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SABRINA DA CONCEIÇÃO PEREIRA

EFEITOS DO TRATAMENTO COM RESVERATROL E/OU COM O FATOR DE CRESCIMENTO DE FIBROBLASTOS 19 SOBRE OS ASPECTOS MORFOLÓGICOS, COMPORTAMENTAIS E MOLECULARES DO SISTEMA NEUROMUSCULOESQUELÉTICO NA PARALISIA CEREBRAL EXPERIMENTAL

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Tese apresentada ao programa de Pós-graduação em Neuropsiquiatria e Ciências do Comportamento da Universidade Federal de Pernambuco, como requisito parcial para a obtenção do título de Doutora em Neurociências.

Área de concentração: Neurociências.

Orientador (a): Profª. Drª. Ana Elisa Toscano Meneses da Silva

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RESUMO

A paralisia cerebral (PC) é considerada a principal desordem motora da infância. Os tratamentos atuais visam minimizar as limitações funcionais e comorbidades, mas não revertem seus distúrbios primários no sistema neuromusculoesquelético. Dentre as perspectivas terapêuticas destaca-se o fator de crescimento de fibroblastos 19 (FGF19) por seu efeito hipertrófico no musculoesquelético, e o resveratrol, por seus efeitos neuroprotetores e antioxidantes, porém ambos ainda escassos em modelos de PC. O objetivo do presente estudo foi investigar os efeitos do tratamento com FGF19 e/ou Resveratrol sobre os aspectos morfológicos, comportamentais e moleculares do sistema neuromusculoesquelético de ratos submetidos à PC. Trata-se de um estudo experimental, aprovado pelo comitê de ética em experimentação animal CCB-UFPE (CEUA:0009/2020). Filhotes ratos machos Wistar foram divididos nos grupos experimentais (n=10-13 por grupo) conforme a indução da PC e o tratamento com FGF19 0,1mg/kg subcutânea do 22º ao 28º dia de vida: Controle+Veículo (CV); Controle+FGF19 (CF); PC+Veículo (PCV); PC+FGF19 (PCF); ou o tratamento com Resveratrol 10mg/kg via intraperitoneal do 3º ao 21º dia de vida: Controle+Salina (CS), PC+Salina (PCS); Controle+Resveratrol (CR) e PC+Resveratrol (PCR). O modelo de PC associou a anóxia perinatal à restrição sensório-motora. Animais submetidos à PC apresentaram atraso na ontogênese de reflexos primitivos, redução do crescimento somático, da atividade locomotora, da coordenação motora e da força muscular ($p<0.05$), além de redução no peso muscular, da área, perímetro, distorção no padrão de distribuição das fibras musculares e alterações na expressão de genes relacionados ao desenvolvimento muscular e junções neuromusculares ($p<0.05$). Nos ratos PC foram observados, no osso tíbia, a redução do peso, espessura cortical, área medular e volume trabecular e aumento da porosidade ($p<0.05$), e no cerebelo, houve aumento da densidade de micróglia e do percentual de micróglia ativada. O tratamento com FGF19 na PC experimental aumentou o peso corporal, a atividade locomotora e a força muscular, o peso dos músculos sóleo e extensor longo dos dedos (EDL), área e perímetro das fibras musculares e reverteu as alterações na expressão gênica muscular ($p<0.05$). A tíbia do grupo PCF apresentou aumento da espessura cortical e do volume trabecular, e na sua epífise proximal houve redução da porosidade comparado aos animais PCV ($p<0.05$). Os animais PC tratados com resveratrol recuperaram os

danos na ontogênese dos reflexos, na atividade locomotora, na distribuição de tipos de fibras musculares, na densidade microglial e na porcentagem de microglia ativada no cerebelo comparado ao PCS ($p<0.01$). Conclui-se que o tratamento com FGF19 reverteu a atrofia musculoesquelética e as alterações na expressão de genes relacionados à imaturidade muscular, melhorando a locomoção e a força muscular em modelo de PC. E o tratamento com resveratrol recuperou a morfologia muscular e reduziu a neuroinflamação cerebelar, beneficiando o neurodesenvolvimento e a aquisição de habilidades motoras como a locomoção na PC experimental.

Palavras-chave: modelos animais; lesões encefálicas; músculo esquelético; locomoção.

ABSTRACT

Cerebral palsy (CP) is considered the main motor disorder in childhood. The current treatments aim to minimize functional limitations and comorbidities, but do not reverse their primary disorders in the neuromusculoskeletal system. Among the therapeutic perspectives, the fibroblast growth factor 19 (FGF19) stands out for its non-musculoskeletal hypertrophic effect, and resveratrol, for its neuroprotective and antioxidant effects, both of which are still scarce in CP models. The objective of the present study was to investigate the effects of treatment with FGF19 and/or Resveratrol on the morphological, behavioral and molecular aspects of the neuromusculoskeletal system at times subjected to PC. This is an experimental study, approved by the CCB-UFPE animal experimentation ethics committee (CEUA:0009/2020). The Wistar male samples were divided into experimental groups (n=10-13 per group) according to CP induction and treatment with FGF19 0.1mg/kg subcutaneously from the 22nd year to the 28th day of life: Control+Vehicle (CV); Control+FGF19 (CF); CP+Vehicle (CPV); CP+FGF19 (CPF); or treatment with Resveratrol 10mg/kg intraperitoneally from the 3rd year or 21st day of life: Control+Saline (CS), CP+Saline (CPS); Controle+Resveratrol (CR) and PC+Resveratrol (CPR). The CP model associated with perinatal anóxia and sensory-motor restriction. Animais submetidos à PC show delay in the ontogenese of primitive reflexes, reduction in somatic growth, locomotor activity, motor coordination and muscle strength ($p<0.05$), as well as reduction in muscle weight, area, perimeter, distortion in the pattern of distribution of muscle fibers and alterations in the expression of genes related to muscle development and neuromuscular junctions ($p<0.05$). In the observed PC foram moments, in the osso tibia, a reduction in weight, cortical thickness, medullary area and trabecular volume and increase in porosity ($p<0.05$), in the cerebellum, there was an increase in the density of microglia and the percentage of activated microglia. The treatment with FGF19 in experimental CP increased body weight, locomotor activity and muscular strength, weight of the soleus and extensor longus digitorum (EDL) muscles, area and perimeter of muscle fibers and reverted as alterations in muscle genetic expression ($p< 0.05$). The CPF group showed an increase in cortical thickness and trabecular volume, and its proximal epiphyse had reduced porosity compared to the CPV animals ($p<0.05$). The CP animals treated with resveratrol will recover the damage in the ontogenesis of the

reflexes, in the locomotor activity, in the distribution of muscle fiber types, in the microglial density and in the percentage of activated microglia in the cerebellum compared to the CPS ($p<0.01$). It is concluded that treatment with FGF19 reverts to musculoskeletal atrophy and alterations in the expression of genes related to muscular immaturity, improving locomotion and muscular strength in the CP model. The treatment with resveratrol recovers muscle morphology and reduces cerebellar neuroinflammation, benefiting neurodevelopment and the acquisition of motor skills as well as locomotion in experimental CP.

Keywords: models, animal; brain injuries; muscle, skeletal; locomotion.

LISTA DE ABREVIATURAS E SIGLAS

CR	Grupo controle resveratrol
CS	Grupo controle salina
CV	Grupo controle veículo
EDL	*Extensor digitorum longus* (Extensor longo dos dedos)
EVA	Etil Vinil Acetato
FGF19	Fator de crescimento de fibroblastos 19
HE	Hematoxilina-eosina
MyHC	Miosina de cadeia pesada
PBS/BSA	Solução tampão fosfato-salino com albumina de soro bovino
PC	Paralisia Cerebral
PCF	Grupo paralisia cerebral FGF19
PCR	Grupo paralisia cerebral resveratrol
PCS	Grupo paralisia cerebral salina
PCV	Grupo paralisia cerebral veículo
SNC	Sistema Nervoso Central

SUMÁRIO

1 INTRODUÇÃO

Lesões cerebrais ocorridas durante o desenvolvimento podem levar a uma desordem permanente do movimento na infância, a paralisia cerebral (PC) (Gulati; Sondhi, 2017; Marret; Vanhulle; Laquerriere, 2013). As crianças acometidas apresentam diversas alterações do sistema neuromusculoesquelético que resultam na deterioração funcional, em que a sobrevivência está relacionada à severidade da incapacidade (Gulati; Sondhi, 2017; Liptak et al., 2004). A PC não tem cura e poucas intervenções são modificadoras da doença onde a gestão dos sintomas é a base do tratamento (Wimalasundera; Stevenson, 2016). Assim, fazem-se necessários estudos que investiguem propostas terapêuticas para as disfunções primárias da PC como os prejuízos musculoesqueléticos e dano no sistema nervoso central que influenciam o desenvolvimento motor.

Perspectivas terapêuticas que abranjam tanto as lesões do sistema nervoso central como as repercussões no sistema musculoesquelético têm se destacado. Dentre elas, está o fator de crescimento de fibroblastos 19 (FGF19) que tem potencial para atuar em modelos de atrofia muscular induzido por obesidade e envelhecimento (Benoit et al., 2017), sendo promissor também em estudos que utilizam atrofia muscular de origem neurológica, como em modelos de PC. Além disso, os alimentos funcionais tem ganhado destaque como o polifenol chamado resveratrol devido aos seus efeitos antioxidantes e neuroprotetores promissores em modelos de lesão cerebral precoce como em modelo de hipóxico-isquemia perinatal (ArteagA et al., 2014, 2015; Pan et al., 2016) e em modelos de atrofia muscular induzida por desnervação e obesidade (Asami et al., 2018; Bai et al., 2020; Huang et al., 2019). Dessa forma, o resveratrol torna-se uma possível alternativa de tratamento em modelos de PC porém os estudos ainda são escassos.

Estudos experimentais de PC são ferramentas para elucidar o mecanismo subjacente a esta patologia e investigar potenciais terapias farmacológicas já que reproduzem os prejuízos neuromusculares semelhantes a crianças com CP (Costa-De-Santana et al., 2023; Silva et al., 2016c; Strata et al., 2004a). Neste contexto, o objetivo deste estudo foi investigar os efeitos do tratamento com FGF19 e/ou Resveratrol sobre os aspectos morfológicos, comportamentais e moleculares do sistema neuromúsculoesquelético de ratos submetidos à PC.Os dados provenientes

deste estudo irão contribuir para o esclarecimento do potencial papel do FGF19 e Resveratrol nos danos resultantes da PC experimental, e consequentemente sua atuação no desenvolvimento motor. Assim, possibilitará também novas janelas de atuação terapêutica, visto que a utilização de modelo experimental de PC, obedecendo às normas éticas, permite extrapolar informações relevantes com os devidos cuidados para a criança acometida por essa doença, que afeta milhares delas no mundo.

2 REFERENCIAL TEÓRICO

2.1 PARALISIA CEREBRAL

A Paralisia Cerebral (PC) foi inicialmente descrita com base em observações do tônus muscular e deformidades em crianças que apresentavam dificuldades ao nascer, com evidências pós-morte de lesão cerebral e medular, sendo referida como uma desordem cérebro-espinhal pelo Dr. William John Little em 1862 (Brandenburg; Fogarty; Sieck, 2019).

Como o envolvimento da medula espinhal na gênese dos sinais clínicos foi sendo abandonado, assim surgiu o termo ‘paresia cerebral’ (Brandenburg; Fogarty; Sieck, 2019; Rosenbaum et al., 2007) Esse termo buscava esclarecer que o comprometimento precoce do cérebro seria o condutor dos sintomas físicos. As dificuldades relacionadas ao parto foram caracterizados como o fator etiológico para a ‘paresia cerebral’, levantando a hipótese de que esse distúrbio era devido à asfixia neonatal ou anóxia, apesar das evidências limitadas nesta época (Brandenburg; Fogarty; Sieck, 2019). Atualmente, o termo utilizado é a ‘paralisia cerebral’ (PC) que foi introduzido em 1889 por Sir William Osler quando descrevia, através de suas palestras, os pacientes com sintomas de PC com histórico de adversidades durante o nascimento (Brandenburg; Fogarty; Sieck, 2019).

Dessa forma, foram criados conceitos referentes à PC, sendo que a mais recente define: “A paralisia cerebral é um grupo de distúrbios permanentes do desenvolvimento do movimento e da postura, causando limitação da atividade, que são atribuídos a distúrbios não progressivos que ocorreram no desenvolvimento do cérebro fetal ou infantil. Os distúrbios motores da paralisia cerebral são frequentemente acompanhados por distúrbios de sensação, percepção, cognição, comunicação e comportamento, por epilepsia e por problemas musculoesqueléticos secundários” (Brandenburg; Fogarty; Sieck, 2019; Rosenbaum *et al.,* 2007).

Essa definição que se utiliza até hoje considera a heterogeneidade dos distúrbios abrangidos pelo termo PC incluindo as deficiências do desenvolvimento neurológico não motor onde os prejuízos sensoriais, cognitivos e comportamentais muitas vezes incapacita o indivíduo de forma significativa (Rosenbaum *et al.,* 2007).

Apesar do progresso no consenso clínico de PC, a sua definição continua com foco no cérebro sem reconhecimento de disfunção da medula espinhal durante o desenvolvimento como foi hipotetizado inicialmente (Brandenburg; Fogarty; Sieck, 2019).

A prevalência global geral de PC é estimada ser 2 a 3 por 1000 nascidos vivos, variando conforme o acesso aos cuidados obstétricos e neonatais da região sociodemográfica (Gulati; Sondhi, 2017; Maenner *et al.,* 2016; Oskoui *et al.,* 2013). A etiologia da PC é diversificada, resultando da lesão cerebral antes, durante ou após o nascimento (Gulati; Sondhi, 2017). Nesse sentido, enquanto os países desenvolvidos lidam com prematuridade e as morbidades relacionadas ao baixo peso ao nascer, os países subdesenvolvidos lidam com infecções no período pré ou pós-natal, a asfixia perinatal e a hiperbilirrubinemia neonatal (Gulati; Sondhi, 2017; Wimalasundera; Stevenson, 2016). Notadamente, a prematuridade contribui de forma significativa para o aumento da prevalência de PC, mesmo em países desenvolvidos, acometendo entre 40-100 por 1000 nascidos vivos para aqueles nascidos com menos de 28 semanas de gestação (Oskoui *et al.,* 2013; Wimalasundera; Stevenson, 2016)

Em geral um conjunto de fatores predisponentes pré-natais, eventos perinatais agudos ou subagudos e fatores agravantes pós-natais agem juntos no cérebro fetal e do recém-nascido em desenvolvimento para alterar maturação do cérebro e levar à PC (Marret; Vanhulle; Laquerriere, 2013). Os fatores de risco pré-natais podem estar relacionados à condições maternas, fetais ou fatores sociais (Graham; Paget; Wimalasundera, 2019), onde existe janelas de vulnerabilidade do sistema nervoso central ao dano conforme a idade gestacional (Marret; Vanhulle; Laquerriere, 2013).

Até a 24ª semana de gestação está ocorrendo a neurogênese cortical que é caracterizada por proliferação, migração e organização de células precursoras neuronais, e depois de neurônios. Esta fase é amplamente vulnerável a alterações genéticas ou adquiridas como infecções virais ou toxicidade, resultando em malformações como lissencefalia, heterotopias nodulares, displasia cortical, etc (Marret; Vanhulle; Laquerriere, 2013). A partir desse período, o sistema nervoso central está atravessando a fase de crescimento e diferenciação incluindo o

crescimento axonal e dendrítico, formação de sinapses e mielinização, bem como processos de estabilização como a poda sináptica, e especialização de circuitos, que persistem após o nascimento e alcançam seu máximo durante os primeiros 2 anos de vida. Nesta etapa do desenvolvimento do cérebro, eventos perinatais agudos ou subagudos como a asfixia ao nascimento são determinantes nas lesões de substância branca e cinzenta geralmente observadas em crianças com PC (Marret; Vanhulle; Laquerriere, 2013).

Com o passar do tempo esses fatores etiológicos foram sendo identificados e a paralisia cerebral passou a receber classificações com base no fenótipo motor e sua distribuição (Brandenburg; Fogarty; Sieck, 2019; Wimalasundera; Stevenson, 2016). Quanto à distribuição, pode ser unilateral ou bilateral. Quanto às anormalidades de tônus e movimentos ela pode ser espástica, acometendo aproximadamente 85%–90% dos indivíduos com paralisia cerebral, sendo um terço desses unilateral e dois terços bilaterais (Wimalasundera; Stevenson, 2016).

A forma espástica é caracterizada pelo aumento do tônus dependente da velocidade, com hiperreflexia e sinais do neurônio motor superior (Wimalasundera; Stevenson, 2016). Quando o fenótipo é visto como movimentos recorrentes, descontrolados e involuntários que podem ser estereotipados, classificamos a PC como discinético, que acomete aproximadamente 7% dos indivíduos com PC, geralmente bilateral (Wimalasundera; Stevenson, 2016). Nas formas mistas, espasticidade com discinesia, nenhuma anormalidade de tônus é única e nenhum distúrbio de movimento predomina (Wimalasundera; Stevenson, 2016). Outros prejuízos musculoesqueléticos podem estar associados, como a redução da área das fibras musculares e desorganização do tecido muscular provavelmente induzida por atrofia relacionada à inatividade física e insuficiência da ativação neural, como visto em estudo anterior onde foi realizada a biopsia de indivíduos com PC (Borg *et al.,* 2019).

Antes dos 12 ou 24 meses, a identificação da PC com precisão era considerada difícil de ser realizada, quando acreditava-se que esse seria o período latente da PC. Hoje em dia, isso está desatualizado, e a PC ou o alto risco de PC já podem ser previstos com precisão antes dos 6 meses de vida (Novak, 2017). Dessa forma, o diagnóstico da PC é realizado através de escalas que se baseiam na

observação de movimentos espontâneos, reflexos, atenção e comportamento das crianças, em conjunto com exames de neuroimagem, além do raciocínio clínico.

A disfunção motora é considerada o critério diagnóstico essencial neste processo de identificação da PC precocemente, onde observa-se redução da qualidade dos movimentos e atividades motoras abaixo do esperado para a idade cronológica, incluindo atrasos no aparecimento de reflexos primitivos (Chandradasa; Rathnayake, 2020; Kobesova; Kolar, 2014; Novak *et al.,* 2016). Os reflexos primitivos são movimentos involuntários automáticos que ocorrem em resposta a um estímulo. Nos seres humanos, a maturidade cerebral satisfatória é essencial para permitir a inibição de reflexos primitivos e o aparecimento de respostas posturais para a progressão normal das funções psicomotoras; isso requer uma transição de um tronco cerebral involuntário resposta reflexa a outra controlada pelo córtex. Uma criança com atraso psicomotor desenvolvimento, como acontece na PC, pode demonstrar dificuldades no desempenho motor como locomoção e coordenação de movimentos, estando estes relacionados com o maior risco para desenvolvimento anormal (Chandradasa; Rathnayake, 2020; Kobesova; Kolar, 2014). O controle desses reflexos primitivos é realizado em níveis de controle sensório-motor dentro do SNC com o cerebelo envolvido em todos níveis de integração, atuando na regulação do tônus muscular, manutenção postural e do equilíbrio (Kobesova; Kolar, 2014). Isto demonstra um papel importante também do cerebelo no estudo dos danos motores da PC.

Além dos distúrbios motores do desenvolvimento, a história clínica que indica risco de paralisia cerebral inclui riscos de pré-concepção, riscos na gravidez, no parto, neonatal e até os 24 meses é considerada critério adicional neste processo diagnóstico (Novak *et al.,* 2017). Outro critério adicional importante para o diagnóstico da PC enfoca as anormalidades neuroanatômicas. Dentre as alterações que podem ser visualizadas no exame de neuroimagem a lesão de substância branca é a mais frequente ocorrendo em aproximadamente 50% dos casos (leucomalácia periventricular cística ou infartos hemorrágicos periventriculares), a lesão de substância cinzenta cortical e profunda (gânglios da base ou lesões do tálamo, encefalomalácia multicística ou acidente vascular cerebral) e o desenvolvimento deficiente do cérebro (lisencefalia, paquigiria, displasia cortical, polimicrogiria ou esquizencefalia) também podem estar presentes (Novak et al.,

2017). Além disso, achados de neuroimagem do cerebelo em crianças com PC indicam menor área do vermis e hemorragia cerebelar fortemente relacionados aos prejuízos no neurodesenvolvimento (Kim *et al.,* 2023; Wu *et al.,* 2019).

A evolução nas ferramentas de identificação precoce como ocorre através neuroimagem na PC é fundamental para o manejo clínico, inclusive das crianças categorizadas ainda como um caso suspeito. Em que essas crianças requerem e se beneficiam de intervenções precoces específicas diferentes das crianças em risco de atraso no desenvolvimento, ou de autismo, ou em risco social, por exemplo (Novak et al., 2017). A paralisia cerebral não tem cura e poucas intervenções são modificadoras da doença. A gestão dos sintomas é a base do tratamento (Wimalasundera; Stevenson, 2016). Assim, faz-se necessário estudo de propostas terapêuticas para as disfunções primárias da PC, como o dano precoce no sistema nervoso central e os prejuízos musculoesqueléticos que influenciam no desenvolvimento motor da criança.

2.2 MODELOS EXPERIMENTAIS DE PARALISIA CEREBRAL

Sabe-se que a PC é uma disfunção heterogênea e complexa resultante de um dano cerebral durante o período crítico de desenvolvimento. Os modelos de PC tentam reproduzir esses danos, e consequentemente buscam reproduzir as repercussões neurofuncionais no desenvolvimento (Da Conceição Pereira *et al.,* 2021).

Assim, modelos experimentais tornaram-se ferramentas fundamentais na elucidação dos mecanismos fisiopatológicos da PC e nos estudos que utilizam propostas terapêuticas para os prejuízos da PC à curto e a longo prazo (Da Conceição Pereira et al., 2021). A partir dos dados obtidos através de modelos experimentais, podemos extrapolar os resultados, dentro dos limites éticos, para seres humanos e embasar futuras pesquisas na área.

Modelos de PC surgiram nas últimas décadas e foram se expandindo e, atualmente, temos uma variedade de modelos de PC. Esses modelos são diversificados quanto a estratégia de indução da lesão cerebral precoce, o momento de indução da PC e a capacidade de manutenção dos prejuízos funcionais ao longo

da vida, sendo que esses modelos podem ser utilizados de forma isolada ou combinada. Vejamos a seguir os principais modelos de PC descritos na literatura.

Alguns modelos de PC que utilizam insultos durante o pré-natal estão bem estabelecidos. Dentre eles, está a utilização da injeção de Lipopolissacarídeo (LPS), também chamado de endotoxina bacteriana, que é uma molécula que se encontra abundantemente na membrana externa das bactérias gram-negativas. Então, neste modelo a PC é induzida nos animais pela inflamação materna que causa um desequilíbrio no ambiente intra-útero. Neste modelo, foi demonstrado que os animais com PC apresentaram aumento de citocinas pró inflamatórias em diversas regiões do cérebro levando à danos pela neuroinflamação (Shi *et al.,* 2019; Zhang *et al.,* 2016). Sendo considerado um modelo interessante para desfechos relacionados ao sistema nervoso central. Por outro lado, os estudos observaram pouca ou nenhuma repercussão sobre o desenvolvimento neurofuncional, como os prejuízos na locomoção ou coordenação motora como se esperava de um modelo que reproduzisse o fenótipo motor da PC (ShI *et al.,* 2019; Zhang *et al.,* 2016).

Outro modelo bastante difundido é a isquemia pré-natal, onde a indução da PC ocorre devido à ligação unilateral da artéria uterina que irriga o útero materno. Dessa forma, a isquemia afeta os filhotes ainda intra-útero, resultando em anormalidades estruturais no cérebro desde a infância até a fase adulta, incluindo, por exemplo, a redução do tamanho e redução da densidade neuronal no hipocampo (Ruff *et al.,* 2017), a lesão de substância branca abaixo do córtex motor primário, e também da substância branca do córtex cingular e entorrinal e do hipocampo (Delcour *et al.,* 2012). Este modelo, consequentemente, resulta em restrição de crescimento, prejuízos sensoriais, motor e cognitivo nos animais com PC (Delcour *et al.,* 2012; Ruff *et al.,* 2017).

Modelos de PC perinatais e pós-natais também são bastante conhecidos na literatura, como o modelo de hipóxico-isquemia (HI), que induz a PC geralmente no 3º ou 7º dias de vida pós-natal, através da oclusão unilateral da artéria carótida comum. E em seguida, os animais passam por uma hipóxia entre 30 e 120min, com exposição de baixo percentual de oxigênio, em geral de 6 a 8% (Alexander *et al.,* 2014; Sanches *et al.,* 2015, 2019). A HI é um dos modelos mais utilizados na literatura atualmente devido à extensão da lesão cerebral provocada, mas quanto às repercussões funcionais a literatura demonstra resultados divergentes (Da Conceição Pereira et al., 2021). Alguns estudos com este modelo mostram poucos

prejuízos na locomoção ou coordenação motora, já em outros estudos não são observadas alterações neurofuncionais ou até mesmo observa-se uma hiperatividade motora (Arteni *et al.,* 2010; Sanches *et al.,* 2015).

Modelos pós-natais combinados têm se destacado por sua efetividade, pois a combinação de insultos aumentou a capacidade de reproduzir o fenótipo motor da PC semelhante aos humanos (Da Conceição Pereira *et al.,* 2021). Dentre eles, está o modelo que realiza a privação de oxigênio, chamada anóxia ou asfixia, associada a restrição sensório-motora.

A anóxia perinatal foi um dos primeiros mecanismos a ser apresentado como modelo de PC em animais (Windle; Becker, 1943). É capaz de reproduzir os danos no sistema nervoso central como a lesão de substância branca periventricular e decréscimo da densidade neuronal, resultantes do evento isquêmico no cérebro imaturo e consequente estresse oxidativo (Blomgren; Hagberg, 2006; Coq et al., 2016). Mas este modelo, por si só, apresenta uma limitação considerável, por não ocasionar incapacidade crônica característica da PC (Johnston *et al.,* 2005), sendo observadas apenas alterações discretas no tônus muscular e nas habilidades motoras em ratos submetidos apenas à anóxia perinatal (Strata *et al.,* 2004).

A restrição sensório motora contribui para a manutenção dos danos motores, semelhante à PC em humanos a longo prazo, devido à imobilização dos membros posteriores dos animais semelhante à falta de movimento das crianças com PC (Buratti *et al.,*; Delcour *et al.,* 2018; Dos Santos *et al.,* 2017; Marcuzzo *et al.,* 2010). Isso ocorre possivelmente devido a redução das aferências sensoriais e leva à degradação da organização do córtex motor na representação de membros posteriores, além da atrofia muscular e alterações nos tipos de fibras musculares por desuso em músculos importantes para o desenvolvimento da locomoção, simulando assim uma paralisia cerebral diplégica, onde há predominância de comprometimento de membros inferiores (Stigger *et al.,* 2011; Strata *et al.,* 2004). Isso foi observado inclusive através de metanálise, onde os modelos que incluíram a restrição sensório-motora obtiveram nos animais pior desempenho na locomoção e coordenação motora (Johnston *et al.,* 2005).

Estudos utilizando este modelo de PC que associa anóxia e restrição sensório-motora dos membros posteriores mostram que os animais apresentam alterações estruturais e desenvolvimentais devido à lesão cerebral precoce e manutenção da incapacidade motora. Sendo assim, os estudos mostram alterações

no tônus muscular (Strata *et al.,* 2004), desorganização na representação cortical dos membros inferiores no córtex somatossensorial (S1) (Coq *et al.,* 2008), redução na atividade locomotora e coordenação motora (Da Conceição Pereira *et al.,* 2021; Silva *et al.,* 2016), prejuízos na mastigação (Lacerda *et al.,* 2017), além de habilidades motoras prejudicadas, semelhantes às ocorridas em crianças com PC (Marcuzzo *et al.,* 2010).

Por esta razão, este modelo torna-se uma ferramenta relevante para elucidação da patogênese da PC, incluindo como esta afeta o desenvolvimento principalmente no sistema neuromusculoesquelético, e evidencia a importância de estudos que se propõem a agregar novas perspectivas de tratamento para esta patologia.

2.3 RESVERATROL: NEUROPROTEÇÃO E ATUAÇÃO MUSCULOESQUELÉTICA

O uso de polifenóis têm sido evidenciados pois estão incluídos na categoria de alimentos funcionais importantes para a proteção e prevenção de doenças crônicas (Adefegha, 2017).

O resveratrol está entre os polifenóis que são utilizados em modelos de neurodegeneração (doença de Alzheimer, Parkinson ou Huntington) e modelos de hipóxia-isquemia como estratégia neuroprotetora (Arteaga et al., 2014, Juul; Ferriero, 2014., 2014; Shulin Pan, 2016). Os efeitos neuroprotetores do resveratrol resultam de sua atividade antioxidante que permite eliminar uma variedade de radicais livres e espécies reativas de oxigênio, por sua capacidade de induzir a expressão de várias enzimas antioxidantes (Arteaga *et al.,* 2014).

Estudos têm demonstrado efeitos benéficos do resveratrol na lesão cerebral hipóxico-isquêmica e hemorrágica em modelo animal (Arteaga *et al.,* 2014; CAI *et al.,* 2018). Este polifenol pode reduzir a intensidade do processo inflamatório e apoptose celular, através da inibição de citocinas pró inflamatórias TNFa e IL-6, sendo capaz de diminuir sua expressão e secreção, além de aumentar a secreção da citocina anti-inflamatória IL-10 (Palackz-Wrobel; SHulin Pan, 2016).

O resveratrol reduz significativamente a lesão cerebral, preservando áreas cerebrais neocorticais e subcorticais como o córtex sensório-motor e reduz o dano à substância branca (Arteaga *et al.,* 2014), e também foi demonstrado ser capaz de

reduzir a perda de tecido cerebral após lesão hipóxica-isquêmica em ratos neonatais (SHULIN PAN, 2016).

De forma semelhante, o resveratrol foi destacado em estudos anteriores por seus efeitos antioxidantes no cerebelo que podem estar diretamente relacionados ao controle muscular e motricidade. O resveratrol mostrou um efeito anti apoptótico em modelos apoptóticos em neurônios granulares cerebelares de ratos (CGNs) em modelo in vitro da doença de Parkinson (Alvira *et al.,* 2007), bem como um efeito neuroprotetor em neurônios granulares cerebelares em modelos de intoxicação por amônia (Bobermin *et al.,* 2015) e neurotoxicidade do etanol (Kumar *et al.,* 2011). Em modelos de doença neurológica, o pós-tratamento com resveratrol atuou na proteção das células de Purkinje com melhora no desempenho motor e atividade muscular de ratos atáxicos (Ghorbani et al., 2018).

Estes achados sugerem que o resveratrol é uma estratégia terapêutica promissora para a PC, já que os estudos demonstram o seu papel na proteção contra lesão do sistema nervoso central neonatal, resultante da PC experimental (Shulin Pan, 2016; Cai *et al.,* 2018), sendo, citado como um dos melhores candidatos farmacológicos a ser utilizado nas lesões ocorridas tanto na fase pré-natal como na pós natal (Juul; Ferriero, 2014.). Consequentemente, melhora os déficits comportamentais de curto e longo prazo induzidos por hipóxia-isquemia e melhora o desempenho motor (Arteaga *et al.,* 2014). , além de benefícios do modelo de PC na postura e comportamento (Calado *et al.,* 2023; Da Silva Souza *et al.,* 2023).

Além de sua atuação relevante no SNC, o resveratrol tem sido implicado como recurso terapêutico em modelos de atrofia e dano muscular. Estudos evidenciam efeitos protetores do tratamento com resveratrol atenuando a atrofia das fibras musculares em modelos animais de obesidade, de atrofia induzida por envelhecimento e por denervação (Asami *et al.,* 2018; Bai *et al.,* 2020; Huang *et al.,* 2021).

Em modelos experimentais de obesidade, o resveratrol parece atuar na prevenção da perda de massa muscular. Huang et al. (2020) demonstraram que o efeito protetor do resveratrol se deve a redução da disfunção mitocondrial e estresse oxidativo no sistema musculoesquelético e consequente melhora do metabolismo das proteínas mediado pela via PKA/LKB1/AMPK.

O efeito protetor do resveratrol no músculo também está relacionado à sua atuação contra a inflamação e redução da apoptose de células musculoesqueléticas não somente em camundongos com sarcopenia induzida por obesidade, mas também na sarcopenia relacionada à idade (Bai *et al.,* 2020). Adicionalmente, músculos submetidos à desnervação e ao tratamento preventivo com resveratrol também se beneficiaram. Foi observado que a suplementação de resveratrol na dieta durante uma semana preveniu a atrofia muscular por desnervação através da diminuição da atrogin-1 e da sinalização dependente de p62 (Asami *et al.,* 2018)

Quanto à atuação terapêutica do resveratrol em modelos de danos musculares, estudos também evidenciam seu potencial papel no reparo muscular. Na injúria muscular induzida por contusão o resveratrol pode contribuir na regeneração de células musculares satélites através do aumento da proteína desmina no músculo gastrocnêmio de camundongos (Hsu *et al.,* 2020). No retardo do crescimento intrauterino, os filhotes de porcos que foram submetidos ao tratamento com resveratrol aumentaram a atividade de enzimas antioxidantes e também do complexo V mitocondrial do músculo longuíssimo do dorso, reduzindo a disfunção mitocondrial e dano oxidativo muscular (Cheng *et al.,* 2020)

Entretanto, a literatura ainda é escassa quanto aos efeitos do resveratrol sobre o músculo esquelético e desenvolvimento neurofuncional em animais submetidos à PC experimental.

2.4 FATOR DE CRESCIMENTO DE FIBROBLASTOS 15/19: ATUAÇÃO MUSCULOESQUELÉTICA

Os Fatores de Crescimento de Fibroblastos (FGFs) constituem um grupo de 22 proteínas, sendo nomeadas sequencialmente. Existem 6 subfamílias definidas conforme a homologia de sequência e a filogenia da seguinte forma: 1) FGF1 e FGF2; 2) FGF3, FGF7,FGF10, FGF22; 3) FGF4, FGF5 e FGF6; 4) FGF8, FGF17 e FGF18; 5) FGF9, FGF16 e FGF20; 6) FGF19,FGF21 e FGF23 (BEENKEN, 2009). Como pode-se observar alguns FGFs não são incluídos nessas subfamílias apesar de serem numerados. Mesmo com a sua homologia aos FGFs anteriores, com identidade de sequências semelhantes, eles não ativam receptores de FGF (FGFRs), sendo eles FGF11 — FGF14. O FGF15 e o FGF19 são proteínas ortólogas

apresentando sequências homólogas e função similar, sendo o FGF15 encontrado em ratos e o FGF19 encontrado em humanos (Markan, Potthof, 2016).

As cinco primeiras famílias são consideradas fatores parácrinos com atuação intercelular participando da organogênese durante o período embrionário. A sexta subfamília são considerados fatores endócrinos atuando através de co-receptores em tecidos alvo como, por exemplo, o FGF19 que atua através das proteínas b-klotho regulando a homeostase metabólica (Beenken, 2009; Potthof, 2012). A natureza endócrina do FGF19, FGF21 e FGF23 é determinada pela interação do seu sítio de ligação com heparan sulfato glicosaminoglicano, em que quando liberados na corrente sanguínea irão atuar em todo corpo (Beenken, 2009; Potthof, 2012; Fernandes-Freitas, 2015). Como a interação com o heparan sulfato é restrita, os FGFs endócrinos requerem membros da família de proteínas Klotho que os ligam aos seus FGFRs cognatos para aumentar a afinidade ligante receptor no tecido alvo (Potthoff; Kliewer; Mangelsdorf, 2012).

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

O FGF15/19 destaca-se por seu papel em diversos tecidos atuando no desenvolvimento do sistema nervoso central (SNC), na homeostase metabólica ou no crescimento do músculo lesado. No SNC, o FGF15/19 é expresso apenas durante o período embrionário e fetal, desempenhando ações de supressão da proliferação e promovendo a diferenciação de precursores neurais (Potthoff; Kliewer; Mangelsdorf, 2012; Zhang *et al.,* 2015). Ademais, em cultura de células humanas também foi demonstrado que o FGF19 é expresso dentro da placa de crescimento fetal, contribuindo assim para o desenvolvimento da cartilagem junto com outros FGFs que regulam a proliferação dos condrócitos (Krejci et al., 2007).

No adulto e em condições fisiológicas o FGF15/19 não atravessa a barreira hematoencefálica o que restringe sua ação aos órgãos periféricos (Fernandes-Freitas; Owen, 2015; Hsuchou; Pan; Kastin, 2013). Ele é expresso no íleo terminal, de onde irá atuar conforme a disponibilidade de βKlotho no tecido alvo (Tomiyama et al., 2010). Tem um papel essencial na regulação da homeostase metabólica em que através da ativação do FGFR4 gera uma transdução de sinal subsequente e regula a síntese de ácido biliar (Fernandes-Freitas; Owen, 2015; Tomiyama *et al.,* 2010).

Uma função primária do FGF15/19 no adulto é regular a homeostase dos ácidos biliares, sendo um regulador negativo de síntese e transporte do ácido biliar (Potthoff; Kliewer; Mangelsdorf, 2012; Zhang et al., 2015). O FGF15/19 entra na circulação porta e atua no fígado através do receptor FGF-4 (FGFR4) em complexo com a proteína transmembrana essencial βKlotho (Fernandes-Freitas; Owen, 2015). Além dos efeitos sobre a homeostase dos ácidos biliares, há efeitos metabólicos pós-prandiais mais amplos no fígado (Fernandes-Freitas; Owen, 2015). Semelhante à insulina, estimula a síntese de proteína e de glicogênio e inibe a gliconeogênese (Fernandes-Freitas; Owen, 2015). No entanto, existem diferenças importantes. Os níveis de pico pós-prandiais de FGF15/19 ocorrem no sangue substancialmente mais tarde, e duram mais tempo, que os da insulina (Fernandes-Freitas; Owen, 2015) e não estimula a lipogênese (Potthoff; Kliewer; Mangelsdorf, 2012).

O sistema musculoesquelético também foi evidenciado como um alvo direto do FGF15/19, devido à presença de FGFR e Klothoβ em vários músculos de camundongos como o sóleo e o tibial anterior, além de miofibras isoladas e em cultura primária de miotubos humanos (Benoit *et al.,* 2017). Foi demonstrado que o tratamento com FGF15/19 promoveu efeito hipertrófico em modelos animais de atrofia muscular induzida por dexametasona, atrofia relacionada ao envelhecimento e em modelo de obesidade (Benoit *et al.,* 2017). Dentre os mecanismos deste resultado de recuperação ao dano muscular de origem metabólica, pode estar o aumento da área dos miotubos durante o processo de diferenciação dos mioblastos em miotubos como observado em células musculares humanas primárias (Benoit et al., 2017).

O FGF15/19 tem sido implicado também na atenuação dos danos musculares em doença neurológica, como a paralisia cerebral, onde a principal característica é a atrofia por desuso decorrente de uma lesão no SNC em desenvolvimento (Da

Conceição Pereira *et al.,* 2021). O tratamento com FGF19 foi capaz de atuar inclusive influenciando a expressão de genes relacionados ao desenvolvimento muscular e junções neuromusculares, levando ao aumento dos níveis de mRNA de *Igfbp5*, *Kcnn3*, *Gdf8*, e *MyH4* e decresceu a expressão de *Myog*, *Ucp2* e *Lpl*) (Da Conceição Pereira *et al.,* 2021). Além de que reduziu os danos sobre o desenvolvimento somático, favoreceu a locomoção e a força muscular (Da Conceição Pereira *et al.,* 2021). Demonstrando assim que o tratamento com FGF19 também tem implicações sobre as funções musculoesqueléticas na PC experimental.

2.5 PLASTICIDADE FENOTÍPICA E SISTEMA NEURO-MUSCULOESQUELÉTICO

Os organismos vivos possuem uma notável capacidade de responder a insultos ambientais com mudanças na forma e função. Ajustes fenotípicos podem ocorrer em diferentes períodos, duráveis e às vezes mudanças irreversíveis (Giudice, 2015). Dependendo de como a palavra “fenótipo” é definida (por exemplo, evento desenvolvimental, ajuste fisiológico, mudança de comportamento, expressão gênica dependente do ambiente, etc.) todos processos biológicos são, de alguma forma, influenciados pelo ambiente e, consequentemente, qualquer modificação resultante pode ser categorizada como plasticidade fenotípica (Kelly; Panhuis; Stoehr, 2012).

Dessa forma, a plasticidade fenotípica pode ser amplamente definida como a capacidade de um genótipo de produzir diferentes fenótipos em resposta às condições ambientais levando à alterações na forma, estado, movimento ou taxa de atividade (Kelly; Panhuis; Stoehr, 2012; Turcotte; Levine, 2016; West-Eberhard, 1986). Pode ser reversível, chamada de flexibilidade fenotípica, ou irreversível, como a plasticidade desenvolvimental (Turcotte; Levine, 2016). Dependendo dos fatores que induzem a plasticidade fenotípica e do tempo de indução, pode afetar múltiplas gerações através de efeitos maternos ou de fatores epigenéticos (Turcotte; Levine, 2016).

Vários fatores bióticos e abióticos podem induzir a plasticidade fenotípica como fatores ambientais locais, alterações químicas, sociais e hormonais (Turcotte;

Levine, 2016). Sendo a plasticidade uma variação intraespecífica (West-Eberhard, 1998), em que alguns indivíduos são mais suscetíveis a esses fatores. Isso pode ser atribuído às diferenças nos mecanismos precoces envolvidos na coleta de informações do meio ambiente e traduzindo-o em efeitos fenotípicos, que são mediadores da plasticidade no nível precoce (Giudice, 2015). Estudos mostram que o período perinatal corresponde ao período de maior plasticidade e representa a janela crítica de desenvolvimento (Hübener; Bonhoeffer, 2014; Jensen, 2002) em que insultos precoces podem levar à repercussões permanentes nos sistemas fisiológicos (Barker, 1995; Toscano *et al.,* 2010).

Neste contexto, destaca-se o conceito das Origens do Desenvolvimento da Saúde e da Doença (*Developmental Origins of Health and Disease* - DOHaD) que se refere a ideia de que fatores ambientais no início da vida fetal são capazes de influenciar a expressão dos genes, com efeitos na saúde e na doença posteriores (Suzuki, 2018). Considera-se que o DOHaD iniciou através dos estudos de Barker e Osmond, 1986 que hipotetizou que a má nutrição no início da vida aumenta a susceptibilidade aos efeitos de uma dieta abundante, resultando num aumento da mortalidade por doenças coronárias na vida adulta (Barker, 2007; Barker; Osmond, 1986; Suzuki, 2018).

À medida que os estudos foram se desenvolvendo a hipótese 'DOHaD' estendeu-se desde o período fetal original de Barker até o período pós-natal, desde a infância até adolescência (Suzuki, 2018). Além disso, os fatores causais de desfechos DOHaD evoluíram para incluir vários fatores ambientais externos e internos que afetam o organismo, como o ambiente físico, químico e biológico, além do estresse mental e físico que podem modular diversa vias neuroendócrinas em todas as fases do desenvolvimento (Suzuki, 2018).

Sabe-se que o sistema nervoso central é um tecido altamente plástico capaz de se adaptar ao meio ambiente, é o que chamamos de neuroplasticidade (Kourosh-Arami; Hosseini; Komaki, 2021). O cérebro é modulado constantemente pela plasticidade neuronal, principalmente, durante um período crítico do desenvolvimento, o período pós-natal precoce (Kourosh-Arami; Hosseini; Komaki, 2021).

Ao longo da vida, existem mecanismos homeostáticos que estabilizam a atividade dos neurônios em desenvolvimento (Tien; Kerschensteiner, 2018). Então, o

cérebro pode mostrar plasticidade em resposta a demandas fisiológicas, mudanças na atividade neural ou danos no tecido nervoso (Bernhardi; Bernhardi; Eugenín, 2017). Conforme o aumento ou diminuição da atividade neuronal ocorre a regulação homeostática da força pré e pós sináptica e/ou da excitabilidade intrínseca a fim de restaurar a atividade normal (Tien; Kerschensteiner, 2018).

Isso ocorre por uma variedade de processos biológicos relacionados à plasticidade neural incluindo a neurogênese, migração celular, mudanças na excitabilidade neuronal e neurotransmissão, a geração de novas conexões e modificação das já existentes (Bernhardi; Bernhardi; Eugenín, 2017). Remodelação e refinamento de conexões usam formação e eliminação de sinapses, expansão e retração de arborização dendrítica e brotação e poda axonal (Bernhardi; Bernhardi; Eugenín, 2017).

Além da plasticidade está envolvida na formação da rede neuronal durante o desenvolvimento, ela também está relacionada à aquisição de novos comportamentos motores ou aprendizagem ao longo da vida (Bernhardi; Bernhardi; Eugenín, 2017). Estudos evidenciam que diversas intervenções em patologias neurológicas modulam a neuroplasticidade, como as intervenções nutricionais ou farmacológicas (Sasmita; Kuruvilla; Ling, 2018; Zielińska-Nowak *et al.,* 2021).

Existem diversos reguladores neurogênicos que podem ser caracterizados por moléculas individuais, como os produtos naturais ou fatores de crescimento, e também por condições ambientais (Sasmita; Kuruvilla; Ling, 2018). Esses fatores contribuem para a neuroplasticidade, principalmente sobre a regulação da neurogênese, que é uma prioridade fundamental nas intervenções terapêuticas que se propõe a atuar com a plasticidade do sistema nervoso central (Sasmita; Kuruvilla; Ling, 2018), mas abre perspectivas adicionais a outros sistemas do organismo.

Neste contexto, o sistema musculoesquelético também exibe um elevado potencial adaptativo (Fluck; Hoppeler, 2003; Pette, 2002). Esse potencial é resultante da habilidade das fibras musculares em ajustar suas propriedades metabólicas, moleculares e funcionais a fim de acomodar demandas específicas (Bassel-Duby; Olson, 2006; Hoppeler; Flück, 2002; Pette, 2002). Assim, o fenótipo definitivo de uma fibra muscular esquelética adulta é o resultado de eventos que começam no embrião e são continuamente modulados e refinados ao longo da vida do indivíduo (Stockdale, 1992).

Diversos estímulos influenciam o fenótipo musculoesquelético como a atividade contrátil (exercícios de endurance, eletroestimulação, denervação), condições de carga (treinamento de resistência, microgravidade), suprimento de substrato (intervenções nutricionais) ou fatores ambientais (hipóxia) (Fluck; Hoppeler, 2003; Pette, 2002). Ademais, estudos experimentais são capazes de demonstrar em diferentes modelos animais, as alterações resultantes no fenótipo muscular, incluindo alterações nas propriedades biomecânicas do músculo (Toscano et al., 2010) e na proporção dos tipos de fibras (Lacerda; Morais, 2017).

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

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

Dessa forma, a plasticidade fenotípica é um fenômeno comum (Turcotte; Levine, 2016) em que no sistema musculoesquelético diversos fatores podem favorecer, desde insultos ambientais ocorridos precocemente durante o desenvolvimento (Lacerda *et al.,* 2017; Silva *et al.,* 2016) até eventos tardios, como

o exercício, fatores endócrinos e patologias (Baskin *et al.,* 2016; Ward *et al.,* 2006). E estudos experimentais são fundamentais para a compreensão dos mecanismos associados à plasticidade do sistema musculoesquelético, principalmente com intervenções nas patologias que acometem esse sistema.

3 HIPÓTESE

O tratamento com FGF19 reverte a atrofia musculoesquelética e o resveratrol reverte os danos musculares e cerebelares decorrentes da PC experimental, em que ambas intervenções têm consequências benéficas para o neurodesenvolvimento e a locomoção dos ratos.

4 OBJETIVOS

4.1 OBJETIVO GERAL

Investigar os efeitos do tratamento com resveratrol e/ou com o fator de crescimento de fibroblastos 19 sobre os aspectos morfológicos, comportamentais e moleculares do sistema neuromusculoesquelético na paralisia cerebral experimental.

4.2 OBJETIVOS ESPECÍFICOS

Avaliar, em ratos submetidos à paralisia cerebral experimental expostos ao tratamento com o fator de crescimento de fibroblastos 19 e/ou com resveratrol:

- Evolução ponderal;
- Ontogênese dos reflexos;
- Reflexos primitivos;
- Desenvolvimento da atividade locomotora;
- Força muscular;
- Coordenação motora;
- Peso dos músculos sóleo e extensor longo dos dedos e do osso tíbia;
- Área e perímetro das fibras musculares;
- Distribuição dos tipos de fibras musculares;
- Expressão gênica de marcadores relacionadas ao desenvolvimento muscular e junção neuromuscular;
- Imuno-histoquímica para Pax7 no musculoesquelético;
- Análise da neurogênese cerebelar;
- Densidade de micróglia e percentual de micróglia ativada no cerebelo.

5 MATERIAIS E MÉTODOS

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

5.1 ANIMAIS E CONDIÇÕES DE BIOTÉRIO

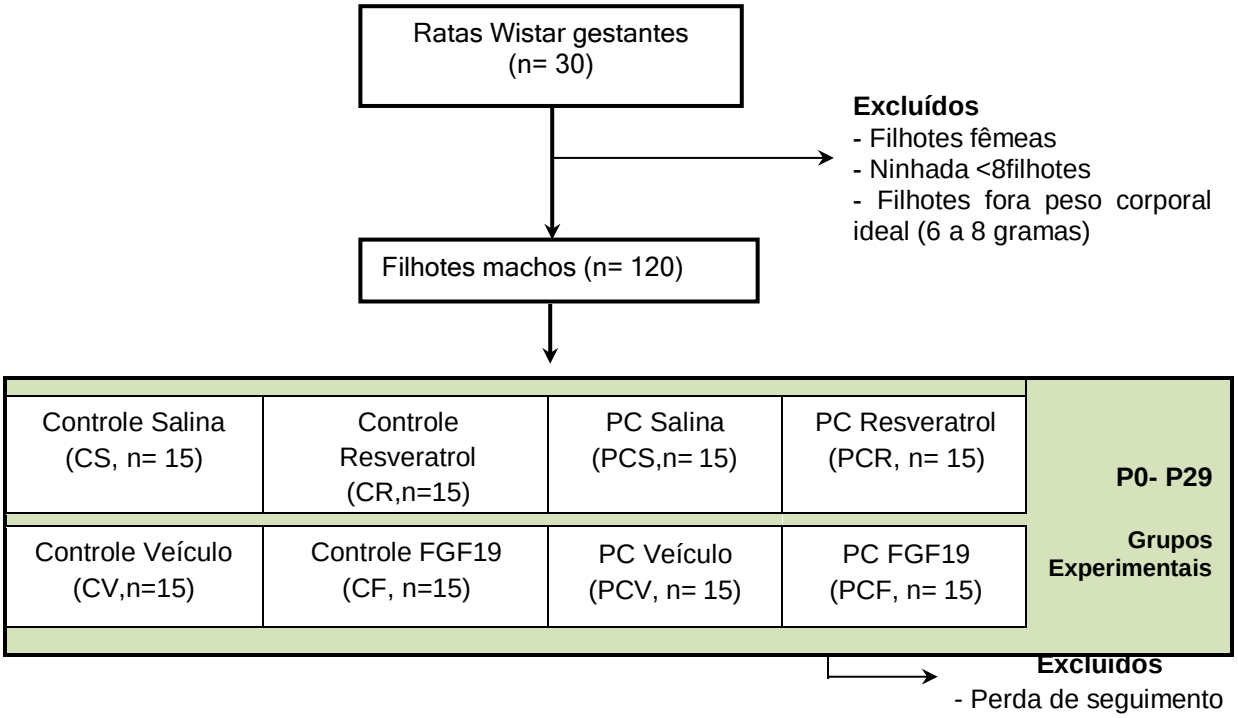
O presente estudo é experimental com animais em que foram utilizados filhotes ratos machos *Rattus Norvegicus Albinus* da linhagem *Wistar* provenientes da colônia do Departamento de Nutrição da UFPE. Os animais foram mantidos em biotério com temperatura de 22 ± 2 ° C, ciclo claro-escuro invertido de 12/12 horas (Ciclo claro - 20:00 as 8:00h; Ciclo escuro - 08:00 às 20:00 h), alojados em gaiolas de polipropileno (46cmx34cmx20cm) forrada com maravalha estéril com acesso livre a água e dieta padrão de biotério. Todos os procedimentos foram realizados em conformidade com as diretrizes do Conselho Nacional de Controle de Experimentação Animal (CONCEA) e com as normas internacionais do *National Institute of Health Guide for Care and Use of Laboratory Animals* (8ª ed). O projeto foi submetido à Comissão de Ética em Uso Animal (CEUA) da UFPE e aprovado nº0009/20 (Anexo A).

5.2 GRUPOS EXPERIMENTAIS

Os filhotes foram obtidos através de 30 ratas nulíparas da espécie *Rattus Norvegicus Albinus* da linhagem *Wistar* provenientes do biotério de criação do Departamento de Nutrição da UFPE que foram acasaladas com machos reprodutores na proporção de duas fêmeas para um macho, não consanguíneos, com idade entre 90 e 120 dias e peso entre 220 e 260g. Após a confirmação da gravidez através de esfregaço vaginal quando observado a presença de espermatozóide na secreção vaginal, as gestantes foram distribuídas em gaiolas individuais e acompanhadas durante todo período gestacional. No dia do nascimento foi realizada uma randomização dos filhotes machos, com um peso corporal ideal (6 a 8 gramas), distribuídos nos grupos experimentais, com base na indução da

paralisia cerebral e na manipulação farmacológica administrada: 1- Controle salina (CS, n=15), constituído por filhotes que receberam solução salina do 3º ao 21º dia; 2- Controle resveratrol (CR, n=15), constituído por filhotes que receberam resveratrol do 3º ao 21º dia; 3- PC Salina (PCS, n=15), constituído por filhotes submetidos à modelo experimental de PC e que receberam solução salina do 3º ao 21º dia; 4- PC resveratrol (PCR, n=15), constituído por filhotes submetidos à modelo experimental de PC e que receberam resveratrol do 3º ao 21º dia; 5- Controle FGF-19 (CF, n=15), constituído por filhotes que receberam solução de FGF-19 do 22º ao 28º dia de vida; 6- Controle veículo (CV, n=15), constituído por filhotes que receberam solução veículo PBS/BSA do 22º ao 28º dia de vida; 7- PC FGF-19 (PCF, n=15), constituído por filhotes submetidos à modelo experimental de PC e que receberam solução FGF-19 do 22º ao 28º dia de vida; 8- PC veículo (PCV, n=15), constituído por filhotes submetidos à modelo experimental de PC e que receberam solução veículo PBS/BSA do 22º ao 28º dia de vida. Cada ninhada foi composta por 8 filhotes, sendo 4 filhotes PCs e 4 filhotes controles, que permanecerm com as mães até o 25^{0} dia pós-natal, quando eles foram desmamados e os machos colocados em gaiolas individuais até a eutanásia por decaptação no 29 dia pós-natal (Figura 1). As filhotes fêmeas foram excluídas da análise mas completaram a ninhada de 8 filhotes até o desmame, caso não houvesse filhotes machos suficientes nascidos na ninhada. Os filhotes machos foram excluídos da análise caso houvesse perda de seguimento, ou seja, caso algum filhotes fosse à óbito durante o período de experimento.

Figura 1 - Representação esquemática da formação dos grupos experimentais de acordo com a indução da paralisia cerebral e manipulação farmacológica, sendo o Resveratrol ou a Salina administrados do 3º ao 21º dia de vida pós natal (P3- P21), ou o FGF-19 ou Veículo administrados do 22º ao 28º dia de vida pós natal (P22-P28).



Fonte: A autora (2024).

5.3 MODELO EXPERIMENTAL DE PARALISIA CEREBRAL

O modelo experimental de PC que foi adotado é baseado nos experimentos que associam a anóxia perinatal e restrição sensório-motora das patas posteriores (Coq *et al.,* 2008; Lacerda *et al.,* 2017b; Silva *et al.,* 2016). Os filhotes machos dos grupos PC foram submetidos a dois episódios de anóxia pós-natal, no dia do nascimento e no primeiro dia de vida (P0 e P1). Os filhotes foram colocados dentro de uma câmara de acrílico parcialmente imersa em água a 37 graus e expostos a nitrogênio (100%) a 9L/min durante 12 minutos. Em seguida, foram devolvidos às suas respectivas mães. Do 2º ao 28º dia de vida (P2 ao P28) foi feita a restrição sensório-motora das patas posteriores durante 16 horas por dia, sendo permitida nas 8 horas restantes, a livre movimentação do animal. Para a restrição sensório-motora foi fixada uma órtese em molde de epóxi, mantendo as patas posteriores

estendidas, sem que a eliminação de urina e fezes e os cuidados maternos fossem prejudicados (Coq *et al.,* 2008) (Figura 2).

Figura 2: Animais submetidos ao modelo de PC. A) Animais em câmara de Anóxia; B) Cuidados maternos com os animais restrição sensório-motora; C) Animal pós desmame em restrição sensório-motora.

Fonte: A autora (2024).

5.4 TRATAMENTO FARMACOLÓGICO COM FGF-19

A manipulação farmacológica foi realizada no período pós natal em que os prejuízos musculoesqueléticos estão instalados. Nos animais foram administrados, diariamente, entre o 22º e 28º dia de vida, por via subcutânea no dorso do animal a solução com FGF19 ou veículo. A administração foi realizada às 8h da manhã após a transição do ciclo claro para o escuro, pois este momento coincide com os níveis de pico pós-prandiais de FGF15/19 nos animais (Fernandes- Freitas; Owen, 2015). De acordo com os grupos experimentais, os animais receberam solução Veículo, que corresponde a uma solução tampão fosfato-salino com albumina de soro bovino (PBS/BSA a 0,1%), ou receberam a solução FGF19 humano recombinante a uma concentração de 0,1 mg/kg na solução Veículo (FGF19 + PBS/BSA a 0,1%) (Benoit *et al.,* 2017).

5.5 TRATAMENTO FARMACOLÓGICO COM RESVERATROL

Durante o período neonatal, do 3º ao 21º dia de vida pós-natal, os filhotes foram administrados com salina ou resveratrol por via intraperitoneal (Girbovan; Plamondon, 2015) a fim de observar seus efeitos sobre os prejuízos do

neurodesenvolvimento e no sistema neuromusculoesquelético decorrentes do modelo de PC. De acordo com os grupos experimentais, os filhotes foram distribuídos em tratados com resveratrol (dose diária, 10mg/kg) ou tratados com salina (0,9% NaCl), o volume de injeção foi de 0,1 ml/100 g peso do rato). Os ratos foram pesados diariamente e o volume de injeção foi ajustado para corresponder ao peso corporal do animal.

5.6 COLETA DOS DADOS

5.6.1 Evolução ponderal

Para monitorar o crescimento dos animais, o peso corporal de todos os filhotes foi verificado a partir do dia do nascimento até o último dia de vida, ou seja até o 29^{o} dia de vida pós-natal. A pesagem dos animais, em gramas, foi obtida através de uma balança digital eletrônica (Marte, S-1000 modelo, a capacidade de 1 kg e 0,1 g de sensibilidade).

5.6.2 Avaliação da ontogênese de reflexos

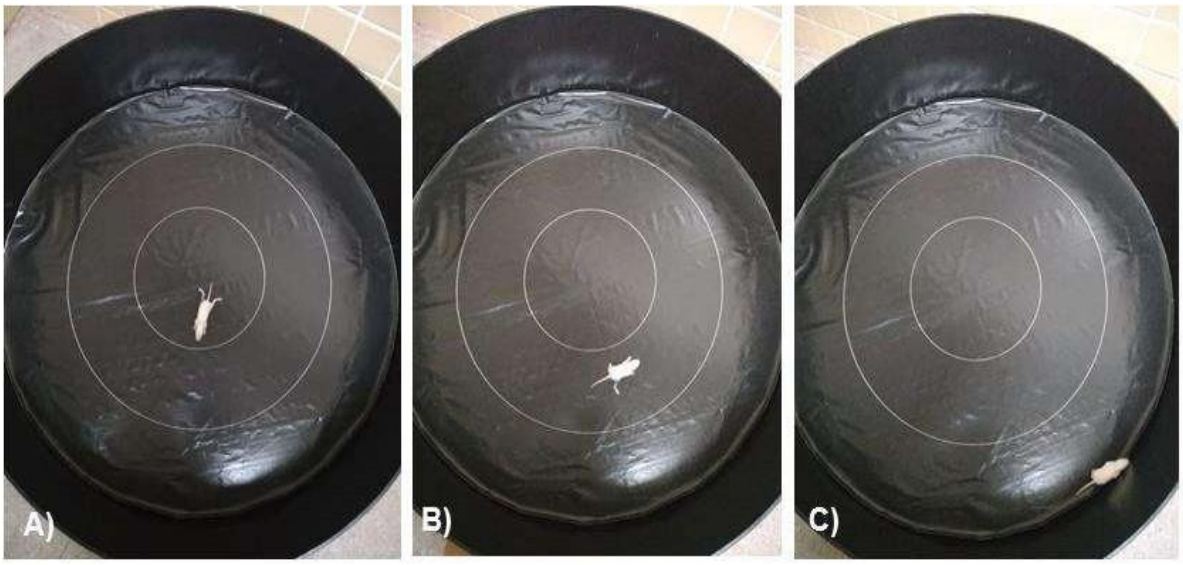
Diariamente, do 1° ao 21° dia pós-natal, os animais PC ou controle submetidos ao tratamento neonatal com resveratrol ou salina foi avaliado o aparecimento e desaparecimento de reflexos primitivos relacionados à maturação motora (Falcão-Tebas *et al.,* 2012; Fox, 1965). O primeiro de uma série de três dias consecutivos em que a resposta esperada ocorreu completamente foi considerado o dia da maturação do reflexo, sendo avaliado o desaparecimento do reflexo preensão palmar-PP (flexão rápida dos dedos após duas leves percussões na palma da pata dianteira) e o aparecimento dos reflexos de recuperação de decúbito-RD (giro do corpo em decúbito dorsal para decúbito ventral em até 10 segundos); reflexo de colocação pelas vibrissas- CV (reflexo de colocar as patas anteriores sobre a mesa, tentando caminhar quando suspenso pela cauda em até 10 segundos); reflexo de aversão ao precipício-AP (deslocamento angular de 45° do animal em até 10 segundos, quando o animal é colocado com as patas dianteiras sobre a margem de uma superfície plana e alta considerada um precipício); reflexo de resposta ao susto-RS (simultânea e rápida retração com a imobilização involuntária do corpo do animal

após estampido agudo); reflexo de queda livre-QL (o giro do animal em queda livre de 30 cm de altura, quando segurado pelas quatro patas em decúbito dorsal, apoiando-se sobre as quatro patas em um leito de algodão); e reflexo de geotaxia negativa-GN (centro de uma rampa de 45º de inclinação, o animal com a cabeça no sentido descendente gira em até 10 segundos e posiciona a cabeça em sentido ascendente) (Falcão-Tebas *et al.,* 2012; Fox, 1965).

5.6.3 Avaliação do desenvolvimento da Locomoção

O desenvolvimento locomotor de todos os animais (n=10 por grupo) foi avaliado nas idades de 8, 14, 17, 21 e 28 dias pós natal em campo aberto. Para a atividade locomotora em campo aberto foi utilizado o software *Anymaze* (San Diego Instruments) em que os animais foram colocados no centro do campo e gravados em vídeo durante um período de 5 minutos cada. O teste ocorreu em sala escura anexa ao biotério de manutenção, durante o ciclo escuro, em que o animal está naturalmente no estado de vigília após três horas de aplicação do fármaco devido ao estresse provocado. No intervalo entre as filmagens o campo foi limpo com hipoclorito 3% e o EVA trocado para que o odor do animal anterior não influencie no teste do animal seguinte. Os parâmetros obtidos foram: Distância percorrida (m); Velocidade média (m/s); Tempo que o animal permaneceu parado(s); Número total de paradas feitas pelo animal(n); Proporção entre o tempo parado/número de paradas(s); Tempo de permanência nas áreas central (zona 1), intermediária (zona 2) e periférica (zona 3) do campo aberto (Figura 3).

Figura 3 - Análise da atividade locomotora em campo aberto. Representação da atividade exploratória espontânea nas áreas central (A), intermediária (B) e periféricado campo aberto.

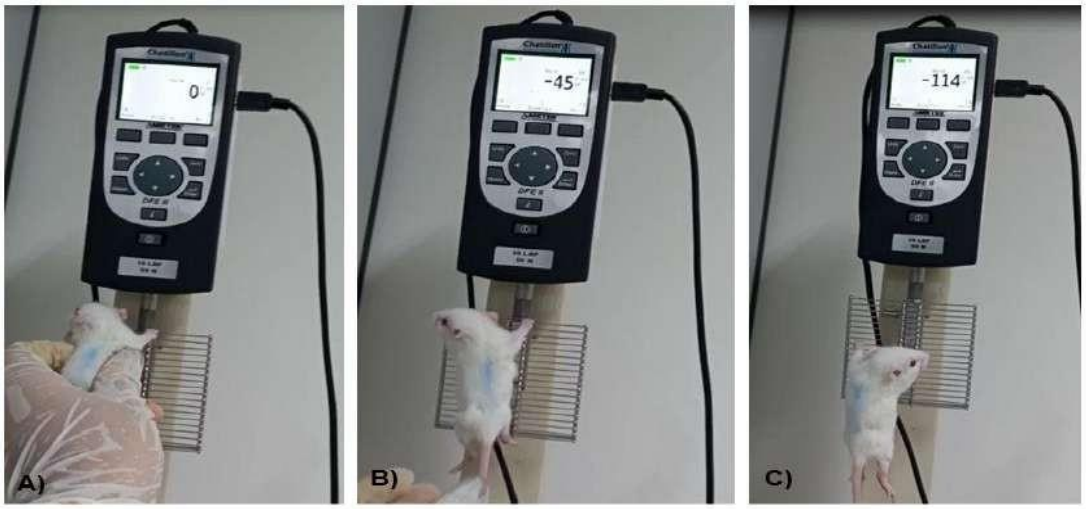


Fonte: A autora (2024).

5.6.4 Avaliação da força muscular

Nas idades de 22 e 28 dias de vida pós-natal foi realizada a análise da força muscular em todos os animais através do teste de força da preensão dos membros anteriores modificado (Taekshita *et al.,* 2017). Cada animal deve agarrar-se a barra de apoio do equipamento exercendo uma força tração sobre ele em posição vertical enquanto o examinador o suspende pela cauda. Este teste foi realizado no equipamento *Animal GripStrength System* (*SD Instruments*) com capacidade de 200Kgf, resolução 0,1Kgf e acurácia de ± 0,2% (n=10 por grupo) (Figura 4).

Figura 4 – Análise da força muscular através do teste de força da preensão dosmembros anteriores modificado. Sequência de realização do teste em A, B e C.

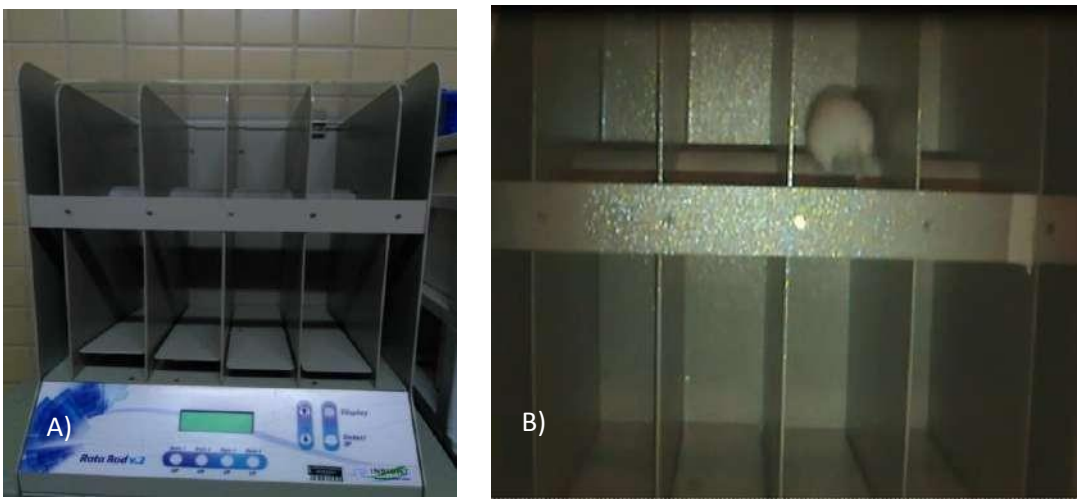


Fonte: A autora (2024).

5.6.5 Avaliação da Coordenação Motora

Para avaliar a coordenação motora dos grupos experimentais, foi realizado o teste de performance no equipamento rotarod (Insight) aos 29 dias de vida pós-natal (n=10 por grupo). Um animal por vez foi colocado no equipamento rotarod sobre uma haste de 60 mm de diâmetro e 75 mm de comprimento em rotação. Inicialmente, os animais passaram por um período de adaptação no equipamento por 2 minutos a uma velocidade de 16 rpm. Após aguardar o período de descanso de 2 minutos, os animais foram colocados individualmente no rotarod por 5 tentativas, respeitando o intervalo de 2 minutos para descanso, com uma velocidade de 25 rpm por no máximo de 3 minutos para que a latência de queda foi registrada (adaptado de Strigger *et al.,* 2011). A análise da latência de quedas foi sintetizada como média das 5 tentativas de acordo com os grupos experimentais (Figura 5).

Figura 5 - Avaliação da coordenação motora através do teste de performance em rotarod. A: Equipamento rotarod; B: Execução do teste em rotarod.



Fonte: A autora (2024).

5.6.6 Eutanásia, coleta e aferição do peso dos músculos sóleo e extensor longo dos dedos

Aos 29 dias de vida pós-natal, metade dos animais foram eutanasiados por decapitação (n=7 por grupo experimental). Em seguida, os músculos sóleo e extensor longo dos dedos (EDL) da pata direita foram dissecados, pesados, congelados em n-hexano (dióxido de carbono pré-arrefecida solidifica -78,5 °C) e armazenados a -80 °C para posterior procedimento histoquímico e análise histomorfométrica em corte transversal. Para pesar os músculos foi utilizada uma balança de precisão (modelo Marte AUW220, capacidade de 220g e 0,1 mg de sensibilidade) e foi obtido o peso absoluto de ambos os músculos e seu peso relativo, normalizando o peso de cada músculo através do peso corporal. Os músculos sóleo e EDL da pata esquerda foram dissecados e imediatamente congelados em dióxido de carbono solidificado -78,5 °C e armazenados a -80 °C para posterior análise molecular.

5.6.7 Análise morfológica dos músculos sóleo e extensor longo dos dedos

Foram realizadas secções transversais (10 µm) em criostato a -30°C, dos músculos sóleo e extensor longo dos dedos, coletados da pata direita no 29º dia de

vida pós eutanásia por decapitação. As secções foram fixadas em lâminas e coradas pela técnica de ATPase miofibrilar, depois de atingir a temperatura ambiente (Brooke; Kaiser, 1970; Lacerda *et al.,* 2017b). As fibras musculares foram coradas em relação aos três tipos de fibras principais (I, IIa, IIb), de acordo com as diferenças na intensidade da coloração ATPase após pré-incubação de ácido (pH 4.3 e 4.55). Na pré-incubação com pH 4.3 as fibras foram coradas em tipo I (mais escura) e tipo II (mais clara) e no pH 4,55 as fibras foram coradas em tipo I (mais escura), tipo IIa (pálida) e tipo IIb (cinza). Os cortes foram analisados com um microscópio óptico (Olympus BX-41, 100x) conectado a um computador com software de captura de imagem *Analysis Get It*. Todas as fibras musculares foram contadas em cada secção histológica, sendo os valores apresentados para os diferentes tipos de fibras como uma percentagem do número total. Também foi feita a análise da área e perímetro de cada fibra muscular, definindo-se a área como a medida da superfície de cada fibra e o perímetro como a soma das medidas de todos os lados de cada fibra. Para contagem e análise da área e perímetro das fibras foi utilizado o software *ImageJ* (versão 1.51p).

5.6.8 Análise de expressão de genes relacionadas ao desenvolvimento muscular e junção neuromuscular

Os músculos sóleo e EDL da pata esquerda coletados pós eutanásia por decapitação seguiram para análise de expressão de genes relacionadas ao desenvolvimento muscular e junção neuromuscular. O RNA total de ambos músculos foi extraído usando TRI Reagent (Sigma Aldrich, Saint-Louis, MO, EUA). As preparações de RNA foram quantificadas usando Nanodrop 2000 (Ozyme) e sua qualidade foi verificada usando *Agilent bioanalyser* 2100. Os cDNAs de primeira fita foram sintetizados a partir de 1 µg de RNA total usando o kit Prime Script RT Reagent (Perfect Real Time) 200X (Ozyme) e uma combinação de oligodT e primers aleatórios. Os níveis de transcrição foram medidos por PCR em tempo real (Rotor-Gene 6000, Qiagen, Courtaboeuf, França) em um volume final de 20 µL usando o kit SYBR qPCR Premix Ex Taq (Ozyme). Cada ensaio foi realizado em duplicata e a validação das corridas de RT-PCR foi avaliada pela avaliação da temperatura de fusão dos produtos e pela inclinação e erro obtidos com a curva padrão. As análises foram realizadas no software Rotorgene (Qiagen) conforme sequência pré-

estabelecida (Tabela 1). Os resultados foram normalizados para a expressão de Tbp (proteína de ligação a TATA), utilizada como padrão interno.

Tabela 1: Sequência de primers utilizados na análise de RT-qPCR.

Gene	**Ortólogo Humano**	**Nome**	**Sens primer (5'-3')**	**Anti-sens primer (5'-3')**
Tbp	TBP	*TATA box binding protein*	TGGTGTGCACAGGAGCCAAG	TTCACATCACAGCTCCCCAC
Myostatin	MSTN	*myostatin*	TGCTGTAACCTTCCCAGGACC	GTGTTCATCACAGTCAAGCCC
Igfbp5	IGFBP5	*insulin-like growth factor binding protein 5*	TACGGCGAGCAAACCAAGAT	GGGTCAGCTTCTTTCTGCGA
Igf1	IGF1	*insulin-like growth factor 1*	GCTCTTCAGTTGGTGTGTGG	GCAACACTCATCCACAATGC
Myod	MYOD1	*myogenic differentiation 1*	TCCAGCCCGCGCTCCAACTGC	TCGACACGGCCGCACTCTTCC
Myogenin	MYOG	*myogenin*	CAATGCACTGGAGTTTGGTC	CATATCCTCCACCGTGATGC
Myf5	MYF5	*myogenic factor 5*	CGTCCCCAATGAGATTAGCA	ACAAGGAAAAGGGGGCTTCA
Lpl	LPL	*lipoprotein lipase*	ATTTGCCCTAAGGACCCCTG	GGGCACCCAACTCTCATACA
Ucp2	UCP2	*uncoupling protein*	CCTGAAAGCCAACCTCATGAC	AGGATCCCAAGCGGAGAAAGG
Ckmt2	CKMT2	*creatine kinase, mitochondrial 2*	CTCATCGATGACCACTTTCTG	ATTCCACATGAACTCCCAGC
Murf1	TRIM63	*tripartite motif-containing 63*	TGCATCTCCATGCTGGTGGC	CTTCTTCTCGTCCAGGATGG
Kcnn3	KCNN3	*potassium calcium-activated channel*	CCTTCCCCAAAGCCAACAAG	GGGCCAACGAAAACATGGAA

		subfamily N member 3		
Nestin	NES	*nestin*	GGAGCAGGAG AAGCAAGGTC	GAGTTCTCAG CCTCCAGCAG
Musk	MUSK	*muscle associated receptor tyrosine kinase*	CACAAAGCCAG GACTCTACAC	GTCTTTCACCA GGACGGCAT
Dmd	DMD	*dystrophin*	TCCCCTGACTG CCTGTGAAA	AGGCTCAAGA GATCCAAGCA
Troponin I1	TNNI1	*troponin I1*	TGAGGAGCGC TATGATATCG	TTCACAGACTT GAGGTTGGC
Nebulin	NEB	*nebulin*	TGCAACCAAAA TAGCGAGCG	TCCAGCTCTAG GTCTCGCTT
Myh4	MYH4	*myosin heavy chain 4*	TGACTCAGCCT GCCTCCTTC	TGTTCTGAGCC TCGATTCGC

Fonte: A autora (2024).

5.6.9 Expressão de Pax7 por imuno-histoquímica no músculo

Para imunocoloração de Pax7 foram utilizadas seções do músculo sóleo, provenientes também da pata esquerda dos animais, que foram marcadas primeiro com anticorpo anti-Pax7 (diluição a 3 µg/mL, Developmental Studies Hybridoma Bank) por 1h, seguido com anti-camundongo de cabra AlexaFluor 555 (1:1000, Invitrogen). Após a lavagem, as lâminas foram incubadas com anticorpo anti-laminina (1:100, Sigma Aldrich) e detectadas com um anti-coelho de cabra AlexaFluor 488 (1:1000, Invitrogen). Em seguida, os músculos sóleos foram contra-corados com um meio de montagem DAPI (Abcam). Cinco a dez campos foram adquiridos com uma ampliação de 20x usando um microscópio Olympus BX63. Pelo menos 500 fibras foram usadas para registrar as células satélite PAX7+/DAPI+ e os dados foram normalizados pelo número de fibras positivas para laminina.

5.6.10 Coleta e aferição do peso da tíbia

O osso da tíbia de ambas as patas posteriores foram dissecadas nos gruposPC e controle tratados com FGF19 ou veículo, pesadas em balança de precisão

(modelo Marte AUW220, capacidade de 220g e 0,1 mg de sensibilidade) no P29. Além disso, foi mensurado o comprimento longitudinal da tíbia utilizando um paquímetro universal analógico (Digimess, 0,02mm), em que foi feita a média de duas medidas.

5.6 11 Eutanásia, coleta do encéfalo e análise da neurogênese do cerebelo

A outra metade dos animais (n=8 por grupo) foi eutanasiada por perfusão transcardíaca (Matos et al., 2011) e receberam previamente duas injeções intraperitoneais de BrdU (200 mg / kg de peso corporal), no 5º e 6º dia de vida pós-natal. Os animais foram anestesiados e perfundidos inicialmente com solução salina seguida de solução com paraformaldeido 4% afim de fixar os tecidos antes da dissecação do encéfalo. Foi realizada uma craniotomia, o encéfalo foi dissecado por inteiro e pós-fixado paraformaldeido 4%, solução de sacarose 30% por 48h e armazenado em solução crioprotetora. Secções de 40um de espessura foram coletadas e submetidas à imuno-histoquímica (Matos *et al.,* 2011). As secções foram tratadas com 50% de formamida e solução de NaCl 0,3 M, Citrato de sódio 0,03 M) durante 2 h a 65 ° C, enxaguado várias vezes em PBS, foram incubadas durante 30 min em HCl 2 M a 37 ° C e enxaguadas 3 a 5 minutos com tampão de ácido bórico 0,1 M a pH 8,5. As secções foram enxaguadas 4 a 5 min com PBS e incubadas 3 a 10 minutos em H2O2 a 3% (em PBS) para bloquear a atividade endógena da peroxidase. Após uma incubação final em tampão de bloqueio durante 1h, as seções foram expostas a um anticorpo monoclonal anti-BrdU de rato (Sigma-Aldrich), durante a noite a 4 ° C. No dia seguinte, as secções foram lavadas de 4 a 5 minutos com PBS e incubadas no escuro durante 2, com anticorpo secundário (Life Technologies). Após as lavagens, as secções foram incubadas com complexo avidina-biotina-peroxidase durante 1h. Após as lavagens, a rotulagem da BrdU foi visualizada por incubação das secções numa solução de PBS contendo 0,5 mg / ml 3,3'-diaminobenzidina e 0,01% de peróxido de hidrogênio. Secções foram montadas em lâminas, desidratadas e cobertas. O número de células marcadas com BrdU no cerebelo foram avaliados com auxílio de microscópio acoplado a microcomputador (Matos *et al.,* 2011).

5.6.12 Análise da densidade da micróglia e micróglia ativada no cerebelo

O cerebelo também foi avaliado quanto ao perfil da micróglia nas áreas Crus 1 e 2. Foi conduzido utilizando seções cerebelares incubadas primeiro em 10% H2O2 em metanol e posteriormente em H2O2 a 10% em tampão fosfato (0,1 M, pH 7,4) contendo 3% de Triton X-100 (PBT). Os cortes foram então incubados a 4°C por

48 horas em anticorpo primário da molécula adaptadora de ligação ao cálcio ionizado 1 (Iba1) (coelho anti-Iba1/IAF1, 1:30.000, Wako), diluído com 5% de soro de cavalo em PBT (Saavedra et al., 2021). As seções foram posteriormente incubado em um anticorpo secundário (anti-coelho biotinilado; 1:750, Sigma- Aldrich) por um período de 2 horas a 4 °C. As seções cerebrais foram então incubadas em soluções do complexo avidina-biotina-peroxidase (ABC Elite Kit; Vector Laboratories, Burlingame, CA, EUA) e uma solução do kit de coloração com diaminobenzidina (Kit DAB; Vector Laboratórios) para corar a micróglia (SAAVEDRA et al., 2021). Seções de cada grupo foram processadas paralelamente para evitar efeitos de coloração inespecíficos (Visco *et al.,* 2022). As seções cerebrais foram então montadas em lâminas gelatinizadas e cobertas com lamínulas Cytoseal (*Thermo Scientific*, EUA). Avaliação de células microgliais Iba1 + no cerebelo (n = 6 por experimental grupo) foi conduzido selecionando dois campos por seção de um total de quatro seções selecionados aleatoriamente por seção cerebral das regiões Crus 1 e Crus 2, dando um total de oito fotografias por área do cerebelo para cada animal. Os campos selecionados foram captados usando um microscópio óptico (Zeiss, Alemanha) com objetiva de 20x. Um pesquisador cego usou o software *ImageJ* para contar o número de células imunorreativas a Iba1 por área e classificar o perfil da microglia de acordo com as descrições anteriores. Células Microgliais com um soma pequeno e poucos ou numerosos processos foram consideradas microglias ramificadas, enquanto aqueles com corpo grande ou amebóide e mais espesso, processos mais curtos foram considerados microglia ativada (Roque; Ochoa-Zarzosa; Torner, 2016; Saavedra et al., 2021). Os dados apresentados foram densidade da microglia (n/mm3 - células totais divididas pela área avaliada) e a porcentagem de ativação da microglia em relação ao total (%) (Visco *et al.,* 2022).

5.7 ANÁLISE ESTATÍSTICA

Os dados obtidos foram inicialmente analisados quanto à distribuição normal (teste de Shapiro-Wilk). Se constatada distribuição normal foram realizados os testes paramétricos adequados como o Anova TwoWay para comparação dos grupos nas análises realizadas em apenas uma idade do animal, ou o Anova TwoWay Medidas repetidas para comparação dos grupos nas análises realizadas em mais de uma idade. Se não for observada distribuição normal foram adotados testes estatísticos não paramétricos como o teste de Kruskal-Wallis e Friedman. Os resultados foram expressos como média ± desvio padrão ou como mediana e valores máximos e mínimos, sendo o nível de significância utilizado de 5%. Foi utilizado o software GraphPadPrism® versão 9 para análises dos dados e construção dos gráficos.

6 RESULTADOS

O presente estudo foi realizado em duas fases, em que a 1ª fase envolveu os dados referentes à intervenção com FGF19 e a 2ª fase envolveu dados referentes à intervenção com resveratrol em modelo de PC. Sendo assim, ambas fases concluídas e publicadas como artigos originais nas revistas internacionais: *Journal of Cachexia, Sarcopenia and Muscle*, fator de impacto 12.910 e Experimental Neurology, fator de impacto 5.3 (Apêndices A e B). Uma revisão sistemática com metanálise também foi desenvolvida e publicada no *Journal of Neuroscience Methods* fator de impacto 2.390 (Apêndice C), além de outros artigos em além de outros artigos em colaboração abrangendo esta linha de pesquisa (Apêndices D, E e F). Assim, serão apresentados a seguir os principais resultados originais provenientes deste projeto.

6.1 PREJUÍZOS NO SISTEMA NEUROMUSCULOESQUELÉTICO DECORRENTES DO MODELO DE PC

O modelo de PC que associa anóxia a restrição sensório-motora confirma os prejuízos funcionais semelhantes a humanos com PC em diversos desfechos do neurodesenvolvimento e do sistema neuromusculoesquelético.

O modelo de PC provocou redução da evolução ponderal dos animais, redução da atividade locomotora e da coordenação motora, além de atraso na aquisição de reflexos primitivos do desenvolvimento e redução da força muscular corroborando estudos anteriores (Costa-de-Santana *et al.*, 2022; Silva *et al.*, 2016; Stigger e*t al.*, 2011).

Prejuízos no crescimento e nas habilidades motoras decorrentes na PC experimental foram associados com a redução do peso muscular e ósseo, redução da área e perímetro das fibras musculares consistente com atrofia muscular. A atrofia muscular é um desfecho esperado e frequente em modelos de PC, estando relacionada ao desuso por imobilidade prolongada como acontece no tipo de modelo utilizado no presente estudo (Strata *et al.*, 2004; Marcuzzo *et al.*, 2010)

Também houve distorção no padrão de tipagem das fibras musculares dos músculos sóleo e EDL. O sóleo é conhecido por ser um músculo constituído predominantemente de fibras do lentas, do tipo I (Komiya *et al*, 2027; Schiaffino *et al*., 2011), porém o modelo de PC reduziu o percentual de fibras do tipo I e aumentou as fibras do tipo II nos animais submetidos à PC. Em contrapartida, o EDL é conhecido por ser um músculo constituído predominantemente de fibras rápidas, do tipo II (Komiya *et al*, 2027; Schiaffino *et al*., 2011), mas também apresentou inversão no padrão dos seus tipos de fibras, por causa do modelo de PC, reduzindo o percentual de fibras do tipo II e aumentando as fibras do tipo I.

Além disso, de forma inédita, evidenciamos em modelo de PC as alterações na expressão de genes relacionadas ao desenvolvimento muscular e a junção neuromuscular semelhante ao que ocorre em músculos de crianças com PC (Smith *et al.*, 2009). Observamos o aumento na expressão de genes relacionados ao desenvolvimento muscular como Igfbp5, Dmd e Kcnn3 geralmente expressos em células musculares imaturas. Além disso, Kcnn3 está envolvido na excitabilidade de membrana e potencial de ação regulando canais de K+ e a transmissão de sinapses químicas (Kessi *et al.,* 2020) O aumento na expressão de Kcnn3 pode justificar as alterações na ativação muscular e consequentemente as deficiências nas habilidades motoras e aprendizado motor.

O modelo de PC também levou ao aumento da expressão de genes relacionados à atrofia muscular como Miostatina (Gdf8) que tem ação catabólica muscular favorecendo a degradação proteica através da autofagia e reduzindo a síntese proteica por bloqueio da via AKT mTOR (Kumagai *et al.,* 2021).

A fim de verificar os prejuízos sobre o desenvolvimento muscular investigamos a expressão de genes envolvidos desde o surgimento das células satélites até a diferenciação dos tipos de fibras musculares. Assim, identificamos que houve um aumento da expressão de pax7 e um aumento de células positivas para pax7 via imunohistoquímica nos animais submetidos PC. A pax7 marca as células satélites, presente, por exemplo, em músculos pós lesão e favorece a diferenciação das células satélites em mioblastos (Rahman *et al.*, 2023) . Houve também a redução da expressão de Miogenina (Myog) destacando o dano sobre a diferenciação muscular já que a Myog influencia a diferenciação de miócitos em

miotubos (Huang *et al.*, 2016), reforçando o estado imaturo das fibras musculares decorrente da PC experimental.

Corroborando nosso resultado de alterações na distribuição dos tipos de fibras musculares, representado pelo aumento de fibras do tipo II no sóleo modificando sua morfologia usual, observamos também através da expressão gênica o aumento de Myh4, fator envolvido na maturação das fibras musculares, especificamente, na diferenciação das fibras musculares do tipo IIb. Assim, nossos achados através de modelo de PC demonstram alterações na expressão de genes semelhante ao que acontece em músculos de punho de crianças com PC (Smith *et al.*, 2009) e evidencia que o modelo é capaz de reproduzir os danos ao desenvolvimento muscular e a junção neuromuscular.

O modelo de PC não influenciou a proliferação celular nas áreas Crus 1 e Crus 2 do cerebelo, porém houve aumento da densidade da micróglia e sua ativação no cerebelo demonstrando a cascata inflamatória instalada decorrente do insulto perinatal. A micróglia são células imunológicas e refletem a neuroinflamação pós insulto, que neste caso foi a anóxia associada a restrição sensório-motora, onde sua ativação prolongada pode prejudicar o sistema nervoso em desenvolvimento (Orso *et al.*, 2023; Lenz *et al.*, 2018; De Pablos *et al.,* 2014).

Esses resultados reforçam a importância do modelo experimental na reprodução dos prejuízos causados pela PC em humanos, e destaca a importância de estudos que desenvolvam novas propostas de atuação terapêutica voltada aos danos neuromusculoesqueléticos com consequentes benefícios às habilidades motoras dos animais.

6.2 EFEITOS DO TRATAMENTO COM FGF19 SOBRE OS DANOS MUSCULOESQUELÉTICOS CAUSADOS PELO MODELO DE PC

No presente estudo, o tratamento com FGF19 recombinante por 7 dias, do 22º ao 28º dia de vida, pôde restaurar a disfunção musculoesquelética e locomotora instalada por modelo de PC. Animais submetidos à modelo de PC e tratados com FGF19 tiveram o peso corporal preservado, aumentaram o peso muscular e ósseo, assim como houve o aumento da área e perímetro das fibras musculares dos

músculos sóleo e EDL, associada a redução do número de fibras musculares muito pequenas (<200 μm²) em ambos os músculos.

O grupo PC tratado com FGF19 também apresentou a reversão das alterações na expressão dos genes relacionados ao desenvolvimento muscular e a junção neuromuscular. Houve redução na expressão gênica de Igfbp5, Igf1, Dmd, e tendência a diminuição da expressão de Kcnn3, demonstrando o potencial do FGF19 em neutralizar o estado imaturo dos músculos esqueléticos induzido pela PC experimental.

Consequentemente, o tratamento com FGF19 em modelo de PC beneficiou aspectos funcionais do animal levando ao aumento da força muscular e da locomoção nos ratos pós tratamento aos 28 dias de vida. Resultado alcançado possivelmente pelos seus efeitos hipertróficos no sistema musculoesquelético (Benoit *et al.*, 2017). O que sugere que o FGF19 pode representar uma estratégia terapêutica potencial para combater os distúrbios musculoesqueléticos e locomotores associados à PC.

6.3 EFEITOS DO TRATAMENTO NEONATAL COM RESVERATROL SOBRE OS DANOS NEUROMUSCULOESQUELÉTICOS CAUSADOS PELO MODELO DE PC

O tratamento com resveratrol no período neonatal beneficiou o neurodesenvolvimento não somente através da recuperação dos danos no sistema musculoesquelético mas também no sistema nervoso central causados pelo modelo de PC.

O tratamento com resveratrol neonatal evitou o atraso na aquisição de reflexos primitivos do desenvolvimento que são importantes para aquisição de habilidades motoras como a locomoção. Reflexos primitivos estão relacionados a maturidade do sistema nervoso em que o cerebelo está envolvido em todos os níveis de integração sensório-motora regulando por exemplo o tônus muscular e a manutenção da postura (Chandradasa *et al.*, 2020; Kobesova *et al.*, 2014) . Como observado no presente estudo, o resveratrol recuperou os reflexos de geotaxia negativa e de queda livre em modelo de PC que são importantes para a maturação de respostas posturais durante o desenvolvimento.

De forma consistente, observamos que o tratamento neonatal com resveratrol melhorou o desempenho locomotor dos ratos afetados pela PC experimental. Houve um aumento da capacidade exploratória dos animais PC tratados com resveratrol, visto pelo maior tempo gasto nas áreas periféricas do campo aberto e diminuição do tempo gasto nas áreas mais centrais do campo. Além de aumento na distância percorrida, velocidade média e diminuição do tempo parado durante o teste em campo aberto.

A recuperação dos reflexos primitivos e da atividade locomotora podem ser justificados pela reversão dos dados no sistema neuromusculoesquelético causados pelo modelo de PC. O resveratrol foi capaz de reverter os prejuízos na morfologia das fibras musculares dos músculos sóleo e EDL, restaurando o padrão de distribuição dos seus tipos de fibras musculares. Apesar de não ter demonstrado efeito hipertrófico em modelo de PC, o resveratrol restaurou o maior percentual de fibras do tipo I no músculo sóleo, assim como restaurou o maior percentual de fibras do tipo II no músculo EDL.

Quanto aos danos no sistema nervoso central, o tratamento neonatal com resveratrol atuou na neuroinflamação das áreas Crus 1 e Crus 2 do cerebelo decorrente da PC experimental, reduzindo a densidade e a ativação da microglia cerebelar. Isso pode ser atribuído aos efeitos antioxidantes e neuroprotetores do resveratrol corroborando estudos anteriores que observaram o mesmo desfecho no próprio cerebelo e em outras áreas encefálicas (Calado *et al.*, 2023; Costa-de-Santana *et al.*, 2022), beneficiando assim as habilidades motoras dos ratos como a aquisição de reflexos primitivos e a locomoção.

6.4 FGF19 e resveratrol em modelo de PC: limitações e perspectivas futuras

Como observado, o tratamento com FGF19 reverteu a atrofia musculoesquelética e as alterações na expressão gênica relacionada à imaturidade muscular beneficiando a força muscular e a locomoção, e o tratamento com resveratrol restaurou a morfologia muscular e reverteu a neuroinflamação cerebelar, beneficiando o neurodesenvolvimento e a locomoção. Porém, nenhuma das duas intervenções reverteu o déficit de coordenação motora causado pela PC

experimental. Podemos justificar este resultado pela complexidade relacionada a coordenação motora que depende de informações sensoriais preservadas e envolve o aprendizado motor e a sinergia de grupos musculares (Singh *et al.*, 2018; Aoi, Funato, 2016) que são prejudicados pelo modelo de PC. Dessa forma, o tratamento farmacológico isolado não foi capaz de reverter a redução da coordenação motora, então, sugere-se a associação de tratamentos multidisciplinares como o enriquecimento ambiental e o exercício físico que também são implicados em modelos animais. Além disso, sugere-se que análises da medula espinal devem ser consideradas em futuros estudos já que os chamados geradores de padrões centrais são responsáveis pela locomoção automática (Baruzzi, Lodi e Storace, 2024; Mantziaris, Bockemühl, Büschges, 2020) e poderiam justificar a melhora da locomoção e não da coordenação motora mesmo após tratamento farmacológico.

Novos estudos também são necessários para elucidar os mecanismos envolvidos na atuação terapêutica do FGF19 e resveratrol já que a literatura ainda é limitada quanto aos seus efeitos na PC experimental, e, inclusive sugerimos a associação do FGF19 e do resveratrol que se mostraram intervenções potenciais para reverter os prejuízos no sistema neuromusculoesquelético e no desenvolvimento motor causado pelo modelo de PC.

7 CONSIDERAÇÕES FINAIS

Concluímos que o tratamento com FGF19 e/ou resveratrol puderam beneficiar as habilidades motoras dos animais, como a locomoção, através de seus diferentes mecanismos de atuação sobre os danos no sistema neuromusculoesquelético causados pelo modelo de PC. Enquanto o tratamento com FGF19 foi capaz de causar efeito hipertrófico muscular e reverteu as alterações na expressão de genes do desenvolvimento muscular, melhorando a força e a locomoção, o resveratrol neonatal atuou na recuperação da morfologia das fibras musculares e reverteu a neuroinflamação cerebelar beneficiando o neurodesenvolvimento e a locomoção dos ratos submetidos ao modelo de PC.

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APÊNDICE A – FIBROBLAST GROWTH FACTOR 19 AS A COUNTERMEASURE TO MUSCLE AND LOCOMOTION DYSFUNCTIONS IN EXPERIMENTAL CEREBRAL PALSY

ORIGINAL ARTICLE

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Fibroblast growth factor 19 as a countermeasure to muscle and locomotion dysfunctions in experimental cerebral palsy

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Abstract

Background Cerebral palsy (CP) associates cerebral function damages with strong locomotor defects and premature sarcopenia. We previously showed that fibroblast growth factor 19 (FGF19) exerts hypertrophic effects on skeletal mus- cle and improves muscle mass and strength in mouse models with muscle atrophy. Facing the lack of therapeutics to treat locomotor dysfunctions in CP, we investigated whether FGF19 treatment could have beneficial effects in an exper- imental rat model of CP.

Methods Cerebral palsy was induced in male Wistar rat pups by perinatal anoxia immediately after birth and by sen- sorimotor restriction of hind paws maintained until Day 28. Daily subcutaneous injections with recombinant human FGF19 (0.1 mg/kg bw) were performed from Days 22 to 28. Locomotor activity and muscle strength were assessed be- fore and after FGF19 treatment. At Day 29, motor coordination on rotarod and various musculoskeletal parameters (weight of tibia bone and of soleus and extensor digitorum longus (EDL) muscles; area of skeletal muscle fibres) were evaluated. In addition, expression of specific genes linked to human CP was measured in rat skeletal muscles.

Results Compared to controls, CP rats had reduced locomotion activity (37.8% of distance travelled, $P < 0.05$), mo- tor coordination (88.9% latency of falls on rotarod, $P < 0.05$) and muscle strength (25.1%, $P < 0.05$). These defects were associated with reduction in soleus (51.5%, $P < 0.05$) and EDL (42.5%, $P \leq 0.05$) weight, smaller area of mus- cle fibres, and with lower tibia weight (38%, $P < 0.05$). In muscles from rats submitted to CP, changes in the expres- sion levels of several genes related to muscle development and neuromuscular junctions were similar to those found in wrist muscle of children with CP (increased mRNA levels of *Igfbp5*, *Kcnn3*, *Gdf8*, and *MyH4* and decreased expression of *Myog*, *Ucp2* and *Lpl*). Compared with vehicle-treated CP rats, FGF19 administration improved locomotor activity (+53.2%, $P < 0.05$) and muscle strength (+25.7%, $P < 0.05$), and increased tibia weight (+13.8%, $P < 0.05$) and so- leus and EDL muscle weight (+28.6% and +27.3%, respectively, $P < 0.05$). In addition, it reduced a number of very small fibres in both muscles ($P < 0.05$). Finally, gene expression analyses revealed that FGF19 might counteract the immature state of skeletal muscles induced by CP.

Conclusions These results demonstrate that pharmacological intervention with recombinant FGF19 could restore mus- culoskeletal and locomotor dysfunction in an experimental CP model, suggesting that FGF19 may represent a potential therapeutic strategy to combat the locomotor disorders associated with CP.

Keywords Fibroblast growth factor 19; Cerebral palsy; Skeletal muscle; Sarcopenia

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Hubert Vidal and Ana Elisa Toscano are co-last authors.

Introduction

Cerebral palsy (CP) is a perinatal disease affecting about 2–3 per 1,000 children worldwide.[1] CP is associated with permanent posture disorders and immobility due to neurofunctional damages of the developing brain.[1,2] Children affected by CP have robust deficiency of gait and movement and develop premature sarcopenia, with high vulnerability to weakness and increased fatigue during activities.[3] In addition, by reducing the load on the developing skeleton, the insufficient functional musculature and immobility impair the healthy development of bones.[4,5] Currently, individuals with CP are mainly treated by physiotherapy, bracing and orthopaedic surgery, which all have limited impacts for the patient's welfare.[6] Facing the reduced quality of life of these children, developing new therapeutic measures are highly warranted.[4,5]

To better understand the pathogenesis of CP and explore novel therapeutic strategies, perinatal anoxia and sensorimotor restriction of hind paws have been used to develop preclinical CP models.[7,8] In rats, this experimental CP model is characterized by reduced body growth, abnormal walking patterns, atrophy of hind limb muscles, extracellular matrix changes and joint degeneration of knee and ankle.[7,9] It is also associated with reduced locomotor activity,[10] increased spasticity,[9] impaired chewing[11] and motor skills, and reduced sarcomere density.[12] In addition, this experimental CP model shows brain alterations, such as an increase in the permeability of the blood–brain barrier[13] and a degraded representation of hind limbs in the primary motor cortex.[9]

We recently discovered that the fibroblast growth factor 19 (FGF19) increases skeletal muscle mass and strength.[14] FGF19 (and its rodent ortholog FGF15) is a member of the atypical endocrine subfamily of FGFs, produced by ileal enterocytes. In mice, treatment with recombinant human FGF19 significantly increases skeletal muscle mass and muscle fibre surface. Furthermore, FGF19 increases the size of human myotubes *in vitro*. At the signalling level, FGF19 binds to FGF receptor/ß-klotho complex and induces its hypertrophic effect by activating an extracellular-signal-regulated protein kinase 1/2 (ERK1/2)/mammalian target of rapamycin (mTOR) pathway.[14] Importantly, FGF19 treatment during 1 or 2 weeks improved muscle wasting and muscle strength in different experimental models including sarcopenic aged mice and glucocorticoid-treated mice,[14] thus supporting the therapeutic potential of FGF19 in pathologies with muscle weakness.

In the present proof-of concept study, we aimed at verifying whether FGF19 could be used as a countermeasure to fight against muscle atrophy and mobility dysfunction in a rat model of CP. We found that daily administration of human recombinant FGF19 between day 22 and day 28 after birth in CP rats, improved locomotion and musculoskeletal parameters such as muscle fibre size and tibia bone mass. In addition, FGF19 treatment restored the muscle expression of several genes that have been previously found altered in wrist muscle of children with CP.[15]

Methods

Animals

The study was approved by the Ethics Committee on Animal Use (protocol 0011/2017) and performed in accordance with the 1964 Declaration of Helsinki and its later amendments. Wistar rats were kept in the maintenance vivarium of the UFPE Department of Nutrition at a temperature of 22 ± 2 °C, inverted light–dark cycle of 12/12 h, housed in polypropylene cages with free access to water and diet. On the day of birth, male pups were randomly distributed in the experimental groups as followed: control + vehicle (V); control + FGF19 (F); CP + vehicle (CPV); CP + FGF19 (CPF). Female pups were used to complete the litter of eight pups until weaning. CP was induced by submitting male pups to two episodes of anoxia (exposure to 100% nitrogen at 9 L/min for 12 min), on the day of birth (P0) and the day after (P1). Afterwards, from P2 to P28, sensorimotor restriction of the hind limbs was performed daily for 16 h, with free movement of the animal in the remaining 8 h of the day.[7,11] Weaning occurred at P25, and after this time, the male pups were placed in individual cages. Treatment with recombinant human FGF19 (R&D System, UK) was performed from P22 to P28. All injections of vehicle solution (phosphate-saline buffer solution with 0.1% bovine serum albumin) or recombinant human FGF19 solution (0.1 mg/kg in the vehicle solution) were performed subcutaneously.[14]

Body weight and locomotor activity

Animals were weighed at P0, P8, P14, P17, P22 and P29 using an electronic digital scale (Marte, S-1000 model with 0.1 g of sensitivity). Locomotor activity was analysed at P22 and P28 in a dark room during the dark cycle when the animals are usually awake. Animals were positioned in the center of an open field and filmed (Ulead Video Studio® software) for a

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period of 5 min. Each video was analysed using the ANY-maze software to obtain the following parameters: total distance travelled (m), average speed (m/s), number of stops, and immobility time (s), as previously described.[10] Representative recordings are shown as supporting information, *Video* S1.

Motor coordination assessment

The rotarod test was performed at P29 by a blinded evaluator. One animal at a time was placed in the rotarod equipment (rod 60 mm in diameter and 75 mm in length). Five attempts were made, with a 2 min rest interval, at a speed of 25 rpm for a maximum of 3 min. The time (latency) before the fall was recorded, and the mean latency time of the five attempts was calculated (adapted from Stigger *et al*.[12]).

Muscle strength assessment

Analysis of muscle strength was performed at P22 and P28, using the suspension test (forelimb grip test), with video recording. Animal was positioned 1 m away from the ground on a coated steel cable (3 mm in diameter) and remained gripped by the forelimbs for a time limit of 60 s while suspended by the tail. Videos were analysed by a blind appraiser, using the Windows Movie Maker program, and the fall latency, expressed in seconds, was measured and the data were further expressed as arbitrary units (adapted from Teo *et al*.[16]).

Tissue sampling

At the time of euthanasia (P29), skeletal muscles [soleus and extensor digitorum longus (EDL)] and tibia bone from the hind limbs were harvested and weighted. Left posterior limb muscles were immediately frozen at −80°C for gene expression analyses. Muscles of the right hind limb were frozen in *n*-hexane (pre-cooled with dry ice) and stored at −80°C for histological analyses. The longitudinal length of the tibia bone was measured using a calliper.

Muscle fibre area measurements

To determine cross-sectional fibre size, 10 µm-thick cryosections taken at the mid-belly of the muscles (soleus and EDL) were processed for immunostaining, as described previously.[14] Briefly, sections were blocked for 1 h at room temperature and incubated overnight at 4°C with a rabbit anti-laminin antibody (Sigma, L9393), followed by incubation with a secondary antibody (AlexFluor Goat anti Rabbit IgG AlexaFluor 594—A11012 ThermoFisher). The 10× magnification images were taken using a Zeiss Axiovert200M microscope. The Axiovision software was configured to take into account only the transverse fibres with a Ferret ratio strictly up to 0.5 and their area was measured in square micrometres (μm^2).

Pax7 expression by immunohistochemistry

For Pax7 immunostaining, soleus muscle sections were first labelled with anti-Pax7 antibody (dilution at 3 µg/mL, Developmental Studies Hybridoma Bank) for 1 h, followed with AlexaFluor 555 goat anti-mouse (1:1000, Invitrogen). After washing, slides were incubated with anti-laminin antibody (1:100, Sigma Aldrich) and detected with an AlexaFluor 488 goat anti-rabbit (1:1000, Invitrogen). Then, soleus muscles were counterstained with a DAPI mounting medium (Abcam). Five to ten fields were acquired with a 20× magnification using an Olympus BX63 microscope. At least 500 fibres were used to record the $PAX7^+/DAPI^+$ satellite cells and the data were normalized by the number of laminin positive fibres.

Gene expression analysis

Total RNA from soleus and EDL muscles was extracted using TRI Reagent (Sigma Aldrich, Saint-Louis, MO, USA). RNA preparations were quantified using Nanodrop 2000 (Ozyme) and their quality was checked using Agilent bioanalyser 2100. First-strand cDNAs were synthesized from 1 µg total RNA using Prime Script RT Reagent kit (Perfect Real Time) 200X (Ozyme) and a combination of oligodT and random primers. Transcript levels were measured by real-time PCR (Rotor-Gene 6000, Qiagen, Courtaboeuf, France) in a final volume of 20 µL using the SYBR qPCR Premix Ex Taq kit (Ozyme). Each assay was performed in duplicate and validation of the RT-PCR runs was assessed by evaluating the melting temperature of the products, and by the slope and error obtained with the standard curve. The analyses were performed using Rotorgene software (Qiagen). The results were normalized to *Tbp* (TATA binding protein) expression, used as internal standard. The list of primer sequences is available in *Table* S1.

Statistics

One-way or two-way analysis of variance tests were performed to determine differences between experimental groups. *Post-hoc* comparisons were performed by Tukey's test, with statistical significance set at $P \leq 0.05$. For gene expression and immunohistochemistry, Mann–Whitney test was used. All statistics were performed using GraphPad Prism 8.4.1 and data are presented as means ± SEM.

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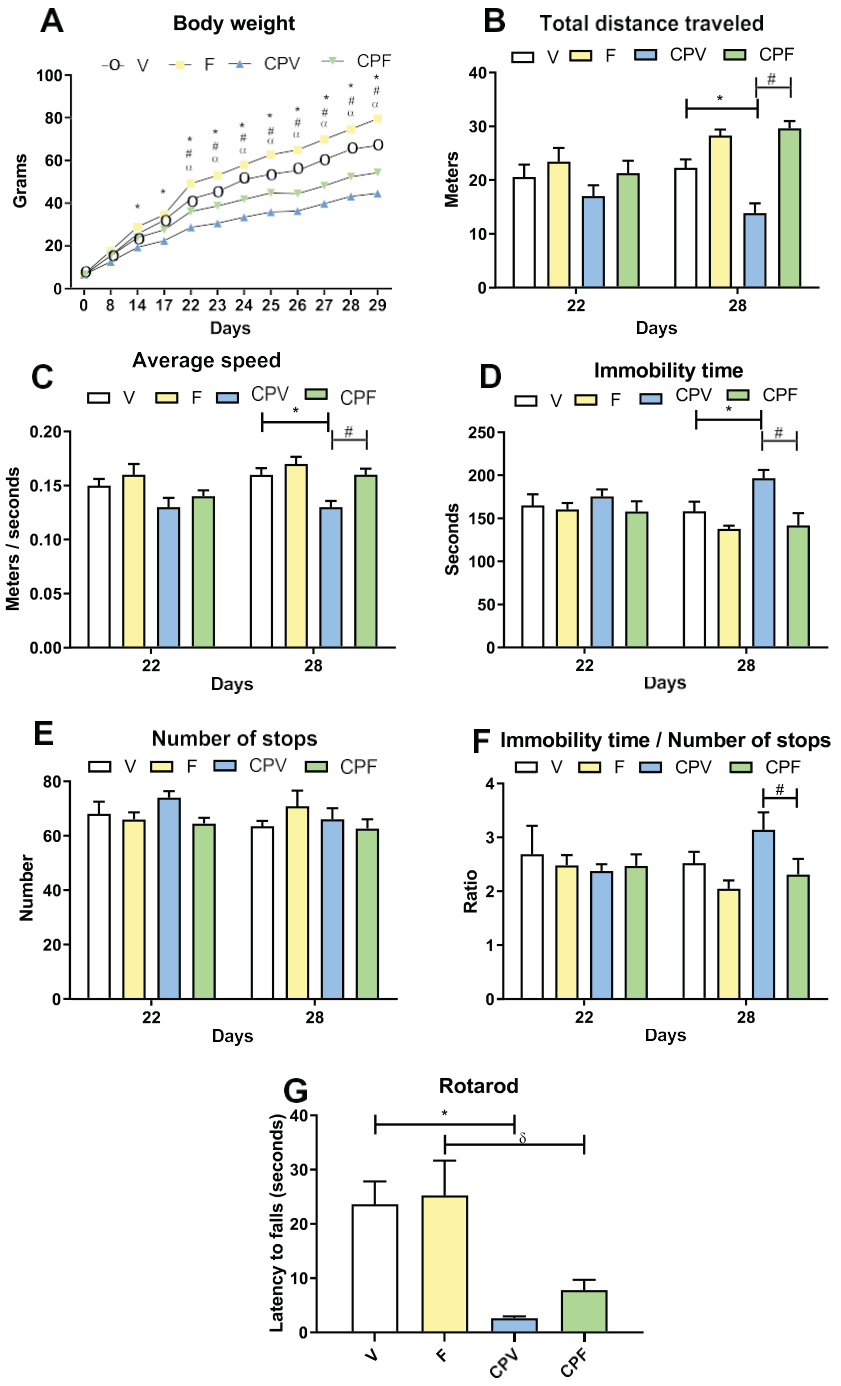

Figure 1 FGF19 treatment increases body weight and preserves locomotor activity, but not motor coordination in cerebral palsy (CP) rats. *(A)* Body weight evolution curves (n = 10–13), *(B)* total distance travelled, *(C)* average speed, *(D)* immobility time, *(E)* number of stops, and *(F)* immobility time/number of stops during locomotor activity tests, before (Day 22) and after (D28) treatment with FGF19 (n = 10 animals per group). *(G)* Motor coordination assessed at Day 29 using the rotarod test (n = 10). V (control + vehicle); F (control + FGF19); CPV (CP + vehicle); CPF (CP + FGF19). Data are expressed as mean ± SEM. $P < 0.05$ for *CPV × V; $^{\#}$CPV × CPF, $^{\delta}$CPF × F, and $^{\alpha}$V × F.

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Results

FGF19 preserves body weight and increases locomotor activity in experimental cerebral palsy

Cerebral palsy rats (CPV and CPF groups) had reduced body weight (*Figure* 1A) and food intake (*Figure* S1) compared with the control non-CP rats (V and F). When treated with recombinant human FGF19, CP animals had higher body weight at the end of the protocol (CPF vs. CPV; *Figure* 1A), but the weight gain during the treatment (D22 to D29) was not significantly different (CPF = 18.2 ± 0.7 vs. CPV = 15.9 ± 1.0 g taken during the treatment period, P = 0.445). The body weight gain in the non-CP groups was increased in the presence of FGF19 (F = 30.3 ± 1.0 vs. V = 25.2 ± 1.0 g during the treatment period, P = 0.008). There was no significant change in food consumption in response to FGF19 in non-CP and CP animals (*Figure* S1).

At P22, open field experiments revealed no locomotion differences between groups (*Figure* 1B–1F). In contrast, open field records obtained at P28 showed that CPV group had a shorter distance travelled (*Figure* 1B), lower average speed (*Figure* 1C) and longer immobility time (*Figure* 1D) compared with the V group (all with $P < 0.05$). No difference was observed between the four groups in terms of the number of stops (*Figure* 1E). Importantly, rats in the CPF group had and almost complete restoration of their locomotor activity, with parameters globally similar to the control animals (V or

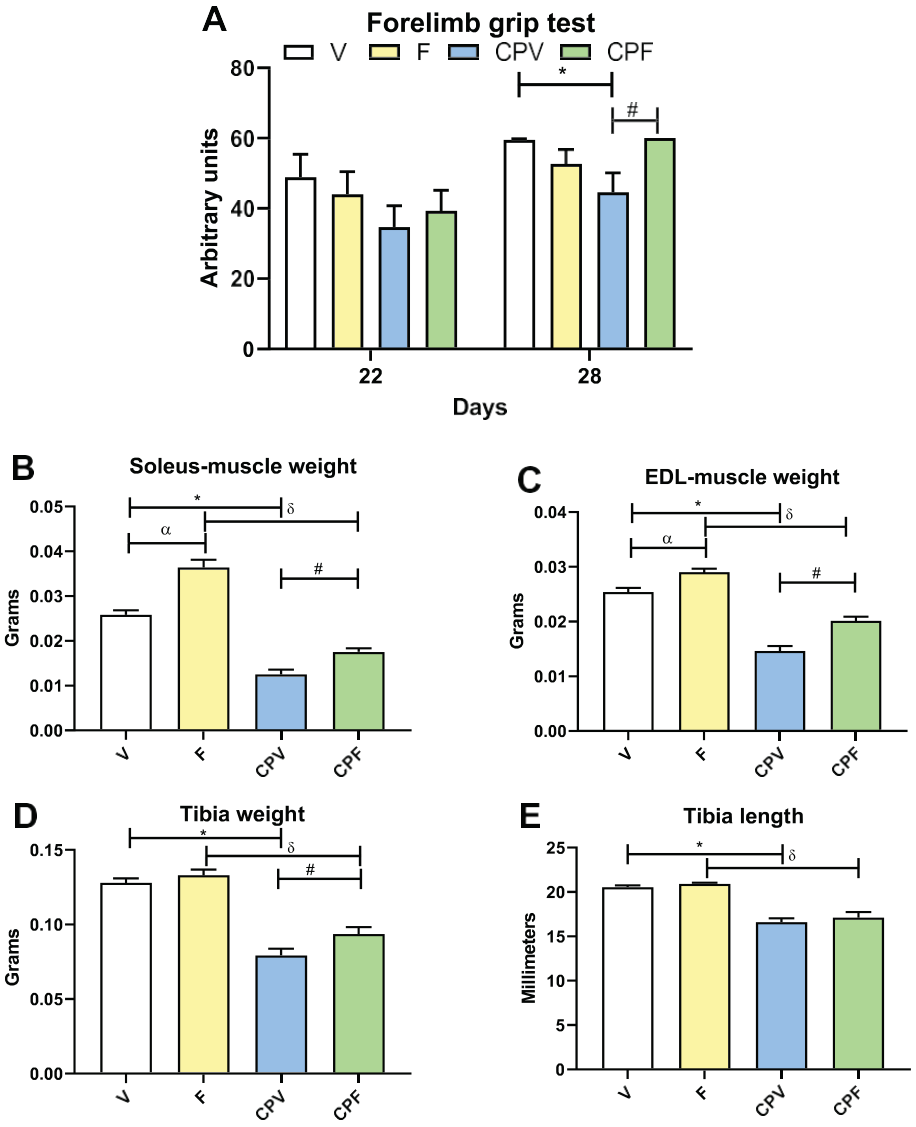

Figure 2 FGF19 treatment increases muscle strength and the weight of skeletal muscles and tibia bone in cerebral palsy (CP) rats. *(A)* Forelimb grip test (n = 10), *(B)* soleus weight, *(C)* extensor digitorum longus (EDL) weight, *(D)* tibia weight, *(E)* tibia length (n = 10–13). V (control + vehicle); F (control + FGF19); CPV (CP + vehicle); CPF (CP + FGF19). Data are expressed as mean ± SEM. $P < 0.05$ for *CPV × V; $^{\#}$CPV × CPF, $^{\delta}$CPF × F, and $^{\alpha}$V × F.

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F groups). A video recording showing representative locomotor activity of groups V, CPV, and CPF is available as supporting information (*Video* S1).

When motor coordination tests were performed with the rotarod, animals submitted to CP (CPV and CPF) stayed less time on the rod and fell more rapidly compared to non-CP rats (V and F). In CP rats, treatment with FGF19 (CPF) did not significantly improve motor coordination assessed with this test as compared to CPV (*Figure* 1G).

FGF19 increased muscle strength in cerebral palsy

Compared with V group, animals of the CPV group showed a reduction in muscle strength already at P22, which reached statistical significance at P28 (*Figure* 2A). Treatment with FGF19 significantly increased muscle strength at P28 in the CPF group compared with CPV, with a muscle grip strength reaching values similar to those obtained from non-CP animals (*Figure* 2A).

At the end of the experiment (P29), weights of soleus (*Figures* 2B) and EDL (*Figures* 2C) muscles were lower in the CPV group compared with the V group. Treatment with FGF19 significantly increased soleus and EDL muscle weight in both control (F) and CP (CPF) groups (*Figure* 2B and 2C).

Further, we found that CP rats (CPV and CPF) had decreased tibia weight and length as compared to non-CP rats (V and F) (*Figure* 2D and 2E). The administration of FGF19 in CP animals slightly, but significantly, increased tibia weight (*Figure* 2D) without affecting tibia length (*Figure* 2E).

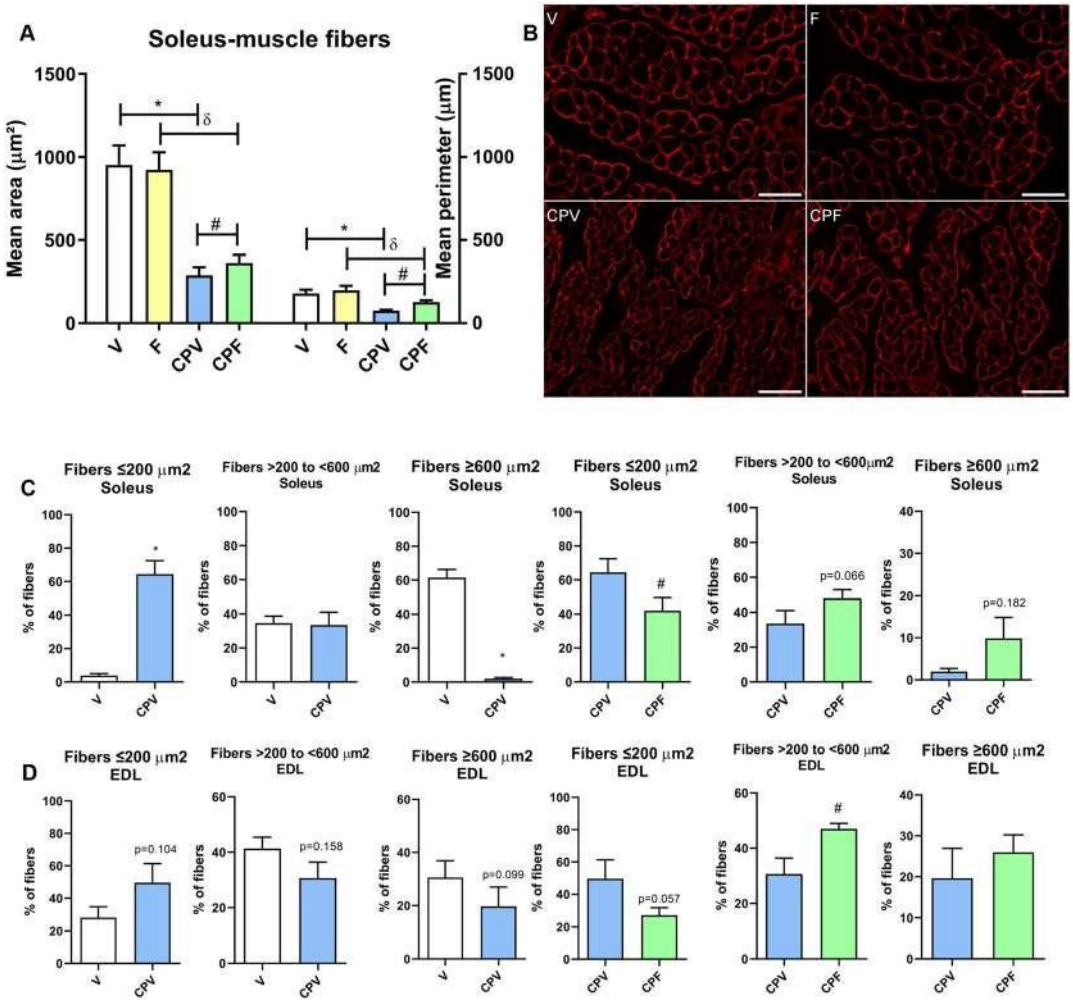

Figure 3 FGF19 treatment affects skeletal muscle fibres size and distribution in cerebral palsy (CP) rats. *(A)* Mean area and perimeter of soleus fibres; *(B)* representative images of laminin-stained soleus muscle (scale bars: 100 µm); *(C)* distribution of cross-sectional soleus muscle fibre area (n = 6–7 animals par group); *(D)* distribution of cross-sectional EDL muscle fibre area (n = 6–7 animals par group). V (control + vehicle); F (control + FGF19); CPV (CP + vehicle); CPF (CP + FGF19). Data are expressed as mean ± SEM. $P < 0.05$ for *CPV × V; $^{\#}$CPV × CPF, $^{\delta}$CPF × F, and $^{\alpha}$V × F.

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FGF19 improves skeletal muscle fibre size in cerebral palsy

In CP animals, soleus muscle fibres were characterized by smaller mean area and perimeter as compared with non-CP animals (*Figure* 3A). In rats submitted to CP, FGF19 treatment increased the mean area and perimeter of the soleus fibres (CPF compared with CPV group, *Figure* 3A and 3B). Distribution of fibre area revealed that rats from the CPV group had a marked increase in very small fibres (<200 μm^2) and a dramatic reduction of fibres higher than 600 μm^2 as compared with V group (*Figures* 3C and S2). Similar tendency was observed in EDL muscle although the difference did not reach statistical significance (*Figures* 3D and S2). When CP animals were treated with FGF19 for 1 week (CPF), the abundance of very small fibres (<200 μm^2) decreased in both muscles, and larger fibres reappeared (*Figure* 3C and 3D). There was no difference in the distribution of fibres between V and F (*Figure* S2).

Table 1 Gene expression in skeletal muscles: comparison between human and rat CP and effects of FGF19 treatment

Studied genes	Modifications in human CP (*wrist muscles data*) from[15]	Modifications in rat CP (*soleus or EDL data*) CPV vs. V	Effects of FGF19 in rat CP (*soleus or EDL data*) CPF vs. CPV
Igfbp5	↗	↗	↘
Igf1	↗	↗(tendency)	↘
Dmd	↗	↗	↘
Kcnn3	↗	↗	↘ (tendency)
Gdf8	↗	↗	=
Myh4	↗	↗	=
Neb	↗	=	=
Ucp2	↘	↘	=
Lpl	↘	↘	=
Myod	=	=	=
Myf5	=	↗	↘
Myog	=	↘	=
Musk	Not reported	↗	↘
Nes	Not reported	↗	↘
Pax7	Not reported	↗	=
Tnni1	Not reported	↘	=
Ckmt2	Not reported	↘	=

FGF19 treatment affected the expression of genes in skeletal muscles

The molecular mechanisms occurring in skeletal muscles during CP remain poorly known, but a transcriptomic study has revealed that the expression of a number of genes coding for important proteins and factors involved in skeletal muscle development, myogenesis, and neuromuscular junctions (NJM) are dysregulated in the wrist muscles of children with CP.[15] We therefore measured the expression of some of these genes in the soleus and EDL muscles, and further evaluated whether FGF19 treatment could affect their expression. We found that several genes (9 over 12 tested) displayed similar expression pattern in rat and in human CP (*Table* 1). Indeed, the mRNA levels of *Igfbp5*, *Igf1*, *Dmd*, and *Kcnn3* were increased in soleus or in EDL in CP rats compared with control animals (*Figure* 4). In addition, like in children with CP (Table 1), *Gdf8* (myostatin) and *Myh4* mRNAs levels were increased (*Figure* S3), whereas *Ucp2* and *Lpl*

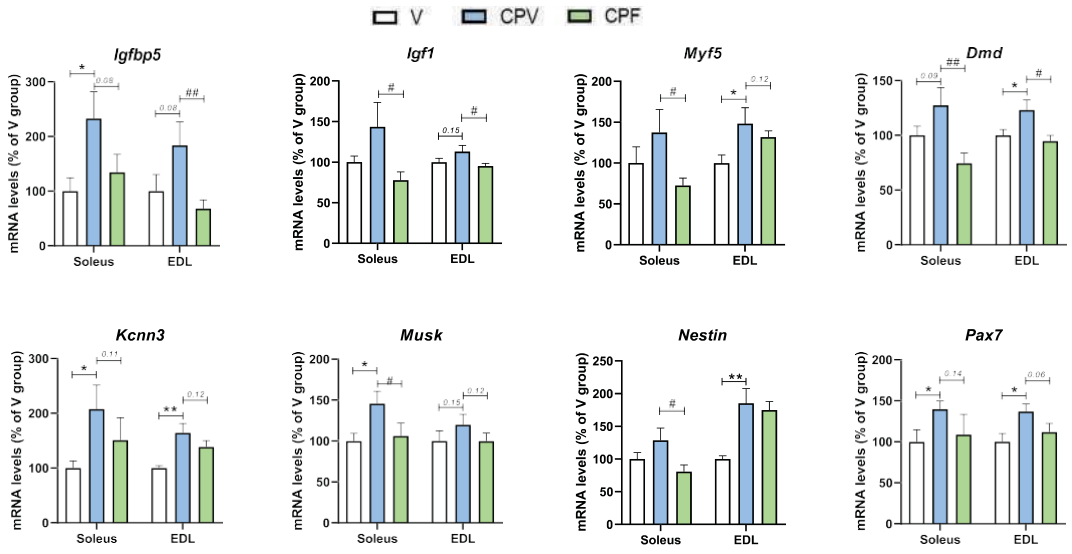

Figure 4 FGF19 treatment regulates the expression of genes altered in human with cerebral palsy (CP) in the soleus and the extensor digitorum longus (EDL) muscle of CP rats. Expression levels of the specific mRNAs were measured by RT-qPCR and normalized to Tbp. The data are presented in % of V group. V (control + vehicle); CPV (CP + vehicle); CPF (CP + FGF19). Data are expressed as mean ± SEM (n = 7–8 different animals per group). $P < 0.05$ for *CPV × V and #CPV × CPF. $P < 0.01$ for **CPV × V and ##CPV × CPF.

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expression levels were decreased in the soleus (these genes were not measured in EDL) (*Figure* S3). In contrast, the change observed in children with CP for nebulin (*Neb*) was not found in rat soleus, and the expression of *Myog* (myogenin) was decreased in soleus of CP rat while it was not affected in patients (*Figures* 4 and S3, Table 1). The other myogenic factors (*Myf5*, *Myod*) were neither modified in children with CP nor in the soleus of CP rats (although *Myf5* was increased in EDL) (*Figure*s 4 and S3, Table 1). From their transcriptomic studies, Smith *et al*. suggested that skeletal muscle were maintained in an immature state during CP, with a possible dysregulation of the NMJ.[15] We found that *Musk* expression, like *Kcnn3*, was increased in the soleus of the CP rat, with a tendency in the EDL (*Figure* 4). We also studied the expression of some additional genes related to muscle differentiation, myogenesis, contraction and metabolism, such as *Pax7*, *Nes* (Nestin), *Tnni1* (Troponin i1), and *Ckmt2* (mitochondrial creatine kinase 2), that were not reported in the human transcriptomic study. Of note, *Nes* and *Pax7* gene expression was increased in both soleus and EDL (*Figure* 4), whereas *Tnni1* and C*kmt2* mRNA levels were decreased in soleus of CP rat compared to control animals (*Figure* S3).

Interestingly, treatment with FGF19 counteracted the CP-associated increased in the expression levels of *Igfbp*, Igf1, *Myf5*, and *Dmd* in the soleus or the EDL muscles (CPF vs. CPV), globally restoring the expression of these 4 genes to levels similar to those observed in the control group (V) (*Figure* 4). Expression of NJM-related genes (*Kcnn3* and *Musk*) and differentiation-associated genes (*Nestin* and *Pax7*) was not significantly affected by FGF19, except for *Musk* and *Nes* mRNA levels that were decreased in soleus only (Figure 4). Other investigated genes in soleus muscle were not modified by FGF19 treatment (*Figure* S3).

The increased mRNA expression of *Pax7* in muscles of CP as compared with V (*Figure* 4) suggested a more immature state of skeletal muscle associated with CP. To confirm these gene expression data, we performed Pax7 immunostaining in soleus muscle samples. As shown in *Figure* 5, muscle of CPV rats showed increased Pax7 staining, confirming the mRNA result. Moreover, treatment with FGF19 did not significantly modify the number of Pax7 labelled cells (*Figure* 5). At the mRNA level, FGF19 tended to reduce *Pax7* gene expression in soleus and EDL, without reaching significance (*Figure* 4). To further investigate whether FGF19 treatment was associated with satellite cell fusion, we evaluated the number of central nuclei in cross-sectional sections of soleus stained with haematoxylin and eosin. Results indicated no significant difference between conditions although there was a tendency (P = 0.12) for a higher number of central nuclei in CP rats (CPV and CPF) as compared to non-CP animals (V and F), with no difference induced by FGF19 treatment (V: 1.1 ± 0.2, F: 1.1 ± 0.4, CPV: 1.5 ± 0.4, and CPF: 2.1 ± 0.4 central nuclei per 100 muscle fibres. Data not shown).

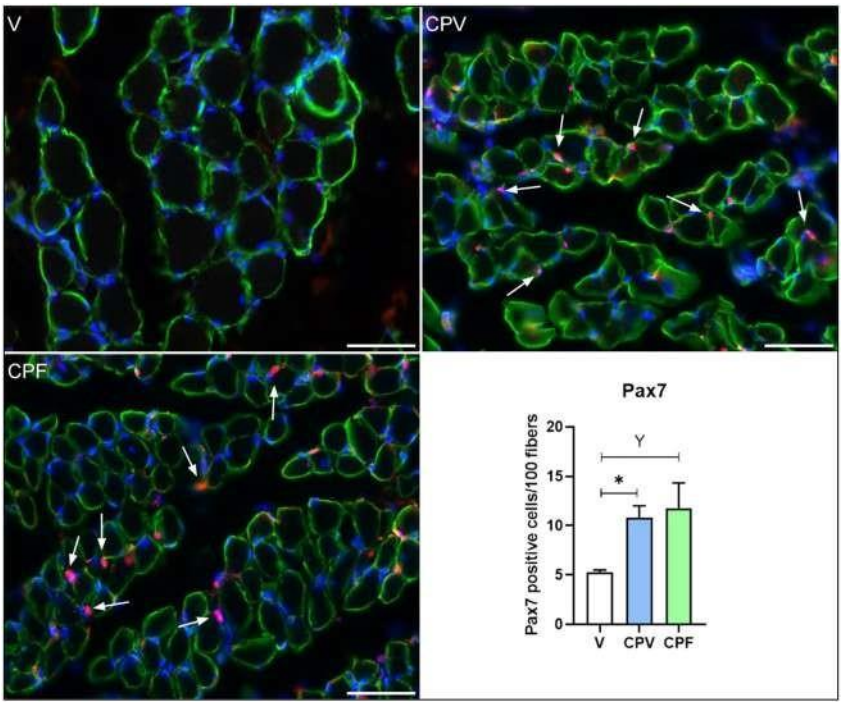

Figure 5 Cerebral palsy (CP) rats have increased number of Pax7 positive cells, which is not affected by FGF19 treatment. Pax7 positive cells were visualized after immunostaining in soleus muscle, counted and normalized by the number of laminin positive fibres (scale bars: 50 μm). V (control + vehicle); CPV (CP + vehicle); CPF (CP + FGF19). Data are expressed as mean ± SEM (n = 7–8 different animals per group). $P < 0.05$ for *CPV × V and ᵞCPF × V.

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Discussion

This proof-of-concept preclinical study aimed at evaluating whether a 1 week treatment with human FGF19 could improve motor functions and muscle alterations in an experimental model of CP. Perinatal anoxia associated to restriction of hind paws in rats has been previously reported as a representative model for CP.[7,8,10,11] Evaluated 29 days after birth, the CP animals presented significant defects in locomotion, motor coordination and muscle strength. These damages were associated with lower body weight, smaller area and perimeter of muscle fibres, and reduced bone mass of the tibia. In addition, our analysis of skeletal muscle gene expression revealed similar pattern of alterations than those reported in a genomic profiling study in wrist muscle of patients with CP.[15] Altogether, these data indicated that the experimental CP rat model used in our study closely mimicked the motor disturbances and muscle alterations observed in affected children.

In this study, our strategy was to administer human recombinant FGF19 by daily subcutaneous injections between Days 22 and 28 after birth in the rat model of CP in order to assess the therapeutic potential of FGF19. We found that this treatment improved locomotor activity as well as several musculoskeletal parameters (i.e. area and perimeter of muscle fibres, number of larger fibres, and tibia bone mass) linked to CP. In addition, the expression of several genes that were previously found altered in children with CP[15] was corrected by FGF19 treatment in CP rats. Our data suggest therefore that FGF19 could be a potential novel therapeutic compound against locomotor activity impairments and skeletal muscle weakness associated with CP.

In agreement with preceding reports,[9,10,12] perinatal anoxia and sensorimotor restriction of the posterior limbs affected the development of the animals, as evidenced by a reduction in body weight and weight of muscles and tibia bone. In children with CP, deficiencies in oral feeding and inadequate nutrition are regarded as a major cause of retarded growth and sub-optimal body fat reserves.[17] Here, FGF19 increased muscle and bone weight without affecting food intake. In adult mice, FGF19 treatment is accompanied by a reduction in body weight in obesity models, due to increased energy expenditure.[18,19] However, FGF19 is also known to preserve energy stores by increasing protein and glycogen synthesis in the liver,[20] and we recently discovered that it can also increase skeletal muscle mass in various mouse models.[14] We did not measure glycogen and other parameters in the liver, but we evidenced significant increase in soleus and EDL muscle weight as well as tibia bone. Mechanisms underlying these effects are not known and a potential effect of FGF19 as trophic factor in very young rats, cannot be excluded and remain to be evaluated.

The main defect in the experimental CP group was a marked impairment of locomotor activity, as evidenced both by a reduction in the distance travelled and average speed and by an increase in immobility in the open field test. These observations were consistent with previous studies showing that experimental CP model promotes physical changes interfering with gait performance.[8–10,12] Furthermore, we found a marked decrease in muscle strength using the forelimb suspension test. Reduction in skeletal muscle weight and strength in experimental CP has been previously reported.[7,8,10,12] During the postnatal period, the mechanical forces directed by the muscles adjacent to the bones were also found critical for bone development.[5] We observed that bones were also affected in experimental CP, with a reduction in weight and length of the tibia.

Importantly, the locomotor activity was improved after 1 week of FGF19 treatment. At the end of the treatment, we found that the animals travelled longer distance, had a higher average speed, and had a reduction in the immobility time. In agreement with the recent discovery that skeletal muscle is a direct target of FGF19,[14] we found that treatment with FGF19 increased the weight of soleus and EDL muscles in CP rats, with reduced proportion of very small muscle fibres and increased number of large fibres, and ultimately improved muscle strength. Furthermore, FGF19 treatment increased tibia weight, suggesting that FGF19 may contribute to the interplay between muscles and bones to sustain the development of the musculoskeletal system. Whether FGF19 acts directly on bone or indirectly through its effect on skeletal muscle[14] remains to be determined. Indeed, the literature is scarce regarding the effects of FGF19 on the musculoskeletal system; FGF19 was found expressed in foetal cartilage[21] and a study suggested a potential contribution to growth plate.[22] Whether an action of FGF19 in cartilage could have contributed to the observed increase in tibia weight in young rats remains to be evaluated.

In addition to locomotion defect, experimental CP was associated with a decrease in coordination, which is in agreement with a previous report.[12] Coordination is related to the control of movements, including muscle synergy, in which the neural command activates the co-contraction of specific muscles resulting in the generation of strength and movement in space. Children with CP have deficits in motor planning and execution that do not resolve over time.[23] Similarly, in experimental CP, impaired central brain networks may be responsible for impaired motor coordination.[24] While FGF19 improved locomotion and muscle weight, it did not significantly improve motor coordination as assessed by the rotarod test. This suggested that possible brain damages associated with experimental CP were not affected by treatment with FGF19.

To further shed light on the mechanism of action of FGF19 in skeletal muscle from rats submitted to CP, we performed specific gene expression analyses, using RT-qPCR, in soleus and EDL muscles. Transcriptional profiles

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of skeletal muscles from CP patients have been published, identifying several sets of genes with altered expression covering different cellular processes.[15,25] Interestingly, the observed adaptations in gene expression in CP were different from those found in other muscle diseases such as Duchenne muscular dystrophy and muscle atrophy induced by immobilization.[15] Furthermore, comparison of transcriptomic profiles in different muscles (wrist muscles and hamstring muscle) revealed increased expression of genes related to muscle immaturity in human CP.[25] In addition to extracellular matrix and fibre type-related genes, the microarray study in wrist muscles revealed an increase in the anabolic IGF1 (insulin like-growth factor 1) pathway (*Igf1* and *igfbp5* up-regulation), together with an increase in *Gdf8* (myostatin) and *Dmd* (dystrophin) mRNA levels.[15] Of note, one of the most up-regulated genes was *Kcnn3*, encoding the small-conductance calcium-activated potassium channel (SK3) protein.[15] These genes have all been associated with states of muscle atrophy or immaturity in the literature. Indeed, increased *Gdf8* expression has been already associated with skeletal muscle atrophy,[26] and *Kcnn3* gene is expressed in immature muscle cells.[27] Although IGF1 is generally viewed as an anabolic and trophic factor favouring myogenesis, its level is increased in denervated or paralyzed skeletal muscle in rats.[28] We therefore decided to investigate the expression of these genes in the experimental rat CP model. Interestingly, we found that CP is associated with an increase in the expression of *Igfbp5*, *Dmd*, and *Kcnn3*, as well as a tendency for an increase of *Igf1*, in soleus and EDL as compared with non-CP animals. Increased expression of *Gdf8* was also ob- served in the soleus muscle. Altogether, these data indi- cated that the molecular characteristics observed in the wrist muscles of patients with CP are conserved in the ex- perimental rat model.

During development, myogenesis is controlled by muscle regulatory factors including myogenin (*Myog*), Myod, and Myf5. Transcriptomic profiling revealed that the expression of these genes was not significantly altered in the muscle of children with CP.[15] In the experimental rat model, we found slightly different results, with no difference in *Myod*, increased expression of *Myf5,* and decreased expression of *Myog*. The myogenic factor Myf5 is among the first signs of myogenesis in mouse embryos and its expression decreases in the late myogenesis stages, when fibres become mature.[29] Myogenin is also involved in the control of the terminal differentiation of myoblasts to myocytes in embryos.[30] These data suggested the presence of more immature muscle cells in the experimental CP. This was also supported by the expression of *Troponin* and of metabolic genes such as *Ucp2*, *Lpl*, and *Ckmt2*, which are generally expressed in mature muscle cells and significantly down-regulated in the soleus muscle of CP rats. Further confirming a retarded development of skeletal muscles in experimental CP, we measured the expression of Pax7, a transcription factor specific of satellite cells and myoblasts, which is classically assessed to estimate the state of differentiation of muscle cells as well as the fusion of myoblasts to form mature fibres.[31,32] Pax7 mRNA levels were increased in both soleus and EDL in rat CP as well as Pax7 immunostaining in soleus supporting therefore a significant increase in the number of satellite cells in skeletal muscles in experimental CP.

Treatment with FGF19 did not modify the number of Pax7 positive cells in the soleus nor the mRNA of *Pax7* gene in the soleus and EDL muscle, indicating therefore that the beneficial effect of FGF19 in muscles was not associated with muscle regeneration or with fusion of satellite cells to form new fibres. This conclusion was also supported by the quantification of the central nuclei in soleus muscle which was not affected by FGF19 treatment, and by the lack of effect on the expression of myogenic factors (*Myog*, MyoD). These results agreed with our previous observations in mouse muscles and in primary culture of human myoblasts showing that FGF19 does not affect myoblast fusion and satellite cells mobilization to sustain its trophic effect on skeletal muscle fibres.[14] In this initial work, we characterized the signalling pathway required by FGF19 to stimulate muscle fibre enlargement. We demonstrated, both *in vitro* and *in vivo*, the involvement of the ERK1/2 mTOR pathway, but we did not identify specific downstream molecular targets in muscle cells.[14] In the present study, focusing on a pathological state with muscle atrophy, we found that the expression levels of several genes that were altered in experimental CP were corrected or restored almost to the control values in response to FGF19 treatment. One of the noticeable observations is that FGF19 significantly decreased *Igf1* and *Igfbp5* expression in the muscles of CP rats, suggesting a possible involvement of an IGF-1 related pathway in the beneficial effects of FGF19. The treatment also decreased the expression of *Dmd* and of *Myf5* in the skeletal muscles, as well of *Nes* and the NMJ-related genes *Kcnn3* and *Musk* in the soleus of CP rats. Increased expression of these different genes have been associated with an immature state of skeletal muscles,[25,26,28,33] and therefore, these data suggested that FGF19 could promote more mature muscles, associated with fibre size enlargement and restoration of muscle strength. However, how FGF19 can interact with these genes and with the IGF1 pathway remains to be investigated, because many overlapping mechanisms could be involved, including central effects increasing locomotor activity in addition to direct action on skeletal muscle.

FGF19 has been suggested to be responsible for growth and invasion of tumours in liver, contributing to hepatocellular carcinoma,[34] thus strongly limiting it therapeutic use in humans.[19] However, a non-mitogenic FGF19 analogue, called Aldafermin (or NGM282) has been developed, and this

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engineered form is not able to activate the signalling pathway essential for FGF19-mediated hepatocellular carcinoma, while retaining its ability to regulate metabolism.[35] Safety of Aldafermin in clinical trials has also been evidenced,[36] and despite nothing has been yet reported regarding its possible action on muscle, it might be interesting to envisage its utilization for indications such as CP.

Some limitations of this proof-of-concept study are the duration and window of the treatment, animals being sacrificed at P29, at the end of 1 week of daily treatment with FGF19. We were, therefore, unable to obtain information regarding the medium-term or long-term effects of the treatment, and we cannot ascertain that the observed improvements of locomotion and musculoskeletal system can be maintained overtime. Other periods or durations of FGF19 treatment could also produce different results. Finally, we explored only male animals and additional studies are required to verify whether the beneficial action of FGF19 is observed in both genders.

In summary, this pre-clinical study demonstrates that human recombinant FGF19 therapy could be a novel countermeasure with beneficial effects on locomotion and the musculoskeletal system in a rat model of CP closely mimicking children with CP. Although a number of additional experiments are needed to understand the precise mechanism of action and to demonstrate the long-term benefit of such treatment, our study opens new directions for establishing a possible novel strategy to fight against the locomotor consequences of CP, a highly debilitating neurological disease without efficient treatment.

Acknowledgements

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Online supplementary material

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Food consumption in the experimental cerebral palsy rat model. A) Food consumption (g). Food consumption was estimated by measuring offered diet minus the rejected diet in each day; B) Daily food consumption (g/day): Total food consumption/days assessed; C) Food efficiency coefficient (g/g): Body weight change/total food consumption. V (Control + Vehicle, n = 11); F (Control + FGF19, n = 10); CPV (CP + Vehicle, n = 12); CPF (CP + FGF19, n = 13). Data are expressed as mean ± SEM. *$p < 0.05$ comparing CPV and V; $^{\delta}$ $p < 0.05$ comparing CPF and F.

Figure S2. Frequency distribution of cross-sectional muscle fiber area from soleus and EDL in the different experimental groups.
V (Control + Vehicle, n = 11); CPV (CP + Vehicle, n = 10); CPF (CP + FGF19, n = 10). Data were expressed as mean ± SEM.

Figure S3. RT-qPCR quantification of the expression of a subset of genes in soleus muscle. Levels of the specific mRNAs were measured by RT-qPCR and normalized to Tbp. The data are presented in % of V group. V (Control + Vehicle, n = 7); CPV (CP + Vehicle, n = 8); CPF (CP + FGF19, n = 8). Mean ± SEM. * $p < 0.05$ (CPV vs. V).

Video S1. Representative video showing the locomotor activity in the open field test of a control rat (Vehicle), a rat subjected to cerebral palsy (CP + V) and a CP rat treated with human recombinant FGF19 (CP + FGF19) at 28 days of postnatal life.

Table S1. Sequences of the primers used for RT-qPCR analysis.

Conflict of interest

The authors declare that they have no conflict of interest.

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APÊNDICE B – NEONATAL RESVERATROL TREATMENT IN CEREBRAL PALSY MODEL RECOVERS NEURODEVELOPMENT IMPAIRMENTS BY RESTORING THE SKELETAL MUSCLE MORPHOLOGY AND DECREASES MICROGLIAL ACTIVATION IN THE CEREBELLUM

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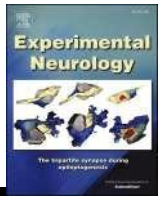

Research paper

Neonatal resveratrol treatment in cerebral palsy model recovers neurodevelopment impairments by restoring the skeletal muscle morphology and decreases microglial activation in the cerebellum

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ABSTRACT

Cerebral Palsy (CP) is the main motor disorder in childhood resulting from damage to the developing brain. Treatment perspectives are required to reverse the primary damage caused by the early insult and consequently to recover motor skills. Resveratrol has been shown to act as neuroprotection with benefits to skeletal muscle. This study aimed to investigate the effects of neonatal resveratrol treatment on neurodevelopment, skeletal muscle morphology, and cerebellar damage in CP model. Wistar rat pups were allocated to four experimental groups (n = 15/group) according CP model and treatment: Control+Saline (CS), Control+Resveratrol (CR), CP + Saline (CPS), and CP + Resveratrol (CPR). CP model associated anoxia and sensorimotor restriction. CP group showed delay in the disappearance of the palmar grasp reflex ($p < 0.0001$) and delay in the appearance of re- flexes of negative geotaxis ($p = 0.01$), and free-fall righting ($p < 0.0001$), reduced locomotor activity and motor coordination ($p < 0.05$) than CS group. These motor skills impairments were associated with a reduction in muscle weight ($p < 0.001$) and area and perimeter of soleus end extensor digitorum longus muscle fibers ($p < 0.0001$), changes in muscle fibers typing pattern ($p < 0.05$), and the cerebellum showed signs of neuro-inflammation due to elevated density and percentage of activated microglia in the CPS group compared to CS group ($p < 0.05$). CP animals treated with resveratrol showed anticipation of the appearance of negative geotaxis and free-fall righting reflexes ($p < 0.01$), increased locomotor activity ($p < 0.05$), recovery muscle fiber types pattern ($p < 0.05$), and reversal of the increase in density and the percentage of activated microglia in the cerebellum ($p < 0.01$). Thus, we conclude that neonatal treatment with resveratrol can contribute to the recovery of the delay neurodevelopment resulting from experimental CP due to its action in restoring the skeletal muscle morphology and reducing neuroinflammation from cerebellum.

1. Introduction

Brain injuries during early development can result in a permanent childhood movement and posture disorder known as cerebral palsy (CP). It affects children around the world differently varying depending on access to obstetric and neonatal care in the sociodemographic region (Graham et al., 2019; Gulati and Sondhi, 2017; Maenner et al., 2016; Oskoui et al., 2013; McIntyre et al., 2022). The Global prevalence of CP is estimated at approximately 1.6 per 1000 live births in high-income countries and its prevalence is markedly higher in low- and middle-

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income countries reaching up to 3.4 per 1000 live births (McIntyre et al., 2022). High-income countries deal with prematurity and morbidities related to low birth weight, while low- and middle-income countries tend to deal with infections in the pre- or post-natal period and perinatal asphyxia (Gulati and Sondhi, 2017; Wimalasundera and Stevenson, 2016).

The early insult that leads to CP occurs during the critical period of development, in other words, the insult occurs at some stage of the window of vulnerability of the central nervous system (CNS) (Marret et al., 2013; Semple et al., 2013). For example, acute or subacute perinatal events such as birth asphyxia are determinants in the white and gray matter lesions generally observed in children with CP (Marret et al., 2013). A window of greatest vulnerability of the white matter occurs between 24 and 34 weeks of gestation and is related to the active growth of brain pathways. It is a phase of high proliferation, migration and maturation of glial cells. At this stage, axonal and dendritic growth, synapse formation and myelination are also occurring, as well as stabilization processes such as synaptic pruning, and specialization of circuits that reach their peak in the postnatal period at around two years of age (Marret et al., 2013; Semple et al., 2013). Thus, isolated white matter damage or combined gray and white matter abnormalities are frequent neuroimaging findings (Korzeniewski et al., 2008) as well as reduced total brain, cerebellum, and gray matter volumes in children with cerebral palsy (Kułak et al., 2016).

Due to early damage to the CNS, children often experience delays in the acquisition of motor skills, including delays in the appearance of primitive reflexes or their inhibition. Primitive reflexes are automatic involuntary movements that occur in response to a stimulus. In humans, satisfactory brain maturity is essential to allow the inhibition of primitive reflexes and the appearance of postural responses for the normal progression of psychomotor functions; this requires a transition from an involuntary brainstem reflex response to one controlled by the cortex. A child with delayed psychomotor development, as happens at CP, may demonstrate difficulties in motor performance such as locomotion and movement coordination, these being related to the greatest risk for abnormal development (Chandradasa and Rathnayake, 2020; Kobesova and Kolar, 2014). The control of these primitive reflexes is carried out at levels of sensorimotor control within the CNS with the cerebellum involved in all levels of integration, acting in muscle tone regulation, postural and balance maintenance (Kobesova and Kolar, 2014). This plays an important role of the cerebellum in the study of CP motor damage. These studies, however, are scarce.

Experimental studies have shown outcomes similar to what occurs in children with CP and its associated mechanisms. Animal models show that after insults during fetal or perinatal development, an inflammatory cascade and cellular apoptosis are triggered, leading to brain tissue damage and consequent deficiency in motor control, delay in appearance or inhibition of primitive reflexes, delay in the development of motor skills, loss of muscle mass (sarcopenia), muscle weakness, change in the distribution of muscle fiber types, dyskinesia, spasticity, hyperreflexia, culminating in functional deterioration (Shi et al., 2019; Zhang et al., 2016; Peterson et al., 2013; Gutman et al., 2011; Costa-de-santana et al., 2023; Lacerda et al., 2017a). Motor disorders are characteristic of children with CP (Peterson et al., 2013; Brandenburg et al., 2019) and they are often accompanied by disturbances of sensation, perception, cognition, communication and behavior (Brandenburg et al., 2019; Rosenbaum et al., 2007). Even the very survival of children is often related to the severity of functional disability (Gulati and Sondhi, 2017) and intellectual disability (Liptak et al., 2004).

In this context, CP models have advanced in the scientific literature to study this disease. Among these models of CP, the model of combined insults anoxia and sensorimotor restriction (SR) stands out for promoting changes similar to CP in children, such as neuromuscular damage (Strata et al., 2004a; da Silva et al., 2016; Calado et al., 2023a; da Silva et al., 2023). The isolated anoxia model determines most of the damage to the central nervous system but it promotes subtle and reversible damage to motor skills, especially locomotion and SR, contributes strongly to the deterioration maintenance of the motor function (da Conceição et al., 2021). Both, anoxia and SR cause damage to the CNS such as increased pro-inflammatory cytokines (Stigger et al., 2013) and oxidative stress in the cerebral cortex (da Silva et al., 2023), decreased neurogenesis in the hippocampus (Calado et al., 2023a; Takada et al., 2016), increased activated microglia in the hippocampus (Calado et al., 2023a) and cerebellum (Costa-de-santana et al., 2023), confirming the inflammatory cascade and excitotoxicity damage installed resulting from experimental model. Consequently, locomotion, motor coordination (da Conceição et al., 2021; Pereira et al., 2021; Coq et al., 2008; Marcuzzo et al., 2010; Strata et al., 2004b) posture, gait pattern (da Silva et al., 2023), masticatory function (Lacerda et al., 2017b; Lacerda et al., 2021), behavior and memory (Calado et al., 2023a; Takada et al., 2016) are impaired, causing a delay in the ontogenesis of developmental reflexes (Costa-de-santana et al., 2023). Thus, experimental models have proven to be important tools for elucidating the mechanisms underlying CP; from these, it is possible to investigate potential therapeutic interventions through phenotypic plasticity.

Phenotypic plasticity can be broadly defined as the ability of a genotype to produce different phenotypes in response to environmental conditions leading to changes in shape, state, movement or rate of activity (West-eberhard, 1986; West-eberhard, 2005). Various biotic and abiotic factors can induce phenotypic plasticity such as local environmental factors, chemical, social and hormonal changes (West-eberhard, 1986; Kelly et al., 2012; Turcotte and Levine, 2016). Among these factors, nutritional agents are strongly involved in recovery of CP model impairments, as observed in previous studies (da Silva et al., 2023; Lacerda et al., 2021; Calado et al., 2023b; Visco et al., 2022) and can be a resource to be used in CP during the critical period of development such as the neonatal period. However, current pharmacological and non-pharmacological treatments aim to minimize the limitation of the child's functionality and improve the comorbidities associated with CP but they are not able to reversing primary neuromuscular disorders.

Therapeutic perspectives that act both CNS and skeletal muscle damage have been highlighted such as resveratrol, included within the group of functional foods named polyphenols (Adefegha, 2018). Resveratrol, administered during the critical period of development, has shown promising effects in models of early brain injury and models of muscular atrophy. Resveratrol is known for its neuroprotective and antioxidant effects in several neurological disease models including models with implications for CP (Arteaga et al., 2014; Arteaga et al., 2015; Girbovan and Plamondon, 2015; Pan et al., 2016; Cai et al., 2018). Pre-treatment with resveratrol has been shown to prevent neuronal damage in the dentate gyrus and parietal cortex, reducing infarct volume, preserving myelination and minimizing the astroglial reactive response in a hypoxic-ischemia model (Arteaga et al., 2015). In post-treatment, resveratrol has also shown similar effects including reduction of the expression levels of key inflammatory factors (Pan et al., 2016). In a model of intracerebral hemorrhage, treatment with resveratrol was also reported to be beneficial in reducing neuronal damage in the hippocampus and decreasing the activation of microglia in the cortex (Cai et al., 2018). Further, in a model of cerebral ischemia reperfusion, a reduction in microglial activation was reported in the hippocampus (Girbovan and Plamondon, 2015). Regarding the effects of resveratrol on skeletal muscle, studies have shown the attenuation of denervation-induced muscle atrophy (Asami et al., 2018) and obesity-related muscle atrophy (Bai et al., 2020; Huang et al., 2019),

On the other hand, resveratrol has been highlighted in previous studies of in vitro models for its antioxidant effects on the cerebellum as well, directly related to muscular control. Resveratrol showed an anti-apoptotic effect in apoptotic models in rat cerebellar granule neurons (CGNs) (an in vitro model of Parkinson's disease) (Alvira et al., 2007), as well as a neuroprotective effect on cerebellar granule neurons in models of ammonia (Bobermin et al., 2015) and ethanol neurotoxicity (Kumar et al., 2011). Resveratrol also appears to act in models of neurological

disease that affect motor performance. Post-treatment with resveratrol evidently improved motor performance as well as muscle activity accompanied by a protection of Purkinje cells in ataxic rats (Ghorbani et al., 2018), in addition to benefits CP model in posture and behavior (Calado et al., 2023a; da Silva et al., 2023).

This makes resveratrol a possible treatment in CP models. Considering that studies on the effects of resveratrol have provided a new window of promising therapeutic action in CP, the present work aimed to investigate the effects of resveratrol neonatal treatment on neurodevelopment, skeletal muscle morphology and cerebellar damage in CP model. The hypothesis of this study was that resveratrol recovers cerebellum and musculoskeletal damage caused by CP model, reversing the damage to motor development.

2. Material and methods

2.1. Animals and experimental groups

This was an experimental study with animals carried out by the Studies in Nutrition and Phenotypic Plasticity Unit, Department of Nutrition, Federal University of Pernambuco, Recife, Pernambuco, Brazil. All procedures were carried out in accordance with the guidelines of the National Council for the Control of Animal Experimentation (CONCEA) and the international standards of the National Institute of Health Guide for Care and Use of Laboratory Animals (8th ed.). The project was approved by the Animal Use Ethics Committee (CEUA) of Federal University of Pernambuco n°0009/20.

Male Wistar rat pups were used. They were kept in standard vivarium conditions with a temperature of 22 ± 2 °C, inverted light-dark cycle of 12/12 h (Light cycle - 8:00 p.m. to 8:00 a.m.; Dark cycle - 08:00 to 20:00 h), housed in polypropylene cages (46cmx34cmx20cm) lined with sterile wood shavings with free access to water and a standard vivarium diet. The offspring were obtained from 30 nulliparous Wistar rats. On the day of birth, male puppies were randomized, with an ideal body weight (6 to 8 g), distributed into experimental groups, based on the induction of cerebral palsy and the neonatal pharmacological manipulation administered: Control Saline (CS, n = 15), consisting of pups that received saline solution; Control Resveratrol (CR, n = 15), consisting of pups that received treatment with resveratrol; CP Saline (CPS, n = 15), consisting of pups subjected to the experimental CP model and which received saline solution; 4- CP resveratrol (CPR, n = 15), consisting of pups subjected to the experimental CP model and which received resveratrol. Each litter consisted of 8 pups. Half of the pups (n = 4) were subjected to the CP model and the other half (n = 4) were controls. All litters were composed of the same number of pups with similar levels of motor function to avoid disproportionality between litters in terms of competitiveness for breast milk. Male animals present were distributed according to pharmacological intervention with four groups in each litter. They remained with their mothers until the 25th postnatal day, when they were weaned and the males were placed in individual cages until euthanasia on the 29th postnatal day. Some animals were euthanized by decapitation and others by transcardiac perfusion.

2.2. Cerebral Palsy model

The CP model that was performed is based on experiments that associate perinatal anoxia and sensorimotor restriction of the hind paws (da Silva et al., 2016; Coq et al., 2008; Lacerda et al., 2017b). The pups in the CP groups were subjected to two episodes of postnatal anoxia, on the day of birth and on the first day of life (P0 and P1). The pups were placed inside an acrylic chamber partially immersed in water at 37° and exposed to nitrogen (100%) at 9 L/min for 12 min. Then returned to their respective mothers when their color and respiratory rate was completely restored. From the 2nd to the 28th day of life (P2 to P28), sensorimotor restriction of the hind paws was carried out for 16 h a day, with the animal being allowed free movement for the remaining 8 h. For sensorimotor restriction, an orthosis was fixed in an epoxy mold, keeping the hind legs extended, without the elimination of urine and fecal material, and maternal care being compromised (Coq et al., 2008).

2.3. Resveratrol treatment

After the birth of the pups, the males were randomly allocated according to the experimental groups. During the neonatal period, from the 3rd to the 21st day of postnatal life, saline or resveratrol were administered intraperitoneally (Girbovan and Plamondon, 2015), were distributed in 1- saline (0.9% NaCl) or 2- treated with resveratrol (daily dose, 10 mg/kg), injection volume was 0.1 ml/100 g rat weight. The rats were weighed daily and the injection volume was adjusted to match the animal's body weight.

2.4. Assessment of neurodevelopment

2.4.1. Ontogenesis of primitive reflexes

Daily, from the 1st to the 21st postnatal day, primitive reflexes related to neuromotor maturation were evaluated from randomized animals (n = 10 per group) (Falcão-Tebas et al., 2012; Fox, 1965). Palmar grasp reflex (PG) was verified by a rapid flexion of the fingers after two light percussions on the front paw palm. The first of a series of three consecutive days in which the expected response disappeared completely was considered the day of PG reflex maturation. Similarly, other primitive reflexes were also assessed, however, the first of a series of three consecutive days in which the expected response appeared completely was considered the day of reflex maturation. The following reflexes were: righting reflex (RR) - turning the body from supine to prone in up to 10 s; vibrissa placing (VP) - reflex of placing the front paws on the table, trying to walk when suspended by the tail within 10 s; cliff avoidance (CA) - 45° angular displacement of the animal in up to 10 s, when the animal is placed with its front paws on the edge of a flat, high surface considered a cliff; auditory startle response (ASR) - simultaneous and rapid retraction with the involuntary immobilization of the animal's body after an acute shock; negative geotaxis (NG) - center of a 45° inclined ramp, the animal with its head in a downward direction turns in up to 10 s and positions its head in an upward direction; free-fall righting (FFR) - the animal's turn in free fall from 30 cm in height, when held by all four paws in dorsal decubitus, resting on four paws on a cotton bed (Falcão-Tebas et al., 2012; Fox, 1965).

2.4.2. Locomotor activity

Randomized animals for locomotor activity were evaluated at the ages of 8, 14, 17, 21 and 28 postnatal days through the automatic evaluation of locomotor parameters using Anymaze® software (San Diego Instruments). The animals executed their trajectory in a circular open field (n = 10 per group). The animals were placed in the center of an open field and recorded by video for a period of 5 min each. The test was performed in a dark room attached to the maintenance vivarium, during the dark cycle, in when the animal was naturally in a state of wakefulness, due to the stress caused by having had application of the drug three hours before. In the interval between filming the different animals, the field was cleaned with 3% hypochlorite and the EVA was changed so that the odor of the previous animal would not influence the test of the next animal. The locomotor parameters obtained were: distance traveled (m); average speed (m/s); time stopped (s); total number of stops made by the animal (n); time spent in the central (zone 1), intermediate (zone 2) and peripheral (zone 3) areas of the open field (s).

2.4.3. Motor coordination assessment

The performance test was carried out with a rotarod apparatus (Insight) at 29 days of postnatal life. Each animal was randomly grouped (n = 10 per group) and placed in the rotarod on a rotating rod measuring 60 mm in diameter and 75 mm in length. Initially, the animals underwent an adaptation period on the apparatus for 2 min at a speed of 16

rpm. After waiting for a 2 min rest period, the animals were placed individually on the rotarod for 5 attempts, respecting the 2 min rest interval, with a speed of 25 rpm for a maximum of 3 min so that the fall latency could be recorded (adapted from (Stigger et al., 2011a)). The fall latency analysis was summarized as the average of the 5 attempts performed by each animal.

2.5. Analysis of skeletal muscle

2.5.1. Euthanasia, collection and weight of the soleus and EDL muscles

At 29 days of postnatal life, part of the animals were euthanized by decapitation ($n = 6$ per experimental group). Then, soleus and extensor digitorum longus muscles of the right paws were dissected, weighed in a precision scale (model Marte AUW220, 220 g capacity and 0.1 mg sensitivity). The absolute weight of both muscles and their relative weight were obtained, normalizing the weight of each muscle by body weight. Both muscles were frozen in n-hexane (pre-cooled carbon dioxide solidifies −78.5 °C) and stored at −80 °C for subsequent histochemical procedures and morphologic analysis.

2.5.2. Morphological analysis of the soleus and EDL muscles

Coronal sections (8 μm) were taken in a cryostat at −30 °C, of the soleus and extensor digitorum longus muscles, collected from the right paw on the 29th day of life. The sections were fixed on slides and stained using the myofibrillar ATPase technique after reaching room temperature (Lacerda et al., 2017b; Brooke and Kaiser, 1970). Muscle fibers were stained for the three main fiber types (I, IIa, IIb), according to differences in the intensity of ATPase staining after acid pre-incubation (pH 4.3 and 4.55). In pre-incubation at pH 4.3 the fibers were stained type I (darker) and type II (lighter) and at pH 4.55 the fibers were stained type I (darker), type IIa (pale) and type IIb (gray). The sections were analyzed with an optical microscope (Olympus BX-41, 40×) connected to a computer with Analysis Get It image capture software. All muscle fibers were counted in each histological section, with the values presented for the different types of fibers as a percentage of the total number. The area and perimeter from muscle fibers were also analyzed, defining the area as the measurement of the surface of each fiber and the perimeter as the sum of the measurements of all sides of each fiber. ImageJ® software (version 1.51p) was used to fiber count and analyze the measure area and perimeter of the fibers.

2.6. Analysis of the cerebellum

2.6.1. Euthanasia and collection of cerebellum

The other part of the animals in the experimental groups ($n = 6$ per experimental group) received an intraperitoneal injection of BrdU (200 mg/kg body weight) at P5 e P6 of postnatal life and were sacrificed at 29 days by transcardiac perfusion, anesthetized and perfused with PBS and a 4% paraformaldehyde solution. Then the brain tissue was dissected, post-fixed, and stored in a cryoprotectant solution. Sections 40um thick were collected from cerebellum and subjected to immunohistochemistry (Visco et al., 2022).

2.6.2. Analysis of cell proliferation in the cerebellum

The cerebellum slices were washed in a phosphate-buffered substance (PB) and incubated in Triton (X-100 0.3%, St. Louis, MO, USA) containing 10% hydrogen peroxide. The sections were then incubated in absolute methanol and washed in PB and incubated in formamide (50% in sodium citrate salt solution, Sigma-Aldrich) at 65 °C for 2 h. After washing, denaturation of DNA was performed in HCl (1 N) at 37 °C. The sections were then incubated in borate buffer solution (pH 8.4) (Ye et al., 2012). The sections were subsequently incubated overnight (4 °C) in a solution of primary anti-BrdU antibody (mouse anti-BrdU, 1: 30,000, Roche Molecular). The following day, the sections were washed for 4 to 5 min in PBS and incubated in the dark for 2 h with biotinylated secondary antibody (1: 750, Vector Laboratories) and developed in an avidin–biotin complex (Elite ABC kit, Vector Laboratories) and diaminobenzidine (DAB staining kit, Vector Laboratories). The cerebellum sections were subsequently washed, dried, and mounted on slides with Cytoseal, and covered with coverslips. Finally, images were captured using a 20× objective optical microscope of 4–5 sections per animal. The number of BrdU + cells in the cerebellum was counted by a blinded researcher for each experimental group (Visco et al., 2022). The boundaries of the Crus 1 and Crus 2 regions of the cerebellum were digitally delineated.

2.6.3. Analysis of microglia density and activated microglia in the cerebellum

The microglia profile was conducted using brain sections incubated first in 10% H2O2 in methanol and subsequently in 10% H2O2 in phosphate buffer (0.1 M, pH 7.4) containing 3% Triton X-100 (PBT). The sections were then incubated at 4 °C for 48 h in primary ionized calcium-binding adapter molecule 1 antibody (Iba1) (rabbit anti-Iba1/ IAF1, 1: 30,000, Wako), diluted with 5% horse serum in PBT (Saavedra et al., 2021). The sections were subsequently incubated in a secondary antibody (biotinylated anti-rabbit; 1:750, Sigma-Aldrich) for a period of 2 h at 4 °C. The brain sections were then incubated in solutions of avidin–biotin-peroxidase complex (ABC Elite Kit; Vector Laboratories, Burlingame, CA, USA) and a solution of the diaminobenzidine staining kit (DAB Kit; Vector Laboratories) to stain the microglia (Saavedra et al., 2021). Sections from each group were processed in parallel to avoid non-specific staining effects (Visco et al., 2022). The brain sections were then mounted on gelatinized slides and covered with Cytoseal coverslips (Thermo Scientific, USA). Evaluation of Iba1 + microglial cells in the cerebellum ($n = 6$ per experimental group) was conducted by selecting two fields per section from a total of four sections randomly selected by brain section from the Crus 1 and Crus 2 regions, giving a total of eight photographs per area of the cerebellum for each animal. The selected fields were captured using a light microscope (Zeiss, Germany) 20 × objective. A blinded researcher used ImageJ software to count the number of cells Iba1-immunoreactive by area and classify the profile of the microglia according to the previous descriptions. Microglial cells with a small soma and few to numerous processes were considered to be branched microglia (types I–III), while those with a large soma or amoeboid body and thicker, shorter processes were considered to be activated microglia (types IV–V) (Saavedra et al., 2021; Roque et al., 2016). The data presented were microglia density (n/mm3—total cells divided by the area assessed. 1000,000. 0.001. 40) and the percentage of microglia activation relative to the total (%) (Visco et al., 2022).

2.7. Statistical analysis

The data obtained were initially analyzed for normal distribution with the Shapiro-Wilk test. If a normal distribution was found, appropriate parametric tests were carried out, such as the Two-Way Anova, to compare groups in analyses, carried out for animals of the same age, or the Two-Way Anova Repeated Measures to compare groups in analyses carried out with animals of different ages. If a normal distribution was not observed, non-parametric statistical tests such as the Kruskal-Wallis and Friedman tests were adopted. The results were expressed as mean ± standard error of mean or as median and maximum and minimum values, with a significance level of 5%. The GraphPadPrism® version 9 software was used to analyze the data and construct the graphs.

3. Results

3.1. Neonatal treatment with resveratrol reverses the delay in reflex ontogenesis caused by experimental CP

The cerebral palsy model caused changes in developmental reflexes. There were delay in the disappearance of the palmar grasp reflex (CPS: 14.7 ± 0.67 days / CS: 10.4 ± 0.79 days, $p < 0.0001$) and a delay in the

appearance of reflexes of negative geotaxis (CPS: 15.4 ± 0.69 days / CS: 12.7 ± 0.3 days, $p = 0.01$) and free-fall righting (CPS: 15.2 ± 0.53 days / CS: 9.0 ± 0.89 days, $p < 0.0001$) compared to the control group. Treatment with resveratrol was able to reverse this delay in the appearance of negative geotaxis reflexes (CPR: 12.2 ± 0.53 days / CPS: 15.4 ± 0.69 days, $p < 0.01$) and free-fall righting (CPR: 12.2 ± 0.55 days / CPS: 15.2 ± 0.53 days, $p < 0.01$) (Fig. 1). No differences were observed between the groups regarding righting, vibrissa placing, cliff avoidance reflex and auditory startle response ($p > 0.05$) (Fig. 1).

3.2. Neonatal treatment with resveratrol reverses impairments in locomotor development

The experimental CP reduced the locomotor activity of the animals. At 21 and 28 days of postnatal life, the CP group performed shorter distance traveled (P21: CPS: 15.30 ± 2.02 m / CS: 26.76 ± 7.42 m, $p = 0.02$ / P28: CPS: 15.45 ± 2.91 m / CS: 40.57 ± 4.03 m, $p < 0.0001$) and average speed (P21: CPS: 0.51 ± 0.06 m/s / CS: 0.89 ± 0.08 m/s, $p = 0.02$ / P28: CPS: 0.52 ± 0.09 m/s / CS: 1.35 ± 0.13 m/s, $p < 0.0001$) compared to control group (Figs.2A and B). The time stopped (CPS: 148.46 ± 14.82 s / CS: 52.03 ± 3.44 s, $p < 0.0001$) and the number of stops (CPS: 24.50 ± 1.78 / CS: 12.88 ± 1.64, $p < 0.001$) was increased in the CP group compared to the control group at 28 days of life (Figs.2C and D).

The animals' exploratory capacity was also affected by the CP model. Animals in the CPS group had more time spent in zones 1 and 2 (central and intermediate area of the open field) at 17 days of life (Zone 1: CPS: 91.05 ± 29.35 s / CS: 25.94 ± 11.79 s, $p < 0.0001$ / Zone 2: CPS: 86.54 ± 20.70 s / CS: 28.87 ± 10.15 s, $p < 0.0001$), and less time spent in zone 3 (peripheral region of the field) at 14 (CPS: 87.75 ± 25,79 s / CS: 187,78 ± 18.83 s, $p < 0.0001$) and 17 (CPS: 129.36 ± 29.65 s / CS: 243.94 ± 20.63 s, $p < 0.0007$) days of postnatal life compared to control group (Figs.2E, F and G).

Treatment with Resveratrol was able to reduce damage in locomotion. CPR group showed an increase in the distance traveled (P21: CPR: 27.37 ± 3.50 m / CPS: 15.30 ± 2.02 m, $p < 0.05$ / P28: CPR: 30.34 ± 4.48 m / CPS: 15.45 ± 2.91 m, $p < 0.01$) and average speed (P21: CPR: 0.92 ± 0.12 / CPS: 0.51 ± 0.07 m/s, $p < 0.05$ / P28: CPR: 1.01 ± 0.15 / CPS: 0.52 ± 0.09 m/s, $p < 0.01$) at 21 and 28 compared to CPS (Fig. 2A and B). Consistently, resveratrol in experimental CP led to a decrease in time stopped (CPR: 89.42 ± 16.76 s / CPS: 148.46 ± 14.82 s, $p < 0.0001$) and the number of stops (CPR: 16.56 ± 2.93 / CPS: 24.50 ± 1.78, $p < 0.001$) made in the open field at 28 days of life compared to the CPS group (Fig. 2C and D).

Similarly, resveratrol also had positive effects on open field exploratory ability. Animals in the CPR group had less time spent in open field zones 1 and 2 at 17 days of life (Zone 1: CPR: 27.45 ± 6.03 s / CPS: 91.05 ± 29.35 s, $p < 0.05$ / Zone 2: CPR: 32.62 ± 5.25 s / CPS: 86.54 ± 20.70 s, $p < 0.05$), and more time spent in zone 3 at 14 (CPR: 176.87 ± 80.91 s / CPS: 87.75 ± 25.79 s, $p < 0.001$) and 17 (CPR: 256.57 ± 8.51 s / CPS: 129.36 ± 29.65 s, $p < 0.0001$) days of postnatal life compared to CPS group (Figs.2E, F and G).

Exposure to neonatal resveratrol in control animals also stimulated locomotion in the initial ages of locomotor development but this was not maintained at the other ages evaluated. CR group showed a decrease in the number of stops at 8 days (CR: 17.5 ± 1.79 / CS: 32.70 ± 2.45, $p < 0.0001$) and greater average speed at 14 days (CR: 0.77 ± 0.07 / CS: 0.39 ± 0.06 m/s, $p < 0.05$) compared to CS group (Figs.2B and D).

3.3. Motor coordination in a CP model is not influenced after neonatal treatment with resveratrol

Motor coordination was assessed using the rotarod performance test at 29 days of age. We observed a reduction in the animals' ability to stay on the moving rod, thus they reduced their fall latency in the rotarod (CPS: 2.66 ± 0.24 s / CS: 21.37 ± 7.06 s, $p = 0.0114$) (Fig. 3).

3.4. Muscle weight is not influenced after neonatal treatment with resveratrol in a CP model

The animals subjected to CP had absolute weight of the soleus muscles (CPS: 0.0091 ± 0.002 g / CS: 0.0313 ± 0.001 g, $p < 0.0001$) and EDL muscles reduced (CPS: 0.0155 ± 0.002 g / CS: 0.0277 ± 0.001 g, $p < 0.0003$) as well as the relative weight of the soleus muscles (CPS: 0.0002 ± 0.000 g / CS: 0.0004 ± 0.000 g, $p < 0.0001$) at 29 days compared to the control group (Fig. 2A, B and C). This demonstrates that the CP model used in this study caused atrophy of the muscles in the animals' hind limbs. However, there was no significant difference between animals in the CPS and CPR groups, which demonstrated that treatment with resveratrol had not affected muscle mass in this CP model (Fig. 4).

3.5. Neonatal resveratrol treatment reverses damage to distribution pattern of muscle fiber types

Corroborating the reduction in muscle weight, the area and perimeters of the soleus and EDL muscle fibers were reduced in animals subjected to the CP model compared to the control (Soleus area: CPS: 1241 ± 108.29 μm^2 / CS: 4058 ± 382.53 μm^2, $p < 0.0001$; Solo Perimeter: CPS: 148 ± 6.08 μm / CS: 263 ± 9.71 μm, $p < 0.0001$; EDL Area: CPS: 885 ± 68.62 μm^2 / CS: 3.326 ± 228.52 μm^2, $P < 0.0001$; EDL Perimeter: CPS: 122 ± 3.79 μm / CS: 238 ± 5.10 μm, $p < 0.0001$). Resveratrol treatment in CP led to an increase in the perimeter of muscle fibers in the EDL muscle compared to the CPS group (CPR: 172 ± 10.50 μm / CPS: 122 ± 3.79 μm, $p < 0.0232$) (Fig. 5A, B and G).

The induction of experimental CP also resulted in a distortion of the distribution pattern of muscle fiber types. The CPS group had a lower percentage of type I fibers in the soleus muscle (pH 4.3 CPS: 30.1 ± 5.40% / CS: 61.6 ± 2.74%, $p = 0.0001$ / pH 4.55 CPS: 26.6 ± 5.40% / CS: 67.6 ± 4.23%, $p < 0.001$) and a higher percentage of type II fibers (pH 4.3 CPS: 69.9 ± 5.40% / CS: 38.4 ± 2.74%, $p = 0.0001$ / pH 4.55 fibers IIa CPS: 36.5 ± 9.05% / CS: 16.4 ± 3.23%, $p = 0.0379$; fibers IIb CPS: 42.1 ± 2.01% / CS: 16.0 ± 3.10%, $p < 0.0004$) compared to the CS group (Figs. 5C and D). In the EDL muscle, the CPS group showed an increase in type I fibers (pH 4.3 CPS: 29.3 ± 5.40% / CS: 12.1 ± 1.37%, $p = 0.0006$ / pH 4.55 CPS: 33.8 ± 4.79% / CS: 12.5 ± 2.35%, $p < 0.001$)

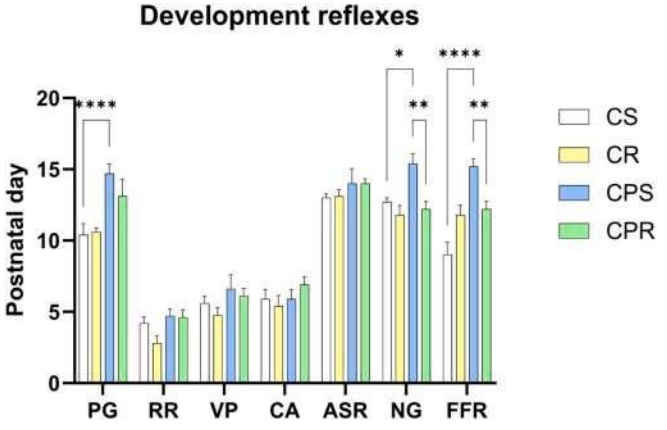

Fig. 1. Effects of neonatal resveratrol treatment on reflex ontogenesis in a CP model. Control Saline (CS, $n = 10$), Control Resveratrol (CR, n = 10), Cerebral Palsy + Saline (CPS, n = 10) and Cerebral Palsy + Resveratrol (CPR, n = 10). Primitive reflex maturation was considered the disappearance of the palmar grasp reflex (PG) and the appearance of righting reflex (RR), vibrissa placing reflex (VP), cliff avoidance (CA), auditory startle response (ASR), negative geotaxis (NG) and free-fall righting (FFR) reflexes. Data were expressed as mean ± SEM. Two-Way ANOVA and Tukey's post hoc test *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

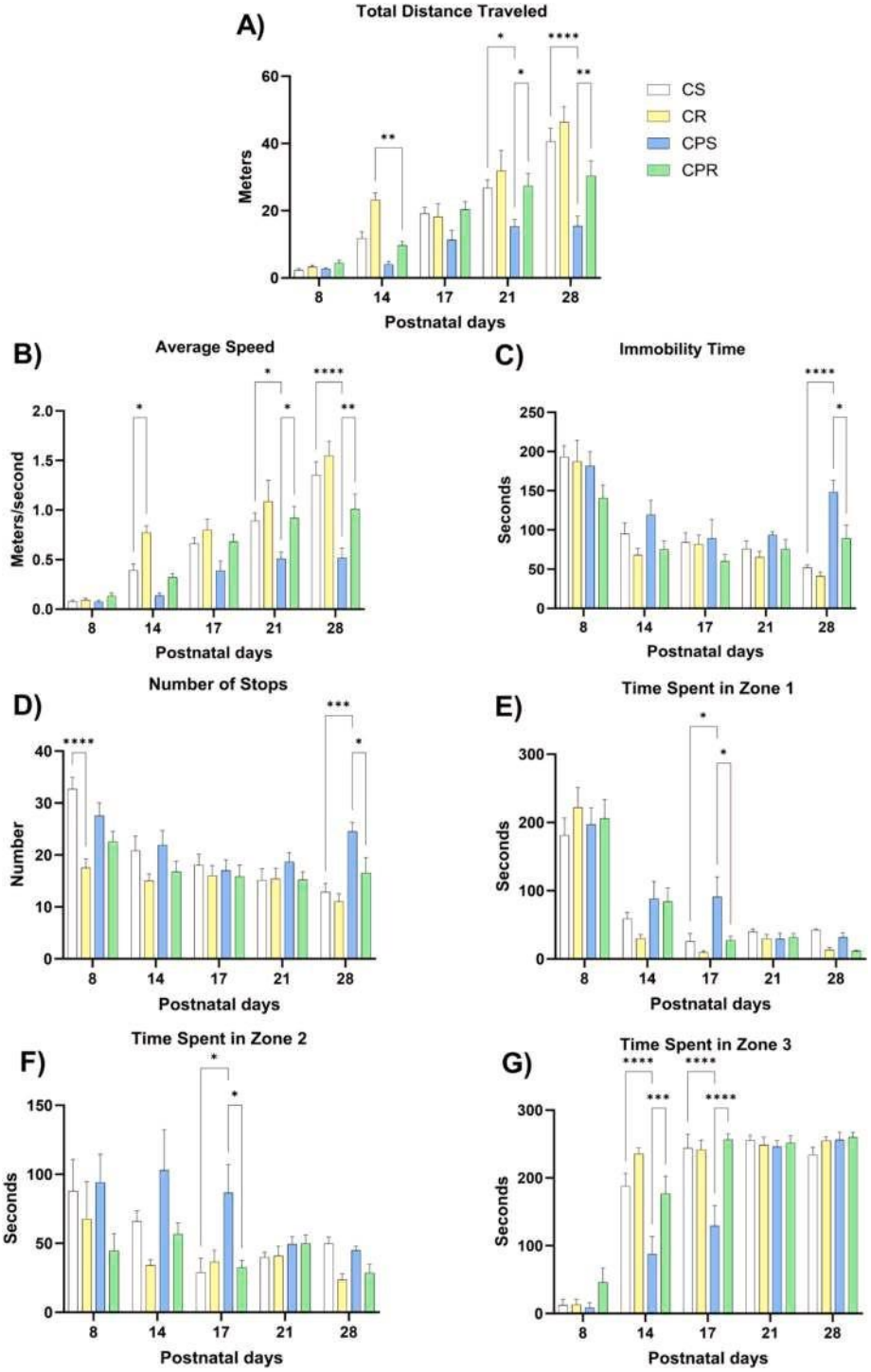

Fig. 2. Effects of neonatal resveratrol treatment on development of locomotor activity in CP model and control animals. Control Saline (CS, $n = 10$), Control Resveratrol (CR, n = 10), Cerebral Palsy + Saline (CPS, $n = 10$) and Cerebral Palsy + Resveratrol (CPR, $n = 10$). A) Distance traveled (m); B) Average speed (m/s); C) Immobility Time (s); D) Total number of stops made by the animal (n); E) Time spent in the central area (zone 1), F) Time spent in the intermediate area (zone 2) and G) Time spent in the peripheral area (zone 3) of the open field. Data were expressed as mean ± SEM. Two-Way ANOVA and Tukey's post hoc test *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

and decrease in type II fibers (pH 4.3 CPS: 70.7 ± 5.40% / CS: 87, 1 ± 1.65%, $p < 0.01$ / pH 4.55 fibers IIa CPS: 29.0 ± 8.36% / CS: 65.8 ± 7.34%, $p = 0.0088$; fibers IIb CPS: 41.2 ± 6 0.34% / CS: 16.3 ± 3.58%, $p = 0.0083$) compared to the CS group (Figs. 5E, F and G).

Treatment with resveratrol led to a reduction in the damage caused by the CP model in the typing of muscle fibers in both muscles. In the soleus muscle, animals in the CPR group compared to the CPS group showed an increase in type I fibers (pH 4.3 CPR: 56.4 ± 4.75% / CPS: 30.1 ± 5.40%, $p = 0.0004$ / pH 4.55 CPR: 62.2 ± 5.13% / CPS: 26.6 ± 5.40%, $p < 0.001$), decrease in type II fibers, specifically type IIb fibers (pH 4.3 CPR: 43.6 ± 4.75% / CPS: 69.9 ± 5.40%, $p < 0.01$ / pH 4.55 fibers IIb CPR: 14.1 ± 2.75% / CPS: 42.1 ± 2.01%, p = 0 0001) (Figs. 5C and D). In the EDL muscle there was a decrease in type I fibers (pH 4.3 CPR: 17.3 ± 1.61% / CPS: 29.3 ± 5.40%, $p < 0.05$/ pH 4.55

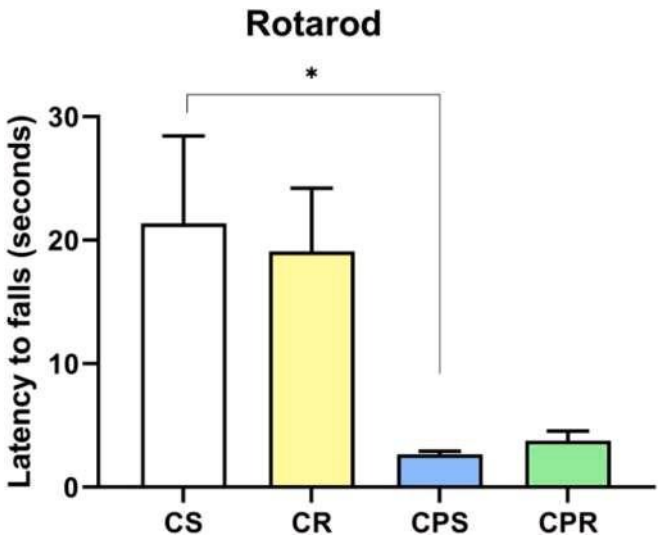

Fig. 3. Effects of experimental CP on motor coordination in rats. Control Saline (CS, *n* = 10), Control Resveratrol (CR, n = 10), Cerebral Palsy + Saline (CPS, n = 10) and Cerebral Palsy + Resveratrol (CPR, n = 10). Data were expressed as mean ± SEM. Two-Way ANOVA and Tukey's post hoc test *p < 0.05, **p < 0.01, ***p < 0.001, ****$p < 0.0001$.

CPR: 17 ± 1.72% / CPS: 33.8 ± 4.79%, $p = 0.0032$) and increase in type II fibers (pH 4.3 CPR: 82.7 ± 1.61% / CPS: 70.7 ± 5.40%, p < 0.05/ pH 4.55 fibers IIa CPR: 73.7 ± 2.50% / CPS: 29.0 ± 8.36%, $p = 0.0012$ / fibers IIb CPR: 9.3 ± 2.95% / CPS: 41.2 ± 6.34%, $p = 0.00122$) (Figs. 5E, F and G).

3.6. Cell proliferation in the cerebellum is not influenced by the CP model or neonatal resveratrol treatment

Neither the CP model used in the present study nor the treatment with resveratrol influenced cell proliferation in Crus 1 areas (CS: 1264.3 ± 100.37 / CR: 1164.84 ± 200.89 / CPS: 960.94 ± 105.05 / CPR: 1291.55 ± 185.54 cells/mm^2, $p > 0.05$) and Crus 2 (CS: 1009.81 ± 78.64 g / CR: 812 ± 152.51 / CPS: 601.74 ± 127.12 / CPR: 845.69 ± 104.53 cells/mm^2, p > 0.05) of the rat cerebellum as observed by immunohistochemical analysis of BrdU+ cells (Figs. 6A, B and C).

3.7. Neonatal treatment with resveratrol reverses damage in the density of microglia and percentage of activated microglia in the cerebellum

Immunohistochemical analysis of the cerebellum Crus 1 and Crus 2 areas revealed changes in the density of microglia and percentage of activated microglia from experimental groups through the presence of Iba$_+$ cells. In the Crus 1 and Crus 2 cerebellum, the density of microglia was increased in rats subjected to CP (Crus 1 CPS: 1157.12 ± 66.68 cells/mm^2 / CS: 601.27 ± 17.21 cells/mm^2, $p < 0.0001$ / Raw 2 CPS: 1006.85 ± 50.29 cells/mm^2 / CS: 560.64 ± 53.80 cells/mm^2, p < 0.0001) (Figs.7A and B), as well as an increase in the percentage of activated microglia in the CPS group compared to the control (Crus 1 CPS: 57.46 ± 1.41% / CS: 42.37 ± 1.66% p < 0.01 / Crus 2 CPS: 63.11 ± 1.58% / CS: 51.24 ± 3, 04% $p < 0.05$) (Figs.7C, D and E). Thus, the CPS group cerebellum showed signs of neuroinflammation due to elevated density of microglia and the percentage of activated microglia compared to CS group.

Treatment with neonatal resveratrol in CP compared to the CP group treated with saline reduced the density of microglia (Crus 1 CPR: 762.94 ± 51.12 cells/mm^2 / CPS: 1157.12 ± 66.68 cells/mm^2, $p < 0.001$ / Crus 2 CPR: 544.08 ± 46.46 cells/mm^2 / CPS: 1006.85 ± 50.29 cells/mm^2, p < 0.0001) and percentage of activated microglia (Crus 1 CPR: 43.79 ± 2.41% / CPS: 57.46 ± 1.41%, $p < 0.01$ / Crus 2 CPR: 32.47 ± 1.71% / CPS: 63.11 ± 1.58%, p < 0.0001), demonstrating a reverse of the damage to the Crus 1 and Crus 2 cerebellum caused by the experimental CP (Fig. 7A, B, C, D and E).

Resveratrol also led to changes in the pattern of cerebellar microglia in control animals. The CR group also had increased microglia density in the cerebellum area Crus 1 (Crus 1 CR: 855.58 ± 40.46 cells/mm^2 / CS: 601.27 ± 17.21 cells/mm^2, p < 0.0001) (Fig. 7A); the percentage of

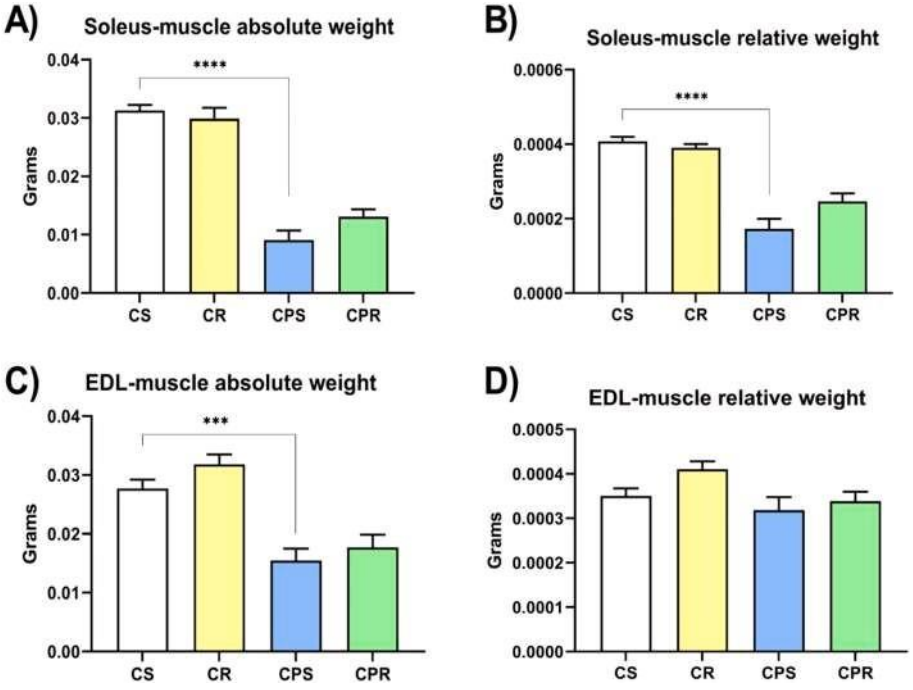

Fig. 4. Effects of experimental CP on soleus and EDL muscle weight in rats. Muscle absolute weight (g) and Muscle relative weight (g) of soleus muscle (A e B) and EDL muscle (C e D). Control Saline (CS, *n* = 8), Control Resveratrol (CR, n = 8), Cerebral Palsy + Saline (CPS, n = 8) and Cerebral Palsy + Resveratrol (CPR, n = 8). Data were expressed as mean ± SEM. Two-Way ANOVA and Tukey's post hoc test *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

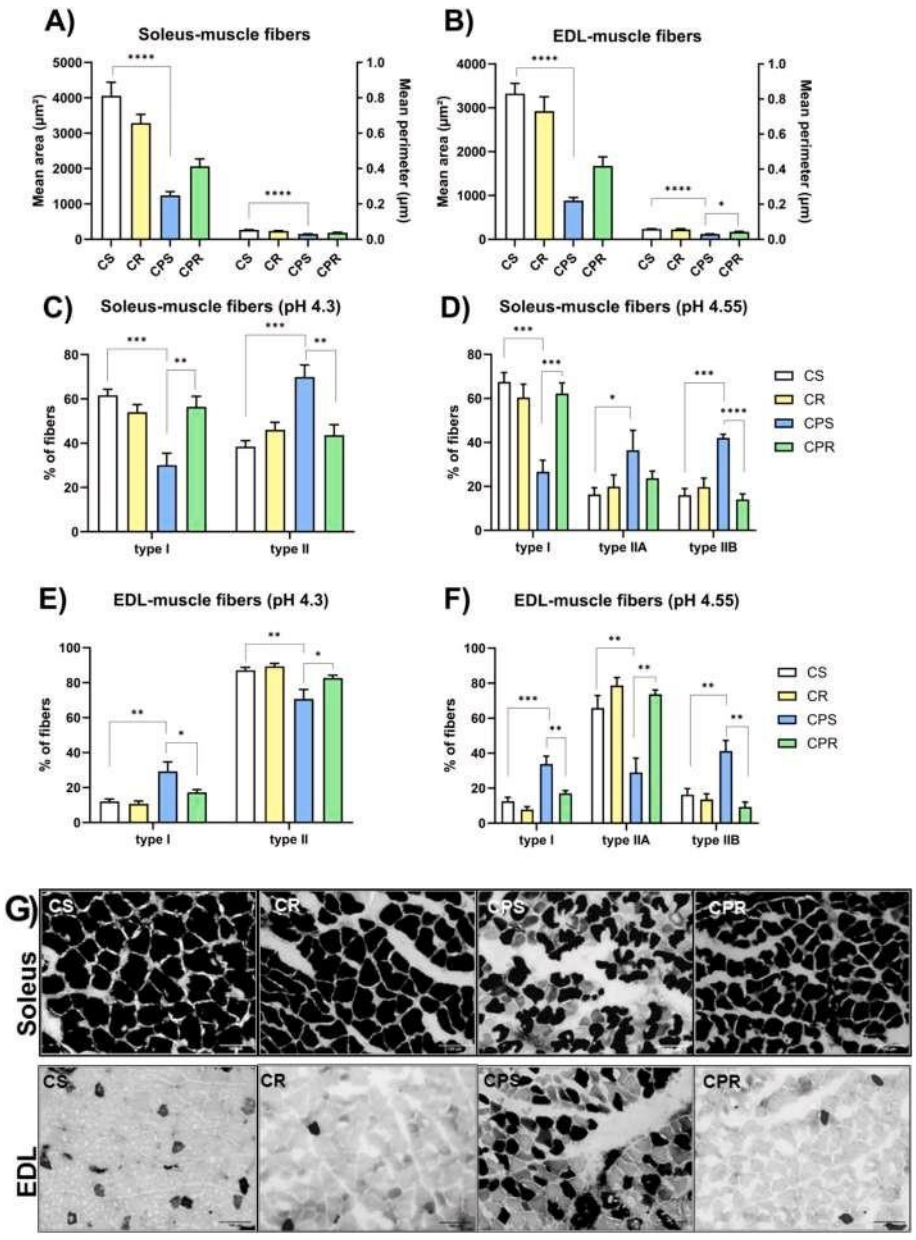

Fig. 5. Effects of neonatal resveratrol treatment on the distribution pattern of muscle fiber types in a CP model. Mean area (µm^2) and perimeter (µm) of soleus muscle (A) and EDL muscle (B); Distribution percentage of fiber types after pre-incubation in a buffer at pH 4.3 (%) in soleus muscle (C) and EDL muscle (D); Distribution percentage of fiber types after pre-incubation in a buffer at pH 4.55 (%) in soleus muscle (E) and EDL muscle (F); (G) Soleus (first row) and EDL (second row) muscle sections stained for myofibrillar ATPase activity after pre-incubation in a buffer at pH 4.55 showing the variability in fiber type content between each experimental group: type I (dark); type IIa (light) and type IIb (gray). Control Saline (CS, n = 6), Control Resveratrol (R, n = 6), Cerebral Palsy + Saline (CPS, n = 6) and Cerebral Palsy + Resveratrol (CPR, n = 6). Data were expressed as mean ± SEM. Two-Way ANOVA and Tukey's post hoc test *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

activated microglia was decreased in Crus area 2 (CR: 35.94 ± 1.15% / CS: 51.24 ± 3.04% $p < 0.05$) compared to the saline control group (Fig. 7D).

4. Discussion

The present study investigated the effects of neonatal resveratrol on neurodevelopment, skeletal muscle morphology and cerebellar damage in experimental CP. The CP model caused impairments in motor skills as seen by the delay in the maturation of neuromotor development reflexes, decreased locomotion and motor coordination. The experimental CP led to a reduction in weight, area and perimeter of muscle fibers in the soleus and EDL muscles, a change in the pattern of fiber types also in both muscles. In the cerebellum, CP increased the density of microglia and the percentage of activated microglia in the areas Crus 1 and Crus 2. Application of neonatal resveratrol showed potential therapeutic effect on the damage caused by the CP model. Resveratrol avoided the delay in the acquisition of neurodevelopment skills with benefits in the ontogenesis of reflexes and locomotion. It restored the phenotype of muscle fibers in the soleus and EDL, and decreased density and activation of cerebellar microglia in rats affect by experimental CP.

As is well established in the literature, CP models lead to impairments in motor development, especially locomotion, corroborating the present study in which we applied a model of combined insults in the perinatal period (da Conceição et al., 2021; Pereira et al., 2021; Strata et al., 2004c; Silva et al., 2016). Neonates exposed to oxygen

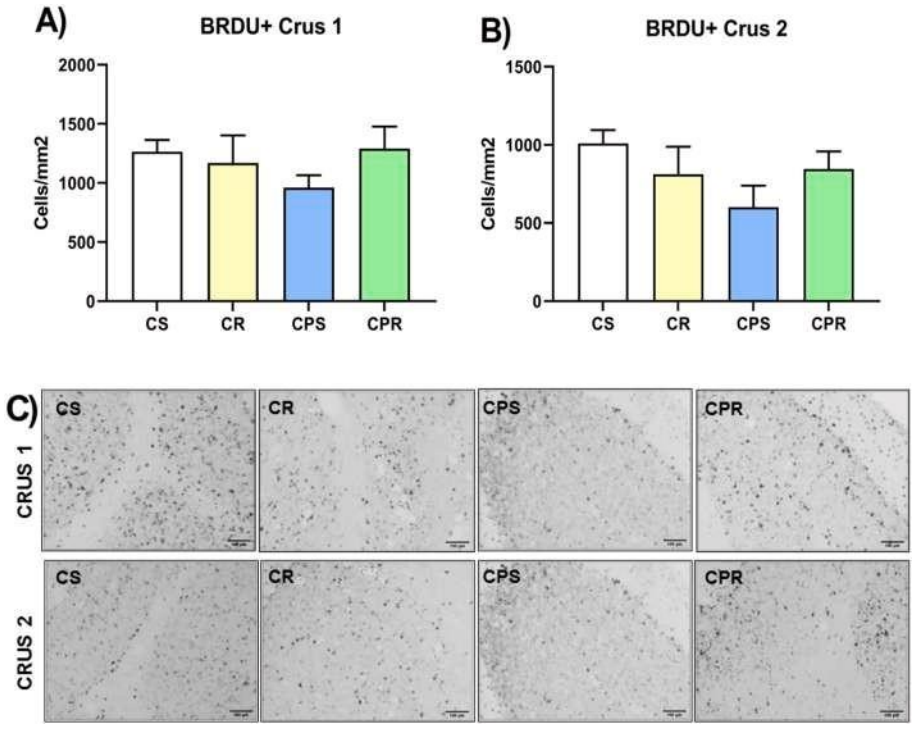

Fig. 6. Effects of the CP model on immunohistochemistry for BrdU+ cells in the cerebellum on the 29th day of postnatal life. BrdU+ cells in Crus 1 (A) and Crus 2 (B) area; (C) Immunohistochemistry for BrdU+ cells in the cerebellum Crus 1 (first row) and Crus 2 (second row) each experimental group. Control Saline (CS, n = 6), Control Resveratrol (CR, n = 6), Cerebral Palsy + Saline (CPS, n = 6) and Cerebral Palsy + Resveratrol (CPR, n = 6). Data were expressed as mean ± SEM. Two-Way ANOVA and Tukey's post hoc test $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

deprivation, as occurs in anoxia, developed early brain damage triggered by a diffuse inflammatory response resulting in an increase in pro-inflammatory cytokines such as IL1 in the cerebral cortex and an increase in glial cells but with reversible changes in gait; thus, it is not used in isolation in CP models (Costa-de-santana et al., 2023; Stigger et al., 2013; Marcuzzo et al., 2010). Complementary sensorimotor restriction leads to an increase in the tone of the muscles of the hind limbs of rats associated with restriction in the range of joint movement and distortion in the representation of the hind limbs in the primary motor cortex and in the S1 somatosensory cortex (Strata et al., 2004a; Coq et al., 2008). Thus, the association of these perinatal insults is fundamental in maintaining the rat's motor phenotype similar to diplegic CP in humans where there is predominant involvement of the lower limbs (Strata et al., 2004a; da Conceição et al., 2021; Coq et al., 2008; Stigger et al., 2011b).

Furthermore, the ontogenesis of primitive reflexes may be impaired in experimental CP, contributing to worse locomotor performance and movement coordination (Costa-de-santana et al., 2023). This is what we demonstrated through the adopted model of experimental CP, which caused an average delay of between three and six days in the acquisition of reflexes for the development of negative geotaxis and free fall, respectively, corroborating previous studies with the same CP model (Costa-de-santana et al., 2023; Marcuzzo et al., 2010). This delay in the ontogenesis of primitive reflexes was directly related to the maturation of ideal locomotor milestones for the animal's age. Thus, locomotion was impaired in animals subjected to experimental CP, which showed less ability to explore the peripheral areas of the open field at 14 and 17 days of postnatal life, ages, coinciding with the delay in the appearance of the negative geotaxis reflex and free fall. Consequently, at more advanced ages, CP animals also exhibited reduced ability to generate movement. The animals in the CPS group showed shorter distance covered, lower average speed, increased time stopped and increased number of stops at 21 and 28 days of life, the period of locomotor development when the animal should be reaching the adult gait pattern (da Conceição et al., 2021; Westerga and Gramsbergen, 1990), reaffirming this relationship between reflex ontogenesis and locomotor activity.

Resveratrol administered during the neonatal period in animals subjected to CP was able to reverse the delay in the appearance of negative geotaxis and free fall reflexes and, consequently, recovered the losses in locomotion, improving exploratory capacity in open field areas and increasing generation of movement. Therefore, resveratrol administered during the neonatal period was deemed to help restore neurodevelopmental impairments, subsequently influencing locomotion and functionality in a CP model. The neonatal period is considered a critical period of brain development in which synaptogenesis and activity-dependent plasticity are at their peak (Jiang and Nardelli, 2016) and the organism has greater susceptibility to environmental agents responding to stimuli imposed by the environment (Jiang and Nardelli, 2016; Chakraborty et al., 2021; Del, 2015). Other authors have already proposed that this critical period may represent a window of opportunity for neuromodulator interventions that are not commonly seen in the adult brain but that can enhance the therapeutic response in the developing brain (Ismail et al., 2017). Therefore, it is suggested that the neonatal period may be the therapeutic window for resveratrol to act, attenuating the damage to neurodevelopment caused by experimental CP.

As we expected, motor coordination was also impaired by the CP model, corroborating previous studies that have used the same model that associates anoxia and sensorimotor restriction as performed on the rotarod test (Pereira et al., 2021; Stigger et al., 2011b) as well in other tests of motor coordination such as Horizontal ladder walking (Marcuzzo et al., 2010), which is similar to what happens with children with CP who present impairments in executive function outcomes (Babik et al., 2023). Motor coordination is a skill that allows broad or refined movements to be carried out based on muscular synergy. Muscular synergy is related to motor control and learning, that is, it depends on the activation of the motor unit resulting in the contraction of specific muscle groups in order to adequately execute the movements required in

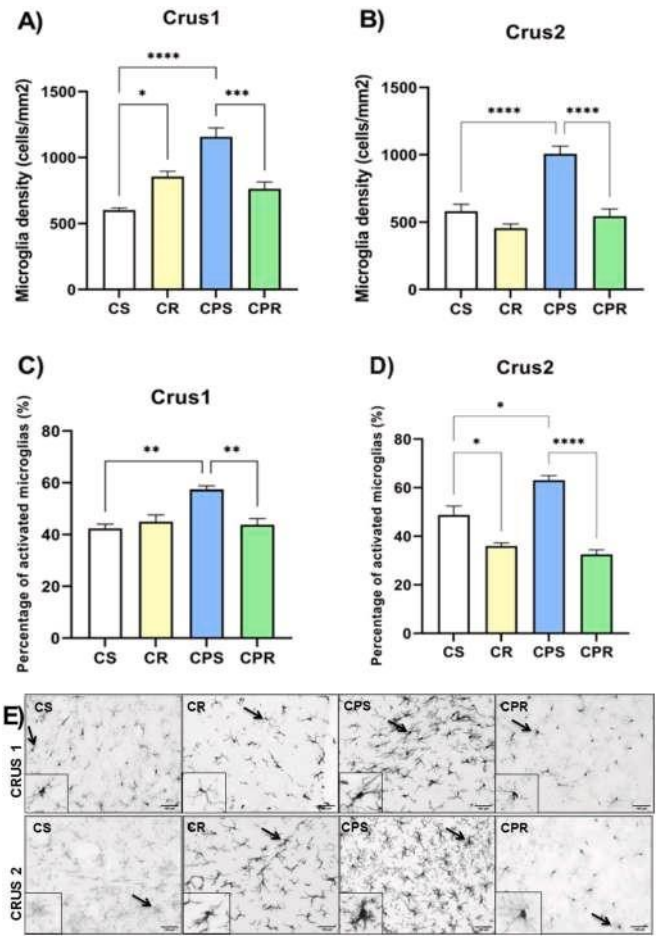

Fig. 7. Effects of neonatal resveratrol treatment on immunohistochemistry for Iba çells in the cerebellum in the Crus 1 and Crus 2 areas on the 29th day of postnatal life. Microglia density (cells/mm^2) in Crus 1 (A) and Crus 2 (B) area; Percentage of activated microglia (%) in Crus 1 (C) and Crus 2 (D) areas; (E) Immunohistochemistry for Iba + cells in the cerebellum - black arrows indicate the activated microglia represented in the inset from Crus 1 (first row) and Crus 2 (second row) each experimental group. Control Saline (CS, *n* = 6), Control Resveratrol (R, n = 6), Cerebral Palsy + Saline (CPS, n = 6) and Cerebral Palsy + Resveratrol (CPR, n = 6). Data were expressed as mean ± SEM. Two-Way ANOVA and Tukey's post hoc test *p < 0.05, **p < 0.01, ***p < 0.001, ****$p < 0.0001$.

an activity (Singh et al., 2018). Furthermore, sensory information also influences motor control. A previous study has suggested that for the swing-to-stance phase transition, sensory regulation based on foot contact information increases the robustness of locomotion (Aoi and Funato, 2016). Thus, this impairment in motor coordination in animals subjected to CP is justified since the insults carried out in the model lead to changes in neuromotor development. Sensorimotor restriction itself leads to sensory deprivation of the hind limbs, associated with anoxia, leading to topographical disorganization of the S1 cortex foot maps (Coq et al., 2008). Therefore, this understanding of the complexity of recovering motor coordination in CP suggests that multidimensional treatment is often necessary, including, in addition to pharmacological interventions, environmental enrichment and treadmill training (Novak et al., 2020). This may explain why we did not observe significant differences with exclusive pharmacological treatment with resveratrol in CP.

The impairments in motor skills highlighted in this study due to experimental CP are the result of primary damage, such as musculoskeletal and neurological damage. Our research showed that the CP model caused a reduction in muscle weight and in the area and perimeter of the soleus and EDL muscle fibers, confirming the muscular atrophy that occurs in the animals' hind legs. Muscle atrophy is linked to the fact that sensorimotor restriction is one of the insults of the CP model, similar to that observed in previous studies (Pereira et al., 2021; Marcuzzo et al., 2010; Stigger et al., 2011b). It is already known that muscular atrophy in an experimental model is not restricted only to the peripheral muscles of the hind limbs and can also affect masticatory muscles, influencing their feeding process and consequently causing gain in muscle mass in different regions of the body (da Silva et al., 2016; Pereira et al., 2021; Lacerda et al., 2021; Lacerda et al., 2017c; Lacerda et al., 2017d). Atrophy is a key outcome, quite evident and known in CP, and is important to be evaluated as confirmation of the effectiveness of the model. Furthermore, we also observed in the analysis of microscopic anatomy polymorphic fibers, disorganization of the muscle tissue with spaced fascicles suggesting loss of continuity of the perimysium in CP animals compared to the control. Therefore, in this study we confirmed the similarity between the model used in this study and the muscle fiber phenotype observed in humans when evaluated in three dimensions by Synchrotron X-ray computed tomography (Borg et al., 2019).

Polyphenols have been demonstrated to have a potential preventive effect on musculoskeletal atrophy (Salucci and Falcieri, 2020). Among them, resveratrol has shown benefits in preventing muscle atrophy induced by denervation (Asami et al., 2018) and obesity (Bai et al., 2020; Huang et al., 2019). Studies in the literature, however, are scarce regarding its effects on muscle atrophy induced by early brain injury, as was researched in the present study. We observed that resveratrol did not appear to act directly on muscle mass gain in our CP model, as it did not recover muscle weight or the fiber area of the soleus and EDL muscles, but it was able to reverse the changes in the distribution pattern of the types of muscle fibers resulting from the CP model.

In this context of musculoskeletal changes, the distribution pattern of fiber types was also impaired by experimental CP. The soleus muscle is known for supporting postural maintenance, with a fatigue-resistant characteristic consisting predominantly of type I (slow-twitch) fibers, unlike the EDL muscle, which is made up of type II fibers (fast-twitch) and is less resistant to fatigue during their recruitment (Komiya et al., 2017; Schiaffino and Reggiani, 2011). CP animals showed distortion of this muscle fiber distribution pattern. Type I muscle fibers in the CPS group were decreased in the soleus and increased in the EDL, and type II were increased in the soleus and decreased in the EDL. Neuromuscular changes in the CP model associated with the decrease in muscle activity due to immobilization may justify the changes in the types of soleus and EDL muscle fibers. Motor nerves have a high potential to influence the composition and properties of the functional elements of the muscle fiber, with the reinnervation mechanism being a key outcome for muscle plasticity (Pette, 2001; Bassel-duby and Olson, 2006). When the slow-twitch soleus muscle became reinnervated by nerve fibers normally supplying the fast-twitch flexor digitorum longus muscle contractile speed increased (Pette, 2001). When the fast-twitch muscle was reinnervated with the soleus nerve it became slower contracting (Pette, 2001). Therefore, distortion in the pattern of muscle fiber typing may be a form of muscular adaptation to the current functional demand. It may be related to the limited resistance to fatigue seen by the lower capacity for locomotion and motor coordination in the present study. Our result corroborates other studies that have used the same CP model; chewing muscles were evaluated, which showed distortion in the pattern of muscle fiber typing (Lacerda et al., 2021; Lacerda et al., 2017c) and reinforcement of the global impairment of the musculoskeletal system to the detriment of the perinatal insult.

In the soleus muscle, treatment with resveratrol restored the percentage of type I fibers, also called slow-twitch, in CP animals, corroborating a study that used an intrauterine growth delayed pig model (Cheng et al., 2020). This may be related to the role of resveratrol in the AMPK/SIRT1/PGC-1α signaling pathway. In bovine myotubes, protein and gene expression of AMPK, SIRT1 and PGC-1α were upregulated by resveratrol; these are proteins involved in metabolic homeostasis,

including protein synthesis that might promote muscle fiber type transition from fast-twitch to slow-twitch as observed in a previous study with bovine myotubes (Zhang et al., 2023). Similarly, in porcine myotubes and mouse C2C12 AMPK, SIRT1 and PGC-1α were also increased with resveratrol treatment and inhibited the expression of miR-22-3p (Wen et al., 2022), suggesting that resveratrol regulates muscle fiber type gene expression through the AMPK signaling pathway and miR-22-3p. However more studies are needed to clarify the mechanisms involved in the differentiation of muscle fibers in experimental CP.

In the EDL muscle, resveratrol restored the fiber typing pattern expected for this muscle; that is, it increased the percentage of type II fibers, also called fast-twitch. Our results raise the hypothesis that the action of resveratrol goes beyond the AMPK signaling pathway and possibly its antioxidant effects may be the mechanism of action of resveratrol on EDL. In diabetic rats, fast-twitch muscle incurs more oxidative stress (Chang et al., 2014), suggesting that fast-twitch muscle fibers may be more susceptible to oxidative stress than slow-twitch muscle fibers (Nonaka et al., 2014). However, further investigation is needed regarding the mechanisms of action of resveratrol on fast-twitch muscle fibers in a CP model.

Damage to the musculoskeletal system was not solely responsible for the impairments in motor and behavioral skills resulting from the CP model. It is known that the central nervous system is damaged by insults in early life (Cioni et al., 2011; Bersani et al., 2020). The brain that undergoes a prenatal CP model is impacted by losses in neurogenesis and curbs generation of neural stem cells, whereas postnatal models show increased proliferation of neural precursor cells, improper migration, and reduced survival of new neurons (Visco et al., 2021). Thus, we observed in our study that cell proliferation in the cerebellum was not influenced by our postnatal CP model since Brdu+cells in the Crus 1 and Crus 2 areas of the cerebellum did not change. However, studies with a CP model using anoxia associated or not with sensorimotor restriction showed that other areas may present damage to neurogenesis such as the hippocampus (Calado et al., 2023a; Takada et al., 2016) and may even benefit from treatment with resveratrol (Calado et al., 2023a). Thus, the literature reports heterogeneous outcomes, but also there is a scarcity of studies showing the effects of resveratrol on the cerebellum. With human subjects, an analysis through neuroimaging examinations of cerebellar lesions in neonates after early brain injury is unclear and often underestimated, requiring further studies in the cerebellar histopathological area (Chalak, 2021; Annink et al., 2021). This makes us hypothesize that the cerebellum may present a different pattern of response to perinatal injury.

Although the cerebellum did not change its neurogenesis due to the experimental CP, it was sensitive to changes in microglia. In a previous study, motor deficits in experimental CP were seen to be associated with the activation of glial cells in the cerebellum, corroborating our study (Costa-de-santana et al., 2023). We showed that there was an increase in the density of microglia and an increase in the percentage of activated microglia in animals subjected to the CP model, corroborating studies that showed that insults in the developing brain (prenatal or early life) provoke the microglial defense response, increasing its density in the hippocampus (Costa-de-santana et al., 2023; Orso et al., 2023) and in the cerebellum (Costa-de-santana et al., 2023). Microglia are primary innate immune cells of the brain that trigger the release of cytokines and chemokines and have phagocytic action on dead neurons. Their sustained activation can be harmful to the developing nervous system (Orso et al., 2023; De Pablos et al., 2014; Lenz and Nelson, 2018). Thus, after oxygen deprivation, as occurred in perinatal anoxia in our CP model, there was intense activation of microglia leading to the production of mitochondrial free radicals and brain injuries due to excitotoxicity (Costa-de-santana et al., 2023), confirming our result.

Resveratrol is known to act as a neuroprotector with effects on neuroinflammation and antioxidants in models of insults to the nervous system (Miguel et al., 2021; Zhou et al., 2014). A similar action was observed in the present study, as the resveratrol reversed the changes in microglia density and percentage of activated microglia in those animals subjected to the CP model. Considering that brain neuroplasticity is modulated during postnatal development (Kourosh-Arami et al., 2021), we have presented an original therapeutic potential of neonatal resveratrol in experimental CP. We show that resveratrol can contribute to the control of neuroinflammation with a consequent reduction in neurodevelopmental damage.

The proposed neonatal treatment with resveratrol at a dose of 10 mg/kg proved to be sufficient to promote benefits in the acquisition of important skills for the animals' motor development and to promote changes in the muscle phenotype and microglial activation. It did not demonstrate adverse effects beyond what was already expected with this route of administration, such as discomfort during intraperitoneal application. However, in the present study we can highlight as limitations the exclusive period of treatment that was carried out, only during the neonatal period, while other studies have demonstrated preventive benefits of pre-insult resveratrol (Arteaga et al., 2015; Zhou et al., 2014). Therefore, new studies are suggested in this therapeutic approach with resveratrol comparing prenatal treatment in pregnant rodent and postnatal care, and even combining resveratrol with other interventions such as environmental enrichment to gain motor coordination. Furthermore, we showed the effects of resveratrol in the short and medium term, suggesting that future research into the long-term effects of resveratrol in adult or elderly animals is also required.

5. Conclusion

A CP model in neonatal rats caused neurodevelopment impairments observed by deficiency in locomotor activity and motor coordination similar to CP in humans, associated with delay in the acquisition of developmental reflexes, muscle atrophy, change in muscle fiber typing pattern and microglia density in the cerebellum. Treatment with resveratrol was shown to act on neurodevelopmental impairments caused by this experimental CP, benefiting the acquisition of motor skills such as locomotion, by recovering the muscle fibers morphology and reversing neuroinflammation in the cerebellum.

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CRediT authorship contribution statement

Sabrina da Conceição Pereira: Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. **Raul Manhães-de-Castro:** Writing – review & editing, Software, Resources, Project administration, Funding acquisition, Conceptualization. **Vanessa da Silva Souza:** Writing – original draft, Project administration, Methodology, Investigation, Formal analysis. **Caio Matheus Santos da Silva Calado:** Writing – original draft, Supervision, Methodology, Investigation, Formal analysis. **Beatriz Souza de Silveira:** Writing – original draft, Supervision, Project administration, Methodology, Formal analysis. **Letícia Nicoly Ferreira Barbosa:** Writing – original draft, Visualization, Supervision, Methodology, Formal analysis. **Luz Torner:** Writing – review & editing, Validation,

Supervision, Conceptualization. **Omar Guzmán-Quevedo:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Funding acquisition. **Ana Elisa Toscano:** Writing – review & editing, Visualization, Validation, Supervision, Software, Resources, Project administration, Funding acquisition, Data curation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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APÊNDICE C – LOCOMOTION IS IMPACTED DIFFERENTLY ACCORDING TO THE PERINATAL BRAIN INJURY MODEL: META-ANALYSIS OF PRECLINICAL STUDIES WITH IMPLICATIONS FOR CEREBRAL PALSY

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Locomotion is impacted differently according to the perinatal brain injury model: Meta-analysis of preclinical studies with implications for cerebral palsy

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ABSTRACT

Background: Different approaches to reproduce cerebral palsy (CP) in animals, contribute to the knowledge of the pathophysiological mechanism of this disease and provide a basis for the development of intervention strategies. Locomotion and coordination are the main cause of disability in CP, however, few studies highlight the quan- titative differences of CP models, on locomotion parameters, considering the methodologies to cause brain le- sions in the perinatal period.

Methods: Studies with cerebral palsy animal models that assess locomotion parameters were systematically retrieved from Medline/PubMed, SCOPUS, LILACS, and Web of Science. Methodological evaluation of included studies and quantitative assessment of locomotion parameters were performed after eligibility screening.

Results: CP models were induced by hypoXia-ischemia (HI), Prenatal ischemia (PI), lipopolysaccharide inflam- mation (LPS), intraventricular haemorrhage (IVH), anoXia (A), sensorimotor restriction (SR), and a combination of different models. Overall, 63 studies included in qualitative synthesis showed a moderate quality of evidence.

16 studies were included in the quantitative meta-analysis. Significant reduction was observed in models that combined LPS with HI related to distance traveled (SMD -7.24 95 % CI [-8.98, -5.51], Z = 1.18, p < 0.00001) and LPS with HI or anoXia with sensory-motor restriction (SMD -6.01, 95 % CI [-7.67, -4.35], Z = 7.11), or IVH (SMD

-4.91, 95 % CI [-5.84, -3.98], Z = 10.31, p < 0.00001) related to motor coordination.

Conclusion: The combination of different approaches to reproduce CP in animals causes greater deficits in locomotion and motor coordination from the early stages of life to adulthood. These findings contribute to methodological refinement, reduction, and replacement in animal experimentation, favoring translational purposes.

1. Introduction

Over the past few years, a variety of experimental models of early brain injury with implications for cerebral palsy (CP) have been pro- posed. These preclinical studies have revealed fundamental importance in elucidating the pathophysiological aspects related to CP and they are based on the ability to reproduce neurofunctional changes observed in CP in humans (Cavarsan et al., 2019; Silva et al., 2016). CP is a multi- factorial pathology that arises from insults on the brain and it can occur prenatally, perinatally, or postnatally (Graham et al., 2019). It is considered a non-progressive neurodevelopmental disorder that permanently impairs motor functions, and it is considered a more

Abbreviations: CP, cerebral palsy; HI, hypoXia-ischemia; LPS, lipopolysaccharide inflammation; IVH, intraventricular haemorrhage; A, anoXia; SR, sensorimotorrestriction.

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common motor disability in childhood (Gulati and Sondhi, 2017; Stavsky et al., 2017).

Experimental models of brain injury with pathophysiology related to cerebral palsy use early insults during the critical period of development too, at the time there is greater temporal and regional vulnerability to brain damage (Jensen, 2002; Jiang and Nardelli, 2016). These models are methodologically diverse, varying as to the time of the injury. or the mechanism of injury. There are models of prenatal inflammation, intrauterine or perinatal oxygen deprivation, prematurity, perinatal intraventricular haemorrhage, and postnatal sensorimotor restriction. In this sense, the injury period is an essential factor for the establishment of functional phenotypes (Marret et al., 2013). Methods and age of the evaluation of locomotion from experimental studies also determine the observation of motor disability according to the developmental milestones to be evaluated (Westerga and Gramsbergen, 1990). Thus, several animal models can represent the neuromotor alterations related to PC induction.

Depending on the objective of the experimental study and the phenotype to be reproduced with a specific model, it may cause more repercussions on motor development than other models, however, this is not elucidated in the literature. The child with CP is characterized by disorders of muscle tone and movement, in addition to the delay in the acquisition of motor skills (Gulati and Sondhi, 2017; Stavsky et al., 2017). Among the outcomes of CP, impairments on locomotion are the most explored in the literature as a striking feature to be reproduced. CP models usually propose to demonstrate how much this neuromotor disease compromises the locomotor functional ability (Aoi and Funato, 2016; Hoppeler and Flück, 2002).

The acquisition of locomotion follows the milestones of the development of the nervous system and depends on its maturation and integration among other systems (Westerga and Gramsbergen, 1990). Preclinical studies demonstrate that early brain injury triggers an inflammatory cascade in the central nervous system that promotes deregulation of the neurogenic niche and, consequently, impairs normal motor development (Dada et al., 2014; Stolp et al., 2012). After perinatal brain damage, neurogenesis response and brain repair mechanism are altered, and this occurrence may contribute to the retardation of development and acquisition of motor skills (Visco et al., 2021). The performance of movements of the animal that was submitted to experimental CP is impaired, including locomotor activity, gait pattern, which is related to impaired motor coordination, balance, and decreased physical capacity (Peterson et al., 2013; Silva et al., 2016). Thus, it is expected that a CP model will be able to reproduce the damage in this fundamental motor task for survival, the locomotion.

Although several CP models are demonstrated in the literature, there is a need for further clarification on which model of cerebral palsy most influences locomotor parameters concerning the relationship between different approaches to reproduce cerebral palsy and the motor damage that is reproduced in animals. In addition to the motor repercussions of the experimental models when combined still derived from greater elucidation. Thus, the objective of this systematic review was to determine the effects of different experimental models of cerebral palsy on locomotion and motor coordination in the short and long term. Also, providing evidence through a systematic review of animal models is valuable to achieve a better translational correlation to human health. This might serve as a guide for the development of the future intervention, contribute to “replacement” “reduction” and “refinement” in animal experimentation, and improve the rigor of the conduction and reporting of preclinical research.

2. Methods

2.1. Systematic review reporting and protocol registration

This systematic review was carried out following the recommendations of the Preferred Reporting Items for Systematic Reviews and Meta-Analyzes (PRISMA). The protocol is registered in the Collaborative Approach to Meta-Analysis and Review of Animal Data from Experimental Studies (CAMARADES) and its full text is available at http://syrf.org.uk/protocols.

2.2. Search strategy

The search in the scientific literature was carried out between July and August 2020 in the databases Medline/PubMed (National Library of Medicine / Medical Literature Analysis and Retrieval System Online: 1946 - Aug / 2020), SCOPUS (2004 - Aug / 2020), LILACS (Latin American and Caribbean Literature in Health Sciences: 1985 - Aug / 2020) and Web of Science (1900 - Aug / 2020). Appropriate terms were used in each database to cover the experimental models of cerebral palsy and locomotion, according to the MeSH (Medical Subject Headings) and DeCS (Health Sciences Descriptors) descriptors (Table 1). The detailed search strategy is available in supplementary data.

During the research from the databases, there was no linguistic restriction, year or, journal publication. The selection of studies took place in two phases, initially by reading the title and abstract, followed by reading the full text of the selected articles, and studies were included according to the eligibility criteria. Besides, the reference lists of the included articles were inspected and the relevant articles were selected. This process was carried out by two independent reviewers (PEREIRA and VISCO) in which any disagreement was resolved by discussion and consensus between the two reviewers, and when necessary, through a third reviewer (TOSCANO).

2.3. Eligibility

The selected studies followed the inclusion criteria: experimental studies with animals that were submitted to any model of CP; that CP has been induced during the prenatal, perinatal, or early postnatal period; which contained a control group in its study design; who evaluated short and/or long-term locomotion after induction of experimental CP. The exclusion criteria were: articles in which the animals were subjected to some treatment or insult concomitant with the CP model were excluded; which included genetically modified animals; that did not describe in detail the methods of the induced experimental CP model, which do not allow replication (Table 2). Discrepancies were resolved after discussion between the two authors (PEREIRA and VISCO) or were referred to a third author (TOSCANO).

2.4. Data extraction

The extraction of data from the included studies was carried out by two independent reviewers using a standardized form developed to collect relevant information. The following details were extracted from each study: 1- the year of publication and name of the first author; 2- Characteristics of the animals used (strain, sex, sample size); 3-

Table 1
Standard terms used in the search strategy.

Search strategy Component	Terms / Booleans operators
Animal model with implications for cerebral palsy	(Cerebral Palsy) OR (Hypoxia-Ischemia, Brain) OR (Lipopolysaccharides/toxicity) OR (Asphyxia Neonatorum) OR (Immobilization) OR (perinatal asphyxia) OR (sensory-motor restriction) OR (leukomalacia, periventricular)
	AND
Locomotor activity	(Gait) OR (Locomotion) OR (Motor Skills Disorders)
	AND
Animals	Laboratory animals search filters and terms (Hooijmans et al., 2010)

Note: The terms used could vary according to the requirement of each database.

Table 2
Inclusion and exclusion criteria.

Inclusion Criteria	Exclusion Criteria
Participants: o Animals.	Participants: o Humans. o Genetically modified animals.
Exposure: o Animal model with implications for cerebral palsy	Exposure: o Brain injury after perinatal/ neonatal period. o Any other intervention.
Control: o Sham.	Control: o Studies without a control group.
Outcomes: o Locomotion; o Motor coordination.	Outcomes o Drug-induced locomotion
Study type: o Original data. o Full-text was available.	Study type: o No original data (e.g reviews, editorial).

Characteristics of the cerebral palsy model (age and type of insult); 4- Analysed Sample; 5- Locomotion assessment (method and age at testing); 6- Outcomes and for each comparison between control and exposed groups we extracted data of mean value and standard deviation; 7- Statistical method used and descriptive results. As a secondary result, the neurodevelopment parameters were extracted like motor activity and postural changes on gait to qualitative descriptions. In the absence of data, we tried to contact the author for more specific information. Otherwise, we performed only qualitative analysis. The characteristics of the studies included in this systematic review are described in Table 3.

2.5. Methodological quality analysis

Methodological evaluation of the included studies was carried out using the SYRCLE risk of bias tool, which is an adapted version of the Cochrane Collaboration risk of bias tool (Hooijmans et al., 2014a, 2014b). It assesses specific issues that may interfere with the methodological quality of experimental animal studies, across 10 domains. These entries are related to selection bias (sequence generation, baseline characteristics, and allocation concealment), performance bias (random housing and blinding of caregivers and researchers), detection bias (random outcome assessment and blind of outcome assessor), attrition bias (incomplete outcome data), reporting bias (selective outcome reporting) and other sources of biases (Hooijmans et al., 2014a, 2014b). A "yes" judgment indicates a low risk of bias; a "no" judgment indicates a high risk of bias; the judgment will be "unclear" if insufficient details have been reported to assess the risk of bias properly (Hooijmans et al., 2014a, 2014b). The entire process was carried out by two independent reviewers in which the discrepancies were resolved after discussion between the two authors (PEREIRA and VISCO) or were referred to a third author (TOSCANO). The Review Manager Software Package 5.3 (RevMan v.5.3) was used to create the risk of bias summary figure and risk of bias graph.

2.6. Statistical analysis

The Kappa statistic for the interobserver agreement of inclusion criteria was performed using GraphPad QuickCalcs Web. The software RevMan v.5.3 was used to calculate separate meta-analytic comparisons were made for each locomotor parameter assessed and reported in > 3 studies. Cross-sectional comparisons between exposed and control animals assessed standardized mean differences (SMDs) calculated from mean and variance data computed into Cohen's d pooled effect sizes (ES) and 95 % confidence intervals (95 % CI). These were weighted for sample size, using a random-effects model (Hooijmans et al., 2014a, 2014b). Sensitivity analyses were conducted to test the effects of removing potentially incomparable studies, comprising: those with insufficiently matched exposed and control animals, factors that may modify the relationship between Cerebral Palsy and locomotion (e.g. age analysis). These data are subsequently reported as "potentially incomparable". The extent of heterogeneity between studies was inspected using I^2, with I^2 values exceeding 60 % indicating important heterogeneity (Hooijmans et al., 2014a, 2014b). Potential sources of statistical heterogeneity were explored; depending on sufficient data availability, variability, and the number of studies, meta-regression was planned. In comparisons that contained at least ten studies, the likelihood of publication bias was also assessed using the observation of funnel plots. Thus, a subgroup analysis was performed referring to different models of CP on each outcome: motor coordination in rotarod, distance traveled, and average speed in the open field test.

3. Results

3.1. Selection of studies

Initially, by searching the databases, 2,020 articles were found, 293 duplicates were excluded, leaving 1,727 articles for analysis of the eligibility criteria in the screening by title and abstract. 90 articles were selected for the complete reading, including 31 eligible articles based on the inspection of the reference list of the articles included in this phase. Of these 90 eligible studies, 27 were excluded and 63 remained for final qualitative analysis and 16 went to meta-analysis (Fig. 1).

3.2. Characteristics of studies included

A systematic narrative synthesis was carried out that summarizes the characteristics of the included studies and their main locomotor results according to the CP models used (Table 3). The animals predominantly used were mammals of the rodent strain. 21 studies included Wistar rats (Alexander et al., 2014; Arteni et al., 2010; Alles et al., 2010; Van De Berg et al., 2003; Bona et al., 1997; Buratti et al., 2019; Durán-Carabali et al., 2016; Jansen and Low, 1996; Lubics et al., 2005; Marcuzzo et al., 2010; Miguel et al., 2015; Misumi et al., 2016; Quinzaños-Fresnedo et al., 2008; Rousset et al., 2013; Sanches et al., 2013, 2015a, 2015b; Sanches et al., 2019; dos Santos et al., 2017; Stigger et al., 2013, 2011; Ueda et al., 2018), 23 studies used Sprague-Dawley Rats (Baharnoori et al., 2012; Balasubramaniam et al., 2006; Balduini et al., 2000; Delcour et al., 2011, 2018a; Delcour et al., 2012b, a, 2018b; Fan et al., 2011; Grow et al., 2003; Hermans et al., 1992; Hoeger et al., 2000; Jantzie et al., 2014; Lekic et al., 2011, 2012; Ohshima et al., 2016; Poggi et al., 2005; Robinson et al., 2005; Strata et al., 2004; Tashima et al., 2001; Wallace et al., 2010; Wang et al., 2013; Weitzdoerfer et al., 2002), 1 study used Lewis Rats (Girard et al., 2009), another study used Long Evans Rats (Ruff et al., 2017), 1 used mice (Zaghloul et al., 2017), 4 used CD-1 mice (Dada et al., 2014; Kadam et al., 2009; Makinson et al., 2017; Wang et al., 2010), 2 used guinea pigs (Hoeger et al., 2003; Windle and Becker, 1943) and 9 used New Zealand white rabbits (Balakrishnan et al., 2013; Chua et al., 2009; Derrick et al., 2004; Georgiadis et al., 2008; Kannan et al., 2011; Saadani-Makki et al., 2008; Shi et al., 2018; Tan et al., 2005; Zhang et al., 2016). Only one study used the piglet as an experimental animal (Aquilina et al., 2007). Of these studies, 18 included only male animals in their sample, 3 used only females and 28 studies included both sexes. Besides, 18 studies do not make clear the sex of the animals studied.

The experimental CP models included in this systematic review were based on hypoxic-ischemia (HI), Prenatal ischemia (PI), lipopolysaccharide inflammation (LPS), intraventricular hemorrhage (IVH), anoxia (A), sensorimotor restriction (SR), and the combination of the different models. HI was the one of most frequent among the CP models being performed in17 studies in the postnatal period through unilateral common carotid artery occlusion followed by a period of anoxia at P3 or P7 (Alexander et al., 2014; Arteni et al., 2010; Balduini et al., 2000; Bona et al., 1997; Durán-Carabali et al., 2016; Grow et al., 2003; Jansen and Low, 1996; Lubics et al., 2005; Miguel et al., 2015; Quinzaños-Fresnedo

Table 3
Characteristics of included studies.

CP MODEL	AUTHOR (YEAR)	ANIMAL	SEX	EXPERIMENTAL GROUPS (n)	CP INDUCTION PERIOD			LOCOMOTION AND MOTOR COORDINATION OUTCOMES
					Prematurity	Antenatal	Postnatal	
COMBINED MODELS	Strata et al. (2004)	Sprague-Dawley Rats	Not described	C (n = 17); A (n = 18); SR (n = 22); A + RS (n = 22).	–	–	A (P0 e P1) + SR (P2 - P28)	↓ Fall latency in rotarod (P24, P31, and P38) ↓ The open-field score (P17, P 24, and P 31) ↓ Suspended bar score (P17, P24, P31, P38, and P45) ↓ Stride, swing, and vertical displacement in Plexiglas walkway (P17, P24, P31, P38, and P45)
	Girard et al. (2009)	Lewis Rats	Both sexes	C; HI; LPS; LPS + HI. *Total: 106 pups	–	LPS (E17 until the end of pregnancy)	HI (P1)	↓ Fall latency in rotarod (P30, P35, and P40) ↓ Distance traveled, Crossings, ↑ time of immobility open-field (P15, P20, and P25)
	Marcuzzo et al. (2010)	Wistar Rats	Male	C (n = 14); A (n = 16); SR (n = 14); A + SR (n = 12).	–	–	A (P0) + SR (P1 - P30)	↑ Step error mean Horizontal ladder walking (P31 and P52) ↓ Beam walking score (P31, P38, P45, and P52)
	Stigger et al. (2011)	Wistar Rats	Male	C; LPS; A; LPS + A; RS; LPS + SR; A + SR; LPS + A + SR. *Total: 57 pups (n = 6–8/group)	–	LPS (E17 until the end of pregnancy)	A (P0) + SR (P2 - P28	↓ Fall latency in rotarod (P29) ↑Step error mean on Horizontal ladder (P29) ↑Step error mean on the suspended bar (P29)
	Stigger et al. (2013)	Wistar Rats	Male	C (n = 13); LPS (n = 14); A (n = 14); LPS + A (n = 13).	–	LPS (E17 until the end of pregnancy)	A (P0)	↓ Fall latency in rotarod (P29) ↓ Distance traveled and Average time in locomotor activity in open-field (P29) ↓ Motor activity score (P1 - 15)
	Jantzie et al. (2014)	Sprague-Dawley Rats	Both sexes	C (n = 18); LPS (n = 13); PI (n = 14); PI + LPS (n = 21).	–	PI + LPS (E18)	–	↓ Stride length, time in the propel phase, paw area at peak stride, consistent with toe-walking on Digigait analyses (P28) ↑Stride variation, stride frequency, and ataxia coefficient on Digigait analyses (P28)
	dos Santos et al. (2017)	Wistar Rats	Male	C (n = 12) LPS + A + SR (n = 12)	–	LPS (E17)	A (P0) + SR (P2 – P30)	↓ Hindlimb motor function on Basso, Beattie e Bresnahan score (BBB score) in adapted open-field (P29 and P45)
LIPOPOLYSACCHARIDE INFLAMM	Poggi et al. (2005)	Sprague-Dawley Rats Male	Not described	Sham (n = 30) LPS (n = 45)	–	LPS (E15)	–	There was no difference between groups in Rotarod and the open-field test (2.5 months old)
	Saadani-Makki et al. (2008)	New Zealand white rabbits	Not described	Sham (n = 30) LPS (n = 18)	–	LPS (E28)	–	↓ Motor activity score (P1)
	Wallace et al. (2010)	Sprague-Dawley Rats	Male	Sham (n = 25) LPS (n = 25)	PT (E20-21)	LPS (E17)	–	↓ Fall latency in rotarod (P30, P60-62)

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Table 3 (*continued*)

CP MODEL	AUTHOR (YEAR)	ANIMAL	SEX	EXPERIMENTAL GROUPS (n)	CP INDUCTION PERIOD			LOCOMOTION AND MOTOR COORDINATION OUTCOMES
					Prematurity	Antenatal	Postnatal	
	Wang et al. (2010)	Mice CD-1	Both sexes	Sham (n = 10) LPS (n = 10)	–	LPS (E15)	–	There was no difference between groups in locomotor parameters evaluated by the open-field (P30- P60) ↑ Peripheral activity and crossings in the open-field (female on P200) ↓Latency Beam walking (female on P200) ↑ Tightrope score (male on P400)
	Kannan et al. (2011)	New Zealand white rabbits	Not described	C (n = 4) Sham (n = 6) LPS (n = 8)	–	LPS (E28)	–	↓ Motor activity score (P1)
	Fan et al. (2011)	Sprague-Dawley Rats	Not described	Sham (n = 20) LPS (n = 20)	–	–	LPS (P5)	↑ Distance traveled (P14, P35, and P42) and rearings (P14 - P49 or P56) in the open-field ↑ Latency in movement initiation test (P21 - P56) ↑ Latency in pole test (P28 - P56) ↑ Slip step ratio and latency in tapered / ledged beam walking test (P21 - P56)
	Baharnoori et al. (2012)	Sprague-Dawley Rats Male	Not described	Sham (n = 5) LPS (n = 5)	–	LPS (E15 e E16)	–	There was no difference between groups in locomotor parameters evaluated by the open-field (P6-P10)
	Wang et al. (2013)	Sprague-Dawley Rats	Female	Sham (n = 18) LPS (n = 18)	–	–	LPS (P5)	↑ Rearing in the open-field (P21 and 49) There was no difference between groups in distance traveled in the open field (P21, P49, and P70)
	Rousset et al. (2013)	Wistar Rats	Both sexes	Sham (n = 12) LPS (n = 10)	–	LPS (E19 e E20)	–	↓ Gait efficiency (P14) There was no difference between groups in Grid walking and foot fault test, Rotarod and Cyclotron Test
	Balakrishnan et al. (2013)	New Zealand white rabbits	Not described	C (n = 10) Sham (n = 6) LPS (n = 13)	–	LPS (E28)	–	↓ Motor activity score (P1)
	Dada et al. (2014)	CD-1 outbred mouse strain	Both sexes	Sham (N = 11) LPS (N = 16)	–	LPS (E17)	–	↑ time to complete the ambulation test (P9) ↑ Peripheral activity in the open-field (P60)
	Zhang et al. (2016)	New Zealand white rabbits	Not described	Sham (n = 8) LPS (n = 8)	–	LPS (E28)	–	↓ Motor activity score (P1)
	Shi et al. (2018)	New Zealand white rabbits	Not described	Sham P1 (n = 18) Sham P1 (n = 128) LPS P1 (n = 128) LPS P11 (n = 33)	–	LPS (E28)	–	↓ Motor activity score (P1 and P11)
	Makinson et al. (2017)	Mice CD-1	Both sexes	Sham (n = 10) LPS (n = 10)	–	LPS (E15)	–	↑ Peripheral activity and rearing in the open-field (12-14 weeks old)

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Table 3 (*continued*)

CP MODEL	AUTHOR (YEAR)	ANIMAL	SEX	EXPERIMENTAL GROUPS (n)	CP INDUCTION PERIOD			LOCOMOTION AND MOTOR COORDINATION OUTCOMES
					Prematurity	Antenatal	Postnatal	
HYPOXIA-ISCHEMIA	Jansen and Low (1996)	Wistar Rats	Both sexes	C (n = 15) HI (n = 19)	–	–	HI (P7)	↓ Fall latency in rotarod (3 - 9 weeks old) ↓ Motor activity score (P42)
	Bona et al. (1997)	Wistar Rats	Both sexes	C (n = 11) Sham operated without hypoxia (n = 5) Ischemia without hypoxia (n = 4) HI (n = 11)	–	–	HI (P7)	Foot fault asymmetric on-grid walking (P42)
	Balduini et al. (2000)	Sprague–Dawley Rats	Male	Sham (n = 8) HI (n = 8)	–	–	HI (P7)	There was no difference between groups on parameters evaluated by the open-field (P90) or in rotarod (P35) ↑ Spontaneous activity in automated motor activity monitor (P21)
	Grow et al. (2003)	Sprague-Dawley Rats	Both sexes	C (n = 8) HI (n = 16)	–	–	HI (P7)	Asymmetry of the paws in cylinder test (5 weeks old)
	Lubics et al. (2005)	Wistar Rats	Both sexes	C (n = 12) HI (n = 12)	–	–	HI (P7)	↓ Gait efficiency (P1 – P9) ↑ Foot fault and on ↓ number of steps on-grid walking (2 and 4 weeks old) ↓ Time to remain a square bridge (2 weeks old) ↑ Distance traveled, speed (2 and 6 weeks old), rearing (6 weeks old), peripheral activity in the open field speed (2 weeks old) There was no difference between groups in rotarod (2 and 5 weeks old)
	Quinzaños-Fresnedo et al. (2008)	Wistar Rats Both sexes	Not described	24 pups distributed in: C Sham HI	–	–	HI (P7)	↓ Crossings in the open field (P42)
	Kadam et al. (2009)	Mice CD-1	Both sexes	Sham (n = 15, 8 Male, 7 Female) HI (n = 15, 7 Male, 8 Female)	–	–	HI (P12)	↓ Distance traveled in the open-field (P40) There was no difference between groups in Rotarod and Cylinder test (P40)
	Arteni et al. (2010)	Wistar Rats	Both sexes	C HI-L HI-R (n = 11-13/ group)	–	–	HI (P7)	↑ Crossings in the open-field (13 weeks old)
	Sanches et al. (2013)	Wistar Rats	Both sexes	Sham HI-R HI-L (n = 8 a 16 / group)	–	–	HI (P3)	There was no difference between groups in locomotor parameters evaluated by the open-field (17 weeks old)
	Alexander et al. (2014)	Wistar Rats	Male	Sham (n = 6) HI P3 (n = 14) HI P7 (n = 14)	–	–	HI (P3 ou P7)	↓ Fall latency in rotarod (HI P7 group on P30-31) There was no difference between the Sham and HI P3 group in the rotarod
	Misumi et al. (2016)	Wistar Rats	Male	Sham(n = 12)	–	–	HI (P3)	

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Table 3 (*continued*)

CP MODEL	AUTHOR (YEAR)	ANIMAL	SEX	EXPERIMENTAL GROUPS (n)	CP INDUCTION PERIOD			LOCOMOTION AND MOTOR COORDINATION OUTCOMES
					Prematurity	Antenatal	Postnatal	
PRENATAL ISCHEMIA	Sanches et al. (2015a, 2015b)	Wistar Rats	Both sexes	HI (n = 16) Sham HI-R HI-L (n = 12 a 18 / group) HI (n = 9)	–	–	HI (P3 ou P7)	↓ Fall latency of falls in rotarod (8 months old) ↑ Crossings in the open field in HI P7 group (P60)
	Miguel et al. (2015)	Wistar Rats	Male	Controle (n = 11)	–	–	HI (P7)	There was no difference between groups in locomotor parameters evaluated by the open-field (P60)
	Durán-Carabali et al. (2016)	Wistar Rats	Both sexes	C (n = 14) HI 120'(n = 11) HI 180' (n = 14) HI 210' (n = 10)	–	–	HI (P3)	↑Foot fault on ladder walking test (P35 and 45) Asymmetric use of the forelimb on cylinder test (P35 and 45)
	Ueda et al. (2018)	Wistar Rats	Male	C (n = 46) Sham (n = 10) HI (n = 62) Sham (n = 25)	–	–	HI (P3)	↓ Locomotor activity score (8–9 weeks old) Swing durations asymmetry on DigiGait (8–9 weeks old) There was no difference between groups on Horizontal Ladder Test (8–9 weeks old)
	Zaghloul et al. (2017)	Mice	Not described	HI (n = 25)	–	–	HI (P5)	↓ Fall latency in rotarod (P60) ↓ Beam break and rearing in the open-field (P60)
	Sanches et al. (2019)	Wistar Rats	Both sexes	Sham (n = 13) HI (n = 19)	–	–	HI (P3)	↑ Crossings in the open field (P45)
	Windle and Becker (1943)	Guinea pig	Not described	C (n = 90) PI (n = 103)	PT (E63 - E65)	PI	–	↓ Motor activity and loss of control of hind limbs
	Tashima et al. (2001)	Sprague- Dawley Rats	Both sexes	C (n = 15, 10 Male e 10 Female) PI (n = 12, 5 e 7 Female)	–	PI (E17 and E22)	–	↓ Crossings in the open-field in male (7 weeks old)
	Derrick et al. (2004)	New Zealand white rabbits	Male	C (n = 129) PI 30 min on E22 (n = 26) PI 37-40 min on E22 (n = 102) PI 37-40 min on E21 (n = 61)	–	PI (E21 ou 22)	–	↓ Motor activity score (P1)
	Tan et al. (2005)	New Zealand white rabbits	Both sexes	C (n = 26) PI (n = 16)	–	PI (E21, 22, ou 25)	–	↓ Motor activity score (P1)
	Robinson et al. (2005)	Sprague- Dawley Rats	Not described	C (n = 24) PI (n = 20)	–	PI (E18)	–	↓ Squares visited and stride length in the open field (4 months old)
	Delcour et al. (2011)	Sprague-Dawley Rats	Both sexes	Sham (n = 42) PI (n = 44)	–	PI (E17)	–	↓ Normalized swing length relative to the tibial size and normalized foot speeds during a swing on both the *x*-axis and the *z*-axis on treadmill 3D kinematics (P65) ↑ Squares visited in the open field (P65)
	Delcour et al. (2012a)	Sprague-Dawley Rats	Both sexes	Sham (n = 18) PI (n = 14)	–	PI (E17)	–	The angles of all joints were significantly different between HI and sham rats indicating both hips over flexion

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Table 3 (*continued*)

CP MODEL	AUTHOR (YEAR)	ANIMAL	SEX	EXPERIMENTAL GROUPS (n)	CP INDUCTION PERIOD			LOCOMOTION AND MOTOR COORDINATION OUTCOMES
					Prematurity	Antenatal	Postnatal	
								and knee and ankle overextension when the foot was at the highest point of the swing.
	Delcour et al. (2012b)	Sprague-Dawley Rats	Both sexes	Sham (n = 26) PI (n = 23)	–	PI (E17)	–	↑ Squares visited in the open-field (P65) ↑ square visiting in the open field (P40- P80)
	Oshima et al. (2016)	Sprague-Dawley Rats	Both sexes	Sham (n = 9 Female and 14 Male) C (n = 24 Female and 24 Male). PI (n = 17 Female and 23 Male)	–	PI (E17)	–	↑ Crossings in females in the open field (P15) ↓ Rearings in males in the open field (P15)
	Ruff et al. (2017)	Long Evans Rats	Both sexes	Sham (n = 12) PI / Growth restricted (mild n = 5; moderate, n = 9)	–	PI (E20)	–	↓ Gait on Catwalk
	Hermans et al. (1992)	Sprague-Dawley Rats	Both sexes	C (n = 15) A (n = 15)	–	A (E15 to E20)	–	↑ Crossings (P19 P44 e 80) and rearings (P19) in the open-field in males ↓ Crossings (P19 P44 e 80 and rearings (P19) in the open-field in females
	Hoeger et al. (2000)	Sprague-Dawley Rats	Female	C A 5' A 10' A 15' A 20' (n = 10/group)	–	–	A (P0)	There was no difference between groups in locomotor parameters evaluated by the open field or motor activity score (3 months old)
	Weitzdoerfer et al. (2002)	Sprague-Dawley Rats	Female	31 pups Female distributed in: C A 10' A 20'	–	–	A (P0)	There was no difference between groups in motor activity score or latency of falls in rotarod or locomotor score
ANOXIA	Hoeger et al. (2003)	Guinea pig	Not described	C A 2' A 3' A 4' A 5' (n = 10/group)	–	–	A (P0)	There was no difference between groups in locomotor parameters evaluated by the open-field or motor activity score (3 months old)
	Van de Berg et al. (2003)	Wistar Rats	Male	C (n = 15) A (n = 12)	–	–	A (P0)	There was no difference between groups in locomotor parameters evaluated by the open-field or footprint test (P21 and P42)
	Ireland et al. (2010)	Spiny mouse	Not described	C (n = 13) PI (n = 13)	–	PI (E38)	–	↑ Fall latency in rotatod (P1 and P2) ↓ Distance traveled and percentage of time stationary in the open field (P5 and P15)
INTRAVENTRICULAR HEMORRHAGE	Balasubramaniam et al. (2006)	Sprague-Dawley Rats	Both sexes	C (n = 17, 9 Male and 8 Female) Sham (n = 19, 6 Male and 13 Female) IVH (n = 21, 11 Male and 10 Female)	–	–	IVH (Po – P1)	↓ Locomotor score (P4, 8, 11 e 15) ↑ Fall latency on the rotarod (6, 7 e 8 weeks old)
	Aquilina et al. (2007)	Piglet		C (n = 3)	–	–	IVH (P0)	

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Table 3 (*continued*)

CP MODEL	AUTHOR (YEAR)	ANIMAL	SEX	EXPERIMENTAL GROUPS (n)	CP INDUCTION PERIOD			LOCOMOTION AND MOTOR COORDINATION OUTCOMES
					Prematurity	Antenatal	Postnatal	
			Not described	14 pups distributed in: IVH autologous blood IVH autologous blood with elevated hematocrit IVH artificial CSF				↓ Locomotion only in the 1st week old
	Georgiadis et al. (2008)	New Zealand white rabbits	Not described	12 litters distributed in: C IVH C (n = 15)	PT (E29)	–	IVH (P0)	↓ Locomotor score (P1 – P3)
	Chua et al. (2009)	New Zealand white rabbits	Not described	C + Glicerol (n = 17) IVH + Glicerol (n = 20)	PT (E29)	–	IVH (P0)	↓ locomotor scores and distance traveled in 60 seconds
				C (n = 12)				↓ Locomotor score (P7 e 11)
	Alles et al. (2010)	Wistar Rats	Male	Salina-Uni (n = 12) Salina-Bi (n = 12) IVH Bi (n = 12) IVH Uni (n = 12) C (n = 8)	–	–	IVH (P6)	↓ Crossings and rearings in the open-field (P30)
	Lekic et al. (2011)	Sprague-Dawley Rats	Not described	Sham (n = 8) IVH (n = 8)	–	–	IVH (P7)	↑ Locomotor activity in the open-field (4 weeks old) ↓ Fall latency of falls in rotarod (4 weeks old)
	Lekic et al. (2012)	Sprague-Dawley Rats	Not described	C (n = 18) Sham (n = 41) IVH Colagenase-0,1 units (n = 10); IVH Colagenase-0,3 units (n = 84) C (n = 19, 10 Female and 9 Male);	–	–	IVH (P7)	↑ Distance traveled in the open field (P8 and P9) ↓ Fall latency in rotarod (2 weeks old) ↑ Foot fault on-grid walking
SENSORIOMOTOR RESTRICTION	Delcour et al. (2018a)	Sprague-Dawley Rats	Both sexes	SR (n = 16, 8 Female and 8 Male) C (n = 18)	–	–	RS (P1 -P28)	Somatosensory hind limb map features were highly correlated to locomotion kinematics impairments, to musculoskeletal histopathology and tended to correlate with neurotransmission findings based on principal components (PC) analysis (PCA) locomotion on a treadmill (P30 and P65) ↑ Swing duration (P30 and P65), ↓ normalized swing length relative to the tibial size, and normalized foot speeds during a swing on both the *x*-axis and the *z*-axis on treadmill 3D kinematics (P65),
	Delcour et al. (2018b)	Sprague-Dawley Rats	Both sexes	SR (n = 16)	–	–	RS (P1 -P28)	↓ feet contact area during weight support (P30) in 3D kinematics during treadmill locomotion.

Abbreviations: P: Postnatal day; C - Control; A - Anoxia; RS - Sensorimotor restriction; LPS - Lipopolysaccharide inflammation; HI: Hypoxic-ischemia; PI: Prenatal Ischemia; L-Left injury; R-Right injury; CSF: cerebrospinal fluid; n - number of animals per group. **Symbols:** ↑- Increased locomotor parameter in the test; ↓- Reduced locomotor parameter in the test.

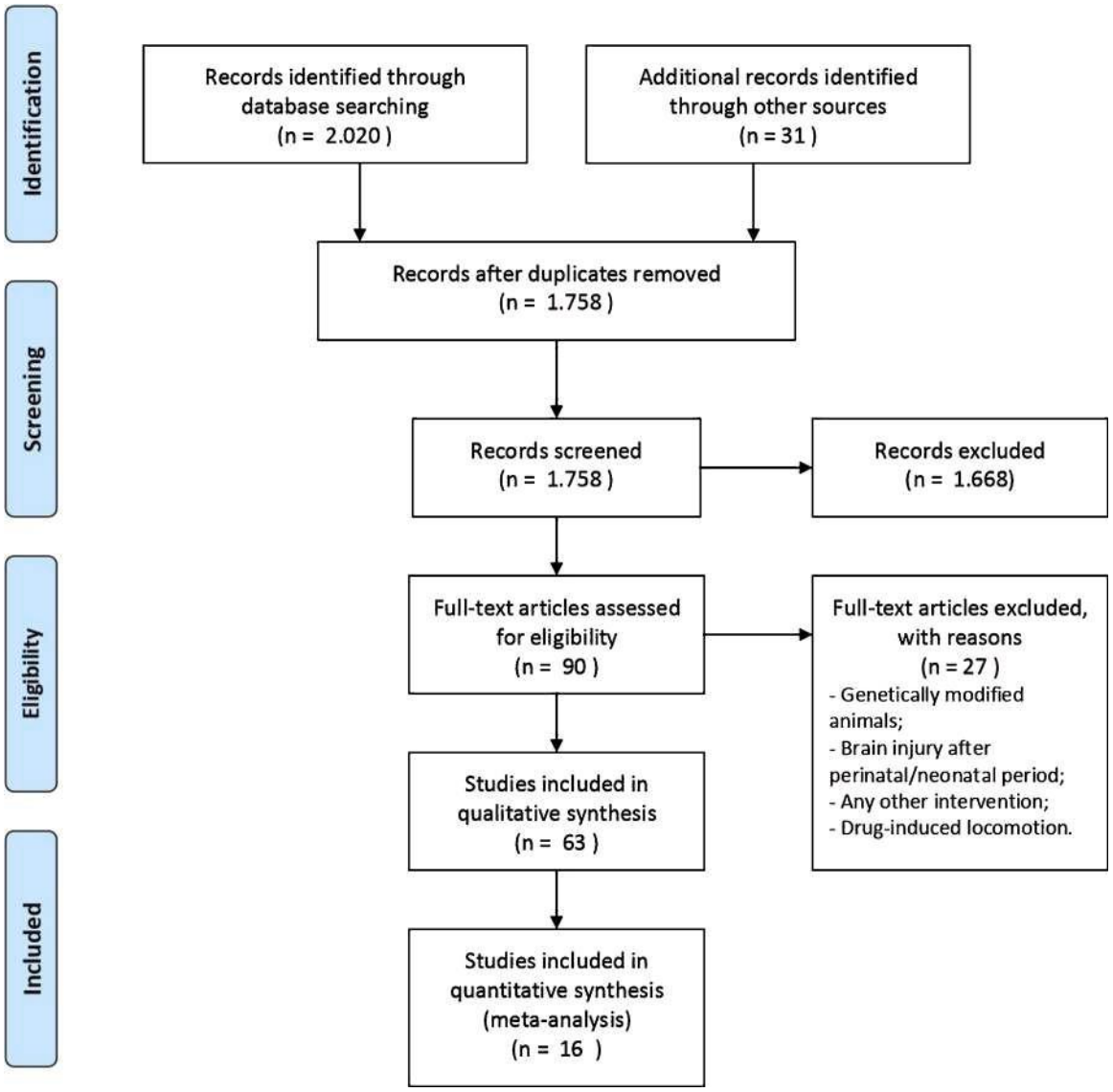

Fig. 1. PRISMA flow diagram of the study selection process.

et al., 2008; Sanches et al., 2013, 2015a, 2015b; Sanches et al., 2019; Ueda et al., 2018; Zaghloul et al., 2017), or it was performed at P12 as observed in only one study (Kadam et al., 2009). PI was performed in 10 studies that used the prenatal period through uterine ischemia (Delcour et al., 2011, 2012a; 2012b; Derrick et al., 2004; Ohshima et al., 2016; Robinson et al., 2005; Ruff et al., 2017; Tan et al., 2005; Tashima et al., 2001; Windle and Becker, 1943).

Another frequent model of CP was exposure to LPS inflammation occurring in the prenatal period through maternal injection of LPS as observed in 12 studies (Baharnoori et al., 2012; Balakrishnan et al., 2013; Dada et al., 2014; Kannan et al., 2011; Makinson et al., 2017; Nance et al., 2017; Poggi et al., 2005; Rousset et al., 2013; Saadani--Makki et al., 2008; Shi et al., 2018; Wallace et al., 2010; Wang et al., 2010; Zhang et al., 2016). The model of LPS inflammation in the postnatal period was performed in only 2 studies through intracerebral injection of LPS in pups at P5 (Fan et al., 2011; Wang et al., 2013).

Among the models that performed insults on the postnatal period to induce CP, the IVH model and the anoxia model occurred earlier. The IVH model occurred in the postnatal period between P0 and P7 by injection of glycerol intraperitoneally in 2 studies (Chua et al., 2009; Georgiadis et al., 2008), or by intracerebral infusion of collagenase in 3 studies (Alles et al., 2010; Lekic et al., 2011) or autologous blood in 2 studies (Aquilina et al., 2007; Balasubramaniam et al., 2006). The anoxia model was performed predominantly as a single event at P0 in 5 studies (Van De Berg et al., 2003; Ireland et al., 2010; Hoeger et al., 2000, 2003; Weitzdoerfer et al., 2002), but it was also performed as repeated events in the prenatal period from E15 to E20 in one study (Hermans et al., 1992). The sensorimotor restriction model was the one that extended the most throughout postnatal development, as observed in 2 studies that covered P1 to P28 (Delcour et al., 2018a; 2018b).

The combination of experimental models to induce CP was diverse and included the combination of prenatal LPS inflammation combined with postnatal HI (Girard et al., 2009), LPS inflammation and PI both in prenatal period (Jantzie et al. 2014), anoxia with sensorimotor restriction both in the postnatal period (Marcuzzo et al., 2010; Strata et al., 2004) and prenatal LPS inflammation combined with anoxia with the

post-sensorimotor restriction natal (Buratti et al., 2019; dos Santos et al., 2017; Stigger et al., 2013, 2011). In addition to these combinations between different CP models, 3 studies induced prematurity in a complementary insult, it was performed in the models of PI (Windle and Becker, 1943), LPS inflammation (Wallace et al., 2010), and IVH (Chua et al., 2009; Georgiadis et al., 2008). In all studies that used an injectable agent or surgical procedure as in the HI, LPS, IVH, or combination of these models, the authors included a simulated control group in which saline was administered or the same surgical procedure was performed, but without inducing CP.

3.3. Main results

3.3.1. Effects of different CP models on locomotion

In general, studies have shown a deficit in the global mobility of animals in which the hind limbs were more affected than the forelimbs in all models. HI impaired the quality of movement of the head, forelimbs, and hind limbs, and it leads to hypertonia and postural deficits impaired the animals' ability to swim (Tan et al., 2005). It was observed that in pups submitted to PI there were changes in the articular angles of the lower limbs, including a slight hyperflexion of the hip compensated by a slight overextension of both knees and ankles during gait, mainly in the maximum swing amplitude before the foot was projected for the front (Delcour et al., 2011). The LPS inflammation model also promoted postural changes in the extremities, such as joint stiffness (Saadani--Makki et al., 2008), the crossing of hind limbs combined with hypertonia leading to poor motor function (Zhang et al., 2016). In animals submitted exclusively to perinatal anoxia, changes in gait were subtle and reversible (Stigger et al., 2013, 2011; Strata et al., 2004), or were not even evident in the short term (Van De Berg et al., 2003; Hoeger et al., 2000, 2003; Weitzdoerfer et al., 2002).

In animals submitted to experimental model combinations, more severe and lasting functional deficits were observed, especially when the sensorimotor restriction was present. It was evidenced in animals submitted to sensorimotor restriction combined with HI, LPS, or Anoxia, reduced movements of the joints, irregular cycles of steps, inability to coordinate the anterior and posterior limbs, hip elevation, external turns of the feet, unstable standing posture with the base of extended support, in addition to that the forelimbs propelled the rats, while the hindlimbs were dragged (dos Santos et al., 2017; Stigger et al., 2011; Strata et al., 2004). Similarly, the combination between PI and LPS led to impairments in cadence, consistency, and coordination between limbs during gait, there was a decrease in the area of the hind legs at the peak of the step consistent with finger-walking (Jantzie et al., 2014).

In addition to the observational assessment of gait patterns and deficiencies in dynamic postural control, locomotor activity was analyzed using devices already established in the literature. The open-field test was the most used to evaluate the animals and it was carried out in 36 studies that included the parameters distance traveled, average speed, mobility and immobility time, and exploratory behavior. Another method frequently used was the gait and mobility score, it was performed in 23 studies from the earliest stages of postnatal development. This score points to the quality of the head, trunk, and limb movements during spontaneous locomotion. 6 studies used the cylinder test to analyze the use of forelimbs and asymmetries in locomotion, 6 studies performed the test on a horizontal ladder to assess the accuracy of placing paws on the steps, 4 carried out the test on a treadmill for capacity analysis of walking at different speeds, 3 performed the gait analysis in Catwalk that evaluates the kinematics in the gait phases and 2 performed the Suspended bar to assess the ability to walk on the bars.

As for the locomotion assessment period, in this review, short-term outcomes were considered for analyses carried out up to the 30th day of postnatal life, and for long-term analyses carried out after this period. Studies that used the LPS inflammation model found divergent effects. Some were able to reproduce the damage to locomotor activity resulting from this insult, but only in the short term in the bar walk test and gait and mobility score (Balakrishnan et al., 2013; Dada et al., 2014; Fan et al., 2011; Kannan et al., 2011; Rousset et al., 2013; Saadani-Makki et al., 2008; Shi et al., 2018; Zhang et al., 2016), others did not show dysfunction in the short or long term in the open field or the gait score and mobility (Baharnoori et al., 2012; Poggi et al., 2005; Wallace et al., 2010; Wang et al., 2013). In contrast, other studies observed long-term hyperactivity in the open field (Dada et al., 2014; Fan et al., 2011; Makinson et al., 2017; Wang et al., 2010).

Similarly, the hypoxic-ischemia e prenatal ischemia models also showed divergences in their results, including some studies that observed opposite effects due to the same model. Most studies have found short or long-term deficits in locomotor activity in the open field, in the horizontal ladder test, or in the treadmill test (Alexander et al., 2014; Bona et al., 1997; Delcour et al., 2011, 2012b; Derrick et al., 2004; Durán-Carabali et al., 2016; Grow et al., 2003; Ireland et al., 2010; Jansen and Low, 1996; Kadam et al., 2009; Lubics et al., 2005; Misumi et al., 2016; Quinzaños-Fresnedo et al., 2008; Robinson et al., 2005; Ruff et al., 2017; Tan et al., 2005; Ueda et al., 2018; Windle and Becker, 1943; Zaghloul et al., 2017). Other studies have observed hyperactivity mainly in the open field and in the bar walk test (Arteni et al., 2010; Delcour et al., 2011, 2012b; Ireland et al., 2010; Lubics et al., 2005; Sanches et al., 2015a, 2015b; Ueda et al., 2018). A PI study demonstrated effects depending on the sex of animals, in which the male pups showed hypoactivity in the open field and the female hyperactivity (Ohshima et al., 2016). Finally, other studies have not been able to show differences between the animals submitted to HI and the control animals in the open field analysis, the cylinder test, the bar walk test, and the horizontal stair test (Balduini et al., 2000; Kadam et al., 2009; Lubics et al., 2005; Miguel et al., 2015; Sanches et al., 2013, 2019).

On IVH model studies, most of them have evidenced the locomotor deficit resulting from this insult, mainly in the short term in the open field analysis, in the bar walk test, and the gait and mobility score (Alles et al., 2010; Balasubramaniam et al., 2006; Chua et al., 2009; Georgiadis et al., 2008). Only one study observed hyperactivity in the open field, occurring in juvenile animals (Lekic et al., 2012). In contrast, studies using perinatal anoxia have not been able to demonstrate deficits in locomotor parameters even in the short term through open field analysis, bar walk test, horizontal ladder test, and gait and mobility score (Van De Berg et al., 2003; Hoeger et al., 2000, 2003; Weitzdoerfer et al., 2002; Ireland et al., 2010). Only one study, which used anoxia as a prenatal insult, observed opposite effects between males and females, in which males showed hyperactivity and females hypoactivity in an open field in the short term (Hermans et al., 1992).

Combined models of CP showed disorders in several locomotor parameters evaluated. The combination of the models, regardless of the period of application of the insult, was effective in demonstrating the worst performance of the animals in the spontaneous locomotion tests in the open field, in the bar walk test, in the horizontal stair test, in the treadmill test, in the suspended bar and gait and mobility score. From the earliest stages of life, the combined models led to a reduction in locomotor activity that extended into adulthood. Thus, the method of insult to which the animals were subjected shows that the models of LPS combined with anoxia (Stigger et al., 2013) and LPS combined with HI or PI (Girard et al., 2009; Jantzie et al., 2014) promote less lasting effects than in models of sensorimotor restriction, where functional degradation remained for a long term (Buratti et al., 2019; Marcuzzo et al., 2010; dos Santos et al., 2017; Stigger et al., 2011; Strata et al., 2004). Studies with isolated sensorimotor restriction also observed a similar effect in the gait analysis in Catwalk (Delcour et al., 2018a; 2018b).

The distance covered and the average speed in the open field test are outcomes widely used in the assessment of spontaneous locomotor activity, and the effects of different models on these parameters are investigated in this review. Through the analysis of subgroups, there was a significant reduction in the distance traveled only in the model that combined LPS with anoxia with SR (Girard et al., 2009) (SMD -7.24 95 % CI [-8.98, -5.51], Z = 1.18) and in the anoxia model (Van De Berg et al.,

2003) (SMD -1.77 95 % CI [-2.66, -0.87], Z = 3.87). Despite this result, in the models in general, the distance traveled showed a high rate of heterogeneity without significant effect size (SMD 1.00, 95 % CI [-0.20, 2.19], Z = 1.64; p < .10; I^2 = 95 %) (Fig. 2). As for the speed meta-analysis, according to eligibility criteria and data availability, only the models of HI and anoxia were included. Similarly, the average speed also showed no significant effect resulting from these models, even with high heterogeneity (SMD 0.96 95 % CI [-1.31, 3.22], Z = 0.83; p < .41; I^2 = 96 %) (Fig. 4). Also, the funnel plots for the analysis of the distance traveled and average speed demonstrated good accuracy and symmetry consistent with low publication bias (Figs. 3 and 5).

3.3.2. *Effects of different CP models on motor coordination*

Considering motor coordination and balance as an essential part of locomotion, many articles have evaluated this outcome. 19 studies performed the performance test on rotarod through the latency of falls, 6 studies the suspension rope test (Suspended bar) (Balasubramaniam et al., 2006; Lubics et al., 2005; Stigger et al., 2011; Strata et al., 2004; Wang et al., 2010; Weitzdoerfer et al., 2002), 4 used the analysis of the animal's ability to walk on a suspended bar (Beam Walking) (Marcuzzo et al., 2010; Misumi et al., 2016; Sanches et al., 2019; Wang et al., 2010), 4 assessed coordination through failure to place paws in the grid walk test (Grid walking and foot fault test) (Bona et al., 1997; Lekic et al., 2012; Lubics et al., 2005; Rousset et al., 2013) as well as in 4 studies on the ladder walk test (Durán-Carabali et al. 2016; Marcuzzo et al., 2010; Stigger et al., 2011; Ueda et al., 2018). Observational assessment of motor incoordination during spontaneous locomotion tests has also been reported in 5 studies (Jantzie et al., 2014; Marcuzzo et al., 2010; Stigger et al., 2013; Strata et al., 2004; Ueda et al., 2018).

The rotarod performance test assesses the latency of the animal's falls from the apparatus, that is, it determines the animal's ability to remain walking on a moving rod. Thus, studies that evaluated animals from P21 were considered for inclusion in the meta-analysis, since at this age the pups already have gait in the adult pattern. 16 studies were included in the meta-analysis, in which most of these were able to cause a decrease in performance during the test (Alexander et al., 2014; Balasubramaniam et al., 2006; Girard et al., 2009; Jansen and Low, 1996; Lekic et al., 2011; Misumi et al., 2016; Stigger et al., 2013, 2011; Strata et al., 2004; Wallace et al., 2010; Zaghloul et al., 2017). But 4 studies did not demonstrate significant differences between the group submitted to the CP model and the control group (Balduini et al., 2000; Kadam et al., 2009; Lubics et al., 2005; Rousset et al., 2013). This reduction in motor coordination in rotarod was confirmed through meta-analysis despite a high level of heterogeneity between studies (SMD -2.47, 95 % CI [-3.43, -1.51], Z = 5.05; p < .00001; I^2 = 97 %) (Fig. 6). Subgroup analysis shows the influence of the different CP models used in this outcome. We observed that the models that combined different insults stood out in reproducing the impairments in the animals' motor coordination (SMD -6.01, 95 % CI [-7.67, -4.35], Z = 7.11; p < .00001; I^2 = 89 %) (Girard et al., 2009; Stigger et al., 2013, 2011; Strata et al., 2004). As well as the IVH model, where considerable effect size with moderate heterogeneity was also observed between the studies (SMD -4.91, 95 % CI [-5.84, -3.98], Z = 10.31; p < .00001; I^2 = 44 %) (Balasubramaniam et al., 2006; Lekic et al., 2011, 2012) (Fig. 6). Also, the funnel plot demonstrates good accuracy of study results and symmetry consistent with low publication bias (Fig. 7).

3.4. *Methodological quality of included studies*

The methodological evaluation of the included studies was carried out through the SYRCLE risk of bias tool by two independent evaluators in which the Kappa test demonstrated agreement Kappa = 0.891 95 % CI

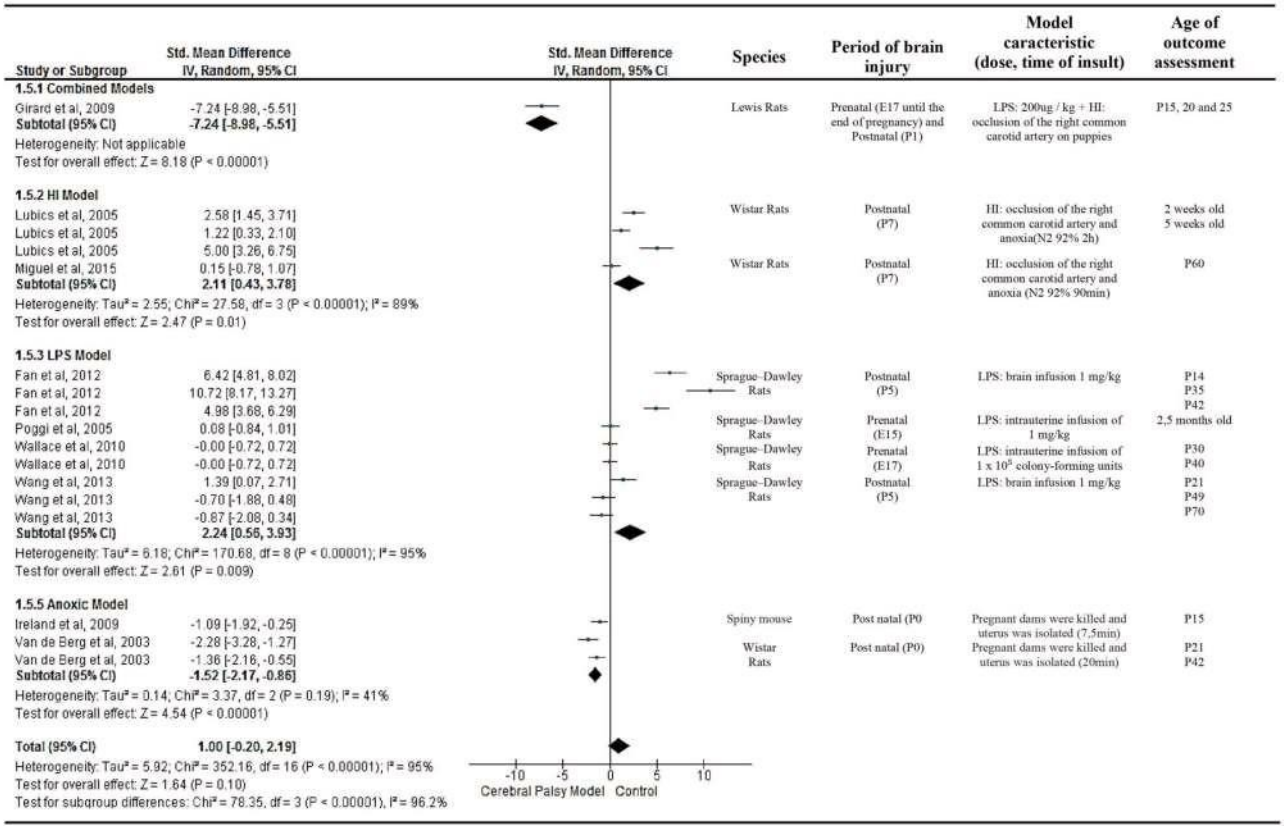

Fig. 2. Forest plot of included studies evaluating distance traveled in open-field (Subgroup: Experimental models of CP). Horizontal lines represent the effect size ± the confidence interval (95 %). The summary effect size is represented by the diamond. P: Postnatal day; C - Control; A - Anoxia; RS - Sensorimotor restriction; LPS - Lipopolysaccharide inflammation; HI: Hypoxic-ischemia.

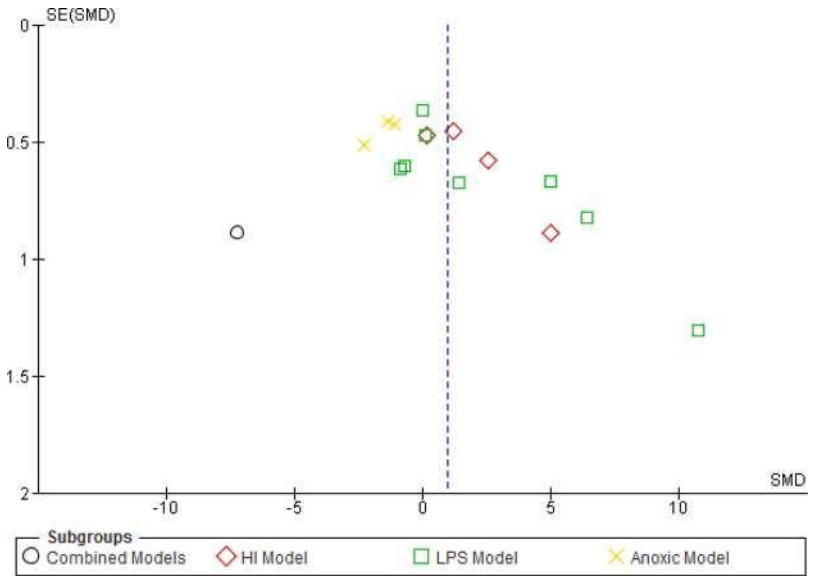

Fig. 3. Funnel plot of standardized mean differences (SMD) of distance traveled in open-field. *SE* = standard error.

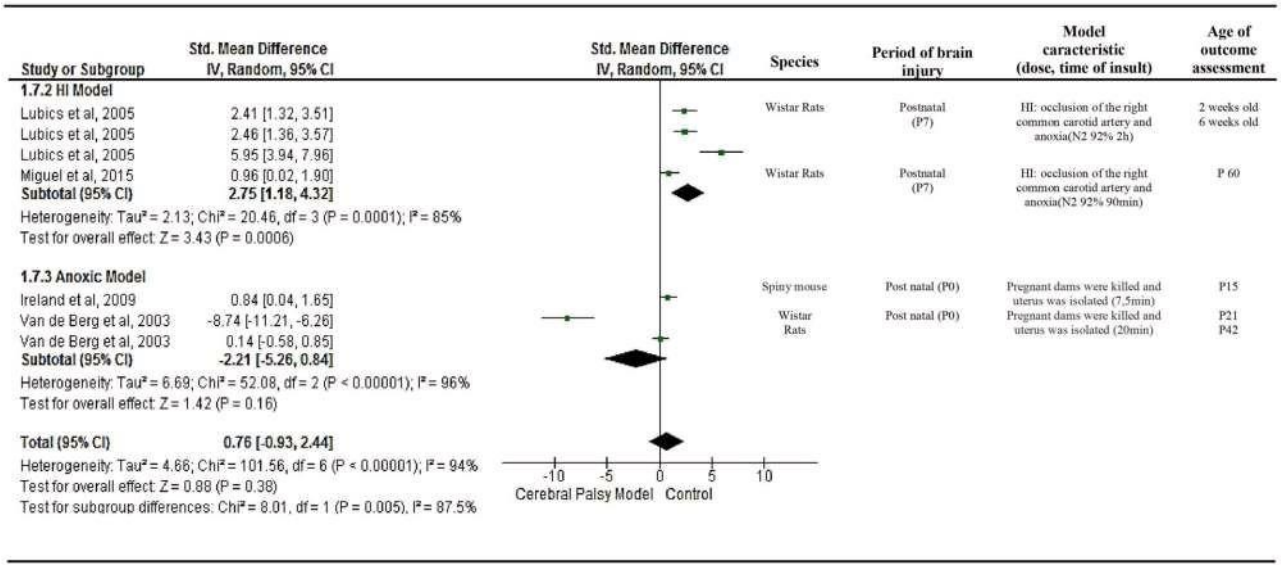

Fig. 4. Forest plot of included studies evaluating average speed in open-field (Subgroup: Experimental models of CP). Horizontal lines represent the effect size ± the confidence interval (95 %). The summary effect size is represented by the diamond. P: Postnatal day; C - Control; A - Anoxia; RS - Sensorimotor restriction; LPS - Lipopolysaccharide inflammation; HI: Hypoxic-ischemia.

(0.770–1.000) for the items of the tools. Kappa agreement was calculated using the GraphPad QuickCalcs Web site: http://www.graphpad.com/quickcalcs/ConfInterval1.cfm (accessed Jan/2021). It was observed that the baseline characteristics of the groups' experimental results were similar in the studies. Most of them did not perform adequate randomization, and of the 67 articles included, 18 performed randomization for groups (Aquilina et al., 2007; Balduini et al., 2000; Van De Berg et al., 2003; Durán-Carabali et al., 2016; Girard et al., 2009; Lekic et al., 2012; Marcuzzo et al., 2010; Miguel et al., 2015; Ohshima et al., 2016; Quinzaños-Fresnedo et al., 2008; Sanches et al., 2013, 2015a, 2015b; Stigger et al., 2013, 2011; Strata et al., 2004; Wallace et al., 2010; Wang et al., 2010; Zhang et al., 2016) and only 2 performed randomization to assess the outcome (Derrick et al., 2004; Kannan et al., 2011). No study has reported housing randomization, allocation concealment, or blinded the researchers. As for the blinding of the evaluators for the outcome, 19 studies performed it (Alles et al., 2010; Balasubramaniam et al., 2006; Chua et al., 2009; Delcour et al., 2018a; Derrick et al., 2004; Hermans et al., 1992; Hoeger et al., 2000, 2003; Jantzie et al., 2014; Kannan et al., 2011; Lekic et al., 2012; Makinson et al., 2017; Marcuzzo et al., 2010; Robinson et al., 2005; Saadani-Makki et al., 2008; Stigger et al., 2013, 2011; Strata et al., 2004; Zhang et al., 2016). Incomplete data and selective description of the outcome were observed in 2 studies (Aquilina et al., 2007; Weitzdoerfer et al., 2002). These data are presented in Fig. 8, which is a summary of the individual articles, and in Fig. 9, through a summary graph that shows the risk of bias in the studies included in percentage. Also, to detect publication bias, funnel plots were created for motor coordination outcomes in rotarod, distance traveled, and average speed in the open-field test. All of these demonstrated good precision and symmetry, consistent with low publication bias (Figs. 3,5, and 7).

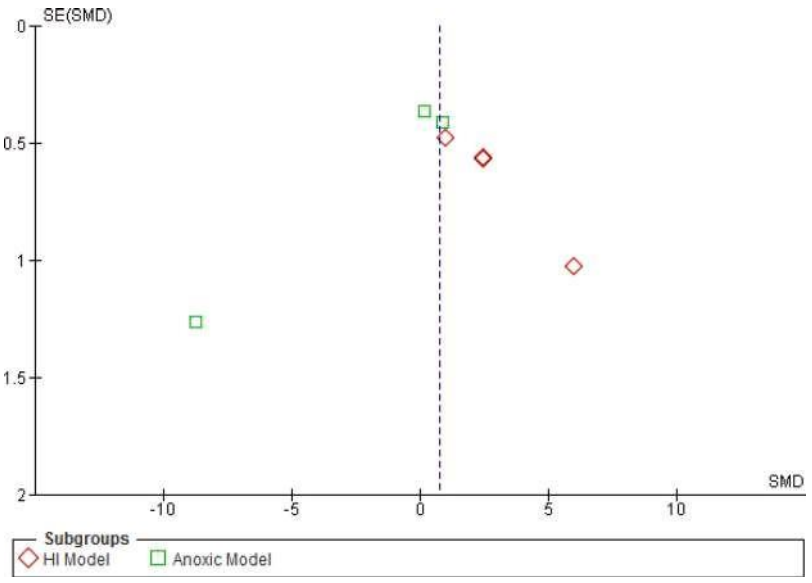

Fig. 5. Funnel plot of standardized mean differences (SMD) of average speed in open-field. *SE* = standard error.

4. Discussion

This systematic review demonstrated the effects of CP models in reproducing the damage to locomotion and motor coordination in animals. The combination of the different experimental models has been demonstrated as the CP model that leads to more severe changes and maintained over the long term, especially when the sensorimotor restriction was present. The combined models potentiated the functional repercussions of the individual models on locomotion and motor coordination, as confirmed through the meta-analysis of distance traveled and motor coordination. As a short-term locomotor dysfunction model, the IVH model stands out in this review. The LPS, PI and HI models were divergent in terms of locomotor outcomes, whereas the isolated anoxia model promoted subtle and reversible damage on locomotion. Additionally, this review evidenced that the hind limbs of the animals were the most affected, regardless of the model adopted in the studies, contributing strongly to the deterioration of the motor function, even if transiently in some models.

As observed in the present review, the combination of different experimental CP models has shown to be able to promote impairments in persistent locomotion and motor coordination. This can be elucidated by understanding the individual effects of each adopted experimental model. The sensorimotor restriction alone or combined with other models was the model that most contributed to the maintenance of motor damage in the long term due to the immobilization of the animals' hind limbs similar to the lack of movement as happens in humans (Buratti et al., 2019; Delcour et al., 2018a; 2018b; Marcuzzo et al., 2010; dos Santos et al., 2017; Stigger et al., 2011; Strata et al., 2004). This result can be elucidated by studies that show that reduction of sensory afferences leads to the degradation of the organization of the primary motor cortex in the representation of posterior limbs, reduction of the cross-sectional area of muscles important to locomotion, such as the anterior tibial and soleus, in addition to changes in types of muscle fibers, thus simulating a diplegic cerebral palsy, where there is a predominant involvement of hindlimbs (Stigger et al., 2011; Strata et al., 2004), leading to worse performance in locomotor outcomes and motor coordination as demonstrated in the meta-analyzes of the present study.

The greatest damage to the lower limbs resulting from experimental CP was not exclusive to SR and often occurred only in the short term. In models of HI, PI and LPS, deficiency in the control of lower limb movements is combined with the inflammatory process established in the central nervous system (Shi et al., 2018), which leads to a decrease in myelination (Saadani-Makki et al., 2008; Zaghloul et al., 2017), increased neuronal apoptosis (Saadani-Makki et al., 2008; Zaghloul et al., 2017) and reduced Purkinje cells in the cerebellum (Wallace et al., 2010), for example. Thus, among the observed changes are stiffness, deficiency in movement control, and postural compensations during gait that impair the animals' ability to move in a stable and coordinated manner (Delcour et al., 2011; Saadani-Makki et al., 2008; Tan et al., 2005; Zhang et al., 2016).

In this context, motor coordination was one of the outcomes that most demonstrated to be impaired in animals submitted to CP as found in our meta-analysis. Considering the neuromotor complexity required of animals, rotarod testing is an established and effective method for this type of assessment in rodents (Dunham and Miya, 1957; Kim et al., 2017). As with other functional tests, the analysis protocols were varied and we observed a high rate of heterogeneity, but it did not interfere with the significant size of the final effect. Thus, we can emphasize that the combination of the models obtained a larger effect size in this case, in which the models that combined LPS with HI (Girard et al., 2009), LPS with Anoxia (Stigger et al., 2013), LPS with Anoxia and SR (Stigger et al., 2011), and Anoxia with SR (Strata et al., 2004), reinforces the potential of this method in reproducing neurofunctional damage due to the confluence of the effects of individual models.

The animals' difficulty in maintaining a satisfactory gait pattern not only interferes with gait on a moving rod, as in the rotarod but also influences the simplest locomotion, that is, it affects spontaneous gait. As observed in the present study, a variety of tests were used in this analysis, including the open field test that allows us to quantify kinematic parameters of spontaneous locomotion such as the distance covered and the average speed (da Silva Aragão et al., 2011). In the meta-analysis of the distance traveled, once again it is evident that combination of models leads to a reduction in the ability to move, but as models of HI, PI and LPS were predominantly included, where the studies were divergent, we did not obtain a significant effect size (Lubics et al., 2005; Miguel et al., 2015; Poggi et al., 2005; Wallace et al., 2010; Wang et al., 2013). Similarly, in the analysis of average speed, only models of HI and anoxia were included, in which the latter has already revealed that it does not lead to motor deficits even in the short term (Van De Berg et al., 2003; Ireland et al., 2010; Lubics et al., 2005; Miguel et al., 2015). Thus, the absence of results models that limit the functionality of the animals contributed to not obtaining a significant effect size for the speed parameter.

We observed in this review that the variability of locomotor or motor

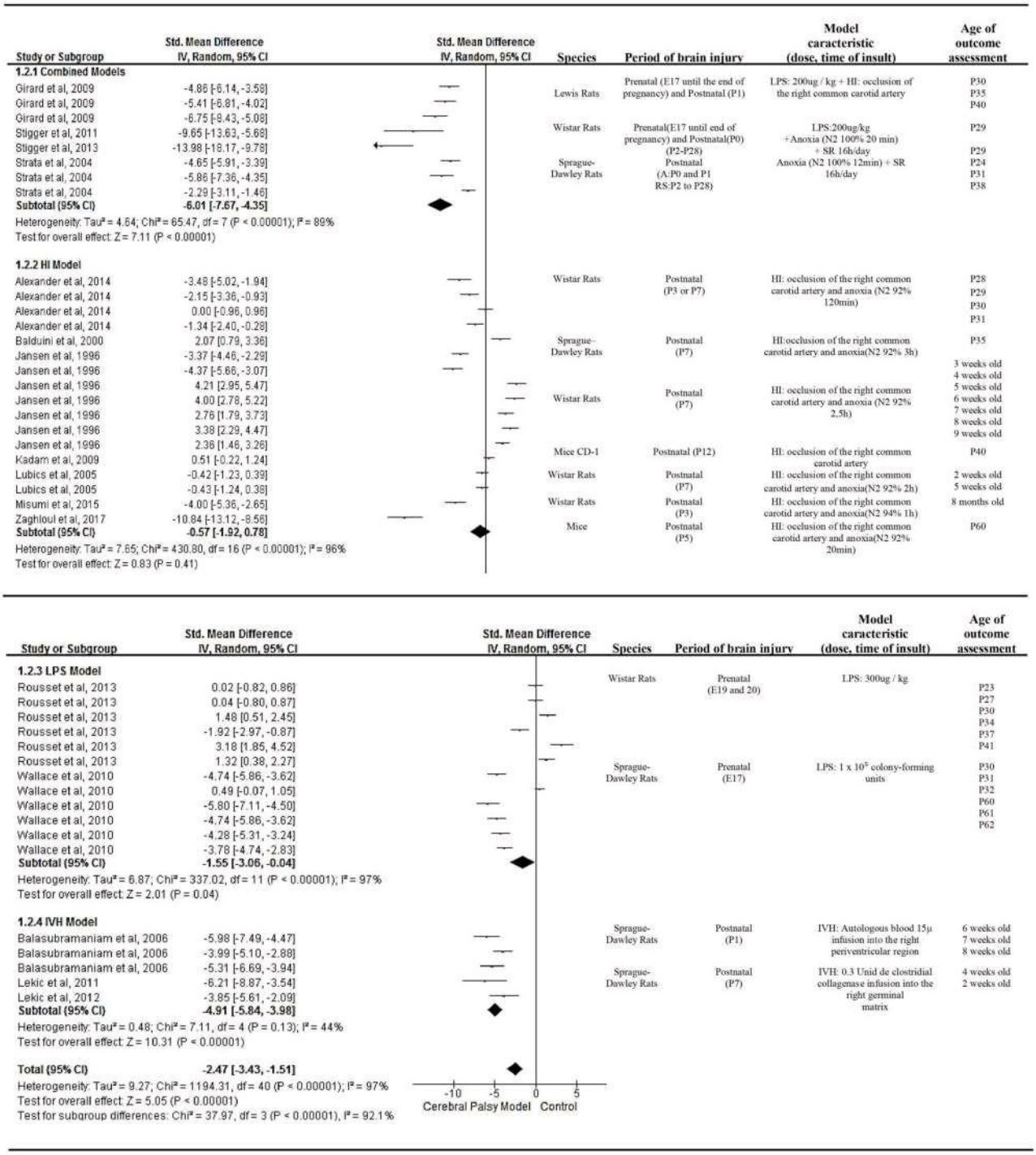

Fig. 6. Forest plot of included studies evaluating motor coordination in rotarod (Subgroup: Experimental models of CP). Horizontal lines represent the effect size ± the confidence interval (95 %). The summary effect size is represented by the diamond. P: Postnatal day; A - Anoxia; RS - Sensorimotor restriction; LPS - Lipopolysaccharide inflammation; HI: Hypoxic-ischemia.

coordination deficits related to the sex of the animals was discrete and punctual and points to the need for discussions on this topic, since the inclusion of animals of both sexes is still a controversial factor, diverging between the authors. Some studies have found no difference between the sexes regarding the parameters evaluated. Others who used only males in their sample seemed to be able to reproduce neurofunctional damage more significantly (Alexander et al., 2014; Marcuzzo et al., 2010; Misumi et al., 2016; Ohshima et al., 2016; dos Santos et al., 2017; Stigger et al., 2013, 2011; Tashima et al., 2001) which did not occur in some studies that used only females (Hoeger et al., 2000; Weitzdoerfer et al., 2002). Females submitted the LPS model (Wang et al., 2010) or HI (Arteni et al., 2010; Ohshima et al., 2016) showed greater evidence of hyperactivity in the open field, so we cannot rule out the effect of the model on these results, and it is necessary to raise the question of the real influence of sex on locomotion in CP models. Therefore, we suggest that further studies use both sexes in their experimental sample to elucidate the mechanisms involved in the particularities of the sexes.

In addition to elucidating the objectives of reproducing neurofunctional damage with a CP model, it is essential to know the reproducibility of the models, including possible adverse events that may

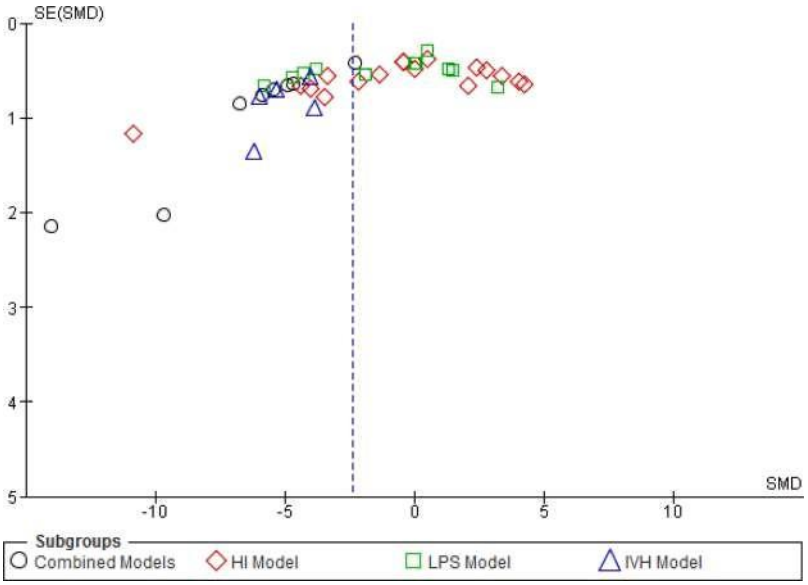

Fig. 7. Funnel plot of standardized mean differences (SMD) of motor coordination in rotarod. *SE* = standard error.

compromise the conduct of the study. In this sense, the principle of 3Rs (Replacement, Reduction, and Refinement) stands out as being a basic requirement for quality science (Kirk, 2018; Russell and Burch, 1959). The tendency is that experimental studies should be increasingly concerned with safer and reproducible methods, not only as an ethical issue but also as a financial one (Kirk, 2018; Percie du Sert et al., 2017). Relevant points to be considered when choosing an experimental model to be adopted: reducing the number of animals and resources needed to obtain information on the research question, and refinement, minimizing the suffering of animals during any experimental procedure (Kirk, 2018; Russell and Burch, 1959). Thus, careful planning is necessary for all phases of the animal care process, including accommodation, acclimatization, anesthesia, analgesia, permanent care of the litters, including mothers and pups, and the use of scales to assess the good. -being of animals (Percie du Sert et al., 2017). In addition to training and awareness of the team about the importance of this care, they are indispensable for improving the quality of evidence from pre-clinical studies (Percie du Sert et al., 2017).

In this way, combined models, in addition to better efficiency in reproducing the damage on locomotion and motor coordination of CP, also demonstrated a low mortality rate of the pups during the application of the model. The mortality rate was below 5% in pups that were submitted to the combination of the postnatal anoxia model with sensorimotor restriction (Strata et al., 2004) and no mortality was observed in pups submitted to inflammation by prenatal LPS with postnatal HI (Girard et al., 2009). In contrast, in the model that associates LPS and PI both on prenatal, although there was no impact on the size of the litter, the early fetal loss was high, reaching 42 % (Jantzie et al., 2014). This suggests that insults combined with the prenatal phase favor more losses related to adverse events, requiring greater resources such as additional skills from researchers and effective preventive measures.

The quality of the studies included in this review was moderate. This can be explained by the lack of detailed information in the articles regarding the methods used to avoid risks of bias, such as, for example, describing the randomization method for experimental groups. Few studies have reported randomization for the groups, randomization for the outcome, and blinding for the assessment of outcomes. This may be due to the publication period of the included studies, in which a large part of them was published before understanding the importance of caring for biases in experimental studies (Hooijmans et al., 2014a, 2014b). Thus, we suggest the adoption of the SYRCLE risk of bias tool (Hooijmans et al., 2014a, 2014b) in future research as a guide from the initial stage of research development, so that better control of the risks of bias and consequently increase the level of available evidence. Additionally, the heterogeneity of the studies regarding the protocols for analyzing the repercussions on locomotion and coordination was one of the limiting points of the present study. This heterogeneity reflects the lack of standardization of methods in the use of the equipment and among research teams that investigate CP, which interferes with the crossing of data and reduces our capacity for inferences. A future alternative would be to homogenize the experimental analysis, for example, by the creation of an online platform for a consultation to protocols or, the strengthening of partnerships between the research groups through the construction of guides of evaluation methods. Finally, another limitation of our study was the generalization of motor impairments results, as they are very much dependent on the timing of insult and species used in the experimental model, and methods and age of the evaluation of locomotion. In this sense, we sought to facilitate the reader's understanding by performing the meta-analysis together with the study characteristics data.

Thus, as noted in this review, there are a variety of experimental CP models that are capable of reproducing neurofunctional changes similar to what happens in humans. Despite the limitations noted above it becomes necessary to reinforce the relevance of preclinical studies of good methodological quality since from them it is possible to clarify the mechanisms related to CP. Consequently, it is possible to outline a range of intervention strategies based on the motor phenotype that is produced with these models and to extrapolate within the ethical limits their implications for humans.

5. Conclusions

The results obtained in this study led us to the following conclusions: (a) The combination of perinatal brain injury models, regardless of the period of insults, enhances the damage on locomotion and motor coordination in the short and long term, especially when the sensorimotor restriction is performed; (b) The locomotor damage phenotype induced by the IVH model is temporary, whereas the isolated anoxia model does not lead to significant motor repercussions even in the short term; (c) New studies are needed to elucidate the effects of the LPS and HI models on locomotion, which presented divergent results in this review, in addition to the fact that new studies may clarify the influence of the sexes in the different models of CP.

Fig. 8. Risk of bias summary of included studies review authors' judgments about each risk of bias item for each included article. + (green) low risk of bias; - (red) high risk of bias; (uncolored) unclear risk of bias.

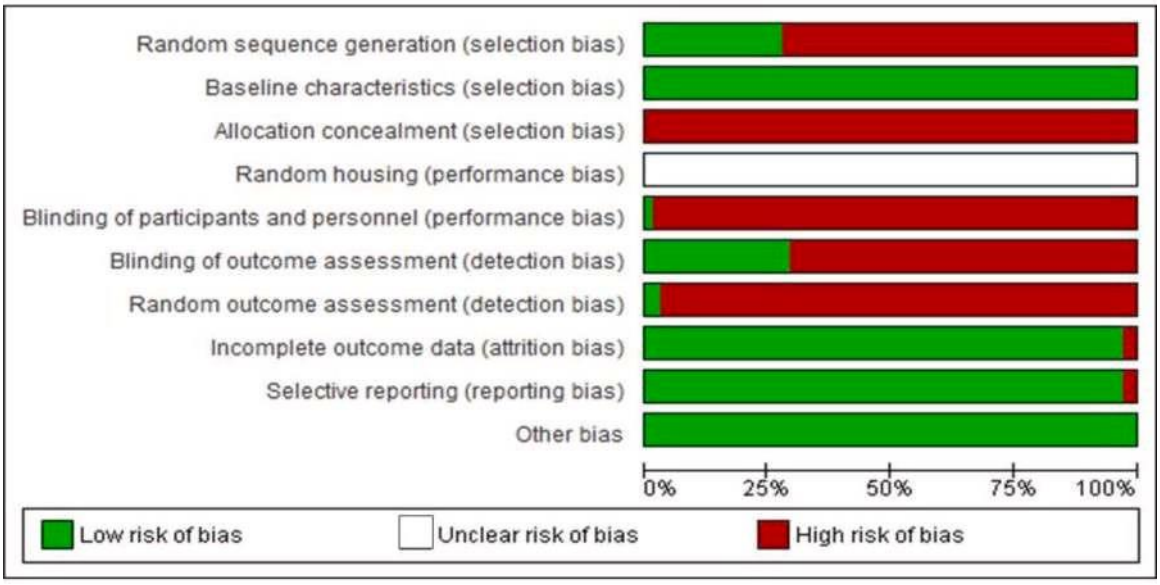

Fig. 9. Risk of bias graph of included studies. Review authors' judgments about each risk of bias item presented as percentages across all included studies. (green) low risk of bias; (red) high risk of bias; (white) unclear risk of bias.

CRediT authorship contribution statement

A.E. Toscano: is the supervisor. **S.C. Pereira, G.L. Albuquerque, C. M.S.S Calado, V.S. Souza, D.B. Visco**, and **A.E. Toscano:** carried out the manuscript preparation process, all of which participated in determining the eligibility criteria and standardizing the data collection forms and analyzing the risk of study bias. **D.B. Visco, R. Manhães-de-Castro**, and **A.E. Toscano:** participated in writing the review and editing for preparation. They contributed their experience regarding intellectual content and guidance. All authors reviewed and agreed with the final manuscript.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jneumeth.2021.109250.

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APÊNDICE D – NEONATAL TREATMENT WITH RESVERATROL DECREASES POSTURAL AND STRENGTH IMPAIRMENTS AND IMPROVES MITOCHONDRIAL FUNCTION IN THE SOMATOSENSORY CORTEX RATS SUBMITTED TO CEREBRAL PALSY

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Neonatal treatment with resveratrol decreases postural and strength impairments and improves mitochondrial function in the somatosensory cortex rats submitted to cerebral palsy

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ABSTRACT

Cerebral palsy is a neurodevelopmental disease characterized by postural, motor, and cognitive disorders, being one of the main causes of physical and intellectual disability in childhood. To minimize functional impairments, the use of resveratrol as a therapeutic strategy is highlighted due to its neuroprotective and antioXidant effects in different regions of the brain. Thus, this study aimed to investigate the effects of neonatal treatment with resveratrol on postural development, motor function, oXidative balance, and mitochondrial biogenesis in the brain of rats submitted to a cerebral palsy model. Neonatal treatment with resveratrol attenuated deficits in somatic growth, postural development, and muscle strength in rats submitted to cerebral palsy. Related to oXidative balance, resveratrol in cerebral palsy decreased the levels of MDA and carbonyls. Related to mito- chondrial biogenesis, was observed in animals with cerebral palsy treated with resveratrol, an increase in mRNA levels of TFAM, in association with the increase of citrate synthase activity. The data demonstrated a promising effect of neonatal resveratrol treatment, improving postural and muscle deficits induced by cerebral palsy. These findings were associated with improvements in oXidative balance and mitochondrial biogenesis in the brain ofrats submitted to cerebral palsy.

1. Introduction

Cerebral palsy (CP) is a heterogeneous group of diseases that result from non-progressive damage to the brain at an early stage of devel- opment. CP symptoms include irregular movement and irregularities in posture (Hadders-algra, 2014). According to the definition, the damage is not progressive, but the general condition can change over the years due to treatment and the plasticity of the maturing nervous system (Mlodawski et al., 2019). People with CP often perform poorly in ac- tivities of daily living due to limited limb, trunk, and head control (Velasco et al., 2017), have abnormal muscle tone and motor control, which contributes to the loss of postural control and motor coordination, compromising functionality and, consequently, quality of life (McNish et al., 2019).

CP is the most common motor development disorder in children (McIntyre et al., 2012), with an incidence of 3,4 per 1000 live births in regions from low- and middle-income countries and 1,5 per 1000 live

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Abbreviations

CP	Cerebral Palsy
ROS	Reactive Oxygen Species
RES	Resveratrol
TBA	Thiobarbituric Acid
MDA	Malondialdehyde
TCA	Trichloroacetic Acid
DNPH	Dinitrophenylhydrazine
SOD	Superoxide dismutase
CAT	Catalase
GST	Glutathione-S-Transferase
GSH/GSSG	Glutathione/Oxidized Glutathione ratio
OPT	o-phthaldialdehyde
DTNB	5,5′-dithio-bis (2-nitrobenzoic acid)
β2M	β-2 microglobulin
RT-PCR	Real-time polymerase chain reaction
LPS	liposaccharide
OS	oxidative stress
PGC-1α	Peroxisome proliferator-activated receptor-gamma coactivator-1α
TFAM	transcription factor A

births in regions from high-income countries (McIntyre et al., 2022). The etiology of CP is diverse and multifactorial, and the risk factors are found in the periods before and around the time of conception, and up to 2 years of age (McIntyre et al., 2022; Pakula et al., 2009). The following risk factors are described as associated with CP: genetic variants, congenital anomalies, neonatal respiratory distress syndrome, meconium aspiration, birth asphyxia, kernicterus, hypoxic-ischemia, and cerebrovascular insults during pregnancy and in infancy, and accidental and non-accidental brain injury (McIntyre et al., 2012, 2022). Among these factors, the mechanisms that cause oxygen and nutrient deprivation to the central nervous system stand out, such as perinatal asphyxia (Hakobyan et al., 2019; Perlman and Shah, 2011), which are associated with the severe neurofunctional consequences of CP (Low and Roland, 2004; Rainaldi and Perlman, 2016; Vannucci et al., 2004; Volpe, 2001).

Oxidative stress (OS) is considered a major contributor to ischemic brain injury (Warner et al., 2004) because it is an important consequence of neurotransmitter-mediated toxicity in combination with hyperoxygenation after hypoxia-ischemia (Ferriero, 2001). The generation of ROS and consequent oxidative stress and mitochondrial dysfunction is related to the onset of neuronal injury, leading to neurodegeneration (Manzanero et al., 2013; Sun et al., 2022). The effects of OS are observed in the pathogenesis of different diseases that affect the brain, such as mitochondrial disorders, cerebral ischemia, epilepsy, and cerebral palsy (Aycicek and Iscan, 2006). Understanding the role of OS in cerebral palsy is important as therapy with agents that increase the body's antioxidant capacity may be a treatment option for preventing the neurological insult (Aycicek and Iscan, 2006).

In this context, experimental models emerge as tools that mimic the pathophysiological mechanisms of CP and its motor disorders similar to those that occur in humans (Lacerda et al., 2017; Pereira et al., 2021a; Pereira et al., 2021b). In this sense, the model that associates perinatal anoxia and sensory-motor restriction of the lower limb reproduces the motor deficits found in children with cerebral palsy, such as reduced locomotor activity (Marcuzzo et al., 2010; Pereira et al., 2021a), reduced sarcomere density (Stigger et al., 2011), changes in oral functions (Lacerda et al., 2017), in addition to impairments in motor performance (Marcuzzo et al., 2010).

Through experimental models, early therapeutic perspectives may emerge for the treatment of CP based on phenotypic plasticity. Phenotypic plasticity is defined as 'the ability of individual genotypes to produce different phenotypes when exposed to different environmental conditions (Pigliucci et al., 2006). This includes the ability of neural synapses and brain pathways to be modified by altered thoughts and emotions, as well as environmental, behavioral, and neural stimuli, thus enabling neural plasticity (Kourosh-Arami et al., 2021). Studies with polyphenols have shown that the consumption of these natural compounds can improve neural plasticity (Bensalem et al., 2018) and resveratrol (RES), a polyphenolic compound present in many plant species (Peng et al., 2022; Song et al., 2014), improves neuroplasticity through a variety of mechanisms (Peng et al., 2022). It is important to highlight the importance of interventions due to their ability to modulate neuronal activity, generating adaptive responses that can cause normal differentiation of motor neurons and consequent ideal neuromuscular performance in adulthood (Stigger et al., 2011).

Several researches have indicated that the use of neuroprotective drugs can improve the prognostic perspectives for patients with cerebral palsy (Sun et al., 2021) and interventions on the central nervous system, mainly in early life, have been suggested as therapies aimed at reducing oxidative stress by interrupting the lesion cascade (Juul and Ferriero, 2014; Visco et al., 2022). Resveratrol has received considerable attention recently for its strong brain protection (Peng et al., 2022). RES is a substance that has shown effective treatment of a wide range of pathologies, including neurodevelopmental disorders and neurocognitive disorders (Baur and Sinclair, 2006). Resveratrol is among the polyphenols that are being used in acute central nervous system injury (Peng et al., 2022) and hypoxia-ischemia models as a neuroprotective strategy and (Juul and Ferriero, 2014; Ortega et al., 2014; Pan et al., 2016). Several lines of evidence have also demonstrated its antioxidant, anti-inflammatory and anti-aggregating properties (Jing et al., 2013; Marques et al., 2009; Pandey and Rizvi, 2009). In addition, a study on early intervention with RES in hypoxia-ischemia brain damage was reported (Karalis et al., 2011), however, the outcomes evaluated in this study have not yet been explored in the literature. Thus, this study aims to investigate the effects of neonatal treatment with resveratrol on postural development, motor function, and oxidative balance in the brain of rats submitted to a cerebral palsy model through neonatal anoxia and sensorimotor restriction.

2. Methodology

2.1. Animals

This study was performed by the guidelines of the National Council for the Control of Animal Experiments (CONCEA) and with the international standards of the National Institute of Health Guide for Care and Use of Laboratory Animals (8th ed) and has been approved by the Ethics Committee for Animal Use (CEUA) of the Federal University of Pernambuco (process number CEUA: 0032/2021). Twenty-one female *Wistar* rats were mated with breeding males in the proportion of two females to one male in a 12h dark-light-cycle (dark 8 a.m.-8 p.m.) at 22 ± 2 °C with free access to water and diet. On the day of birth, male pups were randomly assigned with an ideal body weight (6–8g) and they were divided into four groups, based on resveratrol manipulation and cerebral palsy induction: 1- saline control (CS, n = 12), consisting of pups that received a saline solution from the 3rd to the 21st day of life; 2- resveratrol control (CR, n = 11), consisting of pups that received resveratrol from the 3rd to the 21st day of life; 3- CP salina (CPS, n = 11), constituted by pups submitted to the experimental model of CP and that received a saline solution from the 3rd to the 21st day of life; 4- CP resveratrol (CPR, n = 12), constituted by pups submitted to the experimental model of CP and that received resveratrol from the 3rd to the 21st day of life. Each litter consisted of 8 pups that remained with their mothers until the 25th postnatal day (P25) when they were weaned, and the males were placed in individual cages until euthanasia by decapitation at P29.

2.2. Experimental model of cerebral palsy

The experimental CP model used was the same as described by Strata et al. (2004), Coq et al. (2008), Lacerda et al. (2017), and Pereira (2021a) which associates perinatal anoxia with sensorimotor restriction of the hind paws. The pups in the CP groups underwent two episodes of postnatal anoxia, on the day of birth and the first day of life (P0 and P1). Pups were placed inside an acrylic chamber partially immersed in water at 37 °C and exposed to nitrogen (100%) at 9L/min for 12 min, then recovered in air and at room temperature and returned to their respective mothers. From P2 to P28 sensorimotor restriction of the hind paws was performed for 16 h a day, with the animal being allowed free movement for the remaining 8 h. For the sensorimotor restriction, an orthosis made with an epoxy mold was used, leaving the hind legs extended, without affecting the elimination of urine and feces and maternal care (Strata et al., 2004).

2.3. Administration of resveratrol

After the birth of the animals, the male offspring were randomly allocated according to the experimental groups in the neonatal period, and the drug was administered intraperitoneally from P3 to P21 (Girbovan and Plamondon, 2015). The animals were divided into 1- treated with resveratrol (daily dose, 10 mg/kg) and 2-saline (0.9% NaCl), injection volume was 0.1 ml/100g rat weight). Rats were weighed daily, and the injection volume was adjusted to match the animal's body weight.

2.4. Weight evolution, somatic growth, and maturation of physical features

The weight of the animals was recorded daily from the day of birth to P29. For this, a Marte scale, models S-100, capacity of 1 kg and sensitivity of 0.01g was used.

The somatic growth and physical features were evaluated daily between 7:00 and 9:00h until maturation. For the somatic growth, the animals were evaluated with a digital caliper (JOMARCA®), for the following measurements, in centimeters (H. J. da Silva et al., 2005): lateral skull axis (distance between the ear holes), anteroposterior skull axis (distance between snout and head-neck articulation), tail length (distance from tail tip to tail base) and longitudinal axis (distance between snout and tail base).

The age at the maturation of a particular physical feature was defined as the day when it was first observed (Smart and Dobbing, 1971). The following features were evaluated: auricle opening, auditory conduit opening, eyes opening, upper incisors eruption, and lower incisors eruption.

2.5. Gait analysis on catwalk

Gait analysis was performed using the Catwalk (Noldus), as described by Herold et al. (2016), at P28. Changes in the components of the gait cycle are determined and deficits in locomotion are quantified. The CatWalk System consists of an enclosed walkway (glass plate) that is illuminated by fluorescent light. The system is equipped with a high-speed color camera connected to a computer with the appropriate detection software (CatwalkXT9.1), which can detect various static and dynamic parameters during the rat's spontaneous locomotion (Fig. 1). In this way, the animal is positioned in a 1-m-diameter corridor and individually filmed as it crosses the Catwalk to measure static and dynamic parameters of spontaneous locomotion.

2.6. Forelimb grip strength and postural analysis

Muscle strength analysis was performed using the forelimb grip strength test at P22 and P28. Each animal grabbed the support bar, exerting a traction force on it in a vertical position while suspended by the tail. The force sensor quantifies the peak force of each animal (g). This test was performed on the Animal Grip Strength System (SD Instruments) equipment with a capacity of 200Kgf, resolution 0.1Kgf, and accuracy of ϱ.2% (Takeshita et al., 2017).

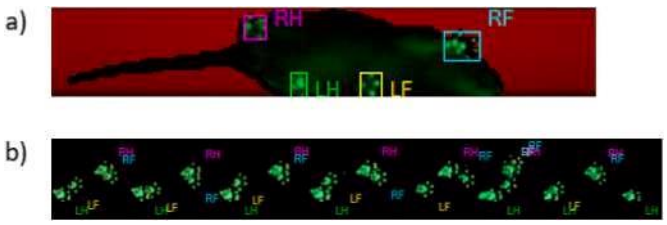

Fig. 1. Evaluation of static and dynamic gait parameters during animal's locomotion by the Catwalk's system. A – Rat walking through the platform. B - Representative images of paw prints during active locomotion of the animals on the platform. LF, left front paw; LH, left hind paw; RF, right front paw; RH, right hind paw.

For the postural analysis, the animals were submitted to the adapted forelimb grip test and evaluated in a horizontal position, at P14, P21 and P28 for the analysis of postural alignment. The performance of the animals during the test was recorded using video. After that, two images of the corresponding video were captured, an image after the initial 5 s of filming, when the animal was still, and another image before the final 5 s, in the same way. We thus determined two moments of analysis to obtain an average of each animal at the ages studied. The parameters evaluated were head and trunk alignment in the horizontal posture. To evaluate the head alignment, an angle will be delimited using the tragus, the occipito-cervical transition, and the midpoint in the transition between the head and the belly of the animal as reference points. To assess trunk alignment, another angle will be defined, the reference points being the transition between the back and tail, the midpoint on the animal's back, and the occipito-cervical transition (adapted from Lelard et al., 2006).

2.7. Extraction, tissue preparation, and protein quantification

At P29, the animals were euthanized by decapitation. The somatosensory cortex (here from now just mention as sample) was removed and kept at −80 °C and prepared for RT-PCR assays and biochemical analysis. The sample was homogenized in cold extraction buffer (100 mM Tris base, pH 7.5; 10 mM EDTA; containing a cocktail of protease inhibitors). After homogenization, samples were centrifuged at 4 °C at 1180 ɡ for 5 min, and supernatants were used for protein quantification according to the Bradford method (BRADFORD, 1976).

2.8. Measurement of citrate synthase activity

Citrate synthase is the first enzyme in the Krebs cycle, its activity capacity is widely used as a marker of cellular and mitochondrial function. Citrate synthase activity was evaluated as previously described (Patel, 1976). Briefly, the reaction was carried out in a mixture containing Tris-HCl (pH = 8.2), magnesium chloride (MgCl), ethylenediamine-tetra-acetic acid (EDTA), 0.2 of 5.5 dithiobis (2-nitrobenzoic acid) (E = 13.6 μmol/(mL.cm), 3 acetyl CoA, 5 oxaloacetate and 0.1 mg/mL of sample. The activity was evaluated by measuring the change in the absorbance rate at 412 nm for 3 min at a temperature of 25 °C, using the EVEN UV-VIS spectrophotometer. The levels are expressed as U/mg of protein.

2.9. Oxidative stress biomarkers

- Evaluation of malondialdehyde production

0.2 mg/ml of protein sample was used to measure the thiobarbituric acid (TBA). In this assay, malondialdehyde (MDA) or MDA-like substances produce a pink pigment with maximum absorption at 535 nm. The reaction was carried out using 30% trichloroacetic acid (TCA) and Tris-HCl (3 mM), followed by centrifugation at 2500×*g* for 10 min. Then, the supernatant was transferred to a tube, mixed with the same volume of 0.8% TBA (v/v), and boiled for 30 min. The absorbance of the organic phase was read at 535 nm in a spectrophotometer, and the results were expressed as mmol per mg of protein (Buege and Aust, 1978).

3. Evaluation of protein oxidation

The carbonyl content is the primary marker for oxidative damage to protein, measured as previously published (Reznick and Packer, 1994). Briefly, 30% TCA was added to the sample (0.2 mg/ml of protein) on ice, mixed, and centrifuged for 15 min at 1180×*g*. The pellet was suspended in 10 mM 2,4-dinitrophenylhydrazine (DNPH) and immediately incubated in a dark room for 1 h with shaking every 15 min. Then, samples were centrifuged, washed three times with ethyl/acetate buffer, and the pellet suspended in 6 M guanidine hydrochloride, followed by incubation for 5 min in a water bath at 30 ˚C. Absorbance was read up to 370 nm, and the results were expressed as mmol per mg of protein.

3.1. Enzymatic antioxidant defense

3.1.1. - Measurement of superoxide dismutase (SOD) activity

The total superoxide dismutase enzyme activity (t-SOD) was determined according to the previously described method (Misra and Fridovich, 1972). Sample (0.2 mg/ml) were incubated with 880 μl sodium carbonate (0.05%, pH 10.2, 0.1 mM EDTA) at 25 ˚C, and the reaction started by 30 mM epinephrine (in 0.05% acetic acid). The kinetics of the inhibition of adrenalin auto-oxidation was monitored for 180 s at 480 nm, and the result was expressed as U/mg protein.

3.1.2. Measurement of catalase (CAT) activity

CAT activity was measured according to the method described by Aebi (Aebi, 1984). The assay consisted of 50 mM-phosphate buffers (pH 7.0), 0.300 mM H_2O_2 and 0.3 mg/mL of sample. The constant rate of the enzyme was determined by measuring the absorbance change at 240 nm for 4 min at 25 ˚C. CAT activity was expressed as U/mg protein.

- Measurement of Glutathione-S-Transferase (GST) activity

GST activity was measured as described previously (Habig et al., 1974). Two hundred micrograms of the sample were added to 0.1 M-phosphate buffer (pH 6.5) containing one mM-EDTA at 25 ˚C. The assay was initiated with 1 mM of 1-chloro-2.4-dinitrobenzene plus 1 mM-GSH. The formation of 2,4-dinitrophenyl-S-glutathione was monitored at 340 nm of absorbance, and the enzymatic activity was defined as the amount of protein required to catalyze the formation of 1 μmol 2, 4-dinitrophenyl S-glutathione. The results were expressed as U/mg protein.

3.2. Non-enzymatic defense

- REDOX State

Reduced Glutathione/Oxidized Glutathione ratio (GSH/GSSG) was evaluated as previously described by (Hissin and Hilf, 1976). The samples were incubated in a 0.1M phosphate buffer containing 5 mM-EDTA (pH 8.0) and with 1 μg/ml o-phthaldialdehyde (OPT) at room temperature (RT) for 15 min and evaluated by fluorescence with wavelengths of 350 nm and 420 nm. GSSG levels were assessed by incubating the same samples with 40 mM N-ethylmaleimide for 30 min in RT with the addition of a 100 mM NaOH buffer. The same steps of the GSH assay were followed to determine the GSSG levels. The ratio of GSH/GSSH determined the REDOX state.

- Quantification of total thiol groups

The quantification of sulfhydryls was based on the reduction of 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB). The sample was mixed in a solution containing Tris-EDTA buffer (pH 7.4), with 10 mM of DTNB and incubated at room temperature for 30 min. The absorbance was measured at 412 nm and results are expressed as mmol/mg protein.

3.3. mRNA evaluation

Total RNA was extracted from cortex tissue using TRIzol reagent and the guanidine isothiocyanate method according to the manufacturer's instructions (Invitrogen, USA). RNA quantification was performed in a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA), and purity was assessed using the ratio of 260/280 nm. Real-time polymerase chain reaction (RT-PCR) experiments were performed using the SuperScript® III Platinum® SYBR® Green One-Step qRT-PCR Kit (Invitrogen, USA). All genes used in the experiment were processed in duplicate, and the cycle threshold (Ct) value of each targeted gene was normalized to the β-2 microglobulin (β2M), and data expressed as $2^{-\Delta\Delta Ct}$ (Silva et al., 2023) (see Table 1).

3.4. Statistical analysis

For data analysis and graph construction we used the statistical software GraphPadPrism® version 9 and the results were expressed as mean ± standard error. A significance level of 95% was assigned. The Kolmogorov-Smirnov normality test was performed. As the normal distribution, for intergroup comparison, the ANOVA two-way parametric test was used with experimental cerebral palsy and pharmacological manipulation, followed by the Tukey post-test. For the variables in which the analysis was performed at several ages, the ANOVA two-way repeated measures followed by the Tukey post-test was used.

4. Results

4.1. Weight evolution, somatic growth and maturation of physical features

Fig. 2 presents the weight evolution of pups. The animals in the CPS group had lower body weight compared to the control saline group, from P8 to the last day of the experiment (P8: CPS: 12.45g ± 2.57g/CS: 18.79g ± 1.52g, $p < 0.01$; P14: CPS: 19.72g ± 4.27g/CS: 31.93g ± 2.65g, $p < 0.001$; P17: CPS: 22.45g ± 4.4g/CS: 37.42g ± 3.67g, $p < 0.001$; P21: CPS: 26.62g ± 4.93g/CS: 47.34g ± 4.68g, $p < 0.001$; P29: CPS: 47.87g ± 12.35g/CS: 78.35g ± 4.78g; $p < 0.001$) (Fig. 1). The treatment with resveratrol in CP attenuated the damage on weight gain only at P21 (P21: CPR: 32.69g ± 4.2g/CPS: 26.62g ± 4.93g, $p < 0.05$).

Somatic growth was also affected by experimental CP, evidenced by

Table 1
Primers sequence used in this study.

Gene Name	Forward Primer Sequence	Reverse Primer Sequence
β2M	TGACCGTGATCTTTCTGGTG	ACTTGAATTTGGGGAGTTTTCTG
PGC-1α	AAC AGC AAA AGC CAC AAA GA	AAG TTG TTG GTT TGG CTT GA
TFAM	TCT CAT GAT GAA AAG CAG GCA	GAG ATC ACT TCG CCC AAC TT
Complex II Subunit (Sdhb)	TTT ACC GAT GGG ACC CGG AC	CGT GTT GCC TCC GTT GAT GT
Complex V Subunit (Atp5f1a)	TCC CTG AAC TTG GAA CCC GA	GGC ATT TCC CAG GGC ATC AA

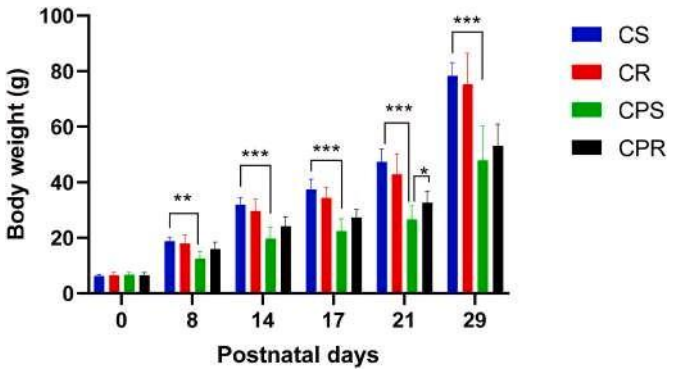

Fig. 2. Weight evolution at P0, P8, P14, P17, P21, and P29 according to the experimental groups: CS: Control Saline (n = 12); CR: Resveratrol Control (n = 11); CPS: CP + Saline (n = 11); CPR: CP + Resveratrol (n = 12). Data were expressed as mean ± standard error, $p < 0.05$ (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

smaller murinometric measurements in the CPS group compared to the CS group. Thus, in the measurements of the skull axes, the smallest lateral skull axis was observed in the CPS group at P12 (CPS: 1.55 cm ± 0.12 cm/CS: 1.79 cm ± 0.15 cm; $p < 0.001$), P15 (CPS: 1.67 cm ± 0.16 cm/CS: 1.82 cm ± 0.09 cm, $p < 0.05$), P21 (CPS: 1.69 cm ± 0.16 cm/CS: 1.90 cm ± 0.05 cm; $p < 0.01$) and P29 (CPS: 1.83 cm ± 0.11 cm/CS: 2.15 cm ± 0.15 cm, $p < 0.001$), as was observed smaller anteroposterior skull axis of the animals submitted to the CP model compared to the control animals at P3 (CPS: 2.23 cm ± 0.16 cm/CS: 1.93 cm ± 0.26 cm; $p < 0.05$), P12 (CPS: 2.85 cm ± 0.27 cm/CS: 3.20 cm ± 0.17 cm, $p < 0.05$), P15 (CPS: 3.09 cm ± 0.24 cm/CS: 3.39 cm ± 0.13 cm, $p < 0.05$), P18 (CPS: 3.24 cm ± 0.3 cm/CS: 3.59 cm ± 0.17 cm; $p < 0.05$), P21 (CPS: 3.32 cm ± 0.23 cm/CS: 3.81 cm ± 0.15 cm; $p < 0.05$) and P29 (CPS: 4.1 cm ± 0.32 cm; $p < 0.05$) (Fig. 3A and B).

Similarly, tail length was also impaired by experimental CP from the 9th postnatal day onwards (P9: CPS: 2.84 cm ± 0.51 cm/CS: 3.5 cm ± 0.36 cm, $p < 0.05$; P12: CPS: 3.31 cm ± 0.47 cm/CS: 4 cm ± 0.28 cm, $p < 0.05$; P15: CPS: 3.66 cm ± 0.64 cm/CS: 4.41 cm ± 0.35 cm, $p < 0.05$; P18: CPS: 4.19 cm ± 0.96 cm/CS: 4.99 cm ± 0.36 cm, $p < 0.05$, P21: CPS: 4.71 cm ± 0.7 cm/CS: 6.07 cm ± 0.47 cm, $p < 0.05$; and P29: CPS: 5.46 cm ± 1.23 cm/CS: 8.21 cm ± 0.91 cm, $p < 0.05$), as well as the longitudinal axis from the 12th postnatal day (P:12 CPS: 7.92 cm ± 0.95 cm/CS: 9.01 cm ± 0.48 cm, $p < 0.05$; P15: CPS: 8.73 cm ± 0.96 cm/CS: 10.51 cm ± 0.98 cm, $p < 0.001$; P18: CPS: 9.49 cm ± 1.12 cm/CS: 11.63 cm ± 0.59 cm, $p < 0.001$; P21: CPS: 10.02 cm ± 1.22 cm/CS: 12.45 cm ± 0.71 cm, $p < 0.001$; and P29: CPS: 12.32 cm ± 1.66 cm/CS: 13.55 cm ± 1.26 cm, $p < 0.01$) when compared to the control group (Fig. 3C and D).

Treatment with resveratrol in animals with CP attenuated the damage in somatic growth. Therefore, the animals in the CP group treated with resveratrol had greater measurements regarding tail length at P29 (CPR: 6.37 cm ± 1.15 cm/CPS: 5.46 cm ± 1.23 cm, $p < 0.05$) and the longitudinal axis at P6 (CPR: 7.49 cm ± 1.05 cm/CPS: 6.27 cm ± 0.42 cm, $p < 0.01$), P9 (CPR: 8.54 cm ± 1.39 cm/CPS: 7.19 cm ± 0.66 cm, $p < 0.01$) and P12 (CPR: 9.16 cm ± 1.32 cm/CPS: 7.93 cm ± 0.95 cm, $p < 0.01$) compared to the CPS group. Additionally, in control animals submitted to resveratrol treatment, it was also possible to observe an increase in the longitudinal axis of the body at P6 (CR: 7.75 cm ± 1.1 cm/CS: 6.38 cm ± 0.38 cm, $p < 0.01$) and P9 (CR: 8.95 cm ± 1.08 cm/CS: 7.66 cm ± 0.46 cm, $p < 0.01$) compared to the CS group (Fig. 3C and D).

Not only the somatic growth was affected by experimental CP, but the maturation of physical features was also impaired. There were a delay in the post-natal day of auricle opening (CPS: 3.41 ± 1.08/CS: 2.0 ± 0.0, $p = 0.0124$), auditory conduit opening (CPS: 14.33 ± 1.87/CS: 12.41 ± 0.51, $p = 0.0003$) and eyes opening (CPS: 14.66 ± 1.23/CS: 12.58 ± 0.51, $p < 0.0001$), in the animals submitted to the CP model compared to the control group. CP model did not cause changes in upper or lower incisor eruption. However, the treatment with resveratrol in the experimental CP did not influence the maturation of the evaluated somatic characteristics (Fig. 4).

4.2. Gait analysis on catwalk

Motor behavior after resveratrol treatment was evaluated. For this, the average speed of the animal during the Catwalk test was used as a kinetic parameter. In this analysis, the CPS group had a lower average speed when compared to the CS group (CS: 21.06 cm/s ± 2.13 cm/s/

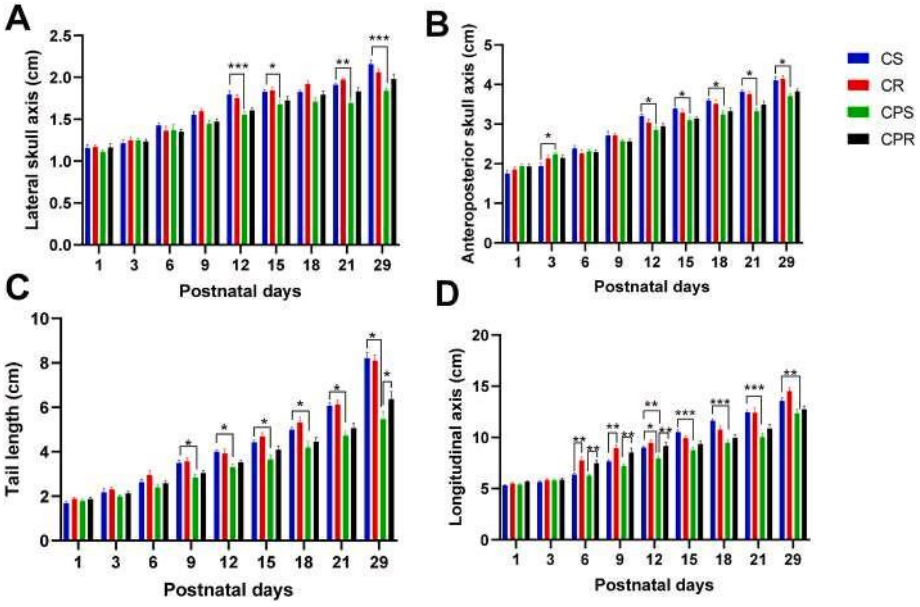

Fig. 3. Somatic growth at P1, P3, P6, P9, P12, P15, P18, P21 and P29 of postnatal life according to the experimental groups: CS: Control Saline (n = 12); CR: Resveratrol Control (n = 10); CPS: CP + Saline (n = 11); CPR: CP + Resveratrol (n = 12). Data were expressed as mean ± standard error, $p < 0.05$ (* = $p < 0,05$; ** = $p < 0,01$; *** = $p < 0.001$).

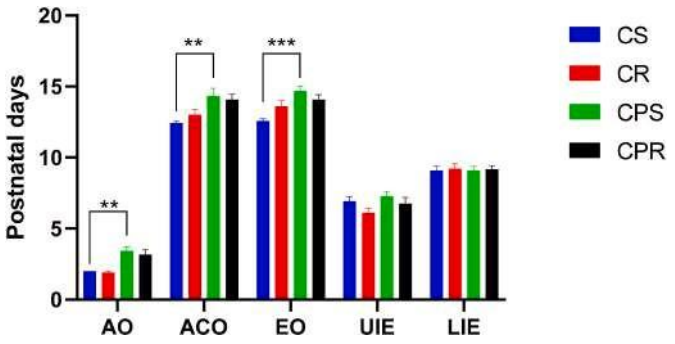

Fig. 4. Physical features between the P1 and P21 according to the experimental groups: CS: Control Saline (n = 12); CR: Resveratrol Control (n = 10); CPS: CP + Saline (n = 11); CPR: CP + Resveratrol (n = 12). AO: auricle opening; ACO: auditory conduit opening; EO: eyes opening UIE: upper incisors eruption; LIR: lower incisors eruption. Data were expressed as mean ± standard error, p < 0.05 (** = p < 0,01; *** = p < 0.001).

CPS: 18.35 cm/s ±3.39 cm/s; p < 0.05) (Fig. 5).

In addition to the average velocity, the evaluation of spatial and temporal parameters showed important changes. Regarding the time of the swing phase, the CPS group had a long time when compared to the CS group (CS: 0.26s ± 0.04s/CPS: 0.32s ± 0.07s; p < 0.05) (Fig. 6B). In the analysis of swing velocity, the CPS group had a lower mean velocity compared to the saline control group (CS: 71.74 cm/s ±3.58 cm/s/CPS: 55.61 cm/s ± 5.52 cm/s; p < 0.05) (Fig. 6C). The analysis of the maximum contact area showed that the CPS group showed a reduction in this area when compared to the CS group (CS: 0.78 cm^2 ± 0.04 cm^2/CPS: 0.52 cm^2 ±0.05 cm^2; p < 0.05) (Fig. 6D). About the print length, the animals in the CPS group had a reduction in this length compared to the CS group (CS: 1.73 cm ±0.04 cm/CS: 1.27 cm ± 0.06 cm; p < 0.0001) (Fig. 6E). The print width analysis showed that the CPS group had a reduced print width when compared to the saline control group (CS: 1.43 cm ± 0.04 cm/CPS: 1.18 cm ±0.06 cm; p < 0.0001), but it showed no statistical difference between the groups CPS and CPR (Fig. 6F).

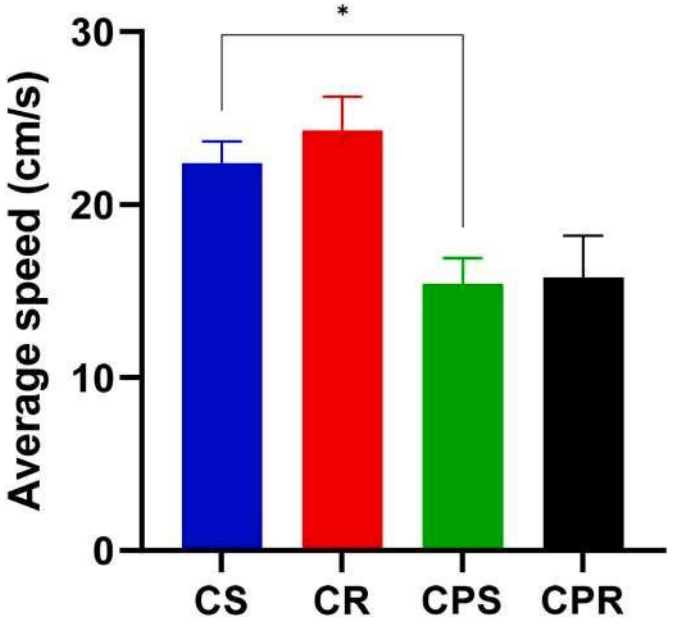

Fig. 5. Average speed during active locomotion on the platform. Experimental groups: CS: Control Saline (n = 12); CR: Resveratrol Control (n = 10); CPS: CP + Saline (n = 10); CPR: CP + Resveratrol (n = 10). Data were expressed as mean ± standard error, p < 0.05 (* = p < 0,05).

4.3. Forelimb grip strength and postural analysis

In the muscle strength analysis with the grip test, at P22 and P28, the CS group showed greater strength compared to the CPS group (P22: CS: 160.97g ±2.22g/CPS: 113.58g ±10.85g; p < 0.01; P28: CS: 235.05g ± 6.51g/CPS: 187.05g ±8.5g; p < 0.01) (Fig. 6A and B). At the same ages, CP animals treated with RES showed greater strength compared to the CPS group (P22: CPR: 175.6g ± 9.17g/CPS: 113.58g ±10.85g; p < 0.001; P28: CPR: 225, 19g ± 11.72g/CPS: 187.05g ±8.5g; p < 0.05) (Fig. 7A and B). No significant difference was seen between the control groups (p > 0.05).

For the postural analysis, at P28 the animals in the CPS group presented greater trunk angulation when compared to the CPR group (CPR: 159.33° ± 1.44°/CPS: 167.38° ± 2.87°; p < 0.05). Likewise, the CS group presented a greater angulation compared to the CR group (CR: 153.98° ± 2.51°/163.95° ± 0.75°; p < 0.05) (Fig. 8A). The analysis of the head alignment showed that, from P14 to P28, the CPS animals presented greater angulation compared to the CS group (P14: CS: 45.34° ± 2.2°/CPS: 54.01° ± 2.28°; P21: CS: 48.4° ± 1.55°/57.65° ± 1.71°; P28: CS: 46.5° ± 1.46°/CPS: 62.06° ± 1.93°; p < 0.05) (Fig. 8B). Also, at P28, the CP animals submitted to the treatment with resveratrol presented smaller head angulation when compared to the pups of the CPS group (P28: CPR: 46.87° ± 2.09°/CPS: 62.06° ± 1.93°; p < 0.05) (Fig. 8B).

4.4. Oxidative state in the somatosensory cortex

After our previous analyzes, with the intent to understand how resveratrol treatment could improve the mechanical properties, we evaluate the oxidative stress biomarkers. Our data showed that treatment with resveratrol decreased MDA and Carbonyl markers in the animals with CP compared to the CPS group (MDA: CPR: 4.66 ± 1.27/CPS: 10.98 ± 1.30 μmol/mg prot; p < 0.001; Carbonyls: CPR: 13.81 ± 2.85/CPS: 29.14 ± 5.08 μmol/mg prot; p < 0.01) (Fig. 9A and B). Also, the control animals treated with resveratrol showed a decrease in MDA compared to the CS group (CR: 5.47 ± 1.04/CS: 12.12 ± 1.61 μmol/mg prot; p < 0.001) (Fig. 9A), while the CPS group showed an increase in Carbonyls compared to the CS group (CPS: 29.14 ± 5.08/CS: 13.55 ± 2.52 μmol/mg prot; p < 0.01) (Fig. 9B).

In addition, we performed several assays to investigate antioxidant capacity. Superoxide dismutase (SOD) and Catalase (CAT) activity in control animals treated with resveratrol were decreased when compared to the CS group (SOD: CR: 45.44 ± 4.34/CS: 72.81 ± 6.19 U/mg prot; p < 0.05; CAT: CR: 223.1 ± 45.78/CS: 405.3 ± 47.02 U/mg prot; p < 0.05) (Fig. 9C and D). No significant difference was seen in the others assays (p > 0.05) (Fig. 9E, F,9G, 9H and 9I).

4.5. Mitochondrial biogenesis and citrate synthase activity in the somatosensory cortex

After the observations in oxidative stress biomarkers, we further analyze some parameters linked with oxidative status. First, we measure the mitochondrial biogenesis mRNA levels. Our data showed a decrease in PGC-1α in the group of animals with cerebral palsy treated with resveratrol compared to the untreated (CPR: 0.6 ± 0.1/CPS: 1.4 ± 0.15; p < 0.01) (Fig. 10A) associated with an increase in TFAM levels (CPS: 0.3 ± 0.1/CPR: 0.9 ± 0.15; p < 0.05). In addition, we observed in CPS decreased levels of TFAM compared to CS (CPS: 0.3 ± 0.1/CS: 1 0.0 ± 0.1; p < 0.05) (Fig. 10B).

When we evaluated the mitochondrial complexes II and V, we observed in CR group an increase in both complexes' subunits compared to the CS group (Complex II: CR: 1.7 ± 0.2/CS: 1.0 ±0.2; p < 0.05; Complex V: CR: 5.3 ± 0.25/CS: 1.0 ± 0.08; p < 0.0001). The CPS group showed an increase in V complex when compared with the CPR and CS groups (CPS: 2.8 ± 0.17/CPR: 0.51 ±0.09/CS: 1.0 ±0.08; p < 0.0001) (Fig. 10C and D).

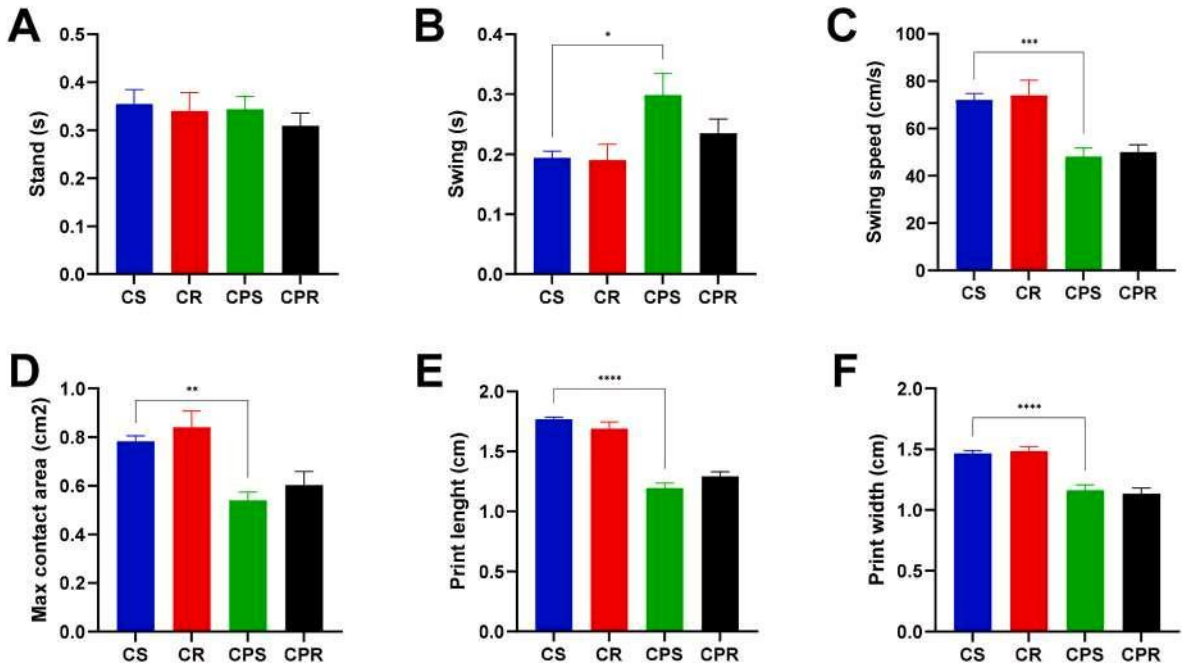

Fig. 6. Evaluation of temporal and spatial parameters of gait at P28. A – Paw support time during locomotion. B – Time during the swing phase on the paws. C – Velocity of the paws during the swing phase. D – Measurement of the area of the paws in contact with the platform during locomotion; E — paw print length during locomotion; F – paw print width during locomotion. Experimental groups: CS: Control Saline (n = 12); CR: Control Resveratrol (n = 10); CPS: CP + Saline (n = 10); CPR: CP + Resveratrol (n = 10). Data were expressed as mean ± standard error, $p < 0.05$ (* = $p < 0,05$; ** = $p < 0,01$; *** = $p < 0.001$; **** = $p < 0,0001$).

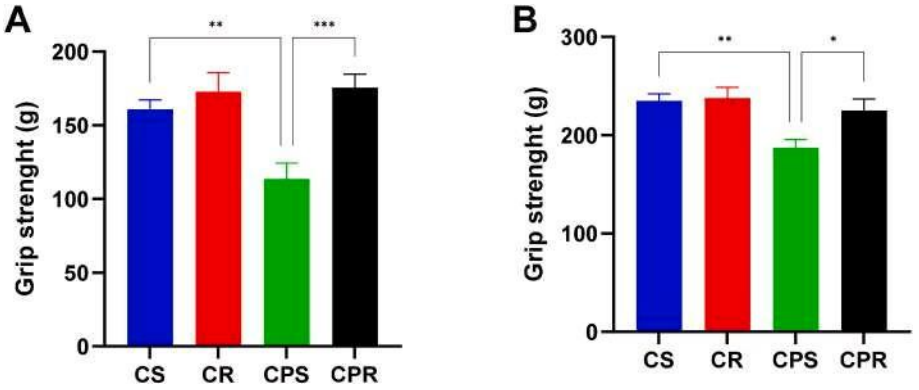

Fig. 7. Grip strength test at P22 (A) and P28 (B) according to the experimental groups: CS: Saline Control (n = 12); CR: Resveratrol Control (n = 10); CPS: CP + Saline (n = 11); CPR: CP + Resveratrol (n = 10). Data were expressed as mean ± standard error, $p < 0.05$ (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

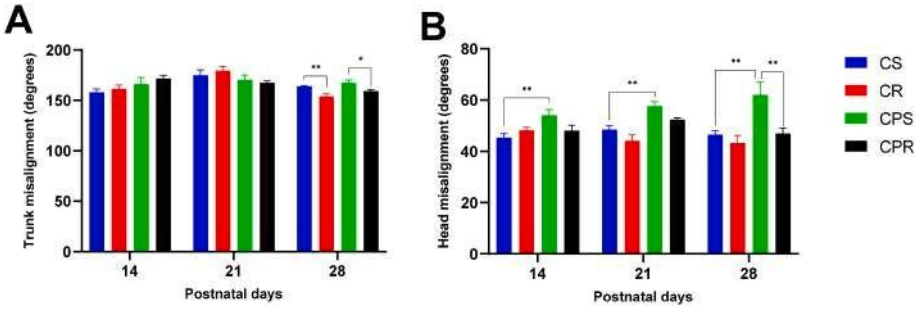

Fig. 8. Analysis of posture based on trunk (A) and head alignment (B) at P14, P21 and P28 according to the experimental groups: CS: Control Saline (n = 12); CR: Resveratrol Control (n = 10); CPS: CP + Saline (n = 10); CPR: CP + Resveratrol (n = 12). Data were expressed as mean ± standard error, $p < 0.05$ (* = $p < 0.05$; ** = $p < 0.01$).

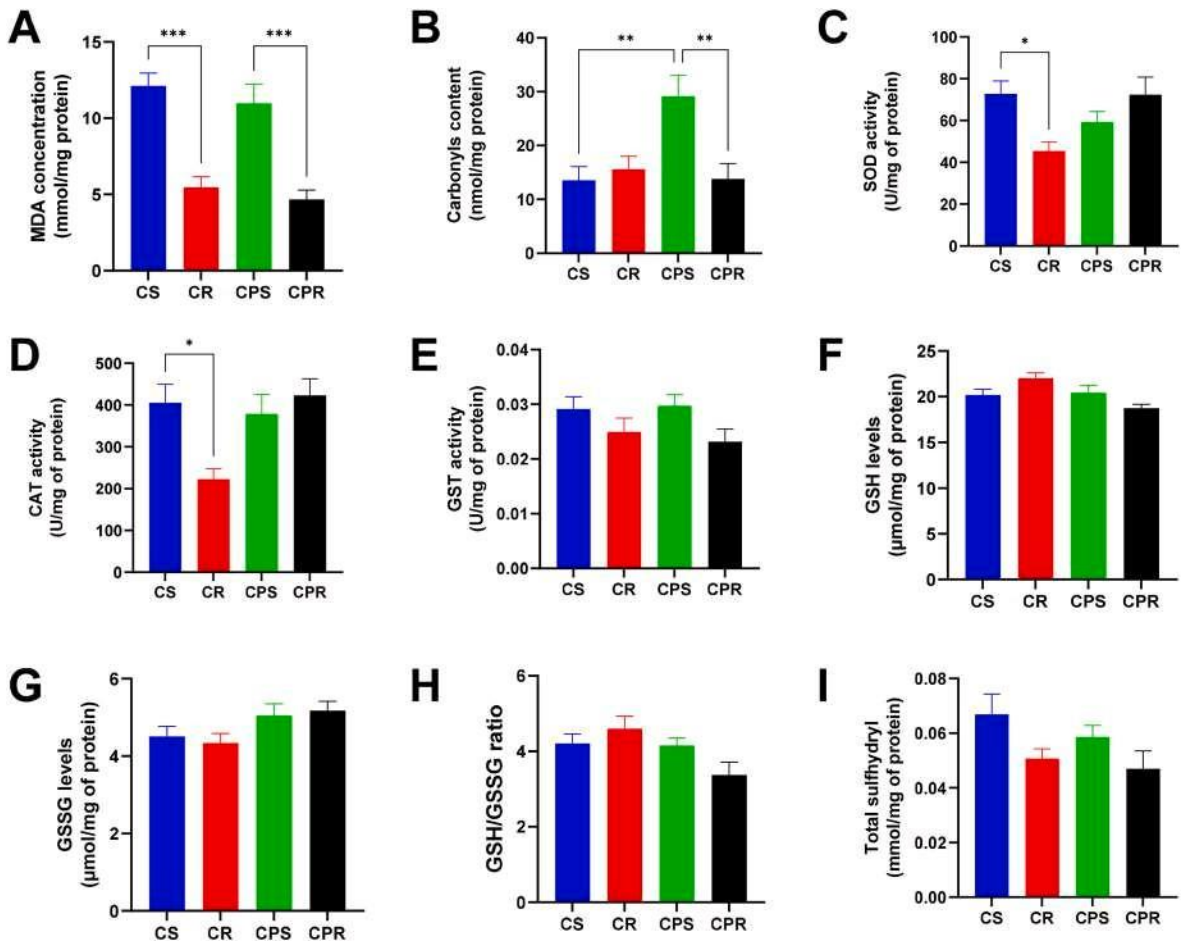

Fig. 9. Analysis of oxidative stress in the somatosensory cortex at P29 according to the experimental groups: CS: Control Saline (n = 8); CR: Resveratrol Control (n = 7); CPS: CP + Saline (n = 7); CPR: CP + Resveratrol (n = 8). Data were expressed as mean ± standard error, $p < 0.05$ (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

After our data related to the mRNA of important genes related to mitochondrial biogenesis, we further analyze the citrate synthase activity, since is the first enzyme in Krebs cycle a crucial enzyme for mitochondrial function. Our data showed an increase in the activity of this enzyme in the animals with CP treated with resveratrol compared to the CPS group (CPR: 21.83 ± 1.14/CPS: 13.43 ± 1.36; $p < 0.05$). Similarly, the CPS group showed a decrease in citrate synthase when compared to the CS group (CPS: 13.43 ± 1.36/CS: 25.0 ± 1.63; $p < 0.01$). Our data also showed that the resveratrol-treated control group had an increase in enzyme compared to the saline-treated control group (CR: 39 ± 3.66/CS: 25.0 ± 1.63; $p < 0.001$) (Fig. 11).

5. Discussion

The findings of this study demonstrated that neonatal treatment with resveratrol was able to improve muscle strength and posture in the animals, in addition to acting as a modulator of brain function, reducing markers of oxidative stress and increasing gene expression related to mitochondrial biogenesis in the somatosensory cortex of rats submitted to cerebral palsy. Furthermore, we observed an increase in the activity of the enzyme citrate synthase in the somatosensory cortex of the CPR group, which demonstrates an increase in mitochondrial function.

We analyzed the body weight of the experimental groups over the 28 days and significant differences were found between the groups from P14, in which the animals submitted to experimental CP showed lower weight gain compared to the control animals. Previous studies with this model of CP observed impairments in the musculoskeletal system, verifying a reduction in muscle fibers in the soleus, EDL muscles, and tibial bone mass associated with impairments in locomotion (Pereira et al., 2021a; Visco et al., 2023). Thus, it is understood that delays in locomotion can impair the animals' eating behavior, which would explain the low weight found in our study. In addition, findings point to a reduction in masseter muscle fibers (Lacerda et al., 2017), which is one of the main muscles involved in mastication, suggesting that there are chewing impairments present in this CP model. In addition, the association of sensorimotor restriction with anoxia exposes the animals to situations of early stress that can delay the weight development of the animals (Pereira et al., 2021a; Strata et al., 2004). However, treatment with resveratrol showed significant changes in the animal's body weight only in the P21. We suggest that more studies be done to better understand how RES influences on weight gain in cerebral palsy.

In addition to body weight analysis, we also evaluated the development of physical features and somatic growth. We observed alterations in the development of the physical features of the animals in the CP group, being possible to notice a delay in the auricle opening, auditory conduit opening and eyes opening in relation to the control animals. In a study carried out by Toso et al. (2005) a delay in eyes opening of animals submitted to a CP model was also observed. However, these authors used a different model of CP through the application of liposaccharides (LPS) during pregnancy, suggesting that brain injuries during pregnancy and after birth can harm the development of animals. Furthermore, the PC model delayed the animals' somatic growth, evidenced by the evaluations of the head axes, longitudinal axis, and tail length. Neonatal exposure to resveratrol was able to attenuate the delay in the growth of the longitudinal axis, from the P6 to the P12, and tail length, in the P29. Visco et al. (2023), using the same CP model, found a reduced body

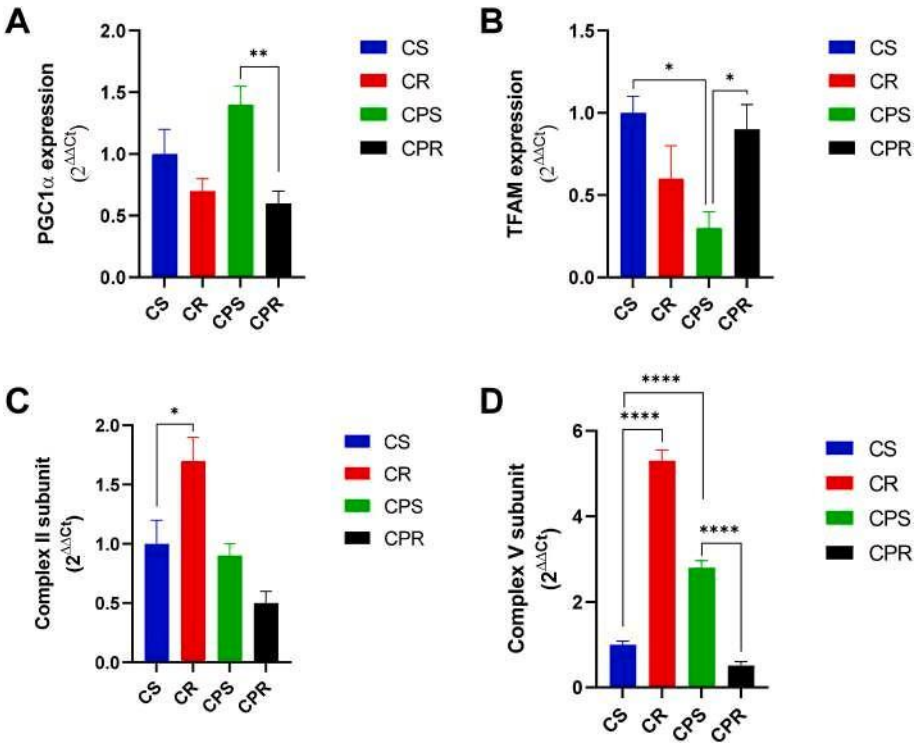

Fig. 10. Analysis of mitochondrial biogenesis genes in the somatosensory cortex at P29 according to the experimental groups: CS: Control Saline (n = 8); CR: Resveratrol Control (n = 7); CPS: CP + Saline (n = 7); CPR: CP + Resveratrol.Data were expressed as mean ± standard error, $p < 0.05$ (* = $p < 0.05$; ** = $p < 0.01$; **** = $p < 0.0001$).

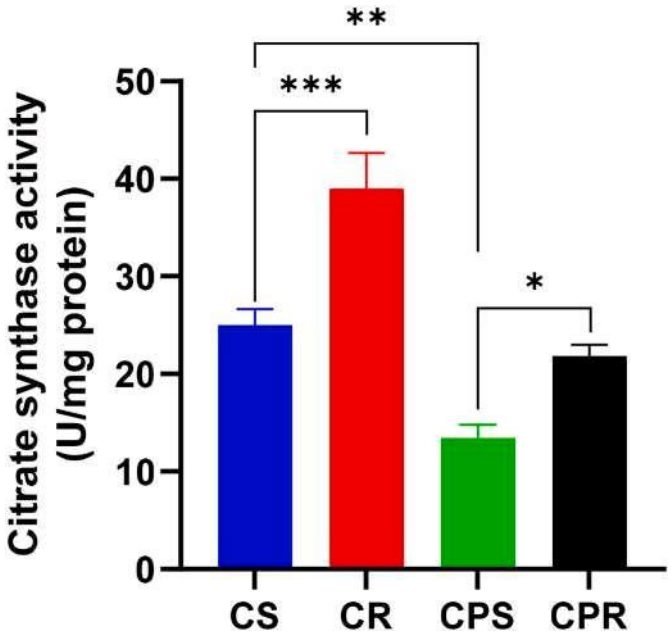

Fig. 11. Analysis of citrate synthase activity in the somatosensory cortex at P29 according to the experimental groups: CS: Control Saline (n = 8); CR: Resveratrol Control (n = 7); CPS: CP + Saline (n = 7); CPR: CP + Resveratrol (n = 8). Data were expressed as mean ± standard error, $p < 0.05$ (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

growth in animals with CP, which was attenuated with a neonatal treatment with kaempferol, another polyphenol. These results show that polyphenols have an important positive action on development. Thus, it is understood that cerebral palsy delays body growth and development, in addition to delaying the motor development of these children. To date, we are not aware of other studies evaluating the effects of resveratrol on these outcomes in models of cerebral palsy. We suggest that future studies be carried out to obtain more information about the effectiveness of RES in improving the somatic growth and physical features of animals with CP.

In the Catwalk analysis, we demonstrated that the induction of CP altered the gait pattern of the animals. Pups submitted to CP model showed lower average speed during walking and lower speed during the swing phase, resulting in a longer duration of this phase. In addition, a decrease in the contact area and the length and width of the paw print of these animals was observed, suggesting dysfunctions in the gait of the animals of the CP group. These findings are similar to those found in children with cerebral palsy, such as incoordination of locomotion, lack of precision in foot positioning, and reduced walking ability (Cappellini et al., 2016; Prosser et al., 2010). A previous study using the same PC model found similar results, in addition to altered gait cadence, with interlimb incoordination, and an increased base of support for the forepaws during active locomotion (Visco et al., 2023). The use of sensorimotor restriction was able to change the gait pattern, reducing the maximum contact area of the rodents' paws (Delcour et al., 2018), corroborating the findings of this study. A recent review showed that models that associate perinatal anoxia and sensorimotor restriction lead to a greater impairment of locomotion and motor coordination in animals, similar to what happens in children with CP (Pereira et al., 2021b). Animals with CP develop locomotor characteristics similar to the equinus foot, possibly because they undergo ankle and knee extension during sensorimotor restriction (Delcour et al., 2018), which explains the abnormal gait pattern observed in our results.

In an unprecedented way, this is the first study to evaluate the postural outcomes present in an experimental model of CP. From these results, postural alterations evidenced by the misalignment of the head and trunk were found. However, neonatal treatment with RES attenuated these damages, where the animals in the CPR group had better postural control, similar to the pups in the control group. Regarding the

evaluation of the muscular strength of the front paws, the animals submitted to the CP model showed a reduction in strength. Treatment with resveratrol was able to reverse these losses, where the animals in the CPR group demonstrated an increase in strength, similar to the animals in the control group. Here, we demonstrate that neonatal treatment with resveratrol prevented the impact of CP on postural development and strength, suggesting that this polyphenol may act by modifying motor function.

It is already well established that muscle weakness is the predominant negative characteristic in people with cerebral palsy (Multani et al., 2019), as observed in the experimental model used in our study. Children with CP have skeletal muscle abnormalities such as weakness and decreased muscle thickness and volume (Elder et al., 2003; Graham and Selber, 2003; Multani et al., 2019). Studies are currently being carried out with natural compounds that can act on skeletal muscle, such as polyphenols (resveratrol, curcumin, urolithin A) and flavonoids (quercetin, apigenin) (Hil et al., 2015; Nikawa et al., 2021; Yadav et al., 2022). These are well known for increasing muscle strength and mass, promoting muscle stem cell differentiation and mitochondrial biogenesis, and reducing hydrogen peroxide production as well as inflammation in skeletal muscles (Yadav et al., 2022). Suggesting possible ways of action of resveratrol in the musculoskeletal system, which may explain the improvement in posture and muscle strength of the animals.

In addition to motor function outcomes, we further investigate the effects of resveratrol on the somatosensory cortex. It is well known in the literature that several injuries are associated to the oxidative stress, because of this we evaluate several parameters, and we notice a significant increase in carbonyls levels, an important OS biomarker, in rats submitted to CP model. Furthermore, we continue analyzing the effect of CP and we observed a significant decrease in MDA and carbonyls in CP rats treated with resveratrol. There are no reports in the literature that show the neuroprotective and antioxidant effects of resveratrol in our model of cerebral palsy. Using a different models, previous authors showed results that indicated that RES had an antioxidant effect, being able to decrease MDA in brain tissue as we observed in CR group (Gao et al., 2018; Orsu et al., 2013). These results are similar to those of our study and support the antioxidant role of resveratrol as evidenced by significantly reducing the levels of MDA and carbonyls. Gao et al. (2018) suggested that RES can scavenge a variety of free radicals owing to its phenol rings and three free hydroxyl group, and thus RES treatment can concomitantly abrogate the ROS production under ischemic conditions.

After the promising finding observed in MDA and carbonyls levels in the CPR group, we further analyzed the antioxidant enzymes, but to our surprise we observed a decrease in SOD and CAT activity in CR group, compared to the control animals. Raising the hypothesis that the antioxidant enzymes had a decrease activities, due to the potent antioxidant effect of RES. Resveratrol is a remarkable antioxidant (Miguel et al., 2021) and here it can act in the healthy body; we hypothesized that it acts preventively against the insults. In this context, we suggest that future studies be carried out to elucidate the molecular and biochemical mechanisms whereby resveratrol beneficially affects the nervous system of healthy animals.

In sequence, with the intent to understand more the mechanistic effect of RES we evaluated mitochondrial biogenesis markers, measuring the levels of transcription factors and mitochondrial complexes subunits. Our data showed that PGC-1α expression is reduced in CPR animals when compared to the CPS group. However, resveratrol-treated CP animals showed upregulation of mitochondrial transcription factor A (TFAM) compared to the CPS group. PGC-1α is mainly expressed in energy-demanding tissues, such as the brain and skeletal muscle (Di et al., 2018), and is an important coordinator of mitochondrial metabolism and function (Finck and Kelly, 2006; Gabrielson et al., 2014). In addition, it participates in the regulation and TFAM transcription (Gabrielson et al., 2014). These data suggest that PGC-1α and TFAM were positively modulated by RES treatment in animals submitted to CP. Therefore, at mRNA levels, we can speculate that RES treatment in CP animals might increase the number of mitochondria. As a result, the increase in the number may decrease the levels of free radicals released by mitochondria that CP damaged. Corroborating our results, Zhou et al. (2021) highlight that RES increased the expression of PGC-1α, NRF1, and TFAM in the temporal cortex in animals submitted to early brain injury after subarachnoid hemorrhage (Zhou et al., 2021), improving the mitochondrial biogenesis.

Oxidative stress is closely linked to mitochondrial function, with the mitochondrial respiratory chain being the main source of ROS (Chen et al., 2021). Dysfunctions in the mitochondrial respiratory chain, such as changes in the protein complexes, which are responsible for the functionality of this system, prevents electrons from being transferred, resulting in increased production of reactive oxygen species (Chen et al., 2021). The lack of oxygen in the developing brain leads to the depletion of cellular energy stores and triggers various pathophysiological responses, including suppression of mitochondrial respiration (Jacobsson and Hagberg, 2004; Thornton and Hagberg, 2015). Here, we observed a marked decrease in complex V subunits and a tendency to reduce the complex II subunits in the CPR group compared to the animals of the CPS group. With our results, RES treatment decreases the levels of these mRNA, which might lead to a decrease in the electron leak and ROS production.

On the other hand, in healthy animals, RES treatment affects the cells differently. In the control group treated with resveratrol, we observed an increase in complexes II and V subunits, compared the CS group, which suggest that RES treatment increase the flow of electrons at mitochondrial respiratory chain, improving the mitochondrial function as a whole. Due to these results, we decided to analyze the activity of the citrate synthase, the first enzyme in Krebs cycle and highly used as an indicator of mitochondrial function. Indeed, in CR group, the citrate synthase activity increased significantly compared to CS, corroborating with our data related to the complex subunits. However as expected in CPS the activity of the citrate synthase was decreased compared to CS, and the treatment with RES could restore the activity capacity in CPR similarly to the levels of CS. Citrate synthase is an important component of energy metabolism: it is necessary for the catalysis and condensation of Acetyl CoA with oxaloacetate for the formation of citrate (Fernandes et al., 2020; Meng et al., 2021). With the increase in citrate synthase activity in the groups treated with resveratrol, we can speculate that this polyphenol was able to substantially improve the mitochondrial function of cells after CP injury.

Finally, our study demonstrated that resveratrol improved posture and muscle strength, reduced carbonyls and MDA, the main markers of oxidative stress, and increased the expression of the TFAM gene and the enzyme citrate synthase in animals submitted to cerebral palsy. The findings of this work give a new perspective on the use of RES in the treatment of cerebral palsy, through its antioxidant properties. More studies are needed to determine the mechanisms of action involved and to establish the best form of administration to obtain a beneficial and prolonged effect.

CRediT authorship contribution statement

Vanessa da Silva Souza: Conceptualization, Formal analysis, Investigation, Writing – original draft. **Raul Manhães-de-Castro:** Resources, Writing – original draft. **Sabrina da Conceição Pereira:** Formal analysis, Investigation, Writing – original draft. **Caio Matheus Santos da Silva Calado:** Formal analysis, Investigation, Writing – original draft. **Beatriz Souza de Silveira:** Formal analysis, Investigation, Writing – original draft. **Eulália Rebeca da Silva Araújo:** Formal analysis, Investigation, Writing – original draft. **Severina Cassia de Andrade Silva:** Formal analysis, Investigation, Writing – original draft. **Osmar Henrique dos Santos Junior:** Formal analysis, Investigation, Writing – original draft. **Claudia Jacques Lagranha:** Formal analysis, Investigation, Writing – original draft, Resources. **Luan Kelwyny Thaywã Marques da Silva:** Formal analysis, Investigation, Writing –

original draft. **Ana Elisa Toscano:** Supervision, Conceptualization.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

Data availability

Data will be made available on request.

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APÊNDICE E – RESVERATROL REDUCES NEUROINFLAMMATION AND HIPPOCAMPAL MICROGLIA ACTIVATION AND PROTECTS AGAINST IMPAIRMENT OF MEMORY AND ANXIETY-LIKE BEHAVIOR IN EXPERIMENTAL CEREBRAL PALSY

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Resveratrol Reduces Neuroinflammation and Hippocampal Microglia Activation and Protects Against Impairment of Memoryand Anxiety-Like Behavior in Experimental Cerebral Palsy

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Abstract

Cerebral palsy (CP) is a neurodevelopmental disorder characterized by motor and postural impairments. However, early brain injury can promote deleterious effects on the hippocampus, impairing memory. This study aims to investigate the effects of resveratrol treatment on memory, anxiety-like behavior, and neuroinflammation markers in rats with CP. Male Wistar rats were subjected to perinatal anoxia (P0-P1) and sensory-motor restriction (P2-P28). They were treated with resveratrol (10 mg/kg, 0.1 ml/100 g) or saline from P3-P21, being divided into four experimental groups: CS (n = 15), CR (n = 15), CPS (n = 15), and CPR (n = 15). They were evaluated in the tests of novel object recognition (NORT), T-Maze, Light–Dark Box (LDB), and Elevated Plus Maze (EPM). Compared to the CS group, the CPS group has demonstrated a reduced dis- crimination index on the NORT (p < 0.0001) and alternation on the T-Maze (p < 0.01). In addition, the CPS group showed an increase in permanence time on the dark side in LDB (p < 0.0001) and on the close arms of the EPM (p < 0.001). The CPR group demonstrated an increase in the object discrimination index (p < 0.001), on the alternation (p < 0.001), on the permanence time on the light side (p < 0.0001), and on the open arms (p < 0.001). The CPR group showed a reduction in gene expression of IL-6 (p = 0.0175) and TNF-α (p = 0.0007) and an increase in Creb-1 levels (p = 0.0020). The CPS group showed an increase in the activated microglia and a reduction in cell proliferation in the hippocampus, while CPR animals showed a reduction of activated microglia and an increase in cell proliferation. These results demonstrate promising effects of resveratrol in cerebral palsy behavior impairment through reduced neuroinflammation in the hippocampus.

Keywords Brain injury · Cognition, Emotional behavior · Episodic memory · Polyphenols

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Introduction

The perinatal period is considered critical for the development of mammals, especially that of the brain [1]. Environmental exposures during gestation and/or the first years of life can thus lead to the development of adaptive or maladaptive phenotypes, favoring the emergence of disorders, such as cerebral palsy (CP), in later stages of life [2–4]. Hypoxia–ischemia and infections in this period are therefore one of the main risk factors for the development of CP [5–7]. CP is a neurodevelopmental disorder with severe repercussions for the organism, characterized by severe impairment of movement and posture [6], and is one of the main causes of physical and cognitive disability in childhood [6, 8, 9].

Although locomotion and motor coordination are impaired by a brain injury [10], studies indicate that the hippocampus is a region sensitive to oxygen deprivation [11] or exposure to inflammation [12, 13]. Different regions of the brain may be affected in CP, depending on the type of injury, time of exposure, and the extent of the injury [14]. Impairment of maturation of the hippocampus of animals with cerebral palsy has thus been observed, and a reduction in the expression of antioxidant enzymatic genes with reduced cell proliferation has also been noted [15]. Studies thus indicate that perinatal brain injuries impair the maturation of the hippocampus, with consequences for behavior and have noted an increase in anxiety-like behaviors [16, 17] and impairment of the formation of new memories in adulthood [18].

Memory is a complex ability associated with learning. Learning can be described as the organism's ability to acquire information, or skills, while memory is defined as the organism's ability to consolidate and recover this information [19]. Memory is associated with synapses and the synthesis of new proteins (or changes in existing proteins) in regions of the brain such as the hippocampus [19–21]. The formation of new memories involves changes in the brain, which are known as engrams [22]. The long-term episodic memories formation involves a cascade of molecular mechanisms such as the expression of CREB-1 (CAMP responsive element binding protein 1) and suppression of CREB-2 (CAMP responsive element binding protein 2) [19, 20, 23–26]. Studies also indicate that BDNF (brain-derived neurotrophic factor) synthesis is involved in the formation of long-term memories [19, 20, 23–26]. Additionally, the hippocampal choline acetyltransferase (ChAT) has been identified as one of the genes that act on memory [27, 28]. The ChAT reduction is also associated with the cognitive deficit present in Alzheimer's disease [27, 28]. Thus, the appropriate development of the hippocampus and synaptic connections plays a crucial role in the formation of new memories [19, 26, 29, 30].

Memory can also, however, be altered, both at the time of information recovery, when this information can be reconsolidated [31], and also by epigenetic mechanisms. Brain injuries and inadequate nutrition at a critical period can lead to memory formation impairment in adulthood [32–34]. Studies show that brain injuries early in life increase the expression of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α in the hippocampus [35, 36]. Neuroinflammation is an important aspect of early brain injuries, such as those caused by CP [37], which can impair maturation of the hippocampus and cause abnormalities relating to memory and behavior [35, 38–40].

By contrast, findings indicate that neonatal polyphenol supplementation is capable of attenuating hippocampal damage by reducing oxidative stress and microglial activation in the hippocampus [15]. This occurs because polyphenols are able to modulate metabolic and brain activity [41, 42]. Polyphenols are natural metabolite compounds commonly found in plants and foods, and their chemical structure contains at least one aromatic ring linked to a hydroxyl [43]. They have antioxidant and anti-inflammatory effects [44–46]. Resveratrol is a polyphenol with anti-inflammatory effects that is found in grapes and red wine and in nuts [47]. Studies indicate promising effects on the brain, improving mitochondrial function and preventing microglia activation and thereby reducing TNF-α, IL-1β, IL-6, and nitric oxide expression in the hippocampus and improving behavior and the capacity to form new memories [47–53].

The present study thus aimed to assess the effects of neonatal resveratrol treatment on memory formation, anxiety-like behavior, pro-inflammatory markers, and the expression of genes involved in memory, microglial activation, and cell proliferation in the hippocampus of rats submitted to a cerebral palsy model. We hypothesized that neonatal exposure to resveratrol reduces the severity of problems present in cerebral palsy related to the formation of new memories and abnormal emotional behavior by reducing neuroinflammation and microglial activation and increasing cell proliferation in the hippocampus.

Materials and Methods

Animals and Animal Housing Conditions

A randomized, controlled, pre-clinical study was conducted using Wistar rats from the colony of the Federal University of Pernambuco's Department of Nutrition. All animals were kept under standard conditions at a temperature of 22 ± 2 °C, with an inverted light–dark cycle of 12/12 h (light cycle—20:00 to 8:00 h; dark cycle—08:00 to 20:00 h), housed in polypropylene cages (46 cm × 34 cm × 20 cm) lined with sterile wood shavings, with free access to food and water.

Experimental Groups

The male pups (n = 60) were obtained from nulliparous female rats (n = 30) placed to mate with reproductive males (n = 15) at the proportion of two females to one male. The rats were consanguineous, aged between 90 and 120 days, with a body weight of between 220 and 260 g. Once pregnancy had been confirmed by detecting spermatozoa in vaginal smear, the pregnant rats were placed in separate individual cages until the birth of the pups. After birth, the male rat pups were randomly distributed into four experimental groups, consisting only of healthy male pups (weighing 6–8 g). The female offspring were used only to complete the litter of eight pups until weaning. The experimental groups were divided accordingly by exposure to cerebral palsy and pharmacological manipulation using saline or resveratrol, as follows: (i) control saline (CS, n = 15); (ii) control resveratrol (CR, n = 15); (iii) cerebral palsy saline (CPS, n = 15); (iv) cerebral palsy resveratrol (CPR, n = 15). Each litter consisted of eight pups that remained with their mothers until P25 (25th postnatal day of life), when they were weaned and the male rats placed in individual cages until subjected to euthanasia (Fig. 1).

Experimental Model of Cerebral Palsy

The CP phenotype was reproduced using a model that combines perinatal anoxia and sensory-motor restriction of the hindlimbs [54–56]. The model is applied to male pups only, because males are more prone to developing CP [57]. This model reproduces phenotypic characteristics of diplegic-spastic CP [10, 54]. The pups underwent two episodes of anoxia, at P0 and P1 (0th and 1st postnatal day of life), during which they were placed in a glass chamber, partially immersed in water at 37 °C, and exposed to nitrogen 100% (White Martins, Brazil) at 9 l/min for 12 min on each occasion. No more than four males were exposed to anoxia in each experiment. The pups were then placed at room temperature for recovery (breathing, skin color, and postural reflexes) and were then returned to their respective mothers [54, 55]. Sensory motor restriction of the hindlimbs was performed from P2 to P28 (2nd to 28th postnatal day of life) for 16 h a day. The procedure was performed from 4 pm until 8 am the next day. This involved fixing an orthosis in such a way as to enable only limited movement of the hip joint, leaving the hindlimbs extended with the support of surgical and adhesive tape, without hindering maternal care or the elimination of urine and feces.

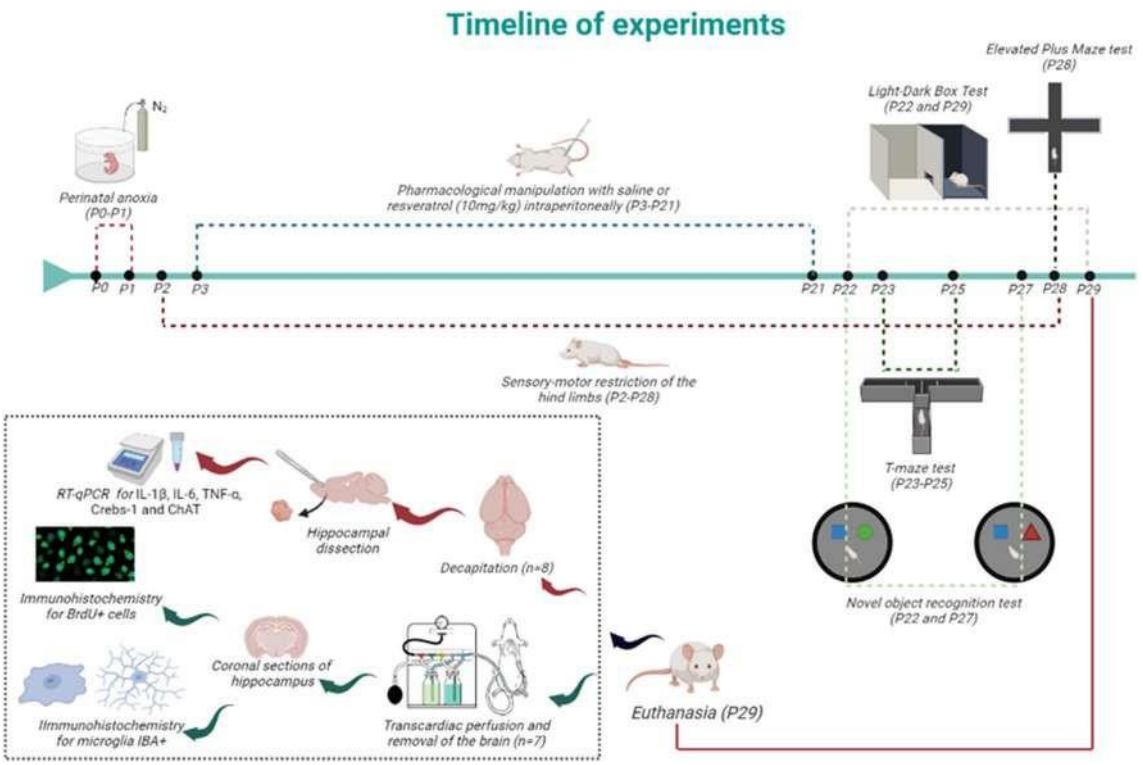

Fig. 1 Summary of all experiments performed on male offspring. The picture created with BioRender

Neonatal Treatment with Resveratrol or Saline

Male pups were randomly divided into groups to assess the effects of the intervention on brain injury, receiving treatment with saline or resveratrol by way of a daily peritoneal injection at 9 am from P3 to P21 (3rd to 21st postnatal day of life). The animals were distributed into the following groups: treatment with resveratrol (daily dose of 10 mg/kg) and treatment with saline (0.9% NaCl), injected at a volume of 0.1 ml/100 g of the rat's weight. Resveratrol (Sigma, St. Louis, MO, USA) was diluted according to Girbovan and Plamondon, 2015. Resveratrol was diluted in 50% ethanol and 50% in a vehicle consisting of 0.9% saline solution (NaCl) containing 20% hydroxypropyl β-cyclodextrin (Sigma, St. Louis, MO, USA) [58]. To 10 mg of resveratrol, 0.25 ml of ethanol was used to dissolve it and 0.25 ml of saline solution was added. The final substance was stored in aliquots in a freezer at − 20 °C. After that, the resveratrol was only unfrozen at the time of application. The rats were weighed daily, and the injection volume was adjusted to match the animal's body weight.

Evaluation of Novel Object Recognition Memory Test

The animals were randomized for the behavioral tests, with at least twelve animals per group for each test. The novel object recognition test (NORT) [53] was used to assess the formation of new episodic memories. The experiment was conducted during the dark period of the cycle. The training session was conducted on the 22nd day of life (P22). The animals were placed one at a time in an entirely black circular open field with two identical objects and allowed to freely explore the objects. After completion of training, each animal was returned to the cage, and the open field and objects were cleaned with ethanol (70%). The familiar object (big rectangular, acrylonitrile butadiene styrene) on the right side was subsequently placed once again in the field, after the open field and objects were cleared, while the object on the left side was replaced with a novel object (small cube, acrylonitrile butadiene styrene). The animal was placed again in the open field and was filmed for 5 min to assess the formation of short-term memories. At P27, the test was repeated with another novel object different (conical object, polyethylene) from the familiar object in order to assess the animals' ability to acquire and retrieve long-term memories [53]. All videos were analyzed by an evaluator blinded to the experimental groups and treatments, and the novel object discrimination index (DI) and recognition index (RI) was calculated. The mathematical formula used to calculate DI is as follows: [DI = (time spent exploring the novel object) - (time spent exploring the familiar object) / (total exploration time)]. This result can vary between + 1 and − 1. The formula used to calculate RI is as follows: [RI = (time spent exploring the novel object) / (total exploration time) × 100 (%)] [59].

Evaluation of Memory Using the T-Maze Test

The animals' spatial memory was assessed using the T-Maze test [60, 61]. The maze consists of a black plexiglass structure shaped like an upper-case letter "T", with sliding doors dividing the paradigm. The experiment was conducted in sets of two trials each to assess the animal's alternation behavior [60, 61]. The test was applied four times daily (two sets of two) for three consecutive days (P24-P27). Thirty minutes prior to the experiment, the animals were placed in the behavior room for adaptation. Each animal was placed at the bottom of the "T" and was allowed to explore the paradigm. Once the animal had chosen one arm of the maze, the sliding door of the chosen arm was closed. The animal was then returned to its cage, and the maze was cleaned. The same procedure was then repeated to complete the first set of two. On conclusion of the test, each animal was returned to its cage for 10 min, the maze was cleaned, and the procedure repeated, giving a total of four runs per day. By the end of the experiment, six sets of tests had been conducted, and the number of new arm choices was calculated [60, 61].

Evaluation of Anxiety-Like Behavior Usingthe Light–Dark Box Test

The Light–Dark Box (LDB) test was used to evaluate the effects of brain injury and treatment on anxiety-like emotional behavior of animals at P22 and P29 (22nd and 29th postnatal day of life). The apparatus consisted of a 40 cm × 40 cm box with a height of 80 cm, with a central wall that divides the box into two sides, one completely white (the light side) and one completely black (the dark side), with only one central entrance allowing the animal to move from one side to the other. The animal was placed in the dark side of the box during the light period of the daily cycle and was allowed to explore the apparatus for a period of 5 min. The test was filmed by a camera attached to the ceiling. The videos were analyzed by a blinded evaluator using Anymaze software to establish the following parameters: permanence time, and number of entries into the light and dark side of the box.

Evaluation of Anxiety-Like Behavior Usingthe Elevated Plus Maze Test

The Elevated Plus Maze (EPM) test consists of a task that assesses anxiety-like behavior in rodents [56]. On P28 (28th postnatal day of life), the pups were transferred to the behavior room for 30 min of adaptation to the environment prior to

commencing the test. Each animal was placed in the central area of the apparatus and allowed to explore it for 10 min. The test was performed in the dark, and the animals were filmed using a camera attached to the ceiling [11, 57]. All videos were analyzed by an evaluator blinded to the experimental groups and treatments using Anymaze software to establish the following parameters: the number of entries and permanence time in the open and closed arms, and permanence time in the central area.

Euthanasia and Body and Brain Weight of Animals

The weight of each of the animals was measured daily from P0-P29 using a precision balance (Marte, capacity of 1 kg and sensitivity of 0.01 g). Half of the animals (n = 8 per experimental group) were euthanized by decapitation at P29 after a 4-h fasting period. The brain was then removed and weighed in an electronic digital balance (Marte AUW220, capacity of 220 g and sensitivity of 0.1 mg). The hippocampus was removed shortly thereafter and stored in a freezer at – 70 °C for gene expression analysis.

RNA Extraction

Total RNA was extracted from dissected hippocampal tissue with TRIzol reagent using the guanidine isothiocyanate [62] method in accordance with the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). RNA pellets were washed in 75% ethanol, centrifuged at 7500 × g for 5 min at 4 °C, air-dried, and boiled in DEPC-treated ultrapure water. RNA quantification was performed using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) and purity was assessed using the 260/280 nm range.

RT–PCR Evaluation of Hippocampal IL-1β, IL-6, TNF-α, Krebs-1, and ChAT Gene Expression

Real-time polymerase chain reaction (RT-PCR) was subsequently performed for β2-microglobulin (β2M) as a normalizer and for the genes of interest, IL-1β, IL-6, TNF-α, Krebs-1, and ChAT (Table 1) using the SuperScript® III Platinum® SYBR® Green One-Step qRT-PCR Kit (Invitrogen, USA). Sequences were obtained from the NCBI database and were designed using Primer3 Input Software (v.0.4.0) [63]. The housekeeping gene GAPDH was used as the internal control for PCR analysis. The relative mRNA expression of the genes was calculated using the 2-ΔΔCt comparative method [64].

Euthanasia and Brain Histology

The remaining animals (n = 7) were euthanized by transcardiac perfusion for evaluation of cell proliferation and microglial activation in the hippocampus. The pups were anesthetized with an intramuscular injection of ketamine (100 mg/kg) and xylazine (12 mg/kg), and the thoracic cavity was opened and transcardiac perfusion was performed using 150 ml of saline (0.9% NaCl) and 200 ml of fixative solution (4% paraformaldehyde). After perfusion, the brain was removed and stored, and post-fixed in the same fixative solution overnight. Sucrose (30%) was added for the 24 h–48 h period. The brains were cryosectioned at a thickness of 30 μm into coronal sections to collect the posterior portion of the hippocampus (between – 1.82 and – 2.18 mm, posterior to the bregma) in a – 30 °C Leica cryostat. The sections were kept in an antifreeze solution for immunohistochemical processing.

Profile of Hippocampal Microglia

Analysis of the microglia profile was conducted using brain sections incubated first in 10% H_2O_2 in methanol and subsequently in 10% H_2O_2 in phosphate buffer (0.1 M, pH 7.4) containing 3% Triton X-100 (PBT). The sections were then incubated at 4 °C for 48 h in primary ionized calcium-binding adapter molecule 1 antibody (Iba1) (rabbit anti-Iba1/IAF1, 1: 30,000, Wako), diluted with 5% horse serum in PBT [65]. The sections were subsequently incubated in a secondary antibody (biotinylated anti-rabbit; 1:750, Sigma-Aldrich) for a period of 2 h at 4 °C. The brain sections were then incubated in solutions of avidin–biotin-peroxidase complex (ABC Elite Kit; Vector Laboratories, Burlingame, CA, USA) and a solution of the diaminobenzidine staining kit (DAB Kit; Vector Laboratories) to stain the microglia [65]. Sections from each group were processed in parallel to avoid non-specific staining effects [15]. The brain sections were then mounted on gelatinized slides and covered with Cytoseal coverslips (Thermo Scientific, USA).

Table 1 Primers used in PCR analysis

Gene	Forward primer	Reverse primer	Amplicon size
B2M	TGACCGTGATCTTTCTGGTG	ACTTGAATTTGGGGAGTTTTCTG	73 bp
1 l-6	AAGGAGTGGCTAAGGACCAA	GTTTGCCGAGTAGACCTCAT	89 bp
TNF-α	AAGCATGATCCGAGATGTGG	AGTAGACAGAAGAGCGTGGT	141 bp
CHAT	CCTGGAAAAGGCTCCCCAAA	CCCAAACCGCTTCACAATGG	178 bp
CREB	GACGGAGGAGCTTGTACCAC	TGGCATGGATACCTGGGCTA	156 bp

Evaluation of Iba1 + microglial cells in the hippocampus (n = 7 per experimental group) was conducted by selecting two fields per section from a total of four sections randomly selected by brain section from the CA1, CA3, and dentate gyrus regions of the posterior hippocampus, giving a total of eight photographs per area of the hippocampus for each animal. The selected fields were examined using a 20 × objective microscope. A blinded researcher used ImageJ software (https://imagej.nih.gov) to count the number of cells/area and classify the profile of the microglia according to the previous descriptions. Microglial cells with a small soma and few to numerous processes were considered to be branched microglia (types I–III), while those with a large soma or amoeboid body and thicker, shorter processes were considered to be activated microglia (types IV–V) [65, 66]. The data presented relate to microglia density (n/mm^3—total cells divided by the area assessed × 1,000,000 × 0.001 × 40) and the percentage of microglia activation relative to the total [15].

Cell Proliferation in the Hippocampus

To investigate cell proliferation in the hippocampus, animals to be euthanized by perfusion (n = 7 per experimental group) received, at P5 and P6, an intraperitoneal injection of 50 mg/kg of BrdU (5-bromo-2′-deoxyuridine- BrdU, Sigma-Aldrich Co. LLC). Cryosections subsequently underwent immunohistochemical analysis. BrdU is a thymidine analog that marks DNA during the S phase of the cell cycle [59], as it is a mitotic marker used to identify newborn neurons [60, 61]. BrdU is therefore used to assess cell proliferation [62]. The brains were cut into 30-µm sections and these were washed in a phosphate-buffered substance (PB) and incubated in Triton (X-100 0.3%, St. Louis, MO, USA) containing 10% hydrogen peroxide. The sections were then incubated in absolute methanol and washed in PB and incubated in formamide (50% in sodium citrate salt solution, Sigma-Aldrich) at 65 °C for 2 h. After washing, denaturation of DNA was performed in HCl (1N) at 37 °C. The sections were then incubated in borate buffer solution (pH 8.4) [63].

The sections were subsequently incubated overnight (4 °C) in a solution of primary anti-BrdU antibody (mouse anti-BrdU, 1: 30,000, Roche Molecular). The following day, the sections were washed for 4 to 5 min in PBS and incubated in the dark for 2 h with biotinylated secondary antibody (1: 750, Vector Laboratories) and developed in an avidin–biotin complex (Elite ABC kit, Vector Laboratories) and diaminobenzidine (DAB staining kit, Vector Laboratories). The brain sections were subsequently washed, dried, and mounted on slides with Cytoseal, and covered with coverslips. Finally, images were captured using a 20 × objective optical microscope of 4–5 sections per animal. The number of BrdU + cells in the hippocampus was counted by a blinded researcher for each experimental group [15]. The boundaries of the dentate gyrus (DG) granular cell layer (GCL) and the subgranular zone (SGZ), and CA1 and CA3 regions of the hippocampus were digitally delineated.

Statistics

The data obtained were tested for normality using the Shapiro–Wilk test. If a normal distribution was identified, appropriate parametric tests, such as the two-way ANOVA test (for comparison of the groups at only one age of the animal) or repeated measures two-way ANOVA (for comparisons of the performance of animals at more than one age) were applied. For data found not to be distributed normally, nonparametric statistical tests, such as the Kruskal–Wallis and Friedman tests, were employed. The results were expressed as mean ± standard error of the mean. The significance level was set at 5%. GraphPadPrism® version 8.0.2 software was used for data analysis and creation of the graph.

Results

Resveratrol Provides Protection Against Object Recognition Memory Impairment

Recognition memory was assessed using the novel object recognition test at P22 and P27. On P22, significant differences were found between the CS and the CPS groups and between the CPS and the CPR animals for exploration time in relation to a novel object [F (3.33) = 29.42; p < 0.0001), exploration time in relation to a familiar object [F (3.33) = 8.723; p = 0.0002)), for the discrimination index [F (3.88) = 7.491; p = 0.0002], and for the recognition index [F (3.88) = 7.491; p = 0.0002]. However, no significant differences in total exploration time were found between any of the experimental groups (p > 0.05). Animals in the CPS group showed reduced exploration time in relation to a novel object (CPS = 26.08 ± 3.342 vs. CS = 61.13 ± 4.380; p = 0.001) (Fig. 2B) and spent more time exploring a familiar object compared to those in the CS group (CPS = 62.41 ± 8.346 vs. CS = 30.06 ± 2.708; p = 0.007) (Fig. 2C). These animals consequently presented a lower discrimination index (CPS = – 0.549 ± 0.041 vs. CS = 0.342 ± 0.054; p < 0.0001) and recognition index (CPS = 22.53 ± 2.03 vs. CS = 67.11 ± 2.67; p < 0.0001) (Fig. 2D and E). This suggests impairment in the object recognition memory of animals submitted to the cerebral palsy model. The pups in the CPR group exhibited increased novel object exploration time (CPR = 63.56 ± 3.915 vs. CPS = 26.08 ± 3.342; p = 0.0006) (Fig. 2B), reduced exploration time for the familiar object (CPR = 33.09 ± 4.906 vs. CPS = 62.41 ± 8.346; p = 0.0106) (Fig. 2C), and an

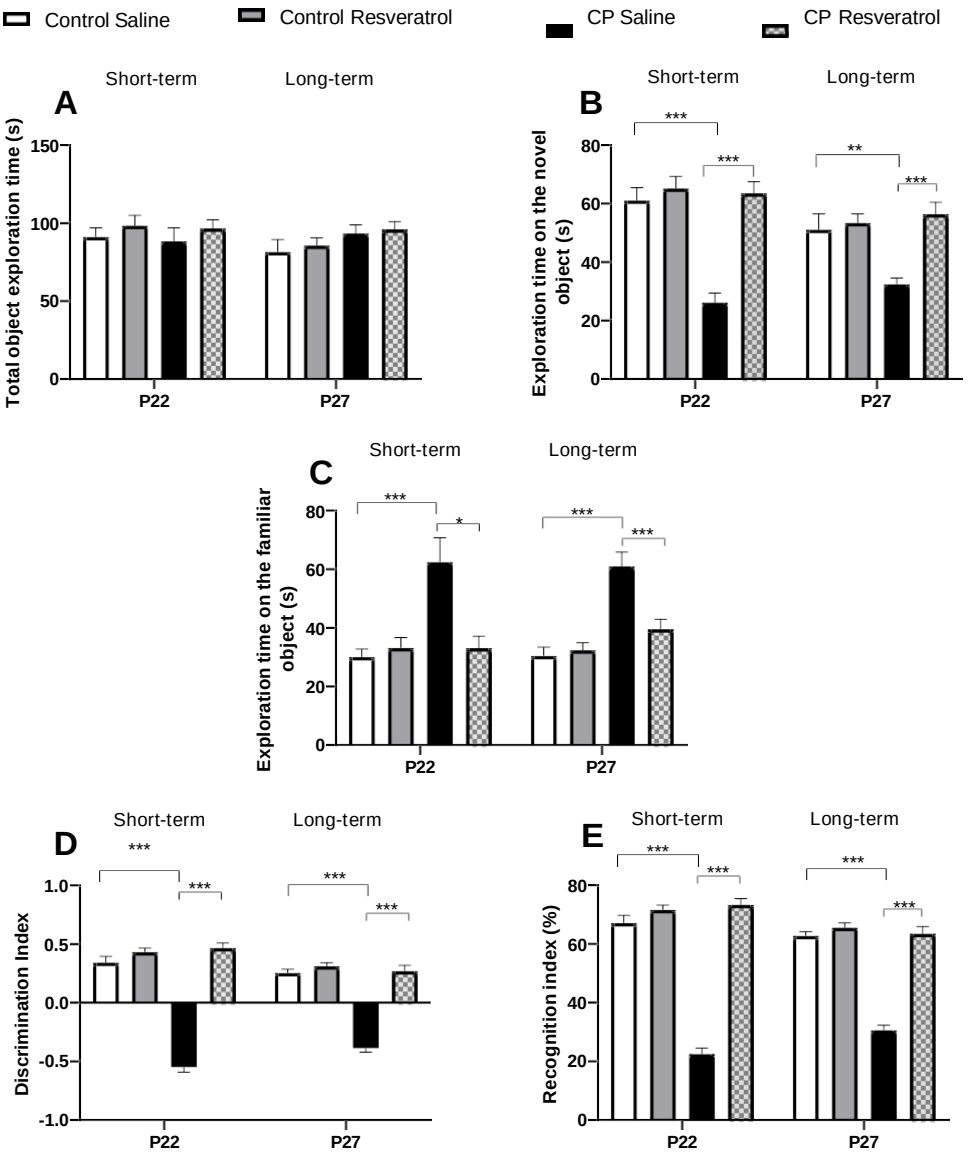

Fig. 2 Novel object recognition test on P22 and P27 in the four experimental groups: CS (n = 12), CR (n = 12), CPS (n = 12), and CPR (n = 12). **A** Total exploration time of the animals, where no significant differences were found. **B** Exploration time on the novel object, where the CPS group showed a shorter time ($p < 0.001$), while the CPR group demonstrated exploration similar to the CS group. **C** Exploration time on the familiar object, where the CPS group showed a longer time compared to the CS group ($p < 0.05$ and $p < 0.001$) and CPR group ($p < 0.0001$ and $p < 0.0001$). **D** Novel object discrimination index where the pups from the CP group showed a worse index ($p < 0.001$) relative to the CS and CPR group. **E** Recognition index where the animals from the CP group showed a worse index relative to the CS and CPR groups. Values are expressed as mean ± standard error of the mean. ANOVA two-way multiple comparisons Tukey's post hoc test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

increase in the discrimination index (CPR = 0.467 ± vs. CPS = − 0.549 ± 0.041; $p < 0.0001$) and recognition index (CPR = 73.36 ± 2.092 vs. CPS = 22.53 ± 2.032; $p < 0.0001$) (Fig. 2D and E) compared to the CPS group.

After 5 days, the novel object recognition test was repeated to check long-term recognition memory. On P27, differences persisted for exploration time in relation to a novel object [F (3.33) = 9.905; $p < 0.0001$], in relation to a familiar object [F (3.33) = 14.24; $p < 0.0001$) and for the novel object discrimination index [F (3, 88) = 7.491; $p = 0.0002$) and recognition index [F (3, 88) = 7.491; $p = 0.0002$]. No significant differences, however, were found in the total exploration time for the objects [F (3.33) = 1.439; $p = 0.2490$) (Fig. 2A). Animals in the CPS group performed less well in relation to novel object exploration (CPS = 32.42 ± 2.147 vs. CS = 51.13 ± 5.371; $p = 0.0028$) (Fig. 2B) and spent more time exploring a familiar object (CPS = 61.04 ± 4.818 vs. CS = 30.44 ± 3.00; $p < 0.0001$) (Fig. 2C), and consequently presented the worst discrimination index (CPS = − 0.387 ± 0.034 vs. CS = 0.254 ± 0.030; $p < 0.0001$) and recognition index (CPS = 30.61 ± 1.68 vs. CS = 62.73 ± 1.52; $p < 0.0001$) (Fig. 2D and E), providing evidence of the presence of episodic memory impairment in animals with cerebral palsy compared to the CS group. The animals in the CPR group, however, presented the longest exploration time for a novel object (CPR = 56.43 ± 4.059 vs. CPS = 32.42 ± 2.147; $p = 0.0001$) and a reduction in exploration time for a familiar object (CPR = 39.58 ± 3.378 vs. CPS = 61.04 ± 4.818; $p = 0.0014$) (Fig. 2B and C), thereby increasing the discrimination index (CPR = 0.270 ± 0.050 vs. CPS = − 0.387 ± 0.034; $p < 0.0001$) and recognition index (CPR = 63.50 ± 2.489 vs. CPS = 30.61 ± 1.683; $p < 0.0001$)

(Fig. 2D and E) indicating that resveratrol improved the long-term recognition memory of animals with cerebral palsy (Fig. 2).

Resveratrol Facilitates Recovery of Novel Spatial Memories

The spatial memory of the animals was assessed using the T-Maze test at ages P25, P26, and P27, using the overall percentage of new arm choices. Significant differences were found in the animals' spontaneous alternation [F (3.33) = 44.30; $p < 0.0001$). The animals in the CPS group made fewer new arm choices in the maze, opting for the same arm of the maze that had already been explored, compared to the CS group (CPS = 25.00 ± 5.618 vs. CS = 81.94 ± 3.815; $p = 0.0016$). However, animals in the CPR group showed a higher number of new arm choices, alternating more often in the maze (CPR = 83.33 ± 5.428 vs. CPS = 25.00 ± 5.618; $p = 0.0003$) (Fig. 3).

The CP Model Increases and Resveratrol Reduces Anxiety-Like Behavior

Anxiety was assessed using the LDB test at P22 and P29. At P22, significant differences were observed between the experimental groups in the time spent on the light side [F (3.88) = 23.68; $p < 0.0001$), the time spent on the dark side [F (3.88) = 23.90; $p < 0.0001$), and the total number of transitions from one side of the box to the other [F (3.88) = 6.129; $p = 0.0008$). At P22, the animals in the CPS group spent less time exploring the light side of the box (CPS = 68.72 ± 8.841 vs. CS = 159.1 ± 10.34; $p < 0.0001$) (Fig. 4A) and more time on the dark side compared to the CS group (CPS = 231.3 ± 8.841 vs. CS = 140.9 ± 10.36; $p < 0.0001$) (Fig. 4B). These outcomes suggest that anxiety-like behavior traits had been induced by the CP model, since the animals spent more time in the dark and explored the novelty of the light side less. Animals in the CPR group, however, exhibited an increase in time spent on the light side (CPR = 181.7 ± 16.75 vs. CPS = 68.71 ± 8.841; $p < 0.0001$) (Fig. 4A) and a decrease in time spent on the dark side (CPR = 118.1 ± 16.75 vs. CPS = 231.3 ± 8.841; $p < 0.0001$) (Fig. 4B), suggesting possible anxiolytic effects of resveratrol in CP. No differences, however, were found between the control groups. Neither were any differences observed between any of the experimental groups in relation to the number of entries into the light and dark sides of the apparatus (Fig. 4C–E).

On P29, the animals in the CPS group were found to spend less time on the light side (CPS = 70.23 ± 7.717 vs. CS = 125.8 ± 13.16; $p = 0.0119$) (Fig. 4A), and more time on the dark side (CPS = 229.8 ± 7.717 vs. CS = 174.2 ± 13.16; $p = 0.0117$) (Fig. 4B), along with a decrease in the number of entries into the light side (CPS = 4.167 ± 0.6945 vs. CS = 7.417 ± 0.6904; $p = 0.0274$) and a reduced total number of entries into the light and dark sides (CPS = 7.417 ± 0.8207 vs. CS = 14.50 ± 1.449; $p = 0.0049$) (Fig. 4C), suggesting anxiety-like behavior in the CPS group. The animals in the CPS group showed a reduction in the number of entries into the light side compared to the CS group (CPS = 3.583 ± 0.5833 vs. CS = 6.125 ± 1.292; $p = 0.0274$) (Fig. 4D). Animals in the CPR group were found to spend more time on the light side (CPR = 158.1 ± 20.50 vs. CPS = 70.23 ± 7.17; $p < 0.0001$) and less time on the

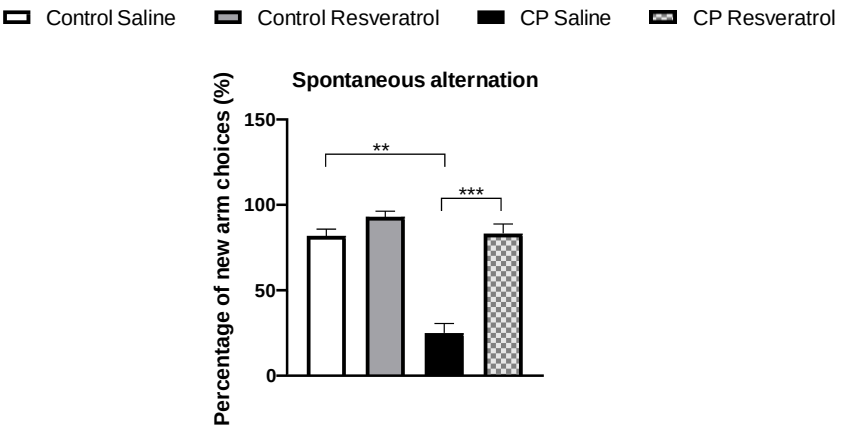

Fig. 3 Percentage of total new arm choices (choices followed by opposite sides of the maze) in the T-Maze test at ages 25, 26, and 27 post-natal day of the four experimental groups: CS ($n = 12$), CR ($n = 12$), CPS ($n = 12$), and CPR ($n = 12$); 4 runs per day were performed. The pups in the CPS group had a higher percentage of the same arm choices in the test ($p = 0.0016$) compared to the CS group, while resveratrol treatment increased the percentage of new arm choices ($p = 0.0003$). There were no significant differences between the CS and CR groups. Values are expressed as mean ± standard error of the mean. ANOVA two-way multiple comparisons Tukey's post hoc test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

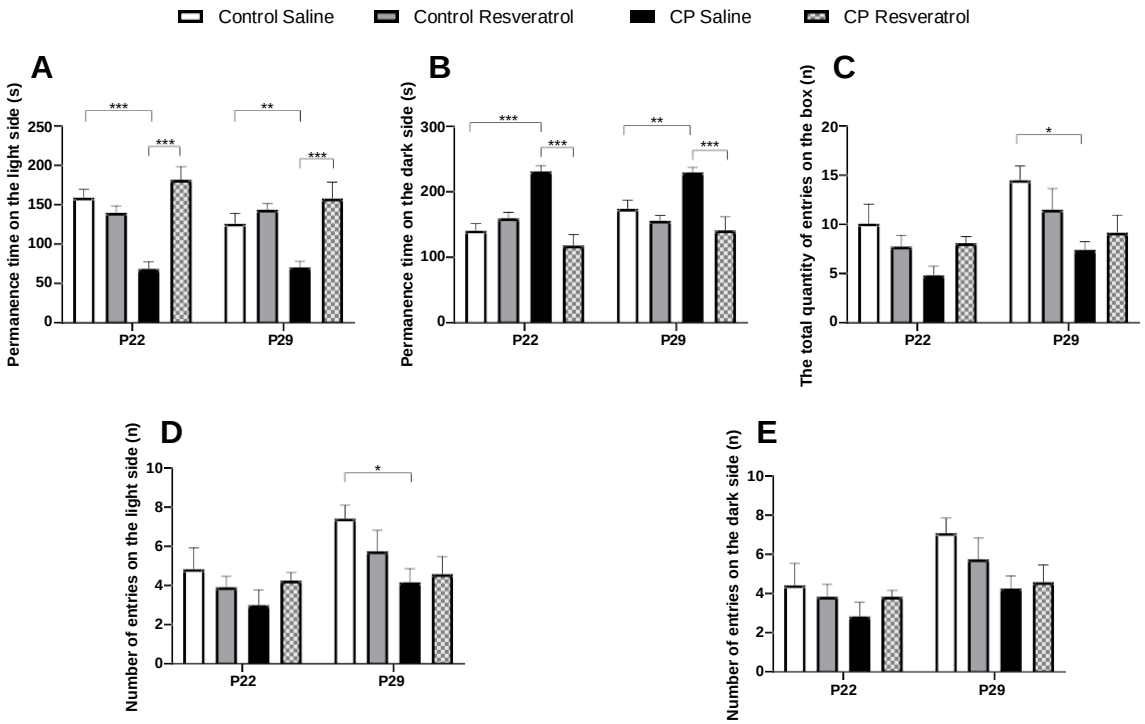

Fig. 4 Spent time and number of transitions between light and dark side in the light–dark box test at P22 and P29 of the four experimental groups: CS ($n = 12$), CR ($n = 12$), CPS ($n = 12$), and CPR ($n = 12$). **A** Spent time on the light side. **B** Spent time on the dark side. **C** Total number of entries on the light and dark side. **D** Number of entries on the light side. **E** Number of entries on the dark side. Values expressed as mean ± standard error of the mean. Tukey's two-way ANOVA repeated measures and multiple comparisons post hoc test. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$

dark side (CPR = 141.4 ± 20.39 vs. CPS = 229.8 ± 7.717; $p = 0.0001$) (Fig. 4A and B), suggesting that the anxiolytic effects of resveratrol persisted beyond P29, restoring the CPR animals to the levels of the control animals. We found no significant differences in the number of entries for the light and the dark sides, or in the total number of entries into the light and dark sides between the CPR and CPS groups ($p > 0.05$) (Fig. 4C–E). There were also no differences between control groups for any of the parameters evaluated (Fig. 4).

On P28, anxiety was also assessed using the Elevated Plus Maze and the results demonstrated differences in permanence time [F (3.33) = 21.29; $p < 0.0001$) and percentage of permanence time spent in the open arms [F (3.33) = 21.29] (Fig. 5A and B); permanence time [F (3.33) = 13.12; $p < 0.0001$] (Fig. 5D), the percentage of the permanence time [F (3.33) = 13.12; $p < 0.0001$] (Fig. 5E), the number of entries [F (3.33) = 7.051; $p = 0.0009$] (Fig. 5F) and the number of fecal boluses (Fig. 5I) in the closed arms [F (3.33) = 35.66; $p < 0.0001$). Similar to the LDB test, animals in the CPS group presented a shorter total permanence time (CPS = 135.4 ± 12.69 vs. CS = 275.4 ± 9.751; $p < 0.0001$) and a shorter percentage of time (CPS = 22.56 ± 2.114 vs. CS = 45.90 ± 1.625; $p < 0.0001$) (Fig. 5A and B) in the open arms of the maze compared to the CS group. They consequently also showed an increased permanence time (CPS = 322.4 ± 23.01 vs. CS = 196.7 ± 11.35; $p = 0.0004$), an increased percentage of time (CPS = 53.74 ± 3.835 vs. CS = 32.79 ± 1.892; $p = 0.0004$), and a larger number of fecal boluses (CPS = 5.667 ± 0.6435 vs. CS = 0.6667 ± 0.3553; $p < 0.0001$) in the closed arms (Fig. 5D, E, and I), indicating the presence of anxiety-like behavior in the cerebral palsy animals. Animals in the CPR group, however, showed an increase in permanence time (CPR = 301.9 ± 26.21 vs. CPS = 135.4 ± 12.69; $p < 0.001$) and, consequently, an increase in the percentage of permanence time (CPR = 50.32 ± 4.369 vs. CPS = 22.56 ± 2.114; $p < 0.0001$) in the open arms compared to the CPS group (Fig. 5A and B). Animals in the CPR group also spent less time in the closed arms (CPR = 172.4 ± 23.55 vs. CPS = 322.4 ± 23.01; $p < 0.001$) (Fig. 5D) and, consequently, presented a lower percentage for permanence time (Fig. 5E) (CPR = 28.73 ± 3.924 vs. CPS = 53.74 ± 3.835; $p < 0.0001$) and a reduction in the number of entries (CPR = 12.67 ± 1.281 vs. CPS = 22.42 ± 1.990; $p = 0.0009$) (Fig. 5F) and fecal boluses (CPR = 1.167 ± 0.3218 vs.

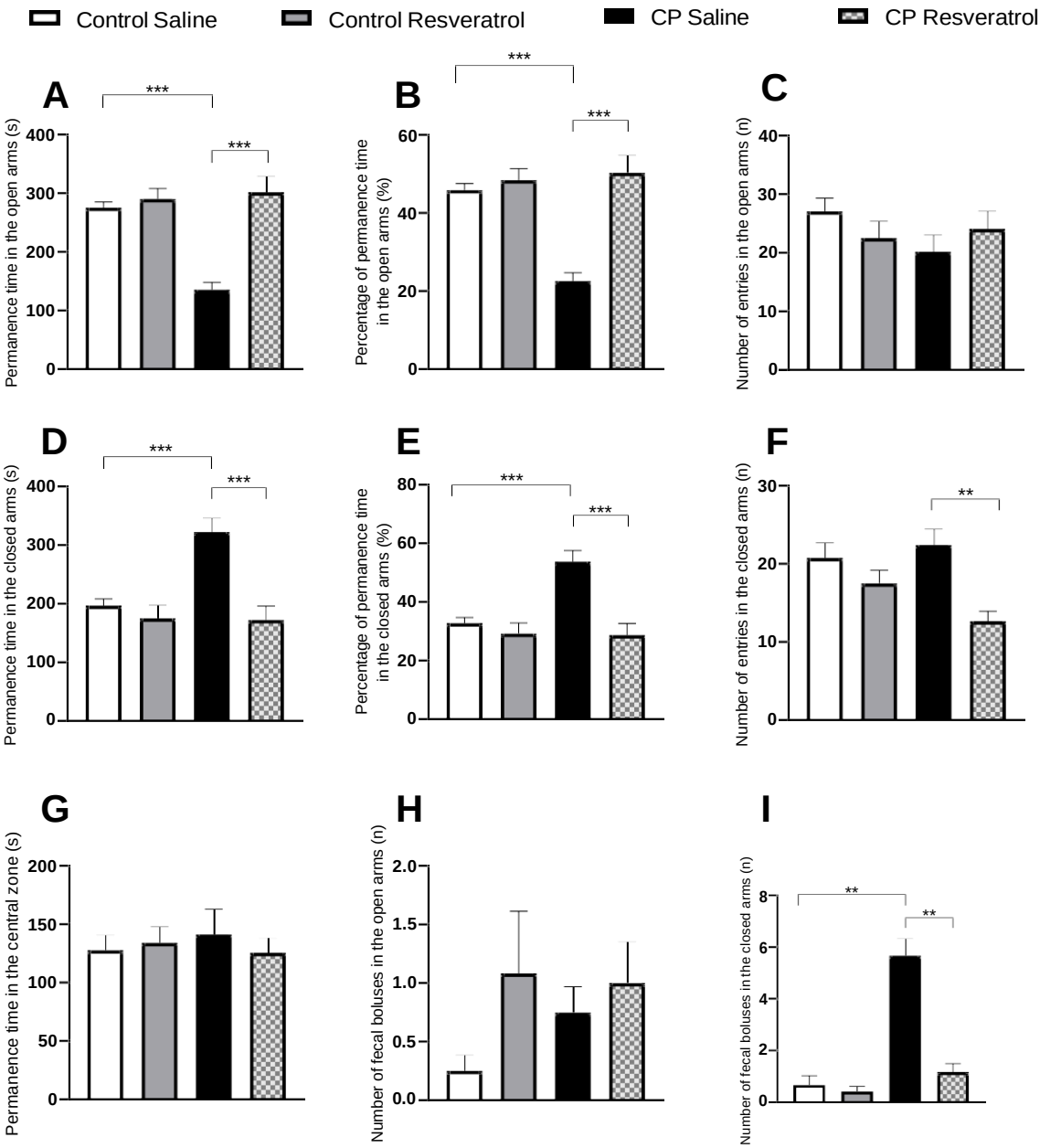

Fig. 5 Performance of animals in the Elevated Plus Maze at P28 of the four experimental groups: CS (n = 12), CR (n = 12), CPS (n = 12), and CPR (n = 12). **A**–**C** The permanence time, the percentage of permanence time, and the number of entries in the open arms, respectively. **D**–**F** The permanence time, the percentage of permanence time, and the number of entries in the closed arms, respectively. **G** The permanence time in the central zone. **H**, **I** The number of fecal boluses in the open and closed arms, respectively. Animals in the CPS group show anxiety-like behavioral traits, while the CPR group showed behaviors similar to controls, suggesting therapeutic effects. Values are expressed as mean ± standard error of the mean. ANOVA two-way multiple comparisons Tukey's post hoc test or Kruskal–Wallis statistic. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

CPS = 5.667 ± 0.6434; p = 0.0038) (Fig. 5I) in the closed arms. No significant differences were found between animals in the CS and CR groups, nor in the number of entries (Fig. 5C) and fecal boluses (Fig. 5H) in the open arms and the permanence time in the central zone between any of the experimental groups (Fig. 5G).

Treatment with Resveratrol Protects Against Body Weight Loss But Does Not Prevent Brain Weight Loss in Cerebral Palsy

On P0, prior to the initiation of experiments, the animals presented no differences in body weight. By P29, at the end of the experiments, however, significant

differences were found between the experimental groups [F (3.44) = 144.9; $p < 0.0001$). Animals in the CPS group exhibited lower body weight compared to the CS group (CPS = 37.65 ± 1.528 vs. CS = 80.04 ± 0.6119; $p < 0.0001$). Treatment with resveratrol attenuated this weight loss in animals in the CPR group compared to animals in the CPS group (CPR = 55.05 ± 2.093 vs. CPS = 37.65 ± 1.528; $p < 0.0001$). No significant differences were found between the animals in the CS group and those in the CR group (CS = 80.04 ± 0.6119 vs. CR = 78.66 ± 1.994; $p > 0.055$), suggesting that resveratrol did not alter the body weight of the control animals. In relation to *in* vivo brain weight, significant differences were found [F (3.21) = 14.66; $p < 0.0001$), with animals in the CP saline group showing a reduction in brain weight in comparison to those in the CS group (CPS = 1.259 ± 0.04726 vs. CS = 1.533 ± 0.04471; $p < 0.001$). Animals in the CPR group showed no significant changes in the absolute weight of the brain (CPR = 1.318 ± 0.03006 vs. CPS = 1.259 ± 0.04726; $p > 0.05$). No differences were observed between animals in the CS group and those in the CR group nor in relation to relative brain weight (brain weight/body weight) [F (3, 21) = 2.084; $p = 0.1330$) (Table 2).

Resveratrol Reduces Gene Expression Associated with Neuroinflammation in Animals with Cerebral Palsy But Not in Healthy Animals

Gene expression analysis of proinflammatory genes revealed significant differences between experimental groups in the expression of the IL-6 [F (1.19) = 10.99; $p = 0.0036$) and TNF-α [F (1.17) = 61.62; $p < 0.0001$). Neonatal treatment with resveratrol-reduced IL-6 expression in the CPR group compared to that of animals in the CPS group (CPR = 0.609205 ± 0.581588 vs. CPS = 1.392811 ± 0.404912; $p = 0.0175$) (Fig. 6A). TNF-α expression was also reduced in the animals in the CPR group (CPR = 0.1 ± 0.5 vs. CPS = 2.4 ± 1; $p = 0.0007$) (Fig. 6B). There was, however, an increase in expression of IL-6 (CS = 1 ± 0.260384 vs. CR = 2.084932 ± 0.204809; $p = 0.0006$) and TNF-α (CS = 1 ± 0.8 vs. CR = 6.6 ± 0.6; $p < 0.0001$) in the animals in the CR group compared

Table 2 Body and brain weight. The table showed animal weight at P0 and P29 of the four experimental groups: CS ($n = 12$), CR ($n = 12$), CPS ($n = 12$), and CPR ($n = 12$). Before anoxia, sensory motor restriction, and pharmacological manipulation, the animals did not show differences in body weight. On the last day of the experiment, animals in the CPS group showed the lowest body weight

Group	Body weight (g) P0	Body weight (g) P29	Brain absolute weight (g) P29	Brain relative weight (g) P29	p value
Control saline	6.003 ± 0.155	80.038 ± 0.612[b***]	1.533 ± 0.04471[b***]	0.02226 ± 0.001208	$p < 0.0001$
Control resveratrol	5.838 ± 0.189	78.655 ± 1.994	1.547 ± 0.02074	0.02039 ± 0.0009867	
CP saline	5.938 ± 0.135	37.647 ± 1.528[c]	1.259 ± 0.04726	0.02526 ± 0.002199	$p < 0.0001$
CP resveratrol	5.902 ± 0.103	55.046 ± 2.093	1.318 ± 0.03006	0.02471 ± 0.001131	

Values expressed as mean ± standard error of the mean. Tukey's two-way ANOVA repeated measures and multiple comparisons post hoc test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. [a]CS vs. VR; [b]CS vs. CP; [c]CP vs. CR

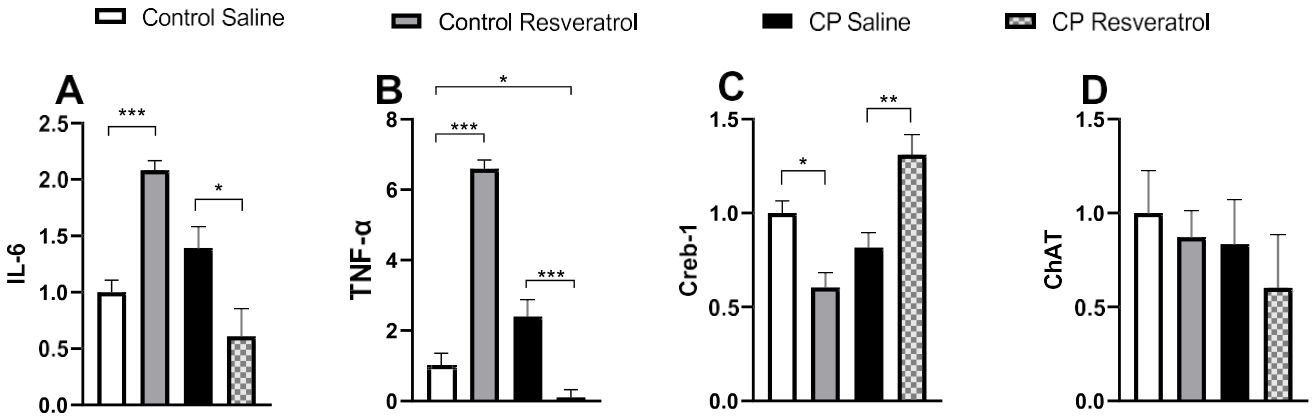

Fig. 6 RT-PCR of IL-6 (CS $n = 6$; CR $n = 6$; CPS $n = 5$; CPR $n = 6$), TNF-α (CS $n = 5$; CR $n = 6$; CPS $n = 5$; CPR $n = 5$), Creb-1 (CS $n = 6$; CR $n = 6$; CPS $n = 6$; CPR $n = 6$), and ChAT (CS $n = 6$; CR $n = 6$; CPS $n = 5$; CPR $n = 6$) genes from the hippocampus of animals sacrificed by decapitation at P29. Values expressed as mean ± standard error of the mean. ANOVA two-way multiple comparisons Tukey's post hoc test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

with those in the CS group (Fig. 6A and B). Analyses of gene expression related to long-term memory consolidation revealed significant differences in Creb-1 (Fig. 6C) gene expression [F (1. 20) = 10.20; p = 0.0046), but no differences in that of ChAT [F (1.19) = 0.9161; p = 0.3505) (Fig. 6D). The animals in the CPR group presented an increase in Creb-1 expression compared to those in the CPS group (CPR = 1.311908 ± 0.257985 vs. CPS = 0.817147 ± 0.185024; p = 0.0020) (Fig. 6C). Animals in the CR group, however, presented a reduction in Creb-1 levels compared to the CS group (CR = 0.604299 ± 0.188108 vs. CS = 1 ± 0.160555; p = 0.0137). No significant differences were observed in levels of ChAT (Fig. 6D).

Neonatal Resveratrol Treatment Reduces Microglia Activation in the Hippocampus in the Cerebral Palsy Model

Immunohistochemistry analysis to confirm the presence of Iba + cells in the hippocampus revealed significant differences in the density of microglia in the DG [F (3. 15) = 9.998; p = 0.0007), CA1 [F (3.15) = 9.683; p = 0.0008), and CA3 [F (3.15) = 7.388; p = 0.0029). Significant differences were also observed in the percentage of activated microglia in the DG [F (3.15) = 46.17; p < 0.0001] and in CA1 [F (3.15) = 48.10; p < 0.0001) and CA3 [F (3.15) = 147.9; p < 0.0001]. The animals in the CPS group exhibited a reduction in the density of microglia in the DG (CPS = 361.2 ± 14.09 vs. CS = 479.1 ± 22.04; p = 0.0020) (Fig. 7A), CA1 (CPS = 631.4 ± 36.30 vs. CS = 951.1 ± 56.80; p = 0.0013) (Fig. 7B) and in CA3 (CPS = 522.3 ± 76.30 vs. CS = 803.0 ± 57.55; p = 0.0350) (Fig. 7C) compared to the CS group. Despite this reduction in cell density, the CPR group presented an increase in the number of microglia in the DG compared to CPS group (CPR = 461.8 ± 15.10 vs. CPS = 361.2 ± 14.09; p = 0.0074) (Fig. 7A). In relation to the profile of microglia in the hippocampus, animals in the CPS group showed an increase in the percentage of activated microglia in the DG (Fig. 7C) (CPS = 35.30 ± 0.6264 vs. CS = 28.54 ± 1.080;

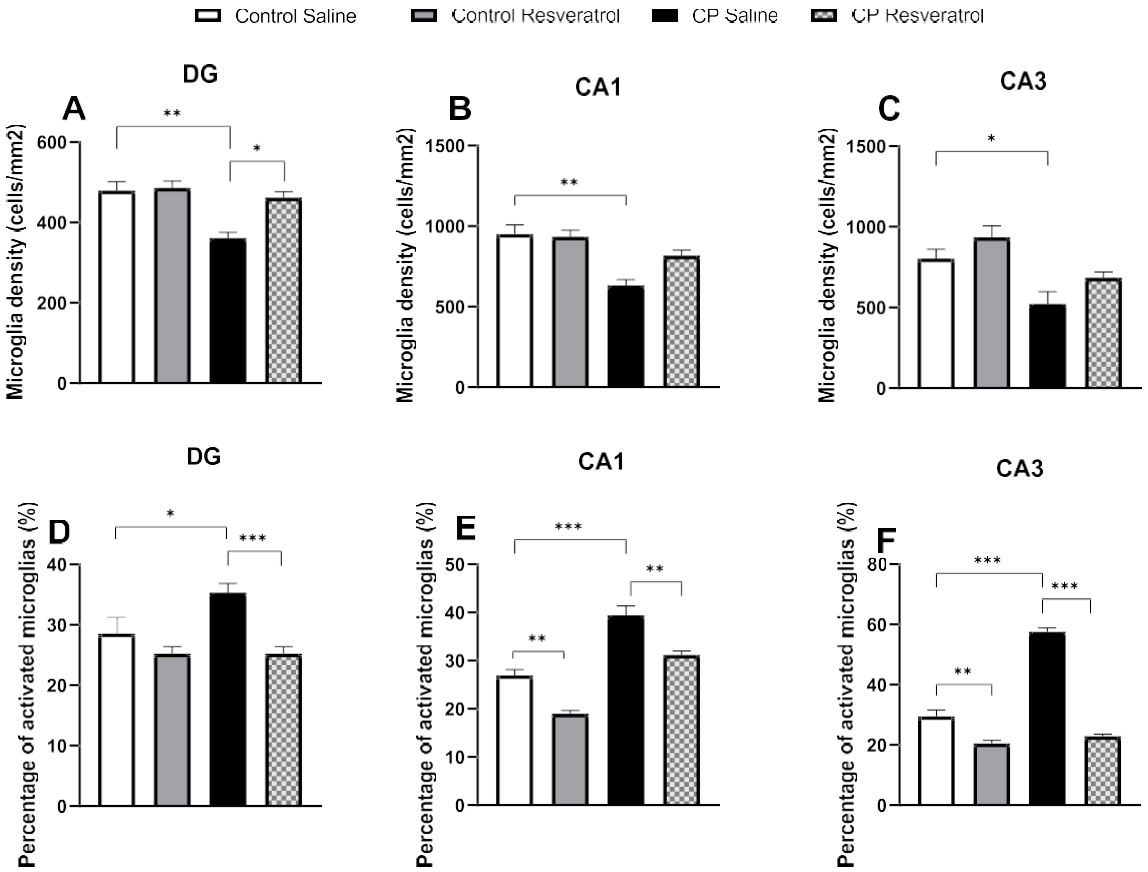

Fig. 7 Immunohistochemistry for Iba1 + cells in the hippocampus. **A**, **B**, **C** The cell density in DG, CA1, and CA3, respectively. **D**, **E**, **F** The percentage of activated microglia in DG, CA1, and CA3, respectively. The experimental groups were randomly divided according to the cerebral palsy model or control with resveratrol or saline treatment: control saline (n = 6), control resveratrol (n = 6), CP saline (n = 6), and CP resveratrol (n = 6). The values expressed as mean ± standard error of the mean. ANOVA two-way multiple comparisons Tukey's post hoc test. *p < 0.05; **p < 0.01; ***p < 0.001

$p < 0.0001$), in CA1 (CPS = 39.38 ± 1.964 vs. CS = 26.93 ± 1.227; $p < 0.0001$) (Fig. 7D), and in CA3 (CPS = 57.58 ± 1.286 vs. CS = 29.50 ± 2.130; $p < 0.0001$) (Fig. 7E) compared to the CS group. Nevertheless, animals in the CPR group showed a reduced percentage of activated microglia in the DG (CPR = 25.24 ± 0.4723 vs. CPS = 35.30 ± 0.6264; $p < 0.0001$), CA1 (CPR = 31.18 ± 0.8415 vs. CPS = 39.38 ± 1.964; $p = 0.0014$), and CA3 (CPR = 22.93 ± 0.6704 vs. PCS = 57.58 ± 1.286; $p < 0.0001$) compared to CPS group (Fig. 7D–F). The pups in the CR group also exhibited a reduction in the percentage of activated microglia in CA1 (CR = 18.98 ± 0.6863 vs. CS = 26.93 ± 1.227; $p = 0.0018$) (Fig. 7E) and CA3 (CR = 20.48 ± 1.024 vs. CS = 29.50 ± 2.130; $p = 0.0020$) (Fig. 7F) compared to the CS group (Fig. 8).

Resveratrol Treatment Increases Cell Proliferation in the Hippocampus

Immunohistochemistry analysis to identify BrdU + cells revealed significant differences in the number of cells in the dentate gyrus [F (3.18) = 17.87; $p < 0.000$), CA1 [F (3.18) = 69.96; $p < 0.0001$), and CA3 [F (3.18) = 56.75; $p < 0.0001$). The pups in the CPS group had fewer cells marked by BrdU + in the dentate gyrus (CPS = 1139 ± 40.50 vs. CS = 2489 ± 140.2; $p < 0.0001$) (Fig. 8A), CA1 (CPS = 314.6 ± 20.96 vs. CS = 1491 ± 72.70; $p < 0.0001$) (Fig. 8B), and CA3 (CPS = 287.9 ± 19.93 vs. CS = 1361 ± 82.06; $p < 0.0001$) (Fig. 8C) compared to the CS group. Despite this, animals in the CPR group presented an increased number of BrdU + cells in the CA1 (CPR = 766.4 ± 66.71 vs. CPS = 314.6 ± 20.96; $p = 0.0003$) (Fig. 8B) and CA3 (CPR = 698.9 ± 70.14 vs. CPS = 287.9 ± 19.93; $p = 0.0008$) (Fig. 8C) compared to the CPS group. Healthy animals treated with resveratrol, however, exhibited the opposite effect, with a reduction in the numbers of cells in the dentate gyrus (CR = 1668 ± 206.2 vs. CS = 2489 ± 140.2; $p = 0.0020$) (Fig. 8A), CA1 (CR = 563.0 ± 41.97 vs. CS = 1491 ± 72.70; $p < 0.0001$) (Fig. 8B), and CA3 (CR = 520.7 ± 19.81 vs. CS = 1361 ± 82.06; $p < 0.0001$) (Fig. 8C) compared to animals that received saline (Fig. 9).

Discussion

The results of the present study demonstrate the negative impact of the cerebral palsy model through the association of perinatal anoxia at ages 1 and 2 days of postnatal life and sensorimotor restriction at ages 2 to 28 days of postnatal life on episodic memory and anxiety-like behavior associated with developmental impairments in

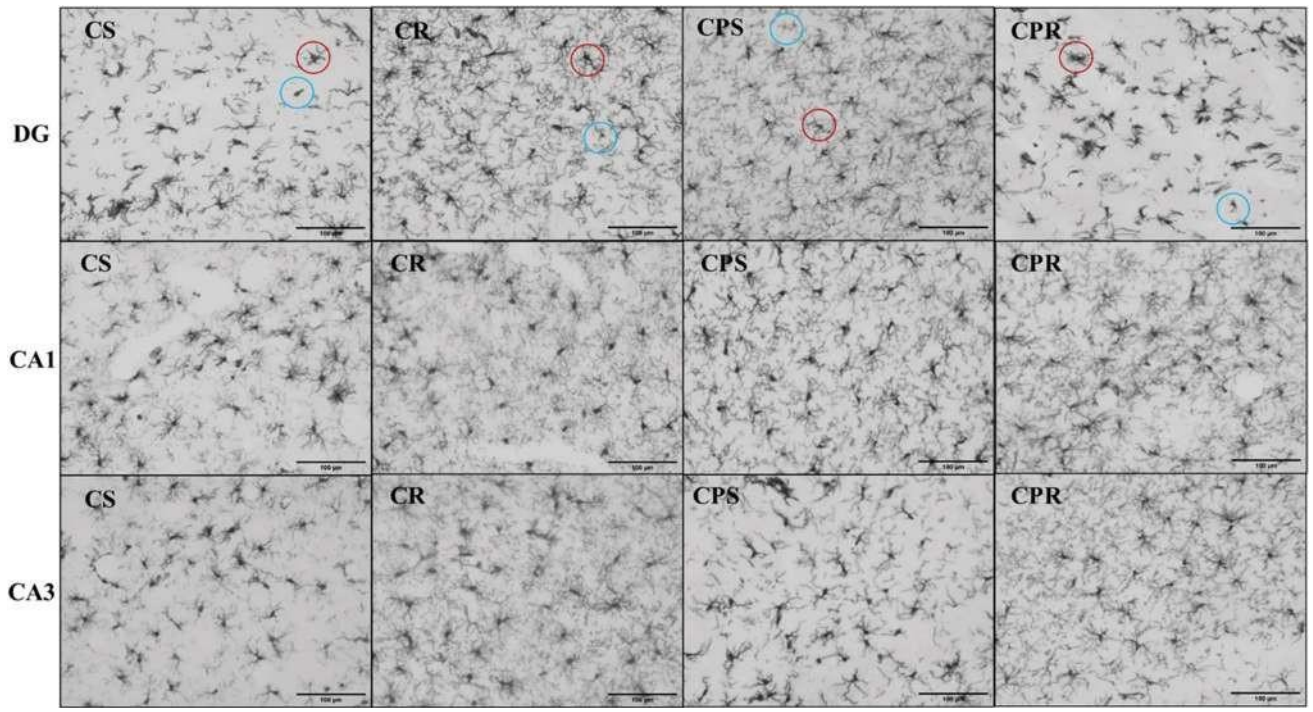

Fig. 8 Immunohistochemistry for Iba1 + cells in the hippocampus. Encircled in red are activated microglia, while encircled in blue are inactivated microglia. Each column represents an experimental group in the following order: CS, CR, CPS, and CR. Each row of the image represents a region of the hippocampus in the following order: DG, CA1, and CA3

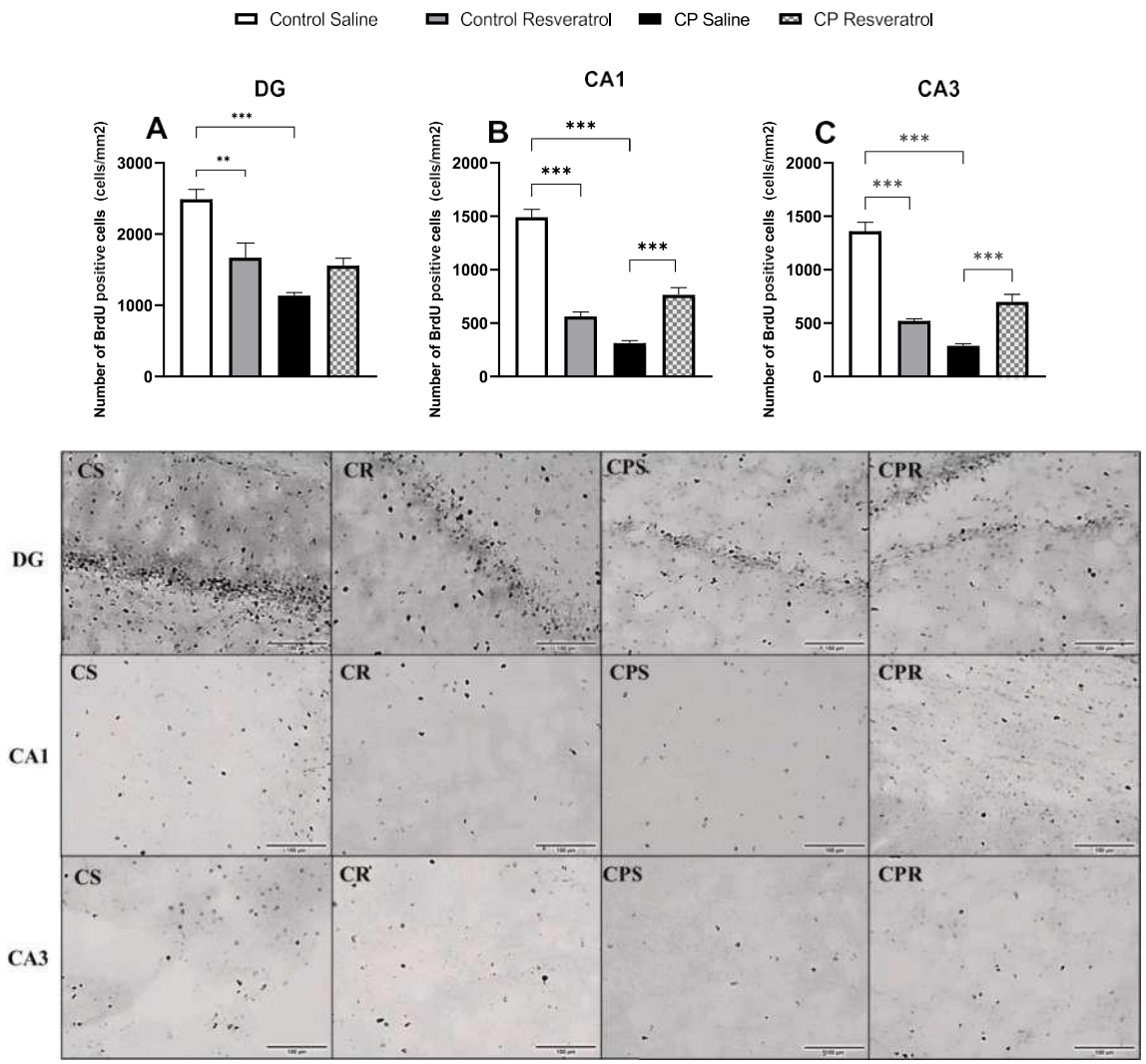

Fig. 9 Immunohistochemistry for BrdU + cells in the hippocampus. **A** The number of cells in DG. **B** The number of cells in CA1. **C** The number of cells in CA3. The experimental groups were randomly divided according to the cerebral palsy model or control with resveratrol or saline treatment: control saline ($n = 7$), control resveratrol ($n = 7$), CP saline ($n = 7$), CP resveratrol ($n = 7$). The values expressed as mean ± standard error of the mean. ANOVA two-way multiple comparisons Tukey's post hoc test. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$

the hippocampus of young rats. The behavioral analyses in young animals at 21st to 29th postnatal day of life demonstrated impairments on visuospatial memory for object recognition as well as short- and long-term spatial localization. The animals also presented disturbances in relation to anxiety-like emotional behavior in paradigms assessing anxiety. It is interesting to note that these behavioral disturbances are correlated with increased expression of pro-inflammatory genes and microglial activation, leading to a reduction in neuroplastic mechanisms such as cell proliferation and Creb-1 levels, in the hippocampus. Nevertheless, neonatal treatment with resveratrol demonstrated promising results in relation to attenuation of this impairment, facilitating the formation and recovery of novel episodic memories and reducing hippocampal damage.

Previous studies using models of CP have demonstrated promising results for the use of polyphenols in the critical period of development, such as resveratrol and kaempferol, in the neuromusculoskeletal sequelae present in CP [15, 67, 68]. The neonatal treatment with resveratrol has demonstrated its effectiveness in reducing biomarkers of oxidative stress and mitochondrial biogenesis in the somatosensory cortex, promoting an evolution in the strength and postural development of animals submitted to CP [67]. Similarly, supplementation with kaempferol in the neonatal period has been demonstrated to reduce biomarkers of oxidative stress

and activated microglia in the hippocampus [15], thus promoting an improvement in locomotor activity by increasing the distance traveled in the open field, speed, and a reducing of the number of immobile episodes [15]. These data suggest a possible improvement in the motor deficits present in CP by neonatal treatment with polyphenols.

Despite these studies revealing a negative impact of cerebral palsy on the somatosensory cortex and hippocampus [15, 67, 68], which are regions involved in cognitive and behavioral processes [18, 20, 22, 23, 29], there are still lacunae in the literature about the effects of polyphenols on these sequelae. Furthermore, studies indicate that early brain injury can impact the brain by increasing markers of oxidative stress and neuroinflammation causing impairments in episodic memory [18, 69, 70]. This occurs because the formation of new memories involves a series of changes in synaptic connections and biochemical modifications such as the synthesis of new proteins or alterations in preexisting proteins in the brain [20, 22, 23, 29]. However, the memory should thus be seen as an ability that is related to adequate brain functioning and that can be modulated by environmental factors such as brain injuries early in life [11, 71–73]. The findings of the present study indicate that brain injury can impact the formation and retrieval of episodic memories.

The animals submitted to a cerebral palsy model presented a reduced novel object discrimination index for novel objects in the NORT and a reduction in the number of a novel choices in the T-Maze test. The impaired performance of these animals suggests impairments in spatial memory and in short-term and long-term object recognition memory. The early injury can result in a deficiency of oxygen and glucose in the brain, impairing neurodevelopment [74, 75]. In view of its high level of functioning and consequent need for a greater supply of oxygen and glucose supply, the hippocampus is one of the regions most affected by brain injury, resulting in abnormal development of this region [75–77]. Animals subjected to CP in the present study were found to have short-term memory impairment and this corroborates previous findings indicating that such injuries cause disorders in the dorsal hippocampus and a synaptic disequilibrium that impacts the formation and retrieval of short-term memory [11, 78]. An insult in the critical period can lead to metabolic changes in the brain that persist into adulthood, impairing the maturation of the hippocampus and the formation of new long-term memories [79].

Although CP causes memory formation impairments in young animals, neonatal treatment with resveratrol was found to attenuate impairments in object recognition and spatial recognition memory. Neonatal treatment with polyphenols has demonstrated promising effects in reducing hippocampal impairment resulting from early brain injury [15]. Resveratrol is a polyphenol that has antioxidant and anti-inflammatory properties in the brain, and also improves synaptic plasticity [80, 81]. Synaptic plasticity is the molecular basis of memory and learning [20, 23, 82]. Treatment with resveratrol at a dose of 100 mg/kg has been shown in previous studies to improve spatial memory after brain injury by increasing synaptic plasticity, leading to increased numbers of dendritic spines in the dentate gyrus of the hippocampus and increased expression of synapsin protein [82]. This indicates the possible mechanisms underlying the memory impairment reduction found in the present study.

In addition to memory impairment, the animals in the cerebral palsy group also exhibited an increase in anxiety-like behaviors in the LDB Test and EPM Test. Previous findings have likewise indicated that brain injury impacts the hippocampus in diverse ways, affecting both spatial memory and anxiety-like behavior [83]. This occurs because the ventral and dorsal regions of the hippocampus are connected to different brain structures and consequently perform different behavioral functions [84]. The ventral region of the hippocampus seems to be associated with emotional behavior because it has connections with areas such as the amygdala and hypothalamus [84, 85]. Interesting findings indicate that in cerebral palsy there is an increased activation of microglia in the hypothalamus, suggesting negative impacts of the injury on regions involved in emotional responses [86]. Moreover, early hypoxia–ischemia increases anxiety-like behavior in association with sensory-motor, tactile, and memory alterations [87]. The model adopted in the present study, however, uses two different insults to mimic cerebral palsy sequelae, exposing the animals to early aggressions [54, 55]. Stress early in life can produce anxiety-like behaviors, as observed in the results of the present study. It should, however, be borne in mind that the animals exposed to this model underwent severe alterations in relation to locomotion [10, 15] and this may have affected the results, despite the fact that the animals performed similarly on two different apparatuses.

Neonatal treatment with resveratrol was found to attenuate anxiety-like behaviors. The animals spent less time on the dark side of the box at P22 and P29. On the EPM, animals in the CP resveratrol group spent less time in the closed arms. These results suggest that the anxiolytic effects of the treatment persisted even after the pharmacological manipulation was completed. Resveratrol is a natural compound capable of crossing the blood–brain barrier and modulating brain function, improving memory and mood, and producing antidepressant and anxiogenic effects [88]. Hyperactivation and dysregulation of the limbic hypothalamic–pituitary–adrenal axis seem to be associated with anxiogenic behaviors in a rat model of post-traumatic stress disorder [89]. Treatment with resveratrol would seem, however, to exert an impact on this axis in so far as it increases permanence time in the open arms of the EPM, as observed in the present study(89), and in addition, reduces the frequency of

freezing behavior by increasing Creb-1 and BDNF levels in the brain [89]. The anxiolytic effect of resveratrol appears to be associated with its ability to activate SIRT1, with animals subjected to a model of chronic normobaric hypoxia showing a reduction in anxiety-like behavior in the LDB, EPM, and open field tests after treatment with resveratrol (20 mg/kg) [90]. This is similar to the results of the present study, which also show that lower doses (10 mg/kg) of resveratrol produce favorable outcomes in juvenile rats.

The present study found that animals with CP exhibited a reduction in the weight of both the body and the brain. Previous studies have reported that animals with cerebral palsy experienced impaired somatic development [10, 15, 37, 91]. This occurs because the animals are subjected to early-life stress factors and the use of sensory-motor restriction may hinder the animal's ability to move about [54, 55], thereby impairing feeding behavior. The literature reports severe deficits in the locomotor activity of such animals [86], which may make it difficult for the animals to feed. Previous studies have also found a deficit in the masseter and digastric muscles involved in mastication [92], which would also hinder feeding behavior and reduce body weight.

The present study conducted an intervention during the critical period and observed that neonatal treatment with resveratrol was capable of attenuating somatic damage. Although we did not evaluate specific aspects of the animals' anthropometric measurements, a systematic review with meta-analysis has observed that resveratrol supplementation can reduce body weight by reducing the quantity of fat and abdominal circumference and promoting an increase in muscle tissue [93]. Findings indicate that resveratrol is capable of regulating cellular energetic metabolism by increasing Sirt1 expression and regulating mitochondrial functions, thereby protecting against lipid accumulation [94, 95]. Although the present study did not observe the effects of resveratrol in malnutrition models, resveratrol may thus affect metabolic disorders by reducing lipid accumulation and increasing glycogen storage in muscle and liver cells [96]. This provides some indication of which mechanisms may underlie the effect of resveratrol on metabolic alterations that affect body weight in cerebral palsy.

The dorsal portion of the hippocampus is connected to brain regions involved in the process of formation, storage, and recovery of episodic memories, while the ventral portion is connected to regions involved in emotional regulation [84]. Alterations in the development of the hippocampus may thus cause significant behavioral impairments. Cytokines, such as tumor necrosis factor and interleukins, are small glycoproteins that act as communication vectors between cells and are involved in the control of crucial cellular functions, such as development, differentiation, and cell death [97]. When released into the nervous system, however, these cytokines can have deleterious effects [76, 97] and are the cells involved in basal and inflammatory conditions in the brain [98, 99]. Alterations in interleukin expression can also affect brain function and behavior. Findings indicate that dysregulations of IL-6 expression produce behavioral impairment in cases of brain injury [100]. This occurs because neuroinflammation is one of the main factors contributing to neurological disorders and impaired behavior [101] and has been associated with the development of cerebral palsy [6, 102].

The mechanisms underlying hypoxia–ischemia are not yet fully understood, but findings indicate that low supplies of oxygen and glucose during hypoxia–ischemia negatively impact the energy reserve needed for cell survival and cause increased excitotoxicity [103–105]. After this, a dysregulation of levels of free radicals and production of reactive oxygen species occur in the brain, leading to oxidative stress and neuroinflammatory responses and causing tissue damage [105, 106]. Nevertheless, neonatal treatment with resveratrol (10 mg/kg) has been shown to reduce the expression of IL-6 and TNF-α in the hippocampus of animals with CP. Studies suggest that resveratrol is a compound with powerful anti-inflammatory properties [48, 52, 107] deriving from activation of the Sirt-1/NF-κB signaling pathway [108, 109]. Previous findings suggest that resveratrol is capable of inhibiting neuroinflammation in ischemia models by inhibiting miR-155 gene expression and, consequently, inhibiting M2-type microglia [110].

Previous studies demonstrate that abnormalities in neuroplasticity mechanisms in the dentate gyrus after brain injury, such as reduced cell migration, may be associated with long-term memory impairment [111]. The results of the present study provide novel evidence that, in cerebral palsy, there is an increase in microglial activation, associated with a reduction in cell proliferation in the DG, CA1, and CA3 regions of the hippocampus, indicating the neural correlates that may be involved in the cognitive and behavioral impairments observed in CP. In this context, neonatal supplementation with resveratrol was shown to be capable of reducing activation of microglia in the DG, CA1, and CA3. Previous studies have found that overexpression of IL-6 and activation of microglia in the hypothalamus, cerebellum, and hippocampus are associated with the motor and behavior sequelae of CP [37, 86]. However, the reducing microglia activation in the hippocampus promoted a reduction in motor impairment [15]. Microglia play a crucial role in brain development by eliminating excess synapses during postnatal life, operating out the process of synaptic elimination [112–114]. However, the hippocampus is a region marked by neurogenesis, resulting in synaptic reorganization [115]. Thus, microglia act in synaptic pruning and are essential in synaptic reorganization induced by neurogenesis [114], to acting in the natural process of forgetting [114]. Besides, the microglia are the first cells to respond to insults by mediating inflammatory

responses [116–118]. The activation of microglia in the hippocampus can occur in excess in response to brain injury impairing the adequate function of the hippocampus [37], and alteration of microglial metabolism and profile contributes to neurotoxicity stage [119]. For this reason, we suggest that reduced microglial activation in the hippocampus is related to improved memory impairment, as observed in our study. Previously, we verified that reducing microglial activation in the hippocampus reduced the motor sequelae of CP [15, 37].

Additionally, the neonatal treatment with resveratrol caused an increased cell proliferation in the CA1 and CA3 regions of the hippocampus, providing evidence of promising effects in relation to attenuating CP sequelae. Previous evidence has observed that early insult (prenatal or postnatal) directly impacts the generation of new neurons in the hippocampus, causing a decline in the production of neural stem cells [76]. Furthermore, the reduction of neuroplastic mechanisms such as CREB-1 gene expression, BDNF, and the neurogenesis process are associated with memory impairment in early brain injuries [18]. Therefore, we suggest that reduced microglial activation associated with increased cell proliferation in the dorsal hippocampus is associated with a reduction in episodic memory impairment in young animals. Despite the beneficial effects in relation to cerebral palsy, the control animals treated with resveratrol showed an increase in the expression of IL-6 and TNF-α genes and a reduction in numbers of $BrdU^+$ cells in the DG, and the CA1 and CA3 regions. This occurs because healthy animals do not necessarily need this supplementation during the neonatal period, different from animals with CP which have been exposed to an early brain injury and present a series of developmental dysfunctions.

Nevertheless, the control animals treated with resveratrol did not present memory impairments. This occurs because memory is a complex cognitive process and various neural structures and biochemical mechanisms participate in this process, for example, the prefrontal cortex, in particular the dorsolateral prefrontal cortex, which is related to the ability to process and manipulate sensory information before it is stored as a long-term memory [120, 121]. The prefrontal cortex is a region involved in executive attention, working memory, decision-making, and emotional regulation [122, 123]. These abilities directly affect the process of formation of long-term memories. Besides, previous studies with polyphenols have demonstrated that supplementation during the critical period has beneficial effects on the brain and behavior of animals with cerebral palsy, but has contrary effects on control animals [15, 81]. For this reason, histological, molecular, and behavioral tests to obtain a more comprehensive response have been performed. Thus, we did not obtain impairments in memory and anxiety-like behavior in the CR animals. Therefore, neonatal treatment with resveratrol would thus appear to have beneficial effects on the neuroinflammatory response present in young rats with cerebral palsy, but additional studies are needed to better comprehend the long-term effects of polyphenols.

The present study observed impairments in the formation and recovery of visuospatial episodic object recognition and spatial location memory and an increase in anxiety-like behaviors in young animals with cerebral palsy. Increased neuroinflammation and reduced cell proliferation in the dentate gyrus, and the CA1 and CA3 regions of the animals' hippocampus were also observed. Neonatal treatment with resveratrol was able to reduce neuroinflammation in the hippocampus and promote an increase in cell proliferation and reduction in episodic memory impairment and in anxiety-like behavior.

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Author Contribution All authors contributed to the study conception and design. C.M.S.S.C: conceptualization, data collection, analysis of result and decided on the manuscript's structure. R.M.C: intellectual supervision and analysis of results. S.C.P, V.S.S, and L.N.F.B: data collection, results analysis, and writing. O.H.S.J and C.J.L: conducting the gene expression experiments and analysis of results. P.A.R.J, O.G.Q, and L.T: conducting the immunohistochemistry procedure and intellectual supervision. A.E.T: supervision, conceptualization, formal analysis, and decided on the manuscript's structure.

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Data Availability The datasets analyzed during the study are available from the corresponding author on reasonable request.

Declarations

Ethics Approval The present study was conducted in accordance with the recommendations of the National Council for Animal Control and Experimentation (CONCEA-Brazil) and was not initiated until approval had been obtained from the Ethics Committee on Animal Use (process number CEUA- 0082/2022) of the Federal University of Pernambuco (UFPE). All animal experiments were conducted in accordance with the "National Institute of Health Guide for Care and Use of Laboratory Animals" [124].

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Competing Interests The authors declare no competing interests.

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APÊNDICE F – THERAPEUTIC ADVANCES FOR TREATING MEMORY IMPAIRMENTS IN PERINATAL BRAIN INJURIES WITH IMPLICATIONS FOR CEREBRAL PALSY: A SYSTEMATIC REVIEW AND META-ANALYSIS OF PRECLINICAL STUDIES

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Review Article

Therapeutic advances for treating memory impairments in perinatal braininjuries with implications for cerebral palsy: a systematic review and meta-analysis of preclinical studies

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ABSTRACT

Cerebral palsy (CP) is a neurodevelopmental disorder caused by damage to the immature brain. CP is considered the main cause of physical disability in childhood. Studies have shown that memory function and emotional behaviour are significantly impaired in CP. Current thought is that interventions for neuromotor damaged play a prominent role, but neglects the memory acquisition problems that affect the functioning and quality of life of these children. This systematic review aims to map and analyse pre-clinical interventions used *to treat* memory formation problems resulting from CP. For this, a search was carried out in the Pubmed, Web of Science, Scopus and Lilacs databases. Then, eligibility, extraction date and evaluation of the methodological quality of the studies were determined. 52 studies were included in this review, and 27 were included in a meta-analysis. Assessing memory performance as a primary outcome, and structural and biochemical changes in the hippocampus as a secondary outcome. CP models were reported to be induced by hypoxia-ischemia, oxygen deprivation and lip- osaccharide (LPS) exposure, resulting in impairments in the formation of short-term and long-term memory in adult life. A reduction in escape latency and dwell time were observed in the target quadrant as well as an in- crease in the time needed for the rodents to find the platform in the Morris Water Maze (MWM). Brain injuries during the perinatal period are considered an insult that negatively impacts hippocampus maturation and causes impairment in memory formation in adult life. Some studies reported that regions of the hippocampus such as the dentate gyrus and *cornu ammonis* 1 were impaired in CP, noting an increase in oxidative stress enzymes and pro- inflammatory cytokines, associated with a reduction in BDNF and neurogenesis levels. These were reported to cause a reduction in the number of neurons and the volume of the hippocampus, in addition to an increase in astrogliosis and apoptosis of neurons and difficulties in forming new memories similar to those that occur in children with CP. Interventions that reduced neuroinflammation and the presence of free radicals were high- lighted as a therapy for the memory disturbance present in CP. Preclinical studies registered treatments with oxygen interventions, resveratrol and erythropoietin, which were able to reduce the damage to the hippocampus and promote improvements in memory and behaviour. In the meta-analysis of selected studies, we observed favorable results, through effect size, for the use of oxygen interventions (SDM -6.83 95% CI [—7.91, —5.75], Z = 12.38, $p = 0.03$; I2 = 71%), erythropoietin (SDM -3.16 95% CI [—4.27, —2.05], Z = 5.58, $p = 0.002$; I2 = 82%) and resveratrol (SDM -2.42 95% CI [—3.19, — 1.66], Z = 6.21, $p = 0.01$; I2 = 77%), stimulating plastic responses in the hippocampus and facilitating the memory formation, with these presenting positive effects in general(SDM -2.84 95% CI [—3.10, —2.59], Z = 22.00; $p < 0.00001$; I2 = 92.9%). These studies demonstrate possible

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avenues of intervention for memory alterations in experimental models of early brain injuries, highlighting promising interventions that can facilitate the maturation of the hippocampus and memory formation and, consequently, minimize functional problems that arise during development.

1. Introduction

Cerebral palsy (CP) is a neurodevelopmental disorder with a number of functional repercussions in the organism (Pereira et al., 2021; Fragopoulou et al., 2019; Jacobsson and Hagberg, 2004). Due to the complexity of its aetiology, its causes are not fully understood. Brain injuries in the neonatal and perinatal period are known to contribute to CP development, including asphyxia, hypoxia-ischemia and infections (Fragopoulou et al., 2019; McIntyre et al., 2022; Jacobsson and Hagberg, 2004; Blair and Stanley, 2002). These trigger a cascade of functional consequences (Fragopoulou et al., 2019)1. Given the alterations and damages present in CP, it has been the subject of a number of studies: pre-clinical studies have pointed to a relationship between the lesions and brain alterations present in CP and their impact on behaviour, especially in locomotion (Pereira et al., 2021), where it is possible to observe a delay in the acquisition of motor skills (Krigger, 2006; Ward et al., 2006; Mockford and Caulton, 2010; Peterson et al., 2013; Coq et al., 2008) and in motor coordination and movement (Coq et al., 2008; Pereira et al., 2021), causing functional impairments of various aspects of locomotion (Pereira et al., 2021).

Thus, the CP is understood as a set of syndromes that cause impairments in the movement and posture of affected children (Marret et al., 2013; Rumajogee et al., 2016). Nevertheless, sensory-perceptual abnormalities are also found in association with CP, impairing cognitive abilities such as memory (Hirsh et al., 2010; Al-Nemr and Abdelazeim, 2017; Pirila et al., 2004; Pueyo et al., 2009; White and Christ, 2005). For this reason, a high prevalence of learning problems is observed, in approximately about 40% of children with CP (Himmelmann et al., 2006; Van Rooijen et al., 2015). Memory and fine motor movement impairments are associated with low school performance in children with CP (Van Rooijen et al., 2015).

The development of CP is multifactorial and may occur after brain lesions in prenatal, perinatal and postnatal periods (Graham et al., 2019). The main risk factors for the emergence of CP are closely linked to events that occur in the foetal and neonatal period, such as foetal growth restriction, multiple pregnancies, birth asphyxia and, above all, premature birth, which is still considered the main risk factor for the development of CP (Graham et al., 2019; Oskoui et al., 2013; Stavsky et al., 2017). Due to the number and complexity of events that can trigger the onset of CP, it is considered the most common cause of physical disability in childhood (Himmelmann, 2013; Yeargin-Allsopp et al., 2008), affecting about 3,4 per 1000 live births in regions from low-income countries and 1,5 per 1000 live births in regions from high-income countries (McIntyre et al., 2022), thus pointing to the relevance of obstetric care.

However, recent preclinical studies have pointed to impairments beyond the neuromotor sequelae, observing an increase in depressive and anxiogenic behaviours (Herrera et al., 2018; Granja et al., 2021) and a reduction in memory performance (Granja et al., 2021; Matsuda et al., 2021) in early brain lesion models. This difficulty in forming and retrieving new memories can be associated with reported damages in the brain areas related to emotional behaviour and memory, such as the frontal cortex and hippocampus (Granja et al., 2021; Visco et al., 2021; Basilious et al., 2014; Matsuda et al., 2021). The hippocampus can be understood as a structure sensitive to neuroinflammation and asphyxia in the perinatal period, showing a reduction in neurogenesis and a significant increase in neurodegeneration, accompanied by severe behavioural and memory alterations (Granja et al., 2021; Visco et al., 2021; Basilious et al., 2014; Matsuda et al., 2021). Among the most present alterations, there is damage to the *cornu ammonis* 1 (CA1) and 3 (CA3) and the dentate gyrus (DG) of the hippocampus, reflected in changes in memory formation especially short-term spatial memory and reference memory (Matsuda et al., 2021; Takada et al., 2016). Also, in these previous studies, the literature reported that perinatal brain damage affected neurogenesis after birth in key brain regions such as the hippocampus and subventricular zone (Visco et al., 2021). For this reason, early brain injury affects hippocampal maturation and impairs memory formation.

The first years of life, this perinatal period, represent a crucial phase for development, with consequences on both the physiological systems and behaviour (Kelly et al., 2012; Anderson et al., 2011). The effects of hypothermia on memory are not yet fully understood, because memory have not yet matured in this early period; there are some disagreements about its long-term repercussion (Cainelli et al., 2021). In this context, various types of interventions are currently being studied to mitigate functional impairments in individuals with brain injuries, hypothermia is commonly used in children with brain injuries because can impede the extent of brain infarct (Wagner et al., 2002). Hypothermia can reduce oxidative stress and inhibit the release of pro-inflammatory cytokines after brain injury (Talma et al., 2016), as well as reduce neurodevelopmental damage in newborns (Azzopardi et al., 2014) and also reduce the risk of mortality in neonates, becoming an acute therapeutic strategy investigated in clinical and preclinical studies (Cainelli et al., 2021; Azzopardi et al., 2014; Shankaran et al., 2012).

In this context, despite advances in perinatal care to reduce mortality and functional consequences, there are still few therapeutic strategies that address the central problem of CP as a brain injury for clinical use. As well, there are reported few strategies to attenuate the damage and memory formation disturbances in subjects with brain lesions. Given the annual increase in cases of CP and brain lesions cases, we questioned: what are the treatment prospects for memory formation problems during lifespan in subjects with CP? Thus, the aim of this review is to map and analyse the effects of the main used in the treatment of memory formation problems and hippocampal damage present in adult animals that were subjected to brain damage during the perinatal period with implications for the development of cerebral palsy.

2. Materials and methods

2.1. Systematic review report and description of protocol

The present systematic review was carried out according to the recommendations of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement for transparent reporting of systematic reviews and meta-analyses (Rethlefsen et al., 2021; Liberati et al., 2009). The protocol used for this systematic review was registered with the International Prospective Register of Systematic Review (PROSPERO) (Registration n. CRD42022298566).

2.2. Search strategy

The search in the scientific literature was carried out between November 2021 and January 2022 in the Medline/Pubmed databases (National Library of Medicine / Medical Literature Analysis and Retrieval System Online: 1943- Jan/2022), Web of Science 1991- Dec/2021), SCOPUS (1953-Jan/2022) and LILACS (Latin American and Caribbean Literature in Health Sciences: 1990-Dec/2021). Appropriate terms were used in each database to cover experimental models of cerebral palsy and interventions aimed at memory performance according to MeSH (Medical Subject Headings), DeCS Descriptors (Health Science

Descriptors) and appropriate keywords and synonyms for each database, using the following terms: cerebral palsy AND memory AND treatment (Table 1).

2.3. Eligibility

No linguistic preference or year of publication restrictions were applied to the database search. The selection of studies was in two phases: the first, by title and abstract. Where there was any doubt whether the method within the defined inclusion and exclusion criteria, the methodology section was read in full. The second phase consisted of a complete reading of the selected articles, being considered for inclusion in the present review according to the eligibility criteria. In addition, the reference lists of included articles were inspected and relevant articles were selected and submitted to the eligibility criteria. This process was carried out by two independent reviewers (Calado and Pereira). Any disagreement or conflict was resolved by discussion and consensus between the two reviewers and, when necessary, a third reviewer was consulted (Toscano).

The studies selected in this review carefully followed the inclusion criteria. The preclinical studies followed the following criteria: i) rodents that were submitted to any experimental model of CP in the prenatal, perinatal or postnatal period or rodents submitted to brain injuries in the prenatal, perinatal or postnatal period that could result in CP (up to day 10 of postnatal life); ii) studies that assessed memory through behavioural tests; iii) animals that had been exposed to any intervention with an effect on memory after the brain insult, where the type of intervention, exposure time and dosage were detailed; iv) studies that assessed the impact of brain injury and intervention on the hippocampus and the method used to assess; v) studies that had a control group in their experimental design. On the other hand, the following exclusion criteria were adopted: i) non-original article; ii) did not use a cerebral palsy model; iii) did not assess memory; iv) used genetically modified animal species; v) reported brain damage in adult animals; vi) in vitro studies; vii) did not assess memory; viii) did not assess the hippocampus; ix) reported intervention before brain injury; x) did not report the age of the animals when they were submitted to brain injury; (Table 2 and Fig. 1). Discrepancies in the inclusion of both preclinical studies were resolved after consensus between the two authors (Calado and Pereira) or were referred for evaluation by a third reviewer (Toscano).

Table 1
Standard terms used in the search strategy.

Search strategy		
Component		Terms/Boolean operators
Cerebral palsy	OR	(Perinatal asphyxia) OR (anoxia) OR (hypoxic-ischemic) OR (ischemic) OR (hypoxic) OR (lipopolysaccharide maternal exposure) OR (brain paralysis) OR (encephalopathia infantilis) OR (neonatal stroke) OR (white matter damage) OR (encephalopathia) OR (brain inflamatory)
AND Memory	OR	(Memory disorders) OR (memory impairment) OR (cognitive performance) OR (neurobehavior) OR (cognition) OR (executive function) OR (system executive)
AND Treatment	OR	(behaviour therapies) OR (cognition therapies) OR (neuromodulation) OR (cognitive technique) OR (non-invasive techniques) OR (invasive techniques) OR (electrical stimulation) OR (direct current stimulation) OR (transcranial magnetic stimulation) OR (neurofeedback) OR (attenuates) OR (drug) OR (pharmacological treatment) OR (nutritional intervention)
AND NOT review	OR	(meta-analysis) OR (case study) OR (case report) OR (Alzheimer) OR (Alzheimer's disease)

Note: the terms used in the search strategy may have varied according to the specific needs of each database.

Table 2
Inclusion and exclusion criteria in pre-clinical studies.

Inclusion criteria	Exclusion criteria
Participants:	Participants:
o Animals.	o Genetically modified animal species;
Exposure:	o Non-rodents;
o Animal model of brain injury with implications for the development of cerebral palsy and the treatment of memory disorders resulting from the injury.	Exposure:
Control:	o Brain injury after the gestational, perinatal, or neonatal period;
o Sham	o Intervention started before brain injury;
Outcomes:	o Unclear treatment protocol;
o memory performance;	o Studies that do not report the age of rodents during brain injury induction.
o Changes in the hippocampus.	o Control:
Study type:	o Studies without a control group
o Original data;	Outcomes:
o o Full-text was available.	o Studies that do not mention the test used to assess memory;
	o In vitro evaluation of the hippocampus.
	Study type:
	o Non-original data (e.g., reviews, editorial).

2.4. Data extraction

Data extraction from the studies selected was performed by two independent researchers (Calado and Pereira) using a specific form that was developed to collect the results relevant to the review. The following information for clinical trials was extracted from each study: i) surname of the first and second author; ii) year of publication; iii) information regarding subjects in preclinical studies (species, sex and number of animals per group); iv) brain injury with implications for CP (the date the injury occurred); v) intervention used (dosage, treatment period and route of administration); vi) memory assessment as a primary outcome (type of memory assessed, method used and age during the task); vii) repercussions of interventions on the hippocampus and technique used to assess structural and biochemical parameters as a secondary outcome; viii) results for each comparison between control and exposed, extracting the mean value and standard deviation; ix) statistical method used and descriptive results, as a secondary result. All characteristics of the included studies are summarized in Table 3.

2.5. Assessment of methodological quality

Three researchers (Calado, Pereira and Souza) independently assessed the methodological quality of the studies included in this review using Syrcle's Risk of Bias (RoB) tool (Hooijmans et al., 2014a, 2014b). This tool is guided by the Cochrane Risk of Bias tool, adjusted for aspects of bias that play a specific role in animal studies (Hooijmans et al., 2014a, 2014b). This tool is composed of 10 items that assess selection bias, performance bias, detection bias, attrition bias, reporting bias and other sources of bias, evaluating the following items: i) sequence generation; ii) baseline and concealment characteristics; iii) allocation concealment; iv) housing for random allocation of animals; v) blinding caregivers and researchers involved in the research; vi) random outcome assessment; vii) blinding of outcome advisor; viii) incomplete outcome data; ix) selective reporting of results; x) other sources of bias (Hooijmans et al., 2014a, 2014b).

A "yes" judgment indicates a low risk of bias; a "no" judgment indicates a high risk of bias; the "uncertain" judgment indicates that insufficient detail was reported to provide an adequate assessment of the

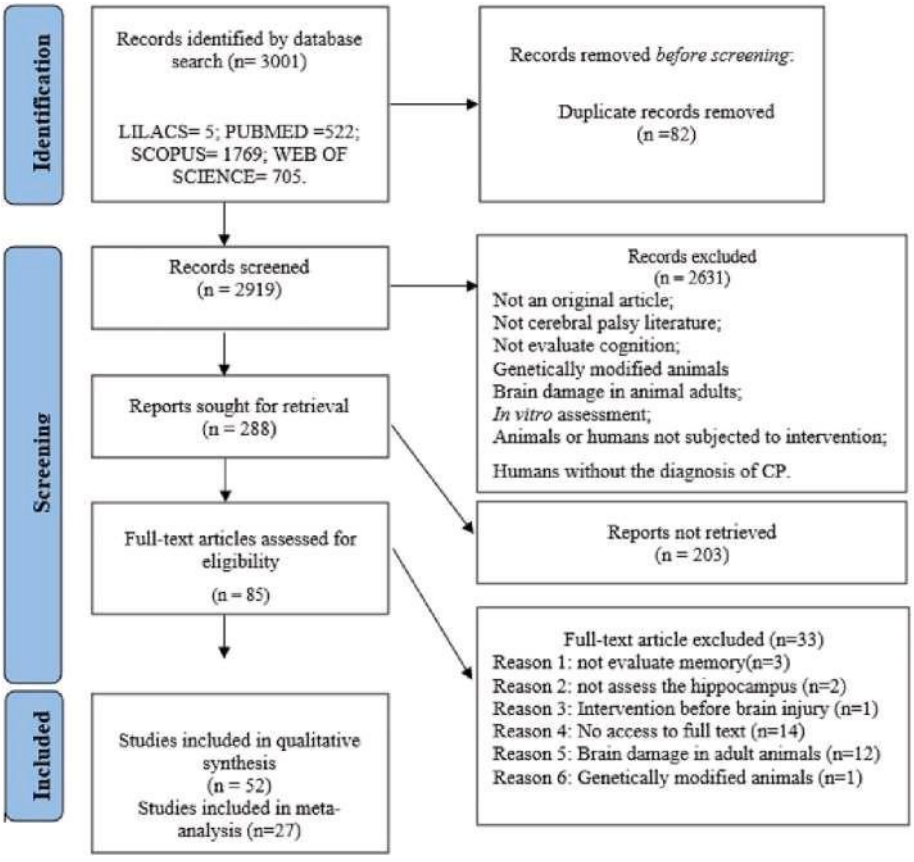

Fig. 1. PRISMA flow diagram of the study selection process.

risk of bias (Hooijmans et al., 2014a, 2014b). Conflicts and disagreements during the evaluation were resolved in a consensus among the three researchers (Calado, Pereira and Souza), in the absence of consensus, a fourth author was consulted (Toscano). RevMan v.5.3 was used to create the "summary risk of bias" and "risk of bias" graphs.

2.6. Statistical analysis

The software RevMan v.5.4 was used to calculate the meta-analytic comparisons for each behavioural parameter in the Morris water maze test, as assessed and reported in >3 studies. Cross-sectional comparisons between exposed and control animals assessed standardized mean differences (SMDs) calculated from mean and variance data computed with Cohen's d pooled effect sizes (ES) and 95% confidence intervals (95% CI). These were weighted for sample size, using a random-effects model (Hooijmans et al., 2014a, 2014b). Sensitivity analyses were conducted to test the effects of removing potentially incomparable studies. Incompatible studies were those with insufficiently matched exposed and control animals, factors that could have modified the relationship between Cerebral Palsy and behaviour (e.g. age analysis). These data are subsequently reported as "potentially incomparable". The extent of heterogeneity between studies was inspected using I^2, with I^2 values exceeding 60% indicating important heterogeneity (Hooijmans et al., 2014a, 2014b). Potential sources of statistical heterogeneity were explored; depending on sufficient data availability, variability, and the number of studies, meta-regression was planned. In comparisons that contained at least ten studies, the likelihood of publication bias was also assessed using the observation of funnel plots. Thus, a subgroup analysis was performed referring to different models of CP related to each outcome in the Morris water maze test: escape latency and time spent in the target quadrant.

3. Results

3.1. Selection of studies

Initially, the search in the databases with the appropriate terms found a total of 3001 articles subject to analysis; 82 duplicate studies were found, which were immediately removed. After that, 2919 studies were left to be analysed according to the screening eligibility criteria by reading the title and abstract of the article. As a result, 2631 studies presented one or more exclusion criteria, leaving 288 articles for full-text reading. After reading the full text, we detected 33 studies that had one or more exclusion criteria, such as not evaluating memory through tests, not evaluating the effects of the insult on the hippocampus, the intervention starting before the brain injury or because we did not have access to the full text. For this reason, 52 studies were included in the final qualitative analysis, and 27 were included in the meta-analysis. (Fig. 1).

3.2. Characteristics of studies included

A systematic synthesis of the characteristics of the 52 studies included is presented in Table 3. Predominantly, of these 52 studies, 28 used Sprague Dawley rats (Berger et al., 2019; Chen et al., 2021; Cho et al., 2018; Fan et al., 2008; Gonzalez et al., 2009; Gao et al., 2020; Gou et al., 2020; Holubiec et al., 2018; Huang et al., 2021; Jung and Kim, 2017; Kim et al., 2017; Li et al., 2019b; Liu et al., 2013; Lowe et al., 2017; Niu et al., 2021; Pan et al., 2012; Pak et al., 2018; Ren et al., 2017; Sampath et al., 2020; Sun et al., 2014; Wagner et al., 2002; Wang et al., 2002; Wei et al., 2015; Wei et al., 2017; Xiao et al., 2016; Xu et al., 2015; Zhang et al., 2014; Zhao et al., 2014), 16 used Wistar rats (Dell'Anna et al., 1997; Farfán et al., 2020; Greggio et al., 2011; Griva et al., 2017; Halis et al., 2019; Karalis et al., 2011; Kumral et al., 2004; Iuvone et al.,

Table 3
Characteristics of included rodent studies.

CP MODEL	AUTHOR (YEAR)	STRAIN	EXPERIMENTAL GROUPS (n)	INTERVENTION	HIPPOCAMPAL OUTCOMES	MEMORY OUTCOMES
H-I	Berger et al., 2019	Sprague Dawley rats Both sexes CP I: P7	i) HI + Mel; ii) Sham + Mel; iii) HI + DMSO; iv) Sham + DMSO; v) HI + PBS; vi) Sham + PBS. *n* = 6–11 animals per group	Intraperitoneal injections of melatonin (10 mg/kg) or DMSO at 3 time points: immediately, 6 h and 25 h after the hypoxia	↓infarct volume at 1 day after HI ($P < 0,05$) in the HI+ Mel group compared with the HI+ DSMO group.	The novel object recognition test was performed at 12 and 41 days after HI ↓ Exploration time of the novel object in the HI group compared to the sham group. No significant differences were found in the HI+ Mel group compared to the HI group
Ischemia	Chen et al., 2014	Male ICR mice CP I: 20 ± 2 g	i) Sham; ii) Model control; iii) AS 20 mg/kg; iv) AS 40 mg/kg; v) AS 60 mg/kg. n = not specified	Mice were treated orally with 20, 40, or 60 mg/kg of Asiaticoside (AS) 24 h after the operation, and then once a day for a week. The sham-operated control group and model control group were treated with distilled water.	↓ iNOS activity in AS-treated HI animals compared to HI and placebo-treated animals with 40 and 60 mg/kg AS ($p < 0.05$, $p < 0.01$) ↓ microglial overactivation and p38 MAPK phosphorylation observed in the HI + AS treatment group (20 mg/kg or 40 mg/kg or 60 mg/kg) compared to the model control group ($p < 0.05$, $p < 0.05$ and $p < 0.01$ respectively).	Morris water maze test began 8 days after brain damage ↓ Escape latency in the HI+ AS 60 mg/kg treatment group and HI+ AS 40/mg/kg compared to the model control group ($p <$ 0.01 and $0 < 0,01$, respectively)
H-I	Chen et al., 2015	Rats Both sexes CP I: P3	i) HI + PBS; ii) HI + OPC; iii) Sham + OPC; iv) Sham + PBS n = 6–8 animals per group	Transplantation of oligodendrocyte progenitor cells (OPC) in animals 2 h after brain damage, injected into the left lateral ventricles.	↑ The proliferation of neural stem cells in the dentate gyrus of the hippocampus at 2, 3, 7, and 14 days after HI ($p < 0.01$) in the HI+ OPC group compared to the HI+ PBS group. ↓ apoptosis of endogenous neurons following HI injury in the dentate gyrus of the hippocampus at 7 days after HI ($p < 0.01$) in the HI+ OPC group compared to the HI+ PBS group.	Morris water maze at P36–P40 ↓escape latency ($p < 0.01$), ↑ platform crossings ($p < 0.05$), ↑ percentage of time spent in the platform quadrant ($p < 0.01$), ↑ percentage of distance traveled in the platform quadrant ($p < 0.05$) in the HI+ OPC group compared to the HI + PBS group.
Oxygen deprivation	Chen et al., 2021	Rats Both sexes CP I: P3	i) control saline; ii) Hypo + saline; iii) Hypo + Pre-Dex; iv) Hypo + Dex *n* = 12 animals per group	Dexmedetomidine (25 μg/kg, i.p.) 30 min pre (Hypo+Pre-Dex group) or post (Hypo+Post-Dex group) hypoxia injected intraperitoneally.	↑ Synapse density ($p < 0.05$) and ↑ PSD95 and synaptophysin protein expression ($p < 0.05$) in the Hypo +Dex group compared to Hypo+ saline group. ↓ NOX2 protein expression ($p < 0.05$) and ↓ NF-κB activation and proinflammatory cytokines, including IL-1β, IL-6, and TNF-α ($p < 0.05$)) in the Hypo +Dex group compared to Hypo+ saline group.	Morris water maze at P31-P36, approximately. ↓ escape latency ($p < 0.05$), ↑ platform crossings ($p < 0.05$) and ↑ time spent in the platform quadrant ($p < 0.05$) in the Hypo +Dex group compared to Hypo+ saline group.
LPS	Cho et al., 2018	Sprague-Dawley rat Both sexes CP I: Antenatal (maternal LPS injection at E15, E17 and E20)	i) Control; ii) Control TM; iii) CP; iv) CP + TM. *n* = 8 animals per group	Treadmill running: 5 weeks after birth, for 30 min, 5 times a week during 6 weeks.	0.05), ↑ p-PI3K/ ↑ cell proliferation in DG ($p <$ PI3K ratio ($p < 0.05$), ↑ p-Akt/Akt ratio ($p <$ 0.05) and ↑ Wnt-3 expression ($p < 0.05$) in CP + TM group compared to CP group. ↓ p-GSK-3β/GSK-3β ratio ($p < 0.05$) and β-catenin expression ($p < 0.05$) in CP + TM group compared to CP group.	Step-down avoidance task P35-P77, approximately. ↑ Step-down latency time ($p < 0.05$)
Anoxia	Dell'Anna et al., 1997	Wistar rats Both sexes CP I: P2	i) Sham Saline; ii) Anoxia Saline; iii) Sham ALC; iv) Anoxia ALC.	The animals received intraperitoneal injections of ALC (50 mg/kg) or saline (50 mg/kg) of a 0.5 ml volume, daily from P2 (1h after brain injury) until P60.	Histological analysis of the brain and hippocampus did not show changes that could be related to ALC treatment	The Maze Test at P30-P35 ↓ time to reach the food compared to the Anoxia Saline group (p ⟨0,001) Morris water maze at P50-P60

(*continued on next page*)

Table 3 (*continued*)

CP MODEL	AUTHOR (YEAR)	STRAIN	EXPERIMENTAL GROUPS (n)	INTERVENTION	HIPPOCAMPAL OUTCOMES	MEMORY OUTCOMES
LPS	Fan et al., 2008	Sprague–Dawley rats Both sexes CP I: P5	i) Saline; ii) Saline+PBN; iii) LPS; iv) LPS + PBN. n = 12 animals per group.	PBN (100 mg/kg) or vehicle alone was administered 5 min after the LPS injection	↓ Enlargement of ventricles comparing the LPS+ PBN treatment group with the LPS+ Saline ↓ Neuronal loss; improved the associated neurological dysfunctions ($p < 0.05$) in the LPS+ PBN treatment group compared to the LPS+ Saline.	↓ Escape latency (p ⟨0,01) in anoxia ALC compared to the Anoxia saline group. Passive avoidance test P20-P21 ↓ The number of shocks applied ($p < 0,05$) in the LPS+ PBN treatment group with the LPS+ Saline.
Oxygen deprivation	Farfán et al., 2020	Wistar rats Both sexes CP I: P0	i) control; ii) A; iii) A + DFX + MSC; iv) A+ TNF-α + IFN-γ-MSC-S *n* = 5–8 animals per group	MSC-S, preconditioned with either deferoxamine (a hypoxia-mimetic) or TNF-α + IFN-γ (pro-inflammatory cytokines) intranasally administered 16 µL 2 h post-asphyxia or in two doses, 2 h and 7 days post-asphyxia	↓ GSSG/GSH Ratio in the hippocampus at P7 ($p < 0.005$) and P60 ($p < 0.0005$) Hippocampus at P7: ↓ cytoplasmic NRF2 protein levels ($p < 0.05$) ↑ nuclear NRF2 protein levels ($p < 0.005$) ↑ NRF2 effector NQO1 protein levels ($p < 0.05$) ↓ nuclear p65 protein levels ($p < 0.0001$) ↑ microglial primary ($p < 0.005$) and secondary ($p < 0.05$) process length in hippocampal CA1 region (only A+ TNF-α + IFN-γ-MSC-S versus A) ↓ cleaved-caspase-3 protein level	Novel object recognition test at P30-P31 ↑ Memory index ($p < 0.05$)
H-I	Gao et al., 2020	Sprague-Dawley rat Both sexes CP I: P7	i) C + S; ii) Hypo/Reox; iii) Hypo/Reox DI; iv) Hypo/Reox D2; v) Hypo/Reox D3 *n* = 34 animals per group	Dexmedetomidine was injected intraperitoneally with 25 mg/kg (D1), 50 mg/kg (D2), and 75 mg/kg (D3)	↑ mean density and relative expression level of neuroglobin at 2, 24, 48, and 72 h after Hypo/Reox ($p < 0.05$) ↑ Nissl staining positive cells at 2 h and 24 h after Hypo/Reox in the hippocampal CA1 region ($p < 0.05$) ↓apoptosis of hippocampal neurons and ↓ relative expression level of cytosolic Cyt-c, Apaf-1, and caspase-3 at 2 and 24 h after Hypo/Reox.	Morris water maze at P28-P33: ↓ escape latency ↑ platform crossings In Hypo/Reox D2 group and Hypo/Reox D3 group compared to Hypo/Reox ($p < 0.05$).
Ischemia	Gonzalez et al., 2009	Sprague Dawley rats Both sexes CP I: P10	i) MCAO + single dose vehicle; ii) MCAo +3 dose vehicle; iii) MCAO + single dose EPO; iv) MCAO +3 dose EPO; v) Sham + single dose vehicle; vi) Sham + single dose EPO; vii) Sham +3 dose vehicle; viii) Sham +3 dose EPO *n* = 6–12 animals per group	Intraperitoneal injections of one dose (after brain injury) or three doses (after injury, 24 h and 7 days after injury) of erythropoietin (5 U/g/kg) or vehicle (5 U/g/kg) according to the experimental group	↓ Brain tissue loss in MCAO +3 dose EPO ($P < 0,05$) compared to MCAO +single dose EPO and MCAO + single dose or three-dose vehicle groups	Morris water maze at P65-P70 ↓ escape latency ($p < 0.05$) in MCAO+ single dose EPO and MCAO +3 dose EPO groups compared to sham groups ↑ time spent in the platform quadrant in MCAO+ 3 dose EPO compared to other groups MCAO (P ⟨0,02).
H-I	Gou et al., 2020	Sprague-Dawley rats Both sexes CP I: P7	i) Sham; ii) HI; iii) HI + DMSO treatment; iv) HI + Mel (10 mg/kg) treatment; v) HI + treatment + RSL3 treatment (5 mg/kg); vi) HI + Mel treatment + *n* = 587 animals in total	Rats with HI were treated with intraperitoneal injections of melatonin (10 mg/kg) after brain injury	↑ NueN cell number ($p < 0,01$ and $p < 0,05$) in HI + Mel (10 mg/kg) and HI+ Mel (5 mg/g) group compared to the HI group. ↓ inhibits neuron ferroptosis in the hippocampus ($p < 0,05$)	Morris water maze at 28–33 ↓ Escape latency ($p < 0,05$) and ↑ platform crossing ($p < 0,05$) in HI + Mel (10 mg/kg) and HI+ Mel (5 mg/g) group compared to the HI group.
H-I	Greggio et al., 2011	Wistar rats Males	i) Sham operated; ii) HI + Vehicle; iii) HI + NAP treatment	intraperitoneal injection of the NAP solution was administered at 3 µg/g body weight (0.1	↓ Reduced frequency and rate of DNA damage in the HI+ NAP group compared to the HI Vehicle group ($p < 0,001$)	Morris water maze at P60-P64 ↓ Escape of latency ($p < 0,001$) and ↑ Time

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Table 3 (*continued*)

CP MODEL	AUTHOR (YEAR)	STRAIN	EXPERIMENTAL GROUPS (n)	INTERVENTION	HIPPOCAMPAL OUTCOMES	MEMORY OUTCOMES
		CP I: P7	n = 5–9 animals per group	mL) immediately (0 h) and 24 h after the HI insult.	↓ Oxidative stress markers such as lipid peroxidation (TBA) and glutathione (GSH) ($P < 0.001$) in the HI+ NAP group compared to the HI Vehicle Group	spent in the target quadrant ($p < 0.05$) in the HI+ NAP group compared to the HI Vehicle group.
H-I	Griva et al., 2017	Wistar rats Both sexes CP I: P7	i) Control + standard environment; ii) Control + EE; iii) Control + GCSF + EE; iv) HI + standard environment; v) HI + EE; vi) HI + GCSF + EE *n* = 7–9 animals per group	Rats submitted to the HI model were treated from P7 to P11 with GCSF (50 μg/kg daily) or EE at P21 or were treated with GCSF + EE	↓ Expression of BDNF in HI + EE ($p < 0.01$) and HI + GCSF + EE ($p < 0.05$) groups compared to control + EE group in the right hippocampus,	Morris water maze at P50-P55 ↑ Escape latency ($p < 0.05$), ↓ time to locate the platform ($P < 0,01$), and ↑ spent more time ($p < 0.05$) and performing more entries ($p < 0.01$) in the reference quadrant and HI+ EE group compared to the HI + standard environment.
H-I	Halis et al., 2019	Wistar rats Male CP I: P7	i) Control; ii) Sham; iii) HI; iv) HI+ PTX60; v) HI+ PTX100. *n* = 11–20 animals per group.	Pentoxifylline immediately after and again 2 h after HI with 60 mg/kg/dose (HI+ PTX60) or100 mg/kg/dose (HI+ PTX100)	↓ Relative weight of the right hippocampus in the HI group compared to the HI+ PTX60 group ($p < 0.05$).	Morris water maze at P77 −P85 No difference was observed between control or sham groups and HI+ PTX60 on escape latency, path length and time spent in target quadrant.
H-I	Holubiec et al., 2018	Male Sprague Dawley CP I: P7	i) Sham-Vehicle; ii) Sham-O2; iii) Sham-O10; iv) Sham-P2; v) Sham-P10; vi) AI-Vehicle; vii) AI-O2; viii) AI-O10; ix) AI-P2; x) AI-P10 (*n* = 9) n = 7–9 animals per group	An hour after the brain damage was finished pups were injected intraperitoneally with a DMSO solution or OEA (2 mg/kg or 10 mg/kg) or PEA (2 mg/kg or 10 mg/kg).	↑- The group administered with 10 mg/kg of PEA, showed a significantly higher number of Iba-1 positive cells in the CA1 hippocampal area compared with sham groups ($p < 0.05$) ↑- PEA treatment was able to prevent neuroinflammation and ↓ astrogliosis and preserve cognitive functions in the AI O2, AI O10, AIP2 and AIP10 groups compared to the AI-vehicle group ($p < 0,05$)	Object Recognition, Morris Water Maze and Passive Avoidance tests when rats were 30 days old ↑ HI was associated with a spatial reference memory deficit that was prevented by the treatment with PAE (10 mg/kg)
H-I	Huang et al., 2021	Sprague-Dawley rat Male CP I: P7	i) Sham; ii) HI; iii) HI + Dex; iv) HI + Dex + mimic NC. *n* = 15 animals per group	Immediately intraperitoneally injected with 0.1 mg/kg Dex after HI induction (HI + Dex).	↓ hippocampal tissue and ↑ pro-inflammatory cytokines in the HI group compared to the Sham group ($p < 0,05$). ↓ IL-6, IL-8 e TNF-a in HI + Dex and HI + Dex + mimic NC groups compared to HI ($P < 0,05$)	Water maze test ↓ escape latency and ↑ platform crossing
Oxygen deprivation	Iuvone et al., 1996	Wistar rats Both sexes CP I: P2	i) Sham; ii) Sham + anoxia; iii) Sham + EE treatment; iv) Anoxia + EE treatment *n* = 6 animals per group	Pups were weaned at P21 and, housed by the experimental group, in simple cages or with an enriched environment until P60.	↓ expression of cytosolic proteins such as the calcium binding protein parvalbumin in the hippocampus ($p > 0,05$) in the group Anoxia+ EE (p ⟨0,03) compared to Sham+ anoxia group.	Water maze test at P58-P60 ↑ platform crossing velocity was significantly increased in the enriched treatment group ($p < 0,03$) compared to the Sham+ anoxia group.
LPS	Jung and Kim, 2017	Sprague-Dawley rat Male CP I: Antenatal (maternal LPS injection at E15, E17 and E20)	i) Control; ii) TM; iii) CP; iv) CP + TM; v) CP + TM + SR. n = 8 animals per group	Treadmill running: 5 weeks after birth, for 30 min, 5 times a week during 4 weeks.	↑ cell proliferation in DG, ↑ Synapsin I, ↑ PSD-95, ↑ p-PI3K, ↑ p-Akt time and ↓ GSK-3β in CP + TM and CP+ TMsr group compared to the CP group ($p < 0,05$)	Step-down avoidance task (Short-term memory) ↑ Step-down latency time (CP + TM and CP + TM + SR versus CP, $p < 0.05$)

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Table 3 (*continued*)

CP MODEL	AUTHOR (YEAR)	STRAIN	EXPERIMENTAL GROUPS (n)	INTERVENTION	HIPPOCAMPAL OUTCOMES	MEMORY OUTCOMES
H-I	Karalis et al., 2011	Wistar rats Both sexes CP I: P7	i) Sham-operated; ii) HI+ Resveratrol-treated; iii) HI-treated-vehicle *n* = 15–10 animals per group	Rats submitted to the HI model treated immediately after hypoxia with RES (90 mg/kg).	↓ Extension of injury observed in the ipsilateral cerebral hemisphere of resveratrol-treated rats compared to HI-treated rats ($p < 0.05$) .	Morris water maze from P51 to P55 ↑- Escape latency ($p = 0.016$; $p = 0$; p = 0; $p = 0.012$), and ↑ swimming time to locate the platform in CP animals ($p < 0.01$) in compared to control animals.
Ischemia	Kim et al., 2006	Male ICR mice CP I: 25-30 g	i) Sham; ii) Ischemia+ Oroxylin treatment; iii) Ischemia control	Mice were treated orally with 1.25, 2.5, or 5 mg/kg of oroxylin A 60 min after reperfusion, and then once a day for a week. The last treatment was completed 1 h before each test	↓ Cresyl violet from CA1 and DG indicated loss of cells in the CP group compared to the sham animals ($p < 0.05$), and reduction in the number of pyramidal cells of CA1 ($p < 0.01$) ↑ Number of cells in DG and CA1 in the Ischemia+ Oroxylin group compared to the Ischemia control group ($p < 0,05$)	Y-maze task ↓ spontaneous alternation ($p < 0,05$) in the ischemia group compared to the sham group. Morris Water Maze ↑ Escape latency and the time searching the platform ($p < 005$) in the ischemia group compared to the sham group. ↓Escape latency and the time searching the platform ($p < 005$) in the Ischemia+ Oroxylin group compared to the Ischemia control group ($p < 0,05$)
H-I	Kim et al., 2017	Sprague Dawley rats Both sexes CP I: P7	i) Sham; ii) HI + Saline; iii) HI+ LCM pre-treatment; iv) HI + LCM treatment *n* = 13–25 animals per group	Animals received treatment with lacosamide (50, 100, 200, and 300 mg/kg) in two doses administered with an orogastric tube immediately, before and after brain injury or immediately after brain injury and two hours after brain injury.	↓ Infarcted areas found in several brain regions including the hippocampus ($p < 0.005$) in the HI+ LCM treatment compared to the HI+ saline group.	Morris water maze at P56-P61 ↓ Escape latency ($p < 0,001$) in the HI+ LCM treatment compared to the HI+ saline group.
H-I	Kumral et al., 2004	Wistar rats Both sexes CP I: P10	i) HI + EPO; ii) HI + Saline; iii) Sham operated; iv) Normal control *n* = 7 animals per group	After HI or after the sham operation, each animal received a single intraperitoneal injection of EPO (1000 unit/kg) or saline (1000 unit/kg) according to the experimental group.	↑ treatment with Epo significantly protected CA1 neurons against neonatal HI insult as compared to the HI+ Saline group ($p < 0,05$)	Morris water maze at P28-P32 and P147-P152 ↓ escape latency ($p < 0,05$) and ↑ time spent in the target quadrant ($p < 0,05$) in the HI+ EPO group compared to the HI + saline group.
H-I	Li et al., 2019b	Sprague Dawley rats Males CP I:´P7	i) Sham; ii) CP; iii) CP + vitB1 and vitB2- HA *n* = 6 animals per group	The animals were treated with Vitamin B1 (300 mg) and B12 (1 mg) injected for 0.1 ml in one acupoint one time per day and lasted for 5d in each course.	↑ increase expression of MALAT1, miR-1, and BDNF in the hippocampus and ↑ The PI3K and Akt phosphorylation was enhanced in the CP+ VitB1+ VitB2-HA compared to the CP group.	Step-down avoidance task ↑ Latency to fall in seconds ($p < 0,05$) in the CP+ VitB1+ VitB2-HA compared to the CP group. ↑ Step-down latency time ($p < 0,05$ in the CP+ VitB1+ VitB2-HA compared to the CP group.
H-I	Li et al., 2019aLi et al., 2019	C57BL/6 mice Both sexes CP I: P7	i) Control; ii) HI model; iii) HI+ RES 10 mg/kg; iv) HI+ RES 40 mg/kg *n* = 4–10 animals per group	Treatment mice received high (40 mg/kg) or low (10 mg/kg) Resveratrol. HI model and control group received an equal volume vehicle.	↑ Proliferation of neural stem cells and increase neuronal differentiation in the hippocampal DG region ($p < 0.01$) in the HI-RES 10 mg/kg and HI+ RES 40 mg/kg group compared to the HI model group.	Morris water maze at P28-P33, approximately. ↓ Receiving groups both RES dosages showed remarkable shorter escape latency

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Table 3 (*continued*)

CP MODEL	AUTHOR (YEAR)	STRAIN	EXPERIMENTAL GROUPS (n)	INTERVENTION	HIPPOCAMPAL OUTCOMES	MEMORY OUTCOMES
						compared with the HI model group ($p < 0.001$)
H-I	Liu et al., 2013	Sprague–Dawley rats Both sexes CP I: P7	i) HI with HBO treatment; ii) HI; iii) Sham. *n* = 36 animals per group	At 60 min after the end of hypoxia, pups in the HBO-treated group were placed in the HBO chamber (100% oxygen, while under 2.5 absolute atmosphere) for 90 min and returned to their cages after treatment.	↓ Extent of brain damage was observed ($p < 0.01$) in the HI+ HBO group compared to the HI group. ↓ Infarcted areas in several brain regions of 24.50% and 29.83% in the cerebral cortex and the hippocampus ($p < 0.01$). in the HI+ HBO, the group compared to the HI group.	Morris water maze test from P56 to P60 ↑- escape latency and crossing platform in the HI group. ↓ escape latency and crossing platform in the HI + HBO group ($P < 0,01$) compared to the HI group.
H-I	Lowe et al., 2017	Sprague Dawley rats Both sexes CP I: ´P7	i) Sham; ii) HI+ Hypothermia with saline; iii) HI+ Hypothermia + NAC; iv) HI+ Hypothermia + NAC + VitD *n* = 10–12 per sex per group	Hypothermia started together with the application of NAC (50 mg/kg/day) OR VitD (0.1 μg/kg/day) or NAC (50 mg/kg/day) and VitD (0.1 μg/kg/day) 1 h after injury brain and at P8-P14 by intraperitoneal injections and continued by gavage at P15-P21.	↑ CYP24A1 increased in both hippocampi after HNAC+VitD treatment in females *($p = 0.03$)* compared to HI+ hypothermia in the saline group. ↓ in males, CYP24A1 protein levels *decreased* after treatment with HNAC+VitD in the ipsilateral hippocampus ($p < 0,029$) compared to HI+ hypothermia in the saline group.	Morris water maze at P35-P48 ↓ time to locate the platform, ↑ swimming speed ($p < 0.001$) and ↓ distance traveled in the hypothermia+ NAC + VitD group compared to Hypothermia with saline group ($p < 0,05$)
H-I	Miguel et al., 2020	Wistar rats Male CP I: P7	i) CS; ii) C + MPH; iii) HI + S; iv) HI + MPH *n* = 11–13 animals per group	Methylphenidate (MPH) was injected intraperitoneally (dose of 2.5 mg/kg, volume of 1 mL/kg) 30 min prior to each behavioural session (from P30 to P45).	↑ BDNF levels in the hippocampus at P45 in the HI + MPH group compared to CS group ($p < 0.05$).	Novel-object recognition and Morris water maze between P30 and P45: No difference between groups was observed in the assessed parameters
Oxygen deprivation	Morales et al., 2009	Wistar rats Both sexes CP I: P0	i) Control Vehicle; ii) Control Nicotamine; iii) Asphyxia vehicle; iv) Ashphyxia Nicotamine *n* = 4–9 animals per group.	Nicotinamide [0.8 mmol/kg, i.p. (100 mg/kg)] or vehicle administered to pups exposed to asphyxia or Caesarean section, with the following administration schedule: intraperitoneal injections at 24 h, 48 h, and 72 h after brain injury.	↓ the number of apoptotic nuclei. ($p > 0,05$) in the Asphyxia nicotinamide group compared to the Asphyxia vehicle group.	The novel object recognition test (NORT) ↑ exploration of the new object compared to the Asphyxia Vehicle group ($p < 0,05$) Y Maze test no effect was observed on spontaneous behavioural alternation in all groups.
H-I	Morán et al., 2017	C57BL6/CNr Mice Male CP I: P9	i) Sham; ii) HI-S; iii) HI-C3a. *n* = 18–19 animals per group.	C3a 8 μl, i.e. 1.6 pmol (4 μl /nostril; corresponding to ca. 2.56 μg/kg body weight) was given intranasal 1 h after HI induction every 24 h for three days (P9, P10, and P11)	↓ astrocyte activation in CA1 e DG of the dorsal hippocampus at P55 in HI-C3a compared to the HI-S group ($p < 0,05$) No difference between Sham and HI- C3a.	Object recognition test at P53-P54: ↑ time exploring the novel object ($p < 0.01$) ↑ total object exploration time ($p < 0.05$)
H-I	Mori et al., 2015	Wistar rats Male CP I: P7	i) Sham; ii) HI; iii) HI + S; iv) HI + SDF-1α 60; v) HI + SDF-1α 600 *n* = 12–15 animals per group.	Intracranial injections stromal cell-derived factor-1α (SDF-1α) in 0.1% 60 or 600 μg/kg	Total area of the infarct in the hippocampus was high in the HI group (64.1% ± 2.9%) and was not affected by 60 or 600 μg/kg SDF-1α (66.2% ± 3.0%)	Morris water maze at P18-P22: ↓ escape latency ↑ platform crossings (HI + SDF-1α 600 versus H + S $p < 0.05$).
H-I	Mishima et al., 2004	Wistar rats Both sexes CP I: P7	i) sham-control; ii) normothermic; iii) hypothermic; iv) hyperthermic *n* = 3–5 animals per group	The HI group was further divided into three groups by the chamber temperature: hypothermic group (27 °C), hyperthermic group (37 °C), and normothermic control group (33 °C) during 2 h of hypoxia.	↓- In each section of ipsilateral hemispheres, the normothermic group showed a decrease in each area in the 18th week following HI insult, and hyperthermia significantly increased the brain injury areas in all sections compared with the normothermic group ($p < 0.01$)	Morris Water Maze P112-P117, approximately ↑- HI insult (normothermia group) severely impaired spatial learning, and hypothermia reduced the spatial learning in the water maze test, increasing the platform crossing ($p < 0.01$).
H-I	Niu et al., 2021	Sprague-Dawley rat Male CP I: P7	i) Sham; ii) HI; iii) HI + Tuina *n* = 10 animals per group	Spinal Tuina: On the governor vessel, bladder meridian and acupoints Baihui, Yintang, Shenting, Sishencong, Quchi, Waiguan, Hegu, Yanglingquan, Zusanli, Sanyinjiao, and	↓ expression of NLRP3 (no difference between Sham and HI- C3a) ↓ cleaved caspase-1, ↓ IL-1β, ↓ IL-18, and ↓ GSDMD ($p < 0.05$)	Morris Water Maze at P50 ↓ time finding the platform ($p < 0.05$) and ↑ time spent in the target quadrant ($p < 0.05$)

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Table 3 (*continued*)

CP MODEL	AUTHOR (YEAR)	STRAIN	EXPERIMENTAL GROUPS (n)	INTERVENTION	HIPPOCAMPAL OUTCOMES	MEMORY OUTCOMES
				Taichong. Once a day from P7 to P49 and lasted for approximately 20 min each time.		in the HI+ Tuína group compared to the HI group.
H-I	Odorcyk et al., 2016	Wistar rat Both sexes CP I: P7	i) Sham; ii) HI + S; iii) HI + V; iv) HI + HqAE n = 10–15 animals per group	Huperzia quadrifariata alkaloid extract (HqAE) was injected intraperitoneally with 10 mg/kg administered 1, 24, 48 and 72 h after HI	↓ AChE Activity in the HI group ↓ The number of apoptotic cells in the HI + HqAF group.	Morris Water Maze (between P30 and P45) ↓ time spent in finding the platform ($p < 0.05$) Step down inhibitory avoidance (between P30 and P45) ↑ Step-down latency time (HI + HqAE Short-term test (Central bar) and long-term test (Right bar) compared to the training day, $p < 0.05$)
H-I	Pak et al., 2018	Sprague-Dawley rat Male CP I: P7	i) C; ii) HI; iii) HI + EA; iv) HI + TM; v) HI + EA + TM n = 10 per group	Electroacupuncture (EA): Electrical stimulation (2 Hz, 1 mA) at two acupoints, Baihui (GV20) and Zusanli (ST36) at a frequency of 2 Hz and an intensity of 1 mA for 20 min. Treadmill exercise: running at 5 m/min for 10 min in the first week and 15 min in the second week. In the third week, they ran at 5 m/min for 5 min, then increased to 7 m/min for 10 min and returned to 5 m/min for 5 min. 5 days per week. EA and TM were performed from 3 to 5 weeks after HI induction.		Passive avoidance test (P45): ↑ entry latency in the HI + TM and HI + EA + TM groups compared to the HI group ($p < 0.05$)
H-I	Pan et al., 2012	Sprague Dawley rats Both sexes CP I:´P7	i) Sham; ii) HI + Vehicle; iii) HI + SP n = 3–9 animals per group	The animals received intraperitoneal injections of SP or vehicle in a single dose at 5 min after brain injury in a dose of 500 mg/kg	↑ hippocampal tissue compared to the HI Vehicle group 48 h and 7 weeks after injury ($p < 0,001$)	Morris water maze at P56-P60, approximately ↓ latency time ($p < 0,05$) and ↑ time spent in the platform quadrant ($p < 0,05$) in HI + SP group compared to the HI+ Vehicle group.
H-I	Peng et al., 2022	Mice Both sexes CP I: P7	i) Sham; ii) HI + PBS; iii) HI + RES *n* = 8 animals per group	Mice were treated with RES (100 mg/kg) immediately after hypoxia and 24 and 48 h after the insult.	↑- the morphology and staining of hippocampal neurons in the RES treatment group ($p < 0.05$) compared to the HI+ PBS group. ↑-the number of neuronal dendrites, the density of dendritic spines, and the percentage of mushroom-shaped spines ($p < 0.05$) in the RES treatment group.	Morris water maze at P35-P40, approximately. ↓ Search time ($p < 0.01$), ↓ platform search time, ($p < 0.001$), ↑ platform crossing, and time in the target quadrant ($p < 0.05$) in the RES treatment group compared to HI + vehicle group.
H-I	Potter et al., 2018	Wistar rats Male CP I: P6	i) Sham saline normothermic; ii) Sham saline hypothermic; iii) Sham caffeine normothermic; iv) Sham caffeine hypothermic; v) HI saline normothermic; vi) HI caffeine normothermic; vii) HI saline hypothermic; viii) HI caffeine hypothermic *n* = 6–10 animals per group.	After brain injury, the animals received caffeine citrate (20 mg/kg) or saline through intraperitoneal injections. After that, the animals were placed in normothermic or hypothermic baths for 180 min according to experimental group.	For Caffeine Treatment, right cortices and hippocampal volumes for HI Caf did not differ from either Sham or HI groups, indicating in-between scores ($p > 0.05$).	Morris water maze at P90 ↓ performance in hypothermic as compared to normothermic shams, regardless of caffeine status ($p < 0.05$). No group differences (Hypothermia/Caffeine) effects were seen for the water escape task ($p = 0.05$) Data from the combined (Caffeine + Hypothermia aka "CafCool") groups could not be interpreted, and those findings are not included.

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Table 3 (*continued*)

CP MODEL	AUTHOR (YEAR)	STRAIN	EXPERIMENTAL GROUPS (n)	INTERVENTION	HIPPOCAMPAL OUTCOMES	MEMORY OUTCOMES
Oxygen deprivation	Presti et al., 2006	C57/BL6J mice Both sexes CP I: P7 or P8	i) sham; ii) CP + *Re*-O2; iii) CP+ Re-Air *n* = 112 animals in total	Immediately after hypoxic exposure, pups were exposed either to room air (RA) or 100% O2 for 30 min at 25 °C and then returned to their dams.	↓ loss of the hippocampal tissue volume ($p < 0.01$) in CP+ Re−O2 compared to CP + Re-Air. ↑ Hippocampal atrophy in Re-Air in granular zones CA1 – CA4, compared to Re−O2 mice.	Morris water maze at P63-P68, approximately. ↑- Navigational learning ($p < 0.01$), spatial memory ($p < 0.0001$), and spatial orientation skills (($p < 0.01$)) were significantly better preserved in Re-O2mice compared to Re-Air counterpart.
H-I	Ren et al., 2017	Sprague Dawley rats Both sexes CP I: P2	i) Sham; ii) HI; iii) HI-EPO (immediately); iv) HI + EPO (48H after brain injury) *n* = 12–19 animals per group	Immediately or 48 h after brain injury, the animals received a single dose of EPO (5000 U/kg) or saline (0.1 ml/kg) according to the experimental group.	↓ significant reduction of MBP expression in the HI-injured brain at P9 ($p = 0.006$) and P16 ($p = 0.01$) compared with the sham group. No significant difference was found in the two EPO groups compared with the sham group at both P9 and P16 ($p > 0.05$).	Morris water maze at P33-P37, approximately ↓ platform search time in HI + EPO treatment immediately or 48 h after brain injury (p = 0.00).
H-I	Rodríguez-Fanjul et al., 2017	Wistar rats Both sexes CP I: P10	i) Sham intervention (Control); ii) HI; iii) HI + Hypothermia; iv) HI + Allopurinol; v) HI + Dual therapy (hypothermia and allopurinol; HIHA) *n* = 7–8 animals per group.	All treatment groups received a single intraperitoneal injection of allopurinol at 135 mg/kg (volume: 0.01 ml/g) or saline, 15 min after hypoxia, depending on the randomization, before beginning hypothermia or normothermia protocol.	↓ The hippocampus in the HI group when compared to the others ($p < 0.05$) ↓- Cleaved caspase 3 expression in the hippocampus in the HI and HI + Allopurinol group. When sex was considered, there were no differences in the hippocampal volume between HIA, HIH, and HIHA among the females ($p = 0.398$; $p = 1$; p = 1)	Morris Water maze at P25-P35, approximately. ↑- All groups acquired the task and improved their performance over time ($p < 0.0001$), but with significant differences in the learning process, with the HI group being the worst. ↑- In females, global learning was also different between groups ($p = 0.001$). Control, HIH ($p = 0.999$), HIHA ($p = 0.991$), and HIA ($p = 0.719$) groups showed normal learning performance.
H-I	Roumes et al., 2020	Wistar rats Both sexes CP I: P7	i) Sham; ii) HI; iii) HI-L; iv) HI-3 L *n* = 10–17 animals per group.	Intraperitoneal injection(s) of lactate (40 µmol) after hypoxia (HI-L group) or three consecutive (150 min, 24 h and 48 h post-HI event, HI-3 L group)	Magnetic resonance imaging (P7) ↑ ipsilateral ADC in the hippocampus (HI-L versus HI on P7, $p < 0.01$; HI-3 L versus HI on P7, P8 and P9, $p < 0.05$; HI-3 L versus HI-L on P8 and P9, $p < 0.05$	Novel object recognition (P45): ↑ Discrimination index in the HI-3 L group compared to the HI group ($p < 0.01$)
H-I	Sampath et al., 2020	Sprague-Dawley rats Male CP I: P7	i) Sham + Vehicle; ii) Sham + Flupirtine; iii) HI + Vehicle; iv) HI + Flupirtine. *n* = 5–15 animals per group.	Pups were administered a single dose of 25 mg/kg flupirtine maleate or an equivalent volume of vehicle every 24 h for 4 days after HI induction (total 4 doses).	↓ tissue hippocampal in the HI group ($p < 0,05$) compared to the sham vehicle group. ↑ tissue hippocampal and cortical in the HI+ flupirtine group ($p < 0,05$) compared to the HI group.	Morris water maze at P118-P138 ↑- The performance of sham rats in the HI + flupirtine group on all trial days was very similar to sham rats treated with vehicle ($p < 0.05$)
H-I	Sun et al., 2014	Sprague-Dawley rats Both sexes CP I: P7	i) Sham-operated (SO); ii) HI; iii) HI + Polydatin *n* = 40–58 animals per group	Polydatin dry powder was dissolved in physiological saline and injected intraperitoneally into the rats from the Polydatin group (10 mg/kg body weight) once a day for 10 consecutive days.	↑ BDNF expression in the hippocampus of the rats in the HI + Polydatin group ($p < 0,05$) compared to the HI group in P10 and P21. At 24 h after the HI induction, there was no significant difference in BDNF expression in the left hippocampal CA1 ($P > 0.05$)	Y-maze learning test at P17 ↓ reaction time in HI + Polydatin and Sham group compared to the HI group ($P < 0.01$) ↑ correct avoidance rate in the rats in the Polydatin group, compared to the HI group ($P < 0.01$).

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Table 3 (*continued*)

CP MODEL	AUTHOR (YEAR)	STRAIN	EXPERIMENTAL GROUPS (n)	INTERVENTION	HIPPOCAMPAL OUTCOMES	MEMORY OUTCOMES
H-I	Wagner et al., 2002	Sprague-Dawley rats Both sexes CP I: P7	i) HI + hypothermia; ii) HI + normothermia; iii) Control + hypothermia; iv) Control + normothermia. *n* = 14–17 animals per group.	Two hours after the HI insult, one group of animals was maintained at 37 °C (normothermic temperature) measured rectally for another 26 h, whereas the second group of animals was cooled to 30 °C (hypothermic temperature) measured rectally and kept at this temperature for 26 h.	↓ size of cerebral infarction by 23% at 6 weeks after the insult in the HI+ Hypothermia compared to the HI+ normothermia group($p < 0.05$).	Morris water maze at P42-P47, approximately. ↓ escape latency in HI+ Hypothermia compared to the HI+ normothermia group ($p < 0.05$).
H-I	Wang et al., 2002	Sprague–Dawley rats Both sexes CP I: P7	i) HI; ii) HI + Sline-treated; iii) HI + HupA 0.05; vi) HI + HupA 0.1 mg/kg. *n* = 10–12 animals per group	Daily i.p. HupA (0.05 mg/kg or 0,1 mg/kg) or saline started on P7 immediately after hypoxia-ischemia and terminated before the day of sacrifice (P42).	↓ hippocampal atrophy in HI+ HupA 0.1 mg/kg. group compared to the HI group ($p < 0,01$).	Morris water maze at P36-P41 ↓ escape latency in HI+ HupA 0.1 mg/kg. group compared to the HI group ($p < 0,01$).
H-I	Wang et al., 2021	Rats Both sexes CP I: P7	i) Sham; ii) HI; ii) HI + Sevoflurane *n* = 18 animals per group	Neonatal rats immediately inhaled 2% sevoflurane and received 30% O2 and 70% N2 humidified mixed gas for 30 min after induction of HI.	↓ The neuronal density in the HI group compared to the Sham group ($p < 0,001$) and ↓inflammation markers in the HI + Sevoflurane group compared to the HI group ($p < 0.001$) ↑ The protein levels of G9a and H3K9me2 in the HI group compared to the Sham group ($p < 0,05$) and ↓ in HI + Sevoflurane group ($p < 0,001$).	Morris Water Maze at P36-P41 ↓ escape latency in HI + Sevoflurane group compared to the HI group ($p < 0.001$).
H-I	Wei et al., 2015	Sprague-Dawley rats Both sexes CP I: P7	i) Sham; ii) HI; iii) HI + HBO n = 40 animals per group	Rats were put into the HBO chamber within 15–30 min after brain injury and spent 15 min in the chamber with pure oxygen. HBO therapy performed once daily for 7 days.	↓ The lesions in CA1, CA3 and DG ($p < 0.05$) in the HI+ HBO group compared to the HI group. ↑ Nestin and BrdU positive cells in the HBO group compared to HI and Sham group ($p < 0,01$)	Morris water maze at P37-P43 ↑ platform crossing in the HI+ HBO group compared to the HI group. ($p < 0,05$). No differences were found between the I+ HBO group and the Sham group ($p > 0,05$).
H-I	Wei et al., 2017	Sprague-Dawley rats Both sexes CP I: P7	i) Sham; ii) HI; iii) HI+ HBO; HI + NGF; iv) HI + HBO + NGF *n* = 8 animals per group	Animals were exposed to a hyperbaric oxygen chamber after brain injury and/or received intraperitoneal injections of 0.5 μg NGF for each rat after 15–30 min of brain damage for 3 days.	↑- Brain and hippocampus tissue in all experimental groups compared to the HI group	Morris water maze at P30-P35 ↑ escape latency in all groups compared to sham group ($p < 0,05$)
Oxygen deprivation	Xiao et al., 2016	Sprague-Dawley rats Male CP I: P0	i) S; ii) Hy; iii) Hy + PPADS; iv) Hy + TNP-ATP *n* = 17 per group	A single dose of PPADS dissolved in saline (5, 10, 20, or 40 mg/kg) or TNP-ATP dissolved in saline (1, 2, 4, or 8 mg/kg) was injected intraperitoneally (i.p.) 2 h after the hypoxia exposure.	↓ IL-1b expression (Hy + TNP-ATP versus Hy 1 day after HYy, $p < 0.05$) ↓ glutamate level (Hy + TNP-ATP versus Hy 4 h and 1 day after HYy, $p < 0.05$) ↓ EAAT2 (Hy + PPADS versus Hy 1 day after HYy, $p < 0.05$)	Y-Maze at P30: ↓ number of days needed to reach the learning criterion, with a peak effect at 10 mg/kg for PPADS and 2 mg/kg for TNP-ATP ($p < 0.01$) Morris water maze at P45: ↑ platform crossings ($p < 0.05$) and ↑ time spent in the target quadrant Hy + TNP-ATP 2 mg/kg versus Hy, $p < 0.05$)
Oxygen deprivation	Xu et al., 2015	Sprague-Dawley rats Male CP I: P0	i) C; ii) P; iii) PA + 0.33 MCP; iv) PA + 1.0 MCP; v) PA + 3.0 MCP. *n* = 15 animals per group	Marine collagen peptides (MCPs) intragastrically administered with 0.33 g/kg, 1.0 g/kg and 3.0 g/kg body weight MCPsfrom postnatal day 0 (P0) till the age of 90-days.	↑ neuron count in the hippocampal CA1 region at 3 months of age (PA + 1.0 MCP and PA + 3.0 MCP versus PA, $p < 0.01$) ↓ hippocampal AChE activity (PA + 1.0 MCP and PA + 3.0 MCP versus PA, $p < 0.01$)	Morris Water Maze P90, approximately ↓ time spent in finding the platform and ↑ platform crossing times ($p < 0.05$)

(*continued on next page*)

Table 3 (*continued*)

CP MODEL	AUTHOR (YEAR)	STRAIN	EXPERIMENTAL GROUPS (n)	INTERVENTION	HIPPOCAMPAL OUTCOMES	MEMORY OUTCOMES
					↑ p-CREB/CREB ratio in hippocampus (PA + 1.0 MCP and PA + 3.0 MCP versus PA, $p < 0.01$; PA + 0.33 MCP versus PA, $p < 0.05$) ↑ BDNF expression. (PA + 1.0 MCP and PA + 3.0 MCP versus PA, $p < 0.05$)	
H-I	Zhang et al., 2014	Sprague Dawley rats Both sexes CP I: ´P7	i) HI; ii) Sham; iii) HI + Carnosine; iv) Normal *n* = 10 animals per group	The carnosine treatment group received an intraperitoneal injection of 250 mg/kg of carnosine at 0 h, 24 h and 48 h after the end of the hypoxic episode.	↓ The level of oxidative stress markers ($p <$ 0,05) in the HI + Carnosine compared to the HI group. ↓ Several TUNEL-positive cells in the CA1 region of the hippocampus and cortex ($p <$ 0.05) in the HI + Carnosine compared to the HI group.	Morris water maze at P28-P33 ↓ swimming time to find the platform ($p <$ 0,05) in the HI + Carnosine compared to the HI group.
H-I	Zhao et al., 2014	Sprague-Dawley rats Both rats CP I: P7	i) Control; ii) Sham + NS + 1.5% isoflurane posttreatment; iii) HI + NS; iv) HI + NS + ISO v) HI + Atr; vi) HI + Atr + ISO vii) HI + CsA; viii) HI + CsA + ISO *n* = 45 animals per group	NS, Atr or CsA were injected into the lateral cerebral ventricle immediately after HI.	↓ Weight and neuronal density in the CA3 hippocampus ($P < 0.05$) in the HI group compared to the sham group. ↓ brain loss was reduced by isoflurane postconditioning, CsA injection in HI + NS+ ISO and HI + CsA + ISO compared to the HI group ($p < 0,05$)	Morris water maze at P36–P41, approximately ↓ escape latency in all groups received ISO treatment ($p < 0,05$) compared to the HI group.

Abbreviations: Acetylcholinesterase (AChE); Acetyl-L-carnitine (ALC); Alpha-phenyl-n-tert-butyl-nitrone (PBN); Apparent Diffusion Coefficient (ADC); Apoptotic Protease activating factor-1 (Apaf-1); Asiaticoside (AS); ATP analogue 20,30–0-(2,4,6-trinitrophenyl)-adenosine 50-triphosphate (TNP-ATP); Anoxia-ischemia (AI); Brain-derived neurotrophic factor (BDNF); cAMP Response Element Binding Protein (CREB); Cerebral Palsy (CP); Cerebral Palsy Induction (CP I); Choice Reaction Time (CRT); Cytosolic cytochrome *c* (Cyt-c); Dentate gyrus (DG); Dexmedetomidine (DEX); Dimethyl Sulfoxide (DMSO); Embryonic day (E); Enriched Environment (EE); Erythropoietin (EPO); Excitatory amino acid transporter 2 (EAAT2); Gasdermin-D (GSDMD); Glycogen synthase kinase-3β (GSK-3β); Granulocyte colony-stimulating factor (G-CSF); Hydro-acupuncture (HA); Hyperbaric oxygen (HBO); Hypoxia/Reoxygenation (Hypo/Reox); Human adipose mesenchymal stem cells (MSC-S); Huperzine A (HupA); Inducible Nitric Oxide Synthase (iNOS); Interleukin-1 beta (IL-1β); Interleukin-6 (IL-6); Interleukin-18 (IL-18); isoflurane (ISO); Lipopolysaccharide (LPS); Maternal lipopolysaccharide (LPS); melatonin (Mel); Minute (min); Mptp Inhibitor Cyclosporin A (CsA); Myelin basic protein (MBP); *N*-acetylcystein (NAC); NADPH oxidase 2 (NOX2); Neural Stem Cells (NSC); Normal saline (NS; Nuclear Erythroid 2-Related Factor 2 (NRF2); Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB); number of animals per group (n); Oleoylethanolamide (OEA); Oligodendrocyte Progenitor Cell (OPC); Opener Atractyloside (Atr); Oxidized Glutathione (GSSG); Palmitoylethanolamide (PEA); Pentoxifylline (PT); Phosphorylated Akt (p-Akt); Phosphorylated CREB (p-CREB); phosphorylated Glycogen synthase kinase-3β (p-GSK-3β); pyridoxalphosphate-6-azophenyl-20,40-disulphonic acid (PPADS); phosphatidylinositol 3-kinas (PI3K); Phosphorylated PI3K (p-PI3K); Postnatal day (P); Postsynaptic density protein 95 (PSD95); Protein Kinase B (Akt); Resveratrol (RES); Saline (S); Sodium pyruvate (SP); Stromal cell-derived; Traditional Chinese medicine (TCM); Transplantation of oligodendrocyte progenitor cells (OPC); Treadmill exercise (TM); Tumor Necrosis Factor-α (TNF-α); V: Vehicle (V); Vitamin B (VitB); Vitamin D (VitD), 4-hydroxynonenal (4-HNE); 8-amino-acid NAP (NAP).
Symbols: ↑- Increased parameter; ↓- Reduced parameter.

1996; Miguel et al., 2020; Mishima et al., 2004; Morales et al., 2009; Mori et al., 2015; Odorcyk et al., 2016; Potter et al., 2018; Rodríguez-Fanjul et al., 2017; Roumes et al., 2020), 3 used C57BL/6 mice (Li et al., 2019a; Morán et al., 2017; Presti et al., 2006), 2 studies used ICR mice (Chen et al., 2014; Kim et al., 2006), 2 studies did not specify the strain of mice (Chen et al., 2015; Wang et al., 2021) and 1 study did not specify the mice lineage (Peng et al., 2022).

13 studies used only males (Chen et al., 2014; Greggio et al., 2011; Halis et al., 2019; Holubiec et al., 2018; Huang et al., 2021; Kim et al., 2006; Li et al., 2019b; Miguel et al., 2020; Morán et al., 2017; Niu et al., 2021; Potter et al., 2018; Sampath et al., 2020; Xu et al., 2015) while 39 used rodents of both sexes (Berger et al., 2019; Chen et al., 2015; Chen et al., 2021; Cho et al., 2018; Dell'Anna et al., 1997; Fan et al., 2008; Farfán et al., 2020; Gao et al., 2020; Gonzalez et al., 2009; Gou et al., 2020; Griva et al., 2017; Jung and Kim, 2017; Karalis et al., 2011; Kim et al., 2017; Kumral et al., 2004; Li et al., 2019a; Liu et al., 2013; Lowe et al., 2017; Luvone et al., 1996; Morales et al., 2009; Mishima et al., 2004; Mori et al., 2015; Odorcyk et al., 2016; Pan et al., 2012; Pak et al., 2018; Peng et al., 2022; Presti et al., 2006; Ren et al., 2017; Rodríguez-Fanjul et al., 2017; Roumes et al., 2020; Sun et al., 2014; Wagner et al., 2002; Wang et al., 2002; Wang et al., 2021; Wei et al., 2015; Wei et al., 2017; Xiao et al., 2016; Zhag et al., 2014; Zhao et al., 2014). All characteristics of the included studies are summarized in Table 3.

3.2.1. CP experimental models and phenotypic characteristics

The experimental models of CP were based on brain lesions in the perinatal period up to the 10th day of postnatal life that might reproduce the CP phenotype in animals. The vast majority of studies used the hypoxia-ischemia model through occlusion of the carotid artery between P3-P7, with 41 studies (Berger et al., 2019; Chen et al., 2014; Chen et al., 2015; Gao et al., 2020; Gonzalez et al., 2009; Gou et al., 2020; Greggio et al., 2011; Griva et al., 2017; Halis et al., 2019; Holubiec et al., 2018; Huang et al., 2021; Karalis et al., 2011; Kim et al., 2006; Kim et al., 2017; Kumral et al., 2004; Li et al., 2019b; Li et al., 2019a; Liu et al., 2013; Lowe et al., 2017; Morán et al., 2017; Mori et al., 2015; Miguel et al., 2020; Mishima et al., 2004; Niu et al., 2021; Odorcyk et al., 2016; Pan et al., 2012; Pak et al., 2018; Peng et al., 2022; Potter et al., 2018; Ren et al., 2017; Rodríguez-Fanjul et al., 2017; Roumes et al., 2020; Sampath et al., 2020; Sun et al., 2014; Wagner et al., 2002; Wang et al., 2002; Wang et al., 2021; Wei et al., 2015; Wei et al., 2017; Zhag et al. al., 2014; Zhao et al., 2014). 3 studies used exposure to lipopolysaccharide at the end of pregnancy or within the first 48 h after birth (Cho et al., 2018; Fan et al., 2008; Jung and Kim, 2017), 6 used oxygen deprivation, inducing delivery by surgery and exposing the pups to nitrogen to induce asphyxia or asphyxia occurred within the first 48 h of full-term birth (Chen et al., 2021; Dell'Anna et al., 1997; Luvone et al., 1996; Presti et al., 2006; Xiao et al., 2016; Xu et al., 2015), and 2 used oxygen deprivation on the last day of pregnancy (Farfán et al., 2020; Morales et al., 2009).

Results reported on movement, coordination or posture reported by type of brain injury were summarized to verify the cerebral palsy phenotype in perinatal brain injury models. Of these 52 included studies, only 20 evaluated or reported these outcomes. 16 models used hypoxia-ischemia and observed a reduction in the fall latency time in the rotarod test or rope suspension test (Griva et al., 2017; Karalis et al., 2011; Li et al., 2019b; Liu et al., 2013; Lowe et al., 2017; Mori et al., 2015; Pak et al., 2018; Potter et al., 2018; Wagner et al., 2002), a decrease in the distance traveled in the open field test (Holubiec et al., 2018; Kim et al., 2017; Li et al., 2019a), an increase in time to cross the bar in the Balance Beam Test (Niu et al., 2021), a reduction in paw pressure in the catwalk test (Pak et al., 2018), a reduction in strength in the grip test (Sampath et al., 2020) and reduced sensorimotor activity in the foot-fault test (Pan et al., 2012; Wei et al., 2015) and one study found no significant differences (Zhao et al., 2014). While 2 studies used oxygen deprivation and observed a reduction in the distance traveled in the open field test (Iuvone et al., 1996; Xu et al., 2015). Of the studies that used exposure to LPS, only 1 reported a reduction in the fall latency time in the rotarod test (Jung and Kim, 2017). None study reported postural changes in their results.

3.2.2. Disturbances in memory formation present in CP models and forms of evaluation

The animals submitted to the experimental models of CP showed various types of memory and behavioural alterations arising from CP in adulthood, exhibited by a poorer performance when compared with the control and/or sham group in tests that evaluated different characteristics of memory. Sprague Dawley rats at 12 and 41 days after brain injury showed reduced exploitation of objects used in the novel object recognition test (NORT) (Berger et al., 2019), as well as a preference for the old object (Li et al., 2019a). These are parameters typical of animals with some memory formation disturbance. The Y-maze demonstrated reduced spontaneous alternation in CP animals (Kim et al., 2006), indicating the presence of deficits in spatial memory in CP. The step-down passive avoidance test, which consists of a tool used to assess memory through aversive electrical stimuli, demonstrated that the animals in the CP group had a poorer performance, showing a reduction in step-down latency and a greater number of errors (Chen et al., 2014; Li et al., 2019b; Liu et al., 2013), demonstrating impairments in memory formation and learning.

The Morris water maze (MWM) test was used to assess the memory performance of the animals, noting that animals with CP needed a longer time to locate the platform (Chen et al., 2014; Griva et al., 2017; Halis et al., 2019; Halis et al., 2019; Karalis et al., 2011; Li et al., 2019a; Lowe et al., 2017), and had a higher mean escape latency (Chen et al., 2015, Gao et al., 2020; Sampath et al., 2020; Gonzalez et al., 2009; Gou et al., 2020; Greggio et al., 2011; Griva et al., 2017; Halis et al., 2019; Karalis et al., 2011; Kim et al., 2006; Kumral et al., 2004; Liu et al., 2013; Lowe et al., 2017). In addition, they took more time in the task of finding the target quadrant (Greggio et al., 2011; Karalis et al., 2011) and presented fewer entries and less time spent in the target quadrant (Chen et al., 2015; Greggio et al., 2011; Griva et al., 2017; Halis et al., 2019; Holubiec et al., 2018; Kim et al., 2006; Kumral et al., 2004). In addition, these animals spent a shorter time at the platform crossing in the MWM test (Chen et al., 2015; Gao et al., 2020; Gou et al., 2020; Holubiec et al., 2018; Huang et al., 2021; Liu et al., 2013) and decreased exploration of the new object in the NORT (Morales et al., 2009), indicating severe disturbance of memory formation in adult animals that suffered brain damage in the perinatal period.

3.2.3. Hippocampus damages present in CP models and forms of evaluation

Magnetic resonance imaging studies of the brains of rats with CP found severe impairments in the morphology of the nervous system, observing a reduction in grey matter volume on the side ipsilateral to the brain lesion and the hippocampus of Sprague Dawley rats 41 days after HI (Berger et al., 2019). Corroborating this, a reduction in the absolute weight of the dissected hippocampus of animals with CP was observed in Wistar rats on the 81th post-natal day (Halis et al., 2019). Histological evaluations with HE point in the same direction, where a reduction in hippocampal tissue was noted (Berger et al., 2019; Gao et al., 2020; Huang et al., 2021), where the dorsal region was severely affected (Griva et al., 2017).

Experimental assays with PCR, Western blot and ELISA analyses found a significant expression of pro-inflammatory cytokines in the brain of CP animals, the most present being IL-1β, IL-6, IL-8 and TNF-α (Chen et al., 2014; Huang et al., 2021) through an increase in the expression of cytosolic Cyt-c, apoptotic protease activating factor-1 (Apaf-1) and caspase-3 in the hippocampus (Gao et al., 2020; Liu et al., 2013) and a reduction in the expression of MiR-29a-3p, MALAT1, miR-1, and BDNF (Huang et al., 2021; Li et al., 2019b). Biomarkers of oxidative stress were also significantly present after brain injury, noting an increased expression of nitric oxide (NO) and nitric oxide synthase (iNOS) activity, TBARS and a reduction in GSH in the hippocampus of

animals with CP (Chen et al., 2014; Greggio et al., 2011). The selected studies reported an increase in the expression of caspase-3 and NeuN+ cells in the dentate gyrus, indicating an increase in apoptotic neurons after brain injury (Chen et al., 2015; Liu et al., 2013). Analysis by the TUNEL method showed a high level of apoptosis in the hippocampal neurons of the animals with CP (Gao et al., 2020; Huang et al., 2021; Liu et al., 2013). In the same vein, histological images of the hippocampus by the crystal violet technique demonstrated a reduction in the number of neurons and an increase in dark and deformed nuclei in the CP (Gao et al., 2020; Gonzalez et al., 2009; Kim et al., 2006; Kim et al., 2017; Kumral et al., 2004).

Immunohistochemistry and immunofluorescence also revealed a lower number and less density of the NeuN+ and Brdu+ cells in the CP group (Gao et al., 2020; Li et al., 2019a), in addition to a reduction in BDNF and synaptophysin (SYN) levels (Griva et al., 2017; Kim et al., 2006), revealing impairments in neurogenesis and neural plasticity present in CP. In addition to an increase in the number of Glial fibrillary acidic protein (GFAP) positive cells in CA1, CA3 and DG (Holubiec et al., 2018), indicating the presence of astrogliosis in CP. Synaptic alterations were also found: a reduction in the number of synapses and an enlargement of the synaptic cleft in the CA1 of the hippocampus was observed through transmission Electron Microscopy (TEM) (Chen et al., 2021), showing that brain injury in the critical period alters the maturation of the hippocampus and consequently impacts the memory formation in adulthood.

3.3. Therapeutic perspectives on memory disturbances and hippocampal damage present in CP models

The experimental models of CP caused alterations in the morphology, functioning and connection of the hippocampus cells, affecting their microarchitecture and causing severe memory implications in the short and long term. To define this problem, this study investigated possible invasive or non-invasive interventions for memory formation disturbances and hippocampal damage present in brain lesions with implications for CP. For the treatment of memory formation alterations and hippocampal damage present in CP, 4 studies used resveratrol or resveratrol precursors as an intervention (Karalis et al., 2011; Li et al., 2019a; Peng et al., 2022; Sun et al., 2014). Treatment with erythropoietin, a drug with anti-inflammatory and antioXidant action, also occupied a prominent place in this review, investigated in 3 studies (Kumral et al., 2004; Gonzalez et al., 2009; Ren et al., 2017). Supplementation with group vitamins was also highlighted in the treatment of memory alterations in CP, vitamins B1 and B2 (Li et al., 2019b), B3 (Morales et al., 2009) and D (Lowe et al., 2017). Physical exercise was also evaluated as a therapeutic strategy in 4 studies (Chen et al., 2021; Farfán et al., 2020; Jung and Kim, 2017; Pak et al., 2018). 3 other studies investigated the repercussions of cell transplant treatment (Chen et al., 2015; Morán et al., 2017; Mori et al., 2015).

Herbal medicine or natural compounds treatment was evaluated for the treatment of CP memory formation disturbances in CP in 3 studies (Odorcyk et al., 2016; Roumes et al., 2020; Xu et al., 2015). Another 3 studies looked at the effects of sedative drugs to treat brain injury (Chen et al., 2021; Gao et al., 2020; Huang et al., 2021). 4 studies investigated the effects of hypothermia or hyperthermia (Mishima et al., 2004; Potter Pak et al., 2018; Rodríguez-Fanjul et al., 2017; Wagner et al., 2002). OXygen therapy was also evaluated in 4 studies (Liu et al., 2013; Presti et al., 2006; Wei et al., 2015; Wei et al., 2017). Another 5 studies looked at the effects of antioXidant drugs such as carnosine (Zhang et al., 2014), NAPS (Greggio et al., 2011), sodium pyruvate (Pan et al., 2012) and acetyl-L-carnitine (Dell'Anna et al., 1997). Four other studies looked at the effects of anti-inflammatory drugs such as flupirtine maleate (Sampath et al., 2020), isoflurane (Zhao et al., 2014), sevoflurane (Wang et al., 2021), palmitoylethanolamide (Holubiec et al., 2018) and alpha-Phenyl-n-tert-butyl-nitrone (Fan et al., 2008). Two other studies have investigated the effects of melatonin treatment (Berger et al., 2019; Gou et al., 2020). The effects of housing in an enriched environment after brain injury were also evaluated in 2 studies (Griva et al., 2017; Luvonne and DellAnna, 1996). Two other studies used herbal medicine with the treatment of asiaticoside (Chen et al., 2014) and huperzine A (Wang et al., 2002). One study used techniques based on Chinese medicine (Niu et al., 2021), 1 study investigated the use of pentoXifylline a peripheral vessel dilator (Halis et al., 2019), 1 article used oroXylin, a flavonoid, and another study evaluated the effects of the use of methylphenidate (Miguel et al., 2020). Other interventions were also evaluated such as TNP-ATP treatment (Xiao et al., 2016).

The question of memory outcome was analysed to elucidate the effect of each intervention on the CP model. In this review, we performed a meta-analysis that included HI-type brain injury or oXygen deprivation in the period from P0 to P10. Related to memory performance, the results of animals submitted to the MWM test from P28 to P85 were included. Thus, we grouped the studies according to the type of intervention by significant effect size in each subgroup with respect to latency escape in MWM analysis (SDM -2.84 95% CI [−3.10, −2.59], Z = 22.00; $p < 0.00001$; I2 = 92.9%) (Figs. 2 and 3). Among the interventions found, the following stand out: i) oXygen interventions (SDM -6.83 95% CI [−7.91, −5.75], Z = 12.38, $p = 0.03$; I2 = 71%); ii) early administration of resveratrol (SDM -2.42 95% CI [−3.19, −1.66], Z = 6.21, $p = 0.01$; I2 = 77%) and iii) erythropoietin interventions (SDM -3.16 95% CI [−4.27, −2.05],Z = 5.58, $p = 0.002$; I2 = 82%). For the meta-analysis of the length of stay in the target quadrant in the MWM, it was not possible to observe a significant effect size of the interventions compared to the control group (SDM 2.72 95% CI [1.84, 3.60], Z = 6.05; $p < 0.00001$; I2 = 82%) (Figs. 4 and 5). Thus, despite the heterogeneity among the studies, our meta-analysis of latency escape in MWM indicated that there are currently several promising interventions able to reduce impairments in memory formation in the CP model. In addition, a complementary meta-analysis was performed using the results of studies that showed the CP phenotype in an animal model associated with impairments in memory formation in the Morris water maze test (Figs. 6, 7, 8, and 9). These interventions will be detailed below. In addition, a graphic abstract has been added describing the main findings of this study for better understanding (Fig. 10).

3.3.1. Drugs with antioxidant action

A prominent place in the literature has been given to treatment with drugs with antioXidant action towards reducing the memory formation disturbance present in CP, given their ability to eliminate free radicals and exert a neuroprotective effect in the brain (Gonzalez and Ferriero, 2009). Treatment with 8-amino-acid (NAP) increased GSH levels and reduced TBARS levels, resulting in an improvement in memory performance in the MWM, reducing escape latency and time to find the target quadrant, in addition to promoting an increase in time spent in the target quadrant (Greggio et al., 2011). Treatment of the CP group with carnosine (250 mg/kg) was able to reduce the number of cells undergoing apoptosis and the expression of caspase-3. The levels of 8-iso-–PGF2a, an important biomarker of oXidative stress, decreased in the CA1 field of the hippocampus in the CP group (Zhang et al., 2014).

OXygen deprivation during the neonatal period, which can result in the development of CP, promotes a number of toXic events in the brain (McQuillen and Ferriero, 2004). Treatment with melatonin has been reported to be a tool in reducing damage to the nervous system and the memory formation problems present in CP. In this case, melatonin is characterized by being a neurohormone with neuroprotective effects (Berger et al., 2019), making it possible to detect a reduction in the size of the cerebral infarction in the hippocampus, an increase in the number of NeuN neurons, an increase in the expression of GpX4 and a reduction in the expression of 4-HNE (Berger et al., 2019; Gou et al., 2020). Treatment with sodium pyruvate, in another study, promoted the preservation of hippocampal tissue, reducing the size of cerebral infarction in HI (Pan et al., 2012). Treatment with acetyl-L-carnitine (ALC) and huperzine-a, on the other hand, improved the performance of

the animals in the MWM, but the histological analyses did not show significant improvements in the hippocampus resulting from this treatment (Dell'Anna et al., 1997; Wang et al., 2021). Flavonoids were reported to have therapeutic potential for CP, noting a regulation of microglial cells that are disorganized in the hippocampus of animals with CP, in addition to greater survival of neurons in CA1 after brain injury (Kim et al., 2006).

Resveratrol, a polyphenol, has been suggested as a promising tool in the treatment of some brain injuries due to its strong antioXidant capacity. Regarding its use in CP disorders, an increase in the number of neurons and neuronal dendrites was observed, in addition to an increase in the density of dendritic spines and recuperation of the hippocampal neurons from the insult (Peng et al., 2022). In addition, there was increased SIRT1 levels and reduced brain lesion on the ipsilateral side to the lesion in the hippocampus (Peng et al., 2022; Karalis et al., 2011). An increase in cell proliferation and differentiation is also observed in the DG of the hippocampus in treatment with resveratrol (Li et al., 2019). A precursor to resveratrol, polydatin, increased BDNF levels in the hippocampal CA1, increasing brain plasticity (Sun et al., 2014). Regardless of dosage, this protective effect on the hippocampus resulted in some of the following on the MWM test: a reduction in the time the CP animals exposed to treatment took to find the platform (Peng et al., 2022; Karalis et al., 2011); an increase in the number of platform crossings (Peng et al., 2022); an increase of time spent in the target quadrant (Li et al., 2019); reduction in escape latency (Peng et al., 2022; Li et al., 2019; Karalis et al., 2011). On the maze-y test, there was increased alternation in animals treated with polydatin (Sun et al., 2014). In our meta-analysis, we observed significant effect size from the use of resveratrol (SDM -2.42 95% CI [−3.19, −1.66], $Z = 6.21$, $p = 0.01$; $I2 = 77\%$) with reduced escape latency in the MWM test. These demonstrate promising effects of resveratrol use on CP memory formation disturbance.

The attenuation of damage to the nervous system, especially in the hippocampus, through the reduction of free radicals and toXic events, triggered behavioural changes that corresponded to improvements in the memory performance of the animals, through the reduction in escape latency (Griggio et al., 2011; Zhang et al., 2014; Dell'Anna et al., 1997; Gou et al., 2020; Wang et al., 2021; Kim et al., 2006), a decrease in the time to find the platform (Dell'Anna et al., 1997; Kim et al., 2006), a reduction in platform crossing (Gou et al., 2020) and a decrease in finding and an increase in permanence in the target quadrant (Greggio et al., 2011) in the MWM test. Regarding recognition memory, there was an increase in the exploration of the new object (Berger et al., 2019).

3.3.2. Drugs with anti-inflammatory action

Animals with CP showed an increase in inflammatory cytokines, indicating neuroinflammation, in addition to the presence of astrogliosis and increased PeroXisome Proliferator-Activated Receptor Alpha expression in the hippocampus (Holubiec et al., 2018). These changes are related to the memory formation disturbance found in CP (Jiang et al., 2018). From this perspective, interventions that aim to minimize neuroinflammation and its consequences become a target in the treatment of secondary disorders of CP, such as memory alteration. Treatment with palmitoylethanolamide 10 mg/kg reduced the number of Glial fibrillary acidic protein (GFAP) positive cells in hippocampal areas CA1, CA3 and DG, as well as a decrease in ionized calcium-binding adapter molecule cells in the CP group (Holubiec et al., 2018). However, treatment with flupirtine maleate or isoflurane or a-Phenyl-n-tert-butyl-nitrone, or erythropoietin exerted a neuroprotective factor by preventing the loss of hippocampal tissue and reducing the size of the infarct in the hippocampus of CP animals (Sampath et al., 2020; Zhao et al., 2014; Fan et al., 2008; Gonzalez et al., 2009).

Erythropoietin treatment promoted a greater protective effect on CA1 (Kumral et al., 2004) and reduced myelin basic protein (MBP) expression (Ren et al., 2017) in CP animals. Our meta-analysis showed that improvement in the memory performance of the animals showed that the use of erythropoietin was able to reduce the escape latency in the MWM test in CP animals (SDM 5.2% 95% CI [−4.27, −2.05], $Z = 5.58$, $p = 0.002$; $I2 = 82\%$). Furthermore, a-Phenyl-n-tert-butyl-nitrone prevented the enlargement of cerebral ventricles, improving the learning of rats (Fan et al., 2008), while pentoXifylline reduced the atrophy of the hippocampus present in CP, increasing the absolute weight (Halis et al., 2019). However, isoflurane attenuated the effects of inflammation and reduced the levels of G9a and H3K9me2 proteins that were elevated in CP (Wang et al., 2021). There was a reduction in glutamate levels and IL-1b expression in adenosine 50-triphosphate (TNP-ATP) treatment in CP animals (Xiao et al., 2016).

The use of asiaticoside (AS) isolated from *Centella Asiatica* in CP significantly reduced microglial overactivation and phosphorylation of p38 MAPK in the hippocampus, and reduced markers of neuroinflammation in the hippocampus (Chen et al., 2014). Improvements with reduced neuroinflammation were reported to have therapeutic repercussions on behaviour, increasing cross-platform crossing (Holubiec et al., 2018; Xiao et al., 2016; Chen et al., 2014); reducing escape latency and time to find the platform (Zhao et al., 2014; Wang et al., 2021; Gonzalez et al., 2009; Kumral et al., 2004; Ren et al., 2017; Chen et al., 2014; Chen et al., 2014), decreasing swimming time (Zhao et al., 2014) and increasing time in the target quadrant (Gonzalez et al., 2009; Kumral et al., 2004; Xiao et al., 2016; Chen et al., 2014; Kumral et al., 2004; Xiao Takada et al., 2016; Chen et al., 2014; Chen et al., 2014) in the MWM test. Spatial memory in the maze-y test in the CP group was improved (Xiao et al., 2016).

3.3.3. Neurotransmitter reuptake inhibitors

Methylphenidate is a drug used in psychiatric disorders by inhibiting the reuptake of dopamine and norepinephrine. The use of methylphenidate in CP was able to stimulate an increase in BNDF levels, altering brain plasticity and promoting an attenuation of damage in the hippocampus. However, no differences were found in the assessment of memory function (Miguel et al., 2020).

3.3.4. Herbal medicine or natural compounds treatment

Nutrition is an essential part of brain and memory development, especially in neurodevelopmental disorders. Vitamin B1 and B2 supplementation increased the expression levels of BDNF and MALAT1, in addition to enhancing the phosphorylation of PI3K and Akt enzymes (Li et al., 2019b). While treatment with nicotinamide, a form of vitamin B3, reduced apoptosis in the hippocampus (Morales et al., 2009). In contrast, vitamin D treatment modulated the expression of CYP24A1, causing different effects in males and females (Lowe et al., 2017). Supplementation with several vitamins increased the latency time in the Step-down avoidance task (Li et al., 2019b), reduced the search time for the platform and increased swimming speed in the MWM (Lowe et al., 2017); vitamin supplementation increased exploration of the new object at NORT (Morales et al., 2009).

Other nutritional interventions, such as treatment with Huperzia quadrifariata alkaloid extract (HqAE), modulated the cholinergic system by reducing AChE activity, which was increased in animals with CP, and reduced the number of T-cells and apoptosis (Odorcyk et al., 2016). Supplementation with Marine collagen peptides (MCPs) also reduced AChE activity in addition to increasing the number of neurons and BDNF expression in the CA1 region (Xu et al., 2015). MRI images show that lactate reduced the volume of brain injury through metabolic changes in CP animals (Roumes et al., 2020). These studies provide evidence that nutrition is a key factor in memory formation, reducing the search time for the platform in the MWM test (Odorcyk et al., 2016; Xu et al., 2015) and increasing object discrimination and exploration time in the new object in the NORT of CP animals (Roumes et al., 2020).

3.3.5. Drugs with sedative action

Anaesthetics applied before or after brain injury have implications for the nervous system. Dexmedetomidine (DEX) treatment after brain injury attenuated hippocampal damage by regulating the number and

density of synapses, also preventing synaptic cleft enlargement and increasing the activity of the postsynaptic protein PSD-95 and the presynaptic protein synaptophysin and reducing the expression of NOX2 in microglia, of ROS and 4-HNE in the hippocampus (Chen et al., 2021). In addition to promoting the regulation of neuroglobin levels that were elevated in CP, it increased the number of neurons and reduced the number of cells with apoptosis (Gao et al., 2020; Huang et al., 2021). DEX treatment reduced IL-6, IL-8, and TNF-a levels and increased IL10 levels in the hippocampus of CP animals (Huang et al., 2021). These changes in the hippocampus resulted in changes in behaviour, such as a reduction in escape latency and an increase in platform crossing in the MWM test (Chen et al., 2021; Gao et al., 2020; Huang et al., 2021). Corroborating this, the meta-analysis findings highlight the effect size of sedatives on memory formation impairments present in CP (SDM -2.63 95% CI [−3.39, 1.88], Z 6.86, p 0.0006; I2 87%), demonstrating promising effects in reducing escape latency in the MWM test.

3.3.6. Treatment with cell implants

Treatment with transplantation of stem cells, mesenchymal cells or differentiated oligodendrocyte progenitor cells has demonstrated strong positive therapeutic evidence in brain injury and behavioural disorders present in CP. This was observed by an increase in the number of neurons in the subventricular zone of the hippocampus, and a reduction in apoptosis through decreased expression of caspase3 and NeuN in the hippocampus of CP animals (Chen et al., 2015). Intranasal administration of human adipose mesenchymal stem cells (MSC-S), preconditioned with either deferoxamine or TNF-α+IFN-γ, was reported to reduce the levels of cleaved caspase-3 proteins, cytoplasmic NRF2, nuclear NRF2, NRF2 effector NQO1 protein nuclear p65, promoting an increase in the length of the primary and secondary microglial process in the CA1 region of the hippocampus (Farfan et al., 2020). Intranasal administration of the peptide derived from complement activation C3a reduced the activation of astrocytes in the hippocampal areas CA1 and DG of CP animals (Morán et al., 2017). Infarct size was also reduced in the treatment with cells, this reduction observed in intracranial administration of the stromal cell-derived factor-1α injections in the hippocampus of CP animals (Mori et al., 2015). This type of intervention was able to reduce latency escape (Chen et al., 2015; Mori et al., 2015), increase dwell time in the target quadrant (Chen et al., 2015) and increase platform crossing (Chen et al., 2015; Mori et al., 2015) in the MWM test of CP animals, as well as promote an increase in the exploration and recognition time of the new object in the NORT (Farfán et al., 2020; Morán et al., 2017).

3.3.7. Non-pharmacological interventions

Non-pharmacological interventions are also used to attenuate behavioural and memory disturbances resulting from brain injuries. An enriched environment is one of these therapeutic interventions. It is possible to observe by Cresyl Violet staining an increase in the number of neurons of CP rats housed in an enriched environment in the neonatal period of P5-P20 (Luvone and DellAnna, 1996), as well as a reduction in the size of the lesion in the hippocampus (Gonzalez et al., 2009). From these improvements in the hippocampus, behavioural changes were also observed in MWM, where animals showed better memory performance through a reduction in escape latency (Luvone and DellAnna, 1996; Griva et al., 2017), a decrease in time to find the target quadrant (Luvone and DellAnna, 1996) and to find the platform (Griva et al., 2017), and an increase in the number of entries in the target quadrant (Griva et al., 2017) in CP group.

Physical exercise was also able to promote improvements in motor and memory function in rats with CP. Treadmill running in CP rats was seen to facilitate neurogenesis and suppress apoptosis, increase cell proliferation in DG and synapsin levels (Cho et al., 2018; Jung and Kim, 2017), promoting therapeutic effects on memory through the PI3K-Akt-Wnt activation pathway, improving short-term memory by increasing the step-down latency time in the Step-down avoidance task (Cho et al., 2018; Jung and Kim, 2017). Improvement in memory performance was also observed when physical exercise was associated with electroacupuncture (Pak et al., 2018) or with a physical therapy based on Chinese medicine, Tuína, which was able to reduce the expression of cleaved caspase-1, IL-1ß, IL-18 and cause a reduction in escape latency and an increase in dwell time in the target quadrant in MWM (Niu et al., 2021).

Hypothermia is a classic intervention in CP causing a reduction in brain damage (Mishima et al., 2004; Wagner et al., 2002; Wei et al., 2017; Rodríguez-Fanjul et al., 2017). However, one study reviewed did not observe significant differences between animals exposed to room temperature and those exposed to hypothermia in MWM (Mishima et al., 2004). In another experimental design, however, hypothermia showed behavioural deficits in the parameters evaluated in the MWM test (Potter et al., 2018). On the other hand, hyperbaric oxygenation soon after exposure was able to also reduce brain injury, in addition to decreasing the degeneration of the hippocampus fields CA1, CA2, CA3 and CA4 (Liu et al., 2013; Presti et al., 2006). An increase in the number of Bdru-positive cells was noted, increasing cell proliferation (Wei et al., 2015). These improvements were observed through an increase in platform crossing and improved spatial learning (Wei et al., 2015; Mishima et al., 2004; Wagner et al., 2002). As seen in the meta-analysis, interventions with oxygen had the largest effect size in reducing the escape latency of animals with CP on the MWM test (SDM -6.83 95% CI [—7.91, —5.75], Z = 12.38, p = 0.03; I2 = 71%).

4. Study quality assessment

An evaluation of the methodological quality of the studies included in the review was performed using the SYRCLE risk of bias tool by three independent evaluators (Calado, Pereira and Souza). Item assessment of the baseline characteristics of the results of the experimental groups was similar for all 52 included studies in that they presented the participants' baseline characteristics. Regarding the random sequence generation item, 33 studies showed adequate performance (Berger et al., 2019; Chen et al., 2015; Chen et al., 2021; Dell'Anna et al., 1997; Farfán et al., 2020; Gao et al., 2020; Gonzalez et al., 2009; Gou et al., 2020; Greggio et al., 2011; Griva et al., 2017; Halis et al., 2019; Holubiec et al., 2018; Karalis et al., 2011; Kim et al., 2017; Li et al., 2019b; Li et al., 2013; Lowe et al., 2017; Luvone and DellAnna, 1996; Miguel et al., 2020; Moran et al., 2017; Niu et al., 2021; Odorcyk et al., 2016; Pan et al., 2012; Potter et al., 2018; Ren et al., 2017; Rodríguez-Fanjul et al., 2017; Sampath et al., 2020; Sun et al., 2014; Wang et al., 2002; Wei et al., 2015; Wei et al., 2017; Zhang et al., 2014; Zhao et al., 2014). Regarding allocation concealment, random housing and blinding of participants and personnel items, all studies showed a high risk of bias. With respect to the blinding of the participants, in the experimental studies of CP, this was not possible due to the phenotypic characteristics that enabled the identification of which animal was part of the group with CP or the control group.

19 studies presented adequate blinding of outcome assessment (Berger et al., 2019; Chen et al., 2015; Fan et al., 2008; Holubiec et al., 2018; Huang et al., 2021; Karalis et al., 2011; Kumral et al., 2004; Liu et al., 2013; Lowe et al., 2017; Miguel et al., 2020; Morán et al., 2017; Potter et al., 2018; Presti et al., 2006; Roumes et al., 2020; Sampath et al., 2020; Wang et al., 2002; Wang et al., 2021; Xiao et al., 2016; Zhao et al., 2014) and 15 studies presented adequate random outcome assessment (Chen et al., 2015; Gonzalez and Ferriero, 2009; Gou et al., 2020; Greggio et al., 2011; Holubiec et al., 2018; Kumral et al., 2004; Li et al., 2019b; Li et al., 2013; Lowe et al., 2017; Peng et al., 2022; Ren et al., 2017; Rodríguez-Fanjul et al., 2017; Wang et al., 2021; Xiao et al., 2016; Zhao et al., 2014). The reviewed studies were similar regarding the incomplete outcome data item, with only 3 studies showing a high risk of bias (Berger et al., 2019; Sun et al., 2014; Wagner et al., 2002). Selective reporting items were observed in only 4 studies that showed a high risk of bias in this evaluation item (Kim et al., 2017; Li et al., 2019b;

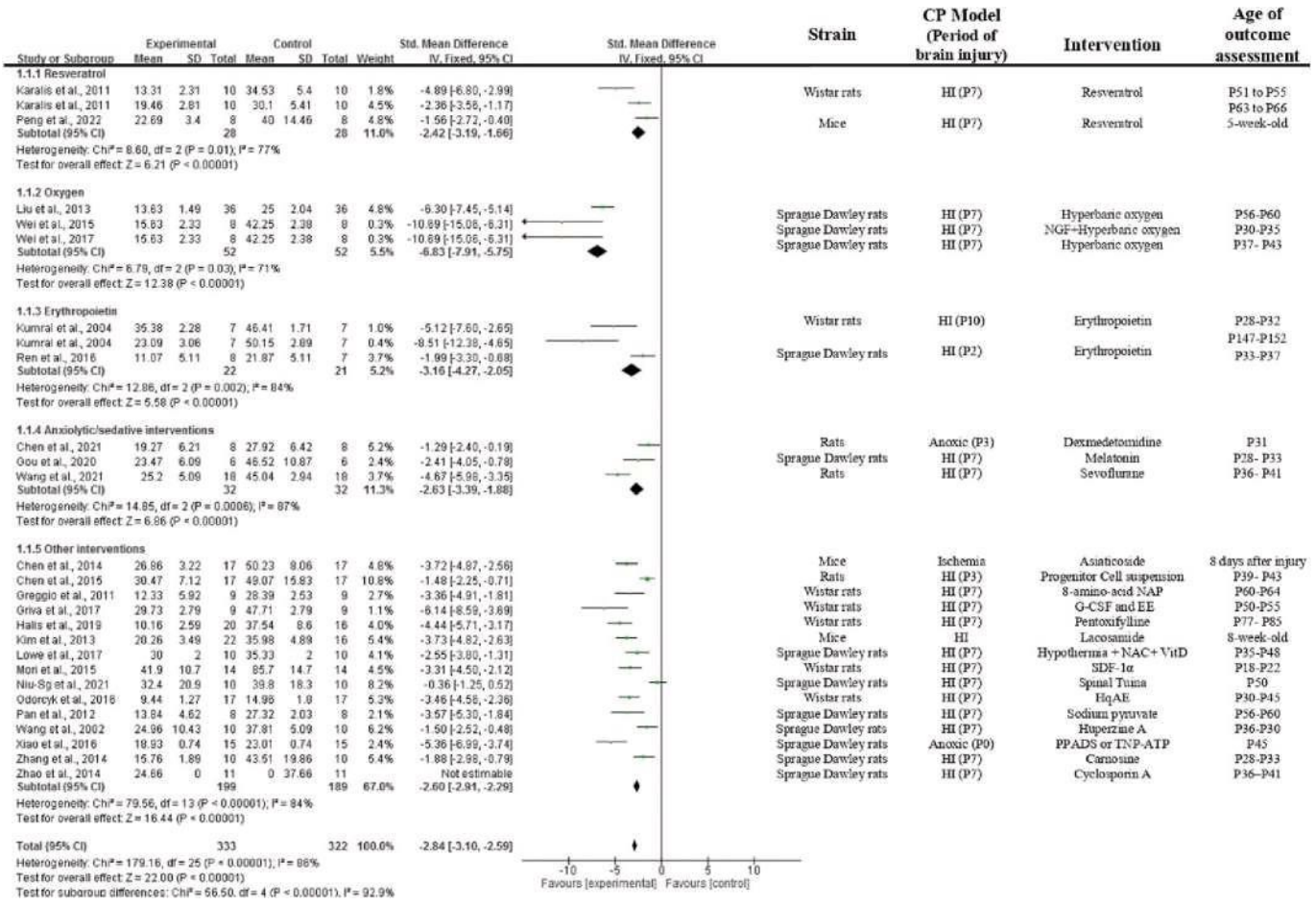

Fig. 2. Forest plot of included studies evaluating the escape latency in Morris water maze test (Subgroup: Intervention on models of CP). Horizontal lines represent the effect size ± the confidence interval (95%). The summary effect size is represented by the diamond. P: Postnatal day; HI: Hypoxic-ischemia; G-CSF: Granulocyte colony-stimulating factor; EE: Enriched environment; NAC: *N*-acetylcysteine; VitD: D Vitamin; HqAE: Huperzia quadrifariata alkaloid extract; PPADS: pyridoxal phosphate-6 azophenyl-20,40-disulphonic acid; TNP-ATP: ATP analogue 20,30–0-(2,4,6-trinitrophenyl)-adenosine 50-triphosphate.

Morales et al., 2009; Pan et al., 2012). These results are presented in Fig. 11, which is a general summary of the results presented in percentages and the individual results of the evaluation of each article. These data indicate that consistency and methodological rigor were present in the experimental designs and the presentation of results in most of the included studies.

5. Discussion

This is the first study to systematically review and summarize promising treatment findings for memory formation alterations present in perinatal brain lesions with consequences for cerebral palsy, bringing new treatment perspectives that can be studied in the future as clinical interventions. We analysed how memory is affected and the morphological, biochemical and functional changes present in the hippocampus of animals submitted to early brain injuries associated with the aetiology of CP. Through behavioural parameters, we observed memory formation alteration in these models. Our analysis includes the main outcomes, the effects of interventions on the improvement of these damages. Through a qualitative analysis, associated with a meta-analysis, we observed the potential therapeutic effects of the use of oXygen interventions, resveratrol, anxiolytic/sedative interventions, erythropoietin and physical exercise on memory formation deficits and hippocampal damage present in CP through the reduction of free radicals and pro-inflammatory cytokines that trigger a cascade of neurotoXic events in the hippocampus.

Overall, the studies presented here indicate that spatial and recognition memory are impaired in CP, through increased escape latency, reduced permanence in the target quadrant and longer time to find the platform in the MWM test, reduced spontaneous switching in the Y-Maze test and T-Maze test, and decreased discrimination of new objects in the NORT. These memory alterations are related to the damage in the animals' hippocampus. A reduction in brain volume and relative weight was observed, while immunohistochemical markers and histological stains pointed to a reduction in the number of new neurons with an increase in astrogliosis, of oXidative stress enzymes and pro-inflammatory cytokines in regions such as CA1 and DG of the hippocampus. This set of adverse events can increase apoptosis and consequently the degradation of the hippocampus, signalling possible ways of understanding the memory formation disturbance present in perinatal brain lesions with consequences for CP. Thus, it can be understood that brain injury early in life promotes damage to the maturation of the hippocampus and consequently impairs memory formation in the adult life of animals. On the other hand, treatments that reduced neuroinflammation and free radicals showed promising effects on memory, causing a protective effect on the hippocampus, preserving the number of neurons, increasing tissue volume and reducing astrogliosis, resulting in an improvement in the performance of animals on tests to assess memory.

It was possible to observe a heterogeneity of results independent of the model used in the hippocampus and the memory performance in animals with CP. A large number of studies reported a reduction in the

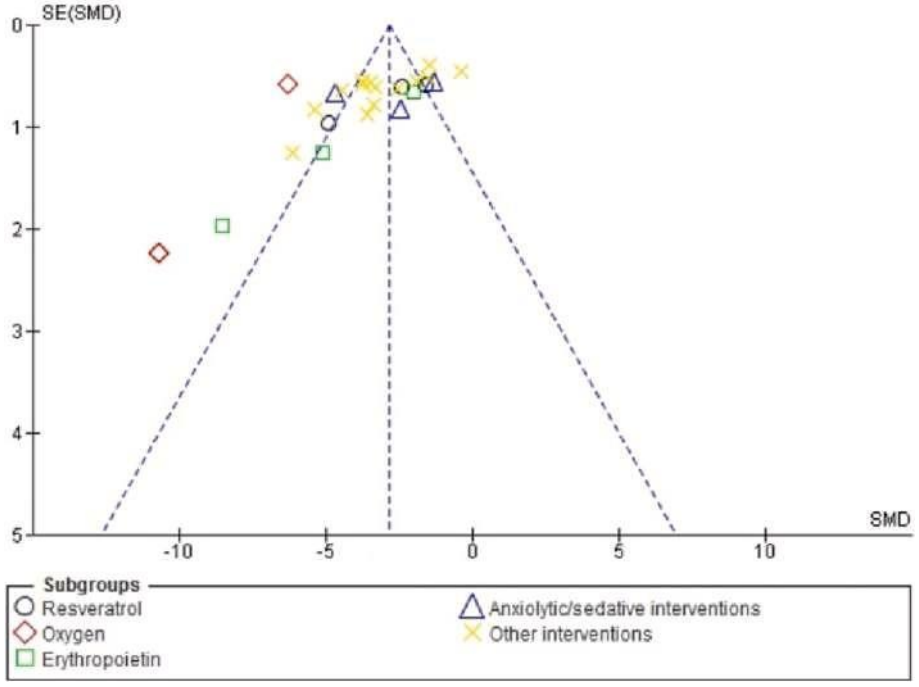

Fig. 3. Funnel plot of standardized mean differences (SMD) of the escape latency in the Morris water maze test. SE = standard error.

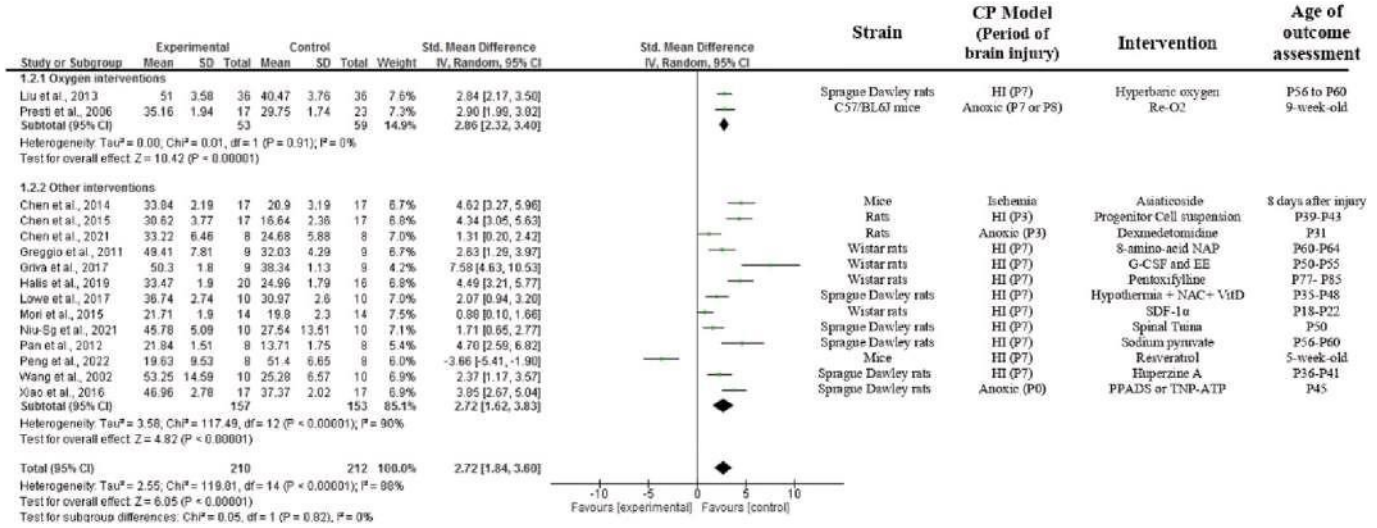

Fig. 4. Forest plot of included studies evaluating the time spent in the target quadrant in Morris water maze test (Subgroup: Intervention on models of CP). Horizontal lines represent the effect size ± the confidence interval (95%). The summary effect size is represented by the diamond. P: Postnatal day; HI: Hypoxic-ischemia; *Re*-O2: Reoxygenation; G-CSF: Granulocyte colony-stimulating factor; EE: Enriched environment; NAC: *N*-acetylcysteine; VitD: D Vitamin; PPADS: pyridoxal phosphate-6-azophenyl-20,40-disulphonic acid; TNP-ATP: ATP analogue 20,30–0- (2,4,6-trinitrophenol)-adenosine 50-triphosphate.

number of neurons and the volume of areas such as CA1 and DG and an increase in the number of glial cells in the hippocampus. In memory assessment tests on brain-injured subjects, a reduction was reported in target quadrant time and an increase in escape latency. Although CP is characterized by neuromotor and postural damaged, impairing motor skills (Krigger, 2006; Ward et al., 2006; Mockford and Caulton, 2010; Peterson et al., 2013; Coq et al., 2008; Pereira et al., 2021), many studies reported disturbances in memory and behaviour in experimental models of CP. In the present review, we observed that the hippocampus is a structure sensitive to oXygen deprivation in the prenatal, perinatal, or postnatal period (Matsuda et al., 2021) triggering a cascade of toXic events in the hippocampus, increasing levels of free radicals and inflammatory cytokines (Chen et al., 2014; Greggio et al., 2011; Huang et al., 2021) and triggering a cascade of behavioural and memory changes (Greggio et al., 2011; Zhang et al., 2014; Dell'Anna et al., 1997; Gou et al., 2020; Wang et al., 2021; Kim et al., 2006; Peng et al., 2022).

Memory and learning are two processes that are closely linked. Learning is the process by which organisms acquire information, while memory can be defined as the ability to store this information (Bekinschtein et al., 2007). The formation of new memories involves a series of synaptic changes, in addition to changes in pre-existing proteins or the synthesis of new proteins in hippocampal neurons (Kandel, 2001). The formation of new memories causes changes in the brain, forming new synaptic connections, or strengthening connections related to the acquired information, which can be observed at the time of memory formation, known as engrams (Josselyn et al., 2015). For the proper

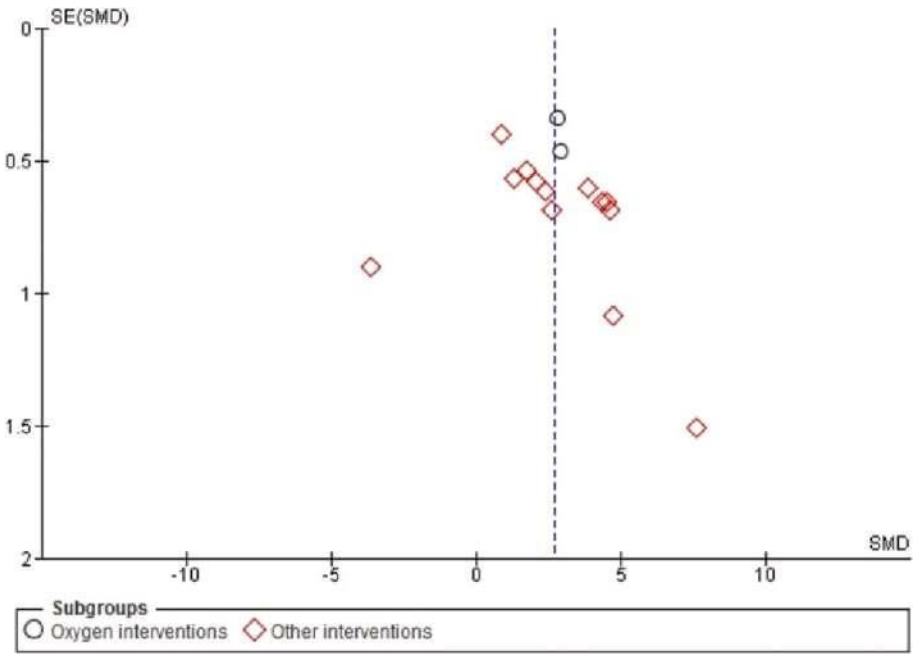

Fig. 5. Funnel plot of standardized mean differences (SMD) of the time spent in the target quadrant in Morris water maze test. SE = standard error.

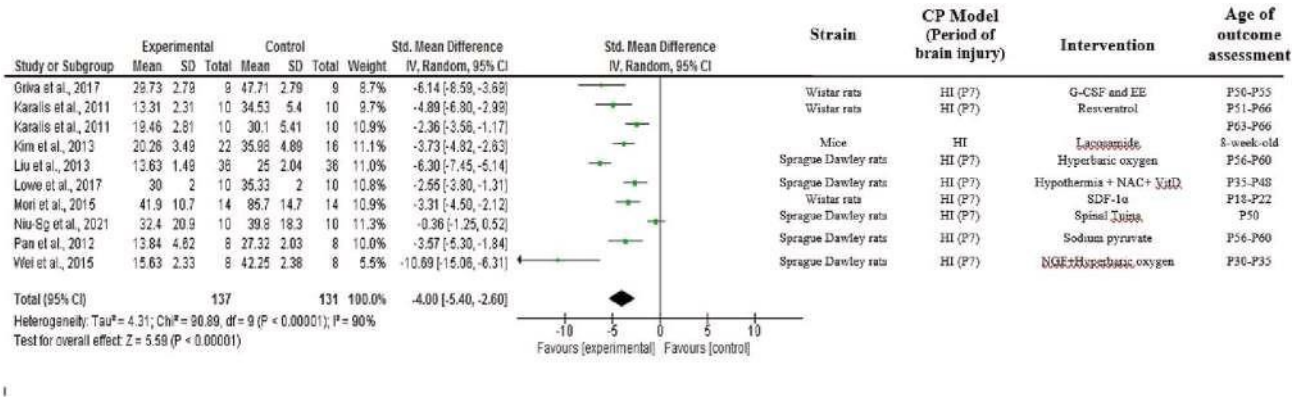

Fig. 6. Forest plot of included studies with CP experimental phenotype evaluating the escape latency in Morris water maze test. Horizontal lines represent the effect size ± the confidence interval (95%). The summary effect size is represented by the diamond. P: Postnatal day; HI: Hypoxic-ischemia; G-CSF: Granulocyte colony-stimulating factor; EE: Enriched environment; NAC: *N*-acetylcystein; VitD: D Vitamin.

functioning of memory and learning, some essential steps are encoding, storing and recalling this information. A number of neural structures act on the proper functioning of memory, such as the amygdala in conditioned fear learning, the hippocampus in declarative memories, the caudate nucleus, medial septum and other cortical structures such as the prefrontal, entorhinal, parietal cortex (Izquierdo et al., 2013; Izquierdo et al., 1992). In our review, we studied the effects of perinatal brain injury models on memory and the hippocampus because of this relationship.

For the proper functioning of memory, certain proteins and molecules are essential, such as the brain-derived neurotrophic factor (BDNF) which is essential in neuronal plasticity in the growth of the dendritic spines of the hippocampus and in the storage of information, which is essential for the formation of long-term memories (Bekinschtein et al., 2007; Bekinschtein et al., 2008). In addition, other molecular bases of plasticity act in the hippocampus for memory formation where CREB levels participate in the transcription of long-term memories (Bevilaqua et al., 1999). The role of BDNF in the hippocampus is essential in the transformation of short-term memories into long-term, also acting on neurotransmission systems and synaptic plasticity. Some findings also indicate that this protein actively participates in declarative memories, more specifically in memory recognition of objects (Gonzalez et al., 2019a; Gonzalez et al., 2019b). In our study, we identified declines in BDNF levels in the hippocampus of animals with perinatal brain lesions that may have implications for CP, explaining the memory formation disturbance in adult life that is associated with these lesions in the perinatal period.

Regardless of the experimental model used, by oxygen deprivation, maternal exposure to LPS, or occlusion of the carotid artery, an increase in the number of free radicals, pro-inflammatory cytokines, and glial cells was observed, while there was a reduction in the volume of the hippocampus associated with a memory formation deficit (Chen et al., 2014; Berger et al., 2019; Sampath et al., 2020; Zhao et al., 2014). In our study, we observed that this series of alterations in the hippocampus, caused by environmental insults in the critical period of development, is capable of causing repercussions on the behaviour of animals in adult life, with impairments in episodic memory being observed through poor performance in recognition objects tests and spatial location tests. Memory formation alteration can be understood as the proper functioning of the hippocampus, especially synapses, associated with

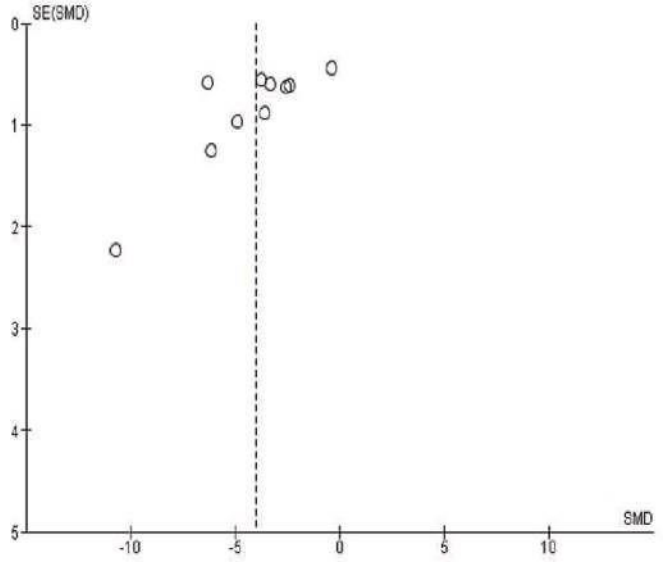

Fig. 7. Funnel plot of standardized mean differences (SMD) of the escape latency in Morris water maze test of included studies with CP experimental phenotype. *SE* = standard error.

memory (Zhang et al., 2015). Although memories are not reduced to the function of a single brain area, it is known that the hippocampus participates in the initiation of memories (Izquierdo et al., 2013; Izquierdo et al., 1992), which suggests why memories are impaired in hippocampal malformation.

Despite the scarcity of studies that evaluate memory in subjects diagnosed with CP, encouraging findings to indicate that children with CP are at a higher risk of developing changes in memory and executive functions that can lead to impaired learning (Jenks et al., 2009; Schenker et al., 2005). Studies observe impaired learning in children with CP due to disturbance in memory formation and executive functions, compromising mathematical skills and school performance (Feldberg et al., 2021; Pereira et al., 2018; Stadskleiv et al., 2017). Recent clinical research has found changes in brain wave oscillations, such as alpha-beta, in cortical regions of adults with CP, for example in prefrontal and temporal areas, during the codification of information, associated with deficits in working memory (Hoffman et al., 2021). This suggests possible memory formation problems present during adulthood in subjects with CP as a result of dysfunctions in brain regions. In the same sense, we observed in our studies that the environmental insult in the perinatal period disrupts the functioning of the brain, promoting disturbances in the memory in rodents, in a similar way as observed in humans, making possible the investigation of interventions that can act in the brain injury and its consequences.

In our review, experimental models of perinatal brain injury with rodents proved to be an essential tool, within the limits of preclinical studies, enabling an understanding of the relationship between the extent of damage in the brain areas present in perinatal lesions with consequences for CP to the development of memory acquisition problems that can last into adulthood. This provides a tool for the investigation of interventions that act on the attenuation of neurological and memory sequelae, as used in our meta-analysis. Therapies that used oXygen, however, such as exposure to a hyperbaric oXygen chamber, had an excellent outcome in the treatment of memory formation deficits present in the brain-damaged model. Corroborating this, in our meta-analysis this intervention presented the best effect size among the analysed interventions (Figs. 2 and 3).

In many studies, excellent outcomes were observed in the exposure to an oXygen chamber before the first hour after brain injury, reported to reduce the size of the brain lesion and the degeneration of neurons in the CA1, CA2, and DG of the hippocampus (Liu et al., 2013; Wei et al., 2015; Wei et al., 2017). These interventions in the perinatal period had repercussions on permanent improvements in the animals, by an observed reduction in the escape latency at P30–35 (Wei et al., 2017) and at P37–43, the end of the adolescence period of the rodents of the Sprague-Dawley rats. In addition, improvements in this treatment were also observed in the perinatal period during the beginning of the adult life of these animals, with a reduction in the escape latency in P56–60 (Liu et al., 2013) and P60-P68 (Presti et al., 2006). This suggests that oXygen therapies are promising in the treatment of memory formation disturbance present in the perinatal brain lesions and that the treatment causes permanent repercussions on the organism. However, these

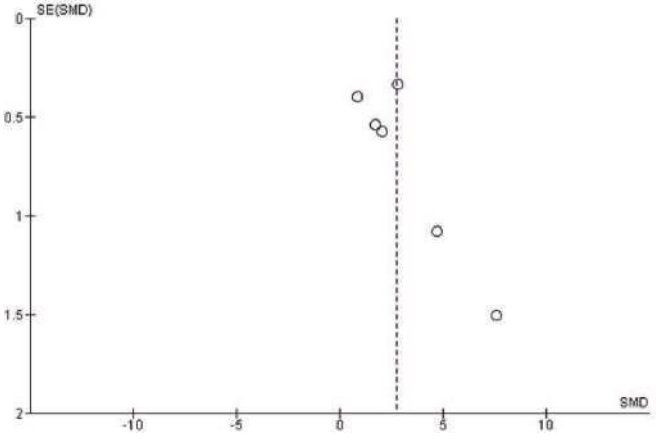

Fig. 9. Funnel plot of standardized mean differences (SMD) of the time spent in the target quadrant in Morris water maze test of included studies with CP experimental phenotype. *SE* = standard error.

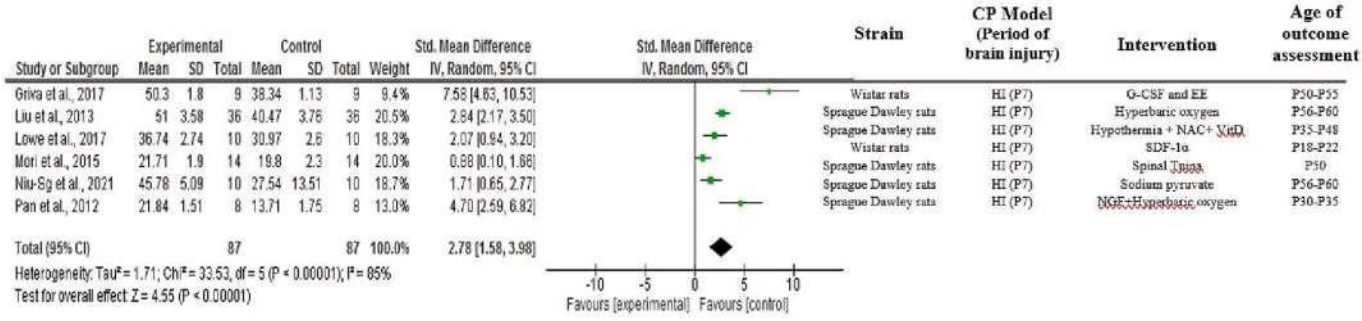

Fig. 8. Forest plot of included studies with CP experimental phenotype evaluating the time spent in the target quadrant in the Morris water maze test. Horizontal lines represent the effect size ± the confidence interval (95%). The summary effect size is represented by the diamond. P: Postnatal day; HI: Hypoxic-ischemia; G-CSF: Granulocyte colony-stimulating factor; EE: Enriched environment; NAC: *N*-acetylcysteine; VitD: D Vitamin.

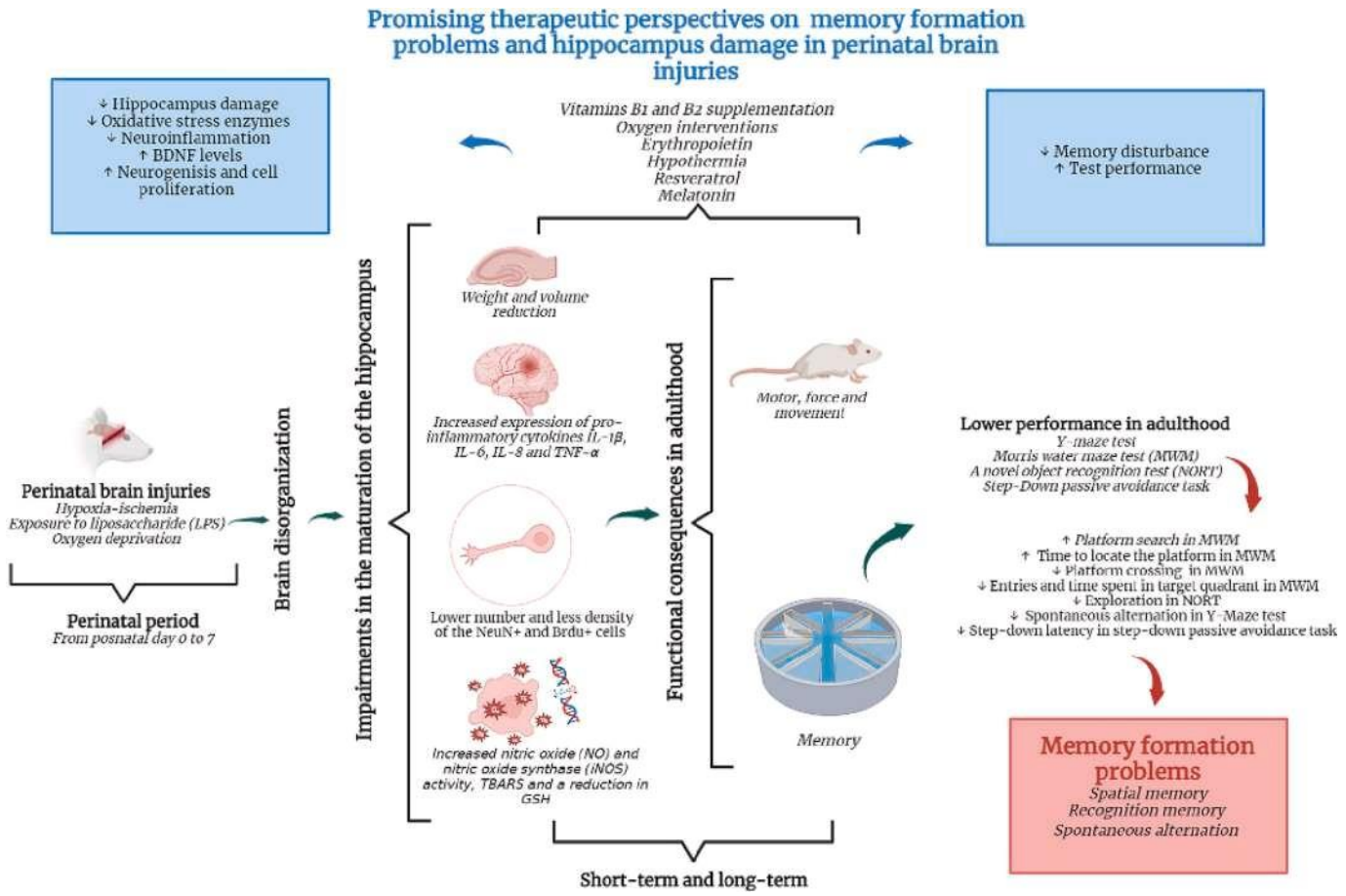

Fig. 10. Graphic abstract with the main findings of the study. Brain injuries in the perinatal period cause long-term and short-term memory, behavioural and motor changes in adulthood. Treatment with vitamin B1 and B2 supplementation, interventions with oxygen, Erythropoietin, Hypothermia, Resveratrol, and Melatonin show promising findings in hippocampal disorganization and memory alterations.

findings are reflections of studies in experimental models with rodents and recent findings point to side effects in the use of hyperbaric oxygen in children with CP (Novak et al., 2020; Novak and Badawi, 2012).

Therapeutic interventions that attenuate the effects of inflammation and oxidative stress in the hippocampus occupied a prominent place in the studies selected for review, together with sensory stimulation, physical exercise, and supplementation with some specific foods that increased brain plasticity and its associated mechanisms, such as expression of BDNF (Sun et al., 2014; Li et al., 2019; Xu et al., 2015). Some organic compounds, such as polyphenols, which are natural compounds present in some plants and foods, have therapeutic properties by increasing BDNF levels in some cases of brain injuries such as ischemia and hypoxia, based on the relationship between metabolic activity, food consumption, and neuronal activity. Some polyphenols, such as resveratrol, act by increasing levels of BDNF in the brain that is related to memory functioning (Ma et al., 1997; Gomez-Pinilla and Nguyen, 2012; Khanna et al., 2020). These results emphasize the importance of multidisciplinary interventions in all impaired skills in CP, starting in childhood. Among the interventions analysed, treatment with erythropoietin was also highlighted, being able to increase neurogenesis and cell differentiation in brain development and to increase the number of cells such as neurons, astrocytes and endothelial cells, exerting a neuroprotective factor in brain lesions (Bernaudin et al., 1999; Siren et al., 2001; Mu et al., 2005), and modulating immune and anti-inflammatory responses (Villa et al., 2003; Agnello et al., 2002; Arvin et al., 1996). Treatment with erythropoietin improved the memory performance of animals with CP by reducing damage to the hippocampus (Gonzalez et al., 2009; Kumral et al., 2004; Ren et al., 2017).

Neither type of intervention presented deleterious effects, opening new perspectives for the treatment of brain injuries and memory formation disturbance present in subjects with CP. Furthermore, in our meta-analysis, regardless of the CP model, studies were included that evaluated the memory performance of animals in the Morris water maze, (Figs. 2 and 4). It is possible to observe promising effects of oxygen interventions, resveratrol and erythropoietin on MWM reducing latency escape. But it was not possible to find significant differences in the interventions in the length of stay on the target platform. Escape latency is a sensitive parameter for elucidation of the memory formation deficits present in neurological diseases, which may explain the results found for this parameter in most studies, rather than time spent in the target quadrant.

Treatment with cell transplantation is presented as an innovative intervention in neurological lesions, where intranasal or intraventricular administration have demonstrated beneficial effects on the analysed parameters, reducing apoptosis, caspase-3 expression and cerebral infarction size (Chen et al., 2015; Farfán et al., 2020; Mori et al., 2015). Thus, cell transplant represents a prominent treatment in this review for its positive effects on the learning of animals. Increase in the exploration time of a new object in the NORT test and reduction of escape latency in the MWM test in CP animals (Chen et al., 2015; Farfán et al., 2020; Mori et al., 2015). This is a very invasive intervention, however, that can have side effects, making it necessary to carefully evaluate its repercussions and whether possible therapeutic effects exist through other less invasive routes of administration before it can be considered a safe intervention in the field of paediatrics.

In this study, it was possible to observe promising results of

environmental stimulation in the improvement of memory and brain damage in preclinical studies, as it occurs in an enriched environment, making it an important tool for use in memory formation disturbance. Preclinical studies (Luvone and DellAnna, 1996; Griva et al., 2017; Gonzalez et al., 2009) have demonstrated that enriched environment improved the memory performance of animals with CP in the MWM test, reduced the escape latency and the time to find the platform; clinical trials (Løhaugen et al., 2014; Lieto et al., 2020) have demonstrated the positive repercussions of environmental stimulation on various type memory. Physical exercise and supplementation with specific vitamins were reported to be strong allies in the treatment of CP reinforcing the importance of multidisciplinary interventions in mitigating the limitations that can arise along with CP in childhood (Cho et al., 2018; Jung and Kim, 2017; Li et al., 2019).

Thus, in preclinical studies, memory formation disturbance similar to those that occur in children with CP can be observed, by behavioural deficits in object discrimination, reduced spatial alternation, shorter time spent in the target quadrant, increased escape latency, and in the time to find the platform in the behavioural tests performed, indicating impairments in recognition memory and spatial memory, in long and short-term assessment protocols. Although the literature is still sparse regarding therapeutic perspectives in the clinic, pre-clinical studies have shown promising effects in the treatment of memory formation deficits present in CP, with improvements in the analysed parameters, such as

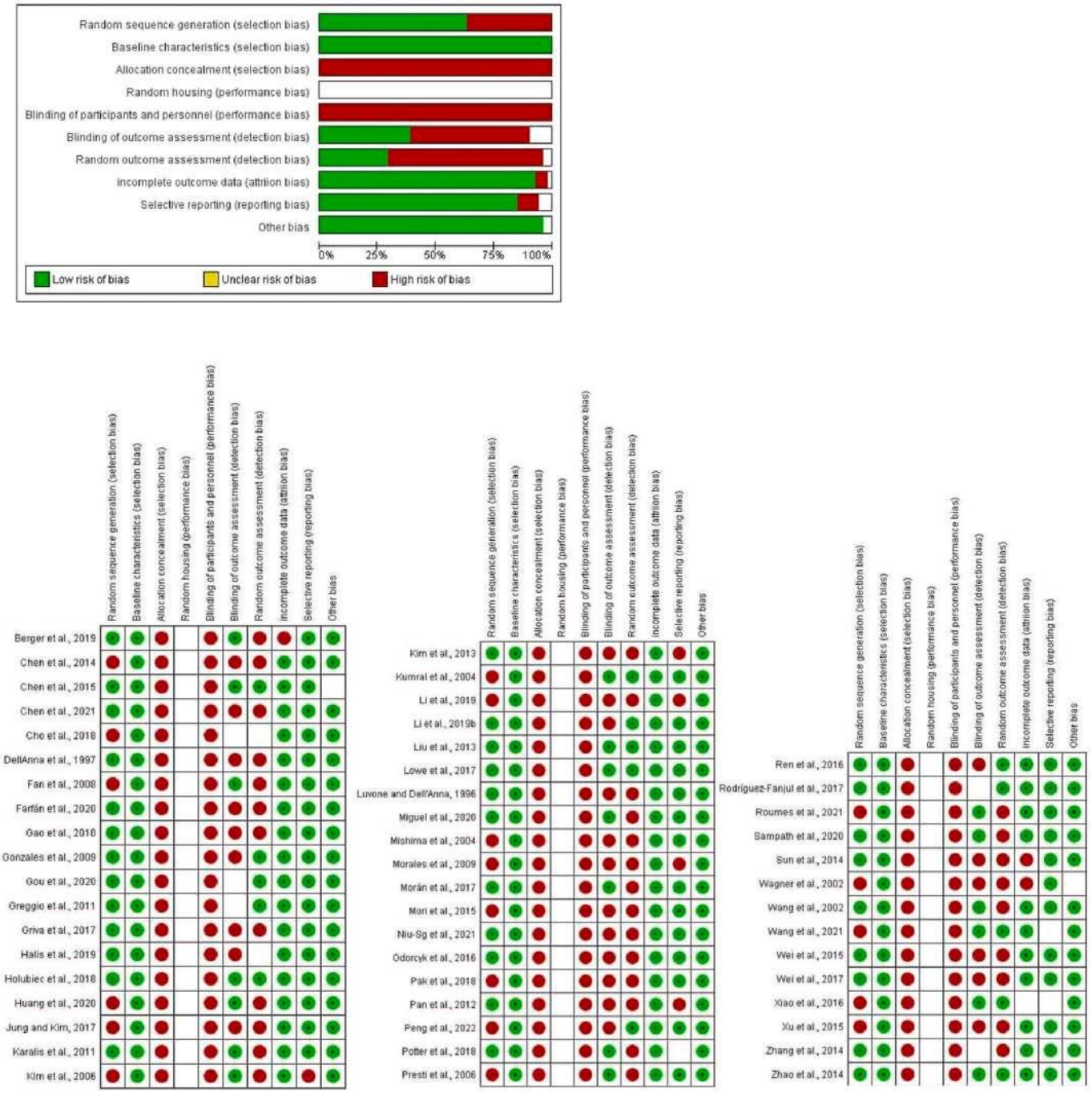

Fig. 11. Risk of bias summary and graph of included studies. Review authors' judgments about each risk of bias item presented as percentages across all included studies. (green) low risk of bias; (red) high risk of bias; (white) unclear risk of bias. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

reduced escape latency and time to find the platform in MWM. The experimental perinatal brain lesions models showed biochemical and morphological changes in the hippocampus, in regions such as the dentate gyrus and CA1, an increase in pro-inflammatory cytokines such as IL-1β, IL-6, IL-8, and a reduction in levels and expression of BDNF. Furthermore, there was a higher expression of nitric oxide (NO) and nitric oxide synthase (iNOS) activity, TBARS, and a reduction in GSH in the hippocampus, indicating the presence of oxidative stress in the hippocampus of animals with CP.

On the other hand, there were reports of therapeutic effects from treatments that attenuated the deleterious effects on the hippocampus caused by neuroinflammation and oxidative stress, which are the main processes that affect the maturation of the hippocampus in CP, bringing improvements in spatial, recognition, short-term and long-term memory. Among the interventions analysed, were the use of oxygen interventions, resveratrol, erythropoietin, stem cells, herbal medicine or natural compounds treatment, and the use of physical exercise, all attenuating damage to the hippocampus and memory formation of animals with CP. These improvements were observed during adulthood even after the end of treatment.

5.1. Strengths and limitations

This is the first systematic review with meta-analysis that investigates therapeutic advances in the treatment of memory formation problems in animals that suffered brain injury in the perinatal period. We systematically evaluated changes in memory formation resulting from environmental insults such as hypoxia-ischemia, maternal exposure to LPS and oxygen deprivation. We observed that the hippocampus is one of the brain structures affected by these insults in the critical period of development in the prenatal and neonatal phases, causing a disturbance in episodic memory formation during adulthood in various behavioural tests. We systematically investigated the literature and summarized the main interventions used in memory formation disturbances present in perinatal brain injuries and their effects on hippocampal damage. For this, we followed strict methodological criteria throughout this review. From the moment of elaborating a search protocol in the databases until the presentation of the results, reducing the risks of possible bias to the maximum. We provide a series of results regarding alterations in the hippocampus maturation present in animals that exposure to brain injuries in the perinatal period, since it is difficult to obtain results regarding morphological, biochemical and functional alterations in children with CP. In the experimental models, it was possible to observe an increase in oxidative stress, neuroinflammation, apoptosis and a reduction in neuroplastic mechanisms such as neurogenesis and a decrease in BDNF levels associated with episodic memory formation disturbance, making it possible to understand. Additionally, we present the outcomes of interventions used after brain injury and their effects on memory formation and hippocampus disturbances, performing a qualitative analysis of these results and a quantitative analysis through a meta-analysis. to verify the effect size of the interventions.

This study has some limitations, that are related to the original studies limitations. First, the studies included in this study only covered brain injuries with implications for CP of environmental origin, being developed from insults such as hypoxia-ischemia, early exposure to LPS or oxygen deprivation (Fragopoulou1 et al., 2019; Jacobsson and Hagberg, 2004). Due to the complexity of factors involving the CP, there are no reliable models that reproduce all the characteristics of the CP in animals. There are models that use insults during the perinatal period that reproduce some dimensions of the CP phenotype, affecting behaviour and the nervous system in different ways (Coq et al., 2008; Pereira et al., 2021, Visco et al., 2021). This limitation of our study is related to the limitations of the original studies, we were unable to detect genetic models of CP, limiting the study only to CP of environmental origin. That mimics brain damage acquired early in life. Regarding the methodological quality of the included studies, few studies blinded the animals to reduce possible biases in the presentation of the results due to the observable phenotypic characteristics of the CP that allow the identification of the animals, but the blinding for the pharmacological manipulation and analysis of the results are possible to be carried out, but few studies have described having done this blinding. In addition, it is only possible to evaluate episodic memory, due to the limitations of evaluating other types of memory in animal models. In addition, due to the large scope of this review and the heterogeneity of the methods used to assess memory in animals, it was only possible to carry out the meta-analysis of studies that used the MWM test.

In addition, trying to verify the antecedents of memory formation problems present in CP is complex, due to the number of environmental factors that influence, through epigenetic mechanisms, the adequate development of memory, such as brain injuries, adequate nutrition and sleep (De Souza et al., 2011; Fesser et al., 2021; Potter Pak et al., 2018; Presti et al., 2006). Memory formation alterations are heterogeneous because they manifest themselves in different ways depending on the severity and extent of the brain injury and its sequelae. Different types of CP diagnosis can present a distinct neuropsychological profile, where some cognitive skills are more deficient than others (Stadskleiv et al., 2017). Although we found memory formation disturbances in all studies included in our review, memory formation problems are not present in all subjects diagnosed with CP. Moreover, they are findings from preclinical studies, and the results found are not necessarily often observed in humans with CP. Some subjects with CP present deficits on memory acquisition due to early brain injuries, but these are only noticed with the development of the organism and the emergence of functional demands of everyday or school life present in their reality (Pascal et al., 2018; Pak et al., 2018). Although not present in all cases of CP, memory formation impairment is reported in recent studies with children and adults with CP (Fesser et al., 2021; Hoffman et al., 2020), highlighting the importance of investigating interventions that act such as neurological sequelae and memory formation disturbance in adulthood.

5.2. Implications for future investigation

Overall, this study provides evidence of how brain damage in early life affects hippocampal development and promotes problems in the formation of new memories during adulthood in rodents. Furthermore, we provide quantitative and qualitative evidence of the effects of several promising interventions in alleviating the memory formation problems present in animals. These pieces of evidence serve as a guide for future studies to investigate the effects of promising interventions such as the administration of Melatonin or Erythropoietin, or the supplementation of vitamins, such as B1 and B2, or polyphenols, such as resveratrol, as they have strong antioxidant and anti-inflammatory effects, attenuating deficits in hippocampal maturation and consequently reducing memory formation problems in adulthood. Future studies investigating the effects of these interventions on the maturation of other damaged brain structures in CP may promote a better understanding of treatments for the primary problems of CP, such as neuromusculoskeletal sequelae, and the secondary problems such as alterations in memory formation, allowing future investigations of the use these interventions in clinical practice.

This systematic review can also open the way for the investigation of other interventions that act by reducing reactive oxygen species and neuroinflammation and promoting an increase in neuroplastic mechanisms, such as an increase in the levels of BDNF, CREBS and consequently an increase in neurogenesis and cell proliferation in the hippocampus, considering that the interventions that reduced memory formation problems in early brain injury acted through these mechanisms. For this reason, nutritional interventions are present promising due to the relationship between food intake and brain activity.

6. Conclusions

In our study, we observed difficulties in the formation of new episodic memories, a type of declarative memory, from a low performance in tests assessing object recognition and spatial location, in the adult life in animals that were exposed to brain lesions in the perinatal period with implications for the development of cerebral palsy. Our findings indicate that early brain injury impairs the development and maturation of brain structures such as the hippocampus, leading to difficulties in memory formation in adult life, highlighting the importance of the perinatal period in the development of problems in memory formation and acquisition that may be observed during life. These memory formation problems are associated with damage to the hippocampus from brain injury, noting an increase in oXidative stress, neuroinflammation, apoptosis; and a reduction in neuroplastic mechanisms such as neurogenesis and decrease BDNF levels. Interventions that reduced neuroinflammation and the presence of free radicals were highlighted as a therapy for the memory formation problems present in CP. Preclinical studies registered treatments with oXygen interventions, resveratrol, erythropoietin, melatonin and physical exercise, which were able to reduce the damage to the hippocampus and promote improvements in memory and behaviour in experimental models of perinatal brain lesions. These reviewed studies, then, suggest possible avenues of intervention for memory formation disturbance present in perinatal brain lesions, highlighting promising interventions that can mitigate memory problems.

Author contributions

A.E. Toscano was the supervisor and third reviewer. C. M.S.·S Calado and S.C. Pereira were the first and second reviewers. C. M.S.S. Calado, S. C. Pereira, V.S. Souza, D.B. Visco, B.S. De Silveira, S.L. Souza, and A.E. Toscano carried out the manuscript preparation process, all of whom participated in determining the eligibility criteria and standardizing the data collection forms and analysing the risk of study bias. R. Manhães-de- Castro, S.L. Souza, and A.E. Toscano participated in the review and editing for preparation. They contributed their experience regarding intellectual content and guidance. All authors reviewed and agreed with the final manuscript.

Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. Reporting no conflict of interest.

Data availability

No data was used for the research described in the article.

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

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Recife, 05 de maio de 2020

Ofício nº 16/20

Da Comissão de Ética no Uso de Animais (CEUA) da UFPE
Para: Prof. Ana Elisa Toscano Meneses da Silva de Castro
Centro Acadêmico de Vitória
processo nº0009/20

Certificamos que a proposta intitulada " **Efeitos do tratamento com resveratrol e/ou com o fator de crescimento de fibroblastos 19 sobre o sistema neuro-músculo-esquelético de ratos submetidos à paralisia cerebral" .** registrado com o **nº0009/20** sob a responsabilidade da **Prof. Ana Elisa Toscano Meneses da Silva de Castro** o que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo CONSELHO NACIONAL DE CONTROLE DE EXPERIMENTAÇÃO ANIMAL (CONCEA), e foi aprovada pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) DA UNIVERSIDADE FEDERAL DE PERNAMBUCO (UFPE), em reunião de 28/04/2020

Finalidade	() Ensino (x) Pesquisa Científica
Vigência da autorização	05/05/2020 á 28/02/2024
Espécie/linhagem/raça	Rato heterogênico Wistar
Nº de animais	165 wistar
Peso/Idade	220-250g 90-120 dias/ 5-80g 1-29 dias, Adultos e neonatos
Sexo	Fêmeas = 30 e Machos = 15 e 120 neonatos machos.
Origem: Biotério de Criação	Biotério de criação do Departamento de Nutrição da UFPE.
Destino: Biotério de Experimentação	Biotério de criação do Departamento de Nutrição da UFPE.

Atenciosamente

[redacted]

Prof. Sebastião R. F. Silva
Presidente CEUA/UFPE
SIAPE 2345091