UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM NEUROCIÊNCIAS

O PAPEL DA SUPLEMENTAÇÃO COM ÁCIDO FÓLICO COMO ESTRATÉGIA PREVENTIVA E NEUROPROTETORA: UM ESTUDO AVALIANDO OS SEUS EFEITOS *IN VIVO* E *IN VITRO*.

TESE DE DOUTORADO

Jaqueline Vieira Carletti

Porto Alegre, RS, Brasil

2016

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Jaqueline Vieira Carletti

Orientadora: Prof.ª. Dr.ª Lenir Orlandi Pereira Silva

Tese apresentada ao curso de Pós-graduação em Neurociências como requisito parcial para a obtenção do grau de Doutora em Neurociências.

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"(...) Quem cultiva a semente do amor

segue em frente e não se apavora (...)"

Xande De Pilares; Gilson Bernini; Carlinhos Madureira

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RESUMO

A hipóxia-isquemia neonatal (HI) é um dos principais motivos de mortalidade e morbidade perinatal. Diferentes causas estão associadas à HI, tais como: interrupção do fluxo sanguíneo, ineficientes trocas gasosas pela placenta, prematuridade, pré-eclâmpsia (PE), entre outras. Achados prévios do nosso grupo, utilizando o ácido fólico (AF) como estratégia terapêutica na HI, mostraram que o tratamento foi efetivo em amenizar alguns danos em animais submetidos ao modelo de encefalopatia hipóxico-isquêmica (EHI). Os objetivos do presente estudo foram investigar os efeitos/consequências do tratamento/suplementação com AF durante: (i) a EHI em ratos submetidos ao modelo Rice-Vannucci, e (ii) o tratamento com tert-butil-hidroperóxido (TBH) em células trofoblásticas humanas (linhagem BeWo e HTR-8 SV/neo), simulando o estresse oxidativo ocasionado por PE. Para a realização do experimento *in vivo*, aos sete dias de vida ratos Wistar foram divididos em quatro grupos experimentais: controle salina (CT-S), controle ácido fólico (CT-AF), hipóxico-isquêmico salina (HI-S) e hipóxico-isquêmico ácido fólico (HI-AF). Para o experimento *in vitro* células das linhagens BeWo e HTR-8 SV/neo foram cultivadas, suplementadas com três distintas concentrações de AF (deficiente, fisiológica e supra-fisiológica) e expostas durante 24 horas ao TBH. Nossos resultados demonstraram que a EHI dificultou a memória e aprendizado nos testes do labirinto aquático de Morris e do ox-maze, reduziu a atividade da Na^+, K^+-ATPase em animais adultos, causou atrofia em importantes áreas encefálicas como estriado e hipocampo, contribuiu para o desequilíbrio das enzimas antioxidantes SOD e CAT, além de causar um aumento da reatividade astrocitária em diferentes períodos do desenvolvimento animal. No entanto, quando observado o efeito do tratamento com AF nos diferentes parâmetros analisados foi constatado que este contribuiu para o déficit cognitivo no teste do labirinto aquático, enquanto que esse efeito foi amenizado no teste do ox-maze. Também, observou-se que, enquanto o tratamento com AF contribuiu para a inibição da enzima Na^+, K^+ -ATPase nos animais HI adolescentes, este foi efetivo em reverter essa inibição nos adultos. Além do mais, o tratamento com AF promoveu a recuperação parcial da atrofia hipocampal, aumentou a atividade das enzimas SOD e CAT e atrasou a formação da cicatriz glial. No modelo *in vitro* com exposição ao TBH, o tratamento com AF foi responsável pelo aumento da peroxidação lipídica, da carbonilação proteica e dos níveis de glutationas. Além disso, houve também um efeito antiproliferativo, antimigratório e citotóxico. Interessantemente, todos esses resultados foram intensificados quando observados nas células cultivadas sob a concentração supra-fisiológica de AF. Tendo em vista que cerca de 1 a 8% das gestantes desenvolvem pré-eclâmpsia e essas normalmente estão expostas a altas dosagens de AF devido à suplementação e à ingestão de alimentos fortificados, mais atenção é necessária especialmente na população de risco para essa doença. Os resultados obtidos neste estudo contribuem para o avanço das pesquisas nesta área e destacam a importância de mais investigações a fim de esclarecer até que ponto a suplementação com AF e sua dosagem adequada trariam benefícios para neonatos com EHI e gestantes com PE.

ABSTRACT

Hypoxia-ischemia (HI) is one of the main causes of perinatal death and morbidity. There are many imbalances related to the onset of HI, such as: interrupted blood flow, placenta gas exchange insufficiency, prematurity, preeclampsia (PE), among others. Previously, our group have investigated folic acid (FA) as a therapeutic agent of HI and showed it was able to reduce some deficits observed in animals submitted to the hypoxic-ischemic encephalopathy model (HIE). The aim of this study was the investigation of the effects and consequences of the treatment/supplementation with FA during (i) the HIE in rats subject to the Rice-Vannucci model, and (ii) the treatment of human trophoblastic cells (BeWo and HTR-8SV/neo) with tert-butyl-hydroperoxide (TBH) chemical agent, which mimics the oxidative stress during PE. During the in vivo experiment, seven-days Wistar rats were divided into four groups, as follows: control saline (CT-S), control folic acid (CT-FA), hypoxia-ischemia saline (HI-S) and hypoxia-ischemia folic acid (HI-FA). During the in vitro experiment BeWo and HTR-8 SV/neo cells were cultured under three distinct FA concentrations (deficient/physiological/supra-physiological) and exposed to TBH during 24 hours. Results showed HIE promoted memory and learning impairments as observed during the Moris water maze and ox-maze tests, reduced the activity of Na^+, K^+ -ATPase in adult rats, promoted atrophy in important areas as striatum and hippocampus, contributed to the imbalances in the oxidative enzymes SOD and CAT, and promoted the increase of astrocytic reactivity in distinct periods of development. Nevertheless, the treatment with FA contributed to the cognitive deficits observed in the water maze, while this effect was reduced in the ox-maze test. It was also observed that while FA treatment contributed to the inhibition of Na^+, K^+ -ATPase in HI adolescent rats, it was effective to recover this activity in HI adult rats. Moreover, FA treatment was able to partially recover hippocampal atrophy, to increase the activity of SOD and CAT enzymes and to delay the glial scar. The FA treatment in the in vitro model, where cells were challenged with TBH, was responsible for the increase in lipid peroxidation, protein cabonilation and glutathione levels. Moreover, it was observed antiproliferative, antimigration and cytotoxic effect. Interestingly, these outcomes were more intense when cells were cultured under a supra-physiological concentration of FA. Considering that about 1-8% of pregnant women suffer from PE and are often exposed to high doses of FA due to the supplementation and the ingestion of fortified food, more attention should be given to this group of risk. Results obtained during this work contribute to the improvement of research in this field and highlight the need of further studies to better understand to each extent the FA supplementation and daily dose intake are beneficial to EHI newborns and pregnant women with PE.

LISTA DE ABREVIATURAS E FÓRMULAS

5-MTHF: 5-metil-tetrahidrofolato

AF: ácido fólico

AMPA: ácido α-amino-3-hidroxi-5-metil-4-isoxazolepropionico

Ca: cálcio

CAT: catalase

CDC: *Centers for Disease Control*

Cl^-: íon cloreto

CT-FA: controle ácido fólico

CT-S: controle salina

EHI: encefalopatia hipóxico-isquêmica

ERN: espécies reativas de nitrogênio

EROs: espécies reativas de oxigênio

FAP: fator de ativação plaquetária

FDA: *Food and Drugs Association*

Fe: ferro

FEBRASGO: Federação Brasileira das Associações de Ginecologia e Obstetrícia

FR: receptor de folato

GFAP: proteína ácida fibrilar glial

GPx: glutationa peroxidase

H_2O_2: peróxido de hidrogênio

HI: hipóxia-isquemia

HI-FA: hipóxico-isquêmico ácido fólico

HI-S: hipóxico-isquêmico salina

m-GLU-r: receptor metabotrópico de glutamato

Na: sódio

NMDA: *N*-metil-*D*-aspartato

$NO^{\bullet}$: Óxido Nítrico

$O_2^{\bullet -}$: radical superóxido

OH^-: Radical hidroxil

OMS: Organização mundial da saúde

$ONOO^-$: Peroxinitrito

PCFT: transportador de folato acoplado a próton

PE: pré-eclâmpsia

RFC: transportador de folato reduzido

SAM: s-adenosilmetionina

SNC: sistema nervoso central

SOD: superóxido dismutase

TBH: tert-butil-hidroperóxido

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1. Introdução

1.1 Hipóxia-Isquemia encefálica neonatal

A hipóxia-isquemia (HI) encefálica neonatal é definida como um insulto ao sistema nervoso central (SNC) do feto, ocasionada por diversas complicações no período perinatal que resultam em hipóxia cerebral parcial ou completa juntamente com reduzido fluxo sanguíneo (Dammann *et al.*, 2011; Wachtel e Hendricks-Munoz, 2011). Essas complicações acarretarão em dano cerebral e no desenvolvimento da encefalopatia hipóxico-isquêmica (EHI), a qual é caracterizada por uma série de complicações e sequelas neuropsicológicas tais como: atraso cognitivo (dificuldade de aprendizado), déficit de atenção e hiperatividade, disfunção visual, paralisia cerebral, hidrocefalia, epilepsia e disfunções motoras (Vannucci e Perlman, 1997; Glass *et al.*, 2011; Juul e Ferriero, 2014; Ahearne *et al.*, 2016; Placha *et al.*, 2016). De acordo com estudos recentes, a EHI ocorre em 2-3/1000 nascidos vivos a termo e em 22/1000 nascidos prematuros; e é uma das grandes causas de mortalidade e morbidade entre os neonatos (Kurinczuk *et al.*, 2010; Lai e Yang, 2011; Davidson *et al.*, 2015; Rumajogee *et al.*, 2016). Além do mais, de acordo com a Organização mundial da saúde (OMS), é estimado que em países emergentes o índice de asfixia neonatal esteja em torno de 7 para cada 1000 nascidos vivos (WHO, 2006). No Brasil, um recente estudo demonstrou que houveram 32,38 óbitos neonatais para cada 1000 nascidos vivos (3,2%) e este desfecho foi associado com hipóxia intrauterina, asfixia durante o nascimento, aspiração de mecônio, além de baixo peso ao nascer (de Almeida *et al.*, 2015).

1.2 Patogênese da encefalopatia hipóxico-isquêmica

1.2.1 Etiologia e diagnóstico da EHI

Diferentes situações estão associadas à falta de oxigenação para o feto e consequentemente ao surgimento da EHI, tais como: interrupção do fluxo sanguíneo umbilical por compressão, ineficientes trocas gasosas pela placenta, perfusão placentária inadequada, prematuridade, angústia respiratória do recém-nascido e síndrome da aspiração meconial (Volpe, 2001b; Esquiliano *et al.*, 2004; Rumajogee *et al.*, 2016). Além das situações já descritas, o desenvolvimento da pré-eclâmpsia durante a gestação também se destaca por estar associada à restrição de crescimento do feto e hipóxia intrauterina, uma vez que podem culminar em um quadro de EHI (Gaccioli e Lager, 2016).

A pré-eclâmpsia (PE) é uma doença multissistêmica que afeta em torno de 1-8% das gestações e é caracterizada por hipertensão e proteinúria após a 20ª semana de gestação, sendo uma importante causa de morbidade e mortalidade maternal e neonatal (Gathiram e Moodley, 2016; Paauw *et al.*, 2016; Phipps *et al.*, 2016). Embora os mecanismos envolvidos na fisiopatologia da PE não estejam completamente elucidados, a principal hipótese é de que haja uma falha do citotrofoblasto em remodelar as artérias espirais uterinas (inadequada invasão trofoblástica e placentação), o que acaba levando à hipoperfusão e isquemia da placenta (Lambert *et al.*, 2014; Gathiram e Moodley, 2016). A placenta isquêmica por sua vez liberará fatores angiogênicos, anti-angiogênicos e pró-inflamatórios na corrente sanguínea materna que causarão disfunção endotelial e estresse oxidativo, contribuindo para as manifestações clínicas da

doença (Chaiworapongsa *et al.*, 2014; Gathiram e Moodley, 2016; Lamarca *et al.*, 2016). Dentre as manifestações clínicas graves estão o aparecimento de convulsões maternas, disfunção renal e hepática, além de restrição de crescimento, hipóxia, asfixia do feto e desenvolvimento de EHI (Redman, 2011; Ventolini, 2011; Chaiworapongsa *et al.*, 2014; Miller *et al.*, 2016; Rakotomalala *et al.*, 2016) (Figura 1).

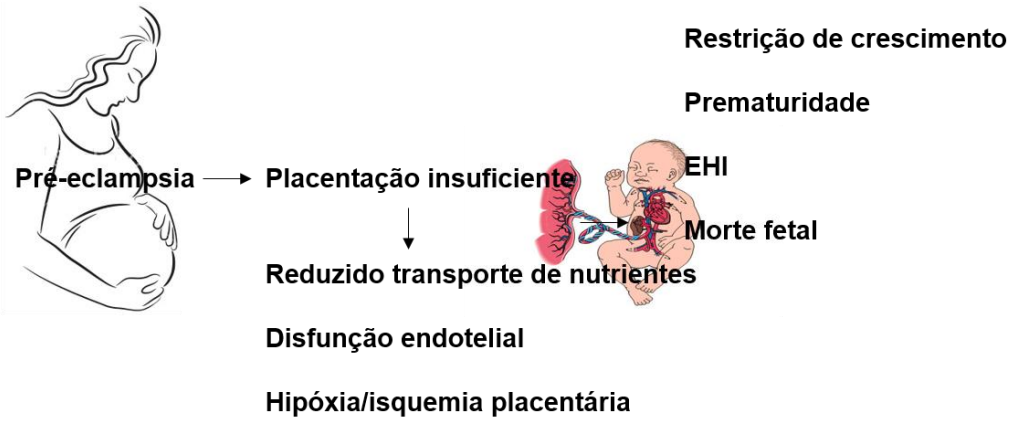

Figura 1. Desenho esquemático representando as consequências maternais e fetais da pré-eclâmpsia (Adaptado de Gaccioli e Lager (2016)).

De fato, qualquer situação que leve o feto à hipoxemia, hipercapnia e à hipoperfusão tecidual culminarão em imediatas consequências e danos neurológicos (Volpe, 2001b; Bouiller *et al.*, 2016). Normalmente o diagnóstico da EHI é realizado através de alguns critérios pré-estabelecidos como: convulsões, acidose metabólica (pH<7), baixo escore de Apgar (<7) no primeiro e no quinto minuto de vida (Tabela 1), dano sistêmico, anormalidades no eletroencefalograma e em neuroimagens produzidas por ressonância magnética (Procianoy, 2001; Perlman, 2006; Pisani e Spagnoli, 2016).

Tabela 1. Sistema de pontuação APGAR

Sinais	**0**	**1**	**2**
Cor	Azul, pálido	Extremidades acrocianóticas	Completamente rosado
Frequência cardíaca	Ausente	<100 bpm	>100 bpm
Tônus Muscular	Flácido	Algumas flexões nas extremidades	Movimento ativo
Respiração	Ausente	Choro fraco, hipoventilação	Boa, chorando
Reflexo de irritabilidade	Sem resposta	Careta	Choro ativo

Adaptado de Watterberg *et al.* (2015); Academia Americana de Pediatria.

Além do mais, a gravidade da asfixia perinatal e sua evolução podem ser também avaliadas e classificadas em diferentes graus: leve, moderada e grave, por meio dos critérios descritos na escala de Sarnat e Sarnat (1976) (Tabela 2).

1.2.2. Mecanismos celulares e moleculares envolvidos na EHI

Diversas modificações moleculares como falha energética, excitotoxicidade glutamatérgica, inflamação e formação de radicais livres ocorrem durante e após o período de hipoxemia e isquemia, os quais contribuirão para a morte neuronal levando ao dano encefálico caracterizado pela EHI (Volpe, 2001b; Mclean e Ferriero, 2004) (Figura 2). Em nível metabólico, inicialmente a hipóxia leva à diminuição do aporte de ATP celular, através da falha do sistema mitocondrial, ciclo do ácido cítrico e da cadeia transportadora de elétrons, acelerando a glicólise anaeróbica (Perlman, 2007).

Tabela 2. Classificação de EHI de acordo com o grau de Sarnat.

Sinais	Leve	Moderado	Grave
Nível de consciência	Hiperalerta	Letárgico	Coma
Tônus muscular	Normal	Hipotonia leve	Flácido
Postura	Flexão distal leve	Flexão distal forte	Descerebração intermitente
Reflexos tendinosos	Aumentados	Aumentados	Ausentes/Diminuídos
Mioclonias	Presente	Presente	Ausente
Convulsões	Ausente	Frequente (focal ou multifocal)	Variável
Sucção	Ativa/pouco fraca	Fraca/ausente	Ausente
Moro	Exacerbado	Incompleto	Ausente
Oculovestibular	Normal	Exagerado	Fraco/Ausente
Tônus do pescoço	Leve	Forte	Ausente
Funções autonômicas	Simpáticas generalizadas	Parassimpática generalizada	Ambos sistemas diminuídos
Pupila	Midríase/reativas	Miose/reativas	Variável/ pouco reativas
Batimento cardíaco	Taquicardia	Bradicardia	Variável/Bradicardia
Respirações	Espontâneas	Periódicas	Apneia
Secreções de vias aéreas	Escassa	Profusa	Variável
Motilidade gastrointestinal	Normal	Aumentada	Variável
Eletroencefalograma	Normal (acordado)	Baixa voltagem, padrão periódico (desperto)	Periódico ou isoelétrico
Duração dos sintomas	< 24 h	2-14 dias	Horas-semanas

Adaptado de (Sarnat e Sarnat, 1976).

Além disso, a baixa produção de ATP dispara uma série de mecanismos adicionais que iniciam com a falha na atividade da enzima Na^+, K^+-ATPase, o que contribui para aumentados níveis intracelulares de Na^+, Cl^- e água, levando a edema, lise e morte celular por necrose. A enzima Na^+, K^+-ATPase é uma proteína de membrana essencial para a regulação intracelular de íons, que mantem o correto potencial de membrana e excitabilidade neuronal (Mobasheri *et al.*, 2000; Golden *et al.*, 2001). Assim, qualquer mudança na sua atividade pode levar a graves consequências para o funcionamento do SNC (Edwards *et al.*, 2013). Ademais, durante o evento da HI e falha energética, também ocorre um aumento na produção de lactato, o que acaba contribuindo para a acidose tecidual, e inibição da atividade da fosfofrutocinase, a qual por sua vez bloqueia o processo glicolítico colaborando para o dano neuronal e morte celular por necrose (Volpe, 2001b).

Outro importante mecanismo envolvido na morte neuronal é a excitotoxicidade glutamatérgica que ocorre também devido à falha energética. Assim, os astrócitos ficam impossibilitados de remover o excesso de glutamato da fenda sináptica, causando uma super estimulação dos receptores glutamatérgicos (N-metil-D-aspartato (NMDA), ácido α-amino-3-hidroxi-5-metil-4-isoxazolepropionico (AMPA), receptores metabotrópicos de glutamato (m-GLU-r) e cainato) conhecida como excitotoxicidade glutamatérgica. Esta super estimulação leva a um descontrolado influxo de Na^+ e Ca^{2+} desencadeando edema e morte celular por necrose e apoptose. Somado a isso, o encéfalo do neonato apresenta maior expressão do receptor NMDA e em casos de HI isso pode contribuir para um efeito devastador no SNC (Mclean e Ferriero, 2004).

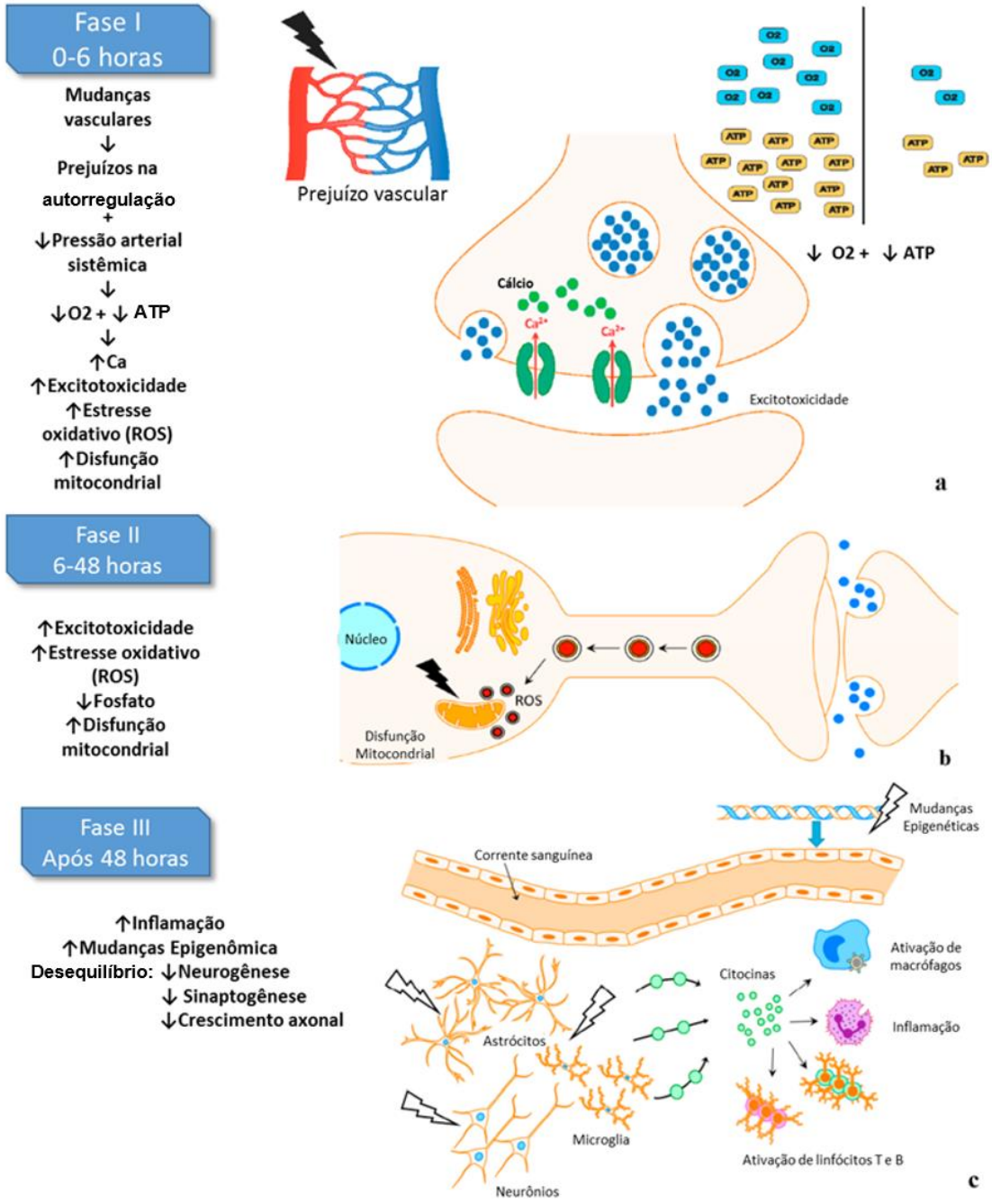

Figura 2. Mecanismos celulares e moleculares envolvidos na patogênese da EHI. Representação da primeira fase da EHI (Fase I). Alterações na vasculatura após EHI levam à grave hipotensão ocasionando menor aporte de oxigênio e consequentemente formação de ATP. Além disso, aumento da excitotoxicidade, cálcio intracelular, estresse oxidativo e disfunção mitocondrial são também observados. **(b)** Representação da segunda fase da EHI (Fase II) mostrando excitotoxicidade contínua, estresse oxidativo e disfunção mitocondrial. **(c)** Representação da terceira fase da EHI (Fase III) enfatizando a inflamação crônica, demonstrada pelo dano à microglia, neurônios e astrócitos. Nessa fase ocorre a liberação de citocinas e outros fatores que contribuem para o processo de inflamação crônica, o qual leva a mudanças epigenéticas, bem como, prejuízo para a sinaptogênese, crescimento neuronal e neurogênese. (Adaptado de Dixon *et al.* (2015))

Espécies reativas de oxigênio (EROs) e nitrogênio (ERN) são as mais importantes causadoras de dano encefálico após a HI. O estresse oxidativo é causado por um desequilíbrio entre a produção de radicais livres ou espécies reativas de oxigênio e a capacidade dos sistemas biológicos de detoxificar as várias formas ativadas dessas espécies (Couto *et al.*, 2012). As maiores consequências do estresse oxidativo é o dano ao DNA, lipídeos e proteínas, comprometendo as funções celulares e levando à morte (Birben *et al.*, 2012). O radical superóxido ($O_2^{\bullet-}$), o qual é o primeiro substrato para a formação de outras espécies reativas juntamente com o peróxido de hidrogênio (H_2O_2), o qual pode reagir com ferro (Fe) presente no SNC e formar o altamente reativo radical hidroxil ($^{\bullet}OH$) via reação de Fenton e Harberweiss (Gill e Perez-Polo, 2008). Além disso, o radical hidroxil pode reagir com o oxido nitríco ($NO^{\bullet}$) formando o potente agende nitrosilativo peroxinitrito ($ONOO^-$) (Markesbery e Lovell, 2007). A fim de conter os efeitos tóxicos das espécies reativas, o SNC conta com três principais enzimas antioxidantes: 1) superóxido dismutase (SOD), a qual catalisa a dismutação de $O_2^{\bullet-}$ em H_2O_2; 2) catalase (CAT), que atua convertendo o H_2O_2 em água e oxigênio e por fim, 3) glutationa peroxidase (GPx), que atua convertendo H_2O_2 em água (Jackson *et al.*, 1991) (Figura 3). Ademais, o encéfalo do neonato é particularmente vulnerável ao dano oxidativo devido as defesas antioxidantes como SOD, CAT e GPx serem imaturas, por possuir as membranas neuronais ricas em ácidos graxos poliinsaturados e também ser rico em ferro livre, contribuindo assim para o dano encefálico pós-HI (Saugstad, 1996; Buonocore *et al.*, 2001; Ferriero, 2001; Volpe, 2001a; Khan e Black, 2003; Mclean e Ferriero, 2004).

Um terceiro mecanismo envolvido na patogênese da EHI é o inflamatório.

Embora a origem da resposta inflamatória ainda não seja completamente conhecida, é bem conceituado o envolvimento da microglia como as primeiras células a reagir ao insulto hipóxico-isquêmico e sua contribuição para o dano encefálico através da liberação de citocinas pró-inflamatórias, glutamato, radicais livres e oxido nítrico (Pocock e Kettenmann, 2007).

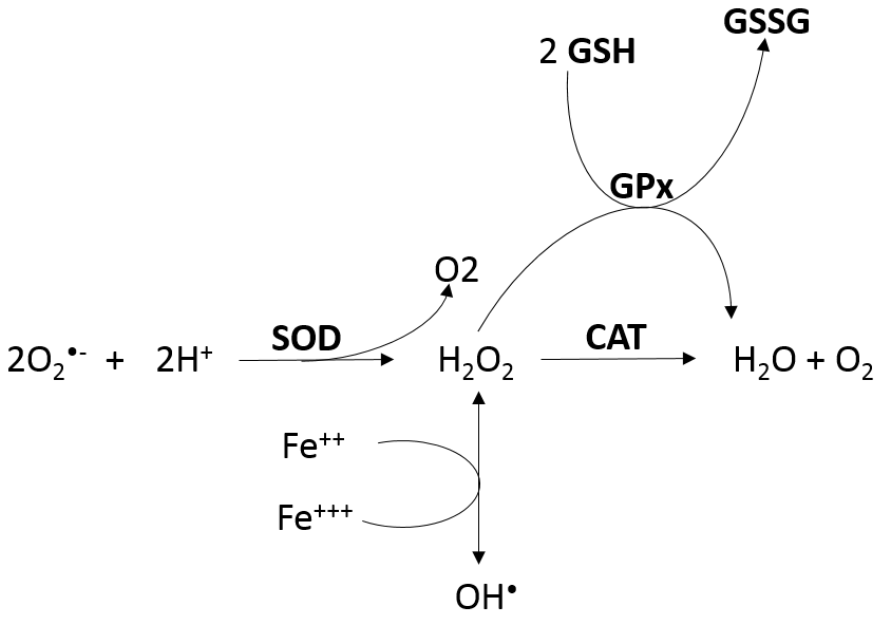

Figura 3. Ilustração do sistema enzimático antioxidante, demonstrando a detoxificação das espécies reativas de oxigênio, representado pelas enzimas superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GPx). Os peptídeos reduzido (GSH) e oxidado (GSSG) da glutationa também são apresentados. (Adaptado de Mclean e Ferriero (2004)).

Citocinas são polipeptídios que atuam sistemicamente ou no local da lesão guiando a resposta inflamatória e são importantes moduladores de dano e recuperação dos tecidos (Mclean e Ferriero, 2004; Titomanlio *et al.*, 2015). As citocinas IL-1beta, TNFα, IL-6, e IL-8 estão envolvidas nos efeitos patológicos da resposta inflamatória encefálica e nesse contexto contribuem também para a alteração da integridade da barreira hematoencefálica (Faustino *et al.*, 2011;

Titomanlio *et al.*, 2015; Rumajogee *et al.*, 2016). Ainda, outros mediadores pró-inflamatórios estão envolvidos na resposta inflamatória e patogênese da EHI, tais como: fator de ativação plaquetária (FAP), ciclooxigenases e ácido araquidônico e seus metabólitos (prostaglandinas e tromboxanos) (Faustino *et al.*, 2011). Em conjunto esses mediadores podem, de fato, contribuir para potencializar o dano ao SNC. Outro tipo celular envolvido na modulação da resposta inflamatória pós-HI são os astrócitos. Os astrócitos participam ativamente em diversos mecanismos patofisiológicos, incluindo regulação do glutamato extracelular, edema, aumento da expressão de aquaporina-4, liberação de citocinas e quimiocinas, além de mudanças na estabilidade da barreira hematoencefálica devido à retração dos pés astrocitários (Faustino *et al.*, 2011; Mae *et al.*, 2011). Somado a isso, após o insulto hipóxico-isquêmico os astrócitos se tornam reativos na tentativa de preservar o tecido adjacente à lesão, aumentando sua proliferação e expressando em maior quantidade uma proteína do filamento intermediário, conhecida como proteína fibrilar ácida glial (GFAP). Esta resposta astrocitária pode estabelecer a cicatriz glial e inibir a regeneração e plasticidade axonal (Anderson *et al.*, 2003; Middeldorp e Hol, 2011; Sullivan, 2014; Filous e Silver, 2016; Revuelta *et al.*, 2016).

1.3. Modelos para estudos de patologias *in vivo* e *in vitro*

1.3.1. O clássico modelo in vivo para estudo da EHI proposto por Rice-Vannucci

Diversos modelos tanto *in vivo* quando *in vitro* têm sido propostos para o estudo dos mais variados tipos de doenças. Um modelo animal ideal, além de mimetizar o estágio de desenvolvimento de um ser humano, deve ser reprodutível e ter pouca variabilidade. Um dos melhores modelos *in vivo* amplamente utilizado para estudar a HI neonatal, há mais 30 de anos, é o descrito por Rice e Vannucci (1981). Este modelo é uma adaptação do modelo proposto por Levine (1960) que permite estudar os mecanismos relacionados aos danos encefálicos durante o desenvolvimento e é um dos mais reprodutíveis modelos de dano cerebral perinatal (Vannucci *et al.*, 1999; Northington, 2006; Rumajogee *et al.*, 2016). No sétimo dia pós-natal do rato são realizados os procedimentos para indução de HI, devido à imaturidade cerebral ser equivalente ao recém-nascido à termo ou prematuro (Clancy *et al.*, 2001; Volpe, 2001b; Rumajogee *et al.*, 2016). O experimento ocorre através da ligação permanente unilateral da artéria carótida comum seguido por hipoxemia sistêmica, que dura por volta de 1-3 h (Vannucci *et al.*, 1999) (Figura 4).

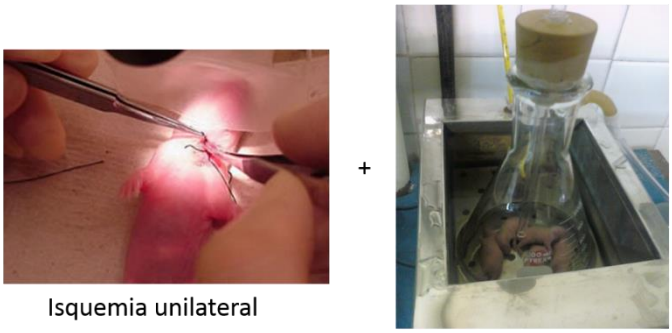

Figura 4. Indução da EHI (modelo Rice-Vannucci) através da isquemia unilateral (ligação permanente da artéria carótida comum) juntamente com hipóxia sistêmica.

Esse procedimento resulta em dano encefálico e atrofia tecidual, principalmente em áreas como estriado, região CA1 do hipocampo, cerebelo, tálamo e neocortex (Levine, 1960; Rice *et al.*, 1981; Vannucci *et al.*, 1988; Volpe, 2001b; Rees e Inder, 2005; Pereira *et al.*, 2007; Miguel *et al.*, 2015; Carletti *et al.*, 2016; Rocha-Ferreira e Hristova, 2016; Schuch, Diaz*, et al.*, 2016) (Figura 5).

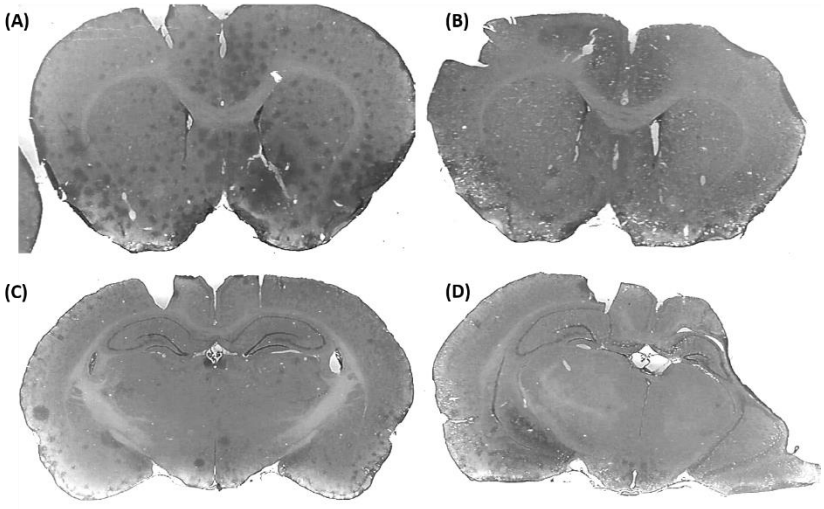

Figura 5. Secções coronais representativas do estriado (bregma +1.20 mm); e hipocampo (bregma -2.92 mm) demonstrando atrofia estrutural de ratos submetidos ao modelo de HI proposto por Rice-Vannuci (B;D). Grupo controle (A;C); grupo hipóxico-isquêmico (B;D).

Destacando-se a atrofia dessas estruturas, principalmente do lado ipsilateral à oclusão arterial, a combinação de hipoxemia e isquemia torna-se uma das mais relevantes situações in vivo para o neonato. A aumentada suscetibilidade do rato neonato ao dano provocado pela hipóxia-isquemia predispõe a sequelas cognitivas, motoras, déficits sensoriais e alterações moleculares vistos em recém-nascidos prematuros e a termo que sofreram hipoxemia e isquemia no período perinatal (Tomimatsu *et al.*, 2002; Arteni *et al.*, 2003; Schuch, Jeffers*, et al.*, 2016). Diversos estudos utilizando o modelo Rice-Vannucci têm ajudado a caracterizar os mais variados déficits cognitivos por meio de testes neurocomportamentais. Por exemplo, é bem estabelecido que roedores expostos ao procedimento de HI apresentam déficit na memória espacial, de trabalho, de reconhecimento e aversiva (Ikeda *et al.*, 2001; Arteni *et al.*, 2003; Pereira *et al.*, 2007; Carletti, J. *et al.*, 2012; Rojas *et al.*, 2013; Carletti *et al.*, 2016). Além disso, vários autores têm descrito que a HI também altera a função de diferentes enzimas como a Na^+, K^+-ATPase, SOD, CAT e GPx (Pereira *et al.*, 2009; Weis *et al.*, 2011; Rojas *et al.*, 2015).

1.3.2. O modelo experimental in vitro para estudo de patologias placentárias representado por trofoblastos humanos (linhagem BeWo e HTR-8 SV/neo)

Além dos modelos animais para estudos das mais variadas patologias humanas, confiáveis modelos *in vitro* estão disponíveis para reproduzir os efeitos dessas patologias de maneira controlada e contribuir para o entendimento dos seus mecanismos e consequências (Schweiger e Jensen, 2016). Um dos modelos amplamente utilizado é a utilização de células humanas do

sinciciotrofoblasto da linhagem BeWo, as quais derivam de um coriocarcinoma gestacional humano (Orendi *et al.*, 2011). Essa linhagem celular foi primeiramente isolada por Hertz (1959) de uma metástase cerebral de um coriocarcinoma humano. Então, foram transferidas para a bochecha de hamster e mantidas em subsequentes inoculações/transfecções por um período de oito anos. Finalmente, Pattillo e Gey (1968) conseguiram estabelecer a cultura removendo as células dos hamsters e cocultivando com tecidos deciduais. Essa linhagem fornece um bom sistema de modelo *in vitro* e apresenta morfologia, marcadores bioquímicos e secreção de hormônios de trofoblastos normais como a gonadotrofina coriônica humana, além de estar bem caracterizada e ser amplamente utilizada para investigar transportes e metabolismo placentário (Takeuchi *et al.*, 1990; Bode *et al.*, 2006). Além disso, estas células apresentam muitas das características dos trofoblastos presentes no terceiro trimestre da gestação e são similares às culturas primárias de células trofoblásticas e placenta humana a termo (Friedman e Skehan, 1979; Liu *et al.*, 1997; Rama Sastry, 1999). Recentemente, estudos utilizando a linhagem BeWo expostas durante 24 horas ao tert-butil-hidroperóxido (TBH), um potente agente oxidante, demonstraram alterações similares às vistas em placentas de mães que sofreram PE, apresentando um efeito citotóxico e antiproliferativo, além de níveis aumentados de biomarcadores relacionados ao estresse oxidativo (malondialdeído, carbonilação proteica, glutationa total e oxidada) (Watson *et al.*, 2012; Araújo *et al.*, 2013).

Outro modelo *in vitro* muito utilizado para estudos placentários inclui a linhagem celular HTR-8 SV/neo. Essa linhagem celular foi estabelecida na década de 90 por Graham *et al.* (1993) através da transfecção de trofoblastos de

primeiro trimestre com o plasmídeo contendo o gene do vírus símio 40 (SV40). Essas células transfectadas preservam o fenótipo epitelial e as características proliferativas e invasivas de trofoblastos humanos, desse modo, sendo aceito como modelo *in vitro* para estudos das mais diversas patologias envolvidas com a placentação, incluindo a PE (Correia-Branco *et al.*, 2015; Graham *et al.*, 1993; Wolfe, 2006; Orendi *et al.*, 2011; Wang *et al.*, 2015).

1.4. Ácido Fólico

O dano encefálico produzido pela EHI é um processo complexo cujo desenvolvimento ocorre ao longo de horas a dias promovendo uma janela única para tratamentos neuroprotetores (Buonocore *et al.*, 2012). Com isso, na busca por amenizar os devastadores danos, como morte neuronal, causados pela hipóxia-isquemia, diferentes estratégias terapêuticas têm sido estabelecidas, tais como hipotermia, enriquecimento ambiental, eritropoietina, alopurinol, xênon, melatonina, estatinas, entre outros (Giuseppe *et al.*, 2012; Rojas *et al.*, 2015; Schuch, Diaz*, et al.*, 2016). Recentemente, nosso grupo de pesquisa demonstrou que o tratamento com ácido fólico, como estratégia neuroprotetora, atenuou o prejuízo causado na memória aversiva e normalizou a atividade da enzima Na^+, K^+-ATPase (córtex e estriado) de ratos Wistar submetidos ao procedimento hipóxico-isquêmico proposto por Rice-Vannucci (Carletti *et al.*, 2012).

O ácido fólico (AF) é uma forma sintética e completamente oxidada dos folatos, que faz parte das vitaminas hidrossolúveis, sendo conhecido também como vitamina B9 (Hyland *et al.*, 2010) (Figura 6). Como nós humanos somos incapazes de sintetizar folatos, esses devem ser obtidos da dieta através do

consumo de vegetais de folhas verdes, frutas cítricas, extrato de levedura, aspargos e brócolis ou ainda através de suplementos e alimentos fortificados (Dary, 2009; Kotecha *et al.*, 2016). Os folatos naturais ou pteroilpoliglutamatos são hidrolisados a pteroilglutamatos antes de serem absorvidos pela parte apical da borda em escova dos enterócitos (Iyer e Tomar, 2009; Visentin *et al.*, 2014).

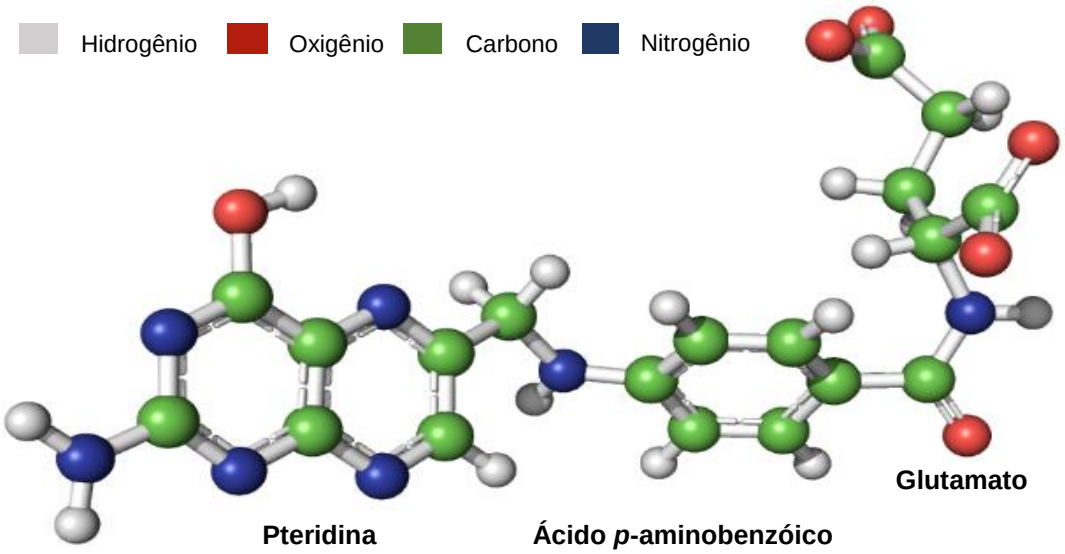

Figura 6. Estrutura química 3D do ácido fólico; ácido pteroilglutâmico (Pubchem).

Já a sua forma sintética apresenta-se na forma de monoglutamatos e ambas as formas podem ser absorvidas diretamente via um específico processo dependente de pH mediado pelo transportador de folato acoplado a próton (PCFT) e do transportador de folato reduzido (RFC) (Couto *et al.*, 2012; Visentin *et al.*, 2014). Após a absorção, os folatos chegam à circulação sanguínea via veia porta e artéria hepática e então são estocados no fígado como poliglutamatos (Visentin *et al.*, 2014) (Figura 7).

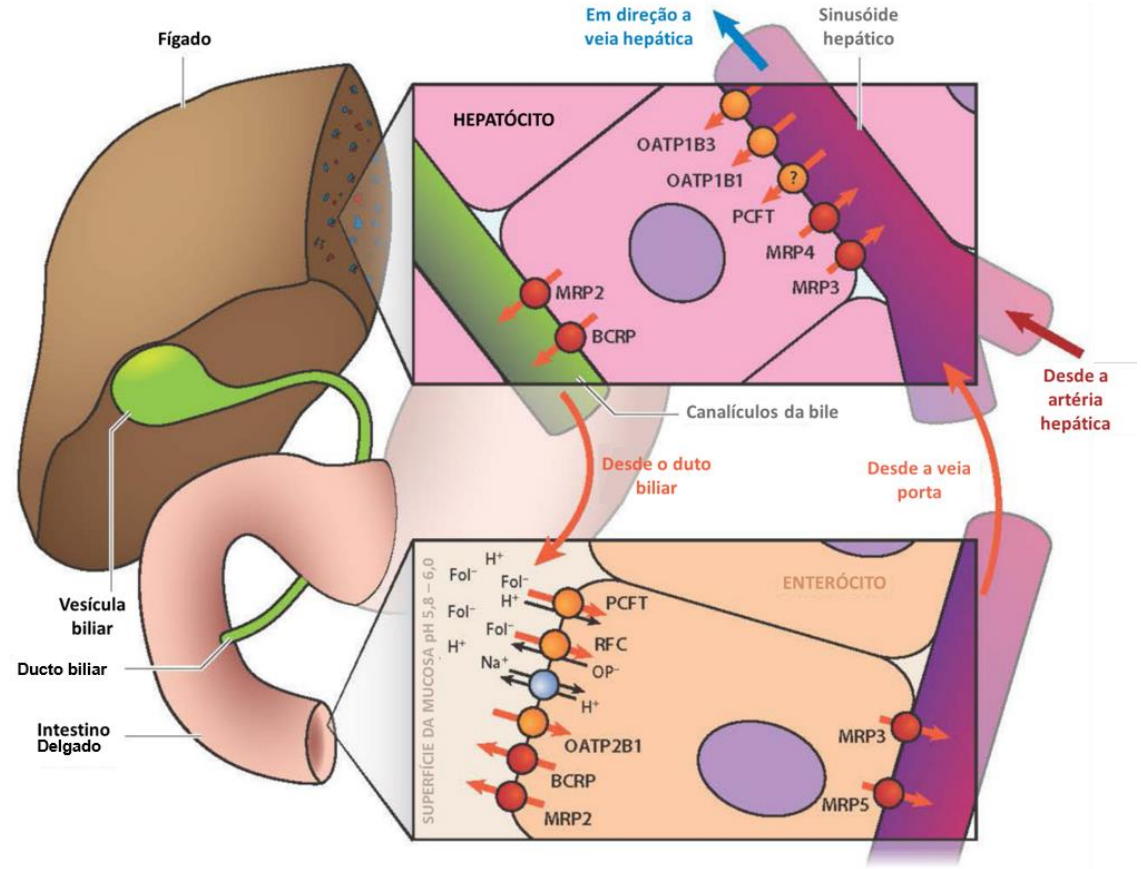

Figura 7. Absorção intestinal e circulação enterohepática dos folatos. Polipetídeo transportador de ânion orgânico (OATP); transportador de folato próton acoplado (PCFT); transportador de folato reduzido (RFC); fosfato orgânico (OP); proteína resistente ao câncer de mama (BCRP); proteína associada à resistência multidrogas (MRP) (Adaptado de Visentin *et al.* (2014)).

Dentro das células esses monoglutamatos voltam à forma de poliglutamatos e são convertidos nas mais variadas formas de folato que estão envolvidas em reações biológicas (Hyland *et al.*, 2010). Uma das mais importantes e predominantes formas de folato encontradas no plasma e no SNC é o 5-metil-tetrahidrofolato (5-MTHF). O 5-MTHF é transportado para o encéfalo através do plexo coroide e este transporte acontece por três diferentes sistemas que incluem o RFC, receptor de folato 1 e 2 (FR-1 e FR-2) e transporte celular ativo (Hyland *et al.*, 2010).

O AF é particularmente importante durante a gestação, quando uma ótima captação da circulação materna é necessária para o normal e correto desenvolvimento e crescimento da placenta e do feto (Keating *et al.*, 2007). Além disso, é um micronutriente essencial para a manutenção do crescimento, desenvolvimento e função celular, atuando na transferência de unidades de 1-carbono, síntese de purinas e pirimidinas (precursores de nucleotídeos), aminoácidos (metionina, serina, glicina e histidina) e S-adenosilmetionina (SAM) (Fenech, 2010; 2012). SAM é uma das mais importantes doadoras de grupamentos metila para reações de metilação no organismo. Especificamente no encéfalo, essas reações são essenciais para a síntese de neurotransmissores (serotonina, dopamina, norepinefrina e acetilcolina), hormônios (melatonina), fosfolipídios de membrana e também para o controle epigenético da expressão gênica (Reynolds, 2006; Hughes *et al.*, 2013; Araujo *et al.*, 2015). Diversos estudos destacam a importância dessa vitamina, visto que a deficiência materna está associada com várias complicações, tais como: altos níveis sanguíneos de homocisteína, anemia megaloblástica, baixo peso ao nascer, aumentado risco de aborto espontâneo, defeitos no tubo neural e anencefalia (Lucock, 2000; Picciano, 2003; Keating *et al.*, 2007; Dary, 2009). Devido a isso, a OMS, o *Centers for Disease Control* and *Prevention* (CDC) e a Federação Brasileira das Associações de Ginecologia e Obstetrícia (Febrasgo) recomendam que mulheres em idade gestacional e gestantes recebam suplementação com AF na dosagem de 0,4 mg/dia pelo menos 1 mês antes da gestação e a mantenham até o fim do primeiro trimestre de gravidez (WHO, 2012; Krawinkel *et al.*, 2014; CDC, 2016; Febrasgo, 2016; Gomes *et al.*, 2016). Além disso, a dose de 4-5 mg/dia de AF está recomendada para mulheres com alto risco de desenvolver

fetos com defeitos no tubo neural (WHO, 2012; Gomes *et al.*, 2016). A fim de prevenir os efeitos ocasionados pela deficiência de AF, desde a década de 90 políticas públicas em vários países têm incentivado a suplementação de farinhas e cereais com essa vitamina. Em 1996 o *Food and Drugs Administration* (FDA) emitiu um regulamento para que, a partir de 1998, os Estados Unidos da América iniciassem a fortificação de farinhas e cereais com AF na tentativa de reduzir a incidência dos defeitos no tubo neural de fetos em que as mães não tinham acesso ao aporte correto dessa vitamina (Choumenkovitch *et al.*, 2002; D'anci e Rosenberg, 2004). Essa mesma fortificação iniciou-se no Brasil em 2002 após a Agência de Vigilância Sanitária (ANVISA) lançar a RDC Nº 344 de 2002 determinando a adição de Fe e AF em farinhas de trigo e milho. Toda essa fortificação alimentar somado à ingesta de AF por mulheres em idade reprodutiva, que têm a intenção de engravidar e grávidas, pode resultar em níveis aumentados dessa vitamina nessa população específica (Tam *et al.*, 2012; Ahmed *et al.*, 2016; Orozco *et al.*, 2016).

Nos últimos anos diversas pesquisas têm estudado o efeito protetor associado à suplementação com AF em outras patologias que não os defeitos no tubo neural. Estes estudos têm focado na prevenção da Doença de Alzheimer, estresse oxidativo-nitrosativo, melhora do quadro de disfunção endotelial, memória espacial e aversiva em modelo animal de homocistinúria e de EHI, efeito antidepressivo em modelo animal de estresse por restrição e prevenção do risco de desenvolver pré-eclâmpsia (Matté *et al.*, 2007; Kolling *et al.*, 2011; Carletti, J. *et al.*, 2012; Budni *et al.*, 2013; Li *et al.*, 2015; Martinussen *et al.*, 2015; Shen e Ji, 2015; Singh *et al.*, 2015; Cheng *et al.*, 2016b; Hua *et al.*, 2016; Sayyah-Melli *et al.*, 2016; Shim *et al.*, 2016; Wen *et al.*, 2016). Muitos

desses efeitos foram associados a um importante papel antioxidante apresentado pelo AF. Ademais, diferentes pesquisas mostram que baixos níveis de AF e elevados níveis de homocisteína contribuem para um quadro de disfunção endotelial que pode levar à hipertensão, doenças vasculares, isquemia e outros eventos trombóticos (Dehkordi *et al.*, 2016; Sobczynska-Malefora *et al.*, 2016; Toda e Okamura, 2016).

Se por um lado várias pesquisas têm demonstrado efeitos benéficos da suplementação com AF, por outro lado na era pós fortificação alimentar diferentes estudos têm focado na super ingestão dessa vitamina e estudado seus efeitos deletérios. Pesquisadores utilizando tanto modelos *in vivo* quanto *in vitro* têm observado os mais variados efeitos da super dose de AF, tais como: desequilíbrio metabólico, como resistência à insulina, em mães e filhos, dano na memória espacial, hiperexcitabilidade neuronal, limite diminuído para epilepsia e diminuição da formação de sinapses e defeitos cardíacos no septo ventricular e paredes do ventrículo esquerdo (Sittig *et al.*, 2012; Girotto *et al.*, 2013; Mikael *et al.*, 2013; Keating *et al.*, 2015; Tomizawa *et al.*, 2015; Pannia *et al.*, 2016; Wiens *et al.*, 2016). Além desses efeitos negativos, altas doses de AF podem aumentar o risco de câncer e a progressão da doença em pessoas sob certas condições (Weissenborn, 2017).

Considerando que até o momento os resultados da suplementação com AF, como estratégia neuroprotetora, no modelo de hipóxia-isquemia neonatal são benéficos e interessantes, é de extrema importância o desenvolvimento de estudos que busquem conhecer os efeitos da suplementação com AF tanto na EHI, quanto na prevenção da PE, a qual traz um grande risco para a EHI. Tais análises são de extrema relevância, uma vez que a suplementação é realizada

por quase todas as gestantes e que possíveis benefícios poderiam contribuir para melhores desfechos na EHI e na PE. Ainda, faz-se necessária também uma abordagem mais cautelosa para entender se o uso de diferentes doses, como resultado da soma da suplementação e da fortificação dos alimentos, poderia trazer benefícios e proteção para a mãe e o feto em desfechos como a PE e a EHI.

2. Objetivos

2.1. Objetivo Geral

Investigar o efeito da suplementação com ácido fólico como estratégia protetora em ratos submetidos à hipóxia-isquemia encefálica neonatal e em células trofoblásticas humanas (linhagem BeWo e HTR-8 SV/neo) submetidas ao estresse oxidativo ocasionado por tert-butil-hidroperóxido.

2.2. Objetivos Específicos

2.2.1. Verificar o possível efeito neuroprotetor do tratamento com AF através da avaliação do desempenho cognitivo (memória espacial e de trabalho), atividade enzimática da Na^+, K^+- ATPase, além de mensurar o volume e a área de estruturas encefálicas (hipocampo e estriado) em ratos submetidos ao modelo de EHI neonatal;

2.2.2. Avaliar o efeito do tratamento com AF: no aprendizado e memória no período da pré-puberdade através do teste do ox-maze, na atividade locomotora e na atividade das enzimas superóxido dismutase e catalase no hipocampo de animais submetidos à EHI em diferentes períodos do desenvolvimento (P22 e P42);

2.2.3. Observar e estudar o comportamento de células trofoblásticas humanas (linhagem BeWo e HTR-8 SV/neo) expostas a um modelo de estresse oxidativo provocado por tert-butil-hidroperóxido e cultivadas sob distintas concentrações de AF (deficiente, fisiológica e supra-fisiológica).

3. Capítulo 1

"Folic Acid Can Contribute to Memory Deficit and Na+, K+- ATPase Failure in the Hippocampus of Adolescent Rats Submitted to Hypoxia- Ischemia".

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Folic Acid Can Contribute to Memory Deficit and Na^+, K^+- ATPase Failure in the Hippocampus of Adolescent Rats Submitted to Hypoxia-Ischemia

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Abstract: Recent findings have demonstrated a dual effect of the folic acid (FA) supplementation on the nervous system of rats. We found that FA treatment prevented memory impairment and Na^+, K^+-ATPase inhibition in the striatum and cortex in adult rats that suffered neonatal hypoxia-ischemia (HI). However, spatial memory deficit has been associated with FA supplementation. In the present study we investigated the role of FA supplementation on spatial memory and Na^+, K^+-ATPase activity in the hippocampus, as well as on morphologic alterations in adolescent rats submitted to neonatal HI. Wistar rats of both sexes at postnatal day (PND) 7 were submitted to Levine-Rice HI procedure. Intraperitoneal doses of FA were administered immediately before HI and repeated daily until the maximum PND 40. Hippocampal volume and striatum area were estimated and Na^+, K^+-ATPase activity in the hippocampus was measured at PND 31. Also, the performance of the animals in the water maze was assessed and Na^+, K^+-ATPase activity measured again at PND 52. Interestingly, HI and FA resulted in spatial memory deficits in the Morris water maze and the Na^+, K^+-ATPase activity was impaired at PND 31 in HI rats which received FA. Additionally, Na^+, K^+-ATPase activity in adulthood showed a decrease after HI and a recovery in supplemented animals. Hippocampal and striatal atrophy were partially reversed by FA. To conclude, the present results support the hypothesis that FA supplementation during development contributes to memory deficits caused by HI and Na^+, K^+-ATPase failure in adolescent rats, although, in adulthood, FA has been effective in reversing enzymatic activity in the hippocampus.

Keywords: Behavior, brain damage, folate, perinatal asphyxia, spatial memory, vitamin supplementation.

INTRODUCTION

Folic acid (FA), a water-soluble B vitamin, is a cofactor in one-carbon metabolism required for DNA synthesis and methylation processes and it is also critical to ensure the closure of the neural tube [1-3]. Recent studies have associated folic acid supplementation with improvement in the oxidative-nitrative stress in cardiac tissue [4], recovery in memory deficits in rats submitted to hyperhomocysteinemia model [5] and in the prevention of depressive-like effects and hippocampal antioxidant imbalance in acute restraint stress in mice [6]. Furthermore, some studies have shown that folate deficiency increases postischemic brain damage in mice submitted to ischemia-reperfusion [7] and may represent a risk factor for hypoxic-ischemic encephalopathy in newborns because it increases plasma homocysteine [8]. The present group previously described the use of this vitamin as a possible treatment for neonatal hypoxic-ischemic brain injury, preventing aversive memory impairment and Na^+, K^+-ATPase activity inhibition [9]. These effects were attributed to its antioxidant potential.

Neonatal hypoxia-ischemia (HI) is an event which occurs due to complications in the perinatal period. Energetic failure, inflammation process, glutamate excitotoxicity as well as oxidative stress imbalance are the main mechanisms involved in brain damage after HI, particularly affecting the hippocampus, striatum and prefrontal cortex [10, 11]. It is well established that hippocampus and striatum are important structures implicated in the process of memory formation [12-14]. Previous studies demonstrated spatial memory impairment in Morris water maze task [15, 16] and aversive memory deficit in inhibitory avoidance task in hypoxic-ischemic adult rats [15].

As already mentioned, it was shown that FA supplementation reversed aversive memory deficit and Na^+, K^+-ATPase activity impairment (in cortex and striatum) due to neonatal HI. Curiously, current studies have associated FA supplementation in rats with negative aspects, such as:

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deficits in spatial memory, hypothyroidism [17], motor coordination difficulties [18], development of ventricular septal defects and thinner left and right ventricular walls [19]. Additionally, Girotto *et al*. [20] attributed the increase in neuronal excitability in cell cultures and seizure susceptibility in offspring to the use of high-dose FA. These findings strongly suggest the presence of a dual effect of the FA supplementation and justify more studies to identify and understand its role in the prevention potential in neurological diseases.

Using the above considerations, the aim of this study has been to investigate the effect of FA supplementation on memory performance in the water maze task and Na^+,K^+-ATPase activity in the hippocampus, as well as on striatal and hippocampal atrophy in rats submitted to the neonatal HI.

MATERIALS AND METHODS

Animals

In this study, a total of hundred Wistar rats were used. For all the procedures, the animals were randomly separated for gender with similar number between them. The animals were obtained from the reproduction and experimentation center of the Institute of basic health science, at the Federal University of Rio Grande do Sul, Porto Alegre, Brazil. The animals were housed with a 12/12h light/dark cycle in a temperature-controlled room (22 ± 1ºC), with food and water available ad libitum. Pups were randomly assigned to four experimental groups: control treated with 0.9% saline solution (CT-S), control treated with FA (CT-FA), hypoxicischemic treated with saline solution (HI-S) and hypoxicischemic treated with FA (HI-FA). All the procedures were in accordance with the 8th Guide for the Care and Use of Laboratory Animals, adopted by The National Institute of Health (USA), with the Federation of Brazilian Societies for Experimental Biology. The experimental protocol was approved by the Ethics Committee of the Federal University of Rio Grande do Sul, Brazil (n. 17401). The timeline of theexperiments is depicted in Fig. (**1**).

Induction of Hypoxia-Ischemia

The Levine rat model [21] was adopted, as modified by Rice *et al*. [22]. On the 7th postnatal day (PND), the animals were anesthetized with halothane and the right common carotid artery was identified, isolated and permanently occluded with surgical silk thread. After this procedure, the pups remained for 15 minutes under a lamp and returned to their dams for recovery for two hours. Subsequently, the pups were exposed to hypoxic atmosphere (nitrogen 92% with oxygen 8%) for 90 min. During the hypoxic procedure, the animals remained in the chamber partially immersed in a 37 ºC water bath. The controls were sham-operated, i.e., without artery ligation or hypoxia-period [9, 16].

Folic acid Chronic Treatment

FA solution (0.011µmol folic acid/g body weight) was injected intraperitoneally once a day from the 7th to the maximum 40th PND [5, 23]. In the previous studies, these doses showed protective effects of FA administration on damaged brain structures and cognitive function in young rats [5, 9, 24]. The control animals received the same volume of saline solution. FA was obtained from Sigma Chemical Co. (St. Louis, MO, USA.).

Morris Water Maze

Twenty-four hours after FA treatment (PND 41), forty-three rats (21 females and 22 males) were submitted to behavioral testing in the Morris water maze. The maze consisted of a black 200 cm diameter circular pool filled with water. It was temperature controlled (23°C) with a 2 cm submerged platform and visual cues on the walls. The pool was virtually divided into four quadrants with four points designed as starting positions (N, S, W or E). Two behavioral protocols were evaluated for reference and working memory, as described below.

In the reference memory protocol, the animals received 5 training days (acquisition sessions) and a probe trial on the 6th day. In the acquisition phase, the animals were placed in water at one of the four starting positions and allowed to swim to locate the platform for a maximum of 60 seconds. Each session consisted of four trials with a 15 min intertrial interval. Latency in finding the platform was measured in each trial and the mean latency for every training day was calculated. The probe trial was performed on the 6th day without a platform and latency to find the original platform position, number of crossings and time spent at the target as well as in the opposite quadrants was measured [15, 16].

After 48 hours following the probe trial, the animals were also evaluated in the working memory protocol. During a four day period, the animals performed in four trials per day in which the platform location was changed on a daily basis.

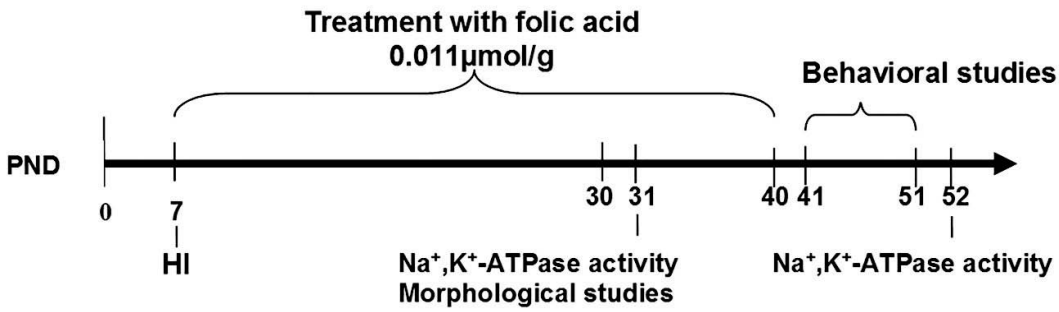

Fig. (1). Timeline of experiments.

The intertrial interval was 5 min. The latency to find the platform in each trial was registered and the mean latency for each trial was calculated [15, 16].

Na^+, K^+-ATPase Activity

Biochemical analysis was performed to assay the Na^+, K^+-ATPase activity. Forty-one animals (21 females and 20 males) were euthanized on postnatal day 31 or 52 (24 h after the water maze task) by decapitation without anesthesia. Right and left hippocampi were quickly dissected in a Petri plate on ice and instantaneously placed in liquid nitrogen and stored at -70°C until the biochemical assays. The hippocampi were homogenized in 10 volumes (1:10, w/v) of 0.32 M sucrose solution containing 5.0 mM HEPES and 0.1 mM EDTA, pH 7.4. The reaction mixture for Na^+, K^+-ATPase activity assay contained 5.0 mM $MgCl_2$, 80.0 mM NaCl, 20.0 mM, KCl and 40.0 mM Tris-HCl, pH 7.4, in a final volume of 200 mL. ATP substrate was added to react with the other chemicals. The controls received the same conditions with the addition of 1.0 mM ouabain. Na^+, K^+-ATPase activity was calculated by the difference between the assays, as described elsewhere [9, 25] and released inorganic phosphate (Pi) was measured according to the method described by Chan *et al*. [26]. Specific enzyme activity was expressed as nmol Pi released per min per mg of protein. All the experiments were performed in duplicate.

Morphological Analysis

Four rats (8 females and 8 males) from each group were euthanized at PND 31. They were anesthetized with ketamine and xylazine (100mg/kg and 10mg/kg body weight, respectively, i.p.) and perfused through the left cardiac ventricle with 0.9% saline followed by 4% paraformaldehyde with phosphate buffer. The brains were quickly removed and placed in 4% paraformaldehyde buffered solution. After 4 hours, the samples were immersed in a solution of 30% sucrose for cryoprotection for two days. Following this, the samples were sectioned using a cryostat (CM1850, Leica, São Paulo-SP, Brazil) in coronal slices of 40 µm thickness. Coronal sections containing the entire hippocampus and striatum area were placed on gelatinized glass slides and stained with hematoxylin and eosin. Images were then captured and digitalized using the Adobe Photoshop software and the areas of each hemisphere were measured using NIH, USA—Image J software.

Hippocampal Volume

The entire hippocampal and dentate gyrus area, in accordance with Paxinos and Watson [27], was delineated in all sections. The hippocampus volume was calculated by the sum of the areas multiplied by the section interval, according to the Cavalieri method [16, 28, 29]. The anterior limit was set to find the first slice containing the hippocampus and the posterior one in which the ventral hippocampus first appeared (i.e. between coordinates - 1.60 mm and - 6.84 mm of the Paxinos Atlas). Ammon's horn volume was calculated as the difference between the entire hippocampus and dentate gyrus volumes [30].

Striatal Area

To estimate striatal atrophy, one slice per rat at the level +1.20 mm from Bregma [27] was used and the striatal area was delineated using Image J program -NIH, USA [31].

Statistical Analysis

Behavioral performance in the training days was analyzed using a two-way repeated-measures analysis of variance (ANOVA) with *lesion* and *treatment* as the independent variables and session as the repeated measure. Two-way ANOVA was performed, with *lesion* and *treatment* as factors, followed by Tukey's *post hoc* to analyze data from the probe trial and working memory in the water maze and Na^+,K^+-ATPase activity and morphological analysis. As a previous data analysis indicated no significant difference between males and females, they were all included in the same group. All the statistical tests were performed using the Statistica© software package and differences were considered statistically significant when $p < 0.05$.

RESULTS

Water Maze Task - Reference Memory

Hypoxia-ischemia caused spatial memory impairment in the acquisition phase regardless of the treatment (Fig. **2**). Two-way repeated measures revealed significant main effect for *lesion* factor [$F(1,42) = 10.4$; $p < 0.05$] but non-significant effects for *treatment* [$F(1,42) = 0.80$; $p > 0.05$] and *lesion*treatment* interaction [$F(1,42) = 0.79$; $p > 0.05$] on latency to find the platform. Greater latency was observed in both HI groups during the learning phase and in the HI group treated with FA in comparison with the CT-S group in the 5th session.

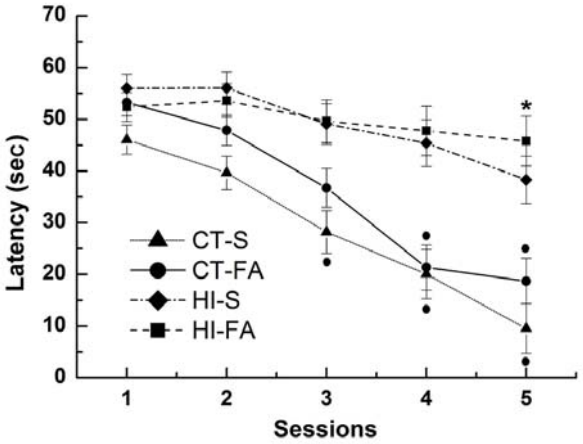

Fig. (2). Acquisition phase of the reference memory protocol in the Morris water maze. Data shows latency in finding the platform and is expressed as mean ± S.E.M. for 10-12 animals per group. ●Difference from first session in each respective group; *Difference of the HI treated with FA in comparison with the CT-S group (Repeated measures ANOVA followed by Tukey's test; $p<0.05$).

The probe trial was performed 24h after the acquisition phase when four parameters were evaluated: the number of crossings over and the latency to cross the platform location, the time spent in the target and the opposite quadrants (Fig. **3A-D**). Two-way ANOVA presented a significant effect of *lesion* [$F(1,42) = 12.5$; $p < 0.05$] and *treatment* factor [$F(1,42) = 7.47$; $p < 0.05$] but non-significant effect for *lesion*treatment* interaction [$F(1,42) = 1.09$; $p > 0.05$], for the number of crossings over (Fig. **3A**). Both HI groups and CT-FA group presented a reduced number of crossings. The analysis of latency to cross the platform (Fig. **3B**) was also indicated as an effect of *lesion* [$F(1,42) = 11.38$; $p < 0.01$] and *treatment* factor [$F(1,42) = 6.46$; $p < 0.05$] but non-significant effect for *lesion*treatment* interaction [$F(1,42) = 0.06$; $p > 0.05$]. It was found that the CT-FA and HI groups had increased latency to cross the platform than the CT-S group. There was a significant main effect for *treatment* factor when time spent at the target [$F(1,42) = 8.54$; $p < 0.05$] and time spent in the opposite quadrant was observed [$F(1,42) = 6.71$; $p < 0.05$] (Fig. **3C**, **D**). The control group treated with FA spent less time in the target quadrant comparing with the HI-S group. Concerning the time spent in the opposite quadrant (Fig. **3D**), no differences were found between the groups. These results indicate that either HI or FA supplementation *per se* contributed to damage in the spatial memory.

Water Maze Task - Working Memory

As seen in the results of reference memory, HI harmed working memory in the Morris water maze, with no protective effect from the use of FA treatment (Fig. **4**). There were significant differences in the escape latency for the *lesion* factor on the 1st [$F(1,42) = 18.2$; $p < 0.05$], 2nd [$F(1,42) = 20.0$; $p < 0.05$], 3rd [$F(1,42) = 37.1$; $p < 0.05$] and 4th trials [$F(1,42) = 31.4$; $p < 0.05$] but non-significant effects were observed for treatment factor and *lesion*treatment* interaction. The rats submitted to neonatal HI showed increased latency to find the platform than both control groups.

Na^+, K^+-ATPase Activity

Na^+, K^+-ATPase activity was measured in the right (ipsilateral) and left hippocampus (contralateral to arterial occlusion) of young rats, at PND 31 (Fig. **5A**). Two-way

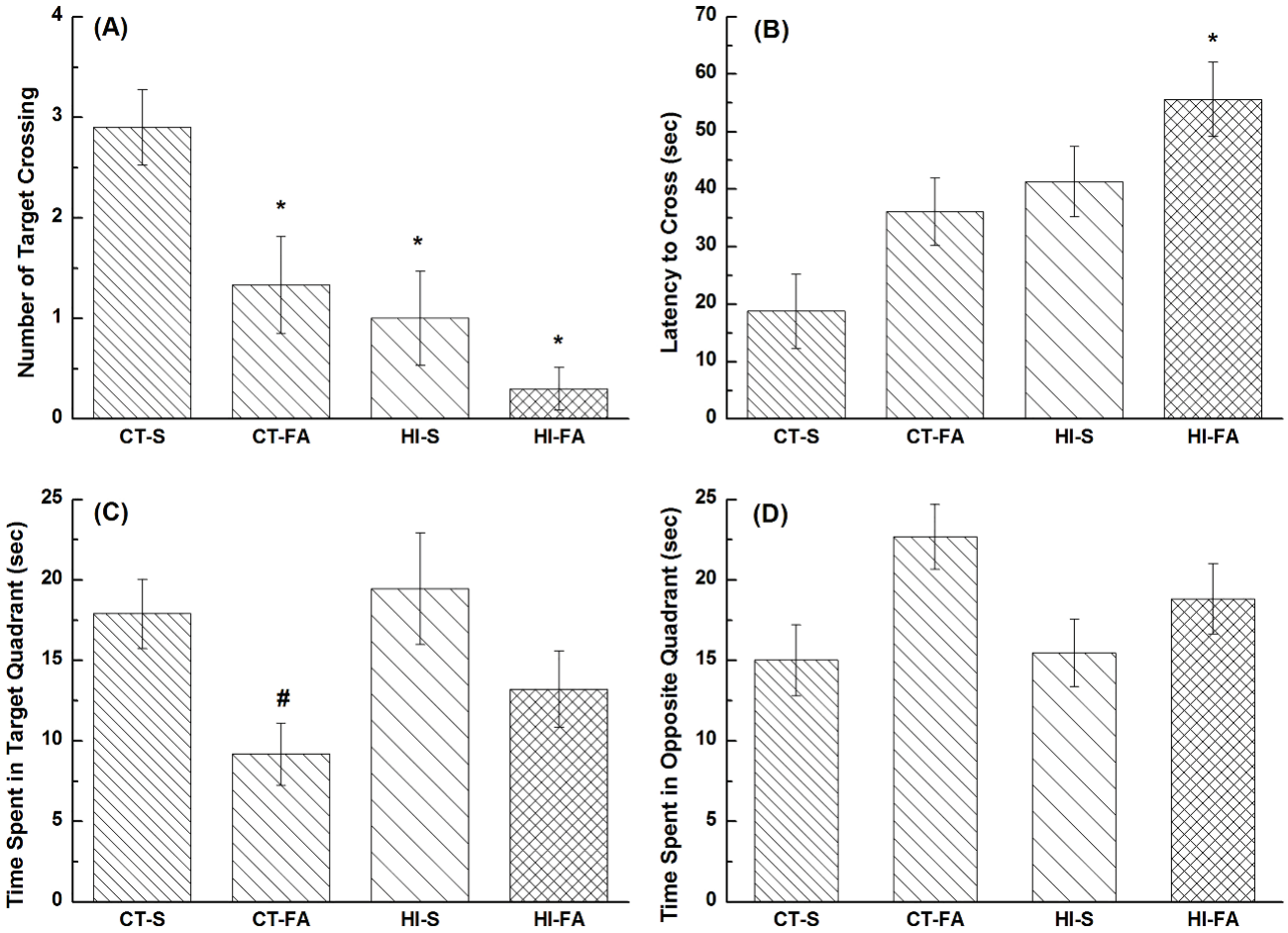

Fig. (3). Probe trial of the reference memory protocol in the Morris water maze. (**A**) Number of target crossings, (**B**) latency to cross the location of the platform, (**C**) time spent in the target quadrant, and (**D**) time spent in the opposite quadrant. Data is expressed as mean ± S.E.M. *Different from the CT-S group; #different from the HI-S group (Two-way ANOVA followed by Tukey's test; $p < 0.05$).

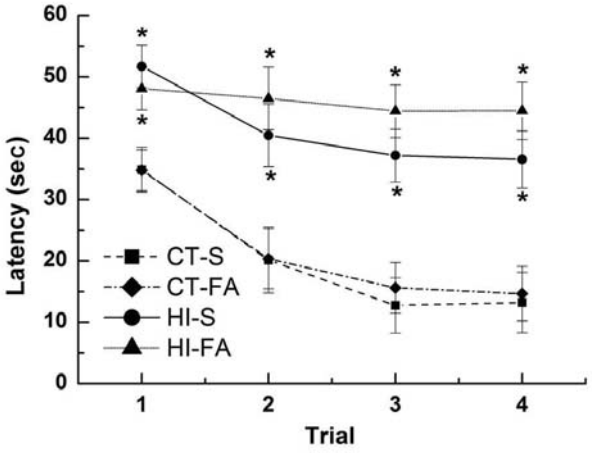

Fig. (4). Working memory protocol in the Morris water maze. Data is expressed as mean ± S.E.M and each line represents latency in finding the platform on each trial over 4 days for 10-12 animals per group. *Different from the control groups (ANOVA followed by Tukey's test; $p < 0.05$).

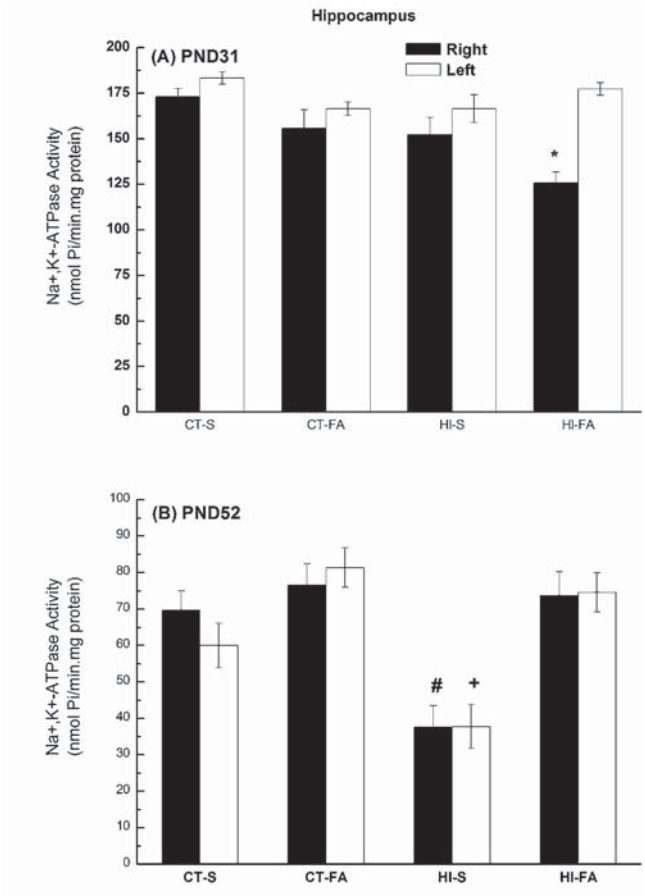

Fig. (5). Na^+,K^+-ATPase activity in ipsilateral (right) and contralateral (left) hippocampus. (**A**) Activity measured at PND 31. (**B**) Activity measured at PND 52. Data is expressed as mean ± S.E.M. for 4-6 animals per group. *Different from CT-S group, #difference from all the others groups and +difference from CT-FA and HI-FA (ANOVA followed by Tukey's test; $p < 0.05$).

ANOVA showed significant differences on the *lesion* [$F(1,18) = 10.3$; $p < 0.05$] and *treatment* factors [$F(1,18) = 7.74$; $p < 0.05$] without *lesion*treatment* interaction [$F(1,18) = 0.34$; $p > 0.05$] on enzyme activity in the ipsilateral hippocampus. Tukey's test demonstrated Na^+, K^+-ATPase activity decrease in HI-FA compared to the CT-S group. When the contralateral hippocampus was analyzed there was a *lesion*treatment* interaction [$F(1,20) = 8.07$; $p < 0.05$], but the *post hoc* test did not show a significant difference between the groups. With regard to enzyme activity in the hippocampus in adulthood (PND 52), ANOVA demonstrated a significant effect associated with the *lesion* (right: [$F(1,19) = 8.85$; $p < 0.05$]; left: [$F(1,17) = 6.35$; $p < 0.05$]) and *treatment* factors (right: [$F(1,19) = 13.4$; $p < 0.05$]; left: [$F(1,17) = 25.5$; $p < 0.05$]). *Lesion*treatment* interaction was identified in the right hippocampus [$F(1,19) = 6.20$; $p < 0.05$]. Tukey's *post hoc* indicated a decrease in Na^+, K^+-ATPase activity in the ipsilateral hippocampus in the HI-S group in comparison with all the other groups. Also, in the contralateral hippocampus, the HI-S group had decreased Na^+, K^+-ATPase activity when compared to the CT-FA and HI-FA groups (Fig. **5B**).

MORPHOLOGICAL ANALYSIS

Total Hippocampal Volume

As shown in Figs. (**6C**, **7A**), HI caused atrophy of the right (ipsilateral), as demonstrated for *lesion* effect [$F(1,15) = 17.9$; $p < 0.05$] and *lesion*treatment* interaction [$F(1,15) = 9.01$; $p < 0.05$] was shown in the left (contralateral) hippocampus. The Hypoxic-ischemic animals (HI-S) showed a decrease in volume when compared to the CT-S and HI-FA groups.

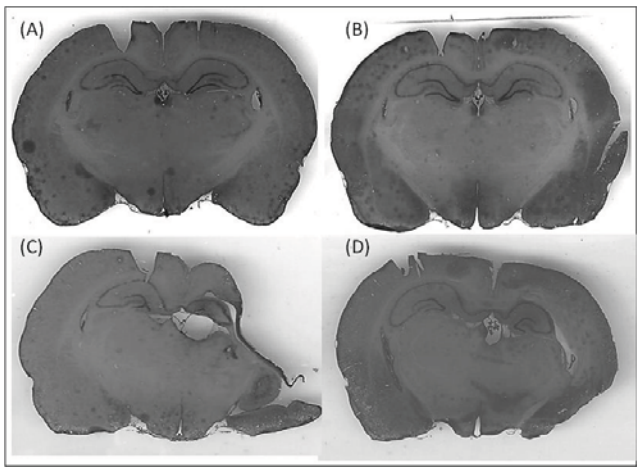

Fig. (6). Representative coronal brain sections from rat euthanized on PND 31. **A**) CT-S, **B**) CT-FA, **C**) HI-S and **D**) HI-FA.

Ammon's Horn Volume

Similar results were found when Ammon's horn volume was analyzed (Fig. **7B**). There was a *lesion* effect [$F(1,15) = 9.94$; $p < 0.05$] and a *treatment* effect [$F(1,15) = 4.94$; $p < 0.05$] in the ipsilateral hemisphere. In the contralateral there was a significant *lesion*treatment* interaction of the

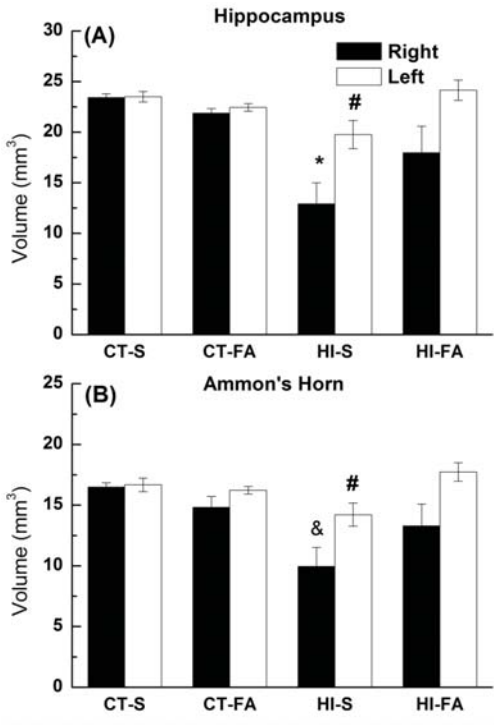

Fig. (7). Measurement of hippocampal volume (**A**) and Ammon's horn (**B**), ipsilateral and contralateral to arterial occlusion. Data is expressed as mean ±S.E.M. for four animals per group. *Difference from the CT-S group, &difference from CT-S and CT-FA #difference from HI-FA (ANOVA followed by Tukey's test; $p < 0.05$).

Table 1. Effect of hypoxic-ischemic damage in area of the striatum.

	Ipsilateral	Contralateral
Control Saline	8.8±0.2	9.7±0.1
Control Folic Acid	9.1±0.3	9.3±0.6
HI Saline	5.8±0.1*	9.1±0.3
HI Folic Acid	5.6±1.4*	9.6±0.4

Data are expressed (mm^2) as mean ±S.E.M. *Difference from CT-S group (ANOVA followed by Tukey's test; $p<0.05$).

left (contralateral) [$F(1,15) = 8.25$; $p < 0.05$]. The HI-S animals showed a decrease of Ammon's horn volume when compared to all the other groups.

Striatal Area

Table **1** shows a decrease in the ipsilateral striatal area in the hypoxic-ischemic animals, demonstrating an effect of the lesion factor [$F(1,15) = 18.5$; $p < 0.05$] when compared to the control groups. Two-way ANOVA did not identify a difference between the groups in the left striatum area (contralateral).

DISCUSSION

In the present study we investigated the effects of FA supplementation on memory performance, Na^+, K^+-ATPase activity and hippocampal and striatal atrophy in animals submitted to HI. Major findings demonstrated that both procedures, HI and FA supplementation, caused spatial memory impairment in the Morris water maze task and Na^+, K^+-ATPase activity failure in the critical moment of puberty (PND 31). Interestingly, in adulthood (PND 52), HI resulted in a decrease of the Na^+, K^+-ATPase activity, which was reversed by FA treatment. Additionally, FA was able to recover hippocampal atrophy in the HI rats.

It is well established that HI causes impairment of the spatial and working memory in the water maze task [15, 16, 31]. Corroborating with these studies, these results indicate that HI induced a significant spatial memory deficit in the Morris water maze. Curiously, it was also observed that supplemented groups, control (CT-FA) and hypoxic-ischemic animals (HI-FA), showed memory deficits. In agreement with the present data, Sittig *et al*. [17] demonstrated that FA intakes for 30 days in adolescence rats caused deficits in spatial memory in the Morris water maze. In addition, they found suppressed thyroid function with T3 and T4 hormones decreased and a diminished expression of thyroid receptor-α1 and -α2 in the hippocampus. This may be a possible explanation for the findings of the present study. Additionally, Brocardo *et al*. [32] observed that FA was able to inhibit *N-methyl-D-aspartate* (NMDA) receptors. If one considers that NMDA receptors induce long term potentiation, which is associated with memory formation in the hippocampus [33, 34], we can suppose that there may be another possible mechanism by which FA interferes with memory formation in rats. Vonder Haar *et al*. [35] also demonstrated that low and high FA doses caused behavioral dysfunction in the water maze task and neuronal loss in the traumatic brain injury model. These authors suggest that increases in methionine may lead to DNA methylation, which effectively silences genes by binding itself to and blocking sections of coding DNA. It is therefore reasonable to consider that poorer behavioral performance in chronic treatment with FA during developmental may indicate potential toxic effects of this vitamin due to excess methylation.

Na^+, K^+-ATPase is an essential membrane protein which plays a role in the maintenance of membrane potential in excitable cells [36]. The previous study to this one, stated for the first time that Na^+, K^+-ATPase activity was inhibited in the striatum and cerebral cortex in HI animals; additionally rats treated with FA showed recovered Na^+, K^+-ATPase activity [9]. In the present study, aiming to correlate with the hippocampal dependent task, Na^+, K^+-ATPase activity in the hippocampus was analyzed in two periods of the animals' development. It is clear that there were time-dependent differences: in adolescent rats (PND 31) Na^+, K^+-ATPase activity inhibition was only found in HI rats supplemented with FA and in adulthood (PND 52), a decrease was observed in enzyme activity only in the non-supplemented HI animals. This finding can be partly associated with poor performance in the water maze. Some studies have reported an acute decrease in Na^+, K^+-ATPase activity after a hypoxic-ischemic event in animals [29, 37-40]. Golden *et al*. [39] investigated mechanisms of striatal neuron death in HI piglets and found a decrease in Na^+, K^+-ATPase activity at 3, 12 and 24 hours after HI and associated this finding to the oxidative damage mediated by NMDA receptor activation.

Also, Weis *et al*. [37] showed reduced activity of the Na^+, K^+-ATPase activity as well as a late increase in malondialdehyde levels and superoxide dismutase activity in the hippocampus in the acute post-HI phase.

In the present study, FA supplementation seems to potentiate deleterious effects of the hypoxic-ischemic event in adolescent rats. Considering that FA has been found to decrease homocysteine content [41-43] and that decreased homocysteine levels have been associated with increasing nitric oxide production [44, 45], it is reasonable to propose that Na^+, K^+-ATPase activity impairment in adolescent rats treated with FA could be related to nitrosative-oxidative stress, particularly in the acute phase after HI. Interestingly, in hypoxic-ischemic adult rats, Na^+, K^+-ATPase activity was recovered by FA, suggesting that chronic treatment produces different effects when administrated from infancy until adulthood. As oxidative damage is alleviated in this phase, FA can be expected to provide a protective role. Furthermore, in the early phase of development, FA supplementation can be the cause of excessive levels of this vitamin [17]. This data supports the idea that special attention is necessary to adopt a therapeutic strategy using FA; the dose and period of treatment appears to be critical for cerebral tissue effects.

The present data also indicates that there is hippocampal and striatal atrophy ipsilateral in the carotid occlusion in the HI animals, which corroborates with previous studies [15, 16, 30]. Since these are crucial structures for memory formation [14, 46], this tissue damage can be partially responsible for spatial memory impairment identified in rats submitted to neonatal HI. Interestingly, HI rats supplemented with FA showed partial recovery of the hippocampal volume. It is a surprising result because this morphological recovery was not accompanied by functional improvement. Cerebral structures volume, especially after a damaged event, should be carefully interpreted. The previous studies demonstrated improved cognitive function without morphological recuperation [16, 31] and the present data showed the contrary. Thus, it is possible to consider that recovery of the hippocampal volume cannot represent an efficient morphological alteration since no protective effect was observed; maybe the volume preservation is result of non-functional increased dendritic arborization and spine density or severe reactive astrogliosis. Considering that HI, as well as other injuries of the central nervous system, may lead to reactive gliosis [47, 48] and that FA acts in cell division through de novo synthesis of purine nucleotides and thymidylate [49], it could be that these effects can be contributed to the stimulation of astrocytic cell division, leading to the formation of gliosis scar. Similar results have been found by Briones *et al*. [50] which suggests that ischemia-induced increased expression of reactive astrocytes may play a role in tissue remodeling rather than providing neuroprotection. Moreover, Martin *et al*. [51] identified an increase in astrocyte proliferation in animals subjected to HI and they stated that activation of these cells has an important functional consequence in situations such as stroke but does not necessarily contribute to recovery cognitive deficits. Additionally, it has been identified that reactive astrogliosis occurs associated to variable degrees of hypertrophy of cell body and stem processes and dispersed astrocyte proliferation [52]. And it is also established that in the severe reactive astrogliosis the potential for resolution and return to normal brain structure is reduced [53]. Thus, these findings must be carefully interpreted. Firstly, atrophy measure is one morphological variable and these are the first data about the effects of folic acid on brain damage in the rats submitted to neonatal hypoxia-ischemia. Then, it is reasonable to propose that other morphological aspects should be investigated to better understand what represents this tissue preservation.

CONCLUSION

Our findings showed that either HI or FA supplementation *per se* in adolescent rats impairs memory and Na^+, K^+-ATPase activity in the hippocampus. Conversely, in adult rats the decrease in Na^+, K^+-ATPase activity and hippocampal atrophy was reversed after folic acid treatment. Taking together, these data suggest a dual effect of FA in different stages of development; additionally, with the aim of understanding, at least in part, the possible effects of FA supplementation on neurological recovery, more studies are necessary. Therefore, considering the dietary supplementation of FA, mainly in industrialized countries, and the cognitive deficits arising from possible overdose during the puberty period, this study has highlighted the importance of a balanced and rational intake of folic acid during a lifespan.

LIST OF ABBREVIATIONS

CT-AF	=	Control Treated with Folic Acid
CT-S	=	Control Treated with Saline Solution
FA	=	Folic Acid
HI	=	Hypoxia-Ischemia
HI-FA	=	Hypoxic-Ischemic Treated with Folic Acid
HI-S	=	Hypoxic-Ischemic Treated with Saline Solution
NMDA	=	N-Methyl-D-Aspartate
PND	=	Postnatal Day

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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4. Capítulo 2

“Cognitive deficit and redox imbalance in rats submitted to neonatal hypoxia-ischemia and folic acid treatment”.

A ser submetido ao periódico *Nutritional Neuroscience*.

"Cognitive deficit and antioxidant response in rats submitted to neonatal hypoxia-ischemia and folic acid treatment".

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Abstract

Background: Our previous studies demonstrated that treatment with folic acid (FA), a crucial vitamin for the central nervous system development, improved aversive memory deficit and curiously contributed to the spatial memory impairment in a neonatal hypoxia-ischemia (HI) model.

Objective: (i) to verify the effects on learning and memory in the ox-maze task and in the rota-rod on motor coordination; (ii) to analyze the antioxidant potential of SOD and CAT defenses and (iii) to evaluate the reactive gliosis on hippocampus at different stages of development in rats submitted to HI and treated with FA.

Methods: at postnatal day 7th (P7) pups were submitted to Rice-Vannucci model and treated with FA (P7-P41). In addition, at P22 rats were evaluated in Rota-rod and Ox-maze tasks. Also, antioxidant enzymes (SOD and CAT) and astroglial marker (GFAP) were observed over the development (P22 and P42).

Results: There were increased latency, time to complete task and incorrect number of nose pokes and decreased number of correct nose pokes more marked in the HI animals untreated than treated compared to controls on ox-maze task. No evident locomotor deficit was found on rota-rod test. SOD and CAT outcomes showed decreased activity at P22 and increased activity at P42 in both treated and untreated HI and control FA groups. Additionally, increased GFAP-positive astrocytes were found in both HI groups in the ipsilateral hippocampus, although FA treatment had contributed to slow down the glial scar formation at P22.

Conclusions: Taken together, these results suggest that folic acid treatment partially prevents the spatial memory failure and reactive gliosis promoted by hypoxic-ischemic injury. Although more studies are needed, such neuroprotective effect seems to not be related to antioxidant properties.

Keywords: folate, vitamin B_9, ox-maze, neonatal stroke, behavior, brain damage, oxidative stress, memory

Introduction

Perinatal hypoxia-ischemia (HI) has been associated with neurodevelopmental disorders that frequently lead to disabilities and morbidity in preterm and full-term infants (1, 2). Neonatal encephalopathy, which is associated to cerebral palsy, epilepsy and cognitive impairments, occurs approximately as 2-3/1000 live births (1, 3). Its cause can be established as a consequence of several events in the antepartum and intrapartum periods that result in intrauterine hypoxia (4). Consequently, the reduction in oxygen and glucose leads to a cascade of metabolic imbalances as ATP depletion, glutamatergic excitotoxicity, cytotoxic edema and oxidative stress inducing cell death and brain damage (5, 6). Additionally, preterm infants exhibit cerebral vulnerability to oxidative stress due to neuronal membranes are rich in polyunsaturated fatty acids and to have incomplete development of the antioxidant defenses, such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) (7-9). Neuronal injury is predominant in areas such as of the CA1 region of the hippocampus, striatum, cerebellum and cerebral cortex (10-13) and clear atrophy

has been identified in the hippocampus consequent to hypoxic-ischemic events (14, 15). Moreover, glial scar has been described as a matter of response to the insult by astrocytes (16, 17). It is also well described that HI rodent model (13, 18), which is widely used in experimental research due to high reproducibility of human disabilities, causes not only cognitive impairments, on Morris water maze, inhibitory avoidance, novel-object recognition and ox-maze task (14, 15, 19-21) but also motor disabilities (22, 23).

In our previous studies folic acid (FA), a water-soluble vitamin, has been employed as a pharmacological therapeutic agent in order to minimize the brain damage caused by the HI (14, 24). FA has a critical role in the synthesis of nucleic acids, phospholipids and neurotransmitters, also participating in the development, function and repair of the central nervous system (25, 26). Additionally, it has been reported in previous study that FA treatment ameliorated aversive memory impairment and Na^+, K^+-ATPase inhibition in frontal cortex and striatum of adolescent rats submitted to the HI model (24). On the other hand, the treatment contributed to the worse spatial memory in adult rats as assessed on the Morris water maze and to the failure in hippocampal Na^+, K^+-ATPase activity in adolescent rats submitted to HI (14, 24). Due to the high relevance of the hippocampus for memory processes, these unexpected results deserve more investigation.

In order to shed some light on the mechanisms behind the vulnerability (and lack of efficacy of FA treatment) of the hippocampus in early stages of the HI events we investigate: (i) the motor coordination and learning and memory on ox-maze task in adolescent rats, (ii) the status of antioxidant defenses through the

analyzing of SOD and CAT enzyme activities, and, (iii) the reactive astrogliosis on hippocampus at different stages of animal development (P22 and P42).

Materials and methods

Animals and study design

Subjects were male and female Wistar rats with similar number between then, obtained from Centro de Reprodução e Experimentação de Animais de Laboratório (CREAL), from the Universidade Federal do Rio Grande do Sul (UFRGS), Brazil. Animals were maintained in acclimatized rooms (22°C) with food and water *ad libitum* and controlled 12/12 light/dark cycle. Pups, at postnatal day 7 (P7), were randomly assigned to four experimental groups: saline control (CT-S), folic acid control (CT-FA), saline hypoxic-ischemic (HI-S) and folic acid hypoxic-ischemic (HI-FA). To evaluated HI effect and FA treatment, biochemistry, immunohistochemistry and behavioral experiments were performed at different stages of the development (P22 and P42). Three distinct group of 11-12 animals were used for: (1) biochemical and immunohistochemical assays at P22; (2) biochemical and immunohistochemical assays at P42 and (3) behavioral evaluation at P22, totaling 146 animals. Time line of experiments is depicted in Figure 1. All animal procedures were approved by the Ethics in Research Committee at the UFRGS, Brazil (number 23564) and were in compliance with NIH Guide for the Care and Use of Laboratory Animals and with the AROUCA Brazilian Law (number 11.794) for animal care and ethical use of animals.

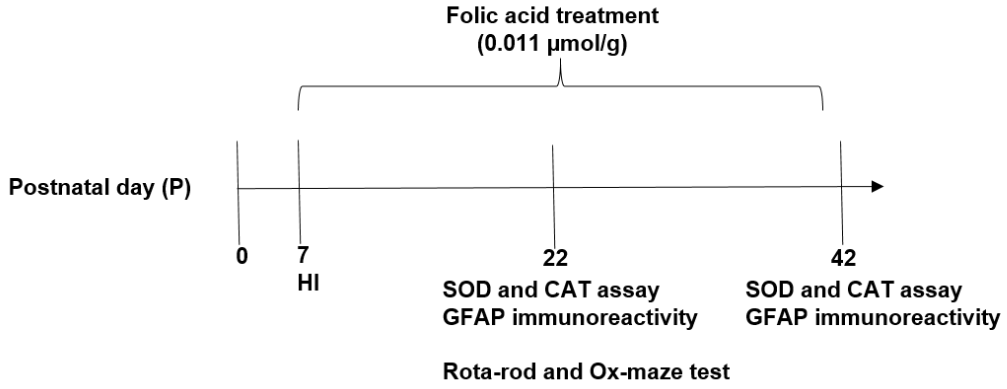

Figure 1. Time line of experimental procedures.

Hypoxia-ischemia procedure

Seven-days-old pups were randomly assigned to HI or CT groups and its treatment (saline or FA) and the Rice-Vannucci procedure was performed (13, 27-29). Animals were anesthetized with halothane (2-4%) and through a neck incision right common carotid artery was occluded permanently with surgical silk thread. Pups were observed for 15 min under a heating lamp and returned to its dams for two hours to recuperation. Then, animals were exposed to hypoxic atmosphere (8% oxygen and 92% nitrogen, 5L/minute flow) for 90 minutes in a chamber partially submerse in water bath with controlled temperature (37°C). Control animals were manipulated, anesthetized and suffered a neck incision, but did not expose to hypoxic environment and arterial occlusion (15, 24, 30).

Folic acid treatment

Animals were weighed daily before treatment and injected intraperitoneally with FA solution (0.011µmol/g body weight). Treatment carried out immediately before and after HI procedure once a day (from 7th to the maximum 41st day of life) (24, 31). CT-S and HI-S groups received saline solution in the same rate of FA groups.

Behavioral assessment

Rota-Rod test

At P22 animals were submitted to rota-rod test (*Insight©, Brazil)* to evaluate the motor coordination (*n*=11-12 animals per group). Carefully rats were disposed to the apparatus firstly, during an adaptation training session (1 min), and subsequently for 5 min with the velocity accelerating rate 5 rpm every 15 seconds into to 40 rpm (32, 33). Latency of the first downfall, number of falls and time of permanence in the apparatus were evaluated.

Ox-maze task

Animals were evaluated on ox-maze task to learning and memory at P25 during 10 consecutive days (*n* = 11-12 animals per group). The apparatus consists of a black square box (60 cm x 60 cm x 30 cm high) divided into 12 equal squares that had four independents black blocks (10 cm x 10 cm x 10 cm high). Each block side had a hole and draw symbol (O,X,=,‖) (30, 34). There was one reward (fruit loops Kellogg's®) in each of block only in the symbol (O). Two days before tasking rats received fruit loops as a form of tasty habituation and also, were

submitted to food restriction (24 hours before test), in which received amount of diet was about 90% of the food intake, and its weight was measured every day (30, 35).. Animals were placed at the center of the maze and allowed to explore it for 10 min or until they found four rewards in each black box. Rewards always remained in the same symbol (O), but the block position was changed, clockwise, every day. Variables analyzed were: time to find the first reward (latency), time to complete task, percentage of correct and incorrect nose pokes. After each session the arena and blocks was cleaned with alcohol 20% v/v.

Biochemistry assay

Tissue preparation

Animals were decapitated and hippocampi were dissected at P22 and P42 (*n* = 6-7 animals per group) and homogenized in 10 volumes (1:10 w/v) in a 20 mM sodium phosphate buffer solution, pH 7.4, including 140 mM potassium chloride. The homogenate was centrifuged at 800g for 10 min at 4°C. The pellet was discarded and the supernatant was used for the biochemistry assays (36, 37).

Superoxide Dismutase (SOD)

Superoxide dismutase activity was determined spectrophotometrically at 420 nm using Marklund's method (38) which consists on the capacity of pyrogallol to autoxidize, a process highly dependent on superoxide, which is a substrate for SOD (36, 37). The results were expressed as SOD units/mg protein.

Catalase (CAT)

The Aebi method was employed (39) in which a reaction is based on the disappearance of H_2O_2 at 240 nm. It was used a reaction medium containing 20 mM H_2O_2, 0.1 % Triton X-100, 10 mM potassium phosphate buffer pH 7.0, and 0.1–0.3 mg protein/mL. One CAT unit was defined as 1 µmol of hydrogen peroxide consumed per minute, and the results were represented as CAT units/mg protein (36, 37).

Protein determination

Protein content was determined by the method of Lowry et al. (40).

Imunohistochemistry

Glial fibrillary acidic protein (GFAP)

Animals were profoundly anesthetized, at P22 and P42, with ketamine and xylazine (80mg/kg and 10mg/kg body weight, respectively, i.p.), heparinized and then perfused transcardiacally with NaCl solution followed by 4% paraformaldehyde with phosphate buffer (*n* = 5-6 animals per group). Brains were removed and fixed for four hours in the same solution at 4°C. They were firstly placed in 15% of the sucrose solution and afterwards in 30% sucrose solution until to sink. Following this, brains were frozen at -20ºC using a cryostat (CM1850, Leica, São Paulo-SP, Brazil) and coronal serial sections (40µm thick) were collected on the dorsal hippocampus at bregma -3.36 mm according to Paxinos

and Watson (41). The sections were rinsed in PBS solution and pre-incubated with 3% bovine serum albumin solution contents 0,4% Triton-X-100 (PBS-Tx), during 30 minutes. Then, samples were incubated with monoclonal rabbit anti-GFAP antibody (DAKO, UK) diluted (1:1000) in 0.3% PBS-Tx) at 4°C overnight. Sections were rinsed in PBS and the endogen peroxidase was inactived with 3% H_2O_2 diluted in PBS and after, washed with PBS-Tx. Slices were incubated with secondary antibody rabbit anti-mouse IgG conjugated with peroxidase (Sigma, USA) diluted (1:500) in PBS-Tx at room temperature for 2 hours. To visualize immunoreactivity a solution 0.06% 3, 3-diaminobenzidine (DAB; Sigma Aldrich, USA) and 10% H_2O_2 were employed for 10 minutes. Finally, the tissues were washed in PBS, dehydrated in ethanol, cleared with xylene and covered to synthetic Canada Balsam (Chemical Reaction, Brazil) and then cover slipped. Antibody primary was omitted in the negative control.

GFAP immunoreactivity – Optical densitometry

To measure the optical densitometry it was utilized a semi-quantitative method described to Xavier et al.(42); Saur et al.(43) and Marques et al.(44). Three images were captured of each hippocampus section (4-6 sections per animal) between the stratum radiatum and lacunosum molecular layers (using an Optiphot-2 microscope (400×, Nikon, Japan) coupled to a CMOS camera (518CU, Micrometrics, USA). For estimate astrocytic density images were converted to an 8-bit gray scale and one AOI (174,03 μm^2) was placed over the astrocyte on each image by Image Pro Plus Software 6.0 (Media Cybernetics, USA). All lighting conditions were constant and the background was corrected in accordance to Xavier et al.(42).

Statistical analysis

Data obtained from biochemistry, immunohistochemistry and behavioral performance (rota-rod and means of the each variable in all days on the ox-maze task) were analyzed using Two-way analysis of variance (ANOVA). Results from ox-maze during 10 testing days were performed by repeated measures. Tukey's *post hoc* teste for multiple comparisons was run when indicated. All data were expressed as mean±S.E.M and performed using Statistica Software package (Statsoft®). Differences were considered statistically significant if $p \leq 0.05$.

Results

Hypoxia-ischemia caused cognitive impairment in the ox-maze task but not in motor coordination

Motor coordination was evaluated on the rota-rod test but there are no significant effect on lesion or treatment (Table 1). Parameters analyzed were latency to the first downfall (lesion [$F(1,43)=1.99;p>0.05$] and treatment [$F(1,43)= 0.12;p>0.05$], number of falls (lesion [$F(1,43)=0.04;p>0.05$] and treatment [$F(1,43)= 0.04;p>0.05$] and total time of permanence in the apparatus (lesion [$F(1,43)=0.09;p>0.05$] and treatment [$F(1,43)=0.66;p>0.05$].

Table 1. Rota-rod Test (seconds)

Group	Latency 1st downfall	Number of falls	Time of permanence
CT-S	165.5±19.7	2.5±0.26	248.0±13.3
CT-FA	174.0±26.1	2.0±0.35	229.1±22.2
HI-S	145.8±29.7	2.0±0.35	239.3±18.2
HI-FA	119.0±29.2	2.3±0.33	225.6±25.1

Values are expressed as mean ± S.E.M per group. No significant differences were seen among the experimental groups. (Two-way ANOVA followed Tukey's test; p>0.05).

In order to assess the effect of HI and FA treatment on the learning and memory four parameters were evaluated in the ox-maze task: latency to find the first reward, time to complete task and percentage of correct and incorrect number of nose pokes. When evaluated latency to find the 1st reward, the repeated measures ANOVA showed main effects of lesion [$F(1,43)=5.94;p<0.05$], day [$F(9,43)=62.9;p<0.05$] and lesion*day interaction [$F(9,387)= 3.19; p<0.05$]. Tukey's *post hoc* indicated that HI-S group presented greater latency to find the reward on the 1st testing day compared to CT-S group (Fig. 2A); also, all groups showed a learning curve from the 2nd testing day. Additionally, means of latencies throughout all sessions were analyzed by Two-way ANOVA (Fig. 2B). There was a significant main effect of lesion [$F(1,43)=5,94; p<0.05$], confirming that HI groups had lower latencies compared to control groups.

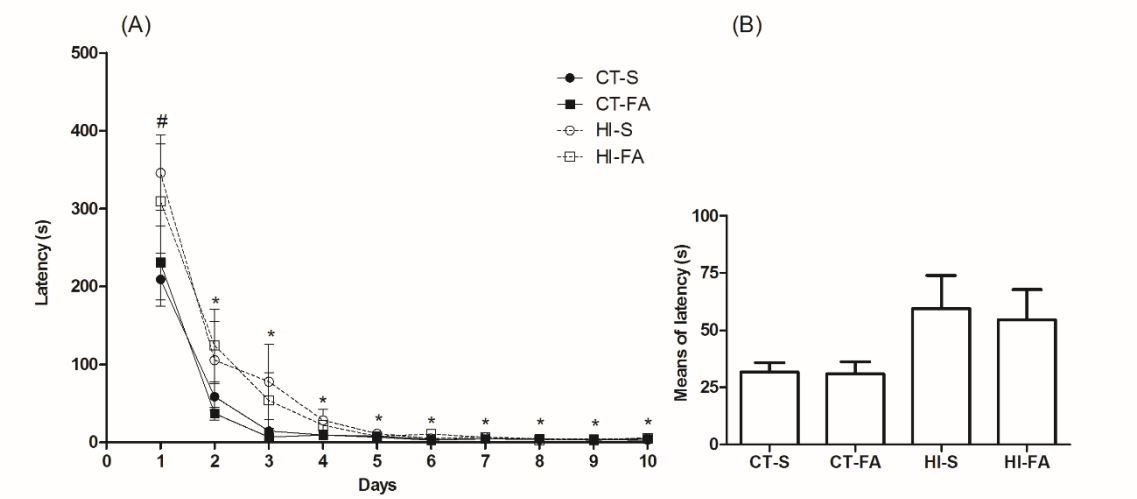

Figure 2. Latency to find the first reward. (A) Repeated measures ANOVA followed by Tukey's test showed greater latency from #HI-S compared to CT-S group and a *Significant difference from 1st day. (B) Means of latency. Two-way ANOVA showed lesion effect (higher latency in HI groups). Results are expressed as mean ± S.E.M.; p≤0.05).

Similar results were found by repeated measures ANOVA when analyzing time to complete the task. There was a significant main effect of lesion [$F(1,43)=23.1;p<0.05$], treatment [$F(1,43)=5.0;p<0.05$], day [$F(9,387)=213.8;p<0.05$] and lesion*day interaction [$F(9,387)= 3.4; p<0.05$]. The HI-S group presented greater time to complete the task on the 2nd, 3rd and 4th day compared to CT-FA group. On the other hand, HI-FA group showed this effect only on the 2nd day when compared to CT-FA group (Fig.3A). The learning curve was also observed from the 2nd day for all groups. Observing the means, Two-way ANOVA showed significant main effect of lesion [$F(1,43)=23.1;p<0.05$] and

treatment [F(1,43)=5.0;p<0.05]. Both HI groups presented significant differences when compared to CT-S and CT-FA groups (Fig.3B).

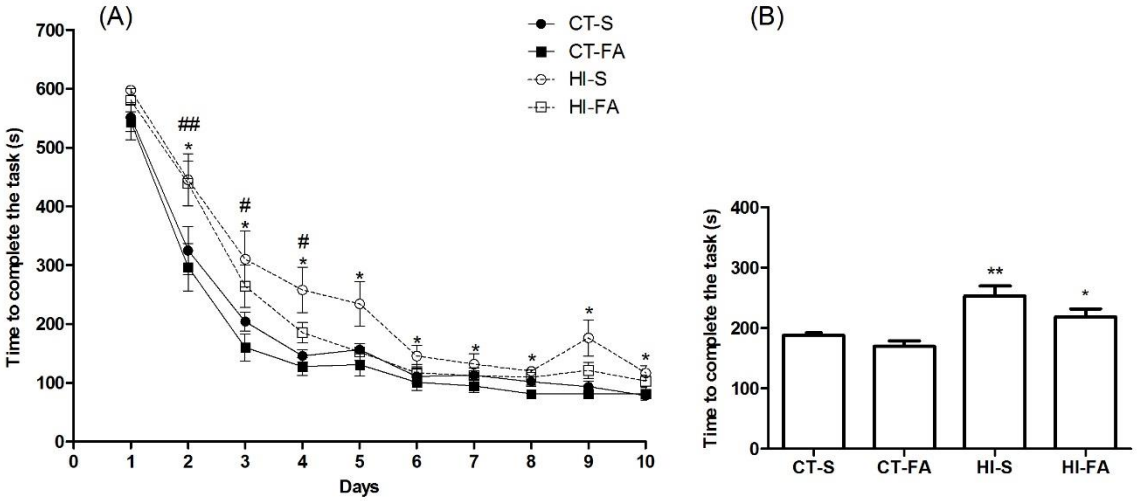

Figure 3. Time to complete task (A) Repeated measures ANOVA followed by Tukey's test showed ##greater time to complete task in the HI-S and HI-FA compared to CT-FA at 2nd day. At 3rd and 4th days, the #HI-S group spent more time compared to CT-FA. *All groups showed decrease in the total time from 1st day. Means of time to complete task (B). Two-way ANOVA followed by Tukey's test presented **significant difference when compared to both controls and * when compared to CT-FA. Results are expressed as mean ± S.E.M.; p≤0.05).

The percentage of correct nose pokes (Fig. 4A) showed mains effect of lesion [F(1,43)=13.9;p<0.05] and day [F(9,387)=4.1;p<0.05] but did not difference between groups it was found by post-test. owever, two-way ANOVA for means of percentage of correct nose pokes (Fig. 4B) identified significant effect of the lesion [F(1,43)=13.9;p<0.05] and Tukey's demonstrated lower correct options for both HI groups comparing with CT-FA group. The percentage of incorrect nose pokes (Fig. 4C) presented significant results on lesion [F(1,43)=7.98;p<0.05], day

[F(9,387)=3.39;p<0.05] and lesion*day interaction [F(1,387)=2.92;p<0.05] but there was no difference between groups. Means of percentage of incorrect responses (Fig. 4D) presented significant effect of lesion [F(1,43)=7.98; p<0.05], with increased incorrect nose pokes in HI-S group compared to CT-AF.

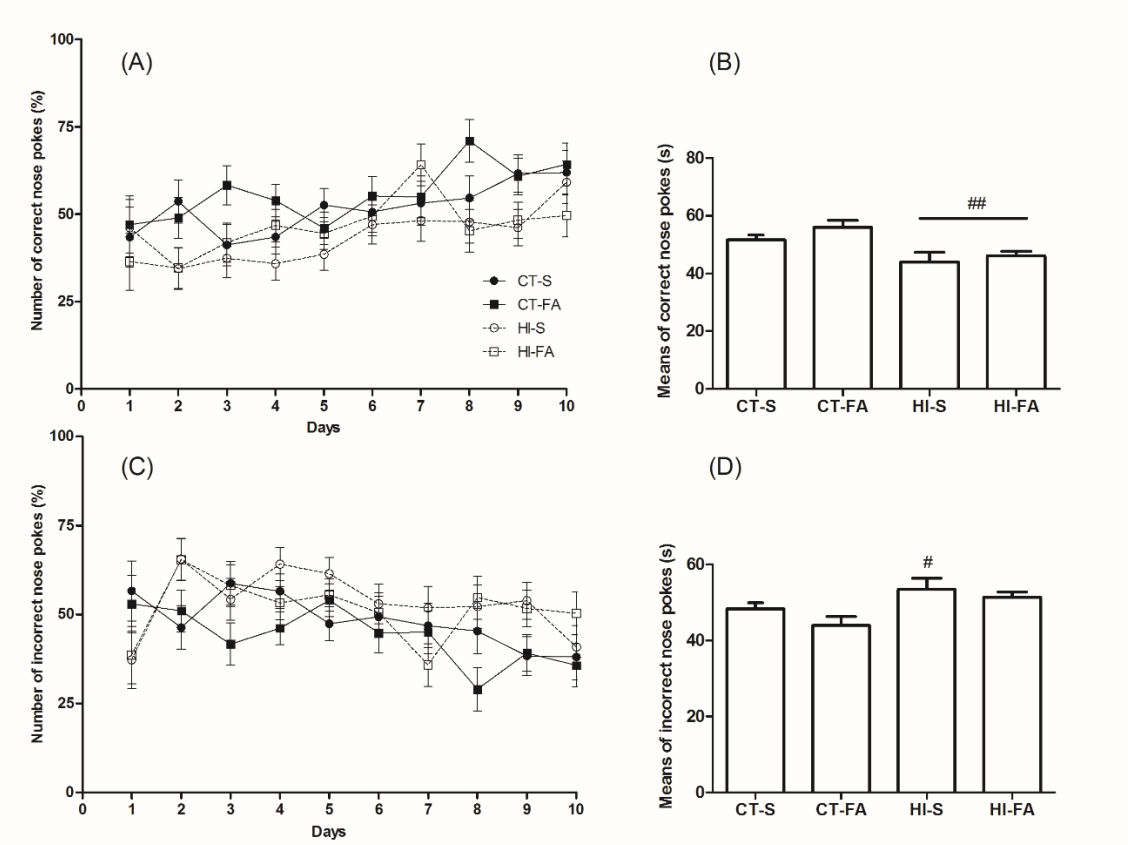

Figure 4. Percentage of number correct (A), means of correct (B), incorrect (C) and means of incorrect (D) nose pokes. Repeated measures ANOVA followed by Tukey's showed effect of lesion and day. Both HI groups presented lower number of correct (A) and higher number of incorrect (C) nose pokes. Similarly, the Tukey's test showed a significant difference in both ##HI groups compared to CT-FA when the means of correct (B) was assessed. *Significant difference when compared to CT-FA when the means of correct was assessed (D). Results are expressed as mean ± S.E.M.; p≤0.05).

Hypoxia-ischemia increased GFAP immunoreactivity in the hippocampus at P22 and P42.

Analyzing the astrocyte optical density two-way ANOVA showed a main effect of lesion in the right hippocampus (ipsilateral to lesion), in both time points, P22 [$F(1,16)=101.5;p<0.05$] and P42 [$F(1,20)=174.4;p<0.05$]. In addition, Tukey's test showed increased immunoreactivity only in HI-S rats at P22 (Fig. 5A). However, at P42 both HI groups presented greater astrocytic optical density compared to controls (Fig. 5B). Folic acid seems to exert a partial modulator effect on the GFAP expression only at P22.

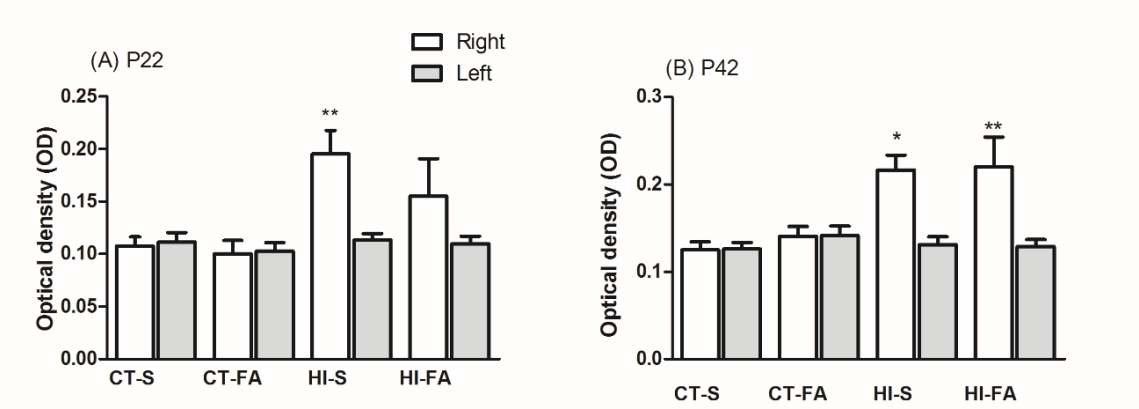

Figure 5. Effects of HI and HI-FA folic acid on hippocampal astrocytic optical density measurements at P22 (A) and P42 (B). **Significant difference when compared to control groups (A and B). *HI-S is different from CT-S. (Two-way ANOVA followed Tukey's test; $p \leq 0.05$).

Effect of hypoxia-ischemia and folic acid treatment on the superoxide dismutase and catalase activities in the hippocampus of rats

The effects of antioxidant defenses in the hippocampus were investigated by analysis of superoxide dismutase (SOD) and catalase (CAT) enzymes activities. At P22, SOD activity presented significant lesion [$F(1,24)=24.8;p<0.05$] and

treatment effects [$F(1,24)=12.0;p<0.05$] in the ipsilateral hippocampus. Post-test verified a decreased SOD activity in the HI-S and HI-FA when compared to control groups. In the contralateral hippocampus we found main effect of lesion [$F(1,24)=7.04$; $p<0.05$], treatment [$F(1,24)=11.4;p<0.05$] and lesion*treatment interaction [$F(1,24)=10.5$; $p<0.05$]. There was lower SOD activity in the HI-S group, presented by Tukey's test, compared to the others groups. In this case, HI-FA group showed partial decrease of enzymatic activity presented by the recovery activity in the contralateral hippocampus (Table 2).

Additionally, at P42 SOD activity presented main effects of lesion [$F(1,23)=5.9;p<0.05$], treatment [$F(1,23)=17.8;p<0.05$] and lesion*treatment interaction [$F(1,23)=10.4;p<0.05$]. Contralateral side presented similar effects of lesion [$F(1,23)=11.9;p<0.05$], treatment [$F(1,23)=24.4;p<0.05$] and lesion*treatment interaction [$F(1,23)=5.2;p<0.05$]. There was higher SOD activity observed only ipsilateral hippocampus from HI groups compared to CT-S indicated by post-test. Moreover, CT-FA group also presented higher SOD activity than others groups in both hippocampi (Table 2).

Table 2. Superoxide dismutase (SOD) and Catalase (CAT) activity at P22 and P42.

Group	SOD				CAT			
	P22		P42		P22		P42	
	Right	Left	Right	Left	Right	Left	Right	Left
CT-S	26.5 ± 2.8	26.7 ± 1.6	56.3 ± 2.1	60.6 ± 2.6	3.34 ± 0.4	3.41 ± 0.2	4.88 ± 0.3	5.81 ± 0.2
CT-FA	32.5 ± 1.2	26.9 ± 1.8	73.9 ± 1.9$^{\&}$	77.4 ± 1.9$^{\#}$	5.71 ± 0.2$^{\#}$	6.25 ± 0.4$^{\#}$	6.91 ± 0.2$^{\&}$	5.52 ± 0.5
HI-S	17.6 ± 0.6**	17.4 ± 0.9$^{\#}$	69.7 ± 0.9$^{\&}$	57.9 ± 1.6	2.36 ± 0.3*	3.16 ± 0.1	7.41 ± 0.3$^{\&}$	6.83 ± 0.3
HI-FA	23.9 ± 1.4*	27.8 ± 1.6	72.0 ± 3.3$^{\&}$	64.0 ± 2.6	3.55 ± 0.2*	3.65 ± 0.2*	7.48 ± 0.4$^{\&}$	6.26 ± 0.3

Table 2. *Data are expressed as Mean ± S.E.M. Superoxide dismutase (SOD) and Catalase (CAT) activity are expressed as U/mg protein. **HI-S is different from both controls; #different from other groups; &different from CT-S and *different from CT-FA. (Two-way ANOVA followed Tukey's test; p≤0.05).*

At P22 Catalase results showed main effect of lesion [F(1,22)=26.5;p<0.05] and treatment [F(1,22)=33.7;p<0.05] without interaction between factors regarding to ipsilateral side of brain. The contralateral side presented main effect of lesion [F(1,22)=21.5;p<0.05], treatment [F(1,22)=29.2;p<0.05] and lesion*treatment interaction [F(1,22)=14.7;p<0.05]. Tukey *post hoc* revealed in both hippocampus an increase in the catalase activity in the CT-FA group compared to all other groups in both brain sides and consequent decreased activity in the HI-S and HI-FA groups (Table 2).

At P42 there was, in the ipsilateral hippocampus, significant effect in the factors lesion [F(1,22)=25.5;p<0.05] and treatment [F(1,22)=11.6;p<0.05] with lesion*treatment interaction effects [F(1,22)=10.1;p<0.05] on catalase activity. Tukey's *post hoc* showed that HI-S, HI-FA and CT-FA had increase in the catalase activity on the ipsilateral side of brain compared to CT-S. Contralateral

hippocampus demonstrated a main lesion effect [$F(1,21)=5.7;p<0.05$], with no significant difference between groups after Tukey's test (Table 2).

Discussion

The present study investigated the effect of FA treatment in animals submitted to HI injury and evaluated at different ages of development/treatment (P22 and P42) in the behavioral performance, as well as reactive astrogliosis and antioxidant enzymes activities (SOD and CAT) in the hippocampus. As expected, the current results demonstrated that HI injury induced cognitive dysfunction (memory deficit) on the ox-maze task and increased astrocyte density, which were alleviated by FA treatment. Additionally, HI damage and FA treatment by itself (*per se*) resulted in an antioxidant enzymatic imbalance in the hippocampus.

In several studies, FA treatment has been shown to enhance neurodevelopment and to provide neuroprotection in different animal models (31, 36, 45, 46). We previously reported that HI animals had impaired aversive memory compared to HI treated with FA for a short period (23 days) (24). On the other hand, recently we demonstrated that a prolonged FA treatment (33 days) contributed to spatial memory damage in the HI animals probably due to hypermethylation, NMDA receptor inhibition and diminished expression of thyroid hormone receptor (14). In order to clarify such findings, in this work animals were treated for a shorter time (15 days) and cognitive dysfunction (spatial memory) was evaluated in the ox-maze task. The ox-maze task was initially developed in a mouse model of Huntington's disease to test visual discrimination and learning and memory, which involve little movement and proximal visual cues, without an aversive

component (34). Recently, an adapted version of the ox-maze model was used to demonstrate spatial memory (hippocampus-dependent) damage in adult rats that suffered neonatal stroke (30). Here we also demonstrated that HI animals had worse performance to find the first reward, spent more time to complete the task and made less correct and more incorrect choices. These results can be associated to hippocampal atrophy and reactive astrogliosis. Corroborating this, many studies related memory and learning impairments in the hippocampus-dependent tasks after HI injury (14, 19, 47, 48). Furthermore, it was observed that treated animals had alleviated deficits in some parameters as latency and total time in the task compared to untreated animals (HI-S) throughout testing days. Tomizawa and coworkers (49) demonstrated the importance of methyl-donor as FA for the learning and memory and the expression of the genes in the mouse hippocampus. However, we hesitate to conclude that animals that received FA treatment had better performance than untreated animals due to the fact that this recovery was only found in some variables. We excluded the possibility of motor function to impair the performance in the ox-maze, evaluating rats in the rota-rod test. There was no gross motor impairment between groups accordingly as previously described (24, 32). Attempting to understand the results found in the behavioral test and its possible mechanisms, the GFAP reactivity and redox parameters were evaluated. GFAP is a monomeric intermediate filament protein predominant in astrocytes throughout the nervous system and is largely involved in its structure and function (50). It is well established that astrocytes can be activated under hypoxic conditions and other injuries to the CNS, resulting in their proliferation and morphological changes surrounding the damage tissue, leading to reactive astrogliosis and inhibition of

the axonal regeneration and plasticity (50-54). Present results show a clear increase in hippocampal reactive astrocytes ipsilateral to arterial occlusion in both HI groups. Although, FA treatment appears to have contributed to slow down the glial scar formation in the HI-FA group (P22). These findings are in agreement to previous study using cerebral stroke model, which describe greatest GFAP expression and special hippocampal, striatal and brainstem vulnerability following acute brain injury (50, 51, 55-57). In this study, we chose to administer FA immediately before and after ischemic injury to target the therapeutic window and to alleviate damage process. Recent published study using aged rats, supplemented with different FA doses, verified lower number of neurons and astrocytes in the strata of the hippocampus (stratum lucidum, stratum radiatum and stratum lacunosum-moleculare) in the folic acid-deficient diet group compared to FA supplemented animals, indicating that an adequate FA level can modulate the GFAP expression (58). This observation reinforce the idea that FA could have contributed to a slight improvement of the performance observed on ox-maze task and the late increased GFAP immunoreactivity in the hypoxic-ischemic rats treated with FA. However, there is a lack of information on the influence of different dietary folic acid conditions on hippocampus functioning and its association to astrocyte metabolism. Additionally, more studies are necessary to confirm this hypothesis.

In the present study, it was also found that there were a lower activity of SOD and CAT at P22 and highest activity at P42 in both HI groups, suggesting an involvement of redox mechanisms and a possible contribution of the oxidative stress for the brain damage. Curiously, the control group supplemented with FA presented higher enzyme activity in both periods. Commonly, HI causes a

reduction in cerebral blood flow to the fetal brain due to energy failure and increase in the reactive oxygen species resulting in brain injury (18). Superoxide dismutase and catalase are enzymes that catalyze the dismutation/neutralization of the superoxide radical ($O_2^{\cdot-}$) and detoxifies the hydrogen peroxide (H_2O_2), respectively (59). Furthermore, theses enzymes are reported to alleviate cell injury caused by oxidants. Several studies have proposed that HI injury could be disrupting the antioxidant enzyme activities (60-62). Our group previously described increase in SOD activity in the ipsilateral hippocampus in adult animals submitted to neonatal HI, but no in CAT and glutathione peroxidase (GPx) activities (20, 63). It is important for neuroprotective strategies, as antioxidants, to slow the evolution of the HI injury and promote repair to support normal development. Several studies have investigated neuroprotective and antioxidant effects of FA supplementation in different rodent and cellular models (24, 36, 45, 64-66). Our results revealed that there was a moderate increase of the enzymes activities in the HI-FA group at P22, which can have contributed to slow down glial scar formation in this same period. On the other hand, FA treatment *per se* seems to have elevated the CAT at P22 and SOD and CAT activities at P42, then a pro-oxidant effect may be attributed to the treatment. Considering that there are several studies involving FA treatment as antioxidant and controversial results have been obtained in our studies, we suggest that more studies are necessary to clarify the potential neuroprotective by FA, as an antioxidant in the HI model.

In conclusion, the present work showed that hypoxic-ischemic injury produced a spatial memory failure and reactive gliosis, and that folic acid treatment was partially able to prevent these effects. The redox evaluation showed an imbalance in the enzymes activities in treated and untreated HI animals, besides FA also

contributed to this effect suggesting a dual effect associated with this vitamin. However, the present findings indicate that their neuroprotective effect is not only due to their antioxidant properties, at least in HI model and more studies are needed to understand the mechanisms involved.

Disclaimer statements

Contributors

Carletti, JV; Deckmann, I; Deniz, FB; Schuch, CP; Rojas, JJ; Diaz, R; Barbosa, S; Kolling, J and dos Santos, TM performed the experiments. Carletti, JV; Deniz, BF and Schuch, CP analyzed the data. Carletti, JV, Wyse, AT and Pereira LO wrote the manuscript.

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Conflicts of interest None

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5. Capítulo 3

"The effect of oxidative stress induced by tert-butylhydroperoxide under distinct folic acid conditions: an in vitro study using human syncytiotrophoblast cell lines".

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"The effect of oxidative stress induced by tert-butylhydroperoxide under distinct folic acid conditions: an in vitro study using human syncytiotrophoblast cell lines".

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Abstract

Preeclampsia (PE) is a pregnancy disorder and an important cause of maternal and fetal morbidity and mortality. It is characterized by symptoms varying from high maternal blood pressure to maternal seizures in severe cases, fetal growth restriction, intrauterine hypoxia and prematurity. Folic acid (FA) is an important vitamin required during pregnancy for the correct development of neural tube and central nervous system and the growth of placenta and fetus. Nevertheless, the effects of FA supplementation in pregnancy on preeclampsia disorder is not fully understood. In the present study it was investigated the effects of distinct FA supplementation (deficient/low, physiological, and supra-physiological) on oxidative stress induced by tert-butylhydroperoxide (TBH) (biomarker levels, antioxidant levels, antioxidant enzyme activity, and antiproliferative, antimigration and cytotoxic effects) in the BeWo and HTR-8/SVneo human trophoblast cell lines. Altogether, results showed a clear enhancement in the oxidative cell damage when cells where cultured with supra-physiological doses of folic acid and challenged with TBH. Considering that preeclampsia affects 1-8% of all pregnancies, the impact of this results are of high relevance as pregnant women are commonly subjected to higher doses of FA due to extra supplementation in food. This study suggests more attention on the dose administered, and ultimately, on the overall folic acid levels during pregnancy and preeclampsia risk.

Keywords: folate, preeclampsia, placenta, reactive oxygen species, hypoxia

Introduction

Preeclampsia (PE) is a pregnancy disorder characterized by high maternal blood pressure and proteinuria at ≥ 20 weeks of gestation [1, 2]. PE remains as an important cause of maternal and fetal morbidity and mortality, affecting 1-8% of all pregnancies through clinical manifestations of the disease as maternal hypertension and proteinuria [3-5]. In severe cases, PE complications may include maternal seizures, cerebrovascular accident, thrombocytopenia, renal failure, fetal growth restriction, intrauterine hypoxia, prematurity and death [2, 6-9]. Placenta is a materno-fetal complex organ formed during pregnancy, which is responsible for nutrient transport, gas exchange, hormone synthesis and embryo protection [10, 11]. An inadequate placentation process, including inadequate trophoblast invasion and insufficient remodeling of the uterine spiral artery, results in persistent placental hypoxia/ischemia. This in turn results in an imbalance in the production of pro-angiogenic and anti-angiogenic factors into the maternal circulation [2, 4, 8]. Although the pathophysiology of PE remains unclear, it is accepted that this imbalance in favor of anti-angiogenic factors is associated with the onset of maternal endothelial dysfunction, affecting all maternal organ systems [2, 12]. Also, an increased oxidative stress imbalance has been described during the pathogenesis and progression of PE, which can promote fetal programming leading to deleterious effects such as cardiovascular complications and metabolic syndrome in adulthood [13-16].

Folic acid (FA) is the synthetic form of folate, a member of B_9 vitamin family, which is essential for the biosynthesis of nucleotides, amino acids and s-adenosyl-L-methionine [17, 18]. Supplementation with FA (0.4 mg/day) has been recommended to all women within childbearing age from the beginning of

pregnancy and until the end of the first trimester of pregnancy, while 4-5 mg/day is advised for women at high risk of neural tube disease [19, 20]. This is because it is well established that an adequate intake of FA is required for the correct development of neural tube and central nervous system and the growth of placenta and fetus [21-23]

In the last decades, motivated by public health policies, many countries introduced FA-fortified foods, which may result in upper intake levels in women at childbearing age and in pregnant women [24-26]. Surprisingly, recent results from *in vivo* and *in vitro* models have shown that high FA supplementation is associated with adverse effects and metabolic imbalance in both mothers and their offspring including predisposition to an insulin-resistant state, memory failure, development of ventricular septal defects and thinner left and right ventricular walls, neuronal hyper-excitability, diminished seizure threshold and synapse formation [27-34]. On the other hand, researchers have studied the protective effects of FA supplementation over the risk of developing PE [35-40]. The possible beneficial effects are associated with adequate placental development and growth, lower homocysteine levels and endothelial function improvement with reduction in the gestational PE incidence [37, 41-44].

Considering that PE is associated with increased oxidative stress levels and the lack of knowledge on the relationship between PE and the intake of different doses of FA during pregnancy [36, 38, 40], we decided to investigate the effect of oxidative stress in human trophoblast cells lines cultured upon deficient/low, physiological and supra-physiological FA levels. For this purpose, it was evaluated some oxidative stress parameters namely: lipid peroxidation, protein

carbonyl content, glutathione levels, glutathione peroxidase activity, as well as cellular viability, proliferation and migration.

2. Materials and methods

2.1. Materials

The following reactants were used: [^{3}H]-thymidine ([methyl-^{3}H]-thymidine; specific activity 79 Ci/mmol) (GE Healthcare GmbH, Freiburg, Germany), fetal calf serum (Gibco, Life Technologies Corporation, CA, USA), albumin from bovine serum, penicillin/streptomycin/amphotericin B solution, *tert*-butylhydroperoxide (TBH), decane, folic acid, folic acid-free RPMI 1640 medium, glutathione reductase from baker's yeast (*S. cerevisiae*), RPMI 1640 medium, malondialdehyde (MDA), β-NADH-Na_2 (nicotinamide adenine dinucleotide reduced disodium salt hydrate), β-NADPH (β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate), hydrochloric acid, ethyl acetate, ethanol, paraformaldehyde, sodium hydroxide, sodium pyruvate, sulforhodamine B (SRB), 2-thiobarbituric acid (TBA), trichloroacetic acid sodium salt (TCA), 2,4-dinitrophenylhydrazine (DNPH), guanidine hydrochloride, trypsin-EDTA solution, ethylenediamine tetraacetic acid (EDTA) N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), 2-vinylpyridine (Sigma, St. Louis, MO, USA); L-glutamine, perchloric acid and triton X-100 (Merck, Darmstadt, Germany) and L-asparagine (British Drug House LTD).

2.2. Cell culture

BeWo cell line was obtained from the Deutsche Sammulung von Mikroorganismen and Zellkulturen (Braunschwieg, Germany) and HTR-8/SVneo cell line was a gift by Charles H. Graham [45] (Department of Anatomy & Cell Biology, Queen's University at Kingston, Canada). The cell lines were cultured in RPMI 1640 medium (containing L-asparagine (0.05 g/L) and L-glutamine (0.3 g/L)) supplemented with 20 mM HEPES, 15% heat-inactivated fetal calf serum (BeWo cells) or 5% heat-inactivated fetal calf serum (HTR-8/SVneo) and 1% penicillin/streptomycin/amphotericin B (100 U/ml, 100 µg/ml and 0.25 µg/ml, respectively). BeWo and HTR-8/SVneo cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C and were used between passage numbers 17-39 and 117-130, respectively [46-48]. Cells were divided into three experimental groups, which were cultivated under three different medium concentration of FA: a deficient/low concentration of FA (cells cultured in RPMI medium containing 1 nM FA), a physiological (cells cultured in RPMI medium containing 20 nM FA) and a supra-physiological (cells cultured in standard RPMI 1640 medium, which contains a high concentration of FA - 2.3 µM) [49-53]. Culture medium was changed every 2 to 3 days and split was realized every 7 days. For subculturing, BeWo and HTR-8SV/neo cells were removed enzymatically with trypsin 0.25% or trypsin-EDTA 0.05%, respectively. Afterwards, split 1:3 (BeWo) or 1:6 (HTR-8SV/neo) was realized and cells were transferred to plastic culture dishes (21 cm^2; ø 60 mm; TPP®, Trasadingen, Switzerlan). Cell culture in physiological and deficient/low FA concentrations were obtained after an adaptation period of 4 weeks, as described previously by Tavares et al [54]. For the experiments, BeWo and HTR-8SV/neo cells were seeded on collagen-coated 24-well plastic cell

culture clusters (2 cm^2; ø 16 mm; TPP®, Trasadingen, Switzerlan) and cultured for 8 and 3 days, respectively.

2.3. Induction of oxidative stress with tert-butylhydroperoxide (TBH)

In order to induce oxidative stress in BeWo and HTR-8SV/neo cells, they were treated with TBH (50 µM, 100 µM or 300 µM) dissolved in decane, during 24h, in serum-free culture medium. At these concentrations TBH were able to produce the desired level of oxidative stress without compromise cell viability during experiments. Similar concentrations were previously used in BeWo cells [46]. Control cells were exposed to an identical concentration of decane.

2.4. Evaluation of oxidative stress

2.4.1. Quantification of lipid peroxidation levels assay

The formation of thiobarbituric acid-reactive substances (TBARS assay) is used as a lipid peroxidation marker. At the end of the 24h-treatment period of BeWo cells (cultivated at three different FA conditions) with TBH (or the respective solvent), the reaction was started by addition of 50 % acetic acid to each sample, followed by a centrifugation for 2 min at 6000 rpm. Then, 1% TBA was added to the supernatant and the reaction was carried out in a boiling water bath for 40 min. A pink-colored complex was quantified spectrophotometrically at 535 nm [55, 56]. Results were presented as percentage of control.

2.4.2. Quantification of protein carbonyl content

At the end of the 24h-treatment period of BeWo cells (growing under three different FA conditions) with TBH (or the respective solvent), protein carbonyl content was measured by a colorimetric reaction with 10 mM DNPH for 1 h. Afterwards, 20% TCA was added for 15 min and samples were centrifuged for 2 min. Samples were then rinsed with ethanol and ethyl acetate (1:1) and the pellet was solubilized with 6 M guanidine hydrochloride solution [57]. Absorbance of the yellow compound was verified spectrophotometrically at 340 nm. Results were represented as percentage of control.

2.4.3. Evaluation of total (GSX), oxidized (GSSG), and reduced (GSH) glutathione levels

At the end of the 24h-treatment period of BeWo cells (growing under three different FA conditions) with TBH (or the respective solvent), cells were scraped and proteins were precipitated with perchloric acid 5 %, and centrifuged for 10 min at 4 °C. Pellet was discarded and the supernatant was collected and neutralized with an equimolar solution of $KHCO_3$. GSX content was determined by adding GSH reductase (0.4 U) and NADPH (0.24 mM) and observing the colorimetric change of nitrobenzoic acid (0.7 mM) at 415 nm. GSSG content was quantified using 2-vinylpiridine to block free SH groups. Afterwards, GSH levels also were calculated according to the following mathematical formula: GSX = GSH + 2GSSG. All experiments were performed as previously described [58, 59] and the results are represented as percentage of control.

2.4.4. Evaluation of glutathione peroxidase (GPx) activity

At the end of the 24h-treatment period of BeWo cells (growing under three different FA conditions) with TBH (or the respective solvent), GPx activity was measured using an indirect method based on coupled reaction of glutathione reductase, as described elsewhere [60, 61]. Each sample was homogenized and centrifuged at 12.000 g for 5 min. The pellet was discarded and supernatant was used for GPx activity evaluation. Afterwards, it was added 30 µl of the glutathione reductase (2.4 U/ml), 30 µl of reduced glutathione (10 mM) and 30 µl of NADPH (1.5 mM). Then, samples were incubated for 10 min at room temperature. Subsequently, 30 µl of cumene hydroperoxide (7 mM) was added and the kinetic curve was observed spectrophotometrically at 340 nm. Results were expressed as percentage of control.

2.5. Cellular viability assessment

2.5.1. Lactate dehydrogenase (LDH) assay

Cellular viability was quantified in BeWo and HTR-8SV/neo cells (growing under three different FA conditions) after treatment with TBH (or the respective solvent) during 24h, by measuring the activity of extracellular lactate dehydrogenase (LDH). LDH was determined spectrophotometrically at 340 nm by measuring the decrease in absorbance of the NADH during pyruvate reduction to lactate, as described elsewhere [55, 62]. Results were expressed as percentage of control.

2.6. Cellular proliferation assessment

2.6.1. [^{3}H]-thymidine incorporation assay

BeWo cells cultivated under three different FA conditions and treated with TBH (or the respective solvent) during 24h were incubated with [^{3}H]-thymidine (0.025 µCi/ml) during the last 5h of the 24h-treatment period. After this period, cells were fixed by incubation with 10% TCA. Fixed cells were rinsed twice with 10% TCA, air-dried for 30 min and then lysed with 1 M NaOH. HCl was added of the lysate and scintillation fluid. Radioactivity in the samples was quantified by liquid scintillation counting [48, 54]. Cellular DNA synthesis rate was expressed as percentage of control.

2.6.2. Sulphorhodamine B (SRB) assay

To assess culture growth, BeWo cells were cultured under three different FA conditions and treated with TBH (or the respective solvent) during 24h. Then, cells were incubated with 50% TCA for 1h at 4°C. Following, each well was washed with tap water to remove TCA and air-dried for 30 min. 0.4% SRB dissolved in 1% acetic acid was added and maintained for 15 min. Afterwards, cell cultures were washed with 1% TCA to remove residual dye and again air-dried. Stained cells were then solubilized 10 mM Tris.NaOH solution (pH 10.5) and the absorbance of each well was determined at 540 nm [54, 55]. Results were expressed as percentage of control.

2.7. Migration rate assessment

To assess the migration rate of BeWo cells, cultures growing under three different FA conditions were scratched with a 10 µl pipette tip before treatment with TBH (or the respective solvent) for 24h. Images were obtained using a microscope coupled to a camera at 0 and 24 h of treatment with TBH and quantification was performed by using the ImageJ software. Cell migration rate was determined as described previously [48, 63, 64]. Results were expressed as percentage of control.

2.8. Protein determination

Protein content was determined according to Bradford [65] using bovine serum albumin as a standard.

2.9. Statistical analysis

Data obtained from the difference between various groups were analyzed by one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls *post hoc* test. Statistical analysis of the difference between two groups was evaluated by Student's *t*-test. The results were expressed as mean±S.E.M and differences were considered to be significant when $p < 0.05$. All statistics analysis was performed using GraphPad Prism® (Graphpad Software, Inc., La Jolla, CA, USA). Value of *n* indicates the number of experiments.

3. Results

3.1. Effect of TBH on oxidative stress biomarkers and antioxidant status of BeWo cells cultured under distinct FA concentrations

Initially, oxidative stress biomarkers and antioxidant status were evaluated by determination of TBARS, protein carbonyl content, glutathione peroxidase activity and total, reduced and oxidized glutathione levels. Figure 1A shows that the increase in TBARS levels induced by 100 µM TBH was higher in cells cultured at supraphysiological FA concentration ($F(1,23)=10.8;p<0.05$) that in cells cultured under physiological and deficient/low FA-conditions ($F(1,23)=3.5;p<0.05$). Also, the increase in TBARS levels induced by 300 µM TBH was higher in cells cultured at supraphysiological FA concentration than in cells cultured under physiological FA conditions (Fig. 1A).

In contrast, the increase in protein carbonyl content (Fig 1B) in response to TBH (300 µM) was not statistically different in the three groups of cells ($F(1,34)=0.2;p>0.05$) (Fig. 1B).

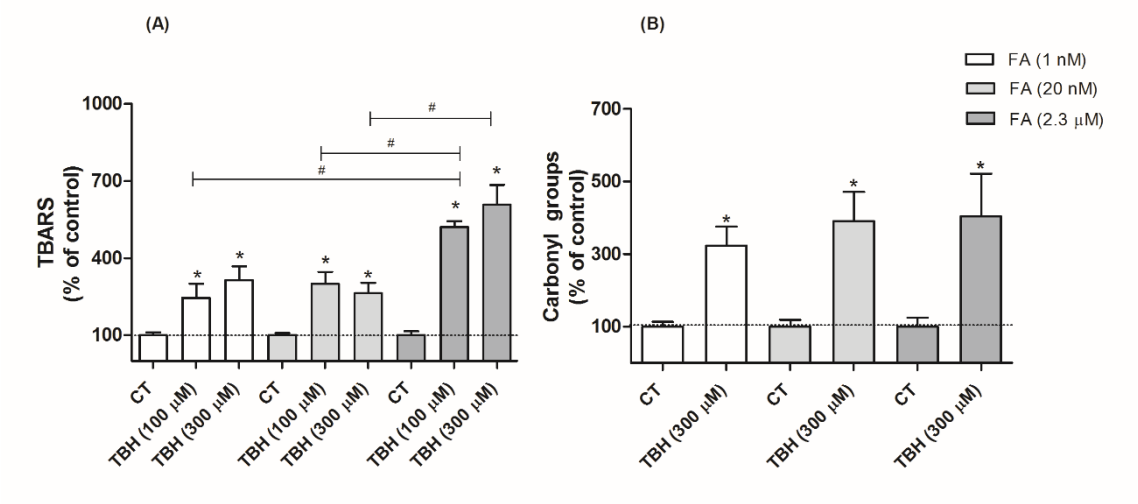

Figure 1. Effect of TBH on TBARS levels (A) (*n*=8) and protein carbonyl content (B) (*n*=10-13) of BeWo cells cultured under distinct medium FA concentrations. BeWo cells cultured under distinct medium FA concentrations were exposed for 24h to TBH (100-300 µM). *Significantly different from the respective control ($p<0.05$) and #significantly different from the respective TBH treatment ($p<0.05$). Data are show as mean ± S.E.M.

In addition, antioxidant defenses were evaluated by quantification of total (GSX), oxidized (GSSG) and reduced (GSH) glutathione levels and of glutathione peroxidase (GPx) activity (Figure 2A-D). The results showed that TBH (300 µM) treatment increased GSX ($F(1,11)=3844.0;p<0.05$) and GSH ($F(1,11)=387.5;p<0.05$) levels in the order: supra-physiological FA>physiological FA>deficient/low FA (Fig. 2A-B). As regarding GSSG analysis, data show higher levels in TBH-treated cells cultured under supra-physiological and physiological FA conditions than under deficient/low FA conditions ($F(1,11)=9.3;p<0.05$) (Fig. 2C). Finally, as shown in Fig. 2D, the activity of GPx was increased after exposure to TBH in cells cultured under supra-physiological and physiological FA conditions ($F(1,32)=4.7;p<0.05$), but not in cells cultured under deficient/low FA

conditions. As a result, the effect of TBH (300 µM) upon GPx activity was significantly different in cells cultured under supra-physiological and physiological FA concentrations when compared with cells cultured under deficient/low FA-conditions (Fig. 2D).

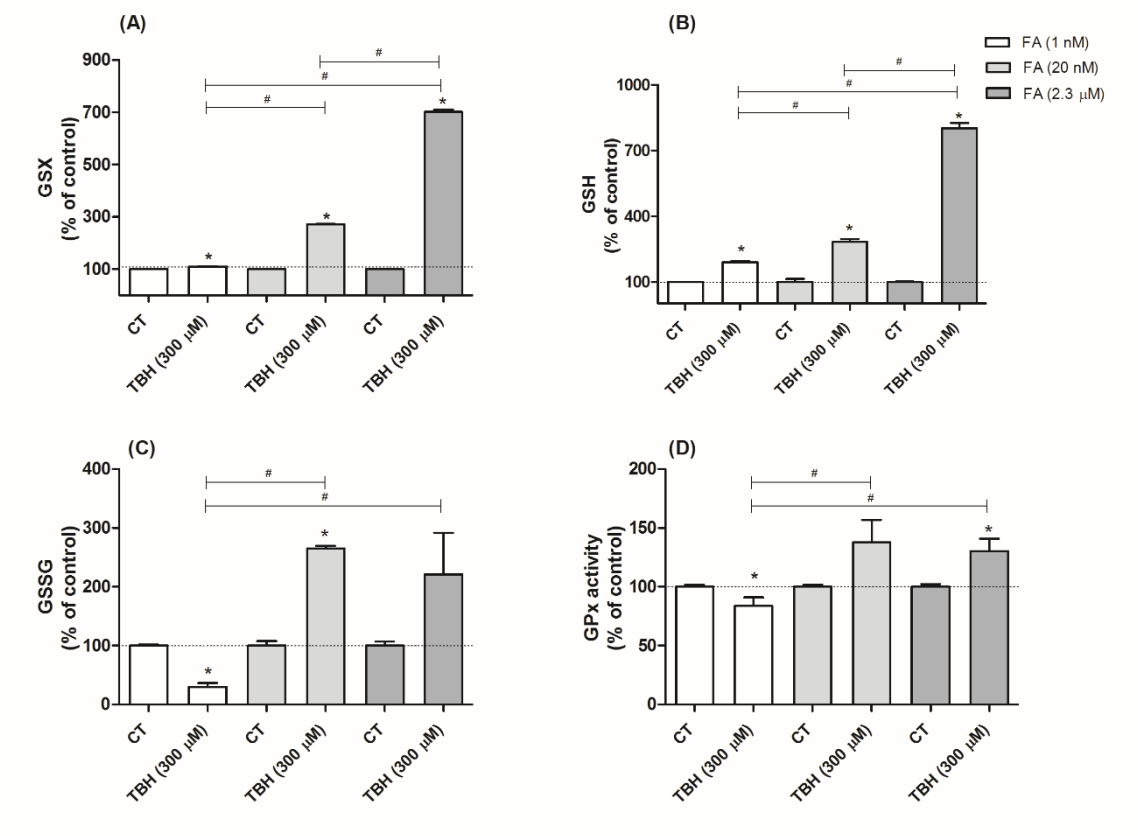

Figure 2. Effect of TBH (300 µM) on total (GSX) (*n*=4) (A), reduced (GSH) (*n*=4) (B) and oxidized (GSSG) (*n*=4) glutathione (C) levels, and glutathione peroxidase activity (GPX) (*n*=11) (D), in BeWo cells cultured at three different FA concentrations. *Significantly different from the respective control ($p<0.05$) and #significantly different from the respective TBH treatment ($p<0.05$). Data are show as mean ± S.E.M.

3.2. Effect of TBH on BeWo and HTR-8SV/neo cell viability under distinct medium FA concentrations

In order to evaluate the cytotoxicity of TBH at distinct medium FA concentrations, the LDH assay was performed with BeWo and HTR-8SV/neo cells. In BeWo cells, the results showed that the cytotoxic effect of TBH (100 µM) was significantly higher in cells cultured under supra-physiological FA concentration, when compared with cells cultured under both physiological FA and low-FA conditions (Fig. 3A) ($F(1,47)=5.7;p<0.05$). The same tendency was observed when cells were treated with TBH 300 µM ($F(1,47)=3.6;p>0.05$), although the difference was not significant (Fig. 3A). Similar results were found with HTR-8SV/neo cells (Fig. 3B) treated with TBH 50 µM ($F(1,35)=3.5;p<0.05$) and 100 µM ($F(1,47)=3.6;p<0.05$). Indeed, results show an increased cytotoxic effect of TBH (50 and 100 µM) in cells cultured under supra-physiological FA concentration than under physiological FA concentration.

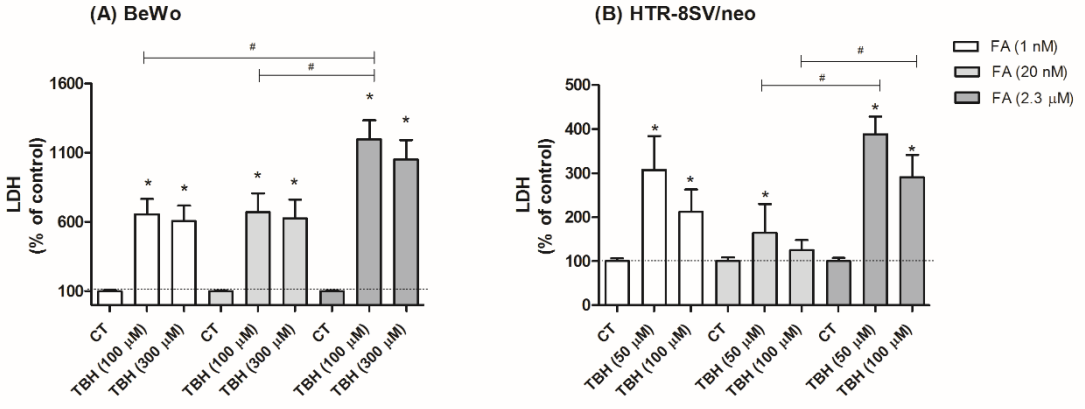

Figure 3. Effect of TBH on the viability of BeWo (Fig. A) and HTR-8SV/neo (Fig. B) cells cultured under distinct medium FA concentrations. BeWo (n=15-16) and HTR-8SV/neo (n=12-24) cells cultured under three different FA concentrations

were exposed for 24h to TBH (100-300 µM and 50-100 µM, respectively) and cell viability (extracellular LDH activity assay) was evaluated. *Significantly different from the respective control ($p<0.05$) and #significantly different from the respective TBH treatment ($p<0.05$). Data are show as mean ± S.E.M.

3.3. Effect of TBH on BeWo cell proliferation under distinct medium FA concentrations

We also determined the effect of TBH upon BeWo cellular proliferation by using two distinct assays: measurement of [^{3}H]-thymidine incorporation and quantification of culture grow (SRB assay). As shown in Fig. 4, exposure of BeWo cells to TBH for 24h (100 and 300 µM) caused a significant decrease in [^{3}H]-thymidine incorporation (Fig. 4A) and culture growth (Fig. 4B). Interestingly enough, the effect of TBH (100 and 300 µM) on [^{3}H]-thymidine incorporation was more marked when cells were cultured with supra-physiological concentrations of FA, when compared with deficient/low FA group ($F(1,29)=4.1;p<0.05$) and ($F(1,20)=3.9;p<0.05$) (Fig, 4A). Corroborating this observation, the effect of TBH (100 and 300 µM) on cell culture growth (SRB assay) was also more marked under supra-physiological and physiological FA medium concentrations than under deficient/low FA conditions ($F(1,47)=8.7;p<0.05$) and ($F(1,43)=4.1;p<0.05$) (Fig. 1B).

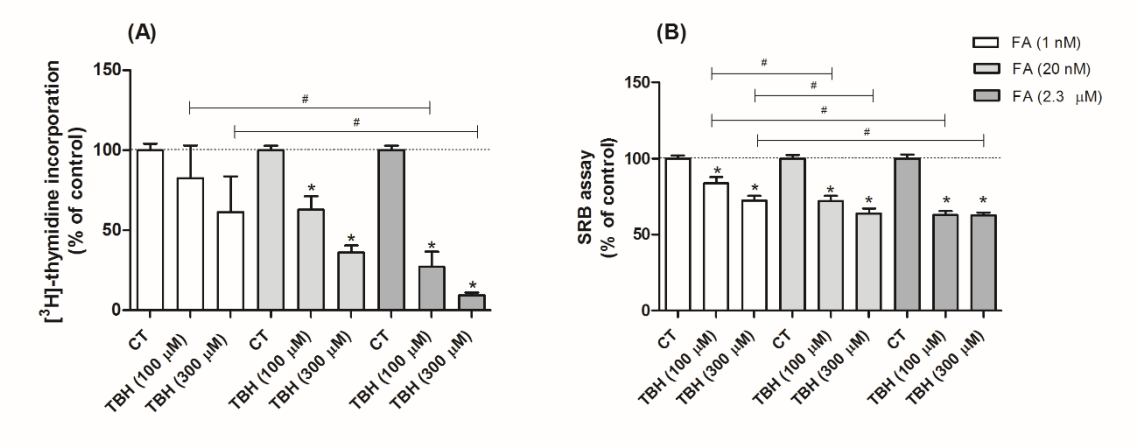

Figure 4. Effect of TBH on proliferation of BeWo cells cultured under distinct medium FA concentrations. BeWo cells cultured under three different FA concentrations were exposed for 24h to TBH (100-300 µM) and cell proliferation (^{3}H-thymidine incorporation assay) (*n*=7-10) and culture growth (SRB assay) (*n*=12-16) were then evaluated. *Significantly different from the respective control ($p<0.05$) and $^{\#}$significantly different from the respective TBH treatment ($p<0.05$). Data are show as mean ± S.E.M.

3.4. Effect of TBH on BeWo cell migration under distinct medium FA concentrations

Under the same conditions, we analyzed BeWo cell migration rates, by performing the injury assay. Curiously, TBH (300 µM) was able to cause a significant decrease in the migration area only when cells were cultured under supra-physiological FA conditions (Fig.5). However, the effect of TBH was no statistically different between groups ($F(1,21)=0.8;p>0.05$), as shown in Fig. 5.

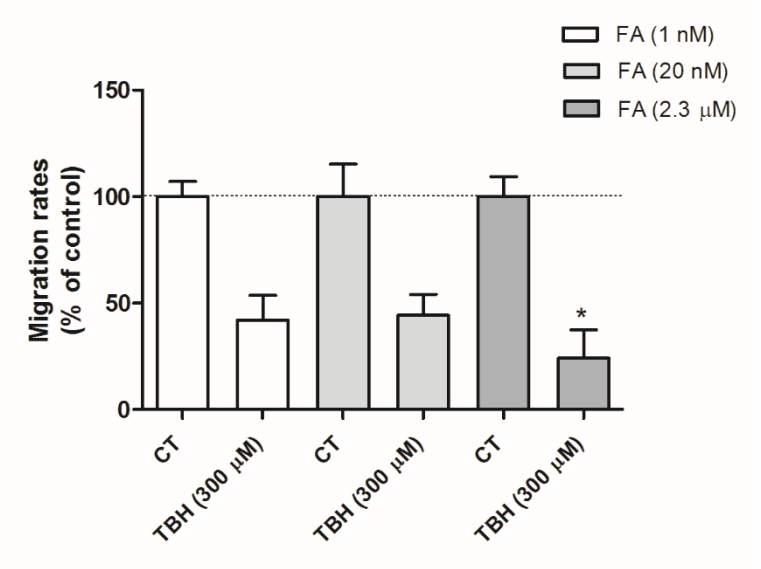

Figure 5. Effect of TBH (300 µM) on migration rates of BeWo cells cultured under distinct medium FA concentrations after injury (24 h) (*n*=6-11). BeWo cells cultured under three different FA concentrations were exposed for 24h to TBH (300 µM). *Significantly different from the respective control ($p<0.05$). Data are show as mean ± S.E.M.

Discussion

PE is a serious pregnancy complication characterized by hypertension and proteinuria, and it is a major cause of maternal and fetal morbidity and mortality [1]. Although its pathophysiology remains unclear, it is established that poor placentation, creating an ischemic placenta, entails excessive release of anti-angiogenic factors that contribute to endothelial dysfunction and oxidative stress [66, 67].

Recently, studies using a chemical cellular model of oxidative stress in human trophoblast cells (BeWo) induced by 24h-exposure to TBH, have demonstrated similar alterations as found in placentas obtained from PE, ie, an increased

cytotoxic and antiproliferative effect and increased levels of oxidative biomarkers (malondialdehyde, carbonyl groups, GSX and GSSG) [46, 68]. Trophoblast cell lines such as HTR-8/SVneo and BeWo cells are the most extensively useful tool for investigations of placental function as they show morphological characteristics and hormonal secretions similar to normal first and third-trimester trophoblast cells *in vivo*, respectively [45, 69-71].

In the present study, we investigated the onset of the oxidative stress response induced by TBH and its deleterious effects on human trophoblast cell models (BeWo and HTR-8 SV/neo) cultured upon three distinct folic acid conditions (deficient/low (1 nM), physiologic (20 nM) and supra-physiological (2.3µM). Our results showed that a 24 h-exposure of BeWo cells to TBH (100 or 300 µM) triggered oxidative stress (increasing its biomarkers), antiproliferative (decreased cellular proliferation and culture growth), antimigration and cytotoxic (increasing extracellular LDH activity) effects. Moreover, HTR-8 SV/neo cells also showed cytotoxicity (increase in extracellular LDH activity) when exposed for 24 h to TBH (50 or 100 µM) treatment. Interestingly, the effects in both cell lines were more marked in the respective order: supra-physiological FA> physiological FA> deficient/low FA.

Initially, the effect of TBH-induced oxidative stress on two oxidative stress biomarkers (lipid peroxidation and protein carbonylation) was investigated. Results showed that TBH (100 or 300 µM) treatment increased malondialdehyde and protein carbonyl levels, suggesting lipoperoxidation and cell protein damage in BeWo cells. Surprisingly, the effect on lipid peroxidation was markedly higher in the following order: supra-physiological cell> physiological FA> deficient/low FA, suggesting that higher FA levels contributed to higher MDA levels in response

to TBH. The present data corroborate previous findings reported by Roy et al. [72], which observed, in female Wistar rats with high intake of FA (8 mg/kg), increased MDA levels in mother plasma and brain pups. This interesting result suggests that FA excess may contribute to an increased lipid peroxidation in response to oxidative stress.

Next, we evaluated the effect of the 24h-exposure of BeWo cells to TBH (300 µM) on levels of the important cellular antioxidant, glutathione, which plays key roles in the cellular control of reactive oxygen species [73]. Results showed that a 24h-exposure of BeWo cells to TBH increased total (GSX), reduced (GSH) and oxidized (GSSG) glutathione levels more markedly in cells cultured upon supra-physiological FA conditions. As expected, glutathione peroxidase (GPx) analysis on BeWo cells treated with TBH (300 µM) also presented an increase on supra-physiological FA supplementation. Several studies have suggested a link between low and high FA supplementation and reduced risk of PE through improved placental and endothelial function by lowering plasma homocysteine [36, 37, 74-76]. Indeed, studies using *in vitro* and *in vivo* models have reported a protective effect of FA supplementation in relation to oxidative stress, associated to its antioxidant properties, improvement in the endothelial dysfunction and reduction of homocysteine levels [43, 77-81]. However, many studies analyzing clinical trials have been inconclusive in demonstrating the effectiveness of FA supplementation in reducing gestational hypertension or PE incidence [35, 38, 82-84]. Surprisingly, the present results indicate that the consequences of oxidative stress (in terms of antioxidant levels (GSH and total glutathione levels) and activity of the antioxidant enzyme GPx) induced by a 24h-exposure of BeWo cells to TBH was markedly higher in cells cultured under supra-physiological FA

conditions, suggesting an over-production of reactive oxygen species (ROS) associated to higher FA levels. These interesting results suggest for the first time, as we known, that excessive FA caused a negative impact on the cellular response to oxidative stress in the trophoblast cell model (BeWo cells) induced by TBH.

Since ROS are the main responsible for DNA, proteins and lipid damage leading to cell death under increased oxidative stress levels [85, 86], we next quantified cellular viability (through LDH measurement) in BeWo and HTR-8 SV/neo cells cultured under different FA levels and exposed for 24h to TBH. Our results showed that a 24h-exposure to TBH (50- 300 µM) induced cytotoxicity in both cellular lines (BeWo and HTR-8 SV/neo), as indicated by the increased extracellular LDH activity. Of importance, this effect was markedly higher in cells cultured under supraphysiological FA levels. Interestingly enough, these findings corroborate a recent study published by Ahmed and colleagues [25], in which it was observed reduced viability in BeWo cells cultured upon high FA supplementation. These results strongly suggest that under an increased oxidative stress condition such as observed in PE, a higher FA concentration may help compromise membrane and cellular structure.

Next, we investigated the effect of distinct FA levels on cellular proliferation and culture growth when cells were challenged with TBH. Our results showed that a 24h-exposure to TBH decreased proliferation and growth culture of BeWo cells. These observations are consistent with previous work of our group, where TBH was found to have an anti-proliferative effect in BeWo cells [46]. Similarly to other parameters already discussed, these effects were higher in cells cultured upon supra-physiological FA supplementation.

In the last part of this work, we evaluated the effect of TBH treatment upon cellular migration after injury. It was observed that BeWo cells treated with TBH presented a decreased cellular migration rate 24h after injury, statistically significant only in cells cultured under supra-physiological FA conditions. These findings are in agreement with our previous results demonstrating that supra-physiological FA condition contribute to the oxidative damage induced by TBH.

In the present study, folic acid status and its eventual unmetabolized levels were not investigated. As mentioned by Swaine and colleagues [87], a high FA supplementation could result in unmetabolized FA and cellular dihydrofolate (DHF) accumulation and consequently chromosome breaks. Although theses authors have not confirmed their hypothesis analyzing chromosome damage in mice erythrocyte progenitor cell, we believe that either unmetabolized FA or DHF excess may be contributing to excessive cell damage and death in response to oxidative stress. In this study, two established trophoblast cellular models, the BeWo and the HTR-8SV/neo cell lines, were used to assess the effect of FA supplementation over TBH cytotoxicity as a model of PE. Our results clearly showed that supra-physiological FA supplementation contribute to oxidative cell damage caused by TBH exposure (as evaluated by oxidative stress biomarker levels, antioxidant levels, antioxidant enzyme activity, and antiproliferative, antimigration and cytotoxic effects. Considering that pregnant women are subject to higher doses of FA due to extra supplementation in food and that the PE development affects 1-8% of all pregnancies, these results suggests more attention on the dose administered, and ultimately, on the overall FA levels during pregnancy.

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Conflict of interest

None.

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6. Discussão

Sabe-se que a HI acarreta em consequências graves para o neonato levando-o muitas vezes à morte. Frequentemente, os desfechos encontrados em casos de EHI são déficits de memória e aprendizado, epilepsia, além de disfunções motoras, entre outros (Kurinczuk *et al.*, 2010; Davidson *et al.*, 2015; Coq *et al.*, 2016). Ademais, normalmente os prejuízos são condicionados ao dano encefálico, principalmente em estruturas importantes para a função cognitiva como o estriado e o hipocampo (Rice *et al.*, 1981; Volpe, 2001a; Rees e Inder, 2005). Dentre os mecanismos envolvidos na patogênese da EHI, e que levam a morte celular neuronal e glial por necrose e apoptose, pode-se citar: o estresse oxidativo, a inflamação e a excitotoxicidade glutamatérgica (Perlman, 2007; Gill e Perez-Polo, 2008).

Diversos estudos têm buscado amenizar o dano encefálico e os resultados causados pela HI neonatal utilizando diferentes estratégias neuroprotetoras. Nos últimos anos o AF tem se destacado como uma vitamina promissora, não somente por prevenir os defeitos no fechamento do tubo neural e anencefalia, mas também por ter um importante papel neuroprotetor na prevenção da Doença de Alzheimer, de declínios cognitivos e da disfunção endotelial causada por altos níveis de homocisteína (Matté *et al.*, 2007; Matté *et al.*, 2009; Li *et al.*, 2015; Shen e Ji, 2015; Gernand *et al.*, 2016). Um estudo recente de nosso grupo sugeriu que a suplementação com AF (5 mg/kg) resulta em melhora na memória aversiva, ansiedade e também na atividade da enzima Na^+,K^+-ATPase (córtex frontal e estriado) em animais tratados diariamente, durante 23 dias, imediatamente antes e após a lesão hipóxico-isquêmica (Carletti *et al.*, 2012). Nesse trabalho, os efeitos sobre a atividade da Na^+,K^+-ATPase foram atribuídos a um potencial efeito antioxidante do AF, também previamente

descrito por Matté *et al.* (2007) onde a mesma dosagem foi utilizada em um modelo animal de homocisteinemia crônica.

No presente estudo, investigou-se o possível efeito protetor da suplementação com AF nas consequências produzidas: a) pela EHI em ratos submetidos ao modelo Rice-Vannucci e B) pelo tratamento com tert-butil-hidroperóxido em células trofoblásticas humanas (linhagem BeWo e HTR-8 SV/neo), simulando o estresse oxidativo ocasionado por PE. Os resultados obtidos durante o desenvolvimento deste trabalho foram apresentados nesta tese na forma de manuscritos e correspondem aos capítulos 1, 2 e 3.

No primeiro capítulo buscou-se investigar, aprofundar e melhor compreender os efeitos da suplementação com AF no modelo de EHI investigando-se o efeito do tratamento com AF sobre o déficit cognitivo apresentado na memória espacial e de trabalho em um teste dependente de hipocampo e estriado (labirinto aquático de Morris), além de mensurar a atividade enzimática da Na^+, K^+- ATPase e a atrofia nessas mesmas estruturas encefálicas de ratos submetidos ao modelo de EHI neonatal. Como esperado, os resultados demonstraram um déficit importante de aprendizado relacionado à memória espacial e de trabalho no teste do labirinto aquático de Morris devido à HI neonatal. Curiosamente, negando nossa hipótese de neuroproteção, para os animais que foram submetidos à EHI, o tratamento com AF exacerbou o déficit de aprendizado e de memória nesses animais. Além disso, observou-se que o tratamento em si prejudicou a memória nos animais que apenas receberam AF como suplementação. Corroborando esses achados, diversos estudos têm observado que a EHI causa prejuízo na aquisição da memória espacial e de trabalho no teste do labirinto aquático e este déficit frequentemente está

associado ao dano estrutural em importantes áreas encefálicas como estriado e região CA1 do hipocampo (Arteni *et al.*, 2003; Ikeda *et al.*, 2006; Pereira *et al.*, 2007; Huang *et al.*, 2016). Também, em um trabalho recente Sittig *et al.* (2012) observaram que ratos adolescentes suplementados diariamente, durante 30 dias, com 8 mg/kg de FA apresentaram déficit de memória no teste do labirinto aquático. Estes autores atribuíram esse prejuízo ao fato de que, neste caso, houve um efeito supressor da suplementação sobre o hormônio da tireoide, demonstrado pelo diminuído conteúdo de seus receptores (α1 e α2) no hipocampo, além de menores níveis plasmáticos de tiroxina (T4) e triiodotironina (T3). É bem estabelecido que a disponibilidade correta dos hormônios da tireoide é essencial para o desenvolvimento normal do hipocampo e consequentemente à formação da memória (Argumedo *et al.*, 2012; Sanchez-Huerta *et al.*, 2016; Vasilopoulou *et al.*, 2016) e diferentes estudos têm demonstrado o efeito do hipotireoidismo no declínio cognitivo em seres humanos e em animais (Wheeler *et al.*, 2015; Vasilopoulou *et al.*, 2016).

Sabe-se também que o correto funcionamento dos receptores glutamatérgicos do hipocampo, como o NMDA, é importante para a formação da memória. A relação dos receptores NMDA e AF também foi abordada em um estudo utilizando um modelo animal de depressão; neste trabalho, o AF apresentou um efeito antidepressivo, reduzindo a imobilidade no teste do nado forçado (Brocardo *et al.*, 2008) e os autores atribuíram esse efeito à inibição dos receptores NMDA pelo AF. Desta forma, considerando que o encéfalo do neonato apresenta alta expressão de receptores NMDA e sabendo-se da sua importância na formação da memória, sugere-se que a inibição destes receptores possa ter contribuído para o prejuízo da memória observado no teste

do labirinto aquático. Portanto, é interessante sugerir que o tratamento crônico com AF especialmente durante o desenvolvimento pode contribuir com efeitos tóxicos e deletérios através de diferentes mecanismos que ainda precisam ser melhor averiguados.

Com o objetivo de correlacionar a atividade enzimática no hipocampo com a performance no teste de memória dependente da mesma estrutura, a atividade da Na^+, K^+- ATPase foi avaliada em dois diferentes períodos do desenvolvimento (P31 e P52). Curiosamente, foi observado um resultado tempo-dependente envolvendo a atividade dessa enzima; demonstrando que o tratamento com AF somado à HI contribuiu para o prejuízo encontrado na atividade enzimática nos animais adolescentes (P31). No entanto, quando os animais foram observados por mais tempo (33 dias) foi averiguada a diminuição da atividade enzimática nos animais HI não tratados e esta atividade foi recuperada, como esperado, pelo tratamento com AF. É relevante observar que a lesão hipóxico-isquêmica ocorre durante dias à semanas e que um dos principais contribuintes para a morte celular é o estresse oxidativo-nitrosativo (Mclean e Ferriero, 2004; Perlman, 2007). Embora neste estudo nós não tenhamos avaliado parâmetros relacionados ao estresse oxidativo-nitrosativo é interessante considerar que o tratamento com AF pode contribuir para diminuir os níveis de homocisteína e por sua vez aumentar a produção de óxido nítrico e assim, estar disponível para a formação do peroxinitrito causando maior desequilíbrio na fase aguda da lesão. Adicionalmente, com o passar dos dias a lesão se estabelece e o organismo se adapta na tentativa de recuperar os danos celulares. Nesta fase, o estresse oxidativo pode amenizado e então o AF disponível poderia contribuir com seu

potencial antioxidante para a melhora na atividade da enzima Na^+, K^+- ATPase, como visto nos animais HI adultos (P52) tratados com essa vitamina.

Ainda, foi observado uma grave atrofia hipocampal e estriatal, no lado ipsilateral à lesão, nos animais submetidos ao procedimento hipóxico-isquêmico, assim como já descrito previamente (Arteni *et al.*, 2003; Pereira *et al.*, 2007; Pereira *et al.*, 2008; Miguel *et al.*, 2015). Esses resultados confirmam que o dano estrutural é, sem dúvida, um dos responsáveis pelo prejuízo cognitivo observado no labirinto aquático. Surpreendentemente, quando observado o volume hipocampal dos animais tratados com AF esses apresentaram recuperação parcial na dimensão dessa estrutura. Entretanto, apesar dessa recuperação não houve melhora cognitiva nesses animais. Para compreender esta interação de resultados, a seguinte fundamentação é apresentada: é bem descrito na literatura que uma das consequências pós-isquemia são os astrócitos tornarem-se reativos e hipertróficos com aumento da expressão do GFAP a fim de participar do isolamento da lesão, plasticidade e inibição da regeneração axonal (Anderson *et al.*, 2003; Middeldorp e Hol, 2011; Sullivan, 2014; Filous e Silver, 2016; Revuelta *et al.*, 2016). Devido ao fato de que o AF participa ativamente na síntese de nucleotídeos e divisão celular, acredita-se que a administração deste possa ter favorecido a replicação astrocítica e consequentemente o aumento no volume da estrutura no lado ipsilateral à lesão nos animais que receberam esse tratamento. Com base no exposto, sugere-se cautela na interpretação desses dados, bem como esses resultados nos apontam que é necessária a realização de mais estudos, visto que o comportamento astrocitário pode variar de acordo com o grau da lesão, e essa por sua vez também apresenta grande variabilidade, e em casos graves o potencial para a preservação da estrutura está reduzido.

No ***capítulo 2,*** visando a melhor compreensão do tratamento agudo e crônico com AF no modelo de EHI e seus efeitos no hipocampo em diferentes períodos do desenvolvimento, foi investigado o efeito do tratamento com AF sobre (i) os parâmetros redox, como a atividade das enzimas SOD e CAT, (ii) a astrogliose reativa em hipocampo, e (iii) os parâmetros funcionais, como atividade locomotora e memória dependente de hipocampo no teste do ox-maze. A fim de esclarecer os resultados controversos e negativos previamente encontrados no teste do labirinto aquático de Morris, novos experimentos foram realizados onde os animais foram tratados com AF por um período curto de tempo (15 dias) e avaliados no P22 em um novo paradigma desenvolvido para avaliar a memória espacial e discriminação visual, o teste do ox-maze. Este teste foi inicialmente elaborado por Wood *et al.* (2011) para avaliar a memória e a discriminação visual, sem um componente aversivo, em um modelo animal de Doença de Huntington. Nossos resultados demonstraram que a EHI causa um prejuízo na memória dos animais, observado pela maior latência para encontrar a primeira recompensa, maior tempo para completar a tarefa e maior número de escolhas incorretas. Esses achados corroboram os achados anteriores do nosso grupo, os quais também verificaram pior performance em uma versão adaptada dessa tarefa nos animais HI mantidos em ambiente padrão durante nove semanas (Rojas *et al.*, 2015). No entanto, quando observados os animais HI tratados com AF foi identificado que esses tiveram o prejuízo no aprendizado amenizado somente nos parâmetros de latência e tempo total ao longo dos dias quando comparados aos animais não tratados. Diversos estudos têm relacionado déficits na memória espacial com dano hipocampal pós-isquemia (Pereira *et al.*, 2007; Pereira *et al.*, 2008). Algumas pesquisas demonstram que

nutrientes relacionados ao metabolismo de 1-carbono, como AF, metionina e colina, são importantes mediadores da metilação ao DNA (Ishii *et al.*, 2014; Tomizawa *et al.*, 2015). Embora neste estudo não é possível afirmar que o AF contribuiu de fato para aliviar o prejuízo na memória, suspeita-se que seu potencial efeito sobre a metilação do DNA poderia estar relacionado à modulação de genes hipocampais envolvidos na formação da memória. Contudo, esta hipótese precisa ser melhor investigada frente à variabilidade dos resultados e uma vez que a melhora ocorreu somente em alguns parâmetros observados ao longo dos dez dias de teste.

A fim de verificar a atividade locomotora dos diferentes grupos experimentais os animais foram avaliados no teste do rota-rod. Nenhuma alteração na coordenação motora foi verificada nos diferentes grupos, esclarecendo que as alterações vistas no teste do ox-maze não podem ser atribuídas a um dano na função motora nesses animais.

Com relação aos achados morfológicos os resultados destacam um aumento da imunorreatividade astrocitária, verificada através do aumento da densidade óptica de GFAP, nos animais HI avaliados nos dois diferentes períodos do desenvolvimento. Tais achados são esperados, uma vez que a isquemia cerebral aumenta a expressão de GFAP, como resposta à lesão, especialmente no hipocampo, estriado e tronco cerebral (Wilhelmsson *et al.*, 2006; Middeldorp e Hol, 2011; Tskitishvili *et al.*, 2014; Parmar e Jones, 2015; Revuelta *et al.*, 2016). Além disso, alguns autores destacam a ocorrência de neurogênese e gliogênese principalmente nessas áreas pós-isquemia e sugerem que este é um potencial mecanismo que colabora para o reparo da lesão e de suas consequências (Darsalia *et al.*, 2005; Jin *et al.*, 2006; Wright *et*

al., 2016). Recentemente, Partearroyo *et al.* (2013) observaram que animais que receberam uma dieta deficiente em AF apresentaram menor número de neurônios e astrócitos no estriado e hipocampo comparados aos suplementados. Esses autores sugerem que o AF é uma vitamina indispensável para a manutenção celular e que níveis adequados são essenciais para modular a expressão de GFAP e outras proteínas do SNC. Durante nosso trabalho, não foram observadas diferenças no processo de reatividade astrocitária entre os grupos HI tratados por 33 dias. Contudo, observou-se que o processo de reatividade astrocitária foi mais lento no grupo hipóxico-isquêmico tratado com AF, conforme análises realizadas aos 15 dias de tratamento. Estes achados, associados ao menor déficit de memória observado no teste do ox-maze aos 15 dias, sugerem uma associação direta entre o processo de gliose reativa/atrofia e o dano à memória, através de um mecanismo regulatório dependente do tempo. No trabalho anterior, foi observado que o AF contribuiu para a recuperação parcial da atrofia hipocampal dos animais HI, e esta melhora foi associada a um possível aumento da proliferação astrocítica. Aqui, os novos resultados demonstram que existe hiper-reatividade astrocítica, mas infelizmente não deixam claro se nesse caso houve maior proliferação celular ou apenas hipertrofia dessas células. Assim, em conjunto estes resultados demonstram as variações no efeito do AF associados a distintos períodos de tratamento e por isso evidenciam a necessidade de mais estudos com o intiuto de elucidar as consequências do tratamento no hipocampo bem como o seu envolvimento na reatividade astrocitária.

Com a finalidade de melhor compreender o mecanismo de ação molecular do AF, seu efeito sob o sistema enzimático associado ao estresse oxidativo

desenvolvido durante a HI foi avaliado. Como descrito anteriormente, a hipoxemia associada à isquemia leva à falha energética e esta é via mais comum para formação de radicais livres, neste caso (Patel *et al.*, 2014). Para isso, o encéfalo conta com enzimas antioxidantes que atuam para detoxificar esses radicais e diminuir seus efeitos deletérios. Duas importantes enzimas que atuam em conjunto para a neutralização do radical superóxido e do peróxido de hidrogênio são a SOD e a CAT (Maksimenko, 2016).

Este estudo observou que, como esperado, a HI causou um desequilíbrio enzimático apresentado por menor atividade da SOD e CAT no P22 e maior atividade no P42, destacando o envolvimento dessas enzimas na tentativa de detoxificar o tecido lesado. Estudos anteriores realizados por nosso grupo destacaram o desequilíbrio nas enzimas detoxificantes pós-HI (Pereira *et al.*, 2009; Rojas *et al.*, 2015). No presente trabalho foi avaliada a variação na atividade enzimática da SOD e CAT a fim de estudar o efeito do AF e seu potencial antioxidante no modelo Rice-Vannucci. Várias pesquisas têm atribuído ao AF um efeito neuroprotetor e antioxidante através de diferentes modelos *in vivo* e *in vitro* (Matté *et al.*, 2009; Kolling *et al.*, 2011; Carletti *et al.*, 2012; Beltagy *et al.*, 2016; Cheng *et al.*, 2016a; Huang *et al.*, 2016). Nossos resultados demonstram um aumento moderado nas atividades enzimáticas no grupo HI suplementado com AF por um período curto (P22). Este resultado pode estar associado ao aparecimento lento da cicatriz glial nos animais HI-AF (P22), conforme descrito acima. Interessantemente, o grupo controle suplementado apresentou elevada atividade enzimática em ambos os períodos, sugerindo que a disponibilidade de AF em si pode ter um efeito pró-oxidante.

Juntos, os resultados observados neste capítulo sugerem o efeito pró-oxidante de altas doses de AF associados à HI. Contudo, devido a limitações do modelo utilizado, mais estudos com o enfoque molecular serão necessários para esclarecer até que ponto o tratamento com AF terá potencial antioxidante ou pró-oxidante.

No ***capítulo 3*** com o intuito de investigar se a suplementação com diferentes doses de AF poderia contribuir de fato para amenizar os efeitos causados pelo estresse oxidativo gerado durante a PE, nosso objetivo foi: investigar o efeito do estresse oxidativo causado pelo TBH em trofoblastos humanos cultivados sobre três distintas concentrações de AF (deficiente, fisiológica e supra-fisiológica). A pré-eclâmpsia é uma doença hipertensiva grave que normalmente envolve extenso estresse oxidativo e disfunção endotelial, os quais levam à hipoperfusão e isquemia placentária, resultados estes que podem levar o feto ao sofrimento, hipóxia, restrição do crescimento e por consequência à EHI. Ademais, torna-se interessante investigar a contribuição do AF nessa situação visto que a suplementação indicada para gestantes muitas vezes ocorre durante toda a gravidez somada à demanda vitamínica advinda da fortificação dos alimentos.

Neste experimento, os trofoblastos das linhagens BeWo e HTR-8 SV/neo foram cultivados em três distintas concentrações de AF e expostos ao agente oxidante TBH, simulando o estresse oxidativo gerado pela PE. Nossos resultados demonstraram que a exposição das células BeWo ao TBH causou um desequilíbrio nos marcadores de estresse oxidativo (TBARS, carbonilas, enzimas antioxidantes e peptídeos da glutationa), além de um efeito antiproliferativo, antimigratório e citotóxico nas culturas. Adicionalmente, as

células HTR8-SV/neo também demonstraram efeito citotóxico quando expostas ao TBH. Curiosamente, esses efeitos foram maiores em ambas as linhagens quando as células foram cultivadas em uma concentração supra-fisiológica de AF.

Diferentes pesquisas têm associado a suplementação com AF ao menor risco de desenvolvimento da PE devido ao seu potencial antioxidante, melhora dos níveis de homocisteína e função endotelial (Wen *et al.*, 2013; Hekmati Azar Mehrabani *et al.*, 2015; Lopez-Alarcon *et al.*, 2015; Martinussen *et al.*, 2015; Singh *et al.*, 2015; Cheng *et al.*, 2016a; Hua *et al.*, 2016; Sayyah-Melli *et al.*, 2016; Shim *et al.*, 2016; Wen *et al.*, 2016). Com o intuito de entender a contribuição das diferentes concentrações de AF durante o estresse oxidativo, diferentes parâmetros bioquímicos foram avaliados neste trabalho. É bem estabelecido que as EROs são as principais responsáveis pelo dano ao DNA, proteínas e lipídios de membrana, os quais levam à morte celular; e um desequilíbrio entre a produção dessas espécies reativas e a capacidade celular da detoxificação é conhecido como estresse oxidativo (Halliwell, 2007; Davalli *et al.*, 2016). Neste experimento, foram observados elevados níveis de malondialdeído e carbonilação proteica, sugerindo que o TBH induziu lipoperoxidação celular e dano proteico nas células BeWo. Curiosamente, os níveis de peroxidação lipídica foram maiores nas células cultivadas na condição supra-fisiológica de AF, sugerindo que a elevada concentração dessa vitamina contribuiu para esse desfecho juntamente com o TBH. Essa alteração corrobora com os resultados encontrados na literatura onde elevados níveis de malondialdeído foram encontrados em plasma de ratas, suplementadas com uma alta dosagem de AF (8 mg/Kg) e em encéfalo de seus filhotes, reforçando

a hipótese de que o excesso dessa vitamina contribui para a lipoperoxidação frente ao estresse oxidativo.

Outro importante detoxificante presente no organismo são a glutationa e seus peptídeos, as quais participam diretamente do controle das EROs, convertendo o peróxido de hidrogênio em água e oxigênio (Couto *et al.*, 2016). Como pode ser observado no presente estudo, houve também um desequilíbrio dos níveis de glutationa (total, reduzida, oxidada) e da enzima GPx causado pelo TBH e um aumento marcado quando observado esses níveis na condição supra-fisiológica de cultivo com AF. Ainda, foi demonstrado um efeito citotóxico, visto pelo nível aumentado de LDH extracelular, consequente ao tratamento com TBH tanto nas células BeWo quanto nas HTR-8 SV/neo. Como esperado, esses níveis foram maiores nas células mantidas nas condições supra-fisiológicas de AF. Esses resultados estão de acordo com um recente estudo publicado por Ahmed *et al.* (2016), o qual verificou viabilidade reduzida celular em trofoblastos humanos da linhagem BeWo cultivadas em alta dosagem de AF (2000 ng/mL). Em conjunto com os resultados anteriores, este experimento mostra pela primeira vez na literatura, que a suplementação supra-fisiológica com AF contribuiu para a formação do estresse oxidativo e seus efeitos deletérios em trofoblastos humanos desafiados com TBH, um modelo apropriado para o estudo da PE.

Estudos demonstraram que o tratamento com TBH durante 24 horas em culturas de trofoblastos humanos (BeWo) causa um aumento dos níveis de marcadores de estresse oxidativo, como TBARS, carbonilas glutationas e seus peptídeos, além de um efeito citotóxico e antiproliferativo, similar ao encontrado em placentas advindas de mães que sofreram PE (Watson *et al.*, 2012; Araújo

et al., 2013). Neste estudo foi observado claramente que o excesso de AF diminuiu a proliferação, o crescimento da cultura e a migração celular, muito provavelmente devido ao seu efeito citotóxico e pró-oxidante.

Embora neste trabalho nós não tenhamos investigado os níveis de AF não metabolizado, a alta disponibilidade dessa vitamina pode facilmente saturar a enzima dihidrofolato redutase (DHFr), a qual converte o ácido fólico em dihidrofolato (DHF) e este em tetrahidrofolato (Swayne *et al.*, 2012). A disponibilidade do DHF pode contribuir para quebras cromossômicas, dano ao DNA e por sua vez levar à citotoxicidade por excesso de AF e consequente morte celular (Bailey e Ayling, 2009; Swayne *et al.*, 2012; Selhub e Rosenberg, 2016).

Fazendo um apanhado geral desta tese, observou-se efeitos contraditórios associados à suplementação com ácido fólico nos mais diversos parâmetros avaliados tanto *in vivo* quanto *in vitro,* nos testes comportamentais, morfológicos e bioquímicos. Não obstante, esses efeitos claramente podem ser associados a dosagem período de tratamento/suplementação e período do desenvolvimento animal. Além disso, nota-se que a suplementação atua por meio de diferentes mecanismos que precisam ser melhor esclarecidos, em importantes estruturas encefálicas como córtex pré-frontal, estriado e hipocampo. Ainda, considerando que 1-8% das gestantes desenvolvem pré-eclâmpsia e essas normalmente estão expostas a altas dosagens de AF devido à suplementação e à ingestão de alimentos fortificados, mais atenção é necessária especialmente na população de risco para essa doença. Tal estudo traz relevante contribuição para o avanço nesta área do conhecimento e destaca que mais pesquisas devem ser demandadas para esclarecer até que ponto a

suplementação com AF e sua dosagem adequada traria benefícios para neonatos com EHI e gestantes com PE.

7. Conclusões

Com base em nossos achados podemos concluir que:

A EHI resultou no modelo in vivo:

- em déficit cognitivo nos testes do labirinto aquático de Morris e no ox-maze;

- redução na atividade da enzima Na^+, K^+ -ATPase em animais adultos;

- atrofia hipocampal e estriatal;

- desequilíbrio na atividade das enzimas antioxidantes SOD e CAT e aumento da reatividade astrocitária vista em diferentes períodos do desenvolvimento animal;

E o tratamento com AF nesse modelo:

- por si só causou déficit cognitivo além de exacerbar o prejuízo funcional dos animais HI tratados por um longo período no teste do labirinto aquático de Morris;

- suavizou o dano no aprendizado e memória no teste do ox-maze;

- inibiu a atividade da Na^+, K^+ -ATPase em animais HI adolescentes e recuperou nos animais HI adultos;

- recuperou parcialmente a atrofia hipocampal;

- per se aumentou a atividade das enzimas antioxidantes SOD e CAT;

- atrasou a formação da cicatriz glial durante o desenvolvimento nos animais HI.

O tratamento com TBH ocasionou no modelo in vitro:

- aumento nos níveis dos marcadores de estresse oxidativo (lipoperoxidação e carbonilação proteica);

- aumento nos níveis das glutationas (GSX e GSH) e glutationa peroxidase;

- menor nível da glutationa reduzida;

- diminuição da viabilidade celular observado pela elevação dos níveis de LDH extracelular;

- efeito antiproliferativo e antimigratório;

E a suplementação com AF nesse modelo:

- aumentou a peroxidação lipídica nos grupos tratados com TBH 100-300 µM, sendo que um maior efeito foi encontrado no grupo suplementado com a concentração supra-fisiológica de AF. Não foi observada diferença entre os grupos fisiológico e deficiente de AF;

- aumentou os níveis das glutationas (GSX e GSH) nas células tratadas com TBH 300 µM sendo mais pronunciado na seguinte ordem de suplementação: supra-fisiológica > fisiológica > deficiente;

- aumentou os níveis de glutationa oxidada (GSSG) nas células tratadas com TBH 300 µM nas concentrações supra e fisiológica de AF, mas não condição deficiente;

- aumentou os níveis da enzima GPx nas células tratadas com TBH 300 µM nas condições supra e fisiológica de AF, mas não na condição deficiente;

- promoveu um efeito citotóxico visto pelo aumento dos níveis LDH extracelular nas culturas BeWo e HTR-8 SV/neo tratadas com TBH 50 e 100 µM na condição supra-fisiológica de AF;

- diminuiu a proliferação celular e o crescimento nas culturas tratadas com TBH 100 e 300 µM na condição supra-fisiológica de AF.

8. Perspectivas

Tendo em vista os resultados dessa tese, nossas perspectivas são investigar:

- o efeito de diferentes dosagens do tratamento com AF no modelo de EHI, através de uma curva dose-resposta;

- níveis de AF não-metabolizado, homocisteína e DHF em diferentes estruturas encefálicas no modelo de EHI;

- a expressão dos diferentes receptores dos hormônios tireoideanos em hipocampo e seus níveis plasmáticos no modelo de EHI;

- o efeito de diferentes dosagens da suplementação com AF em trofoblastos humanos linhagem HTR-8 SV/neo;

- a morte celular por apoptose em trofoblastos humanos (linhagem BeWo e HTR-8 SV/neo) submetidos ao modelo de estresse oxidativo por TBH e suplementados com diferentes concentrações de AF;

- o envolvimento das diferentes enzimas antioxidantes (CAT, SOD, GPx) e outros parâmetros envolvidos no estresse oxidativo e nitrosativo em trofoblastos humanos submetidos ao modelo de estresse oxidativo por TBH e suplementados com diferentes concentrações de AF;

- a captação de AF e os receptores de folato envolvidos nesse processo em trofoblastos humanos submetidos ao modelo de estresse oxidativo por TBH e suplementados com diferentes concentrações de AF.

9. Referências

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10. Anexo

Prezado Pesquisador LENIR ORLANDI PEREIRA SILVA,

Informamos que o projeto de pesquisa ADMINISTRAÇÃO DE ÁCIDO FÓLICO, COMO ESTRATÉGIA NEUROPROTETORA, EM RATOS SUBMETIDOS À HIPÓXIA-ISQUEMIA NEONATAL, encaminhado para análise em 20/07/2012, foi aprovado pelo Comissão de Ética no Uso de Animais com o seguinte parecer:

Projeto Nº: 23564Titulo: ADMINISTRAÇÃO DE ÁCIDO FÓLICO, COMO ESTRATÉGIA NEUROPROTETORA, EM RATOS SUBMETIDOS À HIPÓXIA-ISQUEMIA NEONATAL

Coordenador: Lenir Orlandi Pereira Silva
Projeto de doutoramento
Paracer circunstanciado favorável do PPG Ciencias Biológicas – Neurociências

Objetivo: Investigar o efeito do tratamento com ácido fólico em parâmetros bioquímicos, morfológicos e funcionais de ratos submetidos à hipóxia-isquemia encefálica neonatal.Numero amostral calculado e baseado na experiência previa do grupo. 14 animais por grupo para análise motora, 7 para a avaliação bioquímica e 7 para avaliação imunoistoquímica. 4 grupos experimentais: Controle tratado com salina; Controle tratado com ácido fólico; Hipóxia-isquemia tratado com salina; Hipóxia-isquemia tratado com ácido fólico. 3 tempos experimentais. Total de 270 ratos machos e fêmeas, Wistar nascidos de 34 ratas provenientes do CREAL da UFRGS. Alojamento, alimentação, água, ciclo de luz e temperatura adequados.Aos 7dias, os animais serão anestesiados com halotano 2-4%, e submetidos incisão na linha média da face anterior da região cervical, para fazer a oclusão da artéria carótida direita com fio cirúrgico de seda 4.0. Após os animais permanecerão em recuperação sob temperatura controlada durante 15 minutos antes de serem devolvidos às caixas moradia, aí permanecerão por duas horas para recuperação. Após, em grupos de 5, serão colocados em uma câmara (1500 ml) e expostos à atmosfera hipóxica (8% de oxigênio e 92 % de nitrogênio, com fluxo de 5L/min) durante 90 min. A câmara de hipóxia permanecerá parcialmente submersa em banho-maria a 370C a fim de que seja mantida a temperatura corporal dos ratos. Ao final da hipóxia, os animais serão retirados da câmara e devolvidos à sua caixa de origem. Os animais controle serão submetidos à incisão cirúrgica, porém sem haver a oclusão arterial e sem a exposição ao ambiente hipóxico. Os animais permanecerão com a mãe até o 21º dia e então serão separados por sexo e mantidos em caixas padrão de biotério. A utilização de analgésicos nestes animais é evitada por poder influenciar nos res ultados do estudo. Moderado A solução de ácido fólico será preparada na concentração 0,011µmol/g de peso corporal e ajustado o pH para 7,4 com NaOH. A solução será injetada intraperitoneal imediatamente antes do procedimento de HI e, uma vez ao dia, a partir do 8º até o 42º dia pós-natal. Severidade ModeradaA morte acontecerá após anestesia, por via intraperitoneal com cetamina e xilazina, injetados com 1000 UI de heparina no ventrículo esquerdo e perfundidos por via trans-cardíacaNo 22º e 42º dias os animais serão avaliados quanto a sua coordenação motora e equilíbrio no teste de rota Rod e no teste de suspensão em barra, onde será avaliada a força de preensão dos animais. Destino das carcaças, produtos químicos e demais materiais adequado.Prezada Coordenadora, para aprovação solicitamos que seja inserido no projeto que a solução de ácido fólico a ser injetada IP terá o local de aplicação alternado a cada dia para minimizar o sofrimento dos animais.

CEUA/UFRGS

Parecer 30/08/2012

Os métodos são apropriados para o alcance dos objetivos propostos. Recomendamos que o presente projeto seja aprovado com base na Lei de Procedimentos para o Uso Científico de Animais - Lei No 11.794 (08.10.2008).

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Atenciosamente,

Comissão de Ética no Uso de Animais