nevertheless, findings from studies with KF and the KF-closely related flavonol, quercetin, could suggest that KF modulates both parameters. First, da-Silva et al., (2007) found that both KF and quercetin produce a huge increase in T3-dependent EE in muscle cells, with a stronger effect of KF [37]. Interestingly, a quercetin-rich onion peel extract promoted in 3T3-L1 preadipocytes a browning effect, which consists of the passage from a white adipocyte phenotype to a thermogenic brown adipocyte. In the same study, mice fed on HFD supplemented with 0.5% of quercetin-rich extract for 8 weeks exhibited the above referred browning effect. It was demonstrated by up-regulation of the central thermogenic genes peroxisome proliferator-activated receptor gamma coactivator

1α (PGC-1α) and uncoupling protein 1 (UCP1) in both retroperitoneal and subcutaneous adipose tissues [39]. In the same line, rutin, a quercetin-glycoside, promotes a brown adipose tissue (BAT)-dependent thermogenesis and white adipose tissue (WAT) browning effect in both HFD-induced and genetically obese mice [40]. These results obtained in muscle cells, BAT, and WAT highlight the ability of these compounds to act in different tissues. In this sense, a potential activation of EE by KF at the central level, where it can enter [41] and act [42], is equally possible. Consistent with that, other flavonoids including catechins have been shown to activate sympathetic nervous system (SNS)-dependent thermogenesis [43]. Future studies using indirect calorimetry, the only validated method to measure EE, may clarify the possible induction of EE by KF in vivo.

High fasting glucose, glucose intolerance, and reduced insulin sensitivity are characteristic alterations of obesity. Findings of this study show that obese mice subjected to chronic KF ameliorated their fasting glucose and trend to improve insulin sensitivity. Similar effects have been already observed in other studies using KF or other flavonoids [19,20,44]. The regulatory effects of KF and familiar compounds on glucose control involve a wide array of mechanisms. Indeed, KF improves hyperglycemia through suppressing hepatic glucose production along with increasing insulin sensitivity [20]. Studies *in vitro* revealed the ability of KF to increase both viability and insulin secretion of pancreatic beta-cells and human islets [45]. Thus, KF could improve glucose control through its effects on liver, muscle and pancreas. However, KF could also improve glucose control, through a suppression of hypothalamic insulin resistance, known to alter glucose homeostasis in obesity [46] and susceptible to being improved by the consumption of catechins [47]. The beneficial effect of KF on fasting glucose was not consistent with glucose tolerance in this study. The absence of an effect on glucose tolerance may be a matter of time of treatment, as observed in fasting blood glucose, which was decreased on day 38 but not on day 32 of treatment. It is likely that if the study is prolonged, the effects on insulin

sensitivity and glucose tolerance could be significant. Consistent with that, it seems that the effects on glucose control depend on BW loss, since fasting glucose was only different after obtaining differences in body weight.

It is well established that the consumption of HFDs is related to hypothalamic inflammation, which disrupts the regulation of energy balance by the ARC, leading to obesity [48]. At the hypothalamic level, saturated fatty acids (SFAs), predominant in the HFD, are able to reach the ARC, where they interfere with the POMC-depending anorexigenic signals of insulin and leptin promoting a positive EB and then BW gain [9,48,49]. SFAs selectively activate microglia and through TLR4 pathway induces the synthesis of pro-inflammatory factors including TNF-α, IL-1β, and IL-6 [11,49]. This inflammatory program related to HFD intake necessarily depends on the activation of the microglia [9,12,13,49]. Mice in our study exhibited typical obesity-related hypothalamic inflammation in the ARC (**Fig. S2**). When the ARC of obese mice was examined we found that KF, administrated chronically, reduced the levels of microglia activation. Several studies have demonstrated that KF is a bioactive compound with anti-inflammatory and neuroprotective properties. In fact, mice pre-treated for 7-d with KF and then exposed to LPS showed neuroprotection verified by reduced expression of TLR4, IL-1β, IL-6, and TNF-α in brain tissue [42]. Effects directly of KF regulating the LPS-induced production of pro-inflammatory factors have been also observed in cultured microglia [21]. We propose that the reduced microglia activation in the ARC observed in our study is due to local actions of KF. Supporting this idea, previous studies have reported a significant concentration and distribution of KF (orally administrated) in the rat brain [41]. Furthermore, it was shown that peripheral KF is able to modulate gene expression in the hypothalamus [50]. Thus, the low inflammatory phenotype promoted by KF in the ARC could restore the impaired EB regulation, improving in turn, BW and glucose control.

Furthermore, to confirm whether KF is able to modulate centrally EB regulation, obese mice received acute KF icv. This treatment led to identical findings observed in obese mice administrated chronically with KF ip. In fact, KF icv promoted a reduction in BW gain (without changes in FI) as well as decreased FE, suggesting an increase in EE. Thus, the effects of KF on EB could be mediated, at least partially, by the thyroid system, since KF promotes Dio2 activity and further thyroid activation [37]. By acting in the hypothalamus, this hormone critically regulates EB through sympathetic nervous system-dependent peripheral EE via thermogenesis [51]. In this line, we recently reported a hypothalamic-induced thermogenesis involving the bile acid receptor TGR5 (Takeda G-protein-coupled receptor 5) [26], an activator of Dio2 [52] and recognized receptor for naturally-occurring compounds [53,54]. We can also speculate about a regulatory action of KF on EB through the melanocortin system, considering its ability to modulate POMC expression [50]. However, such claims need to be supported by further experimental studies. Furthermore, even if the effects observed with central and peripheral administration show some similarity and suggest common mechanisms, further analysis comparing chronic effects of centrally and peripherally administered KF are necessary. In order to generate mechanistic evidence of the central effects of KF, in this work we evaluate the effect of its central administration on the expression of Tnf-α and Il-1β. The icv treatment did not lead to any change in the mRNA expression of these cytokines. Given that the same protocol led to a negative energy balance, the lack of effect on these inflammation mediators suggests that it is weight loss that leads to a decrease in microglial activation and not *vice versa*. However, complementary studies are necessary in different experimental conditions and focusing on other targets to rule out what is stated above.

5. Conclusion

The present work shows the anti-obesity effects of KF in mice exposed to a high-fat diet. Specifically, KF promoted body weight loss and improved glucose control. Likewise, it shows

the original finding regarding the restorative effect of KF on hypothalamic inflammation associated with obesity. Furthermore, we show, to my knowledge, for the first time that KF is capable of regulating EB by acting at the brain level. The results suggest that the anti-obesity effects of KF could occur at the central level, particularly in the hypothalamus, the key brain structure in the regulation of BE, involving anti-inflammatory effects. The findings of this work give a new perspective on the use of this natural compound to combat obesity, through its anti-neuroinflammatory properties. Further studies are necessary to determine the precise mechanisms of action involved in the brain effects with particular focus on the melanocortin and thyroid system. Establishing the best way of administration to achieve the levels of bioavailability necessary to obtain a sustained central effect, in perspective of use in humans, is among the next challenge.

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Disclosure statement

No potential conflict of interest was reported by the authors.

Data Availability statement

The data that support the findings of this study are available from the corresponding author, OGQ, upon reasonable request.

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Table and Figure legends

Table 1. Energy and composition of the standard and high-fat diet.

Figure 1. Experimental design. Selection of the dose of kaempferol (A), effect of chronic administration of kaempferol on diet-induced obesity (B), and effect of intracerebroventricular administration of kaempferol on energy balance (C). KF: kaempferol; ip: intraperitoneal; FI: food intake; BW: body weight; FE: feed efficiency; HFD: high-fat diet; GTT: glucose tolerance test; ITT: insulin tolerance test; icv: intracerebroventricular.

Figure 2. Effect of the acute treatment with kaempferol on energy balance. 24-h body weight change (A), cumulative (at 1, 2, 4, and 24-h) food intake (B), 24-h cumulative food intake normalized to body weight (C), and feed efficiency (D) were evaluated in mice fed on standard diet injected with vehicle (white bar), 0.5 (gray bar) or 1 mg/kg (black bar) kaempferol (KF, ip). Before KF injection mice were subjected to 24-h of fasting. Data are presented as mean ±

S.E.M. and were analyzed using a One-way ANOVA (A, C and D) or RM Two-way ANOVA (B), followed by the Bonferroni post-hoc test. *p<0.5 vs Vehicle. n=6 mice/group.

Figure 3. Effect of the chronic treatment with kaempferol on energy balance in obese mice. HFD-induced obese mice received daily a Vehicle solution (circle line or white bar) or 0.5 mg/kg (square line or black bar) kaempferol (KF, ip) for 40 days. % Body weight change (A), cumulative food intake (B), average/day of food intake normalized to body weight (C), and feed efficiency (D) were determinate at the end of the treatment. Data are presented as mean ± S.E.M. and were analyzed using a Student t-test. *p<0.05 vs Vehicle. n=6-7 mice/group.

Figure 4. Effect of the chronic treatment with kaempferol on glucose tolerance and insulin sensitivity in obese mice. Glucose tolerance test (GTT) and insulin tolerance test (ITT) were conducted on the day 32 and 38 of treatment with kaempferol (KF, ip), respectively. Vehicle (circle line) and KF (square line) mice with 6-h of fasting were subjected to a GTT (A) with an exogenous (ip) glucose load (2 mg/kg). The area under the curve (AUC) (B) and fasting glucose (time=0) (C) were calculated from GTT values of the Vehicle (white bar) and KF (black bar) groups. ITT (D) was carried out in 6-h fasted mice receiving 1 U/kg insulin (ip). From ITT values the AUC (E) and fasting glucose (F) were calculated. Data are presented as mean ± S.E.M. and were analyzed using a RM Two-way ANOVA (A and D), followed by the Bonferroni post-hoc test, or using a Student t-test (B, C, E, and F). *p<0.05. n=6 mice/group.

Figure 5. Effect of the chronic treatment with kaempferol on hypothalamic microglia activation in obese mice. Immunohistochemical analyses were performed on brain sections obtained from obese mice treated with Vehicle or 0.5 mg/kg kaempferol (KF, ip). Representative pictures and magnification inset of Iba1-immunoreactive cells in the ARC of Vehicle (A) or KF (B) groups are presented. Scale bar=100 μm. The effect of the treatment with Vehicle (white bar) or KF (black bar) on density (total microglial cells) (C), number of microglial cells with activated

phenotype (D), and percentage of activation (E). Data are presented as mean ± S.E.M. and were analyzed using a Student t-test. *p<0.05, ****p<0.0001 vs Vehicle. n=6 mice/group.

Figure 6. Effect of the acute intracerebroventricular treatment with kaempferol on energy balance in obese mice. 24-h body weight change (A), cumulative (at 1, 2, 4, and 24-h) food intake (B), 24-h cumulative food intake normalized to body weight (C), and feed efficiency (D) were evaluated in obese mice fed on high-fat diet injected with vehicle (white bar) or 5 mg/kg (black bar) kaempferol (KF, icv). Before KF injection mice were subjected to 24-h of fasting. Data are presented as mean ± S.E.M. and were analyzed using a Student t-test (A, C, and D) or RM Two-way ANOVA (B), followed by the Bonferroni post-hoc test. *p<0.5 vs Vehicle. n=6 mice/group.

Figure 7. Effect of the acute intracerebroventricular treatment with kaempferol on hypothalamic cytokine expression in obese mice. mRNA expression of Tnf-α (A) and Il-1β (B) in the hypothalamus of HFD-fed obese mice that were injected with Vehicle (white bar) or a single doses (5µg) of Kaempferol (KF) (black bar) via icv and euthanatized 2h after. The relative expression was calculated using the comparative method $2^{-\Delta\Delta CT}$. Data are presented as mean ± S.E.M. n=5 mice/group.

Figure S1. Effect of the chronic HFD intake on body weight change (A) and basal blood glucose of mice subjected to 6-h fasting (B). Mice were exposed for 12 weeks to HFD or SD. HFD-exposed groups (square line and black bar) showed body weight gain and higher blood glucose compared to SD group (circle line and white bar). Data are presented as mean ± S.E.M. and were analyzed using a RM Two-way ANOVA (A), followed by the Bonferroni post-hoc test or Student t-test (B). **p<0.01 vs SD, ****p<0.0001 vs SD. n=6-7 mice/group.

Figure S2. Effect of high-fat diet-induced obesity on Microglia activation in the arcuate nucleus of the hypothalamus. Immunohistochemical analyses were performed on brain sections

obtained from mice fed on standard diet (SD) or high-fat diet (HFD) for 12 weeks. Representative pictures of Iba1-immunoreactive cells in the ARC of SD (A) or HFD (B) groups are presented. Scale bar=100 µm. Body weight of both groups (C). The effect of the SD (white bar) or HFD (black bar) on density (total microglial cells) (D), number of microglial cells with activated phenotype (E), and percentage of activation (F). Data are presented as mean ± S.E.M. and were analyzed using a Student t-test. **p<0.01, ***p<0.001, ****p<0.0001 vs SD. n=6 mice/group.

Figure S3. Body weight change during 7 days of follow-up in mice subjected to daily handling and restraint. During this time mice of the Vehicle (circle line) or Kaempferol (KF) (square line) assigned groups underwent both handling and restraint daily until body weight stabilization.

Figure and tables

Graphical abstract

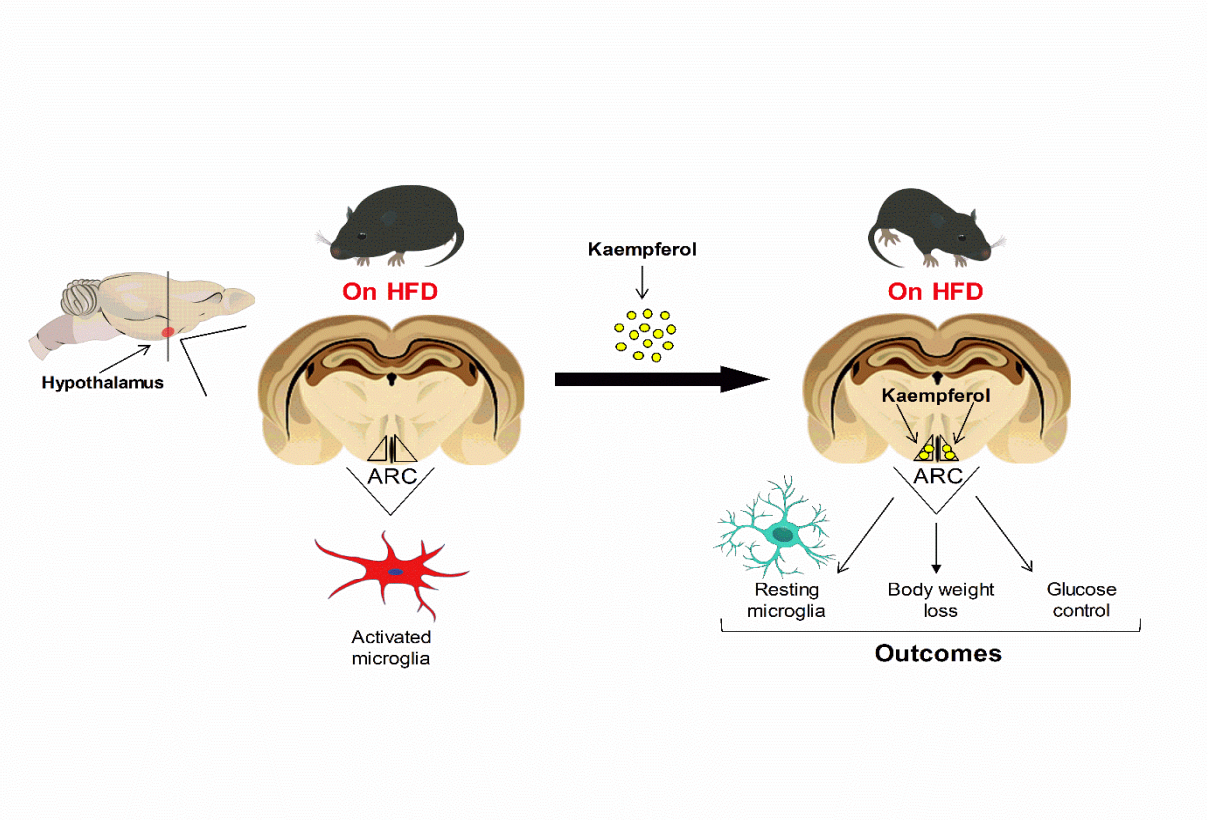


Figure 1.

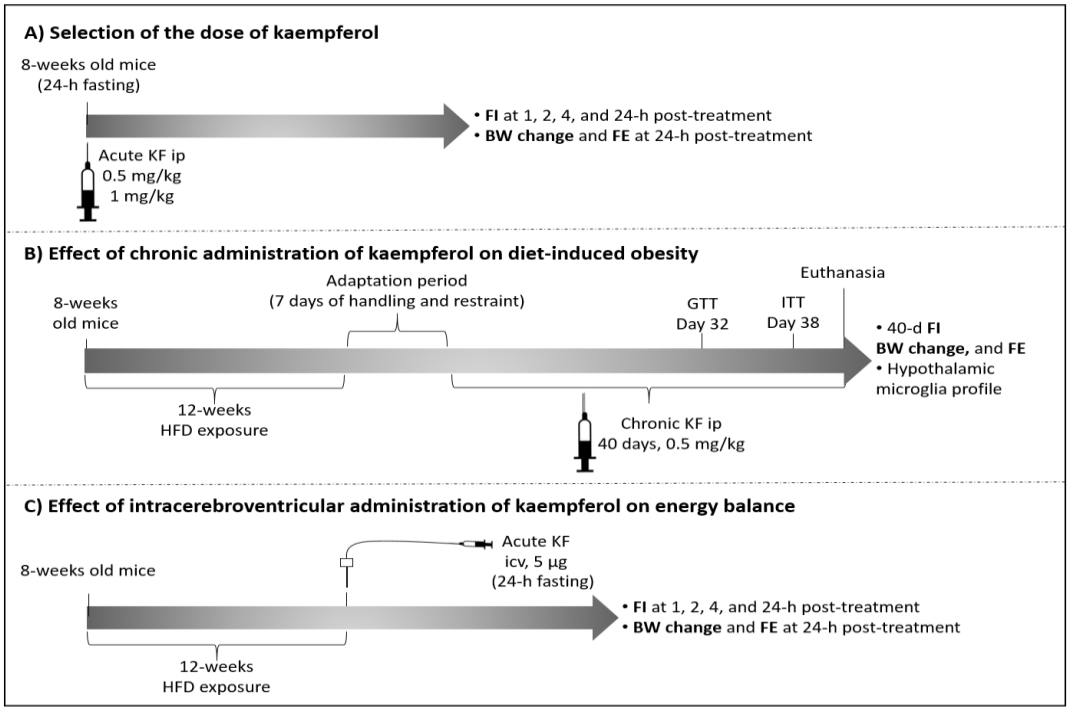


Figure 2.

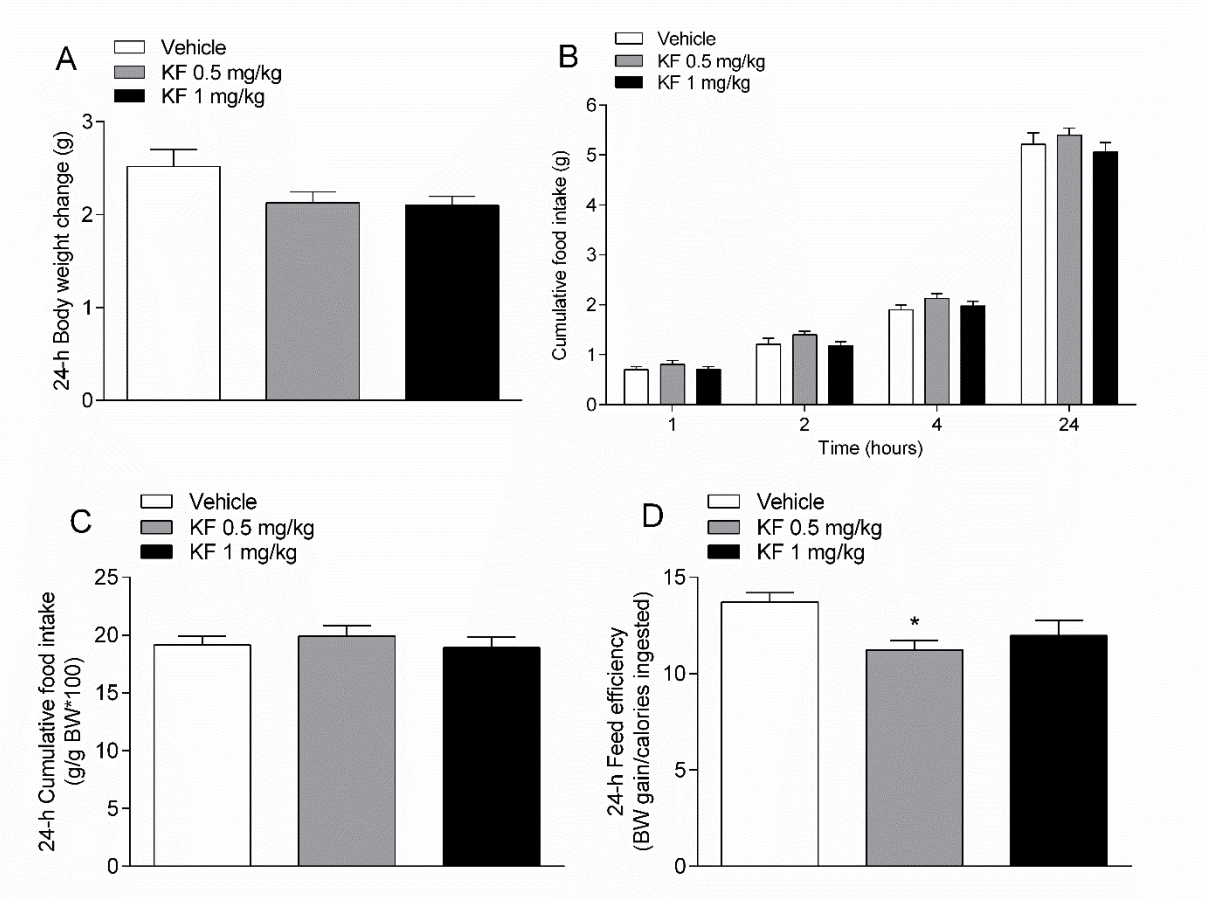
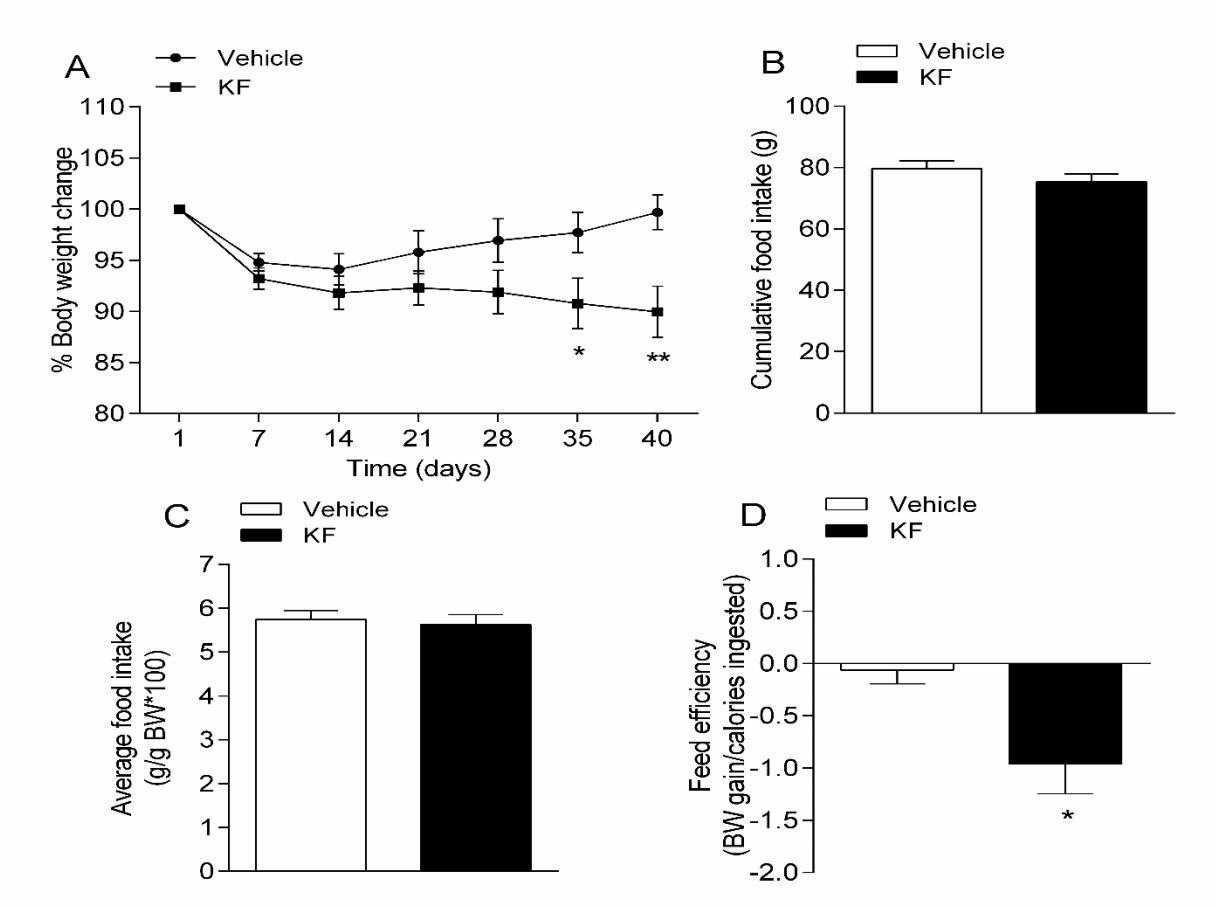

A
Vehicle
KF 0.5 mg/kg
KF 1 mg/kg
24-h Body weight change (g)
0
1
2
3
B
Vehicle
KF 0.5 mg/kg
KF 1 mg/kg
Cumulative food intake (g)
0
1
2
3
4
5
6
1
2
4
24
Time (hours)
C
Vehicle
KF 0.5 mg/kg
KF 1 mg/kg
24-h Cumulative food intake (g/g BW*100)
0
5
10
15
20
25
D
Vehicle
KF 0.5 mg/kg
KF 1 mg/kg
24-h Feed efficiency (BW gain/calories ingested)
0
5
10
15
*

Figure 3.



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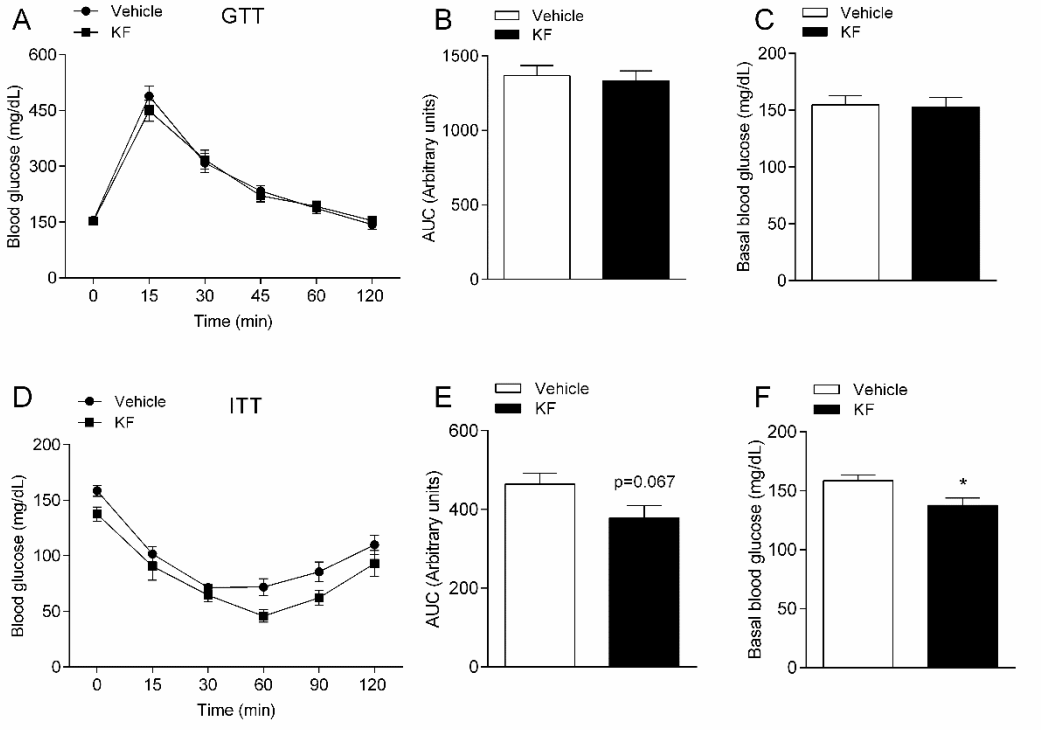

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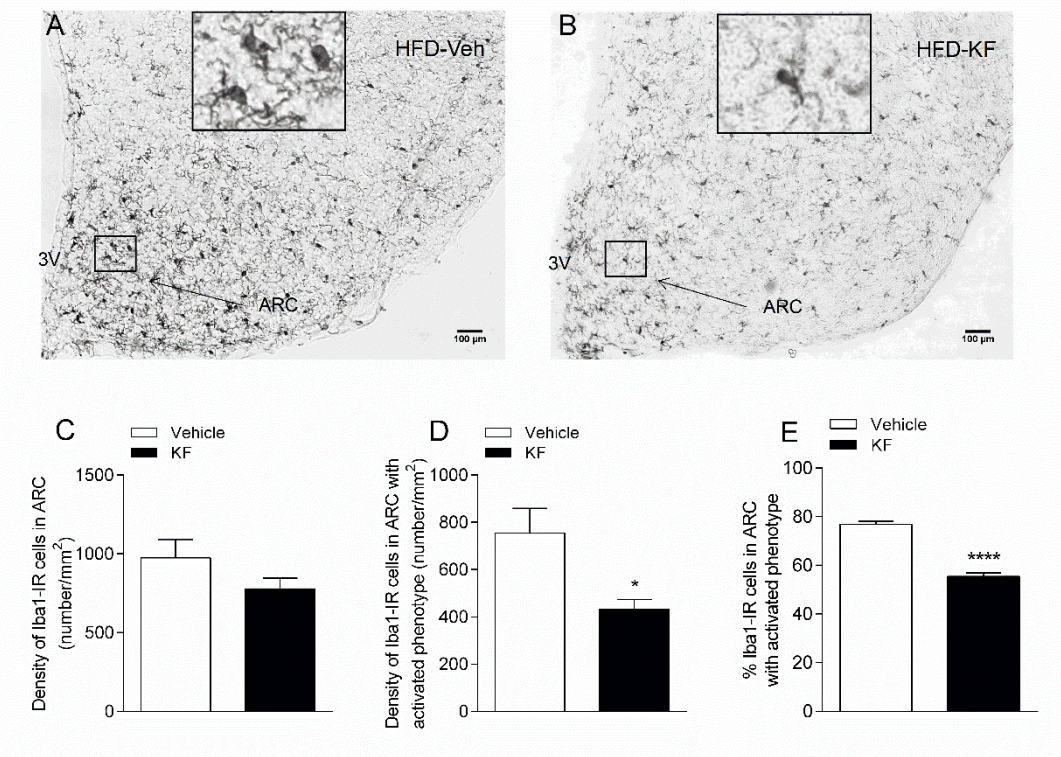
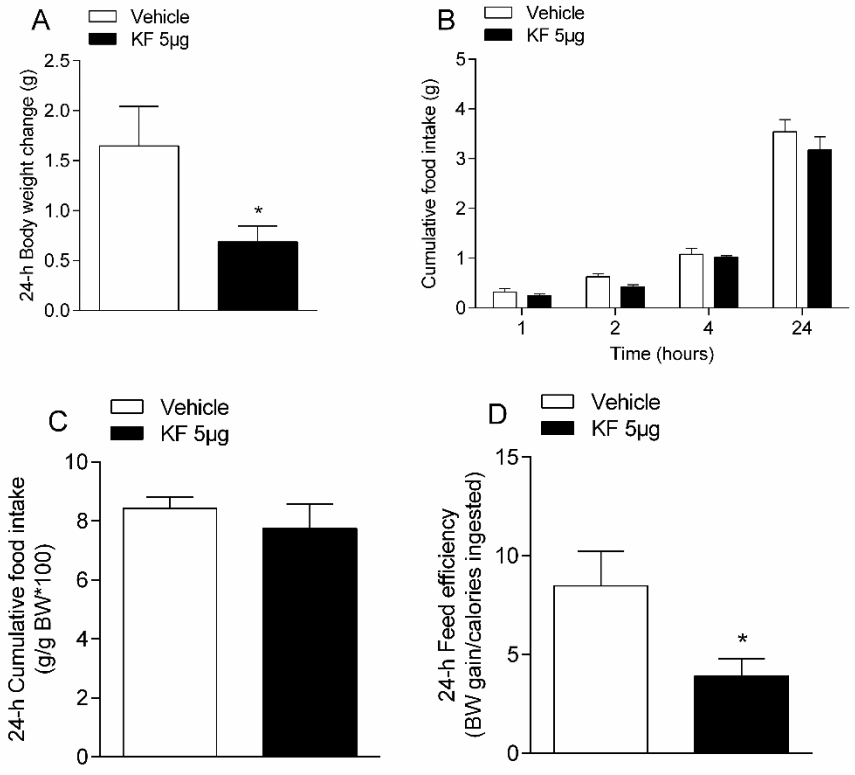


Figure 6



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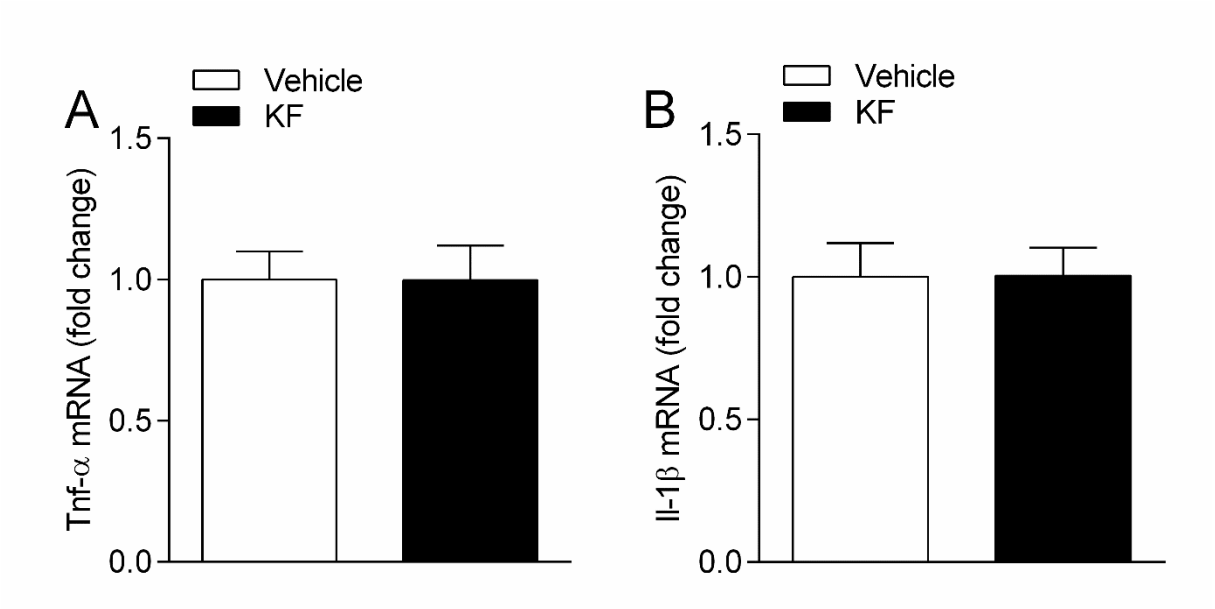
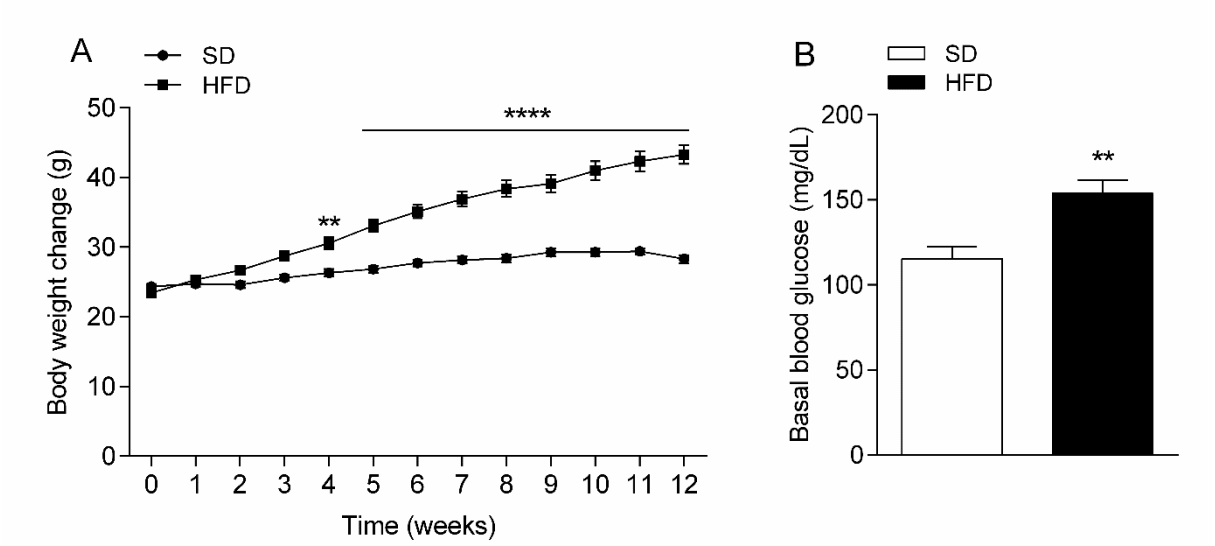
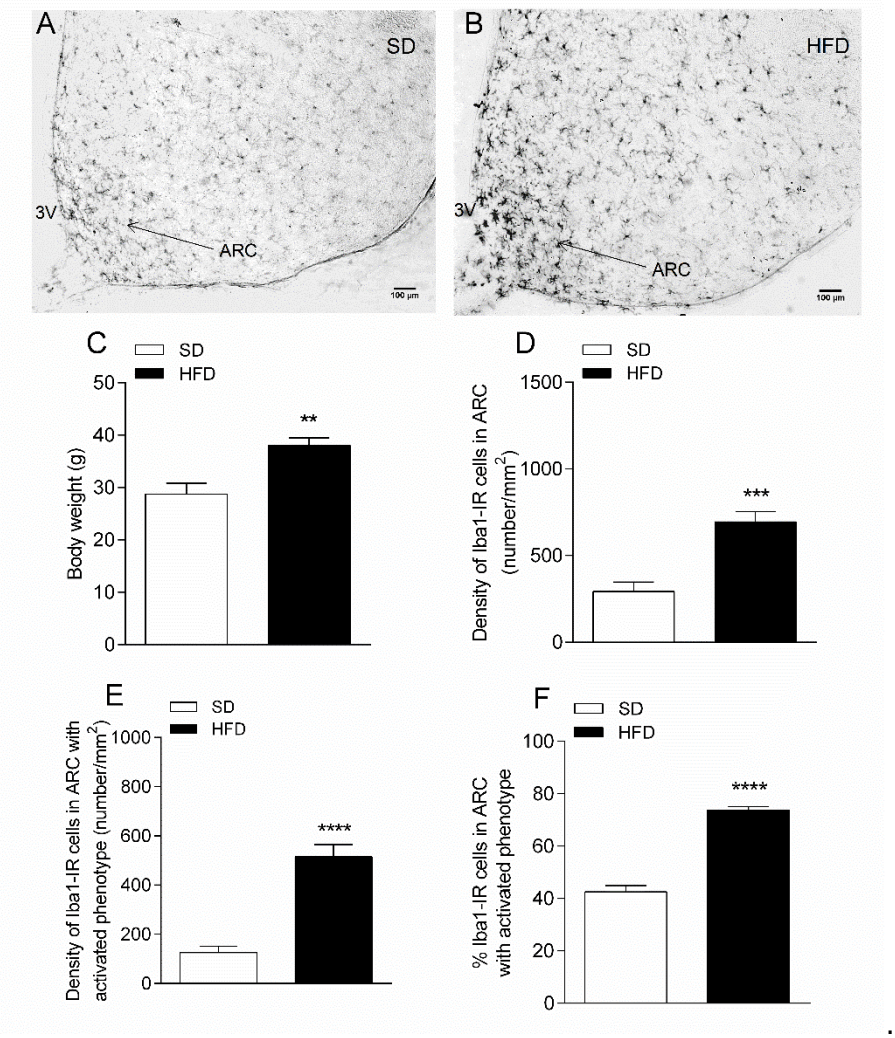


Supplementary figure 1.



Supplementary figure 2



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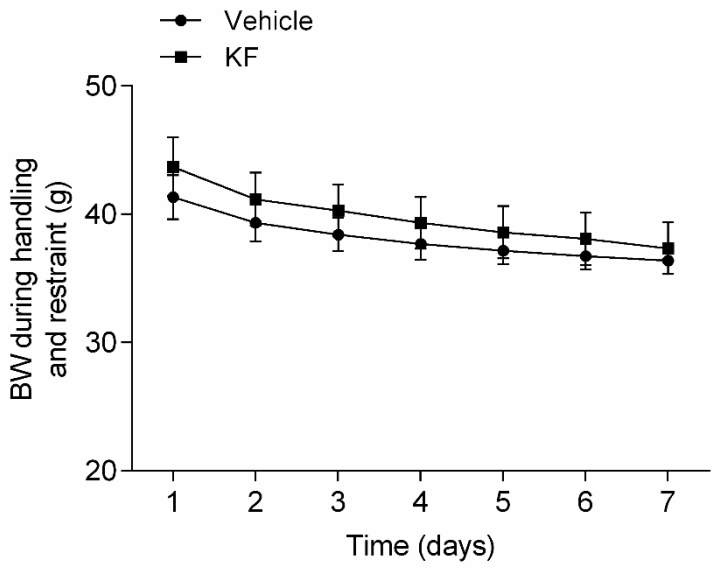

Table 1. Energy and composition of the standard and high-fat diet.

Ingredient	SD	HFD
Cornstarch (g/kg)	610	256
Sucrose (g/kg)	100	100
Casein (g/kg)	140	180
BCellulose (g/kg)	50	50
Guar (g/kg)	15	15
Soybean oil (g/kg)	40	40
Lard (saturated fat) (g/kg)	-	314
Mineral Mix (g/kg)	35	35
Vitamin Mix (g/kg)	10	10
%kcal from carbohydrates	75.5	26.72
%kcal from proteins	14.9	13.51
%kcal from lipids	9.6	59.77
kcal/g	3.76	5.33

SD = Standard diet; HFD = High-fat diet; kcal = kilocalorie.

Table S1. Body weight during the chronic treatment with kaempferol.

Vehicle group	**Day 1**	**Day 7**	**Day 14**	**Day 21**	**Day 28**	**Day 35**	**Day 40**
Mouse 1	38.20	36.80	34.50	35.00	36.20	37.22	37.45
Mouse 2	39.68	36.75	35.00	36.10	36.05	36.95	37.75
Mouse 3	38.02	35.10	34.75	33.90	34.60	35.50	36.80
Mouse 4	34.90	32.95	33.50	34.15	35.05	33.00	34.20
Mouse 5	34.52	33.05	33.60	33.85	33.40	33.85	34.65
Mouse 6	33.64	33.20	33.15	35.35	35.95	36.40	36.60
Mouse 7	33.50	31.30	32.50	32.75	32.80	33.25	33.75
Mean	**36.07**	**34.16**	**33.86**	**34.44**	**34.86**	**35.17**	**35.89**
KF group							
Mouse 1	42.40	39.26	37.54	38.86	38.38	37.70	39.00
Mouse 2	42.54	40.42	40.35	40.32	36.85	34.64	33.50
Mouse 3	38.14	35.70	35.20	35.35	36.95	36.40	36.10
Mouse 4	33.04	29.40	28.95	29.55	29.85	30.75	29.78
Mouse 5	32.46	31.30	31.80	31.95	32.30	31.90	31.05
Mouse 6	31.12	28.85	27.90	27.05	27.20	27.25	27.58
Mean	**36.62**	**34.16**	**33.62**	**33.85**	**33.59**	**33.11**	**32.84**

KF=kaempferol

ANEXO A- APROVAÇÃO DA COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA).

Universidade Federal de Pernambuco
Centro de Biociências
Av. Prof. Nelson Chaves, s/n
50670-420 / Recife – PE – Brasil
Fones: 2126 8842
ceua@ufpe.br

Recife, 12 de novembro de 2018

Ofício nº 100/18

Da Comissão de Ética no Uso de Animais (CEUA) da UFPE
Para: **Prof. Ana Elisa Toscano da Silva**
Centro Acadêmico de Vitoria (CAV)
Universidade Federal de Pernambuco
Processo nº 0052/2018

Certificamos que a proposta intitulada **"Efeitos do tratamento neonatal com kaempferol sobre o desenvolvimento neuromúsculo-esquelético em modelo de paralisia cerebral experimental."** registrada com o **nº0052/2018,** sob a responsabilidade de **Prof. Ana Elisa Toscano da Silva** que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo CONSELHO NACIONAL DE CONTROLE DE EXPERIMENTAÇÃO ANIMAL (CONCEA), e foi aprovada pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) DA UNIVERSIDADE FEDERAL DE PERNAMBUCO (UFPE), em reunião de 02/10/2018.

Finalidade	() Ensino (X) Pesquisa Científica
Vigência da autorização	12/11/2018 a 12/03/2022
Espécie/linhagem/raça	Ratos heterogenico
Nº de animais	100
Peso/Idade	220-250g/ 90-120 dias
Sexo	Macho (80) e (20) Femea
Origem	Bioterio de criação do Departamento de Nutrição (UFPE).

Atenciosamente,

Prof. Sebastião R. F. Silva
Vice-Presidente CEUA/UFPE
SIAPE 2345691