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DIETA MATERNA DURANTE A GESTAÇÃO: IMPACTOS COMPORTAMENTAIS E NEUROQUÍMICOS NA PROLE

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RESUMO

A dieta materna consumida durante a gestação pode afetar o neurodesenvolvimento, o comportamento, o metabolismo, a homeostase redox e o estado inflamatório da prole. Durante a gestação a demanda de folato aumenta para a manutenção das reações de transferência de um carbono, que participam da síntese de DNA, e reações de metilação biológica importantes para o crescimento uterino, placentário e fetal. Além disso, as proteínas consumidas pela dieta são uma fonte de aminoácidos que são cruciais para o crescimento e desenvolvimento fetal adequado. No entanto, estudos têm demonstrado que alterações no consumo materno durante a gestação de macronutrientes ou micronutrientes podem causar prejuízos na prole. Baseado nisto, o objetivo dessa tese foi investigar os efeitos da suplementação de ácido fólico ou do consumo de dieta hiperproteica durante a gestação sobre parâmetros comportamentais e neuroquímicos no córtex cerebral e soro da prole de ratos machos. No capítulo I, as ratas prenhes foram randomizadas em dois grupos, de acordo com a dieta: grupo 1, (controle) dieta padrão (2 mg/kg dieta de ácido fólico) e grupo 2, dieta suplementada com 4 mg/kg dieta de ácido fólico. No capítulo II, as ratas prenhes foram divididas aleatoriamente em dois grupos, de acordo com a dieta: grupo 1, (controle) dieta padrão contendo 20% de proteína e grupo 2, dieta hiperproteica contendo 50% de proteína. Durante todo o período gestacional as ratas prenhes receberam as dietas experimentais. Em ambos os capítulos, a prole de 21 dias pós-natal a foi submetida a uma bateria de testes comportamentais, a saber: qualidade do cuidado materno, preferência olfatória da prole pelo odor maternal, campo aberto, reconhecimento de objetos, labirinto em cruz elevado, transição claro-escuro, teste de depressão relacionada à alimentação, escada horizontal, trave de equilíbrio e *rotarod*. Após a realização dos testes comportamentais, a prole foi eutanasiada e o soro e o córtex cerebral total foram imediatamente coletados para análise dos parâmetros neuroquímicos (homeostase redox, neuroinflamação e níveis de aminoácidos teciduais e séricos). Os resultados do capítulo I demonstram que a dieta suplementada com 4 mg/kg de ácido fólico durante toda a gestação prejudicou a memória e a motricidade da prole quando comparada ao controle. Também foi observado um aumento no comportamento do tipo ansioso e depressivo neste grupo, bem como os níveis de nitritos que aumentaram no córtex cerebral da prole. Entretanto, o imunoconteúdo e a expressão de óxido nítrico sintase induzível não foram alterados. Além disso, identificamos um aumento na expressão gênica do fator de necrose tumoral-*α*, interleucina-1β, interleucina-6, interleucina-10 e da proteína quimioatraente de monócitos-1 no córtex cerebral da prole suplementada com ácido fólico ao longo da gestação. Em relação ao capítulo II, os resultados evidenciaram um aumento nos níveis de homocisteína e mediadores neuroinflamatórios no córtex cerebral da prole de ratas prenhes que consumiram dieta hiperproteica durante toda a gestação. Além de diminuir os níveis de histidina no soro da prole. Os resultados também revelaram

prejuízos na memória e motricidade e um aumento do comportamento do tipo ansioso na prole suplementada com dieta hiperproteica durante a gestação. Os resultados dessa tese indicam que a suplementação com ácido fólico ou o consumo de dieta hiperproteica durante a gestação podem causar prejuízos neuroquímicos e comportamentais na prole de ratos machos. Em conjunto, esses achados fornecem uma nova base para a compreensão dos resultados bioquímicos e comportamentais associados à suplementação de ácido fólico ou o consumo de dieta hiperproteica durante todo o período gestacional.

Palavras-chave: Ácido fólico; Aminoácidos; Ansiedade; Comportamento, Depressão; Dieta hiperproteica; Estresse oxidativo; Gestação; Homocisteína; Memória; Motricidade; Neuroinflamação; Óxido nítrico; Prole.

ABSTRACT

Pregnancy diet can impact in offspring's neurodevelopment, behavior, metabolism, redox homeostasis, and inflammatory status. In pregnancy, folate demand increases due to the requirement for one-carbon transfer reactions required for DNA synthesis and biological methylation reactions to uterine, placental, and fetal growth. Moreover, proteins in the diet are a source of amino acids that are crucial for proper fetal growth and development. However, studies have a shown that changes in maternal macronutrients or micronutrients consumption during pregnancy may cause behavioral impairments in the offspring. Based on this, the objective of the thesis was to investigate the effects of folic acid supplementation or high-protein diet consumption throughout pregnancy on neurochemical and behavioral parameters in male offspring. For this, in the chapter I, pregnant rats were randomly divided into two groups, according to the diet: group 1, (control) standard diet (2 mg/kg diet of folic acid) and group 2, diet supplemented diet with 4 mg/kg diet of folic acid. In the chapter II, pregnant rats were randomly divided into two groups, according to the diet: group 1, (control) standard diet containing 20% protein, and group 2, the high-protein diet containing 50% protein. Throughout the gestational period, the pregnant rats received experimental diets. In both chapters, offspring at 21 postnatal days were submitted to behavioral tests, namely: maternal behavior, olfactory preference test, open field, object recognition, plus maze, light-dark transition, eating-related depression test, ladder walking, beam balance, and rotarod. After conducting behavioral tests, offspring were euthanized and serum and total cerebral cortex were immediately collected for the analysis of neurochemical parameters (redox homeostasis, neuroinflammation, and tissue and serum amino acid levels, including homocysteine). The results of the chapter I showed that the supplemented diet with 4 mg/kg diet of folic acid throughout pregnancy impaired memory and motricityof the offspring when compared with control (standard diet). It was also observed an increase in anxiety- and depression-like behavior in this group. Nitrite levels increased in cerebral cortex of the offspring. In contrast, inducible nitric oxide synthase expression and immunocontent were not altered. Moreover, we identify an increase in tumor necrosis factor-α, interleukin-1β, interleukin-6, interleukin-10, and monocyte chemoattractant protein-1 gene expression in the cerebral cortex of the offspring supplemented with folic acid throughout pregnancy. In relation to chapter II, Results showed an increase in homocysteine levels and neuroinflammatory mediators in the offspring's cerebral cortex from pregnant rats supplemented with a high-protein diet throughout pregnancy. Besides decreasing histidine levels in offspring's serum. The results also revealed impairment in memory and motricity and an increase in anxiety-like behavior in the offspring supplemented with a high-protein diet throughout pregnancy. The results of this thesis indicate that folic acid supplementation or maternal high-protein diet consumption throughout pregnancy may cause biochemical and behavioral impairments in the offspring. Together, these

findings provide a new basis for understanding the biochemical and behavioral outcomes associated with folic acid supplementation and high-protein exposure throughout pregnancy.

Keywords: Amino acid; Anxiety; Behavior, Depression; Folic acid; Gestation; High-protein diet; Homocysteine; Memory; Motricity; Neuroinflammation; Offspring; Nitric oxide; Oxidative stress.

LISTA DE ABREVIATURAS

5-MTHF: 5-metiltetrahidrofolato
5,10-METHF: 5,10-metilenotetrahidrofolato
ANVISA: Agência Nacional de Vigilância Sanitária
ATP: Adenosina trifosfato
BDNF: Fator neurotrófico derivado do cérebro
CAT: Catalase
DHF: Dihidrofolato
DHFR: Dihidrofolato redutase
DOHaD: Origens Desenvolvimentistas da Saúde e da Doença (do inglês, Developmental Origins of Health and Disease)
Egr2: Gene 2 de resposta ao crescimento precoce
eNOS: Óxido nítrico sintase endotelial
ERN: Espécies reativas de nitrogênio
EROs: Espécies reativas de oxigênio
GPx: Glutationa peroxidase
GSH: Glutationa reduzida
GSSG: Glutationa oxidada
iNOS: Óxido nítrico sintase induzível
IL: Interleucina
JAK: Janus cinase
LCR: Líquido cefalorraquidiano
MCP-1: Proteína quimioatraente de monócitos-1
MTHFR: Metilenotetrahidrofolato redutase
NGF: Fator de crescimento neural
NMDA: N-metil-d-aspartato
nNOS: Óxido nítrico sintase neuronal
NOS: Óxido nítrico sintase
Nrf2: Fator nuclear relacionado ao eritróide 2
OMS: Organização Mundial da Saúde
PrxR: Peroxiredoxina
RDC: Resolução da Diretoria Colegiada
RF: Receptor de folato
SAM: S-adenosilmetionina
SLC: *Solute carrier*
SNC: Sistema nervoso central
SOD: Superóxido dismutase
STAT: Transdutor de sinal e ativador da transcrição
TBARS: Substâncias reativas ao ácido tiobarbitúrico
THF: Tetrahidrofolato
TNF-*α*: Fator de necrose tumoral-*α*
TNFR1: Receptor do fator de necrose tumoral 1

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PARTE I

1 INTRODUÇÃO

1.1 Dieta materna durante a gestação

A teoria das Origens Desenvolvimentistas da Saúde e da Doença (DOHaD), do inglês *Developmental Origins of Health and Disease* sugere que a exposição materna durante a gestação a componentes externos pode ser um importante fator de risco para o desenvolvimento de doenças metabólicas durante a vida da prole. A teoria DOHaD descreve como componentes externos o estilo de vida, exposição ao estresse, prática de atividade física, doenças, adição a drogas/álcool e a nutrição materna (Suzuki, 2017; Almeida et al., 2019).

A qualidade e a quantidade de nutrientes consumidos pela mulher durante a gestação é considerado um dos aspectos mais relevantes para a saúde materno-fetal. Durante a gestação, as necessidades de energia e nutrientes aumentam para permitir o crescimento e o desenvolvimento do feto, aumento do tamanho do útero, hipertrofia das glândulas mamárias e o acúmulo de reservas de gordura (Puszko et al.,2017; Garcia-Casal et al., 2018).

O consumo de uma dieta nutricionalmente inadequada durante o desenvolvimento intrauterino do feto, principalmente devido à quantidade e à qualidade dos nutrientes consumidos pelas mães, pode afetar o crescimento e o metabolismo, a função imunológica e cerebral e o desenvolvimento cognitivo da prole (Bordeleau et al., 2020; Lecorguillé et al., 2021; Mirpuri, 2021). Neste contexto, a deficiência ou o excesso do consumo de micronutriente e macronutrientes durante a gestação vêm sendo descritas como importantes fatores de risco para transtornos do neurodesenvolvimento, tais como transtorno do espectro autista,

transtorno de déficit de atenção e hiperatividade e esquizofrenia (Bordeleau et al., 2021), cujos mecanismos não são totalmente compreendidos.

Baseado nisto, é importante investigar os efeitos da suplementação de ácido fólico e o consumo da dieta hiperproteica durante toda a gestação sobre o comportamento e a neuroquímica da prole.

1.2 Folatos

O folato (vitamina B9) é uma vitamina do complexo B e é solúvel em água. O folato tem uma estrutura central de pteridina e alta atividade metabólica devido à sua capacidade de aceitar elétrons e reagir com ligações O-, S-, N-, C- (Relton et al., 2004; Shulpekova et al., 2021). O folato é inerentemente conjugado com para-aminobenzoil-glutamato como mono-, di-, tri- e poliglutamatos. O folato natural possui a forma de poliglutamato (Thöny et al., 2000). Na natureza o folato pode ser encontrado em alimentos como fígado, rim, frutas cítricas, cogumelos, espinafre, fermentos e folhas verdes (Engevik et al., 2019).

O folato catalisa reações bioquímicas essenciais, especialmente por meio de seu papel central no ciclo da metionina, participa dos processos de metilação, regula os níveis de homocisteína e está envolvido no metabolismo de purinas, pirimidinas, serina, glicina ehistidina (Iyer et al., 2009). Essa vitamina é uma molécula que existe fisiologicamente como tetrahidrofolato (THF) e 5-metiltetrahidrofolato (5-MTHF). O THF e o 5-MTHF são metabólitos intermediários no ciclo do folato e são relacionados ao metabolismo de um carbono. Juntos, o ciclo do folato e o metabolismo de um carbono são responsáveis por gerar grupos metila e coordenar os processos envolvidos nas modificações epigenéticas e *imprinting* (Crider et al., 2012). Assim, manter o equilíbrio adequado de substratos e cofatores

no ciclo do folato é crucial para manter a homeostase, pois a interrupção de uma etapa enzimática em qualquer parte do ciclo pode ter consequências prejudiciais significativas. A homeostase do ciclo do folato é crucial durante a gestação, onde a deficiência de folato tem impacto no fechamento do tubo neural, neurodesenvolvimento e no crescimento placentário e fetal (Crider et al., 2014).

Folato é um termo genérico que engloba o ácido fólico e seus compostos derivados dihidro-, tetra-hidro-, metil-, formil com atividade metabólica (Thöny et al., 2000). O ácido fólico é uma molécula quimicamente sintetizada que é usada como suplemento dietético e na fortificação de alimentos (Bailey et al., 2009). É totalmente oxidado e mais estável e biodisponível que o folato natural. A fórmula química do ácido fólico é $C_{19}H_{19}N_7O_6$. O núcleo da molécula consiste em uma estrutura heterocíclica de pteridina, com um grupo metila na sexta posição ligada a ácidos para-aminobenzóico e glutâmico para que o ácido fólico apresente ácido pteroilglutâmico. O ácido fólico possui a forma de monoglutamato (Shulpekova et al., 2021). A pteridina é composta por anéis de pirimidina e pirazina com substituição dos grupos ceto e amino na segunda e quarta posições. Estruturas heterocíclicas aromáticas fornecem uma capacidade de aceitação reversível de elétrons (Mahendran et al., 2018). A figura 1 demonstra a estrutura molecular do ácido fólico.

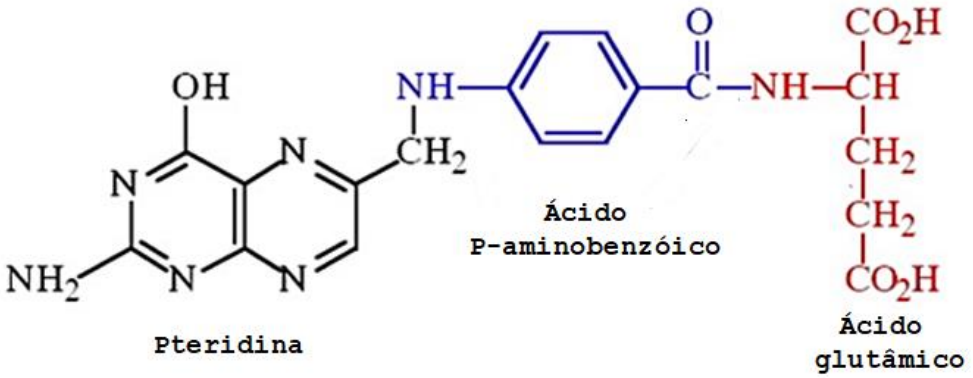

Figura 1: Estrutura molecular do ácido fólico (Menezo et al., 2022, modificado).

Após o consumo, o ácido fólico é absorvido principalmente no jejuno proximal (Aufreiter et al., 2009). O ácido fólico em si não é ativo como coenzima e passa por várias etapas metabólicas dentro da célula para ser convertido na forma metabolicamente ativa. Sua atividade biológica depende da ação da enzima dihidrofolato redutase (DHFR). Nos enterócitos e hepatócitos, o ácido fólico absorvido inicialmente é reduzido pela DHFR em dihidrofolato (DHF) e THF. O THF é convertido em 5,10- metilenotetrahidrofolato (5,10-METHF) pela enzima serina hidroximetiltransferase dependente de piridoxina e após é reduzido a 5-MTHF pela metilenotetrahidrofolato redutase (MTHFR) (Menezo et al., 2022). A figura 2 ilustra o metabolismo do ácido fólico.

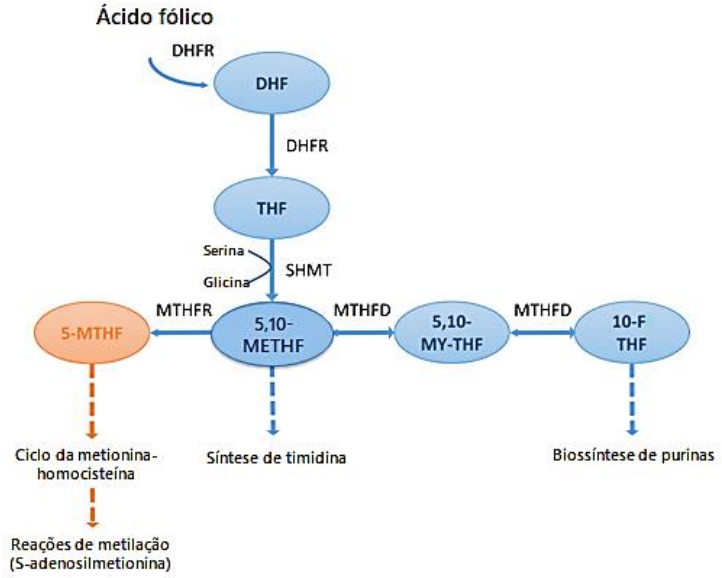

Figura 2: Metabolismo do ácido fólico. 5-MTHF: 5-metiltetrahidrofolato; 5,10-METHF: 5,10-metilenotetrahidrofolato; 5,10-MITHF; 5,10-meteniltetrahidrofolato; 10-FTHF: 10-formiltetrahidrofolato; DHF: dihidrofolato; DHFR: dihidrofolato redutase; MTHFD: metilenotetrahidrofolato desidrogenase; MTHFR: metilenotetrahidrofolato

redutase; SHMT: serina hidroximetiltransferase; THF: tetrahidrofolato. (Lubout et al., 2020, modificado).

O 5-MTHF é transportado para o sistema nervoso central (SNC) principalmente através do plexo coroide para então chegar às células nervosas (Wollack et al., 2008). As células epiteliais do plexo coroide expressam altos níveis de receptores de folato *α* (RF*α*), sugerindo que essas células desempenham um papel importante no transporte de folato para o SNC e na manutenção de seus níveis no líquido cefalorraquidiano (LCR), o qual é mantido em uma concentração de 2,5 a 3,5 vezes maior do que o plasma (Zhao et al., 2013).

1.3 Suplementação com ácido fólico durante a gestação

A Organização Mundial da Saúde (OMS) recomenda que todas as mulheres que estão tentando engravidar realizem a suplementação com 400 µg de ácido fólico diariamente (Organização Mundial da Saúde, 2013). A suplementação é recomendada antes da concepção e não somente após a confirmação da gestação, pois o fechamento do tubo neural pode ocorrer antes de muitas mulheres estarem cientes de sua gravidez (Greene et al., 2014). Visando a redução de defeitos no tubo neural, muitos países com deficiência nutricional de folato, os produtos a base de farinha são fortificados com ácido fólico na tentativa de reduzir essa deficiência (Crider et al., 2011). No Brasil, a fortificação mandatória dos alimentos iniciou com a publicação da Resolução da Diretoria Colegiada (RDC) nº 344 de 13 de dezembro de 2002. Nesta publicação a Agência Nacional de Vigilância Sanitária (ANVISA) recomenda que as farinhas de milho e trigo sejam fortificadas com 150 µg de ácido fólico para cada 100 g de farinha (Brasil, 2002).

É amplamente aceito que a suplementação de ácido fólico no período perinatal protege contra o desenvolvimento de defeitos do tubo neural. Os defeitos do tubo neural resultam da falha do tubo neural em fechar aproximadamente na terceira a quarta semana de gestação em humanos e nos dias gestacionais 10,5 a 11 em ratos, podendo resultar em mortalidade ou deficiências em longo prazo (Rice et al., 2000; Greene et al., 2014; Flores et al., 2015). A maioria dos defeitos do tubo neural é evitável pela ingestão suficiente de folato ou de sua forma sintética, o ácido fólico (Greene et al., 2014; Czeizel et al., 2013). O folato desempenha um papel importante no fechamento do tubo neural, regulando processos de biossíntese de nucleotídeos e reações de metilação (Greene et al., 2014). Além disso, o folato é reconhecido como um dos principais nutrientes necessários para a formação e desenvolvimento do encéfalo fetal devido ao seu envolvimento na proliferação e crescimento de células neuronais e na síntese de neurotransmissores (Naninck et al., 2019).

1.4 Transferência de folato para o feto através da placenta

A concentração de folato no soro fetal é muito mais elevada do que a encontrada no soro materno, provavelmente devido ao transporte ativo de folato (Obeid et al., 2005). Estudos experimentais *in vivo* mostram que há transporte placentário ativo de folato e concentrações totais elevadas de folato são encontradas no cérebro durante o desenvolvimento fetal precoce (Yasuda et al., 2008). Três mecanismos específicos de transporte de folato operam na placenta, incluindo o RFα e RFβ, o carreador de folato reduzido e o transportador de folato acoplado a prótons/proteína transportadora de heme 1 (Zhao et al., 2009; Solanky et al., 2010). A transferência de folato da circulação da mãe para o feto é resultado da ação

coordenada desses transportadores (Castaño et al., 2018). O envolvimento de membros da superfamília de transportadores cassete de ligação de adenosina trifosfato (ATP) na homeostase celular de folato na placenta humana também foi relatado (Joshi et al., 2016). A placenta é um órgão que sofre modificações e adaptações no decorrer da gestação (Zeng et al., 2017). O transporte de folato placentário é estabelecido no início da gestação, mas os transportadores de folato podem mudar durante o curso da gravidez. Os genes do carreador de folato reduzido e do transportador de folato acoplado a prótons/proteína transportadora de heme 1 diminuem entre o primeiro e o último trimestre de gravidez, enquanto o RFα não se altera durante todo o período gestacional. Isso sugere que o RFα pode ser o principal regulador para o aumento do transporte de folatos da mãe para o feto à medida que a gestação progride (Yasuda et al., 2005; 2008; Castaño et al., 2018).

1.5 Efeitos na prole da suplementação com ácido fólico durante a gestação

Além da proteção contra defeitos no fechamento do tubo neural, estudos também relatam que a suplementação com ácido fólico durante a gestação melhora a neurogênese, sinaptogênese, reflexos primitivos, aprendizagem espacial e motricidade (McGarel et al., 2015; Wang et al., 2018; 2019; 2021; Harlan et al., 2021). Entretanto, recentemente a suplementação de ácido fólico durante a gestação está sendo relacionada com alterações patológicas na prole (Deniz et al., 2018; Li et al., 2019). Variações no consumo de ácido fólico durante a gestação podem alterar a atividade de DNA metiltransferases e histonas metiltransferases (Joubert et al., 2016). Além disso, o folato pode alterar as respostas inflamatórias ao estimular a metilação aberrante e alterar a atividade de proliferação celular (Jones et al., 2019).

Estudos experimentais têm demonstrado que a suplementação com ácido fólico durante o período gestacional pode causar na prole intolerância à glicose (40 mg de ácido fólico/kg dieta), câncer de mama (5 mg de ácido fólico/kg de dieta), disfunção na tireoide (8 mg de ácido fólico/kg de dieta) e diminuição do peso e tamanho dos embriões (20 mg de ácido fólico/kg de dieta) (Yajnik et al., 2008; Ly et al., 2011; Sittig et al., 2012; Mikael et al., 2013; Huang et al., 2014; Khot et al., 2015; Boyles et al., 2016). Além disso, estudos identificaram que a suplementação de ácido fólico durante toda a gestação pode causar atraso no neurodesenvolvimento, diminuição no imunoconteúdo de neurotrofinas (fator de crescimento neural [NGF] e fator neurotrófico derivado do cérebro [BDNF]) e comprometimento da memória espacial na prole de roedores (Sable et al., 2012;2014; Barua et al., 2014), cujos mecanismos ainda são desconhecidos.

Bahous e colaboradores (2017) observaram que a ingestão de folato durante a gestação leva à deficiência de pseudo-metilenotetrahidrofolato redutase, causando distúrbios no metabolismo da acetilcolina, diminuição do volume hipocampal e déficit de memória na prole. Neste contexto, um estudo pré-clínico demostrou que camundongos que ingeriram água suplementada com ácido fólico (3,75 mg/L) tiveram uma aprendizagem motora deficiente (Chu et al., 2019).

Em humanos, os resultados de uma metanálise demonstraram que a cada 100 mg/dia de aumento na ingestão de folato materno há um P de 0,02% mais risco do desenvolvimento de asma infantil (Li et al., 2019). Além disso, um estudo de coorte prospectivo mostrou que crianças de um ano de idade nascidas de mães que relataram consumir doses de ácido fólico de 5000 µg/dia ou mais tiveram um desenvolvimento neuropsicomotor mais lento em comparação com mães que

consumiram doses de 400-1000 µg/dia (Valera-Gran et al., 2014). Embora conflitantes, evidências também sugerem que a ingestão de folato pode estar associada ao aumento do risco de transtorno do espectro do autista (Choi et al., 2014; Neggers, 2014).

Assim, o uso crescente de ácido fólico durante a gestação cria a necessidade de melhor investigar as consequências de seu uso em longo prazo no comportamento, homeostase redox e estado inflamatório da prole.

1.6 Metabolismo de aminoácidos

Os aminoácidos têm importância fisiológica essencial. Além da sua participação na síntese de proteínas, os aminoácidos participam da sinalização celular e regulam a expressão gênica e a cascata de fosforilação de proteínas. Os aminoácidos são precursores chave para a síntese de hormônios, neurotransmissores e outras substâncias nitrogenadas de baixo peso molecular, sendo todas de grande importância biológica (Wu et al., 2009; 2014).

Há mais de 700 aminoácidos conhecidos na natureza, mas apenas 20 deles (alanina, arginina, asparagina, aspartato, cisteína, glutamato, glutamina, glicina, histidina, isoleucina, leucina, lisina, metionina, fenilalanina, prolina, serina, tirosina, treonina, triptofano e valina) participam da síntese proteica nas células (Hu et al., 2021). Os aminoácidos são tradicionalmente classificados como essenciais ou não essenciais. Os aminoácidos essenciais, que não são sintetizados em eucariotos, devem estar presentes na dieta. Os aminoácidos essenciais descritos são a histidina, isoleucina, leucina, lisina, metionina, fenilalanina, treonina, triptofano e valina (Wu et al., 2009).

A síntese de aminoácidos depende da disponibilidade de substrato, espécie,

estágio de desenvolvimento, a microbiota no lúmen do trato gastrointestinal, fatores ambientais e estados patológicos (Wu et al., 2013). Conforme descrito anteriormente, concentrações fisiológicas de aminoácidos e seus derivados como óxido nítrico, poliaminas, glutationa, taurina, hormônios tireoidianos e serotonina são necessários para a manutenção da homeostase no organismo (Wu, 2009; Hu et al., 2021). No entanto, níveis elevados de aminoácidos e seus produtos como a amônia, dimetilarginina, homocisteína e outros, podem desregular a homeostase redox e inflamatória e estão relacionados à fisiopatologia de doenças cardiovasculares, danos cerebrais, distúrbios neurometabólicos e psiquiátricos (Fabregues et al.,2019; Leite et al., 2021; Ikenaka et al., 2022). Assim, um equilíbrio ideal entre o consumo de aminoácidos na dieta e sua concentração na circulação é crucial para a homeostase de todo o organismo.

1.7 Ciclo da metionina-homocisteína-folato

A homocisteína é um aminoácido sulfurado que não pode ser obtido por meio da dieta e é gerada no metabolismo do aminoácido metionina. A metionina é adquirida através da dieta, por meio do consumo de alimentos ricos em proteínas ou pela degradação de proteínas endógenas (Martínez et al., 2017; Geltink et al., 2019). A metionina é principalmente metabolizada no fígado pela enzima metionina adenosiltransferase que transfere o grupamento adenosil do ATP para a metionina, formando S-adenosilmetionina (SAM) (Skovierová et al., 2016). A SAM é responsável pela metilação de diversas moléculas, doando seu grupamento metila para o DNA, por exemplo, e formando S-adenosilhomocisteína. A enzima S-adenosilhomocisteína hidrolase é responsável pela hidrólise da S-adenosilhomocisteína em homocisteína (Sanderson et al., 2019). A homocisteína

pode ser metabolizada por duas diferentes vias: a via da remetilação e a via da transulfuração. Na via da remetilação, o 5-MTHF, proveniente do metabolismo do folato, doa seu grupamento metila para a homocisteína formando novamente metionina. Esse processo é catalizado pela enzima metionina sintase e utiliza vitamina B12 como cofator (Sanderson et al., 2019). Na via da transulfuração, a homocisteína é condensada com uma serina para formar cistationina através da ação da enzima Cistationina β-sintase e utiliza vitamina B6 como cofator. A enzima cistationina γ-liase converte cistationina em α-cetobutirato e cisteína (Martínez et al., 2017; Geltink et al., 2019). A figura 3 esquematiza as principais rotas de formação e degradação da metionina e da homocisteína.

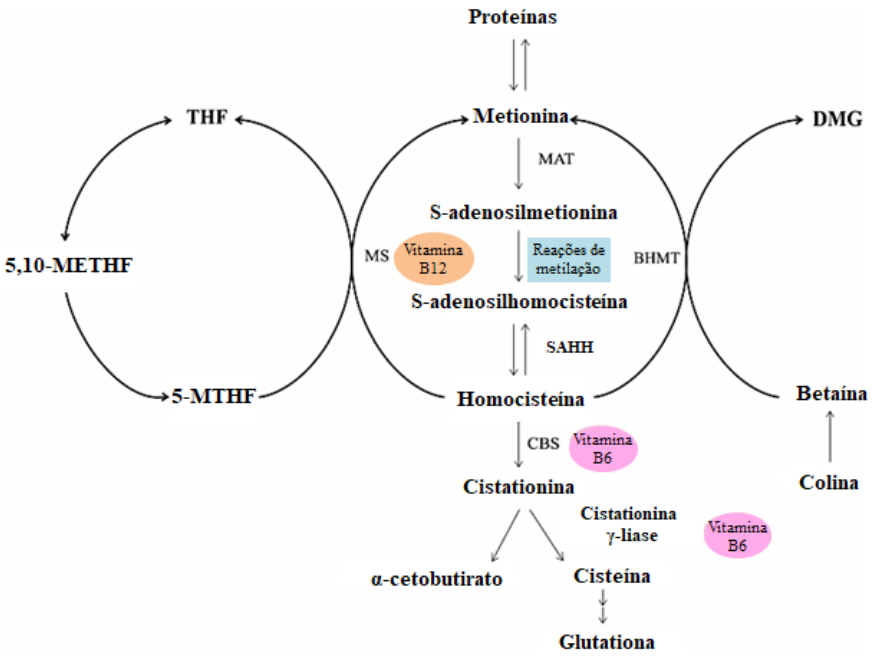

Figura 3: Rota da metionina-homocisteína-folato. 5-MTHF: 5-metiltetrahidrofolato; 5,10- METHF: 5,10-metilenotetrahidrofolato; BHMT: betaína-homocisteína-metiltransferase; CBS: cistationina β-sintase; MAT: metionina adenosiltransferase; MS: metionina sintase; SAHH: S-adenosilhomocisteína hidrolase; THF:

tetrahidrofolato. (Schweinberger et al., 2016, modificado).

Níveis plasmáticos de homocisteína entre 5 e 12 µmol/L (µM em mulheres) e 5 e 15 µM (em homens) são considerados normais. Níveis deste aminoácido superiores a 13-16 µM caracterizam uma condição denominada hiper-homocisteinemia (Herrmann 2006), que pode ser leve, moderada ou grave (severa), sendo que a hiper-homocisteinemia grave é característica da homocistinúria clássica, que é um erro inato do metabolismo. Os níveis de homocisteina nos pacientes afetados por esta doença podem atingir a 500 µM (hiper-homocisteinemia grave). Entretanto, causas não genéticas também podem elevar os níveis deste aminoácido, como por exemplo, a deficiência das vitaminas do complexo B (principalmente B6, B9 e B12), dieta rica em proteínas, principalmente de origem animal, insuficiência renal crônica e uso de medicamentos como fenitoína, L-DOPA e metotrexato (De Bree et al. 2002; Selhub 2002; Liew et al., 2014; Yin et al. 2017). As hiper-homocisteinemas, leve ou moderada, vêm sendo relacionada a fatores de risco para várias doenças, especialmente doenças neuropsiquiátricas e neurodegenerativas (Tinelli et al., 2019; Yu et al., 2022). Neste contexto, estudos mostram que a homocisteína pode induzir comprometimento da homeostase redox, disfunção no metabolismo energético, aumento da acetilcolinesterase, neuroinflamação e alterações na sinalização celular no SNC (Arutjunyan et al., 2020; dos Santos et al., 2021; Ramires-Junior et al., 2022). Considerando todas essas alterações neuroquímicas causadas pela hiper-homocisteinemia, prejuízos comportamentais como déficit de memória, ansiedade, depressão, alterações motoras tem sido relatadas (Jadavji et al., 2019; Esnafoglu et al., 2020; Wyse et al., 2020).

1.8 Transferência de aminoácidos para o feto através da placenta

A transferência placentária fornece os aminoácidos necessários para o crescimento, desenvolvimento e metabolismo fetal. O feto requer aminoácidos para a síntese de proteínas, processos metabólicos e vias biossintéticas (Cleal et al., 2018). As concentrações de aminoácidos são maiores no plasma fetal em comparação com o plasma materno, indicando um processo de transferência ativo através da placenta (Cetin et al., 1996). Transportadores de aminoácidos são expressos nas microvilosidades voltadas para a mãe e nas membranas basais voltadas para o feto do sinciciotrofoblasto da placenta (Cleal et al., 2018). O transporte de aminoácidos através da placenta é um processo complexo que é determinado por múltiplos fatores, incluindo as características do transportador, estrutura placentária, fluxo sanguíneo e asconcentrações de aminoácidos dentro do compartimento materno, placentário e fetal (Panitchob et al. 2016). O tráfego de proteínas transportadoras de aminoácidos para a membrana plasmática também é um fator importante na regulação da transferência de aminoácidos (Chen et al. 2015; 2017). Além disso, o metabolismo materno, placentário e fetal influenciará as concentrações de aminoácidos no plasma arterial e, portanto, no gradiente que determina a captação e o efluxo. A síntese proteica placentária reduz a quantidade de aminoácidos disponíveis para transporte para o feto enquanto a quebra de proteínas aumentará sua disponibilidade (Bonninet al., 2011; Lofthouse et al. 2016).

A atividade dos transportadores de aminoácidos depende da concentração total de aminoácidos disponíveis para transporte e em suas concentrações relativas (Cleal et al., 2018). Todos os transportadores de aminoácidos na placenta são membros da superfamília de *Solute carrier* (SLC) (Hediger et al., 2013). Para

transportar os aminoácidos para o feto, a placenta requer as diferentes classes de transportador de aminoácidos para trabalhar em conjunto tanto nas microvilosidades como na membrana basal do sinciciotrofoblasto. Os transportadores de aminoácidos são divididos em três classes funcionais com base em seu modo de funcionamento: transportadores acumulativos, de troca e facilitadores (Cleal et al., 2011). Os transportadores acumulativos como SLC1 e SLC7 promovem a absorção líquida de aminoácidos específicos através das microvilosidades para o sinciciotrofoblasto. Este mecanismo cria um gradiente de aminoácidos transmembrana através das microvilosidades que conduzem a captação de outros aminoácidos extracelulares via transportadores de troca de aminoácidos (Cleal et al., 2008; Fotiadis et al., 2013). Os transportadores de troca nas microvilosidades incluem a família do SLC7 e trocam aminoácidos intracelulares por outros aminoácidos específicos no plasma materno. Portanto, os transportadores de troca de aminoácidos alteram a concentração de aminoácidos dentro da placenta, mas não modificam a quantidade de aminoácidos (Widdows et al., 2015). Os transportadores acumulativos e trocadores de aminoácidos devem trabalhar em conjunto para fornecer a placenta todos os aminoácidos de que ela necessita (Cleal et al., 2018). Os transportadores facilitadores promovem o transporte de soluto em ambas às direções através da membrana (Cleal et al., 2018). O transporte líquido de aminoácidos ocorrerá a favor do gradiente de concentração até se atinja uma mesma concentração de aminoácidos em ambos os lados da membrana (Cleal et al., 2018).

1.9 Dieta hiperproteica

A proteína é um componente da estrutura corpórea, uma fonte de energia e um nutriente essencial que promove o crescimento e o desenvolvimento (Wu et al.,

2017). O consumo de dietas ricas em proteínas aumentou consideravelmente em todo o mundo nos últimos anos (Acheson, 2013). Neste contexto, devido ao aumento da obesidade e sobrepeso da população nas últimas décadas, dietas ricas em proteínas têm sido desenvolvidas como uma intervenção nutricional para diminuir massa corporal excessiva e prevenir o seu ganho (Wycherley et al., 2012; Moon et al., 2020). Atualmente, a hipernutrição é considerada uma importante questão de saúde pública. Já é demonstrado na literatura que a ingestão dietética de proteínas excede os níveis recomendados entre os adultos jovens (Delimaris et al., 2013).

Dietas ricas em proteínas promovem uma maior saciedade pós-prandial, aumento da termogênese, gliconeogênese, cetogênese, lipólise e inibição da lipogênese de novo (Martens et al., 2013; Azzout-Marniche et al., 2007 Drummen et al., 2018). Evidências demonstram que consumir mais proteína do que a quantidade recomendada na dieta não apenas reduz o peso corporal, mas também melhora a composição corporal, diminuindo a massa gorda e aumenta a hipertrofia muscular. Assim, devido aos seus efeitos metabólicos que contribuem para o controle de ganho de massa corporal, dietas ricas em proteínas vêm sendo ingeridas por mulheres em idade fértil e durante a gravidez (Andreasyan et al., 2007; Halldorsson et al., 2021).

Apesar dos benefícios no metabolismo causados pela ingesta de dietas hiperproteicas, o consumo em longo prazo de uma dieta rica em proteínas pode estar relacionado a problemas metabólicos e clínicos, como perda de massa óssea e disfunção renal em pessoas com alteração renal prévia (Kerstetter et al., 2003; Huang et al., 2008; Farhadnejad et al., 2019; Björnson et al., 2020). Assim, dietas

ricas em proteínas podem ser apropriadas para algumas pessoas, mas devem ser consumidas com cautela por outras. Portanto, necessidades individuais específicas, bem como potenciais consequências negativas, devem ser consideradas com cautela antes que tal dieta seja adotada (Cuenca-Sánchez et al., 2015).

1.10 Dieta hiperproteica na gestação

As proteínas da dieta são uma fonte de aminoácidos essenciais que são cruciais para crescimento e desenvolvimento adequados do feto (Wu et al., 2017). Neste contexto, o perfil de aminoácidos e a capacidade de transporte da mãe para o feto é determinada pela quantidade de proteínas consumidas pela mãe durante a gestação (Cleal et al., 2018). A ingestão adequada de proteínas durante a gravidez é importante para garantir desfechos maternos e neonatais saudáveis (Herring et al., 2018).

Embora o consumo de proteínas pela mãe durante a gestação contribua para um adequado crescimento e desenvolvimento fetal, estudos tem demonstrado que a utilização de uma dieta hiperproteica durante a gestação pode ser prejudicial à prole. A ingestão de dieta rica em proteínas pode aumentar os níveis de derivados da metionina (homocisteína e sulfeto de hidrogênio) e amônia, que podem prejudicar a sobrevivência e o crescimento embrionário e fetal (Taylor, 2016; Wu, 2016). Evidências têm demonstrado que o consumo de dieta hiperproteica durante a gestação está associadas ao crescimento fetal reduzido, níveis mais elevados de pressão arterial, morfologia renal alterada, modificações da microbiota intestinal, redução da imunidade humoral, elevação da produção de espécies reativas de oxigênio (EROs), cortisol e à resistência a insulina (Campbell et al., 1996; Shiell et al., 2001; Sloan et al., 2001; Thone-Reineke et al., 2006; Andreasyan et al., 2007;

Reynolds et al., 2007; Mitchell et al., 2009; Tuchscherer et al., 2012; Maredsous et al., 2015). Estudos experimentais realizados em animais também mostram que uma dieta hiperproteica durante a gestação pode levar a um crescimento fetal reduzido e subsequentemente desencadeiam aumento da massa de gordura na idade adulta (Maredsous et al., 2016).

O consumo de uma dieta hiperproteica durante a gestação também está relacionado ao aumento da resposta inflamatória na prole. Neste contexto, foi demonstrado que o consumo materno de uma dieta rica em proteínas (40%) durante a gravidez e a lactação aumenta os níveis de IL-6 no tecido adiposo marrom da prole (Maurer et al., 2011). Além disso, uma dieta rica em proteínas (40%) durante a gestação afetou significativamente os genes de resposta aguda, do sistema complemento e da Janus cinase/transdutor de sinal e ativador da transcrição (JAK/STAT) no fígado da prole de camundongos (Vanselow et al., 2011). Evidências também mostram que uma dieta rica em proteínas (30%) durante a gestação altera a expressão de genes que determinam a ação dos glicocorticoides no hipotálamo fetal de porcos causando um desequilíbrio no eixo hipotálamo-hipófise-adrenal com consequências duradouras na prole (Kanitz et al., 2012). A desregulação do eixo hipotálamo-hipófise adrenal pode predispor a doenças psiquiátricas como a ansiedade (Sheng et al., 2021). Um estudo de coorte prospectivo demonstrou que uma maior ingestão de proteínas de origem animal durante o segundo trimestre de gestação está ligada ao aumento do risco de diabetes mellitus gestacional (Yong et al., 2021).

Assim, acreditamos ser crucial investigar os efeitos de uma dieta rica em proteínas sobre os possíveis efeitos sobre o comportamento e neuroquímica da

prole, já que há um número limitado de estudos em relação ao tema.

1.11 Estresse oxidativo e nitrativo

Os principais locais de geração de espécies reativas nas células são as mitocôndrias e os peroxissomos (García-Caparrós et al., 2021). As espécies reativas produzidas resultam do metabolismo celular normal e são essenciais para muitas funções biológicas como em processos de sinalização celular, defesa imunológica e plasticidade sináptica (Halliwell al., 2007; Rajendran et al., 2014). As espécies reativas são geradas a partir de fontes endógenas e fontes exógenas. Ativação de células imunes, inflamação, isquemia, infecção, câncer, exercício excessivo, estresse mental, e envelhecimento são exemplos de produção endógena de espécies reativas. A produção exógena de espécies reativas pode ocorrer como resultado da exposição à poluição, algumas drogas, solventes químicos, fumaça de cigarro, álcool e radiações (Pizzino et al., 2017). O estresse oxidativo/nitrativo é um fenômeno causado por um desequilíbrio entre a produção de espécies reativas e as defesas antioxidantes (Halliwell, 2012). As espécies reativas podem induzir lesões celulares pela oxidação de proteínas, peroxidação lipídica e danos ao DNA, que podem resultar em morte celular (Akter et al., 2015; He et al., 2018; Ozgur et al., 2018).

As EROs são moléculas reativas e radicais livres derivados do oxigênio molecular (Halliwell, 2012). As principais EROs produzidas são o ânion superóxido ($O_2^{\bullet-}$), o oxigênio singlet ($^1O_2$), peróxido de hidrogênio (H_2O_2) e o radical hidroxil ($OH^{\bullet}$). Entre as defesas antioxidantes enzimáticas podemos destacar a superóxido dismutase (SOD), a catalase (CAT), a glutationa peroxidase (GPx) e a peroxiredoxina (PrxR). Como defesas não enzimáticas podemos citar as vitaminas

A, C, E, glutationa reduzida (GSH), flavonoides, carotenoides, polifenóis entre outros (Gutteridge et al., 2010). Em resposta ao estresse oxidativo/nitrativo, as células aumentam suas defesas antioxidantes através da ativação do fator nuclear relacionado ao eritróide 2 (Nrf2), um importante fator de transcrição de enzimas antioxidantes (Baird et al., 2020).

As reações que envolvem as principais enzimas antioxidantes e as EROs são:

SOD – $2O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2$

CAT – $2H_2O_2 \rightarrow 2H_2O + O_2$

GPx – $2GSH + H_2O_2 \rightarrow GSSG$ (glutationa oxidada, dímero de glutationa) + $2H_2O$

As espécies reativas de nitrogênio (ERN) são representadas principalmente pelo óxido nítrico (NO) e o peroxinitrito ($ONOO^-$) (Halliwell et al, 2007; Halliwell, 2012). O NO atua como uma importante molécula de sinalização celular e é crucial para uma adequada modulação do fluxo sanguíneo, eliminação de patógenos intracelulares e células tumorais e para a atividade neural normal (Genestra; Pacher et al., 2007). A formação de NO ocorre por meio da enzima óxido nítrico sintase (NOS). Há três isoformas de NOS: NOS neuronal (nNOS), NOS endotelial (eNOS) e a NOS induzível (iNOS) (Cinelli et al., 2020).

O folato pode regular a produção de NO (Stanhewicz et al., 2017). A ativação da NOS depende da presença do cofator tetrahidrobiopterina (BH_4) e substrato disponível para acoplar a oxidação da l-arginina à redução do oxigênio molecular para produzir NO (Montezano et al., 2012). A enzima DHFR dependente de folato tem um papel essencial na redução de BH_2 novamente a BH_4 através da via de salvamento BH_4 (Crabtree et al., 2011). Quando ocorre uma diminuição na formação de BH_4, há um desacoplamento enzimático da NOS e aumenta a produção de EROS

(Crabtree et al., 2009; 2011). O 5-MTHF pode ligar-se ao sítio ativo da NOS e mimetizar a ação da BH_4 e aumentar a produção de NO e reduzir a produção de EROs (Chalupsky et al., 2015). O folato regula a produção de EROs indiretamente através da prevenção do desacoplamento da NOS (Joshi et al., 2001).

O cérebro é vulnerável aos efeitos estresse oxidativo/nitrativo, principalmente devido à grande quantidade de oxigênio consumido neste órgão, alto conteúdo de lipídios insaturados, presença de metais de transição reativos, neurotransmissores que sofrem auto-oxidação e baixos níveis de defesas antioxidantes (Halliwell, 2012; Cobley et al., 2018). O cérebro humano consome 20% do quantitativo total de oxigênio basal para suprir ATP para a intensiva atividade neuronal. Assim, danos na cadeia transportadora de elétrons, causadas pelas EROs/ERN, podem levar a redução da síntese de ATP e contribuir para a fisiopatologia da neurodegeneração (Cobb et al., 2015; Wilkins et al., 2017).

1.12 Neuroinflamação

O SNC é considerado um local imunoprivilegiado. Entretanto, apesar de ser protegido por barreiras físicas especializadas, o SNC não é inerte e nem imunologicamente separado do sistema imunológico periférico (Pape et al., 2019). A neuroinflamação se refere a uma resposta inflamatória que ocorre dentro do SNC que pode ter várias causas como infecções, trauma, isquemia, toxinas, entre outros. O processo é marcado principalmente pela produção de interleucinas (IL) pró-inflamatórias, incluindo IL-1β, IL-6, IL-18 e fator de necrose tumoral-*α* (TNF-*α*), e quimiocinas como a proteína quimioatraente de monócitos-1 (MCP-1) (DiSabato et al., 2016). A resposta imune do SNC envolve células e mediadores locais bem como mediadores transportados da periferia através da barreira cérebro-sangue. As

células envolvidas são principalmente a micróglia e os astrócitos, mas células endoteliais capilares e células sanguíneas também contribuem no processo neuroinflamatório (Heneka et al., 2014; DiSabato et al., 2016).

O aumento da produção das citocinas pró-inflamatórias IL-1β, IL-6 e TNF-*α* fora do SNC afetam o cérebro principalmente pelas vias neurais vagais, pela interação com receptores de citocinas nas células endoteliais cerebrais e/ou por meio da ativação microglial (Becher et al., 2017). Citocinas pró-inflamatórias podem ter efeitos neurotóxicos aumentando a produção de EROs, reduzindo a transmissão das monoaminas e potencializando a transmissão glutamatérgica. O aumento da produção de citocinas pró-inflamatórias pode também causar disfunção sináptica, morte neuronal e inibição da neurogênese (Mishra et al., 2012; Lyman et al., 2014).

A citocina IL-1β induz disfunção sináptica por aumentar a produção de prostaglandina E2, o que leva liberação pré-sináptica de glutamato e ativação pós-sináptica do receptor de N-metil-d-aspartato (NMDA) (Mishra et al., 2012). A citocina TNF-*α* causa morte neuronal ao ativar o receptor do fator de necrose tumoral 1 (TNFR1) e recrutar caspase 8 (Micheau et al., 2003). Essa citocina também reduz a captação de glutamato pelos astrócitos podendo causar excitotoxicidade (Clark et al, 2016). A quimiocina MCP-1 neuronal é essencial para a quimioatração de monócitos para o local da inflamação no SNC (Howe et al., 2017). Citocinas anti-inflamatórias como a IL-10, também são produzidas durante o processo neuroinflamatório fazendo parte de um mecanismo de controle da inflamação excessiva (Calsolaro et al., 2016). Diversos estudos já demonstraram um aumento na produção de marcadores pró-inflamatórios como IL-6, IL-1β, TNF-*α* e MCP-1 em doenças psiquiátricas e neurodegenerativas (D'Mello et al., 2017; Howes et al., 2017; Dani et al., 2018).

1.13 Efeitos da dieta no comportamento da prole

O SNC necessita de suprimento de nutrientes suficiente para seu desenvolvimento morfológico, neuroquímico e neurofisiológico (Cusick et al., 2016). Especialmente durante a gestação, uma nutrição adequada é importante, porque nesse período o cérebro cresce mais rapidamente e as bases para o desenvolvimento cognitivo, motor e habilidades sociais e emocionais são estabelecidas (Prado et al., 2014). Neste contexto, estudos têm demostrado que a dieta materna consumida durante a gestação pode impactar na cognição da prole (Borge et al., 2017; Freitas-Vilela et al., 2018; Mahmassani et al., 2022).

Há evidências de que uma dieta materna com excesso ou déficit de nutrientes pode prejudicar a memória, a motricidade e o aprendizado bem como aumentar a susceptibilidade à ansiedade e à depressão na prole (Fisch et al., 2019; Silveira et al., 2022). Padrões alimentares maternos inadequados durante a gestação também estão associados a pontuações em testes de inteligência mais baixos, desregulação no comportamento emocional e aumento da probabilidade de sintomas de hiperatividade e desatenção (Pina-Camacho et al., 2015; Freitas-Vilela et al., 2018; Galera et al., 2018). Já a ingestão materna de uma dieta de melhor qualidade durante a gravidez foi associada a melhores habilidades visuais espaciais na primeira infância e uma melhor inteligência e função executiva na prole na metade da infância (Mahmassani et al., 2022). Assim, considerando os achados descritos acima, a nutrição materna durante a gestação impacta na cognição da prole, reforçando a importância do estudo deste tópico.

2 JUSTIFICATIVA E HIPÓTESE

Considerando que: a) a suplementação de ácido fólico durante a gestação é amplamente recomendada e seu excesso pode ser prejudicial à prole e b) dietas hiperproteicas estão sendo altamente consumidas por mulheres em fase reprodutiva e os efeitos do seu consumo em longo prazo no SNC da prole ainda não são totalmente compreendidos, é de extrema importância avaliar o efeito da exposição de ratas Wistar a estes nutrientes durante toda a gestação avaliando o seu impacto no comportamento e neuroquímica da prole. Nossa hipótese é que o consumo de uma dieta hiperproteica ou a suplementação com ácido fólico durante toda a gestação causará alterações comportamentais e neuroquímicas de maneira duradora na prole de ratos machos, pois durante a gestação ocorre o transporte placentário de ácido fólico e de aminoácidos para o feto, e estes nutrientes podem chegar ao cérebro fetal em uma fase crítica do seu neurodesenvolvimento.

3 OBJETIVOS

3.1 Objetivo geral

O objetivo geral da tese foi avaliar as possíveis alterações causadas pela suplementação de ácido fólico ou consumo de dieta hiperproteica durante toda a gestação de ratas Wistar sobre: parâmetros comportamentais (relação mãe-filhote, memória, comportamento do tipo ansioso/depressivo e motricidade), bem como analisar parâmetros neuroquímicos no córtex cerebral e soro da prole de ratos machos.

3.2 Objetivos específicos

Os objetivos específicos da presente tese foram subdivididos em dois (2) capítulos, os quais correspondem a 2 artigos científicos, respectivamente:

3.2.1 Capítulo I

Avaliar os efeitos da suplementação com ácido fólico durante toda a gestação de ratas Wistar sobre parâmetros comportamentais e bioquímicos no córtex cerebral da prole de ratos machos, analisando:

- a qualidade do cuidado materno;
- a preferência olfatória da prole pelo odor maternal;
- o comportamento exploratório e locomotor por meio do teste de campo aberto;
- a memória de longa duração utilizando o teste de reconhecimento de objetos;
- o comportamento do tipo ansioso através dos testes de labirinto em cruz elevado e transição claro-escuro;

- o comportamento do tipo depressivo realizando o teste de depressão relacionada à alimentação;a motricidade pelos testes da escada horizontal, da trave de equilíbrio e *rotarod*;
- plasticidade sináptica por meio do imunoconteúdo da sinapsina I;
- alguns parâmetros de estresse oxidativo, a saber: níveis de EROS, níveis de substâncias reativas ao ácido tiobarbitúrico (TBARS), conteúdo de sulfidrilas e de GSH;
- a atividade de algumas enzimas antioxidantes, a saber: SOD, CAT e GPx;
- alguns parâmetros de estresse nitrativo, a saber: níveis de nitritos, imunoconteúdo e expressão da iNOS;
- a expressão de citocinas inflamatórias incluindo TNF-α, IL-1β, IL-6 e a quimiocina MCP-1, bem como a citocina anti-inflamatória IL-10.

3.2.2 Capítulo II

Avaliar os efeitos do consumo de uma dieta hiperproteica durante toda a gestação de ratas Wistar sobre parâmetros comportamentais e bioquímicos no córtex cerebral da prole de ratos machos, analisando:

- a qualidade do cuidado materno;
- a preferência olfatória da prole pelo odor maternal;
- o comportamento exploratório e locomotor por meio do teste de campo aberto;
- a memória de longa duração utilizando o teste de reconhecimento de objetos;

- o comportamento do tipo ansioso através dos testes de labirinto em cruz elevado e transição claro-escuro;
- o comportamento do tipo depressivo realizando o teste de depressão relacionada à alimentação;
- a motricidade pelos testes da escada horizontal, da trave de equilíbrio e *rotarod*;
- a concentração de aminoácidos (aspartato, glutamato, serina, histidina, glutamina, glicina, alanina, tirosina, triptofano, metionina, valina, fenilalanina, isoleucina, leucina, ornitina e lisina) no soro e córtex cerebral;
- os níveis de homocisteína no soro e córtex e cerebral;
- alguns parâmetros de estresse oxidativo, a saber: níveis de EROS, níveis de TBARS, conteúdo de sulfidrilas e GSH;
- a atividade de algumas enzimas antioxidantes, a saber: SOD, CAT e GPx;
- parâmetros de estresse nitrativo, a saber: níveis de nitritos e a expressão de iNOS;
- a expressão de citocinas inflamatórias incluindo TNF-α, IL-1β, IL-6 e a quimiocina MCP-1, bem como a citocina anti-inflamatória IL-10.

PARTE II

4 METODOLOGIA E RESULTADOS

A metodologia e os resultados serão apresentados em 2 capítulos. Os capítulos I e II correspondem a artigos científicos que serão apresentados posteriormente. No artigo científico do capítulo I foi utilizado o modelo experimental de suplementação com ácido fólico durante a gestação. Já no artigo científico do capítulo II, utilizamos o modelo de dieta hiperproteica gestacional.

4.1 Modelo de suplementação com ácido fólico durante a gestação

Após a confirmação do acasalamento, o qual foi considerado o dia zero de gestação (G0), as fêmeas prenhes foram randomizadas em 2 grupos experimentais: 1) grupo controle e 2) grupo suplementado com ácido fólico. A dieta padrão continha 2 mg de ácido fólico por kg de dieta e a dieta suplementada teve a adição de 2 mg de ácido fólico por kg de dieta (totalizando 4 mg de ácido fólico por kg de dieta) (Deniz et al., 2018a; 2018b). As ratas prenhes receberam as dietas experimentais somente durante a gestação. Após o nascimento dos filhotes, as mães e a prole receberam somente dieta padrão (2 mg de ácido fólico por kg de dieta). Nos primeiros 10 dias de vida da prole foi realizado diariamente a avaliação da qualidade do cuidado materno. Aos 10 dias pós-natal os filhotes foram submetidos ao teste de preferência olfatória da prole pelo odor maternal. Aos 21 dias pós-natal, os filhotes foram submetidos a testes comportamentais a fim de analisar a atividade locomotora, memória, comportamento do tipo ansioso e depressivo e a motricidade. Após a realização dos testes comportamentais, os animais foram eutanasiados e o córtex cerebral total foi coletado para a realização das análises bioquímicas.

4.2 Modelo de consumo de dieta hiperproteica durante a gestação

Após a confirmação do acasalamento, o qual foi considerado o dia zero de gestação (G0), as fêmeas prenhes foram randomizadas em dois grupos experimentais: 1) grupo controle e 2) grupo dieta hiperproteica. A dieta padrão continha 20% de proteína e a dieta hiperproteica foi composta de 50% de proteína (Maredsous et al., 2016). As ratas prenhes receberam as dietas experimentais somente durante a gestação. Após o nascimento dos filhotes, as mães e a prole receberam somente dieta padrão (20% de proteína). Nos primeiros 10 dias de vida da prole foi realizado diariamente a avaliação da qualidade do cuidado materno. Aos 10 dias pós-natal os filhotes foram submetidos ao teste de preferência olfatória da prole pelo odor maternal. Aos 21 dias pós-natal, os filhotes foram submetidos a testes comportamentais a fim de analisar a atividade locomotora, memória, comportamento do tipo ansioso e depressivo e a motricidade. Após a realização dos testes comportamentais, os animais foram eutanasiados e o córtex cerebral total e soro foram coletados para a realização das análises bioquímicas.

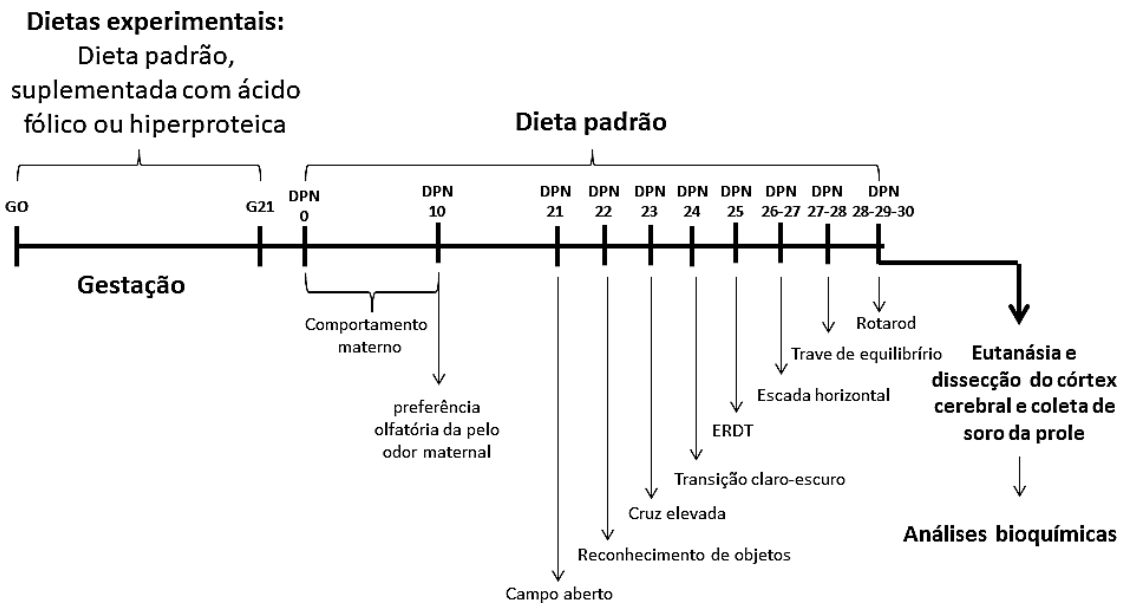

Figura 4: Linha do tempo dos modelos e das análises experimentais. DPN: dia pós-natal, ERDT: teste de depressão relacionada à alimentação, G: dia gestacional.

4.3 Capítulo I

Folic acid supplementation during pregnancy alters behavior in male rat offspring: nitrative stress and neuroinflammatory implications

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Folic acid supplementation during pregnancy alters behavior in male rat offspring: nitrative stress and neuroinflammatory implications

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Abstract
Pregnancy diet can impact offspring's neurodevelopment, metabolism, redox homeostasis, and inflammatory status. In pregnancy, folate demand is increased due to the requirement for one-carbon transfer reactions. The present study was proposed to investigate the effect of folic acid supplementation throughout pregnancy on a battery of behavior tests (olfactory preference, motor activity, exploratory capacity, habituation, memory, anxiety- and depression-like behavior). Redox homeostasis and neuroinflammatory status in cerebral cortex were also investigated. After pregnancy confirmation, the pregnant rats were randomly divided into two groups, according to the diet: group 1, (control) standard diet (2 mg/kg diet of folic acid) and group 2, supplemented diet with 4 mg/kg diet of folic acid. Throughout the gestational period, the pregnant rats received experimental diets. Results show that the supplemented diet with 4 mg/kg diet of folic acid throughout pregnancy impaired memory and motricity of the offspring when compared with control (standard diet). It was also observed an increase in anxiety- and depression-like behavior in this group. Nitrite levels increased in cerebral cortex of the offspring, when compared to control group. In contrast, iNOS expression and immunocontent were not altered. Moreover, we identify an increase in TNF-α, IL-1β, IL-6, IL-10, and MCP-1 gene expression in the cerebral cortex. In conclusion, our study showed that the supplemented diet with 4 mg/kg diet of folic acid throughout pregnancy may cause behavioral and biochemical changes in the male offspring.

Keywords Folic acid · Offspring · Behavior · Oxidative stress · Nitric oxide · Neuroinflammation

Abbreviations

5-MTHF	5-Methyltetrahydrofolate
Actb	β-Actin gene
AIN	American Institute of Nutrition
BH4	Tetrahydrobiopterin
CAT	Catalase
cDNA	Complementary DNA
Ct	Cycle threshold
DCF	Dichlorofluorescein
DHFR	Dihydrofolate reductase
DTNB	5,5′-Dithio-bis(2-nitrobenzoicacid)
EDTA	Ethylenediamine tetraacetic acid
eNOS	Nitric oxide synthase endothelial
ERDT	Eating-related depression test
GPx	Glutathione peroxidase
GSH	Reduced glutathione
iNOS	Inducible nitric oxide synthase
IL	Interleukin
MCP-1	Monocyte chemoattractant protein 1
NADPH	Nicotinamide adenine dinucleotide phosphate
NIH	National Institute of Health
nNOS	Nitric oxide neuronal synthase
PCR	Polymerase chain reaction
ROS	Reactive oxygen species
RT-qPCR	Real-time quantitative PCR reactions
SAM	S-adenosylmethionine

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SEM	Standard error of the mean
SDS	Sodium dodecyl sulfate
SOD	Superoxide dismutase assay
TBARS	Thiobarbituric acid reactive substances
TBST	Tris-buffered saline plus 0.05% Tween 20
WHO	World Health Organization

Introduction

Folate plays an important role in DNA synthesis, stability, integrity, and repair. It is an essential cofactor for the biosynthesis of nucleotides [1]. Moreover, folate is important to early neuronal development, cell growth, gene expression, and neural stem cell differentiation and proliferation via epigenetic modifications [2].

Pregnancy diet can impact offspring's neurodevelopment, metabolism, redox homeostasis, and inflammatory status [3–5]. In pregnancy, folate demand increases due to the requirement for one-carbon transfer reactions required for DNA synthesis and biological methylation reactions to uterine, placental, and fetal growth [6]. It has been known that folate deficiency during gestation increases the risk of congenital malformations, especially neural tube defects [7]. On the other hand, an excess folate intake during pregnancy has also been associated with long-term offspring phenotype changes [8, 9]. To prevent congenital abnormalities, such as neural tube defects, many countries, including Brazil, Canada, and USA, have recommended folic acid supplementation (400 ug/day), the synthetic oxidized and most stable form of folate, during the periconceptional period and the first trimester of the pregnancy [10–13]. However, many pregnant women still receive daily treatment with 5 mg of folic acid that is a dose 10 times higher than recommended, or receive the dose of 400 µg/day throughout gestation instead of the first trimester as recommended. Moreover, many countries, such as Brazil, supplement the flours with folic acid to provide a sufficient amount of this vitamin at the beginning of pregnancy in most women [12, 14–16]. Due to the folic acid supplementation and flours fortification, high levels of folic acid and unmetabolized folic acid (UMFA) are found in most maternal and fetal circulation, as well as in breast milk [17–21]. In this context, potential adverse effects of excess folic acid intake need to be investigated.

Evidence has shown that there is active placental transport of folate and elevated total folate concentrations in the brain during early fetal development [22]. Variations in the consumption of folic acid in pregnancy can alter the activities of DNA methyltransferases and histone methyltransferases [23]. In addition, folate may alter inflammatory responses by encouraging aberrant methylation and cell proliferation activity [24]. Experimental studies have also shown that gestational folic acid supplementation may cause offspring glucose intolerance (40 mg folic acid/kg diet), breast cancer (5 mg folic acid/kg diet), thyroid dysfunction (8 mg folic acid/kg diet), and decrease weight and size of the embryos (20 mg folic acid/kg diet) [25–31]. In this context, Li and colleagues showed in a meta-analysis that each 100 mg/day increment in maternal folate intake was associated with a 0.02% higher risk of infant asthma [32]. Moreover, studies have identified that folic acid supplementation during gestation may cause neurodevelopment delay, alteration in the gene expression, being many of them involved in the neurodevelopment, decreased in neurotrophins immunocontent, and spatial memory impairment in rodents offspring [8, 33, 34]. Although conflicting, reports also suggest that folate intake might be associated with the increased risk for autism spectrum disorder in infants [35, 36]. On the other hand, studies also showed that folic acid supplementation during gestation improves neurogenesis, synaptogenesis, early reflexes, spatial learning, and motricity [37–41]. Thus, the increasing use of folic acid during pregnancy or the use of the dose of 400 µg/day throughout gestation instead of the first trimester as recommended creates the need to further investigate the long-term consequences of its use on brain development, behavior, redox homeostasis, and inflammatory status of offspring.

Considering that in some situations, folic acid may be supplemented throughout pregnancy and its excess may be harmful to the offspring, we decided to investigate its use throughout pregnancy and its potential effect on behavior, redox homeostasis, and neuroinflammation in male offspring. Thus, we hypothesize that the supplementation of folic acid at 4 mg/kg/day (which correspond to the dose of 400 ug/day recommended for humans only for the first trimester of gestation) throughout pregnancy in rats could cause lasting manner behavioral and neurochemical impairments in the male offspring, whereas that there is active placental transport of folic acid and reaches the fetus's brain at an important stage of its neurodevelopment. We found that consumption of folic acid throughout pregnancy may cause behavioral and biochemical changes in the male offspring.

Experimental procedures

Animals and treatment with folic acid in pregnancy

Female and male adult Wistar rats (70–90 postnatal days) were acquired from Centro de Reprodução e Experimentação de Animais de Laboratório (Institute of Basic Health Science, Federal University of Rio Grande do Sul, Brazil). Animals were maintained in a temperature-controlled room (22 ± 1 °C) on a 12/12 light/dark cycle, with free access to food and water available ad libitum. Female rats were submitted to daily collect vaginal smears and when the proestrus

phase was detected, they were put in a new cage with one male rat. Pregnancy was confirmed by the presence of sperm in the vaginal smear. After pregnancy confirmation, the pregnant rats were randomly divided into two groups, according to the diet: standard diet and supplemented diet with 4 mg/kg diet of folic acid. Throughout the gestational period (around 21 days), the pregnant rats received experimental diets. The standard diet contained 2 mg/kg diet of folic acid needed for proper animal maintenance and the supplemented diets had an addition of 2 mg/kg diet of folic acid (totalizing 4 mg/kg diet) [42, 43]. The supplementation with 4 mg/kg diet of folic acid, for rats, is considered the equivalent quantity recommended by the WHO [12] for pregnant women (400 µg/day) [15]. This amount was chosen because in humans folic acid is prescribed at a dose of 400 µg/day to pregnant women. The supplementation with folic acid extra occurred only in the pregnancy. The diets were isocaloric and isoproteic diets (20%) followed the American Institute of Nutrition (AIN) 93 Purified Diets for Laboratory Rodents (American Institute of Nutrition). The diets were purchased from a commercial supplier (PragSoluções®, Brazil). After the birth of the offspring, all rat dams and offspring received the standard diet until the end of the experiments. Offspring male at 10 postnatal days was submitted to olfactory preference test for familiar odors. Male pups were kept with the dams until 21 postnatal days. After weaning, they were rehoused in boxes containing up to four animals. Male pups at 21 postnatal days were submitted to behavioral tests in order to evaluate exploratory abilities, locomotor activity, memory, anxiety, depression, and motricity. Behavioral tests were conducted in the following order: open field test, object recognition memory, elevated plus maze, light–dark transition test, eating-related depression test (ERDT), ladder walking test, balance test, and rotarod. After conducting behavioral tests, animals were euthanized by decapitation without anesthesia, and encephalon was immediately removed followed by dissection of the cerebral cortex. Altogether ten pregnant rats were used in the study, five pregnant females to each experimental diet. A total of 70 male rat offspring from 10 litters were used. Litter effects were controlled by assigning not more than two subjects from a litter to a particular group. We used 11–14 animals per group for behavioral analysis. In the biochemical analyzes, we used in the redox homeostasis analyzes 9 animals per group, 8 animals per group for western blot analysis, and 5–6 animals per group for gene expression. Sample sizes were based on standard error of the mean values of previous studies, with power estimation of 0.80 and alpha = 0.05. The female animals of the offspring, dams after weaning, and the males used in the mating were euthanized with inhaled isoflurane and discarded respectfully. Figure 1 shows the summary of the experimental design.

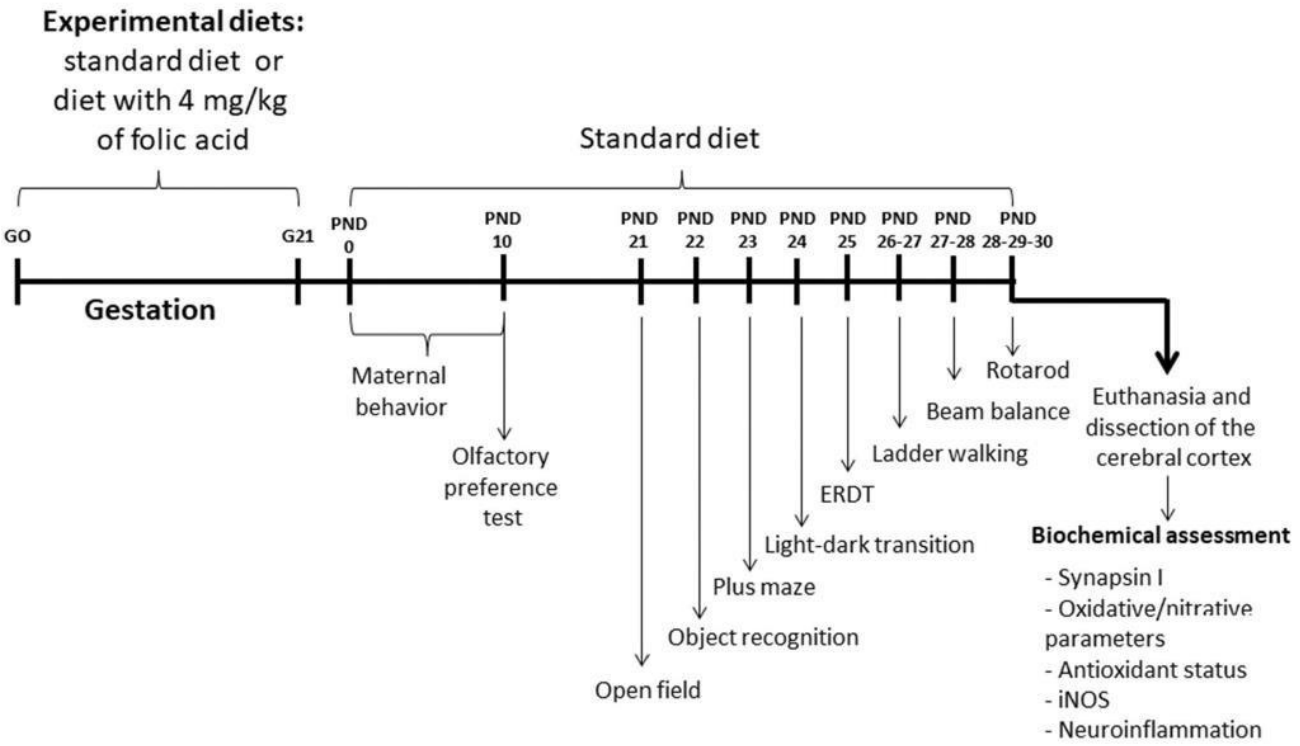

Fig. 1 Timeline of experimental procedures. *ERDT* eating-related depression test, *G* gestational day, *iNOS* inducible nitric oxide synthase, *PND* post-natal day

Ethics statement

The present study was approved by the Ethics Committee of the Federal University of Rio Grande do Sul, Brazil (No.36416) and followed institutional guidelines, Brazilian Society of Science in Laboratory Animals, and the Guide for the Care and Use of Laboratory Animals adopted by the National Institute of Health (NIH, USA). We further attest that all efforts were made to minimize the number of animals used and their suffering.

Behavioral assessments

Before the testing, animals were acclimatized for 20 min in the behavioral room. The apparatus were cleaned up with a 40% ethanol solution after each animal.

Maternal behavior

Maternal behavior after the birth of the offspring was observed during 1 to 10 postnatal days of pups. This test was recorded once a day, during the light cycle phases (16:00 h). Within each observation session, the behavior of each dam at that specific moment was scored every 3 min (11 observations/dam/day). The following behaviors were recorded: dam nursing pups, licking the pups (licking the surface of their bodies and their anogenital regions), and mother off the nest (the lactating female is out of the nest). The total maternal care score was calculated by summing the behaviors directed towards the pups (licking + breastfeeding postures) in all recording periods. The data are reported as the number of observations or frequency that the behavior was recorded (maximum of 110 times/dam) [44–46].

Olfactory preference test for familiar odors in the offspring

The olfactory preference test for the odor of the home cage sawdust (mother and littermates) was performed according to Raineki and colleagues [47] with modifications. The test comprised a two-odor choice between areas with a nest or fresh bedding. A plastic box was divided in the center, using scotch tape, into two areas (fresh bedding area and nest bedding area). A portion of nest bedding (approximately 300 mL) was placed in one corner, and a similar portion of fresh sawdust was placed at the other corner. Pups at 10 postnatal days were placed on the middle line of the testing box (neutral zone). The test was performed in 2 consecutive sessions, which lasted 3 min each with a 2 min interval between each session. At the end of each session, the box was cleaned with 40% ethanol, and the positions of the nest bedding and fresh bedding were switched. Time in fresh bedding area, time in nest bedding area, time in fresh bedding, time in nest bedding, and latency to find nest bedding were recorded. We performed this test in pups of 10 postnatal days because pups gain mobility and leave the nest after this age [48], they easily learn to avoid the nest and the former fail to produce preferences [49].

Open field test

The rats were individually placed in the center of the open field (50 × 50 × 50 cm), and they were filmed for 10 min. The distance traveled, mobile time on the periphery, time in center, crossings number, crossings number in center, crossings number in periphery, and crossings number in periphery were analyzed. The data were recorded and analyzed with an automated activity-monitoring system (Any-maze) [50].

Object recognition memory

This test was performed on the open field apparatus and all of the objects were constructed with plastic LEGO blocks. The test consists of two phases, familiarization and test. In the familiarization session, the animal was placed in the arena containing two identical objects (A1 and A2) for 10 min and total exploration time and the time spent in the exploration of each object were recorded. After 24 h, to evaluate the long-term memory, the animals were returned to the arena for 10 min with two objects: the familiar object (A1) and a novel object (B). Total exploration time and the time devoted to each object were analyzed. Recognition index was determined through the division of the time devoted to the novel object by the total time spent exploring both objects (B/A1 + B) [51].

Elevated plus maze

We released elevated plus maze test in order to evaluate anxiety-like behavior. The apparatus consisted of two open arms and two closed arms (65 cm long and 14 cm wide arranged) such that the two arms of each type were opposite to each other. For this test, each rat was placed in the center of the maze and facing one of the open arms. Rat behavior was registered for 5 min. Total number entries, entries in open arms, the time spent in open arms, percentage of the time spent in open arms, the time spent in closed arms, entries in closed arms, time spent in neutral zone, and time spent in open arms/entries in open arms ratio were recorded [52].

Light–dark transition test

The apparatus used for the light–dark transition test comprised a box (21 × 42 × 25 cm) divided into two compartments of equal size by a partition with a small opening. One chamber was brightly illuminated, whereas the other chamber was dark. Rats were placed into the light side its

behavior was registered for 10 min. Time spent in light, time spent in dark, and number of cross were recorded [53].

Eating-related depression test (ERDT)

This test examines the levels of depression and motivation to eat after food deprivation. Chow was removed from the animal cages for 24 h. After, four chow pellets were placed on a piece of filter paper positioned at one of the four corners of the home cage. Each rat was placed in a corner opposite to the chow and its behavior was registered for 5 min. The latency of the first bite was analyzed [54].

Ladder walking test

Animals were placed on a horizontal ladder. The apparatus used for this experiment consists of two transparent acrylic walls (diameter: 1 m. height: 20 cm) with metal rungs (diameter: 3 mm) inserted into the walls. The space between the two side walls is 55 mm. The ladder remained 30 cm above the floor, and a dark cage was located at the end of the device. All animals were trained for one day using a regular pattern (keeping the same distance between steps). Throughout each training session, the rats walked the ladder three times, and they could explore the refuge cage for 10 s. On the second day, the animals' walk was assessed using an irregular pattern, that is, the steps were spaced at intervals of 1 to 3 cm. The difficulty of the task was modified by varying the position of the metal rungs. Three templates of irregular rung patterns were used so that the same patterns were applied to all animals. Unsuccessful steps are as defined by Metz and colleagues [55], where a hind limb or forelimbs either misses a rung completely or initially makes contact with a rung but slips during weight-bearing. The time spent for the animal to cross the ladder, percentage of correct steps (successful steps/total steps), the total number of errors, the total number of errors/number of steps, and percentage of the total number of errors from forelimbs and hind limb [(number of errors/number of steps) × 100] were recorded [56].

Beam balance test

The test consists of two phases: training and test (three trials each). Rats were trained to traverse a narrow wooden beam (width: 2.5 cm, length: 100 cm). The beam was elevated 50 cm above the floor by two acrylic boxes. The animals were placed on one side, with a safe place (box with nest bedding) on the other side, encouraging the animals to walk on the beam. The number of hind paw slips and average time to cross a beam (three trials) was counted [57].

Rotarod

Rats were exposed to one habituation session for 3 min in the apparatus (Insight®, Brazil) at slow velocity (20 rpm). After, two test days were performed: rats were placed on rotating drums with acceleration from 16 to 40 rpm over 5 min. The number of falls (maximum 3), the latency of the first downfall, and time of permanence in rotarod were analyzed [58].

Biochemical assessments

Assay of redox homeostasis parameters

For evaluation of redox homeostasis parameters, the cerebral cortex was homogenized (1:10, w/v) in 140 mM potassium chloride and 20 mM sodium phosphate buffer, pH 7.4. The homogenates were centrifuged at 3000 rpm for 10 min at 4 °C. The supernatant was immediately collected and used for the measurements of analysis. Biochemical assessments were measured in SpectraMax M5 Microplate Reader (Molecular Devices, USA).

2′7′-Dichlorofluorescein fluorescence assay

Reactive oxygen species (ROS) production in cerebral cortex was determined by the technique based on the oxidation of the 2′7′-dichlorofluorescein diacetate (H2DCF-DA) described by LeBel and colleagues [59]. The samples were incubated in a medium containing 100 μM H2DCF-DA. Then the reaction produces a fluorescent compound, dichlorofluorescein (DCF), which was determined at 488 nm of excitation and 525 nm of emission. The data were plotted as nM DCF per milligram of protein.

Nitrites levels assay

Nitrite is a product of the autoxidation of nitric oxide and is more stable in an aqueous solution [60]. Nitrites levels were measured using the Griess reaction. Cerebral cortex supernatant was mixed with Griess reagent (1:1 mixture of 1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride). The absorbance was measured at a wavelength of 543 nm. Nitrite levels were expressed as μM per milligram of protein [61].

Thiobarbituric acid reactive substances (TBARS) assay

Lipid peroxidation was measured through the determination of TBARS assay described by Ohkawa and colleagues [62]. The samples were incubated in a solution containing 8.1% sodium dodecyl sulfate (SDS), 20% acetic acid, and

0.8% thiobarbituric acid. The resulting pink-stained TBARS were determined at a wavelength of 535 nm. Results were expressed as nM of TBARS per milligram of protein.

Sulfhydryl content

This assay was performed as described by Aksenov and colleagues [63], which is based on the reduction of 5,5′-dithiobis(2-nitrobenzoicacid) (DTNB) by sulfhydryl groups present in samples, which in turn become oxidized (disulfide), generating a yellow derivative (TNB). The sulfhydryl content is inversely correlated to oxidative damage to the protein. Initially, samples were incubated in a solution containing phosphate buffer saline and 1 mM ethylenediamine tetraacetic acid (EDTA), pH 7.4, and 10 mM DTNB. Yellow derivative (TNB) formed were determined a wavelength of 412 nm. Results were presented as nM of TNB per milligram of protein.

Reduced glutathione (GSH) content

Firstly, samples were mixed with metaphosphoric acid and centrifuged at 3000 rpm for 10 min to deproteinize samples. After, deproteinized samples were incubated solution containing 100 mM sodium phosphate buffer and 5 mM EDTA and O-phthaldialdehyde diluted in methanol (1 mg/mL). The fluorescence was determined at a wavelength of 420 nm emission and 350 nm excitation. Results were presented as nM of GSH per milligram of protein [64].

Superoxide dismutase assay (SOD) activity

The SOD analysis in cerebral cortex was based on the technique of the self-oxidizing ability of pyrogallol, a highly superoxide-dependent process that is a substrate for SOD [65]. Inhibition of the self-oxidation of pyrogallol happens in the presence of SOD. The samples were mixed with 1 mM EDTA, 50 mM Tris, 80U/mL CAT, and 0.8 mM pyrogallol. The SOD activity was indirectly measured at 420 nm. The SOD activity was plotted as units of SOD per milligram of protein.

Catalase assay (CAT) activity

The CAT analysis was based on the measure of the decrease in hydrogen peroxide consumption at 240 nm [66]. The samples were incubated in a solution containing 20 mM hydrogen peroxide, 0.1% of Triton X-100, and 10 mM potassium phosphate, pH 7.4. One CAT unit was defined as hydrogen peroxide 1 μM consumed per minute. The CAT activity was presented as units of CAT per milligram of protein.

Glutathione peroxidase (GPx) activity

The GPx analysis was determined according to Wendel [67]. The samples were mixed in medium containing 2 mM GSH, 0.15 U/mL glutathione reductase, 0.4 mM azide, 0.5 mM tert-butylhydroperoxide, and 0.1 mM nicotinamide adenine dinucleotide phosphate (NADPH). The decay of the NADPH was monitored at 340 nm. One GPx unit was defined as 1 μM of NADPH consumed per minute. The GPx activity was presented in GPx units per milligram of protein.

Western blot analyses

Initially, cerebral cortex was homogenized in lysis solution (2 mM EDTA, 50 mM tris-hydrochloric acid (HCl), pH 6.8, and 4% SDS). Then, samples were dissolved 1:1 in Laemmli buffer 2×containing 40% glycerol, 5% 2-mercaptoethanol, 50 mM tris–HCl, pH 6.8, and 10% SDS and boiled for 5 min. Proteins (30 μg) were separated in polyacrylamide gel (10%) by electrophoresis. Proteins were transferred (Trans-Blot SD Semi-Dry Transfer Cell, Bio-Rad) to a nitrocellulose membrane (1 h at 15 Voltz) in a transfer buffer (48 mM trizma, 39 mM glycine, 20% methanol, and 0.25% SDS). The blot was washed for 10 min in Tris-buffered saline plus 0.05% Tween 20 (TBST). After, the blot was blocked with blocking buffer (milk diluted in TBS plus 0.05% tween 20) for 2 h. The blot was rewashed for 10 min in TBST. Afterward, the blot was incubated with anti-iNOS (1:500; Sigma-Aldrich), anti-synapsin I (1:1000; sigma), and anti-β-actin (1:1000, Cell Signaling Technology) in TBST overnight at 4 °C. Finally, it was incubated with secondary antibodies, horseradish peroxidase anti-rabbit IgG (1:1000, Cell Signaling Technology) or anti-mouse IgG (1:1000; Cell Signaling Technology) in TBST for 2 h. The blot was developed using a chemiluminescence kit (Thermo Fisher) and detected by ImageQuant LAS 4000 (GE Healthcare Life Sciences). The bands were normalized by β-actin using Image J [68].

RNA isolation and real-time quantitative polymerase chain reaction (PCR)

The gene expression of TNF-α, IL-1β, IL-6, IL-10, MCP-1, and iNOS in the cerebral cortex was evaluated. For the extraction of total RNA from the cerebral cortex, the TRIzol® reagent (Thermo Fisher Scientific) was used according to the manufacturer's instructions. RNA samples were quantified, and the purity was assessed in a NanoDrop BioChrom apparatus (Harvard Bioscience). The synthesis of complementary DNA (cDNA) was performed with 1 μg of total RNA using the High Capacity Reverse Transcription Kit with RNase Inhibitor (Thermo Fisher Scientific) according to the manufacturer's instructions in a Master Cycler Gradient thermocycler (Eppendorf). The real-time

Table 1 Primer sequences for real-time quantitative polymerase chain reaction (PCR) analysis

Gene		Sequence (5′-3′)
Actb	Sense	ACC CAC ACT GTG CCC ATC TAT G
Actb	Antisense	AGA GTA CTT GCG CTC AGG AGG A
TNF-α	Sense	GGT CCC CAA AGG GAT GAG AAG TTC
TNF-α	Antisense	CCA CTT GGT GGT TTG CTA CGA CG
IL-1β	Sense	TTC AGG CAG GCA GTA TCA CTC
IL-1β	Antisense	CCA CGG GAA AGA CAC AGG TAG
IL-6	Sense	CTG CAA GAG ACT TCC ATC CAG
IL-6	Antisense	AGT GGT ATA GAC AGG TCT GTT GG
IL-10	Sense	GCT GGA CAA CAT ACT GCT AAC C
IL-10	Antisense	ATT TCC GAT AAG GCT TGG CAA
MCP-1	Sense	CCA ACC ACC AGG CTA CAG G
MCP-1	Antisense	GCG TCA CAC TCA AGC TCT G
iNOS	Sense	CCT CCT CCA CCC TAC CAA GT
iNOS	Antisense	CAC CCA AAG TGC TTC AGT CA

Actb actin β, *IL* interleukin, *iNOS* inducible nitric oxide synthase, *MCP*-1 monocyte chemoattractant protein, *TNF-α* tumor necrosis factor

quantitative PCR reactions (RT-qPCR) were performed using the Master Mix SYBR Green PCR (Applied Biosystems) in a StepOnePlus real-time PCR system (Applied Biosystems). The reactions were performed in a final volume of 10 µL, using 2 µL of diluted cDNA (1:10) and 300 nM of each of the primer forward and reverse. The relative gene expression was calculated using the comparative cycle threshold (Ct) method (ΔΔCt). The β-actin gene (Actb) was used as the endogenous control. The results are presented as relative expression of the gene of interest/Actb. The primer sequences are provided in Table 1.

Protein determination

The protein concentration was determined by the method of Lowry and colleagues [69] using bovine serum albumin as the standard.

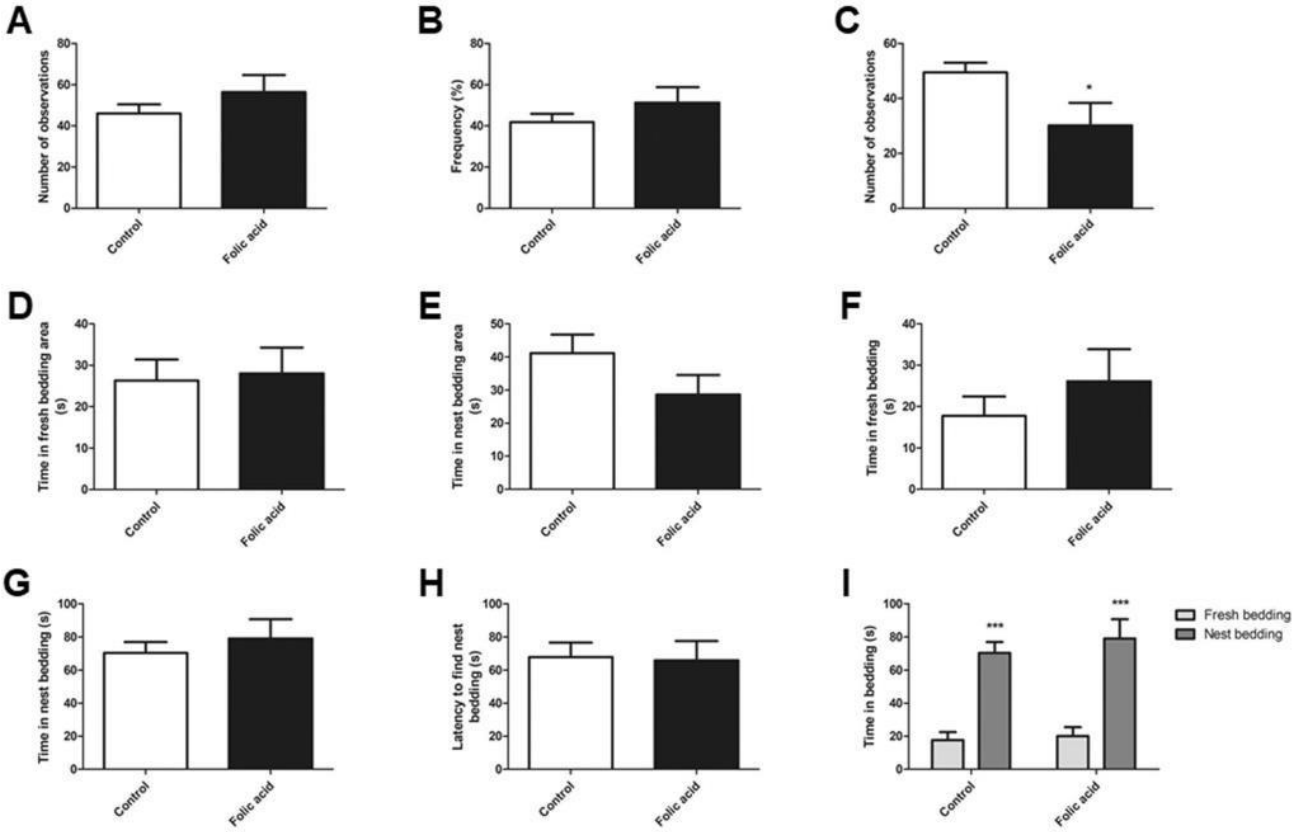

Fig. 2 Effect of folic acid supplementation during pregnancy on maternal and offspring behavior: maternal care score (**A**), frequency of maternal care (**B**), and observations of the dams off the nest (**C**). Olfactory preference test for familiar odors in the offspring: time in fresh bedding area (**D**), time in nest bedding area (**E**), time in fresh bedding (**F**), time in nest bedding (**G**), latency to find nest bedding (**H**), and time in bedding (**I**). Results are expressed as mean ± SEM for 11–14 animals in each group. Different from control, $*p < 0.05$ and $***p < 0.001$ (Student's *t* test)

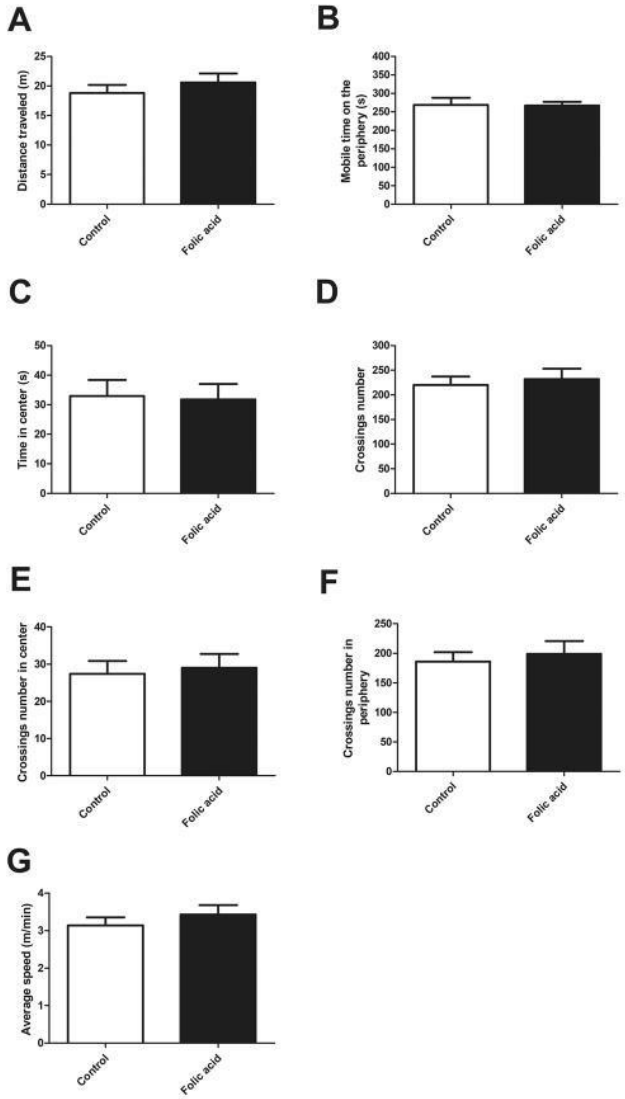

Fig. 3 Effect of folic acid supplementation during pregnancy on open field test: distance traveled (**A**), mobile time on the periphery (**B**), time in the center (**C**), crossings number (**D**), crossings number in the center (**E**), crossings number in the periphery (**F**), and average speed (**G**). Results are expressed as mean ± SEM for 11–14 animals in each group. Different from control, $p > 0.05$ (Student's t test)

Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM). The Shapiro–Wilk normality test was used to evaluate the normal distribution of the data. The Student's t test was used to evaluate the different parameters, and the adopted significance level was $p \leq 0.05$. Statistical analysis and graphs were performed using GraphPad Prism Software, version 5 (GraphPad Software, San Diego).

Results

Behavioral assessments

To identify possible influences of folic acid supplementation in maternal care, maternal behavior was analyzed. We did not observe differences between control and folic acid groups in total maternal care score [$t(12) = 1.965$; $p > 0.05$] (Fig. 2A). Also, there were no significant differences in the frequency of maternal care [$t(12) = 1.964$; $p > 0.05$] (Fig. 2B). However, dams that received supplementation with folic acid during pregnancy were seen less often outside the nest when compared to dams that received a standard diet [$t(13) = 2.689$; $p < 0.05$] (Fig. 2C).

Afterward, we evaluated the effect of folic acid supplementation during pregnancy on the olfactory preference of the pups at 10 postnatal days. Pups rodents naturally learn to identify and prefer the nest odor [70]. We did not observe differences between control and folic acid groups in time in fresh bedding area [$t(48) = 1.265$; $p > 0.05$] (Fig. 2D), time in nest bedding area [$t(48) = 1.670$; $p > 0.05$] (Fig. 2E), time in fresh bedding [$t(49) = 1.350$; $p > 0.05$] (Fig. 2F), time in nest bedding [$t(49) = 1.573$; $p > 0.05$] (Fig. 2G), and latency to find nest bedding [$t(49) = 1.126$; $p > 0.05$] (Fig. 2H). On the other hand, we identify an increase in time spent in nest bedding (Fig. 2I) in the control and folic acid groups [$F(1) = 57{,}95$, $p < 0.001$], but no difference among the groups.

Open field test was performed at 21 postnatal days to evaluate the locomotion, exploratory activity, and habituation of animals. No differences between the control and folic acid groups were observed in the parameters analized in this test (Fig. 3A–G).

Next, we evaluated the performance of animals on long-term object recognition memory. No differences were found between control and folic acid on the training day in total exploration time [$t(21) = 1.953$; $p > 0.05$] (Fig. 4A) and time spent with each object [$t(2) = 128.3$; $p > 0.05$] (Fig. 4B). Furthermore, the results showed that the animals from the folic acid group showed a longer total exploration time of objects [$t(21) = 1.262$; $p < 0.05$] (Fig. 4C) on the test day when compared to the control group. We did not verify differences between control and folic acid groups in time spent with each object on the test day [$t(2) = 78.98$; $p > 0.05$] (Fig. 4D). Finally, we observed a reduction in the object recognition index [$t(21) = 1.504$; $p > 0.05$] (Fig. 4E) in the folic acid group when compared to the control group. Afterward, we decided to analyze synapsin I immunocontent in the cerebral cortex of offspring. Synapsin I is associated with synaptogenesis regulation and modulates neurotransmitter release [71]. We did not identify differences between control and folic acid groups in synapsin I immunocontent [$t(13) = 1.327$; $p > 0.05$] (Fig. 4F).

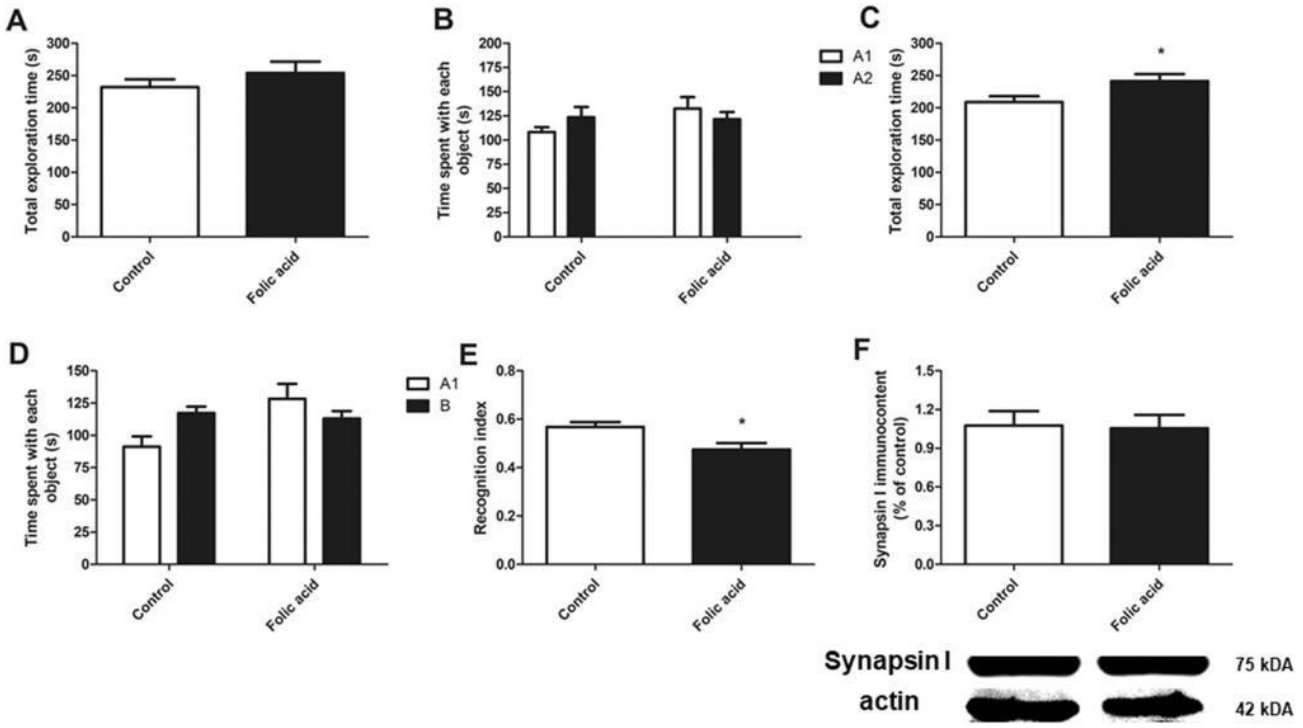

Fig. 4 Effect of folic acid supplementation during pregnancy on object recognition memory: total exploration time during the training (**A**), time spent with each object during the training (**B**), total exploration time during the test (**C**), time spent with each object during the test (**D**), object recognition index (**E**). Synapsin immunocontent (**F**) in the cerebral cortex: all lanes received equivalent amounts (30 μg) of total protein from tissue extract. The blots were developed using an ECL kit. Uniformity of gel loading was confirmed with β-actin as the standard since its level is not affected by the experimental treatment. Representative immunological reactions are shown below. Results are expressed as mean ± SEM for 11–14 animals (object recognition task) or 8 animals (synapsin immunocontent) in each group. Different from control, $*p < 0.05$ (Student's *t* test)

Subsequently, we analyzed anxiety-like behavior in the elevated plus maze. We observed that folic acid group presented a decrease in total number entries [$t(38) = 1.287$; $p < 0.01$] (Fig. 5A), entries in open arms [$t(38) = 1.190$; $p < 0.001$] (Fig. 5B), time spent in open arms [$t(38) = 1.050$; $p < 0.001$] (Fig. 5C), and percentage of the time spent in open arms [$t(38) = 1.055$; $p < 0.001$] (Fig. 5D) when compared to the control group. Moreover, we showed that the folic acid group presented an increase in time spent in closed arms [$t(21) = 1.667$; $p < 0.01$] (Fig. 5E) when compared to the control group. No differences were found between control and folic acid groups in entries in closed arms [$t(38) = 1.317$; $p > 0.05$] (Fig. 5F), time spent in the neutral zone [$t(38) = 1.682$; $p > 0.05$] (Fig. 5G), and time spent in open arms/entries in open arms [$t(38) = 2.143$; $p > 0.05$] (Fig. 5H).

Light–dark transition test, a tool to analyze anxiety-like behavior, is based on the innate aversion of rodents to brightly illuminated areas and their tendency to exhibit spontaneous locomotor behavior in response to novel environments. We did not find differences between the groups in this behavioral test (Fig. 6A–C).

In the ERDT, a test to examine the levels of depression and motivation to eat after food deprivation, we identify an increase in latency for the first bite [$t(15) = 2.455$; $p < 0.01$] (Fig. 6D) in the folic acid group when compared to control group.

Subsequently, the ladder walking test was used to assess walking ability and to measure both forelimb and hind limb placement, stepping, and inter-limb coordination of animals. We showed that the folic acid group presented an increase in time to the cross ladder [$t(14) = 27.37$; $p < 0.01$] (Fig. 7A) on training when compared to control group. We also observed an increase in folic acid group in time to cross ladder [$t(15) = 9.142$; $p < 0.05$] (trial 2; Fig. 7C), average time to cross ladder [$t(15) = 1.520$; $p < 0.05$] (average of the 3 trials; Fig. 7E), number of errors [$t(15) = 1.091$; $p < 0.05$] (Fig. 7G), and percentage of errors [$t(15) = 1.186$; $p < 0.05$] (Fig. 7I) on testing when compared to control group. No differences were found between control and folic acid groups in time to cross ladder [$t(14) = 1.567$; $p > 0.05$] (trial 1; Fig. 7B), time to cross ladder [$t(15) = 1.087$; $p > 0.05$] (trial 3; Fig. 7D), percentage of correct steps [$t(15) = 1.645$; $p > 0.05$] (Fig. 7F), and number of errors/number of steps

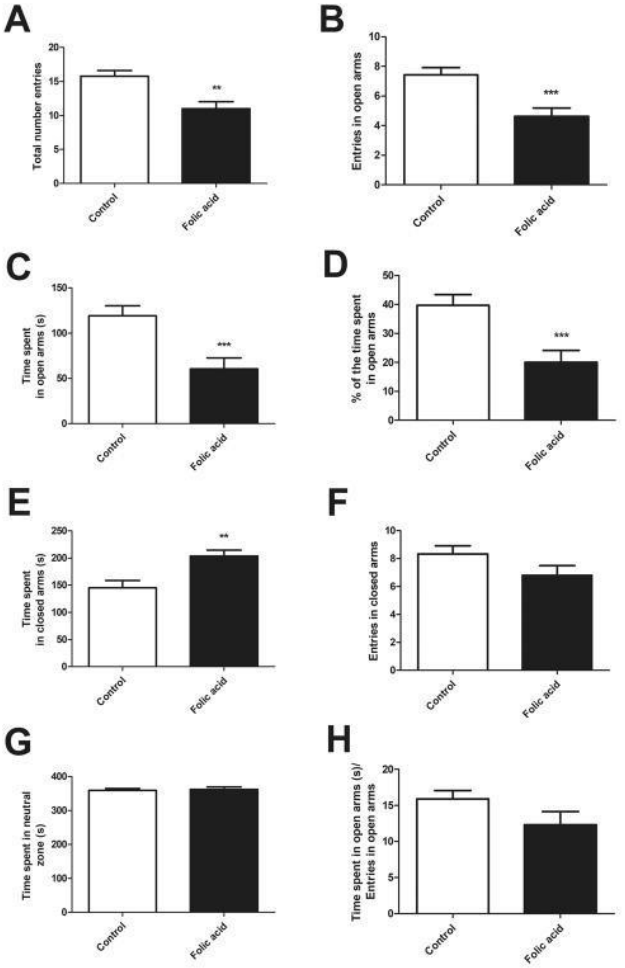

Fig. 5 Effect of folic acid supplementation during pregnancy on elevated plus maze: total number entries (**A**), entries in open arms (**B**), time spent in open arms (**C**), percentage of the time spent in open arms (**D**), time spent in the closed arms (**E**), entries in the closed arms (**F**), time spent in the neutral zone (**G**), and time spent in the open arms/entries in the open arms (**H**). Results are expressed as mean ± SEM for 11–14 animals in each group. Different from control, $^{**}p < 0.01$ and $^{***}p < 0.001$ (Student's *t* test)

[$t(15) = 1.384$; $p > 0.05$] (Fig. 7H) on testing. Likewise, no differences were detected between control and folic acid groups in the beam balance test (Fig. 8A–D), which is used to assess gait abnormality, gross motor ability, and gross vestibular motor function.

Finally, the rotarod test was performed to evaluate the coordinate walking, balance abilities, and vestibulomotor function of animals. We verify that the folic acid group presented an increase in the number of falls [$t(14) = 3.550$; $p < 0.01$] (testing: day 1) (Fig. 8F) when compared to control group. Results did not evidence differences between control and folic acid groups in number of falls [$t(15) = 1.486$; $p > 0.05$] (training) (Fig. 8E), number of falls [$t(15) = 0.1385$; $p > 0.05$] (testing: day 2) (Fig. 8G), the latency of the first downfall [$t(15) = 1.496$; $p > 0.05$] (testing: day 1) (Fig. 8H), the latency of the first downfall [$t(15) = 0.8507$; $p > 0.05$] (testing: day 2) (Fig. 8I), time on the rotarod [$t(15) = 1.229$; $p > 0.05$] (testing: day 1) (Fig. 8J), and time on the rotarod [$t(15) = 0.5284$; $p > 0.05$] (testing: day 2) (Fig. 8K).

Biochemical assessments

To evaluate the presence of oxidative stress caused by the treatment with folic acid in pregnancy, we measured oxidative/nitrative and antioxidant parameters in the cerebral cortex of offspring. Initially, we demonstrated in the folic acid group a decrease in reactive species levels [$t(15) = 3.346$; $p < 0.01$] (Fig. 9A) in the cerebral cortex when compared to control group. We also did not identify differences between control and folic acid groups in TBARS levels [$t(16) = 0.7543$; $p > 0.05$] (Fig. 9B) and sulfhydryl content [$t(16) = 0.6635$; $p > 0.05$], an indicator of lipid peroxidation and protein damage, respectively (Fig. 9C). No differences were found between control and folic acid groups in GSH content [$t(16) = 1.454$; $p > 0.05$] (Fig. 9D) in the cerebral cortex. Next, we analyze the antioxidant enzyme activities. No differences were found between control and folic acid groups on the activity of SOD [$t(16) = 1.509$; $p > 0.05$] (Fig. 9E), CAT [$t(16) = 0.6592$; $p > 0.05$] (Fig. 9F), GPx [$t(16) = 0.3296$; $p > 0.05$] (Fig. 9G), and SOD/CAT ratio [$t(16) = 0.6592$; $p > 0.05$] (Fig. 9H).

Afterward, we analyze nitrites levels and iNOS expression and immunocontent in the cerebral cortex of offspring. We showed an increase in nitrites levels [$t(16) = 6.391$; $p < 0.001$] (Fig. 10A) in the folic acid group when compared to control group. In contrast, treatment with folic acid during pregnancy did not alter iNOS expression [$t(10) = 0.2769$; $p > 0.05$] (Fig. 10B) and immunocontent [$t(14) = 0.2529$; $p > 0.05$] (Fig. 10C) in cerebral cortex of offspring.

Finally, to verify the presence of neuroinflammation caused by the treatment with folic acid in pregnancy, we evaluated cytokines and a chemokine expression in the cerebral cortex of offspring. We identify an increase in TNF-α [$t(9) = 2.794$; $p < 0.05$] (Fig. 11A), IL-1β [$t(10) = 2.804$; $p < 0.05$] (Fig. 11B), IL-6 [$t(9) = 8.264$; $p < 0.001$] (Fig. 11C), IL-10 [$t(9) = 4.426$; $p < 0.01$] (Fig. 11D), and MCP-1 [$t(16) = 6.391$; $p < 0.05$] (Fig. 11E) gene expression in the cerebral cortex.

Discussion

The present study was proposed to investigate the effects of folic acid supplementation throughout pregnancy on maternal care, olfactory preference, motor activity, exploratory capacity, habituation, memory, anxiety-like behavior,

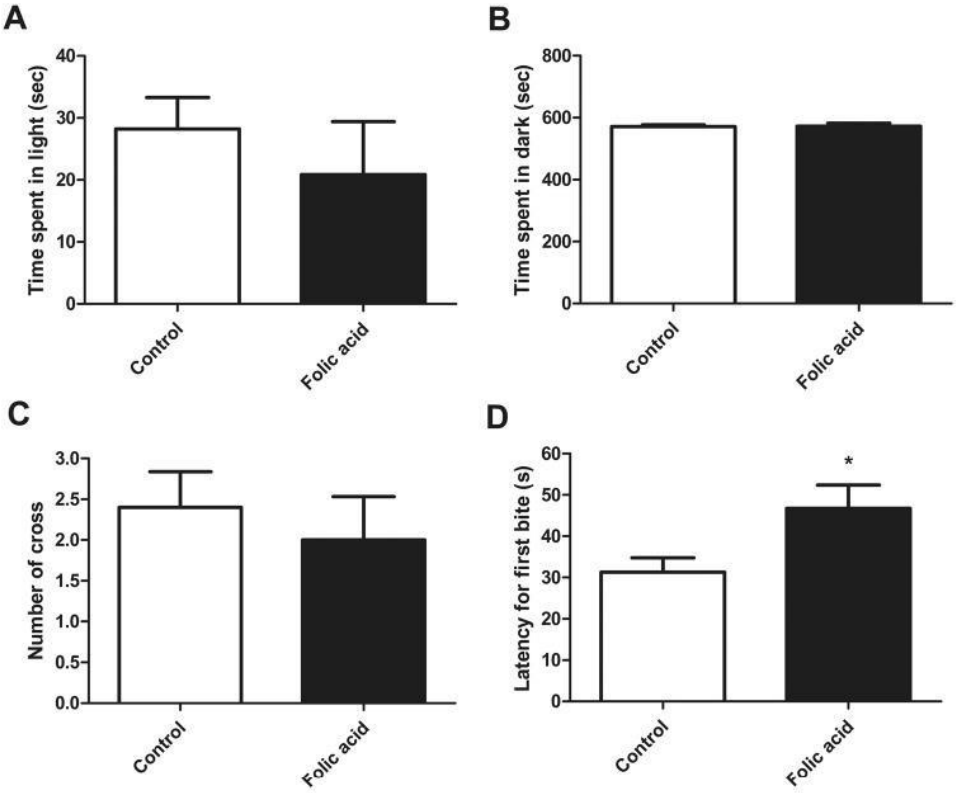

Fig. 6 Effect of folic acid supplementation during pregnancy on light–dark transition test: time spent in the light box (**A**), time spent in the dark box (**B**), and number of cross in the light–dark box (**C**). Eating-related depression test: latency for first bite (**D**). Results are expressed as mean ± SEM for 11–14 animals in each group. Different from control, $*p < 0.05$ (Student's *t* test)

depression, motricity, redox homeostasis, and neuroinflammatory status of the male rat offspring. Folic acid supplementation did not impair the mother–pup relationship. Our findings revealed an impairment in memory, motricity, and deficient motor learning of the offspring supplemented with folic acid throughout pregnancy. We also showed an increase in anxiety- and depression-like behavior in this group. We observed that intake of folic acid by pregnant rats resulted in an increase in nitrative stress and neuroinflammation in the cerebral cortex of the offspring. These results are socially relevant due to their correlation with pregnant women who widely supplement with folic acid throughout pregnancy.

Evidence has shown that diet during gestation may alter maternal care and consequently impact the neurodevelopment of offspring [72–75]. We did not observe differences between control and folic acid groups in total maternal care score and frequency of maternal care. However, dams of the folic acid group spent less time off the nest when compared to control group, indicating that they spent more time with the offspring. Evidence has shown the important role of smell in the onset, maintenance of maternal care, and offspring survival [76]. The performance of the offspring on the olfactory preference test was not affected by folic acid supplementation during gestation. According to previous studies, maternal care influence significantly nest odor preference [46, 77], we hypothesize that we did not observe an alteration in nest odor preference due to maternal care have not being harmed. Thus, in our study, we showed that folic acid supplementation throughout pregnancy did not impair the mother–pup relationship.

Nutritional status during pregnancy has a significant impact on the offspring's neurological development and cognitive functions [78–81]. We observed that folic acid supplementation throughout pregnancy caused long-term memory impairment. Bahous and colleagues [9] showed that high folate intake during pregnancy leads to pseudo-methylenetetrahydrofolate reductase (MTHFR) deficiency, disturbed choline/methyl metabolism, disturbance in acetylcholine metabolism, decreased hippocampal volume, and memory impairment in offspring. In this context, studies have identified that supplementation of folic acid during pregnancy causes a decrease in the levels of neurotrophins such as neural growth factor (NGF) and brain-derived neurotrophic factor (BDNF) in the offspring [33, 34]. Moreover, Brocardo and colleagues [82] observed that folic acid is capable of inhibiting N-methyl-D-aspartate (NMDA) receptors. The NMDA receptors are involved in the formation of long-term memory [83, 84], we can presuppose that supplementation of folic acid during pregnancy can cause inhibition of these receptors and affect the memory of the offspring. Based upon the novel-object recognition test results and in order to better understand the memory deficit observed in the offspring, we decided to analyze synapsin I immunocontent in the cerebral cortex. We did not identify differences between

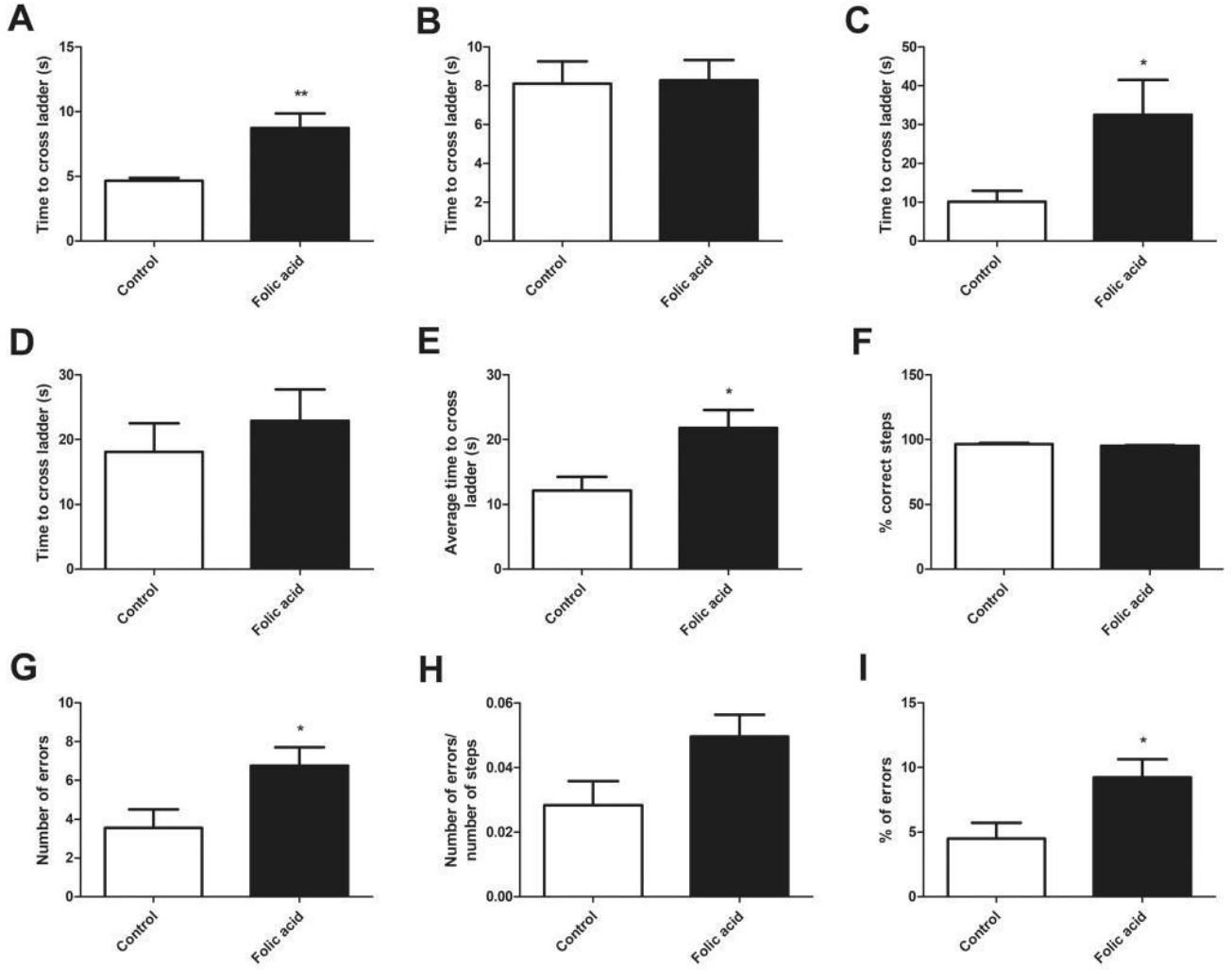

Fig. 7 Effect of folic acid supplementation during pregnancy on ladder walking test: time to cross ladder during the training (**A**), time to cross ladder during the test — trial 1 (**B**), time to cross ladder during the test — trial 2 (**C**), time to cross ladder during the test-trial 3 (**D**), average time to cross ladder during the test — average of the 3 trials (**E**), percentage of correct steps (**F**), number of errors (**G**), number of errors/number of steps (**H**), and percentage of errors (**I**). Results are expressed as mean ± SEM for 11–14 animals in each group. Different from control, $*p<0.05$ (Student's *t* test)

control and folic acid groups in synapsin I immunocontent. Thus, we showed that folic acid supplementation during pregnancy impairs the long-term memory independent of synapsin I immunocontent in the offspring.

Epidemiological studies in humans have shown that individuals subjected to an adverse environment during pregnancy have an increased risk of developing psychiatric disorders [85, 86]. We identified that folic acid group presented anxiety-like behavior in the elevated plus-maze test but did not in the light–dark transition test. Similar to our results, Matsumoto and colleagues [87] demonstrated that the offspring of rats treated with folic acid presented an increase in anxiety-like behavior, evidenced by the decrease in the number of entries in the open arms. In contrast to our results, Barua and colleagues [8] did not demonstrate significant alterations in the elevated plus-maze test in the offspring of mice that received folic acid during pregnancy. Curiously some studies in humans have observed that folate deficiency during pregnancy has been associated with anxiety [88, 89]. Our results of the assessment of anxiety-like behavior are in agreement with previous studies that showed that different anxiety tests seem to reflect different levels of anxiety [90].

Exogenous factors such as a diet enriched or deficient in nutrients, such as amino acids and vitamins can modify the synthesis of neurotransmitters and can contribute to the development of depression [91]. We identify an increase in latency for the first bite in the folic acid group when compared to control group. This test assesses anhedonia in a situation where there is a conflict between food reward and anxiety [92]. Zheng and colleagues [93] identify that total folate

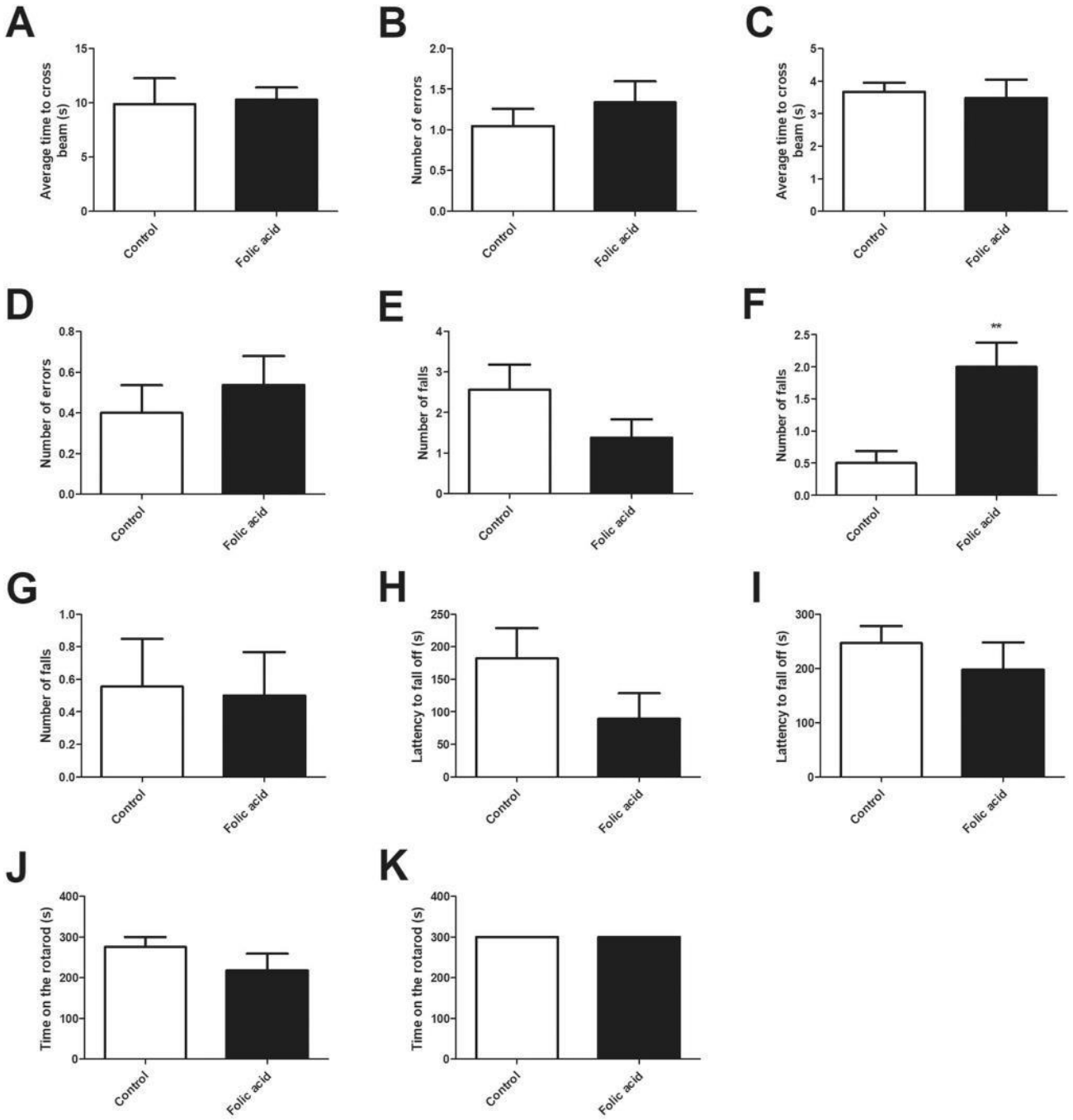

Fig. 8 Effect of folic acid supplementation during pregnancy on beam balance test: average time to cross beam during the training (**A**), number of erros during the training (**B**), average time to cross beam during the test (**C**), and number of errors during the test (**D**). Rota rod test: number of falls during the training (**E**), number of falls during test — day 1 (**F**), number of falls during test — day 2 (**G**), latency of the first downfall during test — day 1 (**H**) latency of the first downfall — day 2 (**I**), time on the rotard during the test — day 1 (**J**), and time on the rotard during the test — day 2 (**K**). Results are expressed as mean ± SEM for 11–14 animals in each group. Different from control, $^{**}p < 0.01$ (Student's *t* test)

intake and natural folate intake were inversely associated with depressive symptoms, while synthetic folic acid was not. In contrast to our results, Matsumoto and colleagues [87] demonstrated that the offspring of rats supplemented with folic acid during gestation did not alter the motivation for food intake. Studies in patients with neuropsychiatric disorders, folate deficiency has been associated with low levels of serotonin in the cerebrospinal fluid and folic acid

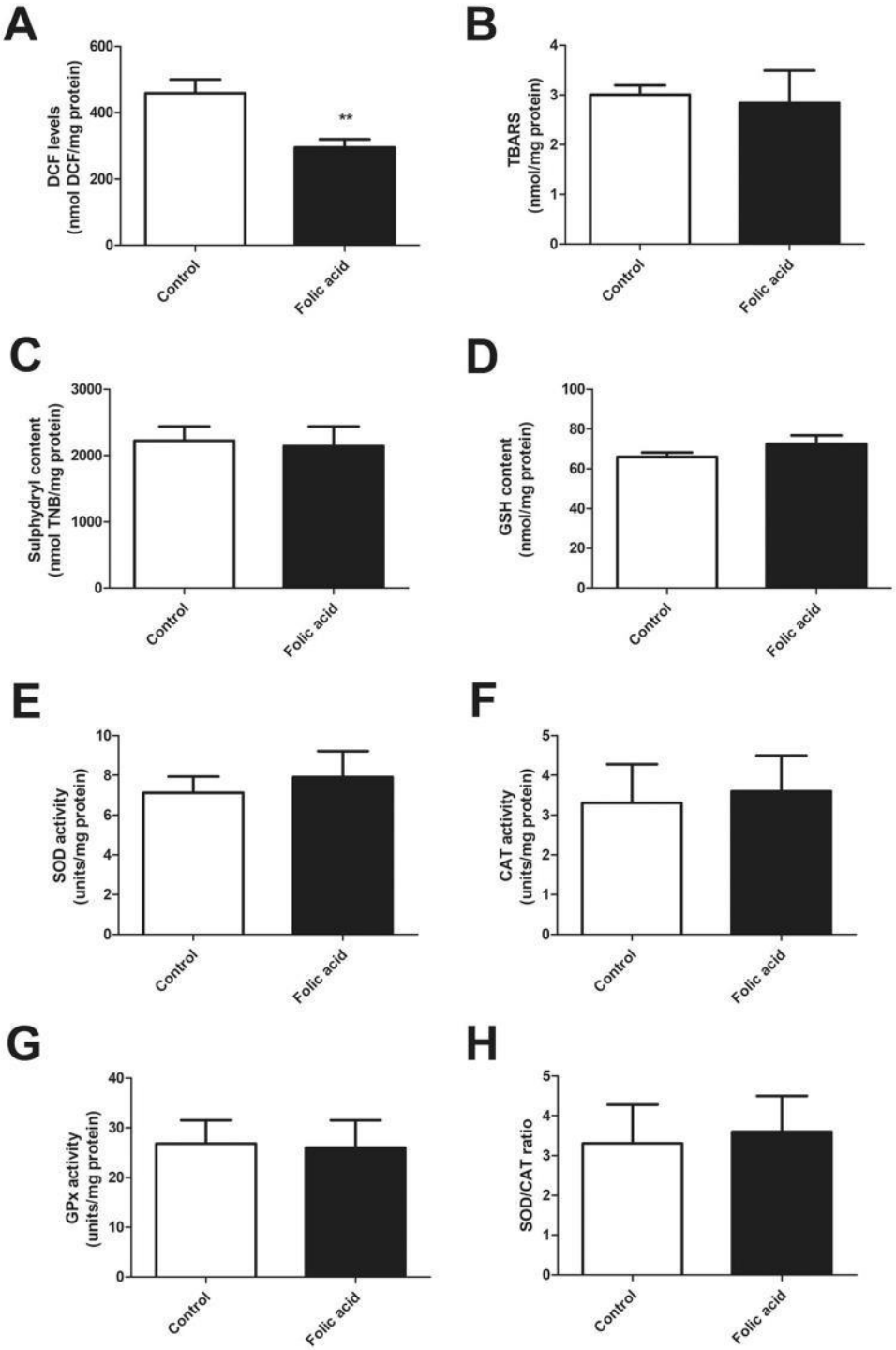

Fig. 9 Effect of folic acid supplementation during pregnancy on oxidative stress: DCF levels (**A**), TBARS levels (**B**), sulphydryl content (**C**), GSH content (**D**), SOD activity (**E**), cat activity (**F**), GPx activity (**G**), SOD/CAT ratio (**H**). Results are expressed as mean ± SEM for 9 animals in each group. Different from control, $p > 0.05$ (Student's *t* test). *CAT* catalase, *DCF* dichlorofluorescein, *GPx* glutathione peroxidase, *GSH* reduced glutathione, superoxide dismutase, *TBARS* Thiobarbituric acid reactive substances

supplementation restored levels of this neurotransmitter [94, 95]. In summary, results in the literature about folic acid supplementation and depression are conflicting, and further studies should be performed about this topic mainly during pregnancy.

Clinical and preclinical studies have shown that pregnancy diet can impact offspring's motor function [5]. In the ladder walking test, we observed an increase in folic acid group in time to cross ladder number of errors, and percentage of errors when compared to control group. In the rotarod test, we verified that the folic acid group presented an increase in the number of falls (testing: day 1) when compared to control group. However, we did not observe differences between control and folic acid groups in the number

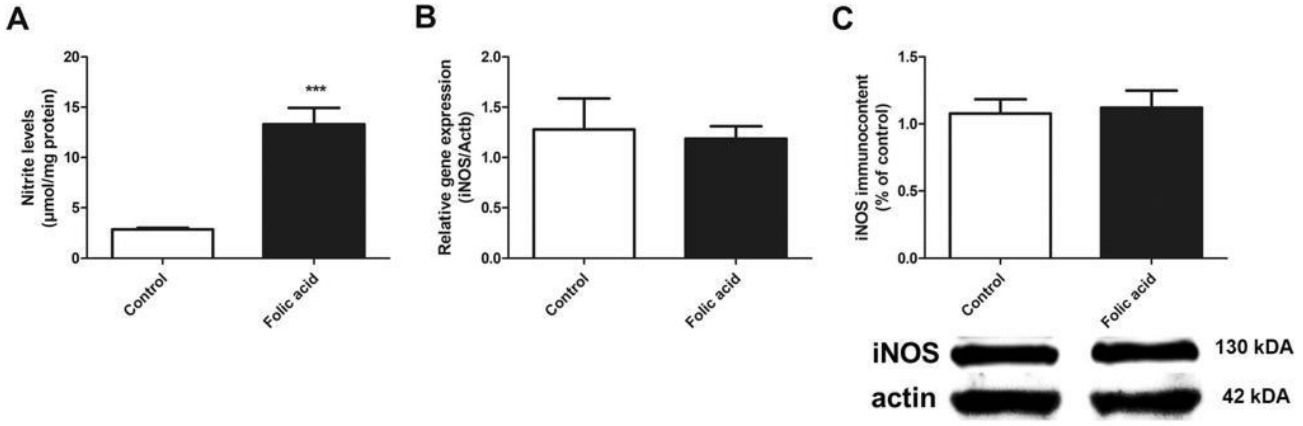

Fig. 10 Effect of folic acid supplementation during pregnancy in the cerebral cortex on nitrite levels (**A**). Relative gene expression of iNOS (**B**). The β-actin was used as the endogenous control, and the results are expressed as relative expression of the gene of interest/Actb. iNOS immunocontent (**C**). All lanes received equivalent amounts (30 µg) of total protein from tissue extract. The blots were developed using an ECL kit. Uniformity of gel loading was confirmed with β-actin as the standard since its level is not affected by the experimental treatment. Representative immunological reactions are shown below. Results are expressed as mean ± SEM for 9 animals (nitrite levels), 6 animals (iNOS gene expression), or 8 animals (iNOS immunocontent) in each group. Different from control, $***p<0.001$ (Student's *t* test). *Actb*:actin β, *iNOS* inducible nitric oxide synthase

of falls (testing: day 2); these results showed that the animals could learn when performing this test. Agreement our results, a prospective cohort study showed that children one year of age born to mothers that reported consuming doses of folic acid of 5000 µg/day or more had less psychomotor development compared to mothers who consumed doses of 400–1000 µg/day [96]. Chu and colleagues [81] showed that male offspring of mice that drank water supplemented with folic acid (3.75 mg/L) had deficient motor learning and an increase in the expression of early growth response 2 (Egr2). Egr2-deficient mice showed improved performance in motor learning in the rotarod [97].

Oxidative/nitrative stress during pregnancy is a condition that occurs mainly because the placenta is rich in mitochondria. During gestation, placental changes from a hypoxic environment to an oxygen-rich environment. Thereat, defense mechanisms against free radical damage increase during pregnancy progresses [98, 99]. We decided to perform biochemical analyses in the cerebral cortex because this region is responsible for several cognitive functions such as memory, decision-making, learning, emotions, and motricity [100]. Firstly, we demonstrated in the folic acid group a decrease in reactive oxygen species levels in the cerebral cortex when compared to control group. Afterward, we showed an increase in nitrites levels in the folic acid group when compared to control group. However, treatment with folic acid in pregnancy did not alter iNOS expression and immunocontent in the cerebral cortex of offspring. An increase in nitrite levels suggests that there may is an increase in the production of reactive nitrogen species. The increase in nitrites observed in the cerebral cortex of animals may be related to the activity of other enzymes that produce nitric oxide, such as nitric oxide neuronal synthase (nNOS) and nitric oxide synthase endothelial (eNOS). Folate can regulate nitric oxide and reactive oxygen species production [101]. The iNOS formation depends on the presence of the cofactor tetrahydrobiopterin (BH4) and available substrate to couple the oxidation of L-arginine to the reduction of molecular oxygen to produce nitric oxide [24]. Folate-dependent dihydrofolate reductase (DHFR) has a role in reducing BH2 back to BH4 via the BH4 salvage pathway [102]. Decrease in BH4 formation, leading to enzymatic uncoupling of iNOS and increased reactive oxygen species production [102, 103]. The 5-methyl-tetrahydrofolate may bind to the active site of iNOS and mimic the BH4's action in inducing oxid nitric production and reducing ROS production [104]. Folate regulates ROS production indirectly via the prevention of iNOS uncoupling. Moreover, folate may reduce ROS production by acting directly as an antioxidant [105, 106]. These mechanisms may partially explain the increase in the nitrite levels and decrease in the ROS production in the cerebral cortex of the offspring of rats that consumed folic acid during pregnancy.

Folic acid supplementation may affect the methylation of anti-inflammatory and pro-inflammatory genes [107]. Thereat, studies have shown that the relationship between folate and inflammation is dependent on the timing of intervention, dosage, methylated genes, and interaction with the environment [24]. We identify an increase in TNF-α, IL-1β, IL-6, IL-10, and MCP-1 gene expression in the cerebral cortex in the folic acid group when compared to control group. The inflammatory cytokines TNF-α, IL-1β, and

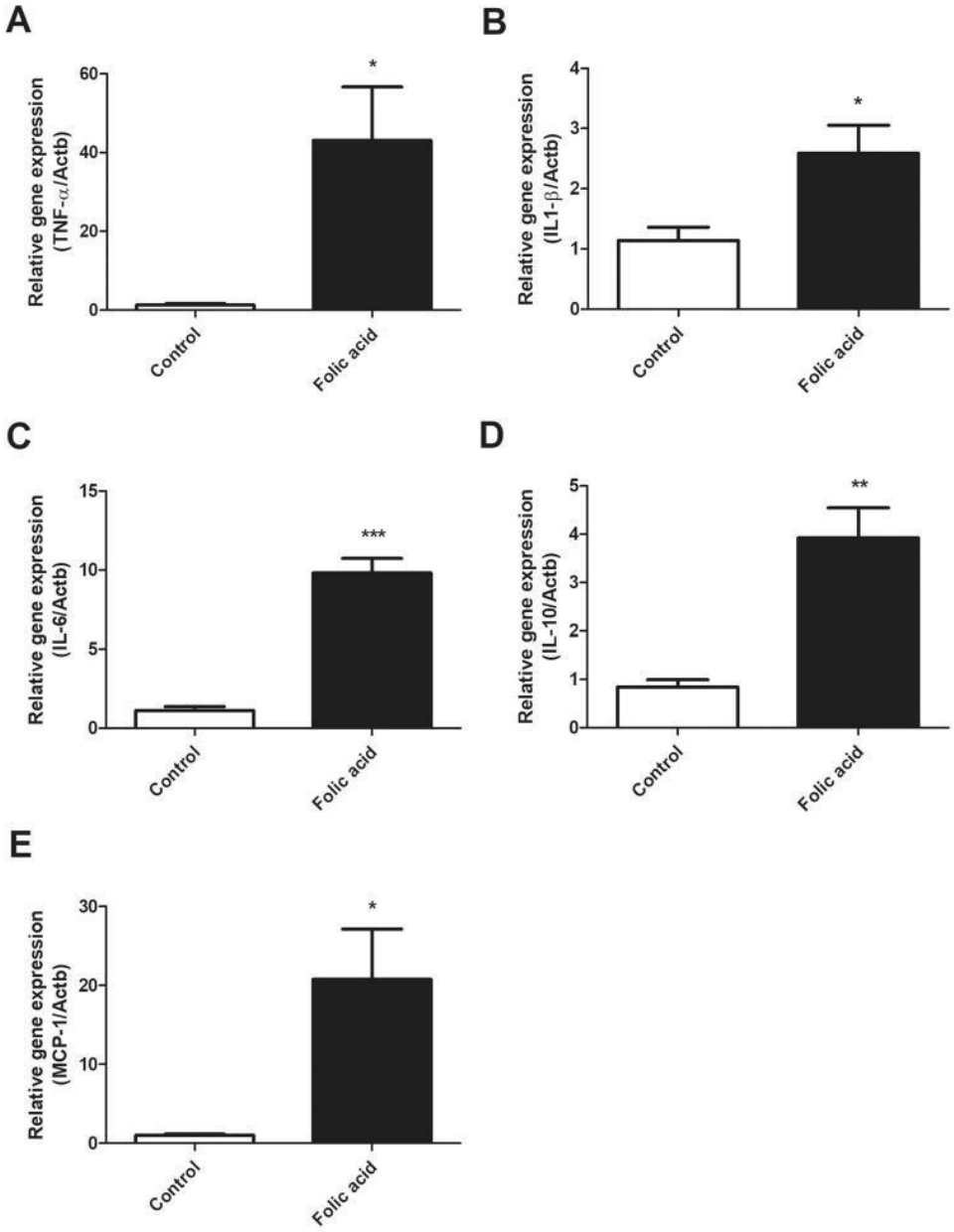

Fig. 11 Effect of folic acid supplementation during pregnancy in the cerebral cortex on neuroinflammation: relative gene expression of TNF-α (**A**), relative gene expression of IL-1β (**B**), relative gene expression of IL-6 (**C**), relative gene expression of IL-10 (**D**), and relative gene expression of MCP-1 (**E**). The Actb was used as the endogenous control, and the results are expressed as relative expression of the gene of interest/Actb. Results are expressed as mean ± SEM for 5–6 animals in each group. Different from control, $*p<0.05$, $**p<0.01$, and $***p<0.001$ (Student's *t* test). *Actb* actin β, *IL* interleukin, *MCP*-1:monocyte chemoattractant protein, *TNF-α* tumor necrosis factor

IL-6 are produced by mainly microglia cells in response to homeostatic unbalance in central nervous system [108]. The cytokine TNF-α decreases uptake of glutamate by astrocytes and may increase excitotoxicity. The cytokine IL-1β secretion is strongly related to the exacerbation of neurodegenerative disease [109]. Neuronal MCP-1 is essential for the chemoattraction of monocytes to the site of inflammation in central nervous system [110]. In our study, we observed an increase in inflammatory as well as anti-inflammatory cytokines. An increase in the anti-inflammatory cytokine IL-10 can be due to a compensatory mechanism in order to decrease the inflammatory process. Based on our results, we suggest that the increase in neuroinflammation observed in the cerebral cortex can be associated with behavioral impairment of the offspring.

Some limitations should be taken into account in this study. First and foremost, due to the species difference, our results should not be directly extrapolated to humans. Secondly, we used only one dose of folic acid. Higher doses could cause other changes not observed in this study. Thirdly in our study, we analyze only on the male offspring. We choose to perform our study with male rats because female rats are more variable than males due to the fluctuation of hormones across their estrus cycle which typically lasts 4 days in rats [111]. Moreover, several studies have shown that the estrus cycle can affect behavior tests [112–115]. In this context, female offspring may present different

responses to folic acid consumption during the gestational period.

In conclusion, the results of the present study showed that changes in maternal micronutrients, such the consumption of folic acid throughout pregnancy, may cause biochemical and behavioral impairments in the offspring. Our findings revealed impairment in memory, motricity, and an increase in anxiety- and depression-like behavior in the offspring supplemented with folic acid throughout pregnancy. We also observed an increase in nitrative stress and neuroinflammation in the cerebral cortex of the offspring. These results reinforce the importance of using folic acid in the dose and period recommended by WHO and consuming a balanced diet during the gestational period, especially nutrients methyl group donors such as folic acid, which may impact the development of the offsprings. This study represents an important step in exploring the effects of folic acid intake by pregnant rats in the offspring since it provides additional evidence that exposure to folic acid can have complex effects on the offspring. In addition, it gives a new basis for understanding the biochemical and behavioral outcomes associated with folic acid exposure throughout pregnancy, which may conduct future studies. Future studies need to focus on analyzing epigenetic changes, sexual dimorphism, and alterations throughout life in the offspring supplemented with folic acid throughout pregnancy.

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Author contribution JSS and ATSW designed the studies. JSS, OVRJ, FS, FSF, FCR, LEBS, and RCS contributed to the data collection. JSS and ATSW analyzed and interpreted the work. JSS and ATSW wrote the manuscript. All authors have read, revised, and approved the final version of this manuscript.

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Data availability The data presented in this study are available on request from the corresponding author.

Declarations

Ethics approval The present study was approved by the Ethics Committee of the Federal University of Rio Grande do Sul, Brazil (No.36416) and followed institutional guidelines, Brazilian Society of Science in Laboratory Animals, and the Guide for the Care and Use of Laboratory Animals adopted by the National Institute of Health (NIH, USA).

Consent to participate Not applicable.

Consent for publication All the authors consent to publish this manuscript in Molecular Neurobiology.

Conflict of interest The authors no competing interest.

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4.4 Capítulo II

High-protein nutrition during pregnancy increases neuroinflammation and homocysteine levels and impairs behavior in male adolescent rats offspring

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High-protein nutrition during pregnancy increases neuroinflammation and homocysteine levels and impairs behavior in male adolescent rats offspring

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ABSTRACT

Aims: Throughout gestation, proteins in the diet are a source of essential amino acids that are crucial for proper healthy fetal growth and development. The present study was proposed to investigate the effect of high-protein diet consumption throughout pregnancy on redox homeostasis, neuroinflammatory status and amino acid levels, including homocysteine, in the male adolescent rats offspring's cerebral cortex. We also performed a battery of behavioral tests to evaluate maternal care, olfactory preference, exploratory capacity, habituation, memory, anxiety- and depression-like behavior motor activity in the offspring.

Main methods: After pregnancy confirmation, the pregnant rats were randomly divided into two groups, according to the diet: group 1, (control) standard diet containing 20 % protein, and group 2, the high-protein diet containing 50 % protein. Throughout the gestational period, the pregnant rats received experimental diets.

Key findings: Results showed an increase in homocysteine levels and neuroinflammatory mediators in the offspring's cerebral cortex from pregnant rats supplemented with a high-protein diet throughout pregnancy. Besides decreasing histidine levels in offspring's serum. The results also revealed an impairment in memory and motricity and an increase in anxiety-like behavior in the offspring supplemented with a high-protein diet throughout pregnancy. Our findings showed a significant effect of high-protein diet consumption throughout pregnancy on offspring's neurobiochemistry, which can negatively impact behavioral performance.

Significance: Our results reinforce the importance of consuming a balanced diet during the gestational period, especially macronutrients such as proteins since the fetus is sensitive to the mother's diet during pregnancy which may impact the development of the offspring.

Abbreviations: Actb, β-Actin gene; AIN, American Institute of Nutrition; CAT, Catalase; cDNA, Complementary DNA; Ct, Cycle threshold; DCF, Dichlorofuorescein; DOHaD, Developmental Origins of Health and Disease; DTNB, 5,5′-Dithio-bis(2-nitrobenzoicacid); EDTA, Ethylenediamine tetraacetic acid; GPx, Glutathione peroxidase; GSH, Reduced glutathione; H2DCF-DA, 2′7′ dichlorofluorescein diacetate; HPLC, High-Performance Liquid Chromatography; IL, Interleukin; iNOS, Inducible nitric oxide synthase; MCP-1, Monocyte chemoattractant protein 1; NADPH, Nicotinamide adenine dinucleotide phosphate; NIH, National Institute of Health; PCR, Polymerase chain reaction; ROS, Reactive oxygen species; RT-qPCR, Real-time quantitative PCR reactions; SAM, S-adenosylmethionine; SDS, Sodium dodecyl sulfate; SEM, Standard error of the mean; SOD, Superoxide dismutase; TBARS, Thiobarbituric acid reactive substances; TNF-α, Tumor necrosis factor-α.

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1. Introduction

The Developmental Origins of Health and Disease (DOHaD) theory proposes that the perinatal period is critical for mammals. A hostile uterine environment during critical fetal phases of growth and development can impair the offspring's short- and long-term health [1,2]. An imbalanced diet during gestation, both in amount and composition, significantly aggravates the developing fetus as the mother is the sole source of nutrients [3,4]. Environmental influences such as maternal nutrition during pregnancy may induce epigenetic modifications and cause lifelong consequences in offspring, a phenomenon known as fetal programming [5].

Proteins in the diet are a source of essential amino acids that are crucial for proper growth and development [6]. In this context, amino acid profile and transport capacity from mother to fetus is determined by the amount of maternal dietary proteins [6,7]. Adequate protein intake throughout pregnancy is important to ensure healthy maternal and neonatal outcomes [8,9].

High-protein diet intake has increased considerably worldwide [10]. Due to the increase in obesity and overweight population in the last decades, high-protein diets have been developed as a nutritional intervention to decrease excessive body mass and prevent body mass gain [11,12]. High-protein diets cause longer postprandial satiety, increased thermogenesis, gluconeogenesis, ketogenesis, lipolysis, and inhibition of *de novo* lipogenesis [13–15]. Thus, due to its metabolic effects helping to control body mass gain, high protein diets have been ingested by women in childbearing age and throughout pregnancy [16]. On the other hand, although dietary protein is crucial for healthy fetal development, some researchers have shown that a maternal high-protein diet is deleterious for the fetus. A high protein diet consumption during gestation has been related to low birth weight [17,18], increased blood pressure [19,20], and cortisol levels [21]. Previous studies have observed in animal studies that a gestational diet high in protein can lead to fetal growth retardation and subsequently trigger increased fat mass in adulthood [18]. Reports on the impact of high-protein diet intake throughout gestation on offspring's brain and behavior are scarce. This topic should be further investigated.

Considering that a high-protein diet during gestation on the neurological phenotype of the offspring is still unclear, we decided to investigate the effects of maternal high-protein diet consumption throughout pregnancy on neurochemical (redox homeostasis, neuroinflammation and amino acid levels, including homocysteine) and behavioral performance parameters in male adolescent rats offspring. The objective was to test the hypothesis that exposure to maternal high-protein diet consumption throughout pregnancy could cause persistently imprinted effects on brain homeostasis and consequently impairs offspring's behavior.

2. Material and methods

2.1. Ethics statement

The present study was approved by the Ethics Committee of the Federal University of Rio Grande do Sul, Brazil (No.36406) and followed institutional guidelines, Brazilian Society of Science in Laboratory Animals, and the Guide for the Care and Use of Laboratory Animals adopted by the National Institute of Health (NIH, USA). We further attest that all efforts were made to minimize the number of animals used and their suffering.

2.2. Animals and treatment with high-protein in pregnancy

Female and male adult Wistar rats (70–90 postnatal days) were acquired from Centro de Reprodução e Experimentação de Animais de Laboratório (Biochemistry Department, Institute of Basic Health Science, Federal University of Rio Grande do Sul, Brazil). Animals were maintained in a temperature-controlled room (22 ± 1 °C) on a 12/12 light/dark cycle, with free access to food and water available *ad libitum*. Female rats were submitted to daily collect vaginal smears and when the proestrus phase was detected, they were put in a new cage with one male rat. Pregnancy was confirmed by the presence of sperm in the vaginal smear. After pregnancy confirmation, the pregnant rats were randomly divided into two groups, according to the diet: standard diet containing 20 % protein and high-protein diet containing 50 % protein [18]. During the gestational period (around 21 days), the pregnant rats received experimental diets. The supplementation with high-protein diet containing 50 % occurred only in the pregnancy. The diets were isocaloric followed the American Institute of Nutrition (AIN) 93 Purified Diets for Laboratory Rodents (American Institute of Nutrition). After the birth of the offspring, all rat dams and offspring received the standard diet (20 % protein) until the end of the experiments. Offspring male at 10 postnatal days was submitted to olfactory preference test for familiar odors. Male pups were kept with the dams until 21 postnatal days. After weaning, they were re-housed in boxes containing up to four animals. Male pups at 21 postnatal days were submitted to behavioral tests in order to evaluate exploratory abilities, locomotor activity, memory, anxiety, depression, and motricity. Behavioral tests were conducted in the following order: open field test, object recognition memory, elevated plus maze, light-dark transition test, eating-related depression test, ladder walking test, beam balance test, and rotarod. After conducting behavioral tests, animals were euthanized by decapitation without anesthesia, and encephalon was immediately removed followed by dissection of the total cerebral cortex. A total of 70 male rat offspring were used. We used 11–14 animals per group for behavioral analysis. In the biochemical analyzes, we used in the redox homeostasis analyzes 9 animals per group, 5–6 animals per group for gene expression, and 7–8 animals for determination of amino acid and total homocysteine levels. The female animals of the offspring, dams after weaning, and the males used in the mating were euthanized with inhaled isoflurane and discarded respectfully. Fig. 1 shows the summary of the experimental design.

2.3. Biochemical assessments

2.3.1. Determination of amino acid concentrations

Free amino acids in the serum and cerebral cortex were analyzed by High Performance Liquid Chromatography (HPLC) method. Initially, the cerebral cortex was previously homogenized in 0.9 % saline solution (1:5, *w/v*), centrifuged at 800 ×*g* for 10 min at 4 °C, and the supernatant was taken to amino acid determination. Amino acids were quantitatively measured by relating their chromatographic peak area to those obtained from a known standard mixture and to that of internal standard peak area (homocysteic acid) [22].

2.3.2. Total homocysteine levels determination

Total homocysteine levels in serum and cerebral cortex were analyzed as using liquid chromatography electrospray tandem mass spectrometry (LC-MS/MS). Initially, the cerebral cortex was previously homogenized in 0.9 % saline solution (1:5, *w/v*), centrifuged at 800 ×*g* for 10 min at 4 °C, and the supernatant was taken to Total homocysteine determination. After samples reduction and deproteinization, the total homocysteine levels were detected through the transition from the precursor to the production ion (m/z136tom/z90). Homocysteine-d was added as an internal standard [23].

2.3.3. Assay of redox homeostasis parameters

For evaluation of redox homeostasis parameters, the cerebral cortex was homogenized (1:10, *w/v*) in 140 mM potassium chloride and 20 mM sodium phosphate buffer, pH 7.4. The homogenates were centrifuged at 1000 ×*g* for 10 min at 4 °C. The supernatant was immediately collected and used for the measurements of analysis. Biochemical assessments were measured in SpectraMax M5 Microplate Reader (Molecular

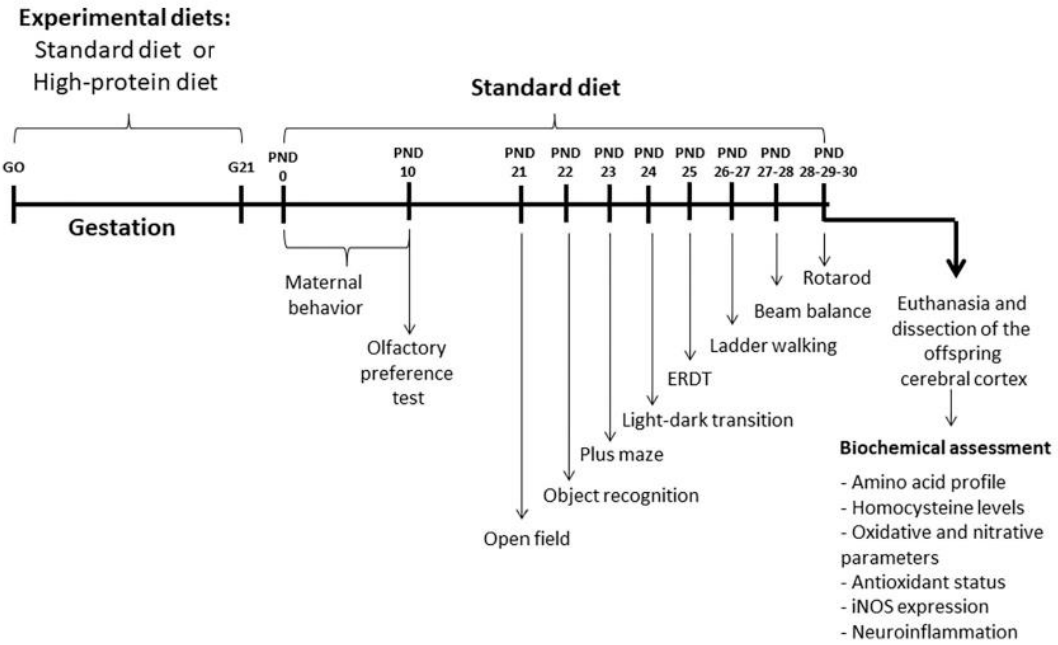


Fig. 1. Timeline of experimental procedures. ERDT: eating-related depression test, G: gestational day, iNOS: inducible nitric oxide synthase, PND: post-natal day.

Devices, USA).

2.3.3.1. 2′7′-Dichlorofluorescein fluorescence assay. Reactive oxygen species (ROS) production in cerebral cortex was determined by the technique based on the oxidation of the 2′7′ dichlorofluorescein diacetate (H2DCF-DA) described by LeBel and colleagues [24]. The samples were incubated in a medium containing 100 μM H2DCF-DA. Then the reaction produces a fluorescent compound, dichlorofluorescein (DCF), which was determined at 488 nm of excitation and 525 nm of emission. The data were plotted as nM DCF per milligram of protein.

2.3.3.2. Nitrites levels assay. Nitrite is a product of the autoxidation of nitric oxide and is more stable in an aqueous solution [25]. Nitrites levels were measured using the Griess reaction. Cerebral cortex supernatant was mixed with Griess reagent (1:1 mixture of 1 % sulfanilamide and 0.1 % naphthylethylenediamine dihydrochloride). The absorbance was measured at a wavelength of 543 nm. Nitrite levels were expressed as μM per milligram of protein [26].

2.3.3.3. Thiobarbituric acid reactive substances (TBARS) assay. Lipid peroxidation was measured through the determination of TBARS assay described by Ohkawa and colleagues [27]. The samples were incubated in a solution containing 8.1 % sodium dodecyl sulfate (SDS), 20 % acetic acid, and 0.8 % thiobarbituric acid. The resulting pink-stained TBARS were determined at a wavelength of 535 nm. Results were expressed as nM of TBARS per milligram of protein.

2.3.3.4. Sulfhydryl content. This assay was performed as described by Aksenov and colleagues [28], which is based on the reduction of 5,5′-dithio-bis(2-nitrobenzoicacid) (DTNB) by sulfhydryl groups present in samples, which in turn become oxidized (disulfide), generating a yellow derivative (TNB). The sulfhydryl content is inversely correlated to oxidative damage to the protein. Initially, samples were incubated in a solution containing phosphate buffer saline and 1 mM ethylenediamine tetraacetic acid (EDTA), pH 7.4, and 10 mM DTNB. Yellow derivative (TNB) formed were determined a wavelength of 412 nm. Results were presented as nM of TNB per milligram of protein.

2.3.3.5. Reduced glutathione (GSH) content. Firstly, samples were mixed with metaphosphoric acid and centrifuged at 3000 rpm for 10 min to deproteinize samples. After, deproteinized samples were incubated solution containing 100 mM sodium phosphate buffer and 5 mM EDTA and O-phthaldialdehyde diluted in methanol (1 mg/mL). The fluorescence was determined at a wavelength of 420 nm emission and 350 nm excitation. Results were presented as nM of GSH per milligram of protein [29].

2.3.3.6. Superoxide dismutase assay (SOD) activity. The SOD analysis in cerebral cortex was based on the technique of the self-oxidizing ability of pyrogallol, a highly superoxide-dependent process that is a substrate for SOD. Inhibition of the self-oxidation of pyrogallol happens in the presence of SOD. The samples were mixed with 1 mM EDTA, 50 mM Tris, 80 U/mL CAT, and 0.8 mM pyrogallol. The SOD activity was indirectly measured at 420 nm. The SOD activity was plotted as units of SOD per milligram of protein [30].

2.3.3.7. Catalase assay (CAT) activity. The CAT analysis was based on the measure of the decrease in hydrogen peroxide consumption at 240 nm. The samples were incubated in a solution containing 20 mM hydrogen peroxide, 0.1 % of Triton X-100, and 10 mM potassium phosphate, pH 7.4. One CAT unit was defined as hydrogen peroxide 1 μM consumed per minute. The CAT activity was presented as units of CAT per milligram of protein [31].

2.3.3.8. Glutathione peroxidase (GPx) activity. The GPx analysis was determined according to Wendel [32]. The samples were mixed in medium containing 2 mM GSH, 0.15 U/mL glutathione reductase, 0.4 mM azide, 0.5 mM tert-butylhydroperoxide, and 0.1 mM nicotinamide adenine dinucleotide phosphate (NADPH). The decay of the NADPH was monitored at 340 nm. One GPx unit was defined as 1 μM of NADPH consumed per minute. The GPx activity was presented in GPx units per milligram of protein.

2.3.3.9. RNA isolation and real-time quantitative polymerase chain reaction (RT-qPCR). The gene expression of tumor necrosis factor-α (TNF-α), Interleukin (IL)-1β, IL-6, IL-10, monocyte chemoattractant protein 1 (MCP-1), and inducible nitric oxide synthase (iNOS) in the cerebral cortex was evaluated. For the extraction of total RNA from the cerebral

cortex, the TRIzol® reagent (Thermo Fisher Scientific) was used according to the manufacturer's instructions. RNA samples were quantified, and the purity was assessed in a NanoDrop BioChrom apparatus (Harvard Bioscience). The synthesis of complementary DNA (cDNA) was performed with 1 μg of total RNA using the High Capacity Reverse Transcription Kit with RNase Inhibitor (Thermo Fisher Scientific) according to the manufacturer's instructions in a Master Cycler Gradient thermocycler (Eppendorf). The RT-qPCR was performed using the Master Mix SYBR Green PCR (Applied Biosystems) in a StepOnePlus real-time PCR system (Applied Biosystems). The reactions were performed in a final volume of 10 μL, using 2 μL of diluted cDNA (1:10) and 300 nM of each forward and reverse primer. The relative gene expression was calculated using the comparative cycle threshold (Ct) method (ΔΔCt). The β-actin gene (Actb) was used as the endogenous control. The results are presented as relative expression of the gene of interest/Actb. The primer sequences are provided in Table 1.

2.3.3.10. Protein determination. The protein concentration was determined by the method of Lowry and colleagues [33] using bovine serum albumin as the standard.

2.4. Behavioral assessments

Before the testing, animals were acclimatized for 20 min in the behavioral room. The apparatus were cleaned up with a 40 % ethanol solution after each animal.

2.4.1. Maternal behavior

Maternal behavior after the birth of the offspring was observed during 1 to 10 postnatal days of pups. This test was recorded once a day, during the light cycle phases (16:00 h). Within each observation session, the behavior of each dam at that specific moment was scored every 3 min (11 observations/dam/day). The following behaviors were recorded: dam nursing pups; licking the pups (licking the surface of their bodies and their anogenital regions); and mother off the nest (the lactating female is out of the nest). The total maternal care score was calculated by summing the behaviors directed towards the pups (licking+breastfeeding postures) in all recording periods. The data are reported as the number of observations or frequency that the behavior was recorded (maximum of 110 times/dam) [34,35].

2.4.2. Olfactory preference test for familiar odors in the offspring

The olfactory preference test for the odor of the home cage sawdust (mother and littermates) was performed according to Raineki and colleagues [36] with modifications. The test comprised a two-odor choice between areas with a nest or fresh bedding. A plastic box was divided in the center, using scotch tape, into two areas (fresh bedding area and nest bedding area). A portion of nest bedding (approximately 300 mL) was placed in one corner, and a similar portion of fresh sawdust was placed at the other corner. Pups at 10 postnatal days were placed on the middle line of the testing box (neutral zone). The test was performed in 2 consecutive sessions, which lasted 3 min each with a 2 min interval between each session. At the end of each session, the box was cleaned with 40 % ethanol, and the positions of the nest bedding and fresh bedding were switched. Time in fresh bedding area, time in nest bedding area, time in fresh bedding, time in nest bedding, and latency to find nest bedding were recorded. We performed this test in pups of 10 postnatal days because pups gain mobility and leave the nest after this age [37], they easily learn to avoid the nest and the former fail to produce preferences [38].

Table 1
Primer sequences for real-time quantitative polymerase chain reaction (PCR) analysis.

Gene		Sequence (5′-3′)
Actb	Sense	ACC CAC ACT GTG CCC ATC TAT G
Actb	Antisense	AGA GTA CTT GCG CTC AGG AGG A
TNF-α	Sense	GGT CCC CAA AGG GAT GAG AAG TTC
TNF-α	Antisense	CCA CTT GGT GGT TTG CTA CGA CG
IL-1β	Sense	TTC AGG CAG GCA GTA TCA CTC
IL-1β	Antisense	CCA CGG GAA AGA CAC AGG TAG
IL-6	Sense	CTG CAA GAG ACT TCC ATC CAG
IL-6	Antisense	AGT GGT ATA GAC AGG TCT GTT GG
IL-10	Sense	GCT GGA CAA CAT ACT GCT AAC C
IL-10	Antisense	ATT TCC GAT AAG GCT TGG CAA
MCP-1	Sense	CCA ACC ACC AGG CTA CAG G
MCP-1	Antisense	GCG TCA CAC TCA AGC TCT G
iNOS	Sense	CCT CCT CCA CCC TAC CAA GT
iNOS	Antisense	CAC CCA AAG TGC TTC AGT CA

Abbreviations: Actb: actin β; IL: interleukin; iNOS: inducible nitric oxide synthase; MCP-1: monocyte chemoattractant protein; TNF-α: tumor necrosis factor.

2.4.3. Open field test

The rats were individually placed in the center of the open field (50 × 50 × 50 cm), and they were filmed for 10 min. The distance traveled, mobile time on the periphery, time in center, crossings number, crossings number in center, crossings number in periphery, and crossings number in periphery were analyzed. The data were recorded and analyzed with an automated activity-monitoring system (Any-maze) [39].

2.4.4. Object recognition memory

This test was performed on the open field apparatus and all of the objects were constructed with plastic LEGO blocks. The test consists of two phases: familiarization and test. In the familiarization session, the animal was placed in the arena containing two identical objects (A1 and A2) for 10 min and total exploration time and the time spent in the exploration of each object were recorded. After 24 h, to evaluate the long-term memory, the animals were returned to the arena for 10 min with two objects: the familiar object (A1) and a novel object (B). Total exploration time and the time devoted to each object were analyzed. Recognition Index was determined through the division of the time devoted to the novel object by the total time spent exploring both objects (B/A1 + B) [40].

2.4.5. Elevated plus maze

We released elevated plus maze test in order to evaluate anxiety-like behavior. The apparatus consisted of two open arms and two closed arms (65 cm long and 14 cm wide arranged) such that the two arms of each type were opposite to each other. For this test, each rat was placed in the center of the maze and, facing one of the open arms. Rat behavior was registered for 5 min. Total number entries, entries in open arms, the time spent in open arms, % of the time spent in open arms, the time spent in closed arms, entries in closed arms, time spent in neutral zone, and time spent in open arms/entries in open arms ratio were recorded [41].

2.4.6. Light-dark transition test

The apparatus used for the light-dark transition test comprised a box (21 × 42 × 25 cm) divided into two compartments of equal size by a partition with a small opening. One chamber was brightly illuminated, whereas the other chamber was dark. Rats were placed into the light side its behavior was registered for 10 min. Time spent in light, time spent in dark, and number of cross were recorded [42].

2.4.7. Eating-related depression test

This test examines the levels of depression and motivation to eat after food deprivation. Chow was removed from the animal cages for 24 h. After, four chow pellets were placed on a piece of filter paper positioned at one of the four corners of the home cage. Each rat was placed in a corner opposite to the chow and its behavior was registered for 5 min. The latency of the first bite was analyzed [43].

2.4.8. Ladder walking test

Animals were placed on a horizontal ladder. The apparatus used for this experiment consists of two transparent acrylic walls (Diameter: 1 m. Height: 20 cm) with metal rungs (diameter: 3 mm) inserted into the walls. The space between the two side walls is 55 mm. The ladder remained 30 cm above the floor and a dark cage was located at the end of the device. All animals were trained for one day using a regular pattern (keeping the same distance between steps). Throughout each training session, the rats walked the ladder three times and they could explore the refuge cage for 10 s. On the second day, the animals' walk was assessed using an irregular pattern, that is, the steps were spaced at intervals of 1 to 3 cm. The difficulty of the task was modified by varying the position of the metal rungs. Three templates of irregular rung patterns were used so that the same patterns were applied to all animals. Unsuccessful steps are as defined by Metz and colleagues [44], where a hindlimb or forelimbs either misses a rung completely or initially makes contact with a rung but slips during weight-bearing. The time spent for the animal to cross the ladder on training and test and % control errors/steps [(average of High-protein group animals/average of controls) x100]/steps on test were recorded. Average number of errors made per step compared to 100 % control values in animals [45].

2.4.9. Beam balance test

The test consists of two phases: training and test (three trials each). Rats were trained to traverse a narrow wooden beam (width 2.5 cm, length 100 cm). The beam was elevated 50 cm above the floor by two acrylic boxes. The animals were placed on one side, with a safe place (box with nest bedding) on the other side, encouraging the animals to walk on the beam. The number of hind paw slips and average time to cross a beam (three trials) was counted [46].

2.4.10. Rotarod

Rats were exposed to one habituation session for 3 min in the apparatus (Insight®, Brazil) at slow velocity (20 rpm). After, two test days were performed: rats were placed on rotating drums with acceleration from 16 to 40 rpm over 5 min. The number of falls (maximum 3), the latency of the first downfall, and time of permanence in rotarod were analyzed [47].

2.5. Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM). The Shapiro-Wilk normality test was used to evaluate the normal distribution of the data. The student's *t*-test was used to evaluate the different parameters and the adopted significance level was $p \leq 0.05$. Statistical analysis and graphs were performed using the GraphPad Prism Software, version 5 (GraphPad Software, San Diego).

3. Results

3.1. Biochemical assessments

Firstly, we evaluated the effect of supplementation with a high-protein diet in pregnancy on amino acid profile and homocysteine levels in the in the male adolescent offspring's serum and cerebral cortex. Table 2 shows that the high-protein diet caused a significant decrease in histidine levels in serum ([t(14) = 2.283; $p < 0.05$) when compared to the control group. Although the ornithine levels in the cerebral cortex (Table 3) were not significantly different from control, the results showed a tendency to increase ($p = 0.058$) in the high-protein diet group. The levels of other amino acids examined (aspartate, glutamate, serine, glutamine, glycine, alanine, tyrosine, tryptophan, methionine, valine, phenylalanine, isoleucine, leucine, and lysine) were not altered ($p > 0.05$) (Tables 2 and 3). Afterward, we observed that high-protein diet caused a significant increase in homocysteine levels ([t(9) = 2.444; $p < 0.05$) in the male adolescent offspring's cerebral cortex when compared to the control group (Fig. 2A). However, no differences were found between control and high-protein diet groups in homocysteine levels in serum ($p > 0.05$; Fig. 2B).

Table 2
Effect of supplementation with a high-protein diet in pregnancy on amino acid profile in the male adolescent offspring's serum (μmol/L).

Amino acid	Control	High-protein diet
Aspartate	31.41 ± 2.665	26.50 ± 2.628
Glutamate 164.4 ± 7.654	183.8 ± 9.391	
Serine	253.4 ± 9.272	222.6 ± 12.54
Histidine	22.83 ± 2.061	17.14 ± 1.400*
Glutamine	1644 ± 57.72	1492 ± 54.58
Glycine	591.9 ± 16.43	585.4 ± 31.71
Alanine	538.0 ± 15.33	572.6 ± 33.26
Tyrosine	98.88 ± 5.970	89.90 ± 4.928
Tryptophan	114.9 ± 5.692	120.6 ± 7.185
Methionine	50.66 ± 1.718	46.80 ± 2.488
Valine	196.3 ± 7.877	178.6 ± 11.17
Phenylalanine	71.19 ± 2.067	64.03 ± 4.975
Isoleucine	83.83 ± 3.076	78.33 ± 5.009
Leucine	165.8 ± 4.708	164.7 ± 6.895
Ornithine	76.36 ± 3.039	73.68 ± 3.415
Lysine	363.0 ± 10.07	336.6 ± 8.371

Results are expressed as mean ± Standard error of the mean for 8 animals in each group. Different from control: *$p < 0.05$ (Student's *t*-test).

Table 3
Effect of supplementation with a high-protein diet in pregnancy on amino acid profile in the male adolescent offspring's cerebral cortex (μmol/L).

Amino acid	Control	High-protein diet
Aspartate	1029 ± 49.89	961.6 ± 34.93
Glutamate	1724 ± 80.57	1702 ± 93.67
Serine	256.7 ± 16.76	239.2 ± 19.84
Histidine	3.357 ± 0.3169	4.950 ± 0.9969
Glutamine	2487 ± 92.39	2555 ± 144.6
Glycine	422.9 ± 27.11	408.7 ± 27.25
Alanine	221.9 ± 12.18	235.1 ± 15.32
Tyrosine	668.3 ± 25.91	748.6 ± 42.85
Tryptophan	11.09 ± 0.7614	9.714 ± 1.048
Methionine	32.96 ± 2.699	30.21 ± 2.795
Valine	46.29 ± 3.335	45.71 ± 3.582
Phenylalanine	39.26 ± 4.267	35.94 ± 4.107
Isoleucine	26.73 ± 2.606	24.50 ± 2.251
Leucine	53.70 ± 12.66	56.04 ± 5.283
Ornithine	6.143 ± 1.014	11.08 ± 2.251 $P = 0.0588$
Lysine	93.33 ± 7.850	75.95 ± 5.526

Results are expressed as mean ± Standard error of the mean for 7 animals in each group. Different from control: $p < 0.05$ (Student's *t*-test).

To evaluate the presence of oxidative stress caused by the supplementation with high-protein diet in pregnancy, we measured oxidative/nitrative and antioxidant parameters in the male adolescent offspring's cerebral cortex. We did not identify differences between control and high-protein diet groups in oxidative stress parameters evaluated (reactive species levels, nitrite levels, TBARS levels, sulfhydryl content, GSH content, SOD activity, CAT activity, GPx activity, and SOD/CAT ratio, $p > 0.05$) in the cerebral cortex (Table 4). Moreover, no differences were found between control and high-protein diet groups in iNOS expression ($p > 0.05$) (Table 4).

Finally, to verify the presence of neuroinflammation caused by the supplementation with a high-protein diet in pregnancy, we evaluated cytokines and a chemokine expression in the male adolescent offspring's cerebral cortex. We identify an increase in TNF-α ([t(9) = 3.505; $p < 0.01$), IL-1β ([t(10) = 3.453; $p < 0.01$), IL-6 ([t(9) = 4.580; $p < 0.01$), IL-10 ([t(9) = 6.563; $p < 0.001$), and MCP-1 ([t(9) = 6.875; $p < 0.001$) gene expression in the high protein diet group (Fig. 3A-E).

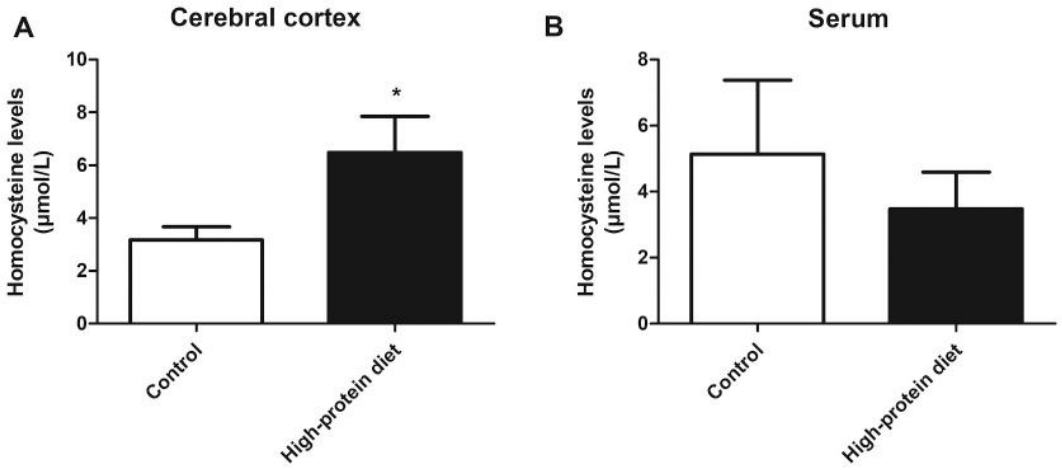


Fig. 2. Effect of high-protein diet consumption throughout pregnancy on homocysteine levels in male adolescent offspring: homocysteine levels in cerebral coxtex (A), homocysteine levels in serum (B). Results are expressed as mean ± SEM for 7–8 animals in each group. Different from control, $^*p < 0.05$ (Student's *t*-test).

Table 4
Effect of supplementation with a high-protein diet in pregnancy on redox homoeostasis in the male adolescent offspring's cerebral cortex.

Parameters	Control	High-protein diet
DCF levels (nmol DCF/mg protein)	458.9 ± 40.75	414.4 ± 37.41
Nitrite levels (µmol/mg protein)	2.868 ± 0.162	3.418 ± 0.272
iNOS expression (iNOS/Actb)	1.278 ± 0.307	1.122 ± 0.235
TBARS levels (nmol/mg protein)	3.009 ± 0.060	3.239 ± 0.196
Sulphydryl content (nmol TNB/mg protein)	2224 ± 70.58	2058 ± 107.3
GSH content (nmol/mg protein)	66.06 ± 2.047	72.37 ± 3.845
SOD activity (units/mg protein)	7.124 ± 0.2717	7.429 ± 0.284
CAT activity (units/mg protein)	3.305 ± 0.3255	3.257 ± 0.335
GPx activity (units/mg protein)	26.83 ± 1.565	29.10 ± 2.223
SOD/CAT ratio	3.305 ± 0.325	3.257 ± 0.335

Results are expressed as mean ± Standard error of the mean for 5–6 animals per group for iNOS relative expression and 9 animals in each group in the other parameters. Different from control: $p < 0.05$ (Student's *t*-test). Abbreviations: Actb: β-Actin gene, CAT: catalase, DCF: dichlorofuorescein, GPx: glutathione peroxidase, GSH: reduced glutathione, iNOS: inducible nitric oxide synthase SOD: superoxide dismutase, TBARS: thiobarbituric acid reactive substances.

3.2. Behavioral assessments

Maternal behavior was analyzed to identify possible influences of a high-protein diet in maternal care. We did not observe differences between control and high-protein diet groups in total maternal care score ($p > 0.05$), frequency of maternal care ($p > 0.05$), and observations of the dams outside the nest ($p > 0.05$) (Fig. 4A-C).

Afterward, we evaluated the effect of a high-protein diet during pregnancy on the olfactory preference of the pups at 10 postnatal days. Pups rodents naturally learn to identify and prefer the nest odor [48]. We did not observe differences between control and high-protein diet groups in time in fresh bedding area ($p > 0.05$), time in nest bedding area ($p > 0.05$), time in fresh bedding ($p > 0.05$), time in nest bedding ($p > 0.05$), and latency to find nest bedding ($p > 0.05$) (Fig. 5A-E). On the other hand, we identify an increase in time spent in nest bedding in the control and high-protein diet groups ([$t(2) = 9.959$; $p < 0.01$), but no difference between the groups (Fig. 5F).

Open field test was performed at 21 postnatal days to evaluate the locomotion, exploratory activity, and habituation of animals. The results show that there were no differences between the control and high-protein diet groups in distance traveled ($p > 0.05$), mobile time on the periphery ($p > 0.05$), time in the center ($p > 0.05$), crossings number ($p > 0.05$), crossings number in the center ($p > 0.05$), crossings number in the periphery ($p > 0.05$), and average speed ($p > 0.05$) (Table 5).

Next, we evaluated the performance of animals on long-term object recognition memory. No differences were found between control and high-protein diet groups on the training and test days in total exploration time ($p > 0.05$) and time spent with each object ($p > 0.05$; Fig. 8A-D). However, we observed a decrease in the object recognition index ([$t(25) = 3.059$; $p < 0.01$) in the high-protein diet group when compared to the control group (Fig. 6E).

Subsequently, we analyzed anxiety-like behavior in the elevated plus-maze. No differences were found between control and high-protein diet groups in total number of entries ($p > 0.05$), entries in open arms ($p > 0.05$), time spent in open arms ($p > 0.05$), percentage of the time spent in open arms ($p > 0.05$), time spent in closed arms ($p > 0.05$), entries in closed arms ($p > 0.05$), time spent in the neutral zone ($p > 0.05$), and time spent in open arms/entries in open arms ($p > 0.05$) (Table 5).

Light-dark transition test, a tool to analyze anxiety-like behavior, is based on the innate aversion of rodents to brightly illuminated areas and their tendency to exhibit spontaneous locomotor behavior in response to novel environments. We did not find differences in time spent in the light box ($p > 0.05$) (Fig. 7A) and time spent in the dark box ($p > 0.05$) (Fig. 7B) between the groups. However, we observed that high-protein diet group presented an increase in number of cross in the light-dark box ([$t(40) = 2.069$; $p < 0.05$) (Fig. 7C).

In the eating-related depression test, we identify a decrease in latency for the first bite ([$t(15) = 3.000$; $p < 0.01$) in the high-protein diet group when compared to control group (Fig. 7D).

Subsequently, the ladder walking test was used to assess walking ability and to measure both forelimb and hind limb placement, stepping, and inter-limb coordination of animals. No differences were found between control and high-protein diet groups in time to the cross ladder on training and test ($p > 0.05$) (Fig. 8A,B). We showed that the high-protein diet group presented an increase in percentage control errors/steps ([$t(17) = 2.147$; $p < 0.05$) on the test when compared to control group (Fig. 8C).

After, the beam balance test was used to assess gait abnormality, gross motor ability, and gross vestibular motor function. We observed that the high-protein diet group presented an increase in average time to cross a beam on training ([$t(17) = 2.145$; $p < 0.05$) (Fig. 8D). No differences were found between control and high-protein diet groups in the average time to cross a beam ($p > 0.05$) on the test and the number of hind paws slips on training and test ($p > 0.05$) (Fig. 8E-G).

Finally, rotarod test was performed to evaluate the coordinate walking, balance abilities, and vestibulomotor function of animals. Results did not evidence differences between control and high-protein diet groups in number of the falls (training and testing: day 1 and 2) ($p > 0.05$), the latency of the first downfall (testing: day 1 and 2) ($p > 0.05$), and time on the rotarod ($p > 0.05$) (testing: day 1 and 2) (Fig. 9A-G).

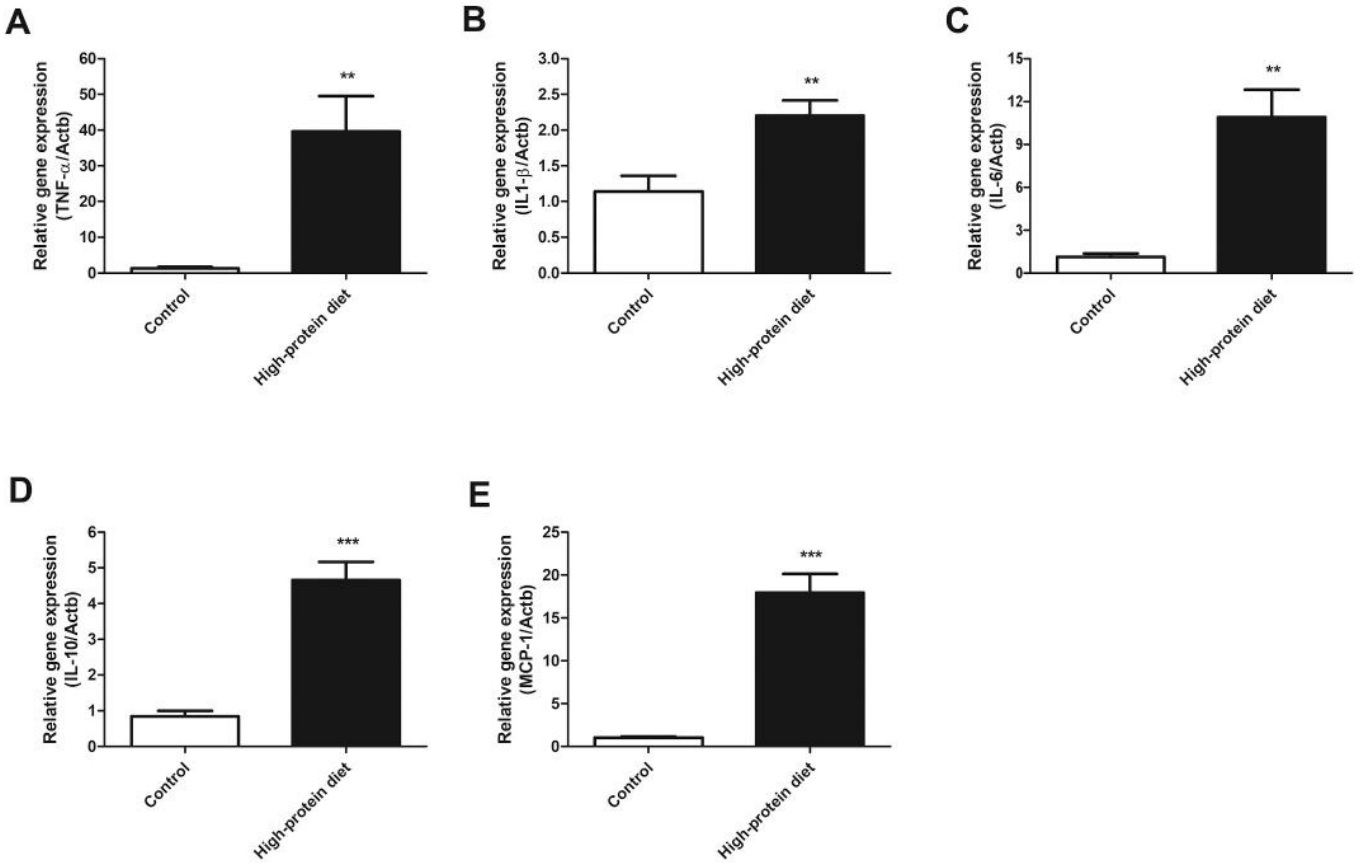


Fig. 3. Effect of high-protein diet consumption throughout pregnancy on neuroinflammatory status in the male adolescent offspring's cerebral cortex: relative gene expression of TNF-α (A), relative gene expression of IL-1β (B), relative gene expression of IL-6 (C), relative gene expression of IL-10 (D), and relative gene expression of MCP-1 (E). The Actb was used as the endogenous control, and the results are expressed as relative expression of the gene of interest/Actb. Results are expressed as mean ± SEM for 5–6 animals in each group. Different from control, $^{**}p < 0.01$ and $^{***}p < 0.001$ (Student's *t*-test). Actb: actin β, IL: interleukin, MCP-1: monocyte chemoattractant protein, TNF-α: tumor necrosis factor.

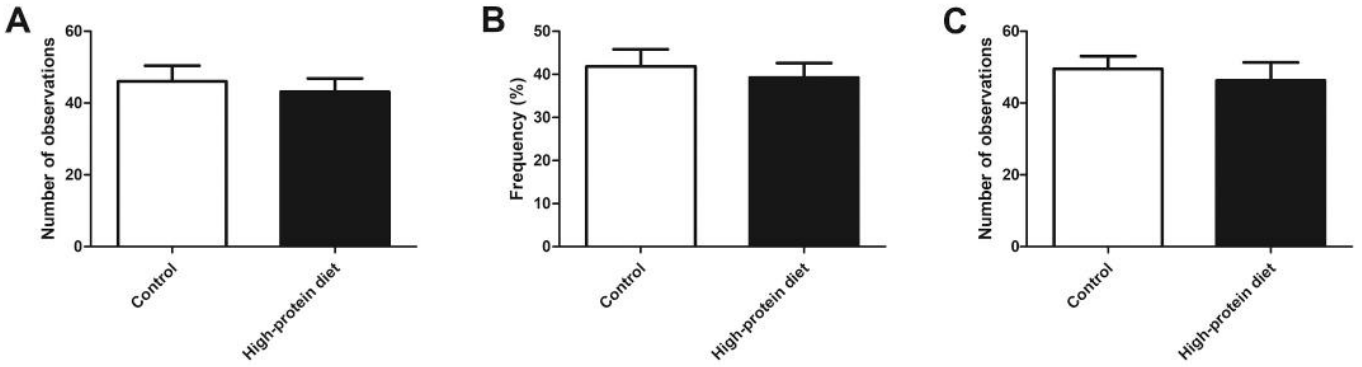


Fig. 4. Effect of high-protein diet consumption throughout pregnancy on maternal behavior: maternal care score (A), frequency of maternal care (B), and observations of the dams of the nest (C). Results are expressed as mean ± SEM for 11–14 animals in each group. Different from control, $^{*}p > 0.05$ (Student's *t*-test).

4. Discussion

The present study was proposed to investigate the effects of a high-protein diet throughout pregnancy on neurochemical parameters and behavioral performance in male adolescent rats offspring. We observed an increase in homocysteine levels and cytokine expression in the male adolescent rats offspring's cerebral cortex from pregnant rats supplemented with a high-protein diet throughout pregnancy. The total cerebral cortex was chosen because is a structure that plays an important role in diverse behaviors. The cerebral cortex controls behaviors such as perception, motor function, cognition, memory, and processing of learning [49,50]. Besides decreasing histidine levels in the male adolescent offspring's serum. High-protein did not impair the mother-pup relationship. Our results also revealed an impairment in memory and motricity of the male adolescent offspring supplemented with a high-protein diet throughout pregnancy. We also showed an increase in anxiety-like behavior in this group.

The gestational period was chosen for the administration of the high-protein diet in pregnant rats because this is a period in which the fetal brain is particularly vulnerable to changes in the maternal environment

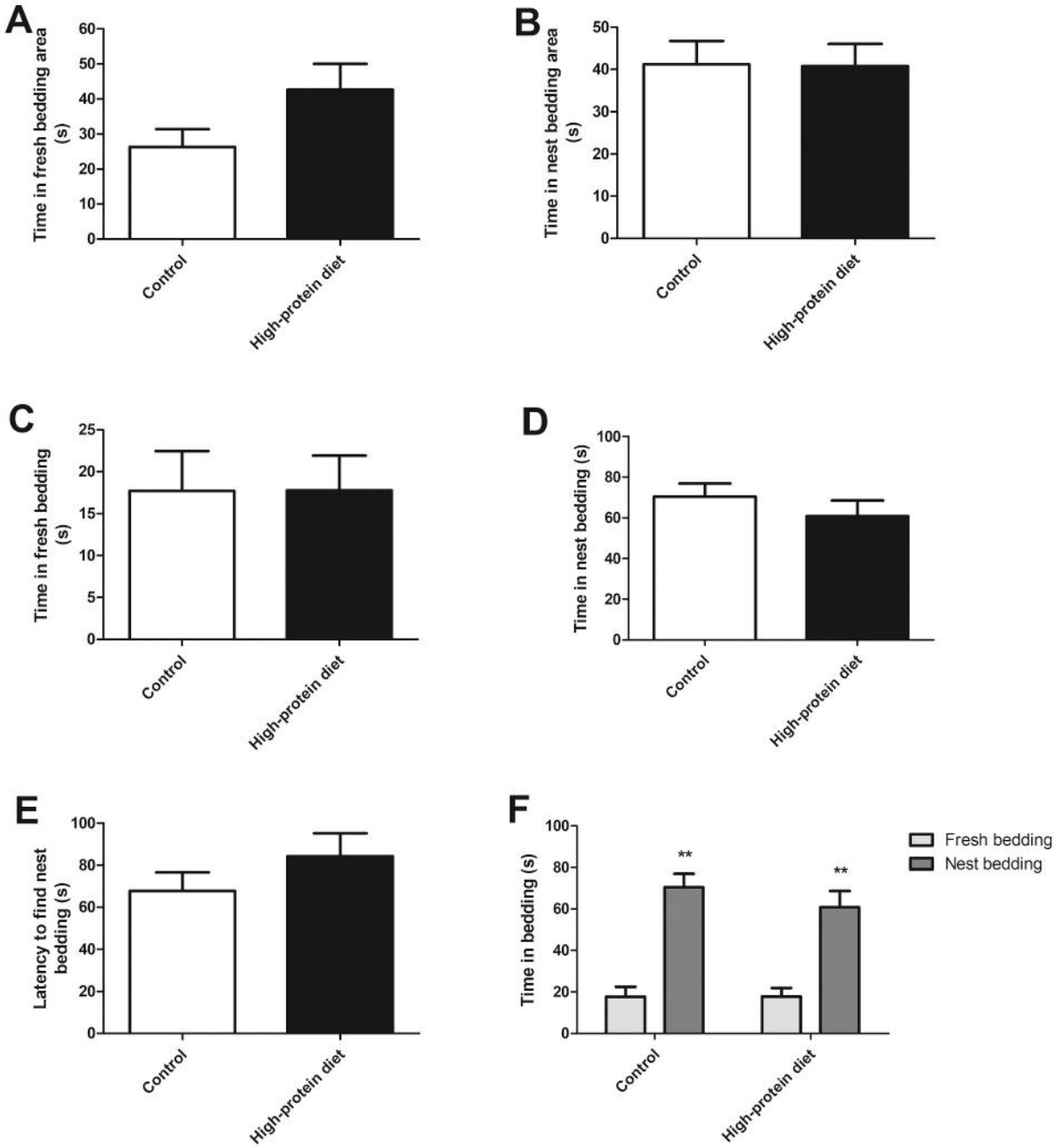


Fig. 5. Effect of high-protein diet consumption throughout pregnancy male adolescent offspring on olfactory preference test for familiar odors: time in fresh bedding area (A), time in nest bedding area (B), time in fresh bedding (C), time in nest bedding (D), latency to find nest bedding (E), and time in bedding (F). Results are expressed as mean ± SEM for 11–14 animals in each group. Different from control, $^{**}p < 0.01$ (Student's *t*-test).

since the development of the brain is a process that commences early in pregnancy [51]. However, brain development in rodents is not completed at birth [52]. In childhood, adolescence, and early adulthood an intense maturation and refinement of synaptic and neural circuitry occur, which are crucial for the gradual enhancement of neurocognitive performance that matures by adulthood [53–55]. In rodents, neurogenesis begins at gestational day 9.5 and is completed only in PND 15 [56,57]. Moreover, the critical period of synaptogenesis occurs between PND 10 and PND 30 [56,58]. Gliogenesis occurs in fetal and postnatal periods in rodents [59]. During the first few postnatal weeks, the astrocytes undergo a period of maturation that involves changes in their connectivity, morphology, and electrophysiology [60]. Myelination in the rat brain begins at approximately PND 10, reaching its peak at PND 20 [61]. In our study, we chose the endpoint PND 30 (adolescent rats) because in the first weeks during the postnatal period rats are characterized by intense synaptogenesis, myelination, and gliogenesis since brain development in rodents is not completed at birth [52,56].

Amino acids are important for protein synthesis and other molecules such as catecholamines, creatine, dopamine, nitric oxide, polyamines, and thyroid hormones [7]. High-protein diet intake increases amino acid metabolites formation, such as homocysteine, hydrogen sulfide, and ammonia, which may also impair embryonic/fetal survival and growth [62,63]. This study showed that a high-protein diet throughout pregnancy decreases histidine levels in the male adolescent offspring's serum. Histidine is an essential amino acid that participates in protein composition and is a precursor of histamine [64]. Brain histamine is

Table 5
Effect of supplementation with a high-protein diet in pregnancy in male adolescent offspring performance in the open field test and elevated plus maze.

Parameters	Control	High-protein diet
Open field		
Distance traveled (m)	18.83 ± 1.320	19.14 ± 0.749
Mobile time on the periphery (S)	268.6 ± 19.48	247.7 ± 9.964
Time in the center (S)	32.96 ± 5.451	39.95 ± 5.706
Crossings number	219.9 ± 17.40	234.2 ± 14.55
Crossings number in the center	27.38 ± 3.474	29.40 ± 2.894
Crossings number in the periphery	186.1 ± 15.86	207.7 ± 13.22
Average speed (m/min)	2.750 ± 0.286	2.860 ± 0.2193
Elevated plus maze		
Total number entries	15.76 ± 0.869	16.20 ± 0.7681
Entries in open arms	7.429 ± 0.495	7.720 ± 0.595
Time spent in open arms (S)	119.2 ± 11.16	101.7 ± 10.35
Percentage of the time spent in open arms	39.71 ± 3.735	33.88 ± 3.454
Time spent in closed arms (S)	145.0 ± 13.58	157.2 ± 7.362
Entries in closed arms	8.333 ± 0.5828	8.400 ± 0.3786
Time spent in the neutral zone (S)	359.5 ± 5.289	373.4 ± 4.501
Time spent in open arms (S)/ entries in open arms	15.90 ± 1.177	13.08 ± 0.8425

Results are expressed as mean ± Standard error of the mean for 11–14 animals in each group in the other parameters. Different from control: $p < 0.05$ (Student's *t*-test).

synthesized from histidine and plays a role as a neurotransmitter in the brain to control mood, the sleep cycle, learning, memory, alertness, regulation of appetite, and the perception of pain [65]. We also have observed that a high protein diet caused a significant increase in homocysteine levels in the male adolescent offspring's cerebral cortex. Homocysteine is a sulfur-containing amino acid derived from methionine metabolism [66]. An increase in homocysteine levels has been linked to oxidative stress, exacerbation of inflammation, and varied metabolic changes as well as behavioral damage [67–70].

Maternal immune activation during gestation can be a risk factor for the neurological alterations observed in the offspring that later manifest as behavioral disorders [71,72]. We verify the presence of neuroinflammation in the cerebral cortex of the male adolescent offspring's caused by the supplementation with a high-protein diet throughout pregnancy. We have shown an increase in TNF-α, IL-1β, IL-6, IL-10, and MCP-1 gene expression in the high protein diet group. The activated microglia produce proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 which coactivate the astrocytes, resulting in neuronal dysfunction and death and participating in the pathogenesis of neurodegenerative disorders [73,74]. The MCP-1 chemoattractant protein induces the accumulation of monocytes and microglia around blood vessels [75]. In agreement with our study, maternal consumption of a high-protein diet (40 % *w*/w) during pregnancy and lactation increased IL-6 levels in offspring's brown adipose tissue at PND 35 [76]. Curiously, in our study, we have shown an increase in the anti-inflammatory cytokine IL-10 that can be due to a compensatory mechanism to decrease the inflammatory process caused by the increase of TNF-α, IL-1β, IL-6, and MCP-1. Based on our results, we suggest that the increase in neuroinflammation identified in the cerebral cortex can be associated with the behavioral impairment of the male adolescent offspring.

Maternal nutrition during pregnancy influences the neurodevelopment of offspring, particularly cognitive processes such as memory [77,78]. We observed that high-protein diet consumption throughout pregnancy caused long-term memory impairment in the male adolescent offspring. We suggest that the memory impairment observed in the offspring can be associated in part with the decrease in histidine levels and an increase in homocysteine levels in the cerebral cortex. Histidine is a neurotransmitter that controls a wide variety of neurobiological and behavioral functions such as memory. Histamine is synthesized from histidine through the catalysis of histidine decarboxylase [64]. Low histamine levels can cause behavioral damage such as depression, poor motivation, motor functions, learning, and memory

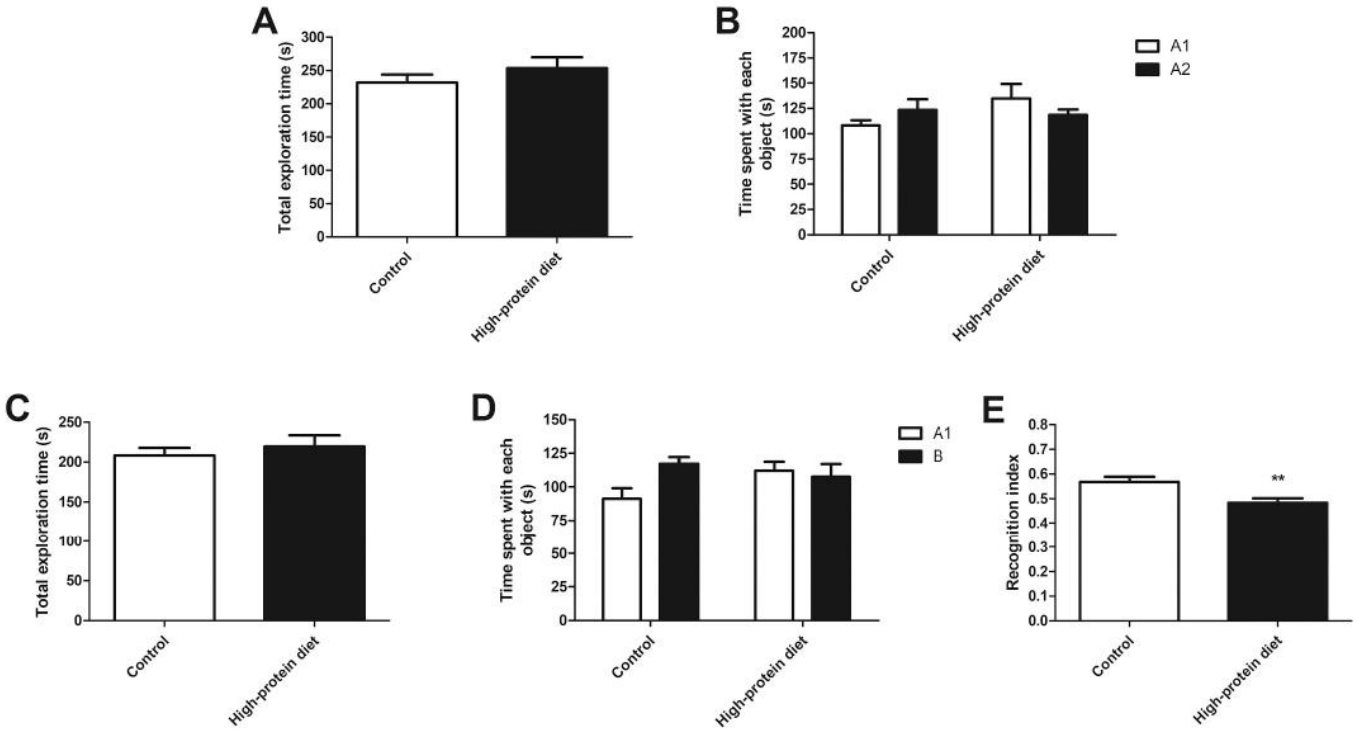


Fig. 6. Effect of high-protein diet consumption throughout pregnancy in male adolescent offspring on object recognition memory: total exploration time during the training (A), time spent with each object during the training (B), total exploration time during the test (C), time spent with each object during the test (D), object recognition index (E). Results are expressed as mean ± SEM for 11–14 animals in each group. Different from control, $^{**}p < 0.01$ (Student's *t*-test).

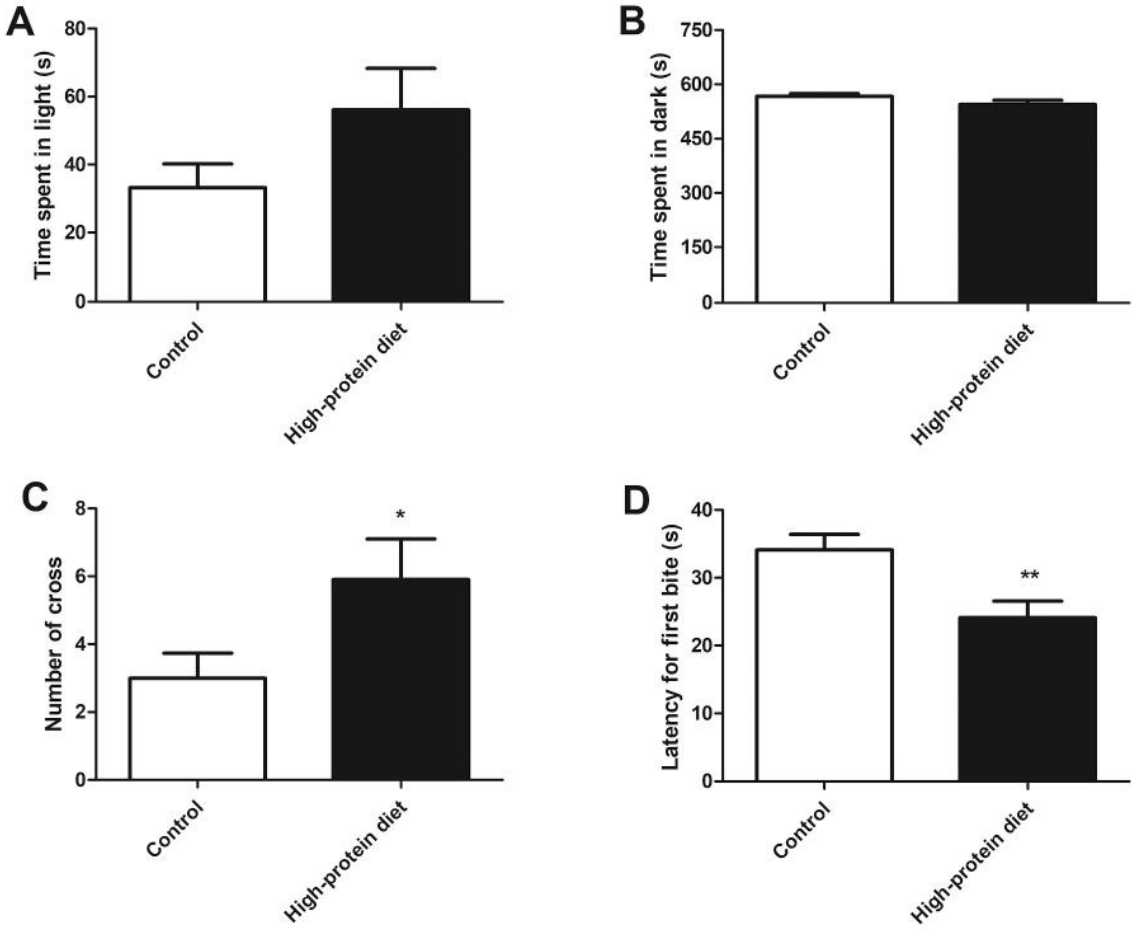


Fig. 7. Effect of high-protein diet consumption throughout pregnancy male adolescent offspring on light-dark transition test: time spent in the light box (A), time spent in the dark box (B), and number of cross in the light-dark box (C). Eating-related depression test: latency for first bite (D). Results are expressed as mean ± SEM for 11–14 animals in each group. Different from control, $^{*}p < 0.05$ and $^{**}p < 0.01$ (Student's *t*-test).

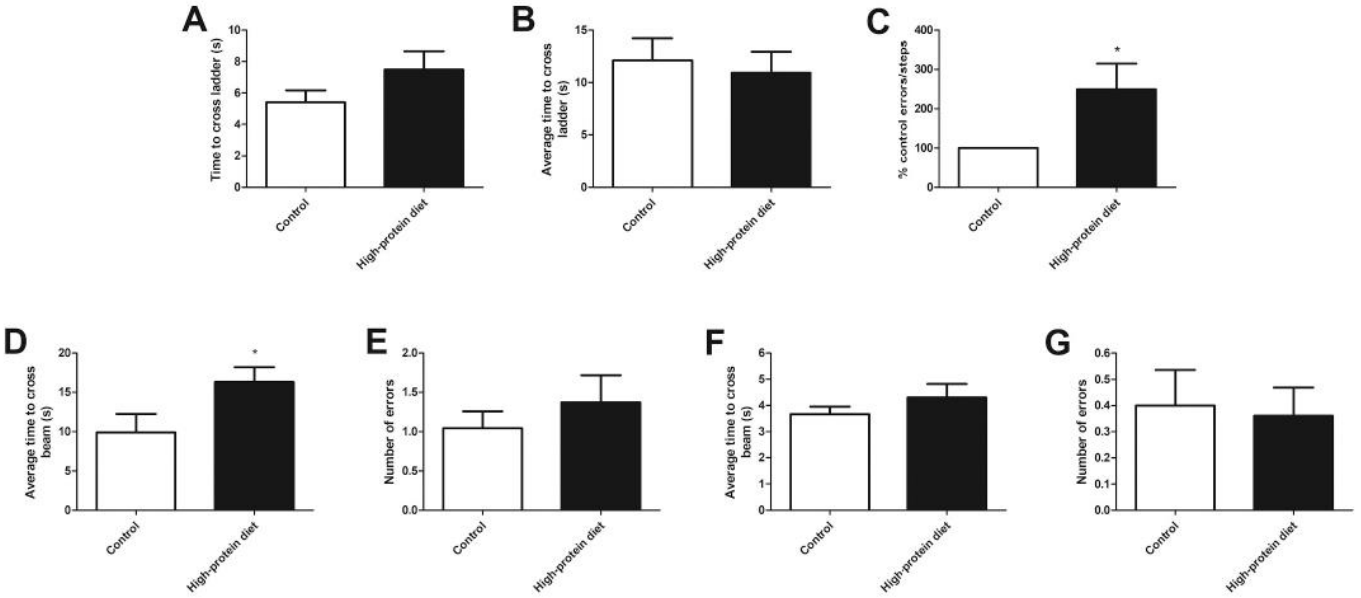


Fig. 8. Effect of high-protein diet consumption throughout pregnancy in male adolescent offspring on ladder walking test: time to cross ladder during the training (A), average time to cross ladder during the test - average of the 3 trials (B), % control error/steps [average number of errors made per step compared to 100 % control values in animals] (C). Beam balance test: average time to cross beam during the training (D), number of errors during the training (E), average time to cross beam during the test (F), and number of errors during the test (G). Different from control, $^{*}p < 0.05$ (Student's t-test).

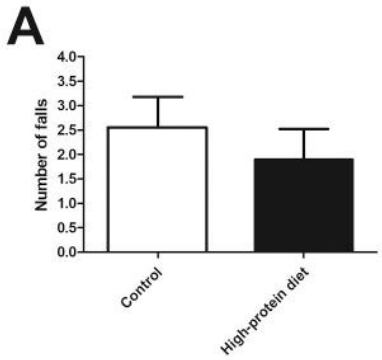
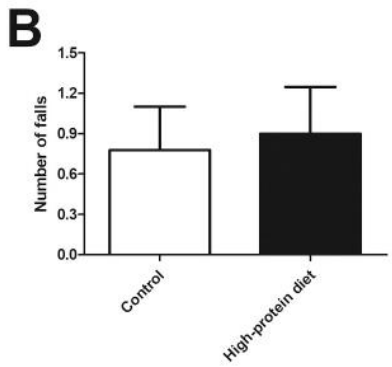
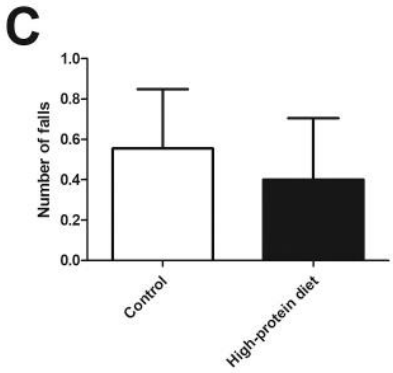
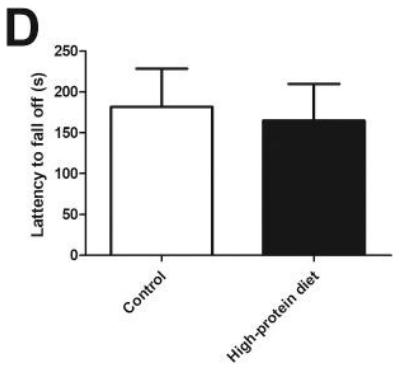
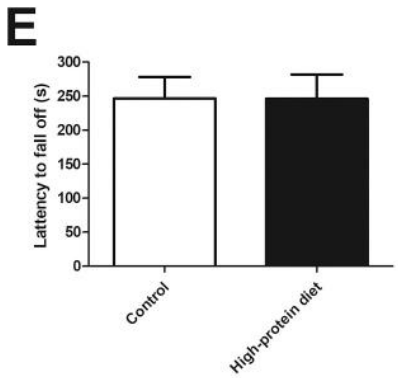


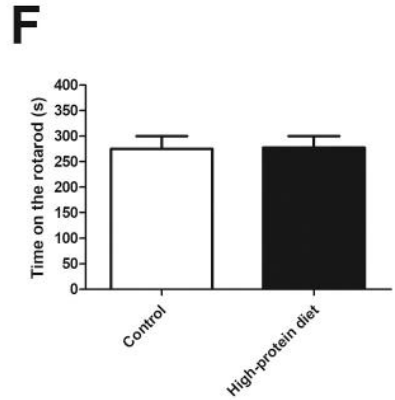
Fig. 9. Effect of high-protein diet consumption throughout pregnancy in male adolescent offspring on rotarod test: number of falls during the training (A), number of falls during test - day 1 (B), number of falls during test - day 2 (C), latency of the first downfall during test - day 1 (D), latency of the first downfall - day 2 (E), time on the rotarod during the test - day 1 (F), and time on the rotarod during the test - day 2 (G). Results are expressed as mean ± SEM for 11–14 animals in each group. Different from control, $p > 0.05$ (Student's t-test).

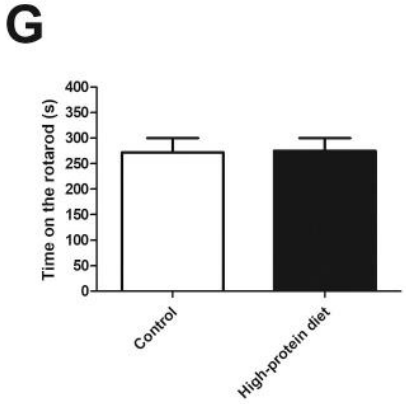












deficits [79–81]. Histamine deficit can also be seen in Alzheimer's brain disease [82]. This suggests that a high-protein diet throughout pregnancy decreases histidine levels in the offspring and consequently histamine production, which could contribute to the memory impairment observed in this study.

Homocysteine is an important risk factor for neurodegenerative diseases such as Alzheimer's disease [83]. Recently, Ramires-Junior and colleagues [84] and colleagues showed that the chronic mild hyperhomocysteinemia model in rats at PND 60 caused cognitive deficits in working memory and decreased synapsin I levels in the hippocampus. Thus, due to neurochemical changes caused by homocysteine that may impair memory, we suggest that a high-protein diet throughout pregnancy increases homocysteine levels in male adolescent offspring and consequently cause memory impairment.

Diet during pregnancy enriched or deficient in nutrients, such as amino acids and vitamins can modify the synthesis of neurotransmitters and can contribute to the development of psychiatric disorders [85,86]. Our results revealed an increase in anxiety-like behavior of the male adolescent offspring supplemented with a high-protein diet throughout pregnancy evidenced in the light-dark transition test. The maternal diet also may influence offspring feeding behavior. We identify a decrease in latency for the first bite in the high-protein diet group evidenced in eating-related depression test, a test to examine the levels of depression and motivation to eat after food deprivation. This test assesses anhedonia in a situation where there is a conflict between food reward and anxiety [87]. Kanitz and colleagues [21] observed that the high-protein diet (30 % *w*/w) during gestation alters the expression of genes encoding key determinants of glucocorticoid hormone action in the fetal pig hypothalamus causing an unbalance in the hypothalamic-pituitary-adrenal axis with potentially long-lasting consequences. The dysregulation of the hypothalamic-pituitary-adrenal axis may cause neuropsychological diseases such as anxiety [88]. We hypothesized that an increase in anxiety-like behavior observed in the high-protein diet group was partly due to increased homocysteine levels in the offspring's cerebral. Since, an increase in homocysteine levels has been strongly associated with neuropsychiatric disorders such as schizophrenia, bipolar disorder, autism, depression, and anxiety [69,89–93] The damage in the neuronal histamine is involved in several neurological diseases including narcolepsy, eating disorders, Tourette syndrome, depression, and anxiety [82]. Our second hypothesis is that decreased histidine levels observed in male adolescent offspring exposed to a high-protein diet during gestation disturbed the histaminergic system and consequently caused anxiety-like behavior. Thus an increase in homocysteine concentration and decrease in histidine levels in the male adolescent offspring's cerebral cortex may have contributed to an increase in anxiety-like behavior observed in the high-protein diet group.

Evidence has observed that pregnancy diet can impact offspring's motor function [94]. Our findings revealed impairment in motricity of the male adolescent offspring supplemented with a high-protein diet throughout pregnancy evidenced in the ladder walking test and beam balance test. In part, an increase in homocysteine levels in offspring supplemented with a high-protein diet throughout pregnancy may have contributed to the motor impairment. An increase in homocysteine levels is related to muscular malfunction, loss of motor coordination, and locomotion [95,96]. Studies have observed that diet composition may alter muscle biochemistry [97,98]. We have shown that the high-protein diet throughout pregnancy decreases histidine levels in the male adolescent offspring's cerebral cortex. Histidine is required as a precursor of carnosine [99]. Carnosine constitutes an integral part of skeletal muscle contractility and homeostasis, through its role as an antioxidant, pH-buffering, and calcium regulator [100,101]. We suggest that the impairment in motricity observed in the offspring can be associated in part with the decrease in histidine levels and probable decrease in carnosine levels.

Some limitations should be taken into account in this study. Firstly, due to the species difference, our results should not be directly extrapolated to humans. Rodents are in many respects biologically and psychologically similar to humans, such as characteristics of pain, fear, and suffering. However, evidence demonstrates that species differences in physiology and genetics, as well as exposure to laboratory procedures can result in markedly different outcomes in humans [102]. Moreover, we used only one concentration of high-protein diet during pregnancy. High-protein diet supplementation throughout the perinatal period or only during lactation could cause other effects that did not show in this study. For example, high-protein diet supplementation throughout the perinatal period (prenatal, gestation, and lactation period) could cause more severe impairment of offspring's neurobiochemistry and behavioral performance. Since the offspring would be exposed to a high-protein diet for a longer period. In our study, we investigate the effect of high-protein diet consumption throughout pregnancy in adolescent rat offspring. Adolescence is a period of substantial brain development in rodents, and exposure to gonadal hormones during this period of life could have a long-term impact on neurochemical and behavior [103,104]. Thus, offspring of other ages should also be evaluated, such as during the fetal, neonatal, and adult period. In our study, we analyze only the male offspring. We choose to perform our study with male rats because female rats are more variable than males due to the fluctuations of hormones across their estrus cycle which typically lasts 4 days in rats [105]. Moreover, several studies have shown that the estrus cycle can affect behavior tests [106–109]. In this context, female offspring could present different responses to high-protein diet consumption during the gestational period. Since male placentas are more dependent on the mother's diet even if the nutrient transfer of their placentas is more efficient because they possess lower storage capacities, which increases their vulnerability to undernutrition [110]. Thus, considering female placentas are less dependent on the mother's diet, female offspring could present less impairment caused by maternal exposure to a high-protein diet throughout pregnancy.

5. Conclusion

In conclusion, the results of the present study showed that changes in maternal macronutrients, such as the consumption of a high-protein diet throughout pregnancy, may cause neurochemical and behavioral impairments in male adolescent rats offspring's. Our findings revealed an increase in neuroinflammation in the male adolescent offspring's cerebral cortex, besides disturbing amino acid homeostasis in the cortex cerebral of the offspring. We also observed an impairment in memory and motricity, and an increase in anxiety-like behavior in male adolescent offspring supplemented with a high-protein diet throughout pregnancy. This study represents an important step in exploring the effects of high-protein diet intake by pregnant rats on the offspring since it provides additional evidence that exposure can have complex effects on the offspring. In addition, it gives a new basis for understanding the biochemical and behavioral outcomes associated with high-protein exposure throughout pregnancy, which may conduct future studies.

CRediT authorship contribution statement

JSS and ATSW designed the study. JSS, OVRJ, FS, and FSF contributed to the most data collection. FCR, RCS and LEBS contributed to the RT-qPCR technical and MD, GR, and CRV to the amino acid and homocysteine determination technical. JSS and ATSW analyzed and interpreted the work, as well as wrote the manuscript. ATSW contributed to funding acquisition and project administration. The author-s'responsibilities read revised and approved the final version of this manuscript.

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2551–0001684-0] and Conselho Nacional de Desenvolvimento Científco e Tecnológico (CNPq)/Edital Universal [Grant number 405128/2021-5], Brazil.

Ethics approval

The present study was approved by the Ethics Committee of the Federal University of Rio Grande do Sul, Brazil (No.36406) and followed institutional guidelines, Brazilian Society of Science in Laboratory Animals, and the Guide for the Care and Use of Laboratory Animals adopted by the National Institute of Health (NIH, USA).

Consent to participate

Not applicable.

Consent for publication

All the authors consent to publish this manuscript in Molecular Neurobiology.

Declaration of competing interest

The authors no competing interest.

Data availability

The data presented in this study are available on request from the corresponding author.

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PARTE III

5 DISCUSSÃO

Na presente tese, demonstramos que a suplementação com ácido fólico e o consumo de dieta hiperproteica durante toda a gestação causaram prejuízos comportamentais e neuroquímicos no córtex cerebral da prole de ratos machos adolescentes, confirmando nossa hipótese inicial. Escolhemos realizar as análises experimentais em ratos adolescentes, pois as primeiras semanas do período pós-natal são caracterizadas por intensa sinaptogênese, mielinização e gliogênese, uma vez que o desenvolvimento do cérebro em roedores não é concluído ao nascimento (Rice et al., 2000; Gilmore et al., 2018). Para a realização das análises bioquímicas a estrutura escolhida foi o córtex cerebral total, uma vez que tal região desempenha um importante papel em diversos comportamentos, tais como percepção, função motora, cognição, memória e aprendizagem (Essen et al., 2018; Natale et al., 2021).

No artigo I, demonstramos que a suplementação com ácido fólico durante toda a gestação não prejudicou a relação mãe-filhote, mas causou déficit de memória, alterações na motricidade e prejudicou a aprendizagem motora, bem como aumentou o comportamento do tipo ansioso e depressivo na prole. A ingestão de ácido fólico por ratas prenhes resultou também na indução de estresse nitrativo e de neuroinflamação no córtex cerebral da prole. Esses resultados são relevantes devido à sua correlação com gestantes que amplamente recebem suplementação com ácido fólico durante a gestação.

Evidências mostraram que a dieta durante a gestação pode alterar o cuidado materno e, consequentemente, impactar no neurodesenvolvimento da prole (Fairbanks et al., 2010; Purcell et al., 2011; Tang et al., 2014; Bstista et al., 2017). Não observamos diferenças entre os grupos controle e ácido fólico no cuidado

materno total e na frequência de cuidados maternos. No entanto, as mães do grupo ácido fólico passaram menos tempo fora do ninho quando comparado ao grupo controle, indicando que elas passaram mais tempo no ninho com os seus filhotes. Evidências mostram o olfato no início da vida desempenha um importante papel na sobrevivênciada prole, bem como na manutenção dos cuidados maternos (Meyer et al., 2016). O desempenho da prole no teste de preferência olfatória pelo odor maternal não foi afetado pela suplementação com ácido fólico durante a gestação. De acordo com estudos anteriores, o cuidado materno influencia significativamente nos resultados do teste de preferência pelo odor maternal (Uriarte et al., 2007; Kojima et al., 2009). Acreditamos que não foram observadas alterações no teste de preferência pelo odor maternal devido aos cuidados maternos não terem sido prejudicados. Assim, em nosso estudo, a suplementação com ácido fólico durante a gestação não prejudicou a relação mãe-filhote.

O estado nutricional durante a gravidez tem um importante impacto no desenvolvimento neurológico e nas funções cognitivas da prole (Gresham et al., 2014; Grieger et al., 2014; Chu et al., 2019; Neves et al., 2020). No presente estudo, foi observado que a suplementação com ácido fólico durante toda a gestação causou um déficit de memória de longo prazo na prole. Bahous e colegas (2017) observaram que ingestão de folato durante a gestação levou à deficiência de pseudo-metileno tetrahidrofolato redutase, causando distúrbios no metabolismo da acetilcolina, diminuição do volume hipocampal e déficit de memória na prole. Nesse contexto, estudos identificaram que a suplementação de ácido fólico durante a gestação pode provocar na prole uma diminuição nos níveis de neurotrofinas como NGF e BDNF (Sable et al., 2012; 2014). Além disso, Brocardo e colegas (2008)

observaram que o ácido fólico é capaz de inibir os receptores NMDA. Os receptores NMDA estão envolvidos na formação da memória (Crair et al., 1995; Feldman et al., 1999). Sugerimos que a suplementação com ácido fólico durante toda a gestação pode causar inibição destes receptores e afetar a memória da prole. Baseado nos resultados encontrados no teste de reconhecimento de objetos, decidimos analisar o imunoconteúdo da proteína sinapsina I no córtex cerebral da prole. Não identificamos diferenças entre os grupos controle e ácido fólico no imunoconteúdo da sinapsina I. Assim, nossos achados demonstram que a suplementação com ácido fólico durante gestação prejudica a memória de longo prazo da prole independente do imunoconteúdo de sinapsina I.

Estudos epidemiológicos evidenciam que a exposição a um ambiente adverso durante a gravidez eleva o risco de desenvolvimento de transtornos psiquiátricos na prole (Degroote et al., 2016; Tuovinen et al., 2021). Neste contexto, foi observado que a prole do grupo ácido fólico apresentou um aumento do comportamento do tipo ansioso no teste do labirinto em cruz elevado. Entretanto, não observamos diferenças estatísticas entre os grupos no teste de transição claro-escuro. Semelhante aos nossos resultados, Matsumoto e colaboradores (2016) demonstraram que a prole de ratas suplementadas com ácido fólico durante a gestação apresentou um aumento no comportamento do tipo ansioso, evidenciado pela diminuição do número de entradas nos braços abertos. Contrário aos nossos resultados, Barua e colegas (2014) não demonstraram alterações no teste do labirinto em cruz elevado em camundongos que receberam ácido fólico durante a gestação. Curiosamente, alguns estudos clínicos realizados em humanos observaram que a deficiência de folato durante a gravidez tem sido associada à

ansiedade (Steenweg et al., 2012; Anjos et al., 2013). Nossos resultados da avaliação do comportamento do tipo ansioso estão em concordância com estudos anteriores que mostraram que diferentes testes de ansiedade parecem refletir diferentes níveis de ansiedade (Holmes et al., 2001).

Fatores exógenos, como uma dieta enriquecida ou deficiente em nutrientes, como aminoácidos e vitaminas podem modificar a síntese de neurotransmissores podendo contribuir para o desenvolvimento de depressão (Marx et al., 2021). No teste de depressão relacionada à alimentação, identificamos um aumento na latência para a primeira mordida no grupo ácido fólico quando comparado ao grupo controle. Este teste avalia a anedonia em uma situação onde há um conflito entre a recompensa alimentar e a ansiedade (Ansorge et al., 2004). Zheng e colegas (2020) identificaram que a ingestão total de folato natural foi inversamente associada com sintomas depressivos, enquanto o ácido fólico sintético não foi. Contrário aos nossos resultados, Matsumoto e colegas (2016) demonstraram que a prole de ratas suplementada com ácido fólico durante a gestação não apresentou alteração da motivação para ingestão de alimentos, isto pode ser devido a uma menor dose de ácido fólico utilizada neste estudo (3mg/kg).

Estudos em pacientes com doenças neuropsiquiátricas, a deficiência de folato tem sido associada a baixos níveis de serotonina no LCR e a suplementação de ácido fólico restaura os níveis desse neurotransmissor (Hyland et al., 2010; Pan et al., 2017). Em resumo, os resultados encontrados na literatura sobre a suplementação de ácido fólico e depressão são conflitantes, e estudos devem ser realizados sobre este tema principalmente durante o período gestacional.

Estudos clínicos e pré-clínicos mostraram que a suplementação da dieta

durante a gravidez pode impactar na função motora da prole (Georgief et al., 2018). No teste da escada horizontal, observamos um aumento no tempo para atravessar a escada, no número de erros, bem como na porcentagem de erros no grupo ácido fólico quando comparado ao grupo controle. No *rotarod*, verificamos um aumento no número de quedas (teste: dia 1) no grupo ácido fólico quando comparado ao grupo controle. No entanto, não observamos diferenças entre os grupos controle e ácido fólico no número de quedas (teste: dia 2). Esses resultados mostraram que os animais do grupo ácido fólico apresentaram a capacidade de aprendizagem motora preservada. De acordo com os nossos resultados, um estudo de coorte prospectivo mostrou que crianças de um ano de idade nascidas de mães que relataram consumir doses de ácido fólico superiores a 5000 µg/dia tiveram um prejuízo no desenvolvimento psicomotor em comparação com mães que consumiram doses de 400-1000 µg/dia (Valera-Gran et al., 2014). Chu e colegas (2019) observaram que camundongos machos que consumiram água suplementada com ácido fólico (3,75 mg/L) tiveram uma aprendizagem motora deficiente e um aumento na expressão do gene 2 de resposta ao crescimento precoce (Egr2). Camundongos deficientes em Egr2 mostraram melhor desempenho na aprendizagem motora no *rotarod* (Poirier et al., 2007).

O estresse oxidativo/nitrativo durante a gravidez é uma condição que ocorre principalmente porque a placenta é rica em mitocôndrias. Durante a gestação, a placenta sofre alterações de um ambiente hipóxico para um ambiente rico em oxigênio. Com isso, mecanismos de defesas contra os danos dos radicais livres aumentam à medida que a gravidez progride (Lymperaki et al., 2015; Wu et al., 2016). Primeiramente, demonstramos no grupo ácido fólico uma diminuição nos

níveis de EROs no córtex cerebral da prole quando comparado ao grupo controle. Observamos também um aumento nos níveis de nitritos no grupo ácido fólico quando comparado ao grupo controle. No entanto, a suplementação com ácido fólico durante a gestação não alterou a expressão e o imunoconteúdo da iNOS no córtex cerebral da prole. Um aumento nos níveis de nitritos sugere que pode haver um aumento na produção de ERN. O aumento nos níveis de nitritos observado no córtex cerebral da prole pode estar relacionado com a atividade de outras enzimas que produzem óxido nítrico, tais como a nNOS e a eNOS. O folato pode regular a produção de NO e de EROs (Stanhewicz et al., 2017). A formação de iNOS depende da presença do cofator BH_4 e de substrato disponível para acoplar a oxidação da l-arginina à redução do oxigênio molecular para produzir NO (Jones et al., 2019). A enzima DHFR dependente de folato tem um papel na redução de BH_2 a BH_4 através da via de salvamento de BH_4 (Crabtree et al., 2011). Uma diminuição na formação de BH_4, leva ao desacoplamento enzimático da iNOS e aumento da produção de EROs (Crabtree et al., 2009; 2011). O 5-MTHF pode ligar-se ao sítio ativo da iNOS e mimetizar a ação da BH_4, promovendo a síntese de óxido nítrico e uma redução na produção de EROs (Chalupsky et al., 2015). O folato regula a produção de EROs indiretamente através da prevenção do desacoplamento da iNOS. Além disso, o folato pode reduzir a produção de EROs agindo diretamente como antioxidante (Joshi et al., 2001; Rezk et al., 2003). Tais mecanismos citados acima podem explicar parcialmente o aumento dos níveis de nitritos e a diminuição da produção de EROs no córtex cerebral da prole de ratas que foram suplementadas com ácido fólico durante toda a gestação.

A suplementação com ácido fólico pode afetar a metilação de genes anti-

inflamatórios e pró-inflamatórios (O'Neill et al., 2014). A partir disso, estudos têm mostrado que a relação entre folato e inflamação depende do momento da suplementação, da dosagem, dos genes metilados e da interação com o meio ambiente (Jones et al., 2019). Identificamos um aumento na expressão gênica de TNF-α, IL-1β, IL-6, IL-10 e MCP-1 no córtex cerebral no grupo ácido fólico quando comparado ao grupo controle. As citocinas inflamatórias TNF-α, IL-1β e IL-6 são produzidas principalmente por células da microglia em resposta ao desequilíbrio homeostático do sistema nervoso central (Becher et al., 2017). A citocina TNF-α diminui a captação de glutamato pelos astrócitos e pode aumentar a excitotoxicidade. A secreção da citocina IL-1β está fortemente relacionada à exacerbação de doenças neurodegenerativas (Clark et al., 2016). A quimiocina MCP-1 neuronal é essencial para a quimioatração de monócitos para o local da inflamação no sistema nervoso central (Howe et al., 2017).

Em nosso estudo, observamos um aumento da citocina anti-inflamatória IL-10 que pode ser devido a um mecanismo compensatório para tentar diminuir o processo neuroinflamatório. Com base em nossos resultados, podemos sugerir que o aumento da neuroinflamação observado no córtex cerebral pode estar associado às alterações comportamentais observadas na prole.

Os resultados obtidos no artigo I reforçam a importância do uso do ácido fólico na dose e período recomendados pela OMS, além do consumo de uma dieta balanceada durante todo o período gestacional, principalmente de nutrientes doadores de grupo metila, como o ácido fólico, o qual pode afetar o comportamento e a neuroquímica da prole.

Em relação ao artigo II, observamos um aumento nos níveis de homocisteína

no córtex cerebral, bem como uma redução dos níveis de histidina no soro da prole de ratas que consumiram dieta hiperproteica durante toda a gestação. Demonstramos também um aumento da expressão de citocinas pró-inflamatórias no córtex cerebral deste grupo. O consumo da dieta hiperproteica não prejudicou a relação mãe-filhote. Nossos achados também evidenciaram prejuízos na memória e motricidade, bem como aumento do comportamento do tipo ansioso na prole de ratas suplementadas com dieta hiperproteica durante toda a gestação.

Os aminoácidos são importantes para a síntese de proteínas e outras moléculas como catecolaminas, creatina, dopamina, óxido nítrico, poliaminas e hormônios (Wu et al., 2017). A ingestão de uma dieta rica em proteínas aumenta a formação de metabólitos de aminoácidos como homocisteína, sulfeto de hidrogênio e amônia, que podem prejudicar a sobrevivência e o crescimento embrionário e fetal (Taylor et al., 2016; Wu, 2016). Nosso estudo demostrou que o consumo de uma dieta hiperproteica durante a gestação diminui os níveis de histidina no córtex cerebral da prole. A histidina é um aminoácido essencial que participa da síntese de proteínas e é um precursor da histamina (Moro et al., 2020). A histamina desempenha um importante papel como neurotransmissor regulando o humor, o ciclo do sono, a aprendizagem, a memória, o estado de alerta, o apetite e a percepção da dor (Carthy et al., 2021). Foi observado que a dieta hiperproteica causou um aumento significativo nos níveis de homocisteína no córtex cerebral da prole. A homocisteína é um aminoácido que contém enxofre e é derivada do metabolismo da metionina (Skovierová et al., 2016). Um aumento nos níveis de homocisteína tem sido associado ao estresse oxidativo, exacerbação da inflamação, alterações metabólicas e comportamentais (Loureiro et al., 2010; Pierozan et al.,

2018; Wyse et al., 2020; dos Santos et al., 2021; Ramires-Junior et al., 2022).

A ativação imune materna durante a gestação pode ser um fator de risco para alterações neurológicas observadas na prole que posteriormente se manifestam como distúrbios comportamentais (Knuesel et al., 2014; Ziats et al., 2015). Verificamos a presença de neuroinflamação no córtex cerebral da prole causada pela suplementação com dieta hiperproteica durante toda a gestação. Demonstramos um aumento da expressão gênica de TNF-α, IL-1β, IL-6, IL-10 e MCP-1 na prole de ratas que consumiram dieta hiperproteica. A microglia ativada produz citocinas pró-inflamatórias como TNF-α, IL-1β, e IL-6 que ativam os astrócitos, podendo resultar em morte e disfunção neuronal e estão envolvidas na patogênese de doenças neurodegenerativas (Jacobs et al., 2012; Wang et al., 2015). A proteína MCP-1 neuronal é uma quimiocina que induz o acúmulo de monócitos no local da inflamação (Guzman-Martinez et al., 2019). Corroborando com os nossos resultados, Maurer e colegas (2011) mostraram que o consumo materno de uma dieta rica em proteínas (40%) durante a gravidez e a lactação aumentaram os níveis de IL-6 no tecido adiposo marrom da prole.

Curiosamente, em nosso estudo, observamos um aumento da expressão da citocina anti-inflamatória IL-10 na prole de ratas que consumiram dieta hiperproteica. Esse aumento de IL-10 pode ser devido a um mecanismo compensatório para controlar o processo neuroinflamatório causado pelo aumento de TNF-α, IL-1β, IL-6 e MCP-1. Com base em nossos resultados, sugerimos que o aumento da neuroinflamação observado no córtex cerebral pode estar associado as alterações comportamentais identificadas na prole de ratas que consumiram dieta hiperproteica durante toda a gestação.

A nutrição materna durante a gravidez influencia o neurodesenvolvimento da prole, especialmente os processos cognitivos, como a memória (Ge et al., 2021; Cortés-Álvarez et al., 2022). Nossos achados mostram que o consumo de uma dieta hiperproteica durante a gestação prejudicou a memória de longo prazo da prole, sugerimos que o comprometimento da memória observada na prole pode estar associado em parte com a diminuição dos níveis de histidina e aumento dos níveis de homocisteína no córtex cerebral. A histidina é um neurotransmissor que controla uma ampla variedade de processos neurobiológicos e funções comportamentais, como a memória. A histamina é sintetizada a partir da histidina através da catálise da histidina descarboxilase (Moro et al., 2020). Níveis baixos de histamina podem causar prejuízos comportamentais, como depressão, redução da motivação, prejuízos motores, dificuldade na aprendizagem e déficits de memória (Leppik et al., 2018; Solís-Ortiz et al., 2021; Nakamura et al., 2022). Uma redução dos níveis de histamina também é observada na Doença de Alzheimer (Haas et al., 2008). Esses resultados sugerem que o consumo de uma dieta hiperproteica durante toda a gestação diminui os níveis de histidina na prole e consequentemente pode reduzir a produção de histamina, o que pode contribuir para o comprometimento da memória na prole.

O aumento dos níveis de homocisteína é um importante fator de risco para doenças neurodegenerativas como a doença de Alzheimer (Cordaro et al., 2021). Recentemente, Ramires-Junior e colegas (2022) demostraram déficit de memória de trabalho e diminuição da proteína sinapsina I no hipocampo em um modelo de hiper-homocisteinemia leve crônica em ratos. Assim, devido às alterações neuroquímicas causadas pela homocisteína que podem prejudicar a memória,

sugerimos que o consumo de uma dieta hiperproteica durante a gestação aumenta os níveis de homocisteína na prole e consequentemente, pode ser um dos fatores associados ao déficit de memória observado na prole.

A dieta materna enriquecida ou deficiente em nutrientes durante a gravidez, como aminoácidos e vitaminas pode modificar a síntese de neurotransmissores e contribuir para o desenvolvimento de doenças psiquiátricas (Marx et al., 2021; Siveira et al., 2022). Na análise do teste de transição claro-escuro, nossos resultados revelam um aumento do comportamento do tipo ansioso na prole de ratas suplementadas com dieta hiperproteica durante toda a gestação. A dieta materna também pode influenciar o comportamento alimentar da prole. No teste de depressão relacionada à alimentação, identificamos uma diminuição na latência para a primeira mordida no grupo dieta hiperproteica. Esse teste analisa os níveis de depressão e motivação para comer após privação alimentar e a anedonia em uma situação onde há um conflito entre recompensa alimentar e ansiedade (Ansorge et al., 2004). Kanitz e colegas (2012) observaram que uma dieta rica em proteínas (30%) durante a gestação altera a expressão de genes que controlam a produção do hormônio glicocorticóide no hipotálamo fetal de porcos, causando um desequilíbrio no eixo hipotálamo-hipófise-adrenal com consequências duradouras na prole. A desregulação do eixo hipotálamo-hipófise-adrenal pode causar doenças neuropsiquiátricas como a ansiedade (Sheng et al., 2021).

O aumento nos níveis de homocisteína tem sido fortemente associado a distúrbios neuropsiquiátricos, como esquizofrenia, transtorno bipolar, autismo, depressão e ansiedade (Kinoshita et al., 2013; Nabi et al., 2013; Vuksan-Cusa et al., 2013; Hrncic et al., 2016; Józefczuk et al., 2017; Wyse et al., 2020). Uma das

causas do aumento do comportamento do tipo ansioso observado no grupo dieta hiperproteica pode ter sido o aumento nos níveis de homocisteína no córtex cerebral da prole. O déficit de histamina neuronal está envolvida em várias doenças neurológicas, incluindo narcolepsia, distúrbios alimentares, síndrome de Tourette, depressão e ansiedade (Haas et al., 2008). A diminuição dos níveis de histidina observada na prole exposta a uma dieta hiperproteica durante a gestação pode ter alterado o sistema histaminérgico no SNC e, consequentemente, pode ter contribuído para o desenvolvimento do comportamento do tipo ansioso. Assim, um aumento na concentração de homocisteína e uma diminuição dos níveis de histidina observados em nosso estudo pode ter contribuído para o aumento do comportamento do tipo ansioso na prole.

Evidências mostram o tipo de dieta consumida durante a gravidez pode afetar a motricidade da prole (Georgief et al.,, 2018). Nossos resultados demonstram um prejuízo na motricidade da prole de ratas suplementadas com dieta hiperproteica durante a gestação evidenciada nos testes da escada horizontal e da trave de equilíbrio. Em parte, o aumento nos níveis homocisteína na prole pode ter contribuído para o comprometimento motor. Estudos têm relacionado o aumento dos níveis de homocisteína a alterações na função muscular, prejuízos na coordenação motora e locomoção (Veeranki et al., 2015; Gales et al., 2018). Estudos demonstram que a composição da dieta pode alterar a bioquímica muscular (Braggion et al., 2016; Sohel et al., 2020). Em nosso estudo mostramos que a dieta hiperproteica consumida durante toda a gestação diminui os níveis de histidina no córtex cerebral da prole. A histidina é precursor da carnosina (Brosnan et al., 2020). A carnosina contribui para a contratilidade e homeostase do músculo esquelético, através de seu

papel como antioxidante, tampão de pH e regulador de cálcio (Caruso et al., 2012; Boldyrev et al., 2013). Portanto, o prejuízo na motricidade observado na prole pode ser associado em parte com a diminuição dos níveis de histidina e a provável diminuição dos níveis de carnosina. Novos estudos são necessários para elucidar tal mecanismo.

Algumas limitações devem ser consideradas nos achados dessa tese. Em primeiro lugar, devido à diferença entre espécies, nossos resultados não devem ser diretamente extrapolados para humanos. Os roedores são, em muitos aspectos, biologicamente e psicologicamente semelhantes aos humanos, tais como em características de dor, medo e sofrimento. No entanto, as evidências demonstram que as diferenças entre as espécies na fisiologia e genética, bem como a exposição a procedimentos laboratoriais podem resultar em desfechos expressivamente diferentes em humanos (Akhtar et al., 2015). Outra limitação refere-se ao período o qual foi realizada a suplementação com ácido fólico ou o consumo de dieta hiperproteica durante a gestação. A suplementação com ácido fólico ou o consumo de dieta hiperproteica durante todo o período perinatal ou apenas durante a lactação pode causar outros efeitos que não foram encontrados nesse estudo. Em nosso estudo realizamos as análises experimentais na prole de ratos machos. No presente estudo, optamos por realizar os experimentos somente com a prole de ratos machos pois as fêmeas são suscetíveis às flutuações hormonais que ocorrem durante o ciclo estral (Beery et al., 2011). Além disso, vários estudos mostraram que o ciclo estral pode afetar o resultado dos testes comportamentais (Meziane et al., 2007; Prendergast et al., 2014; Kokras et al., 2015; Chari et al., 2020). Nesse contexto, a prole feminina poderia apresentar diferentes respostas a suplementação de ácido

fólico e dieta hiperproteica durante o período gestacional. Uma vez que placenta da prole masculina é mais dependente da dieta materna e a transferência de nutrientes é mais eficiente porque possui uma menor capacidade de armazenamento comparada com a placenta da prole de fêmeas (Eriksson et al., 2010).

Os achados observados nos capítulos I e II desta tese nos permitem concluir que a suplementação com ácido fólico ou o consumo de dieta hiperproteica durante toda a gestação de ratas Wistar causaram prejuízos comportamentais e neuroquímicos na prole de ratos machos. Nosso estudo representa um importante passo na exploração dos efeitos da composição da dieta materna consumida por ratas prenhes durante toda a gestação, uma vez que fornece evidências de desfechos desfavoráveis sobre a prole.

6 CONCLUSÕES

Os resultados da presente tese permitem concluir que:

CAPÍTULO I:

A dieta suplementada com 4 mg/kg de ácido fólico durante toda a gestação prejudicou a memória e a motricidade da prole quando comparada ao controle. Também foi observado um aumento no comportamento do tipo ansioso e depressivo neste grupo, bem como os níveis de nitritos aumentaram no córtex cerebral da prole. Entretanto, o imunoconteúdo e a expressão de iNOS não foram alterados. Além disso, identificamos um aumento na expressão gênica de TNF-*α*, IL-1β, IL-6, IL-10 e da MCP-1 no córtex cerebral da prole suplementada com ácido fólico ao longo da gestação.

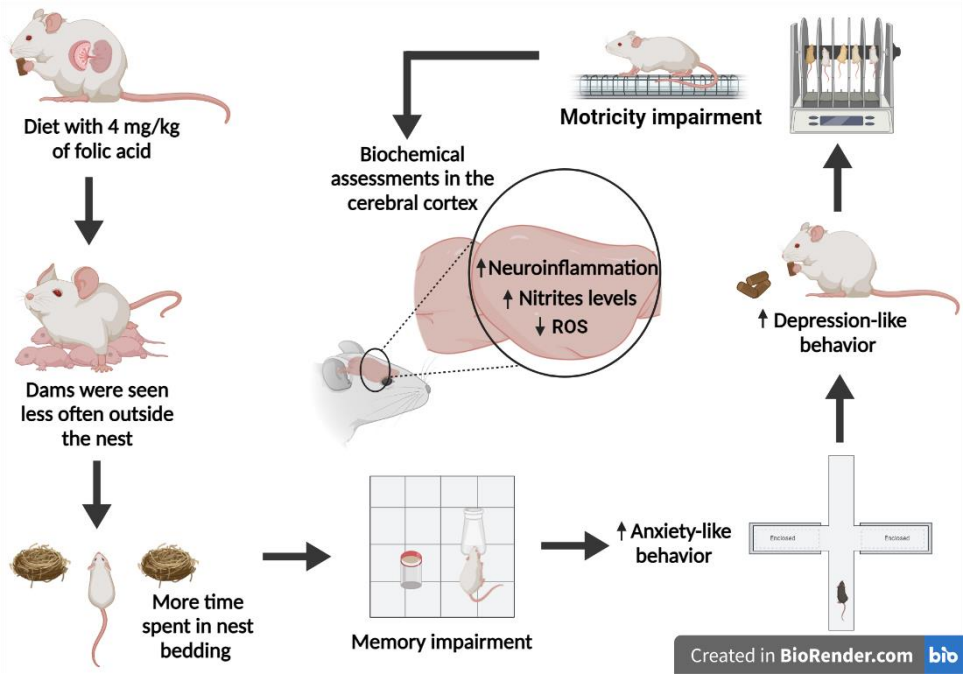

Figura 5: Resumo dos resultados do capítulo I (Silveira et al., 2022).

CAPÍTULO II:

Os resultados evidenciam um aumento nos níveis de homocisteína e na expressão gênica de TNF-*α*, IL-1β, IL-6, IL-10 e da MCP-1 no córtex cerebral da prole de ratas prenhes que consumiram dieta hiperproteica (50%) durante toda a gestação. Além de diminuir os níveis de histidina no soro da prole. Os resultados também revelaram prejuízos na memória e motricidade e um aumento do comportamento do tipo ansioso na prole suplementada com dieta hiperproteica durante a gestação.

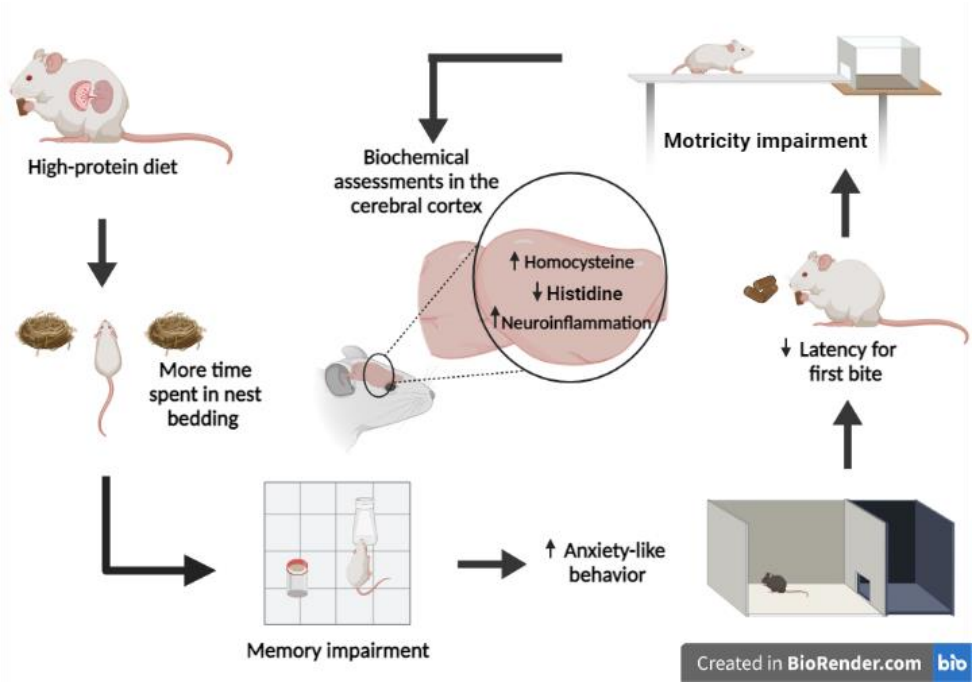

Figura 6: Resumo dos resultados do capítulo II.

Em conjunto, nossos resultados demonstram que alterações na dieta materna tais como suplementação com ácido fólico e consumo de dieta hiperproteica durante todo o período gestacional, podem provocar alterações comportamentais e neuroquímicas na prole. Nossos resultados reforçam a importância do consumo de uma dieta equilibrada durante o período gestacional, onde o consumo de nutrientes,

principalmente de doadores de grupo metila, pode impactar no desenvolvimento da prole. Nossos achados fornecem uma base para a melhor compreensão de que a exposição do feto a um ambiente intrauterino adverso pode provocar alterações duradouras na prole. Além disso, fornece evidências adicionais para a compreensão dos resultados bioquímicos e comportamentais associados à suplementação de ácido fólico ou à exposição à dieta hiperproteica durante toda a gestação. Assim, mais estudos são necessários, principalmente relacionados à análise de mudanças epigenéticas, dimorfismo sexual e alterações ao longo da vida da prole de mães suplementadas com ácido fólico ou dieta hiperproteica durante toda a gestação.

7 PERSPECTIVAS

Avaliar na prole de ratas Wistar submetidas à suplementação de ácido fólico ou ao modelo de dieta hiperproteica gestacional:

1. o metabolismo energético e parâmetros relacionados à função mitocondrial;
2. o metabolismo da glicose;
3. avaliação das modificações epigenéticas;
4. avaliar se as alterações observadas nesta tese persistem na prole em ratas fêmeas.

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ANEXO

CARTA DE APROVAÇÃO DO COMITÊ DE ÉTICA UFRGS (CEUA/UFRGS)

U F R G S
UNIVERSIDADE FEDERAL
DO RIO GRANDE DO SUL

PRÓ-REITORIA DE PESQUISA
Comissão De Ética No Uso De Animais

CEUA UFRGS

CARTA DE APROVAÇÃO

Comissão De Ética No Uso De Animais analisou o projeto:

Número: 36406
Título: AVALIAÇÕES COMPORTAMENTAIS, BIOQUÍMICAS E HISTOLÓGICAS AO LONGO DO DESENVOLVIMENTO DA PROLE DE RATAS WISTAR SUBMETIDAS À HIPERMETIONINEMIA E À DIETA HIPERPROTEICA DURANTE O PERÍODO GESTACIONAL: EFEITO

Vigência: 01/02/2019 à 01/10/2022

Pesquisadores:

Equipe UFRGS:

ANGELA TEREZINHA DE SOUZA WYSE - coordenador desde 01/02/2019
Josiane Silva Silveira - Aluno de Doutorado desde 01/02/2019

Comissão De Ética No Uso De Animais aprovou o mesmo , em reunião realizada em 18/03/2019 - Auditório Plenarinho- Saguão do Prédio da Reitoria- Campus Centro da UFRGS- Bairro Farroupilha - Porto Alegre, em seus aspectos éticos e metodológicos, para a utilização de 77 machos e 77 fêmeas Wistar , com 70 a 90 dias de idade (progenitores), além de mais 777 filhotes (machos e fêmeas) oriundos da prole dos progenitores, com idades experimentais de 07, 21 e 60 dias, todos provenientes do CREAL-UFRGS; de acordo com os preceitos das Diretrizes e Normas Nacionais e Internacionais, especialmente a Lei 11.794 de 08 de novembro de 2008, o Decreto 6899 de 15 de julho de 2009, e as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), que disciplinam a produção, manutenção e/ou utilização de animais do filo Chordata, subfilo Vertebrata (exceto o homem) em atividade de ensino ou pesquisa.

Porto Alegre, Sexta-Feira, 29 de Março de 2019

ALEXANDRE TAVARES DUARTE DE OLIVEIRA
Coordenador da comissão de ética